The relationship between pre-diabetic hyperglycemia and markers of nitric oxide bio-availability in a cohort of Africans and Caucasians: the SABPA-study

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Innovation through diversity

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CONTRIBUTIONS OF THE AUTHORS

The following researchers contributed to this study:

Mrs. ASE Koegelenberg

Responsible for conducting the literature searches, statistical analyses, processing of data, design, planning and writing of the manuscript.

Prof. AE Schutte

Supervisor:

Supervised the writing of the manuscript, collecting and interpretation of data, guidance regarding statistical analyses, initial planning and design of the manuscript.

Dr. W Smith

Co-supervisor:

Gave recommendations regarding the writing and construction of the manuscript and contributed to the interpretation of the results.

This is a statement from the co-authors confirming their individual roles in the study and giving their permission that the manuscript may form part of this dissertation.

Prof. AE Schutte **Dr. W Smith**

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SUMMARY

Motivation

Cardiovascular disease (CVD) is becoming an eminent health problem worldwide. Several studies have implicated that type 2 diabetes mellitus, together with other risk factors including hypertension, contributes significantly to the development of CVD. Vascular endothelial dysfunction is one of the most common characteristics of diabetes, and involves alterations such as a decrease in nitric oxide (NO) bio-availability. However, endothelial dysfunction is already present in individuals suffering from impaired fasting glucose, more commonly known as pre-diabetes. The International Diabetes Federation estimates that adults living with pre-diabetes by 2030 in sub-Saharan Africa will comprise 9.6% of the population, whereas adults living with diabetes will comprise of 4.3% of the population. The excessive amount of pre-diabetics in sub-Saharan Africa and its association with vascular endothelial dysfunction motivated this study. In order to gain a better understanding of this relationship, we wanted to explore the relationship between pre-diabetic hyperglycemia and markers of NO bio-availability in Africans and Caucasians residing in South Africa.

Aim

Our aim was firstly to determine whether glucose measures (fasting glucose and glycated hemoglobin (HbA1c)) and markers of NO bio-availability (namely L-arginine, asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), L-citrulline and reactive oxygen species (ROS)) differ between African and Caucasian individuals. Secondly, we aimed to determine the relationship of glucose measures with markers of NO bio-availability;

blood pressure and renal function. Thirdly we aimed to establish whether these associations are ethnic-specific.

Methodology

This study forms part of the SABPA *(Sympathetic Activity and Ambulatory Blood Pressure in Africans*) study which included a total of 409 urbanised African and Caucasian teachers between the ages of 25 and 65 years. Exclusion criteria were an elevated ear temperature, psychotropic substance dependence or abuse, the use of α- and β-receptor blockers, being blood donors or having been vaccinated during the previous three months. For this sub-study participants were excluded due to missing data on HbA1c and markers of NO bio-availability (n=16), participants infected with the human immunodeficiency virus *(*HIV) (n=19), participants with HbA1c levels greater than or equal to 6.5% (n=29), and participants using diabetes medication (n=5). The overall sample of this study therefore consisted of 340 participants divided into African (n=148) and Caucasian (n=192) sub-groups. All participants signed informed consent forms. The Ethics Review Board of the North-West University approved the study. General health questionnaires were used to determine medication use and lifestyle habits. Anthropometric measurements such as weight, height, and waist circumference were determined. Ambulatory blood pressure measurements (ABPM) and physical activity were monitored during a normal working day. Blood samples were taken after subjects were requested to fast overnight. Biochemical analyses of HbA1c, glucose, L-arginine, ADMA, L-citrulline, SDMA, ROS, ferric reducing antioxidant power (FRAP), urea, lipid profile (high-density lipoprotein cholesterol, total cholesterol), γ-glutamyl transferase (GGT), cotinine, high sensitivity C-reactive protein (CRP), and creatinine were performed. HIV testing was performed with First Response HIV Card Test 1-2.0 (PMC Medical, India Pvt Ltd) and confirmed with Pareekshak HIV Triline (UCB Pharma).

Results

In our study we found higher levels of L-arginine ($p<0.001$) in Africans. L-citrulline ($p=0.053$) levels tended to be higher in Africans who also presented with higher levels of HbA1c ($p<0.001$), blood pressure ($p<0.001$) and albumin-to-creatinine ratio (ACR) (1.04 [0.35; 3.95] vs. 0.34 [0.09; 1.88] for Africans and Caucasians, respectively). The Caucasians presented higher levels of SDMA ($p<0.001$) whereas the groups had similar ADMA and ROS levels.

Another finding was the disparate manner in which components of the NO biosynthesis pathway correlated with glucose and HbA1c in both the Africans and Caucasians. In Africans alone, L-citrulline was independently associated with fasting glucose ($R^2=0.21$; $\beta=0.19$; $p=0.017$) and HbA1c ($R^2=0.21$; $\beta=0.19$; $p=0.018$) whereas in Caucasians alone, ADMA was independently associated with fasting glucose ($R^2=0.13$; $\beta=0.39$; $p<0.001$) and HbA1c ($R^2=0.06$; $\beta=0.17$ $p=0.03$). Independent variables included: age, gender, BMI, physical activity, cotinine, GGT, CRP, triglycerides, fasting glucose or HbA1c and systolic blood pressure.

Secondly, blood pressure and estimated creatinine clearance were differentially associated with glucose and HbA1c among the two ethnic groups. Independent variables for this model included: age, gender, BMI, physical activity, cotinine, GGT, CRP, triglycerides, fasting glucose or HbA1c and anti-hypertensive medication. Systolic blood pressure was positively associated with fasting glucose (borderline significant relationship $p=0.06$) and HbA1c ($p=0.04$) in Caucasians. Glucose was also positively associated with diastolic blood pressure in Caucasians ($p=0.008$). In Africans alone, estimated creatinine clearance was negatively associated with glucose (borderline significant relationship $p=0.088$) and HbA1c ($p=0.019$). A further analysis also showed an independent relationship between estimated creatinine clearance and L-citrulline ($R^2=0.55$; $\beta=-0.20$; $p=0.0015$) in Africans.

Conclusion

Regardless of the unfavourable cardiovascular, glucose and renal profile in Africans, they demonstrated a more favourable profile regarding markers of NO bio-availability. Still the main finding remained the lack of associations between markers of NO bio-availability and hyperglycemia in both ethnic groups, with the exception of a positive independent association between L-citrulline and glucose measures in Africans and a positive independent association between ADMA and glucose measures in Caucasians. In the African group, the relationship was probably driven by an unfavourable renal profile, which is characterised by an ACR that is approximately three times higher in Africans than Caucasians. In the Caucasian group, who presented a more favourable cardiovascular profile, our findings support the literature in which hyperglycemia had a significant positive independent association with both ADMA and blood pressure.

Die verwantskap tussen pre-diabetiese hiperglisemie en merkers van stikstofoksied bio-beskikbaarheid in ‘n swart en wit proefgroep: die SABPA-studie

OPSOMMING

Motivering

Kardiovaskulêre siektes is toenemend besig om 'n oorheersende, wêreldwye gesondheids-probleem te word. Verskeie studies veronderstel dat tipe 2 diabetes mellitus tesame met ander risiko faktore, insluitende hipertensie, betekenisvol bydra tot die ontwikkeling van kardiovaskulêre siektes. Vaskulêre endoteel disfunksie is een van die mees algemene karaktereienskappe van diabetes, en sluit verandering soos die afname in stikstofoksied (NO) bio-beskikbaarheid in. Endoteel disfunksie kom alreeds voor in pasiënte met ingekorte vastende glukose, meer algemeen bekend as pre-diabetes. Die Internasionale Diabetes Federasie beraam dat 9.6% van die totale populasie in sub-Sahara Afrika teen 2030 aan pre-diabetes sal ly, terwyl volwassenes met diabetes 4.3% van die populasie sal uitmaak.

Die oormatige voorkoms van pre-diabete in sub-Sahara Afrika, en die assosiasie tussen pre-diabetes en vaskulêre endoteel disfunksie, het hierdie studie gemotiveer. Ten einde 'n beter begrip van hierdie assosiasie te verkry wou ons die verhouding tussen pre-diabetiese hiperglisemie en merkers van NO bio-beskikbaarheid in swart en wit individue wat in Suid-Afrika woonagtig is ondersoek.

Doel

Ons doel was om eerstens vas te stel of glukose metings (vastende glukose en HbA1c) en merkers van NO bio-beskikbaarheid (naamlik L-arginien, asimmetriese dimetielarginien

(ADMA), simmetriese dimetielarginien (SDMA), L-sitrulien en reaktiewe suurstof spesies) verskil tussen swart en wit individue. Tweedens het ons daarop gemik om die verhouding tussen glukose metings en merkers van NO bio-beskikbaarheid, asook bloeddruk en nierfunksie te bepaal. Derdens het ons daarop gemik om vas te stel of hierdie assosiasies etnisiteit-spesifiek is.

Metode

Hierdie studie vorm deel van die SABPA *(Sympathetic Activity and Ambulatory Blood Pressure in Africans)* studie wat in totaal uit 409 verstedelike swart en wit onderwysers tussen die ouderdomme 25 to 65 jaar bestaan het. Die uitsluitingskriteria vir deelname aan die studie was deelnemers met ‘n verhoogde oor temperatuur, wat van psigotropiese substanse afhanklik was of dit misbruik, wat van α- en β-reseptor blokkeerders gebruik maak, bloedskenkers en individue wat binne die laaste drie maande ingeënt is. Vir hierdie sub-studie is 16 deelnemers uitgesluit as gevolg van ontbrekende data rakende HbA1c en merkers van NO bio-beskikbaarheid. Verder is deelnemers wat geïnfekteer is met die menslike immuniteitsgebrekvirus (MIV) (n=19), deelnemers met ‘n HbA1c waarde gelyk aan of hoër as 6.5 % (n=29), en deelnemers wat van diabetiese medikasie (n=5) gebruik maak, uitgesluit. Die totale steekproef van hierdie studie bestaan uit 340 deelnemers wat onderverdeel is in ‘n wit (n=192) en swart (n=148) proefgroep. Elke deelnemer het ‘n ingeligte toestemmingsvorm onderteken, en die studie is goedgekeur deur die Noordwes-Universiteit se Etiekkomitee. Algemene gesondheidsvraelyste is gebruik om medikasie gebruik en lewenstyl gewoontes te bepaal. Antropometriese metings geneem sluit in gewig, lengte en middelomtrek. Ambulatoriese bloeddruk metings asook fisieke aktiwiteit was tydens ‘n normale werksdag gemonitor. Bloedmonsters is geneem nadat deelnemers versoek was om oornag te vas. Biochemiese ontleding van HbA1c, glukose, L-arginien, ADMA, L-sitrulien, SDMA, reaktiewe suurstof spesies, ferri-reduserende antioksidantpotensiaal, ureum, lipied profiel (hoë-digtheid lipoproteien cholesterol, totale cholesterol), γ-glutamiel transferase, kotinien, hoë sensitiwiteit C-reaktiewe proteïen en

kreatinien was uitgevoer. Elke deelnemer se MIV status is vasgestel deur die First Response MIV toetskaart (PMC Medical, India Pvt Ltd) te gebruik. Indien die deelnemer positief getoets het was ‘n tweede toets (Pareekshak, HIV Triline) uitgevoer om te verseker vals positiewe toetse word uitgesluit.

Resultate

In ons studie is gevind dat L-arginien ($p<0.001$) vlakke betekenisvol hoër is in die swart proefgroep. L-sitrulien ($p=0.053$) vlakke het geneig om hoër te wees in die swart groep - ‘n groep wat ook hoër HbA1c ($p<0.001$), bloeddruk ($p<0001$) asook ‘n hoër albumien-tot-kreatinien verhouding (1.04 [0.35; 3.95] teenoor 0.34 [0.09; 1.88] vir swart en wit proefpersone, afsonderlik) voorstel. Die wit proefgroep het hoër vlakke van SDMA getoon ($p<0.001$), terwyl beide groepe dieselfde vlakke van ADMA en reaktiewe suurstof spesies getoon het.

Nog 'n bevinding is die ongelyksoortige wyse waarop komponente betrokke in NO-sintese weg gekorreleer het met glukose en HbA1c tussen die swart en wit individue. In die swart proefgroep word L-sitrulien geassosieer met vastende glukose ($R^2=0.21$; $\beta=0.19$; $p=0.017$) en HbA1c ($R^2=0.21$; $\beta=0.19$; $p=0.018$), terwyl die wit proefgroep se ADMA verband hou met beide vastende glukose ($R^2=0.13$; $\beta=0.39$; $p<0.001$) en HbA1c ($R^2=0.06$; $\beta=0.17$ $p=0.03$). Onafhanklike veranderlikes sluit in: ouderdom, geslag, liggaamsmassa-indeks, fisieke aktiwiteit, kotinien, γ-glutamiel transferase, hoë sensitiwiteit C-reaktiewe proteïen, trigliseriedes, vastende glukose of HbA1c en sistoliese bloeddruk.

Tweedens het bloeddruk en die geraamde kreatinienopruiming verskillend geassosieer met glukose en HbA1c in die twee etniese groepe. Onafhanklike veranderlikes vir hierdie model sluit in: ouderdom, geslag, liggaamsmassa-indeks, fisieke aktiwiteit, kotinien, γ-glutamiel transferase, hoë sensitiwiteit C-reaktiewe proteïen, trigliseriedes, vastende glukose of HbA1c en anti-hipertensiewe medikasie. ‘n Positiewe verwantskap het voorgekom tussen

sistoliese bloeddruk en beide vastende glukose (p=0.06) en HbA1c (p=0.04) in die wit proefgroep. Glukose het ook ‘n positiewe assosiasie getoon met diastoliese bloeddruk in hierdie groep (p=0.008). ‘n Negatiewe verwantskap tussen beraamde kreatinienopruiming en glukose (p=0.088) sowel as HbA1c (p=0.019), het voorgekom onder die swart proefgroep. Verdere analises in die groep het ook ‘n negatiewe korrelasie getoon tussen beraamde kreatinienopruiming en L-sitrulien (R^2=0.55; β=-0.20; p=0.0015).

Gevolgtrekking

Ongeag van die ongunstige kardiovaskulêre, glukose en nier profiel in die swart proefgroep, het ons steeds gevind dat hul ‘n meer gunstige profiel ten opsigte van merkers vir NO bio-beskikbaarheid getoon het. Steeds bly die hoofresultaat die gebrek aan assosiasies tussen merkers vir NO bio-beskikbaarheid en hiperglisemie in beide die wit en swart proefgroep, behalwe vir die positiewe onafhanklike assosiasie tussen L-sitrulien en glukose metings in die swart groep, asook die positiewe onafhanklike assosiasie tussen ADMA en glukose metings in die wit proefgroep. Ons bevinding in die swart proefgroep was waarskynlik gedryf deur ‘n ongunstige nier profiel, wat gekarakteriseer word deur die albumien-tot-kreatinien verhouding wat ongeveer drie keer hoër is in swart proefgroep as in wit proefgroep. Ons bevinding betreffende die wit proefgroep, wat ‘n meer gunstige kardiovaskulêre profiel besit, stem ooreen met die literatuur wat toon dat hiperglisemie ‘n positiewe onafhanklike assosiasie getoon het met beide ADMA en bloeddruk.

PREFACE

For this dissertation the article-format was chosen. This is a format approved and recommended by the North-West University, consisting basically of a motivation, literature review, a manuscript ready for submission to a peer reviewed journal and a concluding chapter. The chosen journal for the article as part of the dissertation is *Diabetes & Vascular Disease Research.*

The layout of this dissertation is as follows:

- Chapter 1 consists of a background and motivation that led to this study.
- Chapter 2 consists of a complete literature review together with the aims, objectives, and hypotheses.
- Chapter 3 consists of the research article, which includes the author's instructions for the journal *Diabetes & Vascular Disease Research*, an abstract, introduction, the methodology, results, discussion, conclusion and acknowledgements of the research study.
- Chapter 4 consists of concluding remarks, a critical discussion of the findings and recommendations.

Relevant references are given at the end of each chapter, according the Vancouver referencing style, as prescribed by the journal *Diabetes & Vascular Disease Research.*

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LIST OF ABBREVIATIONS

ABPM	=	Ambulatory blood pressure measurements
ADMA	=	Asymmetric dimethylarginine
AGE	=	Advance glycation end-product
BH_4	=	Tetrahydrobiopterin
BMI	=	Body mass index
cGMP	=	Cyclic gaunosine-3', 5-monophosphate
CRP	=	C-reactive protein
CVD	=	Cardiovascular disease
DBP	=	Diastolic blood pressure
DDAH	=	Dimethylarginine dimethylhydrolase
ECG	=	Electrocardiogram
FG	=	Fasting glucose
FRAP	=	Ferric reducing antioxidant power
GGT	=	Gamma-glutamyl transferase
GTP	=	Gaunosine-3', 5-monophosphate
HbA1c	=	Glycated hemoglobin
HDL	=	High density lipoprotein
HIV	=	Human Immunodeficiency Virus
NADPH	=	Nicotinamide adenine dinucleotide phosphate
NIDDM	=	Non-insulin dependent diabetes mellitus
NO	=	Nitric oxide
NOS	=	Nitric oxide synthase
PKC	=	Protein kinase C
PP	=	Pulse pressure
PRMTs	=	Protein L-arginine N-methyltransferase
ROS	=	Reactive oxygen species
SABPA	=	Sympathetic Activity and Ambulatory Blood Pressure in Africans
SBP	=	Systolic blood pressure
SDMA	=	Symmetric dimethylarginine
WC	=	Waist circumference

Chapter 1

Background and motivation.

The cardiovascular disease (CVD) burden is growing rapidly in sub-Saharan Africa, with diabetes mellitus being a major contributor.[1] In 2011 approximately 366 million people of the world's population were suffering from diabetes, and according to estimations this will rise to 552 million in 2030.[2] The adoption of a Western lifestyle, which is characterised by nutritional, lifestyle, psychological well-being and health transitions,[3-5] has recently emerged as one of the major contributors to this increased prevalence of CVD and type 2 diabetes in South Africa.[6-9] Currently, large proportions of the population of sub-Saharan Africa are residing in rural areas, but according to estimations, more than 70% of the population will reside in urban areas by 2025, thus adopting a more Western lifestyle.[10] These lifestyle changes also contribute to a higher prevalence of both type 2 diabetes and hypertension found in Africans compared to Caucasians.[6]

Diabetes is accompanied by an array of cardiovascular risk factors including hypertension,[11] obesity,[12] dyslipidemia,[13] smoking[14] and physical inactivity.[15] For instance, a diabetic patient has a twofold higher risk of developing hypertension, than an age-matched healthy subject.[11] Hypertensive patients are also at higher risk of developing diabetes than normotensive patients.[11] The most common links between hypertension and diabetes mellitus are obesity,[16] autonomic dysfunction,[16] insulin resistance,[17] and hyperinsulinemia.[18,19] Both hypertension and diabetes cause micro- and macrovascular complications.[16] The microvascular complications result in renal disease, eye disease and sexual dysfunction, whereas macrovascular complications include cardiac disease, cerebrovascular disease and peripheral vascular disease.[16]

The adverse effects of hyperglycemia play a crucial role in the development of endothelial dysfunction in patients suffering from type 2 diabetes,[10] which involves a number of functional

alterations of the vascular endothelium, including impaired vasodilation.[12] Nitric oxide (NO) is one of the most important substances responsible for vasodilation,[20] and endothelial dysfunction is associated with decreased NO bio-availability in hyperglycemic patients.[12] Several studies have indicated that the association between hyperglycemia and endothelial dysfunction are present even before the onset of diabetes, in the pre-diabetic patient.[21-23] In a study by Su et al. patients with impaired fasting glucose, also known as pre-diabetes, had impaired flow-mediated dilatation, indicating endothelial dysfunction.[22]

The prevalence of pre-diabetes in sub-Saharan Africa is increasing rapidly and according to the International Diabetes Federation approximately 35.2 million more people will suffer from pre-diabetes, than from diabetes by 2030.[2] Due to the fact that pre-diabetes are an eminent health problem in sub-Saharan Africa, and the significant association between hyperglycemia and endothelial dysfunction, we want to explore the relationship between blood glucose and markers of NO bio-availability in Africans and Caucasians residing in South Africa.

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Chapter 2

Literature overview.

1) Introduction

Cardiovascular disease (CVD) is a major health concern in individuals with diabetes mellitus.[1-3] In sub-Saharan Africa, the prevalence of diabetes and CVD are increasing dramatically.[4-6] According to the estimations of the International Diabetes Federation in 2012, the number of adults living with diabetes in sub-Saharan Africa is ever increasing and will expand from 14.7 million in 2011 to 28.0 million in 2030. On the other hand, the amount of adults with impaired glucose tolerance (IGT), also known as pre-diabetes, will increase from 32.8 million in 2011, to 63.2 million in 2030,[4,7] which is in fact much higher than the number of patients suffering from diabetes.

Hyperglycemia is associated with endothelial dysfunction which already develops in a pre-diabetic patient and progress linearly with increasing glycemia.[8-11] According to the Study to prevent Non-Insulin-Dependent Diabetes Mellitus (NIDDM), a decrease in postprandial hyperglycemia is associated with a decrease in the risk for hypertension and cardiovascular disease, supporting the notion that elevated glucose is a risk factor for cardiovascular disease.[12]

One of the key features of hyperglycemia-induced endothelial dysfunction is that the arteries and arterioles are unable to dilate in response to stimuli.[13] The cause of this maladaptation might be twofold: 1) due to reduced bio-availability of vasodilators including nitric oxide (NO), or due to 2) increased production of vasoconstrictors such as angiotensin II and endothelin-1.[13,14] It is important to take into consideration that NO bio-availability might also be influenced by its biosynthesis.[13]

For the purpose of this dissertation, the relationship between blood glucose and markers contributing to the bio-availability of NO, will be discussed in the subsequent sections of this literature review.

The various pathways responsible for the regulation of NO bio-availability are depicted in Figure 2.1.

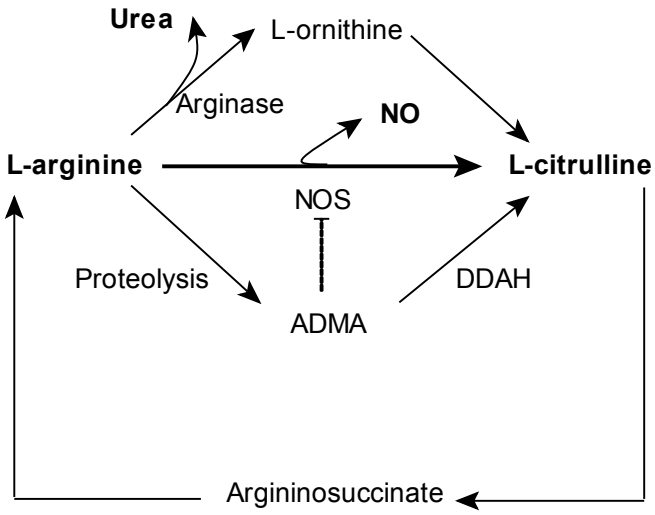

Figure 2.1: Proposed model for the regulation of nitric oxide (NO) bio-availability (adapted from Leiper et al).[15] L-arginine is metabolised to and L-citrulline by nitric oxide synthase (NOS). Asymmetric dimethylarginine (ADMA) is formed during proteolysis of methylated proteins. ADMA is a NOS inhibitor, and therefore inhibits the synthesis of NO. ADMA is metabolised to L-citrulline by dimethylarginine dimethylaminohydrolase (DDAH). L-arginine also forms part of the urea cycle in which L-arginine is converted to L-citrulline which, in turn, can be reconverted to L-arginine, in the presence of various enzymes. Inactivation of NO can result when NO binds to superoxide anions, to form peroxynitrate (not shown in figure).

NO, nitric oxide; NOS, nitric oxide synthase; ADMA, asymmetric dimethylarginine; DDAH, dimethylarginine dimethylaminohydrolase.

2.1) The L-arginine nitric oxide pathway

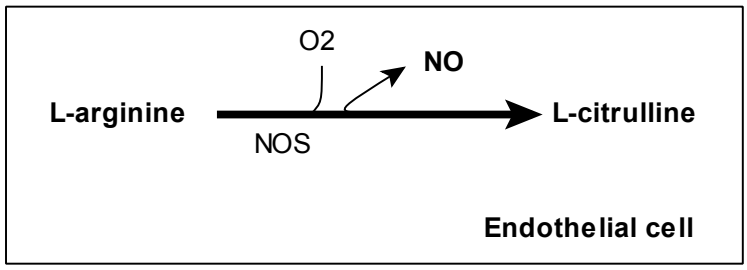

Figure 2.2: Nitric oxide synthesis (adapted from Leiper et al).[15]

NO, nitric oxide; NOS, nitric oxide synthase.

The endothelium, which forms the inner layer of the vascular surface, regulates vascular function and structure through various vasoactive molecules secreted by the endothelium.[16,17] One of the most critical vasoactive molecules is NO.[18] NO is secreted by the endothelium in response to a variety of physical and biochemical stimuli. These include shear stress,[19] hypoxemia,[20] receptor dependent agonists (e.g. acetylcholine) and receptor independent agonists (e.g. calcium-ATPase inhibitors).[21] NO is synthesised whenever the enzyme nitric oxide synthase (NOS) convert free L-arginine to L-citrulline as shown in Figure 2.2.[18] Several co-factors such as flavin mononucleotide, flavin adenine dinucleotide, nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH_4), and calmodulin are required for NO biosynthesis.[22] When NO is synthesised; it has a half–life of a few seconds.[23] Due to its binding to the heme moiety of hemoglobin or the enzyme gaunylyl cyclase, NO is rapidly rendered biologically inactive.[24,25] Therefore, once NO is synthesised, NO diffuses into the blood where it binds to hemoglobin and is rapidly oxidised to nitrite and nitrate.[22,25] NO also diffuses across the endothelial cell membrane into the vascular smooth muscle cells were it activates gaunylate cyclase. This will result in an increase in intracellular cyclic gaunosine-3', 5-monophosphate

(cGMP) levels.[22,26] cGMP acts as a second messenger to mediate the biological effect of NO, by inducing smooth muscle relaxation (Figure 2.3).[26-28]

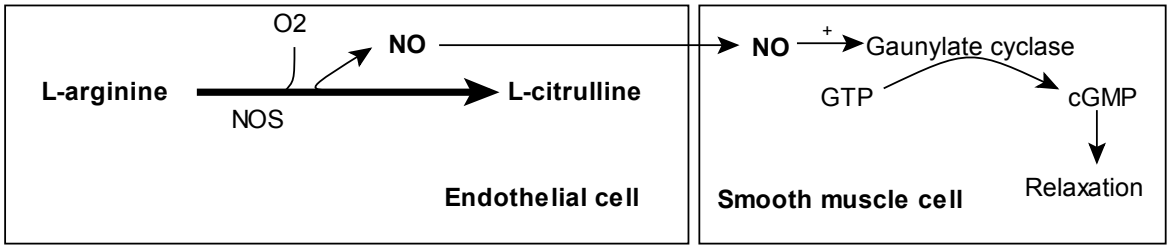

Figure 2.3: The vascular effect of nitric oxide(adapted from Leiper et al).[15]

NO, nitric oxide; NOS, nitric oxide synthase; GTP, gaunosine triphosphate; cGMP, cyclic gaunosine-3',5-monophosphate.

cGMP induces smooth muscle relaxation through multiple mechanisms: firstly, an increase in cGMP inhibits calcium entry into the cell, thus resulting in a decrease in intracellular calcium concentrations.[26,29] Secondly, cGMP activates K^+ channels which promotes hyperpolarisation and relaxation of the smooth muscle cell.[26] cGMP also stimulates a cGMP-dependent protein kinase that activates myosin light chain phosphatase, the enzyme responsible for the dephosphorylation of myosin light chains, resulting in smooth muscle relaxation.[26,30]

Vascular effects of nitric oxide

NO is mainly responsible for vasodilation, either through the second messenger cGMP, or alternatively by inhibiting vasoconstrictor influences such as angiotensin II and sympathetic induced vasoconstriction.[31] Apart from its vasodilatory effect , NO also has an anti-thrombotic effect due to the inhibition of platelet aggregation,[32-34] an anti-inflammatory effect through the inhibition of leukocyte adhesion to the vascular endothelium,[35,36] and an anti-proliferative effect through the inhibition of smooth muscle hyperplasia.[37] Therefore vasoconstriction,[38]

thrombosis,[32-34] inflammation[35,36] and vascular hypertrophy might result from reduced NO bio-availability,[39] emphasising the importance of NO in the maintenance of vascular function and structure.[40,22]

Reduced NO bio-availability

A reduction in NO bio-availability can either be a result of: a) a decrease in NO biosynthesis, or b) the inactivation of NO by oxygen derived free radicals.

a) Reduced nitric oxide biosynthesis

Several studies have reported that the accumulation of endogenous inhibitors of NOS; such as asymmetric dimethylarginine (ADMA),[41] might be a contributing factor to reduced nitric oxide synthesis (Figure 2.4).

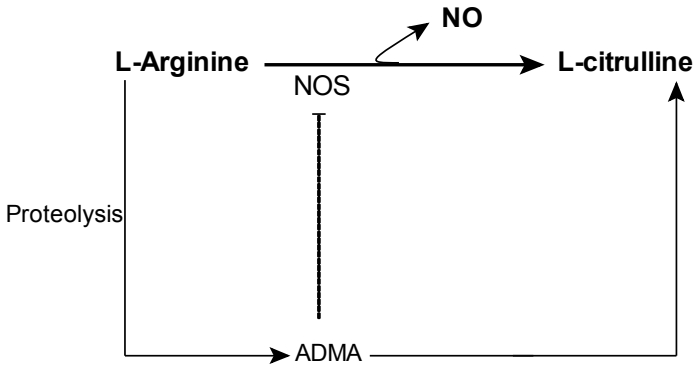

Figure 2.4: Asymmetric dimethylarginine as an endogenous inhibitor of nitric oxide synthase (adapted from Leiper et al).[15]

NO, nitric oxide; NOS, nitric oxide synthase; ADMA, asymmetric dimethylarginine.

ADMA is generated by the endothelial cells from the methylation of arginine residues that are incorporated into protein synthesis.[42] The methylation process is catalysed by a group of enzymes, named protein L-arginine N-methyltransferase (PRMTs). When these proteins

undergo proteolysis, free methylarginines are released.[42] Two distinguishable PRMTs have been classified. Type I catalyses an asymmetrically dimethylation of L-arginine to produce ADMA, an endogenous inhibitor of NOS. Type II catalyses a symmetrical dimethylation of L-arginine to form symmetrical dimethylarginine (SDMA).[42] In contrast to ADMA, SDMA does not inhibit NOS,[43] but rather competes with L-arginine for cellular uptake,[44] which will consequently reduce NO synthesis.[45] SDMA also increases the production of reactive oxygen species (ROS).[46] SDMA's counterpart, ADMA, is partially cleared by renal excretion.[47] The production of ADMA is, however, balanced by its metabolism through the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which in turn, accounts for most of the clearance of ADMA.[48] DDAH metabolises ADMA to L-citrulline and dimethylamine.[49]

Circulating ADMA is increased in certain diseases including hyperglycemic environments, such as with impaired fasting glucose (IFG), IGT, type 1 and type 2 diabetes mellitus.[50-53] One study has even suggested that ADMA levels might be elevated in diabetics prior to the onset of vascular dysfunction. Alev et al. studied a group of 40 patients with uncomplicated type 1 diabetes. Plasma ADMA concentration was elevated and L-arginine levels were lower in the diabetic group, compared with controls. In the diabetic group ADMA levels correlated positively with fasting blood glucose.[54] In another study on 41 pre-diabetic women, aged 35-55 years, a positive correlation between ADMA and both 2-hours postprandial blood glucose ($p=0.003$) and glycated hemoglobin (HbA1c) ($p<0.001$) were found.[55] In contrast to these studies, Mahfouz et al. only found this positive correlation with ADMA and HbA1c in diabetic patients that experienced cardiovascular complications.[56]

The above mentioned studies allude to a level of interaction between circulating glucose and ADMA. Indeed it has been shown that hyperglycemia impairs DDAH activity as a result of

oxidative stress.[50] This in turn contributes to elevated endothelial ADMA levels.[50] ADMA binds to NOS, and through this binding ADMA is capable of competing with the binding of L-arginine to the enzyme, thereby reducing NO synthesis.[50] There is limited evidence indicating that elevated levels of ADMA are caused by increased methylation of L-arginine residues.[57] Elevation in ADMA levels are largely due to impaired activity of DDAH,[58] as DDAH has a sulfhydryl functional group in the active site of the enzyme, that is required for the metabolism of ADMA. This sulfhydryl moiety can either be inhibited by NO as a form of negative feedback, or oxidised, which will deactivate the enzyme.[59]

In addition to the mechanism by which hyperglycemia increase ADMA levels, hyperglycemia also reduces NO synthesis through activating the polyol pathway,[60] which reduces unused glucose to sorbitol through aldose reductase. This reaction oxidises NADPH, an important cofactor in the biosynthesis of NO.[60] Therefore activation of the polyol pathway decreases NO production. Furthermore activation of the polyol pathway also increase concentrations of ROS,[60] which will be discussed briefly in section 2.1.b.

b) Oxidative inactivation of nitric oxide

NO bio-availability is reduced in several conditions due to oxidative inactivation which result from an excessive production of superoxide anions in the vascular wall.[61] Both NO and superoxide are highly reactive and unstable. They react together in a rapid manner to form peroxinitrate, which is a more stable oxidant.[62] This reaction occurs approximately three times faster than the dismutation of superoxide by superoxide dismutase.[63] In addition, peroxynitrite oxidizes the NOS co-factor BH_4, and this reaction uncouples the enzyme, which then increases superoxide anion production over the production of NO. Therefore, it is not surprising that superoxide reduces the bio-availability of NO.[64]

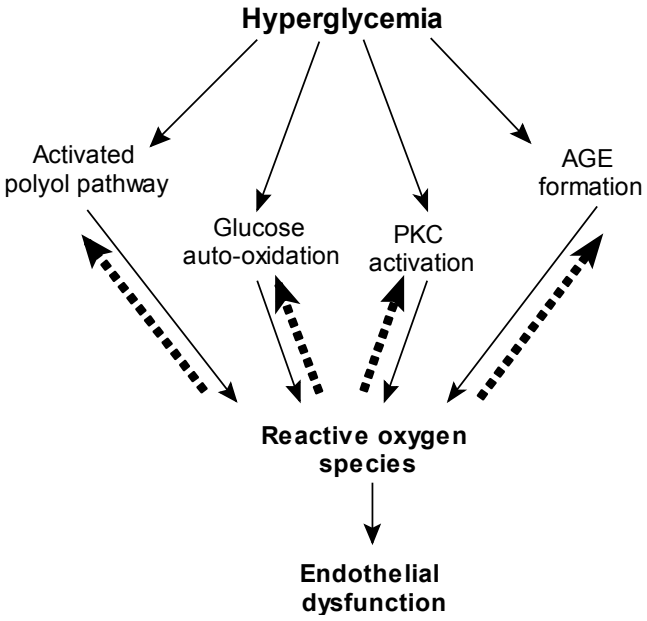

Figure 2.5: Hyperglycemia-induced formation of reactive oxygen species (adapted from Bash et al.).[65]
PKC, protein kinase C; AGE, advance glycation end product.

High levels of glucose in the circulation can divert glucose into various metabolic pathways (Figure 2.5) resulting in: 1) the activation of the polyol pathway;[13,60] 2) increased advanced glycation end product formation;[13] 3) the activation of protein kinase C (PKC) isoforms;[13,66] and 4) glucose auto-oxidation.[67] This in turn will result in the formation of ROS, resulting in oxidative stress. ROS has the ability to worsen endothelial function by activating the metabolic pathways (dotted arrows in Figure 2.5) that were initially responsible for its production.[65] The consequence is an additional increase in ROS production.[65] As previously mentioned superoxide anions has the ability to react with NO, and therefore down-regulate NO bio-availability.[67]

Not only does hyperglycemia increase the formation of free radicals, but also inhibits antioxidant systems namely the interacting glutathione and thioredoxin.[68] Therefore, elevated glucose is typically associated with the increased generation of free radicals in the form of ROS, and/or the

inhibition of antioxidant systems which will result in tissue damage and therefore in cardiovascular dysfunction.[67]

Another mechanism through which NO biosynthesis/bio-availability is reduced in diabetics is via an altered arginase activity; an enzyme which forms part of the urea cycle.[69] A detailed discussion will be conducted in the next section.

2.2) The urea cycle

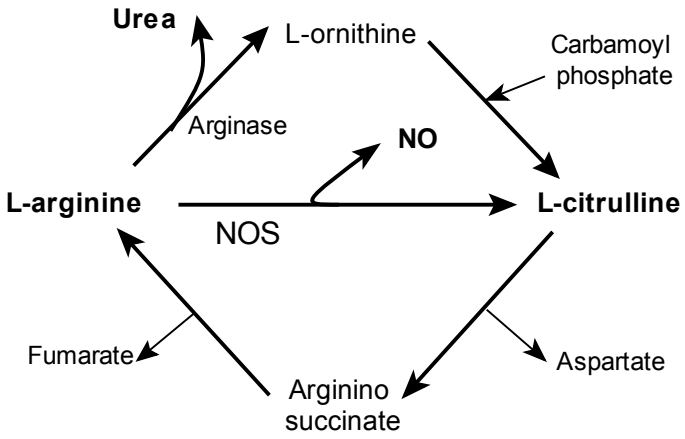

Figure 2.6: The urea cycle (adapted from Romero et al.).[70]

NO, nitric oxide; NOS, nitric oxide synthase.

Carbamoyl phosphate synthetase, mainly found in the mitochondria of the liver and intestine, catalyses the formation of carbamoyl phosphate (Figure 2.6).[70,71] Carbamoyl phosphate reacts with L-ornithine to form L-citrulline in the mitochondria. This reaction is catalysed by ornithine transcarbamoylase.[70,72] The product L-citrulline is transported across the mitochondrial membrane into the cytosol in exchange for cytoplasmic ornithine.[72] In the cytosol, argininosuccinate synthetase catalyzes the reaction between L-citrulline and aspartate to

produce argininosuccinate.[70,73] Argininosuccinate lyase catalyses the breakdown of argininosuccinate to form L-arginine and fumarate. The amount of L-arginine converted from L-citrulline is equivalent to 75% of the L-citrulline taken up in the cytosol.[13] L-arginine is then cleaved by arginase to produce urea and regenerate L-ornithine. The reaction in which L-citrulline is converted to L-arginine, after which L-arginine is cleaved to produce urea and L-ornithine, occurs in the cytosol.[70] The product ornithine is then transported into the mitochondria in exchange for L-citrulline.[70]

Reduced availability of L-arginine to NOS due to elevated arginase levels, may result in vascular dysfunction.[69] Two forms of arginase are present in humans, arginase I and II. Arginase I, a cytoplasm enzyme is mostly expressed in the liver and forms part of the urea cycle, whereas arginase II, located in the mitochondria, is expressed abundantly in the kidney.[74] Arginase competes with NOS for L-arginine.[74] Therefore, altered arginase activity decreases tissue and cellular L-arginine levels, reducing its availability to NOS.[69] This may result in a decrease of NO production or an increase in superoxide by NOS.[75] Increased levels of L-citrulline inhibit the activity of arginase, and L-citrulline is therefore used as a supplementation in diseases associated with reduced levels of L-arginine for NO production, either to increase the synthesis of L-arginine or to inhibit the activity of arginase.[70]

Increased arginase activity has been implicated in certain diseases characterised by vascular dysfunction.[76,77] As stated earlier, vascular dysfunction is associated with oxidative stress in diabetic patients, which has been shown to be associated with an altered arginase activity in various studies.[78,79] In a study done on 12 diabetic patients with good glycemic control (mean HbA1c=6.8%), plasma arginase levels were increased by 50% in the diabetic group when

compared to controls. Arginase also correlated positively with both HbA1c and plasma glucose.[76]

3) Ethnic differences regarding cardiovascular disease, diabetes and markers of NO bio-availability.

In South Africa the prevalence of diabetes mellitus and hypertension varies significantly between the different ethnic groups, with a higher prevalence found in the Africans compared to Caucasians.[80] In the African communities the rising prevalence of diabetes and hypertension is associated with aging and changes in lifestyle habits, especially during urbanisation and westernisation.[81-83] The urban lifestyle in Africa is characterised by physical inactivity and diets that involve an increased consumption of refined sugars, saturated fat, and a low fibre intake.[82,83] Rural populations, on the other hand, are known to be more physically active.[84] Therefore the prevalence of what is 1.5-4-fold higher in urban than in rural populations.[85] It is also known that Africans are more sodium sensitive than Caucasians, which bring about an increase in the risk for cardiovascular consequences of hypertension.[86]

Studies done in South Africa to determine the ethnic differences regarding markers of NO bio-availability are limited. In a study done by Glyn et al, they found that L-arginine levels were significantly higher in Caucasian men when compared to their black counterparts with lower socio-economic status.[87] Ethnic differences in ADMA levels are quite controversial, as mentioned before. A recent study found that ADMA levels were significantly higher in Africans compared to Caucasians,[88] while Glyn et al. found similar ADMA levels in Africans and Caucasians.[87] Schutte et al. found similar ADMA levels in African and Caucasian men; however ADMA levels were significantly higher in Caucasian women compared to African women.[89] To

the best of our knowledge no previous studies have explored the relationship between glucose levels and markers contributing to NO bio-availability in the black South African population.

4) Summary

Pre-diabetes is a growing health problem in sub-Saharan African, and is associated with endothelial dysfunction. Hyperglycemia alters both ADMA and arginase activities, and increases the production of ROS. All these effects of hyperglycemia lead to a decrease in NO bio-availability, resulting in endothelial dysfunction. The extent to which pre-diabetic hyperglycemia contributes to these changes in Africans and Caucasians in South Africa, is not yet clear.

5) Aims, objectives and hypotheses

The aim of this study is therefore to explore blood glucose and glycated hemoglobin levels of a bi-ethnic population of school teachers with specific reference to their relationship with markers of NO bio-availability.

The detailed objectives are:

1. to determine whether glucose measures (fasting glucose and HbA1c) and markers of NO bio-availability (L-arginine, ADMA, SDMA, L-citrulline and ROS) differ between African and Caucasian participants;
2. to determine the relationship of glucose measures with markers of NO bio-availability, blood pressure and renal function; and
3. to establish whether these associations are ethnic-specific.

With regards to the literature, the hypotheses are:

1. Concentrations of glucose measures and markers of NO bio-availability differ between Africans and Caucasians, with Africans displaying an unfavourable profile.
2. Hyperglycemia, as characterised by elevated fasting glucose and HbA1c levels, is negatively associated with L-arginine, L-citrulline and estimated creatinine clearance. Additionally hyperglycemia is positively correlated with ADMA, SDMA, ROS and blood pressure.
3. With respect to hypothesis 2, stronger associations will be evident in the African participants.

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Chapter 3

The relationship between pre-diabetic hyperglycemia and markers of nitric oxide bio-availability in a cohort of Africans and Caucasians: the SABPA-study

INSTRUCTIONS FOR AUTHORS

Journal: Diabetes and Vascular Disease Research

The paper should include:

- Title page
- Abstract of not more than 200 words. It should outline the purpose of the study, key methods, the main results and the main conclusion. A maximum of six keywords should be included.
- Introduction: Short description of background and a clear statement of the purpose of the study.
- Methods: This section should contain a brief description of the study design, procedures, analytical techniques and statistical analyses.
- Results: The results section should include a clear account of the study findings using quantitative language and cross-references to tables and figures.
- Discussion: This section should be an interpretation of the study placed within the context of current knowledge leading to conclusions where possible.
- Acknowledgements should appear first at the end of an article prior to the declaration of conflicting interests.
- Declaration of conflicting interests: Any declarations should be included at the end of the manuscript after acknowledgements and prior to the references, under the heading “Conflict of Interest Statement”.
- References should follow Vancouver format. In the text they should appear as numbers starting at one and at the end of the paper they should be listed in numerical order corresponding to the order of citation in the text. Up to three authors may be listed; for papers with more than three authors, the first three only should be quoted, followed by et al. Where et al. is used, it should be upright, not italic in both references and textual citations. Example: Gasowski J, Fagard RH, Staessen JA,et al. Pulsatile blood pressure component as predictor of mortality in hypertension: a meta-analysis of clinical trial control groups. *J Hypertens* 2002; **20**: 145–51.

The relationship between pre-diabetic hyperglycemia and markers of nitric oxide bio-availability in a cohort of Africans and Caucasians: the SABPA-study

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Abstract

Cardiovascular disease and diabetes are eminent health problems in Sub-Saharan Africa. We aimed to examine the relationship of glucose measures (fasting glucose and glycated hemoglobin (HbA1c)) with markers of nitric oxide (NO) bio-availability (L-arginine, asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), L-citrulline and reactive oxygen species (ROS)), blood pressure and renal function in pre-diabetic Africans (n=148) and Caucasians (n=192). We determined circulating levels of glucose, HbA1c, L-arginine, ADMA, SDMA, L-citrulline, ROS and creatinine. Ambulatory blood pressure and urinary albumin and creatinine were determined. Africans presented significantly higher HbA1c ($p<0.001$), blood pressure ($p<0.001$) and higher albumin-to-creatinine ratio ($p<0.001$) than Caucasians. In Africans, L-citrulline was positively and independently associated with glucose measures ($p=0.017$ and $p=0.018$ for glucose and HbA1c, respectively). An independent negative association was also found between estimated creatinine clearance and L-citrulline in Africans. To conclude, no clear links between hyperglycemia and markers of NO bio-availability were found in Africans and Caucasians, except for an unexpected association between L-citrulline and glucose measures in Africans, possibly driven by an unfavourable renal profile. In Caucasians our findings support the literature in which pre-diabetic hyperglycemia had a positive independent association with ADMA and blood pressure.

Keywords: fasting glucose, glycated hemoglobin, NO bio-availability, blood pressure, renal function, ethnicity.

Introduction

Cardiovascular disease (CVD) is an increasing burden worldwide with the rising prevalence of type 2 diabetes mellitus being one of the major contributors.[1] According to the International Diabetes Federation the number of adults suffering from diabetes will increase from 366 million in 2011 to 552 million in 2030. [2] In sub-Saharan Africa the prevalence of diabetes will rise with 13.3 million people from 2011 until 2030.[2] Rapid urbanisation, which is common among the black African communities,[3] contributes to this rising prevalence of diabetes and CVD in sub-Saharan Africa.[4,5] Typical changes associated with urbanisation include lifestyle, psychological well-being and health transitions, as well as changes in nutritional behaviour which involves diets with less fibre but high levels of total calories and fat.[6]

Cardiovascular complications of diabetes are mainly a result of hyperglycemia in association with other cardiovascular risk factors, including hypertension, obesity, dyslipidemia, smoking and physical inactivity.[6,7] Hyperglycemia-induced endothelial dysfunction involves a number of alterations to the vascular endothelium, including impaired vasodilation.[8] Impaired vasodilation in diabetic patients can be a result of reduced bio-availability of vasodilators including nitric oxide (NO),[8,9] which in turn is caused by either a decrease in NO biosynthesis or an increase in free radical production secondary to hyperglycemia.[10,11]

In addition to various studies indicating an association between diabetes and endothelial dysfunction,[8,12,13] several other studies have found that alterations of the vasculature already exist in the pre-diabetic state.[14-16] Su et al. published two studies in which they found that endothelial dysfunction is already present in subjects with impaired fasting glucose, also known as pre-diabetes.[14,15] Pre-diabetes is an eminent health problem in sub-Saharan Africa and according to the International Diabetes Federation, 63.2 million adults are estimated to

have pre-diabetes by 2030, whereas 28.0 million adults are estimated to have diabetes.[2] Data with regards to markers of NO bio-availability and their relationship with pre-diabetic hyperglycemia in black South Africans is limited. Therefore, the aim of this study was to explore fasting glucose and glycated hemoglobin (HbA1c) levels in a bi-ethnic population from South Africa with specific reference to their relationships with markers of NO bio-availability, blood pressure and renal function.

Methods

Study population

This study forms part of the SABPA (Sympathetic Activity and Ambulatory Blood Pressure in Africans) study conducted between February and May 2008 and 2009. A total of 409 urbanised African and Caucasian teachers working in the Dr. Kenneth Kuanda Education District in the North West Province, South Africa were recruited. The reason for this selection was an attempt to attain a homogenous sample from a similar socio-economic class. We invited all eligible participants between the ages of 25 and 65 years to participate. Exclusion criteria were participants with an elevated ear temperature, making use of α- and β-receptor blockers, psychotropic substance dependence or abuse, blood donors, and individuals vaccinated in the past three months. Power calculations based on the largest standard deviation of 24h systolic blood pressure have shown that 50 participants per group in this type of study are more than sufficient to show significant differences in biological profiles.[17] For this sub-study 16 participants were excluded due to missing data on glycated hemoglobin (HbA1c) or markers of NO bio-availability. Human immunodeficiency virus (HIV) infected participants (n=19), participants with HbA1c levels equal or greater than 6.5% (n=29),[18] and participants that made use of diabetes medication (n=5), were also excluded. The overall sample of this sub-study consisted of 340 participants divided into an African (n=148) and Caucasian (n=192) group. Participants were fully informed about the objectives

and procedures of the study prior to their inclusion. All participants signed an informed consent form. The study complied with all applicable requirements of U.S. and international regulations, in particular the Helsinki declaration of 1975 (as revised in 2008) for investigation of human participants. The Ethics Review Board of the North-West University (Potchefstroom Campus) approved the study.

Organisational Procedures

Each morning at approximately 08h00, postgraduate students attached an ambulatory blood pressure monitor (ABPM) device on the participants' non-dominant arm at their workplace. At 16h30 participants were transported to the Metabolic Unit Research Facility of the North-West University. This facility consists of 10 bedrooms, two bathrooms, a living room and kitchen. Participants received a standardised dinner and had their last beverages (tea/coffee) and two biscuits at 20h30. Thereafter they relaxed by reading, watching television, or social interaction and refrained from consuming alcohol, caffeine, smoking and doing exercise. They were encouraged to go to bed at around 22h00. Urine was collected over night. At 06h00, subjects were woken at which time the ABPM apparatus was removed and subsequent measurements commenced. After the collection of a urine sample and anthropometric data, nurses obtained a fasting venous blood sample. Actical® accelerometers (Montréal, Québec) were attached around the hip of each participant before they leave for work, in order to assess physical activity during a normal working day.

Questionnaires

General health questionnaires were used to determine lifestyle habits and medication use.

Anthropometric measurements

Height (stature) and weight of participants were measured while being in their underwear. Measurements were taken in triplicate using standard methods with calibrated instruments (Precision Health Scale, A & D Company, Japan; Invicta Stadiometer, IP 1465, UK).[19]

Waist circumference (WC) was measured over the abdomen between the costal margin and the iliac crest. Measurements were taken to the nearest 0.1 cm using a non-stretchable standard tape (Lufkin, Cooper Tools, Apex, NC). Body mass index (BMI) was calculated by weight divided by height squared (kg/m^2).

Blood Pressure Measurements

An ABPM apparatus (Meditech CE120® Cardiotens; Meditech, Budapest, Hungary) was attached on the participant at their workplace. The ABPM apparatus was programmed to measure blood pressure at 30 minute intervals during the day (08h00-22h00) and every hour during night time (22h00-6h00). Blood pressure data were downloaded onto a database using the CardioVisions 1.9.0 Personal Edition (Meditech, Budapest, Hungary).

Blood Sampling and Biochemical Analyses

A registered nurse obtained a blood sample with a sterile winged infusion set from the antebrachial vein branches. Fasting glucose (collected in NaF tubes) and serum samples for high density lipoprotein (HDL) cholesterol, gamma-glutamyl transferase (GGT), urea, and high-sensitivity C-reactive protein (CRP) were analysed using two sequential multiple analyzers (Konelab 20i; Thermo Scientific, Vantaa, Finland (2008); Unicel DXC 800 - Beckman and Coulter®, Germany(2009)). The intra- and inter-coefficients of variation for all assays were below 10%. Serum cotinine levels were determined with a homogeneous immunoassay (Automated Modular Roche, Switzerland). Serum reactive oxygen species (ROS) were determined by an improved assay system based on the principle of the

derivatives of reactive oxygen metabolites test, which is recognised as an efficient method for evaluating oxidative stress in the body. The Bio-Tek FL600 Microplate Fluorescence Reader (Bio-Tek, Instruments, Inc., Highland Park, Winooski, VT, USA) was used to measure ROS levels, where 1.0 mg/L H_2O_2 represents one unit of ROS.[20] Serum ferric reducing antioxidant power (FRAP) was also measured using the Bio-Tek FL600 Microplate Fluorescence Reader (Bio-Tek, Instruments, Inc., Highland Park, Winooski, VT, USA). EDTA plasma samples were used to determine L-arginine, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) by using a fully validated high throughout liquid chromatography – tandem mass spectrometry (MS)/MS assay, which is commercially available (DLD Diagnostika, Hamburg, Germany).[21,22] L-citrulline was determined with an electrospray ionization tandem mass spectrometry (ESI-MS/MS) method. The percentage of glycated hemoglobin (HbA1c) was determined by means of the turbidimetric inhibition immunoassay on whole blood using the Roche Integra 400 (Roche, Basil, Switzerland). In urine, creatinine was determined with a calorimetric method and albumin with the measurement of immunoprecipitation enhanced by polyethylene glycol at 450nm, with sequential multiple analyzer computer (Konelab 20iTM, Thermo Scientific, Vantaa, Finland) with a coefficient of variation of1.7–3.3%. Albumin-to-creatinine ratio (ACR) was measured in an 8-h overnight urine sample which is highly correlated with 24-hour urine albumin excretion.[23,24] Estimated creatinine clearance was calculated using the Cockcroft–Gault formula.[25] HIV testing was performed with the First Response HIV Card Test 1-2.0 (PMC Medical, India Pvt Ltd) and confirmed with the Pareekshak HIV Triline test (UCB Pharma).

Statistical Analyses

African and Caucasian sub-groups were compared with independent T-tests (for continuous data) and Chi-square tests (for categorical data). Variables that were not normally distributed were logarithmically transformed (BMI, L-arginine, ADMA, ROS, FRAP, fasting glucose,

HbA1c, triglycerides, albumin-to-creatinine ratio (ACR), estimated creatinine clearance, CRP, physical activity, cotinine, GGT). The associations of HbA1c with both L-arginine and ADMA were tested for interaction with gender by introducing appropriate interaction terms. The association of glucose measures (fasting glucose and HbA1c) with markers of NO bio-availability (L-arginine, ADMA, SDMA, L-citrulline and ROS) were assessed with linear, partial and multiple regression analyses. Additional forward stepwise analyses were used to assess the associations between blood pressure and estimated creatinine clearance with glucose measures.

Results

Table 1 lists the characteristics of the African and Caucasian participants. The two ethnic groups were not stratified by gender, due to insignificant interactions determined by univariate ANCOVA analyses to test for main effects of gender on the association between HbA1c and both L-arginine and ADMA ($p=0.70$ and $p=0.33$ for L-arginine and ADMA, respectively). The BMI ($p<0.001$) of Africans was significantly higher compared to Caucasians, supporting by higher HbA1c levels ($p<0.001$). Fasting glucose ($p<0.001$) was higher in Caucasians. Blood pressure and ACR were also significantly higher in the Africans. Despite the Africans displaying a more unfavourable profile, they had higher L-arginine ($p<0.001$) levels and L-citrulline ($p=0.053$) levels tended to be higher than in Caucasians. On the other hand, Caucasians had higher SDMA ($p<0.001$) and FRAP levels ($p<0.001$). ADMA ($p=0.098$) and ROS ($p=0.18$) levels were similar between the groups. Africans presented higher GGT ($p<0.001$) and cotinine ($p=0.004$) levels with lower levels of physical activity ($p<0.001$) than their Caucasian counterparts.

Table 1: Characteristics of the African and Caucasian participants.

	African	**Caucasian**	***p value***
n	148	192	
Age (yrs)	43.7 ± 8.42	45.3 ± 10.5	0.12
Gender (men/women)	69/79	86/106	0.74
Anthropometric measurements			
Body mass index (kg/m^2)	29.2 (20.5; 41.0)	26.7 (19.9; 38.7)	**<0.001**
Waist circumference (cm)	92.3 ± 14.7	91.9 ± 16.0	0.81
Biochemical measures			
Fasting glucose (mmol/L)	5.08 (3.92; 6.24)	5.58 (4.70; 6.8)	**<0.001**
Glycated hemoglobin (%)	5.70 (5.10; 6.30)	5.45 (5.00; 6.10)	**<0.001**
L-arginine (µmol/L)	67.7 (21.0 ; 136)	52.6 (19.0; 149)	**<0.001**
Urea (mmol/L)	4.50 ± 1.08	4.60 ± 1.18	0.43
Asymmetric dimethylarginine (µmol/L)	0.65 (0.43; 0.96)	0.69 (0.46; 0.94)	0.098
Symmetric dimethylarginine (µmol/L)	0.41 ± 0.11	0.53 ± 0.12	**<0.001**
L-citrulline (µmol/L)	99.7 ± 26.6	94.4 ± 23.3	**0.053**
Reactive oxygen species (U)	91.2 (57.1; 147)	87.4 (56.9; 140)	0.18
Ferric reducing antioxidant power (µM)	381 (284; 507)	416 (274; 627)	**<0.001**
HDL cholesterol (mmol/L)	1.16 ± 0.33	1.22 ± 0.39	0.13
Triglycerides (mmol/L)	1.07 (0.49; 2.77)	0.98 (0.42; 2.79)	0.17
C-reactive protein (mg/L)	4.49 (0.65; 33.0)	2.00 (0.99; 9.00)	**<0.001**
Albumin-to-creatinine ratio (mg/mmol)	1.04 (0.35; 3.95)	0.34 (0.09; 1.88)	**<0.001**
Estimated creatinine clearance (ml/min)	115 (73.9; 192)	116 (72.6; 196)	0.53
Cardiovascular measurements			
Ambulatory systolic blood pressure (mmHg)	132 ± 16.4	123 ± 11.7	**<0.001**
Ambulatory diastolic blood pressure (mmHg)	82.4 ± 10.8	76.4 ± 7.91	**<0.001**
Ambulatory pulse pressure (mmHg)	49.6 ± 9.19	47.0 ± 7.20	**0.004**
*Hypertensive (%)	64.19	47.40	**0.026**
Lifestyle measures			
Physical activity (Kcal/d)	2552 (1700; 4021)	2885 (1915; 4377)	**<0.001**
Cotinine (ng/ml)	0.02 (0.01; 122)	0.01 (0.001; 237)	**0.008**
Gamma-glutamyl transferase (µ/L)	44.5 (20.1; 177)	18.6 (7.00; 74.0)	**<0.001**
Intake of medication			
Anti-hypertensive medication, n (%)	25 (16.9)	17 (8.85)	**0.026**
Lipid-lowering medication, n (%)	2 (1.35)	8 (4.17)	0.13
Anti-inflammatory medication, n (%)	10 (6.76)	12 (6.25)	0.85
Anti-oxidant intake, n (%)	0 (0)	5 (2.60)	**0.048**

Data are arithmetic mean ± SD or geometric mean (5th and 95th percentile intervals) for logarithmically transformed variables.

n, number of participants; HDL-cholesterol, high-density lipoprotein cholesterol.

* 24hr classification as hypertensive: ≥125/80 mmHg.

Unadjusted analyses

In Africans and Caucasians (Table 2), ambulatory blood pressure measurements showed significant positive associations with HbA1c as well as glucose. L-arginine correlated positively with HbA1c in both groups. A significant positive association of ADMA with HbA1c and glucose was evident only in Caucasians. L-citrulline correlated strongly with both HbA1c and glucose in Africans, but in Caucasians this relationship only existed between L-citrulline and HbA1c. Another similarity between the two groups was the positive relationship of urea and FRAP with HbA1c and glucose. Significant positive associations of ROS with HbA1c and glucose were found only in Caucasians.

Table 2: Unadjusted associations of blood pressure and markers of nitric oxide bio-availability with HbA1c and glucose respectively.

	Africans (n=148)		Caucasians (n=192)	
	HbA1c	Glucose	HbA1c	Glucose
Ambulatory SBP (mmHg)	r=0.18; p=0.029	r=0.23; p=0.005	r=0.33; p<0.001	r=0.34; p<0.001
Ambulatory DBP (mmHg)	r=0.16; p=0.046	r=0.26; p=0.001	r=0.29; p<0.001	r=0.39; p<0.001
L-arginine log (µmol/L)	r=0.20; p=0.014	-	r=0.22; p=0.003	-
ADMA log log (µmol/L)	-	-	r=0.20; p=0.007	r=0.32; p<0.001
SDMA log (µmol/L)	-	-	-	-
L-citrulline (µmol/L)	r=0.28; p=0.001	r=0.30; p<0.001	r=0.24; p=0.001	-
Urea log (mmol/L)	r=0.17; p=0.041	r=0.18; p=0.029	r=0.23; p=0.002	r=0.18; p=0.015
ROS log (U)	-	-	r=-0.14; p=0.048	r=-0.22; p=0.003
FRAP log (µM)	r=0.24; p=0.003	r=0.26; p=0.002	r=0.18;p=0.012	r=0.24; p=0.001

ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; ROS, reactive oxygen species; FRAP, ferric reducing antioxidant power.

Adjusted analyses

After adjusting for age, gender, BMI, cotinine, GGT, and systolic blood pressure/anti-hypertensive medication, the positive correlations of L-citrulline with HbA1c (r=0.19; p=0.023) and glucose (r=0.19; p=0.023) were the only associations that remained in

Africans. With similar adjustments applied to the Caucasian group, the positive correlations of ADMA with HbA1c (r=0.15; p=0.03) and glucose (r=0.32; p<0.001) were confirmed. However, no further significant associations were observed in the Caucasians.

Multivariate regression analyses

We performed multivariate forward stepwise regression analyses with L-citrulline (Table 3.1) or ADMA (Table 3.2) as our dependant variables in Africans and Caucasians.

These analyses confirmed the significant positive associations of L-citrulline with HbA1c and glucose in Africans. The positive relationship between ADMA and both HbA1c and glucose was again evident in the Caucasians. Other markers of NO bio-availability (SDMA, L-arginine, and ROS) had no independent associations with HbA1c and glucose (data not shown).

Table 3.1: Independent associations between L-citrulline and either glucose (model 1) or HbA1c (model 2).

	L-citrulline		L-citrulline	
	Africans	Caucasians	Africans	Caucasians
	R^2=0.21	R^2=0.18	R^2=0.21	R^2=0.18
Age (years)		β=0.28; p<0.001		β=0.27; p<0.001
Gender(men/women)	β=-0.42; p<0.001	β=-0.30; p<0.001	β=-0.44; p<0.001	β=-0.28; p<0.001
Body mass index log (kg/m^2)				
Cotinine log (ng/ml)		β=0.18; p=0.01		β=0.17; p=0.012
γ-GT log (U/L)	β=-0.20; p=0.014		β=-0.20; p=0.013	
Triglycerides log (mmol/L)		β=-0.23; p=0.003		β=-0.21; p=0.004
Fasting glucose log (mmol/L) model 1	**β=0.19; p=0.017**		**-**	-
HbA1c log (%) model 2	-	-	**β=0.19; p=0.018**	β=0.14; p=0.057

γ-GT, gamma-glutamyl transferase; HbA1c, glycated hemoglobin.
Independent variables included: age, gender, BMI, physical activity, cotinine, GGT, CRP, triglycerides, fasting glucose (model 1), HbA1c (model 2) and systolic blood pressure.

Table 3.2: Independent associations between ADMA and either glucose (model 1) or HbA1c (model 2).

	log ADMA		log ADMA	
	Africans	Caucasians	Africans	Caucasians
	R^2=0.11	R^2=0.13	R^2=0.12	R^2=0.06
Age (years)				
Gender (men/women)				
Body mass index log (kg/m^2)	β=0.30; p<0.001		β=0.30; p<0.001	
Cotinine log (ng/ml)				
γ-GT, log (U/L)				
Triglycerides log (mmol/L)		β=-0.19; p=0.010		β=-0.17; p=0.037
Fasting glucose log (mmol/L) model 1		**β=0.39; p<0.001**	-	-
HbA1c log (%) model 2	-	-		**β=0.17; p=0.03**

ADMA, asymmetric dimethylarginine; γ-GT,gamma-glutamyl transferase;HbA1c, glycated hemoglobin.
Independent variables included: age, gender, BMI, physical activity, cotinine, GGT, CRP, triglycerides, fasting glucose (model 1), HbA1c (model 2) and systolic blood pressure.

To establish whether the aforementioned associations between L-citrulline or ADMA with glucose measures (fasting glucose and HbA1c) also reflect on the cardiovascular system, we determined whether independent associations existed between ambulatory blood pressure or estimated creatinine clearance with glucose and HbA1c. (Independent variables for this model included: age, gender, BMI, physical activity, cotinine, GGT, CRP, triglycerides, fasting glucose or HbA1c and anti-hypertensive medication). Lastly, we also assessed whether blood pressure and estimated creatinine clearance relate to ADMA and L-citrulline using a similar regression model.

With blood pressure measurements (SBP or DBP) as dependent variables both HbA1c (R^2=0.33; β=0.13; p=0.04) and glucose (R^2=0.33; β=0.12; p=0.06) correlated positively with SBP in Caucasians, but not in Africans. Glucose (R^2=0.33; β=0.18; p=0.008) was also

associated with DBP in Caucasians only. No association existed between ADMA and blood pressure in Caucasians (data not shown).

With estimated creatinine clearance as dependant variable a negative association was evident with HbA1c (R^2=0.53; β=-0.15; p=0.019) and glucose (R^2=0.53; β=-0.11; p=0.088) in Africans, but not in Caucasians. Furthermore, we found a strong negative association between estimated creatinine clearance (R^2=0.55; β=-0.20; p=0.0015) and L-citrulline in Africans, using the similar independent variables were used as shown in Table 3.1 and 3.2.

Discussion

The aim of this study was to compare Africans and Caucasians in terms of glucose measures, markers of NO bio-availability, blood pressure and renal function and to explore the associations of these factors with each other.

Despite Africans demonstrating a more favourable profile regarding their markers of NO bio-availability, they were more likely to be hypertensive and additionally displayed an ACR that was significantly higher than that of the Caucasian group. Furthermore, long term glucose control (HbA1c) was worse in the African group, supported by higher levels of obesity (BMI). However, the main finding of the present study remained the lack of associations between glucose measures and markers of NO bio-availability in both ethnic groups. Nevertheless, we found a positive independent association between glucose measures and L-citrulline in the African group and ADMA in the Caucasian group. However, due to the high prevalence of obesity and cardiovascular disease among urban black South Africans,[26] as confirmed in our study, we expected to find more associations between the various markers of NO bio-availability and glucose measures in our pre-diabetic (HbA1c = 5.7%) African sub-group.

The Africans showed higher levels of blood pressure and HbA1c than their Caucasian counterparts. This was not surprising, since various risk factors for hypertension and type 2 diabetes[27-30] such as obesity, smoking, alcohol use, and physical inactivity, were significantly higher among the Africans. Supporting our findings, various other studies found that the prevalence of hypertension in South Africa is significantly higher among Africans than in Caucasians.[31-34]

In Caucasians, higher levels of fasting glucose were found. But according to the American Diabetes Association (ADA), HbA1c is a more reliable measurement than fasting glucose in the diagnosis of diabetes, since HbA1c reflects the average endogenous exposure to glucose over a 3 month period, including postprandial spikes in blood glucose levels, and low intra-individual variability, particularly in individuals with pre-diabetes.[35] Selvin and colleagues also measured HbA1c from 11 092 white and black adults with no history of diabetes or CVD, and found that the black participants were more likely to have higher HbA1c levels than the white participants,[36] which support our findings.

Moreover, we also found independent associations between glucose and blood pressure measurements in Caucasians, highlighting the results of previous studies that indicated glucose to play an important role in blood pressure control.[37,38] This link was absent in the Africans, which was unexpected since both blood pressure and HbA1c were significantly higher in Africans. However, according to Prahan et al. the weak association between HbA1c and incident cardiovascular events are largely attributable to coexistent risk factors.[39] Risk factors such as obesity,[27] excessive use of alcohol and smoking,[29,30] and physical-inactivity[28] might have had stronger associations with blood pressure than glucose in Africans.

Except for an unfavourable cardiovascular and glucose profile in Africans they also present an unfavourable renal profile, since their ACR was significantly higher than in Caucasians. Although Africans could not be classified as having renal dysfunction, we might speculate that they are displaying signs of early renal impairment.

With regards to markers of NO bio-availability, the African sub-group presented higher levels of the beneficial markers L-arginine and L-citrulline, whereas ADMA and ROS were similar in the two groups. Furthermore, higher levels of SDMA were found in Caucasians than in Africans. This favourable profile in the Africans regarding makers of NO bio-availability was unexpected, since the Africans demonstrated an unfavourable cardiovascular and glucose profile. In contrast to our findings, several studies indicated that L-arginine levels will decrease in individuals with hyperglycemia due to an increased arginase activity, an enzyme responsible for the metabolism of L-arginine to urea and L-ornithine.[40,41] Alev et al. also found L-arginine levels to be reduced in diabetics.[42] In another study comparing African men from a low socio-economic status with Caucasian men from higher socio-economis status, L-arginine levels were found to be lower in African men who also presented higher blood pressure.[43]

In addition, hyperglycemia also impairs the enzyme dimethylarginine dimethylaminohydrolase (DDAH),[44] which is responsible for the metabolism of ADMA to L-citrulline and dimethylamine. ADMA, a NO synthase inhibitor, consequently increases in hyperglycemic patients which results in reduced NO and L-citrulline production.[45] Since Africans presented higher levels of HbA1c, we would expect L-citrulline levels to be significantly lower, and ADMA levels to be significantly higher in Africans than Caucasians.

Studies investigating ethnic differences in ADMA levels in South Africa are quite controversial. Some studies found ADMA levels to be significantly higher in Africans than Caucasians. Our findings confirmed the findings of Glyn et al.[43] and Schutte et al.[46] who also found similar ADMA levels in African and Caucasian men. Contradictory to our findings Melikian et al. found that ADMA levels were significantly lower in healthy white European men than in black African men.[47]

Africans and Caucasians had similar ROS levels which was again unexpected, due to various studies indicating that hyperglycemia will increase ROS production.[14,48-51] Our findings might be due to the specific method of ROS measurements found in our study, which was perhaps not sensitive enough.

Overall it appeared that the Africans presented with a more favourable profile regarding markers of NO bio-availability. This is a strange finding in our primarily hypertensive African group considering that NO bio-availability itself has been reported to be reduced among hypertensive patients.[52] We may however speculate that in Africans, a counter-regulatory response may be at work which is aimed to compensate for the unfavourable cardiovascular and glucose profile.

In pre-diabetic Africans the only link found between the various markers of NO bio-availability and glucose measures was an independent positive association between L-citrulline and both glucose and HbA1c. This result is contrary to what we expected since various studies have reported the impairment of mechanisms responsible for L-citrulline synthesis in hyperglycemic patients.[42,44,53] The nature of this relationship is unclear; however, we proposed the following explanation. Microvascular complications of prolonged hyperglycemia may include renal dysfunction,[54,55] and studies have implicated that renal

failure in rats,[56] and renal diseases in African Americans,[57] result in an increase in plasma L-citrulline levels. The African participants had elevated HbA1c and indicated early signs of impaired renal function compared to Caucasians. Furthermore, Africans had higher L-citrulline levels than their Caucasian counterparts. To strengthen this as a possible explanation for the link between L-citrulline and glucose we found a negative association between estimated creatinine clearance and glucose measures in this group, supporting the notion that the pre-diabetic Africans already show signs of renal impairment possibly due to hyperglycemia. Furthermore, a negative association was evident between estimated creatinine clearance and L-citrulline which confirms that reduced renal function is associated with an increase in L-citrulline levels. We therefore speculate that the link found between glucose and L-citrulline in Africans is possibly driven by an unfavourable renal and cardiovascular profile. The Caucasian group on the other hand showed no signs of impaired renal function, which might explain lower levels of L-citrulline and also the absence of an association between glucose measures and both L-citrulline and estimated creatinine clearance.

The second main finding of our study was the independent positive association between ADMA and glucose measures in Caucasians. This result was found despite Africans presenting higher levels of HbA1c and Africans and Caucasians presenting similar ADMA levels. In the Caucasian group our results confirm previous findings in which ADMA was positively associated with hyperglycemia.[42,53,] Notwithstanding the link found between glucose and blood pressure measurements in Caucasians, no associations were found between ADMA and blood pressure. Although we expect ADMA to be a mediator of endothelial dysfunction in hyperglycemic patients,[58] it was not yet evident in this pre-diabetic group. It is also unclear why we found no associations between ADMA and glucose

measures in the African group, since Africans had an unfavourable cardiovascular and glucose profile.

Furthermore, it is unclear why we found no associations between the other NO bio-availability markers (L-arginine, SDMA, and ROS) with glucose measures in either Africans or Caucasians. According to various studies L-arginine is negatively associated with glucose,[40,41] which was absent in our study. Moreover, we expected to find a positive association between SDMA and glucose measures, since both SDMA and ADMA are related to vascular diseases.[59] To our knowledge studies investigating the link between glucose and SDMA are limited.

This study should be viewed in the context of its strengths and limitations. The participants consisted of urban African and Caucasian teachers, and the results can therefore not be extrapolated to the rest of South Africa. The main strengths of this well-designed study include the availability of ambulatory blood pressure measurements and various markers of NO bio-availability in a relatively large bi-ethnic group of participants.

To conclude, despite our pre-diabetic African sub-group demonstrating an unfavourable cardiovascular, glucose and renal profile, they displayed a beneficial profile regarding markers of NO bio-availability. Our most prominent finding is a general lack of associations between markers of NO bio-availability and glucose measures. The only exception was a positive independent association between L-citrulline and glucose in Africans, which may be driven by an unfavourable renal profile. In Caucasians we found a positive independent association between ADMA and glucose measures, supporting the notion that hyperglycemia has an adverse effect on NO bio-availability and ultimately vascular dysfunction.

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Conflict of Interest Statement

None declared.

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Chapter 4

Summary of main findings and recommendations for future research.

1) Introduction

This chapter is a summary of the main findings from the study, which will be discussed and compared to the relevant literature, after which recommendations will be made for future studies investigating the link between pre-diabetic hyperglycemia and markers of nitric oxide (NO) bio-availability.

2) Discussion of the main findings and comparison to the relevant literature

In Chapter 2 the following hypotheses were set based on the literature:

Hypothesis 1: Concentrations of glucose measures and markers of NO bio-availability differ between Africans and Caucasians, with Africans displaying an unfavourable profile.

In our study, the African sub-group presented higher levels of ambulatory blood pressure than the Caucasian sub-group (132/82.4 mmHg vs. 123/76.4 mmHg in Africans and Caucasians, respectively). This finding was expected since several studies have reported that the prevalence of hypertension in black South Africans is significantly higher than in Caucasians.[1-4] Furthermore, we also found that risk factors for hypertension such as smoking,[5] excessive alcohol use[5,6] and glycated hemoglobin (HbA1c)[5] were significantly higher in Africans than Caucasians. Africans were also less active.

With regards to glucose measures, we found that fasting glucose ($p<0.001$) was higher in Caucasians, whereas in Africans we found higher levels of HbA1c ($p<0.001$), which was accompanied by a higher body mass index (BMI). HbA1c is a more dependable measurement for the diagnosis of pre-diabetes as well as diabetes according to the American Diabetes Association, since it reflects the average blood glucose concentration over a 3 month period.[7]

Data available on the difference in blood glucose levels between Africans and Caucasians include a study by Schutte et al. who compared various metabolic syndrome criteria of age-matched urban African and Caucasian women, participating in the POWIRS study, and found that the African group had significantly higher fasting glucose than the Caucasian group.[8] However, in the SAfrEIC study, which consisted of 750 Africans and Caucasians from urban areas, Africans and Caucasians had similar fasting glucose levels.[9]

Africans in the present study also demonstrated an unfavourable renal profile, due to their albumin-to-creatinine ratio (ACR) which was approximately three-fold higher than in Caucasians (1.04 [0.35; 3.95] vs. 0.34 [0.09; 1.88]). We could not classify them as having renal dysfunction, but we can only speculate that they are displaying signs of early renal impairment.

With regards to markers of NO bio-availability, we found that L-citrulline ($p=0.053$) and L-arginine ($p<0.001$) levels were significantly higher in Africans, who presented an unfavourable cardiovascular profile, when compared to Caucasians. To the best of our knowledge no data is available regarding the difference in L-citrulline levels between Africans and Caucasians residing in South Africa. Studies investigating ethnic-specific differences of L-arginine levels in South Africa are limited. In contrary to what we found, Glyn et al. compared profiles of L-arginine in African and Caucasian men and found that L-arginine levels were significantly lower in African men, who presented higher levels of blood pressure.[10] It is also important to note that these were African men with lower socio-economic status than participants from our study group.[10]

It is noteworthy to mention that Perticone et al. found that L-arginine levels were significantly higher in essential hypertensive subjects than in normotensive subjects, and speculated that L-arginine is increased as a response to compensate for the increase in blood pressure.[11] It is also known that elevated L-citrulline levels will inhibit the activity of arginase, an enzyme responsible for the

production of L-ornithine and urea from L-arginine.[12] As result of this inhibition L-arginine levels will increase,[12] which may be a possible explanation for the higher L-arginine levels found in Africans, since their L-citrulline levels are also higher. In the study by Glyn et al. higher levels of reactive oxygen species (ROS) were found in Africans,[10] whereas in our study ROS levels were similar in Africans and Caucasians.

An unexpected finding of this study was that Caucasians had higher symmetric dimethylarginine (SDMA) levels (0.41 ± 0.11 µmol/L) than Africans (0.53 ± 0.12 µmol/L), whereas asymmetric dimethylargnine (ADMA) concentrations were similar. Supporting our finding is that Schutte et al. also found that SDMA levels were significantly higher in Caucasian women even though the African women presented higher blood pressure.[9] But according to the literature SDMA and ADMA levels usually increase in patients with hypertension[13,14] and diabetes.[15,16] As previously discussed, ethnic differences regarding ADMA levels are quite controversial.[9,10,17]

We therefore partially accept our first hypothesis in which the Africans presented an unfavourable profile regarding blood pressure and HbA1c. We also partially reject our hypothesis in which the Africans presented a favourable profile regarding L-arginine, L-citrulline, and SDMA. Furthermore no differences regarding ADMA and ROS levels were found between the two ethnic groups.

Hypothesis 2: Hyperglycemia, as characterised by elevated fasting glucose and HbA1c levels, is negatively associated with L-arginine, L-citrulline and estimated creatinine clearance. Additionally hyperglycemia is positively correlated with ADMA, SDMA, ROS and blood pressure.

In our study we found a lack of associations between markers of NO bio-availablity in both Africans and Caucasians. We did find an independent positive association between L-citrulline and glucose

measures (p=0.017 and p=0.018 for glucose and HbA1c, respectively) in Africans. In Caucasians we found an independent positive association between ADMA and glucose measures (p<0.001 and p=0.03 for glucose and HbA1c, respectively).

Studies investigating the relationship between L-citrulline and glucose measures are limited. The link in the African group was surprising since we expected the opposite due to several studies reporting mechanisms in which L-citrulline synthesis is impaired in the presence of hyperglycemia.[18,19] L-citrulline is synthesised through various mechanisms of which the L-arginine NO pathway is one. L-arginine, the precursor and metabolic product of L-citrulline, is converted to NO and L-citrulline in the presence of nitric oxide synthase (NOS).[18] However, several studies have reported the accumulation of ADMA in diabetic patients,[15,16] as supported by our findings in which ADMA correlated positively with glucose in Caucasians. ADMA inhibits the synthesis of L-citrulline via the L-arginine NO pathway.[18] ADMA could also be metabolised to L-citrulline and dimethylamine through the enzyme dimethylarginine dimethylaminohydrolase (DDAH), but diabetes impairs DDAH activity and therefore impairs the metabolism of ADMA to L-citrulline.[19]

With Africans presenting higher levels of HbA1c and L-citrulline, accompanied by early signs of renal impairment, the following might be a possible explanation for the association between L-citrulline and glucose measures. In the African sub-group we found a negative association between estimated creatinine clearance and HbA1c (R^2=0.53; β=-0.15; p=0.019), which confirms the literature that hyperglycemia is associated with renal impairment.[20,21] Furthermore, Lou et al. did found that African Americans with renal disease had elevated L-citrulline levels.[22] According to a number of studies L-citrulline levels increase in patients with renal disease, due to up-regulation of re-absorptive transporters of L-citrulline and down-regulation of secretory transporters of L-citrulline in the kidney.[23,24] Supporting this notion we found a negative association between estimated creatinine clearance (R^2=0.55; β=-0.20; p=0.0015) and L-citrulline in Africans. Therefore, we

speculate that the link between glucose and L-citrulline is possibly driven by an unfavourable renal profile found in the African group.

In Africans we also expected a negative correlation between L-arginine and glucose measures, since hyperglycemia is associated with an increase in arginase activity which will consequently reduce the availability of L-arginine to NOS.[25] However, it is unclear why this correlation was absent.

No correlations were found between SDMA and ROS with glucose measures. According to the literature increased levels of ROS play a key role in the development of endothelial dysfunction in diabetic patients.[26-28] Excessive production of ROS could result in oxidative stress, and is linked to various exposures besides diabetes and include smoking,[29] alcohol,[30] obesity,[31] hypertension,[32] and hypercholesterolemia.[33] Therefore, a wide variety of conditions could have contributed to the disturbance in the normal redox state of cells in the Africans and Caucasians, which may have caused an underestimation of the association between ROS and glucose measures in our study.

We therefore partially accept our hypothesis since we found a positive correlation between ADMA and glucose measures in Caucasians. We also partially reject our hypothesis since we found a positive relationship between L-citrulline and glucose measures. We did not find relationships between L-arginine, SDMA or ROS with glucose measures.

Hypothesis 3: With respect to hypothesis 2, stronger associations will be evident in the African participants.

We expected stronger associations between markers of NO bio-availability and glucose measures in the African population due to an unfavourable cardiovascular profile, together with higher HbA1c

levels. The only independent association in Africans were between L-citrulline and glucose measures. In Caucasians only, ADMA was independently associated with both fasting glucose and HbA1c. Supporting this link, several studies have reported an association between ADMA and insulin resistance in Caucasians.[34,35] According to Schutte et al. ADMA seems to be a suitable risk factor for cardiovascular and metabolic disease in Africans,[9] whereas our results in a different African population do not support this notion.

We therefore reject this hypothesis in which Africans only presented one unexpected association between L-citrulline and glucose measures.

3) Chance and confounding

It is important to reflect on factors that might have influenced the results of this study. Although included in the multiple regression models, confounding factors such as age, gender, obesity, inflammation, the lipid profile, smoking, alcohol intake and physical activity could have influenced the results by causing over- or underestimation of the associations between markers of NO bio-availability and glucose measures investigated in this study.

Dietary differences among participants may also have influenced levels of L-arginine and L-citrulline, since high amounts of L-arginine and L-citrulline enter the body by eating certain foods such as milk, grains, meat and peanuts.[36]

Salt sensitivity is associated with an increase in blood glucose,[37,38] and since salt sensitivity is more common in Africans[39] it may have influenced our findings.

Although our results were of statistical significance, this does not necessarily indicate physiological significance.

4) Recommendations

Recommendations for future studies include:

- More variables, such as NO metabolites, DDAH, and arginase could be analysed to better understand the underlying mechanisms that influence the bio-availability of NO and how these mechanisms respond to hyperglycemia.
- A specific and well controlled diet for a longer period could be included which will minimise the effect of diet on plasma L-arginine and L-citrulline levels.

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Appendix A

NORTH-WEST UNIVERSITY

POTCHEFSTROOM CAMPUS

SCHOOL FOR PHYSIOLOGY, NUTRITION AND CONSUMER SCIENCES

PARTICIPANT 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 Ridder (Anthropometry), Marié Wissing (Psychology), Linda Brand & Brian Harvey (Pharmacology), Kobus Mentz (Education), Francois van der Westhuizen (Biochemistry), Hester Klopper (Nursing), Nancy Frasure-Smith & Francois Lespérance (Psychology, Canada), Alaa Alkerwi (Epidemiology, Luxembourg), Yackoob Seedat (ECG, Kwazulu Natal), Paul Rheeder (Sonar, Pretoria Univeristy), Drs. Johan Potgieter & Michael Temane& Mr Thumi 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 the 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 planned 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 system 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 can not 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 to 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 (15min in the morning and 2½ hours in the evening) on Day I; and 2 hours on Day II):

DAY I

- 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 min, 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 c-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 an 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 into 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 (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 the blood flow created by small arteries), central 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 (45ml) 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 include:

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 Celcius). As the cold can make you hold your breath you must quietly count to yourself during cold exposure to breath more rythmic.

- 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 values 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 Metabolic Unit facility of the NWU is fully equipped, and in case of an emergency which could not be handled by the registered nurse, the supervising medical doctor Emile Kotzé will be contacted. Dr. Kotzé was notified before the study commenced that this study will be taking place, and that there is a slight possibility that he may be contacted. Supporting medical treatment care facilities will be at hand anytime if needed.

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.

Incentives

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. Neethling 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 this 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 tests will be labelled only with a code number, and will be stored 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, 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 interests of people who agree to participate in human research studies.

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

Further Information or Any Problems

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

Dr Leoné Malan (018-299 2438)

Sr. Chrissie Lessing (018-299 2480)

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 known 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.**

PART 3

Consent

Title of the project:

"THE SABPA STUDY (**S**YMPATHETIC ACTIVITY AND **A**MBULATORY **B**LOOD **P**RESSURE IN **A**FRICANS)".

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.

(Signature of the subject)

Signed at ..... on ……2008/2009

Witnesses

1. .....

2. .....

Signed at _____ on _____2008/2009

Appendix B

SABPA Project

General Health and Sociodemographic Questionnaire

2008

PARTICIPANT NUMBER ☐☐☐☐☐☐☐

Gender ☐

White Black Indian Coloured

RACE ☐☐☐☐

Date of BIRTH ☐☐☐☐☐☐☐

HOUSE N: P.BOX N: .....

STREET: .....

Post Code: .....TOWN.....

MOBILE phone number.....

P_DUR ☐☐☐ Number of years staying in Potchefstroom.

Question 1:

Marital status.

MS_SI ☐ Unmarried

MS_SIP ☐ Unmarried, living with partner

MS_MA ☐ Married, living with "legal" wife/husband

MS_MAP ☐ Married, partner other than "legal" husband/wife

MS_DI ☐ Divorced, not living with new partner

MS_DIP ☐ Divorced, living with new partner

MS_WW ☐ Widow or widower, not living with new partner

MS_WWP ☐ Widow or widower, living with new partner

Question 2: Education

Still attending school?

SC_NOW ☐Now ?

SC_LOC ☐☐☐☐☐ School or institution

EDU DI ☐ Completed DIPLOMA

EDU DE ☐ Completed DEGREE

Question 3: Past occupation.

P_HINS ☐ Long-lasting health problems

P_DUR ☐☐☐ Number of years

P_P_LOC ☐☐☐☐☐☐ Address

Question 4:

SALARY ☐ Employee receiving salary

S_FULL ☐ Full-time basis

S_PART ☐ Part-time basis

S_SUBE ☐☐☐ Persons subordinated to you

Question 5:

EDU DI ☐ DIPLOMA

EDU DE ☐ DEGREE

EDU WW ☐ Hours of work per week

Question 6: (Family members alive)

FH_F ☐ Father

FH_GFf ☐ Grandfather (father's side)

FH_GMf ☐ Grandmother (father's side)

FH_M ☐ Mother

FH_GFm ☐ Grandfather (mother's side)

FH_GMm ☐ Grandmother (mother's side)

FH_Ch ☐ Children

FH_GCh ☐ Grandchildren

FH_BSf ☐ Brothers or sisters of your father

FH_BSm ☐ Brothers or sisters of your mother

FH_BS ☐ Own brothers or sisters

Question 7:

C_DIS ☐ Disease affecting your heart or blood vessels

Disease Starting date

C_COD1 ☐☐☐☐☐☐ C_BMY1 ☐☐☐☐

Date of cure Treating physician

C_EMY1 ☐☐☐☐ C_NAGP1 .....*…….

Disease Starting date

C_COD2 ☐☐☐☐☐☐ C_BMY2 ☐☐☐☐

Date of cure Treating physician

C_EMY2 ☐☐☐☐ C_NAGP2 .....*…….

Disease Starting date

C_COD3 ☐☐☐☐☐☐ C_BMY3 ☐☐☐☐

Date of cure Treating physician

C_EMY3 ☐☐☐☐ C_NAGP3 .....*…….

Disease Starting date

C_COD4 ☐☐☐☐☐☐ C_BMY4 ☐☐☐☐

Date of cure Treating physician

C_EMY4 ☐☐☐☐ C_NAGP4 .....*…….

Question 8:

K_DIS ☐ Disease affecting your kidneys or urinary tract

Disease Starting date

K_COD1 ☐☐☐☐☐☐ K_BMY1 ☐☐☐☐

Date of cure Treating physician

K_EMY1 ☐☐☐☐ K_NAGP1 .....…*……

Disease Starting date

K_COD2 ☐☐☐☐☐☐ K_BMY2 ☐☐☐☐

Date of cure Treating physician

K_EMY2 ☐☐☐☐ K_NAGP2.....…*……..

Disease Starting date

K_COD3 ☐☐☐☐☐☐ K_BMY3 ☐☐☐☐

Date of cure Treating physician

K_EMY3 ☐☐☐☐ K_NAGP3.....…*……..

Disease Starting date

K_COD4 ☐☐☐☐☐☐ K_BMY4 ☐☐☐☐

Date of cure Treating physician

K_EMY4 ☐☐☐☐ K_NAGP4 .....…*……..

Question 9:

L_DIS ☐ Kidney stones or stones in you urinary tract

L_REPC ☐ Repeated pain attacks

L_EVAC ☐ Passed a stone with urine

L_OPER ☐ Surgical treatment

L_NOW ☐ Still suffering from kidney stones or stones in urinary tract

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Question 10:

DIABET ☐ Diabetes

D_DIET ☐ Diet and avoiding sweet foodstuffs

D_ORAL ☐ Pills

D_INS ☐ Insulin

Question 11:

HYPERT ☐ Hypertension

HY_MY ☐☐☐☐When ?

HY_Th ☐ Treatment

Question 12:

DISEAS ☐ Currently in good health

Disease Starting date

DS_CD1 ☐☐☐☐☐☐ DS_BMY1 ☐☐☐☐

Date of cure Treating physician

DS_EMY1 ☐☐☐☐ DS_NAGP1.....……*……..

Disease Starting date

DS_CD2 ☐☐☐☐☐☐ DS_BMY2 ☐☐☐☐

Date of cure Treating physician

DS_EMY2 ☐☐☐☐ DS_NAGP2 .....……*……

Disease Starting date

DS_CD3 ☐☐☐☐☐☐ DS_BMY3 ☐☐☐☐

Date of cure Treating physician

DS_EMY3 ☐☐☐☐ DS_NAGP3 .....……*……..

Disease Starting date

DS_CD4 ☐☐☐☐☐☐ DS_BMY4 ☐☐☐☐

Date of cure Treating physician

DS_EMY4 ☐☐☐☐ DS_NAGP4 .....……*……

Disease Starting date

DS_CD5 ☐☐☐☐☐☐ DS_BMY5 ☐☐☐☐

Date of cure Treating physician

DS_EMY5 ☐☐☐☐ DS_NAGP5.....……*……..

Disease Starting date

DS_CD6 ☐☐☐☐☐☐ DS_BMY6 ☐☐☐☐

Date of cure Treating physician

DS_EMY6 ☐☐☐☐ DS_NAGP6.....……*……..

Question 13:

D_HYPT ☐ Drugs to lower blood pressure

DH_NOW ☐Now ?

Name of drug Tablets/day/dosage

DH_CD1 ☐☐☐☐☐☐ DH_DO1 ☐☐ ☐

Name of drug Tablets/day/dosage

DH_CD2 ☐☐☐☐☐☐ DH_DO2 ☐☐ ☐

Name of drug Tablets/day/dosage

DH_CD3 ☐☐☐☐☐☐ DH_DO3 ☐☐ ☐

Name of drug Tablets/day/dosage

DH_CD4 ☐☐☐☐☐☐ DH_DO4 ☐☐ ☐

Question 14:

D_DIUR ☐ Diuretics

DD_NOW ☐Now ?

	Name of drug		Tablets/day/dosage
DD_CD1	☐☐☐☐☐☐	DD_DO1 ☐☐	☐
	Name of drug		Tablets/day/dosage
DD_CD2	☐☐☐☐☐☐	DD_DO2 ☐☐	☐
	Name of drug		Tablets/day/dosage
DD_CD3	☐☐☐☐☐☐	DD_DO3 ☐☐	☐
	Name of drug		Tablets/day/dosage
DD_CD4	☐☐☐☐☐☐	DD_DO4 ☐☐	☐

Question 15:

D_ANAL ☐ Taking pain-killers

DA_YE ☐☐ How many years ?

DA_SAL ☐ Salicylic acid (Disprin)

DA_PAR ☐ Paracetemol (Grand-Pa)

DA_OTH ☐ Analgesic drugs for arthritis (Brufen)

	Name of drug		Units/week
DA_CD1	☐☐☐☐☐☐	DA_DO1 ☐☐	☐
	Name of drug		Units/week
DA_CD2	☐☐☐☐☐☐	DA_DO2 ☐☐	☐
	Name of drug		Units/week
DA_CD3	☐☐☐☐☐☐	DA_DO3 ☐☐	☐
	Name of drug		Units/week
DA_CD4	☐☐☐☐☐☐	DA_DO4 ☐☐	☐

Question 16:

DR_2WK ☐ Medication during last 2 weeks

	Name of medicine		Units/day
DR_CD1	☐☐☐☐☐☐☐☐	DR_DO1 ☐☐	☐
	Name of medicine		Units/day
DR_CD2	☐☐☐☐☐☐☐☐	DR_DO2 ☐☐	☐
	Name of medicine		Units/day
DR_CD3	☐☐☐☐☐☐☐☐	DR_DO3 ☐☐	☐
	Name of medicine		Units/day
DR_CD4	☐☐☐☐☐☐☐☐	DR_DO4 ☐☐	☐
	Name of medicine		Units/day
DR_CD5	☐☐☐☐☐☐☐☐	DR_DO5 ☐☐	☐
	Name of medicine		Units/day
DR_CD6	☐☐☐☐☐☐☐☐	DR_DO6 ☐☐	☐

Question 17:

T_NOW ☐ Currently smoking

T_CTf ☐☐ Cigarettes with filter per day

T_CT ☐☐ Cigarettes without filter per day

T_CTgt ☐☐☐ Grams of tobacco per day

T_Plgt ☐☐☐ Grams of tobacco per week for pipe

T_SCgr ☐☐ Small cigars per week

T_Cgar ☐☐ Cigars per week

T_AGE ☐☐ When started smoking ? (age)

T_INHA ☐ Inhalation

Question 18:

T_P_PAST ☐ Smoking in the past

T_P_1CDY ☐ At least one cigarette per day during one year

T_P_AGE ☐☐ Age at which participant quitted smoking

T_P_CTf ☐☐ Cigarettes with filter per day

T_P_CT ☐☐ Cigarettes without filter per day

T_P_CTgt ☐☐☐ Grams of tobacco per day

T_P_PIgt ☐☐☐ Grams of tobacco per week for pipe

T_P_SCgr ☐☐ Small cigars per week

T_P_Cgar ☐☐ Cigars per week

T_P_WHY ☐☐ Reason to stop smoking

Question 19:

E_NOW ☐ Current consumption alcoholic beverages

E_BEER ☐☐ Glasses of beer per day

E_TBEER ☐☐ Glasses of traditional beer per day

E_WINE ☐☐ Bottles of wine per week

E_THLOKd ☐☐ Boxes of Thlokwe per day

E_THLOKw ☐☐ Boxes of Thlokwe per week

E_SPIRITt ☐☐ Tot Spirits per day

E_SPIRITb ☐☐ Bottle of Spirits per week

E_LIQR ☐☐ Bottle of Liquor per week

E_AGE ☐☐ When started drinking alcohol regularly ? (age)

Question 20:

E_P_PAST ☐ Consumption of alcoholic beverages in the past

E_P_AGE ☐☐ When stopped ? (age)

E_BEER ☐☐ Glasses of beer per day

E_TBEER ☐☐ Glasses of traditional beer per day

E_WINE ☐☐ Bottles of wine per week

E_THLOKd ☐☐ Boxes of Thlokwe per day

E_THLOKw ☐☐ Boxes of Thlokwe per week

E_SPIRITt ☐☐ Tot Spirits per day

E_SPIRITb ☐☐ Bottle of Spirits per week

E_LIQR ☐☐ Bottle of Liquor per week

E_P_WHY ☐☐ Why stopped consuming alcoholic beverages ?

Question 21:

C_NOW ☐ Now consumption of caffeine-containing beverages

C_REG ☐☐ Cups of coffee

C_COKE ☐☐ Glasses of Coca-cola

C_OTH ☐☐ Other

C_TEA ☐ Tea

C_DECAF ☐ Decaffeinated coffee

C_DECA_N ☐☐ Number of cups of decaffeinated coffee per day

Question 22:

M-NOW ☐ Periods

Question 23:

DCCET ☐ Ever taken "the pill" ?

DC_NOW ☐ “The pill” now ?

DC_COD ☐☐☐☐☐☐ Name of "the pill"

DC_YE ☐☐☐☐ How long ? (years, months)

Question 24:

PR_PST ☐ Pregnant before

PR_N ☐☐ Number of pregnancies

PR_ABO ☐ Number of miscarriages

PR_LIB ☐ Children born alive

PR_STB ☐ Children stillborn

Question 25:

M_NOW ☐ Still periods

M_IRYE ☐☐☐☐ Since when irregular periods ?

M_DISYE ☐☐☐☐ Since when periods completely disappeared ?

M_P_SPON ☐ Spontaneous disappearance

M_P_HYST ☐ Removal of only womb

M_HYSTYE ☐☐☐☐ Date (month/year)

M_P_OVRR ☐ Removal of only right ovary

M_OVRRYE ☐☐☐☐ Date (month/year)

M_P_OVRL ☐ Removal of only left ovary

M_P_OVR2 ☐ Removal of both ovaries

M_OVR2YE ☐☐☐☐ Date (month/year)

M_P_ORHR ☐ Removal of right ovary together with womb

M_ORHRYE ☐☐☐☐ Date (month/year)

M_P_OLHR ☐ Removal of left ovary together with womb

M_OLHRYE ☐☐☐☐ Date (month/year)

M_P_HRT ☐ Removal of both ovaries and womb

M_HRTstart ☐☐☐☐ Date (month/year)

MS_COD1 ☐☐☐☐ Underlying disease 1

MS_COD2 ☐☐☐☐ Underlying disease 2

MS_COD3 ☐☐☐☐ Underlying disease 3

M_P_DRUG ☐ Periods suppressed by taking "the pill"

MD_P_COD ☐☐☐☐☐☐ Name of "the pill"

MD_P_MN ☐☐☐ Number of months

Question 26:

E_EXCS ☐ Results sent only to yourself

R-EXGP ☐ Results sent only to your family doctor

E_S_GP ☐ Results sent to yourself and your family doctor

Question 27:

C-GP ☐ Consent to contact the subject's physician(s)

Appendix C

Subject Group Physiology

AMBP card 2008/2009

SURNAME ----------

NAME ----------

CONTACT NAME AND NUMBER ----------

DBIRTH Day☐☐ Month☐☐ Year☐☐☐☐ date of birth

CPNBR ☐☐☐☐☐☐☐☐☐☐ identification number

DATABP_S ☐☐☐☐☐☐☐☐ date of ABP recording

Please indicate if you experience/do any of the following during the 24 hour recording:

C_DIZ ☐ **dizziness** **1 = yes, 2 = no**

TDIZ_S1 ☐☐☐☐ time of start of dizziness 1

TDIZ_E1 ☐☐☐☐ time of end of dizziness 1

TDIZ_S2 ☐☐☐☐ time of start of dizziness 2

TDIZ-E2 ☐☐☐☐ time of end of dizziness 2

C_FAT ☐ fatigue 1 = yes, 2 = no

TFAT_S1 ☐☐☐☐ time of start of fatigue 1

TFAT_E1 ☐☐☐☐ time of end of fatigue 1

TFAT_S2 ☐☐☐☐ time of start of fatigue 2

TFAT_E2 ☐☐☐☐ time of end of fatigue 2

C_VIS ☐ **visual problems** **1 = yes, 2 = no**

TVIS_S1 ☐☐☐☐ time of start of visual problems 1

TVIS_E1 ☐☐☐☐ time of end of visual problems 1

TVIS_S2 ☐☐☐☐ time of start of visual problems 2

TVIS_E2 ☐☐☐☐ time of end of visual problems 2

C_HEAD ☐**headache** **1 = yes, 2 = no**

THEAD_S1 ☐☐☐☐ time of start of headache 1

THEAD_E1 ☐☐☐☐ time of end of headache 1

THEAD_S2 ☐☐☐☐ time of start of headache 2

THEAD_E2 ☐☐☐☐ time of end of headache 2

C_HOT ☐ **hot flushes** **1 = yes, 2 = no**

THOT_S1 ☐☐☐☐ time of start of flushes 1

THOT_E1 ☐☐☐☐ time of end of flushes 1

THOT_S2 ☐☐☐☐ time of start of flushes 2

THOT_E2 ☐☐☐☐ time of end of flushes 2

C_VOM ☐ **nausea and/or vomiting** **1 = yes, 2 = no**

TVOM_S1 ☐☐☐☐ time of start of nausea 1

TVOM_E1 ☐☐☐☐ time of end of nausea 1

TVOM_S2 ☐☐☐☐ time of start of nausea 2

TVOM_E2 ☐☐☐☐ time of end of nausea 2

C_PALP ☐ **palpitations/fast heart beat** **1 = yes, 2 = no**

TPALP_S1 ☐☐☐☐ time of start of palpitations 1

TPALP_E1 ☐☐☐☐ time of end of palpitations 1

TPALP_S2 ☐☐☐☐ time of start of palpitations 2

TPALP_E2 ☐☐☐☐ time of end of palpitations 2

C_SYNC ☐ **syncope/faint feeling** **1 = yes, 2 = no**

TSYNC_S1 ☐☐☐☐ time of start of syncope 1

TSYNC_E1 ☐☐☐☐ time of end of syncope 1

TSYNC_S2 ☐☐☐☐ time of start of syncope 2

TSYNC_E2 ☐☐☐☐ time of end of syncope 2

C_OTHICD ☐☐☐☐☐ ICD code for other symptoms if present

TOTH_S1 ☐☐☐☐ time of start of other 1

TOTH_E1 ☐☐☐☐ time of end of other 1

TOTH_S2 ☐☐☐☐ time of start of other 2

TOTH_E2 ☐☐☐☐ time of end of other 2

A_LPH ☐ **light physical activity** **1 = yes, 2 = no**

TLPH_S1 ☐☐☐☐ time of start of LPH 1

TLPH_E1 ☐☐☐☐ time of end of LPH 1

TLPH_S2 ☐☐☐☐ time of start of LPH 2

TLPH_E2 ☐☐☐☐ time of end of LPH 2

TLPH_S3 ☐☐☐☐ time of start of LPH 3

TLPH_E3 ☐☐☐☐ time of end of LPH 3

A_PH ☐ **physical effort** **1 = yes, 2 : no**

TPH_S1 ☐☐☐☐ time of start of physical effort 1

TPH_E1 ☐☐☐☐ time of end of physical effort 1

TPH_S2 ☐☐☐☐ time of start of physical effort 2

TPH_E2 ☐☐☐☐ time of end of physical effort 2

TPH_S3 ☐☐☐☐ time of start of physical effort 3

TPH_E3 ☐☐☐☐ time of end of physical effort 3

S-SST ☐ **slightly stressed** **1 = yes, 2 = no**

TSST_S1 ☐☐☐☐ time of start of slightly stressed 1

TSST-E1 ☐☐☐☐ time of end of slightly stressed 1

TSST_S2 ☐☐☐☐ time of start of slightly stressed 2

TSST-E2 ☐☐☐☐ time of end of slightly stressed 2

TSST_S3 ☐☐☐☐ time of start of slightly stressed 3

TSST-E3 ☐☐☐☐ time of end of slightly stressed 3

S_ST ☐ **stress** **1 = yes, 2 = no**

TST_S1 ☐☐☐☐ time of start of stress 1

TST_E1 ☐☐☐☐ time of end of stress 1

TST_S2 ☐☐☐☐ time of start of stress 2

TST_E2 ☐☐☐☐ time of end of stress 2

TST_S3 ☐☐☐☐ time of start of stress 3

TST_E3 ☐☐☐☐ time of end of stress 3

TSLEEP ☐☐☐☐**time of sleep**

TGUP ☐☐☐☐ **time of getting-up**

THSLEEP ☐☐☐☐ **hours of sleep per night**

TASLEEP ☐☐☐☐ **hours awake/can't sleep per night**

TMEAL ☐☐☐☐ **time of main meal**

DR_CD1 ☐☐☐☐☐☐ drug 1 (coded as in the standard questionnaire)

DR_DO1 ☐☐ amount of tablets taken

TIMDR1 ☐☐☐☐ time at which the medication was taken

DR_CD2 ☐☐☐☐☐☐ drug 2 (coded as in the standard questionnaire)

DR_DO2 ☐☐ amount of tablets taken

TIMDR2 ☐☐☐☐ time at which the medication was taken

DR_CD3 ☐☐☐☐☐☐ drug 3 (coded as in the standard questionnaire)

DR_DO3 ☐☐ amount of tablets taken

TIMDR3 ☐☐☐☐ time at which the medication was taken

DR_CD4 ☐☐☐☐☐☐ drug 4 (coded as in the standard questionnaire)

DR_DO4 ☐☐ amount of tablets taken

TIMDR4 ☐☐☐☐ time at which the medication was taken

DR_CD5 ☐☐☐☐☐☐ drug 5 (coded as in the standard questionnaire)

DR_DO5 ☐☐ amount of tablets taken

TIMDR5 ☐☐☐☐ time at which the medication was taken

DR_CD6 ☐☐☐☐☐☐ drug 6 (coded as in the standard questionnaire)

DR_DO6 ☐☐ amount of tablets taken

TIMDR6 ☐☐☐☐ time at which the medication was taken.