Comparing markers of the nitric oxide cycle and their association with ambulatory blood pressure and end organ damage in a bi-ethnic population: The SABPA-study

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Dissertation submitted in partial fulfilment of the requirements for the degree Master
of Science in Physiology at the
Hypertension in Africa Research Team (HART),
Potchefstroom Campus of the North-West University.

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November 2012

Innovation through diversity

NORTH-WEST UNIVERSITY
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POTCHEFSTROOM CAMPUS

ACKNOWLEDGEMENTS

With great appreciation, the author would like to thank to following people regarding their input:

Prof. AE Schutte, for being there for me throughout the year, for helping me with my dissertation and for all the patience.

Dr. CMC Mels, for all the help and advice.

Isabel Swart, for the language editing.

The SABPA participants, for your participation and the permission to use your information.

My mother, grandmother and boyfriend for all the support and encouragement.

Last, but not least, God, for giving me the opportunity and endurance to complete this project successfully.

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AUTHOR CONTRIBUTIONS

The following researchers contributed to this study:

Ms. I Loots

Responsible for literature search, statistical analyses, handling and processing of data, design, planning and writing of the dissertation.

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Supervised the writing of the manuscript, oversaw the collection of cardiovascular data, critically evaluated the manuscript, made recommendations and gave professional input.

Dr. CMC Mels: Co-supervisor

Supervised the writing of the manuscript, critically evaluated the manuscript, made recommendations and gave professional input.

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.

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SUMMARY

Comparing markers of the nitric oxide cycle and their association with ambulatory blood pressure and end organ damage in a bi-ethnic population: The SABPA-study

Aims

There is a high prevalence of hypertension in the African population and it is known that vascular dysfunction (including nitric oxide (NO) bio-availability markers) play an important role in the development of cardiovascular diseases. Since very little is known regarding the role of markers of NO bio-availability in Africans, the aim of this study was to compare markers of NO bio-availability (namely L-arginine, L-citrulline, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA)), ambulatory blood pressure (BP) and markers of end organ damage between African and Caucasian school teachers. Additionally, we also aimed to determine whether these markers of NO bio-availability are associated with ambulatory BP and markers of end organ damage in both ethnic groups.

Methods

The SABPA (Sympathetic activity and Ambulatory Blood Pressure in Africans) study was a cross-sectional study, including urbanised African (N=181) and Caucasian (N=209) men and women, between the ages of 25 and 65 years. Cardiovascular measurements included ambulatory blood pressure, pulse wave velocity (PWV), electrocardiographic Cornell product and carotid intima media thickness (cIMT). Anthropometric measurements included height, weight and waist circumference.

Various bio-markers were analysed, including glucose, L-arginine, ADMA, SDMA, L-citrulline, reactive oxygen species, albumin-to-creatinine ratio (ACR) and estimated creatinine clearance (eCCR).

Characteristics of groups were compared with independent T-tests and Chi-square tests. Single and partial analyses were used to investigate associations between NO bio-availability markers with ambulatory BP measurements and markers of end organ damage. Analyses of covariance (ANCOVA) were used for comparison of variables between groups to determine significant differences, while adjusting for age, body mass index and anti-hypertensive medication. Forward stepwise multiple regression analyses were performed to determine if independent associations exist between ambulatory BP measurements or markers of end organ damage with either- L-arginine, L-citrulline, ADMA or SDMA as the main independent variable.

Results and conclusion

The Africans and Caucasians were of similar ages. However, the Africans had higher blood pressure therefore their cardiovascular profile was unfavourable compared to that of the Caucasians.

The inhibitors of NO biosynthesis, ADMA and SDMA, were significantly lower in the Africans ($p=0.046$; $p<0.001$, respectively). However, the NO bio-availability markers, L-arginine and L-citrulline, were higher in the African compared to the Caucasian participants (all p values <0.05) regarded as significant.

When performing unadjusted analyses, we found significant negative associations between eCCR and L-citrulline in all four subgroups: African men ($r=-0.27$; $p=0.013$), African women

(r=-0.24; p=0.021), Caucasian men (r=-0.21; p=0.044) and Caucasian women (r=-0.28; p=0.003). The association of eCCR with L-citrulline was confirmed to be independent of confounders in all groups: African men (R^2=0.46; β=-0.23; p=0.006), African women (R^2=0.68; β= -0.12; p=0.046), Caucasian men (R^2=0.62; β= -0.24; p<0.001) and Caucasian women (R^2=0.72; β= -0.13; p=0.029). This implicates that renal function may be detrimentally affected by L-citrulline concentrations.

In the Caucasian men and women negative correlations between eCCR and SDMA were found before adjustments (r=-0.33; p=0.003 and r=-0.26; p=0.006, respectively). This phenomenon was confirmed in the forward stepwise multiple regression analysis in Caucasian men (R^2=0.75; β= -0.27; p<0.001) and women (R^2=0.73; β= -0.21; p<0.001), while no associations were found in the Africans. This result is not unexpected, since SDMA can only be eliminated by the kidneys and is therefore an important risk marker for the early detection of renal dysfunction.

In Caucasian men we found that ADMA correlated with ACR (r=0.36; p=0.001), night-time SBP (r=0.34; p=0.002) and night-time DBP (r=0.25; p=0.023) with single linear regression analyses. A similar trend was shown in African men with night-time SBP (r= 0.20; p=0.089) and night-time DBP (r= 0.21; p=0.078) respectively, but this association was absent in the Caucasian and African women. After adjustments for age and body mass index, the associations with ADMA, ACR and SBP in the Caucasian men remained. However, a negative association between eCCR and ADMA also became evident in the African men (r=-0.24; p=0.025) and remained significant in the forward stepwise multiple regression analysis (R^2=0.44; β= -0.18; p=0.034). It is, however, not clear why our results were gender specific, but we could speculate that the female sex hormones may play a part in protecting the vascular endothelium.

Apart from the associations described above, there were no significant independent associations between the markers of the NO cycle (such as L-arginine) and PWV, cIMT, eCCR, ACR or Cornell product.

In conclusion, although Africans presented a more vulnerable cardiovascular profile, we found a consistent negative association between renal function and L-citrulline in all participants, which has only been reported previously in patients with chronic renal disease. Additionally we found a gender-specific link between renal function and ADMA in African and Caucasian men. Our results may indicate that in the general population, markers of NO bio-availability may be associated with early changes in renal function, accompanying elevated blood pressure.

Key words: L-citrulline, L-arginine, ADMA, SDMA, renal function

OPSOMMING

’n Vergelyking van merkers van die stikstofoksied siklus, en die assosiasies daarvan met ambulatoriese bloeddruk en eindorgaan-skade in twee etniese groepe: Die SABPA-studie

Doelstellings

Daar is ’n hoë voorkoms van hipertensie in die swart populasie en vaskulêre disfunksie (insluitende stikstofoksied (NO) bio-beskikbaarheidsmerkers) speel ’n belangrike rol in kardiovaskulêre siektes en die ontwikkeling van eindorgaan skade. Omdat daar min inligting is oor die rol wat NO bio-beskikbaarheidsmerkers in die swart populasie speel, is die doelstellings van die studie om die merkers van NO bio-beskikbaarheid (naamlik L-arginien, L-sitrulien, asimmetriese dimetiel arginien (ADMA) en simmetriese dimetiel arginien (SDMA)), ambulatoriese bloeddruk (BD) en merkers van eindorgaan skade in swart en wit onderwysers te vergelyk. Daar is verder ondersoek ingestel om te bepaal of hierdie merkers van NO bio-beskikbaarheid verband hou met ambulatoriese BD en merkers van eindorgaan skade.

Metodes

Die SABPA (“*Sympathetic activity and Ambulatory Blood pressure in Africans*”) studie is ’n dwarsdeursnee studie wat stedelike swart (N=181) en wit (N=209) mans en vrouens, tussen die ouderdomme van 25 en 65 jaar ingesluit het. Kardiovaskulêre metings het ambulatoriese bloeddruk-metings (ABDM), polsgolf-snelheid (PGS), elektrokardiografiese Cornell-produk en karotis intima-media dikte ingesluit. Antropometriese metings het lengte, gewig en middel omtrek ingesluit.

Verskeie bio-merkers is geanaliseer, insluitende glukose, L-arginien, ADMA, SDMA, L-sitrulien, reaktiewe suurstof spesies, albumien-kreatinien verhouding (ACR) en berekende kreatinien opruiming (eCCR).

Eienskappe van die groepe is vergelyk deur van onafhanklike T-toetse en Chi-kwadraat-toetse gebruik te maak. Enkel en parsiële korrelasies is gebruik om die verbande tussen ambulatoriese BD en eindorgaan skade met merkers van NO bio-beskikbaarheid te ondersoek. Kovariansie-analises (ANKOVA) is gebruik om die merkers tussen groepe te vergelyk, terwyl daar korreksies gemaak is vir ouderdom, liggaamsmassa-indeks en anti-hipertensiewe medikasie. Voorwaartse meervoudige regressie-analises is gedoen om te bepaal of daar onafhanklike assosiasies bestaan tussen ABDM of merkers van eindorgaan skade met L-arginien, L-sitrulien, ADMA of SDMA as die hoof onafhanklike veranderlike.

Resultate en gevolgtrekking

Die swart en wit proefgroepe is van dieselfde ouderdoms-groepe, maar die swart populasie het hoër bloeddruk getoon, dus was hulle kardiovaskulêre profiel ongunstig teenoor die wit proefgroep.

Inhibeerders van NO bio-beskikbaarheid, ADMA en SDMA, was betekenisvol laer in die swart populasie ($p=0.046$; $p<0.001$, onderskeidelik), terwyl die merkers van NO bio-beskikbaarheid, L-arginien en L-sitrulien, hoër is in die swart populasie (alle waardes <0.05) as in die wit proefgroep.

Betekenisvolle negatiewe korrelasies is tussen eCCR en L-sitrulien in al vier groepe gevind: Swart mans ($r=-0.27$; $p=0.013$), swart vrouens ($r=-0.24$; $p=0.021$), wit mans ($r=-0.21$;

p=0.044) en wit vrouens (r=-0.28; p=0.003). Hierdie assosiasies is onafhanklik van ander koveranderlikes in al die groepe: Swart mans (R^2=0.46; β=-0.23; p=0.006), swart vrouens (R^2=0.68; β= -0.12; p=0.046), wit mans (R^2=0.62; β= -0.24; p<0.001) en wit vrouens (R^2=0.72; β= -0.13; p=0.029). Dit kan daarop dui dat nierfunksie negatief beïnvloed word deur L-sitrulien konsentrasies.

In die wit mans en vrouens is ’n korrelasie gevind tussen eCCR en SDMA voor korreksies aangebring is (r=-0.33; p=0.003 en r=-0.26; p=0.006, onderskeidelik). Hierdie verskynsel is bevestig in die voorwaartse meervoudige regressie-analises in die wit mans (R^2=0.75; β= -0.27; p<0.001) en vrouens (R^2=0.73; β= -0.21; p<0.001), terwyl geen assosiasies in die swart populasie gevind is nie. Hierdie resultaat is nie teen ons verwagtinge nie, want SDMA kan net deur die niere uitgeskei word en is ook daarom ’n belangrike risiko faktor vir vroeë waarneming van nierskade.

In die wit mans het ADMA gekorreleer met ACR (r=0.36; p=0.001), nag sistoliese BD (r=0.34; p=0.002) en nag diastoliese BD (r=0.25; p=0.023). ’n Soortgelyke verskynsel is aangetoon in die swart mans met nag sistoliese BD (r=0.20; p=0.089) en nag diastoliese BD (r=0.21; p=0.078), onderskeidelik, maar hierdie assosiasie is nie teenwoordig in die wit en swart vrouens nie. Nadat korreksies vir ouderdom en liggaamsmassa-indeks aangebring is, het ‘n negatiewe assosiasie tussen eCCR en ADMA na vore gekom in die swart mans (r=-0.24; p=0.025) wat betekenisvol gebly het in die voorwaartse meervoudige regressie-analise (R^2=0.44; β= -0.18; p=0.034). Dit is onduidelik hoekom die resultate geslag-spesifiek is, maar ons spekuleer dat die vroulike geslagshormone moontlik ’n beskermde effek op die vaskulêre endoteel het.

Afgesien van die assosiasies wat beskryf is hier bo, was daar geen betekenisvolle onafhanklike assosiasies tussen die merkers van die NO-siklus (soos L-arginien) en PGS, karotis intima-media dikte, eCCR, ACR of Cornell-produk nie.

Samevattend: alhoewel die swart populasie ’n kwesbaarder kardiovaskulêre profiel getoon het, het ons deurgaans ’n onafhanklike negatiewe assosiasie gevind tussen nierfunksie en L-sitrulien in al die deelnemers. Dit is vantevore nog net in pasiënte met kroniese nierskade gevind. Ons het ook ’n nadelige geslag-spesifieke verwantskap gevind tussen nierfunksie en ADMA in die swart en wit mans. Ons resultate dui daarop dat in die algemene populasie merkers van NO bio-beskikbaarheid assosieer met vroeë veranderinge in nierfunksie, wat gepaard gaan met verhoogde BD.

Sleutelwoorde: L-sitrulien, L-arginien, ADMA, SDMA, nierfunksie

PREFACE

This dissertation is presented in the article format. This is a format approved and recommended by the North-West University, consisting amongst others of a manuscript, which is ready for submission to a peer-reviewed journal.

This dissertation contains four chapters with a reference list after each chapter. Chapter 1 contains the motivation and background of the study. Chapter 2 provides a literature overview on the topic, as well as aims, objectives and hypotheses to clarify the purpose of the study. Chapter 3 provides the author's instructions for the Journal: *Hypertension Research*. It also contains the manuscript to be submitted to *Hypertension Research*. Chapter 4 includes the main findings of this study as well as recommendations for future research. References throughout the dissertation were indicated according to the style of *Hypertension Research*.

LIST OF ABBREVIATIONS

ACR	---	Albumin-to-creatinine ratio
ADMA	---	Asymmetric dimethyl arginine
ABPM	---	Ambulatory blood pressure measurement
AngII	---	Angiotensin II
ANCOVA	---	Analysis of covariance
AR	---	Arginase
ASS	---	Argininosuccinate synthase
ASL	---	Argininosuccinate lyase
BH_4	---	Tetrahydrobiopterin
BMI	---	Body mass index
BP	---	Blood pressure
CRP	---	C-reactive protein
CAT	---	Cationic amino acid transporters
CVD	---	Cardiovascular disease
cGMP	---	Cyclic guanosine-3’, 5-monophosphate
cIMT	---	Carotid intima media thickness
DBP	---	Diastolic blood pressure
DDAH	---	Dimethylarginine dimethylaminohydrolase
eCCR	---	Estimated creatinine clearance
EDRF	---	Endothelium derived relaxing factor
eNOS	---	Endothelial nitric oxide synthase
ET	---	Endothelin
ESI-MS/MS	---	Electrospray ionisation tandem mass spectrometry
FMD	---	Flow mediated dilation
FMS	---	Finapres Medical Systems
FRAP	---	Ferric reducing antioxidant power
GC	---	Guanylate cyclase
GFR	---	Glomerular filtration rate

GGT	---	Gamma glutamyl transferase
GTP	---	Guanosine triphosphate
HART	---	Hypertension in Africa Research Team
HDL	---	High density lipoprotein
HIV	---	Human immunodeficiency virus
iNOS	---	Inducible nitric oxide synthase
LDL	---	Low density lipoprotein
LVH	---	Left ventricular hypertrophy
NADP	---	Nicotinamide adenine dinucleotide phosphate
nNOS	---	Neuronal nitric oxide synthase
NO	---	Nitric oxide
NOS	---	Nitric oxide synthase
OAT	---	Organic anion transporters
OCT	---	Organic cation transporters
PRMT	---	Protein arginine methyltransferases
PWV	---	Pulse wave velocity
SABPA	---	Sympathetic activity and Ambulatory Blood Pressure in Africans
SBP	---	Systolic blood pressure
SDMA	---	Symmetric dimethyl arginine
RNS	---	Reactive nitrogen species
ROS	---	Reactive oxygen species

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CHAPTER 1

Introduction

MOTIVATION AND PROBLEM STATEMENT

Cardiovascular disease (CVD) results in morbidity and mortality worldwide (1, 2) and hypertension is the most important cardiovascular risk factor contributing to CVD (3). In 1929, Dennison *et al*. found increased blood pressure in Europeans but not in Africans (4). Greater mental stress in the Europeans was blamed for their higher blood pressure (4). However, 75 years later, changes have taken place in Africa and several studies show that today chronic diseases have become more prevalent in low-income countries, such as sub-Saharan Africa (5). The increased prevalence of CVD seems to be a result of urbanisation and globalisation (5). The rural black community is also developing more chronic disease risk factors compared to urban South Africans (5). A total of 6 million of the 41 million South Africans were hypertensive based on statistics from 1998 (6).

In South Africa, hypertension is more prevalent in Africans than Caucasians and the diagnosis and management of hypertension in Africans is poor (7). Africans are therefore at higher risk for the development of CVD (8).

Disruption of normal endothelial function may lead to the development of CVD (9, 10). The endothelium plays an important role in maintaining vascular tone and structure (9). Nitric oxide (NO) is released from the endothelium to promote vasodilatation (11). NO also regulates thrombosis, platelet function, leukocyte migration (9, 12) and prevents endothelial dysfunction (13). In contrast, in vascular diseases when blood flow is too low, less NO is released, which leads to vasoconstriction (14).

The bio-availability of NO is determined by its rate of biosynthesis and degradation. Regarding synthesis, NO is synthesised from L-arginine through nitric oxide synthase (NOS) (8, 15, 16), and yields L-citrulline as a by-product (17, 18). Eighty five percent of the intestinal L-citrulline is taken up by the kidneys and is used to produce L-arginine (19-22).

This conversion increases when cells are stimulated to produce NO (23). Thus the L-arginine and L-citrulline homeostasis is important for L-arginine production (22).

NO biosynthesis is reduced by increased reactive oxygen species (ROS) (8). ROS, which are produced by the macrophage cells and the mitochondria in the eukaryotic cells (24, 25) lead to endothelial dysfunction and increased asymmetric dimethylarginine (ADMA) (26, 27). ADMA is an inhibitor of NO biosynthesis (12, 28-30) and directly inhibits eNOS (8, 18, 31-33), while symmetric dimethylarginine (SDMA) inhibits or competes with L-arginine for cellular uptake (12, 34). Both ADMA and SDMA therefore reduce NO bio-availability and are associated with endothelial dysfunction, and are recognised as risk markers for vascular disease (28, 29, 35, 36).

Endothelial dysfunction is also a key phenomenon in chronic renal failure and increased ADMA levels were found in patients with chronic renal failure (37). In contrast, although ADMA is partially excreted by the kidneys, no associations between markers of renal function and ADMA were found in a study done by Melikian *et al*. (8) SDMA can also increase in patients with impaired kidney function (38,39), since SDMA can only be eliminated by the kidneys (40).

As mentioned previously, ethnicity is an important risk factor, which contributes to the development of CVD (8). Additionally, it has been demonstrated that African men with higher blood pressure than Caucasians, have lower L-arginine levels (10). It therefore seems as if Caucasians can regulate NO better than Africans, although increasing ADMA levels and therefore NO bio-synthesis inhibition are also associated with cardiovascular disease in Caucasians (9). Furthermore, higher ADMA levels were found in Africans compared to Europeans, with no evidence of increased oxidative stress or inflammation in early stages of vascular dysfunction in the Africans (8).

Age and gender are also important risk factors for the development of CVD (18, 37, 41, 42). Modifiable risk factors such as obesity, smoking and alcohol intake also tend to influence the association of CVD with L-arginine, ADMA and SDMA in Africans negatively (10).

To summarise: until now data regarding chronic diseases and associated risk factors in black populations of South Africa is rare, especially regarding knowledge on the role of NO and its bio-availability in the development of hypertension (5, 7). NO bio-availability markers, such as L-arginine, L-citrulline, ADMA and SDMA play an important role in cardiovascular disease and the development of end organ damage. It is therefore necessary to obtain a better understanding of the underlying mechanisms and functioning of NO markers to increase our understanding of the development of CVD, especially in black South Africans.

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CHAPTER 2

Literature study

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1. Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality (1). Endothelial dysfunction is one of the main underlying mechanisms of CVD development (1). Various risk factors such as ethnicity, gender, increasing age, westernised lifestyle and metabolic factors increase the risk for the development of CVD (2). In addition, hypertension is one of the most prevalent and most poorly controlled risk factors in patients with CVD (3). Endothelial dysfunction leads to hypertension and stroke (4-7) and is associated with end organ damage and renal failure (8). Since endothelial dysfunction is more prevalent in Africans than in Caucasians (4-7), Africans are at higher risk for the development of CVD (9).

In CVD when blood flow is diminished, reduced nitric oxide (NO) bio-availability results in endothelial dysfunction (9,10) and reduces the capacity for blood vessels to dilate appropriately (11). This favours vasoconstriction and results in increased blood pressure (11).

The endothelium plays an important role in maintaining vascular tone and structure (12). There are several vasodilators important in endothelial function (13). However, the main focus of this dissertation is on the precursor of NO, L-arginine and the associated key urea/NO cycle intermediate L-citrulline. NO is a vasodilator and is found in the cerebral, pulmonary, renal and coronary vasculature (10). Endothelium derived relaxing factor (EDRF) was identified as NO and mediates relaxing actions of acetylcholine (14).

2. *The L-citrulline/nitric oxide cycle and the urea cycle*

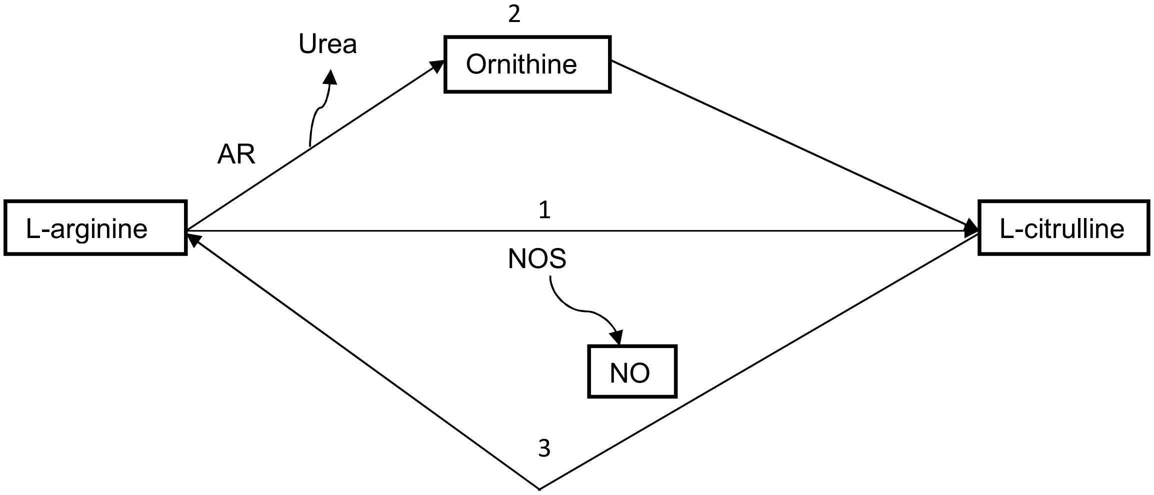

Figure 1: L-arginine and its role in nitric oxide production and the urea cycle. [Adapted from Mori *et al*. 1998 (15)] NO, nitric oxide; NOS, nitric oxide synthase; AR, arginase.1=Synthesis of NO from L-arginine. 2=The Urea cycle. 3=Formation of L-arginine.

As indicated in Figure 1, NO is synthesised in the endothelial cells (16) from L-arginine (9,17), mostly in the kidneys (18-21) in a reaction that requires oxygen (9,14,17), reduced nicotinamideadenine dinucleotide phosphate (NADP), and essential cofactors, including tetrahydrobiopterin (BH_4) (9,22) by the enzyme endothelial nitric oxide synthase (eNOS) (16,23,24), yielding L-citrulline as a by-product (20,23). Eighty five percent of the intestinal citrulline, (which is also an amino acid product of glutamine metabolism) produced in the intestines and liver (1), is taken up by the kidneys, which express argininosuccinate lyase (ASL) activity for L-arginine production (1,18,19,25,26). L-arginine production increases when cells are stimulated to produce NO (27). Thus the L-arginine/L-citrulline homeostasis is important for L-arginine production (1).

There are two pathways by which L-citrulline can be formed, with each process involving a cycle (15). Firstly, L-arginine can be converted to NO and L-citrulline through nitric oxide synthase (NOS) (15). NO synthesis in the endothelial cells regulates blood vessel dilatation and is an important marker in endothelial function (12). Enzymes such as argininosuccinate synthase (ASS) and ASL, synthesize L-arginine from L-citrulline in the kidneys, as seen in Figure 1 number 3 (28). Secondly, the urea cycle begins where L-arginine produces ornithine and urea through arginase (AR) in the liver (29). Ornithine is converted to L-citrulline, which in turn is transformed into L-arginine again (29). Although the main purpose of the urea cycle is still to eliminate excess nitrogen from the system (30), it is also a pathway for L-arginine recycling (15).

2.1. The L-arginine – L-Citrulline cycle

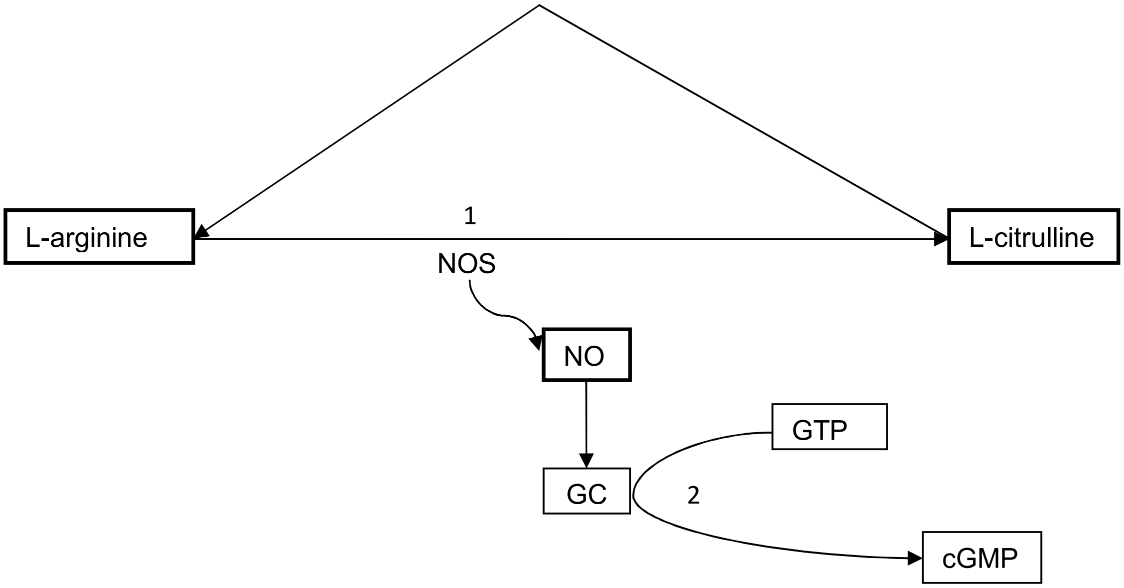

Figure 2: L-arginine – L-citrulline cycle.

[Adapted from Mori *et al*. 1998 (15) and Valance *et al. 2001* (31).] NO, nitric oxide; NOS, nitric oxide synthase; GC, guanylate cyclase; GTP, guanosine triphosphate; cGMP, cyclic guanosine-3’, 5-monophosphate. 1=Synthesis of NO. 2=Actions for vasodilatation.

When blood flow increases, NO is released through eNOS to promote vasodilatation (24) (Figure 2, nr.2). NO activates cytosolic guanylate cyclase, which increases cyclic guanosine monophosphate (cGMP) in the vascular smooth muscle cell (24). There are three forms of NOS: the endothelial isoform (eNOS) and neuronal isoform (nNOS) are present in healthy cells and the inducible isoform (iNOS), which is only identified in conditions of infection and inflammation (10). eNOS is a candidate of endothelial dysfunction because it undergoes functional regulation through Ca/calmodulin regulation and tyrosine phosphorylation that have been linked to cardiovascular phenotypes (32). NOS uncoupling through inhibitory factors, which will be discussed later, also plays a role in the production of reactive oxygen species (ROS), which results in endothelial dysfunction (12,22).

cGMP mediates the effects of NO, including the control of vascular tone (10,33), platelet function, leukocyte migration, low density lipoprotein (LDL) oxidation and cellular adhesion to the endothelium (10,12). Other functions of NO include the regulation of homeostasis and thrombosis, and the prevention of various vascular pathologies, especially, atherosclerosis (34).

In addition, shear stress can also increase NO through receptors that are stimulated on the endothelial surface, which activates NO synthase to release NO (27). Shear stress decreases NADPH oxidation, which results in lower ROS and decreased NO deactivation, thus increased NO and endothelial function (35).

After a local increase in shear stress, which results in vasodilatation of the brachial artery, (36) flow mediated dilation could be determined by ultrasound analysis of brachial artery diameter, induced by a 5 min forearm ischemia (37). Flow mediated dilation is a non-invasive tool known to represent endothelial health (34,37), while decreased flow mediated dilation predicts future development of CVD, which again highlights the importance of NO bio-availability in CVD development (38,39).

2.1.1. L-arginine supplementation

L-arginine is a conditionally essential amino acid and is the primary source for NO biosynthesis (12,40,41). The majority of studies reporting on L-arginine focused on the effects of L-arginine supplementation. Various studies investigated the use of L-arginine as treatment for high BP, since L-arginine increases NO bio-availability (14,42-44). Maintaining basal blood pressure, an important role of NO, indicates that the NO pathway may be abnormal in hypertensive subjects (14). L-arginine supplementation can increase NO in hypertensive patients through decreased superoxide production (44). In addition it can also “recouple” the electron transport in uncoupled NOS to increase NO (43).

On the other hand, the response to treatment with L-arginine depends on the severity and duration of hypertension (45). In moderate or mild hypertension, L-arginine decreases blood pressure and renovascular resistance (46). It also lowers vasoconstrictors, such as angiotensin (Ang II) and endothelin-1 (ET-1), causing hypotension (46,47). However, in adults with malignant hypertension, L-arginine has no hypotensive effect (45). It was found that lower pressure with L-arginine infusion is also more prevalent in salt-sensitive humans, since they are more hypertensive (42).

Thus L-arginine reverses hypertension by restoring endothelium-dependant vasodilatation and decreasing peripheral vascular resistance (14,48). In a study by Böger *et al.* it was demonstrated that 1.5 g L-arginine twice a day improves vasodilatation in patients with elevated asymmetric dimethylarginine (ADMA) levels, with ADMA as a NOS inhibitor. However, this phenomenon is not present in patients with low ADMA levels (12). In disease states with endothelial dysfunction, L-arginine is found to be normal (12). Only a few patients show low L-arginine levels (12). The explanation for the low or “normal” L-arginine could be because of the high ADMA levels present in patients with endothelial dysfunction (12).

L-arginine supplementation can also enhance inhibition of platelet aggregation, inhibition of monocyte adhesion, and reduced vascular smooth muscle proliferation (12,49-51). In addition, L-arginine can restore renovascular homeostasis (33,44). Thus, L-arginine also seems to have an important role in modulating renovascular NO production (52), and it has been shown that L-arginine enhances kidney function (53). In contrast, there are several studies that have reported no benefit with L-arginine supplementation (54-56).

2.1.2. L-citrulline supplementation

L-citrulline supplementation on the other hand could be used as a substitute for L-arginine in conditions such as hypertension, heart failure and diabetes, where L-arginine has been reported to have beneficial effects (1,57). If L-citrulline is given orally it bypasses the hepatic metabolism and is therefore more effective than L-arginine (1). Therefore, use of L-citrulline can treat CVD by increasing NO and improving vascular dysfunction (1).

2.1.3. Pathophysiology

Endothelial dysfunction leads to the disruption of vasoactive substances, which in turn results in changes of the vascular structure and function (58) and plays an important role in regulating endothelial function (24,59). NO is an important marker for resting peripheral vascular resistance and blood pressure (14). Endothelium dependant vasodilatation can predict cardiovascular events and it is therefore a risk for hypertension and CVD (60).

2.1.3.1. Hypertension

Hypertension is one of the main consequences of endothelial dysfunction (12). Hypertension, which is characterised as blood pressure ≥140 mmHg or/and ≥90 mmH(61), is the most important cardiovascular risk factor worldwide, (22) also in black South Africans (62-64). Antihypertensive therapy decreases the mortality rate, although most hypertensive patients still do not achieve optimal blood pressure (33).

In patients with hypertension, acetylcholine induced vasodilatation is impaired (10,33,60). Deficiencies of L-arginine occur (65), which decreases the availability of NO and diminishes endothelial function which can lead to the development of hypertension and CVD (10,44,66). Superoxide generation increases in hypertension and impairs endothelium-dependant vasodilatation, as seen in hypertensive Caucasians (6).

Despite the beneficial effects of L-arginine, Chirinos *et al.* found a positive correlation between L-arginine and systolic blood pressure (20). This could be due to abnormalities in L-arginine transport via system y+, which may limit L-arginine availability (67). L-arginine can also impair vascular function (20). It is possible that increased L-arginine also results in increased arginine metabolites, such as ornithine, which may have unfavourable vascular effects (20). On the other hand, in one study done on middle-aged (<55 years) Finnish men, 6g/day L-arginine intake did not correlate with blood pressure or cardiovascular risk (68).

2.1.3.2. Atherosclerosis and arterial stiffness

Atherosclerosis is a condition where lipid deposits on the arterial surface progress to form plaques (69). These plaques block the artery, thus limiting blood flow (69,70). The classification of plaques differs (69). The early lesions are known as fatty streaks and the raised lesions are known as thrombosis and calcification (69). Atherosclerosis is mainly an intimal disease, whose major effect is flow limitation (70). The media may only be secondarily weakened (70).

Atherosclerosis is therefore a well-known risk for CVD (35). Impaired endothelial dependant vasodilatation also occurs in subjects with atherosclerosis (10,60), but only impairment of the L-arginine: NO pathway was seen (10).

Kals *et al.* found an association between coronary atherosclerosis and arterial stiffness (70). Arterial stiffness describes the distensibility of the arterial wall (71). According to Kinlay *et al.* endogenous NO regulates local arterial elasticity in the human brachial artery, iliac artery as well as both aortic and systemic arterial stiffness (72-74). Increased arterial stiffness is seen in the whole arterial tree in hypertensive patients (70). According to van Popele *et al.* increased aortic PWV is also seen in patients with peripheral arterial disease (75). Therefore arterial stiffness is an important risk marker for CVD and is also involved in atherogenesis, but it is uncertain if arterial stiffness is a predictor of atherosclerosis (70,75).

2.1.3.3. Renal dysfunction

Several studies indicated impaired L-arginine metabolism in end-stage renal disease patients (64,76,77). However, in a study done by Kilhlberg *et al.* in the 1980's L-arginine and ornithine did not change in rats with renal failure (78). Another study found increased NO in end-stage renal disease patients (79).

L-citrulline, which forms part of the L-arginine cycle, is also associated with renal function in several studies (79-83). Eighty precent of L-citrulline is eliminated by the kidneys, (82) which explains why circulating L-citrulline is increased in subjects with chronic renal failure (78,79,81,84). There are two mechanisms, which may contribute to this elevation of L-citrulline in renal failure: up regulation of reabsorptive transporters and down regulation of secretary transporters (80). Organic anion transporters (OATs) and organic cation transporters (OCTs) play an important role in the uptake of L-citrulline (85). However, when renal dysfunction occurs, these transporters are reduced (85,86). Thus, down regulation of OAT's and OCT's increases L-citrulline in individuals with renal disease (80).

Another explanation for increased L-citrulline could be a peripheral adaptation, enabling a decreased mass of functional tissue in the kidneys for maintenance of arginine synthesis (82). The rate of L-citrulline appearance and disappearance is 4 to 5 times higher in end stage renal disease subjects than in healthy subjects (87).

2.1.3.4. Left ventricular hypertrophy

Left ventricular hypertrophy (LVH) is a measurement of end organ damage in the heart (88,89). LVH is a condition where enlargement of the cardiac muscle, thus increased myocardial thickness, takes place (90) to compensate for continuous stress placed on the heart (91). Increased myocardial stress due to pressure overload causes LVH (90) and it therefore reflects that hypertension is a major cause of LVH (90), thus also a predictor of cardiovascular events (92).

There are several methods to determine LVH (90). It can be evaluated by echocardiography as the most ideal method. Electrocardiography can also be used (90). Electrocardiography can be evaluated by using various methods of the standard voltage criterion e.g. by Sokolow and Lyon (93) or the Cornell product (CP) (94). Both of these measures are associated with stroke and cardiovascular events (95). The Cornell product is known to have higher sensitivity for the detection of LVH than the Sokolow and Lyon method (96).

2.2. *The urea cycle*

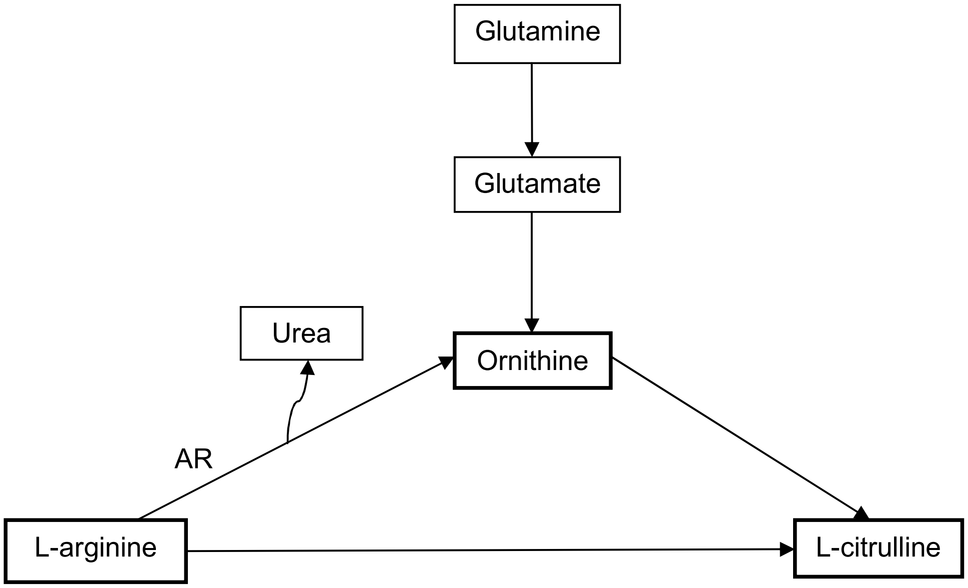

Figure 3: The urea cycle.

[Adapted from Mori *et al*. 1889 (15)] AR, arginase.

As previously mentioned, L-arginine is involved in two pathways (15). The first pathway was explained earlier and results in NO production (15). As shown in Figure 3, L-arginine is also involved in what is known as the urea cycle (23). A link therefore exists between the NO cycle and the urea cycle. The formation of L-citrulline begins with the transformation of glutamine to glutamate, thereafter ornithine forms, which is converted to L-citrulline (1). Most of the L-arginine derived from dietary protein is used for the other pathway of L-arginine metabolism, which passes through the gastrointestinal tract and hepatic portal, where it is converted to ornithine and urea by AR (19,29,97). Urea is water-soluble and can easily be excreted from the body (30). This forms the urea cycle, which is the only pathway capable of removing excess nitrogen (30).

AR, the enzyme responsible for converting L-arginine into L-ornithine and urea, plays an important role in modulating L-arginine bio-availability (98). The inhibition of AR can increase NOS activity, resulting in more NO production (99). However, increased AR results in decreased L-arginine levels, thus attenuates NO production (100). Decreased L-arginine results in decreased NO dependant vasodilatation, which is evident in African Americans with hypertension (101). Hypertension in African Americans is also associated with salt sensitivity (101). In salt-sensitive rats with hypertension, increased AR was evident. This was also accompanied by a decrease in vascular function (102).

2.3. *Endogenous inhibitors of L-arginine*

2.3.1. Oxidative stress

NO bio-availability is principally determined by a reduction in its biosynthesis by ROS (9). NO biosynthesis is reduced by increased ROS (9). ROS, which are produced by various sources, including the macrophage cells (103) and the mitochondria in the eukaryotic cells (104,105), lead to endothelial dysfunction (35,106). ROS prevent ADMA clearance through the inhibition of dimethyarginine dimethylaminohydrolase (DDAH) (107). Alternatively, ADMA may act as an eNOS inhibitor, by inhibition of L-arginine oxidation (9,12,31,107), leading not only to the loss of NO in patients with coronary artery disease, but also an increase in superoxide anion production in the vascular endothelium (31,44,106,107).

ROS normally exist in all aerobic cells in balance with tightly controlled antioxidant defences and repair mechanisms (108). These include antioxidant enzymes, such as superoxide dismutase and catalase and antioxidant scavengers, such as glutathione, vitamins C and E (24,108). If antioxidant enzymes and ROS scavengers cannot cope with the continuous ROS production, a steady state of oxidative stress, which is always present in cells, can increase (increased oxidative stress status) (108) and reduces the biological effects of NO (10,14,109). ROS cause the formation of oxidized low-density lipoprotein and activate redox-sensitive pro-inflammatory signalling pathways (106).

This produces a vicious cycle where superoxide anion production further decreases NO bio-availability by binding to NO to form peroxynitrite, a reactive nitrogen species (RNS) (22). Increased levels of all these different ROS and RNS further increase oxidative stress and nitrosative stress, which in turn inhibit endothelium dependent vasodilatation (12,110,111), resulting in increased peripheral vascular resistance (20,33).

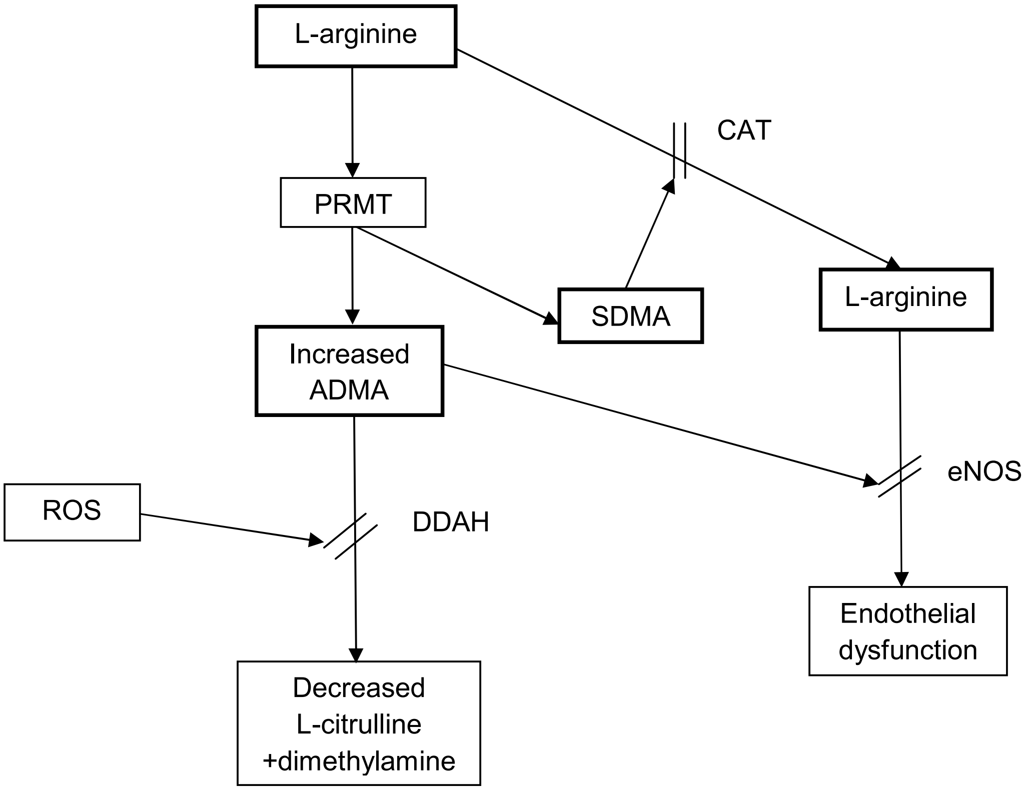

Figure 4: Endogenous inhibitors of L-arginine (asymmetric (ADMA) and symmetric dimethylarginine (SDMA)).

[Adapted from Böger *et al. 2003* (112) and Teerlink *et al. 2009* (113).] PRMT, protein arginine methyltransferases; ROS, reactive oxygen species; DDAH, dimethylarginine dimethylaminohydrolase; CAT, cationic amino acid transporters; eNOS, endothelial nitric oxide synthase.

2.3.2. Dimethylarginines

As indicated in Figure 4, ADMA and SDMA are inhibitors of the NO biosynthesis pathway (107). They are synthesised by redox-sensitive methylating enzymes such as S-adenosylmethionine-dependant protein arginine methyltransferases (PRMT) (12,107,114). The enzyme methylates protein which is broken down during normal protein turnover to release ADMA and SDMA (12,107,114).

There are two pathways for ADMA clearance from the plasma (114). In the first pathway ADMA is metabolised to form L-citrulline and dimethylamine through DDAH (110,114) of which there are two isoforms (31,115) found in all human tissues and biological fluids (116). In the second pathway, a minor portion of ADMA is excreted through the kidneys (114,117). ADMA, which escaped from cells, is exported through cationic amino acid transporters (CAT) from the cell to the plasma (31,114,115).

On the other hand, SDMA interferes with L-arginine uptake. It does not directly inhibit eNOS (31,118). SDMA was found in the human brain tissue in 1971 by Nakajima *et al.* (119), and is produced at a constant rate (120). Raised SDMA indicates higher rates of protein turnover or increased PRMT2, which generates SDMA (31). SDMA inhibits y+ transporters that mediate the intracellular uptake of L-arginine (118) and inhibits renal tubular L-arginine absorption (121). Therefore SDMA is known to interfere with NO synthesis indirectly (122) and additionally it stimulates the production of ROS (123). Since SDMA does not directly inhibit NOS, limited research focused on this isomere and its role in CVD (124-129).

Increased levels of inhibitors of the NO biosynthesis pathway (such as ADMA and SDMA) can reduce NO synthesis and are associated with endothelial dysfunction (10,20,59,107,130-133). These inhibitors are therefore risk markers for vascular disease (130,131,133,134).

2.3.2.1. Asymmetric dimethylarginine (ADMA)

ADMA acts as an eNOS inhibitor, by inhibition of L-arginine oxidation (9,107,135,136). However, only the free ADMA intracellular, formed during proteolysis inhibits NOS. (114). ADMA therefore has an effect on eNOS function in a variety of CVDs (9,20,59,107) even in the presence of normal circulating levels of L-arginine (59). ADMA levels are higher in patients with CVD, signifying that ADMA induces CVD, contributes to higher ADMA levels (117). ADMA increases in disease states, such as hypertension (9,20,135,137), chronic renal failure and atherosclerosis (10,135) and could be a predictor of future development of CVD (9,20,117).

2.3.2.1.1. ADMA and endothelial dysfunction

Increased oxidative stress leads to higher ADMA levels which in turn enhances atherogenesis. (31,59). Thus oxidative stress is a key factor in the pathogenesis of atherosclerosis (31,59). ADMA is associated with coronary artery calcification, a marker for atherosclerosis (117,131). ADMA may be directly involved in the regulation of the vascular redox state in atherosclerosis by affecting superoxide generation and NO bio-availability (9,107). Increased ADMA levels are seen in hypercholesterolemic subjects. In hypercholesterolemia, vascular NO is reduced, leading to impaired endothelium-dependent vasodilatation (77), increased platelet aggregability, (138) and monocyte adhesiveness of the endothelium (139).

Carotid intima media thickness is a marker of the thickness of the arterial wall (71). Intima media thickness increases with increasing ADMA levels (135). However, another study found this only in subjects older than 40 years of age (127). In contrast, another study found raised ADMA levels and decreasing intima media thickness in middle-aged individuals (140).

2.3.2.1.2. ADMA and hypertension

Increased ADMA levels inhibit endothelium dependant vasodilatation (111,135), which leads to vasoconstriction, increased peripheral vascular resistance (20,33), increased systemic blood pressure (33) and increased arterial stiffness (141,142). However, mean arterial pressure increases only slightly with elevated ADMA levels (20). In healthy individuals, ADMA levels associate positively with vascular resistance and mean arterial pressure, and negatively with cardiac output and plasma cGMP concentration, which are important factors for vasodilatation (33).

Oxidative stress and ADMA elevation were indicated in hypertensives, who had reduced NO (135,137). In salt-sensitive animals, as well as humans with hypertension, blood pressure increased with higher ADMA levels (33). In a hypertensive condition, ADMA reduces the heart rate and increases systemic vascular resistance in association with a fall in cardiac output, which results in a rise in blood pressure (20,59). However, it is unknown if the change in cardiac output was secondary to the change in blood pressure or whether it represents a direct effect of NOS inhibition on cardiac function (143).

2.3.2.1.3. ADMA and renal dysfunction

Kidney function is a major risk factor for mortality (117). According to Böger *et al.* patients with chronic kidney disease have the highest risk for the development of CVD (144).

Decreased glomerular filtration rate (GFR), a marker of renal function, indicates impaired kidney function and correlates with the risk for CVD and even death, according to Go *et al.* (145). Thus it is important to detect these GFR changes for early detection of acute kidney injury (120). Creatinine clearance is often used to determine GFR, because creatinine is eliminated through the glomerulus (144).

Endothelial dysfunction is a key phenomenon in chronic renal failure (117). The explanation for this may be due to the increased ADMA levels in patients with chronic renal failure (117). ADMA is mainly eliminated from the body by enzymatic degradation through DDAH (Figure 4), which is present in the kidneys (141). During decreased renal excretory function, DDAH activity also decreases, which results in increased ADMA levels (141). That could be the reason why ADMA is correlated with renal function markers in some studies (141). Thus L-arginine and NO regulation may be affected by severe chronic kidney disease (7,146). Chirinos *et al.* stated that ADMA levels correlate with reduced ejection fraction (20) in patients with renal failure and are elevated in chronic renal failure, which leads to the assumption that ADMA may be responsible for increased cardiovascular risk and hypertension (59,117) and predicts mortality in patients with chronic kidney disease (117).

ADMA correlates with cystatin-C, which is a measurement of kidney function and a better predictor of GFR than creatinine (117). However, there is also a strong relation between serum creatinine and the risk for cardiovascular diseases (117).

Although ADMA is also partially excreted by the kidneys, several studies show no associations between eGFR, creatinine clearance and ADMA (9,141). Nevertheless, ADMA also accumulates in many other diseases in which renal function is normal, and it is ADMA that rises rather than SDMA (147).

2.3.2.2. Symmetric dimethylarginine (SDMA)

SDMA also plays an important role in endothelial dysfunction (144). SDMA impairs L-arginine uptake from the loop of Henle (121), thus SDMA is involved in reducing NOS and in limiting the availability of L-arginine to NOS (121,144). SDMA also reduces NO synthesis and increases ROS formation (144). ROS formation could be contributed by reduced L-arginine, which uncouples NOS (116,144). This leads to increased oxidative stress and causes endothelial dysfunction and hypertension (116,144). The role of SDMA in hypertension was confirmed by Pullamsetti *et al*. where he found increased SDMA in patients with hypertension (148).

2.3.2.2.1. SDMA and renal dysfunction

SDMA can only be eliminated by the kidneys (149) and can therefore increase in patients with impaired kidney function (144,150). Several studies show correlations between SDMA and renal markers, such as serum creatinine, GFR and creatinine clearance (120,141,151,152). However, it is uncertain how fast SDMA increases after GFR decreases (120,141,152). It was first found in children with hypertension (152). Kielstein *et al.* found that if GFR decreases by 50%, SDMA increases significantly and creatinine increases within 6 hours after the removal of one kidney (120). Although there is a strong correlation between SDMA and GFR, it is not known if SDMA fulfils all criteria for an ideal GFR marker, i.e. stable production rate not affected by other diseases, free glomerular filtration and lack of tubular re-absorption (153).

SDMA is an important risk marker for early detection of impaired kidney function (120), but also correlates with total organ failure in patients in the intensive care unit (154).

Contradictory to the above, several studies did not find an association between SDMA and renal function (28,146). Yu *et al.* state that other cardiovascular risk factors and renal dysfunction can influence SDMA levels, because they found that animals that had a high-fat and high-cholesterol diet, had increased SDMA (28). In a study done by Zoccali *et al*. SDMA did not predict cardiovascular diseases in end-stage renal disease patients (146).

The study done by Kiechl *et al*. provided the first evidence that ADMA was not better than SDMA in predicting CVD risk in the general population (116). They found that renal function markers (creatinine and cystatin-C) have a much stronger relationship with SDMA than ADMA (116). SDMA is either a more sensitive marker of renal dysfunction (153) or SDMA itself is biologically active, i.e. it has been suggested that high concentrations of SDMA might compete with cellular L-arginine uptake (118,155).

3. Traditional risk factors for cardiovascular disease in the context of NO bio-availability

Modifiable risk factors, such as alcohol and smoking, can contribute to CVD, such as hypertension and atherosclerosis (9,60,70). Non-modifiable risk factors, such as increased age (10,70), genetic factors and ethnicity (8) also result in high blood pressure (6). The effects of these risk factors on CVD may be via modulation of the NO cycle, among various other mechanisms.

3.1. Age

Age-related endothelial dysfunction explains the increased cardiovascular risk in the elderly (10,70). Aging is a series of morphological and functional changes, which take place over time (156). In addition to disease states, endothelium dependant vasodilatation is also impaired in old age (60,157), and ADMA levels are also increased in the elderly (20).

3.2. *Ethnicity*

Hypertension is the most common cardiovascular risk factor in black South Africans (158). In sub-Saharan Africa, infectious diseases and malnutrition have been the main causes of morbidity and mortality until now. (158) About 80 years ago, in the south of Kavirondo in Kenya, Donnison *et al.* admitted 1800 patients, in whom there was no elevated blood pressure present and no diagnosis of arteriosclerosis or chronic nephritis was made (158).

Donnison *et al.* found increased blood pressure in Europeans up to end of age 40 years, however not in Africans, and blamed greater mental stress for their higher blood pressure (158).

However, 75 years after Donnison *et al.* changes have taken place in Africa (159). Several studies show that chronic diseases have become more prevalent in low-income countries, such as sub-Saharan Africa (159) and are of greater importance with increasing age and are increasing worldwide as a result of urbanisation and globalisation (160). The rural black community has already been developing chronic disease risk factors compared to urban South Africans (160). Chronic diseases in the urban black population of South Africa include stroke, hypertension and type two diabetes (4). Steyn *et al.* stated that the duration of urbanisation is an independent predictor of hypertension in the Africans of Cape Town (161).

Poor blood pressure control is seen through high systolic blood pressure and diastolic blood pressure in rural South Africans (160). However, this is also present in the high-income countries (4). Barriers, such as lack of knowledge and health insurance, unemployment, alcohol abuse and cost of care and medication to hypertension control exist and it is important to address these barriers in preventing cardiovascular risk (158,162,163). In sub-Saharan Africa the management of hypertension is a socio-economic problem as well as a therapeutic problem (163).

Until now data regarding chronic diseases and associated risk factors in rural and urban black populations in South Africa are rare (4,160). Lifestyle changes, such as dietary changes, increased obesity, decrease physical activity, high levels of stress and increased alcohol and tobacco use increase the risk for chronic diseases (160,164-166). More African women are obese compared to African women in the USA and Canada (160,167). Central obesity, which is more prevalent in women, is associated with hypertension, diabetes, CVD and stroke (168,169). Higher cholesterol levels are also more prevalent in the rural black communities nowadays (160).

As mentioned, there are several studies indicating that CVD is a problem in South-Africa and it is necessary to address these risk factors to prevent future development of CVD.

3.2.1. *Ethnicity and hypertension*

Hypertension and stroke (4-7) associated with end organ damage and renal failure (8) are more prevalent in Africans than in Caucasians (4,5,7). An explanation for this phenomenon in Africans is because of salt sensitivity and abnormal hemodynamic reactivity which is characterised by increased peripheral resistance in response to stress (9). Another explanation could be the high alcohol and smoking intake in Africans compared to Caucasians (6).

3.2.2. *Ethnicity and dimethylarginines*

The roles of ADMA and SDMA can be different in Africans and Caucasians (5,7). Increasing ADMA levels are associated with CVD in Caucasians (5,137,148). According to Sydow *et al*. ADMA levels were lower in African Americans and non-Hispanics than in whites (117). Caucasians tend to have a stronger relation between L-arginine and ADMA than Africans, stating that the Caucasians regulate NO better than Africans (7).

In contrast, two other studies found higher ADMA levels in Africans compared to Europeans and there was no evidence of increased oxidative stress or inflammation in the early stage of vascular dysfunction in the Africans (9), thus contributing to higher risk for CVD in Africans (5,9). Glyn *et al.* also found lower L-arginine in African men with higher blood pressure (7).

A correlation between pulse wave velocity (PWV), a marker of arterial stiffness, and ADMA was found in Africans, thus ADMA also has a potential role in regulating arterial stiffness (5). Africans tend to have higher PWV compared to Caucasians (5). Since endothelial dysfunction is also more prevalent in Africans than in Caucasians, it may be a key step in the initiation of arterial stiffness and atherosclerosis (33,52,170,171). Even young, healthy, normotensive Africans show endothelial dysfunction (9).

There is limited literature available on ethnicity and the relationship with L-arginine and L-citrulline.

3.3. Gender

Gender is an important risk factor relating to cardiovascular function, probably due to the influence of sex hormones. Both low and high levels of testosterone are associated with cardiovascular risk (172). Guarner-Lans *et al.* stated that hypertensive men have lower serum testosterone levels than normotensive men of the same age (2). In a study done including three ethnic groups (African Americans, Hispanics and Mexican Americans), men had higher blood pressure than women, independent of ethnicity (173). According to Guarner-Lans *et al.* normotensive men also have higher blood pressure than women (2). Peripheral arterial disease prevalence also increased with age and is normally higher in men than in women (174).

In hypertensive men, increased oxidative stress and BP are found (174). Hypertension also increases more in aging women than in aging men (172). However, Palmer *et al.* stated that African men seem to develop hypertension at an earlier stage compared to women (175).

The influence of the metabolic syndrome on increased atherosclerosis is also different between men and women (176). It seems that in women, the metabolic syndrome is more related to atherosclerosis (176). Arterial stiffness is more common in women and can be explained by the typically smaller body size of women. Boys, however, have a higher average waist circumference when they have elevated blood pressure (177).

3.4. Alcohol and smoking

Alcohol and smoking tend to influence the association between L-arginine, ADMA and SDMA in Africans (7).

In smokers, endothelial dysfunction is seen (6,70), since the endothelium is the main target of the toxic compounds of cigarettes (178). Even small amounts of nicotine can cause endothelial dysfunction (179). Smoking can accelerate aging through the overproduction of ROS, which leads to impaired endothelium dependant vasodilatation (60).

Smokers, especially Caucasians, have high LDL-cholesterol and triglycerides and low HDL-cholesterol and smoking is therefore a great risk factor for the metabolic syndrome (180). Africans tend to smoke cheaper tobacco that contains more nicotine, which may explain why vascular dysfunction and arterial stiffness are more prevalent in Africans (181). Furthermore, more men with a low income tend to smoke (181).

Smoking seems to be the strongest CVD risk factor associated with peripheral arterial disease, according to Criqui *et al.* (174). Nicotine elevates heart rate and cardiac output, which increases blood pressure (181,182). Nicotine also accelerates lipid (99) breakdown that leads to weight loss in smokers (183). Smoking can increase inflammation and coagulation, which are associated with atherosclerosis and CVD (184).

Alcohol abuse, on the other hand, is a modifiable risk factor that can be limited to help prevent cardiovascular diseases. Alcohol consumption is responsible for a great portion of cardiovascular morbidity and mortality (185). The relationship between alcohol and blood pressure is a J- or U- shaped dose-response (186). The risk for the development of high blood pressure is lower when alcohol consumption is moderate and is high when alcohol consumption is high (186).

Moderate consumption is beneficial, since ethanol increases eNOS activity, decreases fibrinolytic activity, increase prothrombotic activity, has an anti-inflammatory role by enhancing the uptake of interleukin 6, fibrinogen, and an increase in adhesion molecules, followed by the elevation of proinflammatory cytokines (185). Chronic consumption of alcohol lowers von Willebrand Factor levels, independently of the type of alcoholic beverage (185). Alcohol abuse reduces glutathione production, and increases H_2O_2 levels, which in turn reacts with transition metals in the mitochondria of the cells and leads to increased oxidative stress (187). Glutathione in the mitochondria is the only protection available to metabolise H_2O_2 (187). Therefore, excessive alcohol intake depletes cells of glutathione in the mitochondria because of a damaged process of the carrier responsible for transport of glutathione from the cytosol into the mitochondrial matrix (187).

One study showed that if alcohol intake is more than 200 g/week it led to larger waist circumference, higher blood pressure and lower HDL-cholesterol (180). Heavy drinkers show increases in weight and increased conversion of acetyl Coenzyme-A to triglycerides, which is then secreted in the blood as very low-density lipoproteins (180). The excess triglycerides cause fat accumulation in the liver and raise serum gamma-glutamyl transferase (GGT) independent of sex and ethnicity (188,189). Elevated GGT is a biomarker that predicts future coronary heart disease and stroke (188,189).

4. Summary

CVD is the leading cause of morbidity and mortality, especially in South-Africa (1,4,160). Endothelial dysfunction is one of the main underlying mechanisms of CVD development (1). Various risk factors, such as ethnicity, gender, increasing age, westernised lifestyle and metabolic factors increase the risk for the development of CVD (2). The effects of these risk factors on CVD may be via modulation of the NO cycle, among various other mechanisms. In addition, hypertension is one of the most prevalent and most poorly controlled risk factors in patients with CVD (3). Endothelial dysfunction leads to hypertension and stroke (4-7) and is associated with end organ damage and renal failure. (8). Since endothelial dysfunction is more prevalent in Africans than in Caucasians (4-7), Africans are at higher risk for the development of CVD (9). Until now there is little data regarding chronic diseases and associated risk factors in black populations in South Africa (160), especially regarding possible contributing vascular mechanisms, such as the NO cycle.

5. AIMS, OBJECTIVES AND HYPOTHESES

The general aim of this study is to compare markers of NO bio-availability (namely L-arginine, L-citrulline, ADMA and SDMA), ambulatory BP and markers of end organ damage between African and Caucasian school teachers. The secondary aim is to determine whether these markers of NO bio-availability are associated with ambulatory BP and markers of end organ damage.

Objectives

- To compare L-arginine, L-citrulline, ADMA and SDMA levels along with ambulatory BP and markers of end organ damage, namely carotid intima media thickness, Cornell product and renal function (albumin-to-creatinine ratio and estimated creatinine clearance) between Africans and Caucasians;
- to determine if L-arginine, L-citrulline, ADMA and SDMA are associated with ambulatory BP and markers of end organ damage; and
- to establish whether these associations are ethnic- or gender specific.

Hypotheses

- L-arginine, L-citrulline, ADMA and SDMA levels differ between Africans and Caucasians, with Africans having lower L-arginine and L-citrulline and higher ADMA and SDMA levels than Caucasians;
- Africans have higher ambulatory BP and end organ damage compared to the Caucasians;
- L-arginine levels associate negatively with BP, while L-citrulline associates negatively with renal function;
- ADMA levels associate positively with BP and end organ damage, with stronger associations in the African population, while SDMA correlates with renal function.

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CHAPTER 3

Comparing markers of the nitric oxide cycle and their association with ambulatory blood pressure and end organ damage in a bi-ethnic population: The SABPA-study

(Prepared for submission to

Hypertension Research)

INSTRUCTIONS TO AUTHORS: *Hypertension Research*

Manuscript style

The article should be no more than 5 000 words, double-spaced with wide margins and should include an abstract of not more than 250 words and 3 to 5 keywords. A title page should give the title, the first and last names and other initials of all authors. A short running head consisting of not more than 50 characters should be included. There should be fewer than 10 co-authors. Full contact details should be provided for the corresponding author.

Main text

The manuscript should start on a new page and should include an introduction, methods, results and discussion. Abbreviations should be defined on their first appearance in the text. The discussion should not recapitulate the results.

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References should be listed in numerical order at the end of the article. References should include the names of all authors when 6 or less; when 7 or more, list only the first 3 names and add *et al.* Each reference should be numbered and listed at the end of the manuscript. Example: 1) Glodny B, Pauli G. Medullopressin: a new pressor activity from the renal medulla. *Hypertens Res* 2005; **28**: 827-836.

Tables and figures

Table footnote references should be made by means of Arabic numerals and they should consist of at least 2 columns. Figures should be sequentially labelled.

Acknowledgments

These should be brief and should include sources of financial support, material and personal assistance.

Comparing markers of the nitric oxide cycle and their associations with ambulatory blood pressure and end organ damage in a bi-ethnic population: The SABPA-study

Running head: Nitric oxide bio-availability markers and endothelial dysfunction

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Disclosure: All authors declare no conflict of interest.

ABSTRACT

Research regarding cardiovascular disease, associated risk factors and contributing vascular mechanisms in the South African black populations is rare. We therefore performed a cross-sectional study to compare markers of nitric oxide (NO) bio-availability (L-arginine, L-citrulline, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA)), ambulatory blood pressure and markers of end organ damage between African and Caucasian teachers (N=390); and to determine whether these markers of NO bio-availability are associated with ABPM and markers of end organ damage. We found significant negative associations between estimated creatinine clearance (eCCR) and L-citrulline in all four subgroups before and after adjustments for confounders: African men (R^2=0.46; β=-0.23; p=0.006), African women (R^2=0.68; β= -0.12; p=0.046), Caucasian men (R^2=0.62; β= -0.24; p<0.001) and Caucasian women (R^2=0.72; β= -0.13; p=0.029). ADMA associated with markers of renal function only in men: In Caucasian men with albumin-to-creatinine ratio (ACR) (r=0.36; p=0.001) and in African men with eCCR (R^2=0.44; β= -0.18; p=0.034). Multiple regression analyses indicated an ethnic-specific link between eCCR and SDMA only in Caucasians: men (R^2=0.75; β= -0.27; p<0.001) and women (R^2=0.73; β= -0.21; p<0.001). Our results suggest that markers of NO bio-availability may be associated with early changes in renal function, accompanying elevated blood pressure.

Key words: L-citrulline, L-arginine, ADMA, SDMA, renal function

INTRODUCTION

Cardiovascular disease (CVD) is the number one cause of morbidity and mortality worldwide (1,2) and hypertension is regarded as one of the most important cardiovascular risk factors (3). As a result of rapid urbanisation and globalisation CVD has become more prevalent in the low-income countries of sub-Saharan Africa (4,5).

Environmental and lifestyle changes associated with urbanisation seem to be the causes of hypertension development (4,5). On the vascular level it is important to explore the possible mechanisms for increased cardiovascular risk. It is well known that endothelial dysfunction leads to hypertension and ultimately stroke (6). Additionally, endothelial dysfunction is also associated with end organ damage, such as renal failure (7-9). In the South African context it is also important to note that endothelial dysfunction is more prevalent in Africans than in Caucasians (6,10,11), placing Africans at higher risk for the development of CVD (12).

There are several factors, which are involved in the complex control of endothelial dysfunction (13). Endothelial dysfunction occurs when functions of nitric oxide (NO), an endothelium derived relaxing factor, are impaired (14). As indicated in Figure 1, various substances such as L-arginine, L-citrulline, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) influence endothelial function via their influence on the production of NO and NO bio-availability (14,15). NO is formed in an enzyme reaction catalysed by nitric oxide synthase (NOS) from L-arginine as substrate (16). L-citrulline is also formed during this reaction as a secondary product (16) (Figure 1). L-arginine is also a substrate for the enzyme arginase (AR), where L-arginine is converted to ornithine to release excessive urea (17). Ornithine can in turn be converted to L-citrulline, which is subsequently converted back to L-arginine to complete the urea cycle (17).

Reduced availability of L-arginine, the substrate, for NO biosynthesis, as well as inhibitors of this reaction, such as the dimethylarginines can therefore reduce NO synthesis (14,18-26). SDMA reduces NO synthesis by impairing the uptake of L-arginine into the mitochondria, while ADMA competes with L-arginine for binding to NOS (14).

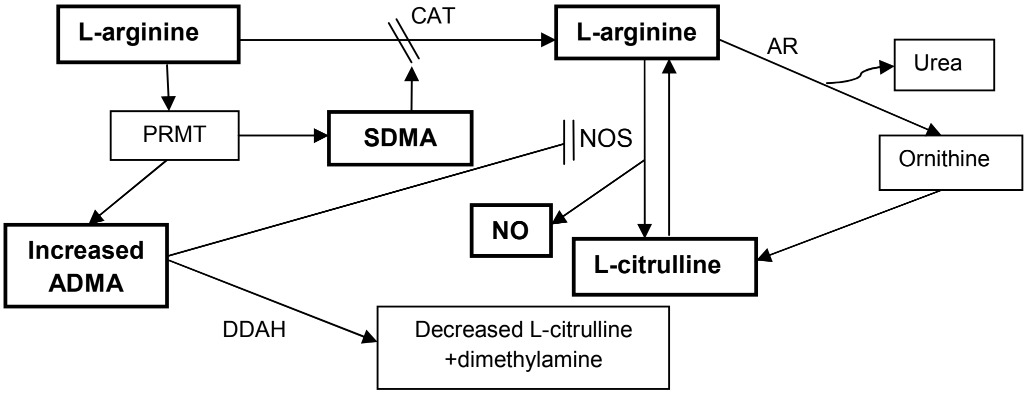

Figure 1: Mechanisms of NO bio-availability.
[Adapted from Böger *et al.* 2005 (14) Teerlink *et al*. 2009 (27) and Mori *et al. 1998* (16)] NO, nitric oxide; AR, arginase; PRMT, protein arginine methyltransferases; DDAH, dimethylarginine dimethylaminohydrolase; CAT, cationic amino acid transporter; eNOS, endothelial nitric oxide synthase

Until now data regarding chronic diseases and associated risk factors in black populations in South Africa is rare (4,5), especially regarding possible contributing vascular mechanisms. The aims of this study are therefore to compare markers of NO bio-availability (including bio-synthesis), namely L-arginine, L-citrulline, ADMA and SDMA, ambulatory blood pressure and markers of end organ damage between African and Caucasian school teachers. In addition, we aim to determine whether these markers of NO bio-availability are associated with ambulatory blood pressure and markers of end organ damage.

METHODS

The Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study was conducted between February 2008 and May 2009. This cross-sectional study included urbanised African (N=200) and Caucasian (N=209) men and women, between the ages of 25 and 65 years. For the purpose of this sub-study individuals infected with human immunodeficiency virus (HIV) were excluded, leaving 181 Africans and 209 Caucasians. Measurements of all parameters were not necessarily available for all participants. The participants were school teachers recruited from the Kenneth Kaunda Education district, Potchefstroom in the North-West Province, South Africa. The reason for this selection was to attempt a homogenous sample from a similar socio-economic class. Exclusion criteria were an ear temperature above 37 °C, psychotropic substance dependence or abuse, blood donors, users of α- and β-blocking agents and individuals vaccinated in the past 3 months. The participants were fully informed about the objectives and procedures of the study and they signed an informed consent form. The study complied with all applicable requirements of international regulations, in particular the Helsinki declaration of 1975 (as revised in 2008) for investigation on human participants. The Ethics Review Board of the North-West University (Potchefstroom Campus) approved the study.

Questionnaires

A general health and lifestyle questionnaire was completed by all the participants.

Clinical measurements

The experimental procedure for each participant followed a 2-day protocol. Ambulatory blood pressure measurements (ABPM) were conducted during the working week. At approximately 08:00, an ABPM apparatus (Meditech CE1201 Cardiotens, Budapest, Hungary) was attached to the participant's non-dominant arm. The ABPM apparatus was programmed to measure blood pressure at 30 min intervals during the day (08:00–22:00) and every hour during night-time (22:00–06:00). The participants continued with their daily activities and were asked to record any abnormalities such as nausea, headache, physical activity and stress on their ambulatory diary cards. The ambulatory blood pressure and electrocardiogram data were downloaded onto a database using the CardioVisions 1.9.0 Personal Edition software (28). Participants were admitted at the Metabolic Research Unit of the North-West University at 16:30. They received a standardised dinner and had their last beverages (tea/coffee) and two biscuits at 20:30. They were requested to go to bed at around 22:00. At 06:00, the ABPM apparatus was removed. Hypertension was defined as a mean ambulatory systolic blood pressure of at least 130 mmHg or/ and diastolic blood pressure of at least 80 mmHg according to the European Society of Hypertension (ESH) 2007 guidelines (29). A standard 12-lead ECG was recorded during resting conditions (PC 1200, v5.030, Norav Medical, Yokneam, Israel). ECG left ventricular mass was determined using the Cornell product (30). Thereafter, the same observer monitored BP continuously by making use of the Finometer device (FMS, Finapres Medical Systems, Amsterdam, Netherlands) (31). This entailed a 5-min recording of each participant's BP under resting, yet awake, conditions. After the first 2 min, the finger pressure was calibrated with the upper arm (brachial) pressure (that is, return-to-flow systolic calibration). This optimised the accuracy of the readings taken.

The SonoSite Micromaxx ultrasound system (SonoSite Inc., WA, USA) and a 6–13 MHz linear array transducer were used to determine the carotid intima media thickness (cIMT). Images from at least two optimal angles of the left and right common carotid artery were obtained. Following previously prescribed protocols these segments were imaged and measured and were imported into the Artery Measurement Systems automated software for dedicated analysis of cIMT (32). A maximal 10 mm segment with good image quality was chosen for analysis. The program automatically identifies the borders of the intima-media of the near and far wall, and the inner diameter of the vessel, and calculates the cIMT and diameter from around 100 discrete measurements through the 10 mm segment.

Anthropometrical measurements and physical activity

All measurements were standardised and taken in triplicate using standard methods with calibrated instruments. Height was measured to the nearest 0.1 cm with a stadiometer (Invicta Stadiometer, IP1465, UK) (33) and weight to the nearest 0.1 kg using a Krups scale (Precision Health Scale, A & D Company, Japan) (33) with participants wearing only their underwear. Body mass index (BMI) was calculated using these measurements. Waist and hip circumferences were measured to the nearest 0.1 cm using a metal tape. To assess physical activity, participants wore Actical accelerometers (Montre´al, Que´bec, Canada) around their hip during a normal working day.

Biochemical analyses

A registered nurse collected blood samples with a sterile winged infusion set from the ante-brachial vein branches of the participant. Serum and plasma samples were prepared and stored at -80°C. Sodium fluoride plasma was used for glucose analysis. High density lipoprotein (HDL) cholesterol, triglycerides, gamma glutamyl transferase (GGT) and high sensitivity C-reactive protein (CRP) were analysed in serum using two sequential multiple analyzers (Konelab 20i; Thermo Scientific, Vantaa, Finland; and Unicel DXC 800 – Beckman and Coulter®, Germany). Serum cotinine levels were determined with a homogeneous immunoassay (Automated Modular, Roche, Basel, Switzerland). The intra- and inter-coefficients of variation for all assays were below 10%. Mass spectrometric determinations of L-arginine, ADMA and SDMA were performed as described elsewhere by using a fully validated high throughput LC-MS/MS assay, which is commercially available (DLD

Diagnostika, Hamburg, Germany) (34,35). L-citrulline was determined with an electrospray ionisation tandem mass spectrometry (ESI-MS/MS) method. Reactive oxygen species (ROS) were measured using a spectrophotometric assay, where 1.0 $mg.l^{-1}$ H_2O_2 represents one unit of reactive oxygen species (36). Ferric reducing antioxidant power (FRAP), as an indication of antioxidant capacity, was determined with the method as described by Benzie and Strain (37), and total glutathione levels were determined with the BIOXYTECH _ GSH/GSSG-412TM kit supplied by OxisResearch TM, a division of OXIS Health Products (Foster City, CA, USA).

Urinary creatinine and albumin were determined with calorimetric and immunoprecipitation methods, respectively on a sequential multiple analyzer computer (Konelab 20i TM, Thermo Scientific, Vantaa, Finland). The albumin-to-creatinine ratio (ACR) was calculated in an 8-h overnight urine sample. We calculated the estimated creatinine clearance (eCCR) by using the Cockcroft–Gault formula (38).

Statistical analysis

Statistical analyses were performed using Statistica version 10 (Statsoft, Inc., Tulsa, OK, 2009). Statistical results are presented as means ± standard deviation. Data that were not normally distributed were log transformed and presented as means with 5% and 95% percentile boundaries. Characteristics of groups were compared with independent T-tests and Chi-square tests. Single and partial analyses were used to investigate associations between L-arginine, L-citrulline, ADMA and SDMA with ambulatory BP measurements and markers of end organ damage (while adjusting for confounders). We plotted quartiles of eCCR (log) against L-citrulline, separately for gender and ethnicity. Analysis of covariance (ANCOVA) was used for comparison of variables between groups to determine significant differences, while adjusting for confounders. Forward stepwise multiple regression analyses were performed to determine if independent associations exist between ambulatory BP measurements or markers of end organ damage with either L-arginine, L-citrulline, ADMA or SDMA as main independent variable. Other independent variables included: age, BMI, ambulatory systolic BP, GGT, cotinine, CRP, glucose, cholesterol, physical activity energy expenditure and anti-hypertensive medication.

RESULTS

Characteristics of the study population are shown in Table 1. Overall, the cardiovascular profile of the Africans was unfavourable compared to that of the Caucasians. This was reflected by their higher ambulatory BP, pulse pressure and Cornell Product (all p values ≤0.05). Additionally, this profile also seems to affect renal function, as ACR was also higher in the Africans (p <0.001) with no difference in eCCR between the groups. In contrast, the Africans had a better lipid profile (total cholesterol/HDL (p=0.003)), but their exposure to modifiable risk factors was higher as seen in the significantly higher GGT and cotinine levels (all p values <0.001).

The NO bio-availability markers such as L-arginine and L-citrulline were higher in the Africans (all p values <0.05), while the inhibitors of NO biosynthesis, such as ADMA and SDMA were significantly lower in the Africans (p=0.046; p<0.001, respectively). Separate analysis for normotensive, hypertensive, male, female and ethnic grouping in gender, as well as unadjusted correlations of age and BP with markers of NO bio-availability were performed in Appendix A.

Table 1: Characteristics of the study population

	Africans (n=181)	Caucasians (n=209)	P
Age, years	44.3 ± 8.21	44.9 ± 10.9	0.53
Gender, men/women	88/93	101/108	0.95
Anthropometric measurements			
Stature, m	1.64 ± 0.09	1.74 ± 0.10	<0.001
Body mass, kg	79.8 (56.2; 113)	81.4 (55.9; 125)	0.14
Body mass index, kg/m^2	30.3 ± 7.09	27.6 ± 5.94	<0.001
Waist circumference, cm	92.5 (70.0; 120)	91.6 (70.7; 122)	0.59
Cardiovascular measurements			
Ambulatory systolic BP, mmHg	133 ± 16.5	124 ± 12.0	<0.001
Ambulatory diastolic BP, mmHg	83.2 ± 11.0	76.6 ± 8.04	<0.001
Ambulatory pulse pressure, mmHg	50.0 ± 9.19	47.5 ± 7.40	0.004
Night-time systolic BP, mmHg	124 ± 17.7	113 ± 13.6	<0.001
Night-time diastolic BP, mmHg	73.8 ± 12.2	66.5 ± 8.68	<0.001
Ambulatory heart rate, mmHg	79.7 ± 10.7	73.6 ± 10.2	<0.001
Stroke volume, mL	101 ± 28.0	97.7 ± 24.3	0.14
Cardiac output, L/min	6.83 ± 1.86	6.43 ± 1.94	0.039
Total peripheral resistance, mmHg/ml/s	1.02 ± 0.39	1.04 ± 0.52	0.60
Windkessel compliance, mL/mmHg	1.87 ± 0.42	2.09 ±0.53	<0.001
Carotid intima media thickness, mm	0.69 ± 0.13	0.64 ± 0.12	0.001
Cornell product, mV.ms	58.6 (19.1; 146)	42.3 (15.5; 102)	<0.001
Hypertensive, n (%)	137 (68.5)	102 (48.8)	<0.001
Basic biochemical measurements			
C-reactive protein, mg/L	4.60 (0.65; 33.8)	2.03 (0.99; 9.00)	<0.001
Glucose, mmol/L	5.42 (3.92; 10.5)	5.62 (4.70; 6.90)	0.08
Cholesterol, mmol/L	4.50 (2.93; 6.47)	5.40 (3.80; 8.10)	<0.001
HDL cholesterol, mmol/L	1.09 (0.66; 1.72)	1.14 (0.64; 1.97)	0.21
Total cholesterol/HDL	4.44 ± 2.10	4.99 ± 1.62	0.003
Triglycerides, mmol/L	1.41 ± 1.31	1.20 ± 0.76	0.042
Serum creatinine, µmol/L	74.2 (54.2; 102)	72.6 (54.0; 100)	<0.001
Urinary creatinine, mmol/L	8.37 (2.85; 20.6)	10.4 (3.00; 30.7)	0.002
Markers of NO bio-availability			
L-citrulline, µmol/L	99.8 ± 26.7	94.4 ± 23.6	0.032
L-arginine, µmol/L	67.7 (19.0; 140)	53.2 (19.0; 146)	<0.001
ADMA, mmol/L	0.64 (0.43; 0.95)	0.67 (0.46; 0.94)	0.046
SDMA, mmol/L	0.42 ± 0.12	0.53 ±0.12	<0.001
Reactive oxygen species, Units^1	91.6 (57.1; 147)	86.7 (57.4; 137)	0.054
FRAP, µM	389 ± 73.7	437 ± 108	<0.001
Glutathione, µM	906 ± 185	820 ± 173	<0.001
Renal function			
Albumin-to-creatinine ratio, mg/mmol	1.10 (0.36; 5.06)	0.33 (0.09; 1.88)	<0.001
Estimated creatinine clearance, mL/min	116 (73.5; 189)	120 (72.7; 205)	0.29
Medication use			
Anti-hypertensive medication, n (%)	43 (21.5)	18 (8.61)	<0.001

	Africans (n=181)	Caucasians (n=209)	P
Statin, n (%)	2 (1.00)	9 (4.31)	0.039
Anti-inflammatory medication, n (%)	16 (8.00)	12 (5.74)	0.37
Lifestyle			
Physical activity, kcal	2580 (1710; 4067)	2942 (1917; 4419)	<0.001
Physical activity index, n (%)			
Low	147 (73.5)	104 (50.0)	<0.001
Moderate	42 (21.0)	78 (37.5)	<0.001
Vigorous	11 (5.50)	26 (12.5)	<0.001
Cotinine, ng/mL	0.02 (0.00; 151)	0.01 (0.00; 210)	<0.001
Current smoking, n (%)	34 (17.0)	29 (13.9)	0.39
Gamma glutamyl transferase, U/L	46.7 (19.9; 184)	19.3 (7.00; 76.0)	<0.001
Current drinking, n (%)	44 (24.3)	102 (49.0)	<0.001

Values are arithmetic mean ± SD, geometric mean (5th to 95th percentile interval). [1](1 unit=1.0mg/L H_2O_2). BP, blood pressure; HDL-C, High-density lipoprotein cholesterol; ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine; FRAP, Ferric reducing antioxidant power.

Unadjusted correlations were performed between markers of NO bio-availability (L-arginine, L-citrulline, ADMA and SDMA) and cardiovascular measurements (Table 2).

Renal function and L-citrulline

We found significant unadjusted negative associations between L-citrulline and eCCR in all four subgroups: African men (r=-0.27; p=0.013), African women (r=-0.24; p=0.021), Caucasian men (r=-0.21; p=0.044) and Caucasian women (r=-0.28; p=0.003) (Table 2). In exploratory analyses (Figure 2) we plotted eCCR by quartiles of L-citrulline, while adjusting for age, body mass index and anti-hypertensive medication. The African women and Caucasian men showed significantly lower eCCR with increasing levels of L-citrulline (p=0.017; p=0.037, respectively). Additionally, eCCR of the lowest L-citrulline quartile was significantly higher than the eCCR of the fourth quartile for African men (p<0.001), African women (p=0.010) and Caucasian women (p<0.001). We further examined this association in a forward stepwise multiple regression analysis (Table 4) with eCCR as the dependant variable. This association of eCCR with L-citrulline was confirmed to be independent in all groups: African men ($R^2=0.46$; $\beta=-0.23$; $p=0.006$), African women ($R^2=0.68$; $\beta= -0.12$; $p=0.046$), Caucasian men ($R^2=0.62$; $\beta= -0.24$; $p<0.001$) and Caucasian women ($R^2=0.72$; $\beta=0.13$; $p=0.029$).

Table 2: Unadjusted correlations of blood pressure and markers of end organ damage with markers of NO bio-availability

	L-arginine	**L-citrulline**	**ADMA**	**SDMA**
African men (N= 74)				
Night-time systolic BP, mmHg		0.29^{1}	0.20^{2}	
Night-time diastolic BP, mmHg		0.23^{1}	0.21^{2}	
Carotid intima media thickness, mm				
eCCR (log), mL/min		-0.27^{1}		
ACR (log), mg/mmol		0.10^{2}		
Cornell product (log), mV.ms				
African women (N=75)				
Night-time systolic BP, mmHg				
Night-time diastolic BP, mmHg				
Carotid intima media thickness, mm		0.30^{1}		
eCCR (log), mL/min		-0.24^{1}	0.20^{2}	
ACR (log), mg/mmol				
Cornell product (log), mV.ms				
Caucasian men (N=81)				
Night-time systolic BP, mmHg	0.34^{1}		0.34^{1}	
Night-time diastolic BP, mmHg	0.27^{1}		0.25^{1}	0.22^{1}
Carotid intima media thickness, mm			0.19^{2}	
eCCR (log), mL/min	0.26^{1}	-0.21^{1}	0.19^{2}	-0.30^{1}
ACR (log), mg/mmol			0.36^{1}	
Cornell product (log), mV.ms				
Caucasian women (N=85)				
Night-time systolic BP, mmHg				
Night-time diastolic BP, mmHg				
Carotid intima media thickness, mm		0.35^{1}		0.18^{2}
eCCR (log), mL/min		-0.28^{1}		-0.26^{1}
ACR (log), mg/mmol				
Cornell product (log), mV.ms		0.20^{2}		

$^{1}p<0.05$ and $^{2}p=0.05$-0.10. ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; BP, blood pressure; eCCR, estimated creatinine clearance; ACR, albumin-to-creatinine ratio.

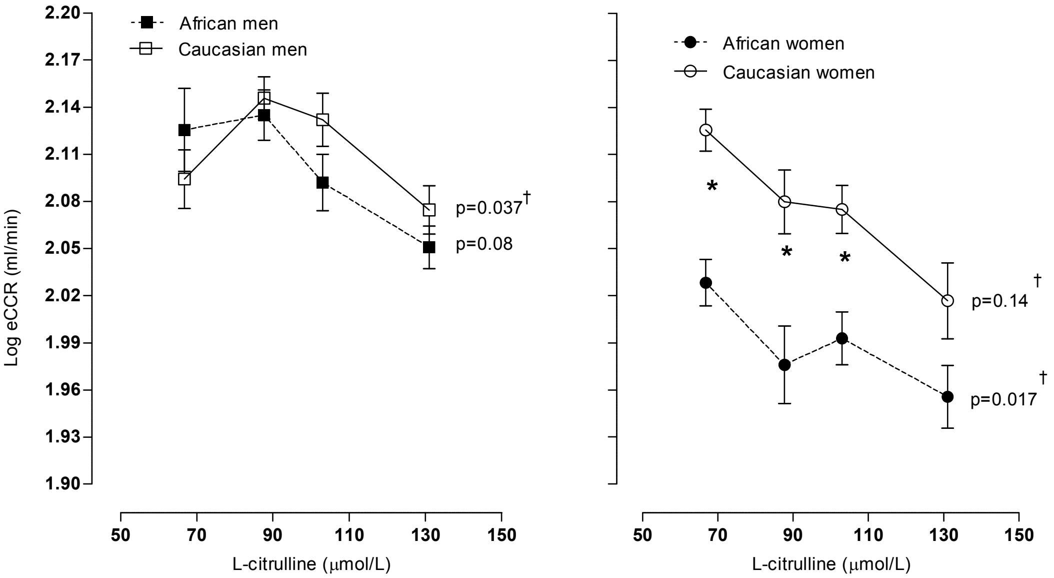

Figure 2: Estimated creatinine clearance according to quartiles of L-citrulline (adjusted for age, body mass index and hypertensive medication).
*Significant difference ($p<0.05$) between African and Caucasian participants within the same quartile.
†Significant difference ($p<0.05$) between quartile 1 and 4 within the same ethnic group.

Positive associations between night-time blood pressure and L-citrulline in the African men (night time SBP (p=0.011) and night-time DBP (p=0.048)) and cIMT in the African and Caucasian women (p=0.004; $p<0.001$) were only present before adjustments were made (Table 2).

Various other significant associations were also present in our unadjusted analyses, including an association in the Caucasian men between night-time DBP and L-arginine (p=0.014) and ADMA (p=0.023) and eCCR with L-arginine (p=0.013). After full adjustments all of these associations lost significance. However, the positive correlation between night-time SBP and L-arginine (p=0.002) and the correlation between night-time DBP and SDMA (p=0.044) in the Caucasian men remained in our forward stepwise multiple regression model (data not shown).

Renal function and ADMA

In Caucasian men we found that ADMA correlated with ACR (r=0.36; p=0.001), night-time SBP (r=0.34; p=0.002) and night-time DBP (r=0.25; p=0.023) with single linear regression analyses (Table 2). A similar trend was shown in African men with night-time SBP (p=0.089) and night-time DBP (p=0.078), respectively. In Caucasian men these associations remained after adjustments for age and BMI (Table 3), (except for night-time DBP). After these adjustments a negative association between eCCR and ADMA became evident in the African men (r=-0.24; p=0.025) (Table 3) and remained significant in the forward stepwise multiple regression analysis (R^2=0.44; β= -0.18; p=0.034) (Table 4). These associations were absent in women.

Table 3: Partial correlations of blood pressure and markers of renal function with ADMA (adjusted for age and body mass index)

ADMA	Night-time SBP	Night-time DBP	eCCR	ACR
African men	r=0.11; p=0.31	r=0.11; p=0.30	r=-0.24; p=0.025	r=0.06; p=0.58
Caucasian men	r=0.23; p=0.028	r=0.18; p=0.09	r=-0.13; p=0.22	r=0.32; p=0.002
African women	r=0.06; p=0.58	r=-0.02; p=0.87	r=0.17; p=0.11	r=-0.16; p=0.14
Caucasian women	r=0.02; p=0.85	r=0.08; p=0.41	r=-0.10; p=0.31	r=0.02; p=0.83

ADMA, asymmetric dimethylarginine; NSBP, night-time systolic blood pressure; NDBP, night-time diastolic blood pressure; eCCR, estimated creatinine clearance and ACR, albumin-to-creatinine ratio.

Renal function and SDMA

In the Caucasian men and women unadjusted correlations indicated that eCCR linked negatively with SDMA before adjustments (r=-0.33; p=0.003 and r=-0.26; p=0.006, respectively) (Table 2). This phenomenon was confirmed in partial and forward stepwise multiple regression analysis in Caucasian men (R^2=0.75; β= -0.27; p<0.001) and women (R^2=0.73; β= -0.21; p<0.001), while no associations were found in the Africans (Table 4).

Table 4: Forward stepwise multiple regressions with estimated creatinine clearance as dependant variable.

	Estimated creatinine clearance		
Main independant variable	**Adjusted R^2**	**β-value (±S.E)**	**p-value**
L-Citrulline, µmol/L			
African men	0.46	-0.23 ± 0.08	0.006
Caucasian men	0.68	-0.12 ± 0.06	0.046
African women	0.62	-0.24 ± 0.07	<0.001
Caucasian women	0.72	-0.13 ± 0.06	0.029
ADMA, mmol/L			
African men	0.44	-0.18 ± 0.08	0.034
Caucasian men	-	-	-
African women	-	-	-
Caucasian women	-	-	-
SDMA, mmol/L			
African men	-	-	-
Caucasian men	0.75	-0.27 ± 0.05	<0.001
African women	-	-	-
Caucasian women	0.73	-0.21 ± 0.05	<0.001

ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine. Variables included in the models were: age, body mass index, ambulatory systolic blood pressure, gamma glutamyl transferase, cotinine, C-reactive protein, glucose, cholesterol physical activity energy expenditure and anti-hypertensive medication with either L-citrulline, asymmetric dimethylarginine or symmetric dimethylarginine in the model as main independent variables.

DISCUSSION

In this study we compared markers of nitric oxide bio-availability (including L-arginine, L-citrulline, ADMA and SDMA) and cardiovascular measurements in African and Caucasian school teachers from South Africa. We also explored the associations of nitric oxide bio-availability markers with ambulatory blood pressure and markers of end organ damage.

The first key finding of our study was a significant negative independent relationship between estimated creatinine clearance and L-citrulline in all four sub-groups. This implicates that renal function may be detrimentally affected by L-citrulline concentrations. Substances that are eliminated by the kidney are based on renal creatinine clearance in renal dysfunction patients (39). However, since creatinine is mainly eliminated via the glomeruli, creatinine clearance is often used to estimate the glomerular filtration rate (GFR) (40). There are several studies confirming a negative association between L-citrulline and renal function (40-46).

L-citrulline is synthesised in the intestines (47), thereafter reabsorbed by the kidneys via organic anionic and organic cationic transporters (40), where L-arginine is released through enzymes such as argininosuccinate synthetase and argininosuccinate lyase, exclusively in the proximal convoluted tubule. This transformation is increased when GFR falls below 60 ml/min (48-50). Thus, impaired kidney function will result in increased L-citrulline (51). L-arginine is known to reverse hypertension by restoring endothelium dependant vasodilatation and to enhance kidney function (9,14). L-citrulline supplementation can also be used as a substitute for L-arginine, since orally L-citrulline bypasses the hepatic metabolism and is therefore more effective than L-arginine in treating cardiovascular diseases (52). Thus, from the literature it seems that increased L-citrulline should be beneficial.

However, as previously said, L-citrulline is reabsorbed by the kidneys via organic anionic and organic cationic transporters (40). These transporters are important for uptake of substances from the blood to the epithelial cells, thus in the direction of the tubular secretion (53,54). When renal dysfunction occurs, the expression of proteins and mRNA of these organic anion transporters and organic cation transporters, at the basolateral membranes of tubular epithelial cells decreases and this may contribute to the increase in plasma concentrations of L-citrulline, since L-citrulline cannot be absorbed by the kidneys from the blood (40,55-57). This was typically found in chronic renal disease patients (40,55-57). Bouby *et al.* explain this increased L-citrulline turnover by peripheral adaptation, which maintains a constant rate of arginine synthesis by enabling a reduced mass of tissue in the kidneys (43).

The individuals of our study had estimated creatinine clearance rates of 116 (73.5; 189) and 120 (72.7; 205) ml/min for Africans and Caucasians, respectively. Despite being in normal ranges of >90 ml/min (58), 69% of African and 49% of Caucasian participants were hypertensive, based on ABPM. This may indicate that a large percentage may have early stages of subclinical renal dysfunction and that the negative association between glomerular filtration rate and L-citrulline represents weakened endothelial function in the glomeruli. This may especially be true in the African group who had significantly increased albumin-to-creatinine ratio compared to their Caucasian counterparts.

Our second key finding is a link between renal function and the endogenous NOS inhibitor, ADMA. Albumin-to-creatinine ratio showed a significantly positive correlation with ADMA in the Caucasian men and negatively with estimated creatinine clearance in the African men. This finding is not surprising, since ADMA is known for its inhibitory effect on nitric oxide synthase in a variety of cardiovascular diseases and renal dysfunction (12,20,25,26). However, this trend was absent in the African and Caucasian women.

Several studies have found increased ADMA levels in patients with chronic renal failure (59,60). Then again, they found associations with ADMA and cystatin C (another marker of renal disease/failure) (59), whereas in our study we found associations of ADMA with eCCR and ACR. According to Böger *et al.* patients with chronic kidney disease have the highest risk for the development of cardiovascular disease (61). This leads to the assumption that ADMA may contribute to endothelial dysfunction (59), hypertension and renal dysfunction (25,59,62). In addition, there is a strong relation between serum creatinine and the risk for the development of cardiovascular diseases (59). As mentioned earlier, this is confirmed in our results where the Africans had higher albumin-to-creatinine ratio and higher blood pressure, thus indicating that the Africans in our study may be more likely to develop organ damage, despite having lower ADMA levels than the Caucasians. In spite of this, our Caucasian men also showed increased ADMA levels in association with renal dysfunction and this is confirmed by Wang *et al.* who found increased ADMA levels in hypertensive Caucasian subjects (63).

Although ADMA is partially excreted by the kidneys, several studies show no associations between GFR, creatinine clearance and ADMA (12,64-66). ADMA is mainly eliminated from the body by enzymatic degradation through dimethylarginine dimethylaminohydrolase (DDAH), which is present in the kidneys (67). During decreased renal excretory function, DDAH activity also decreases, which results in increased ADMA levels (67). This could be the reason for the unexpectedly lower ADMA levels in our African participants compared to the Caucasians (67). Africans also had a significantly higher albumin-to-creatinine ratio, which indicates glomerular endothelial dysfunction (68). It is, however, not clear why our results were gender specific and absent in women, but we could speculate that the female sex hormones may play a part in protecting the endothelium (69).

Our last prominent finding was a strong negative association between estimated creatinine clearance and SDMA, which was only present in the Caucasian men and women. This phenomenon is confirmed by several studies where it was found in both hypertensive children and adults, especially in Caucasians (63,70). SDMA can only be eliminated by the kidneys (61). SDMA is therefore an important risk marker for the early detection of renal dysfunction (71), since it is increased in patients with chronic renal failure, (71-75) despite the fact that some studies found no associations (76,77).

No associations between renal function and SDMA were seen in the Africans and this is also confirmed in a study done by Schutte *et al.* (78). It is not clear why this relationship was absent in Africans. It seems that other mechanisms than the effects of SDMA may be responsible for the more unfavourable cardiovascular profile of the African population.

The limitations of this study were firstly, the relatively small sizes of the subject groups, but the groups were still large enough to ensure statistical reliability. Our population is not representative of the entire South African population, since we only included teachers from the North-West Province. Secondly, although the results were consistent after multiple adjustments, we cannot exclude residual confounding. Thirdly, nitric oxide and ornithine (part of the urea cycle) measurements would have strengthened our results. Another marker, which would have been useful, is Cystatin-C, to represent renal function more clearly. Lastly, because of the cross-sectional study design, all results are based on associations: therefore we cannot predict cause and effect.

In conclusion, although Africans presented a more vulnerable cardiovascular profile (elevated blood pressure and impaired renal function), we found a consistent negative association between renal function and L-citrulline in all participants, which had only been reported previously in patients with chronic renal disease. Additionally we found a gender-specific link between renal function and ADMA in African and Caucasian men. Our results may indicate that markers of NO bio-availability may be associated with early changes in renal function, accompanying elevated blood pressure.

ACKNOWLEDGEMENTS

The Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study was possible due to the voluntary participation of the teachers and the Department of Education, North-West Province, South Africa. The authors thank and acknowledge the technical assistance of Mrs. Tina Scholtz, Sr. Chrissie Lessing and Dr. Szabolcs Péter. Research included in the present study was partially funded by the National Research Foundation, South Africa; the North-West University, South Africa; and the Metabolic Syndrome Institute, France.

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CHAPTER 4

General findings and conclusions

INTRODUCTION

The main findings of this study are summarised in this chapter. The results will be compared to relevant literature, discussed and recommendations will be made for future investigation on the relationship between markers of nitric oxide (NO) bio-availability, ambulatory blood pressure (AMBP) and end organ damage in Africans and Caucasians.

DISCUSSION OF MAIN FINDINGS AND COMPARISON WITH THE LITERATURE

This study aimed to compare markers of NO bio-availability (L-arginine, L-citrulline, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA)) with ABPM and markers of end organ damage (carotid intima media thickness (cIMT), Cornell product and renal function (albumin-to-creatinine ratio and estimated creatinine clearance)) in African and Caucasian school teachers.

Our main results consisted of three key findings, which will be discussed when viewing our four original hypotheses, as indicated in Chapter 2, page 36.

First hypothesis: *L-arginine, L-citrulline, ADMA and SDMA levels differ between Africans and Caucasians, with Africans having lower L-arginine and L-citrulline and higher ADMA and SDMA levels than Caucasians.*

If deficiencies of L-arginine occur (1), NO availability decreases which results in diminished endothelial function (2). This can lead to the development of hypertension and cardiovascular disease (2-4). The Africans in our study had a more vulnerable blood pressure profile, thus we expected that they should have lower L-arginine and L-citrulline and higher ADMA and SDMA levels than Caucasians. However, we were surprised to find higher L-arginine and L-citrulline and lower ADMA and SDMA in the African population, which is in contrast to many other studies. In an African population with lower socio-economic status, Glyn *et al*. found lower L-arginine in African men, with higher blood pressure than in the Caucasian men (5). Another study found higher ADMA levels in Africans when compared to Europeans (6).

NO is formed from an enzyme reaction catalysed by nitric oxide synthase (NOS) from L-arginine as substrate (7). L-citrulline is also formed during this reaction as a secondary product (7) (Figure 1 Chapter 2). L-arginine is also a substrate for the enzyme arginase (AR), where L-arginine is converted to ornithine to release excessive urea (8). This may have unfavourable vascular effects (9). Ornithine can in turn be converted to L-citrulline, which is subsequently converted back to L-arginine to complete the urea cycle (7) (Figure 1 Chapter 2).

AR, the enzyme responsible for converting L-arginine into L-ornithine and urea, plays an important role in modulating L-arginine bio-availability (10). The inhibition of AR can increase NOS activity, resulting in more NO production (11). However, increased AR activity results in decreased L-arginine levels, thus attenuates NO production (12). Our African population possibly have increased AR activity, explaining their vulnerable cardiovascular profile. Decreased L-arginine results in decreased NO dependant vasodilatation, which is evident in African Americans with hypertension (13). Hypertension in African Americans is also associated with salt sensitivity (13). In salt-sensitive individuals with hypertension, increased AR activity was evident. This was also accompanied by a decrease in vascular function (14).

We speculate that our result is possibly due to the African population being more likely to form L-citrulline through the urea cycle, than the L-arginine - L-citrulline cycle, since they may have higher levels of the enzyme AR activity than the Caucasians. It is uncertain whether the results from an African American population are applicable to ours, but similarities have been shown in the past (15). Thus, less L-arginine is available for NO production, contributing to their more unfavourable cardiovascular profile.

Higher L-arginine and L-citrulline levels and lower ADMA and SDMA levels were found in the African population. We therefore reject our first hypothesis as our results indicated the opposite.

Second hypothesis: *Africans have higher ambulatory BP and end organ damage compared to the Caucasians.*

Hypertension is the most common cardiovascular risk factor in black South Africans (16). Hypertension and stroke (5, 17-19) associated with end organ damage and renal failure (20) are more prevalent in Africans than in Caucasians (5, 17-19).

Impaired endothelial dependant vasodilatation occurs in subjects with atherosclerosis and left ventricle hypertrophy (LVH) (2, 21), which is also more common in Africans (22, 23).

Our study indicated the same phenomenon. The African cohort of our study population showed significantly higher ABPM and markers of end organ damage, such as Cornell product, renal dysfunction (elevated albumin-to-creatinine ratio (ACR)) and subclinical atherosclerosis (cIMT). Therefore our second hypothesis is accepted.

Third hypothesis: *L-arginine levels associate negatively with BP, while L-citrulline associates negatively with renal function.*

The above hypothesis was formulated based on previous research on renal function and L-citrulline (24-29). We found an independent negative association between estimated creatinine clearance (eCCR) and L-citrulline in all our subgroups, which is our first key finding. Others found this result only in chronic renal disease patients (28, 30-32). In our study 69% of the African and 49% of the Caucasian participants were hypertensive. This negative association between renal function and L-citrulline may therefore indicate early stages of renal dysfunction and impaired endothelial function.

L-citrulline is taken up from the blood by the kidneys via organic anion transporters and organic cation transporters (28, 30-32). These transporters are important for uptake of substances from the blood to the epithelial cells, thus in the direction of the tubular secretion to eliminate L-citrulline through the kidneys (33, 34). When renal dysfunction occurs, the expression of proteins and mRNA of these organic anion transporters and organic cation transporters, at the basolateral membranes of tubular epithelial cells decreases (28, 30-32). Thus, L-citrulline cannot be absorbed from the blood by the kidneys for elimination (28). Therefore, this may contribute to the increase in plasma concentrations of L-citrulline (28).

We also found a positive correlation between blood pressure and L-arginine in the Caucasian men, confirmed with a forward stepwise multiple regression analysis. This result is contrary to our expectations, since L-arginine increases NO bio-availability to promote vasodilatation (4, 35-37). Thus L-arginine is known to reverse hypertension by restoring endothelium-dependent vasodilatation and decreases peripheral vascular resistance (35, 38). However, there was one study by Chirinos *et al*. who found a positive correlation between systolic blood pressure and L-arginine (9). L-arginine is predominantly transported by the y+, system in human platelets (39). This system y+ mediates high-affinity, Na+-independent cationic and Na+-dependent neutral amino acid transport (39). According to the authors this could be due to abnormalities in L-arginine transport via system y+ which may limit L-arginine availability for NO production (40, 41) In humans and rats, it was found that this transport system is abnormal in the blood cells of hypertensive subjects and animals (42, 43).

There could also be abnormalities in the degradation of L-arginine, because the activity of AR may be altered in hypertensive subjects (43). AR competes with NOS for L-arginine; therefore changes in AR activity can limit NO production (40). In rats, active AR resulted in decreased NO activity (44, 45). Increased AR activity was also found in rats, which showed a decline in NOS activity, therefore L-arginine cannot be used for the production of NO (11). In a study done by Huyns *et al.* they found increased NO production with decreased AR activity (46). However, NO bio-availability was decreased as a result of increased conversion to peroxynitrite, which also results in endothelial dysfunction (46). Additionally, various eNOS gene polymorphisms are associated with endothelial dysfunction, since the carriers of these polymorphisms are increased in CVD (47, 48). Lastly, deficiencies of cofactors required for NO production, such as tetrahydrobiopterin (BH_4) could also result in decreased NO production, thus L-arginine levels increase, since it cannot be utilized (49).

Apart from the links found with renal function, we found no associations between NO bio-availability markers and the other markers of end organ damage, namely Cornell product and cIMT. This was an unexpected finding, because functions of the NO bio-availability cycle include the regulation of homeostasis and thrombosis, and the prevention of various vascular pathologies, especially atherosclerosis (50). cIMT, a marker of atherosclerosis and LVH are directly related to endothelial dysfunction (51, 52). Based on our results it therefore seems that by using the measurement techniques for assessing early organ damage, the measures assessing renal function may be most sensitive to detect possible endothelial dysfunction.

Since we found associations of NO bio-availability markers with renal function, but not with Cornell product and cIMT, our third hypothesis is only partially accepted.

Fourth hypothesis: *ADMA levels associate positively with BP and end organ damage, with stronger associations in the African population, while SDMA correlates with renal function.*

Increased ADMA levels inhibit endothelium dependent vasodilatation (53, 54), which leads to vasoconstriction and increased systemic blood pressure (55). This is also found in our study in the Caucasian men.

ADMA is known for its inhibitory effect on NOS function in a variety of cardiovascular diseases (6, 9, 56, 57). ADMA is mainly eliminated from the body by enzymatic degradation through dehydro dimethylaminohydrolase (DDAH), which is present in the kidneys (58). During decreased renal excretory function, DDAH activity also decreases, which results in increased ADMA levels (58). That could be the reason why ADMA is correlated with renal function markers in some studies (58), especially in our African and Caucasian men (our second key finding).

The following question evolves when we look at the involvement of NO bio-availability markers in vascular and renal function: Could it be that impairment of renal function due to decreased NO production results in increased blood pressure? Or is it perhaps increased ADMA levels, which results in endothelial dysfunction, contributing to higher blood pressure, which results in renal dysfunction?

No associations between ADMA with Cornell product and cIMT were found. This was another unexpected finding. ADMA is known for its interference with NO production (59). Since NO modulates the growth of the myocardium, ADMA has a connection with the pathogenesis of LVH (59). ADMA is also related to atherosclerosis as seen in several studies (9, 60, 61). However, there was one study done on individuals younger than 40 years who reported a negative correlation between ADMA and cIMT (62). The authors speculated that ADMA could have a protective effect in the early stages of atherosclerosis, since it inhibits inducible NOS, which is involved in inflammation and atherosclerosis (62).

Our final key finding was a negative correlation between SDMA and eCCR in the Caucasian men and women. Several studies show correlations between SDMA and renal markers such as serum creatinine, glomerular filtration rate and creatinine clearance (58, 63-65). This phenomenon is confirmed by several studies where it was found in both hypertensive children and adults, especially in Caucasians. However, we are not certain why this phenomenon is absent in our African group (64, 66). SDMA can only be eliminated by the kidneys (67) and can therefore increase in patients with impaired kidney function (68, 69).

As seen, ADMA correlated positively with BP and renal function, however not with cIMT and Cornell product. SDMA was also associated with renal function. Our fourth hypothesis is therefore partially accepted.

In summary, the characteristics of our study population showed that the Africans were more obese and had an unfavourable cardiovascular profile compared to that of the Caucasians. This was reflected by their higher ambulatory BP, pulse pressure and Cornell Product. Although 69% of our Africans were hypertensive, only 22% were using anti-hypertensive medication. Additionally, the Africans also had higher ACR levels therefore they might have a higher possibility of renal dysfunction compared to the Caucasians. All these results indicate that the Africans are at higher risk for the development of CVD and end organ damage.

However, this study also yielded unexpected findings. The Africans had higher L-arginine and lower ADMA levels. Another unexpected phenomenon was increased L-arginine, which associated with increased blood pressure. Therefore, since it is a cross sectional study, we cannot predict cause and effect. However, it is speculated that an imbalance or a dysregulation within the NO cycle, between role players such as L-arginine, L-citrulline, ADMA and SDMA exists in the African population.

CHANCE AND CONFOUNDING

It is of the utmost importance to reflect on some of the factors that may have affected the results of this study, such as some methodological issues that could have weakened the outcomes of this study.

Firstly, the relatively small sizes of the subject groups could be a problem. However, the groups were still large enough with 74 African men, 75 African women, 81 Caucasian men and 85 Caucasian women, to ensure statistical reliability; also when compared to other studies on this topic. Our population is not representative of the entire South African population, since we only included teachers from the Potchefstroom district of the North-West Province. However, this was a well designed study conducted under controlled conditions. All participants were school teachers, which was done in order to achieve a homogenous group.

Since some of the results from our study were unexpected, it is also necessary to confirm quality control regarding the biochemical analyses of our main independent variables. L-arginine, ADMA and SDMA were all analysed in Hamburg, Germany, in the laboratory of the expert group of Prof. Rainer Böger. This method is standard (70) and many analyses have been done in this specialist laboratory. L-citrulline analyses were done by a biochemist in South Africa, using a careful specialist method of electrospray ionisation tandem mass spectrometry (ESI-MS/MS). Blood pressure was assessed with the golden-standard ambulatory technique. It is therefore doubtful that there were measurement errors in the data.

The possibility of chance ought to be taken into account in relation to the results. Statistics indicate that one out of twenty significant correlations might be due to chance, by using partial and forward multiple regression analyses. Although the results were consistent after multiple adjustments for age, body mass index, hypertensive medication, gamma glutamyl transferase, cotinine, physical activity energy expenditure, C-reactive protein, glucose, cholesterol and ambulatory systolic blood pressure, we cannot exclude residual confounding. A physiological perspective was necessary to investigate all the statistical results, which entails that all statistical significance does not necessarily indicate physiological significance.

CONCLUSION

In conclusion, we found a consistent negative association between renal function and L-citrulline in all participants, although Africans presented a more vulnerable cardiovascular profile. This negative association has only been reported previously in patients with chronic renal disease. Additionally, a gender-specific link between renal function and ADMA in African and Caucasian men was evident. Our results may indicate that in the general population, markers of NO bio-availability may be associated with early changes in renal function, accompanying elevated blood pressure.

RECOMMENDATIONS

The following is recommended for future research:

- Our study had a cross-sectional design. However, it is recommended that in future longitudinal studies should be done to confirm long-term effects of impaired NO cycle.
- The sample population should be larger to investigate the associations between markers of NO bio-availability, ABPM and end organ damage.
- Random selection of participants is important, in order to have representation by participants with low and high socio-economic status.
- Future research should include NO and L-ornithine measurements, since it is of the greatest importance in the NO-L-citrulline and urea cycle.
- Another marker, such as Cystatin-C would be useful to represent renal function more clearly.

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Appendix A

Table 1: Characteristics of the normotensive study population

	Africans (n=60)	**Caucasians (n=107)**	**P**
Age, years	41.9 ± 7.13	43.5 ± 11.4	0.32
Gender, men/women	20/40	31/76	0.56
Anthropometric measurements			
Stature, m	1.62 ± 0.08	1.71 ± 0.09	<0.001
Body mass, kg	73.2 (54.4; 99.8)	72.3 (53.6; 99.8)	0.71
Body mass index, kg/m^2	28.5 ± 6.41	25.0 ± 3.99	<0.001
Waist circumference, cm	83.7 (66.1; 104)	84.6 (68.6; 105)	0.62
Cardiovascular measurements			
Ambulatory systolic BP, mmHg	117 ± 5.31	116 ± 5.53	0.33
Ambulatory diastolic BP, mmHg	73.1 ± 4.60	71.3 ± 4.59	0.017
Ambulatory pulse pressure, mmHg	43.5 ± 4.77	44.5 ± 5.30	0.23
Night-time systolic BP, mmHg	107 ± 6.40	105 ± 7.06	0.09
Night-time diastolic BP, mmHg	63.2 ± 5.49	61.6 ± 4.79	0.050
Ambulatory heart rate, mmHg	79.2 ± 8.68	71.6 ± 9.09	<0.001
Stroke volume, mL	98.5 ± 29.0	91.6 ± 17.4	0.06
Cardiac output, L/min	6.49 ± 1.77	5.97 ± 1.42	0.041
Total peripheral resistance, mmHg/ml/s	0.95 ± 0.26	1.01 ± 0.27	0.21
Windkessel compliance, mL/mmHg	1.99 ± 0.37	2.05 ±0.47	0.41
Carotid intima media thickness, mm	0.62 ± 0.10	0.60 ± 0.11	0.15
Cornell product, mV.ms	43.7 (17.5; 104)	35.5 (13.4; 98.0)	0.05
Hypertensive, n (%)	137 (68.5)	102 (48.8)	<0.001
Basic biochemical measurements			
C-reactive protein, mg/L	4.25 (0.27; 26.3)	1.79 (0.99; 7.50)	<0.001
Glucose, mmol/L	4.87 (3.72; 6.06)	5.35 (4.60; 6.20)	<0.001
Cholesterol, mmol/L	4.20 (2.60; 6.19)	5.30 (3.60; 7.80)	<0.001
HDL cholesterol, mmol/L	1.14 (0.66; 1.72)	1.26 (0.75; 2.10)	0.040
Total cholesterol/HDL	3.81 ± 1.00	4.41 ± 1.39	0.004
Triglycerides, mmol/L	0.97 ± 0.70	0.98 ± 0.62	0.90
Serum creatinine, µmol/L	76.4 (58.8; 103)	69.2 (55.0; 93.0)	<0.001
Urinary creatinine, mmol/L	8.84 (3.06; 23.9)	8.59 (2.74; 28.0)	0.79
Markers of NO bio-availability			
L-citrulline, µmol/L	94.9 ± 26.4	91.9 ± 22.8	0.43
L-arginine, µmol/L	65.6 (12.0; 141)	50.1 (15.0; 155)	0.009
ADMA, mmol/L	0.62 (0.41; 1.00)	0.66 (0.41; 0.90)	0.13
SDMA, mmol/L	0.40 ± 0.12	0.51 ±0.10	<0.001
Reactive oxygen species, Units^1	91.1 (50.9; 157)	87.9 (57.8; 145)	0.47
FRAP, µM	366 ± 57.5	419 ± 109	<0.001
Glutathione, µM	908 ± 171	795 ± 170	<0.001
Renal function			
Albumin-to-creatinine ratio, mg/mmol	0.86 (0.31; 2.61)	0.39 (0.11; 2.11)	<0.001
Estimated creatinine clearance, mL/min	103 (64.3; 145)	109 (75.0; 174)	0.18
Medication use			
Anti-hypertensive medication, n (%)	8 (13.3)	7 (6.54)	0.14

	Africans (n=60)	**Caucasians (n=107)**	**P**
Statin, n (%)	0 (0.00)	5 (4.67)	0.09
Anti-inflammatory medication, n (%)	3 (5.00)	6 (5.61)	0.87
Lifestyle			
Physical activity, kcal	2400 (1700; 3313)	2626 (1780; 3946)	0.06
Physical activity index, n (%)			
Low	46 (76.7)	49 (45.8)	<0.001
Moderate	13 (21.7)	41 (38.3)	<0.001
Vigorous	1 (1.67)	17 (15.9)	<0.001
Cotinine, ng/mL	0.02 (0.00; 174)	0.00 (0.00; 144)	0.07
Current smoking, n (%)	4 (6.67)	17 (15.9)	0.08
Gamma glutamyl transferase, U/L	45.4 (16.7; 89.8)	14.6 (6.00; 144)	<0.001
Current drinking, n (%)	9 (15.0)	49 (45.8)	<0.001

Values are arithmetic mean ± SD, geometric mean (5th to 95th percentile interval). [1](1 unit=1.0mg/L H_2O_2). BP, blood pressure; HDL-C, High-density lipoprotein cholesterol; ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine; FRAP, Ferric reducing antioxidant power.

Table 2: Characteristics of the hypertensive study population

	Africans (n=121)	Caucasians (n=102)	P
Age, years	45.5 ± 8.48	46.4 ± 10.1	0.46
Gender, men/women	68/53	70/32	0.06
Anthropometric measurements			
Stature, m	1.65 ± 0.09	1.76 ± 0.10	<0.001
Body mass, kg	83.2 (57.2; 120)	92.0 (64.1; 137)	<0.001
Body mass index, kg/m^2	31.2 ± 7.25	30.3 ± 6.42	0.33
Waist circumference, cm	97.2 (76.5; 128)	99.6 (77.8; 131)	0.24
Cardiovascular measurements			
Ambulatory systolic BP, mmHg	141 ± 13.9	132 ± 10.8	<0.001
Ambulatory diastolic BP, mmHg	88.2 ± 9.70	82.2 ± 7.07	<0.001
Ambulatory pulse pressure, mmHg	53.0 ± 9.24	50.5 ± 8.02	0.035
Night-time systolic BP, mmHg	132 ± 15.6	122 ± 13.7	<0.001
Night-time diastolic BP, mmHg	79.1 ± 11.2	71.7 ± 8.85	<0.001
Ambulatory heart rate, mmHg	79.9 ± 11.7	75.7 ± 10.8	0.006
Stroke volume, mL	103 ± 27.5	104 ± 28.5	0.82
Cardiac output, L/min	7.00 ± 1.88	6.91 ± 2.28	0.72
Total peripheral resistance, mmHg/ml/s	1.04 ± 0.43	1.07 ± 0.69	0.76
Windkessel compliance, mL/mmHg	1.81 ± 0.43	2.14 ±0.57	<0.001
Carotid intima media thickness, mm	0.71 ± 0.13	0.69 ± 0.12	0.11
Cornell product, mV.ms	66.3 (25.2; 157)	50.0 (16.6; 109)	<0.001
Hypertensive, n (%)	137 (68.5)	102 (48.8)	<0.001
Basic biochemical measurements			
C-reactive protein, mg/L	4.78 (0.67; 33.8)	2.31 (0.99; 9.00)	<0.001
Glucose, mmol/L	5.70 (4.26; 11.7)	5.92 (5.00; 7.40)	0.24
Cholesterol, mmol/L	4.64 (3.19; 6.51)	5.51 (4.10; 8.10)	<0.001
HDL cholesterol, mmol/L	1.07 (0.66; 1.70)	1.03 (0.61; 1.79)	0.34
Total cholesterol/HDL	4.74 ± 2.40	5.60 ± 1.62	0.002
Triglycerides, mmol/L	1.62 ± 1.47	1.42 ± 0.83	0.20
Serum creatinine, µmol/L	73.3 (53.8; 95.2)	76.2 (53.0; 103)	<0.001
Urinary creatinine, mmol/L	8.14 (2.85; 20.4)	12.6 (3.67; 33.0)	<0.001
Markers of NO bio-availability			
L-citrulline, µmol/L	102 ± 26.6	97.0 ± 24.4	0.12
L-arginine, µmol/L	68.7 (20.7; 138)	56.6 (25.0; 128)	0.017
ADMA, mmol/L	0.65 (0.46; 0.95)	0.69 (0.51; 1.01)	0.06
SDMA, mmol/L	0.43 ± 0.12	0.56 ±0.13	<0.001
Reactive oxygen species, Units[1]	91.9 (57.1; 142)	85.5 (56.9; 130)	0.042
FRAP, µM	400 ± 78.1	455 ± 104	<0.001
Glutathione, µM	905 ± 191	846 ± 174	0.019
Renal function			
Albumin-to-creatinine ratio, mg/mmol	1.25 (0.38; 5.80)	0.28 (0.09; 1.54)	<0.001
Estimated creatinine clearance, mL/min	122 (78.7; 195)	131 (72.6; 233)	0.08
Medication use			
Anti-hypertensive medication, n (%)	32 (26.5)	11 (10.8)	0.003

	Africans (n=121)	**Caucasians (n=102)**	**P**
Statin, n (%)	2 (1.65)	4 (3.92)	0.30
Anti-inflammatory medication, n (%)	10 (8.26)	6 (5.88)	0.49
Lifestyle			
Physical activity, kcal	2672 (1747; 4170)	3314 (2307; 4679)	<0.001
Physical activity index, n (%)			
Low	87 (71.9)	55 (54.5)	0.03
Moderate	27 (22.3)	37 (36.6)	0.03
Vigorous	7 (5.79)	9 (8.91)	0.03
Cotinine, ng/mL	0.03 (0.00; 145)	0.01 (0.00; 243)	0.007
Current smoking, n (%)	25 (20.7)	12 (11.9)	0.08
Gamma glutamyl transferase, U/L	53.3 (20.5; 188)	25.9 (10.0; 90.0)	<0.001
Current drinking, n (%)	35 (28.9)	53 (52.5)	<0.001

Values are arithmetic mean ± SD, geometric mean (5th to 95th percentile interval). [1](1 unit=1.0mg/L H_2O_2). BP, blood pressure; HDL-C, High-density lipoprotein cholesterol; ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine; FRAP, Ferric reducing antioxidant power.

Table 3: Characteristics of the male study population

	Africans (n=88)	**Caucasians (n=101)**	**P**
Age, years	43.1 ± 8.24	45.0 ± 11.1	0.19
Anthropometric measurements			
Stature, m	1.70 ± 0.06	1.81 ± 0.07	<0.001
Body mass, kg	78.6 (56.9; 113)	93.8 (69.3; 135)	<0.001
Body mass index, kg/m^2	27.6 ± 5.75	29.0 ± 5.20	0.07
Waist circumference, cm	92.3 (70.0; 119)	101 (80.9; 131)	<0.001
Cardiovascular measurements			
Ambulatory systolic BP, mmHg	137 ± 16.6	128 ± 10.4	<0.001
Ambulatory diastolic BP, mmHg	87.8 ± 11.2	79.5 ± 7.44	<0.001
Ambulatory pulse pressure, mmHg	49.6 ± 8.56	48.4 ± 6.98	0.27
Night-time systolic BP, mmHg	128 ± 18.7	117 ± 11.6	<0.001
Night-time diastolic BP, mmHg	78.5 ± 12.8	68.6 ± 8.28	<0.001
Ambulatory heart rate, mmHg	78.9 ± 11.4	72.0 ± 11.1	<0.001
Stroke volume, mL	100 ± 26.0	103 ± 20.3	0.42
Cardiac output, L/min	6.60 ± 1.76	6.56 ± 1.82	0.88
Total peripheral resistance, mmHg/ml/s	1.08 ± 0.32	1.07 ± 0.68	0.86
Windkessel compliance, mL/mmHg	1.87 ± 0.43	2.32 ± 0.52	<0.001
Carotid intima media thickness, mm	0.70 ± 0.15	0.68 ± 0.12	0.35
Cornell product, mV.ms	69.4 (24.6; 170)	58.3 (28.2; 119)	0.032
Hypertensive, n (%)	68 (77.3)	70 (69.3)	0.28
Basic biochemical measurements			
C-reactive protein, mg/L	2.75 (0.27; 16.1)	1.80 (0.99; 8.00)	0.003
Glucose, mmol/L	5.82 (4.47; 11.7)	5.92 (5.00; 7.40)	0.57
Cholesterol, mmol/L	4.07 (3.34; 6.50)	5.46 (4.00; 8.10)	<0.001
HDL cholesterol, mmol/L	1.02 (0.59; 1.64)	0.96 (0.61; 1.43)	0.22
Total cholesterol/HDL	5.05 ± 2.57	5.88 ± 1.49	0.006
Triglycerides, mmol/L	1.81 ± 1.67	1.51 ± 0.87	0.11
Serum creatinine, µmol/L	76.3 (55.1; 102)	83.0 (68.0; 107)	<0.001
Urinary creatinine, mmol/L	9.34 (5.06; 21.1)	15.0 (6.41; 33.0	<0.001
Markers of NO bio-availability			
L-citrulline, µmol/L	109 ± 24.7	101 ± 25.1	0.020
L-arginine, µmol/L	73.3 (24.0; 136)	57.0 (24.0; 149)	0.002
ADMA, mmol/L	0.60 (0.43; 0.88)	0.69 (0.49; 1.01)	<0.001
SDMA, mmol/L	0.43 ± 0.13	0.57 ± 0.12	<0.001
Reactive oxygen species, Units[1]	80.5 (50.9; 114)	75.2 (55.2; 106)	0.034
FRAP, µM	421 ± 72.4	503 ± 89.0	<0.001
Glutathione, µM	950 ± 189	859 ± 180	<0.001
Renal function			
Albumin-to-creatinine ratio, mg/mmol	1.06 (0.34; 7.80)	0.23 (0.08; 1.15)	<0.001
Estimated creatinine clearance, mL/min	122 (79.2; 182)	131 (87.9; 217)	0.07
Medication use			
Anti-hypertensive medication, n (%)	17 (19.3)	9 (8.91)	0.038

	Africans (n=88)	**Caucasians (n=101)**	**P**
Statin, n (%)	1 (1.14)	6 (5.94)	0.08
Anti-inflammatory medication, n (%)	6 (6.82)	1 (0.99)	0.034
Lifestyle			
Physical activity, kcal	2585 (1725; 4021)	3478 (2547; 4679)	<0.001
Physical activity index, n (%)			
Low	63 (71.6)	50 (50.0)	0.01
Moderate	20 (22.7)	35 (35.0)	0.01
Vigorous	5 (5.68)	15 (15.0)	0.01
Cotinine, ng/mL	0.05 (0.00; 151)	0.01 (0.00; 243)	0.010
Current smoking, n (%)	26 (29.6)	16 (15.8)	0.02
Gamma glutamyl transferase, U/L	62.3 (23.6; 280)	27.3 (11.0; 90.0)	<0.001
Current drinking, n (%)	33 (37.5)	55 (54.5)	0.020

Values are arithmetic mean ± SD, geometric mean (5th to 95th percentile interval). [1](1 unit=1.0mg/L H_2O_2). BP, blood pressure; HDL-C, High-density lipoprotein cholesterol; ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine; FRAP, Ferric reducing antioxidant power.

Table 4: Characteristics of the female study population

	Africans (n=93)	Caucasians (n=108)	P
Age, years	45.5 ± 8.04	44.9 ± 10.7	0.67
Anthropometric measurements			
Stature, m	1.58 ± 0.06	1.67 ± 0.06	<0.001
Body mass, kg	80.9 (54.9; 121)	71.2 (53.6; 105)	<0.001
Body mass index, kg/m^2	33.0 ± 7.26	26.3 ± 6.29	<0.001
Waist circumference, cm	92.6 (68.8; 123)	84.0 (68.4; 112)	<0.001
Cardiovascular measurements			
Ambulatory systolic BP, mmHg	129 ± 15.4	121 ± 12.4	<0.001
Ambulatory diastolic BP, mmHg	78.8 ± 8.87	73.9 ± 7.67	<0.001
Ambulatory pulse pressure, mmHg	50.1 ± 9.79	46.6 ± 7.71	0.005
Night-time systolic BP, mmHg	119 ± 15.0	110 ± 14.7	<0.001
Night-time diastolic BP, mmHg	69.2 ± 9.75	64.6 ± 8.65	<0.001
Ambulatory heart rate, mmHg	80.5 ± 10.1	75.2 ± 8.95	<0.001
Stroke volume, mL	103 ± 29.8	92.8 ± 26.7	0.012
Cardiac output, L/min	7.05 ± 1.93	6.13 ± 2.05	0.010
Total peripheral resistance, mmHg/ml/s	0.95 ± 0.43	1.01 ± 0.30	0.25
Windkessel compliance, mL/mmHg	1.87 ± 0.42	1.88 ± 0.44	0.85
Carotid intima media thickness, mm	0.67 ± 0.11	0.61 ± 0.11	<0.001
Cornell product, mV.ms	50.0 (18.7; 111)	30.0 (10.3; 78.4)	<0.001
Hypertensive, n (%)	53 (57.0)	32 (29.6)	<0.001
Basic biochemical measurements			
C-reactive protein, mg/L	7.56 (0.81; 35.7)	2.26 (0.99; 14.3)	<0.001
Glucose, mmol/L	5.06 (3.69; 6.89)	5.34 (4.60; 6.40)	0.032
Cholesterol, mmol/L	4.31 (2.60; 6.27)	5.34 (3.60; 8.00)	<0.001
HDL cholesterol, mmol/L	1.17 (0.74; 1.74)	1.34 (0.78; 2.26)	0.001
Total cholesterol/HDL	3.85 ± 1.26	4.16 ± 1.26	0.08
Triglycerides, mmol/L	1.03 ± 0.61	0.90 ± 0.48	0.11
Serum creatinine, µmol/L	72.3 (54.0; 95.2)	63.9 (51.0; 84.0)	<0.001
Urinary creatinine, mmol/L	7.54 (2.55; 20.4)	7.33 (2.74; 21.4)	0.76
Markers of NO bio-availability			
L-citrulline, µmol/L	90.9 ± 25.5	88.3 ± 20.6	0.43
L-arginine, µmol/L	62.6 (16.0; 141)	50.2 (15.0; 125)	0.016
ADMA, mmol/L	0.68 (0.64; 1.05)	0.66 (0.46; 0.90)	0.047
SDMA, mmol/L	0.41 ± 0.11	0.50 ± 0.11	<0.001
Reactive oxygen species, Units^1	104 (63.1; 157)	99.2 (66.9; 149)	0.24
FRAP, µM	358 ± 60.6	376 ± 86.2	0.08
Glutathione, µM	863 ± 171	784 ± 159	0.001
Renal function			
Albumin-to-creatinine ratio, mg/mmol	1.14 (0.36; 3.81)	0.47 (0.13; 2.27)	<0.001
Estimated creatinine clearance, mL/min	110 (64.3; 192)	110 (72.0; 184)	0.95
Medication use			
Anti-hypertensive medication, n (%)	23 (24.7)	9 (8.33)	0.002

	Africans (n=93)	Caucasians (n=108)	P
Statin, n (%)	1 (1.08)	3 (2.78)	0.39
Anti-inflammatory medication, n (%)	7 (7.53)	11 (10.2)	0.51
Lifestyle			
Physical activity, kcal	2574 (1700; 4257)	2516 (1780; 3962)	0.53
Physical activity index, n (%)			
Low	70 (75.3)	54 (50.0)	<0.001
Moderate	20 (21.5)	43 (39.8)	<0.001
Vigorous	3 (3.23)	11 (10.2)	<0.001
Cotinine, ng/mL	0.01 (0.00; 145)	0.00 (0.00; 165)	0.023
Current smoking, n (%)	3 (3.23)	13 (12.2)	0.020
Gamma glutamyl transferase, U/L	35.3 (16.7; 117)	13.9 (6.00; 39.0)	<0.001
Current drinking, n (%)	11 (11.8)	47 (43.9)	<0.001

Values are arithmetic mean ± SD, geometric mean (5th to 95th percentile interval). [1](1 unit=1.0mg/L H_2O_2). BP, blood pressure; HDL-C, High-density lipoprotein cholesterol; ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine; FRAP, Ferric reducing antioxidant power.

Table 5: Characteristics of the African study population

	Male (n=88)	**Female (n=93)**	**P**
Age, years	43.1 ± 8.24	45.5 ± 8.04	0.047
Anthropometric measurements			
Stature, m	1.70 ± 0.06	1.58 ± 0.06	<0.001
Body mass, kg	78.6 (56.9; 113)	80.9 (54.9; 121)	0.37
Body mass index, kg/m^2	27.6 ± 5.75	33.0 ± 7.26	<0.001
Waist circumference, cm	92.3 (70.0; 119)	92.6 (68.8; 123)	0.90
Cardiovascular measurements			
Ambulatory systolic BP, mmHg	137 ± 16.6	129 ± 15.4	<0.001
Ambulatory diastolic BP, mmHg	87.8 ± 11.2	78.8 ± 8.87	<0.001
Ambulatory pulse pressure, mmHg	49.6 ± 8.56	50.1 ± 9.79	0.70
Night-time systolic BP, mmHg	128 ± 18.7	119 ± 15.0	<0.001
Night-time diastolic BP, mmHg	78.5 ± 12.8	69.2 ± 9.75	<0.001
Ambulatory heart rate, mmHg	78.9 ± 11.4	80.5 ± 10.1	0.31
Stroke volume, mL	100 ± 26.0	103 ± 29.8	0.51
Cardiac output, L/min	6.60 ± 1.76	7.05 ± 1.93	0.11
Total peripheral resistance, mmHg/ml/s	1.08 ± 0.32	0.95 ± 0.43	0.026
Windkessel compliance, mL/mmHg	1.87 ± 0.43	1.87 ± 0.42	0.92
Carotid intima media thickness, mm	0.70 ± 0.15	0.67 ± 0.11	0.14
Cornell product, mV.ms	69.4 (24.6; 170)	50.0 (18.7; 111)	<0.001
Hypertensive, n (%)	68 (77.3)	53 (57.0)	0.004
Basic biochemical measurements			
C-reactive protein, mg/L	2.75 (0.27; 16.1)	7.56 (0.81; 35.7)	<0.001
Glucose, mmol/L	5.82 (4.47; 11.7)	5.06 (3.69; 6.89)	<0.001
Cholesterol, mmol/L	4.07 (3.34; 6.50)	4.31 (2.60; 6.27)	0.024
HDL cholesterol, mmol/L	1.02 (0.59; 1.64)	1.17 (0.74; 1.74)	0.002
Total cholesterol/HDL	5.05 ± 2.57	3.85 ± 1.26	<0.001
Triglycerides, mmol/L	1.81 ± 1.67	1.03 ± 0.61	<0.001
Serum creatinine, µmol/L	76.3 (55.1; 102)	72.3 (54.0; 95.2)	0.016
Urinary creatinine, mmol/L	9.34 (5.06; 21.1)	7.54 (2.55; 20.4)	0.0015
Markers of NO bio-availability			
L-citrulline, µmol/L	109 ± 24.7	90.9 ± 25.5	<0.001
L-arginine, µmol/L	73.3 (24.0; 136)	62.6 (16.0; 141)	0.09
ADMA, mmol/L	0.60 (0.43; 0.88)	0.68 (0.64; 1.05)	0.005
SDMA, mmol/L	0.43 ± 0.13	0.41 ± 0.11	0.13
Reactive oxygen species, Units[1]	80.5 (50.9; 114)	104 (63.1; 157)	<0.001
FRAP, µM	421 ± 72.4	358 ± 60.6	<0.001
Glutathione, µM	950 ± 189	863 ± 171	0.001
Renal function			
Albumin-to-creatinine ratio, mg/mmol	1.06 (0.34; 7.80)	1.14 (0.36; 3.81)	0.59
Estimated creatinine clearance, mL/min	122 (79.2; 182)	110 (64.3; 192)	0.009
Medication use			
Anti-hypertensive medication, n (%)	17 (19.3)	23 (24.7)	0.38

	Male (n=88)	**Female (n=93)**	**P**
Statin, n (%)	1 (1.14)	1 (1.08)	0.97
Anti-inflammatory medication, n (%)	6 (6.82)	7 (7.53)	0.85
Lifestyle			
Physical activity, kcal	2585 (1725; 4021)	2574 (1700; 4257)	0.92
Physical activity index, n (%)			
Low	63 (71.6)	70 (75.3)	0.69
Moderate	20 (22.7)	20 (21.5)	0.69
Vigorous	5 (5.68)	3 (3.23)	0.69
Cotinine, ng/mL	0.05 (0.00; 151)	0.01 (0.00; 145)	0.07
Current smoking, n (%)	26 (29.6)	3 (3.23)	<0.001
Gamma glutamyl transferase, U/L	62.3 (23.6; 280)	35.3 (16.7; 117)	<0.001
Current drinking, n (%)	33 (37.5)	11 (11.8)	<0.001

Values are arithmetic mean ± SD, geometric mean (5th to 95th percentile interval). [1](1 unit=1.0mg/L H_2O_2). BP, blood pressure; HDL-C, High-density lipoprotein cholesterol; ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine; FRAP, Ferric reducing antioxidant power.

Table 6: Characteristics of the Caucasian study population

	Male (n=101)	**Female (n=108)**	**P**
Age, years	45.0 ± 11.1	44.9 ± 10.7	0.96
Anthropometric measurements			
Stature, m	1.81 ± 0.07	1.67 ± 0.06	<0.001
Body mass, kg	93.8 (69.3; 135)	71.2 (53.6; 105)	<0.001
Body mass index, kg/m^2	29.0 ± 5.20	26.3 ± 6.29	<0.001
Waist circumference, cm	101 (80.9; 131)	84.0 (68.4; 112)	<0.001
Cardiovascular measurements			
Ambulatory systolic BP, mmHg	128 ± 10.4	121 ± 12.4	<0.001
Ambulatory diastolic BP, mmHg	79.5 ± 7.44	73.9 ± 7.67	<0.001
Ambulatory pulse pressure, mmHg	48.4 ± 6.98	46.6 ± 7.71	0.09
Night-time systolic BP, mmHg	117 ± 11.6	110 ± 14.7	<0.001
Night-time diastolic BP, mmHg	68.6 ± 8.28	64.6 ± 8.65	<0.001
Ambulatory heart rate, mmHg	72.0 ± 11.1	75.2 ± 8.95	0.021
Stroke volume, mL	103 ± 20.3	92.8 ± 26.7	0.003
Cardiac output, L/min	6.56 ± 1.82	6.13 ± 2.05	0.35
Total peripheral resistance, mmHg/ml/s	1.07 ± 0.68	1.01 ± 0.30	0.45
Windkessel compliance, mL/mmHg	2.32 ± 0.52	1.88 ± 0.44	<0.001
Carotid intima media thickness, mm	0.68 ± 0.12	0.61 ± 0.11	<0.001
Cornell product, mV.ms	58.3 (28.2; 119)	30.0 (10.3; 78.4)	<0.001
Hypertensive, n (%)	70 (69.3)	32 (29.6)	<0.001
Basic biochemical measurements			
C-reactive protein, mg/L	1.80 (0.99; 8.00)	2.26 (0.99; 14.3)	0.047
Glucose, mmol/L	5.92 (5.00; 7.40)	5.34 (4.60; 6.40)	<0.001
Cholesterol, mmol/L	5.46 (4.00; 8.10)	5.34 (3.60; 8.00)	0.49
HDL cholesterol, mmol/L	0.96 (0.61; 1.43)	1.34 (0.78; 2.26)	<0.001
Total cholesterol/HDL	5.88 ± 1.49	4.16 ± 1.26	<0.001
Triglycerides, mmol/L	1.51 ± 0.87	0.90 ± 0.48	<0.001
Serum creatinine, µmol/L	83.0 (68.0; 107)	63.9 (51.0; 84.0)	0.38
Urinary creatinine, mmol/L	15.0 (6.41; 33.0	7.33 (2.74; 21.4)	<0.001
Markers of NO bio-availability			
L-citrulline, µmol/L	101 ± 25.1	88.3 ± 20.6	<0.001
L-arginine, µmol/L	57.0 (24.0; 149)	50.2 (15.0; 125)	0.13
ADMA, mmol/L	0.69 (0.49; 1.01)	0.66 (0.46; 0.90)	0.15
SDMA, mmol/L	0.57 ± 0.12	0.50 ± 0.11	<0.001
Reactive oxygen species, Units[1]	75.2 (55.2; 106)	99.2 (66.9; 149)	<0.001
FRAP, µM	503 ± 89.0	376 ± 86.2	<0.001
Glutathione, µM	859 ± 180	784 ± 159	0.002
Renal function			
Albumin-to-creatinine ratio, mg/mmol	0.23 (0.08; 1.15)	0.47 (0.13; 2.27)	<0.001
Estimated creatinine clearance, mL/min	131 (87.9; 217)	110 (72.0; 184)	<0.001
Medication use			
Anti-hypertensive medication, n (%)	9 (8.91)	9 (8.33)	<0.001

	Male (n=101)	**Female (n=108)**	**P**
Statin, n (%)	6 (5.94)	3 (2.78)	0.039
Anti-inflammatory medication, n (%)	1 (0.99)	11 (10.2)	0.37
Lifestyle			
Physical activity, kcal	3478 (2547; 4679)	2516 (1780; 3962)	<0.001
Physical activity index, n (%)			
Low	50 (50.0)	54 (50.0)	<0.001
Moderate	35 (35.0)	43 (39.8)	<0.001
Vigorous	15 (15.0)	11 (10.2)	<0.001
Cotinine, ng/mL	0.01 (0.00; 243)	0.00 (0.00; 165)	0.12
Current smoking, n (%)	16 (15.8)	13 (12.2)	0.39
Gamma glutamyl transferase, U/L	27.3 (11.0; 90.0)	13.9 (6.00; 39.0)	<0.001
Current drinking, n (%)	55 (54.5)	47 (43.9)	<0.001

Values are arithmetic mean ± SD, geometric mean (5th to 95th percentile interval). [1](1 unit=1.0mg/L H_2O_2). BP, blood pressure; HDL-C, High-density lipoprotein cholesterol; ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine; FRAP, Ferric reducing antioxidant power.

Table 7: Unadjusted correlations of age, blood pressure with markers of NO bio-availability

	L-arginine	**L-citrulline**	**ADMA**	**SDMA**
African men (N= 74)				
Age			0.15^2	0.22^1
Systolic BP, mmHg			0.18^2	
Diastolic BP, mmHg				
Day-time systolic BP, mmHg				
Day-time diastolic BP, mmHg				
African women (N=75)				
Age		0.42^1		
Systolic BP, mmHg				
Diastolic BP, mmHg				
Day-time systolic BP, mmHg				
Day-time diastolic BP, mmHg				
Caucasian men (N=81)				
Age				
Systolic BP, mmHg	0.33^1		0.32^1	
Diastolic BP, mmHg	0.25^1		0.28^1	
Day-time systolic BP, mmHg	0.32^1		0.28^1	
Day-time diastolic BP, mmHg	0.21^1		0.20^2	
Caucasian women (N=85)				
Age	0.23^1	0.41^1	0.16^2	0.32^1
Systolic BP, mmHg		0.19^1		0.18^2
Diastolic BP, mmHg				
Day-time systolic BP, mmHg		0.18^2		0.20^1
Day-time diastolic BP, mmHg				

$^1p<0.05$ and $^2p=0.05\text{-}0.10$. ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; BP, blood pressure.