

Plasminogen activator inhibitor-1 in black South Africans: methodological and clinical considerations

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Thesis submitted for the degree Doctor *Philosophiae* in Dietetics
at the Potchefstroom Campus of the North-West University

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November 2016

ACKNOWLEDGEMENTS

*All the honour and praise to our heavenly **Father**, because without Him nothing is possible.*

I would like to thank the following people for their valuable contribution during this study:

Prof Marlien Pieters, my Promoter, who taught me so much about research and challenged me every step of the way. Thank you for your valuable inputs, guidance, motivation and patience.

Dr Cornelia Nienaber-Rousseau and **Dr Zelda De Lange**, my two Co-Promoters, for their continuous encouragement and inputs throughout this study.

Prof Salome Kruger, for her time, advice and support in writing up the Sarcopenic Obesity and Non-communicable Disease Risk in African Adults (SONDRAA) data.

Prof Dingeman Rijken, for his assistance in interpreting the plasminogen activator inhibitor-1 (PAI-1) assay data and for his valuable guidance in writing the article.

Prof Du Toit Loots, for his time and effort in assisting with the beta thromboglobulin (β TG) analysis and critical reading of the related paper.

Me Ellenor Rossouw, for her time and effort spent in analysing of the PAI-1 antigen (PAI-1_{ag}) and β TG data.

Sister Chrissie Lessing, for her time and support in helping with the data collection of the sub-study.

Harman Myburgh for his help with collecting the data for the sub-study and for the **Toinet Cronje**, for her help in analysing the data.

Marika Cockeran, for assisting with the statistical analysis of the study's data.

Gerda Beukman, from the Potchefstroom Campus library, for her help in searching and obtaining the needed research articles.

Mary Hoffman and **Dr Isabella Swart**, for the language editing of this thesis.

National Research Foundation (NRF), for supporting this research project by awarding me with an Innovation Doctoral Scholarship (Grant UID 94213) in 2015 and 2016.

My **husband** and **family**, who I love dearly, thank you the continuous understanding, support and motivation.

ABSTRACT

Introduction and aim

Black South Africans are experiencing an increase in the prevalence of cardiovascular disease (CVD). Obesity and abnormal haemostasis are among the underlying risk factors associated with CVD development in the African populations. Fibrinolysis and, in particular, one of its main inhibitors, plasminogen activator inhibitor-1 (PAI-1), have been found to play an important regulating role in the development of abnormal haemostasis and consequently, increase the risk of CVD development. Most of the studies investigating PAI-1 provide data on individuals of European descent. Much less information is available regarding PAI-1 in other ethnicities. From these initial studies, two issues were identified that requires investigation in African participants and formed the aim of this thesis. The first is a methodological issue relating to the influence of platelets in plasma on PAI-1 and PAI-1 related assays as a possible explanation for inconsistencies in ethnic differences reported for PAI-1. The second is a clinical issue focusing on the relationship between PAI-1 and body fat distribution in Africans. Preliminary evidence suggests this relationship may differ from what has been observed in European populations. The specific objectives were to determine the effect of residual platelets in plasma on different plasma PAI-1 and PAI-1 related assays, to investigate the relationship of PAI-1 with body fat distribution patterns in Africans and also to review the literature reporting on the association between body fat distribution and plasma PAI-1 levels.

Participants and methods

Methodological issues were addressed in the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study that included 151 teachers. In addition, data was also used from a follow-up study where 20 academic staff members of a tertiary institution were recruited. As for the clinical issues, available data from the Sarcopenic Obesity and Non-communicable Disease Risk in African Adults (SONDRRAA) study were used in which 246 African women were included. Fasting blood samples were collected from the participants' antebrachial vein branches with a sterile winged infusion set before 10:00. Samples were centrifuged at different centrifugation speeds, as stipulated by the respective study protocols and stored at -82°C until further analysis. The following variables were analysed in the SABPA and follow-up study: PAI-1 activity (PAI-1_{act}), antigen (PAI-1_{ag}), tissue plasminogen activator (tPA)/PAI-1 complex, clot lysis time (CLT), beta thromboglobulin (β TG), and plasma platelet count and size. In the SONDRRAA study, PAI-1_{act}, insulin, glucose, HIV status, high sensitivity C-reactive protein, urinary albumin and creatinine were analysed. In order to determine different body fat

distribution patterns in the SONDRRA study population, measurements of body weight, waist circumference, abdominal and supraspinal skinfolds, as well as body composition by means of dual-energy x-ray absorption were analysed. The identified clinical issues were also addressed by undertaking an overview of *ex vivo* and *in vivo* studies investigating the association between body fat distribution and plasma PAI-1 levels in order to contextualise apparent disparate findings.

Results

Results from the methodological issues that were addressed indicated that a marker of platelet α -granule release (plasma β TG) associated significantly with total PAI-1 content (PAI-1_{ag}) levels and demonstrated weak associations only with active PAI-1 (PAI-1_{act}) and the functional marker, clot lysis time (CLT). In the follow-up study it was indicated that plasma PAI-1_{ag} was also strongly affected by platelet count in a concentration-dependent manner, and that plasma PAI-1_{ag} levels increased even further after complete platelet degradation. Regarding the association of PAI-1 with different body fat distribution patterns (clinical considerations), the research determined that the PAI-1_{act} of sarcopenic obese women did not differ significantly from that of the non-sarcopenic obese women ($p=0.8$) in this study population. Body fat distribution patterns and degree of obesity influenced the relationship of PAI-1_{act} with body fat percentage, insulin, triglycerides and appendicular skeletal mass (ASM). It was also established that PAI-1_{act} was higher (1.65 vs 0.16 U/ml; $p=0.001$) in women with proportionally higher visceral adipose tissue (VAT), compared to women with proportionally higher abdominal subcutaneous adipose tissue (SCAT) in the total study population. This was, however, not the case in the centrally obese sub-group (1.72 vs 0.83 U/ml; $p=0.5$). By applying multiple regression models, it was established that body fat percentage as such, did not contribute markedly to the PAI-1_{act} variance in women with increased fat mass, but that other factors associated with obesity such as inflammation and endothelial damage contributed to a larger extent. Additionally, in the overview of the literature, factors that influence the relationship between body fat distribution and plasma PAI-1 levels were identified, while the relative contribution of adipose tissue compared to other PAI-1 source tissue was also put into perspective.

Conclusion

With regard to methodological issues, it was concluded that residual platelets in plasma significantly influence plasma PAI-1_{ag} levels mainly by increasing latent PAI-1 levels with limited effects on PAI-1_{act}, tPA/PAI-1 complex or CLT and that this is done in a platelet concentration dependent manner. Platelet concentration should therefore be strictly monitored specifically

when measuring PAI-1_{ag}. In terms of the clinical data, it was found that patterns of fat distribution and the degree of obesity influenced the association of PAI-1_{act} with insulin, triglycerides, ASM and body fat percentage in African women and that in conditions of extreme obesity, abdominal SCAT contributes equally to plasma PAI-1_{act} than does VAT. It was furthermore established that the relationship between VAT and plasma PAI-1 levels is not fixed; it rather seems to be regulated by a number of other factors such as the size of the subcutaneous adipose tissue depot, ethnicity, possibly genetics and other obesity-related metabolic abnormalities. Lastly, it was also determined that body fat percentage *per se* contributes less to PAI-1_{act} variance in African women than other obesity related derangements such as endothelial dysfunction and inflammation which should be taken into account when investigating the relationship between body composition and PAI-1.

Key terms: Plasminogen activator inhibitor-1; platelets; beta thromboglobulin; visceral adipose tissue; subcutaneous adipose tissue; body fat distribution

OPSOMMING

Inleiding en doel

Swart Suid-Afrikaners ervaar tans 'n toename in die voorkomsyfer van kardiovaskulêre siekte (KVS). Vetsug en abnormale hemostase is van die onderliggende faktore wat met KVS-ontwikkeling in die Afrika-bevolkingsgroepe geassosieer word. Daar is bevind dat fibrinolise en veral een van sy hoofinhibeerders, plasminogeen aktiveerder inhibitor-1 (PAI-1) 'n belangrike regulerende rol speel in die ontwikkeling van abnormale hemostase en gevolglik die toename in die risiko van KVS-ontwikkeling. Die meeste van die studies wat PAI-1 ondersoek, voorsien data oor individue van Europese afkoms. Baie minder inligting is beskikbaar ten opsigte van PAI-1 in ander etnisiteite. Uit hierdie aanvanklike studies is twee sake geïdentifiseer wat ondersoek vereis onder Afrikane en wat die doel van hierdie tesis ten grondslag lê. Die eerste is 'n metodologiese kwessie wat verband hou met die invloed van plaatjies in plasma op PAI-1 en PAI-1-verwante-analises as 'n moontlike verduideliking vir die teenstrydighede in etniese verskille wat vir PAI-1 gerapporteer is. Die tweede is 'n kliniese kwessie wat fokus op die verhouding tussen PAI-1 en liggaamsvetverspreiding in Afrikane wat volgens voorlopige bewyse, mag verskil van wat in Europese bevolkings waargeneem is. Die spesifieke doelstellings was om die uitwerking van residuele plaatjies in plasma op verskillende plasma PAI-1 en PAI-1-verwante-analises te ondersoek; om die verhouding van PAI-1 met liggaamsvetverspreidingspatrone onder Afrikane te ondersoek en ook om 'n oorsig te doen van die literatuur wat verslag doen oor die verwantskap tussen liggaamsvetverspreiding en plasma PAI-1-vlakke.

Deelnemers en metodes

Metodologiese kwessies is aangespreek in die studie van die Simpatiese Aktiwiteit en Ambulente Bloeddruk in Afrikane (SABDA) wat 151 onderwysers ingesluit het. Daarby is data ook gebruik uit 'n opvolgstudie waarin 20 akademiese personeellede van 'n tersiêre instelling gewerf is. Wat betref die kliniese kwessies is data van die studie oor Sarkopeniese Vetsug- en Nie-oordraagbare Siekterisiko in Afrikaanvolwassenes (SVNORAV) gebruik waarin 246 Afrika-vroue ingesluit is. Vastende bloedmonsters is voor 10:00 uit deelnemers se antebagiale are versamel. Monsters is gesentrifugeer teen verskillende sentrifugeringsnelhede soos gestipuleer deur die onderskeie studieprotokolle en gestoor by -82°C tot verdere analise. Die volgende veranderlikes is in die SABDA en opvolgstudie geanaliseer: PAI-1-aktiwiteit ($\text{PAI-1}_{\text{act}}$), antigeen (PAI-1_{ag}), weefsel plasminogeen aktiveerder (tPA)/PAI-1-kompleks, stol-lisetyd (SLT), beta tromboglobulien (βTG), en plasmaplaatjietelling en -grootte. In die SVNORAV-studie is $\text{PAI-1}_{\text{act}}$,

insulien, glukose, MIV-status, hoë sensitiviteit C-reaktiewe proteïen, urinêre albumien en kreatien gemeet. Om verskillende liggaamsvetverspreidingspatrone te bepaal in die SVNORAV-studie, is mates van liggaamsgewig, middelomtrek, buik- en supraspinale velvoue, asook liggaamsamestelling deur middel van dubbel-energie x-straalabsorpsie geanaliseer. Die geïdentifiseerde kliniese kwessies is ook aangespreek deurdat 'n oorsig gedoen is van *ex vivo* en *in vivo* studies wat die verwantskap tussen liggaamsvetverspreiding en plasma PAI-1-vlakke ondersoek het om oënskynlik uiteenlopende bevindings te kontekstualiseer.

Resultate

Resultate van die metodologiese kwessies wat aangespreek is, het getoon dat 'n merker van plaatjies α -granulevrystelling (plasma β TG) betekenisvol geassosieer het met totale PAI-1-antigeen (PAI-1_{ag}) vlakke alleenlik en swak geassosieer het met die aktiewe vorm van PAI-1 (PAI-1_{act}) en die funksionele merker, SLT. In die opvolgstudie is daar getoon dat plasma PAI-1_{ag} ook sterk beïnvloed is deur plaatjietelling op 'n konsentrasie-afhanklike wyse en dat plasma PAI-1_{ag} vlakke nog meer toegeneem het na algehele plaatjie-afbreking. Wat betref die verwantskap van PAI-1 met verskillende liggaamsvetverspreidingspatrone (kliniese kwessies), het die navorsing vasgestel dat die PAI-1_{act} van sarkopenies-vetsugtige vroue nie aansienlik verskil het van dié van die nie-sarkopenies-vetsugtige vroue ($p=0.8$) in hierdie studie se bevolking nie. Liggaamsvetverspreidingspatrone en vetsuggraad het die verhouding van PAI-1_{act} met liggaamsvetpersentasie, insulien, trigliseriede en appendikulêre skeletmassa (ASM) beïnvloed. Daar is ook vasgestel dat PAI-1_{act} hoër was (1.65 vs 0.16 U/ml; $p=0.001$) in vroue met proporsioneel hoër visserale vetweefsel (VWV), vergeleke met vroue met proporsioneel hoër abdominale onderhuidse vetweefsel (OVW) in die totale studiebevolking. Dit was egter nie die geval met die sentraal-vetsugtige sub-groep (1.72 vs 0.83 U/ml; $p=0.5$) nie. Deur die toepassing van veelvoudige regressiemodelle is daar vasgestel dat liggaamsvetpersentasie as sodanig nie noemenswaardig bygedra het tot die PAI-1_{act} variansie in vroue met verhoogde vetmassa nie, maar dat ander faktore wat verbind word met vetsug, soos inflammasie en endoteelskade tot 'n groter mate bydra. Daarby is daar in die oorsig faktore geïdentifiseer wat die verhouding tussen liggaamsvetverspreiding en PAI-1-vlakke beïnvloed, terwyl die relatiewe bydra van vetweefsel vergeleke met ander PAI-1 bronweefsel ook in perspektief geplaas is.

Gevolgtrekking

Met betrekking tot metodologiese kwessies, is daar tot die gevolgtrekking gekom dat residuele plaatjies in plasma, plasma PAI-1_{ag} vlakke aansienlik beïnvloed deur hoofsaaklik latente PAI-1-

vlakke te verhoog met 'n kleiner effek op PAI-1_{act}, tPA/PAI-1 kompleks of SLT en dat dit gedoen word op 'n plaatjekonsentrasie-afhanklike manier. Plaatjekonsentrasie moet derhalwe streng gemoniteer word, veral wanneer PAI-1_{ag} gemeet word. Ten opsigte van die kliniese data is daar bevind dat patrone van vetverspreiding en die graad van vetsug die verhouding van PAI-1_{act} met insulien, trigliseriede, ASM en liggaamsvetpersentasie in Afrika-vroue beïnvloed het en dat in toestand van uiterste vetsug, abdominale OVW in gelyke mate tot plasma PAI-1_{act} bydra as VVW. Daar is verder vasgestel dat die verhouding tussen VVW en plasma PAI-1-vlakke nie vas is nie; dit wil eerder voorkom dat dit gereguleer word deur 'n aantal ander faktore, soos die grootte van onderhuidse vetweefselstore, etnisiteit, moontlik genetica, asook ander vetsugverwante metaboliese abnormaliteite. Laastens is daar ook vasgestel dat liggaamsvetpersentasie *per se* minder bydra tot PAI-1_{act} variansie in Afrika-vroue as ander vetsugverwante versteurdhede, soos endoteeldisfunksie en inflammasie wat in ag geneem moet word wanneer die verhouding tussen liggaamsamestelling en PAI-1 ondersoek word.

Sleuteltermes: Plasminogeen aktiveerder inhibitor-1; plaatjies; beta tromboglobulien; visserale vetweefsel; onderhuidse vetweefsel; liggaamsvetverspreiding

LIST OF ABBREVIATIONS

a	Activated
A	alanine
ACE	angiotensin-converting enzyme
ANCOVA	analysis of co-variance
ANOVA	analysis of variance
ASM	appendicular skeletal muscle mass
bp	base pair
BMI	body mass index
βTG	beta thromboglobulin
CA	cytosine-adenine
CI	confidence interval
CLSI	Clinical and Laboratory Standards Institute
CLT	clot lysis time
CRP	C-reactive protein
CTAD	citrate-theophylline, adenosine, dipyridamole
CVD	cardiovascular disease
DBP	diastolic blood pressure
DXA	dual-energy x-ray absorption
ECLIA	electrochemiluminescence immunoassay
ELISA	enzyme-linked immunosorbent assay
EWGSOP	European working group on Sarcopenia in Older people
F	Factor

FNIH	Foundation for the National Institutes of Health
<i>g</i>	gravitational acceleration
G	guanine
HDL-CHOL	high-density lipoprotein-cholesterol
HIV	human immunodeficiency virus
HREC	Health Research Ethics Committee
HRT	hormone replacement therapy
HOMA-IR	homeostatic model assessment of insulin resistance
HUVECs	human umbilical vein endothelial cells
IgG	immunoglobulin G
IL-1	interleukin-1
IL-6	interleukin-6
Indel	insertion/deletion
IR	insulin resistance
ISAK	International Society for the Advancement of Kinantropometry
kb	Kilo bases
LD	linkage disequilibrium
LDL-CHOL	low-density lipoprotein-cholesterol
MetS	metabolic syndrome
MI	myocardial infarction
Min	minutes
MPV	mean platelet volume
mRNA	messenger ribonucleic acid

NCDs	non-communicable diseases
NWU	North-West University
PA	plasminogen activator
PAI-1	plasminogen activator inhibitor type-1
PAI-1 _{act}	plasminogen activator inhibitor type-1 activity
PAI-1 _{ag}	plasminogen activator inhibitor type-1 antigen
PF4	platelet factor 4
PPP	platelet-poor plasma
PRP	platelet-rich plasma
PURE	Prospective Urban and Rural Epidemiology
RFLP	restriction fragment length polymorphism
SABPA	Sympathetic activity and Ambulatory blood pressure in Africans
SANHANES	South African National Health and Nutrition Examination Survey
SBP	systolic blood pressure
SCAT	subcutaneous adipose tissue
SD	standard deviation
SEM	standard error of mean
Serpin	serine proteinase inhibitor
SNP	single-nucleotide polymorphism
SO	sarcopenic obesity
SONDRAA	Sarcopenic obesity and non-communicable disease risk in African adults
T-CHOL	total cholesterol
TF	tissue factor

TFPI	tissue factor pathway inhibitor
TGF- β 1	transforming growth factor- β 1
THUSA	transition and Health during Urbanisation
TNF- α	tumour necrosis factor- α
tPA	tissue plasminogen activator
TRAIN	Trial of Angiotensin-Converting Enzyme Inhibition and Novel Cardiovascular Risk factors
uPA	urokinase-type plasminogen activator
VAT	visceral adipose tissue
VLDL	very low-density lipoprotein
WC	waist circumference
WHR	waist-to-hip ratio
WHtR	waist-to-height ratio
Yrs	years

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CHAPTER 1 INTRODUCTION

1.1 Background

South Africa is a fast growing third world country in which the effects of urbanisation are evident, especially in the black African population. This process of rapid urbanisation has recently been associated with a double burden of disease with an accelerating shift from infectious to non-communicable diseases, specifically cardiovascular diseases (CVD) (Vorster, 2002; Kadiri, 2005). These findings are reflected in the mortality patterns recorded by Statistics South Africa from 2009 to 2011. Cerebrovascular diseases accounted for 5.1% of the total mortality (505 803) in 2011. It was also the most important cause of death after tuberculosis (10.7%), influenza and pneumonia (6.6%) (StatsSA, 2011). There also seems to be an increase in the mortality rates of South Africans above the age of 60 years, due to the rise in vascular diseases (stroke, ischaemic heart disease and hypertensive diseases) (Tollman *et al.*, 2008). In addition, it would seem that the prevalence of obesity and associated lifestyle diseases, such as diabetes, are increasing in South Africa (Ng *et al.*, 2014). South African women have a substantial prevalence of overweight and obesity (24.8% and 39.2%, respectively), which is significantly higher compared to men (20.1% and 10.6%, respectively) (Shisana, 2014).

Elevated plasminogen activator inhibitor-1 (PAI-1) is regarded as a risk factor for the development of CVD and metabolic syndrome (MetS), which is associated with dyslipidaemia, hypertension, glucose intolerance and increased abdominal fat distribution (Mutch *et al.*, 2001; Mertens *et al.*, 2006; Alessi & Juhan-Vague, 2008). This could in part be explained by the fact that PAI-1 is an important regulator (inhibitor) of fibrinolysis (Hoekstra *et al.*, 2004), which refers to the fibrin clot lysis process. More specifically, PAI-1 inhibits the binding of tissue type plasminogen activator (tPA) to plasminogen by binding to and inactivating tPA. The binding of tPA to plasminogen is necessary for the formation of plasmin, which cleaves fibrin clots into soluble fibrin fragments. PAI-1, therefore, plays a regulating role in the development of abnormal haemostasis and consequently, increased CVD risk. PAI-1 is a glycoprotein with a relative molecular weight of 50 000 Dalton and is a member of the serine proteinase inhibitor (serpin) family (Pannekoek *et al.*, 1986; Kruithof, 1988). Plasma PAI-1 exists either in an active or latent form, or in complex with tPA (Hekman & Loskutoff, 1985; Sprengers & Kluft, 1987; Gils & Declerck, 2004). PAI-1 is produced by various cells such as endothelial cells, hepatocytes, smooth muscle cells, adipocytes, and platelets (Sprengers & Kluft, 1987; Alessi *et al.*, 1997). In platelets, PAI-1 is stored in the alpha granules and released during platelet activation and aggregation (Erickson *et al.*, 1984; Sprengers & Kluft, 1987; Bastard & Pieroni, 1999).

Most of the studies investigating PAI-1 provide data on individuals of European descent. Much less information is available regarding PAI-1 in other ethnicities. Only a few publications exist investigating PAI-1 in Africans (Jerling *et al.*, 1994; Greyling *et al.*, 2007; Naran *et al.*, 2008; Pieters *et al.*, 2010; De Lange *et al.*, 2013; Eksteen *et al.*, 2015). From these initial studies, two issues were identified which will be addressed in this thesis. The first is a methodological issue relating to the influence of platelets in plasma on PAI-1 and PAI-1 related assays. The second is a clinical issue focusing on the relationship between PAI-1 and body fat distribution in Africans. Preliminary evidence suggests this relationship may differ from what has been observed in European populations. This will be discussed below.

Methodological: Previous research measuring PAI-1 levels in African and white ethnic groups has shown inconsistent ethnic differences (Jerling *et al.*, 1994; Festa *et al.*, 2003; Matthews *et al.*, 2005; Lutsey *et al.*, 2006; Greyling *et al.*, 2007; Naran *et al.*, 2008; Perry *et al.*, 2008). This inconsistency may be related to the specific PAI-1 assays used in the different studies as well as differences in sample handling procedures and in particular centrifugation speed. Different centrifugation speeds will result in different platelet counts in the plasma, which may in turn influence plasma PAI-1 levels. While it is known that platelets produce PAI-1, it was traditionally believed that platelets store and release mostly latent PAI-1, since, only approximately 5-10% of PAI-1 antigen was shown to be active in lysed platelet-rich plasma (Declerck *et al.*, 1988a). More recent studies, however, suggest that platelets also release a substantial amount of active PAI-1 (Nordenhem & Wiman, 1997; Brogren *et al.*, 2004; Brogren *et al.*, 2011). These observed differences could be the result of different approaches used to prepare the platelet lysates as well as other experimental conditions (Sancho *et al.*, 1994; Brogren *et al.*, 2011), and for now remains an issue of debate. As mentioned previously, PAI-1 exists in several forms in plasma and different assays exist to measure the different forms. Active PAI-1, for instance, is the PAI-1 that is functionally able to bind tPA and to form the tPA/PAI-1 complex and can be measured by functional assays recognising this active form only. Additionally, the tPA/PAI-1 complex itself can be measured separately and lastly the total PAI-1 content in plasma can be determined by assays measuring total PAI-1 antigen (this includes active PAI-1, the tPA/PAI-1 complex as well as the inactive, latent form). It is, however, not clear how the PAI-1, released from the alpha granules of residual platelets in plasma, affect the plasma levels of the different forms of PAI-1 (activity, antigen and tPA/PAI-1 complex) and whether such altered levels will result in functional effects by affecting the plasma fibrinolytic potential. This will be investigated in detail in this thesis in two studies which comprised of 151 participants who formed part of the Sympathetic

activity and Ambulatory Blood Pressure in Africans (SABPA) study and a further 20 participants who were recruited in an additional follow-up study.

Clinical: It is known that PAI-1 is synthesised in, among others, the adipose cells. Several clinical studies have associated obesity with impaired fibrinolysis (Giltay *et al.*, 1998; Bastard *et al.*, 2000; Lindeman *et al.*, 2007). Reviews on the association between obesity and PAI-1 (Mutch *et al.*, 2001; Skurk & Hauner, 2004) report a difference in PAI-1 secretion by adipose tissue depending on the region of fat distribution. Visceral adipose tissue (VAT) is accepted to be the most important source of adipose derived PAI-1 (Bastard *et al.*, 2000). However, contradictory results were reported by Eriksson *et al.* (2000) who found greater *PAI-1* messenger ribonucleic acid (mRNA) expression in subcutaneous adipose tissue (SCAT) compared to VAT, while, Lindeman *et al.* (2007) found comparable *PAI-1* mRNA expression between SCAT and VAT. In addition, Mavri *et al.* (2001) found abdominal SCAT to produce more PAI-1 than peripheral SCAT. Contradicting evidence furthermore also exists regarding the influence of the different fat depots on plasma PAI-1 levels, with limited information only in non-white ethnicities. Previous research has shown differences in the relationship of PAI-1 with body composition between ethnicities (Solano *et al.*, 2003; Greyling *et al.*, 2007). There are also differences in the body fat distribution patterns between different ethnic groups. African women seem to have significantly less VAT compared to white women, despite similar waist circumference (Micklesfield *et al.*, 2010). It is thus not possible to simply extrapolate findings from studies investigating individuals of European descent, to African populations.

Furthermore, the relationship of PAI-1 with specific forms of obesity, such as sarcopenic obesity (SO) is not well defined (Cesari *et al.*, 2005; Cesari *et al.*, 2010). Sarcopenic obesity is considered to be an age-related increase in fat mass, associated with loss of skeletal muscle and fat infiltrating the muscle (Cruz-Jentoft *et al.*, 2010; Kohara, 2014). As SO has been demonstrated to be associated with the development of MetS, insulin resistance and cardiovascular risk through the development of atherosclerosis (Kohara, 2014), it is possible that plasma PAI-1 levels will be higher in individuals with SO compared with non-sarcopenic obese individuals. It is, therefore, necessary to increase our understanding of the association between PAI-1 and body fat distribution, particularly in the understudied African population. This will be addressed in this thesis by providing experimental data on PAI-1 and different body fat distribution patterns in 246 urban dwelling African women who took part in the Sarcopenic Obesity and Non-communicable Disease Risk in African Adults (SONDRAA) study. In addition, the thesis includes a published review of the related literature. The purpose of this review is to

provide an overview of studies investigating the association between body fat distribution and plasma PAI-1 levels. It furthermore identifies factors that influence this relationship and also considers the contribution of other tissue to plasma PAI-1 levels.

1.2 Aim and objectives

The aim of this study is, therefore, to determine two unresolved methodological and clinical issues related to PAI-1, as identified in the black South African population.

In order to reach this aim the following objectives were identified:

- 1) To determine the effect of residual platelets, present in plasma, on different plasma PAI-1 assays (total PAI-1 content, active PAI-1 and tPA/PAI-1 complex) as well as a functional marker namely clot-lysis time (CLT);
- 2) to explore the relationship of PAI-1 with body fat in African women with special focus on excessive obesity, sarcopenic obesity and body fat distribution patterns; and
- 3) to review *ex vivo* and *in vivo* studies investigating the association between body fat distribution and plasma PAI-1 levels, in order to identify factors that may potentially influence the relationship and to determine the contribution of body fat to plasma PAI-1 levels relative to that of other PAI-1 producing tissue.

1.3 Structure of this thesis

This thesis will be presented in article format. Following this introduction, Chapter 2 will provide an overview of the literature relevant to this research topic focusing on: the haemostatic system and the regulation of PAI-1 in plasma; the various forms in which PAI-1 can be measured in the plasma; and lastly the relationship between PAI-1 and obesity as well as SO. Chapter 2 provides the background information that will be required to interpret the research papers presented in this thesis.

Chapter 3 is an article with the title: “*The effects of residual platelets in plasma on plasminogen activator inhibitor-1 and plasminogen activator inhibitor-1-related assays*”. This article has been published in *PLOS ONE* (<http://dx.doi.org/10.1371/journal.pone.0171271>). This article addresses the controversial evidence with respect to the form of PAI-1 present in platelets, by relating the effects of residual platelets present in plasma (a potential pre-analytical variable) to various PAI-1 and PAI-1-related assays.

Chapter 4 is an article with the title: “*Degree of obesity influences the relationship of PAI-1 with body fat distribution and metabolic variables in African women*”. This article has already been published in *Thrombosis Research* (Volume 146 (2016) 95-102 p.). In this article the relationship of PAI-1 with body fat percentage and two different body fat distribution patterns (SO and visceral compared to subcutaneous abdominal obesity) in African women is investigated.

Chapter 5 is a review article with the title: *The contribution of different adipose tissue depots to plasma plasminogen activator inhibitor-1 (PAI-1) levels*”. This article has already been published in *Blood Reviews* and is available in E-pub ahead of print format. This review provides an overview of studies investigating the relationship between body fat distribution and plasma PAI-1 level and factors that can influence this relationship.

Chapter 6 is the final chapter and provides the discussion of results and main conclusions that could be drawn from this study. Recommendations and research possibilities for future studies is also highlighted.

The references of Chapter 1, 2 and 6 are provided according to the mandatory Harvard referencing style of the North-West University at the end of the thesis. Whereas, the references of Chapter 3, 4 and 5 are provided at the end of each chapter, as specified by the authors instructions of each journal in which the article was / will be published.

1.4 Research team and contributions to articles presented as part of this thesis

The contributions of the researchers involved in the study presented in this thesis are given in the table below.

Table 1.1: List of members in the research team and their contributions to this study.

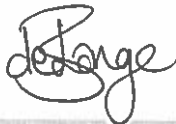
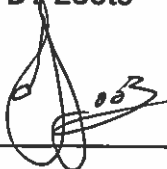
Initial, surname and signature*	Affiliation	Role in the study
SA Barnard (PhD candidate) 	CEN, NWU, Potchefstroom campus, South Africa	Responsible for ethical application, literature review, statistical analysis, data interpretation and writing up of articles and thesis.
Prof M Pieters (Promoter) 	CEN, NWU, Potchefstroom campus, South Africa	Promoter of SA Barnard, analysis and interpretation of results and co-authored Chapters 3-6.
Dr C Nienaber-Rousseau (Co-Promoter) 	CEN, NWU, Potchefstroom campus, South Africa	Co-promoter of SA Barnard and co-authored Chapter 4.
Dr Z De Lange (Co-Promoter) 	CEN, NWU, Potchefstroom campus, South Africa	Co-promoter of SA Barnard and co-authored Chapter 5.
Prof S Kruger 	CEN, NWU, Potchefstroom campus, South Africa	Principle investigator for the SONDRRA study and co-authored Chapter 4.
Prof DT Loots 	Centre of Human Metabolomics, NWU, Potchefstroom campus, South Africa	Assisted with the β TG analyses and co-authored Chapter 3.

Table 1.1 (continues): List of members in the research team and their contributions to this study

Prof DC Rijken 	Department of Hematology, Erasmus University Medical Center, Rotterdam, Netherlands	Assisted with data interpretation and co-authored Chapter 3.

*with my signature I declare that I approved the above-mentioned articles, that my role in the study as indicated above, is representative of my actual contribution and that I hereby consent that it may be published as part of the PhD thesis of Mrs SA Barnard.

βTG, beta thromboglobulin; CEN, Centre of Excellence for Nutrition; NWU, North-West University; SONDRRAA, Sarcopenic Obesity and Non-communicable Disease Risk in African Adults study.

CHAPTER 2 LITERATURE REVIEW

2.1 Introduction

Globally, there is a growing awareness of the need to address the increasing prevalence of non-communicable diseases (NCDs). It is projected that the percentage of people dying from NCDs will increase from 59% in 2002 to 69% in 2030 (Mathers & Loncar, 2006). Cardiovascular disease (CVD), one of the major contributors of NCDs, has until recently been recorded and observed mostly in developed countries (Reddy, 2002). It is estimated that 2150 Americans die of CVD every day (Go *et al.*, 2013). More recently, however, it has become clear that CVD is also affecting the mortality rates of developing countries such as South Africa (Mayosi *et al.*, 2009).

Data from the South African Health and Demographic Survey, which included 52 health districts throughout South Africa, provided evidence on the distribution of CVD in South Africa (Kandala *et al.*, 2014). Cardiovascular diseases such as hypertension and stroke were identified as being concentrated largely in the south-western parts of the country, whereas the northern parts were associated with coronary heart disease and hypercholesterolaemia (Kandala *et al.*, 2014). Other researchers have previously reported that South Africa is experiencing a double burden of disease, with an accelerating shift from infectious diseases to NCDs, especially CVD (Vorster, 2002; Kadir, 2005). Supported by the 2011 statistical release on the mortality and causes of death in South Africa, cerebrovascular diseases were identified as the third highest cause of death in South Africa (StatsSA, 2011). In addition, epidemiology research has shown that, relative to the period from 1992 to 1994, the period from 2002 to 2005 experienced a 65% increase in the mortality rates of rural South Africans above the age of 60 years due to vascular diseases, including stroke, ischaemic heart disease and hypertension (Tollman *et al.*, 2008).

The rise in CVDs has been associated with urbanisation and a change in lifestyle factors (Vorster, 2002; Pieters & Vorster, 2008). For example, black South Africans are undergoing a process of rapid urbanisation that is associated with increased risk factors (such as change in the intake of total fat and animal protein and physical inactivity) for the development of CVD (Vorster, 2002). Steyn *et al.* (2005) reported that lifetime exposure to an unhealthy lifestyle (unhealthy dietary intake, inactivity and smoking) increases the likelihood of developing CVD in the future.

To date, several studies have been undertaken in Sub-Saharan African countries with the objective of determining the possible underlying risk factors associated with CVD in African populations. Data from these studies have identified various risk factors that contribute to the development of CVD, such as smoking, hypertension, diabetes, abdominal obesity, abnormal blood lipids and haemostasis (Vorster, 2002; Steyn *et al.*, 2005; Mensah, 2008). In the context of CVD, abnormal haemostasis is characterised by either hypofibrinolysis or hypercoagulability, or both. Fibrinolysis and, in particular, one of its main inhibitors, plasminogen activator inhibitor-1 (PAI-1), have been found to play an important regulating role in the development of abnormal haemostasis and consequently, increased CVD development (Juhan-Vague & Alessi, 1996; Kohler *et al.*, 2000). PAI-1 inhibits the activation of plasminogen, the fibrin-degrading enzyme, by binding its activators, tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), and is, therefore, a main inhibitor of the fibrinolysis process.

From preliminary investigations of PAI-1 in the black South African population (Jerling *et al.*, 1994; Greyling *et al.*, 2007; Naran *et al.*, 2008; Nienaber *et al.*, 2008; Pieters *et al.*, 2010; De Lange *et al.*, 2012; De Lange *et al.*, 2013; Pieters *et al.*, 2014), two important research issues were identified: i) methodological issues regarding the measurement of the different forms of PAI-1 in plasma, namely antigen (PAI-1_{ag}), activity (PAI-1_{act}) and the tPA/PAI-1 complex; and ii) associations between PAI-1 and obesity as well as the relationship with sarcopenic obesity (SO) in Africans, which will be discussed below.

Both PAI-1_{act} and PAI-1_{ag} have been associated with CVD in populations of European descent (Geppert *et al.*, 1995; Juhan-Vague & Alessi, 1996; Bavenholm *et al.*, 1998; Thogersen *et al.*, 1998; Eržen & Šabovič, 2013). Although PAI-1 levels can be accurately measured by either one of these methods, there is substantial uncertainty regarding the effect of platelets on these PAI-1 assays. The alpha granules of platelets were traditionally thought to contain mostly latent PAI-1 (Declerck *et al.*, 1988a). More recent publications, however, suggest that they also contain active PAI-1 (Nordenhem & Wiman, 1997; Brogren *et al.*, 2004; Brogren *et al.*, 2011). In addition, the effect of residual platelets in plasma on PAI-1 fractions has not been studied in detail and there is emerging evidence suggesting that platelet activation may differ between ethnicities. Previous research measuring PAI-1 levels in African and white ethnic groups has shown inconsistent ethnic differences (Jerling *et al.*, 1994; Festa *et al.*, 2003; Matthews *et al.*, 2005; Lutsey *et al.*, 2006; Greyling *et al.*, 2007; Naran *et al.*, 2008; Perry *et al.*, 2008). We

hypothesise that some of this inconsistency may be related to the specific PAI-1 assays used in the different studies as well as differences in sample handling procedures.

The association between PAI-1 and obesity has been widely studied in populations of European descent, yet only limited information is available in other population groups. This association may be influenced by ethnicity, since body fat distribution has been shown to differ between ethnicities (Conway *et al.*, 1995; Hill *et al.*, 1999; Micklesfield *et al.*, 2010). Increased incidences of CVD in obese and overweight individuals seem to be partially related to impaired fibrinolysis, which may result from increased PAI-1 concentration (Shimomura *et al.*, 1996; Eriksson *et al.*, 1998; Alessi *et al.*, 2000a). Central obesity is also a known component of the metabolic syndrome (MetS) (Alberti *et al.*, 2009). Accordingly, PAI-1 strongly correlates with the MetS and insulin resistance (IR) (Juhan-Vague & Alessi, 1997; Greyling *et al.*, 2007; Chou *et al.*, 2009; Palomo *et al.*, 2009). It is not yet known, however, how PAI-1 relates to SO, a form of obesity that is associated with advanced age, muscle wasting and the presence of IR. It may be assumed that the combined effect of increased PAI-1 plasma levels and SO may potentiate each other, leading to negative health consequence in the elderly. More studies are needed to confirm such an association, however, as very limited data are available on the association of PAI-1 with SO (Cesari *et al.*, 2005; Cesari *et al.*, 2010).

The following sections will provide a brief overview of the haemostasis system, focusing on the coagulation and fibrinolysis systems and including more detail on PAI-1 and the regulation of PAI-1 in the blood. Secondly, issues regarding the measurement of PAI-1 in plasma, either as PAI-1_{ag}, PAI-1_{act}, latent PAI-1 or PAI-1 in complex with tPA, will be discussed and thirdly, the association of PAI-1 and obesity will be considered. In this section special attention will be paid to disagreements in the literature regarding the relationship between PAI-1 and obesity as measured by *ex vivo* and *in vivo* techniques. In the last section, the undefined yet potential relationship between PAI-1, ageing and SO will be discussed.

2.2 Physiology of PAI-1

PAI-1 is a single-chain glycoprotein with a plasma concentration of 0.4 nM (Bastard & Pieroni, 1999; Rijken & Sakharov, 2001). PAI-1 belongs to the family of serine protease inhibitors (serpins) and has a molecular weight of 50 000 Dalton (Pannekoek *et al.*, 1986; Kruithof, 1988). PAI-1 is produced by endothelial cells, hepatocytes, smooth muscle cells and platelets, as

noted in a review by Sprengers and Kluft (1987), as well as adipocytes (Alessi *et al.*, 1997). PAI-1 exists in either an active, latent (or inactive) form or in complex with tPA (Hekman & Loskutoff, 1985; Sprengers & Kluft, 1987; Gils & Declerck, 2004). Normal plasma PAI-1 concentration for active PAI-1 is $<50 \text{ U.mL}^{-1}$ (with 80% of values less than 6 U.mL^{-1}) and $<30 \text{ ng/mL}$ for PAI-1_{ag} (Lijnen, 2005). In pathological conditions, however, plasma PAI-1 levels can increase 50-fold (Juhan-Vague *et al.*, 1987). Active PAI-1 is unstable and has a half-life of approximately two to three hours, after which it will spontaneously convert to its latent form, although it can be reactivated *in vitro* (Sprengers & Kluft, 1987; Loskutoff & Curriden, 1990; Gils & Declerck, 2004). The half-life of PAI-1 can be increased by binding to vitronectin (Zhou *et al.*, 2003) and most of the active PAI-1 in plasma is bound to vitronectin (Declerck *et al.*, 1988b). PAI-1 can also form a stable complex with tPA that is cleared from the circulation by hepatocytes (Owensby *et al.*, 1991). In the active form, PAI-1 can inhibit both tPA and uPA by forming a 1:1 stoichiometric complex with tPA or uPA, whereas latent PAI-1 does not react with these target proteinases (Gils & Declerck, 2004).

PAI-1 is stored in the alpha granules of platelets and is released during platelet activation and aggregation (Erickson *et al.*, 1984; Sprengers & Kluft, 1987; Bastard & Pieroni, 1999). Only approximately 5-15% of PAI-1_{ag} has been found to be active in lysed platelet-rich plasma (Declerck *et al.*, 1988a; Nordenhem & Wiman, 1997); it was believed, therefore, that platelets stored and released mainly inactive (latent) PAI-1 (Declerck *et al.*, 1988a). In contrast, more recent studies have observed that platelets also release a substantial amount of active PAI-1 (Brogren *et al.*, 2004; Brogren *et al.*, 2011). These researchers have concluded that there is a *de novo* synthesis of PAI-1 within platelets and found that when platelets were lysed in the presence of tPA, the majority of the PAI-1 formed tPA/PAI-1 complexes, suggesting that platelets contain mostly active PAI-1 (Brogren *et al.*, 2004; Brogren *et al.*, 2011). Possible explanations for these differences found in the content of platelet lysates could be related to methodological issues, such as the methods used to prepare the platelet lysates (sonification and freezing and/or thawing of the samples), as these were found to affect the detection of PAI-1_{act} (Brogren *et al.*, 2011). It is also known that the conversion of the active form of PAI-1 to the latent is influenced by several conditions such as low temperature, low pH and high salt concentration (Sancho *et al.*, 1994). In addition, it has been suggested that the binding between PAI-1 and Ca^{2+} contributes to the stabilisation of active PAI-1 within platelets (Lang & Schleef, 1996). *In vitro* studies on clot lysis have also shown that PAI-1 released from platelets is an important role player in thrombolysis resistance (Fay *et al.*, 1994; Stringer *et al.*, 1994). Indeed, PAI-1 has an important role in hypofibrinolysis and is also known to be an important inhibitor of the fibrinolytic system (Rijken & Sakharov, 2001). The effect of residual platelets in

plasma on the different PAI-1 assays, therefore, remains an issue of debate. In order to understand PAI-1's inhibiting role in the fibrinolytic system, the formation of a thrombus or blood clot first needs to be understood before its lysis can be discussed.

2.2.1 Blood clot formation: conversion of fibrinogen to fibrin

According to the conventional model of coagulation, blood clot formation takes place either via an intrinsic pathway by activation of the contact system or via an extrinsic pathway (*in vitro* or mechanical damage), e.g. when blood from damaged tissue comes into contact with tissue factor (TF) (Davie & Ratnoff, 1964; Hoffman & Monroe, 2001) (Figure 2.1). A cascade of enzyme reactions then follows, causing the conversion of plasma zymogens of serine proteases to active enzymes or conversion of fibrinogen to fibrin (Colman *et al.*, 2000). After activation [the activated form of the factors (F) is indicated by a small letter "a"] of the intrinsic pathway (contact activation), FXIIa leads to the activation of FXI, FIX, FVIII and FX. The extrinsic pathway, on the other hand, involves TF which activates FVII, which also results in the activation of FX. The formation of TF/FVIIa complex is, however, inhibited by TF pathway inhibitor (TFPI), which also inhibits FXa. Activation of FX is essential for the formation of the prothrombinase complex, which includes FVa and phospholipids and converts prothrombin to thrombin in the common pathway (Colman *et al.*, 2000). In the common pathway, thrombin converts fibrinogen into fibrin.

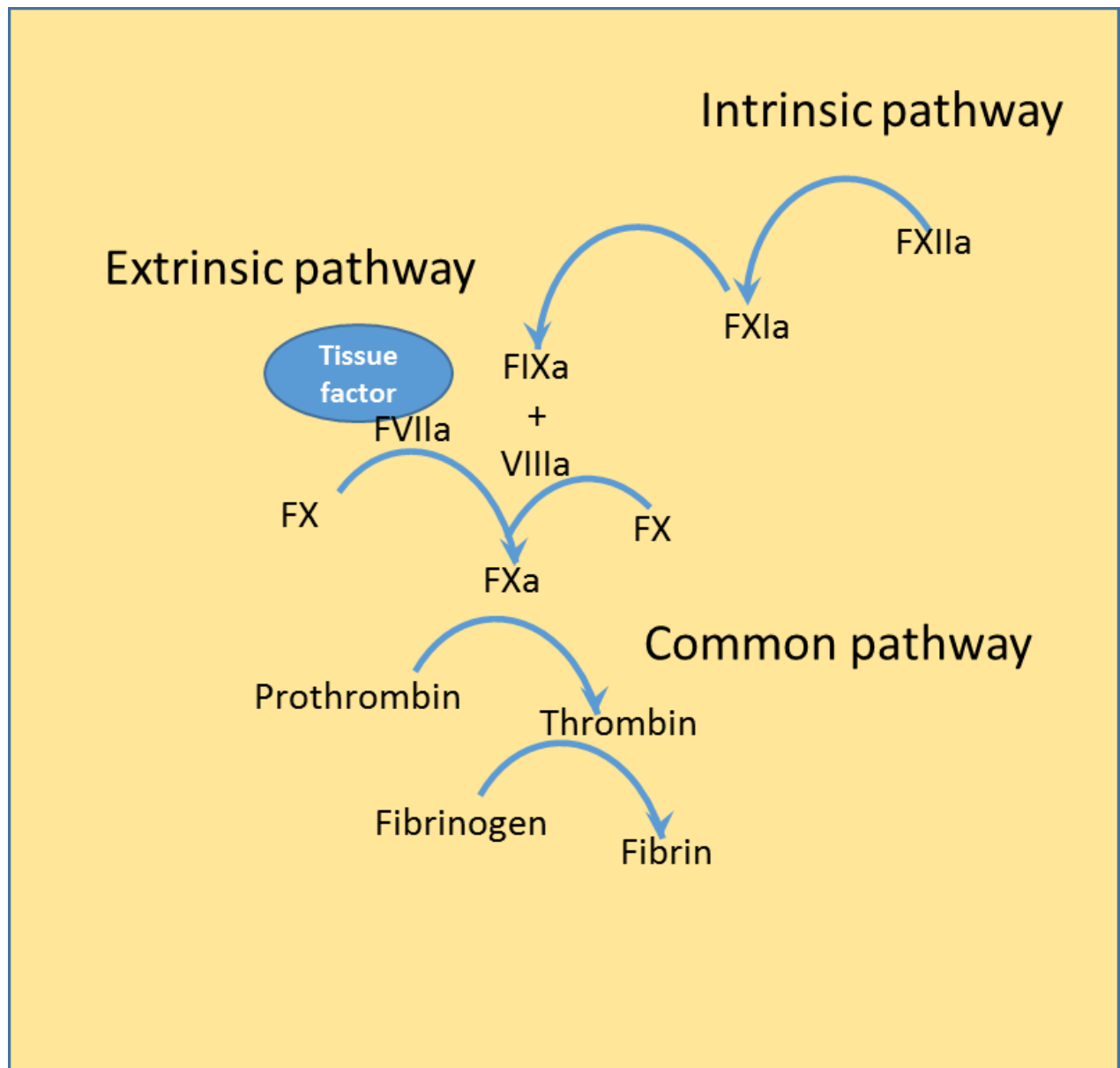


Figure 2.1: Conventional model of coagulation adapted from Hoffman and Monroe (2001) and Davie and Ratnoff (1964).

Arrows indicate activation of zymogens to active enzymes.

However, in a more recent model of coagulation, the cell-based model of coagulation, greater emphasis is placed on the complementary rather than separate roles of the intrinsic and extrinsic pathways (Hoffman & Monroe, 2001; Monroe *et al.*, 2002; Miller, 2005). This model was developed because the conventional model does not explain, for example, why patients with a deficiency of either FIX or FVIII, known as haemophilia, suffer from a serious impaired blood coagulation disorder, despite fully functional extrinsic pathway factors (Miller, 2005). In addition, the cell-based model regards platelets as having a fundamental role in supporting the pro-coagulant and anticoagulant systems (Hoffman & Monroe, 2001; Monroe *et al.*, 2002). The

cell-based model of coagulation postulates that coagulation occurs in three overlapping phases, namely I) the initiation phase, II) the amplification phase and III) the propagation phase (Hoffman & Monroe, 2001; Monroe *et al.*, 2002; Vine, 2009) (Figure 2.2).

The initiation phase is activated when damage to the vessel wall brings plasma into contact with TF-bearing cells, derived from extravascular sources such as fibroblasts (Monroe *et al.*, 2002; Miller, 2005). Activated FVII is required to bind TF in order to activate FIX and FX (Monkovic & Tracy, 1990). Activated FX is, however, quickly inhibited by TFPI or anti-thrombin when leaving the cell (Hoffman & Monroe, 2001), while the FXa that remains on the cell binds to FVa and a small amount of thrombin is generated (Monroe *et al.*, 2002). Although the amount of thrombin formed is too small to cleave fibrinogen throughout the wound, this initially formed thrombin plays an important role in activating platelets and FVII in the second amplification phase of coagulation (Hoffman & Monroe, 2001; Monroe *et al.*, 2002).

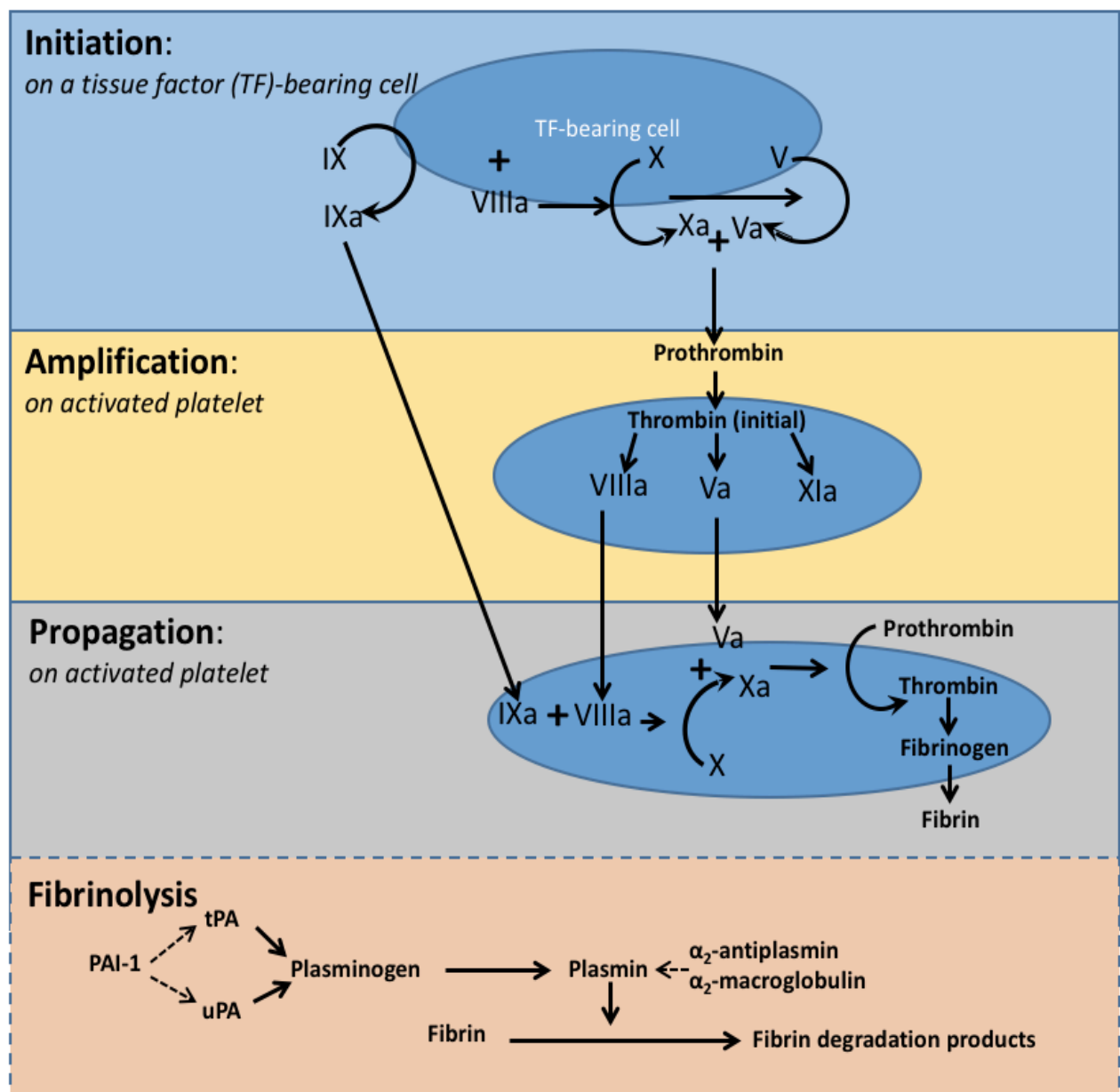


Figure 2.2: Cell-based model of coagulation (Initiation, amplification and propagation) and the main components of the fibrinolytic system as adapted from Rijken and Sakharov (2001), Hoffman and Monroe (2001), and Colman *et al.* (2000).

Solid arrows indicate activation and dashed arrows indicate inhibition.

In the amplification phase, the initially generated thrombin binds to the platelets that have adhered to the extracellular tissue. This binding of the platelets to the extracellular tissue partially activates the platelets and localises them near the site of TF exposure (Hoffman & Monroe, 2001). Thrombin is a potent platelet activator and also activates FV (secreted from the alpha granules of activated platelets), FVIII (after being cleaved from von Willebrand factor) and

FXI (Hoffman & Monroe, 2001). When the platelets are fully activated and FVa and VIIIa are present, the stage is set for large-scale thrombin generation (Hoffman & Monroe, 2001).

In the last phase, propagation, the platelets are fully activated and this is the essential phase for thrombin generation. Activated FIX, released from TF-bearing cells, assembles on the surface of the platelets and forms complexes with FVIIIa (Monroe *et al.*, 2002). Factor IXa is not readily inhibited by anti-thrombin or any other plasma protease inhibitors, making it possible for FIXa to diffuse from the TF-bearing cell onto the platelets (Monroe *et al.*, 2002). Additional FIX can also be provided by FXIa, bound to the surface of the activated platelet (Hoffman & Monroe, 2001). When FX reaches the platelet surface, it is activated by the FIXa/VIIIa complex (Monroe *et al.*, 2002). After activation, FXa binds to its cofactor, FVa, and generates the thrombin needed to convert fibrinogen into a stable fibrin clot (Hoffman & Monroe, 2001).

Fibrinogen is a glycoprotein, composed of two sets of three polypeptide chains ($A\alpha$, $B\beta$ and γ) that are connected by disulphide bonds at the respective N-terminal regions in the E domain (Mosesson *et al.*, 2001). The E domain is the central region of the fibrinogen molecule where all the polypeptide chains are joined together to form two sets of coiled-coils that extend to the two outer D domains (Mosesson *et al.*, 2001). The E and D domains contain binding sites for the conversion of fibrinogen to fibrin, platelet interaction, formation of cross-links and polymerisation sites (Mosesson *et al.*, 2001).

Thrombin converts fibrinogen to fibrin through the cleaving of two pairs of A and B fibrinopeptides from the $A\alpha$ and $B\beta$ chains, respectively (Mutch *et al.*, 2001; Rozenfel'd *et al.*, 2001; Bridge *et al.*, 2014) and expose the EA (des-A fibrin monomer) polymerisation sites (Figure 2.3). Each EA site then combines with a complementary binding pocket (Da) in the D domain of neighbouring molecules (Pratt *et al.*, 1997; Mosesson *et al.*, 2001). The EA:Da association results in the formation of the double-stranded twisting fibrils in which fibrin molecules become aligned in an end-to-middle overlapping domain arrangement (Ferry, 1952; Fowler *et al.*, 1981; Mosesson *et al.*, 2001). Hereafter, protofibrils will form lateral associations and branches to form fibrils and fibres (Mosesson *et al.*, 2001). The clot that has formed is, however, still soluble at acid pH or in urea and unstable. Through the activation of FXIII by thrombin, fibrin forms covalent cross-links and the insoluble fibrin clot is formed (Bridge *et al.*, 2014). This cross-linking occurs through the incorporation of ϵ -(γ -glutamyl) lysine (Lys) bridges between lysine residues on the γ and α chains (Mosesson *et al.*, 2001). These fibrin fibres

contain binding sites where tPA and plasminogen can bind to activate plasminogen (Mosesson *et al.*, 2001), initiating fibrinolysis and degradation of the fibrin clot, as described in the section below.

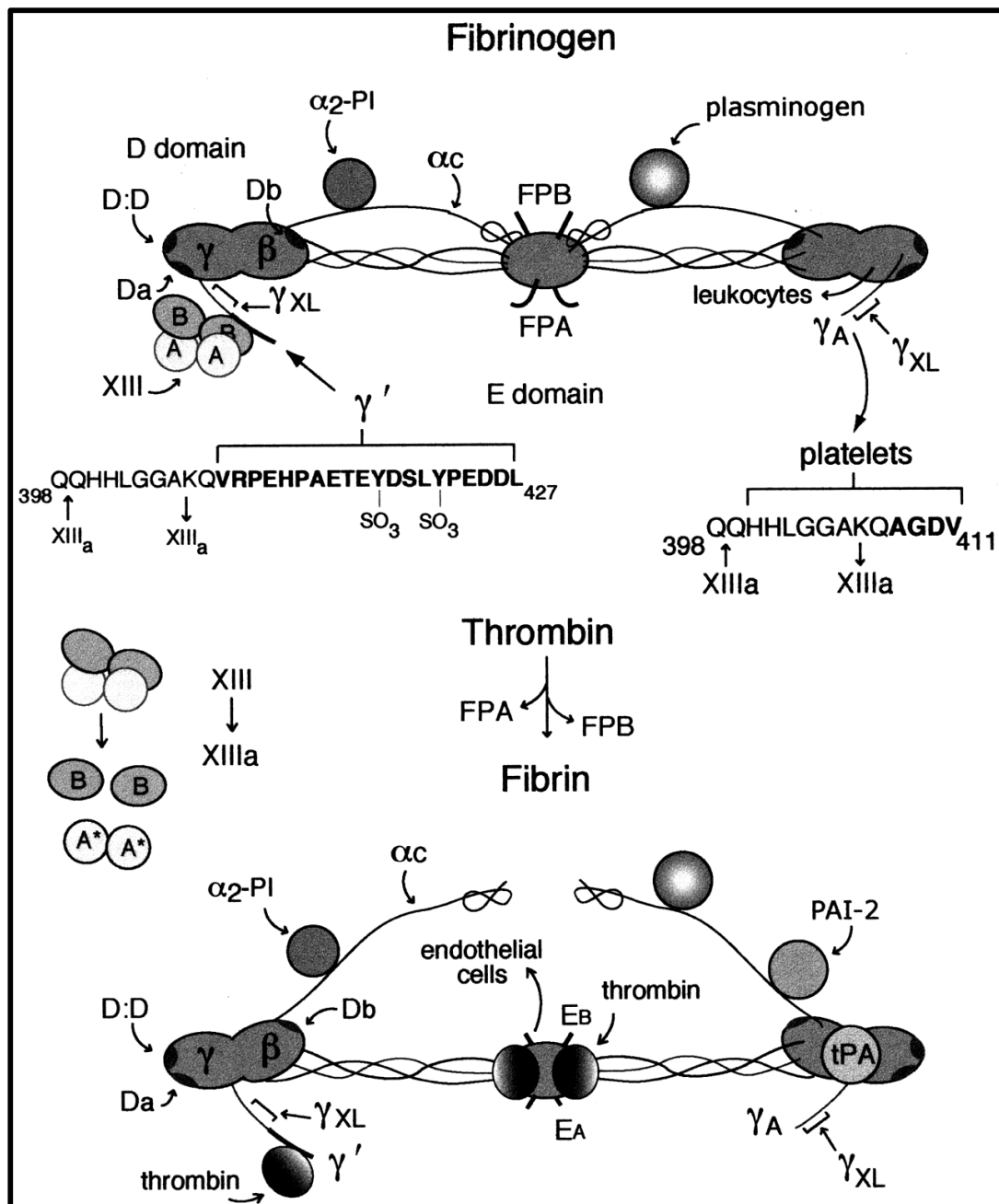


Figure 2.3: Polymerisation of fibrinogen to form fibrin (Mosesson *et al.*, 2001).

2.2.2 The fibrinolytic system

The above-mentioned process of clot formation is necessary for wound healing to occur. However, the fibrin clot cannot remain in the vasculature and should be removed again, through

the process of fibrinolysis. This process involves the binding of plasminogen to fibrin and its subsequent activation by plasminogen activators (PA), tPA and uPA (Thorsen *et al.*, 1988). Plasminogen, an inactive zymogen of plasmin (the active enzyme), circulates in plasma at a concentration of 2 μ M, and a small amount of plasminogen is already bound to fibrin during coagulation (Colman *et al.*, 2000; Rijken & Sakharov, 2001). Plasmin is the main enzyme responsible for the lysis of a fibrin clot (Bridge *et al.*, 2014). The surface of fibrin has binding sites, for both plasminogen and tPA, which help to localise and promote the conversion of plasminogen to plasmin (Rijken & Sakharov, 2001; Bridge *et al.*, 2014). In addition, these binding sites have a regulatory function by ensuring that tPA first binds to fibrin before activating plasminogen, thereby reserving plasminogen in the circulation and preventing systemic fibrinogen lysis (Rijken & Sakharov, 2001). Plasmin bound to lysine residues on fibrin cleaves the fibres transversely, exposing new carboxyl-terminal lysine residues and, therefore, new binding sites for plasminogen (Rijken & Sakharov, 2001). The newly formed carboxyl-terminal lysine residues are important to accelerate the lysis rate of the intact fibrin network by creating a positive feedback mechanism (Suenson *et al.*, 1984).

Two plasmin inhibitors, α_2 -antiplasmin and α_2 -macroglobulin, regulate the rate of fibrinolysis, with α_2 -antiplasmin being the most important inhibitor of plasmin (Rijken & Sakharov, 2001). The C-terminal end of α_2 -antiplasmin binds to the lysine-binding site of plasminogen, where fibrin is also non-covalently bound, thereby inhibiting the binding of fibrin to plasminogen (Carpenter & Mathew, 2008). The main inhibitory mechanism consist of plasmin/ α_2 -antiplasmin complex, while plasmin already bound to fibrin is relatively protected from α_2 -antiplasmin and fibrinolysis can, therefore, still occur (Carpenter & Mathew, 2008). PAI-1, on the other hand, inhibits the conversion of plasminogen to plasmin by inhibiting either tPA or uPA (Thorsen *et al.*, 1988; Rijken & Sakharov, 2001).

Plasminogen activation is regulated by the concentration of tPA and PAI-1 (Van Meijer & Pannekoek, 1995). According to Van Meijer and Pannekoek (1995), PAI-1 has partially overlapping binding sites for tPA and fibrin. In the presence of an excess of PAI-1 over tPA, PAI-1 will bind to the intact fibrin, whereas tPA has a higher affinity for PAI-1 compared with fibrin and will preferentially bind PAI-1 (Van Meijer & Pannekoek, 1995). This will result in the formation of inactive tPA/PAI-1 complex. As tPA and fibrin share a binding site on PAI-1, the tPA/PAI-1 complex will dissociate from fibrin. The inactive tPA/PAI-1 complex competes with the binding of free tPA on fibrin (Van Meijer & Pannekoek, 1995). Consequently, the tPA/PAI-1 complex bound to fibrin will prevent the conversion of plasminogen to plasmin, required for clot

lysis. When plasma PAI-1 is elevated, it can prevent lysis of the fibrin clot. Elevated plasma PAI-1 levels have been identified as a risk factor for the development of CVD (Juhan-Vague & Alessi, 1996; Nordt *et al.*, 2001; Vaughan, 2005; Peng *et al.*, 2008).

2.3 PAI-1 measurement

There are several different principles on which the measurement of PAI-1 in plasma can be based. PAI-1 can be measured as PAI-1_{act}, PAI-1_{ag}, latent (inactive) PAI-1 and PAI-1 in complex with tPA (Booth *et al.*, 1988; Declerck *et al.*, 1988a; Macy *et al.*, 1993). PAI-1_{act} assays measure only the free active form of PAI-1, while PAI-1_{ag} includes active and latent PAI-1 as well as tPA/PAI-1 complex (Juhan-Vague *et al.*, 1987; Booth *et al.*, 1988; Declerck *et al.*, 1988a). It is important to note that not all PAI-1_{ag} kits, however, measure all the forms of PAI-1 with equal affinity, which could result in significant differences in plasma PAI-1_{ag} levels (Kluft & Jie, 1990). Commercial kits are also available for the measurement of tPA/PAI-1 complex, while no commercial kits are available for the measurement of latent PAI-1.

In section 2.2, mention was made of the controversy concerning the main form of PAI-1 released from the alpha granules of aggregated platelets, i.e. latent or active. As PAI-1_{ag} includes the measurement of both active and latent PAI-1, the *in vitro* release of PAI-1 from aggregated platelets (present in plasma) may significantly influence the plasma PAI-1_{ag} level. It is also not yet clear what the effect of residual platelets in plasma is on PAI-1_{act} *per se*. When platelets are activated, the platelets undergo conformational change, aggregate and release the content of their secretory alpha granules (Ikeda *et al.*, 1997; Kamath *et al.*, 2001). There are several parameters that can be used to determine platelet activation. Firstly, there are soluble parameters measured in plasma and/or urine that are released from the platelet granules [e.g. beta thromboglobulin (β TG) and platelet factor 4 (PF4)]; secondly, there are molecules that are exposed on, and then shed from, the platelet surface (e.g. membrane glycoproteins, such as P-selectin, glycoprotein IIb/IIIa, glycoprotein V and glycocalicin); and lastly, metabolic molecules secreted from activated platelets can be measured (e.g. thromboxane and some members of the prostaglandin family) (Kamath *et al.*, 2001; Gurney *et al.*, 2002). Beta thromboglobulin and PF4 are examples of proteins that are released from platelets' alpha granules (Feinberg *et al.*, 1999; Gurney *et al.*, 2002). A debate still exists regarding which of these parameters best reflects *in vivo* platelet activation (Kamath *et al.*, 2001; Gurney *et al.*, 2002). While β TG is frequently used, the plasma levels of β TG can be influenced by several factors such as time of day, age, sample handling and preparation (Gurney *et al.*, 2002). In addition, plasma levels of

β TG could also be raised in patients with renal failure, since the kidneys are responsible for removal of circulating β TG (Kamath *et al.*, 2001). Platelet factor 4, on the other hand, is a heparin-binding protein and can be increased by the presence of an anti-coagulant in the blood (Gurney *et al.*, 2002). It also has a short half-life as it is more rapidly bound to the endothelial cells than β TG (Kamath *et al.*, 2001; Brydon *et al.*, 2006). It is because of the rapid binding of PF4 to the endothelial cells that plasma levels of these two proteins vary considerably, despite the release of similar concentrations of β TG and PF4 from the alpha granules of the platelets (Kamath *et al.*, 2001; Brydon *et al.*, 2006). Therefore, a distinction can be made between *in vivo* and artefactual *in vitro* platelet release since a comparable increase in both PF4 and β TG in blood samples will probably indicate an *in vitro* release, as no endothelial cells are present to bind PF4 (Kaplan & Owen, 1981; Kamath *et al.*, 2001). Although other contents of the platelets' alpha granules are also released, including fibrinogen, α_2 -antiplasmin, albumin, plasminogen and vitronectin, to name a few, β TG and PF4 are preferentially used as parameters as they are platelet-specific and are not secreted by other cells (Gurney *et al.*, 2002). Other parameters such as P-selectin and thromboxane can also be used to measure platelet activation; these parameters, however, are also not platelet-specific (Gurney *et al.*, 2002). Other techniques that can be used to determine platelet activation include functional studies such as the use of aggregometry to measure spontaneous platelet aggregation and the expression of glycoproteins such as P-selection by flow cytometry (Gurney *et al.*, 2002; Brydon *et al.*, 2006).

Plasma PAI-1 levels can, furthermore, vary according to the technique used for sample collection and handling as a result of the presence of aggregated platelets (Macy *et al.*, 1993). It is recommended that collection of blood samples should be done early in the morning after a fasting period to avoid daytime variations (circadian pattern) associated with PAI-1 or an interaction between PAI-1 and nutritional aspects (Macy *et al.*, 1993). In addition, blood should flow freely into the collection container, and prolonged use of a tourniquet or considerable manipulation of the vein by the needle should be avoided to prevent clotting of the sample *in vitro* (Ernst & Ernst, 2003). *In vitro* clot formation can also be prevented by ensuring complete distribution of anticoagulant within the sample, while vigorous shaking should be avoided as this can induce haemolysis or activate platelets (Ernst & Ernst, 2003). Blood samples should also be collected in an anticoagulant mix, such as sodium citrate (3.2 or 3.8%) tubes, to avoid *in vitro* interaction and/or activation of PAI-1 or platelets within the plasma (Funk, 2013). Although either 3.2% or 3.8% sodium citrate tubes are acceptable, it is important to know that clotting times tend to be longer in the 3.8% *versus* the 3.2% sodium citrate tubes (Funk, 2013). Samples should also not be placed on ice before centrifugation as this will enhance platelet activation (Böhm *et al.*, 2006). The platelet content within the sample should ideally also be

determined. According to the guidelines of the Clinical and Laboratory Standard Institute (CLSI), platelet-poor plasma (PPP) i.e. plasma with a platelet count $<10 \times 10^9/L$, can be obtained by centrifuging the blood at no less than 1,500 g for 15 minutes (Arkin *et al.*, 2003). After centrifugation, the middle layer of the plasma should be extracted and stored at a minimum of -70°C (Macy *et al.*, 1993). Plasma can also be collected in tubes containing platelet inhibitors, such as CTAD tubes, to prevent *in vitro* platelet activation and/or degradation, which may influence PAI-1 assays (Funk, 2013).

In reviewing the literature, discrepancies in the plasma PAI-1_{act} and PAI-1_{ag} levels in African (and African American) and white population groups were recognised. Summarised in Table 2.1 is a comparison between the measurements of plasma PAI-1_{act} and PAI-1_{ag} in different ethnic groups (African, African American and European). Special attention was given to sample preparation and handling procedures, method of sample analysis and the resulting effect observed on either plasma PAI-1_{act} or PAI-1_{ag} levels. Studies that investigated plasma PAI-1_{act} levels reported consistently higher plasma PAI-1_{act} levels in white South African participants compared with black South African participants (Jerling *et al.*, 1994; Greyling *et al.*, 2007). Studies that investigated plasma PAI-1_{ag} levels, however, showed inconsistent results, with some studies reporting higher plasma PAI-1_{ag} levels in African Americans (Matthews *et al.*, 2005). Other studies found no significant differences between white American and African American groups (Perry *et al.*, 2008), whereas some reported higher PAI-1_{ag} levels in white compared with African and African American groups (Festa *et al.*, 2003; Lutsey *et al.*, 2006; Naran *et al.*, 2008).

From the summary in Table 2.1 it is evident that a range of different commercial PAI-1 assays and immunological methods is often used to determine plasma PAI-1 levels. Despite the importance of standardised sampling procedures to avoid the presence of platelets in plasma, most studies do not give detailed information on the methods followed during sample collection, handling and analysis (Table 2.1). As no standardised method of PAI-1_{act} and PAI-1_{ag} measurements is being employed, it is difficult to draw conclusions regarding levels of PAI-1_{act} and PAI-1_{ag} in different ethnic groups, and even between different research groups.

Apart from methodological issues, plasma PAI-1 levels are also influenced by genetic and non-genetic or environmental determinants. The different determinants of plasma PAI-1 levels will be the focus of the next section.

Table 2.1: Comparison of plasma PAI-1_{act} and PAI-1_{ag} levels between black (African and African American) and white ethnic groups

Measuring of PAI-1 _{act} or PAI _{ag}	Reference	Participants	Sample preparation	Kit used for analysis	Effect
PAI-1 _{act} (↓ African)	Jerling <i>et al.</i> (1994)	Healthy non-smoking male participants aged 18 to 23 years with a BMI range of 19-24 kg/m ² . Sample consisted of 32 participants, of whom 15 were urban whites and 17 black Venda participants.	1. Sample collection: Fasting blood samples were collected by means of a 21-gauge Johnson & Johnson scalp vein infusion set. Samples were collected between 07:00 and 10:00 AM. 2. Type of tube used for blood sample collected: Citrate tubes 3. Method of sample handling: Samples were centrifuged at 3,000 rpm for 10 min. Sample preparation was done within 30 min of blood collection and thereafter frozen at -72°C.	Plasma PAI-1 _{act} was measured using an indirect enzymatic method. Spectrolyse pL, Biopool, Umea, Sweden, Cat. No. 1012011.	Increased plasma PAI-1 _{act} levels in urban whites compared with black Venda participants were found (12.5 [8.50; 14.60] <i>versus</i> 0.80 [0.00; 5.4] U/mL, p<0.0001).
PAI-1 _{act} (↓ African)	Greyling <i>et al.</i> (2007)	95 African and 114 white healthy urban women, between	1. Sample collection: Fasting (10 hours) venous blood samples were collected by means of	Plasma PAI-1 _{act} was measured using an indirect enzymatic	Increased plasma PAI-1 _{act} levels in white compared

Table 2.1 (continued): Comparison of plasma PAI-1_{act} and PAI-1_{ag} levels between black (African and African American and white ethnic groups

		the ages of 20 and 55 years, were studied.	a 21-gauge scalp vein infusion set. Samples were drawn between 06:00 and 10:00 AM.	method. Spectrolyse pL, Biopool, Umea, Sweden, Cat. No. 1012011.	with African participants were found (10.2 versus 5.2 U/mL, p<0.001).
			2. Type of tube used for blood sample collected: Citrate tubes		
			3. Method of sample handling: Centrifuged at 3500 rpm for 10 min at 10°C. Samples were stored at -82°C until analysis.		
PAI-1 _{ag} [free active and latent PAI-1, not complex PAI-1 (↓ African)]	Festa <i>et al.</i> (2003)	1564 Men and women from three ethnic groups were studied: non-Hispanic whites, Hispanic and African American aged 40 to 69 years.	1. Sample collection: Fasting (24 hours) blood samples were collected. No information provided on the method used to draw blood or the time of day the samples were taken.	Plasma PAI-1 _{ag} was measured by means of a two-site ELISA that is 12 times more sensitive to plasma free active and latent PAI-1, but not to tPA/PAI-1 complex (Declerck & Collen, 1990). This assay utilises the IgG fraction of monoclonal antibody 7D4 or 15H12 as capturing antibody	Plasma PAI-1 _{ag} levels were found to be lower in blacks (SEM 13.38 [0.71] ng/mL) than in non-Hispanic whites (SEM 18.19 [0.80] ng/mL).
			2. Type of tube used for blood sample collected: Citrate tubes		
			3. Method of sample handling: Citrate sample was centrifuged for a minimum of 10 min at 3000 g to ensure no contamination from		

Table 2.1 (continued): Comparison of plasma PAI-1_{act} and PAI-1_{ag} levels between black (African and African American) and white ethnic groups

				platelet PAI-1. Samples were frozen and stored at -70°C, 90 min after blood was drawn.	conjugated to horseradish peroxidase 75F-HRP, 12A4-HRP or 62E8-HRP (Declerck & Collen, 1990).	
PAI-1 _{ag} (↑ African)	Matthews <i>et al.</i> (2005)	2834 Women with CVD risk factors between the ages of 42 and 52 years from five different ethnic groups – White (n=1400), African American (n=729), Hispanic (n=226), Chinese (n=231) and Japanese (n=248) – participated in the study.	1. Sample collection: Fasting (10 hours) blood samples were collected. No indication of the method that was used to draw the blood samples or the time of day samples was collected. 2. Type of tube used for blood sample collected: Type of tube used for blood sampling was not indicated. 3. Method of sample handling: Samples were maintained at 4°C until frozen at -80°C. No indication of sample centrifugation or storage was provided.	Plasma PAI-1 _{ag} was measured by means of a commercial kit from American Diagnostica, IMUBIND®. This kit entails a solid-phase monoclonal antibody and a secondary enzyme-labelled coating antiserum.	African Americans were found to have higher plasma PAI-1 _{ag} levels than white participants. Hispanic participants' plasma PAI-1 _{ag} levels were significantly higher than those of the other groups.	
PAI-1 _{ag} (no significant)	Perry <i>et al.</i> (2008)	The participants were 36 healthy white American	1. Sample collection: Participants were required to remain still for at least five min prior to	A commercial kit from Diagnostic Stago, Inc.	No significant race relationship was observed for	

Table 2.1 (continued): Comparison of plasma PAI-1_{act} and PAI-1_{ag} levels between black (African and African American and white ethnic groups

difference)	and 30 African American overweight and obese (BMI>25 kg/m ²), premenopausal women.	sample collection. Blood was drawn from the antecubital vein after a 12-hour fasting period. Samples were analysed within one week of collection. Time of sample collection was not specified.	(Asserachrom→ PAI-1) was used to determine total circulating PAI-1 levels. This is an ELISA assay using a monoclonal anti-PAI-1 antibody (sandwich method) to capture plasma PAI-1 _{ag} . A second peroxidase-linked monoclonal antibody was used to produce the signal.	plasma PAI-1 _{ag} . In a subgroup of the participants, identified with a VAT measurement $\leq 2.0 \text{ cm}^2$, higher plasma PAI-1 _{ag} level was found in African American women than in the white American women.
PAI-1 _{ag} (↓ African)	Lutsey <i>et al.</i> (2006)	Plasma PAI-1 _{ag} was determined in 996 participants.	Sample collection: Blood collection method was not specified. Type of tube used for blood sample collected: The type of tube use for blood sample collected was not provided. Method of sample handling: No indication of sample centrifugation or storage was	Plasma PAI-1_{ag} was measured with a two-site ELISA in-house method. The IgG fraction of 7D4 monoclonal antibody was utilised as the coating antibody. This monoclonal antibody is five times more sensitive to free PAI-1. 7F5-HRP In males lower plasma PAI-1_{ag} concentration was reported for black than for white participants; however, among females no differences in plasma PAI-1_{ag}

Table 2.1 (continued): Comparison of plasma PAI-1_{act} and PAI-1_{ag} levels between black (African and African American and white ethnic groups

				provided.	was used as secondary antibody.	were observed.
PAI-1 _{ag} (↓ African)	Wong <i>et al.</i> (2006)	924 Participants aged 45 to 85 years without a history of CVD were included. White, black, Hispanic and Chinese ethnic groups were included.	1. Sample collection: Fasting (eight hours) blood samples were used. No further information on sample collection was provided. 2. Type of tube used for blood sample collected: No indication of the type of tube used for sample collection was given. 3. Method of sample handling: The method followed during sample handling procedures was not provided.	It was not specified whether a commercial kit or in-house method was used to measure plasma PAI-1 _{ag} .	Plasma PAI-1 _{ag} levels were higher in white participants than the black participants. The highest plasma PAI-1 _{ag} levels were found in the Chinese group.	
PAI-1 _{ag} (↓ African)	Naran <i>et al.</i> (2008)	Either diabetic or non-diabetic participants were included from three different ethnic groups: Indian (120 non-diabetic and 20 diabetic), White (158 non-diabetic	1. Sample collection: Fasting blood samples were drawn from the antecubital vein (with minimum stasis) between 8:00 and 9:00 AM. 2. Type of tube used for blood sample collected: Samples were collected in pre-cooled tubes.	PAI-1 _{ag} levels were determined by means of a commercial kit (Coaliza PAI-1 kit, Chromogenix, Milano, Italy).	Mean plasma PAI-1 _{ag} concentration was found to be significantly higher in white than in African participants. However, in participants with	

Table 2.1 (continued): Comparison of plasma PAI-1_{act} and PAI-1_{ag} levels between black (African and African American and white ethnic groups

and 55 diabetic) 3.	Method of sample handling:	MetS, no racial
and African (91	Samples were held on ice until	differences were
non-diabetic and 9	centrifugation at 200 x g for 20 min at	found.
diabetic).	4°C and were stored at -70°C until	
	assayed.	
Body mass index (BMI); cardiovascular disease (CVD); enzyme-linked immunosorbent assay (ELISA); gravitational acceleration (g); immunoglobulin G (IgG), minutes (Min); metabolic syndrome (MetS); plasminogen activator inhibitor-1 (PAI-1), PAI-1 antigen (PAI-1 _{ag}); PAI-1 activity (PAI-1 _{act}); standard error of mean (SEM); tissue plasminogen activator (tPA); visceral adipose tissue (VAT)		

2.4 Factors influencing PAI-1 plasma levels

Plasma PAI-1 levels are affected by several genetic and non-genetic determinants, as summarised in Table 2.2. Mechanisms of action are also included in Table 2.2, indicating the possible pathway by which these determinants can modify plasma PAI-1 levels. The human *PAI-1* (*SERPINE*) gene is located at region q21.3-q22 of chromosome 7 with an approximate size of 12.2 kilo bases (kb) and consists of nine exons and eight introns (Strandberg *et al.*, 1988). Several polymorphisms have been identified within the *PAI-1* gene, to name a few, rs2227631 [a -844 G/A single-nucleotide polymorphism (SNP)], rs1799889 [a single-nucleotide insertion/deletion (indel) polymorphism located in the promoter region at -675 4G/5G], a SNP recognised by the *HindIII* restriction enzyme in restriction fragment length (RFLP) assays, a cytosine-adenine (CA)_n dinucleotide repeat and a nine-nucleotide indel polymorphism at 11,320 base pair (bp) (Strandberg *et al.*, 1988; Dawson *et al.*, 1991; Nordt *et al.*, 2001). However, not all polymorphisms within the *PAI-1* gene have been associated with plasma PAI-1 levels (Henry *et al.*, 1997; De Lange *et al.*, 2013).

According to Roche *et al.* (2011), there are three different types of point mutations, namely transition, transversion and indel. Transition is the replacement of one purine or pyrimidine base by the other (adenine↔guanine; thymine↔cytosine) and occurs at a greater frequency than transversion (Roche *et al.*, 2011). Transversion occurs when one purine is replaced by a pyrimidine or *vice versa* (cytosine↔adenine; cytosine↔guanine; thymine↔adenine; thymine↔guanine) (Roche *et al.*, 2011). An example of transition in the *PAI-1* gene is the substitution of a single guanine to an adenine in the -844 G/A bp. Indel refers to the insertion or deletion of a single base into one genome relative to another in such a way that a change takes place in the reading of the nucleotide sequence, as well as in the expression of the amino acids (Roche *et al.*, 2011). This type of mutation is also referred to as frame-shift mutation. The indel mutation within the *PAI-1* gene is located at bp -675 and leads to either the 4G or 5G sequence (Nordt *et al.*, 2001). It has been observed that individuals with the homozygous form (-675 5G/5G) of the indel PAI-1 polymorphism have lower plasma PAI-1 levels than individuals with the heterozygous form (-675 4G/5G) (Sartori *et al.*, 2001; Kathiresan *et al.*, 2005; Bouchard *et al.*, 2010; Incalcaterra *et al.*, 2014). Individuals with the 4G/4G genotype, however, have been found to have significantly higher PAI-1_{act} levels than individuals with the 5G/5G genotype (Pieters *et al.*, 2010). Being located in the promoter region of the *PAI-1* gene, the -675 4G/5G *PAI-1* polymorphism can regulate gene expression and the functional attributes of the *PAI-1* gene (Nordt *et al.*, 2001). Genetic variation in the DNA sequence of the *PAI-1* gene contributes

to interpersonal variants in *PAI-1* expression and plasma PAI-1 level (Lijnen, 2005). The identification of such *PAI-1* polymorphisms is important in identifying individuals that are prone to the development of elevated plasma PAI-1 levels and are, therefore, also at greater risk of fibrinolytic irregularities. Only those *PAI-1* polymorphisms frequently reported in the literature to affect plasma PAI-1 levels were included in Table 2.2.

Apart from the genetic determinants included in Table 2.2, several non-genetic determinants were also identified through the literature. Prospective studies relating to the non-genetic determinants of PAI-1 are still lacking and studies investigating PAI-1 determinants were mostly performed using cross-sectional research methods (Mykkanen *et al.*, 1994; Gray *et al.*, 1995; Margaglione *et al.*, 1998; Morange *et al.*, 1999; Mertens *et al.*, 2006; Asselbergs *et al.*, 2007). Consequently, insufficient information is available on the effect of non-genetic determinants on plasma PAI-1 levels over time.

The purpose of Table 2.2 is to provide a critical overview of the genetic and non-genetic determinants that result in altered plasma PAI-1_{act} and/or PAI-1_{ag} levels. It should be mentioned that associations between PAI-1 and many of these determinants were not consistently found in all studies and there were also studies that found that these determinants had no effect on or association with PAI-1 (Haglund *et al.*, 1994; Prisco *et al.*, 1994; Grubic *et al.*, 1996; Henry *et al.*, 1997; Finnegan *et al.*, 2003; Torres-Carrillo *et al.*, 2008).

Table 2.2: Summary of genetic and non-genetic determinants of plasma PAI-1 levels and possible mechanisms of action

Determinants: Genetic	Effect on plasma PAI-1 _{ag} and/or PAI-1 _{act}	Possible mechanism of action
rs1799889 (PAI-1 -675 4G/5G)	Higher plasma PAI-1 _{act} (Eriksson <i>et al.</i> , 1995; Panahloo <i>et al.</i> , 1995; Henry <i>et al.</i> , 1998; Incalcaterra <i>et al.</i> , 2014) and PAI-1 _{ag} (Margaglione <i>et al.</i> , 1998; Sartori <i>et al.</i> , 2001; Kathiresan <i>et al.</i> , 2005; Ding <i>et al.</i> , 2006) levels associated with the 4G allele.	The -675 4G/5G is a single-nucleotide indel polymorphism located in the promoter region of the <i>PAI-1</i> gene that leads to either the 4G or 5G sequence (Nordt <i>et al.</i> , 2001). In the 5G allele a repressor protein binds to the promoter area of the <i>PAI-1</i> gene, resulting in relatively reduced transcription of PAI-1 and consequently lower plasma <i>PAI-1</i> levels (Dawson <i>et al.</i> , 1993; Eriksson <i>et al.</i> , 1995; Nordt <i>et al.</i> , 2001).
rs2227631 (PAI-1 -844 A/G)	This PAI-1 polymorphism has been associated with higher PAI-1 _{ag} levels (Henry <i>et al.</i> , 1998; Kathiresan <i>et al.</i> , 2005; Morange <i>et al.</i> , 2007; Bouchard <i>et al.</i> , 2010).	The -844 G/A <i>PAI-1</i> SNP is located in the promoter region of the <i>PAI-1</i> gene (Nordt <i>et al.</i> , 2001). Being located in the promoter region, it could be directly responsible for changes in plasma PAI-1 levels as it can influence gene transcription. It is challenging, however, to determine the independent effect of -844 A/G on plasma PAI-1 levels, since it is in strong LD with the -675 4G/5G <i>PAI-1</i> polymorphism (Henry <i>et al.</i> , 1998; Kathiresan <i>et al.</i> , 2005; Bouchard <i>et al.</i> , 2010).
rs2227666	Individuals harbouring the A allele	Both of these <i>PAI-1</i> polymorphisms are located in an intron region of the <i>PAI-1</i> gene,

Table 2.2 (continued): Summary of genetic and non-genetic determinants of plasma PAI-1 levels and possible mechanisms of action

(G/A) rs2227694 (G/A)	for the SNPs at rs2227666 or rs2227694 have higher plasma PAI-1_{ag} (Morange <i>et al.</i>, 2007). rs2227674 has been found to significantly (p=0.03) explain the residual variance in multivariable-adjusted PAI-1_{ag} levels (Kathiresan <i>et al.</i>, 2005).	and it seems that their effect on plasma PAI-1 levels is related to their association with the -844 A allele (Morange <i>et al.</i>, 2007).
rs6465787 (C/T)	This SNP has been observed to explain the residual variance in multivariable-adjusted PAI-1_{ag} levels (Kathiresan <i>et al.</i>, 2005).	This <i>PAI-1</i> polymorphism is located at the 5' flanking region that is 3 kb upstream of the 4G/5G <i>PAI-1</i> indel (Kathiresan <i>et al.</i>, 2005). The reasons for the association between rs6465787 (C/T) and plasma PAI-1 levels are currently not known.
rs6092 (Ala/Thr)	This SNP has been observed to contribute to plasma PAI-1_{ag} levels (Bouchard <i>et al.</i>, 2010).	According to Morange <i>et al.</i> (2007), this <i>PAI-1</i> polymorphism is located in an exon (coding) region of the <i>PAI-1</i> gene. Although the mechanism behind the association of this polymorphism with plasma PAI-1 levels is unknown, it could be related to its functional location on the <i>PAI-1</i> gene.
+12078 G/A	This SNP has been associated with increased plasma PAI-1 levels (Henry <i>et al.</i>, 1998).	Being located in the 3' region, +12078 G/A could possibly be involved in the posttranscriptional regulation of the <i>PAI-1</i> gene (Fattal <i>et al.</i>, 1992). Yet it is also in strong linkage with both the -844G and -675 <i>PAI-1</i> polymorphisms and it is difficult,

Table 2.2 (continued): Summary of genetic and non-genetic determinants of plasma PAI-1 levels and possible mechanisms of action

		therefore, to determine its independent contribution to the variation in PAI-1 levels (Henry <i>et al.</i> , 1998).
(CA) _n	The (CA) _n polymorphic site in the PAI-1 gene has been associated with increased plasma PAI-1 _{act} levels (Dawson <i>et al.</i> , 1991).	(CA) _n is a dinucleotide repeat polymorphism located in intron 3 that may be responsible for alterations elsewhere in the <i>PAI-1</i> gene (Dawson <i>et al.</i> , 1991) and is associated with increased plasma PAI-1 levels.
3' <i>Hind</i> III (RFLP)	The 3' <i>Hind</i> III polymorphic site in the <i>PAI-1</i> gene has been associated with higher plasma PAI-1 _{act} levels (Dawson <i>et al.</i> , 1991).	The <i>Hind</i> III polymorphic site is a polymorphism determined through RFLP with the <i>Hind</i> III. It results in a change in the 3' untranslated region of the <i>PAI-1</i> gene and gene expression.
Determinants: Non-genetic	Effect on plasma PAI-1_{ag} and/or PAI-1_{act}	Possible mechanism of action
Central obesity	Associated with increased plasma PAI-1 _{ag} levels (Shimomura <i>et al.</i> , 1996; Alessi <i>et al.</i> , 1997; Eriksson <i>et al.</i> , 1998; Alessi <i>et al.</i> , 2000b) and plasma PAI-1 _{act} levels (Eriksson <i>et al.</i>	The association between increased plasma PAI-1 levels and central obesity can be explained by the high numbers of stromal cells located in VAT, which seems to be the main source of PAI-1 in adipose tissue (Bastelica <i>et al.</i> , 2002). Another possible mechanism for this association between PAI-1 and central obesity could be related to cytokine (TGF-β1, TNF-α and IL-1) disturbances, often seen in individuals with central obesity, and which are known to increase <i>PAI-1</i> expression (Eriksson <i>et al.</i> , 1998;

Table 2.2 (continued): Summary of genetic and non-genetic determinants of plasma PAI-1 levels and possible mechanisms of action

	<i>al.</i> , 1998).	Cigolini <i>et al.</i> , 1999; Morange <i>et al.</i> , 1999; Alessi <i>et al.</i> , 2000b; Birgel <i>et al.</i> , 2000; Skurk <i>et al.</i> , 2001).
Blood lipids	HDL-chol has a negative association with plasma PAI-1 _{ag} levels (Asselbergs <i>et al.</i> , 2007; Barbato <i>et al.</i> , 2009; Raiko <i>et al.</i> , 2012).	The mechanism behind the association of HDL-chol with PAI-1 is unknown.
Blood lipids	Serum triglyceride levels are associated with increased plasma PAI-1 _{ag} (Margaglione <i>et al.</i> , 1998; Schoenhard <i>et al.</i> , 2008; Barbato <i>et al.</i> , 2009; Raiko <i>et al.</i> , 2012) and PAI-1 _{act} levels (Gray <i>et al.</i> , 1995; Panahloo <i>et al.</i> , 1995; Scarabin <i>et al.</i> , 1998).	The association between PAI-1 and triglyceride could be related to VLDL, as triglycerides are predominantly transported in VLDL (Ramasamy, 2014). A VLDL response element in the PAI-1 promoter area mediates VLDL-induced PAI-1 transcription (Nilsson <i>et al.</i> , 1998) and promotes <i>PAI-1</i> gene up-regulation (Eriksson <i>et al.</i> , 1998; Olufadi & Byrne, 2006). The observed association has been identified to be a result of VLDL stimulating PAI-1 production from endothelial and HepG2 cells (Mussoni <i>et al.</i> , 1992; Allison <i>et al.</i> , 1999).
Insulin resistance	Insulin resistance is positively associated with increased plasma PAI-1 _{act} (Vague <i>et al.</i> , 1989; Bavenholm <i>et al.</i> , 1998; Mavri <i>et al.</i> , 2001; Aubert <i>et al.</i> , 2003; Mertens <i>et al.</i> , 1998).	The mechanism responsible for the association between IR and PAI-1 is largely unknown, although it has been explained by disturbances at adipose tissue level (Juhan-Vague & Alessi, 1996; Juhan-Vague <i>et al.</i> , 1999; Alessi <i>et al.</i> , 2000b; Solano <i>et al.</i> , 2003). Such disturbances include the secretion of IR-related cytokines (TGF- β 1 and TNF- α), which also stimulate <i>PAI-1</i> expression in adipose tissue (Eriksson <i>et al.</i> , 1998;

Table 2.2 (continued): Summary of genetic and non-genetic determinants of plasma PAI-1 levels and possible mechanisms of action

	<i>al.</i>, 2006) and PAI-1_{ag} levels (Sartori <i>et al.</i>, 2001; Aubert <i>et al.</i>, 2003; Nakamura <i>et al.</i>, 2003; Solano <i>et al.</i>, 2003; Brogren <i>et al.</i>, 2004; Coffey <i>et al.</i>, 2011).	Cigolini <i>et al.</i>, 1999; Morange <i>et al.</i>, 1999; Alessi <i>et al.</i>, 2000b).
Hypertension /renin-angiotensin pathway	Associated with increased plasma PAI-1_{act} (Yarnell <i>et al.</i>, 2000; Pieters <i>et al.</i>, 2014) and PAI-1_{ag} (Margaglione <i>et al.</i>, 1998; Poli <i>et al.</i>, 2000; Wang <i>et al.</i>, 2007; Lieb <i>et al.</i>, 2009) levels.	The renin-angiotensin pathway plays an important role in the regulation of blood pressure. Renin activates angiotensin-I, which is converted to angiotensin-II by ACE. This results in vascular constriction and an increase in blood pressure (Swales & Samani, 1993). One possible mechanism for the increase in plasma PAI-1 levels associated with high blood pressure could be related to the stimulating effect of angiotensin-II on PAI-1 production, as seen <i>in vivo</i> (Ridker <i>et al.</i>, 1993) and <i>in vitro</i> (Vaughan <i>et al.</i>, 1995; Mehta <i>et al.</i>, 2002). Also, a polymorphism in the ACE gene loci was found to correlate with increased PAI-1 levels (Margaglione <i>et al.</i>, 1998). Other possible mechanisms could be through accelerated perivascular and media fibrosis (Kaikita <i>et al.</i>, 2001) or increased shear stress or endothelial dysfunction (Poli <i>et al.</i>, 2000).
Gender difference	Higher plasma PAI-1_{ag} (Gebara <i>et al.</i>, 1995; Margaglione <i>et al.</i>, 1998; Lutsey <i>et al.</i>, 2006; Asselbergs <i>et al.</i>, 2007) and PAI-1_{act} (Krishnamurti <i>et al.</i>, 1988; Sundell <i>et al.</i>, 1989;	Oestrogen might increase the clearance rate of plasma PAI-1 levels in pre-menopausal women (Lansink <i>et al.</i>, 1999; Gopal <i>et al.</i>, 2012). The effect could also be related to differences in WHR and fat distribution between men and women (Van Kesteren <i>et al.</i>, 1998; Van Harmelen <i>et al.</i>, 2000). The higher PAI-1_{act} levels in African women are only in part related to the higher prevalence of obesity and the difference in fat deposition

Table 2.2 (continued): Summary of genetic and non-genetic determinants of plasma PAI-1 levels and possible mechanisms of action

	Lacroix <i>et al.</i>, 1996) levels were found in white men compared with white pre-menopausal women. However, in an African population, higher PAI-1_{act} levels were found in the women compared with the men (Pieters <i>et al.</i>, 2010).	patterns between genders (Eksteen <i>et al.</i>, 2015).
Age	Plasma PAI-1_{act} (Hashimoto <i>et al.</i>, 1987; Berge <i>et al.</i>, 2013) and PAI-1_{ag} levels increase with age (Tofler <i>et al.</i>, 2005; Peng <i>et al.</i>, 2008; Raiko <i>et al.</i>, 2012).	Increased plasma PAI-1 levels seem to be induced by a variety of pathogens associated with the ageing process (MI, cerebral infarction, cancer, endothelial dysfunction, obesity, IR, hypertension and low-grade inflammation) (Tofler <i>et al.</i>, 2005; Yamamoto <i>et al.</i>, 2014).
N-3 polyunsaturated fatty acid	Inconsistent findings; some intervention studies report that n-3 fatty acid supplementation increases plasma PAI-1_{act} level (Schmidt <i>et al.</i>, 1990; Spannagl <i>et al.</i>, 1991; Oosthuizen <i>et al.</i>, 1994; Grundt <i>et al.</i>, 1999), whereas other studies show no effect (Prisco <i>et al.</i>, 1994; Toft <i>et al.</i>, 1997; Hansen <i>et al.</i>,	The mechanism of action responsible for the association between plasma PAI-1 levels and n-3 polyunsaturated fatty acids is not fully understood. <i>In vitro</i> studies of cultured cells have suggested this observation could probably be related to the VLDL response element in the <i>PAI-1</i> gene (Nilsson <i>et al.</i>, 1998) or through activation of protein kinase C (Banfi <i>et al.</i>, 1997).

Table 2.2 (continued): Summary of genetic and non-genetic determinants of plasma PAI-1 levels and possible mechanisms of action

	2000; Finnegan <i>et al.</i>, 2003). Observational studies mostly report a positive association between n-3 fatty acids and increased plasma PAI-1_{act} levels (Haglund <i>et al.</i>, 1994; Byberg <i>et al.</i>, 2001). Only a few studies analysed the effect of n-3 fatty acids on plasma PAI-1_{ag} levels, though data are also inconclusive (Haglund <i>et al.</i>, 1994; Prisco <i>et al.</i>, 1994).
Alcohol	Heavy drinking is associated with higher plasma PAI-1_{ag} (Margaglione <i>et al.</i>, 1998; Djoussé <i>et al.</i>, 2000; Mukamal <i>et al.</i>, 2001; Sasaki <i>et al.</i>, 2001) and PAI-1_{act} levels (Hendriks <i>et al.</i>, 1994; Sasaki <i>et al.</i>, 2001; Pieters <i>et al.</i>, 2010). Mechanisms for these associations are not completely understood. A possible pathway could be through the association of PAI-1 with triglyceride, explained by the VLDL response element in the <i>PAI-1</i> promoter area (Nilsson <i>et al.</i>, 1998), as previously mentioned. Increased triglyceride levels are associated with heavy alcohol consumption and, therefore, it is also likely that the increase in PAI-1 levels with alcohol consumption could be a consequence of the distorted triglyceride levels (Scarabin <i>et al.</i>, 1998). On the other hand, <i>in vitro</i> investigations have found that low doses of alcohol down-regulate transcription of PAI-1 in cultured human endothelial cells (Booyse <i>et al.</i>, 1999; Grenett <i>et al.</i>, 2000).

Table 2.2 (continued): Summary of genetic and non-genetic determinants of plasma PAI-1 levels and possible mechanisms of action

Physical activity	Increased plasma PAI-1_{act} and PAI-1_{ag} levels were found in untrained individuals (Francis <i>et al.</i>, 2014). Short-term physical activity* decreases both PAI-1_{ag} (Ribeiro <i>et al.</i>, 2007) and PAI-1_{act} (Eriksson-Berg <i>et al.</i>, 2002). Long-term / regular physical activity** decreases plasma PAI-1_{act} level (De Geus <i>et al.</i>, 1992; El-Sayed, 1996; Fernhall <i>et al.</i>, 1997; Jahangard <i>et al.</i>, 2009; Kupchak <i>et al.</i>, 2013; Francis <i>et al.</i>, 2014) and PAI-1_{ag} (Lira <i>et al.</i>, 2010).	Physical activity has been associated with an increase in plasma tPA_{act} and a decrease in plasma PAI-1_{act} (Fernhall <i>et al.</i>, 1997; DeSouza <i>et al.</i>, 1998), which could result from the formation of tPA/PAI-1 complexes. This observed change in plasma tPA_{act} and PAI-1_{act} levels could be related to changes in liver blood flow and clearance of tPA/PAI-1 complex (Fras <i>et al.</i>, 2004). Decreased PAI-1 levels also possibly reflect exercise-related beneficial changes in factors such as body composition, cardiovascular fitness (El-Sayed, 1996; Francis <i>et al.</i>, 2014) and lipid levels (lower plasma triglyceride; LDL-chol and T-chol concentration) (De Geus <i>et al.</i>, 1992; Lira <i>et al.</i>, 2010).
Circadian pattern	Strong positive effect on plasma PAI-1_{ag} (Johansen <i>et al.</i>, 1991; Van der Bom <i>et al.</i>, 2003) and PAI-1_{act} (Angleton <i>et al.</i>, 1989; Hoekstra <i>et al.</i>, 2002) levels, characterised by	The circadian pattern of PAI-1 is mediated by two heterodimers (CLOCK:BMAL1 and CLOCK:BMAL2) that bind to the promoter area of PAI-1 and thereby regulate <i>PAI-1</i> expression (Schoenhard <i>et al.</i>, 2003).

Table 2.2 (continued): Summary of genetic and non-genetic determinants of plasma PAI-1 levels and possible mechanisms of action

	higher PAI-1 levels in the early morning than in the afternoon.	
Acute-phase response	Plasma PAI-1 _{ag} levels are strongly increased in response to inflammation, as seen in sepsis (Mesters <i>et al.</i> , 1996; Green <i>et al.</i> , 2002; Zeerleder <i>et al.</i> , 2006) and atherosclerosis (Peng <i>et al.</i> , 2008).	TGF-β1, TNF-α and IL-6 are inflammatory mediators that modulate the stimulation of acute-phase proteins such as PAI-1 (Binder <i>et al.</i> , 2002). TNF-α can also be stimulated by lipopolysaccharides, which in turn results in the production of PAI-1 from endothelial cells and leads to an increased plasma PAI-1 level (Binder <i>et al.</i> , 2002). TGF-β1 is released from platelets following vascular injury (Binder <i>et al.</i> , 2002) and, therefore, also contributes to the elevated plasma PAI-1 levels associated with inflammation.
Smoking	Associated with increased plasma PAI-1 _{ag} (Margaglione <i>et al.</i> , 1998; Coffey <i>et al.</i> , 2011) and PAI-1 _{act} levels (Haaland <i>et al.</i> , 1992; Ishizaki <i>et al.</i> , 1996; Yarnell <i>et al.</i> , 2000).	The increase in plasma PAI-1 levels associated with regular smoking might be caused by the damaging effects of cigarette smoke on the vascular endothelium (Sasaki <i>et al.</i> , 2001). This seems likely as endothelial cells, among others, are known to be responsible for PAI-1 production (Kruithof, 1988). In HUVECs, cigarette smoke extract has been found to damage the vascular endothelial cells by the c-J N-terminal kinase pathway, partly by means of oxidative stress (Hoshino <i>et al.</i> , 2005).

Alanine (A); angiotensin converting enzyme (ACE); C-reactive protein (CRP); cytosine-adenine (CA); guanine (G); high-density lipoprotein-cholesterol (HDL-CHOL); human umbilical vein endothelial cells (HUVECs); insertion/deletion (indel); insulin resistance (IR); interleukin 1 (IL-1); interleukin 6 (IL-6); linkage disequilibrium (LD); low-density lipoprotein-cholesterol (LDL-CHOL); myocardial infarction (MI); plasminogen activator inhibitor-1 (PAI-1), PAI-1 antigen (PAI-1_{ag}); PAI-1 activity (PAI-1_{act}); single-nucleotide polymorphism (SNP); tissue plasminogen activator (tPA); total cholesterol (T-CHOL); transforming growth factor-β1 (TGF-β1); tumour necrosis factor-α (TNF-α); visceral adipose tissue

Table 2.2 (continued): Summary of genetic and non-genetic determinants of plasma PAI-1 levels and possible mechanisms of action

(VAT); very low-density lipoprotein (VLDL); waist-to-hip ratio (WHR). *acute submaximal physical exercise of ≤ 30 min; **following exercise programme of 3-5 times per week of 30-45 min per session ≥ 3 months.

2.5 PAI-1 and obesity

The association between PAI-1 and obesity is well established, and has been found in animal as well as human studies (Samad & Loskutoff, 1996; Shimomura *et al.*, 1996; Alessi *et al.*, 1997; Eriksson *et al.*, 1998). Samad and Loskutoff (1996) were the first to describe the expression of *PAI-1* by adipose tissue in genetically modified obese mice. They reported *PAI-1* expression to be five times higher in obese mice than in their lean counterparts (Samad & Loskutoff, 1996). In human studies, the production and expression of *PAI-1* in adipose tissue have been studied through *in vitro* (Alessi *et al.*, 1997; Giltay *et al.*, 1998; Cigolini *et al.*, 1999; Alessi *et al.*, 2000b; Bastard *et al.*, 2000; Bastelica *et al.*, 2002; Lindeman *et al.*, 2007) and *in vivo* studies (Mykkanen *et al.*, 1994; De Pergola *et al.*, 1997; Janand-Delenne *et al.*, 1998; Peverill *et al.*, 2007; Ekström *et al.*, 2012). Obese individuals, specifically those with central obesity, have been shown to have increased plasma PAI-1 levels (Vague *et al.*, 1989; De Pergola *et al.*, 1997; Alessi *et al.*, 2000b). Abdominal fat deposition takes place in two main fat depots [subcutaneous adipose tissue (SCAT) and intra-abdominal or visceral fat tissue (VAT)], which differ regarding *PAI-1* expression. PAI-1 is considered by many to be expressed more in VAT than SCAT (Alessi *et al.*, 1997; Cigolini *et al.*, 1999; Bastelica *et al.*, 2002). This seems to be related to structural and functional differences between VAT and SCAT. Visceral adipose tissue, present in the abdominal viscera in the mesentery and omentum, produces more pro-inflammatory cytokines, such as tumour necrosis factor- α (TNF- α), C-reactive protein (CRP) and interleukin-6 (IL-6) (Ibrahim, 2010), and also contains more stromal cells, the cellular component of adipose tissue that produces PAI-1 (Bastelica *et al.*, 2002). In contrast, SCAT expresses more leptin and adiponectin and is found mainly in the femoral-gluteal regions, back and anterior abdominal wall (Ibrahim, 2010). In the following section, differences in *PAI-1* expression in VAT and SCAT will be discussed by comparing the findings of *in vitro* and *in vivo* studies.

2.5.1 *Ex vivo* and *in vivo* studies on *PAI-1* expression and synthesis in different fat depots (VAT and SCAT)

In *ex vivo* experiments, human abdominal fat tissue samples were used to compare PAI-1 production and secretion in SCAT *versus* VAT (Cigolini *et al.*, 1999; Eriksson *et al.*, 2000; Gottschling-Zeller *et al.*, 2000; Bastelica *et al.*, 2002). Results from many *ex vivo* studies indicate that VAT produces high levels of PAI-1 and has a greater capacity for *PAI-1* messenger ribonucleic acid (mRNA) expression in comparison with SCAT. *PAI-1* mRNA

expression in VAT has been reported to be five times higher than in abdominal SCAT (Alessi *et al.*, 2000b; Bastelica *et al.*, 2002). Supporting studies have shown PAI-1 secretion from adipocytes to be significantly higher in VAT depots when compared with SCAT depots, and as being an independent predictor of plasma PAI-1_{act} in healthy men (Cigolini *et al.*, 1999).

Eriksson *et al.* (2000), however, reported that *PAI-1* expression from abdominal SCAT was approximately two times higher than from abdominal VAT in severely obese white individuals. Additionally, females were found to have a significant depot-specific PAI-1 secretion, with SCAT producing higher levels of PAI-1 than VAT (Eriksson *et al.*, 2000). These depot-specific differences were not observed in men (Eriksson *et al.*, 2000). These results differ from findings from other studies suggesting that VAT is the major source of plasma PAI-1_{act} (Bastelica *et al.*, 2002). Differences in findings could possibly be explained by the cell size of the different adipose tissue depots or even differences in study populations with regard to body mass index (BMI). In contrast with other studies (Alessi *et al.*, 1997; Morange *et al.*, 1999; Eriksson *et al.*, 2000), Eriksson *et al.* (2000) expressed their results as nanograms of *PAI-1* expressed / 10⁷ cells, instead of ng/g adipose tissue. Since the cell size of the SCAT was found to be larger than that of VAT, it could have influenced the results significantly. Other studies included overweight and obese participants with a wider BMI range of 18 to 28 kg/m² (Alessi *et al.*, 1997) and 21 to 40 kg/m² (Morange *et al.*, 1999) as compared with the study population (mean BMI 43.1±1.2 kg/m²) of Eriksson *et al.* (2000), indicating the possibility that degree of obesity might influence regional differences in PAI-1 secretion.

The importance of the contribution of accumulated SCAT to increased plasma PAI-1 levels has also been pointed out in other studies. Morange *et al.* (1999) reported a high correlation between PAI-1 production by VAT and SCAT, which suggested a similar regulatory pathway of PAI-1 in these two adipose depots, despite their different anatomic and metabolic characteristics. A study by Mavri *et al.* (2001) investigated *PAI-1* expression from two subcutaneous territories (abdominal and femoral) in obese and lean individuals. The results of the Mavri *et al.* (2001) study found higher *PAI-1* mRNA content in the abdominal SCAT in obese participants than in lean participants, which was also correlated with plasma PAI-1_{ag} levels, even after the obese participants lost weight. However, *PAI-1* mRNA content in the femoral SCAT was the same in both the lean and obese groups and showed no association

with plasma PAI-1 values (Mavri *et al.*, 2001). This study supports the influence of regional fat distribution on elevated plasma PAI-1 levels in obese participants.

Unlike *ex vivo* studies, *in vivo* studies often use computed tomography to assess the different fat depots (SCAT and VAT) to determine their association with PAI-1 plasma levels. These investigations support the findings of most *ex vivo* studies, indicating that plasma PAI-1 levels are closely related to visceral fat areas and that improved fibrinolytic activity was found with the loss of visceral fat mass (Shimomura *et al.*, 1996; Janand-Delenne *et al.*, 1998). Other studies used anthropometric indicators such as BMI, waist circumference (WC) and waist-to-hip ratio (WHR) to investigate the relationship between plasma PAI-1_{act} and distribution of body fat. PAI-1 was found to correlate positively with BMI and WHR in both males and females (Mykkanen *et al.*, 1994). However, a study by Peverill *et al.* (2007) was unsupportive of the findings of *ex vivo* studies when evaluating body fat distribution by means of BMI and measurements of WC and hip circumference. In their findings, WC and WHR correlated with PAI-1 to a similar degree, whereas BMI correlated more strongly (Peverill *et al.*, 2007). An inverse association between hip circumference and markers of coagulation after adjusting for WC was also found and, therefore, indicates a possible protective effect of gluteal fat distribution as opposed to android obesity (Peverill *et al.*, 2007). Differences in findings could possibly be related to the inclusion of post-menopausal women in the study population of Peverill *et al.* (2007), while other studies included only pre-menopausal women (Vague *et al.*, 1989; De Pergola *et al.*, 1997; Janand-Delenne *et al.*, 1998; Peverill *et al.*, 2007). Post-menopausal women experience a redistribution of fat distribution (increased visceral fat accumulation) that is related to the female sex hormone oestrogen (Mattsson & Olsson, 2007). It should also be noted that therapeutic administration of oral oestrogen has been reported to reduce plasma PAI-1 levels (Ågren *et al.*, 2008).

Nevertheless, WC, in comparison with computerised tomography and ultrasound, is regarded as a useful surrogate for the measurement of visceral fat (Pontiroli *et al.*, 2002), while WHR and BMI seem to be poorly associated with these measures of visceral fat or markers of PAI-1 levels (Giltay *et al.*, 1998; Pontiroli *et al.*, 2002). Others have also found PAI-1_{ag} to be positively correlated with WC in obese women, independently of BMI (De Pergola *et al.*, 1997). Increased WC, as an indication of visceral fat mass, could, therefore, be a marker of elevated PAI-1 plasma levels. However, such interpretations should be

made with caution, especially as WC was not found to be a suitable surrogate for assessing visceral fat mass in African women when these women were on a weight loss diet (Weinsier *et al.*, 2001).

From *ex vivo* investigations it is evident that adipose tissue from the abdominal area, specifically VAT, has the greater capacity to induce *PAI-1* expression and production, which, in part, could affect plasma PAI-1 levels. As *ex vivo* investigations are performed under controlled conditions outside the human body, however, it is difficult to assess the total contribution adipocytes might make to circulating PAI-1 levels when compared with other PAI-1-producing cells such as hepatocytes, platelets and endothelial and vascular smooth muscle cells. Furthermore, the results are inconsistent, as SCAT has also been found to have an acknowledgeable role in increased plasma PAI-1 levels. Therefore, it seems that VAT and PAI-1 levels are concurrently related to abnormal fat metabolism (discussion in section 2.5.2), rather than one bringing about the other (Lindeman *et al.*, 2007). To gain a better understanding of the relationship between increased plasma PAI-1 levels and obesity, the following section focuses on possible mechanisms explaining this association.

2.5.2 Possible mechanisms explaining the role of increased *PAI-1* expression and synthesis in adipose tissue

In section 2.5 it was mentioned that there seems to be an association between the stromal cells found in VAT and increased plasma PAI-1 levels. Nevertheless, other possible mechanisms have also been proposed to explain the expression and synthesis of PAI-1 in VAT *versus* SCAT. Weight loss, the involvement of the systemic inflammation process, the contribution of other PAI-1-producing cells, gender and ethnic differences, among others, have been investigated and will be discussed below.

Ex vivo studies strongly support weight loss as a means of decreasing plasma PAI-1 levels in obese individuals, thereby also lowering their risk for thrombotic events (Mavri *et al.*, 2001; Pardina *et al.*, 2012). Weight loss, especially the loss of SCAT, was shown to result in a decline in PAI-1 levels in comparison with loss in femoral regions (Mavri *et al.*, 2001). This association is also supported by *in vivo* investigation. Bastard *et al.* (2000) studied the changes in plasma PAI-1 levels induced by following a very low calorie diet in obese white

individuals. This study aimed to verify whether decreased *PAI-1* expression in adipose tissue is associated with decreasing plasma PAI-1 levels in obese individuals. Although a decrease in plasma PAI-1 levels was found, *PAI-1* mRNA and protein content increased in SCAT. The researchers concluded that the reduction in plasma PAI-1 levels does not result from a diet-induced decrease in SCAT *PAI-1* expression (Bastard *et al.*, 2000). Even though *PAI-1* mRNA and protein content increased in SCAT, it seems that a very low calorie diet may help clear circulating PAI-1 levels. The exact cause of this relationship is, however, still unexplained (Bastard *et al.*, 2000). On the other hand, Janand-Delenne *et al.* (1998) found a positive correlation between loss of VAT and decreased PAI-1 levels in women.

In a recent study, the ability of the acute systemic inflammatory response to activate gene expression and PAI-1 production in VAT was demonstrated. According to Ekström (2012), during open heart surgery, *PAI-1* mRNA expression was found to increase 27-fold in the omental (visceral) adipose tissue, compared with a threefold increase in the SCAT, followed by an increase in plasma PAI-1_{ag} levels. Cytokines, particularly transforming growth factor- β 1 (TGF- β 1), and interleukin-1 (IL-1), are widely acknowledged inflammatory mediators of PAI-1 (Morange *et al.*, 1999; Birgel *et al.*, 2000; Lindeman *et al.*, 2007). TGF- β 1 seems to regulate *PAI-1* expression as part of an autocrine and paracrine function (Birgel *et al.*, 2000). Particularly in subcutaneous adipocytes, TGF- β 1 has been proposed as a main inducer of *PAI-1* mRNA synthesis (Birgel *et al.*, 2000; Eriksson *et al.*, 2000), although others have reported no significant difference in the effect of TGF- β 1 on PAI-1_{ag} secretion between the two fat depots (VAT and SCAT) (Gottschling-Zeller *et al.*, 2000). Also, VAT has been ruled out as a relevant source of proinflammatory mediators by others, as soluble intercellular adhesion molecule-1, a sensitive marker of proinflammatory activity, is lacking in VAT (Lindeman *et al.*, 2007).

Other sources of PAI-1, such as hepatocytes and endothelial cells, could also contribute to the elevated plasma PAI-1 levels seen in obesity (Eriksson *et al.*, 1998). It is known that adipose tissue contains endothelial cells as well as fibroblasts, smooth muscle cells and macrophages, which are involved in the expression of *PAI-1* mRNA (Shimomura *et al.*, 1996). Others have also identified adipocytes, hepatocytes and endothelial cells as underlying sources of elevated PAI-1 levels in obese individuals (Bastard & Pieroni, 1999; Pardina *et al.*, 2012).

As mentioned, increased PAI-1 levels are often associated with central obesity or android fat distribution (Vague *et al.*, 1989; De Pergola *et al.*, 1997; Alessi *et al.*, 2000b). It is also known that there is a difference in body fat distribution between female and male genders. Female gender is generally associated with a gluteal or gynoid fat distribution (meaning greater fat accumulation on the hips, buttocks and thighs) compared with the android fat distribution of men (greater visceral fat mass accumulation in the abdominal area) (Vague, 1996). Android obesity is also a recognised cause of metabolic disorders like diabetes, gout, uric calculous disease and atherosclerosis (Vague, 1996). On the other hand, gynoid obesity is more often associated with mechanical complications such as trophic disturbances of the venous-lymphatic circulation and respiratory complications (bronchopulmonary infections) (Vague, 1996). Since android fat distribution is more strongly associated with male gender, men would be expected to have higher PAI-1 levels than women, especially as men have been found to have more VAT than women (Grauer *et al.*, 1984). Yet decreased abdominal obesity in men has been shown to be more strongly associated with improvement of fibrinolytic activity (decrease in PAI-1_{act} levels) than in women following an endurance exercise training programme three times a week for six weeks (Kulaputana *et al.*, 2005). Others have also reported that women have greater PAI-1 levels compared with men (Giltay *et al.*, 1998). In a study by Nienaber *et al.* (2008), in a group of black South African girls (average 15 years of age) higher PAI-1_{act} levels were found than in boys even after adjustments for physical activity and body fat percentages were made. Van Harmelen *et al.* (2000), on the other hand, showed that differences in plasma PAI-1_{act} levels between men and women are due mainly to differences in WHR as opposed to gender differences in abdominal SCAT PAI-1 secretion. Possible differences in VAT PAI-1 secretion were not investigated. Toft *et al.* (1997), however, found an independent association between plasma PAI-1_{act} levels and WHR in women, but not in men. In order to explain the somewhat stronger relationship found in women, Mykkanen *et al.* (1994) suggested that over time, women with a gluteal fat distribution also tend to accumulate fat in the abdominal area, leading to a greater WHR and increased PAI-1 levels. Men, on the other hand, seem to have a pre-existing tendency to accumulate fat in the abdominal area and, therefore, a plateau in PAI-1_{act} is seen in men with a higher WHR (Mykkanen *et al.*, 1994).

With regard to ethnicity, dissimilarity between circulating PAI-1 and fat distribution is evident. In a comparison of white and African American women with similar PAI-1 levels and BMI, significant associations between PAI-1_{ag} and VAT were found only in white women and not

in the African American women (Solano *et al.*, 2003). When investigating ethnic differences in PAI-1_{act} and the association of PAI-1_{act} and markers of the MetS in 95 African and 114 white women, significantly lower PAI-1_{act} levels along with a weaker association between PAI-1_{act} and markers of the MetS were found in the African than in the white women (Greyling *et al.*, 2007). In this study WC was found to be the most important independent contributor to plasma PAI-1_{act} in both groups (Greyling *et al.*, 2007). The possibility cannot be excluded, however, that the difference in PAI-1 levels could be related to differences in fat distribution between ethnic groups, despite similar WC. Conway *et al.* (1995) investigated fat distribution and metabolic variables in obese black and white American women and showed that, despite similar WHR, black women had less VAT than white women ($p=0.031$). In a black and white South African sample, black women were also found to have less VAT in comparison with white women ($p<0.001$) after adjusting for age, height, weight and fat mass, despite the fact that there were no ethnic differences in WC, WHR, or dual-energy X-ray absorption (DXA) measurement of the abdominal regions (Micklesfield *et al.*, 2010). Similar to the VAT distribution in women, in a study by Hill *et al.* (1999), significantly lower VAT was found in black men than in white men after adjustment for percentage body fat, total body fat, BMI, WC, WHR or sagittal diameter. However, it is difficult to draw conclusions regarding the relationship of PAI-1 and body fat composition in black African populations as such information is limited and most research investigating these issues has been undertaken in populations of European descent (Eriksson *et al.*, 1998; Hill *et al.*, 1999; Bastard *et al.*, 2000; Eriksson *et al.*, 2000; Gottschling-Zeller *et al.*, 2000). Owing to ethnic differences in body fat composition and fat deposition, the relationship of PAI-1 with body composition in Africans may differ from that observed for other population groups and, therefore, merits further investigation.

It is possible that an association between PAI-1 and other forms of obesity such as SO exist. Sarcopenic obesity refers to a condition often seen in obese elderly who have both a low muscle mass and a high fat mass (Baumgartner, 2000). Recent research suggests that SO could be a high risk factor for the development of MetS and atherosclerosis (Kohara, 2014). It has also previously been hypothesised that the combined effect of sarcopenia and obesity relates to an increased incidence of physical disability, IR and, therefore, associated CVD morbidities such as hypertension, dislipidaemia and diabetes, conditions often found in obese elderly persons (Dominguez & Barbagallo, 2007; Zamboni *et al.*, 2008). Furthermore, a strong independent relationship between PAI-1 and the MetS has been demonstrated and PAI-1 is acknowledged to be a recognised component of the MetS (Juhan-Vague & Alessi,

1997; Mertens *et al.*, 2006; Palomo *et al.*, 2009). Equally important, plasma PAI-1 levels have been shown to increase with age (Chou *et al.*, 2009) as well as with co-morbidities associated with the ageing process, such as hypertension, myocardial infarction (MI) and cerebral infarction, arteriosclerosis and inflammation, to name a few (Yamamoto *et al.*, 2014). In older individuals, obesity, IR and impaired fibrinolysis resulting from increased plasma PAI-1 levels are considered risk factors for the development of thrombotic CVD (McGill *et al.*, 1994). Therefore, it seems reasonable to assume that a relationship exists between increased plasma PAI-1 levels and SO. Since such a relationship has not been extensively researched, our knowledge of SO and its relationship with PAI-1 is still lacking. In the following section, attention is given to the difference between sarcopenia and SO and the underlying mechanisms of development. Following this, the importance of exploring the association between PAI-1 and SO is motivated.

2.6 Sarcopenia and SO

2.6.1 Definition of sarcopenia and SO

In order to gain a better understanding of the term SO, it is first necessary to refer to the meaning of sarcopenia. Sarcopenia is a syndrome of age-related loss of muscle mass and strength, and is often associated with negative outcomes such as physical disability and poor quality of life in the elderly. This loss of muscle mass (35-40% of skeletal mass) occurs as part of the ageing process and gradually increases from 30 to 70 years of age (Micklesfield *et al.*, 2010).

According to the European Working Group on Sarcopenia in Older People (EWGSOP, the Sarcopenia Working Group), the recommended criteria for the diagnosis of sarcopenia are the presence of low muscle mass and a documentation of either low muscle strength or low physical performance (Cruz-Jentoft *et al.*, 2010). Several mechanisms underlying age-related muscle loss and the progression of sarcopenia have been identified. These include, among others, abnormal protein synthesis, proteolysis, proinflammatory cytokine levels (IL-1 β , IL-6, TNF- α), hormone levels (growth hormone, insulin-like growth factor-1, testosterone), and neuromuscular and peripheral vascular integrity, but also malnutrition, lack of exercise and immobility (Morley *et al.*, 2001; Nair, 2005; Cruz-Jentoft *et al.*, 2010). Continuous high-intensity resistance training seems to be the most successful intervention

for increasing muscle mass and strength in elderly (Borst, 2004), and is, therefore, the method of choice for preventing and reversing the development of sarcopenia.

In order to help with the clinical assessment and diagnosis of sarcopenia, different stages (pre-sarcopenia, sarcopenia and severe sarcopenia) have been proposed by the EWGSOP (Cruz-Jentoft *et al.*, 2010). Pre-sarcopenia is characterised by the loss of muscle mass and is different from the sarcopenic stage, which also includes the criteria of either low muscle strength or low muscle performance. The last stage, severe sarcopenia, includes all three of the criteria: the presence of low muscle mass, muscle strength and muscle performance (Cruz-Jentoft *et al.*, 2010). In addition, gender-specific cut-off points to define sarcopenia have been proposed. These cut-off points entail the measurement of appendicular skeletal muscle mass (ASM) from a DXA scan, which defines sarcopenia as ASM (kg) divided by body height² (m) two standard deviations (SD) below the reference population group of younger adults (29 years of age), or less than 7.26 kg/m² in men and 5.45 kg/m² in women (Baumgartner *et al.*, 1998; Cruz-Jentoft *et al.*, 2010). Epidemiological data among elderly persons in New Mexico have estimated that the prevalence of sarcopenia increased from 13%-24% in those less than 70 years of age, to more than 50% in persons 80 years and older, and was slightly greater in Hispanics than in non-Hispanic whites (Baumgartner *et al.*, 1998). In a recent study undertaken to develop cut-off points for sarcopenia in black South African women, ethnic-specific changes in age-related muscle and fat mass were highlighted (Kruger *et al.*, 2015). A cut-off point of ASM index (ASMI) of <4.94 kg/m² was determined in the reference population group, which consisted of young black South African women (Kruger *et al.*, 2015). Accordingly, 9.1% of the older black women in the population were determined to be sarcopenic, compared with 16.7 to 38.7% when using other cut-off points (Kruger *et al.*, 2015).

As with sarcopenia, the prevalence of SO increases with age (Zamboni *et al.*, 2008) and is a condition that deserves attention, since persons who are obese and sarcopenic have worse outcomes than those who are sarcopenic and non-obese (Baumgartner *et al.*, 1998; Morley *et al.*, 2001; Tieland, 2013). SO differs from sarcopenia in that fat mass might be preserved or even increased, along with age-related change in muscle composition (such as fat infiltration in the muscle), leading to loss of muscle quality and performance (Visser *et al.*, 2002; Stenholm *et al.*, 2008; Cruz-Jentoft *et al.*, 2010). There is, however, no consensus on the definitions and diagnostic criteria of SO and this issue has been critically reviewed by

Prado *et al.* (2012b). According to Prado *et al.* (2012b), this could possibly be a result of different opinions on the interpretation of low muscle mass and a high fat mass. It was also highlighted that different combinations of indicators used in previous studies to assess body composition might underestimate the risk prediction of SO, since sarcopenia and obesity were investigated in isolation in many of these studies (Prado *et al.*, 2012a). Despite this fact, it would seem that several studies defined sarcopenia by low muscle mass (ASM < 2 SD for a healthy reference population group) and a fat mass percentage greater than the 60th percentile of a reference population group (Baumgartner *et al.*, 2004; Stenholm *et al.*, 2008; Waters *et al.*, 2008; Rolland *et al.*, 2009).

2.6.2 Pathogenesis and clinical implications of SO

The pathogenesis and clinical implications of SO are still poorly understood (Dominguez & Barbagallo, 2007; Zamboni *et al.*, 2008; Cruz-Jentoft *et al.*, 2010; Prado *et al.*, 2012b; Kohara, 2014). Recently, Kohara (2014) proposed that SO is not merely the combined result of existing obesity and the development of sarcopenia with advanced age, but is related more to cardio-metabolic syndrome and functional abnormalities. According to Dominguez and Barbagallo (2007), cardio-metabolic syndrome is the combined diagnosis of hypertension and dyslipidaemia, often seen in older individuals. It would seem that changes that occur during ageing lead to sarcopenia and obesity (especially VAT) and act synergistically to induce IR, hypertension and dyslipidaemia, which then later on in life increase one's risk of the development of type 2 diabetes mellitus (T2DM) and CVD (Dominguez & Barbagallo, 2007).

It has also been suggested that chronic low levels of inflammation, often accompanying advanced age, are caused by increased production of cytokines (TNF- α and IL-6) and leptin from excess adipose tissue and adipocytes, resulting in IR (Roubenoff, 2004; Dominguez & Barbagallo, 2007; Jensen, 2008; Stenholm *et al.*, 2008). This chronic low level of inflammation might cause the progressive loss of muscle mass and an increase in fat mass (Roubenoff, 2004; Zamboni *et al.*, 2008). As previously mentioned, in SO, fat infiltration in the muscle also takes place (Visser *et al.*, 2002; Stenholm *et al.*, 2008; Cruz-Jentoft *et al.*, 2010). This additionally promotes the progressive loss of muscle mass and the degree of inflammation in a person with SO, but also increases the resistance to leptin in the muscle (Unger, 2005; Zamboni *et al.*, 2008), leading to the reduction of fatty acid oxidation in the muscle, liver and heart (Unger, 2005).

Sarcopenic obesity, therefore, seems on the one hand to be related to the health risks associated with obesity (IR, hypertension, dyslipidaemia, diabetes and CVD) and on the other hand, to depleted muscle mass and functional disability (Baumgartner *et al.*, 2004; Roubenoff, 2004). However, the combined effect seems to create an adverse condition associated with more physical functional decline than obesity alone and also a higher risk of MetS and atherosclerosis due to increased IR (Kohara, 2014). Of specific importance to the outcomes of the present study is the pathway (inflammation, obesity and IR) involved in the pathogenesis of SO, which is also related to the synthesis of PAI-1. In the following section, this issue will be addressed.

2.6.3 Sarcopenic obesity as a risk factor for the development of MetS and CVD and its association with PAI-1

Several studies have found SO to be associated with the MetS and CVD. In a cross-sectional survey conducted in New Mexico, the prevalence of T2DM was higher in the SO group (7.7%) than in the sarcopenic non-obese group (1.0% to 1.4%) (Baumgartner *et al.*, 2004). The difference was, however, not statistically significant owing to the small number of cases. The prevalence of the MetS was found to be highest in the non-sarcopenic obese group, followed by the SO group, and was lowest in the non-obese sarcopenic group (Baumgartner *et al.*, 2004). This study also indicated the likelihood of SO preceding disability in community-dwelling elderly (Baumgartner *et al.*, 2004). Prospective observational studies showed that participants with MetS had a lower muscle-to-fat ratio (i.e. SO as determined by ratio of ASM to central obesity), which was found to be a dependent risk factor for the MetS and was also independently associated with arterial stiffness after adjusting for other risk factors (Kim *et al.*, 2011). Recently, SO was shown to be an independent risk factor for the MetS with a 12-fold higher risk than in those without sarcopenia and obesity (Lu *et al.*, 2013). With regard to CVD, longitudinal data indicated that CVD events were 23% [95% confidence interval (CI) 0.99-1.54; $p=0.06$] higher in those with SO compared either with sarcopenic or obese persons (Stephen & Janssen, 2009).

However, some conflicting findings have also been reported. In a small study comparing the CVD risk factors between SO and obesity in postmenopausal women, SO was found to be associated with a lower risk of CVD (Aubertin-Leheudre *et al.*, 2006). Messier *et al.* (2009),

however, found the metabolic profile (i.e. IR) and quality of life of overweight and SO postmenopausal women to be comparable to that of non-sarcopenic overweight or obese women. Possible explanations for the conflicting results might be related to the use of different methods to determine body composition (such as BMI, WC and DXA measurements), diagnostic criteria and inclusion and exclusion criteria (age, gender, and race) (Stenholm *et al.*, 2009; Prado *et al.*, 2012b).

PAI-1 has also been identified as an independent component of the MetS, which is characterised by hyperglycaemia, hypertension, an abnormal blood lipid profile and central obesity (Juhan-Vague & Alessi, 1997; Alberti *et al.*, 2009; Chou *et al.*, 2009; Palomo *et al.*, 2009). In a cross-sectional study investigating the relationship between inflammatory, prothrombotic markers and the MetS in an Asian population group, Chou *et al.* (2009) showed that PAI-1 levels were elevated in the elderly. It was also evident that PAI-1 was positively associated with components of the MetS, including WC, plasma fasting glucose and triglycerides and negatively associated with high-density lipoprotein-cholesterol (HDL-chol) (Chou *et al.*, 2009). Thus, PAI-1 demonstrates a complex relationship with the MetS that has profound implications, specifically for older obese individuals. This has important health implications as the components of the MetS are known to be conventional risk factors for the development of CVD (Alberti *et al.*, 2009). Also, as shown by Tofler *et al.* (2005), advancing age is associated with elevated levels of haemostatic factors (increased levels of fibrinogen, von Willebrand factor, PAI-1_{ag} and tPA_{ag}) indicative of a prothrombotic state in both ageing men and women, relating to endothelium dysfunction and vascular inflammation. They postulate that these haemostatic factors might provide a plausible mechanistic pathway through which chronic levels of low-grade inflammation, associated with age, lead to CVD (Tofler *et al.*, 2005). It seems likely, therefore, that an important relationship between PAI-1 and SO exists, and that this relationship may differ from the association of PAI-1 with central adiposity, although only limited research has previously investigated this question (Cesari *et al.*, 2005).

In a study that included data from the Trial of Angiotensin-Converting Enzyme Inhibition and Novel Cardiovascular Risk factors (TRAIN), total fat mass and appendicular lean mass, as measured by DXA, were used to determine the effect of sarcopenia and obesity on CRP, IL-6 and PAI-1 (Cesari *et al.*, 2005). This study found no significant relationship between either obesity or sarcopenia and CRP, IL-6 or PAI-1 concentration (Cesari *et al.*, 2005). The fact

that no relationship between PAI-1 and sarcopenia was found in this study population, however, does not exclude the possibility that PAI-1 may have a local rather than a systemic role in determining the loss of muscle mass (Cesari *et al.*, 2005). Others have also suggested that increased PAI-1 induction in atrophic skeletal muscle might result in muscle fibrosis (often seen in sarcopenic individuals) by inhibiting the production of plasmin (Naderi *et al.*, 2009). This highlights the possible relationship between PAI-1 and SO.

Since obesity is positively associated with age, it will in future have an increasingly negative impact on public health and the provision of health care services (Roubenoff, 2004). As the prevalence of obesity increases with age, so does the impact of SO, as well as other CVD risk factors in the older population group (Roubenoff, 2004; Yamamoto *et al.*, 2005; Yamamoto *et al.*, 2014). According to the findings of the Transition and Health during Urbanisation (THUSA) study, the prevalence of obesity increases with age in African women and was found to peak at the age of 35 to 44 years (Kruger *et al.*, 2002). Physical inactivity was found to be the strongest determinant of obesity in this population group (Kruger *et al.*, 2002), underlining the increased risk of SO in the African population. From previous research in our group among young black South Africans (average age of 15 years) it was also observed that overfatness, a low lean mass accompanied by high fat mass, usually coexists in children who are stunted (Nienaber *et al.*, 2008). Therefore, it was hypothesised that overfat children have a higher risk of becoming adults with SO. Furthermore, overfatness, which was observed to be very prevalent (31.5% and 73.5% in boys and girls, respectively), was associated with undesirable levels of several haemostatic variables, including PAI-1_{act} (Nienaber *et al.*, 2008). Understanding the connection between SO, ageing and PAI-1 seems to be crucial and deserves particular attention, seeing that this has not yet been thoroughly investigated.

2.7 Conclusion

This chapter focused on several research questions identified in earlier research on PAI-1 levels in black South Africans. These topics include an overview of the physiology of PAI-1, methodological issues regarding the measurement of the different forms of PAI-1, PAI-1 determinants in Africans and the association between PAI-1 and obesity, with a specific focus on SO.

Inconsistency was found in previous research investigating ethnic differences in plasma PAI-1 levels. This inconsistency may be a result of differences in assays used and/or differences in sample handling and preparation, which are known to influence plasma PAI-1 levels owing to the presence of residual platelets in plasma, among other factors. In the literature, disagreement regarding the form of PAI-1 (latent or active) released from the alpha granules of platelets was also found and the effect of residual platelets in plasma on the different PAI-1 assays remain to be determined. In addition, this literature chapter has also highlighted the importance of standardised methodological procedures to be followed during the measurement of plasma PAI-1 levels.

PAI-1 expression and synthesis in different fat depots (VAT and SCAT) have been studied in both *ex vivo* and *in vivo* studies. It seems that plasma PAI-1 levels are closely related to adipose tissue located within the abdominal area (specifically VAT). However, structural and functional differences between VAT and SCAT, as well as the influence of regional fat distribution and/or differences in body fat composition between genders and different ethnic groups also seem to influence plasma PAI-1 levels, although only limited data on Africans are available. In addition, SO, a specific form of obesity associated with muscle wasting, is a condition that is increasingly present in the elderly and is also associated with the development of the MetS and CVD. This chapter also discussed the possible associations between increased plasma PAI-1 levels, the development of SO and CVD risk factors, such as the presence of IR, obesity, chronic low grade inflammation and hypertension. Since there are only two studies which investigated the possible association between PAI-1 and SO, our understanding and knowledge of SO are limited, and no data on this association exist for Africans. Since, SO is associated with worse health outcomes than either sarcopenia or obesity alone, this issue deserves special attention.

CHAPTER 3: THE EFFECTS OF RESIDUAL PLATELETS IN PLASMA ON PLASMINOGEN ACTIVATOR INHIBITOR- 1 AND PLASMINOGEN ACTIVATOR INHIBITOR-1- RELATED ASSAYS

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This chapter includes:

- 3.1 Authors instructions of the journal, *PLOS ONE* (Impact factor: 3.234);
- 3.2 proof that the article has been accepted for publication in *PLOS ONE*; and
- 3.3 The article titled: “The effects of residual platelets in plasma on plasminogen activator inhibitor-1 and plasminogen activator inhibitor-1-related assays”.

In addition, the published article is provided as Addendum D

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Only one corresponding author can be designated in the submission system, but this does not restrict the number of corresponding authors that may be listed on the article in the event of publication. Whoever is designated as a corresponding author on the title page of the manuscript file will be listed as such upon publication. Include an email address for each corresponding author listed on the title page of the manuscript.

- ! PLOS will soon require an ORCID iD for all corresponding authors. We strongly encourage you to register with ORCID now and add your ORCID to your profile in Editorial Manager if you have not already done so.

▶ How to select a new corresponding author in Editorial Manager



Consortia and group authorship

If a manuscript is submitted on behalf of a consortium or group, include the consortium or group name in the author list, and include the full list of members in the Acknowledgments or in a supporting information file. Read the group authorship policy.

Author Contributions

Enter all author contributions in the submission system during submission. The contributions of all authors must be described using the CRediT Taxonomy of author roles. Read the policy.

Contributions will be published with the final article, and they should accurately reflect contributions to the work. The submitting author is responsible for completing this information at submission, and it is expected that all authors will have reviewed, discussed, and agreed to their individual contributions ahead of this time.

PLOS ONE will contact all authors by email at submission to ensure that they are aware of the submission.

Cover letter

Upload a cover letter as a separate file in the online system. The length limit is 1 page.

The cover letter should include the following information:

- › Summarize the study's contribution to the scientific literature
- › Relate the study to previously published work
- › Specify the type of article (for example, research article, systematic review, meta-analysis, clinical trial)
- › Describe any prior interactions with PLOS regarding the submitted manuscript
- › Suggest appropriate Academic Editors to handle your manuscript (see the full list of Academic Editors)
- › List any opposed reviewers

! **IMPORTANT:** Do not include requests to reduce or waive publication fees in the cover letter. This information will be entered separately in the online submission system.

Read about publication fee assistance.

Title page

The title, authors, and affiliations should all be included on a title page as the first page of the manuscript file.

i Download sample title, author list, and affiliations page (PDF)

Abstract

The Abstract comes after the title page in the manuscript file. The abstract text is also entered in a separate field in the submission system.

The Abstract should:

- › Describe the main objective(s) of the study
- › Explain how the study was done, including any model organisms used, without methodological detail
- › Summarize the most important results and their significance
- › Not exceed 300 words

Abstracts should not include:

- › Citations
- › Abbreviations, if possible

Introduction

The introduction should:

- › Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study
- › Define the problem addressed and why it is important
- › Include a brief review of the key literature
- › Note any relevant controversies or disagreements in the field
- › Conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved

Materials and Methods

The Materials and Methods section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.

We encourage authors to submit detailed protocols for newer or less well-established methods as supporting information. Read the supporting information guidelines.

Human or animal subjects and/or tissue or field sampling

Methods sections describing research using human or animal subjects and/or tissue or field sampling must include required ethics statements. See the reporting guidelines for human research, clinical trials, animal research, and observational and field studies for more information.

Data

PLOS journals require authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception.

Large data sets, including raw data, may be deposited in an appropriate public repository. See our list of recommended repositories.

For smaller data sets and certain data types, authors may provide their data within supporting information files accompanying the manuscript. Authors should take care to maximize the accessibility and reusability of the data by selecting a file format from which data can be efficiently extracted (for example,

spreadsheets or flat files should be provided rather than PDFs when providing tabulated data).

For more information on how best to provide data, read our policy on data availability. PLOS does not accept references to “data not shown.”

Cell lines

Methods sections describing research using cell lines must state the origin of the cell lines used. See the reporting guidelines for cell line research for more information.

New taxon names

Methods sections of manuscripts adding new taxon names to the literature must follow the reporting guidelines below for a new zoological taxon, botanical taxon, or fungal taxon.

Results, Discussion, Conclusions

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled “Results and Discussion”) or a mixed Discussion/Conclusions section (commonly labeled “Discussion”). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn.

Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

PLOS ONE editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the *PLOS ONE* Criteria for Publication for more information.

Acknowledgments

Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution.

Authors are responsible for ensuring that anyone named in the Acknowledgments agrees to be named.

- ❗ Do not include funding sources in the Acknowledgments or anywhere else in the manuscript file. Funding information should only be entered in the financial disclosure section of the submission system.

References

Any and all available works can be cited in the reference list. Acceptable sources include:

- Published or accepted manuscripts
- Manuscripts on preprint servers, if the manuscript is submitted to a journal and also publicly available as a preprint

Do not cite the following sources in the reference list:

- Unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., “unpublished work,” “data not shown”). Instead, include those data as supplementary material or deposit the data in a publicly available database.
- Personal communications (these should be supported by a letter from the relevant authors but not included in the reference list)

References are listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, cite the reference number in square brackets (e.g., “We used the techniques developed by our colleagues [19] to analyze the data”). PLOS uses the numbered citation (citation-sequence) method and first six authors, et al.

Do not include citations in abstracts or author summaries.

Make sure the parts of the manuscript are in the correct order *before* ordering the citations.

Formatting references

- ❗ Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of the references is crucial.

PLOS uses the reference style outlined by the International Committee of Medical Journal Editors (ICMJE), also referred to as the “Vancouver” style. Example formats are listed below. Additional examples are in the ICMJE sample references.

A reference management tool, EndNote, offers a current style file that can assist you with the formatting of your references. If you have problems with any reference management program, please contact the source company's technical support.

Journal name abbreviations should be those found in the National Center for Biotechnology Information (NCBI) databases.

Source	Format
Published articles	Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun B, et al. cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (<i>Ailuropoda melanoleuca</i>). Genet Mol Res. 2011;10: 1576-1588. Devaraju P, Gulati R, Antony PT, Mithun CB, Negi VS. Susceptibility to SLE in South Indian Tamils may be influenced by genetic selection pressure on TLR2 and TLR9 genes. Mol Immunol. 2014 Nov 22. pii: S0161-5890(14)00313-7. doi: 10.1016/j.molimm.2014.11.005 <i>Note: A DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers.</i>
Accepted, unpublished articles	Same as published articles, but substitute “In press” for page numbers or DOI.
Web sites or online articles	Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available: http://www.globalizationandhealth.com/content/1/1/14 .
Books	Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.
Book chapters	Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28.
Deposited articles (preprints, e-prints, or arXiv)	Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity; 1991. Preprint. Available: arXiv:1403.3301v1. Accessed 17 March 2014.
Published media (print or online newspapers and magazine articles)	Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 29 Jan 2014. Available: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html . Accessed 17 March 2014.
New media (blogs, web sites, or other written works)	Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/ .
Masters' theses or doctoral dissertations	Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available: http://cumincad.scix.net/cgi-bin/works/Show?2e09
Databases and repositories (Figshare, arXiv)	Roberts SB. QPX Genome Browser Feature Tracks; 2013. Database: figshare [Internet]. Accessed: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214 .
Multimedia (videos, movies, or TV shows)	Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

Supporting Information

Authors can submit essential supporting files and multimedia files along with their manuscripts. All supporting information will be subject to peer review. All file types can be submitted, but files must be smaller than 10 MB in size.

Authors may use almost any description as the item name for a supporting information file as long as it contains an “S” and number. For example, “S1 Appendix” and “S2 Appendix,” “S1 Table” and “S2 Table,” and so forth.

Supporting information files are published exactly as provided, and are not copyedited.

Supporting information captions

List supporting information captions at the end of the manuscript file. Do not submit captions in a separate file.

The file number and name are required in a caption, and we highly recommend including a one-line title as well. You may also include a legend in your caption, but it is not required.

Example caption

S1 Text. Title is strongly recommended. Legend is optional.

In-text citations

We recommend that you cite supporting information in the manuscript text, but this is not a requirement. If you cite supporting information in the text, citations do not need to be in numerical order.

 Read the supporting information guidelines for more details about submitting supporting information and multimedia files.

Figures and Tables

Figures

Do not include figures in the main manuscript file. Each figure must be prepared and submitted as an individual file.

Cite figures in ascending numeric order upon first appearance in the manuscript file.

 Read the guidelines for figures.

Figure captions

Figure captions must be inserted in the text of the manuscript, immediately following the paragraph in which the figure is first cited (read order). Do not include captions as part of the figure files themselves or submit them in a separate document.

At a minimum, include the following in your figure captions:

- › A figure label with Arabic numerals, and "Figure" abbreviated to "Fig" (e.g. Fig 1, Fig 2, Fig 3, etc). Match the label of your figure with the name of the file uploaded at submission (e.g. a figure citation of "Fig 1" must refer to a figure file named "Fig1.tif").
- › A concise, descriptive title

The caption may also include a legend as needed.

Read more about figure captions.

Tables

Cite tables in ascending numeric order upon first appearance in the manuscript file.

Place each table in your manuscript file directly after the paragraph in which it is first cited (read order). Do not submit your tables in separate files.

Tables require a label (e.g., "Table 1") and brief descriptive title to be placed above the table. Place legends, footnotes, and other text below the table.

 Read the guidelines for tables.

Data reporting

All data and related metadata underlying the findings reported in a submitted manuscript should be deposited in an appropriate public repository, unless already provided as part of the submitted article.

 Read our policy on data availability.

Repositories may be either subject-specific (where these exist) and accept specific types of structured data, or generalist repositories that accept multiple data types. We recommend that authors select repositories appropriate to their field. Repositories may be subject-specific (e.g., GenBank for sequences and PDB for structures), general, or institutional, as long as DOIs or accession numbers are provided and the data are at least as open as CC BY. Authors are encouraged to select repositories that meet accepted criteria as trustworthy digital repositories, such as criteria of the Centre for Research Libraries or Data Seal of Approval. Large, international databases are more likely to persist than small, local ones.

 See our list of recommended repositories.

To support data sharing and author compliance of the PLOS data policy, we have integrated our submission process with a select set of data repositories. The list is neither representative nor exhaustive of the suitable repositories available to authors. Current repository integration partners include Dryad and FlowRepository. Please contact data@plos.org to make recommendations for further partnerships.

Instructions for PLOS submissions with data deposited in an integration partner repository:

- Deposit data in the integrated repository of choice.
- Once deposition is final and complete, the repository will provide you with a dataset DOI (provisional) and private URL for reviewers to gain access to the data.
- Enter the given data DOI into the full Data Availability Statement, which is requested in the Additional Information section of the PLOS submission form. Then provide the URL passcode in the Attach Files section.

If you have any questions, please email us.

Accession numbers

All appropriate data sets, images, and information should be deposited in an appropriate public repository. See our list of recommended repositories.

Accession numbers (and version numbers, if appropriate) should be provided in the Data Availability Statement. Accession numbers or a citation to the DOI should also be provided when the data set is mentioned within the manuscript.

In some cases authors may not be able to obtain accession numbers or DOIs until the manuscript is accepted; in these cases, the authors must provide these numbers at acceptance. In all other cases, these numbers must be provided at submission.

Identifiers

As much as possible, please provide accession numbers or identifiers for all entities such as genes, proteins, mutants, diseases, etc., for which there is an entry in a public database, for example:

- Ensembl
- Entrez Gene
- FlyBase
- InterPro
- Mouse Genome Database (MGD)
- Online Mendelian Inheritance in Man (OMIM)
- PubChem

Identifiers should be provided in parentheses after the entity on first use.

Striking image

You can choose to upload a “Striking Image” that we may use to represent your article online in places like the journal homepage or in search results.

The striking image must be derived from a figure or supporting information file from the submission, i.e., a cropped portion of an image or the entire image. Striking images should ideally be high resolution, eye-catching, single panel images, and should ideally avoid containing added details such as text, scale bars, and arrows.

If no striking image is uploaded, we will designate a figure from the submission as the striking image.

 Striking images should not contain potentially identifying images of people. Read our policy on identifying information.

The PLOS content license also applies to striking images. Read more about the content license.

Additional Information Requested at Submission

Funding statement

This information should not be in your manuscript file; you will provide it via our submission system.

This information will be published with the final manuscript, if accepted, so please make sure that this is accurate and as detailed as possible. You should not include this information in your manuscript file, but it is important to gather it prior to submission, because your financial disclosure statement cannot be changed after initial submission.

Your statement should include relevant grant numbers and the URL of any funder's web site. Please also state whether any individuals employed or contracted by the funders (other than the named authors) played any role in: study design, data collection and analysis, decision to publish, or preparation of the manuscript. If so, please name the individual and describe their role.

 Read our policy on disclosure of funding sources.

Competing interests

This information should not be in your manuscript file; you will provide it via our submission system.

All potential competing interests must be declared in full. If the submission is related to any patents, patent applications, or products in development or for market, these details, including patent numbers and titles, must be disclosed in full.

 Read our policy on competing interests.

Manuscripts disputing published work

For manuscripts disputing previously published work, it is *PLOS ONE* policy to invite input from the disputed author during the peer review process. This procedure is aimed at ensuring a thorough, transparent, and productive review process.

If the disputed author chooses to submit a review, it must be returned in a timely fashion and contain a full declaration of all competing interests. The Academic Editor will consider any such reviews in light of the competing interest.

Authors submitting manuscripts disputing previous work should explain the relationship between the manuscripts in their cover letter, and will be required to confirm that they accept the conditions of this review policy before the manuscript is considered further.

Related manuscripts

Upon submission, authors must confirm that the manuscript, or any related manuscript, is not currently under consideration or accepted elsewhere. If related work has been submitted to *PLOS ONE* or elsewhere, authors must include a copy with the submitted article. Reviewers will be asked to comment on the overlap between related submissions.

We strongly discourage the unnecessary division of related work into separate manuscripts, and we will not consider manuscripts that are divided into "parts." Each submission to *PLOS ONE* must be written as an independent unit and should not rely on any work that has not already been accepted for publication. If related manuscripts are submitted to *PLOS ONE*, the authors may be advised to combine them into a single manuscript at the editor's discretion.

Guidelines for Specific Study Types

Human subjects research

All research involving human participants must have been approved by the authors' Institutional Review Board (IRB) or by equivalent ethics committee(s), and must have been conducted according to the principles expressed in the Declaration of Helsinki. Authors should be able to submit, upon request, a statement from the IRB or ethics committee indicating approval of the research. We reserve the right to reject work that we believe has not been conducted to a high ethical standard, even when formal approval has been obtained.

Subjects must have been properly instructed and have indicated that they consent to participate by signing the appropriate

informed consent paperwork. Authors may be asked to submit a blank, sample copy of a subject consent form. If consent was verbal instead of written, or if consent could not be obtained, the authors must explain the reason in the manuscript, and the use of verbal consent or the lack of consent must have been approved by the IRB or ethics committee.

All efforts should be made to protect patient privacy and anonymity. Identifying information, including photos, should not be included in the manuscript unless the information is crucial and the individual has provided written consent by completing the Consent Form for Publication in a PLOS Journal (PDF). More information about patient privacy, anonymity, and informed consent can be found in the International Committee of Medical Journal Editors (ICMJE) Privacy and Confidentiality guidelines.

Manuscripts should conform to the following reporting guidelines:

- Studies of diagnostic accuracy: STARD
- Observational studies: STROBE
- Microarray experiments: MIAME
- Other types of health-related research: Consult the EQUATOR web site for appropriate reporting guidelines

Methods sections of papers on research using human subjects or samples must include ethics statements that specify:

- **The name of the approving institutional review board or equivalent committee(s).** If approval was not obtained, the authors must provide a detailed statement explaining why it was not needed
- **Whether informed consent was written or oral.** If informed consent was oral, it must be stated in the manuscript:
 - Why written consent could not be obtained
 - That the Institutional Review Board (IRB) approved use of oral consent
 - How oral consent was documented

For studies involving humans categorized by race/ethnicity, age, disease/disabilities, religion, sex/gender, sexual orientation, or other socially constructed groupings, authors should:

- Explicitly describe their methods of categorizing human populations
- Define categories in as much detail as the study protocol allows
- Justify their choices of definitions and categories, including for example whether any rules of human categorization were required by their funding agency
- Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: “Caucasian” should be changed to “white” or “of [Western] European descent” (as appropriate); “cancer victims” should be changed to “patients with cancer.”

For papers that include identifying, or potentially identifying, information, authors must download the Consent Form for Publication in a PLOS Journal (PDF), which the individual, parent, or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. The signed consent form should not be submitted with the manuscript, but authors should securely file it in the individual's case notes and the methods section of the manuscript should explicitly state that consent authorization for publication is on file, using wording like:

The individual in this manuscript has given written informed consent (as outlined in PLOS consent form) to publish these case details.

For more information about *PLOS ONE* policies regarding human subjects research, see the Publication Criteria and Editorial Policies.

Clinical trials

Clinical trials are subject to all policies regarding human research. *PLOS ONE* follows the World Health Organization's (WHO) definition of a clinical trial:

A clinical trial is any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes [...] Interventions include but are not restricted to drugs, cells and other biological products, surgical procedures, radiologic procedures, devices, behavioural treatments, process-of-care changes, preventive care, etc.

All clinical trials must be registered in one of the publicly-accessible registries approved by the WHO or ICMJE (International Committee of Medical Journal Editors). Authors must provide the trial registration number. Prior disclosure of results on a clinical trial registry site will not affect consideration for publication. We reserve the right to inform authors' institutions or ethics committees, and to reject the manuscript, if we become aware of unregistered trials.

PLOS ONE supports prospective trial registration (i.e. before participant recruitment has begun) as recommended by the ICMJE's clinical trial registration policy. **Where trials were not publicly registered before participant recruitment began**, authors must:

- › Register all related clinical trials and confirm they have done so in the Methods section
- › Explain in the Methods the reason for failing to register before participant recruitment

Clinical trials must be reported according to the relevant reporting guidelines, i.e. CONSORT for randomized controlled trials, TREND for non-randomized trials, and other specialized guidelines as appropriate. The intervention should be described according to the requirements of the TIDieR checklist and guide. Submissions must also include the study protocol as supporting information, which will be published with the manuscript if accepted.

Authors of manuscripts describing the results of clinical trials must adhere to the CONSORT reporting guidelines appropriate to their trial design, available on the CONSORT Statement web site. Before the paper can enter peer review, authors must:

- › Provide the registry name and number in the methods section of the manuscript
- › Provide a copy of the trial protocol as approved by the ethics committee and a completed CONSORT checklist as supporting information (which will be published alongside the paper, if accepted). This should be named S1 CONSORT Checklist.
- › Include the CONSORT flow diagram as the manuscript's "Fig 1"

Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and we reserve the right to ask for a copy of the patient consent form.

The methods section must include the name of the registry, the registry number, and the URL of your trial in the registry database for each location in which the trial is registered.

Animal research

We work in consultation with the *PLOS ONE* Animal Research Advisory Group to develop policies. Animal Research Advisory Group members may also be consulted on individual submissions.

All research involving vertebrates or cephalopods must have approval from the authors' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee(s), and must have been conducted according to applicable national and international guidelines. Approval must be received prior to beginning research.

If we note differences between an IACUC-approved protocol and the methods reported in a submitted manuscript, we may report these discrepancies to the relevant institution or committee.

Methods sections of manuscripts reporting results of animal research must include required ethics statements that specify:

- › The full name of the relevant ethics committee that approved the work, and the associated permit number(s). Where ethical approval is not required, the manuscript should include a clear statement of this and the reason why.
- › Relevant details for efforts taken to ameliorate animal suffering

Example ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Permit Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

The organism(s) studied should always be stated in the abstract. Where research may be confused as pertaining to clinical research, the animal model should also be stated in the title.

Where unregulated animals are used or ethics approval is not required, authors should make this clear in submitted articles and explain why ethical approval was not required. Relevant regulations that grant exemptions should be cited in full. It is the authors' responsibility to understand and comply with all relevant regulations.

We reserve the right to reject work that the editors believe has not been conducted to a high ethical standard, even if authors have obtained formal approval or approval is not required under local regulations.

We encourage authors to follow the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines for all submissions describing laboratory-based animal research and to upload a completed ARRIVE Guidelines Checklist to be published as supporting information. Please note that

inclusion of a completed ARRIVE Checklist may be a formal requirement for publication at a later date.

Non-human primates

Manuscripts describing research involving non-human primates must include details of animal welfare, including information about housing, feeding, and environmental enrichment, and steps taken to minimize suffering, including use of anesthesia and method of sacrifice if appropriate, in accordance with the recommendations of the Weatherall report, *The use of non-human primates in research* (PDF).

Humane endpoints

Manuscripts describing studies that use death as an endpoint will be subject to additional ethical considerations, and may be rejected if they lack appropriate justification for the study or consideration of humane endpoints.

Observational and field studies

Methods sections for submissions reporting on any type of field study must include ethics statements that specify:

- › Permits and approvals obtained for the work, including the full name of the authority that approved the study; if none were required, authors should explain why
- › Whether the land accessed is privately owned or protected
- › Whether any protected species were sampled
- › Full details of animal husbandry, experimentation, and care/welfare, where relevant

Paleontology and archaeology research

Manuscripts reporting paleontology and archaeology research must include descriptions of methods and specimens in sufficient detail to allow the work to be reproduced. Data sets supporting statistical and phylogenetic analyses should be provided, preferably in a format that allows easy re-use.

Specimen numbers and complete repository information, including museum name and geographic location, are required for publication. Locality information should be provided in the manuscript as legally allowable, or a statement should be included giving details of the availability of such information to qualified researchers.

If permits were required for any aspect of the work, details should be given of all permits that were obtained, including the full name of the issuing authority. This should be accompanied by the following statement:

All necessary permits were obtained for the described study, which complied with all relevant regulations.

If no permits were required, please include the following statement:

No permits were required for the described study, which complied with all relevant regulations.

Manuscripts describing paleontology and archaeology research are subject to the following policies:

- › **Sharing of data and materials.** Any specimen that is erected as a new species, described, or figured must be deposited in an accessible, permanent repository (i.e., public museum or similar institution). If study conclusions depend on specimens that do not fit these criteria, the article will be rejected under *PLOS ONE*'s data availability criterion.
- › **Ethics.** *PLOS ONE* will not publish research on specimens that were obtained without necessary permission or were illegally exported

Systematic reviews and meta-analyses

A systematic review paper, as defined by The Cochrane Collaboration, is a review of a clearly formulated question that uses explicit, systematic methods to identify, select, and critically appraise relevant research, and to collect and analyze data from the studies that are included in the review. These reviews differ substantially from narrative-based reviews or synthesis articles. Statistical methods (meta-analysis) may or may not be used to analyze and summarize the results of the included studies.

Reports of systematic reviews and meta-analyses must include a completed PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist and flow diagram to accompany the main text. Blank templates are available here:

- › Checklist: PDF or Word document
- › Flow diagram: PDF or Word document

Authors must also state in their “Methods” section whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as supporting information and provide the registry number in the abstract.

If your article is a systematic review or a meta-analysis you should:

- › State this in your cover letter
- › Select “Research Article” as your article type when submitting
- › Include the PRISMA flow diagram as Fig 1 (required where applicable)
- › Include the PRISMA checklist as supporting information

Meta-analysis of genetic association studies

Manuscripts reporting a meta-analysis of genetic association studies must report results of value to the field and should be reported according to the guidelines presented in *Systematic Reviews of Genetic Association Studies* by Sagoo *et al.*

On submission, authors will be asked to justify the rationale for the meta-analysis and how it contributes to the base of scientific knowledge in the light of previously published results. Authors will also be asked to complete a checklist (DOCX) outlining information about the justification for the study and the methodology employed. Meta-analyses that replicate published studies will be rejected if the authors do not provide adequate justification.

Personal data from third-party sources

For all studies using personal data from internet-based and other third-party sources (e.g., social media, blogs, other internet sources, mobile phone companies), data must be collected and used according to company/website Terms and Conditions, with appropriate permissions. All data sources must be acknowledged clearly in the Materials and Methods section.

 Read our policy on data availability.

In the Ethics Statement, authors should declare any potential risks to individuals or individual privacy, or affirm that in their assessment, the study posed no such risks. In addition, the following Ethics and Data Protection requirements must be met.

For interventional studies, which impact participants’ experiences or data, the study design must have been prospectively approved by an Ethics Committee, and informed consent is required. The Ethics Committee may waive the requirement for approval and/or consent.

For observational studies in which personal experiences and accounts are not manipulated, consultation with an Ethics or Data Protection Committee is recommended. Additional requirements apply in the following circumstances:

- › If information used could threaten personal privacy or damage the reputation of individuals whose data are used, an Ethics Committee should be consulted and informed consent obtained or specifically addressed.
- › If authors accessed any personal identifying information, an Ethics or Data Protection Committee should oversee data anonymization. If data were anonymized and/or aggregated before access and analysis, informed consent is generally not required.

Note that Terms of Use contracts do not qualify as informed consent, even if they address the use of personal data for research.

 See our reporting guidelines for human subjects research.

Cell lines

Authors reporting research using cell lines should state when and where they obtained the cells, giving the date and the name of the researcher, cell line repository, or commercial source (company) who provided the cells, as appropriate.

Authors must also include the following information for each cell line:

For de novo (new) cell lines, including those given to the researchers a gift, authors must follow our policies for human subjects research or animal research, as appropriate. The ethics statement must include:

- › Details of institutional review board or ethics committee approval; AND
- › For human cells, confirmation of written informed consent from the donor, guardian, or next of kin

For established cell lines, the Methods section should include:

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The Effects of Residual Platelets in Plasma on Plasminogen Activator Inhibitor-1 and Plasminogen Activator Inhibitor-1-related Assays

Short title: Residual Platelets Influence Plasminogen Activator Inhibitor-1 Assays

Key words: PAI-1, Clot lysis time, Fibrinolysis, Platelets, β TG

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1 **Word count:**

2 Abstract: 297 Text: 3150

3

4 **Abbreviations:**

5 β TG, beta thromboglobulin; CLT, clot lysis time; CTAD, citrate-theophylline, adenosine,
6 dipyridamol; ELISA, enzyme-linked immunosorbent assay; HREC, Health Research Ethics
7 Committee; MPV, mean platelet volume; NWU, North-West University; PAI-1; plasminogen
8 activator inhibitor-1; PAI-1_{act}, PAI-1 activity; PAI-1_{ag}, PAI-1 antigen; PPP, platelet-poor
9 plasma; PRP, platelet-rich plasma; tPA/PAI-1 complex, tissue plasminogen activator/PAI-1
10 complex; SABPA, Sympathetic activity and Ambulatory Blood Pressure in Africans.

11

3.3.1 Abstract

Due to controversial evidence in the literature pertaining to the activity of plasminogen activator inhibitor-1 in platelets, we examined the effects of residual platelets present in plasma (a potential pre-analytical variable) on various plasminogen activator inhibitor-1 and plasminogen activator inhibitor-1-related assays. Blood samples were collected from 151 individuals and centrifuged at 352 and 1500 g to obtain plasma with varying numbers of platelet. In a follow-up study, blood samples were collected from an additional 23 individuals, from whom platelet-poor (2000 g), platelet-containing (352 g) and platelet-rich plasma (200 g) were prepared and analysed as fresh-frozen and after five defrost-refreeze cycles (to determine the contribution of *in vitro* platelet degradation). Plasminogen activator inhibitor-1 activity, plasminogen activator inhibitor-1 antigen, tissue plasminogen activator/plasminogen activator inhibitor-1 complex, plasma clot lysis time, β -thromboglobulin and plasma platelet count were analysed. Platelet α -granule release (plasma β -thromboglobulin) showed a significant association with plasminogen activator inhibitor-1 antigen levels but weak associations with plasminogen activator inhibitor-1 activity and a functional marker of fibrinolysis, clot lysis time. Upon dividing the study population into quartiles based on β -thromboglobulin levels, plasminogen activator inhibitor-1 antigen increased significantly across the quartiles while plasminogen activator inhibitor-1 activity and clot lysis time tended to increase in the 4th quartile only. In the follow-up study, plasma plasminogen activator inhibitor-1 antigen was also significantly influenced by platelet count in a concentration-dependent manner. Plasma plasminogen activator inhibitor-1 antigen levels increased further after complete platelet degradation. Residual platelets in plasma significantly influence plasma plasminogen activator inhibitor-1 antigen levels mainly through release of latent plasminogen activator inhibitor-1 with limited effects on plasminogen activator inhibitor-1 activity, tissue plasminogen activator/plasminogen activator inhibitor-1 complex or plasma clot lysis time. Platelets may however also have functional effects on plasma fibrinolytic potential in the presence of high platelet counts, such as in platelet-rich plasma.

40 **Keywords**

41 Plasminogen activator inhibitor-1, Clot lysis time, Fibrinolysis, Platelets, β -thromboglobulin

42

3.3.2 Introduction

Plasminogen activator inhibitor type-1 (PAI-1) is a serine protease inhibitor (serpin) (1, 2), which acts as a main inhibitor of fibrinolysis (3). Elevated plasma PAI-1 levels have been associated with a risk for developing atherothrombosis (4-6) due to its antifibrinolytic properties, by reducing the clearance of fibrin in plaques (5), and also *via* its influence on cellular migration, matrix remodelling and activation of growth factors (7, 8). Plasma PAI-1 exists either in an active or latent form, or in complex with tissue plasminogen activator (tPA) (9-11). The active form of PAI-1 is unstable, with a half-life of approximately two to three hours, after which it will spontaneously convert to the inactive, latent form (9, 12). PAI-1 is produced by various cells such as endothelial cells, hepatocytes, smooth muscle cells, adipocytes, and platelets (11, 13). In platelets, PAI-1 is stored in the alpha granules and is released during platelet activation and aggregation (11, 14, 15).

Recently, there has been a debate about which form of PAI-1, or at least the relative proportion of each form, is released from the platelet alpha granules. It was traditionally believed that platelets store and release mainly latent PAI-1, since, only approximately 5-10% of PAI-1 antigen (PAI-1_{ag}) was shown to be active in lysed platelet-rich plasma (16). More recent studies however, suggest that platelets release a substantial amount of active PAI-1 (17-19). This is due to the observed *de novo* synthesis of PAI-1 within platelets, which was indicated to remain active for over 24 hours (17). Possible explanations for the contradictory evidence pertaining to platelet PAI-1 activity, could be due to the different approaches used in these experiments for preparing the platelet lysates (sonification and freezing and or thawing of the samples), which have been reported to influence the detection of PAI-1 (18). Furthermore, the conversion of active PAI-1 to its latent state can be influenced by low temperatures, low pH and high salt concentrations (20). It is however not clear as to how the PAI-1, released from the alpha granules of residual platelets in plasma, affects PAI-1 assays and PAI-1-related assays.

The overall aim of the study was therefore to investigate the effect of residual platelets in plasma, on various PAI-1 and PAI-1-related assays: PAI-1 activity (PAI-1_{act}), PAI-1 antigen (PAI-1_{ag}), and tPA/PAI-1 complex, as well as plasma fibrinolytic potential (a functional parameter of fibrinolysis, measured as plasma clot lysis time (CLT)). The study consisted of two sub-studies. In the first, varying centrifugation speeds (352 and 1500 g) were used to prepare platelet-containing plasma, from 151 participants in the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study. The purpose of this sub-study was to determine the effect of residual platelets in plasma on various PAI-1 assay results, by relating these assays to a marker of platelet alpha granule release (beta thromboglobulin (β TG)). In this sub-study, absolute platelet counts were not measured, and additionally it was not possible to calculate to what degree plasma PAI-1 levels were influenced by *in vitro* platelet activation and or degradation. Additionally, Merolla *et al.* (21) found that different centrifugation speeds may result in different platelet populations, which could also have had an effect on our results. The purpose of the second study was, therefore, to determine the influence of actual platelet count on PAI-1_{ag}, as the antigen assay was the assay found to be significantly influenced by plasma platelet content in the first sub-study. In the follow-up study, plasma was collected from 23 additional participants, and platelet count and size, in addition to β TG and PAI-1_{ag} concentrations, were determined from three different plasma preparations: platelet-poor plasma (PPP – 2000 g), platelet-containing plasma (352 g, in keeping with the first sub-study protocol) and platelet-rich plasma (PRP – 200 g). PPP was collected in citrate tubes, containing platelet stabilisers, in order to provide basal plasma PAI-1_{ag} levels without any of the influencing effects of *in vitro* platelet activation and/or degradation. Furthermore, the 352 g and 200 g citrated plasma samples were analysed not only as fresh-frozen, but also after five defrost-refreeze cycles, ensuring complete alpha granule release from the platelets, in order to determine the total platelet PAI-1_{ag} and β TG content.

3.3.3 Materials and Methods

Study population and ethics – SABPA study

The Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study, was a cross-sectional study including 409 (202 men and 207 women) school teachers between the ages of 25 – 60 years, from the North West Province, South Africa. Of these participants 151 individuals were randomly selected for inclusion in the present study. All samples were analysed at the same time. Exclusion criteria were: elevated ear temperature, dependence or abuse of psychotropic substances, regular blood donors, and individuals vaccinated within the previous three months. The study complied with all applicable international regulations and the Helsinki declaration for investigation of human participants. The study was approved by the Health Research Ethics Committee (HREC) of the North-West University (NWU), Potchefstroom Campus (NWU-00016-10-A1).

Study population and ethics – follow-up study

Twenty three individuals from the same socio-demographic profile as the SABPA study participants were recruited by means of a purposive sampling method from the Potchefstroom Campus of the NWU. The same inclusion and exclusion criteria as well as ethical principles were adhered to. The study was approved by the HREC of the NWU, Potchefstroom Campus (NWU-00016-10-A1). All samples were collected and analysed at the same time.

Blood collection – SABPA study

Fasting blood samples with minimum stasis were collected from the antebrachial vein before 10:00 am. 3.2% Citrate samples were used for the analysis of PAI-1 (activity, antigen and

tPA/PAI-1 complex), β TG and CLT. Samples were randomly divided into two groups. One half of the study population samples were centrifuged at 352 *g* and the other half at 1500 *g* for 15 minutes at 20 °C to yield plasma containing a varying number of platelets. Aliquots were snap frozen on dry ice and stored at -82 °C until analysis.

Blood collection – follow-up study

Fasting blood samples with minimum stasis were collected from the antebrachial vein before 10:00 am. Blood was collected into two 3.2% citrated tubes and one CTAD tube (a citrate tube containing platelet stabilisers; theophylline, adenosine and dipyridamol). CTAD plasma was prepared by centrifuging the samples at 2000 *g* for 30 minutes at 20 °C, to yield PPP with platelets protected from *in vitro* activation or degradation. Two types of citrate plasma were prepared by centrifuging one of the citrated tubes at 352 x *g* for 15 minutes at 20 °C, to yield platelet-containing plasma, and the other tube at 200 x *g* for 10 minutes at 20 °C, to yield PRP. These conditions were comparable to that of the SABPA study and also served the purpose to provide information on standard plasma type preparations – PPP and PRP. All samples were centrifuged within 20 min of collection.

Platelet count and size analyses were performed in fresh whole blood samples collected both in citrate and CTAD tubes, as well as in the different plasma preparations described above. The remaining plasma was then aliquoted, snap frozen on dry ice and stored at -82°C. The CTAD plasma samples and half of the aliquots of the two citrate plasma preparations, of each individual, were thawed once only, by placing these in a 37 °C water bath for 10 minutes, immediately prior to PAI-1_{ag} and β TG analyses. The second half of the citrated plasma sample aliquots underwent five freeze-thaw cycles (x 5), once daily, prior to

analysis, to ensure maximum platelet α -granule release. Fig. 3.1 provides a schematic depiction of the study design.

Biochemical analysis

PAI-1_{act} was measured using an indirect enzymatic method (Technozym PAI-1 Actibind, Technoclone, Vienna, Austria), and PAI-1_{ag}, using a two-site enzyme-linked immunosorbent assay (ELISA) (TriniLIZE PAI-1_{ag}, TCoag, Bray Ireland). tPA/PAI-1 complex was analysed using a solid phase enzyme immunoassay, specific to PAI-1 complexed to tPA (Technoclone, Vienna, Austria). An antigenic assay was used to measure β TG levels by means of an ELISA (Asserachrom® β TG Diagnostica Stago, Asnières sur Seine, France). CLT was determined by studying the lysis of a tissue factor-induced plasma clot by exogenous tPA. Changes in turbidity during clot formation and lysis were monitored as described by Lisman *et al.* [24]. Tissue factor and tPA concentrations were slightly modified to obtain comparable CLTs of approximately 60 minutes. The modified concentrations were 17 mmol/L CaCl₂, 60 ng/ml tPA (Actilyse, Boehringer Ingelheim, Ingelheim, Germany) and 10 μ mol/L phospholipids vesicles (Rossix, Mölndal, Sweden). Tissue factor was diluted 3000 times (Dade Innovin, Siemens Healthcare Diagnostics Inc., Marburg, Germany). CLT was defined as the time from the midpoint, from clear to maximum turbidity (representative of the clot formation), to the midpoint in the transition from maximum turbidity to the final baseline turbidity (representative of the lysis of the clot) [24]. Platelet count and mean platelet volume were determined with a Coulter AcT 5-part differential (5 diff) autoloader haematology analyser (Beckman Coulter, Fullerton, CA, USA).

Statistical analysis

The data was analysed with the computer software package Statistica (Statsoft Inc., Tulsa Oklahoma, USA). A p-value of 0.05 or less was regarded as statistically significant. Descriptive data is presented as median (25th; 75th percentiles) as most of the variables were not normally distributed. Kruskal-Wallis analysis of variance (ANOVA) with multiple comparisons of mean post-hoc tests were used to compare differences in the PAI-1 and CLT assays, between population sub-groups divided into quartiles of β TG levels. Correlations between variables were determined both with Spearman Rank order and Pearson (for log transformed data) correlation tests. Only the Spearman data is reported, as both correlation tests provided similar results. Significant differences between correlation coefficients obtained were also calculated. For the follow-up study, Wilcoxon-Matched pairs tests were used to determine significant differences between plasma prepared at 200 and 352 g and also between fresh-frozen and 5 times defrosted-refrozen samples.

3.3.4 Results

SABPA study

The study population included 151 participants, with a mean age of 45.7 ($\pm$ 8.75) years and a BMI of 26.9 ($\pm$ 2.29). When comparing the samples prepared at the two different centrifugation speeds, the 352 g group had significantly higher β TG (3263 vs 355 IU/mL; $p < 0.0001$) and PAI-1_{ag} (33.8 vs 20.8 ng/mL; $p < 0.0001$) levels, compared to the 1500 g group, with borderline significantly higher PAI-1_{act} (2.95 vs 1.91 U/mL; $p = 0.03$) and longer CLT (78.2 vs 74.4 min; $p = 0.04$). No difference in tPA/PAI-1 complex ($p=0.09$) was observed (Table 3.1).

When dividing the study population into quartiles according to plasma β TG levels (Table 3.2), PAI-1_{ag} increased consistently across the β TG quartiles. PAI-1_{act} showed a significant

increase in the highest β TG quartile only, with CLT tending to be longer, without reaching significance. No difference was observed in tPA/PAI-1 complex across the β TG quartiles. β TG was furthermore correlated with PAI-1_{ag} ($r=0.66$; $p<0.0001$), demonstrating statistically weaker correlations with PAI-1_{act} ($r=0.22$; $p=0.008$); tPA/PAI-1 complex ($r=0.12$; $p=0.13$) and CLT ($r=0.20$; $p=0.02$) (Table 3.3). Although CLT correlated with all the PAI-1 assays, it showed the strongest correlation with PAI-1_{act} ($r=0.74$; $p<0.0001$).

Follow-up Study

Table 3.4 presents the descriptive statistics of the follow-up study group. A total of 23 participants were included. Platelet counts in the citrated and CTAD whole blood were similar. The platelet count of the CTAD samples centrifuged at 2000 g was 1.00 (1.00-2.00) $\times 10^3/\mu\text{L}$, confirming that it was indeed platelet poor ($<10 \times 10^3/\mu\text{L}$). The platelet counts of the 352 g and 200 g plasma were 323 (257 – 440) $\times 10^3/\mu\text{L}$ and 523 (389 – 674) $\times 10^3/\mu\text{L}$ respectively. The 352 g plasma had a significantly lower mean platelet volume (7.00 [6.65 – 7.60] fL) than the 200 g plasma (7.80 [7.00 – 8.30] fL), which in turn had a similar mean platelet volume than that of the whole blood. β TG levels increased 60 fold and 150 fold in the 352 g and 200 g plasma respectively, compared to the PPP, while PAI-1_{ag} levels increased 15 and 22 fold respectively. In both the 352 g and 200 g plasma, the β TG levels of the samples that underwent 5 freeze-thaw cycles, prior to analyses, were significantly lower than that of the samples that were defrosted once only, prior to analysis, possibly due to instability of β TG (5 % and 3 % respectively), while PAI-1_{ag} levels showed significant increases of 26% and 27% respectively.

When combining the different types of plasma, β TG had a highly significant correlation with platelet count ($r=0.91$, $p<0.0001$). When investigating the different types of plasma

separately, β TG levels correlated well with platelet count in the 352 g ($r=0.60$, $p=0.002$) and 200 g ($r=0.70$, $p=0.0002$) plasma samples, with a smaller and non-significant correlation ($r=0.40$, $p=0.06$) in the PPP (Table 3.5). For all the types of plasma combined, PAI-1_{ag} and platelet count were highly correlated ($r=0.91$, $p<0.0001$). Compared with β TG, PAI-1_{ag} showed even stronger statistical correlations with platelet count in the 352 g ($r=0.85$, $p<0.0001$) and 200 g ($r=0.81$, $p<0.0001$) plasma, also with a non-significant relationship in the PPP. None of the plasma preparations' β TG levels correlated with whole blood platelet count, while the correlation of PAI-1_{ag} of the 200 g plasma with whole blood platelet count, reached borderline significance ($r=0.4$, $p=0.06$). Furthermore, β TG and PAI-1_{ag} correlated significantly when combining the different types of plasma ($r=0.86$, $p<0.0001$), however, correlated negatively in PPP ($r=-0.61$, $p=0.002$), with significant positive correlations in the 352 g ($r=0.55$, $p=0.006$) and 200 g ($r=0.74$, $p<0.0001$) plasma separately (Table 3.6).

3.3.5 Discussion

In this study we investigated the effect of residual platelets present in plasma, on plasma PAI-1 and PAI-1-related assay results. Our data suggests that the presence of platelets in plasma significantly influences plasma PAI-1_{ag} levels in a concentration dependent manner, likely due to an increase in mainly plasma latent PAI-1. Only in the presence of large amounts of platelets such as in PRP, functional effects in terms of plasma fibrinolytic potential are seen, suggesting the presence of a comparatively lower concentration of active PAI-1 in platelets. It was furthermore demonstrated that platelets present in plasma, do not initially release all of their PAI-1 content and that further release of PAI-1 can occur upon further / complete *in vitro* platelet degradation.

SABPA study

The SABPA study data indicated that β TG levels had a significantly larger effect on PAI-1_{ag} levels than any of the other PAI-1 variables or CLT. When dividing the study population into β TG quartiles, PAI-1_{act} and CLT increased in the highest β TG quartile only, suggesting that there may be a small amount of active PAI-1 present in platelets. In agreement with this, Serizawa *et al.* [25] found longer CLT in PRP than PPP which was ascribed to the presence of active PAI-1 in platelets. Since PAI-1_{ag} is composed of latent PAI-1, active PAI-1 and PAI-1 in complex with tPA, and considering that: 1) only minimal differences were observed in PAI-1_{act}, tPA/PAI-1 complex and CLT across the β TG quartiles, and 2) β TG showed much weaker correlations with PAI-1_{act}, tPA/PAI-1 complex and CLT than with PAI-1_{ag}, the data suggests that platelet alpha granule release largely contributes to increased plasma PAI-1_{ag}, by increasing latent PAI-1. It was unfortunately not possible to measure plasma latent PAI-1 levels directly, as no such commercial assay is currently available. Latent PAI-1 is unable to inhibit tPA; therefore, the presence of latent PAI-1 in plasma may lead to a falsely assumed increased fibrinolytic inhibitory capacity. The lack of increase in CLT across the β TG quartiles, (apart from the highest quartile) confirms this. These results are also in agreement with a study by Juhan-Vague *et al.* [26] who found PAI-1, released from platelets, *in vitro*, to be mainly in the inactive form. Combined, this data suggests that platelets likely contain both latent and active PAI-1, but that a high plasma platelet content (such as in PRP) is required before the active PAI-1 present in platelets has functional effects on plasma fibrinolytic potential.

Follow-up Study

Data from the follow-up study, clearly demonstrated the significant effects of platelets present in plasma, on plasma PAI-1_{ag} levels. Platelet count and β TG and PAI-1_{ag} levels were highly correlated, in the different plasma preparations containing platelets (352 g and

200 g), with no significant associations in the PPP. PAI-1_{ag} levels in PRP already tended to correlate with whole blood platelet count. PAI-1_{ag} levels, were furthermore, up to 22 fold higher in PRP when compared to basal levels in PPP (which was exempted from the possible influence of residual platelet content or *in vitro* platelet α -granule release), highlighting the magnitude of the effect of platelets on plasma PAI-1_{ag} levels, compared to other sources of PAI-1 (endothelial cells, hepatocytes, smooth muscle cells and adipocytes) [13,15]. The additional 1.3 fold increase in plasma PAI-1_{ag}, subsequent to maximal degradation (5 x freeze-thaw cycles), suggests that *in vitro* platelet degradation can contribute to a further increase in plasma PAI-1_{ag} levels, confirming the necessity of the use of the correct plasma preparation protocols to standardise platelet count and to ensure the preparation of platelet-poor plasma ($<10 \times 10^3/\mu\text{L}$). Differences in platelet size were also detected when comparing the 352 g and the 200 g plasma, indicating the presence of different platelet populations in the samples centrifuged at different speeds. Since platelets with larger sizes are known to be more metabolically active than smaller platelets [27], platelet size, in addition to platelet count, most likely influence the relationship between platelets present in plasma and PAI-1_{ag} levels.

Although βTG is extensively used as a marker of platelet alpha granule release, it does have limitations. The sensitivity of βTG as a marker of platelet activation and alpha granule release can be influenced by various factors; including the choice of anticoagulant, and sample handling and preparation procedures, [28,29]. Furthermore, βTG shows diurnal variation and is additionally influenced by age, renal function and the use of various medications, such as beta blockers for instance [28,29]. In order to minimise the possible influence of these confounders, in this study all samples were collected in an identical manner, before 10:00 am and stored at identical temperatures. None of the participants were using beta blockers or had impaired renal function. The significant correlation between platelet count and βTG ($r=0.91$, $p<0.0001$) furthermore supports its use as a proxy marker

for the number of platelets in plasma in our study populations. While PAI-1 activity may be influenced by freeze-thaw cycles, we opted to work with frozen samples as this type of sample is most often used in studies and therefore relevant to a larger audience. Furthermore all plasma preparations were treated similarly making comparison between the different preparations possible. Although samples were not specifically treated to prevent possible *in vitro* conversion of active to latent PAI-1, samples were processed within 20 minutes after collection and snap frozen to limit *in vitro* conversion.

The results from the present study indicate that the content of the alpha granules released from platelets in plasma, significantly influences plasma PAI-1_{ag} levels, with limited effects on PAI-1_{act}, tPA/PAI-1 complex or fibrinolysis rate (measured as CLT). This effect on PAI-1_{ag} is thought to be largely due to an increased release of latent PAI-1 from platelets which is unable to bind tPA and inhibit fibrinolysis. Due to the potential contribution of latent PAI-1 to PAI-1_{ag} levels, PAI-1_{act} may be the more clinically useful assay to determine the fibrinolytic inhibitor capacity of plasma. In plasma with a high platelet count, such as PRP, the component of platelet PAI-1 that is active, may, however have functional effects by decreasing plasma fibrinolytic potential. These results suggest that PAI-1_{ag} is more sensitive to the presence of platelets in plasma, than other PAI-1 assays (PAI-1_{act} and tPA/PAI-1 complex) or CLT but that these assays may also be influenced by platelets when present in high numbers such as in PRP.

3.3.6 Acknowledgements

We would like to thank the SAPBA research team, the field-workers of the North-West University, South Africa, and the SAPBA project leader, Prof Leone Malan

320 **3.3.7 Funding statement**

321 This work was supported by grants from the National Research Foundation (NRF) of South
322 Africa (Grant UID: 94213) (<http://www.nrf.ac.za>) and the South African Medical Research
323 Council (<http://www.mrc.ac.za>). Opinions expressed and conclusions arrived at, are those of
324 the authors and are not to be attributed to the NRF.

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401

Table footnotes and legends to figures

Table 3.1. Comparison of β TG, PAI-1 assays and CLT according to centrifugation speed in SABPA study.

β TG - beta thromboglobulin; CLT - clot lysis time; PAI-1 – plasminogen activator inhibitor-1
PAI-1_{act} – PAI-1 activity; PAI-1_{ag} – PAI-1 antigen; tPA/PAI complex - tissue plasminogen
activator/PAI-1 complex.

Table 3.2. PAI-1_{act}, PAI-1_{ag}, tPA/PAI-1 complex and CLT according to β TG in SABPA study group.

ANOVA, analysis of co-variance; β TG, beta thromboglobulin; CLT, clot lysis time; PAI-1,
plasminogen activator inhibitor-1; PAI-1_{act}, PAI-1 activity; PAI-1_{ag}, PAI-1 antigen; tPA/PAI-
complex, tissue plasminogen activator/PAI-1 complex; * # Means with the same symbol
differ significantly.

Table 3.3. Spearman rank order correlations between β TG, PAI-1 assays and CLT in SABPA study group.

β TG, beta thromboglobulin; CLT, clot lysis time; PAI-1, plasminogen activator inhibitor-1;
PAI-1_{act}, PAI-1 activity; PAI-1_{ag}, PAI-1 antigen; tPA/PAI-complex, tissue plasminogen
activator/PAI-1 complex. * Significantly weaker correlation with β TG than the correlation of
PAI-1_{ag} with β TG.

424 **Table 3.4. Descriptive statistics of follow-up group.**

425 BMI, body mass index; β TG, beta thromboglobulin; CTAD, citrate-theophylline, adenosine,
426 dipyridamol; DBP, diastolic blood pressure; g , gravitational acceleration; PAI-1,
427 plasminogen activator inhibitor-1; PAI-1_{ag}, PAI-1 antigen; SBP, systolic blood pressure;
428 MPV, mean platelet volume; #^\$&5%@** Median with the same symbol differ significantly.

429

430 **Table 3.5. Spearman rank order correlations of β TG and PAI-1_{ag} with whole**
431 **blood, CTAD and citrate plasma platelet count of the follow-up study.**

432 β TG, beta thromboglobulin; CTAD, citrate-theophylline, adenosine, dipyridamol; g ,
433 gravitational acceleration; PAI-1, plasminogen activator inhibitor-1; PAI-1_{ag}, PAI-1 antigen.

434

435 **Table 3.6. Spearman rank order correlations between β TG and PAI-1_{ag} in the**
436 **respective plasma preparations of the follow-up study.**

437 β TG, beta thromboglobulin; CTAD, citrate-theophylline, adenosine, dipyridamol; g ,
438 gravitational acceleration; PAI-1, plasminogen activator inhibitor-1; PAI-1_{ag}, PAI-1 antigen.

439

440 **Fig. 3.1. Design of follow-up study**

Table 3.1 Comparison of β TG, PAI-1 assays and CLT according to centrifugation speed in SABPA study.

Variable	352 g (n=75)	1500 g (n=75)	p-value (Mann-Whitney)
	Median (25 th ; 75 th percentiles)	Median (25 th ; 75 th percentiles)	
β TG (IU/mL)	3263 (2009; 4394)	355 (218; 584)	<0.0001
PAI-1 _{ag} (ng/mL)	33.8 (28.4; 42.4)	20.8 (16.7; 25.8)	<0.0001
PAI-1 _{act} (U/mL)	2.95 (0.69; 8.72)	1.91 (0.25; 4.68)	0.03
tPA/PAI complex (ng/mL)	8.78 (6.59; 11.7)	7.90 (6.01; 10.2)	0.09
CLT (min)	78.2 (69.7; 86.4)	74.4 (69.7; 79.8)	0.04

β TG - beta thromboglobulin; CLT - clot lysis time; PAI-1 – plasminogen activator inhibitor-1
PAI-1_{act} – PAI-1 activity; PAI-1_{ag} – PAI-1 antigen; tPA/PAI complex - tissue plasminogen activator/PAI-1 complex.

Table 3.2: PAI-1_{act}, PAI-1_{ag}, tPA/PAI-1 complex and CLT according to β TG quartiles in SABPA study group.

SAPBA study group									
		β TG 1 st Quartile		β TG 2 nd Quartile		β TG 3 rd Quartile		β TG 4 th Quartile	
		$(\leq 341 \text{ IU/mL})$		$(341 \text{ IU/mL} - 817 \text{ IU/mL})$		$(817 \text{ IU/mL} - 3263 \text{ IU/mL})$		$(>3263 \text{ IU/mL})$	
		Median		Median		Median		Median	
Variable	n	(25; 75% percentile)		n	(25; 75% percentile)		n	(25; 75% percentile)	
PAI-1 _{ag} (ng/L)	37	20.4 (16.0; 25.8) *		38	21.5 (17.0; 26.7) *		37	29.6 (25.1; 39.7) #	
PAI-1 _{act} (U/mL)	34	2.56 (0.31; 4.89)		37	1.89 (0.20; 3.77) *		37	1.37 (0.41; 6.70)	
tPA/PAI-1 complex (ng/mL)	35	8.00 (6.36; 10.2)		37	7.55 (5.26; 10.1)		37	8.65 (6.28; 11.7)	
CLT (min)	34	75.4 (69.7; 79.5)		37	73.9 (69.7; 78.6)		36	76.6 (67.7; 84.1)	
							38	9.06 (7.32; 11.3)	
							35	81.5 (71.6; 96.0)	
									p-value

ANOVA, analysis of co-variance; β TG, beta thromboglobulin; CLT, clot lysis time; PAI-1, plasminogen activator inhibitor-1; PAI-1_{act}, PAI-1 activity; PAI-1_{ag}, PAI-1 antigen; tPA/PAI-complex, tissue plasminogen activator/PAI-1 complex; *# Medians with different symbols differ significantly.

Table 3.3: Spearman rank order correlations between β TG, PAI-1 assays and CLT in SABPA study group.

Variables	β TG	PAI-1 _{ag}	PAI-1 _{act}	tPA/PAI-1 complex
	r (p-value)	r (p-value)	r (p-value)	r (p-value)
PAI-1 _{ag} (ng/mL)	0.66 (<0.0001)	-	-	-
PAI-1 _{act} (U/mL)	0.22 (0.008) *	0.43 (<0.0001)	-	-
tPA/PAI-1 complex (ng/mL)	0.12 (0.13) *	0.30 (0.0002)	0.64 (<0.0001)	-
CLT (min)	0.20 (0.02) *	0.41 (<0.0001)	0.74 (<0.0001)	0.50 (<0.0001)

β TG, beta thromboglobulin; CLT, clot lysis time; PAI-1, plasminogen activator inhibitor-1; PAI-1_{act}; PAI-1 activity; PAI-1_{ag}, PAI-1 antigen; tPA/PAI-complex, tissue plasminogen activator/PAI-1 complex. * Significantly weaker correlation with β TG than the correlation of PAI-1_{ag} with β TG.

Table 3.4: Descriptive statistics of the follow-up study group.

Variable	Study population (n=23)
	Median (25 th ; 75 th percentiles)
Gender: men / women (n)	12 / 11
Ethnicity: black / white (n)	11 / 12
Age (years)	36 (29; 42)
SBP (mm Hg)	120 (110; 130)
DBP (mm Hg)	80 (70; 80)
BMI (kg/m ²)	26.4 (22.0; 28.4)
CTAD whole blood platelet count (x10 ³ /μL)	239 (195; 248)
Citrate whole blood platelet count (x10 ³ /μL)	234 (194; 257)
CTAD plasma 2000 g platelet count (x10 ³ /μL)	1.00 (1.00; 2.00)
Citrate plasma 352 g platelet count (x10 ³ /μL)	323 (257; 440)
Citrate plasma 200 g platelet count (x10 ³ /μL)	523 (389; 674)
Whole blood CTAD MPV (fL)	7.80 (7.40; 8.40)
Whole blood Citrate MPV (fL)	7.80 (7.20; 8.30)
MPV (fL) 352 g plasma	7.00 (6.65; 7.60)%
MPV (fL) 200 g plasma	7.80 (7.00; 8.30)%
βTG (IU/mL) CTAD 2000 g plasma	120 (92; 156)
βTG (IU/mL) 352 g x 1 plasma	7269 (6218; 8902)#
βTG (IU mL) 352 g x 5 plasma	6890 (5770; 7985)#
βTG (IU/mL) 200 g x 1 plasma	17683 (14703; 19089)^
βTG (IU/mL) 200 g x 5 plasma	17182 (14322; 18393)^
PAI-1 _{ag} (ng/mL) CTAD 2000 g plasma	5.16 (3.80; 11.5)
PAI-1 _{ag} (ng/mL) 352 g x 1 plasma	76.7 (64.1; 86.0)\$
PAI-1 _{ag} (ng/mL) 352 g x 5 plasma	96.9 (74.7; 117)\$
PAI-1 _{ag} (ng/mL) 200 g x 1 plasma	114.2 (90.6; 155)&
PAI-1 _{ag} (ng/mL) 200 g x 5 plasma	145 (115; 191)&

BMI, body mass index; βTG, beta thromboglobulin; CTAD, citrate-theophylline, adenosine, dipyridamol; DBP, diastolic blood pressure; g, gravitational acceleration; PAI-1, plasminogen activator inhibitor-1; PAI-1_{ag}, PAI-1 antigen; SBP, systolic blood pressure; MPV, mean platelet volume; # ^ \$ & % @ ** Median with the same symbol differ significantly between the 1x and 5 x frozen and defrosted samples.

Table 3.5: Spearman rank order correlations of β TG and PAI-1_{ag} with whole blood, CTAD and citrate plasma platelet count of the follow-up study.

Variable	Platelet count $\times 10^3/\mu\text{l}$ (n=23)				
	CTAD whole blood	CTAD plasma (2000 g)	Citrate whole blood	Citrate plasma (352 g)	Citrate plasma (200 g)
	r (p-value)	r (p-value)	r (p-value)	r (p-value)	r (p-value)
β TG (IU/mL) CTAD 2000 g plasma	-0.14 (0.5)	0.40 (0.06)	-	-	-
β TG (IU/mL) 352 g x 1 plasma	-	-	0.12 (0.6)	0.6 (0.002)	-
β TG (IU/mL) 200 g x 1 plasma	-	-	0.20 (0.3)	-	0.70 (0.0002)
PAI-1 _{ag} (ng/mL) CTAD 2000 g plasma	0.04 (0.9)	-0.36 (0.09)	-	-	-
PAI-1 _{ag} (ng/mL) 352 g x 1 plasma	-	-	0.34 (0.1)	0.85 (<0.0001)	-
PAI-1 _{ag} (ng/mL) 200 g x 1 plasma	-	-	0.40 (0.06)	-	0.81 (<0.0001)

β TG, beta thromboglobulin; CTAD, citrate-theophylline, adenosine, dipyridamol; g, gravitational acceleration; PAI-1, plasminogen activator inhibitor-1; PAI-1_{ag}, PAI-1 antigen.

Table 3.6: Spearman rank order correlations between β TG and PAI-1_{ag} in the respective plasma preparations of the follow-up study

Variables	β TG - PAI-1 _{ag}
	r (p-value)
CTAD plasma 2000 g	-0.61 (0.002)
Citrate plasma 352 g x 1	0.55 (0.006)
Citrate plasma 200 g x 1	0.74 (<0.0001)

β TG, beta thromboglobulin; CTAD, citrate-theophylline, adenosine, dipyridamol; g, gravitational acceleration; PAI-1, plasminogen activator inhibitor-1; PAI-1_{ag}, PAI-1 antigen.

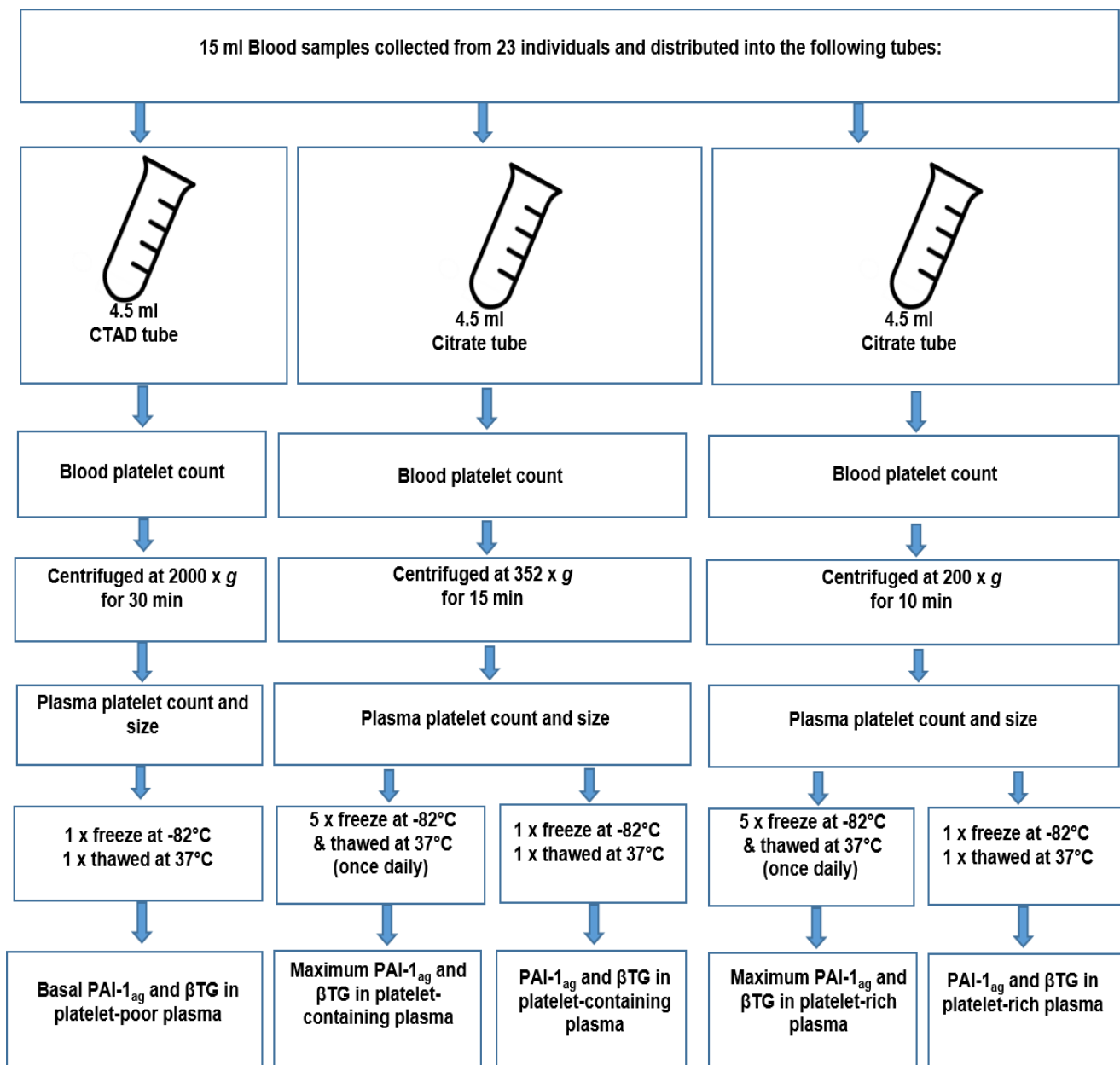


Figure 3.1: Design of follow-up study

CHAPTER 4: DEGREE OF OBESITY INFLUENCES THE RELATIONSHIP OF PAI-1 WITH BODY FAT DISTRIBUTION AND METABOLIC VARIABLES IN AFRICAN WOMEN

Authors: Sunelle A Barnard, Marlien Pieters, Cornelia Nienaber-Rousseau, Herculina S Kruger

This chapter includes:

- 4.1 Authors instructions of the journal, *Thrombosis Research* (Impact factor: 2.320);
- 4.2 proof that the article has been accepted for publication in *Thrombosis Research* and;
- 4.3 the accepted article titled: "Degree of obesity influences the relationship of PAI-1 with body fat distribution and metabolic variables in African women".

In addition, the published article is provided as Addendum E

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(<https://www.elsevier.com/journals/thrombosis-research/0049-3848?generatepdf=true>)

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6 September 2016

4.3 Accepted article submitted to *Thrombosis Research*

Degree of obesity influences the relationship of PAI-1 with body fat distribution and metabolic variables in African women

Running title: Plasminogen activator inhibitor-1 and body fat

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Word count:

Summary: 250

Text: 4622

Abbreviations:

ANCOVA, analysis of co-variance; ANOVA, analysis of variance; ASM, appendicular skeletal muscle mass; BMI, body mass index; CI, confidence interval; CRP, C-reactive protein; DXA, dual-energy x-ray absorptiometry; ECLIA, electrochemiluminescence immunoassay; FNIH, Foundation for the National Institutes of Health; HIV, human immunodeficiency virus; HREC, Health Research Ethics Committee; HRT, hormone replacement therapy; HOMA-IR, homeostatic model assessment of insulin resistance; IR, insulin resistance; ISAK, International Society for the Advancement of Kinanthropometry; MetS, metabolic syndrome; PAI-1, plasminogen activator inhibitor-1; PAI-1_{act}, PAI-1 activity; PURE, Prospective Urban and Rural Epidemiology; SANHANES, South African National Health and Nutrition Examination Survey; SCAT, subcutaneous adipose tissue; SO, sarcopenic obesity; SONDRRA, Sarcopenic Obesity and Non-communicable Disease Risk in African Adults; VAT, visceral adipose tissue; VLDL, very low density lipoproteins; WC, waist circumference; WHtR, waist-to-height ratio.

4.3.1 Abstract

Introduction

Although the relationship of plasminogen activator inhibitor-1 (PAI-1) with obesity has been well established, the relationship of PAI-1 with different body fat distribution patterns is less clear particularly in non-white ethnicities.

Methods

We investigated the cross-sectional association of PAI-1_{act} with body fat % and two different body fat distribution patterns, namely sarcopenic obesity (SO) and visceral (VAT) compared to subcutaneous (SCAT) abdominal obesity, in 246 healthy African women by creating sub-groups according to different body fat distribution patterns.

Results

The PAI-1_{act} level of the SO group did not differ significantly from that of the excessive % body fat, non-sarcopenic group ($p=0.8$). The relationship of PAI-1_{act} with body fat %, insulin, triglycerides and appendicular skeletal mass (ASM) was influenced by body fat distribution patterns and degree of obesity. PAI-1_{act} was higher (1.65 vs 0.16 U/ml; $p=0.001$) in women with a proportionally higher abdominal VAT compared to higher abdominal SCAT compartment in the total study population, but not in the centrally obese sub-group (1.72 vs 0.83 U/ml; $p=0.5$). Multiple regression models indicated that body fat % *per se* did not contribute significantly to PAI-1_{act} variance in women with increased fat mass.

Conclusion

Fat distribution patterns and degree of obesity influenced the association of PAI-1_{act} with insulin, triglycerides, ASM and body fat % in African women. In centrally obese women, abdominal VAT no longer contributed more to plasma PAI-1_{act} than abdominal SCAT. Inflammation and endothelial dysfunction contributed more to PAI-1_{act} variance in obese African women than did body fat % *per se*.

Keywords:

Plasminogen activator inhibitor-1; abdominal obesity; sarcopenic obesity; dual-energy x-ray absorptiometry.

4.3.2 Introduction

Over the last decades the prevalence of obesity has increased dramatically, such that it is currently considered a global pandemic [1]. A world-wide estimate on the prevalence of overweight and obesity has shown that from 1980 to 2013 the proportion of overweight men and women have increased (from 28.8% to 36.9% and 29.8% to 38.0%, respectively) [2]. South Africa is no exception, according to the South African National Health and Nutrition Examination Survey (SANHANES) of 2013, the prevalence of overweight and obesity in women is 24.8% and 39.2%, respectively, which is significantly higher than in men (20.1% and 10.6%, respectively) [3]. Furthermore, females above 45 years of age have a significantly higher prevalence of obesity than younger females [3].

Plasminogen activator inhibitor type 1 (PAI-1), an important inhibitor of fibrinolysis, has previously been reported to be associated with obesity in both *in vitro* [4-7] and *in vivo* studies [8, 9]. Furthermore, PAI-1 has shown strong associations with the metabolic syndrome (MetS) and insulin resistance (IR) [10-12] and other co-morbidities associated with obesity such as hypertension, myocardial and cerebral infarction, atherosclerosis and inflammation [13].

Although the relationship of PAI-1 with obesity and more specifically central obesity (frequently determined by waist circumference (WC) has been well established [4, 14, 15], the relationship of PAI-1 with different central obesity fat distribution patterns is less clear particularly in non-white ethnicities, such as Africans. It is for instance possible that the abdominal visceral adipose tissue (VAT) to abdominal subcutaneous adipose tissue (SCAT) ratio could potentially influence plasma PAI-1 levels, despite similar WC. In general PAI-1 production was found to be higher in VAT (as an ectopic fat depot) than in SCAT due to the higher amount of stromal cells [5, 6], production of pro-inflammatory cytokines and higher macrophage content / infiltration [16, 17], however, the importance of PAI-1 expression in abdominal SCAT has also been demonstrated [18, 19]. It has furthermore been demonstrated that body fat distribution patterns differ between ethnicities, with African women having significantly less VAT compared to white women, despite similar WC [20]. In addition, the association of PAI-1 with body composition also differs between ethnicities [10, 21] necessitating these studies in non-white individuals.

Furthermore, limited information is available regarding the effect of different forms of obesity, such as sarcopenic obesity (SO), on PAI-1 [22, 23]. SO is defined as the co-occurrence of increased fat mass in the presence of age related loss of skeletal muscle mass and strength and is typically associated with fat infiltration in the muscle [24, 25]. SO has also previously been suggested to be strongly associated with the development of MetS and atherosclerosis [24]. Hence, it is possible that plasma PAI-1 levels might be more increased in individuals with SO compared to non-sarcopenic obese individuals.

Understanding the relationship between PAI-1 and different body fat distribution patterns, is important to predict the disease risk conveyed through PAI-1 in individuals who did not escape the overweight and obesity pandemic, especially in the under-studied African population. We consequently aimed to investigate the relationship of PAI-1 with two different body fat distribution patterns, namely SO and visceral compared to subcutaneous abdominal obesity in African women. In addition, we explored the relationship between PAI-1 and body fat % in the different body fat distribution patterns. Data on insulin, triglycerides, appendicular skeletal mass (ASM) and inflammation (C-reactive protein) are also provided as these may act as co-variables in the relationship of PAI-1 with body fat [26-28].

4.3.3 Methods and Methods

Study population

The study population included cross-sectional data collected in 2012 from 246 urban dwelling African women as part of the Sarcopenic Obesity and Non-communicable Disease Risk in African Adults (SONDRAA) study. The SONDRAA study is nested in the South African arm of the longitudinal Prospective Urban and Rural Epidemiology (PURE) study. Detailed information on the study design and participant selection criteria of the PURE study have been previously reported [29]. In brief, the sub-study included participants that were randomly selected from households in the Tlokwe municipality, located in the North West province of South Africa. The inclusion criteria were apparent physically and psychologically healthy African women. Participants were excluded if they presented with any disabilities, serious diseases, taking anabolic steroids or protein supplements, as well as pregnant or lactating women. Twenty-six of the women were premenopausal and only one woman in the cohort used hormone replacement therapy (HRT). Sixty per cent of the women were taking anti-hypertensive drugs and approximately half were current or past tobacco users. Only coded data was used and the

names of the participants remained confidential. The study was approved by the Health Research Ethics Committee (HREC) of the North West University (Potchefstroom Campus) (NWU-00016-10-A1) and all participants gave written informed consent.

Anthropometrical assessment

Body weight was measured to the nearest 0.1 kg on an electronic scale (Seca, Birmingham, UK) and height was measured with a freestanding stadiometer (Seca, Birmingham, UK) to the nearest 0.1 cm. Waist circumference was also recorded to the nearest decimal using a steel measuring tape (Lufkin, Apex, NC, USA). The thicknesses of the abdominal and supraspinal skinfolds were measured with a Harpenden calliper (Baty International, West Sussex, UK) on the landmarks as established by the International Society for the Advancement of Kinanthropometry (ISAK). Skinfolds were measured to the nearest 0.1 mm by gently pulling the skin away from the body, taking care not to include the underlying muscle in the double layer of skin and subcutaneous fat. To improve the accuracy and consistency of all measurements, these measurements were performed twice by the same anthropometrist and the average calculated.

Body composition (fat mass; fat-free soft tissue mass; and body fat %) was measured using DXA (Hologic Discovery W, APEX system software version 2.3.1). Fat mass and fat-free soft tissue mass for the whole body, trunk and limbs were derived using standard DXA cut-off lines. Appendicular skeletal muscle mass (ASM) was derived as the sum of the fat-free mass excluding bone of the arms and the legs [30].

Determination of body fat distribution patterns

The women were categorised into several sub-groups according to distinct body composition patterns, in order to investigate the relationship between PAI-1 and different body fat distribution patterns. These groups were determined by using the available DXA and anthropometrical data. Body fat % instead of body mass index (BMI) was used in this study, as body fat % is considered a more sensitive marker of excessive adiposity than BMI [31].

Sarcopenic obesity

To investigate the relationship between PAI-1 and SO, the study population was divided into four mutually exclusive groups. Group 1 – Excessive body fat % group (n=115), was determined as body fat percentage >35.8% for women aged 30-49 years and body fat percentage >37.7% for women aged 50-84 years [32]. Sarcopenia was an exclusion criterion for this group. Group 2 – Sarcopenic group (n=59), sarcopenia was defined as ASM <15.02 kg based on the guidelines of the Foundation for the National Institutes of Health (FNIH) [33]. The FNIH ASM cut-point was chosen, since it had been found to be sensitive to detect reduced functional ability in the black female South African population [34]. Women with excessive body fat % were excluded from this group. Group 3 – SO group (n=36), this group included individuals who were both sarcopenic and presented with excessive body fat %. Lastly, group 4 included women who were non-sarcopenic and had a normal body fat % (n=36).

Visceral compared to subcutaneous central abdominal obesity

Since the use of sophisticated techniques such as nuclear magnetic resonance or computerised tomography scans was not a financially viable option in our research setting, we created a surrogate marker to differentiate between women with preferential visceral compared to preferential subcutaneous central abdominal obesity. This was done by obtaining the average of the central body skinfolds (abdominal and supraspinal) and dividing it by the WC. We then divided the group into tertiles according to the skinfold : WC ratio variable in order to compare the highest (proportionally more SCAT) with the lowest (proportionally more VAT) tertile group. This was also done separately for women with central obesity (defined as waist-to-height ratio (WHtR) of >0.5) [35] to investigate the effect of the two forms of abdominal fat distribution on PAI-1_{act} levels in women already defined to be centrally obese.

Blood and urine collection

A registered nurse collected fasting blood samples with minimum stasis from the antebraclial vein before 10:00 in the morning. Serum samples were used for C-reactive protein (CRP), triglycerides and insulin analyses, sodium fluoride plasma for glucose and citrated plasma samples for the analysis of PAI-1_{act}. Samples were centrifuged at 2000 x g for 15 minutes and stored at -82°C until further analysis. Spot urine was collected for albumin and creatinine determination.

Biochemical analysis

PAI-1_{act} was measured using an indirect enzymatic method (Technozym PAI-1 Actibind, Technoclone, Vienna, Austria). Serum insulin levels were measured by an electrochemiluminescence immunoassay (ECLIA) with a Roche Elecsys immunoassay analyser kit from Cobas*. Plasma glucose concentrations were determined *via* a hexokinase method using the Synchron→Systems (Beckman Coulter Co., Fullerton, CA, USA). HIV status was determined using the First Response rapid HIV test (PMC Medical, India) and positive results were confirmed with CD4 testing. High sensitivity CRP was determined using the Cobas Integra 400- plus biochemistry analyser (Roche diagnostics, Basel, Switzerland). Triglycerides measurements were done by Sequential Multiple Analyser Computer (SMAC) using the Konelab20iTM auto analyser (Thermo Fisher Scientific Oy, Vantaa, Finland). Urinary albumin and creatinine were determined with the Cobas Integra 400 plus (Roche, Basel, Switzerland) and the ratio calculated. HIV testing, CRP, triglycerides, insulin, glucose, albumin and creatinine measurements were performed to describe the health status of the participants and also since they may act as co-variables in the relationship between PAI-1 and body fat [36, 37].

Calculation of insulin resistance

The estimate of IR as determined by the homeostasis model assessment (HOMA) was calculated using the formula: $\text{HOMA-IR} = [(\text{fasting plasma insulin } (\mu\text{U/ml}) \times \text{fasting plasma glucose (mmol/l)}) / 22.5] \text{ concentration}$ [38].

Statistical analysis

Data was analysed using the computer software package Statistica (Statsoft Inc., Tulsa Oklahoma, USA). A p-value of 0.05 or less was regarded as statistically significant. Since most of the variables were not normally distributed, the data was log-transformed to improve normality and to allow the use of parametric statistical analyses. Descriptive data is presented both as median (25th; 75th percentiles) and mean ($\pm$ standard deviation). As the association of PAI-1 with many of the body fat variables were not linear we decided to follow the approach of sub-dividing the study population rather than to present the data of the total group as continuous data in order to get a better representation of the true relationships. The t-test for independent samples was used to compare differences between two groups. Analysis of variance (ANOVA) with Tukey's honest significant difference *post-hoc* tests were used to

compare differences between more than two groups and analysis of co-variance (ANCOVA) was used when adjustment for confounders was required. Pearson and partial correlations were used to determine correlations between log-transformed variables. Single and forward stepwise multiple regression analyses were used to determine the association of body fat % with PAI-1. The covariates entered into the multiple regression model were: insulin, age, skinfold : WC ratio, tobacco use, alcohol consumption, CRP, body fat %, triglycerides, menopausal status and albumin : creatinine ratio as a marker of endothelial dysfunction. These co-variables were based on their potential influence on the relationship between PAI-1 and body fat [26-28, 37, 39, 40]. Women with CRP >10mg/L were excluded from analysis [41]. Only the variables that entered the stepwise multiple regression are presented in the tables.

4.3.4 Results

Sarcopenic obesity

The study population consisted of 246 women who were grouped according to the following identified fat distribution patterns in order to investigate the relationship between PAI-1_{act} and SO: excessive body fat % (n=115), sarcopenic (n=59), SO (n=36) and non-sarcopenic, normal body fat % (n=36) (Table 4.1). One-way ANOVA indicated no significant age differences between the groups (p=0.5) with the sarcopenic group having the highest prevalence of HIV infection (22%). *Post-hoc* comparison between the groups revealed that for most of the variables (ASM, insulin, glucose, HOMA-IR, body fat %, WC, BMI, WHtR, skinfold : WC ratio), the sarcopenic group had significantly lower values than the other three groups with little difference between the other groups.

PAI-1_{act} of the SO group did not differ significantly from that of the excessive body fat % group (p=0.8) or the non-sarcopenic, normal body fat % (p=0.7). There was also no significant difference between MetS-related markers (insulin, glucose, HOMA-IR and triglycerides) between the SO and excessive body fat % group. The excessive body fat % group did however, have significantly higher BMI, central obesity (WC and WHtR) and a lower central skinfold : WC ratio indicating a proportionally higher central VAT than SCAT fat distribution.

The association of PAI-1_{act} with body fat %, insulin, triglycerides and ASM differed between the different body fat distribution sub-groups (Table 4.2). PAI-1_{act} correlated with body fat % in the

non-sarcopenic, normal body fat % only ($r=0.39$; $p=0.02$) with adjustment for insulin, triglycerides, menopausal status affecting this relationship only marginally ($r=0.34$; $p=0.07$). PAI-1_{act} showed a borderline correlation ($r=0.31$; $p=0.07$) with insulin in the SO group, but not in any of the other sub-groups. It had a significant positive association with triglycerides in the excessive body fat % group ($r=0.27$; $p=0.005$) with an association of similar magnitude in the non-sarcopenic, normal body fat % group ($r=0.3$; $p=0.09$), albeit non-significant probably due to the smaller sample size. PAI-1_{act} correlated with ASM in the non-sarcopenic, normal body fat % group only ($r=0.38$; $p=0.03$).

Visceral compared to subcutaneous central obesity

We divided the study group into tertiles according to the skinfold : WC ratio variable and compared PAI-1_{act}, insulin and HOMA-IR between the first and third tertile to determine the differences in women with proportionally higher abdominal VAT (quartile 1) vs women with proportionally higher abdominal SCAT (quartile 3). PAI-1_{act}, insulin and HOMA-IR were statistically and clinically significantly higher in the group with the proportionally higher abdominal VAT (quartile 1) (Table 4.3). When selecting only women with central obesity (WHTR >0.5), the differences between the first and third quartiles were no longer significant. In addition, PAI-1_{act} correlated significantly (albeit weakly) with the skinfold : WC ratio in the total study population ($r=-0.18$; $p=0.007$), but not in the centrally obese women ($r=-0.12$; $p=0.1$).

Association of PAI-1_{act} with body fat %

In order to determine the relationship of PAI-1, insulin, triglycerides, CRP and skinfold : WC ratio with body fat %, the entire study population was divided into quartiles according to their body fat % (Table 4.4). PAI-1_{act} increased across the body fat % quartiles, but significance was reached in the fourth quartile only (body fat % >45.6%). No increase was observed between the second and third quartile. Insulin and CRP levels in quartile two, three and four were all significantly higher than in quartile one with no difference in triglyceride levels across the four quartiles. Furthermore, CRP had a significant positive correlation with body fat % ($r=0.38$, $p<0.0001$) and correlated negatively with the skinfold : WC ratio ($r=-0.31$, $p<0.0001$) (data not shown). Skinfold : WC ratio levels decreased across the body fat % quartiles, with a significantly lower skinfold : WC ratio in the fourth compared to the first quartile, indicating a proportionally higher VAT vs. SCAT distribution in the fourth quartile.

In order to determine the contribution of body fat % to PAI-1_{act} we performed single and forward stepwise multiple regression for each of the body fat % quartile groups (Table 4.5). In the single regression models, body fat % contributed significantly ($p=0.01$) to the variance in PAI-1_{act} in the third quartile only (>40.5 to $\leq 45.6\%$ body fat), explaining 11% of the variance. In the forward stepwise multiple regression models, insulin made the biggest contribution (12.4%) to the variance in PAI-1_{act} followed by the albumin : creatinine ratio, menopausal status and age, with fat % not entering the model, in the first quartile. In the second quartile, triglycerides, age and insulin entered the model explaining 19.8, 4.9 and 2%, respectively. In the third quartile, in agreement with the single regression, fat % contributed to the variance in PAI-1_{act}, explaining 5.31% of the variance with age (4.07%) and triglycerides (12.7%) also entering the model. In the fourth quartile, the main contributors to PAI-1_{act} variance were the albumin : creatinine ratio explaining 10.7%, CRP 3.58%, menopausal status 2.88% and skinfold : WC .2.99% of the PAI-1 variance. In the most obese group (body fat % $>45.6\%$) the multiple regression models explained the smallest percentage of total PAI-1 variance (16%) compared to the other groups (24, 26.7 and 22%).

4.3.5 Discussion

Although the association between PAI-1 and obesity has been well established, much less is known regarding the relationship of PAI-1 with different body fat distribution patterns, with virtually no information available on non-white ethnicities such as Africans. The association of PAI-1 with VAT [21] and MetS [10] has for instance been shown to differ between black and white individuals. PAI-1 associated significantly with VAT in Caucasians, while no association was found in African Americans [21]. PAI-1 was also found to be more prominently associated with markers of the MetS in white than in black Africans [10]. Furthermore, differences in body fat distribution have also been found, with African women having significantly less VAT compared to white women, despite similar WC [20]. It is, therefore, postulated that due to ethnic differences in body fat composition and fat deposition, the relationship of PAI-1 with body composition in black individuals may differ from that observed for other population groups and, therefore, merits further investigation. Our data demonstrate that in African women, the association of PAI-1_{act} with body fat %, insulin, triglycerides and ASM are influenced by body fat distribution patterns and degree of obesity. It investigates the association of PAI-1_{act} with SO and shows that while increased VAT is associated with increased plasma PAI-1_{act} levels, abdominal SCAT also significantly contributes to PAI-1_{act} levels, in obese women specifically.

Lastly, we demonstrated that body fat % *per se*, does not significantly contribute to increased plasma PAI-1_{act} in women with high fat mass (body fat % >45.6%), but that other factors associated with obesity (known and as of yet unidentified), are more prominently related to the increased PAI-1_{act}.

Sarcopenic obesity

PAI-1 is significantly associated with both obesity and IR [11, 12, 42, 43]. Since IR and MetS appear to be closely related to SO [24], it was postulated that PAI-1_{act} may be even higher in SO individuals than in individuals who are obese without having sarcopenia. There was, however, no difference in PAI-1_{act} between the SO and the excessive body fat % group. This is likely due to the fact that in this study population, insulin-related markers did not differ between the two groups, suggesting that IR was not more prominent among the SO women than among the total excessive body fat % group. The prevalence of HIV infection on the other hand, was significantly higher in the sarcopenic group. It is, therefore, likely that the observed muscle wasting in this group may be the result of HIV infection in those affected. In addition, the excessive body fat % group was on average more centrally obese than the SO group and displayed a proportionally higher abdominal VAT component, two factors that each have the potential to significantly increase PAI-1_{act} [44-46]. These results are also in agreement with Cesari *et al.* [22], who found no significant association between either sarcopenia or obesity and PAI-1 concentration.

When investigating the relationship of PAI-1_{act} with body fat %, insulin, triglycerides and ASM, we found that the relationships differed between the different groups. PAI-1 correlated significantly with body fat % and ASM in the non-sarcopenic, normal body fat % group only, with insulin in the SO group and with triglycerides in the excessive body fat % group. This suggests the association of PAI-1 with these variables is not a constant / fixed relationship, but that their relative contributions to PAI-1 are influenced by body fat distribution patterns.

Visceral compared to subcutaneous central obesity

PAI-1 is considered by many to be expressed more in VAT than in SCAT [5, 6]. This is thought to be related to structural and functional differences between VAT and SCAT. Visceral adipose tissue, a major ectopic fat depot, present in the abdominal viscera in the mesentery and omentum, produces more pro-inflammatory cytokines like tumour necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6) [16], it contains more stromal cells, which is the cellular component of adipose tissue that produces PAI-1 and exhibit a higher macrophage content which contributes to the increased cytokine production [6, 17]. In contrast, SCAT expresses more leptin and adiponectin and is mainly found in the femoral-gluteal regions, back and anterior abdominal wall [16]. Evidence does, however, exist that *PAI-1* mRNA in abdominal SCAT (but not femoral SCAT) correlated positively with plasma PAI-1 levels in obese individuals [19] and that *PAI-1* expression in SCAT is higher than in VAT in obese individuals and in females [18]. This data suggests that the degree of obesity may influence regional differences in PAI-1 secretion. In general agreement with the literature, our data indicated that plasma PAI-1_{act} levels were significantly higher in black African women with a proportionally higher abdominal VAT compared to SCAT compartment. However, in women who were already centrally obese, this was no longer the case. This data together with the literature suggest that in obese women, abdominal SCAT can significantly contribute to the increased plasma PAI-1 levels found in obesity and in so doing, increase CVD risk.

Association of PAI-1_{act} with body fat percentage

In obesity, there are many different factors that can influence PAI-1 levels. These include IR, inflammation and regulation of PAI-1 producing cells by inflammatory cytokines, increased triglycerides, as well as the increased adipocyte mass [42, 43, 47]. We subsequently wanted to determine the relationship of body fat % *per se* with PAI-1. When dividing the study population according to body fat % quartiles, body fat % contributed, in both single and multiple regression models to the PAI-1_{act} variance in quartile 3 only. This suggests that other factors than body fat % itself, which are associated with increased fat mass, are likely responsible for the increased PAI-1_{act} levels in quartile 4. This notion is supported by our data indicating a correlation between PAI-1_{act} and body fat % in the non-sarcopenic, normal body fat % women only while no associations were found in the excessive body fat % or the SO groups. It has also been suggested that PAI-1 is not closely dependent on fat mass, but rather reflects fat redistribution patterns [17, 48].

In order to compare the contribution of body fat % *per se* to other factors associated with obesity on plasma PAI-1_{act}, we also investigated the role of insulin, triglycerides, menopausal status, CRP (as a marker of inflammation), skinfold : WC ratio, age, alcohol consumption, smoking and the albumin : creatinine ratio (as a marker of endothelial dysfunction). Insulin levels entered the stepwise multiple regression as a contributor to PAI-1_{act} variance in model 1 and 2 only, suggesting that it contributes proportionally more to PAI-1_{act} variance in women with a lower body fat %. Triglycerides did not differ across the body fat % quartiles, but it was the most significant contributor to PAI-1_{act} variance in the 2nd and 3rd body fat % quartile. The association between PAI-1 and triglyceride has been previously explained by the fact that very low density lipoproteins (VLDL) triglycerides increase PAI-1 levels [49, 50], through a VLDL response element identified in the promoter region of the PAI-1 gene that mediates VLDL-induced PAI-1 transcription in endothelial cells [51]. Menopausal status entered the stepwise regression models as contributor to PAI-1_{act} variance, but its contribution was also not significant, explaining about 2.8% only. C-reactive protein contributed to the variance in PAI-1_{act} in women with high fat mass (quartile 4) only, although the contribution was relatively minor at 3.7%. Additional analysis confirmed a positive relationship between CRP and body fat %, indicating that inflammation increases as body fat % increase, as is known from the literature [52, 53]. We also found a negative association between CRP and the skinfold : WC ratio, suggesting that inflammation is increased in the presence of proportionally increased abdominal VAT. Next we investigated the possible contribution of the abdominal VAT / SCAT distribution and found that there was a significant proportional increase in the abdominal VAT compartment as body fat % increased, and that it served as a significant contributor to the PAI-1_{act} variance in the most obese women. This proportional increase in VAT is in agreement with the literature indicating that post-menopausal women experience a redistribution of fat distribution with increased visceral fat accumulation, which is thought to be related to the female sex hormone oestrogen [54]. The largest (and only statistically significant) contributor to PAI-1_{act} in the obese individuals was endothelial dysfunction (albumin : creatinine ratio). Evidence exists for the presence of endothelial dysfunction in obesity [37], which is thought to be induced by obesity-associated metabolic abnormalities such as IR, adipokines, oxidative stress, increased free fatty acids, TNF- α as well as activation of innate immunity [55, 56]. At the same time, increased PAI-1 is recognised as an early marker of endothelial dysfunction [57, 58] suggesting endothelial dysfunction as a mechanistically plausible modulator of PAI-1_{act} in obesity. It is also important to note that in the most obese individuals, the above mentioned known PAI-1 associates, explained the smallest percentage of total PAI-1 variance, compared to the other body fat % quartiles, suggesting that other, as of yet unidentified factors, additionally contribute to the increased PAI-1 in obesity. Our results indicate that in African women with increased fat mass,

body fat % *per se* contributes to a lesser extent to plasma PAI-1_{act} than other obesity-related metabolic derangements such as inflammation and endothelial dysfunction.

A limitation of the study, being cross-sectional in design, was that causality could not be determined for PAI-1_{act}. While every attempt has been made to prevent possible selection bias, it is not impossible that it may have occurred in some form. A further limitation of the study was the inability to estimate VAT and SCAT directly using DXA software as this is not available in our laboratory. Also, we acknowledge that the skinfold : WC ratio is a novel and rather crude method to distinguish between abdominal fat distribution patterns. The fact that associations with PAI-1_{act} were found, suggest however that it could potentially be used in settings where access to more sophisticated techniques is limited and it deserves further investigation.

In conclusion, our data indicates that PAI-1_{act} is not higher in SO African women who do not display increased IR, compared to obese, non-sarcopenic women. Furthermore, fat distribution patterns and degree of obesity were found to influence the association of PAI-1_{act} with insulin, triglycerides, ASM and body fat %. Our data also suggests that in centrally obese women, abdominal VAT no longer contributed more than abdominal SCAT to plasma PAI-1_{act}, where no differences were found between the two adipocyte compartments and that abdominal SCAT should be considered a significant contributor to plasma PAI-1 in obese women. Lastly in obese African women, the known PAI-1 associates explained a smaller percentage of total PAI-1_{act} variance compared to the other body fat % quartiles suggesting additional, as of yet unidentified mechanistic pathways in obesity. Also the increased PAI-1 observed in obesity in African women, is more strongly influenced by other obesity-related metabolic abnormalities such as inflammation and endothelial dysfunction than by body fat % *per se*. Whether these varying associations between PAI-1 and body fat distribution patterns in Africans will result in different disease risk profiles of the diseases contingent upon PAI-1 needs to be established in future research.

4.3.6 Acknowledgements

This work was supported by services of the SONDRRA research team and the Centre of Excellence for Nutrition of the North-West University, South Africa. Funding was through the National Research Foundation (NRF) of South Africa (Grant UID: 94213). Opinions expressed and conclusions arrived at, are those of the authors and are not to be attributed to the NRF.

Conflict of Interest

None of the authors have any conflict of interest to declare.

Author Contributions

Sunelle A. Barnard – analysis and interpretation of data; critical writing of manuscript.

Marlien Pieters - analysis and interpretation of data, critical writing of manuscript.

Cornelie Nienaber-Rousseau – anthropometrist that collected the anthropometry data, interpretation of data and critical reviewing the manuscript.

Herculina S. Kruger – interpretation of data, critical reviewing the manuscript and PI of the SONDRRA study.

4.3.7 Reference

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Legends to Tables

Table 4.1 Descriptive statistics of the different body fat distribution groups

ASM, appendicular skeletal muscle mass; HIV, human immunodeficiency virus; HOMA-IR, homeostatic model assessment of insulin resistance; MetS, metabolic syndrome; PAI-1_{act}, Plasminogen activator inhibitor-1 activity; WC, waist circumference; WHtR, waist-to-height ratio. * fat% >35.8% for 30-49 years of age or >37.7% for 50-84 years of age [32]. ** ASM <15.02 kg [33]. # Significantly different from the other three groups. ^\$ Medians/means/percentages with the same symbol differ significantly. & ANCOVA p-value after adjustment for insulin.

Table 4.2 Associations of PAI-1_{act} with body fat percentage, insulin, triglyceride and ASM in the sub-groups

ASM, appendicular skeletal muscle mass; PAI-1_{act}, plasminogen activator inhibitor-1 activity; SO, sarcopenic obesity. * Adjusting for insulin and triglyceride; ** fat% >35.8% for 30-49 years of age or >37.7% for 50-84 years of age[32]. *** ASM <15.02 kg [33].

Table 4.3 Difference in PAI-1_{act}, insulin, HOMA-IR and HOMA-%β between the 1st and 3rd skinfold : WC ratio tertiles

HOMA-IR, homeostatic model assessment of insulin resistance; PAI-1_{act}, Plasminogen activator inhibitor-1 activity; WC, waist-circumference; SCAT, subcutaneous adipose tissue; VAT, visceral adipose tissue. * waist-to-height ratio (WHtR) >0.5.

Table 4.4 PAI-1_{act}, insulin, triglyceride, CRP and skindold : WC ratio according to body fat percentages quartiles

PAI-1_{act}, plasminogen activator inhibitor-1 activity; CRP, C-reactive protein. # Significantly different from the other three groups. ^\$ Medians with the same symbol differ significantly.

Table 4.5 Contribution of body fat percentage to PAI-1_{act} variance: single and multiple regression

Alb:creat, albumin – creatinine ratio; CI, confidence interval; CRP, C-reactive protein; PAI-1_{act}, plasminogen activator inhibitor-1 activity; WC, waist circumference.

Table 4.1: Descriptive statistics of the different body fat distribution groups

Variables	Sarcopenic Obese (n=36)		Excessive body fat %* (n=115)		Sarcopenic** (n=59)		Non-sarcopenic, normal body fat % (n=36)		ANOVA
	Median		Median		Median		Median		p-value
	Mean ($\pm$ Std.)	(25 th ; 75 th percentile)	Mean ($\pm$ Std.)	(25 th ; 75 th percentile)	Mean ($\pm$ Std.)	(25 th ; 75 th percentile)	Mean ($\pm$ Std.)	(25 th ; 75 th percentile)	
Age (years)	60.6 ($\pm$ 11.1)	61.0 (50.5; 68.0)	57.6 ($\pm$ 9.98)	55.0 (49.0; 66.0)	58.6 ($\pm$ 11.4)	57.5 (50.0; 62.0)	58.7 ($\pm$ 9.82)	55.0 (52.0; 65.0)	0.5
PAI-1 _{act} (U/ml)	3.89 ($\pm$ 8.10)	0.78 (0.01; 4.22)	4.00 ($\pm$ 7.23)	1.52 (0.01; 4.13)	1.68 ($\pm$ 3.61)	0.60 (0.01; 1.76)	2.77 ($\pm$ 5.19)	0.01 (0.01; 1.66)	0.09 (0.17) ^{&}
ASM (kg)	13.5 ($\pm$ 1.21)	13.8 (12.7; 14.3) [#]	19.2 ($\pm$ 2.98)	18.5 (16.8; 20.6)	12.5 ($\pm$ 1.49)	12.5 (11.4; 13.4) [#]	18.1 ($\pm$ 2.33)	17.3 (16.3; 19.3)	<0.0001
Insulin (μ U/ml)	18.2 ($\pm$ 13.1)	14.2 (8.94; 20.0)	22.4 ($\pm$ 22.9)	15.0 (10.6; 24.0)	12.7 ($\pm$ 20.0)	6.20 (4.31; 14.0) [#]	26.0 ($\pm$ 24.7)	15.5 (9.30; 25.7)	<0.0001
Glucose (mmol/l)	6.25 ($\pm$ 4.48)	5.00 (4.43; 6.00)	5.86 ($\pm$ 3.19)	5.00 (4.46; 5.86)	4.65 ($\pm$ 1.61)	4.31 (4.01; 4.98) [#]	6.78 ($\pm$ 5.00)	5.09 (4.31; 6.67)	0.002
HOMA-IR	5.86 ($\pm$ 9.21)	3.15 (2.11; 4.68)	6.73 ($\pm$ 13.2)	3.60 (1.90; 6.44)	2.92 ($\pm$ 5.27)	1.19 (0.78; 2.83) [#]	10.9 ($\pm$ 20.2)	4.45 (2.07; 7.76)	<0.0001
Triglyceride(mmol/l)	1.40 ($\pm$ 1.29)	1.23 (0.78; 1.53)	1.22 ($\pm$ 0.84)	0.98 (0.74; 1.33)	1.19 ($\pm$ 0.86)	0.89 (0.67; 1.49)	1.43 ($\pm$ 1.01)	1.09 (0.78; 1.58)	0.3
Fat (%)	42.6 ($\pm$ 3.83)	41.2 (39.7; 45.1)	45.2 ($\pm$ 3.99)	45.2 (42.3; 47.9)	30.2 ($\pm$ 5.22)	30.9 (26.6; 34.5) [#]	33.5 ($\pm$ 4.17) [#]	35.1 (31.6; 36.3)	<0.0001
WC (cm)	86.2 ($\pm$ 10.5)	85.7 (76.8; 95.0)	98.3 ($\pm$ 10.7)	98.5 (92.1; 104) [#]	71.2 ($\pm$ 9.22)	70.3 (65.5; 76.3) [#]	87.1 ($\pm$ 8.79)	86.2 (81.7; 95.3)	<0.0001
WHtR	0.57 ($\pm$ 0.08)	0.57 (0.51; 0.63)	0.63 ($\pm$ 0.07)	0.62 (0.59; 0.66) [#]	0.46 ($\pm$ 0.06)	0.46 (0.42; 0.50) [#]	0.54 ($\pm$ 0.06)	0.53 (0.51; 0.59)	<0.0001

Skinfold : WC ratio	4.60 (±2.15)	4.00 (3.14; 5.18)	3.10 (±1.01)	2.83 (2.47; 3.50) [#]	8.76 (±4.24)	7.70 (5.36; 11.5) [#]	5.64 (±3.28)	4.40 (3.40; 7.54)	<0.0001
Abdominal skinfold (mm)	27.3 (±12.2)	27.6 (17.5; 35.0)	41.5 (±12.1)	41.7 (32.1; 50.5)	13.1 (±7.05)	12.6 (7.10; 18.2)	25.6 (±13.2)	25.2 (14.5; 33.3)	<0.0001
Supra-spinal skinfold (mm)	17.8 (±8.81)	17.4 (11.2; 24.7)	27.5 (±10.2)	26.7 (19.7; 34.8)	7.31 (±3.88)	6.60 (4.10; 9.35)	14.8 (±8.05)	14.1 (8.50; 19.2)	<0.0001
BMI (kg/m ²)	27.9 (±4.82)	26.6 (24.6; 30.5) [^]	34.8 (±5.75)	34.0 (30.8; 38.1) ^{^\$}	20.4 (±2.79)	20.5 (19.1; 21.8) ^{^\$}	26.3 (±3.45)	26.1 (24.0; 29.0) ^{\$}	<0.0001
HIV status n (%)	4 (11%)		5 (4%) [^]		13 (22%) [^]		5 (14%)		0.005
MetS n (%)	16 (44%) ^{\$}		54 (47%) [^]		8 (13.5%) ^{^\$}		13 (36%)		<0.0001
Menopausal status (%)	31 (86.1%)		101 (87.8%)		54 (91.5%)		33 (91.7%)		0.81
Tobacco users (%)	14 (40%)		50 (45%)		30 (57)		35 (60%)		0.2

ASM, appendicular skeletal muscle mass; HIV, human immunodeficiency virus; HOMA-IR, homeostatic model assessment of insulin resistance; MetS, metabolic syndrome; PAI-1_{act}, plasminogen activator inhibitor-1 activity; WC, waist circumference; WHtR, waist-to-height ratio. * fat% >35.8% for 30-49 years of age or >37.7% for 50-84 years of age [32]. ** ASM <15.02 kg [33].

[#] Significantly different from the other three groups. ^{^\$} Medians/means/percentages with the same symbol differ significantly. [&] ANCOVA p-value after adjustment for insulin.

Table 4.2: Associations of PAI-1_{act} with body fat percentage, insulin, triglyceride and ASM in the sub-groups

Variables	Groups	Body fat %			Insulin (μU/ml)	Triglyceride (mmol/l)	ASM (kg)
		n	r (p-value)	Partial r (p-value)*	r (p-value)	r (p-value)	r (p-value)
PAI-1 _{act} (U/ml)	Total	235	0.20 (0.002)	0.20 (0.003)	0.09 (0.2)	0.25 (<0.0001)	0.08 (0.2)
	Sarcopenic Obese	36	0.24 (0.16)	0.16 (0.4)	0.31 (0.07)	0.22 (0.2)	0.002 (0.10)
	Excessive body fat %**	110	0.14 (0.15)	0.14 (0.1)	0.09 (0.4)	0.27 (0.005)	-0.02 (0.8)
	Sarcopenic***	56	-0.01 (0.9)	0.01 (0.9)	-0.13 (0.4)	0.22 (0.10)	-0.22 (0.1)
	Non-sarcopenic, normal body fat %	33	0.39 (0.02)	0.34 (0.07)	0.10 (0.6)	0.30 (0.09)	0.38 (0.03)

ASM, appendicular skeletal muscle mass; PAI-1_{act}, plasminogen activator inhibitor-1 activity; SO, sarcopenic obesity. * Adjusted for insulin, triglyceride and menopausal status; ** fat% >35.8% for 30-49 years of age or >37.7% for 50-84 years of age [32]; *** ASM <15.02 kg [33].

Table 4.3: Difference in PAI-1_{act}, insulin and HOMA-IR between the 1st and 3rd skinfold : WC ratio tertiles

Variables	Total study group					Central obese group*				
	↑VAT ↓SCAT		↑SCAT ↓VAT			↑VAT ↓SCAT		↑SCAT ↓VAT		
	Skinfold : WC ratio		Skinfold : WC ratio			Skinfold : WC ratio		Skinfold : WC ratio		
	tertile 1 (≤ 3.02)	n	tertile 3 (>4.76)	n	p-value	tertile 1 (≤ 2.8)	n	tertile 3 (>3.8)	n	p-value
	Median (25, 75% percentile)		Median (25, 75% percentile)			Median (25, 75% percentile)		Median (25, 75% percentile)		
PAI-1 _{act} (U/ml)	1.65 (0.01; 5.12)	78	0.16 (0.01; 1.40)	78	0.001	1.72 (0.01; 3.76)	59	0.83 (0.01; 4.13)	59	0.5
Insulin (μ U/ml)	16.3 (10.3; 25.3)	76	8.83 (5.19; 16.0)	81	0.0007	15.9 (10.6; 24.8)	58	13.5 (7.54; 23.4)	59	0.3
HOMA-IR	4.03 (2.37; 6.47)	77	1.77 (0.90; 3.48)	81	<0.0001	3.83 (2.31; 6.47)	59	3.00 (1.43; 5.74)	59	0.09

HOMA-IR, homeostatic model assessment of insulin resistance; PAI-1_{act}, Plasminogen activator inhibitor-1 activity; WC, waist-circumference; SCAT, subcutaneous adipose tissue; VAT, visceral adipose tissue. * waist-to-height ratio (WHtR) >0.5.

Table 4.4: PAI-1_{act}, insulin, triglyceride, CRP and skinfold : WC ratio according to body fat percentages quartiles

Variable	Total group								ANOVA
	Body fat% 1 st Quartile		Body fat% 2 nd Quartile		Body fat% 3 rd Quartile		Body fat% 4 th Quartile		
	(≤34.5%)		(>34.5 - ≤40.5%)		(>40.5 - ≤45.6%)		(>45.6%)		
	Median		Median		Median		Median		
	(25, 75% percentile)	n	(25, 75% percentile)	n	(25, 75% percentile)	n	(25, 75% percentile)	n	
PAI-1 _{act} (U/ml)	0.07 (0.01; 1.23) [^]	58	0.82 (0.01; 3.26)	59	0.83 (0.01; 2.83)	59	2.28 (0.01; 5.94) [^]	59	0.005
Insulin (μU/ml)	7.01 (4.43; 14.23) [#]	61	14.9 (9.39; 26.7)	59	15.0 (9.04; 21.44)	59	14.4 (10.0; 23.4)	58	<0.0001
Triglyceride (mmol/l)	0.92 (0.72; 1.37)	62	0.99 (0.73; 1.56)	59	1.03 (0.79; 1.41)	59	0.99 (0.73; 1.27)	59	0.6
CRP (mg/L)	1.81 (0.58; 4.39) [#]	58	3.43 (1.82; 5.93)	47	3.62 (2.23; 5.56)	50	5.09 (3.39; 7.46)	37	<0.0001
Skinfold : WC ratio	8.41 (6.13; 11.48) ^{^\$}	61	4.00 (3.35; 5.16) ^{^\$}	61	3.10 (2.59; 4.02) [^]	61	2.79 (2.38; 3.30) ^{\$}	60	<0.0001

PAI-1_{act}, plasminogen activator inhibitor-1 activity; CRP, C-reactive protein. [#] Significantly different from the other three groups. ^{^\$} Medians with the same symbol differ significantly.

Table 4.5: Contribution of body fat percentage to PAI-1_{act} variance: single and multiple regression

Body fat % Quartiles	Single regression					Forward stepwise multiple regression					
	n	β	95% CI	Total % variance explained	p-value	Co-variables that entered model	β	95% CI	Total % variance explained	% variance explained by each co- variate	p-value
1 st Quartile ($\leq 34.5\%$)	58	-0.65	-3.27; 1.97	0.4	0.6	Insulin	-0.52	-0.89; -0.15	26.5	12.4	0.009
						Alb:Creat	0.32	0.05; 0.59		9.99	0.02
						Menopausal status	-0.78	-2.19; 0.63		2.27	0.2
						Age	1.87	-0.68; 4.41		1.81	0.3
2 nd Quartile ($>34.5 - \leq 40.5\%$)	59	-3.11	-14.0; 7.83	0.6	0.6	Triglyceride	1.13	0.18; 2.08	26.7	19.8	0.02
						Age	-2.19	-5.34; 0.96		4.94	0.2
						Insulin	0.23	-0.49; 0.95		2.00	0.3
3 rd Quartile ($>40.5 - \leq 45.6\%$)	59	16.5	3.79; 29.1	11	0.01	Triglyceride	0.84	-0.05; 1.74	22.0	12.7	0.03
						Body fat %	12.0	-1.96; 26.0		5.31	0.07

						Age	1.86	-1.21; 4.94		4.07	0.2
4 th Quartile (>45.6%)	59	0.35	-10.6; 11.4	<0.1	0.9	Alb:Creat	-0.85	-1.58; -0.12		10.7	0.03
						CRP	-0.55	-1.42; 0.36		3.58	0.2
						Menopausal status	1.78	-0.15; 4.71	20.2	2.88	0.2
						skinfold : WC ratio	-1.44	-4.15; 1.27		2.99	0.3

Alb:creat, albumin – creatinine ratio; CI, confidence interval; CRP, C-reactive protein; PAI-1_{act}, plasminogen activator inhibitor-1 activity; WC, waist circumference.

CHAPTER 5 THE CONTRIBUTION OF DIFFERENT ADIPOSE TISSUE DEPOTS TO PLASMA PLASMINOGEN ACTIVATOR INHIBITOR-1 (PAI-1) LEVELS

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This chapter includes:

- 5.1 The instructions given to authors by the journal *Blood Reviews* (Impact factor: 6.627);
- 5.2 proof that the article has been accepted for publication in *Blood Reviews*; and
- 5.3 the accepted article titled: “The contribution of different adipose tissue depots to plasma plasminogen activator inhibitor-1 (PAI-1) levels”.

In addition, the published article is provided as Addendum F

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Thank you for submitting your work to our journal.

Yours sincerely,

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13 May 2016

5.3 Accepted article submitted to *Blood Reviews*

The contribution of different adipose tissue depots to plasma plasminogen activator inhibitor-1 (PAI-1) levels

Running title: Body fat distribution and plasma plasminogen activator inhibitor-1

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Word count:

Summary: 146 Text: 4566

Abbreviations:

BMI, body mass index; IL-1, interleukin-1; IL-6, interleukin-6; IR, insulin resistance; MetS, metabolic syndrome; mRNA, messenger ribonucleic acid; NRF, National Research Foundation; PAI-1, plasminogen activator inhibitor-1; PAI-1_{act}, PAI-1 activity; PAI-1_{ag}, PAI-1 antigen; PCR, polymerase chain reaction; SCAT, subcutaneous adipose tissue; TGF- β 1, transforming growth factor β ; TNF- α , tumour necrosis factor- α ; VAT, visceral adipose tissue; VLDL, very low density lipoproteins; WC, waist circumference; WHR, waist-to-hip ratio.

5.3.1 Abstract

Increased plasma plasminogen activator inhibitor-1 (PAI-1) level is considered a mechanistic pathway through which obesity contributes to increased cardiovascular disease risk. Abdominal adipose tissue specifically, is a major PAI-1 source with visceral adipose tissue (VAT), an ectopic fat depot, generally considered to produce more PAI-1 than subcutaneous adipose tissue. This does, however, not necessarily lead to increased plasma PAI-1 levels. This review provides an overview of studies investigating the association between body fat distribution and plasma PAI-1 levels. It discusses factors that influence this relationship and also considers the contribution of other tissue to plasma PAI-1 levels, placing the relative contribution of adipose tissue into perspective. In conclusion, the relationship between VAT and plasma PAI-1 levels is not fixed, but can be modulated by a number of factors such as the size of the subcutaneous adipose tissue depot, ethnicity, possibly genetics and other obesity-related metabolic abnormalities.

Keywords

Plasminogen activator inhibitor-1; visceral adipose tissue; subcutaneous adipose tissue; body fat distribution

5.3.2 Introduction

Plasminogen activator inhibitor-1 (PAI-1), the main inhibitor of fibrinolysis, contributes to increased cardiovascular risk in overweight and obese individuals [1]. Elevated plasma PAI-1 levels are considered to be a biochemical marker of obesity [2] and also as a component of the metabolic syndrome (MetS), which is characterised by dyslipidaemia, hypertension, glucose intolerance and increased abdominal fat distribution [3,4]. The association between PAI-1 and obesity, especially central obesity, has been well established in both animal and human studies [5-11] and is largely considered to be the result of PAI-1 production by adipose tissue [5,6,12,13]. PAI-1 is produced by a variety of cells contained in adipose tissue; these include pre-adipocytes, mature adipocytes, stromal cells, endothelial cells, smooth muscle cells and monocytes/macrophages [14].

Abdominal fat deposition, takes place in two main fat depots [subcutaneous adipose tissue (SCAT) and intra-abdominal or visceral fat tissue (VAT)] which is considered to be an ectopic fat depot. Ectopic fat refers to the storage of fat in non-adipose tissue like the liver, skeletal muscle, viscera, pancreas and the heart [15,16]. Although, it is not yet certain how ectopic fat accumulation takes place, it is postulated to be related to an overflow of triglycerides into other organs as SCAT loses the ability to expand and to store excess energy [17]. According to this theory, the expandability of SCAT protects other organs against ectopic fat deposition [18]. In support thereof, SCAT does not seem to be associated with a linear increase in cardiovascular risk factors in obesity [19]. Furthermore, when compared to VAT, abdominal and thigh SCAT were observed to be protective against obesity-associated metabolic complications such as insulin resistance (IR) [20,21].

It is generally accepted that ectopic fat, and for the purpose of this review, VAT specifically, produces more PAI-1 than SCAT [5,22]. Consistently, computed tomography (CT) data from obese individuals have shown higher PAI-1 levels in VAT than SCAT [8,23]. Also, PAI-1 gene expression, determined by means of adipose biopsies, has been shown to be higher in the

omental adipose tissue compared to SCAT during acute systemic inflammation which was also accompanied by increased plasma PAI-1 levels [9]. However, contradicting evidence also exists, as some studies have shown comparable PAI-1 antigen (PAI-1_{ag}) secretion from VAT and SCAT [24], or even higher PAI-1 messenger ribonucleic acid (mRNA) expression and increased rate of PAI-1_{ag} synthesis in SCAT than in VAT [25]. Although adipose tissue PAI-1 concentration and production have important local effects, it is the concentration of PAI-1 in the blood that contributes to the development of CVD [reviewed by 26,27], and therefore, the effect of body fat distribution on plasma PAI-1 levels has important pathophysiological consequences for the development of obesity-related CVD. Evidence regarding the contribution of the different fat depots to plasma PAI-1 levels seems conflicting. While it has been shown that adipose tissue from different body fat depots contributes to plasma PAI-1 levels [6,8,23,28,29], others found PAI-1 expression in different adipose tissue depots not to be directly related to plasma PAI-1 levels [30,31]. What is also often not considered is the relative contribution of the different fat depots to plasma PAI-1 levels, in relation to other PAI-1 producing pathways associated with obesity such as IR, increased triglycerides, inflammation and endothelial dysfunction and their influence on the different PAI-1 producing tissues (adipose tissue, hepatocytes, smooth muscle cells, platelets and endothelial cells) in the body. This review aims to provide an overview of ex vivo and in vivo studies investigating the association between fat distribution and plasma PAI-1 levels. It also discusses the contribution of other PAI-1 producing pathways found in obesity in order to draw conclusions regarding the contribution of different adipose tissue depots to plasma PAI-1 levels.

5.3.3 Ex vivo studies

Several human ex vivo studies have been carried out that show adipose tissue to be an important source of PAI-1 (Table 5.1). These studies can be divided into studies investigating adipocyte PAI-1 content and or gene expression and those investigating plasma PAI-1 levels. Studies investigating adipocyte PAI-1 content and or gene expression strongly support a greater production and expression of PAI-1 mRNA in VAT compared with SCAT [12,13,22]. When

using human adipose tissue in culture from obese individuals, Alessi *et al.* [5] found PAI-1_{ag} concentration to be greater in VAT than SCAT. The association between VAT and PAI-1 seems to be related to structural and functional differences between VAT and SCAT. Visceral adipose tissue, present in the abdominal viscera in the mesentery and omentum, produces more pro-inflammatory cytokines, such as tumour necrosis factor- α (TNF- α), transforming growth factor β (TGF- β 1), interleukin-6 (IL-6) and IL-1 [28,32,33], and also contains more stromal cells, one of the main cellular components of adipose tissue that produces PAI-1 [12] as well as macrophages which also produce PAI-1 [34,35].

However, there are some contradicting findings in human *ex vivo* studies. Eriksson *et al.* [25] for example reported that PAI-1 expression from abdominal SCAT was approximately two times higher than abdominal VAT in severely obese white individuals. Additionally, females were found to have a significant depot-specific PAI-1 secretion, with SCAT producing higher levels of PAI-1 than VAT [25], while these differences were not observed in men. Differences in findings could possibly be explained by the cell size of the different adipose tissue depots or even differences in study populations (as will be discussed below). In contrast with other studies [5,25,28], Eriksson *et al.* [25] expressed their results as nanograms of PAI-1 expressed / 10⁷ cells, and not ng/g adipose tissue. Since the cell size of the SCAT was found to be larger than that of VAT, it could have influenced the results. In a review, Skurk and Hauner [14] even conclude that large fat cells produce more PAI-1 than small fat cells regardless of fat depot. Furthermore, Morange *et al.* [28] reported a high correlation between PAI-1 production by VAT and SCAT and concluded that there could be a similar regulatory pathway of PAI-1 in these two adipose depots, despite their different anatomic and metabolic characteristics. Differences can also be seen between different SCAT depots. When investigating PAI-1 expression from two subcutaneous territories (abdominal and femoral) in obese and lean individuals, the results indicated a higher PAI-1 mRNA content in the abdominal SCAT of the obese participants, which was also correlated with plasma PAI-1_{ag} levels, compared with the lean participants, while no difference in the PAI-1 mRNA content of the femoral SCAT was observed [29].

With regards to ex vivo studies, it should be kept in mind that the results can be influenced by the data collection and analytical method used. For example, data employing cultured human adipose tissue explants, suggest a direct contribution of visceral adipose tissue to plasma PAI-1 levels [8,28,36], while studies using native human adipose tissue found similar or even lower PAI-1 mRNA expression in VAT compared to SCAT [5,36,37]. Lindeman *et al.* [30] suggest that the increased PAI-1 release from adipose tissue explants is likely related to an incubation artefact rather than being a true reflection of the in vivo situation.

An increased adipocyte PAI-1 content does, furthermore not necessarily relate to increased plasma PAI-1 levels. As an example, in SCAT, elevated PAI-1 gene expression was found to be present during very low-calorie diets in obese participants, while plasma PAI-1 levels were decreased [38]. Furthermore, when comparing differences between VAT and SCAT depots, no correlation was found between PAI-1 mRNA and plasma PAI-1 (antigen and activity) in either one of the two fat depots [39]. On the other hand, Morange *et al.* [28] investigated the correlation between plasma PAI-1 and PAI-1 measured in adipose tissue explants by means of real-time polymerase chain reaction (PCR). They found a correlation between plasma PAI-1 (antigen and activity) and the PAI-1_{ag} level measured in cultured SCAT explants. This relationship was not investigated in VAT explants.

Due to the fact that ex vivo investigations are performed under controlled conditions outside the human body, it is also difficult to assess the total contribution adipose tissue might make to plasma PAI-1 levels when compared with other PAI-1 producing cells such as hepatocytes, platelets and endothelial and vascular smooth muscle cells. It is also possible that VAT and PAI-1 levels are concurrently related to abnormal fat metabolism, rather than the one bringing about the other [30]. Notwithstanding the fact that PAI-1 has a strong association with body fat distribution, these results suggest that assumptions regarding plasma PAI-1 levels based on adipose tissue PAI-1 content and gene expression should not be made.

5.3.4 *In vivo* studies

In vivo human studies support the strong association between PAI-1, obesity and body fat distribution and provide more evidence for the influence of adipose tissue depots on plasma PAI-1 levels [9,23,40-43]. *In vivo* studies often use anthropometric indicators [such as body mass index (BMI), waist-to-hip ratio (WHR), waist circumference (WC), weight loss programs etc.] and computerised tomography scan to assess the association of body fat distribution or differences in fat depots (SCAT and VAT) with plasma PAI-1 levels. Whereas, *ex vivo* studies use methods such as mRNA quantification or freshly collected or cultured adipose tissue samples, with or without stimulating factors. When considering the methodological differences between *in vivo* and *ex vivo* studies, it is not surprising that some studies have indicated that *ex vivo* results do not always lead to *in vivo* changes in PAI-1 plasma levels [38,39].

Several studies have used anthropometrical indicators like WC, BMI and WHR to investigate possible associations between plasma PAI-1 levels and body fat distribution [43-45]. BMI, as a general marker of body fat, was found to be strongly associated with PAI-1 [6,29,46] confirming the association of PAI-1 with total obesity. BMI does, however, not reflect body fat distribution and WC, as a measure of central obesity, was demonstrated to be a significant contributor to plasma PAI-1 levels [4,41,43,44] independent of BMI [41], supporting the strong association between plasma PAI-1, VAT and central obesity. Consistently, when compared with computerised tomography scan and ultrasound, WC has been regarded as a useful surrogate for the measurement of visceral fat [47], whereas WHR and BMI seem to be associated with VAT or plasma PAI-1 to a lesser extent [47,48].

On the other hand, a stronger correlation between BMI and plasma PAI-1, as compared with WC has also been demonstrated [42]. However, in this study WC and WHR correlated with plasma PAI-1 to a similar degree. Furthermore, after adjusting for WC, an inverse association between hip circumference and markers of coagulation was found, which possibly indicated a

protective effect of gluteal fat distribution as opposed to android obesity [42]. These anthropometrical differences associated with PAI-1 could be related to the inclusion of different study populations as will be discussed in more detail below. Nevertheless, the use of increased WC, as an indication of visceral fat mass, seems to be a marker of elevated PAI-1 plasma levels. However, such interpretations should be made with caution, particularly in terms of non-European populations as WC was not regarded as a suitable surrogate for assessing visceral fat mass in African women, following a weight loss diet [49].

The main focus of the following section will therefore be to discuss *in vivo* studies investigating the relationship between plasma PAI-1 levels and body fat distribution in relation to factors, identified from the literature, that may influence this relationship namely gender, ethnicity, level or degree of obesity, weight-loss, the 4G/5G PAI-1 polymorphism and other PAI-1 producing tissue in obesity.

5.3.4.1 Gender

It is known that body fat distribution is highly sex specific and that plasma PAI-1 levels differ between men and women [40,50]. Women generally have gluteal-femoral or peripheral depots (lower body obesity consisting of SCAT) compared with the android fat distribution of men (excess of body fat accumulation in the abdominal area that is associated with increased VAT) [51,52]. In layman's terms these are referred to as pear (lower body obesity) and apple (abdominal obesity) shape figures. Furthermore, adipocytes in the VAT depot is considered to be more metabolically active than adipocytes in the SCAT [32]. When considering the strong association between plasma PAI-1 and VAT, men would be expected to have higher plasma PAI-1 levels than women. Previous studies have confirmed this notion by demonstrating a direct correlation between VAT and plasma PAI-1 concentration in obese men, but not in women [53]. VAT has also been suggested to be an independent predictor of plasma PAI-1 activity (PAI-1_{act}) in healthy men [22]. On the contrary, when comparing abdominal VAT in

healthy non-obese, overweight men and women, greater PAI-1_{ag} levels were found in women than men [48]. In agreement, others have also reported a stronger association between WHR and plasma PAI-1 levels in women than men [24,50]. A possible explanation for the stronger relationship between PAI-1 and WHR in women could be related to a tendency of women with a preferential gluteal fat distribution to accumulate fat also in the abdominal area when becoming overweight/obese, leading to a greater WHR (and VAT) and consequently increased PAI-1 levels [40].

Previous research undertaken by our group, involving African participants, found PAI-1_{act} levels to be higher in women than in men, despite adjusting for differences in body composition. Plasma PAI-1_{act} furthermore associated not only more strongly, but also with more anthropometric variables in the men than the women [43]. Plasma PAI-1_{act} had a linear positive relationship with BMI and skinfold thickness in men, whereas in women, PAI-1_{act} plateaued at higher BMI levels and did not change across skinfold categories. Due to the preferential peripheral fat distribution (SCAT) in women and the preferential visceral (abdominal) fat accumulation in men, an obese man will have proportionally more VAT than an obese woman. Consequently, despite the higher PAI-1_{act} levels observed in women, the proportionally lower VAT may explain the overall lower correlations with anthropometric variables compared to the men. Furthermore, since women deposit gynoid fat preferentially, peripheral SCAT likely increased more than VAT in these obese African women, explaining the plateau in plasma PAI-1_{act} at higher BMI. While in men with a preferentially increased VAT deposition in the abdominal area, PAI-1_{act} levels continued to increase in conjunction with the increase in VAT. Similarly, skinfolds represent SCAT and in men, more VAT would have been deposited before an increase in SCAT is observed explaining the rise in PAI-1 levels across skinfold quintiles. Since women preferentially store peripheral SCAT, this adipose compartment will continue to increase in accordance with an increase in skinfold, while their VAT compartment remains proportionally smaller explaining the lack of difference across the skinfold quintiles. There also seems to be important differences in the size of adipocytes between VAT and SCAT depots in

women, as women were found to have larger SCAT adipocytes compared to VAT adipocytes [54] while no differences were observed for men.

According to Eriksson *et al.* [25] obese females seem to have a significant depot-specific PAI-1 secretion, with the SCAT depot producing higher levels of PAI-1 than the VAT depot, while this was not observed in men. Additionally, the authors suggested that SCAT is the most important adipose depot for PAI-1 secretion in obese individuals [25], since it is regarded to be a considerably larger fat depot than VAT [52] (see section on degree of obesity below). In support of this, measurements of abdominal VAT by means of computerised tomography scan have shown that VAT explained 28% only of the variance in plasma PAI-1_{act} in premenopausal women [23].

The menopausal status of women may also influence the relationship between different adipose tissue depots and plasma PAI-1. Compared to pre-menopausal women, post-menopausal women experience a redistribution of fat distribution (increased visceral fat accumulation) that is related to the female sex hormone oestrogen [55]. It should also be noted that therapeutic administration of oral oestrogen has been reported to reduce plasma PAI-1 levels [56]. Therefore, the inclusion of pre- and / or post-menopausal women in a study may influence the outcome of the association of body composition with plasma PAI-1 levels.

5.3.4.2 Ethnicity

When comparing the association between body composition and plasma PAI-1 levels between different ethnic groups, dissimilarities have been found. For instance, significant associations between plasma PAI-1_{ag} and VAT have been observed in white women, but not in African American women, despite similar PAI-1 levels and BMI ranges [57]. African women have also been found to have significantly lower PAI-1_{act} levels as well as a weaker association with markers of the MetS in comparison with white South African women [44]. Previous studies have

indicated that white Americans compared with black Americans have less VAT in spite of similar WC and BMI measurements [45,58]. Similar findings were also observed in studies that included black and white South Africans [59,60]. In a black and white South African sample, black women were found to have less VAT in comparison with white women after adjusting for age, height, weight and fat mass, despite the fact that there were no ethnic differences in WC, WHR, or dual-energy X-ray absorption measurement of the abdominal regions [61]. Similar to the VAT distribution in women, in a study by Hill *et al.* [45], significantly lower VAT was found in black than in white men after adjustment for percentage body fat, total body fat, BMI, WC, WHR or sagittal diameter. However, it is difficult to draw conclusions regarding the relationship of PAI-1 and body fat composition in African populations as such information is limited and most research investigating these issues has been undertaken in populations of European descent [6,13,25,38,45]. Owing to ethnic differences in body fat composition and fat deposition, the relationship of PAI-1 with body composition in Africans may differ from that observed for other population groups and, therefore, merits further investigation.

5.3.4.3 Level or degree of obesity

The general notion that increased plasma PAI-1 levels are associated with central obesity or android fat distribution does not seem to hold true in all circumstances. As mentioned previously, in severely obese individuals, PAI-1 expression from abdominal SCAT was found to be approximately two times higher than from abdominal VAT and it was proposed that the degree of obesity influences regional differences in PAI-1 secretion [25]. As previously explained this might, however, also have been the result of the way in which Eriksson *et al.* [25] reported their results i.e. in terms of cell size.

In support of this finding, when grouping participants according to different body composition patterns, the degree of obesity seems to have a significant influence on regional differences in PAI-1 secretion. African women with a proportionally higher abdominal VAT compartment have

been found to have significantly higher plasma PAI-1_{act} levels than women with a proportionally higher abdominal SCAT compartment (unpublished results). However, in women who were classified to be centrally obese, this was no longer the case. Within reason, it would seem therefore that the degree of obesity have an influence on the association between different body fat depots and plasma PAI-1 levels.

5.3.5 Weight-loss

Weight-loss that is associated with the loss of adipose tissue mass has been shown to be effective in lowering plasma PAI-1 levels in obese individuals [29,38,62-65]. Following gastric bypass in severely obese individuals, plasma PAI-1 levels were observed to normalise one month after surgery and PAI-1 expression, in both SCAT and VAT, was found to be lower than that of non-obese individuals within one year after surgery [62]. Mavri *et al.* [29] found that a three month weight reduction program resulted in a decline in plasma PAI-1 levels, which was only associated with loss of abdominal SCAT and not loss in the femoral adipose tissue. On the contrary, other studies have observed changes in plasma PAI-1 levels that correlated with changes in VAT and not SCAT after weight reduction in healthy pre-menopausal women [23]. Such findings are supported by other studies that observed VAT and plasma PAI-1 levels to decrease significantly after weight loss, in both men and women [53,66]. Yet, the relationship between VAT and PAI-1 disappeared in women after adjustment for total body fat mass [53]. Weight reduction due to surgical treatment or dieting seems to be an effective means to help lower plasma PAI-1 levels and promote a favourable haemostatic profile although whether this reduction is adipose depot specific remains a topic of investigation.

5.3.6 4G/5G PAI-1 polymorphism

Throughout the literature several PAI-1 polymorphisms have been identified, that may influence plasma PAI-1 levels, of these the 4G/5G PAI-1 polymorphism has been shown to exert the greatest impact [67,68] and is also thought to be associated with obesity and increased adipose tissue [11,69-72]. The 4G/5G is a single-nucleotide insertion / deletion polymorphism located at

position -675 in the promoter region of the PAI-1 gene that leads to either a 4G or 5G sequence [67]. In the 5G allele a repressor protein binds to the promoter area of the PAI-1 gene, resulting in relatively reduced transcription of PAI-1, and therefore in lower plasma PAI-1 levels [67,73,74]. Not all studies, however found a significant association between the 4G/5G genotypes and plasma PAI-1 levels and it is postulated that obesity may influence this relationship. The 4G/5G polymorphism is considered a response polymorphism meaning that the difference in PAI-1 levels between 4G and 5G becomes more obvious in the presence of environmental and/or disease factors, that stimulate PAI-1 expression [75], such as obesity. In agreement with this, some studies [11,72,76] found a stronger relationship between plasma PAI-1 levels and the 4G/5G polymorphism in obesity, although there were also some studies that did not find a difference in the strength of the relationship [77-79].

Results regarding the relationship of this polymorphism with obesity are also contradicting, as some studies [11,71,72,80] found carriers of the 4G allele to be more common among obese individuals while others did not [81-84]. Although, the mechanism of action explaining the possible association between the 4G/5G PAI-1 gene and obesity is not yet known, Demiralp *et al.* [85] suggested that overexpression of PAI-1 in 3T3-L1 cells (mouse cell line of fibroblasts that can differentiate into adipocytes) can increase adipocyte differentiation and that the 4G allele was significantly more active than the 5G allele in driving PAI-1 gene transcription, thus contributing to adipogenesis.

Despite possible interaction of plasma PAI-1 levels and the 4G/5G PAI-1 polymorphism with obesity, little is known regarding the relationship with body fat distribution, in particular the difference in VAT versus SCAT. In one study investigating the genetic variations within the PAI-1 gene that are related to the amount of body fat and regional fat distribution, the 4G/5G PAI-1 polymorphism was found to be associated with VAT in postmenopausal women [70]. Homozygotes for the 5G allele had 50% more VAT compared to carriers of the 4G allele. This suggests that it may be involved in modulating adipose tissue distribution at menopause [70]. In

agreement with this, Sartori *et al.* [11] found the 4G/5G polymorphism to influence PAI-1 expression in central obesity (VAT), but not in peripheral obesity (SCAT). Van Harmelen *et al.* [86] furthermore found no effect of the 4G/5G polymorphism on PAI-1 secretion from abdominal SCAT adipose tissue. Although not enough evidence is available to draw firm conclusions, these results suggest that the 4G/5G PAI-1 polymorphism may potentially influence the relationship between plasma PAI-1 levels and body fat distribution, and warrants further investigation.

5.3.7 Other PAI-1 producing tissue in obesity

As mentioned earlier, Janand-Delenne *et al.* [23] found VAT to explain only 28% of the variance in plasma PAI-1_{act} in premenopausal women. Also in a recent study (unpublished results) we found inflammation, endothelial dysfunction and triglycerides to contribute more to PAI-1_{act} variance in obese African women than body fat % *per se*. These results reiterate the concept that plasma PAI-1 levels, in obesity, are influenced by many different factors of which increased adipocyte mass is only one and that plasma PAI-1 in obesity seems to increase owing to a systemic response [87]. Other factors that can influence plasma PAI-1 levels include IR, inflammatory cytokines, oxidative stress as well as increased triglycerides, which can regulate PAI-1 production in all PAI-1 producing tissue and not adipose tissue only [3,88,89]. The association between PAI-1 and triglyceride has been previously explained by the fact that very low density lipoproteins (VLDL) triglycerides increase PAI-1 levels [6,90], through a VLDL response element identified in the promoter region of the PAI-1 gene that mediates VLDL-induced PAI-1 transcription in endothelial cells [91].

The largest contributor to PAI-1_{act} in our recent study was endothelial dysfunction (unpublished results). Evidence exists for the presence of endothelial dysfunction in obesity [92], which is thought to be induced by obesity-associated metabolic abnormalities such as IR, adipokines, oxidative stress, increased free fatty acids, tissue TNF- α as well as activation of innate immunity [93,94]. At the same time, increased PAI-1 is recognised as an early marker of endothelial

dysfunction [95,96] suggesting endothelial dysfunction as a mechanistically plausible modulator of PAI-1_{act} in obesity. Others have also identified hepatocytes and endothelial cells as underlying sources of elevated PAI-1 levels in obese individuals [36,62].

The association between inflammation and PAI-1 has been firmly established with a variety of cytokines such as TNF α , TGF- β 1, IL-1 and IL-6, acting as inflammatory mediators of PAI-1 [7,12,13,30,46,97-100]. Therefore, the chronic low grade pro-inflammatory environment in obesity can significantly contribute to plasma PAI-1 levels in the presence of several PAI-1 inducers [2]. These PAI-1 stimulating effects of pro-inflammatory cytokines are not only limited to adipocytes, but are also present in hepatocytes, smooth muscle cells and endothelial cells [101,102]. In a recent study, the ability of the acute systemic inflammatory response to activate gene expression and PAI-1 production in VAT was demonstrated. According to Ekström [9], during open heart surgery, PAI-1 mRNA expression was found to increase 27-fold in the omental (visceral) adipose tissue, compared with a threefold increase in the SCAT, followed by an increase in plasma PAI-1_{ag} levels. TGF- β 1 seems to regulate PAI-1 expression as part of an autocrine and paracrine function [99]. Particularly in subcutaneous adipocytes, TGF- β 1 has been proposed as a main inducer of PAI-1 mRNA synthesis [25,99], although others have reported no significant difference in the effect of TGF- β 1 on PAI-1_{ag} secretion between the two fat depots (VAT and SCAT) [13].

Apart from adipocytes, adipose tissue furthermore contains endothelial cells as well as fibroblasts, smooth muscle cells and macrophages, which are all involved in the expression of PAI-1 mRNA [8]. Other factors associated with obesity that can influence plasma PAI-1 levels, include oxidative stress, circadian clock proteins, cortisol and angiotensin-converting enzyme [reviewed by 103].

5.3.8 Conclusion

While *ex vivo* evidence indicates VAT to be the main PAI-1 producing adipose depot due to structural and functional differences compared to SCAT, this does not always relate to increased plasma PAI-1 levels as depicted in Figure 5.1. Particularly in obesity and in females where the SCAT depot is often significantly larger than the VAT depot, SCAT becomes a significant contributor to plasma PAI-1 levels and, therefore, also to increased cardiovascular disease risk. Also owing to ethnic differences in body fat composition and fat deposition, the relationship of PAI-1 with body composition in non-European populations may differ from that observed for Europeans and, therefore, merits further investigation. Furthermore, genetic variation such as the 4G/5G PAI-1 polymorphism may influence the relationship between VAT and plasma PAI-1 although more evidence is needed to confirm this. As PAI-1 is produced by many different tissues, the contribution of other tissue such as endothelial cells, particularly in the case of endothelial dysfunction, to plasma PAI-1 levels in obesity should also not be negated and should be considered as a major contributor to plasma PAI-1 levels.

Practice Points

- The contribution of body fat distribution to increased plasma PAI-1 levels has important pathophysiological consequences for the development of obesity-related CVD risk.
- Despite VAT being the main PAI-1 producing adipose depot, due to structural and functional differences compared to SCAT, this does not always relate to increased plasma PAI-1 levels.
- PAI-1 production in adipose tissue explants studied *ex vivo* should not be directly related to the *in vivo* situation due to the possible occurrence of incubation artefact effects, leading to the misinterpretations of results.
- Factors such as gender, ethnicity, possibly genetics, level or degree of obesity, and other PAI-1 producing tissues are important when considering the relative contribution of different

fat depots to plasma PAI-1 levels, since these factors not only influence the plasma PAI-1 levels, but also the relative associated CVD risk.

Research Agenda

- More studies are needed to understand the relationship of PAI-1 with body composition in non-European populations, as most studies included European populations only and data pertaining to non-European populations are limited.
- The relative contribution of endothelium dysfunction to plasma PAI-1 levels in obesity is often overlooked, though could assist in our understanding of the relationship between obesity and PAI-1, and therefore warrants more detailed investigation.

Conflict of interest statement

The authors have no conflict of interest to declare.

Acknowledgements

This work was funded through the National Research Foundation (NRF) of South Africa (Grant UID: 94213). Opinions expressed and conclusions arrived at, are those of the authors and are not to be attributed to the NRF.

5.3.9 References

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Legend to Table

Table 5.1: Summary of studies investigating the association of PAI-1 production and mRNA levels in adipose tissue (VAT versus SCAT)

BMI, body mass index; mRNA, messenger ribonucleic acid; PAI-1, plasminogen activator inhibitor-1; PAI-1_{act}, PAI-1 activity; PAI-1_{ag}, PAI-1 antigen; SD, standard deviation; SCAT, subcutaneous adipose tissue; TGF- β 1, transforming growth factor beta 1; TNF- α , tumour necrosis factor-alpha; VAT, visceral adipose tissue; VLCD, very low calorie diet; yrs., years; $\uparrow$, increased PAI-1 synthesis and/or production.

Legend to Figure

Figure 5.1: The association between VAT and plasma PAI-1 levels and the factors modulating this association

VAT, visceral adipose tissue; PAI-1, plasminogen activator inhibitor-1; SCAT, subcutaneous adipose tissue; $\uparrow$, increased VAT and plasma PAI-1 levels; $\leftrightarrow$ association.

Table 5.1: Summary of studies investigating the association of PAI-1 production and mRNA levels in adipose tissue (VAT *versus* SCAT)

Author, Year	Aims	Participant characteristics (mean±SD)	Adipose tissue measured	PAI-1 measured	Relevant finding
Bastelica <i>et al.</i> , 2002 [12]	Compare the localisation of PAI-1 synthesis between human VAT and SCAT.	n= 26 Twenty-two white women and four men BMI 41±6.3 kg/m ² Age 41±12 yrs.	Cryosections of freshly collected human adipose tissue (in culture). Samples were collected during gastropasty.	Supernatant PAI-1 _{ag} , plasma PAI-1 _{ag} and PAI-1 _{act} and <u>PAI-1</u> mRNA in adipocytes.	↑ PAI-1 synthesis in VAT compared with SCAT. PAI-1 production is related to the presence of stromal cells, which are more numerous in VAT than in SCAT.
Gottschling-Zeller <i>et al.</i> , 2000 [13]	Compare <u>PAI-1</u> expression and secretion in omental fat and SCAT from obese and non-obese participants.	n=11 Obese individuals (eight women and three men) BMI 45±8.7 kg/m ² Age 39±13.8 yrs. n=7 non-obese individuals (two women and five men) BMI 24±2.7 kg/m ² Age 41±13.9 yrs. All participants were of Caucasian origin.	Isolated SCAT and omental abdominal adipocytes (in suspension culture) from severely obese (who underwent vertical gastric banding for	PAI-1 _{ag} and <u>PAI-1</u> mRNA in adipose tissue.	↑ PAI-1 synthesis in VAT compared with SCAT. Omental adipocytes release significantly more PAI-1 <i>in vitro</i> compared to subcutaneous adipocytes from obese and non-obese individuals. Did not find endothelial cells (present in adipose tissue) to contribute to the production of PAI-1. Observed that TGF-β1 contributes to the regulation of PAI-1 secretion.

			weight reduction) and non-obese individuals.		
Cigolini <i>et al.</i> , 1999 [22]	Aimed to determine whether TNF- α influence PAI-1 production in human adipose tissue.	n=35 Non-neoplastic, overweight individuals BMI 28 $\pm$ 5 kg/m ² Seven individuals were surgical patients, for whom both SCAT and VAT were obtained. Twenty-eight individuals were outpatients from which SCAT was obtained by needle biopsies.	Omental and SCAT biopsies (incubated, isolated adipocytes) from obese non-diabetic individuals.	PAI-1 _{ag} and <u>PAI-1</u> mRNA in adipose tissue.	↑ PAI-1 expression and release in VAT compared with SCAT. However, do not support VAT to be the only fat depot that contributes to plasma PAI-1 levels. These two fat depots were found not to be substantially different from each other, with regards to response to TNF- α stimulation. It was concluded that similar factors control expression in VAT and SCAT under these experimental conditions.
Alessi <i>et al.</i> , 1997 [5]	Investigated PAI-1 expression by human adipose tissue and its different cellular fractions, in particular PAI-1 _{ag} production from VAT (omental) <u>versus</u> SCAT.	n=13 women BMI 26-29 kg/m ² Only in seven participants comparison between VAT and SCAT was preformed (four men and three women) BMI 18-28 kg/m ² Age 39-79 yrs.	Tissue was obtained during elective abdominoplasty (incubated).	PAI-1 _{ag} and <u>PAI-1</u> mRNA in adipocytes.	↑ PAI-1 production in VAT compared with SCAT. Supports the important contribution of VAT in determining plasma PAI-1 levels.

Lindeman <i>et al.</i> , 2007 [30]	Systematically evaluate the molecular basis of the association between VAT and plasma PAI-1 in humans.	Men and women were included and grouped according to BMI. BMI 20-32 kg/m ² in one group and morbid obese participants (BMI>40 kg/m ²) in the other group.	Tissue was obtained during elective aneurysm repair (normal weight and overweight individuals) or during gastric banding for morbid obesity.	Plasma PAI-1 _{ag} and <u>PAI-1</u> mRNA from adipose tissue explants.	Data corroborates a strong relationship between VAT and plasma PAI-1 levels. However, low and similar <u>PAI-1</u> mRNA expression in SCAT and VAT excluded VAT as a relevant source of plasma PAI-1. The authors conclude that VAT and plasma PAI-1 are co-correlated rather than causatively related.
Polac <i>et al.</i> , 2001 [39]	To substantiate the link between intra-abdominal VAT and circulating PAI-1 by performing analysis of <u>PAI-1</u> expression in human VAT and SCAT.	n=28 Post-menopausal women BMI 28.29±4.32 kg/m ² Age 53.59±6.35 yrs.	Samples of VAT and SCAT were obtained in the beginning of a gynaecological operation.	Plasma PAI-1 _{ag} and <u>PAI-1</u> mRNA in adipose tissue.	Indicates similar <u>PAI-1</u> mRNA expression in both SCAT and VAT that seems to be influenced by lipid metabolism. No correlation was found between plasma PAI-1 and VAT or SCAT <u>PAI-1</u> mRNA.
Morange <i>et al.</i> , 1999 [28]	Aimed to investigate the relation between the production of PAI-1 by adipose tissue, plasma PAI-1 levels and	n=30 Participants (three men and 27 women). Age 16-70 yrs. (mean = 47) BMI 21-42 kg/m ² (mean = 27) VAT and SCAT were	Tissue was obtained during elective abdominal surgery	Plasma PAI-1 _{ag} and PAI-1 _{ag} in adipose tissue.	Observed a similar correlation between PAI-1 production from VAT and SCAT. Further suggested a similar regulatory pathway of PAI-1 in the two fat depots. Supports the role of adipose tissue in determining plasma PAI-1

	variables related to insulin resistance.	compared in a sub-group of 16 participants that included seven men and nine women Age 35 to 79 yrs. BMI 18-39 kg/m ² (mean = 27).	(incubated explant).		levels and the important contribution of TNF- α and TGF- β in stimulating PAI-1 production.
Dusserre <i>et al.</i> , 2000 [37]	Investigated the difference in gene expression of six proteins secreted by adipocytes obtained from abdominal VAT and SCAT.	n=9 Five women and four men BMI 34 $\pm$ 4 kg/m ² Age 42 $\pm$ 5 yrs.	Adipose tissue biopsies were collected during elective open abdominal surgery.	<u>PAI-1</u> mRNA in adipose tissue.	Observed similar <u>PAI-1</u> mRNA expression in SCAT and VAT.
Bastard <i>et al.</i> , 2000 [38]	Aimed to determine if the changes in SCAT <u>PAI-1</u> expression influence plasma PAI-1 levels during weight loss in obese humans following a VLCD.	n=15 Fourteen women and one male participant, all of Caucasian descent. BMI >30 kg/m ² Age 48 $\pm$ 3 yrs.	Samples were obtained from the abdominal skin of participants by a liposuction mini-cannula attached to a syringe.	Plasma PAI-1 _{ag} and PAI-1 _{ag} and <u>PAI-1</u> mRNA in adipose tissue.	↑ <u>PAI-1</u> mRNA and PAI-1 concentration in SCAT during VLCD. Suggest therefore that the changes in SCAT PAI-1 expression are not related to the decrease of plasma PAI-1 levels observed during VLCD in obese participants.
Eriksson <i>et al.</i> , 2000 [25]	To determine differences in adipose gene	n=22 Obese individuals Fourteen women and eight	Abdominal SCAT and VAT were	PAI-1 _{ag} and <u>PAI-1</u> mRNA in adipose	↑ Secretion of PAI-1 and <u>PAI-1</u> gene expression were found in SCAT compared with

	expression and protein secretion rate of PAI-1 between SCAT and VAT.	men BMI 43.1±1.3 kg/m ² All participants were of Caucasian descent and born in Sweden.	obtained during weight-reduction surgery (adjustable gastric banding).	tissue. Also expressed results as PAI-1 concentration / 10 ⁷ cells.	VAT. PAI-1 secretion rate correlated with cell size in SCAT and SCAT cell size was larger than VAT cell size. The depot specific difference in PAI-1 secretion was confined to obese women and not to men.
Mavri <i>et al.</i> , 2001 [29]	To determine if the changes in <u>PAI-1</u> mRNA content in two SCAT depots (abdominal and femoral) are associated with changes in plasma PAI-1 after weight-loss.	n=40 Obese individuals Twenty-six women and four men BMI >25 kg/m ² Age 40 ± 10 yrs. Also sixteen lean participants, 17 women and seven men. BMI 25 kg/m ² Lean participants had a similar age range as the obese participants.	SCAT biopsies were obtained from the sub-umbilical abdominal region and lateral femoral region by means of a needle.	Plasma PAI-1 _{act} and PAI-1 _{ag} as well as <u>PAI-1</u> mRNA in adipose tissue.	Obese participants had ↑ plasma PAI-1 and <u>PAI-1</u> gene expression (in abdominal SCAT) compared to lean participants. Plasma PAI-1 levels were associated with <u>PAI-1</u> expression in the abdominal adipose tissue, whereas femoral SCAT showed similar <u>PAI-1</u> mRNA in obese and lean participants. The authors concluded that only abdominal and not femoral SCAT <u>PAI-1</u> expression is a contributor to increased plasma PAI-1 levels in obesity. Both plasma PAI-1 and <u>PAI-1</u> mRNA in abdominal SCAT also decreased following weight-loss.
Shimomura <i>et al.</i> , 1996 [8]	To investigate the relationship between plasma PAI-1 levels and the amount of fat tissue	n=101 Sixty-one obese participants (31 men and three women) BMI 31±5 kg/m ²	RNA was extracted from the intra-abdominal SCAT	Plasma PAI-1 _{ag} and <u>PAI-1</u> mRNA in adipose tissue from rats.	Although both VAT and SCAT expressed <u>PAI-1</u> mRNA, <u>PAI-1</u> expression ↑ in VAT but not in SCAT during the development of obesity in rats. In both obese and non-obese humans,

	(VAT <i>versus</i> SCAT) and changes in <i>PAI-1</i> gene expression in adipose tissue during development of obesity in humans and animals.	Age 43±13 yrs. Forty non-obese participants (37 men and 13 women) BMI 23±2 kg/m² Age 44±14 yrs. SCAT and VAT from humans were assessed by means of computerised tomography. n=15 Female Sprague-Dawley rats (12 weeks of age).	and VAT from control rats and rats with a ventromedial hypothalamus lesion.		plasma PAI-1 correlated to VAT, but not SCAT. The authors concluded that enhanced <i>PAI-1</i> gene expression from VAT might contribute to increased plasma PAI-1 levels.

BMI, body mass index; mRNA, messenger ribonucleic acid; PAI-1, plasminogen activator inhibitor-1; PAI-1_{act}, PAI-1 activity; PAI-1_{ag}, PAI-1 antigen; SD, standard deviation; SCAT, subcutaneous adipose tissue; TGF-β1, transforming growth factor-beta 1; TNF-α, tumour necrosis factor-alpha; VAT, visceral adipose tissue; VLCD, very low calorie diet; yrs., years; ↑, increased PAI-1 synthesis and/or production

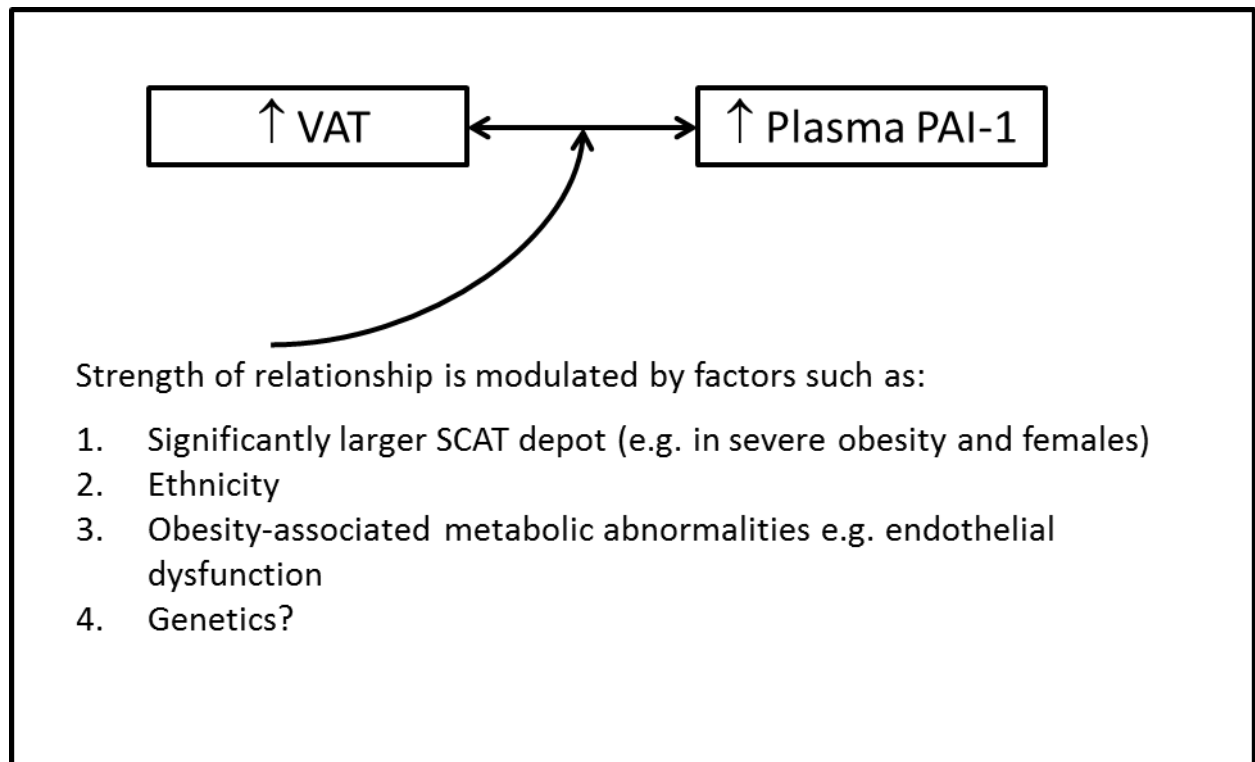


Figure 5.1: The association between VAT and plasma PAI-1 levels and the factors modulating this association

VAT, visceral adipose tissue; PAI-1, plasminogen activator inhibitor-1; SCAT, subcutaneous adipose tissue; ↑, increased VAT and plasma PAI-1 levels; ↔ association.

CHAPTER 6 CONCLUSION AND RECOMMENDATIONS

6.1 Introduction

This chapter provides a summary of the most important findings of the three articles presented in Chapters 3, 4 and 5 of this thesis. In each of these chapters the results were discussed, interpreted and compared with the relevant literature. In this chapter, conclusions based on the research findings and recommendations for future research will be provided. The relevance of these results will also be highlighted against the broader scientific background. The main findings and conclusions drawn are aimed at addressing the objectives of this study. Therefore, the main aim and objectives introduced in Chapter 1 are repeated below, followed by a general discussion and conclusion.

Aim and objectives:

Aim:

The aim of this study was, to determine two unresolved methodological and clinical issues related to PAI-1, as identified in the black South African population.

Objectives:

1. To determine the effect of residual platelets, present in plasma, on different plasma PAI-1 assays (total PAI-1 content, active PAI-1 and tPA/PAI-1 complex) as well as a functional marker namely clot-lysis time (CLT);
2. to explore the relationship of PAI-1 with body fat in African women with special focus on excessive obesity, sarcopenic obesity and body fat distribution patterns; and
3. to review *ex vivo* and *in vivo* studies investigating the association between body fat distribution and plasma PAI-1 levels, in order to identify factors that may potentially influence the relationship and to determine the contribution of body fat to plasma PAI-1 levels relative to that of other PAI-1 producing tissue.

6.2 The effect of residual platelets, present in plasma, on different plasma PAI-1 assays (total PAI-1 content, active PAI-1 and tPA/PAI-1 complex)

Plasminogen activator type-1 (PAI-1) is an important inhibitor of fibrinolysis (Rijken & Lijnen, 2009) and it is also associated with the risk of developing atherothrombosis (Salomaa *et al.*, 1995; Smith *et al.*, 2005; Peng *et al.*, 2008). PAI-1 exists in either an active or latent form or in a complex with tissue plasminogen activator (tPA) (Hekman & Loskutoff, 1985; Sprengers & Kluft, 1987; Gils & Declerck, 2004). PAI-1 is stored in the alpha granules of platelets (Sprengers & Kluft, 1987; Declerck *et al.*, 1988a; Alessi *et al.*, 1997) and is produced by a variety of cells, such as endothelial cells, hepatocytes, smooth muscle cells and adipocytes (Skurk & Hauner, 2004). The accepted view concerning the main form of PAI-1 released from alpha granules of platelets has recently been challenged by controversial evidence. Declerck *et al.* (1988a) stated that the alpha granules of platelets were traditionally thought to contain mostly *latent* PAI-1, while more recent publications suggest that the alpha granules of platelets also contain *active* PAI-1 (Nordenhem & Wiman, 1997; Brogren *et al.*, 2004; Brogren *et al.*, 2011). What is still not clear is how the PAI-1, which is released from the alpha granules of residual platelets in plasma, affects the plasma levels of the different forms of PAI-1 (activity, antigen and tPA/PAI-1 complex) as well as the fibrinolytic potential of plasma. We postulated that residual platelets in plasma might have different effects on apparent PAI-1 levels, which may be the result of different assays used, differences in sample handling such as type of blood collection tube, centrifugation speed and time, additionally also platelet concentration and size. We therefore, investigated the effect of residual platelets in plasma on PAI-1 and PAI-1-related assays by relating a marker of alpha granule release (beta thromboglobulin (β TG)), as well as platelet concentration and size, to PAI-1 activity (PAI-1_{act}), PAI-1 antigen (PAI-1_{ag}), tPA/PAI-1 complex and a functional parameter of fibrinolysis, fibrinolytic potential, measured as plasma clot lysis time (CLT).

We analysed PAI-1_{ag}, PAI-1_{act}, tPA/PAI-1 complex and CLT in platelet-containing plasma prepared at two centrifugation speeds (352 and 1500 g) of 151 participants from the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study. A limitation of the study was that plasma platelet count was not determined and it was not possible to calculate to what degree plasma PAI-1 levels were confounded by *in vitro* platelet activation and or degradation. A follow-up study was, therefore performed to obtain platelet count and size as well as plasma β TG and PAI-1_{ag} concentrations in a similar population group as the original study population. In this follow-up study a variety of plasma types were prepared: platelet-poor plasma (PPP – 2000 g), platelet-containing plasma (352 g) and platelet-rich plasma (PRP – 200

g). Because it is also important for this research to obtain basal plasma PAI-1_{ag} levels not confounded by *in vitro* platelet activation and/or degradation, PPP was collected in citrated tubes containing platelet stabilisers (CTAD tubes). The citrated plasma samples (352 g and 200 g) were analysed in a fresh-frozen state and also after five defrost-refreeze cycles to ensure complete alpha granule release from the platelets; this was done to determine the total platelet PAI-1_{ag} and β TG content. It was determined that plasma β TG had a significant association with PAI-1_{ag} levels and weak associations only with PAI-1_{act} and CLT. Upon dividing the SABPA study population into quartiles based on β TG levels, PAI-1_{ag} increased significantly across the quartiles while PAI-1_{act} and CLT tended to increase in the 4th quartile only, suggesting possible functional effects on plasma fibrinolytic potential in the presence of high platelet counts (such as PRP) only. In addition, the results indicated that platelet count strongly affected plasma PAI-1_{ag} in a concentration dependent manner. Strong correlations between PAI-1_{ag} levels, platelet count and β TG levels in the different plasma preparations containing platelets (325 g and 200 g) were observed. Evidence for the size of the contribution which platelets can make to plasma PAI-1_{ag} was found in that the basal PAI-1_{ag} level in PPP increased up to 22 fold in PRP. With the maximum degradation of the platelets (5x freeze-thaw cycles) a further increase in plasma PAI-1_{ag} was observed. This accentuates the importance of the correct plasma preparation protocol in order to standardise platelet count and to ensure the preparation of PPP ($<10 \times 10^3/\mu\text{L}$). It was unfortunately not possible to determine to what extent the presence of platelet stabilisers can inhibit the effects of platelets in plasma on PAI-1 and related assays, as CTAD tubes were used to prepare PPP only. This should be investigated in future studies on this topic. Also in the first sub-study, the data that was used to compare the effect of the two centrifugation speeds were collected from different individuals. A scientifically stronger design would have been to prepare the samples from the same individuals at two different speeds to prevent inter-individual differences (potentially) confounding the results obtained. This option was unfortunately not available to us and since the inclusion criteria were quite specific, providing a homogenous study population, and the individuals randomly divided into the two sample preparation groups, the results likely reflect a true relationship between β TG and PAI-1_{ag}.

The latter findings suggests that platelets likely contain both latent and active PAI-1, but that a high plasma platelet content (such as in PRP) is required before the active PAI-1 present in platelets has functional fibrinolytic effects. This data therefore aids in settling the debate regarding the main form of PAI-1 present in platelets (Declerck *et al.*, 1988a; Nordenhem & Wiman, 1997; Brogren *et al.*, 2004). This study provides direct evidence of the effect of residual

platelets on plasma PAI-1 and PAI-1 related assays. We conclude that PAI-1_{ag} is more sensitive to the presence of platelets in plasma than is PAI-1_{act} or the functional marker, CLT. We also found that platelets present in plasma do not initially release all of its PAI-1 content and that it releases additional PAI-1 upon further or complete degradation. Our results, consequently, highlight the importance of precise protocol control when measuring plasma PAI-1_{ag}.

6.3 The relationship of PAI-1 with body fat in African women with special focus on excessive obesity, sarcopenic obesity and body fat distribution patterns

In the recent National Health and Nutrition Examination Survey (SANHANES) of South Africa, a significant increase in the prevalence of obesity and overweight in older women compared to men was observed (Shisana *et al.*, 2013). Besides being an important inhibitor of fibrinolysis, PAI-1 plays an important role in obesity, specifically central obesity, the metabolic syndrome (MetS) and insulin resistance (IR) (Juhan-Vague & Alessi, 1997; Mertens *et al.*, 2006; Greyling *et al.*, 2007). Although the association between PAI-1 and obesity is well known, the association between PAI-1 and body fat distribution patterns is less clear, particularly in non-white ethnicities with demonstrated differences in body fat distribution patterns and PAI-1 levels. For instance, the association of PAI-1 with visceral adipose tissue (VAT) and MetS has been shown to differ between black and white South Africans (Greyling *et al.*, 2007). Micklesfield *et al.* (2010) found no association between PAI-1 and VAT in African Americans, but a significant association between PAI-1 and VAT in Caucasians. Work that was done before that of Micklesfield *et al.* (2010) by Vague *et al.* (1989), De Pergola *et al.* (1997) and Alessi *et al.* (2000b) have also shown differences in body fat distribution patterns between ethnicities, with African women having significantly less VAT compared to women of European descent, despite similar waist circumferences (WC). Therefore, it is possible that the association of PAI-1 with WC, which is often used a marker of central adiposity, may differ between ethnic groups, depending on the type of adipose tissue (VAT vs SCAT) deposited in the abdominal area. The main PAI-1 producing adipose tissue is VAT, while, in general, sub-cutaneous adipose tissue (SCAT) produces less PAI-1. According to Alessi *et al.* (1997) and Bastelica *et al.* (2002) this is caused by a higher amount of stromal cells in VAT. Alessi *et al.* (1997) and Ibrahim (2010) indicates that it also the result of the production of pro-inflammatory cytokines and higher macrophage content / infiltration found in VAT. In addition, the importance of PAI-1 expression in abdominal SCAT has also been demonstrated (Eriksson *et al.*, 2000; Mavri *et al.*, 2001). While WC is often used as a surrogate marker for abdominal obesity, the effect of the ratio of abdominal VAT to abdominal SCAT (which seem to differ between ethnicities) despite a similar

WC, on PAI-1 levels, has however, received far less attention. We, therefore, hypothesised that due to ethnic differences in body fat composition and fat deposition, the relationship of PAI-1 with body composition in Africans may differ from that observed for other population groups and, therefore, necessitate its study in non-white individuals. Limited information is also available regarding the effect of specific forms of obesity, such as sarcopenic obesity (SO), on PAI-1 (Cesari *et al.*, 2005; Cesari *et al.*, 2010). Due to the relative close association between IR, MetS and SO (Kohara, 2014) as well as the association between PAI-1, IR and Mets (Alessi & Juhan-Vague, 2008), it was postulated that PAI-1 might be higher in SO individuals than obese individuals, who are not sarcopenic.

The association of PAI-1_{act} with body fat percentage and two different fat distribution patterns, namely SO and preferentially VAT compared to SCAT abdominal obesity in 246 African women were, therefore, investigated by creating sub-groups according to different body fat distribution patterns. The results of this investigation showed that the PAI-1_{act} levels of the SO group did not differ significantly from that of women in the excessive percentage body fat (the non-sarcopenic) group. This can be attributed to the fact that in the population participating in the research, the insulin-related markers and the MetS status of the two groups did not differ. Future studies should focus on the inclusion of SO individuals with confirmed MetS status and / or insulin abnormalities, to better answer the question of whether PAI-1 will be further increased in these individuals. The results of the present study confirmed that the relationship of PAI-1_{act} with body fat percentage, insulin, triglycerides and appendicular skeletal mass (ASM) were influenced by body fat distribution patterns together with the degree of obesity, suggesting that the association of PAI-1 with the mentioned variables is not a constant or fixed relationship, but that the relative contributions of these variables to PAI-1 are influenced by body fat distribution patterns. In comparing preferential VAT to SCAT central obesity, it was established that PAI-1_{act} is higher in women with a proportionally higher abdominal VAT compared to the higher abdominal SCAT compartment in the total study population. This was not the case in the centrally obese sub-group, suggesting that, when central obesity is already present, VAT no longer contributes proportionally more to plasma PAI-1 levels than SCAT. It seems that in the presence of large SCAT depots, such as in women or obese individuals, SCAT becomes a significant contributor to plasma PAI-1 levels. In general this statement is in agreement with the literature in which it was demonstrated that *PAI-1* messenger ribonucleic acid (mRNA) in abdominal SCAT (but not femoral SCAT) is positively correlated with plasma PAI-1 levels in obese individuals (Mavri *et al.*, 2001). It is, furthermore, confirmed by the finding that *PAI-1* expression in SCAT, is higher than in VAT in obese individuals and in females (Eriksson *et al.*,

2000). We also determined the contribution of body fat percentage to plasma PAI-1_{act} taking other metabolic abnormalities associated with obesity into consideration. We demonstrated for the first time that in African women with increased fat mass, body fat percentage contributes to plasma PAI-1_{act} to a lesser extent than other obesity-related metabolic derangements such as inflammation and endothelial dysfunction.

Results from our study make a significant contribution to the limited information available regarding the association of PAI-1 with body composition in Africans. Results obtained from individuals of European descent cannot simply be extrapolated to Africans, due to the known differences in body fat distribution as well as known differences in PAI-1 levels, necessitating these studies in non-white individuals.

6.4 The association between body fat distribution and plasma PAI-1 levels, potential factors influencing this relationship and the contribution of body fat to plasma PAI-1 levels relative to that of other PAI-1 producing tissue

While performing the study mentioned in Section 6.3, it was discovered that there is a large body of evidence on this topic, but with contradicting results. It was, therefore, decided to investigate the apparent discrepant findings between the different studies to provide an overall clearer picture of the association of PAI-1 with body composition. Increased PAI-1 is considered a mechanistic pathway by which obesity contributes to the increased risk of CVD in overweight and obese individuals (Kohler & Grant, 2000). Elevated plasma PAI-1 is considered a biochemical marker of obesity (Phelan & Kerins, 2014) and is also part of the MetS (Mertens *et al.*, 2006; Alessi & Juhan-Vague, 2008). As far as obesity is concerned there are several pathways that contribute to increased plasma PAI-1 production, with abdominal adipose tissue considered a major source. The association between PAI-1 and obesity, especially central obesity, has been well established in research in both humans and animals (Samad & Loskutoff, 1996; Shimomura *et al.*, 1996; Alessi *et al.*, 1997; Eriksson *et al.*, 1998; Alessi *et al.*, 2003; Ekström *et al.*, 2012). This association is considered to be largely the result of PAI-1 produced by adipose tissue (Alessi *et al.*, 1997; Eriksson *et al.*, 1998; Gottschling-Zeller *et al.*, 2000; Bastelica *et al.*, 2002). PAI-1 in adipose tissue is produced by a variety of cells that include pre-adipocytes, mature adipocytes, stromal cells, endothelial cells, smooth muscle cells and monocytes/macrophages (Skurk & Hauner, 2004).

Fat depot specific PAI-1 production has furthermore been identified with VAT, as an ectopic fat depot, generally producing more PAI-1 than SCAT (Alessi *et al.*, 1997; Cigolini *et al.*, 1999). Ectopic fat refers to the storage of fat in non-adipose tissue (such as the liver, skeletal muscles, viscera, pancreas and heart) that seems to accumulate when there is an overflow of triglycerides into other organs as SCAT loses the ability to expand and to store excess energy (Heilbronn *et al.*, 2004; Morelli *et al.*, 2013; Thomas *et al.*, 2013). However, contradicting evidence also exists regarding the main PAI-1 producing depot, as some studies have shown comparable PAI-1_{ag} secretion from VAT and SCAT (Alessi *et al.*, 2000b), or even higher *PAI-1* mRNA expression and increased rate of PAI-1_{ag} synthesis in SCAT than in VAT (Eriksson *et al.*, 2000). Furthermore, an increase in PAI-1 production by a specific fat depot does not necessarily lead to increased plasma PAI-1 levels. Even though several research investigations and reviews have focussed on the relationship between (central) obesity and PAI-1, there is no review investigating the contribution of different body fat depots to plasma PAI-1 levels. Also, despite VAT being the most prominent PAI-1 producing tissue, the relationship with plasma PAI-1 levels is less clear. There is also contradicting conditional evidence indicating SCAT to be the major PAI-1 producing adipose tissue depot instead of VAT.

The aim of this review was, therefore, to provide an overview of *ex vivo* and *in vivo* studies investigating the association between body fat distribution and plasma PAI-1 levels. Factors that seem to influence or regulate this relationship, including gender, ethnicity, level or degree of obesity and weight loss were discussed, while the contribution of other tissue to plasma PAI-1 level in obesity was also considered in order to place the relative contribution of adipose tissue depots into perspective. Although deduction from the literature lead to the conclusion that despite *ex vivo* evidence identifying VAT to be the main PAI-1 producing adipose depot due to structural and functional differences compared to SCAT, this does not always relate to increased plasma PAI-1 levels. SCAT, furthermore, seems to significantly contribute to plasma PAI-1 levels, particularly in obese women who have a greater tendency to accumulate SCAT rather than VAT. This research also highlights the possibility that the increased plasma PAI-1 levels associated with obese women may consequently increase their CVD risk. The need for future studies in non-European populations is demonstrated by the findings on the importance of ethnic differences in plasma PAI-1 and body composition. Further research is also needed to confirm the possible influence of genetic variation, such as the 4G/5G *PAI-1* polymorphism, on the relationship between VAT and plasma PAI-1. This review is the first to highlight the contribution of other tissue to plasma PAI-1 levels, such as endothelial cells, particularly in the case of endothelial dysfunction that is often associated with obesity. Finally, evidence on the

association between body fat distribution and plasma PAI-1 levels is summarised; it is also accentuated that this relationship is not fixed but can be modulated by a number of different factors which include gender, ethnicity, possibly genetics, level or degree of obesity and other PAI-1 producing tissues.

6.5 Recommendations for future research

1. The findings from our study highlight the importance of precise protocol control, especially when measuring plasma PAI-1_{ag}. It is, therefore, recommended that future studies should ensure standardised platelet count and preferentially the use of platelet poor plasma ($<10 \times 10^3/\mu\text{L}$) when measuring PAI-1, and in particular PAI-1_{ag}, to prevent any effect that residual platelets in plasma might have on the measurement of plasma PAI-1 levels. Furthermore, inconsistent ethnic differences as shown by previous research measuring PAI-1 levels in African and white ethnic groups (Jerling *et al.*, 1994; Festa *et al.*, 2003; Matthews *et al.*, 2005; Lutsey *et al.*, 2006; Greyling *et al.*, 2007; Naran *et al.*, 2008; Perry *et al.*, 2008) should be investigated. In such ethnic comparison studies, a standardised PAI-1 assay should be employed as well as standardised sample handling procedures in order to determine true ethnic differences not confounded by analytical differences.
2. Considering our finding that highlighted the important contribution of abdominal SCAT to plasma PAI-1 in obese African women, it is crucial that future studies investigate the contribution of both VAT and SCAT compartments and not only VAT, which is generally regarded as the most important contributor to plasma PAI-1 levels. Furthermore, if available, future studies should use dual-energy x-ray absorption estimates of VAT and SCAT to aid in the understanding of the association of PAI-1 with different body fat distribution patterns, particularly in non-white ethnicities. Nevertheless, the skinfold : WC ratio that was used in our study might potentially be used in settings where such sophisticated techniques are not available. Lastly, our results identified other obesity-related metabolic abnormalities, such as inflammation and endothelial dysfunction, rather than body fat percentage *per se* to have a stronger influence on increased PAI-1 in obesity in African women and these factors should be taken into account when investigating the relationship between obesity and PAI-1.

1. The contribution of body fat distribution to increased plasma PAI-1 levels has important pathophysiological consequences for the development of obesity-related CVD risk. Despite VAT being the main PAI-1 producing fat depot, increased VAT does not always relate to increased plasma PAI-1 levels. Therefore, future studies should consider the relative contribution of factors such gender, ethnicity, possibly genetics, level or degree of obesity and other PAI-1 producing tissues, when considering the influence different fat depots have on plasma PAI-1 levels. Owing to ethnic differences in body fat composition and the fact that existing research is mainly based on European populations only, future research on African populations groups is needed to better understand the relationship of PAI-1 with body composition. Since increased plasma PAI-1 levels in obesity are associated with the increased risk for IR, MetS and CVD, further research investigating the relationship between PAI-1 and body composition will also contribute to the general health and well-being of the black South African populations.

Findings from this study contribute to methodological and clinical considerations relating to PAI-1 in the black South African population. More specifically, our results provide novel insight regarding the measurement of PAI-1 and PAI-1 related assays, the relationship of PAI-1 with different body fat distribution patterns in African women as well as the relative contribution of different fat depots to plasma PAI-1 levels and factors influencing this association. These results indeed answered several research issues and led to new questions in this field of research.

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ANNEXURE A: SABPA INFORMED CONSENT FORM

NORTH-WEST UNIVERSITY

POTCHEFSTROOM CAMPUS

SCHOOL FOR PHYSIOLOGY, NUTRITION AND CONSUMER SCIENCES

PARTICIPATION INFORMATION AND CONSENT FORM

PART 1

PRINCIPAL RESEARCHER: Dr Leoné Malan, Subject Group Physiology

PROJECT LEADER: Dr Leoné Malan, Subject Group Physiology

Associate Researcher(s): The postdoctoral fellow involved in this trial is Dr. Szabolcs Péter. Other persons assisting in the study are Dr. Hugo W. Huisman, Prof. Johannes M. van Rooyen, Prof. Nico T. Malan, Dr. R Schutte, Mrs Carla M.T. Fourie, Mrs. Tina Scholtz (Cardiovascular research group, Physiology), Prof. Salomé Kruger & Dr. Ramoteme Mamabolo, (Physical activity) Proff. Hans de Riddal (Anthropometry), Marié Wissing (Phychology), Linda Brand & Brain Harvey (Pharamacology), Kobus Mentz (Education), Francois van der Westhuizen (Biochemistry), Hester Kloppe (Nursing), Nancy Frasure-Smith & Francois Lespérance (Psychology, Canada), Alaa Alkerwi (Epidemiology, Luxembourg), Yackoob Seedat (ECG, Kwazulu Natal), Paul Rheeder (Sonar, Pretoria University), drs. Johan Potgieter & Michael Temane & Mr Tumi Khumalo (Psychology), Mrs Gedina de Wet (Nursing).

This Participant Information and Consent Form is 7 pages long. Please make sure you have all the pages.

Your Consent

You are invited to take part voluntarily in this research project.

This participant information document contains detailed information about the research project which has been explained to you verbally. Its purpose is to explain to you as openly and clearly as possible all the procedures involved in this project before you decide whether or not to take part.

Please read this *Participant Information Form* carefully. Feel free to ask questions about any information in this document. You may also wish to discuss the project with a relative or friend or your local health worker. Feel free to do this.

Once you understand what the project is about and if you agree to take part in it, you will be asked to sign the Consent Form. By signing the Consent Form, you indicate that you understand the information and that you give your consent to participate in the research project.

You will be given a copy of the Participant Information and Consent Form to keep as a record.

What is the study about?

The aim of this project is to have an impact on the eventual prevention and treatment of lifestyle diseases in Africans from South Africa. New knowledge regarding the relationship between higher nervous system activity implicating cardiovascular metabolic and psychological well-being will improve understanding and change strategies at the roots of treatment and prevention of lifestyle diseases.

Our research has shown that lifestyle diseases in urbanised Africans present higher obesity levels, high blood pressure or hypertension prevalence rates and the experiencing of more stress. This pattern is enhanced during psychosocial stress/urbanisation in participants with a specific coping style.

Hence the planner SABPA project, which is the first study in South Africa where coping and direct markers of nervous system activity in Africans will be measured.

Purpose of study

The purpose of this study is to investigate biological markers associated with higher sympathetic nervous system activity in urbanised teachers with a specific coping style.

To investigate the relationship between blood pressure, inflammation, obesity, stress and coping in more detail we are going to perform this study in 400 men and women from the North-West Province, aged 25-60 years. A comprehensive assessment of the cardiovascular and nervous systems by means of non-invasive painless techniques will be performed and a blood sample will be taken by an experienced research doctor and nurse to determine your blood sugar, cardiovascular, inflammation and stress hormone levels amongst other health markers.

Procedures

All measurements are performed in the Metabolic Unit (lipid clinic) of the University. A researcher has explained the entire procedure in detail and while you are reading this information document you have time to ask questions and to have clarified matters. If you are fine with the explained procedure you are requested to sign a *consent form (at the end of this document). Remember all personal data will be handled with care and remain confidential.

*By consenting to participate in this study, you consent to the storage and later analysis and testing of your stored blood samples for the purposes noted above. Your blood will also be tested for preliminary results on HIV status, since your HIV status may directly influence the main purposes of this study. If you would like to know what your HIV-status is, we will provide it. If tested positive we will refer you to your doctor and he/she will perform the necessary tests which will allow you to apply for chronic medication benefit. Also, the blood cells from your donated blood sample will be used to investigate the molecular genetics of higher nervous activity and type 2 diabetes in order to enable pre-symptomatic diagnosis of hypertension and diabetes in the long term.

Why was I chosen? Teachers are exposed to changing curricula and disciplinary problems whilst living in an urbanised environment adding to higher stress experiencing and nervous system activity.

How was I chosen?

Inclusion criteria:

Phase I: 200 black Africans aged 25-60 years (male=100, female=100)

Phase II: 200 white Africans (N = male, 100 = female) aged 25-60 years.

Exclusion criteria: pregnancy, lactation, any acute/chronic medication (e.g. high blood pressure, TB/tuberculosis, high sugar/diabetes, arthritis, anti-clotting/stroke factors, epilepsy/mental diseases or being treated for it as well-being addicted to the medicine). You cannot be included if you have been vaccinated in the previous 3 months and if you are a regular blood donor.

What will be expected of me?

You, as participant will be screened once by a registered nurse to be eligible complying too the inclusion criteria. The following procedures will be followed:

- Recruitment, screening and informed sessions with all participants will be done two months prior to the study (October – November 2007, Phase I, and November, 2008, Phase II) and informed consent forms will be signed.
- After selection of all participants, the details of the project will be discussed with you in English or your home language, i.e. what the exact objectives of the study are, what procedures will be taken and what will be expected from each of you (e.g. overnight stay, resting blood pressure procedures and fasting urine and blood samples are required, importance of complying with the correct sampling methods, incentives. You will be given the opportunity to ask questions.
- Data collection for each participant will involve two days (15 minutes in the morning and 2 ½ hours in the evening on Day I; and 2 hours on Day II):

DAY 1

- On Day I at 07:00, the blood pressure apparatus, which will measure your blood pressure and heart function as well as a physical activity meter will be applied to your arm and waist at your school and you can then resume your normal daily activities. In the afternoon you must complete the Neethling Brain Instrument questionnaire which measures thought processes of the brain.
- At the end of Day I (+/- 16:30) you will be transported from your schools to overnight in the Metabolic Unit Research Facility of the North-West University. This unit is a research unit for human studies and equipped with 10 well-furnished bedrooms, a kitchen, two bathrooms and a television room. Each of you will be subjected to the following procedures:
 - at the end of Day I between +/- 17:15 and 18:00 you will be welcomed and each of you will receive your own private bedroom.
 - The procedures, which will be done, will be explained again and each of you will then complete a general socio-demographic health questionnaire. Afterwards you will receive dinner.
 - After dinner, psychological questionnaires will be completed under supervision of registered education specialists and psychologists. Completion of questionnaires will take approximately 40 minutes, including a break of 20 minutes with coffee/tea and biscuits. This will be your last meal for Day I as you must be fasting on Day II for obtaining good results.
 - Thereafter, you can relax and watch television or socialise with your co-participants. It will be wise to go to bed not later than 22:00 as the blood

pressure apparatus will take measurements every hour during the night and it can be tiring.

DAY II

- At 06:45 on Day II the AMBP will be removed and a urine sample collected. Once this has been done you will be directed to the anthropometric station where your weight, height and body circumferences will be measured.
- The next station involves the blood pressure measurement station. Whilst in a sitting position your blood pressure will be taken in duplicate with the sphygmomanometer (the same as used at clinics) with a resting period of 5 minutes in between. Our registered research doctor/nurse will take a fasting saliva sample as well as a blood sample of 45ml from a vein in your dominant arm. The infusion set will be left in your arm to lessen the effect of inserting a needle again for blood sampling after exposure to the two stressors. A small amount of diluted heparin will be left in the infusion set in your arm to prevent clotting.

Next the cardiovascular measurements will follow consisting of three separate procedures:

- The 1st measurement involves an ECG apparatus, which measures heart function, with 12 leads, which will be placed in position on your rib cage/front part of the body.
- The 2nd measurements are non-invasive and will be done by means of the Finometer device which also involves the assessment of heart functioning such as pulse (9 beats per minute), stroke volume (blood volume ejected by the heart per beat), cardiac output (blood volume ejected by the heart per minute), total peripheral resistance (resistance against which the heart has to work while ejecting the blood into the aorta) as well as the elasticity of your large arteries (compliance). For this procedure a blood pressure cuff will be placed around your left arm and middle finger which will be inflated and stepwise deflated. You will not have more discomfort than during a common blood pressure measurement. This will take about 5 minutes.
- The stressor application procedure follows: You will now be exposed to a stressor for 1 minute whilst your blood pressure and ECG will still be taken. After exposure a saliva and blood sample (45 ml) will be taken. After 10 minutes another saliva sample will be taken. Then the stressor application procedure will be repeated with the second stressor.
- At another station your 3rd measurement includes the assessment of pulse wave velocity, i.e. how fast your blood travels through your arteries. This measurement gives us an indication about how stiff your vessel walls are. The stiffer your vessel wall is the faster the blood travels from one point of your body to another. These painless measurements will

require two technicians using blunt probes (tonometer) putting light pressure on the neck and on the foot to measure the velocity of the pulse waves. This takes only a few minutes. An ultrasound device will be taken of your arteries in the neck with a blunt probe to indicate the intrinsic thickness of your arteries which contributes to high blood pressure.

The two stressors you will be exposed to for one minute includes:

1. The *Colour-Word-Conflict Chart* (applied for 1 minute) is written in various colours. You must say or select the ink colour rather than the name of the colour spelled out by the word. A sliding scale with monetary incentives (maximum of R55.00) will be given if you can complete reading the chart.
 2. The *Cold Pressor Test (Foot)* (applied for 1 minute): Immersion of your foot up to the wrist in ice water (4 degrees Celsius). As the cold can make you hold your breath you must quietly count to yourself during cold exposure to breath more rhythmic.
- You have reached the end of the sampling phase.
 - **Thank you for your participation! You now will have the opportunity to shower and a take away breakfast will be given.**
 - Immediate feedback on your HIV/AIDS status, obesity, blood pressure and blood glucose/sugar value will be given. HIV/Aids post-test counselling will be arranged if you are tested positive.
 - You are now transported back to your school and after one week you will receive your Neethling Brain Instrument and 24-hour blood pressure reports.

Possible Risks

The measurements performed in our study will include only non-invasive techniques that are not expected to reveal any risks but might cause little discomfort. The taking of blood samples is an invasive procedure with a minimal risk of bleeding. Thus the procedure may cause only a few seconds of light discomfort. All tests will be performed by experienced research nurses of our department. There may be additional unforeseen or unknown risks.

Precautions to protect the participant

The measurements performed in our study will include only non-invasive techniques that are not expected to reveal any risks but might cause little discomfort. The taking of blood samples is an

invasive procedure with a minimal risk of bleeding. Thus the procedure may cause only a few seconds of light discomfort. All tests will be performed by experienced research nurses of our department. There may be additional unforeseen or unknown risks.

Other treatments whilst on study

It is important to tell the research staff about any treatments or medications you may be taking, including non-prescription medications, vitamins or herbal remedies during your participation in the study.

Incentive

1. All teachers will receive feedback on their health profile and if necessary references will be given to physicians/clinics/hospitals.
2. Printout feedback on 24-hour blood pressure monitoring report (normally costing R637.60) sonar of the artery (R1200.00), resting ECG (R600.00) and other variables (R500.00). Your benefit of participation is a comprehensive assessment of the cardiovascular and metabolic condition including investigation of blood pressure, inflammatory status and psychological well-being. These examinations will help us to assess the degree of vascular impairment of the arteries and to predict your risk of possible cardiovascular events such as heart attacks and stroke. The results may assist your doctor in decision making for further treatment or for instituting preventive measures. Our study will also contribute to the identification of possible factors leading to high blood pressure. As 24-hour ambulatory blood pressure monitoring is required for the diagnosis of hypertension, medical aids insist on this method of diagnosis to qualify for chronic medication. Additional testing could also reveal illnesses of a chronic nature and would serve as a motivation to qualify for chronic medication, such as metabolic syndrome, anti-inflammatory and cholesterol-lowering drugs.
3. Monetary incentive on completion of the colour word conflict chart (+/- R55.00).
4. Dinner and breakfast (+/- R24.00).
5. Neetthing Brain Instrument profiles done by registered user of the Whole Brain (normally costing +/- R350.00)
6. Coping skills workshop will be arranged on request.

Privacy, confidentiality and disclosure of information

By consenting to participate in the study, you consent to the storage and later analysis and testing of your stored blood samples for purposes noted above. Your blood samples will be discarded immediately after analysis. All information provided by you and the results of tests will be treated in the strictest confidence and will only be used for the purpose of this research project. It will only be disclosed with your permission, except as required by law. The results of your medical test will be labelled only with a code number, and will be sorted separately from any identifying information. When the results are analysed we will be looking for differences between groups of people, not at the results of individuals. No information that could identify any person taking part in the study will be revealed when the results are reported.

Participation is voluntary

Participation in any research project is voluntary. If you do not wish to take part, you are not obliged to. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage.

Your decision whether to take part or not to take part or to take part and then withdraw, will not affect your routine treatment or your relationship with those treating you or your relationship with the North-West University.

Before you make your decision a member of the research team will be available so that you can ask any questions you have about the research project. You can ask for any information you want. Sign the Consent Form only after you have had a chance to ask your questions and have received satisfactory answers.

If you decide to withdraw from this project, please notify a member of the research team before you withdraw.

Ethical guidelines

This project will be carried out according to Ethical Guidelines of the Helsinki declaration from 2000 with additional notes in 2002. This statement has been developed to protect the interest of people who agree to participate in human research studies.

The ethical aspect of this research project have been approved by the Human Research Ethics Committee of the North-West University Potchefstroom.

Further information or any problems

If you require further information or if you have any problems concerning the project, you can contact the principal researcher or the other researchers responsible for this project:

Dr Leoné Malan (018-299 2438)

Signature:

Sr. Chirssie Lessing (018-299 2480)

Project leader: Dr Leoné Malan

PART 2

To the subject signing the consent as in part 3 of this document

You are invited to participate in a research project as described in paragraph 2 of Part 1 of this document. It is important that you read/listen to and understand the following general principles, which apply to all participants in our research project: participation in this project is voluntary.

1. It is possible that you personally will not derive any benefit from participation in this project, although the knowledge obtained from the results may be beneficial to other people.
2. 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.
3. The nature of the project, possible risk factors, factors which may cause discomfort, the expected benefits to the subjects and the now and the most probable permanent consequences which may follow from your participation in this project, are discussed in Part 1 of this document.
4. 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.
5. We require that you indemnify the university from any liability due to detrimental effects of treatment by University staff or students or other subjects to yourself or anybody else. We also require indemnity from liability of the University regarding any treatment to yourself or another person due to participation in this project, as explained in part 1. Lastly it is required to abandon any claim against the university regarding treatment of yourself or another person due to participation in this project as described in Part 1.

6. If you are married, it is required that your spouse abandon any claims that he/she could have against the University regarding treatment or dearth of yourself due to the project explained in Part 1.

PART 3

Consent

Title of the project: "The SABPA study (Sympathetic activity and Ambulatory Blood Pressure in Africans)".

I, the undersigned..... (full names) read/listen 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.

(Signature of the subject)

Signed at on 2008/2009

Witnesses

1.....

2.....

Signed at on 2008/2009

ANNEXURE B: FOLLOW-UP STUDY INFORMED CONSENT FORM



HREC Stamp

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM FOR ACADEMIC STAFF OF THE NORTH WES PROVINCE, SOUTH AFRICA

TITLE OF THE RESEARCH PROJECT: Platelet PAI-1 content and α -granule release in a black and white South African study group

REFERENCE NUMBERS:

PRINCIPAL INVESTIGATOR: Prof Marlien Pieters

ADDRESS: Centre of Excellence for Nutrition (CEN) (G16), North-West University (NWU), Potchefstroom Campus

CONTACT NUMBER: (018) 299 2462

You are being invited to take part in a research project that forms part of the PhD study of Ms Sunelle Barnard under the supervision of Prof M Pieters. Please take some time to read the information presented here, which will explain the details of this project. Please ask the researcher any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline 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.

This study has been approved by the **Health Research Ethics Committee of the Faculty of Health Sciences of the North-West University (NWU.....)** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki and the ethical guidelines of the National Health Research Ethics Council. It might be necessary for the research ethics committee members or relevant authorities to inspect the research records.

What is this research study all about?

- *This study will be conducted at the Metabolic Unit, building G17 at the NWU, Potchefstroom campus and will involve the drawing of a single blood sample (15ml), by an experienced and trained professional nurse.*
- *The aim of this research is:*
 - *To investigate factors involved in blood clot formation and breakdown*

Why have you been invited to participate?

- *You have been selected to participate because you are from a similar socio-demographic background as participants from the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study and you comply with the*

same inclusion and exclusion criteria. In the SABPA study, a group of 150 participants' blood samples were used to investigate blood clot formation and breakdown. We now need some additional information to explain our initial findings and that is why we have to collect a small amount of blood again.

- *You have complied with the following inclusion criteria:*
 - *you are a healthy black or white academic staff member from the Potchefstroom campus of the North-West University and you are between the ages of 25 to 60 years.*
- *You will be excluded if:*
 - *pregnant or lactating (as these conditions may alter the data.);*
 - *using α or β blockers, psychotropic substances and/or have been vaccinated in the past three months or is regular blood donors (this medication, vaccination or donation of blood might alter the data.); and*
 - *presenting with elevated ear temperature ($>37.5^{\circ}\text{C}$) (to make sure you do not have an infection, which may also alter the data);*

What will your responsibilities be?

- *At the Metabolic Unit (G17), located on the NWU, a professional nurse will take a single blood sample of 15 ml. This will take ± 15 minutes of your time and all blood samples will be taken before 10 am at a pre-specified time slot. Additionally, you will be expected to be in a fasting state when the blood sample is taken. This means that you may not eat or drink anything after 10 pm the night before the sample is taken. You should also avoid alcohol, smoking or exercise the day preceding blood sampling.*

Will you benefit from taking part in this research?

- *The direct benefits for you as a participant will be that you will receive information on your blood clotting profile as well as a full blood count analysis that could help to identify conditions such as inflammation, infection, different types of anaemia and bleeding disorders and includes information on 1) white blood cells (white blood cell count, and differential: neutrophils, lymphocytes, monocytes, eosinophils, basophils); 2) red blood cells (red blood cell count, hemoglobin,*

hematocrit, and red blood cell indices: mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration); and 3) platelets (platelet count). The meaning of all of these will be explained to you and should any of the counts fall outside the normal range, you will be referred to your doctor for follow-up.

- *The indirect benefit will be that the knowledge gained from this study will ultimately lead to the better understanding of the haemostatic profile of the different ethnic groups within South Africa.*

Are there risks involved in your taking part in this research?

- *The risks in this study are:*
 - *Emotional, as you might experience stress (after having blood drawn) that can also possibly result in nausea, sweating and lowering of blood pressure. To prevent this, the blood sampling procedure and what to expect will be explained to you beforehand. You will be warned that you may experience some discomfort and pain when the blood sample is drawn. The blood sample will be taken in a private room and a bed will be available if you feel the need to lie down and in which case you will be kept under observation.*
 - *Physical, as you might experience pain and discomfort when the blood sample is taken. To minimise pain and discomfort, the blood sample will be taken by an experienced professional nurse. Only the amount of blood (15 ml) as stipulated in the protocol will be taken. You will be kept under observation for bleeding. Aftercare will be provided and the necessary procedure will be taken to ensure the bleeding has stopped.*
- *The benefits therefore outweigh the possible risk.*

What will happen in the unlikely event of some form of discomfort occurring as a direct result of your taking part in this research study?

- *Should you have the need for any further assists after the blood sample was taken, a bed will be provided for you to lie down. A professional nurse will also be present to provide all the necessary medical assistance.*

Who will have access to the data?

- *Anonymity will be maintained by assigning a number to each participant, which will be used on the blood samples and tubes, instead of using the participants' name.*
- *Confidentiality will be ensured by only using coded data for all samples, all data storage and analyses.*
- *Reporting of findings will be anonymous by reporting only coded data with no mention of the names of the participants that participated in this study. Only the researchers (Prof Marlien Pieters and Mrs Sunelle A. Barnard) will have access to the data and all researchers working with the data will have to sign a letter of confidentiality, to ensure confidential handling of all data as well as no unethical disclosure of the data.*
- *Data will be kept safe and secure by locking hard copies in locked cupboards in the researcher's office and for electronic data it will be password protected. Data will be stored for seven years.*

What will happen with the data/samples?

- *This is a once off collection and data will be analysed at CEN and physiology department of the North-West University, Potchefstroom campus. After data analysis has taken place all blood samples will be destroyed following the medical waste procedure of the NWU.*

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

- *No, you will not be paid to take part in the study but refreshments (a light healthy snack and something to drink) will be provided. There will be no costs involved for you, if you do take part. A compensation of R50 will be provided for time and effort put into the study.*

Is there anything else that you should know or do?

- *You can contact Prof Marlien Pieters at (018) 299 2462 if you have any further queries or encounter any problems.*

- *You can contact the Health Research Ethics Committee via Mrs Carolien van Zyl at 018 299 2089; carolien.vanzyl@nwu.ac.za if you have any concerns or complaints that have not been adequately addressed by the researcher.*
- *You will receive a copy of this information and consent form for your own records.*

How will you know about the findings?

- *All participants will be offered the results of their blood clotting profile as well as their full blood count. This will be communicated to the participants within a month after data collection at a time and place that is convenient for the participant.*

Declaration by participant

By signing below, I agree to take part in a research study titled: Platelet PAI-1 content and α -granule release in a black and white study group

I declare that:

- I have read this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions to both the person obtaining consent, as well as the researcher and all my questions have been adequately 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 penalised or prejudiced 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.

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

ANNEXURE C: SONDRRA INFORMED CONSENT FORM

PURE-SA PROJECT (Prospective Rural and Urban Epidemiology) and Bone Health Study INFORMED CONSENT

I, the undersigned.....(full names)
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 indemnify the University, also any employee or student of the University, of any liability against
myself, which may arise during the course of the project.

I will not submit any claims against the University regarding personal detrimental effects due to
the project, due to negligence by the University, its employees or students, or any other
subjects.

I agree to be tested for HIV:

YES

NO

I want to know my HIV-status

YES

NO

I give consent that the outcome of results can be disclosed with members of the PURE-SA
research team for effective management and compliance to the project. The test results will be
treated with the highest confidentiality.

(Signature of the subject)

Signed at _____ on _____

Witnesses

1. _____ 2. _____

Signed at _____ on _____

CONFIDENTIALITY

PART 1

1. School/Institute: Africa Unit for Transdisciplinary Health Research (AUTHeR)
2. *Title of project/trial:* PURE: Prospective Urban and Rural Epidemiology and Bone Health Study
3. Full names, surname and qualifications of project leader: Annamarie Kruger, Ph.D. (Nutrition) and Lanthé Kruger, Ph.D. (Physiology)
4. Rank/position of project leader: Research 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.

The aims of the **Bone Health study** is to investigate and improve bone health in women. It is very important to build strong and healthy bones in the childhood and teen years to avoid osteoporosis and other bone problems later in life. Osteoporosis is a condition in which bones are fragile, making them fracture or break much easier. The spine, wrist and hips are particularly vulnerable to fracture.

6. *Evaluation/measurement points:*

Each participant will undergo multiple evaluations at different stations. These include:

- HIV counselling and testing
- Blood sample (18.5 ml)
- Anthropometrics (height, weight, skinfold thickness, circumference of arm, leg and waist)
- Urine sample (spot urine sample)

- Pregnancy test (DXA assessment may not be performed when pregnant)
- Bone density measurements - DXA
- Bone density measurement - Osteometer
- Blood pressure measurement
- Pulse wave velocity
- Carotid IMT scan (Intimal-medial thickness)
- Echo-cardiogram
- Electrocardiogram
- Physical strength test
 - Forearm and hand strength test by squeezing an object
 - Getting up from a chair and walking on straight line for 6 meters
 - Stepping on and off a step
- Questionnaires
 - Eight-year follow-up
 - Bone Health
 - Attitudes towards HIV/AIDS
 - Physical Activity
 - Socio-Demographic
 - Medicine use

7. *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 an hour) to complete the questionnaires and discomfort may be experienced with the taking of blood samples. No measures will have permanent damage or consequences for the participants. If participant is unable to perform a test (especially physical strength test) they will not be forced to take the test.

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 pressure, the information will be communicated to the individual to seek professional help. Since this study is a longitudinal

study, subjects that are high at risk will be identified from the dataset and personal feedback will be given.

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 he/her 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:

- Participation in this project is voluntary.
- It is possible that you personally will not derive any benefit from participation in this project, although the knowledge obtained from the results may be beneficial to other people.
- 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.
- 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.
- 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.
- The University staff will use standardised procedures and take all possible precaution to protect the subject from risks. We require that you indemnify the University from any liability due to detrimental effects of treatment by University staff or students or other subjects to yourself or anybody else. We also require indemnity from liability of the University regarding any treatment to yourself or another person due to participation in this project, as explained in Part 1. Lastly it is required to abandon any claim against the University regarding treatment of yourself or another person due to participation in this project as described in Part 1.

- If you are married, it is required that your spouse abandon any claims that he/she could have against the University regarding treatment or death of yourself due to the project explained in Part 1.
- All information will be kept CONFIDENTIAL.

Signature of project leader

Date

**ANNEXURE D: PUBLISHED ARTICLE - THE EFFECTS OF RESIDUAL
PLATELETS IN PLASMA ON PLASMINOGEN ACTIVATOR INHIBITOR-
1 AND PLASMINOGEN ACTIVATOR INHIBITOR-1-RELATED ASSAYS
(CHAPTER 3)**

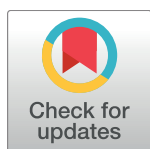
RESEARCH ARTICLE

The effects of residual platelets in plasma on plasminogen activator inhibitor-1 and plasminogen activator inhibitor-1-related assays

Marlien Pieters^{1*}, Sunelle A. Barnard¹, Du Toit Loots², Dingeman C. Rijken³

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OPEN ACCESS

Citation: Pieters M, Barnard SA, Loots DT, Rijken DC (2017) The effects of residual platelets in plasma on plasminogen activator inhibitor-1 and plasminogen activator inhibitor-1-related assays. PLoS ONE 12(2): e0171271. doi:10.1371/journal.pone.0171271

Editor: Pablo Garcia de Frutos, Institut d'Investigacions Biomediques de Barcelona, SPAIN

Received: October 12, 2016

Accepted: January 17, 2017

Published: February 3, 2017

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by grants from the National Research Foundation (NRF) of South Africa (Grant UID: 94213) (<http://www.nrf.ac.za>) and the South African Medical Research Council (<http://www.mrc.ac.za>). Opinions expressed and conclusions arrived at, are those of the authors and are not to be attributed to the NRF. The funders had no role in study design, data collection and

Abstract

Due to controversial evidence in the literature pertaining to the activity of plasminogen activator inhibitor-1 in platelets, we examined the effects of residual platelets present in plasma (a potential pre-analytical variable) on various plasminogen activator inhibitor-1 and plasminogen activator inhibitor-1-related assays. Blood samples were collected from 151 individuals and centrifuged at 352 and 1500 *g* to obtain plasma with varying numbers of platelet. In a follow-up study, blood samples were collected from an additional 23 individuals, from whom platelet-poor (2000 *g*), platelet-containing (352 *g*) and platelet-rich plasma (200 *g*) were prepared and analysed as fresh-frozen and after five defrost-refreeze cycles (to determine the contribution of *in vitro* platelet degradation). Plasminogen activator inhibitor-1 activity, plasminogen activator inhibitor-1 antigen, tissue plasminogen activator/plasminogen activator inhibitor-1 complex, plasma clot lysis time, β -thromboglobulin and plasma platelet count were analysed. Platelet α -granule release (plasma β -thromboglobulin) showed a significant association with plasminogen activator inhibitor-1 antigen levels but weak associations with plasminogen activator inhibitor-1 activity and a functional marker of fibrinolysis, clot lysis time. Upon dividing the study population into quartiles based on β -thromboglobulin levels, plasminogen activator inhibitor-1 antigen increased significantly across the quartiles while plasminogen activator inhibitor-1 activity and clot lysis time tended to increase in the 4th quartile only. In the follow-up study, plasma plasminogen activator inhibitor-1 antigen was also significantly influenced by platelet count in a concentration-dependent manner. Plasma plasminogen activator inhibitor-1 antigen levels increased further after complete platelet degradation. Residual platelets in plasma significantly influence plasma plasminogen activator inhibitor-1 antigen levels mainly through release of latent plasminogen activator inhibitor-1 with limited effects on plasminogen activator inhibitor-1 activity, tissue plasminogen activator/plasminogen activator inhibitor-1 complex or plasma clot lysis time. Platelets may however also have functional effects on plasma fibrinolytic potential in the presence of high platelet counts, such as in platelet-rich plasma.

analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abbreviations: β TG, beta thromboglobulin; CLT, clot lysis time; CTAD, citrate-theophylline, adenosine, dipyridamole; ELISA, enzyme-linked immunosorbent assay; HREC, Health Research Ethics Committee; MPV, mean platelet volume; NWU, North-West University; PAI-1, plasminogen activator inhibitor-1; PAI-1_{act}, PAI-1 activity; PAI-1_{ag}, PAI-1 antigen; PPP, platelet-poor plasma; PRP, platelet-rich plasma; tPA/PAI-1 complex, tissue plasminogen activator/PAI-1 complex; SABPA, Sympathetic activity and Ambulatory Blood Pressure in Africans.

Introduction

Plasminogen activator inhibitor type-1 (PAI-1) is a serine protease inhibitor (serpin) [1, 2], which acts as a main inhibitor of fibrinolysis [3]. Elevated plasma PAI-1 levels have been associated with a risk for developing atherothrombosis [4–6] due to its antifibrinolytic properties, by reducing the clearance of fibrin in plaques [5], and also *via* its influence on cellular migration, matrix remodelling and activation of growth factors [7, 8]. Plasma PAI-1 exists either in an active or latent form, or in complex with tissue plasminogen activator (tPA) [9–11]. The active form of PAI-1 is unstable, with a half-life of approximately two to three hours, after which it will spontaneously convert to the inactive, latent form [9, 12]. PAI-1 is produced by various cells such as endothelial cells, hepatocytes, smooth muscle cells, adipocytes, and platelets [11, 13]. In platelets, PAI-1 is stored in the alpha granules and is released during platelet activation and aggregation [11, 14, 15].

Recently, there has been a debate about which form of PAI-1, or at least the relative proportion of each form, is released from the platelet alpha granules. It was traditionally believed that platelets store and release mainly latent PAI-1, since, only approximately 5–10% of PAI-1 antigen (PAI-1_{ag}) was shown to be active in lysed platelet-rich plasma [16]. More recent studies however, suggest that platelets release a substantial amount of active PAI-1 [17–19]. This is due to the observed *de novo* synthesis of PAI-1 within platelets, which was indicated to remain active for over 24 hours [17]. Possible explanations for the contradictory evidence pertaining to platelet PAI-1 activity, could be due to the different approaches used in these experiments for preparing the platelet lysates (sonification and freezing and or thawing of the samples), which have been reported to influence the detection of PAI-1 [18]. Furthermore, the conversion of active PAI-1 to its latent state can be influenced by low temperatures, low pH and high salt concentrations [20]. It is however not clear as to how the PAI-1, released from the alpha granules of residual platelets in plasma, affects PAI-1 assays and PAI-1-related assays.

The overall aim of the study was therefore to investigate the effect of residual platelets in plasma, on various PAI-1 and PAI-1-related assays: PAI-1 activity (PAI-1_{act}), PAI-1 antigen (PAI-1_{ag}), and tPA/PAI-1 complex, as well as plasma fibrinolytic potential (a functional parameter of fibrinolysis, measured as plasma clot lysis time (CLT)). The study consisted of two sub-studies. In the first, varying centrifugation speeds (352 and 1500 g) were used to prepare platelet-containing plasma, from 151 participants in the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study. The purpose of this sub-study was to determine the effect of residual platelets in plasma on various PAI-1 assay results, by relating these assays to a marker of platelet alpha granule release (beta thromboglobulin (β TG)). In this sub-study, absolute platelet counts were not measured, and additionally it was not possible to calculate to what degree plasma PAI-1 levels were influenced by *in vitro* platelet activation and or degradation. Additionally, Merolla *et al.* [21] found that different centrifugation speeds may result in different platelet populations, which could also have had an effect on our results. The purpose of the second study was, therefore, to determine the influence of actual platelet count on PAI-1_{ag}, as the antigen assay was the assay found to be significantly influenced by plasma platelet content in the first sub-study. In the follow-up study, plasma was collected from 23 additional participants, and platelet count and size, in addition to β TG and PAI-1_{ag} concentrations, were determined from three different plasma preparations: platelet-poor plasma (PPP– 2000 g), platelet-containing plasma (352 g, in keeping with the first sub-study protocol) and platelet-rich plasma (PRP– 200 g). PPP was collected in citrate tubes, containing platelet stabilisers, in order to provide basal plasma PAI-1_{ag} levels without any of the influencing effects of *in vitro* platelet activation and/or degradation. Furthermore, the 352 g and 200 g citrated plasma samples were analysed not only as fresh-frozen, but also after five defrost-refreeze cycles, ensuring complete alpha

granule release from the platelets, in order to determine the total platelet PAI-1_{ag} and β TG content.

Materials and methods

Study population and ethics—SABPA study

The Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study, was a cross-sectional study including 409 (202 men and 207 women) school teachers between the ages of 25–60 years, from the North West Province, South Africa. Of these participants 151 individuals were randomly selected for inclusion in the present study. All samples were analysed at the same time. Exclusion criteria were: elevated ear temperature, dependence or abuse of psychotropic substances, regular blood donors, and individuals vaccinated within the previous three months. The study complied with all applicable international regulations and the Helsinki declaration for investigation of human participants. The study was approved by the Health Research Ethics Committee (HREC) of the North-West University (NWU), Potchefstroom Campus (NWU-00016-10-A1).

Study population and ethics—follow-up study

Twenty three individuals from the same socio-demographic profile as the SABPA study participants were recruited by means of a purposive sampling method from the Potchefstroom Campus of the NWU. The same inclusion and exclusion criteria as well as ethical principles were adhered to. The study was approved by the HREC of the NWU, Potchefstroom Campus (NWU-00016-10-A1). All samples were collected and analysed at the same time.

Blood collection—SABPA study

Fasting blood samples with minimum stasis were collected from the antebrachial vein before 10:00 am. 3.2% Citrate samples were used for the analysis of PAI-1 (activity, antigen and tPA/PAI-1 complex), β TG and CLT. Samples were randomly divided into two groups. One half of the study population samples were centrifuged at 352 g and the other half at 1500 g for 15 minutes at 20°C to yield plasma containing a varying number of platelets. Aliquots were snap frozen on dry ice and stored at -82°C until analysis.

Blood collection—follow-up study

Fasting blood samples with minimum stasis were collected from the antebrachial vein before 10:00 am. Blood was collected into two 3.2% citrated tubes and one CTAD tube (a citrate tube containing platelet stabilisers; theophylline, adenosine and dipyridamol). CTAD plasma was prepared by centrifuging the samples at 2000 g for 30 minutes at 20°C, to yield PPP with platelets protected from *in vitro* activation or degradation. Two types of citrate plasma were prepared by centrifuging one of the citrated tubes at 352 x g for 15 minutes at 20°C, to yield platelet-containing plasma, and the other tube at 200 x g for 10 minutes at 20°C, to yield PRP. These conditions were comparable to that of the SABPA study and also served the purpose to provide information on standard plasma type preparations—PPP and PRP. All samples were centrifuged within 20 min of collection.

Platelet count and size analyses were performed in fresh whole blood samples collected both in citrate and CTAD tubes, as well as in the different plasma preparations described above. The remaining plasma was then aliquoted, snap frozen on dry ice and stored at -82°C. The CTAD plasma samples and half of the aliquots of the two citrate plasma preparations, of each individual, were thawed once only, by placing these in a 37°C water bath for 10 minutes,

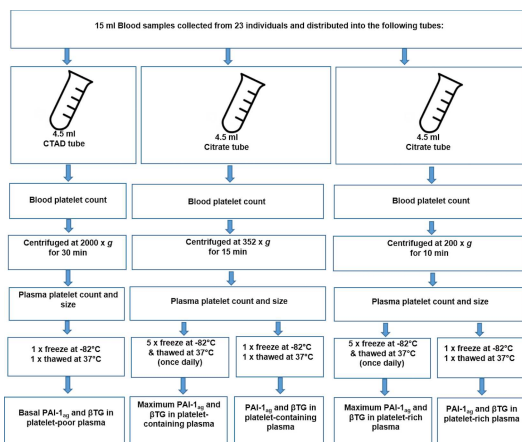


Fig 1. Design of follow-up study.

doi:10.1371/journal.pone.0171271.g001

immediately prior to PAI-1_{ag} and βTG analyses. The second half of the citrated plasma sample aliquots underwent five freeze-thaw cycles (x 5), once daily, prior to analysis, to ensure maximum platelet α-granule release. Fig 1 provides a schematic depiction of the study design.

Biochemical analysis

PAI-1_{act} was measured using an indirect enzymatic method (Technozym PAI-1 Actibind, Technoclone, Vienna, Austria), and PAI-1_{ag}, using a two-site enzyme-linked immunosorbent assay (ELISA) (TriniLIZE PAI-1_{ag}, TCoag, Bray Ireland). tPA/PAI-1 complex was analysed using a solid phase enzyme immunoassay, specific to PAI-1 complexed to tPA (Technoclone, Vienna, Austria). An ELISA assay was used to measure βTG levels (Asserachrom® βTG Diagnostica Stago, Asnières sur Seine, France). CLT was determined by studying the lysis of a tissue factor-induced plasma clot by exogenous tPA. Changes in turbidity during clot formation and lysis were monitored as described by Lisman *et al.* [22]. Tissue factor and tPA concentrations were slightly modified to obtain comparable CLTs of approximately 60 minutes. The modified concentrations were 17 mmol/L CaCl₂, 60 ng/ml tPA (Actilyse, Boehringer Ingelheim, Ingelheim, Germany) and 10 μmol/L phospholipids vesicles (Rossix, Mölndal, Sweden). Tissue factor was diluted 3000 times (Dade Innovin, Siemens Healthcare Diagnostics Inc., Marburg, Germany). CLT was defined as the time from the midpoint, from clear to maximum turbidity (representative of the clot formation), to the midpoint in the transition from maximum turbidity to the final baseline turbidity (representative of the lysis of the clot) [22]. Platelet count and mean platelet volume were determined with a Coulter AcT 5-part differential (5 diff) auto-loader haematology analyser (Beckman Coulter, Fullerton, CA, USA).

Statistical analysis

The data was analysed with the computer software package Statistica (Statsoft Inc., Tulsa Oklahoma, USA). A p-value of 0.05 or less was regarded as statistically significant. Descriptive data is presented as median (25th; 75th percentiles) as most of the variables were not normally distributed. Kruskal-Wallis analysis of variance (ANOVA) with multiple comparisons of mean post-hoc tests were used to compare differences in the PAI-1 and CLT assays, between population sub-groups divided into quartiles of βTG levels. Correlations between variables were determined both with Spearman Rank order and Pearson (for log transformed data) correlation tests. Only the Spearman data is reported, as both correlation tests provided similar

results. Significant differences between correlation coefficients obtained were also calculated. For the follow-up study, Wilcoxon-Matched pairs tests were used to determine significant differences between plasma prepared at 200 and 352 g and also between fresh-frozen and 5 times defrosted-refrozen samples.

Results

SABPA study

The study population included 151 participants, with a mean age of 45.7 (± 8.75) years and a BMI of 26.9 (± 2.29). When comparing the samples prepared at the two different centrifugation speeds, the 352 g group had significantly higher β TG (3263 vs 355 IU/mL; $p < 0.0001$) and PAI-1_{ag} (33.8 vs 20.8 ng/mL; $p < 0.0001$) levels, compared to the 1500 g group, with borderline significantly higher PAI-1_{act} (2.95 vs 1.91 U/mL; $p = 0.03$) and longer CLT (78.2 vs 74.4 min; $p = 0.04$). No difference in tPA/PAI-1 complex ($p = 0.09$) was observed (Table 1).

When dividing the study population into quartiles according to plasma β TG levels (Table 2), PAI-1_{ag} increased consistently across the β TG quartiles. PAI-1_{act} showed a significant increase in the highest β TG quartile only, with CLT tending to be longer, without reaching significance. No difference was observed in tPA/PAI-1 complex across the β TG quartiles. β TG was furthermore correlated with PAI-1_{ag} ($r = 0.66$; $p < 0.0001$), demonstrating statistically weaker correlations with PAI-1_{act} ($r = 0.22$; $p = 0.008$); tPA/PAI-1 complex ($r = 0.12$; $p = 0.13$) and CLT ($r = 0.20$; $p = 0.02$) (Table 3). CLT showed the strongest correlation with PAI-1_{act} ($r = 0.74$; $p < 0.0001$).

Follow-up study

Table 4 presents the descriptive statistics of the follow-up study group. Platelet counts in the citrated and CTAD whole blood were similar. The platelet count of the CTAD samples centrifuged at 2000 g was $1.00 (1.00\text{--}2.00) \times 10^3/\mu\text{L}$, confirming that it was indeed platelet poor ($< 10 \times 10^3/\mu\text{L}$). The platelet counts of the 352 g and 200 g plasma were $323 (257\text{--}440) \times 10^3/\mu\text{L}$ and $523 (389\text{--}674) \times 10^3/\mu\text{L}$ respectively. The 352 g plasma had a significantly lower mean platelet volume (7.00 [6.65–7.60] fL) than the 200 g plasma (7.80 [7.00–8.30] fL), which in turn had a similar mean platelet volume than that of the whole blood. β TG levels increased 60 fold and 150 fold in the 352 g and 200 g plasma respectively, compared to the PPP, while PAI-1_{ag} levels increased 15 and 22 fold respectively. In both the 352 g and 200 g plasma, the β TG levels of the samples that underwent 5 freeze-thaw cycles, prior to analyses, were significantly lower than that of the samples that were defrosted once only, prior to analysis, possibly due to instability

Table 1. Comparison of β TG, PAI-1 assays and CLT according to centrifugation speed in SABPA study.

Variable	352 g (n = 75)	1500 g (n = 75)	p-value(Mann-Whitney)
	Median (25 th ; 75 th percentiles)	Median (25 th ; 75 th percentiles)	
β TG (IU/mL)	3263 (2009; 4394)	355 (218; 584)	<0.0001
PAI-1 _{ag} (ng/mL)	33.8 (28.4; 42.4)	20.8 (16.7; 25.8)	<0.0001
PAI-1 _{act} (U/mL)	2.95 (0.69; 8.72)	1.91 (0.25; 4.68)	0.03
tPA/PAI complex (ng/mL)	8.78 (6.59; 11.7)	7.90 (6.01; 10.2)	0.09
CLT (min)	78.2 (69.7; 86.4)	74.4 (69.7; 79.8)	0.04

β TG—beta thromboglobulin; CLT—clot lysis time; PAI-1—plasminogen activator inhibitor-1 PAI-1_{act}—PAI-1 activity; PAI-1_{ag}—PAI-1 antigen; tPA/PAI complex—tissue plasminogen activator/PAI-1 complex.

doi:10.1371/journal.pone.0171271.t001

Table 2. PAI-1_{act}, PAI-1_{ag}, tPA/PAI-1 complex and CLT according to β TG quartiles in SABPA study group.

Variable	SAPBA study group									ANOVA p-value
	βTG 1 st Quartile(≤341 IU/ mL)		βTG 2 nd Quartile(341 IU/ mL—817 IU/mL)		βTG 3 rd Quartile(817 IU/ mL—3263 IU/mL)		βTG 4 th Quartile(>3263 IU/ mL)			
	n	Median (25; 75% percentile)	n	Median (25; 75% percentile)	n	Median (25; 75% percentile)	n	Median (25; 75% percentile)		
PAI-1 _{ag} (ng/L)	37	20.4 (16.0; 25.8) *	38	21.5 (17.0; 26.7) *	37	29.6 (25.1; 39.7) [#]	37	40.7 (31.0; 42.9) [#]	<0.0001	
PAI-1 _{act} (U/mL)	34	2.56 (0.31; 4.89)	37	1.89 (0.20; 3.77) *	37	1.37 (0.41; 6.70)	36	5.65 (1.28; 10.3) [#]	0.03	
tPA/PAI-1 complex (ng/mL)	35	8.00 (6.36; 10.2)	37	7.55 (5.26; 10.1)	37	8.65 (6.28; 11.7)	38	9.06 (7.32; 11.3)	0.1	
CLT (min)	34	75.4 (69.7; 79.5)	37	73.9 (69.7; 78.6)	36	76.6 (67.7; 84.1)	35	81.5 (71.6; 96.0)	0.06	

ANOVA, analysis of co-variance; β TG, beta thromboglobulin; CLT, clot lysis time; PAI-1, plasminogen activator inhibitor-1; PAI-1_{act}, PAI-1 activity; PAI-1_{ag}, PAI-1 antigen; tPA/PAI-complex, tissue plasminogen activator/PAI-1 complex

* # Medians with different symbols differ significantly.

doi:10.1371/journal.pone.0171271.t002

of β TG (5% and 3% respectively), while PAI-1_{ag} levels showed significant increases of 26% and 27% respectively.

When combining the different types of plasma, β TG had a highly significant correlation with platelet count ($r = 0.91$, $p < 0.0001$). When investigating the different types of plasma separately, β TG levels correlated well with platelet count in the 352 g ($r = 0.60$, $p = 0.002$) and 200 g ($r = 0.70$, $p = 0.0002$) plasma samples, with a smaller and non-significant correlation ($r = 0.40$, $p = 0.06$) in the PPP (Table 5). For all the types of plasma combined, PAI-1_{ag} and platelet count were highly correlated ($r = 0.91$, $p < 0.0001$). Compared with β TG, PAI-1_{ag} showed even stronger statistical correlations with platelet count in the 352 g ($r = 0.85$, $p < 0.0001$) and 200 g ($r = 0.81$, $p < 0.0001$) plasma. None of the plasma preparations' β TG levels correlated with whole blood platelet count, while the correlation of PAI-1_{ag} of the 200 g plasma with whole blood platelet count, reached borderline significance ($r = 0.4$, $p = 0.06$). Furthermore, β TG and PAI-1_{ag} correlated significantly when combining the different types of plasma ($r = 0.86$, $p < 0.0001$), however, correlated negatively in PPP ($r = -0.61$, $p = 0.002$), with significant positive correlations in the 352 g ($r = 0.55$, $p = 0.006$) and 200 g ($r = 0.74$, $p < 0.0001$) plasma separately (Table 6).

Discussion

This study investigated the effect of residual platelets present in plasma, on plasma PAI-1 and PAI-1-related assay results. The presence of platelets in plasma significantly influenced plasma PAI-1_{ag} levels in a concentration dependent manner, likely due to an increase in mainly plasma latent PAI-1. Only in the presence of large amounts of platelets such as in PRP,

Table 3. Spearman rank order correlations between β TG, PAI-1 assays and CLT in SABPA study group.

Variables	β TG r (p-value)	PAI-1 _{ag} r (p-value)	PAI-1 _{act} r (p-value)	tPA/PAI-1 complex r (p-value)
PAI-1 _{ag} (ng/mL)	0.66 (<0.0001)	-	-	-
PAI-1 _{act} (U/mL)	0.22 (0.008) *	0.43 (<0.0001)	-	-
tPA/PAI-1 complex (ng/mL)	0.12 (0.13) *	0.30 (0.0002)	0.64 (<0.0001)	-
CLT (min)	0.20 (0.02) *	0.41 (<0.0001)	0.74 (<0.0001)	0.50 (<0.0001)

β TG, beta thromboglobulin; CLT, clot lysis time; PAI-1, plasminogen activator inhibitor-1; PAI-1_{act}, PAI-1 activity; PAI-1_{ag}, PAI-1 antigen; tPA/PAI-complex, tissue plasminogen activator/PAI-1 complex.

* Significantly weaker correlation with β TG than the correlation of PAI-1_{ag} with β TG.

doi:10.1371/journal.pone.0171271.t003

Table 4. Descriptive statistics of the follow-up study group.

Variable	Study population (n = 23)
	Median (25 th ; 75 th percentiles)
Gender: men / women (n)	12 / 11
Ethnicity: black / white (n)	11 / 12
Age (years)	36 (29; 42)
SBP (mm Hg)	120 (110; 130)
DBP (mm Hg)	80 (70; 80)
BMI (kg/m ²)	26.4 (22.0; 28.4)
CTAD whole blood platelet count (x10 ³ /μL)	239 (195; 248)
Citrate whole blood platelet count (x10 ³ /μL)	234 (194; 257)
CTAD plasma 2000 g platelet count (x10 ³ /μL)	1.00 (1.00; 2.00)
Citrate plasma 352 g platelet count (x10 ³ /μL)	323 (257; 440)
Citrate plasma 200 g platelet count (x10 ³ /μL)	523 (389; 674)
Whole blood CTAD MPV (fL)	7.80 (7.40; 8.40)
Whole blood Citrate MPV (fL)	7.80 (7.20; 8.30)
MPV (fL) 352 g plasma	7.00 (6.65; 7.60) [%]
MPV (fL) 200 g plasma	7.80 (7.00; 8.30) [%]
βTG (IU/mL) CTAD 2000 g plasma	120 (92; 156)
βTG (IU/mL) 352 g x 1 plasma	7269 (6218; 8902) [#]
βTG (IU/mL) 352 g x 5 plasma	6890 (5770; 7985) [#]
βTG (IU/mL) 200 g x 1 plasma	17683 (14703; 19089) [^]
βTG (IU/mL) 200 g x 5 plasma	17182 (14322; 18393) [^]
PAI-1 _{ag} (ng/mL) CTAD 2000 g plasma	5.16 (3.80; 11.5)
PAI-1 _{ag} (ng/mL) 352 g x 1 plasma	76.7 (64.1; 86.0) ^{\$}
PAI-1 _{ag} (ng/mL) 352 g x 5 plasma	96.9 (74.7; 117) ^{\$}
PAI-1 _{ag} (ng/mL) 200 g x 1 plasma	114.2 (90.6; 155) ^{&}
PAI-1 _{ag} (ng/mL) 200 g x 5 plasma	145 (115; 191) ^{&}

BMI, body mass index; βTG, beta thromboglobulin; CTAD, citrate-theophylline, adenosine, dipyridamol; DBP, diastolic blood pressure; g, gravitational acceleration; PAI-1, plasminogen activator inhibitor-1; PAI-1_{ag}, PAI-1 antigen; SBP, systolic blood pressure; MPV, mean platelet volume

[#] [^] ^{\$} [%] [@] ^{**} Median with the same symbol differ significantly between the 1x and 5 x frozen and defrosted samples.

doi:10.1371/journal.pone.0171271.t004

functional effects in terms of plasma fibrinolytic potential are seen, suggesting the presence of a comparatively lower concentration of active PAI-1 in platelets. It was furthermore demonstrated that platelets present in plasma, do not initially release all of their PAI-1 content and that further release of PAI-1 can occur upon further / complete *in vitro* platelet degradation.

SABPA study

The SABPA study data indicated that βTG levels had a significantly stronger association with PAI-1_{ag} levels than any of the other PAI-1 variables or CLT. When dividing the study population into βTG quartiles, PAI-1_{act} and CLT increased in the highest βTG quartile only, suggesting that there may be a small amount of active PAI-1 present in platelets. In agreement with this, Serizawa *et al.* [23] found longer CLT in PRP than PPP which was ascribed to the presence of active PAI-1 in platelets. Since PAI-1_{ag} is composed of latent PAI-1, active PAI-1 and PAI-1 in complex with tPA, the data suggests that platelet alpha granule release largely contributes to increased plasma PAI-1_{ag}, by increasing latent PAI-1. It was unfortunately not possible

Table 5. Spearman rank order correlations of β TG and PAI-1_{ag} with whole blood, CTAD and citrate plasma platelet count of the follow-up study.

Variable	Platelet count $\times 10^3/\mu\text{l}$ (n = 23)				
	CTAD whole blood r (p-value)	CTAD plasma (2000 g) r (p-value)	Citrate whole blood r (p-value)	Citrate plasma (352 g) r (p-value)	Citrate plasma (200 g) r (p-value)
β TG (IU/mL) CTAD 2000 g plasma	-0.14 (0.5)	0.40 (0.06)	-	-	-
β TG (IU/mL) 352 g x 1 plasma	-	-	0.12 (0.6)	0.6 (0.002)	-
β TG (IU/mL) 200 g x 1 plasma	-	-	0.20 (0.3)	-	0.70 (0.0002)
PAI-1 _{ag} (ng/mL) CTAD 2000 g plasma	0.04 (0.9)	-0.36 (0.09)	-	-	-
PAI-1 _{ag} (ng/mL) 352 g x 1 plasma	-	-	0.34 (0.1)	0.85 (<0.0001)	-
PAI-1 _{ag} (ng/mL) 200 g x 1 plasma	-	-	0.40 (0.06)	-	0.81 (<0.0001)

β TG, beta thromboglobulin; CTAD, citrate-theophylline, adenosine, dipyridamol; g, gravitational acceleration; PAI-1, plasminogen activator inhibitor-1; PAI-1_{ag}, PAI-1 antigen.

doi:10.1371/journal.pone.0171271.t005

to measure plasma latent PAI-1 levels directly, as no such commercial assay is currently available. Latent PAI-1 is unable to inhibit tPA; therefore, the presence of latent PAI-1 in plasma may lead to a falsely assumed increased fibrinolytic inhibitory capacity. The lack of increase in CLT across the β TG quartiles, (apart from the highest quartile) confirms this. These results are also in agreement with a study by Juhan-Vague *et al.* [24] who found PAI-1, released from platelets, *in vitro*, to be mainly in the inactive form. Combined, this data suggests that platelets likely contain both latent and active PAI-1, but that a high plasma platelet content (such as in PRP) is required before the active PAI-1 present in platelets has functional effects on plasma fibrinolytic potential.

Follow-up study

Data from the follow-up study, clearly demonstrated the significant effects of platelets present in plasma, on plasma PAI-1_{ag} levels. Platelet count and β TG and PAI-1_{ag} levels were highly correlated in the different plasma preparations containing platelets (352 g and 200 g), with no significant associations in the PPP. PAI-1_{ag} levels in PRP already tended to correlate with whole blood platelet count. PAI-1_{ag} levels, were furthermore, up to 22 fold higher in PRP when compared to basal levels in PPP (which was exempted from the possible influence of residual platelet content or *in vitro* platelet α -granule release), highlighting the magnitude of the effect of platelets on plasma PAI-1_{ag} levels, compared to other sources of PAI-1

Table 6. Spearman rank order correlations between β TG and PAI-1_{ag} in the respective plasma preparations of the follow-up study.

Variables	β TG—PAI-1 _{ag} r (p-value)
CTAD plasma 2000 g	-0.61 (0.002)
Citrate plasma 352 g x 1	0.55 (0.006)
Citrate plasma 200 g x 1	0.74 (<0.0001)

β TG, beta thromboglobulin; CTAD, citrate-theophylline, adenosine, dipyridamol; g, gravitational acceleration; PAI-1, plasminogen activator inhibitor-1; PAI-1_{ag}, PAI-1 antigen.

doi:10.1371/journal.pone.0171271.t006

(endothelial cells, hepatocytes, smooth muscle cells and adipocytes) [11, 13]. The additional 1.3 fold increase in plasma PAI-1_{ag}, subsequent to maximal degradation (5 x freeze-thaw cycles), suggests that *in vitro* platelet degradation can contribute to a further increase in plasma PAI-1_{ag} levels, confirming the necessity of the use of the correct plasma preparation protocols to standardise platelet count and to ensure the preparation of platelet-poor plasma ($<10 \times 10^3/\mu\text{L}$). Differences in platelet size were also detected when comparing the 352 g and the 200 g plasma, indicating the presence of different platelet populations in the samples centrifuged at different speeds. Since platelets with larger sizes are known to be more metabolically active than smaller platelets [25], platelet size, in addition to platelet count, most likely influence the relationship between platelets present in plasma and PAI-1_{ag} levels.

Although βTG is extensively used as a marker of platelet alpha granule release, it does have limitations. The sensitivity of βTG as a marker of platelet activation and alpha granule release can be influenced by various factors; including the choice of anticoagulant, and sample handling and preparation procedures [26, 27]. The significant correlation between platelet count and βTG ($r = 0.91$, $p < 0.0001$) does however support its use as a proxy marker for the number of platelets in plasma in our study populations. While PAI-1 activity may be influenced by freeze-thaw cycles, we opted to work with frozen samples as this type of sample is most often used in studies and therefore relevant to a larger audience. It should be noted that since all samples were frozen at least once, possible effects of freezing on platelet function cannot be excluded. All plasma preparations were however treated similarly making comparison between the different preparations possible. Although samples were not specifically treated to prevent possible *in vitro* conversion of active to latent PAI-1, samples were processed within 20 minutes after collection and snap frozen to limit *in vitro* conversion.

The results from the present study indicate that the content of the alpha granules released from platelets in plasma, significantly influences plasma PAI-1_{ag} levels, with limited effects on PAI-1_{act}, tPA/PAI-1 complex or fibrinolysis rate (measured as CLT). This effect on PAI-1_{ag} is thought to be largely due to an increased release of latent PAI-1 from platelets which is unable to bind tPA and inhibit fibrinolysis. Due to the potential contribution of latent PAI-1 to PAI-1_{ag} levels, PAI-1_{act} may be the more clinically useful assay to determine the fibrinolytic inhibitor capacity of plasma. In plasma with a high platelet count, such as PRP, the component of platelet PAI-1 that is active, may, however have functional effects by decreasing plasma fibrinolytic potential. These results suggest that PAI-1_{ag} is more sensitive to the presence of platelets in plasma, than other PAI-1 assays (PAI-1_{act} and tPA/PAI-1 complex) or CLT but that these assays may also be influenced by platelets when present in high numbers such as in PRP.

Supporting information

S1 Data. SABPA study.

(XLSX)

S2 Data. Follow-up study.

(XLSX)

Acknowledgments

We would like to thank the SAPBA research team, the field-workers of the North-West University, South Africa, and the SAPBA project leader, Prof Leone Malan.

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**ANNEXURE E: PUBLISHED ARTICLE - DEGREE OF OBESITY
INFLUENCES THE RELATIONSHIP OF PAI-1 WITH BODY FAT
DISTRIBUTION AND METABOLIC VARIABLES IN AFRICAN WOMEN
(CHAPTER 4)**



Full Length Article

Degree of obesity influences the relationship of PAI-1 with body fat distribution and metabolic variables in African women



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ARTICLE INFO

Article history:

Received 21 April 2016

Received in revised form 19 August 2016

Accepted 6 September 2016

Available online 13 September 2016

Keywords:

Plasminogen activator inhibitor-1

Abdominal obesity

Sarcopenic obesity

Dual-energy x-ray absorptiometry

ABSTRACT

Introduction: Although the relationship of plasminogen activator inhibitor-1 (PAI-1) with obesity has been well established, the relationship of PAI-1 with different body fat distribution patterns is less clear particularly in non-white ethnicities.

Methods: We investigated the cross-sectional association of PAI-1_{act} with body fat % and two different body fat distribution patterns, namely sarcopenic obesity (SO) and visceral (VAT) compared to subcutaneous (SCAT) abdominal obesity, in 246 healthy African women by creating sub-groups according to different body fat distribution patterns.

Results: The PAI-1_{act} level of the SO group did not differ significantly from that of the excessive % body fat, non-sarcopenic group ($p = 0.8$). The relationship of PAI-1_{act} with body fat %, insulin, triglycerides and appendicular skeletal mass (ASM) was influenced by body fat distribution patterns and degree of obesity. PAI-1_{act} was higher (1.65 vs 0.16 U/ml; $p = 0.001$) in women with a proportionally higher abdominal VAT compared to higher abdominal SCAT compartment in the total study population, but not in the centrally obese sub-group (1.72 vs 0.83 U/ml; $p = 0.5$). Multiple regression models indicated that body fat % *per se* did not contribute significantly to PAI-1_{act} variance in women with increased fat mass.

Conclusion: Fat distribution patterns and degree of obesity influenced the association of PAI-1_{act} with insulin, triglycerides, ASM and body fat % in African women. In centrally obese women, abdominal VAT no longer contributed more to plasma PAI-1_{act} than abdominal SCAT. Inflammation and endothelial dysfunction contributed more to PAI-1_{act} variance in obese African women than did body fat % *per se*.

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1. Introduction

Over the last decades the prevalence of obesity has increased dramatically, such that it is currently considered a global pandemic [1]. A world-wide estimate on the prevalence of overweight and obesity has

shown that from 1980 to 2013 the proportion of overweight men and women have increased (from 28.8% to 36.9% and 29.8% to 38.0%, respectively) [2]. South Africa is no exception, according to the South African National Health and Nutrition Examination Survey (SANHANES) of 2013, the prevalence of overweight and obesity in women is 24.8% and 39.2%, respectively, which is significantly higher than in men (20.1% and 10.6%, respectively) [3]. Furthermore, females above 45 years of age have a significantly higher prevalence of obesity than younger females [3].

Plasminogen activator inhibitor type 1 (PAI-1), an important inhibitor of fibrinolysis, has previously been reported to be associated with obesity in both *in vitro* [4–7] and *in vivo* studies [8,9]. Furthermore, PAI-1 has shown strong associations with the metabolic syndrome (metS) and insulin resistance (IR) [10–12] and other co-morbidities associated with obesity such as hypertension, myocardial and cerebral infarction, atherosclerosis and inflammation [13].

Although the relationship of PAI-1 with obesity and more specifically central obesity (frequently determined by waist circumference (WC)) has been well established [4,14,15], the relationship of PAI-1 with different central obesity fat distribution patterns is less clear particularly in non-white ethnicities, such as Africans. It is for instance

Abbreviations: ANCOVA, analysis of co-variance; ANOVA, analysis of variance; ASM, appendicular skeletal muscle mass; BMI, body mass index; CI, confidence interval; CRP, C-reactive protein; DXA, dual-energy x-ray absorptiometry; ECLIA, electrochemiluminescence immunoassay; FNHI, Foundation for the National Institutes of Health; HIV, human immunodeficiency virus; HREC, Health Research Ethics Committee; HOMA-IR, homeostatic model assessment of insulin resistance; IR, insulin resistance; ISAK, International Society for the Advancement of Kinanthropometry; MetS, metabolic syndrome; PAI-1, plasminogen activator inhibitor-1; PAI-1_{act}, PAI-1 activity; SCAT, subcutaneous adipose tissue; SO, sarcopenic obesity; SONDRRA, Sarcopenic Obesity and Non-communicable Disease Risk in African Adults; SANHANES, South African National Health and Nutrition Examination Survey; PURE, Prospective Urban and Rural Epidemiology; VAT, visceral adipose tissue; VLDL, very low density lipoproteins; WC, waist circumference; WHtR, waist-to-height ratio.

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possible that the abdominal visceral adipose tissue (VAT) to abdominal subcutaneous adipose tissue (SCAT) ratio could potentially influence plasma PAI-1 levels, despite similar WC. In general PAI-1 production was found to be higher in VAT (as an ectopic fat depot) than in SCAT due to the higher amount of stromal cells [5,6], production of pro-inflammatory cytokines and higher macrophage content/infiltration [16, 17], however the importance of PAI-1 expression in abdominal SCAT has also been demonstrated [18,19]. It has furthermore been demonstrated that body fat distribution patterns differ between ethnicities, with African women having significantly less VAT compared to white women, despite similar WC [20]. In addition, the association of PAI-1 with body composition also differs between ethnicities [10,21] necessitating these studies in non-white individuals.

Furthermore, limited information is available regarding the effect of different forms of obesity, such as sarcopenic obesity (SO), on PAI-1 [22, 23]. SO is defined as the co-occurrence of increased fat mass in the presence of age related loss of skeletal muscle mass and strength and is typically associated with fat infiltration in the muscle [24,25]. SO has also previously been suggested to be strongly associated with the development of metS and atherosclerosis [24]. Hence, it is possible that plasma PAI-1 levels might be more increased in individuals with SO compared to non-sarcopenic obese individuals.

Understanding the relationship between PAI-1 and different body fat distribution patterns, is important to predict the disease risk conveyed through PAI-1 in individuals who did not escape the overweight and obesity pandemic, especially in the under-studied African population. We consequently aimed to investigate the relationship of PAI-1 with two different body fat distribution patterns, namely SO and visceral compared to subcutaneous abdominal obesity in African women. In addition, we explored the relationship between PAI-1 and body fat % in the different body fat distribution patterns. Data on insulin, triglycerides, appendicular skeletal mass (ASM) and inflammation (C-reactive protein) are also provided as these may act as co-variables in the relationship of PAI-1 with body fat [26–28].

2. Methods and methods

2.1. Study population

The study population included cross-sectional data collected in 2012 from 246 urban dwelling African women as part of the Sarcopenic Obesity and Non-communicable Disease Risk in African Adults (SONDRRA) study. The SONDRRA study is nested in the South African arm of the longitudinal Prospective Urban and Rural Epidemiology (PURE) study. Detailed information on the study design and participant selection criteria of the PURE study have been previously reported [29]. In brief, the sub-study included participants that were randomly selected from households in the Tlokwe municipality, located in the North West province of the South Africa. The inclusion criteria were apparent physically and psychologically healthy African women. Participants were excluded if they presented with any disabilities, serious diseases, taking anabolic steroids or protein supplements, as well as pregnant or lactating women. Twenty-six of the women were premenopausal and only one woman in the cohort used hormone replacement therapy (HRT). Sixty percent of the women were taking anti-hypertensive drugs and approximately half were current or past tobacco users. Only coded data was used and the names of the participants remained confidential. The study was approved by the Health Research Ethics Committee (HREC) of the North West University (Potchefstroom Campus) (NWU-00016-10-A1) and all participants gave written informed consent.

2.2. Anthropometrical assessment

Body weight was measured to the nearest 0.1 kg on an electronic scale (Seca, Birmingham, UK) and height was measured with a free-standing stadiometer (Seca, Birmingham, UK) to the nearest 0.1 cm.

Waist circumference was also recorded to the nearest decimal using a steel measuring tape (Lufkin, Apex, NC, USA). The thicknesses of the abdominal and supraspinal skinfolds were measured with a Harpenden calliper (Baty International, West Sussex, UK) on the landmarks as established by the International Society for the Advancement of Kinanthropometry (ISAK). Skinfolds were measured to the nearest 0.1 mm by gently pulling the skin away from the body, taking care not to include the underlying muscle in the double layer of skin and subcutaneous fat. To improve the accuracy and consistency of all measurements, these measurements were performed twice by the same anthropometrist and the average calculated.

Body composition (fat mass; fat-free soft tissue mass; and body fat %) was measured using DXA (Hologic Discovery W, APEX system software version 2.3.1). Fat mass and fat-free soft tissue mass for the whole body, trunk and limbs were derived using standard DXA cut-off lines. Appendicular skeletal muscle mass (ASM) was derived as the sum of the fat-free mass excluding bone of the arms and the legs [30].

2.3. Determination of body fat distribution patterns

The women were categorised into several sub-groups according to distinct body composition patterns, in order to investigate the relationship between PAI-1 and different body fat distribution patterns. These groups were determined by using the available DXA and anthropometrical data. Body fat % instead of body mass index (BMI) was used in this study, as body fat % is considered a more sensitive marker of excessive adiposity than BMI [31].

2.4. Sarcopenic obesity

To investigate the relationship between PAI-1 and SO, the study population was divided into four mutually exclusive groups. Group 1 – excessive body fat % group ($n = 115$), was determined as body fat percentage $>35.8\%$ for women aged 30–49 years and body fat percentage $>37.7\%$ for women aged 50–84 years [32]. Sarcopenia was an exclusion criterion for this group. Group 2 – sarcopenic group ($n = 59$), sarcopenia was defined as $ASM < 15.02$ kg based on the guidelines of the Foundation for the National Institutes of Health (FNIH) [33]. The FNIH ASM cut-point was chosen, since it had been found to be sensitive to detect reduced functional ability in the black female South African population [34]. Women with excessive body fat % were excluded from this group. Group 3 – SO group ($n = 36$), this group included individuals who were both sarcopenic and presented with excessive body fat %. Lastly, group 4 included women who were non-sarcopenic and had a normal body fat % ($n = 36$).

2.5. Visceral compared to subcutaneous central abdominal obesity

Since the use of sophisticated techniques such as nuclear magnetic resonance or CT scans was not a financially viable option in our research setting, we created a surrogate marker to differentiate between women with preferential visceral compared to preferential subcutaneous central abdominal obesity. This was done by obtaining the average of the central body skinfolds (abdominal and supraspinal) and dividing it by the WC. We then divided the group into tertiles according to the skinfold:WC ratio variable in order to compare the highest (proportionally more SCAT) with the lowest (proportionally more VAT) tertile group. This was also done separately for women with central obesity (defined as WHtR of >0.5) [35] to investigate the effect of the two forms of abdominal fat distribution on PAI-1_{act} levels in women already defined to be centrally obese.

2.6. Blood and urine collection

A registered nurse collected fasting blood samples with minimum stasis from the antebrachial vein before 10:00 in the morning. Serum

samples were used for C-reactive protein (CRP), triglycerides and insulin analyses, sodium fluoride plasma for glucose and citrated plasma samples for the analysis of PAI-1_{act}. Samples were centrifuged at 2000 × g for 15 min and stored at −82 °C until further analysis. Spot urine was collected for albumin and creatinine determination.

2.7. Biochemical analysis

PAI-1_{act} was measured using an indirect enzymatic method (Technozym PAI-1 Actibind, Technoclone, Vienna, Austria). Serum insulin levels were measured by an electrochemiluminescence immunoassay (ECLIA) with a Roche Elecsys immunoassay analyser kit from Cobas®. Plasma glucose concentrations were determined via a hexokinase method using the Synchron®Systems (Beckman Coulter Co., Fullerton, CA, USA). HIV status was determined using the First Response rapid HIV test (PMC Medical, India) and positive results were confirmed with CD4 testing. High sensitivity CRP was determined using the Cobas Integra 400® plus biochemistry analyser (Roche diagnostics, Basel, Switzerland). Triglycerides measurements were done by Sequential Multiple Analyser Computer (SMAC) using the Konelab20iTM auto analyser (Thermo Fisher Scientific Oy, Vantaa, Finland). Urinary albumin and creatinine were determined with the Cobas Integra 400 plus (Roche, Basel, Switzerland) and the ratio calculated. HIV testing, CRP, triglycerides, insulin, glucose, albumin and creatinine measurements were performed to describe the health status of the participants and also since they may act as co-variables in the relationship between PAI-1 and body fat [36,37].

2.8. Calculation of insulin resistance

The estimate of IR as determined by the homeostasis model assessment (HOMA) was calculated using the formula: $HOMA-IR = \{(\text{fasting plasma insulin } (\mu\text{U/ml}) \times \text{fasting plasma glucose (mmol/l)}) / 22.5\}$ concentration} [38].

2.9. Statistical analysis

Data was analysed using the computer software package Statistica (Statsoft Inc., Tulsa Oklahoma, USA). A p-value of 0.05 or less was regarded as statistically significant. Since most of the variables were not normally distributed, the data was log-transformed to improve normality and to allow the use of parametric statistical analyses. Descriptive data is presented both as median (25th; 75th percentiles) and mean ($\pm$ standard deviation). As the association of PAI-1 with many of the body fat variables were not linear we decided to follow the approach of sub-dividing the study population rather than to present the data of the total group as continuous data in order to get a better representation of the true relationships. The *t*-test for independent samples was used to compare differences between two groups. Analysis of variance (ANOVA) with Tukey's honest significant difference *post-hoc* tests were used to compare differences between more than two groups and analysis of co-variance (ANCOVA) was used when adjustment for confounders was required. Pearson and partial correlations were used to determine correlations between log-transformed variables. Single and forward stepwise multiple regression analyses were used to determine the association of body fat % with PAI-1. The covariates entered into the multiple regression model were: insulin, age, skinfold:WC ratio, tobacco use, alcohol consumption, CRP, body fat %, triglycerides, menopausal status and albumin:creatinine ratio as a marker of endothelial dysfunction. These co-variables were based on their potential influence on the relationship between PAI-1 and body fat [26–28,37,39,40]. Women with CRP > 10 mg/l were excluded from analysis [41]. Only the variables that entered the stepwise multiple regression are presented in the tables.

3. Results

3.1. Sarcopenic obesity

The study population consisted of 246 women who were grouped according to the following identified fat distribution patterns in order to investigate the relationship between PAI-1_{act} and SO: excessive body fat % (*n* = 115), sarcopenic (*n* = 59), SO (*n* = 36) and non-sarcopenic, normal body fat % (*n* = 36) (Table 1). One-way ANOVA indicated no significant age differences between the groups (*p* = 0.5) with the sarcopenic group having the highest prevalence of HIV infection (22%). *Post-hoc* comparison between the groups revealed that for most of the variables (ASM, insulin, glucose, HOMA-IR, body fat %, WC, BMI, WHtR, skinfold:WC ratio), the sarcopenic group had significantly lower values than the other three groups with little difference between the other groups.

PAI-1_{act} of the SO group did not differ significantly from that of the excessive body fat % group (*p* = 0.8) or the non-sarcopenic, normal body fat % (*p* = 0.7). There was also no significant difference between metS-related markers (insulin, glucose, HOMA-IR and triglycerides) between the SO and excessive body fat % group. The excessive body fat % group did however, have significantly higher BMI, central obesity (WC and WHtR) and a lower central skinfold:WC ratio indicating a proportionally higher central VAT than SCAT fat distribution.

The association of PAI-1_{act} with body fat %, insulin, triglycerides and ASM differed between the different body fat distribution sub-groups (Table 2). PAI-1_{act} correlated with body fat % in the non-sarcopenic, normal body fat % only (*r* = 0.39; *p* = 0.02) with adjustment for insulin, triglycerides, menopausal status affecting this relationship only marginally (*r* = 0.34; *p* = 0.07). PAI-1_{act} showed a borderline correlation (*r* = 0.31; *p* = 0.07) with insulin in the SO group, but not in any of the other sub-groups. It had a significant positive association with triglycerides in the excessive body fat % group (*r* = 0.27; *p* = 0.005) with an association of similar magnitude in the non-sarcopenic, normal body fat % group (*r* = 0.3; *p* = 0.09), albeit non-significant probably due to the smaller sample size. PAI-1_{act} correlated with ASM in the non-sarcopenic, normal body fat % group only (*r* = 0.38; *p* = 0.03).

3.2. Visceral compared to subcutaneous central obesity

We divided the study group into tertiles according to the skinfold:WC ratio variable and compared PAI-1_{act}, insulin and HOMA-IR between the first and third tertile to determine the differences in women with proportionally higher abdominal VAT (quartile 1) vs women with proportionally higher abdominal SCAT (quartile 3). PAI-1_{act}, insulin and HOMA-IR were statistically and clinically significantly higher in the group with the proportionally higher abdominal VAT (quartile 1) (Table 3). When selecting only women with central obesity (WHtR > 0.5), the differences between the first and third quartiles were no longer significant. In addition, PAI-1_{act} correlated significantly (albeit weakly) with the skinfold:WC ratio in the total study population (*r* = −0.18; *p* = 0.007), but not in the centrally obese women (*r* = −0.12; *p* = 0.1).

3.3. Association of PAI-1_{act} with body fat %

In order to determine the relationship of PAI-1, insulin, triglycerides, CRP and skinfold:WC ratio with body fat %, the entire study population was divided into quartiles according to their body fat % (Table 4). PAI-1_{act} increased across the body fat % quartiles, but significance was reached in the fourth quartile only (body fat % > 45.6%). No increase was observed between the second and third quartile. Insulin and CRP levels in quartile two, three and four were all significantly higher than in quartile one with no difference in triglyceride levels across the four quartiles. Furthermore, CRP had a significant positive correlation with body fat % (*r* = 0.38, *p* < 0.0001) and correlated negatively with the

Table 1
Descriptive statistics of the different body fat distribution groups.

Variables	Sarcopenic obese (n = 36)		Excessive body fat % ^c (n = 115)		Sarcopenic ^d (n = 59)		Non-sarcopenic, normal body fat % (n = 36)		ANOVA p-Value
	Mean (± Std.)	Median (25th; 75th percentile)	Mean (± Std.)	Median (25th; 75th percentile)	Mean (± Std.)	Median (25th; 75th percentile)	Mean (± Std.)	Median (25th; 75th percentile)	
Age (years)	60.6 (± 11.1)	61.0 (50.5; 68.0)	57.6 (± 9.98)	55.0 (49.0; 66.0)	58.6 (± 11.4)	57.5 (50.0; 62.0)	58.7 (± 9.82)	55.0 (52.0; 65.0)	0.5
PAI-1 _{act} (U/ml)	3.89 (± 8.10)	0.78 (0.01; 4.22)	4.00 (± 7.23)	1.52 (0.01; 4.13)	1.68 (± 3.61)	0.60 (0.01; 1.76)	2.77 (± 5.19)	0.01 (0.01; 1.66)	0.09 (0.17) ^b
ASM (kg)	13.5 (± 1.21)	13.8 (12.7; 14.3) ^a	19.2 (± 2.98)	18.5 (16.8; 20.6)	12.5 (± 1.49)	12.5 (11.4; 13.4) ^a	18.1 (± 2.33)	17.3 (16.3; 19.3)	<0.0001
Insulin (μU/ml)	18.2 (± 13.1)	14.2 (8.94; 20.0)	22.4 (± 22.9)	15.0 (10.6; 24.0)	12.7 (± 20.0)	6.20 (4.31; 14.0) ^a	26.0 (± 24.7)	15.5 (9.30; 25.7)	<0.0001
Glucose (mmol/l)	6.25 (± 4.48)	5.00 (4.43; 6.00)	5.86 (± 3.19)	5.00 (4.46; 5.86)	4.65 (± 1.61)	4.31 (4.01; 4.98) ^a	6.78 (± 5.00)	5.09 (4.31; 6.67)	0.002
HOMA-IR	5.86 (± 9.21)	3.15 (2.11; 4.68)	6.73 (± 13.2)	3.60 (1.90; 6.44)	2.92 (± 5.27)	1.19 (0.78; 2.83) ^a	10.9 (± 20.2)	4.45 (2.07; 7.76)	<0.0001
Triglyceride (mmol/l)	1.40 (± 1.29)	1.23 (0.78; 1.53)	1.22 (± 0.84)	0.98 (0.74; 1.33)	1.19 (± 0.86)	0.89 (0.67; 1.49)	1.43 (± 1.01)	1.09 (0.78; 1.58)	0.3
Fat (%)	42.6 (± 3.83)	41.2 (39.7; 45.1)	45.2 (± 3.99)	45.2 (42.3; 47.9)	30.2 (± 5.22)	30.9 (26.6; 34.5) ^a	33.5 (± 4.17) ^a	35.1 (31.6; 36.3)	<0.0001
WC (cm)	86.2 (± 10.5)	85.7 (76.8; 95.0)	98.3 (± 10.7)	98.5 (92.1; 104) ^a	71.2 (± 9.22)	70.3 (65.5; 76.3) ^a	87.1 (± 8.79)	86.2 (81.7; 95.3)	<0.0001
WhtR	0.57 (± 0.08)	0.57 (0.51; 0.63)	0.63 (± 0.07)	0.62 (0.59; 0.66) ^a	0.46 (± 0.06)	0.46 (0.42; 0.50) ^a	0.54 (± 0.06)	0.53 (0.51; 0.59)	<0.0001
Skinfold:WC ratio	4.60 (± 2.15)	4.00 (3.14; 5.18)	3.10 (± 1.01)	2.83 (2.47; 3.50) ^a	8.76 (± 4.24)	7.70 (5.36; 11.5) ^a	5.64 (± 3.28)	4.40 (3.40; 7.54)	<0.0001
Abdominal skinfold (mm)	27.3 (± 12.2)	27.6 (17.5; 35.0)	41.5 (± 12.1)	41.7 (32.1; 50.5)	13.1 (± 7.05)	12.6 (7.10; 18.2)	25.6 (± 13.2)	25.2 (14.5; 33.3)	<0.0001
Supra-spinal skinfold (mm)	17.8 (± 8.81)	17.4 (11.2; 24.7)	27.5 (± 10.2)	26.7 (19.7; 34.8)	7.31 (± 3.88)	6.60 (4.10; 9.35)	14.8 (± 8.05)	14.1 (8.50; 19.2)	<0.0001
BMI (kg/m ²)	27.9 (± 4.82)	26.6 (24.6; 30.5) [^]	34.8 (± 5.75)	34.0 (30.8; 38.1) ^{^S}	20.4 (± 2.79)	20.5 (19.1; 21.8) ^{^S}	26.3 (± 3.45)	26.1 (24.0; 29.0) ^S	<0.0001
HIV status n (%)	4 (11%)		5 (4%) [^]		13 (22%) [^]		5 (14%)		0.005
MetS n (%)	16 (44%) ^S		54 (47%) [^]		8 (13.5%) ^{S^}		13 (36%)		<0.0001
Menopausal status (%)	31 (86.1%)		101 (87.8%)		54 (91.5%)		33 (91.7%)		0.81
Tobacco users (%)	14 (40%)		50 (45%)		30 (57)		35 (60%)		0.2

ASM, appendicular skeletal muscle mass; HIV, human immunodeficiency virus; HOMA-IR, homeostatic model assessment of insulin resistance; metS, metabolic syndrome; PAI-1_{act}, Plasminogen activator inhibitor-1 activity; WC, waist circumference; WhtR, weight-to-height ratio. ^{^S} Medians/means/percentages with the same symbol differ significantly.

^a Significantly different from the other three groups.

^b ANCOVA p-value after adjustment for insulin.

^c Fat% >35.8% for 30–49 years of age or >37.7% for 50–84 years of age [32].

^d ASM <15.02 kg [33].

skinfold:WC ratio ($r = -0.31$, $p < 0.0001$) (data not shown). Skinfold:WC ratio levels decreased across the body fat % quartiles, with a significantly lower skinfold:WC ratio in the fourth compared to the first quartile, indicating a proportionally higher VAT vs. SCAT distribution in the fourth quartile.

In order to determine the contribution of body fat % to PAI-1_{act} we performed single and forward stepwise multiple regression for each of the body fat % quartile groups (Table 5). In the single regression models, body fat % contributed significantly ($p = 0.01$) to the variance in PAI-1_{act} in the third quartile only (>40.5 to ≤45.6% body fat), explaining 11% of the variance. In the forward stepwise multiple regression models, insulin made the biggest contribution (12.4%) to the variance in PAI-1_{act} followed by the albumin:creatinine ratio, menopausal status and age, with fat % not entering the model, in the first quartile. In the second quartile, triglycerides, age and insulin entered the model explaining 19.8, 4.9 and 2%, respectively. In the third quartile, in agreement with the single regression, fat % contributed to the variance in PAI-1_{act}, explaining 5.31% of the variance with age (4.07%) and triglycerides (12.7%) also entering the model. In the fourth quartile, the main contributors to PAI-1_{act} variance were the albumin:creatinine ratio explaining 10.7%, CRP 3.58%, menopausal status 2.88% and skinfold:WC 2.99% of

the PAI-1 variance. In the most obese group (body fat % >45.6%) the multiple regression models explained the smallest percentage of total PAI-1 variance (16%) compared to the other groups (24, 26.7 and 22%).

4. Discussion

Although the association between PAI-1 and obesity has been well established, much less is known regarding the relationship of PAI-1 with different body fat distribution patterns, with virtually no information available on non-white ethnicities such as Africans. The association of PAI-1 with VAT [21] and metS [10] has for instance been shown to differ between black and white individuals. PAI-1 associated significantly with VAT in Caucasians, while no association was found in African Americans [21]. PAI-1 was also found to be more prominently associated with markers of the metS in white than in black Africans [10]. Furthermore, differences in body fat distribution have also been found, with African women having significantly less VAT compared to white women, despite similar WC [20]. It is, therefore, postulated that due to ethnic differences in body fat composition and fat deposition, the relationship of PAI-1 with body composition in blacks may differ from that observed for other population groups and, therefore, merits further

Table 2
Associations of PAI-1_{act} with body fat percentage, insulin, triglyceride and ASM in the sub-groups.

Variables	Groups	n	Body fat %		Insulin (μU/ml)	Triglyceride (mmol/l)	ASM (kg)
			r (p-value)	Partial r (p-value) ^a	r (p-value)	r (p-value)	r (p-value)
PAI-1 _{act} (U/ml)	Total	235	0.20 (0.002)	0.20 (0.003)	0.09 (0.2)	0.25 (<0.0001)	0.08 (0.2)
	Sarcopenic obese	36	0.24 (0.16)	0.16 (0.4)	0.31 (0.07)	0.22 (0.2)	0.002 (0.10)
	Excessive body fat % ^b	110	0.14 (0.15)	0.14 (0.1)	0.09 (0.4)	0.27 (0.005)	−0.02 (0.8)
	Sarcopenic ^c	56	−0.01 (0.9)	0.01 (0.9)	−0.13 (0.4)	0.22 (0.10)	−0.22 (0.1)
	Non-sarcopenic, normal body fat %	33	0.39 (0.02)	0.34 (0.07)	0.10 (0.6)	0.30 (0.09)	0.38 (0.03)

ASM, appendicular skeletal muscle mass; PAI-1_{act}, plasminogen activator inhibitor-1 activity; SO, sarcopenic obesity.

^a Adjusted for insulin, triglyceride and menopausal status.

^b Fat% >35.8% for 30–49 years of age or >37.7% for 50–84 years of age [32].

^c ASM <15.02 kg [33].

Table 3Difference in PAI-1_{act}, insulin and HOMA-IR between the 1st and 3rd skinfold:WC ratio tertiles.

Variables	Total study group				Central obese group ^a			
	↑VAT ↓SCAT		↑SCAT ↓VAT		↑VAT ↓SCAT		↑SCAT ↓VAT	
	Skinfold:WC ratio tertile 1 (≤3.02) median (25, 75% percentile)	n	Skinfold:WC ratio tertile 3 (>4.76) median (25, 75% percentile)	n	Skinfold:WC ratio tertile 1 (≤2.8) median (25, 75% percentile)	n	Skinfold:WC ratio tertile 3 (>3.8) median (25, 75% percentile)	n
PAI-1 _{act} (U/ml)	1.65 (0.01; 5.12)	78	0.16 (0.01; 1.40)	78	1.72 (0.01; 3.76)	59	0.83 (0.01; 4.13)	59
Insulin (μU/ml)	16.3 (10.3; 25.3)	76	8.83 (5.19; 16.0)	81	15.9 (10.6; 24.8)	58	13.5 (7.54; 23.4)	59
HOMA-IR	4.03 (2.37; 6.47)	77	1.77 (0.90; 3.48)	81	3.83 (2.31; 6.47)	59	3.00 (1.43; 5.74)	59

HOMA-IR, homeostatic model assessment of insulin resistance; PAI-1_{act}, Plasminogen activator inhibitor-1 activity; WC, waist-circumference; SCAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.^a Waist-to-height ratio (WHtR) >0.5.

investigation. Our data demonstrate that in African women, the association of PAI-1_{act} with body fat %, insulin, triglycerides and ASM are influenced by body fat distribution patterns and degree of obesity. It investigates the association of PAI-1_{act} with SO and shows that while increased VAT is associated with increased plasma PAI-1_{act} levels, abdominal SCAT also significantly contributes to PAI-1_{act} levels, in obese women specifically. Lastly, we demonstrated that body fat % *per se*, does not significantly contribute to increased plasma PAI-1_{act} in women with high fat mass (body fat % >45.6%), but that other factors associated with obesity (known and as of yet unidentified), are more prominently related to the increased PAI-1_{act}.

4.1. Sarcopenic obesity

PAI-1 is significantly associated with both obesity and insulin resistance [11,12,42,43]. Since insulin resistance and metS appear to be closely related to SO [24], it was postulated that PAI-1_{act} may be even higher in SO individuals than in individuals who are obese without having sarcopenia. There was, however, no difference in PAI-1_{act} between the SO and the excessive body fat % group. This is likely due to the fact that in this study population, insulin-related markers did not differ between the two groups, suggesting that insulin resistance was not more prominent among the SO women than among the total excessive body fat % group. The prevalence of HIV infection on the other hand, was significantly higher in the sarcopenic group. It is, therefore, likely that the observed muscle wasting in this group may be the result of HIV infection in those affected. In addition, the excessive body fat % group was on average more centrally obese than the SO group and displayed a proportionally higher abdominal VAT component, two factors that each have the potential to significantly increase PAI-1_{act} [44–46]. These results are also in agreement with Cesari et al. [22], who found no significant association between either sarcopenia or obesity and PAI-1 concentration.

When investigating the relationship of PAI-1_{act} with body fat %, insulin, triglycerides and ASM, we found that the relationships differed

between the different groups. PAI-1 correlated significantly with body fat % and ASM in the non-sarcopenic, normal body fat % group only, with insulin in the SO group and with triglycerides in the excessive body fat % group. This suggests the association of PAI-1 with these variables is not a constant/fixed relationship, but that their relative contributions to PAI-1 are influenced by body fat distribution patterns.

4.2. Visceral compared to subcutaneous central obesity

PAI-1 is considered by many to be expressed more in VAT than in SCAT [5,6]. This is thought to be related to structural and functional differences between VAT and SCAT. Visceral adipose tissue, a major ectopic fat depot, present in the abdominal viscera in the mesentery and omentum, produces more pro-inflammatory cytokines like tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [16], it contains more stromal cells, which is the cellular component of adipose tissue that produces PAI-1 and exhibit a higher macrophage content which contributes to the increased cytokine production [6,17]. In contrast, SCAT expresses more leptin and adiponectin and is mainly found in the femoral-gluteal regions, back and anterior abdominal wall [16]. Evidence does, however, exist that PAI-1 mRNA in abdominal SCAT (but not femoral SCAT) correlated positively with plasma PAI-1 levels in obese individuals [19] and that PAI-1 expression in SCAT is higher than in VAT in obese individuals and in females [18]. This data suggests that the degree of obesity may influence regional differences in PAI-1 secretion. In general agreement with the literature, our data indicated that plasma PAI-1_{act} levels were significantly higher in black African women with a proportionally higher abdominal VAT compared to SCAT compartment. However, in women who were already centrally obese, this was no longer the case. This data together with the literature suggest that in obese women, abdominal SCAT can significantly contribute to the increased plasma PAI-1 levels found in obesity and in so doing, increase CVD risk.

Table 4PAI-1_{act}, insulin, triglyceride, CRP and skinfold:WC ratio according to body fat percentages quartiles.

Variable	Total group							
	Body fat% 1st quartile (≤34.5%)		Body fat% 2nd quartile (>34.5–≤40.5%)		Body fat% 3rd quartile (>40.5–≤45.6%)		Body fat% 4th quartile (>45.6%)	
	Median (25, 75% percentile)	n	Median (25, 75% percentile)	n	Median (25, 75% percentile)	n	Median (25, 75% percentile)	n
PAI-1 _{act} (U/ml)	0.07 (0.01; 1.23) [^]	58	0.82 (0.01; 3.26)	59	0.83 (0.01; 2.83)	59	2.28 (0.01; 5.94) [^]	59
Insulin (μU/ml)	7.01 (4.43; 14.23) ^a	61	14.9 (9.39; 26.7)	59	15.0 (9.04; 21.44)	59	14.4 (10.0; 23.4)	58
Triglyceride (mmol/l)	0.92 (0.72; 1.37)	62	0.99 (0.73; 1.56)	59	1.03 (0.79; 1.41)	59	0.99 (0.73; 1.27)	59
CRP (mg/l)	1.81 (0.58; 4.39) ^a	58	3.43 (1.82; 5.93)	47	3.62 (2.23; 5.56)	50	5.09 (3.39; 7.46)	37
Skinfold:WC ratio	8.41 (6.13; 11.48) ^{^S}	61	4.00 (3.35; 5.16) ^{^S}	61	3.10 (2.59; 4.02) [^]	61	2.79 (2.38; 3.30) ^S	60

PAI-1_{act}, plasminogen activator inhibitor-1 activity; CRP, C-reactive protein. ^{^S} Medians with the same symbol differ significantly.^a Significantly different from the other three groups.

Table 5
Contribution of body fat percentage to PAI-1_{act} variance: single and multiple regression.

Body fat % quartiles	Single regression					Forward stepwise multiple regression					
	n	β	95% CI	Total % variance explained	p-Value	Co-variables that entered model	β	95% CI	Total % variance explained	% variance explained by each co-variate	p-Value
1st quartile ($\leq 34.5\%$)	58	-0.65	-3.27; 1.97	0.4	0.6	Insulin	-0.52	-0.89; -0.15	26.5	12.4	0.009
						Alb:Creat	0.32	0.05; 0.59		9.99	0.02
						Menopausal status	-0.78	-2.19; 0.63		2.27	0.2
2nd quartile ($>34.5\%$ – $\leq 40.5\%$)	59	-3.11	-14.0; 7.83	0.6	0.6	Age	1.87	-0.68; 4.41		1.81	0.3
						Triglyceride	1.13	0.18; 2.08	26.7	19.8	0.02
						Age	-2.19	-5.34; 0.96		4.94	0.2
3rd quartile ($>40.5\%$ – $\leq 45.6\%$)	59	16.5	3.79; 29.1	11	0.01	Insulin	0.23	-0.49; 0.95		2.00	0.3
						Triglyceride	0.84	-0.05; 1.74	22.0	12.7	0.03
						Body fat %	12.0	-1.96; 26.0		5.31	0.07
						Age	1.86	-1.21; 4.94		4.07	0.2
4th quartile ($>45.6\%$)	59	0.35	-10.6; 11.4	<0.1	0.9	Alb:Creat	-0.85	-1.58; -0.12	20.2	10.7	0.03
						CRP	-0.55	-1.42; 0.36		3.58	0.2
						Menopausal status	1.78	-0.15; 4.71		2.88	0.2
						Skinfold:WC ratio	-1.44	-4.15; 1.27		2.99	0.3

Alb:Creat, albumin-creatinine ratio; CI, confidence interval; CRP, C-reactive protein; PAI-1_{act}, plasminogen activator inhibitor-1 activity; WC, waist circumference.

4.3. Association of PAI-1_{act} with body fat percentage

In obesity, there are many different factors that can influence PAI-1 levels. These include insulin resistance, inflammation and regulation of PAI-1 producing cells by inflammatory cytokines, increased triglycerides, as well as the increased adipocyte mass [42,43,47]. We subsequently wanted to determine the relationship of body fat % *per se* with PAI-1. When dividing the study population according to body fat % quartiles, body fat % contributed, in both single and multiple regression models to the PAI-1_{act} variance in quartile 3 only. This suggests that other factors than body fat % itself, which are associated with increased fat mass, are likely responsible for the increased PAI-1_{act} levels in quartile 4. This notion is supported by our data indicating a correlation between PAI-1_{act} and body fat % in the non-sarcopenic, normal body fat % women only while no associations were found in the excessive body fat % or the SO groups. It has also been suggested that PAI-1 is not closely dependent on fat mass, but rather reflects fat redistribution patterns [17,48].

In order to compare the contribution of body fat % *per se* to other factors associated with obesity on plasma PAI-1_{act}, we also investigated the role of insulin, triglycerides, menopausal status, CRP (as a marker of inflammation), skinfold:WC ratio, age, alcohol consumption, smoking and the albumin:creatinine ratio (as a marker of endothelial dysfunction). Insulin levels entered the stepwise multiple regression as a contributor to PAI-1_{act} variance in model 1 and 2 only, suggesting that it contributes proportionally more to PAI-1_{act} variance in women with a lower body fat %. Triglycerides did not differ across the body fat % quartiles, but it was the most significant contributor to PAI-1_{act} variance in the 2nd and 3rd body fat % quartile. The association between PAI-1 and triglyceride has been previously explained by the fact that very low density lipoproteins (VLDL) triglycerides increase PAI-1 levels [49,50], through a VLDL response element identified in the promoter region of the PAI-1 gene that mediates VLDL-induced PAI-1 transcription in endothelial cells [51]. Menopausal status entered the stepwise regression models as contributor to PAI-1_{act} variance, but its contribution was also not significant, explaining about 2.8% only. C-reactive protein contributed to the variance in PAI-1_{act} in women with high fat mass (quartile 4) only, although the contribution was relatively minor at 3.7%. Additional analysis confirmed a positive relationship between CRP and body fat %, indicating that inflammation increases as body fat % increase, as is known from the literature [52,53]. We also found a negative association between CRP and the skinfold:WC ratio, suggesting that inflammation is increased in the presence of proportionally increased abdominal VAT.

Next we investigated the possible contribution of the abdominal VAT / SCAT distribution and found that there was a significant proportional increase in the abdominal VAT compartment as body fat % increased, and that it served as a significant contributor to the PAI-1_{act} variance in the most obese women. This proportional increase in VAT is in agreement with the literature indicating that post-menopausal women experience a redistribution of fat distribution with increased visceral fat accumulation, which is thought to be related to the female sex hormone oestrogen [54]. The largest (and only statistically significant) contributor to PAI-1_{act} in the obese individuals was endothelial dysfunction (albumin:creatinine ratio). Evidence exists for the presence of endothelial dysfunction in obesity [37], which is thought to be induced by obesity-associated metabolic abnormalities such as insulin resistance, adipokines, oxidative stress, increased free fatty acids, TNF- α as well as activation of innate immunity [55,56]. At the same time, increased PAI-1 is recognised as an early marker of endothelial dysfunction [57, 58] suggesting endothelial dysfunction as a mechanistically plausible modulator of PAI-1_{act} in obesity. It is also important to note that in the most obese individuals, the above mentioned known PAI-1 associates, explained the smallest percentage of total PAI-1 variance, compared to the other body fat % quartiles, suggesting that other, as of yet unidentified factors, additionally contribute to the increased PAI-1 in obesity. Our results indicate that in African women with increased fat mass, body fat % *per se* contributes to a lesser extent to plasma PAI-1_{act} than other obesity-related metabolic derangements such as inflammation and endothelial dysfunction.

A limitation of the study was that causality could not be determined for PAI-1_{act} being cross-sectional in design. While every attempt has been made to prevent possible selection bias, it is not impossible that it may have occurred in some form. A further limitation of the study was the inability to estimate VAT and SCAT directly using DXA software as this is not available in our laboratory. Also, we acknowledge that the skinfold:WC ratio is a novel and rather crude method to distinguish between abdominal fat distribution patterns. The fact that associations with PAI-1_{act} were found, suggest however that it could potentially be used in settings where access to more sophisticated techniques is limited and it deserves further investigation.

In conclusion, our data indicates that PAI-1_{act} is not higher in SO African women who do not display increased insulin resistance, compared to obese, non-sarcopenic women. Furthermore, fat distribution patterns and degree of obesity were found to influence the association of PAI-1_{act} with insulin, triglycerides, ASM and body fat %. Our data also suggests

that in centrally obese women, abdominal VAT no longer contributed more than abdominal SCAT to plasma PAI-1_{act}, where no differences were found between the two adipocyte compartments and that abdominal SCAT should be considered a significant contributor to plasma PAI-1 in obese women. Lastly in obese African women, the known PAI-1 associates explained a smaller percentage of total PAI-1_{act} variance compared to the other body fat % quartiles suggesting additional, as of yet unidentified mechanistic pathways in obesity. Also the increased PAI-1 observed in obesity in African women, is more strongly influenced by other obesity-related metabolic abnormalities such as inflammation and endothelial dysfunction than by body fat % *per se*. Whether these varying associations between PAI-1 and body fat distribution patterns in Africans will result in different disease risk profiles of the diseases contingent upon PAI-1 needs to be established in future research.

Conflict of Interest

None of the authors have any conflict of interest to declare.

Author contributions

Sunelle A. Barnard – analysis and interpretation of data; critical writing of manuscript.

Marlien Pieters – analysis and interpretation of data, critical writing of manuscript.

Cornelie Nienaber-Rousseau – interpretation of data and critical reviewing the manuscript.

Herculina S. Kruger – interpretation of data, critical reviewing the manuscript and PI of the SONDRRA study.

Acknowledgements

This work was supported by services of the SONDRRA research team and the Centre of Excellence for Nutrition of the North-West University, South Africa. Funding was through the National Research Foundation (NRF) of South Africa (Grant UID: 94213). Opinions expressed and conclusions arrived at, are those of the authors and are not to be attributed to the NRF.

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**ANNEXURE F: PUBLISHED ARTICLE - THE CONTRIBUTION OF
DIFFERENT ADIPOSE TISSUE DEPOTS TO PLASMA PLASMINOGEN
ACTIVATOR INHIBITOR-1 (PAI-1) LEVELS (CHAPTER 5)**



REVIEW

The contribution of different adipose tissue depots to plasma plasminogen activator inhibitor-1 (PAI-1) levels



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ARTICLE INFO

Keywords:

Plasminogen activator inhibitor-1
Visceral adipose tissue
Subcutaneous adipose tissue
Body fat distribution

ABSTRACT

Increased plasma plasminogen activator inhibitor-1 (PAI-1) level is considered a mechanistic pathway through which obesity contributes to increased cardiovascular disease risk. Abdominal adipose tissue specifically, is a major PAI-1 source with visceral adipose tissue (VAT), an ectopic fat depot, generally considered to produce more PAI-1 than subcutaneous adipose tissue. However, this does not necessarily lead to increased plasma PAI-1 levels. This review provides an overview of studies investigating the association between body fat distribution and plasma PAI-1 levels. It discusses factors that influence this relationship and also considers the contribution of other tissue to plasma PAI-1 levels, placing the relative contribution of adipose tissue into perspective. In conclusion, the relationship between VAT and plasma PAI-1 levels is not fixed but can be modulated by a number of factors such as the size of the subcutaneous adipose tissue depot, ethnicity, possibly genetics and other obesity-related metabolic abnormalities.

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1. Introduction

Plasminogen activator inhibitor-1 (PAI-1), the main inhibitor of fibrinolysis, contributes to increased cardiovascular risk in overweight and obese individuals [1]. Elevated plasma PAI-1 levels are considered to be a biochemical marker of obesity [2] and also as a component of the metabolic syndrome (MetS), which is characterised by dyslipidaemia, hypertension, glucose intolerance and increased abdominal fat distribution [3,4]. The association between PAI-1 and obesity, especially central obesity, has been well established in both animal and human studies [5–11] and is largely considered to be the result of PAI-1 production by adipose tissue [5,6,12,13]. PAI-1 is produced by a variety of cells contained in adipose tissue; these include pre-adipocytes, mature adipocytes, stromal cells, endothelial cells, smooth muscle cells and monocytes/macrophages [14].

Abdominal fat deposition takes place in two main fat depots [subcutaneous adipose tissue (SCAT) and intra-abdominal or visceral fat tissue (VAT)], which is considered to be an ectopic fat depot. Ectopic fat refers to the storage of fat in non-adipose tissue like the liver, skeletal muscle, viscera, pancreas and the heart [15,16]. Although, it is not yet certain how ectopic fat accumulation takes place, it is postulated to be related to an overflow of triglycerides into other organs as SCAT loses the ability to expand and to store excess energy [17]. According to this theory, the expandability of SCAT protects other organs against ectopic fat deposition [18]. In support thereof, SCAT does not seem to be associated with a linear increase in cardiovascular risk factors in obesity [19]. Furthermore, when compared to VAT, abdominal and thigh SCAT were observed to be protective against obesity-associated metabolic complications such as insulin resistance [20,21].

It is generally accepted that ectopic fat, and for the purpose of this review, VAT specifically, produces more PAI-1 than SCAT [5,22]. Consistently, computed tomography (CT) data from obese individuals have shown higher PAI-1 levels in VAT than SCAT [8,23]. Also, PAI-1 gene expression, determined by means of adipose biopsies, has been shown to be higher in the omental adipose tissue compared to SCAT during acute systemic inflammation which was also accompanied by increased plasma PAI-1 levels [9]. However, contradicting evidence also exists, as some studies have shown comparable PAI-1 antigen (PAI-1_{ag}) secretion from VAT and SCAT [24], or even higher PAI-1 messenger ribonucleic acid (mRNA) expression and increased rate of PAI-1_{ag} synthesis in SCAT than in VAT [25]. Although adipose tissue PAI-1 concentration and production have important local effects, it is the concentration of PAI-1 in the blood that contributes to the development of CVD

Abbreviations: BMI, body mass index; CT, computed tomography; IL-1, interleukin-1; IL-6, interleukin-6; NRF, National Research Foundation; MetS, metabolic syndrome; mRNA, messenger ribonucleic acid; PAI-1, plasminogen activator inhibitor-1; PAI-1_{act}, PAI-1 activity; PAI-1_{ag}, PAI-1 antigen; PCR, polymerase chain reaction; SCAT, subcutaneous adipose tissue; TGF-β1, transforming growth factor B; TNF-α, tumour necrosis factor-α; VAT, visceral adipose tissue; VLDL, very low density lipoproteins; WC, waist circumference; WHtR, waist-to-height ratio.

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[reviewed by 26,27] and therefore the effect of body fat distribution on plasma PAI-1 levels has important pathophysiological consequences for the development of obesity-related CVD. Evidence regarding the contribution of the different fat depots to plasma PAI-1 levels seems conflicting. While it has been shown that adipose tissue from different body fat depots contributes to plasma PAI-1 levels [6,8,23,28,29], others found PAI-1 expression in different adipose tissue depots not to be directly related to plasma PAI-1 levels [30,31]. What is also often not considered is the relative contribution of the different fat depots to plasma PAI-1 levels, in relation to other PAI-1 producing pathways associated with obesity such as insulin resistance, increased triglycerides, inflammation and endothelial dysfunction and their influence on the different PAI-1 producing tissues (adipose tissue, hepatocytes, smooth muscle cells, platelets and endothelial cells) in the body. This review aims to provide an overview of *ex vivo* and *in vivo* studies investigating the association between fat distribution and plasma PAI-1 levels. It also discusses the contribution of other PAI-1 producing pathways found in obesity in order to draw conclusions regarding the contribution of different adipose tissue depots to plasma PAI-1 levels.

2. *Ex vivo* studies

Several human *ex vivo* studies have been carried out that show adipose tissue to be an important source of PAI-1 (Table 1). These studies can be divided into studies investigating adipocyte PAI-1 content and or gene expression and those investigating plasma PAI-1 levels. Studies investigating adipocyte PAI-1 content and or gene expression strongly support a greater production and expression of PAI-1 mRNA in VAT compared with SCAT [12,13,22]. When using human adipose tissue in culture from obese individuals, Alessi et al. [5] found PAI-1_{ag} concentration to be greater in VAT than SCAT. The association between VAT and PAI-1 seems to be related to structural and functional differences between VAT and SCAT. Visceral adipose tissue, present in the abdominal viscera in the mesentery and omentum, produces more pro-inflammatory cytokines, such as tumour necrosis factor- α (TNF- α), transforming growth factor β (TGF- β 1), interleukin-6 (IL-6) and IL-1 [28,32,33], and also contains more stromal cells, one of the main cellular components of adipose tissue that produces PAI-1 [12] as well as macrophages which also produce PAI-1 [34,35].

However, there are some contradicting findings in human *ex vivo* studies. Eriksson et al. [25] for example reported that PAI-1 expression from abdominal SCAT was approximately two times higher than abdominal VAT in severely obese white individuals. Additionally, females were found to have a significant depot-specific PAI-1 secretion, with SCAT producing higher levels of PAI-1 than VAT [25], while these differences were not observed in men. Differences in findings could possibly be explained by the cell size of the different adipose tissue depots or even differences in study populations (as will be discussed below). In contrast with other studies [5,25,28], Eriksson et al. [25] expressed their results as nanograms of PAI-1 expressed/10⁷ cells, and not ng/g adipose tissue. Since the cell size of the SCAT was found to be larger than that of VAT, it could have influenced the results. In a review, Skurk and Hauner [14] even conclude that large fat cells produce more PAI-1 than small fat cells regardless of fat depot. Furthermore, Morange et al. [28] reported a high correlation between PAI-1 production by VAT and SCAT and concluded that there could be a similar regulatory pathway of PAI-1 in these two adipose depots, despite their different anatomic and metabolic characteristics. Differences can also be seen between different SCAT depots. When investigating PAI-1 expression from two subcutaneous territories (abdominal and femoral) in obese and lean individuals, the results indicated a higher PAI-1 mRNA content in the abdominal SCAT of the obese participants, which was also correlated with plasma PAI-1_{ag} levels, compared with the lean participants, while no difference in the PAI-1 mRNA content of the femoral SCAT was observed [29].

With regards to *ex vivo* studies, it should be kept in mind that the results can be influenced by the data collection and analytical method used. For example, data employing cultured human adipose tissue explants suggest a direct contribution of visceral adipose tissue to plasma PAI-1 levels [8,28,36], while studies using native human adipose tissue found similar or even lower PAI-1 mRNA expression in VAT compared to SCAT [5,36,37]. Lindeman et al. [30] suggest that the increased PAI-1 release from adipose tissue explants is likely related to an incubation artefact rather than being a true reflection of the *in vivo* situation.

Furthermore, an increased adipocyte PAI-1 content does not necessarily relate to increased plasma PAI-1 levels. As an example, in SCAT, elevated PAI-1 gene expression was found to be present during very low-calorie diets in obese participants, while plasma PAI-1 levels were decreased [38]. Furthermore, when comparing differences between VAT and SCAT depots, no correlation was found between PAI-1 mRNA and plasma PAI-1 (antigen and activity) in either one of the two fat depots [39]. On the other hand, Morange et al. [28] investigated the correlation between plasma PAI-1 and PAI-1 measured in adipose tissue explants by means of real-time polymerase chain reaction (PCR). They found a correlation between plasma PAI-1 (antigen and activity) and the PAI-1_{ag} level measured in cultured SCAT explants. This relationship was not investigated in VAT explants.

Due to the fact that *ex vivo* investigations are performed under controlled conditions outside the human body, it is also difficult to assess the total contribution adipose tissue might make to plasma PAI-1 levels when compared with other PAI-1 producing cells such as hepatocytes, platelets and endothelial and vascular smooth muscle cells. It is also possible that VAT and PAI-1 levels are concurrently related to abnormal fat metabolism, rather than the one bringing about the other [30]. Notwithstanding the fact that PAI-1 has a strong association with body fat distribution, these results suggest that assumptions regarding plasma PAI-1 levels based on adipose tissue PAI-1 content and gene expression should not be made.

3. *In vivo* studies

In vivo human studies support the strong association between PAI-1, obesity and body fat distribution and provide more evidence for the influence of adipose tissue depots on plasma PAI-1 levels [9,23,40–43]. *In vivo* studies often use anthropometric indicators [such as body mass index (BMI), waist-hip ratio (WHR), waist circumference (WC), weight loss programs etc.] and CT scan to assess the association of body fat distribution or differences in fat depots (SCAT and VAT) with plasma PAI-1 levels. However, *ex vivo* studies use methods such as mRNA quantification or freshly collected or cultured adipose tissue samples, with or without stimulating factors. When considering the methodological differences between *in vivo* and *ex vivo* studies, it is not surprising that some studies have indicated that *ex vivo* results do not always lead to *in vivo* changes in PAI-1 plasma levels [38,39].

Several studies have used anthropometrical indicators like WC, BMI and WHR to investigate possible associations between plasma PAI-1 levels and body fat distribution [43–45]. BMI, as a general marker of body fat, was found to be strongly associated with PAI-1 [6,29,46] confirming the association of PAI-1 with total obesity. BMI does, however, not reflect body fat distribution and waist circumference, as a measure of central obesity, was demonstrated to be a significant contributor to plasma PAI-1 levels [4,41,43,44] independent of BMI [41], supporting the strong association between plasma PAI-1, VAT and central obesity. Consistently, when compared with CT scan and ultrasound, WC has been regarded as a useful surrogate for the measurement of visceral fat [47], whereas WHR and BMI seem to be associated with VAT or plasma PAI-1 to a lesser extent [47,48].

On the other hand, a stronger correlation between BMI and plasma PAI-1, as compared with WC has also been demonstrated [42]. However, in this study WC and WHR correlated with plasma PAI-1 to a similar degree. Furthermore, after adjusting for WC, an inverse association

Table 1

Summary of studies investigating the association of PAI-1 production and mRNA levels in adipose tissue (VAT versus SCAT).

Author, year	Aims	Participant characteristics (mean $\pm$ SD)	Adipose tissue measured	PAI-1 measured	Relevant finding
Bastelica et al., 2002 [12]	Compare the localisation of PAI-1 synthesis between human VAT and SCAT.	$n = 26$ Twenty-two white women and four men BMI 41 ± 6.3 kg/m ² Age 41 ± 12 yrs.	Cryosections of freshly collected human adipose tissue (in culture). Samples were collected during gastroplasty.	Supernatant PAI-1 _{ag} , plasma PAI-1 _{ag} and PAI-1 _{act} mRNA in adipocytes.	$\uparrow$ PAI-1 synthesis in VAT compared with SCAT. PAI-1 production is related to the presence of stromal cells, which are more numerous in VAT than in SCAT.
Gottschling-Zeller et al., 2000 [13]	Compare PAI-1 expression and secretion in omental fat and SCAT from obese and non-obese participants.	$n = 11$ obese individuals (eight women and three men) BMI 45 ± 8.7 kg/m ² Age 39 ± 13.8 years. $n = 7$ non-obese individuals (two women and five men) BMI 24 ± 2.7 kg/m ² Age 41 ± 13.9 years. All participants were of Caucasian origin.	Isolated SCAT and omental abdominal adipocytes (in suspension culture) from severely obese (who underwent vertical gastric banding for weight reduction) and non-obese individuals.	PAI-1 _{ag} and PAI-1 mRNA in adipose tissue.	$\uparrow$ PAI-1 synthesis in VAT compared with SCAT. Omental adipocytes release significantly more PAI-1 <i>in vitro</i> compared to subcutaneous adipocytes from obese and non-obese individuals. Did not find endothelial cells (present in adipose tissue) to contribute to the production of PAI-1. Observed that TGF- β 1 contributes to the regulation of PAI-1 secretion.
Cigolini et al., 1999 [22]	Aimed to determine whether TNF- α influence PAI-1 production in human adipose tissue.	$n = 35$ non-neoplastic, overweight individuals BMI 28 ± 5 kg/m ² Seven individuals were surgical patients, for whom both SCAT and VAT were obtained. Twenty-eight individuals were outpatients from which SCAT was obtained by needle biopsies.	Omental and SCAT biopsies (incubated, isolated adipocytes) from obese non-diabetic individuals.	PAI-1 _{ag} and PAI-1 mRNA in adipose tissue.	$\uparrow$ PAI-1 expression and release in VAT compared with SCAT. However, do not support VAT to be the only fat depot that contributes to plasma PAI-1 levels. These two fat depots were found not to be substantially different from each other, with regards to response to TNF- α stimulation. It was concluded that similar factors control expression in VAT and SCAT under these experimental conditions.
Alessi et al., 1997 [5]	Investigated PAI-1 expression by human adipose tissue and its different cellular fractions, in particular PAI-1 _{ag} production from VAT (omental) versus SCAT.	$n = 13$ women BMI $26\text{--}29$ kg/m ² Only in seven participants comparison between VAT and SCAT was preformed (four men and three women) BMI $18\text{--}28$ kg/m ² Age $39\text{--}79$ years.	Tissue was obtained during elective abdominoplasty (incubated).	PAI-1 _{ag} and PAI-1 mRNA in adipocytes.	$\uparrow$ PAI-1 production in VAT compared with SCAT. Supports the important contribution of VAT in determining plasma PAI-1 levels.
Lindeman et al., 2007 [30]	Systematically evaluate the molecular basis of the association between VAT and plasma PAI-1 in humans.	Men and women were included and grouped according to BMI. BMI $20\text{--}32$ kg/m ² in one group and morbid obese participants (BMI > 40 kg/m ²) in the other group.	Tissue was obtained during elective aneurysm repair (normal weight and overweight individuals) or during gastric banding for morbid obesity.	Plasma PAI-1 _{ag} and PAI-1 mRNA from adipose tissue explants.	Data corroborates a strong relationship between VAT and plasma PAI-1 levels. However, low and similar PAI-1 mRNA expression in SCAT and VAT excluded VAT as a relevant source of plasma PAI-1. The authors conclude that VAT and plasma PAI-1 are co-correlated rather than causatively related.
Polac et al., 2001 [39]	To substantiate the link between intra-abdominal VAT and circulating PAI-1 by performing analysis of PAI-1 expression in human VAT and SCAT.	$n = 28$ postmenopausal women BMI 28.29 ± 4.32 kg/m ² Age 53.59 ± 6.35 years.	Samples of VAT and SCAT were obtained in the beginning of a gynaecological operation.	Plasma PAI-1 _{ag} and PAI-1 mRNA in adipose tissue.	Indicates similar PAI-1 mRNA expression in both SCAT and VAT that seems to be influenced by lipid metabolism. No correlation was found between plasma PAI-1 and VAT or SCAT PAI-1 mRNA.
Morange et al., 1999 [28]	Aimed to investigate the relation between the production of PAI-1 by adipose tissue, plasma PAI-1 levels and variables related to insulin resistance.	$n = 30$ participants (three men and 27 women). Age $16\text{--}70$ years. (mean = 47) BMI $21\text{--}42$ kg/m ² (mean = 27) VAT and SCAT were compared in a sub-group of 16 participants that included seven men and nine women Age 35 to 79 years. BMI $18\text{--}39$ kg/m ² (mean = 27).	Tissue were obtained during elective abdominal surgery (incubated explant).	Plasma PAI-1 _{ag} and PAI-1 _{ag} in adipose tissue.	Observed a similar correlation between PAI-1 production from VAT and SCAT. Further suggested a similar regulatory pathway of PAI-1 in the two fat depots. Supports the role of adipose tissue in determining plasma PAI-1 levels and the important contribution of TNF- α and TGF- β in stimulating PAI-1 production.
Dusserre et al., 2000 [37]	Investigated the difference in gene expression of six proteins secreted by adipocytes obtained from abdominal VAT and SCAT.	$n = 9$ Five women and four men BMI 34 ± 4 kg/m ² Age 42 ± 5 years.	Adipose tissue biopsies were collected during elective open abdominal surgery.	PAI-1 mRNA in adipose tissue.	Observed similar PAI-1 mRNA expression in SCAT and VAT.

(continued on next page)

Table 1 (continued)

Author, year	Aims	Participant characteristics (mean $\pm$ SD)	Adipose tissue measured	PAI-1 measured	Relevant finding
Bastard et al., 2000 [38]	Aimed to determine if the changes in SCAT <i>PAI-1</i> expression influence plasma PAI-1 levels during weight loss in obese humans following a VLCD.	$n = 15$ Fourteen women and one male participant, all of Caucasian descent. BMI >30 kg/m ² Age 48 ± 3 years.	Samples were obtained from the abdominal skin of participants by a liposuction mini-cannula attached to a syringe.	Plasma PAI-1 _{ag} and PAI-1 _{ag} and <i>PAI-1</i> mRNA in adipose tissue.	$\uparrow$ <i>PAI-1</i> mRNA and PAI-1 concentration in SCAT during VLCD. Suggest therefore that the changes in SCAT PAI-1 expression are not related to the decrease of plasma PAI-1 levels observed during VLCD in obese participants.
Eriksson et al., 2000 [25]	To determine differences in adipose gene expression and protein secretion rate of PAI-1 between SCAT and VAT.	$n = 22$ obese individuals Fourteen women and eight men BMI 43.1 ± 1.3 kg/m ² All participants were of Caucasian descent and born in Sweden.	Abdominal SCAT and VAT were obtained during weight-reduction surgery (adjustable gastric banding).	PAI-1 _{ag} and <i>PAI-1</i> mRNA in adipose tissue. Also expressed results as PAI-1 concentration/10 ⁷ cells.	$\uparrow$ Secretion of PAI-1 and <i>PAI-1</i> gene expression were found in SCAT compared with VAT. PAI-1 secretion rate correlated with cell size in SCAT and SCAT cell size was larger than VAT cell size. The depot-specific difference in PAI-1 secretion was confined to obese women and not to men.
Mavri et al., 2001 [29]	To determine if the changes in <i>PAI-1</i> mRNA content in two SCAT depots (abdominal and femoral) are associated with changes in plasma PAI-1 after weight loss.	$n = 40$ obese individuals Twenty-six women and four men BMI >25 kg/m ² Age 40 ± 10 years. Also sixteen lean participants, 17 women and seven men. BMI 25 kg/m ² Lean participants had a similar age range as the obese participants.	SCAT biopsies were obtained from the sub-umbilical abdominal region and lateral femoral region by means of a needle.	Plasma PAI-1 _{act} and PAI-1 _{ag} as well as <i>PAI-1</i> mRNA in adipose tissue.	Obese participants had $\uparrow$ plasma PAI-1 and <i>PAI-1</i> gene expression (in abdominal SCAT) compared to lean participants. Plasma PAI-1 levels were associated with <i>PAI-1</i> expression in the abdominal adipose tissue, whereas femoral SCAT showed similar <i>PAI-1</i> mRNA in obese and lean participants. The authors concluded that only abdominal and not femoral SCAT <i>PAI-1</i> expression is a contributor to increased plasma PAI-1 levels in obesity. Both plasma PAI-1 and <i>PAI-1</i> mRNA in abdominal SCAT also decreased following weight loss.
Shimomura et al., 1996 [8]	To investigate the relationship between plasma PAI-1 levels and the amount of fat tissue (VAT versus SCAT) and changes in <i>PAI-1</i> gene expression in adipose tissue during development of obesity in humans and animals.	$n = 101$ Sixty-one obese participants (31 men and three women) BMI 31 ± 5 kg/m ² Age 43 ± 13 years. Forty non-obese participants (37 men and 13 women) BMI 23 ± 2 kg/m ² Age 44 ± 14 years. SCAT and VAT from humans were assessed by means of computerized tomography. $n = 15$ female Sprague–Dawley rats (12 weeks of age).	RNA was extracted from the intra-abdominal SCAT and VAT from control rats and rats with a ventromedial hypothalamus lesion.	Plasma PAI-1 _{ag} and <i>PAI-1</i> mRNA in adipose tissue from rats.	Although both VAT and SCAT expressed <i>PAI-1</i> mRNA, <i>PAI-1</i> expression $\uparrow$ in VAT but not in SCAT during the development of obesity in rats. In both obese and non-obese humans, plasma PAI-1 correlated to VAT, but not SCAT. The authors concluded that enhanced <i>PAI-1</i> gene expression from VAT might contribute to increased plasma PAI-1 levels.

BMI, body mass index; mRNA, messenger ribonucleic acid; PAI-1, plasminogen activator inhibitor-1; PAI-1_{act}, PAI-1 activity; PAI-1_{ag}, PAI-1 antigen; SD, standard deviation; SCAT, subcutaneous adipose tissue; TGF- β 1, transforming growth factor-beta 1; TNF- α , tumour necrosis factor-alpha; VAT, visceral adipose tissue; VLCD, very low calorie diet; $\uparrow$, increased PAI-1 synthesis and/or production.

between hip circumference and markers of coagulation was found, which possibly indicated a protective effect of gluteal fat distribution as opposed to android obesity [42]. These anthropometrical differences associated with PAI-1 could be related to the inclusion of different study populations as will be discussed in more detail below. Nevertheless, the use of increased WC, as an indication of visceral fat mass, seems to be a marker of elevated PAI-1 plasma levels. However, such interpretations should be made with caution, particularly in terms of non-European populations as WC was not regarded as a suitable surrogate for assessing visceral fat mass in African women, following a weight loss diet [49].

The main focus of the following section will therefore be to discuss *in vivo* studies investigating the relationship between plasma PAI-1 levels and body fat distribution in relation to factors, identified from the literature, that may influence this relationship namely gender, ethnicity, level or degree of obesity, weight loss, the 4G/5G *PAI-1* polymorphism and other PAI-1 producing tissue in obesity.

3.1. Gender

It is known that body fat distribution is highly sex specific and that plasma PAI-1 levels differ between men and women [40,50]. Women generally have gluteal-femoral or peripheral depots (lower body obesity) consisting of SCAT) compared with the android fat distribution of men (excess of body fat accumulation in the abdominal area that is associated with increased VAT) [51,52]. In layman's terms, these are referred to as pear (lower body obesity) and apple (abdominal obesity) shape figures. Furthermore, adipocytes in the VAT depot are considered to be more metabolically active than adipocytes in the SCAT [32]. When considering the strong association between plasma PAI-1 and VAT, men would be expected to have higher plasma PAI-1 levels than women. Previous studies have confirmed this notion by demonstrating a direct correlation between VAT and plasma PAI-1 concentration in obese men, but not in women [53]. VAT has also been suggested to be an

independent predictor of plasma PAI-1 activity (PAI-1_{act}) in healthy men [22]. On the contrary, when comparing abdominal VAT in healthy non-obese, overweight men and women, greater PAI-1_{ag} levels were found in women than men [48]. In agreement, others have also reported a stronger association between WHR and plasma PAI-1 levels in women than men [24,50]. A possible explanation for the stronger relationship between PAI-1 and WHR in women could be related to a tendency of women with a preferential gluteal fat distribution to accumulate fat also in the abdominal area when becoming overweight/obese, leading to a greater WHR (and VAT) and consequently increased PAI-1 levels [40].

Previous research undertaken by our group, involving African participants, found PAI-1_{act} levels to be higher in women than in men, despite adjusting for differences in body composition. Plasma PAI-1_{act} furthermore associated not only more strongly, but also with more anthropometric variables in the men than the women [43]. Plasma PAI-1_{act} had a linear positive relationship with BMI and skinfold thickness in men, whereas in women, PAI-1_{act} plateaued at higher BMI levels and did not change across skinfold categories. Due to the preferential peripheral fat distribution (SCAT) in women and the preferential visceral (abdominal) fat accumulation in men, an obese man will have proportionally more VAT than an obese woman. Consequently, despite the higher PAI-1_{act} levels observed in women, the proportionally lower VAT may explain the overall lower correlations with anthropometric variables compared to the men. Furthermore, since women deposit gynoid fat preferentially, peripheral SCAT likely increased more than VAT in these obese African women, explaining the plateau in plasma PAI-1_{act} at higher BMI. While in men with a preferentially increased VAT deposition in the abdominal area, PAI-1_{act} levels continued to increase in conjunction with the increase in VAT. Similarly, skinfolds represent SCAT and in men, more VAT would have been deposited before an increase in SCAT is observed explaining the rise in PAI-1 levels across skinfold quintiles. Since women preferentially store peripheral SCAT, this adipose compartment will continue to increase in accordance with an increase in skinfold, while their VAT compartment remains proportionally smaller explaining the lack of difference across the skinfold quintiles. There also seems to be important differences in the size of adipocytes between VAT and SCAT depots in women, as women were found to have larger SCAT adipocytes compared to VAT adipocytes [54] while no differences were observed for men.

According to Eriksson et al. [25] obese females seem to have a significant depot-specific PAI-1 secretion, with the SCAT depot producing higher levels of PAI-1 than the VAT depot, while this was not observed in men. Additionally, the authors suggested that SCAT is the most important adipose depot for PAI-1 secretion in obese individuals [25], since it is regarded to be a considerably larger fat depot than VAT [52] (see section on degree of obesity below). In support of this, measurements of abdominal VAT by means of CT scan have shown that VAT explained 28% only of the variance in plasma PAI-1_{act} in premenopausal women [23].

The menopausal status of women may also influence the relationship between different adipose tissue depots and plasma PAI-1. Compared to premenopausal women, postmenopausal women experience a redistribution of fat distribution (increased visceral fat accumulation) that is related to the female sex hormone oestrogen [55]. It should also be noted that therapeutic administration of oral oestrogen has been reported to reduce plasma PAI-1 levels [56]. Therefore, the inclusion of pre- and/or postmenopausal women in a study may influence the outcome of the association of body composition with plasma PAI-1 levels.

3.2. Ethnicity

When comparing the association between body composition and plasma PAI-1 levels between different ethnic groups, dissimilarities have been found. For instance, significant associations between plasma

PAI-1_{ag} and VAT have been observed in white women, but not in African American women, despite similar PAI-1 levels and BMI ranges [57]. African women have also been found to have significantly lower PAI-1_{act} levels as well as a weaker association with markers of the MetS in comparison with white South African women [44]. Previous studies have indicated that white Americans compared with black Americans have less VAT in spite of similar WC and BMI measurements [45,58]. Similar findings were also observed in studies that included black and white South Africans [59,60]. In a black and white South African sample, black women were found to have less VAT in comparison with white women after adjusting for age, height, weight and fat mass, despite the fact that there were no ethnic differences in WC, WHR or dual-energy X-ray absorption measurement of the abdominal regions [61]. Similar to the VAT distribution in women, in a study by Hill et al. [45], significantly lower VAT was found in black than in white men after adjustment for percentage body fat, total body fat, BMI, WC, WHR or sagittal diameter. However, it is difficult to draw conclusions regarding the relationship of PAI-1 and body fat composition in African populations as such information is limited and most research investigating these issues has been undertaken in populations of European descent [6,13,25,38,45]. Owing to ethnic differences in body fat composition and fat deposition, the relationship of PAI-1 with body composition in Africans may differ from that observed for other population groups and, therefore, merits further investigation.

3.3. Level or degree of obesity

The general notion that increased plasma PAI-1 levels are associated with central obesity or android fat distribution does not seem to hold true in all circumstances. As mentioned previously, in severely obese individuals, PAI-1 expression from abdominal SCAT was found to be approximately two times higher than from abdominal VAT, and it was proposed that the degree of obesity influences regional differences in PAI-1 secretion [25]. As previously explained, this might however also have been the result of the way in which Eriksson et al. [25] reported their results i.e. in terms of cell size.

In support of this finding, when grouping participants according to different body composition patterns, the degree of obesity seems to have a significant influence on regional differences in PAI-1 secretion. African women with a proportionally higher abdominal VAT compartment have been found to have significantly higher plasma PAI-1_{act} levels than women with a proportionally higher abdominal SCAT compartment (unpublished results). However, in women who were classified to be centrally obese, this was no longer the case. Within reason, it would seem therefore that the degree of obesity have an influence on the association between different body fat depots and plasma PAI-1 levels.

3.4. Weight loss

Weight loss that is associated with the loss of adipose tissue mass has been shown to be effective in lowering plasma PAI-1 levels in obese individuals [29,38,62–65]. Following gastric bypass in severely obese individuals, plasma PAI-1 levels were observed to normalise one month after surgery and PAI-1 expression, in both SCAT and VAT, was found to be lower than that of non-obese individuals within one year after surgery [62]. Mavri et al. [29] found that a 3-month weight reduction program resulted in a decline in plasma PAI-1 levels, which was only associated with loss of abdominal SCAT and not loss in the femoral adipose tissue. On the contrary, other studies have observed changes in plasma PAI-1 levels that correlated with changes in VAT and not SCAT after weight reduction in healthy premenopausal women [23]. Such findings are supported by other studies that observed VAT and plasma PAI-1 levels to decrease significantly after weight loss, in both men and women [53,66]. Yet the relationship between VAT and PAI-1 disappeared in women after adjustment for total body fat mass [53]. Weight

reduction due to surgical treatment or dieting seems to be an effective means to help lower plasma PAI-1 levels and promote a favourable haemostatic profile although whether this reduction is adipose depot specific remains a topic of investigation.

3.5. 4G/5G PAI-1 polymorphism

Throughout the literature, several PAI-1 polymorphisms have been identified, which may influence plasma PAI-1 levels; of these, the 4G/5G PAI-1 polymorphism has been shown to exert the greatest impact [67,68] and is also thought to be associated with obesity and increased adipose tissue [11,69–72]. The 4G/5G is a single-nucleotide insertion/deletion polymorphism located at position –675 in the promoter region of the PAI-1 gene that leads to either a 4G or 5G sequence [67]. In the 5G allele, a repressor protein binds to the promoter area of the PAI-1 gene, resulting in relatively reduced transcription of PAI-1, and therefore in lower plasma PAI-1 levels [67,73,74]. Not all studies, however, found a significant association between the 4G/5G genotypes and the plasma PAI-1 levels, and it is postulated that obesity may influence this relationship. The 4G/5G polymorphism is considered a response polymorphism meaning that the difference in PAI-1 levels between 4G and 5G becomes more obvious in the presence of environmental and/or disease factors that stimulate PAI-1 expression [75], such as obesity. In agreement with this, some studies [11,72,76] found a stronger relationship between plasma PAI-1 levels and the 4G/5G polymorphism in obesity, although there were also some studies that did not find a difference in the strength of the relationship [77–79].

Results regarding the relationship of this polymorphism with obesity are also contradicting, as some studies [11,71,72,80] found carriers of the 4G allele to be more common among obese individuals while others did not [81–84]. Although, the mechanism of action explaining the possible association between the 4G/5G PAI-1 gene and obesity is not yet known, Demiralp et al. [85] suggested that overexpression of PAI-1 in 3T3-L1 cells (mouse cell line of fibroblasts that can differentiate into adipocytes) can increase adipocyte differentiation and that the 4G allele was significantly more active than the 5G allele in driving PAI-1 gene transcription, thus contributing to adipogenesis.

Despite possible interaction of plasma PAI-1 levels and the 4G/5G PAI-1 polymorphism with obesity, little is known regarding the relationship with body fat distribution, in particular the difference in VAT versus SCAT. In one study investigating the genetic variations within the PAI-1 gene that are related to the amount of body fat and regional fat distribution, the 4G/5G PAI-1 polymorphism was found to be associated with VAT in postmenopausal women [70]. Homozygotes for the 5G allele had 50% more VAT compared to carriers of the 4G allele. This suggests that it may be involved in modulating adipose tissue distribution at menopause [70]. In agreement with this, Sartori et al. [11] found the 4G/5G polymorphism to influence PAI-1 expression in central obesity (VAT) but not in peripheral obesity (SCAT). Van Harmelen et al. [86] furthermore found no effect of the 4G/5G polymorphism on PAI-1 secretion from abdominal SCAT adipose tissue. Although not enough evidence is available to draw firm conclusions, these results suggest that the 4G/5G PAI-1 polymorphism may potentially influence the relationship between plasma PAI-1 levels and body fat distribution and warrants further investigation.

3.6. Other PAI-1 producing tissue in obesity

As mentioned earlier, Janand-Delenne et al. [23] found VAT to explain only 28% of the variance in plasma PAI-1_{act} in premenopausal women. Also in a recent study (unpublished results) we found inflammation, endothelial dysfunction and triglycerides to contribute more to PAI-1_{act} variance in obese African women than body fat % *per se*. These results reiterate the concept that plasma PAI-1 levels, in obesity, are influenced by many different factors of which increased adipocyte mass is only one and that plasma PAI-1 in obesity seems to increase

owing to a systemic response [87]. Other factors that can influence plasma PAI-1 levels include insulin resistance, inflammatory cytokines, oxidative stress as well as increased triglycerides, which can regulate PAI-1 production in all PAI-1 producing tissue and not adipose tissue only [3,88,89]. The association between PAI-1 and triglyceride has been previously explained by the fact that very low density lipoproteins (VLDL) triglycerides increase PAI-1 levels [6,90], through a VLDL response element identified in the promoter region of the PAI-1 gene that mediates VLDL-induced PAI-1 transcription in endothelial cells [91].

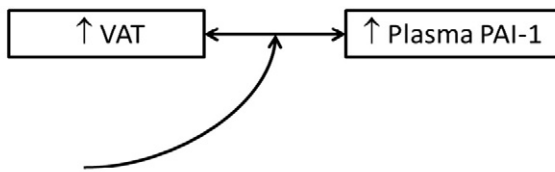
The largest contributor to PAI-1_{act} in our recent study was endothelial dysfunction (unpublished results). Evidence exists for the presence of endothelial dysfunction in obesity [92], which is thought to be induced by obesity-associated metabolic abnormalities such as insulin resistance, adipokines, oxidative stress, increased free fatty acids, tissue TNF- α as well as activation of innate immunity [93,94]. At the same time, increased PAI-1 is recognised as an early marker of endothelial dysfunction [95,96], suggesting endothelial dysfunction as a mechanistically plausible modulator of PAI-1_{act} in obesity. Others have also identified hepatocytes and endothelial cells as underlying sources of elevated PAI-1 levels in obese individuals [36,62].

The association between inflammation and PAI-1 has been firmly established with a variety of cytokines such as TNF α , TGF- β 1, IL-1 and IL-6, acting as inflammatory mediators of PAI-1 [7,12,13,30,46,97–100]. Therefore, the chronic low-grade pro-inflammatory environment in obesity can significantly contribute to plasma PAI-1 levels via the presence of several PAI-1 inducers [2]. These PAI-1 stimulating effects of pro-inflammatory cytokines are not only limited to adipocytes but are also present in hepatocytes, smooth muscle cells and endothelial cells [101,102]. In a recent study, the ability of the acute systemic inflammatory response to activate gene expression and PAI-1 production in VAT was demonstrated. According to Ekström [9], during open heart surgery, PAI-1 mRNA expression was found to increase 27-fold in the omental (visceral) adipose tissue, compared with a threefold increase in the SCAT, followed by an increase in plasma PAI-1_{ag} levels. TGF- β 1 seems to regulate PAI-1 expression as part of an autocrine and paracrine function [99]. Particularly in subcutaneous adipocytes, TGF- β 1 has been proposed as a main inducer of PAI-1 mRNA synthesis [25,99], although others have reported no significant difference in the effect of TGF- β 1 on PAI-1_{ag} secretion between the two fat depots (VAT and SCAT) [13].

Apart from adipocytes, adipose tissue furthermore contains endothelial cells as well as fibroblasts, smooth muscle cells and macrophages, which are all involved in the expression of PAI-1 mRNA [8]. Other factors associated with obesity that can influence plasma PAI-1 levels, include oxidative stress, circadian clock proteins, cortisol and angiotensin-converting enzyme [reviewed by 103].

4. Conclusion

While *ex vivo* evidence indicates VAT to be the main PAI-1 producing adipose depot due to structural and functional differences compared to SCAT, this does not always relate to increased plasma PAI-1 levels as depicted in Fig. 1. Particularly in obesity and in females where the SCAT depot is often significantly larger than the VAT depot, SCAT becomes a significant contributor to plasma PAI-1 levels and therefore also to increased cardiovascular disease risk. Also owing to ethnic differences in body fat composition and fat deposition, the relationship of PAI-1 with body composition in non-European populations may differ from that observed for Europeans and, therefore, merits further investigation. Furthermore, genetic variation such as the 4G/5G PAI-1 polymorphism may influence the relationship between VAT and plasma PAI-1 although more evidence is needed to confirm this. As PAI-1 is produced by many different tissues, the contribution of other tissue such as endothelial cells, particularly in the case of endothelial dysfunction, to plasma PAI-1 levels in obesity should also not be negated and should be considered as a major contributor to plasma PAI-1 levels.



Strength of relationship is modulated by factors such as:

1. Significantly larger SCAT depot (e.g. in severe obesity and females)
2. Ethnicity
3. Obesity-associated metabolic abnormalities e.g. endothelial dysfunction
4. Genetics?

Fig. 1. The association between VAT and plasma PAI-1 levels and the factors modulating this association. VAT, visceral adipose tissue; PAI-1, plasminogen activator inhibitor-1; SCAT, subcutaneous adipose tissue; ↑, increased VAT and plasma PAI-1 levels; ↔ association.

Practice points

- The contribution of body fat distribution to increased plasma PAI-1 levels has important pathophysiological consequences for the development of obesity-related CVD risk.
- Despite VAT being the main PAI-1 producing adipose depot, due to structural and functional differences compared to SCAT, this does not always relate to increased plasma PAI-1 levels.
- PAI-1 production in adipose tissue explants studied *ex vivo* should not be directly related to the *in vivo* situation due to the possible occurrence of incubation artefact effects, leading to the misinterpretations of results.
- Factors such as gender, ethnicity, possibly genetics, level or degree of obesity and other PAI-1 producing tissues are important when considering the relative contribution of different fat depots to plasma PAI-1 levels since these factors not only influence the plasma PAI-1 levels, but also the relative associated CVD risk.

Research agenda

- More studies are needed to understand the relationship of PAI-1 with body composition in non-European populations, as most studies included European populations only and data pertaining to non-European populations are limited.
- The relative contribution of endothelium dysfunction to plasma PAI-1 levels in obesity is often overlooked, though could assist in our understanding of the relationship between obesity and PAI-1, and therefore warrants more detailed investigation.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgements

This work was funded through the National Research Foundation (NRF) of South Africa (grant UID: 94213). Opinions expressed and conclusions arrived at are those of the authors and are not to be attributed to the NRF.

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