# Nitric oxide bioavailability and cardiovascular function in children and young adults

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Thesis accepted in fulfilment of the requirements for the degree Doctor of Philosophy in Sciences Physiology at the North-West University

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## Preface

The article format of this thesis was chosen and approved by the North-West University. This thesis consists of an extensive literature overview and motivation, methodology, three manuscripts (two published and one under review at a peer review journal), and a concluding chapter, which summarises the main findings of this study and recommendations for future studies.

**The layout of the thesis is as follows:**

Chapter I: Literature review, motivation, aims, objectives and hypotheses

Chapter II: Methodology

Chapter III: Manuscript 1 – Nitric oxide-related markers link inversely with blood pressure in black boys and men: The ASOS and African-PREDICT studies
Published in the journal *Amino Acids*

Chapter IV: Manuscript 2 – Central systolic blood pressure relates inversely to nitric oxide synthesis in young black adults: The African-PREDICT study

Chapter V: Manuscript 3 – Urinary albumin-to-creatinine ratio is inversely related to nitric oxide synthesis in young black adults: The African-PREDICT study
Published in the journal *Hypertension Research*

Chapter VI Summary of main findings

The references are provided at the end of each chapter according to the Vancouver reference style (chapters I – VI).

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**Contributions of the authors of this thesis**

The following researchers contributed to the thesis:

**Miss A Craig** Responsible for compiling the background and motivation, literature review, design and planning of the research manuscripts, statistical analyses, interpretation of results and inscription of all sections forming this thesis.

**Prof R Kruger** Promoter of the thesis and principal investigator of the ASOS study. Responsible for intellectual and technical input, evaluation of statistical analyses, design and planning the research manuscripts and thesis.

**Prof CMC Mels** Co-promoter of the thesis and local principal investigator of the African-PREDICT study. Responsible for intellectual and technical input, evaluation of statistical analyses, design and planning the research manuscripts and thesis.

**Prof AE Schutte** Co-promoter of the thesis and principal investigator of the African-PREDICT study. Responsible for intellectual and technical interpretation of data, guidance through statistical analyses, initial planning the research manuscripts and thesis.

The following statement from the researchers confirms their individual involvement in this study and gives their permission that the relevant research manuscript(s) may form part of this thesis.

Hereby, I declare that I approved the abovementioned thesis and that my role in this study (as stated above) is representative of my contribution towards the research manuscript(s) and supervised PhD study. I also give my consent that the research manuscript(s) may be published as part of the thesis of Ms Ashleigh Craig.

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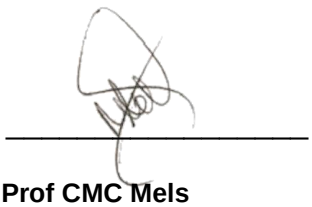
*Promoter*



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**Additional contributors to this thesis**

The following researchers contributed to the prevailing manuscripts that make up chapters III, IV and V of this thesis:

**Prof. Dr. rer. nat. D Tsikas** Co-author of chapters III, IV and V. Responsible for the analyses of the urinary nitric oxide-related data in both the ASOS and African-PREDICT studies that was used in compiling the manuscripts of this thesis.

**Prof. Dr. rer. nat. E Schwedhelm** Co-author of chapters IV and V. Responsible for the analyses of the plasma nitric oxide-related data in the African-PREDICT study that was used in compiling the manuscripts of this thesis.

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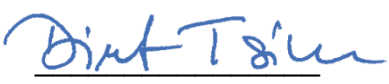
**Dr. rer. nat. A Bollenbach** Co-author of chapter V.

The following statement from the co-authors confirms their individual involvement in co-authoring the manuscripts that make up this thesis and give their permission that the relevant research manuscript(s) may form part of this thesis. I also give my consent that the research manuscript(s) may be published as part of the thesis of Ms Ashleigh Craig.



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**Chapter II: Methodology**

## Summary

### Background and motivation

The global burden of cardiovascular disease (CVD) including hypertension is ever-increasing, especially in the South African context. Nitric oxide (NO) plays a vital role in normal vascular endothelial function with considerable evidence of imbalanced NO bioavailability in the elderly and diseased states such as hypertension. Endothelial dysfunction with attenuated NO bioavailability is central to the pathogenesis of CVD and has been implicated as a possible mechanism in the premature development of hypertension. Due to the prevalence of hypertension being the highest in Sub-Saharan Africa, the need for early identification and risk stratification of vascular abnormalities in arterial hypertension is paramount, especially in understudied black populations.

Due to the nature of NO, measuring markers involved in its bioavailability as possible indicators of a NO profile is warranted. Moreover, further understanding the interactions of NO-related markers with endothelial function may impact the progression of CVD. Conversely, there is limited data surrounding the NO profile and the possible pathophysiological roles thereof in young healthy black and white populations respectively. Therefore, exploring whether an unfavourable NO profile plays a pivotal role in the development of CVD is warranted, especially in the black population who seem to be predisposed to CVD.

### Aim

The aim of this study was to explore the associations of NO-related markers with cardiovascular structure and function in apparently healthy children and young adults. The study also aimed to determine whether the NO profile differed among groups stratified by age, sex and ethnicity and if there are any associations of plasma and urinary NO-related markers with blood pressure (BP), arterial structure and endothelial function in black and white South Africans.

### Methodology

This thesis included data from the Arterial Stiffness in Offspring Study (ASOS) and the African Prospective study on the Early Detection and Identification of Cardiovascular Disease and Hypertension (African-PREDICT). These studies were cross-sectional and included black and white children (ASOS: *n*=81) and young adults (African-PREDICT *n*=1202).

Anthropometric procedures included body height (cm), body weight (kg), waist and hip circumference (cm). Additionally, body mass index (BMI) was calculated.

Biochemical analyses were performed where urinary arginine, homoarginine, asymmetric (ADMA) and symmetric dimethylarginine (SDMA) as well as ornithine/citrulline, malondialdehyde (MDA), creatinine, nitrite and nitrate were determined in both the ASOS and African-PREDICT studies (gas chromatography-mass spectrometry (GC-MS)). The urinary nitrate-to-nitrite ratio ($U_{NOx}R$) was additionally calculated. Plasma arginine, homoarginine, ADMA and SDMA were determined in the African-PREDICT study only (liquid chromatography-tandem mass spectrometry (LC-MS/MS)).

Additional biochemical analyses were performed in the African-PREDICT study which included the lipid profile (total cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and triglycerides), gamma glutamyltransferase, creatinine and high sensitivity C-reactive protein (Cobas Integra 400 plus Roche, Basel Switzerland). Serum cotinine levels were determined with a chemiluminescence method on the Immulite (Siemens, Erlangen, Germany). Creatine kinase (CK) was determined with electrochemiluminescence method on the E411 (Roche, Basel Switzerland). Sodium fluoride plasma glucose (Siemens, Erlangen, Germany) and EDTA whole blood glycated haemoglobin was determined (Cobas Integra 400 plus Roche, Basel Switzerland). Urinary albumin (mg/L) and creatinine (mmol/L) were determined (Cobras Integra® 400plus, Roche, Basel, Switzerland) and the urinary albumin-to-creatinine ratio (uACR) was calculated. Furthermore, the Chronic Kidney Disease Epidemiology (CKD-EPI) formula was utilised to calculate the estimated glomerular filtration rate (eGFR) from serum creatinine values.

Cardiovascular measures in the ASOS study included brachial office BP using the Omron HEM-759-E (750IT) device (Omron Healthcare, Tokyo, Japan) and carotid intima media thickness (cIMT) using B mode ultrasonography (SonoSite MicroMaxx, Bothell, WA). Likewise, the cardiovascular measures in the African-PREDICT study included brachial office BP (Dinamap® ProCare 100 Vital Signs Monitor (GE Medical Systems, Milwaukee, USA)), 24-hour ambulatory BP (CardioXplore, Meditech, Budapest, Hungary, British Hypertension Society (BHS) validated), cIMT (General Electric Vivid E9 device; GE Vingmed Ultrasound A/S, Horten, Norway), central BP (central systolic blood pressure (cSBP)) and pulse wave velocity (PWV) (SphygmoCor XCEL device (AtCor Medical Pty. Ltd., Sydney, New South Wales, Australia).

Statistical analyses included independent T-tests to compare means and chi-square to compare proportions between the groups. Pearson, partial and multiple regression analyses

were performed and adjusted for potential confounders to investigate the associations of both plasma and urinary NO-related markers with cardiovascular and biochemical markers according to the specified focus of each research manuscript.

**Results of each research manuscript**

The objective of the first manuscript (Chapter III) was to compare NO-related markers in plasma and urine between young black and white boys and men and determine whether these markers associated with cardiovascular measures such as BP and cIMT. Black boys and men presented with lower urinary nitrate and $U_{NOx}R$ levels (all $p \leq 0.003$) when compared to their white counterparts. The partial and multivariate regression analyses showed an independent inverse association of diastolic BP in the black boys ($p=0.030$), and systolic BP in black men ($p=0.036$) with urinary nitrate. Carotid intima media thickness associated inversely with $U_{NOx}R$ in the black men ($p=0.023$), but not in the boys. These results suggest that already at young ages, NO bioavailability associates with higher BP in black individuals only.

The second manuscript (Chapter IV) compared NO-related markers in plasma and urine between black and white men and women, along with the NO-related associations with central BP (cSBP) and arterial stiffness (PWV) within these groups. The black men and women had higher cSBP and higher plasma arginine and ADMA, but lower urinary nitrate and $U_{NOx}R$ (all $p \leq 0.003$) than their white counterparts. Multiple regression analyses showed that cSBP associated inversely with plasma homoarginine ($p= 0.006$) in black men and with $U_{NOx}R$ in black women ($p= 0.029$), but positively with urinary ADMA ($p= 0.015$) in white women. Pulse wave velocity associated inversely with plasma ADMA ($p= 0.024$) in the white women. The results indicated that NO synthesis is lower in the black cohort who also had higher cSBP. These results suggest the potential increased risk of the black group for future large artery stiffness and hypertension development.

Since CK may be sensitive to attenuated arginine bioavailability, in the third manuscript (Chapter V), we compared the NO profile in plasma and urine and plasma CK levels between young black and white adults. We also determined the NO-related associations with a marker of systemic endothelial function (uACR). The black group presented with an overall less favourable NO profile as indicated by lower urinary nitrate and $U_{NOx}R$ levels and higher plasma and urinary ADMA. Additionally, the black group also had higher CK and malondialdehyde levels (a biomarker of kidney-associated oxidative stress) when compared to the white group. In multiple regression analysis, uACR associated inversely with both plasma ($p=0.005$) and urinary ($p=0.010$) homoarginine, as well as $U_{NOx}R$ ($p=0.031$) in the black group. The adverse

associations between NO and uACR, suggest that this young black group may already be subjected to early onset endothelial dysfunction.

**Final conclusion**

This study showed that at young ages, in both children and young adults, black individuals are already subjected to early onset CVD related to high BP, arterial stiffness and endothelial dysfunction.

**Key Words:** Nitric oxide; urinary nitrate-to-nitrite ratio; ethnicity; hypertension; central systolic blood pressure; urinary-albumin-to-creatinine ratio; endothelial dysfunction

| | | |
|---|---|---|
| | **%** | Percentage |
| | **°C** | Degrees Celsius |
| | **α** | Alpha |
| | **β** | Beta |
| | **μmol/L** | Micromoles per litre |
| A | **ABPM** | Ambulatory blood pressure measurement |
| | **ADP** | Adenosine diphosphate |
| | **ADMA** | Asymmetric dimethylarginine |
| | **African-PREDICT** | African Prospective study on the Early Detection and Identification of Cardiovascular Disease and Hypertension |
| | **AGAT** | Arginine: glycine amidinotransferase |
| | **ANCOVA** | Analysis of covariance |
| | **ASOS** | Arterial Stiffness in Offspring Study |
| B | **BMI** | Body mass index |
| | **BP** | Blood pressure |
| C | **$Ca^{2+}$** | Calcium |
| | **Calmodulin** | Calcium-modulated protein |
| | **cGMP** | Cyclic guanosine monophosphate |
| | **$CH_2$** | Methylene |
| | **cIMT** | Carotid intima media thickness |
| | **CK** | Creatine kinase |
| | **CKD-EPI** | Chronic Kidney Disease Epidemiology |
| | **cSBP** | Central systolic blood pressure |
| | **CI** | 95% confidence interval |
| | **CRP** | C-reactive protein |

| | | |
|---|---|---|
| | **CVD** | Cardiovascular disease |
| D | **DBP** | Diastolic blood pressure |
| | **DDAH** | Dimethylarginine dimethylaminohydrolase |
| | **DMA** | Dimethylarginine |
| E | **ECG** | Electrocardiogram |
| | **eGFR** | Estimated glomerular filtration rate |
| | **eNOS** | Endothelial nitric oxide synthase |
| | **ESH** | European Society of Hypertension |
| | **ExAMIN Youth SA** | Exercise; Arterial Modulation and Nutrition in Youth South Africa |
| F | **FMD** | Flow mediated dilation |
| | **GC-MS** | Gas chromatography-mass spectrometry |
| G | **GGT** | Gamma glutamyltransferase |
| | **GTP** | Guanosine triphosphate |
| H | **$H_2O_2$** | Hydrogen peroxide |
| | **HART** | Hypertension in Africa Research Team |
| | **HDL-C** | High-density lipoprotein cholesterol |
| | **HIV** | Human immunodeficiency virus |
| I | **IMT** | Intima media thickness |
| | **iNOS** | Inducible nitric oxide synthase |
| | **ISH** | International Society of Hypertension |
| K | **kg** | Kilogram |
| L | **LC-MS/MS** | Liquid chromatography-tandem mass spectrometry |
| | **LDL-C** | Low-density lipoprotein cholesterol |
| | **LNMMA** | L-N monomethyl arginine |
| M | **m** | Metre |
| | **MAP** | Mean arterial pressure |

| | | |
|---|---|---|
| | **MDA** | Malondialdehyde |
| | **mg/dL** | Milligrams per decilitre |
| | **ml** | Millilitre |
| | **mm** | Millimetres |
| | **mmHg** | Millimetres of mercury |
| | **mmol/L** | Millimole per litre |
| | **m/s** | Metres per second |
| N | **n** | Number of participants |
| | **NCD** | Non-communicable disease |
| | **NICI** | Negative-ion chemical ionisation |
| | **nNOS** | Neuronal nitric oxide synthase |
| | **NO** | Nitric oxide |
| | **NOS** | Nitric oxide synthase |
| | **NRF** | National Research Foundation |
| O | **OTC** | Ornithine transcarbamolyase |
| P | **PICI** | Positive-ion chemical ionisation |
| | **PRMT** | Protein arginine methyltransferase |
| | **PURE** | Prospective Urban and Rural Epidemiology |
| | **PWV** | Pulse wave velocity |
| R | **ROS** | Reactive oxygen species |
| S | **SAMRC** | South African Medical Research Council |
| | **SARChI** | South African Research Chairs Initiative |
| | **SBP** | Systolic blood pressure |
| | **SD** | Standard deviation |
| | **SDMA** | Symmetric dimethylarginine |
| | **SHIP** | Strategic Health Innovation Partnerships |

| | | |
|---|---|---|
| | **SIM** | Selected-ion monitoring |
| **U** | **U/L** | Units per litre |
| | **uACR** | Urinary albumin-to-creatinine ratio |
| | **$U_{NOx}R$** | Urinary nitrate-to-nitrite ratio |

## LIST OF APPENDICES



## PUBLICATION STATUS AND CONFERENCE ACCEPTANCE

1. Nitric oxide-related markers link inversely to blood pressure in black boys and men: The ASOS and African-PREDICT studies – **Published *Amino Acids***

Craig A, Mels CMC, Schutte AE, Tsikas D, Kruger R. Nitric oxide-related markers link inversely to blood pressure in black boys and men: The ASOS and African-PREDICT studies. *Amino Acids*. 2020; 52(4): 639-648. doi:10.1007/s00726-020-02842-3.

*2.* Central systolic blood pressure relates inversely to nitric oxide synthesis in young black adults: The African-PREDICT study – **Under review *Journal of Human Hypertension***

Accepted for an oral presentation and Austin Doyle Award nomination at the joint European Society of Hypertension / International Society of Hypertension conference in Glasgow, Scotland, 11-14 April 2021.

*3.* Urinary albumin-to creatinine ratio is inversely related to nitric oxide synthesis in young black adults: The African-PREDICT study – **Published *Hypertension Research***

Craig A, Mels CMC, Schutte AE, Bollenbach A, Tsikas D, Schwedhelm E, Kruger R. Urinary albumin-to-creatinine ratio is inversely related to nitric oxide synthesis in young black adults: The African-PREDICT study. *Hypertens Res.* 2020. doi:10.1038/s41440-020-0514-1. ePub 17 July 2020.

**Chapter I – Literature review, motivation, aims, objectives and hypotheses**



**Chapter II – Methodology**

**Chapter II – Methodology**



**Chapter III – Manuscript 1: Nitric oxide-related markers link inversely to blood pressure in black boys and men: The ASOS and African-PREDICT studies**



**Chapter IV – Manuscript 2: Central systolic blood pressure relates inversely to nitric oxide synthesis in young black adults: The African-PREDICT study**

# Chapter I

## Literature review, motivation, aims, objectives and hypotheses

## 1. Introduction

Cardiovascular disease (CVD) is one of four leading non-communicable diseases (NCDs) reported globally and is the primary cause of morbidity and mortality [1]. The burden of NCDs in Sub-Saharan Africa is increasing [2, 3] and will account for a projected mortality rate of 46% in the Sub-Saharan region by the year 2030 [4]. The increased incidence of NCD is linked to rapid urbanisation, which is accompanied by a change in lifestyle [3, 5]. Lifestyle risk factors that are associated with disease development include obesity, physical inactivity and tobacco and alcohol use [6, 7].

The prevalence of CVD in developing countries, such as South Africa, is twice as high in comparison to developed countries [8]. This is seen together with a high prevalence of hypertension amongst children and adults [9, 10], and a relatively younger age of CVD-related deaths [3]. Therefore, the identification of early predictors for the development of cardiovascular compromise in both children and young adults is warranted.

Nitric oxide (NO) plays a pivotal regulatory role in maintaining vascular homeostasis [11]. A decrease in the synthesis or bioavailability of NO is firstly associated with endothelial dysfunction and secondly implicated in several adverse diseases including hypertension and atherosclerosis. The disruption in vasoactive substances such as NO leads to endothelial dysfunction, which, in turn, leads to structural and functional changes of the vasculature [12]. Therefore, a healthy endothelium is vital in cardiovascular protection and healthy ageing.

Nitric oxide synthesis is regulated via the availability of particular substrates (L-arginine, L-homoarginine), metabolites (L-ornithine/L-citrulline, nitrates and nitrites) and the influence of NO synthesis inhibitors (asymmetric (ADMA) and symmetric dimethylarginine (SDMA)). However, the impact of NO-related markers on CVD in the context of the South African population is limited and controversial. This study therefore aimed to investigate associations of NO-related markers with markers of cardiovascular structure and function in black and white South African children and young adults.

This chapter provides a broad overview of the literature focusing on endothelial function, NO synthesis and bioavailability and the literature surrounding NO and CVD development.

## 2. The endothelium

More than a century ago, physician Rudolf Virchow, once considered the “Pope of Medicine”, spotted a cellular layer within a capillary vessel and referred to it as a simple membrane with

flattened nuclei [13]. Half a century later, Swiss anatomist Wilhelm His invented the word "endothelium" [13] and later, the endothelium was redefined as the inner cellular lining of blood vessels [13, 14].

The endothelium is a unicellular layer comprised of approximately ten trillion cells strategically situated between the lumen of the blood vessel and the vascular smooth muscle cells (**Figure 1**) [15]. For several years after its discovery, the endothelium was deemed an inactive, semi-permeable barrier, the purpose of which was to serve as a protective layer to the underlying tissues from their external environment [16]. However, with years of research, this cellular layer is by no means considered inactive, and is now perceived as a receptor effector organ which responds to a certain stimuli (chemical and physical) via the synthesis and release of a variety of molecules that form part of the regulation of vascular tone, permeability, inflammation, growth and coagulation [17]. In this way, the endothelium regulates vascular homeostasis by retaining a constant balance between a vasodilatory and vasoconstrictor state [18]. During vasodilation, factors such as NO, endothelium-derived hyperpolarising factor and prostacyclin are released by means of the endothelial cells. These factors are commonly associated with anti-inflammatory, anti-oxidant and anti-thrombotic activity [19].

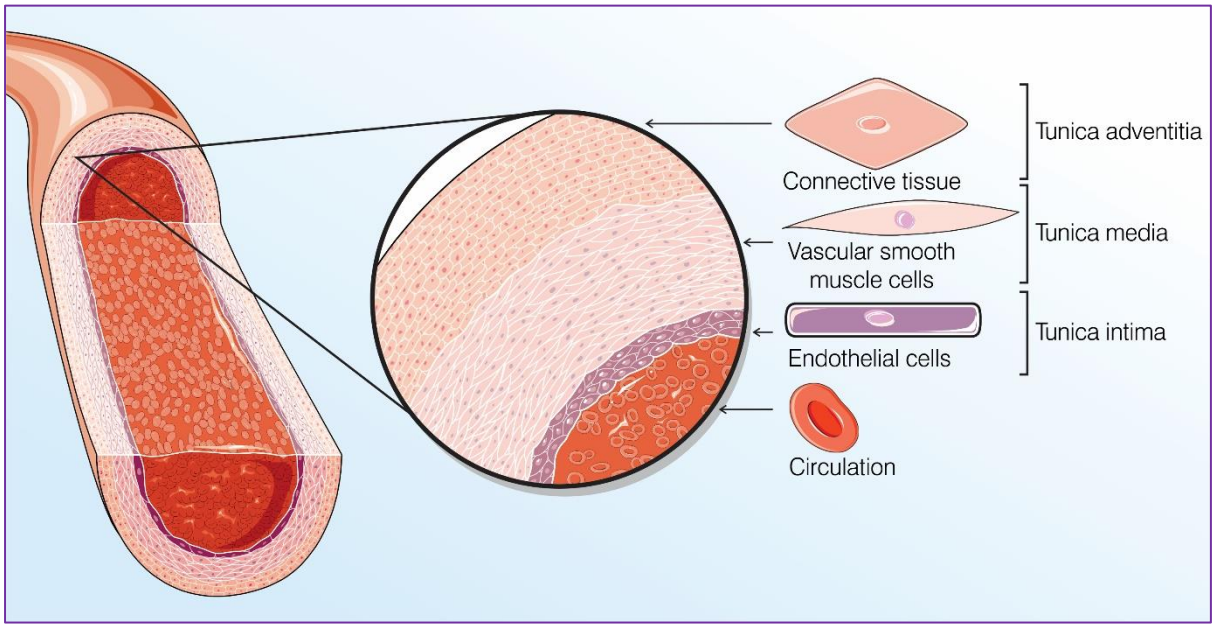


**Figure 1. Location of the endothelium within the arterial wall.**

Alterations within the normal functioning of the endothelium results in endothelial dysfunction, which is considered to be the initial, yet reversible step in the development of CVD [20, 21]. With endothelial dysfunction comes a tendency of pro-inflammatory and pro-thrombotic states as identified by the impairment of endothelium-dependent vasorelaxation [22]. Endothelial

dysfunction is also significantly correlated with cardiovascular risk factors including, amongst others, age, hypertension and tobacco use [17].

The endothelium can be regarded as a gage of cardiovascular health in that risk factors related to CVD hinder endothelial function prior to diseases being detected [23]. Therefore, the assessment and/or measurement of the functional ability of the endothelium is vital for not only the detection of disease development, but also for the evaluation of the effect of lifestyle interventions on endothelial physiology. Numerous methodologies, both invasive and non-invasive, have been acquired to accurately assess endothelial function. While the pathophysiology surrounding the development of endothelial dysfunction is multifaceted, it is currently recognised as a crucial element in attenuated NO, as will be discussed further.

## 3. Endothelial physiology

Under Under normal conditions, the endothelium strives to maintain vascular homeostasis [17]. An important feature of the endothelium is the regulation of vasomotor tone which is primarily regulated by arteries and arterioles. Therefore, the release of certain endothelium-dependent factors such as NO is crucial for endothelial cells to fulfil most of their physiological functions.

### 3.1 Endothelium-dependent factors

#### 3.1.1 Nitric oxide

In 1980, it was first hypothesised that the endothelial lining of blood vessels produces a vaso-relaxing factor termed “endothelium-derived relaxing factor” [24]. Nearly a decade later, endothelium-derived relaxing factor was recognised and confirmed to be NO [24].

Nitric oxide is an extremely volatile gas and potent vasodilator with versatile abilities to protect the vasculature against vascular disease. These protective mechanisms include anti-thrombotic, anti-atherogenic and anti-inflammatory effects [25] that will be described in detail in the forthcoming section. Importantly, NO regulates vascular tone of the endothelium which is vital for the regulation of blood pressure (BP) and blood flow [26]. The bioavailability of NO is preserved through the physical activation of the endothelial cells via specific stimuli such as shear stress and pulsatile flow [27].

#### 3.1.1.1 Nitric oxide synthesis

Due to the short half-life of approximately 3-5 seconds, the biochemical effects of NO are short lived [28]. The synthesis of NO is dependent on the distribution and stimulation of an assembly of enzymes known as NO synthase (NOS). There are three known NOS isoforms: endothelial NOS (eNOS) present in the endothelium, neural NOS (nNOS) present in neurons and inducible NOS (iNOS) present in macrophages, platelets and vascular smooth muscle cells [29, 30]. The distinct NOS isoforms have specific functions and characteristics that are regulated by their site of synthesis, expression and dependency on calcium ($Ca^{2+}$). The action of NO that is produced by the NOS isoforms is highly dependent on both the level of concentration and the location of the isoform.

**The L-arginine-nitric oxide pathway**

L-arginine is an essential amino acid present in the proteins of all life forms [31]. It is the primary substrate for the synthesis of NO. The L-arginine-NO pathway has been thoroughly reviewed and well defined [32-34]. The pathway is initiated by biochemical (acetylcholine, bradykinin, thrombin and adenosine diphosphate (ADP)) and mechanical (shear stress) stimuli which increase the eNOS expression, resulting in an influx of $Ca^{2+}$ from intracellular stores into the endothelial cell [35]. The influx of $Ca^{2+}$ binds to the intermediate calcium-binding messenger protein, calmodulin (calcium-modulated protein) to form a compound which causes eNOS activation [34]. Once calcium-facilitated electron transfer has reduced NOS expression, L-arginine is oxidised to yield NO and L-citrulline in a reaction otherwise referred to as the classical L-arginine-NO pathway (**Figure 2**) [35, 36].

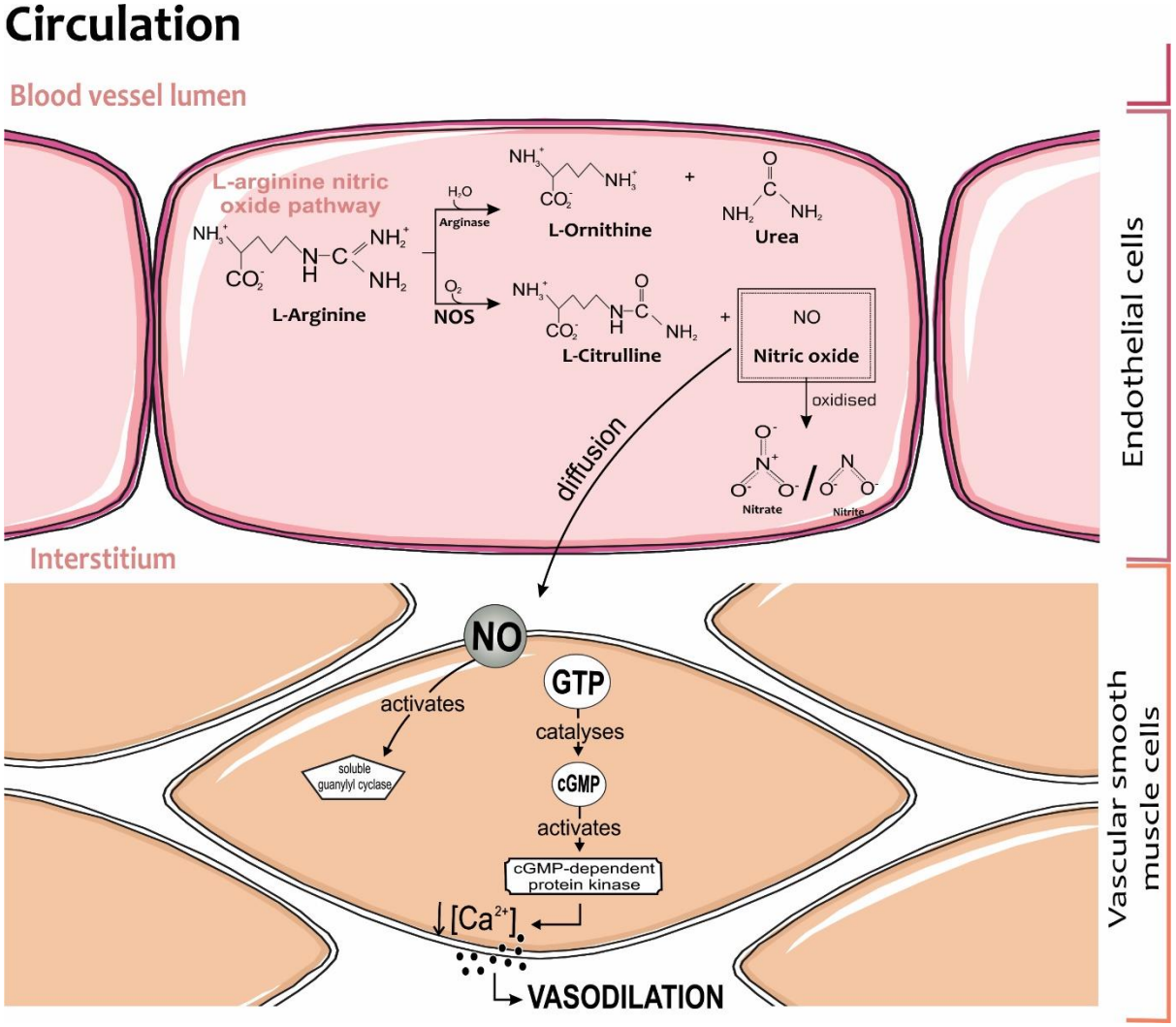


**Figure 2. The L-arginine nitric oxide pathway.**

Within the endothelial cells that line the lumen of the artery, eNOS is released in response to biochemical or mechanical stimuli. The L-arginine-NO pathway is initiated via the conversion of L-arginine to L-ornithine via the enzyme arginase. This process is then followed by L-ornithine being converted to L-citrulline via the enzyme L-ornithine transcarbamoylase (OTC) as part of the urea cycle. Nitric oxide diffuses into the underlying vascular smooth muscle cells where soluble guanylyl cyclase is activated. As a result of the latter, cyclic guanosine monophosphate (cGMP) concentrations increase, which mediates smooth muscle relaxation [36, 37].

In addition, the enzyme arginase is responsible for the catalytic conversion of L-arginine to L-ornithine, yielding the bi-product, urea [38]. This, in turn, is not only a significant process in the urea cycle within the liver, but also in biochemical pathways that are essential for cellular growth and repair [39]. Once synthesised, NO moves rapidly across the endothelial cell membrane via diffusion and activates soluble guanylyl cyclase within the vascular smooth muscle cell (**Figure 2**) by binding to a haemoglobin molecule which causes a rise in the concentration of cGMP. A rise in cGMP and a decrease in $Ca^{2+}$ favours vasorelaxation of the

vascular smooth muscle cell (**Figure 2**). It has been shown that cGMP is accountable for several of the biological effects of NO [40].

A diminished NO substrate availability, such as L-arginine is one of the proposed mechanisms implicated in the pathophysiology of hypertension [41]. It has been shown in a clinical study that the administration of L-arginine improves endothelium-independent vasodilation and subsequently lowers BP [42]. Although the beneficial effects of L-arginine are well documented, a positive association between L-arginine and systolic BP (SBP) has also been reported [43]. This increase in BP and L-arginine levels may be due to an impaired L-arginine transport system which may in itself limit L-arginine availability [44]. It is also possible that an increase in L-arginine may also result in increased NO metabolites, such as L-ornithine due to the classical L-arginine-NO pathway, which may have unfavourable cardiovascular effects [43].

**The nitrate-nitrite nitric oxide pathway**

Since the discovery of the classical L-arginine-NO pathway, an alternative NO pathway, involving nitrates and nitrites, has been explored [45-47]. It was previously thought that inorganic anions such as nitrate and nitrite were inert end products of NO metabolism [48, 49]. However, it has now been reported that these inert anions may possibly be recycled to form NO, demonstrating a secondary source of NO to the classical L-arginine-NO pathway (**Figure 3**) [48, 49].

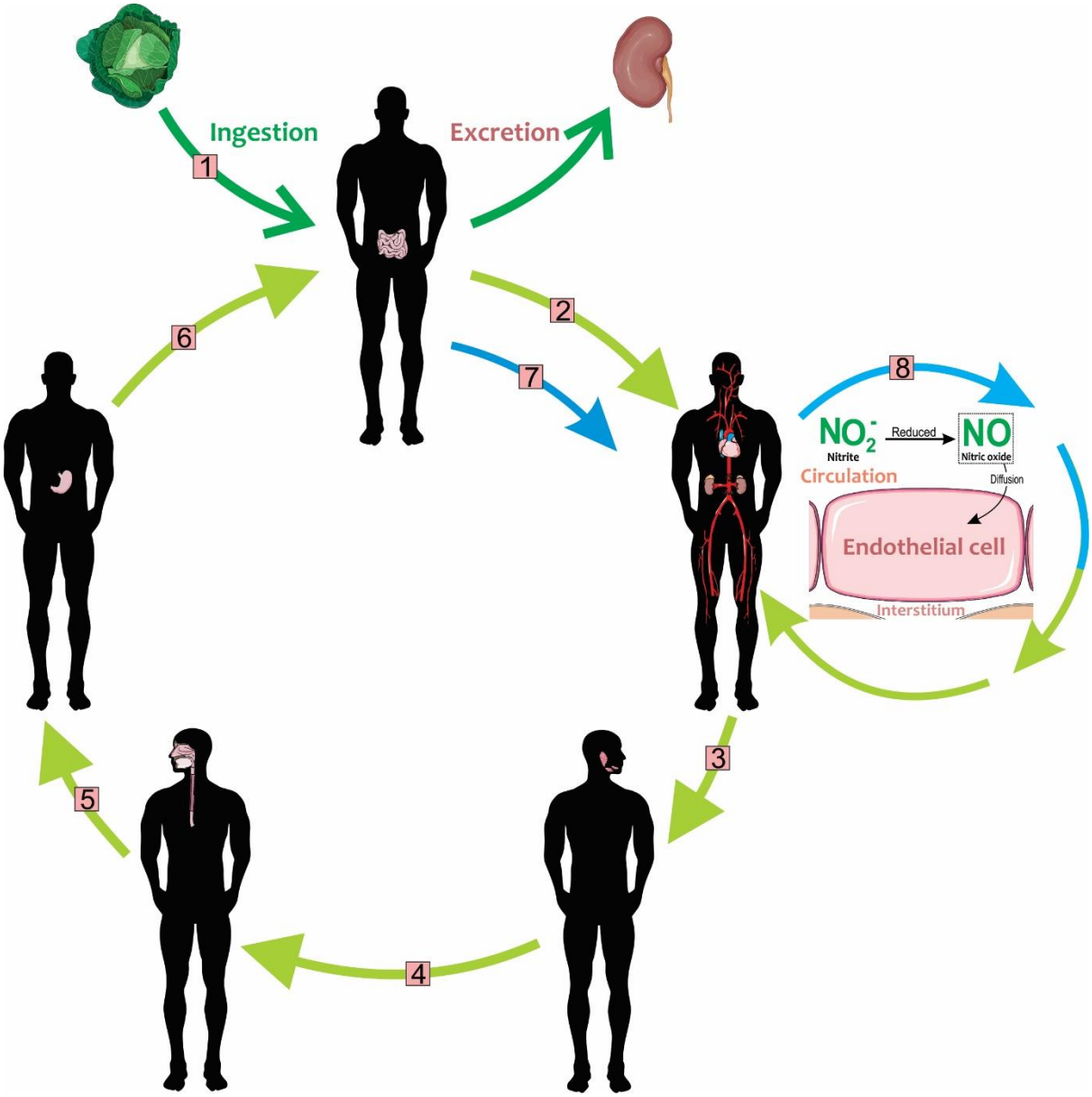


**Figure 3. The nitrate-nitrite nitric oxide pathway.**

After ingestion (1), nitrate is absorbed in the small intestine where it enters the circulation (2). Active uptake by the salivary glands (3) of the remaining nitrate occurs before nitrate reducing bacteria found on the dorsal surface of the tongue (4) reduces nitrate to nitrite. In the stomach some nitrite is reduced to NO (5), and some nitrate is absorbed through the small intestine (6) where it enters the circulation (7). In circulation, nitrite is reduced to NO (8) [45, 50-52].

In the human body, there are two main sources of nitrite and nitrate, these include either end products of the classical L-arginine-NO pathway or through dietary consumption. It has been shown that 80% of dietary nitrate is derived from vegetable consumption [41], while only small amounts of nitrite are ingested. A major source of nitrite is derived from endogenous biochemical pathways that include nitrate being reduced to nitrite by the enterosalivary circulation of nitrate [45, 47, 53] or, to some degree, by nitrate reductases [54].

Saliva secretes concentrated nitrate, where a small amount of nitrate is reduced to nitrite by nitrate-reducing bacteria (oral anaerobic) that are found on the dorsal surface of the tongue [55]. This nitrate-reducing bacteria use the available nitrate as a secondary terminal electron receptor to oxygen, thus yielding the by-product, nitrite. Once swallowed, nitrite is converted to NO in the acidic environment of the stomach, where it stimulates blood flow [51], lowers the possibility of gastrointestinal infection [56] and increases mucous secretion [57]. The remaining nitrite moves into the bloodstream [52], and, collectively with the nitrite that is produced as an end product in the classical L-arginine-NO pathway or by nitrate reductases [54], is promptly dispersed throughout the body where it serves as a source of vasodilatory NO [58]. Therefore, the discovery of this alternative NO pathway could account for the observation that a high-nitrate diet (green-leafy vegetables such as spinach, lettuce and beetroot) is cardiovascular protective [59-61].

The precise mechanism by which nitrite is converted to NO remains unclear; however, this may occur through S-nitrosothiols [62, 63], excess compounds that have been recognised to have the potential to reduce nitrite [45, 64-70] or NOS [71]. Although these precise mechanisms warrant further exploring, it has been shown that when oxygen tensions fall, the L-arginine-NO pathway becomes inactive [72], and this alternative NO pathway (oxygen independent), together with dietary intake of nitrite and nitrate, may be the substitute supply of NO when oxygen is diminished. Therefore, nitrate and nitrite cannot only simply be seen as the end products of NO metabolism, but these anions also exhibit the capability to be converted back to NO. This may explain why NO has certain systemic effects.

Nitric oxide that does not diffuse across the cellular membrane subsequently reacts with both oxy- and deoxy-haemoglobin [73] to form nitrate and iron-nitrosyl haemoglobin respectively. Nitric oxide also reacts with superoxide to form peroxynitrite in a reaction that is highly cytotoxic and responsible for the pathophysiological actions coupled with NO [74]. Peroxynitrite is known to oxidise sulfhydryls to yield hydroxyl radical reactions that induce membrane lipid peroxidation [75]. Physiologically, oxy-radicals form part of the normal regulatory process and are closely controlled by anti-oxidants [76]. However, when free radical levels increase and the anti-oxidant status is lowered, radicals damage the endothelium in a process known as peroxidation [76]. A measure that is used to assess both whole-body lipid peroxidation and oxidative stress is through the measurement of malondialdehyde (MDA) [77]. Malondialdehyde has shown to elevate in association with cardiovascular risk factors [78].

Not only is urinary nitrate considered a major, while urinary nitrite a minor, NO metabolite [79], but urinary nitrate is also considered a useful measure of systemic NOS activity [79]. In

humans, renal carbonic anhydrase isoforms have been implicated in the reabsorption of inorganic nitrite, and, to a lesser extent, the reabsorption of inorganic nitrate [80-83]. As urinary nitrite is an abundant NO reservoir, the proposed function of renal carbonic anhydrase isoforms seems significant to the synthesis of NO via the alternative renal pathway of NO production (nitrate-nitrite-NO pathway). Thus, in theory, urinary nitrite is proposed as a measure of nitrite-dependent renal carbonic anhydrase activity [79]. However, as nitrate and nitrite are dependent on each other, the urinary (u) nitrate-to-nitrite ratio ($U_{NOx}R$) is suggested as a better estimate of carbonic anhydrase-dependent nitrite reabsorption in the kidney [79].

According to Hobbs *et al*. plasma nitrate levels are possible indicators of endothelial dysfunction and significantly correlate with atherosclerotic development [84]. Nitrate has also been linked with endothelial dysfunction, elevated free radical production and the progression of vascular tolerance to other endothelium-dependent vasodilators [85]. It has been shown that urinary nitrate excretion in the alternative renal pathway of NO production (nitrate-nitrite-NO pathway), presented lower in individuals with essential hypertension, thus indicating that systemic NO production may be impaired [86]. However, on the contrary, Goonasekera *et al*. concluded that plasma nitrite and nitrate concentrations increased in children with hypertension [87]. This suggests that a normal or increased NOS activity is present in childhood hypertension in contrast with adult hypertension development for whom it is described as reduced [87].

#### 3.1.1.2 Effects of nitric oxide on the endothelium

The physiological effects of NO on the endothelium are widespread. The presence of the NOS isoform in their specialised location produce NO in response to a specific stimulus in a particular target organ.

In the endothelial cell, the production of NO accounts for endothelium-dependent vasodilation [26]. There is growing evidence surrounding the precise mechanisms by which NO causes the vascular smooth muscle to relax. One mechanism that has been proposed is the release of NO, which participates in the regulation of vascular smooth muscle free $Ca^{2+}$ concentration, which is the primary determinant of contractile tone [88]. Among the other mechanisms proposed is the NO-induced inhibition of $Ca^{2+}$ through L-type $Ca^{2+}$ channels, including their inhibition by cGMP-dependent mechanisms [89], or by membrane hyperpolarisation via direct [90] or indirect cGMP-dependent $Ca^{2+}$-dependent potassium channel activation [91].

In the vascular smooth muscle, NO moves into the vascular smooth muscle cell via diffusion where it targets the protein soluble guanylyl cyclase [92]. Nitric oxide then binds to the soluble

guanylyl cyclase which activates the protein to catalyse guanosine triphosphate (GTP) to covert to cGMP [92]. Cyclic guanosine monophosphate activates cGMP-dependent protein kinase 1 which later activates specific proteins that alter pathways of smooth muscle contraction [93].

Similarly, NO accomplishes its anti-platelet aggregative role by diffusing from the endothelium into platelets [92]. However, it has also been reported that platelets indeed produce their own NO [94]. Within the platelet, NO not only binds to the soluble guanylyl cyclase thus producing cGMP, but also activates cGMP-dependent protein kinase 1. Platelet cGMP-dependent protein kinase 1 phosphorylates specific proteins that decrease intracellular $Ca^{2+}$, thus acting as a deterrent of platelet aggregation [95].

#### 3.1.1.3 Nitric oxide bioavailability

With the knowledge that NO is vital in normal endothelial function, and taking into consideration the fact that its levels are altered in diseased states, has come the necessity to accurately uncover and measure this extremely potent vasodilator. Due to the gaseous nature, free radical structure and short half-life of NO makes the direct measurement analytically challenging [96-98]. Since NO substrates and metabolites are involved in, and nitrate and nitrite are end products of NO metabolism, the focus has been on measuring these molecules as possible indicators of a NO status. However, this does not come without argument [99-101], as these molecules are found in extremely low concentrations [102, 103].

**L-homoarginine**

One substrate with the potential to increase the bioavailability of NO is the cationic amino acid, L-homoarginine [104, 105]. L-homoarginine is structurally similar to the primary substrate L-arginine (**Figure 4**), and is formed by L-lysine [105] in the kidney and liver [106].

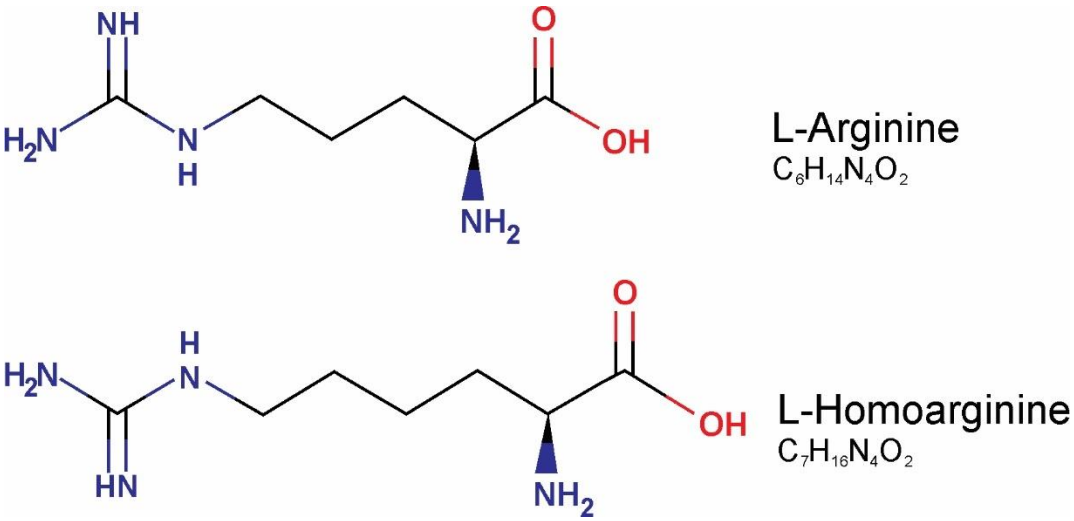


**Figure 4. Structural similarities of L-arginine and L-homoarginine.**

L-homoarginine is a structural homologue to L-arginine by the addition of a methylene group ($CH_2$).

Decades of research has led to the understanding of some of the physiological and biochemical roles of L-homoarginine in humans and animals [107]. Although present in some foods [108], the main dietary source of L-homoarginine is not entirely clear. However, L-homoarginine is produced in small quantities, and suggested to be found in organs such as the kidney, liver, brain, and bodily fluids such as plasma and urine [109]. The likely pathway for the synthesis of L-homoarginine shows arginine: glycine amidinotransferase (AGAT)–a mitochondrial enzyme found in the kidney–responsible for catalysing L-arginine and L-glycine to result in L-ornithine and guanidinoacetate (**Figure 5**). One of the main functions of AGAT is the transfer of an amidino-group from L-arginine to L-glycine, thus leading to guanidino acetic acid formation [110]. At a later stage, guanidino acetic acid is methylated by the enzyme guanidinoacetate methyltransferase to form the energy metabolite, creatine [111]. L-homoarginine is thus produced when AGAT utilises L-lysine instead of L-glycine in this process [104, 112, 113]. An alternative pathway for L-homoarginine synthesis is via the substitution of L-ornithine by L-lysine in the urea cycle [114]. The enzyme OTC is important in this metabolic pathway and catalyses the transamidination of L-lysine which then facilitates L-homoarginine production [104]. L-lysine is utilised by the enzyme OTC, forming homocitrulline in the place of L-citrulline, which is further converted into homoargininosuccinate via argininosuccinate synthase, and subsequently into L-homoarginine by argininosuccinate lyase [115]. The level of L-homoarginine has shown to present higher in children when compared to adults, due to children having a higher level of protein synthesis in order to support growth [116]. In support of this, this cationic amino acid is said to be largely dependent on protein transport in order to cross the cellular membrane [117].

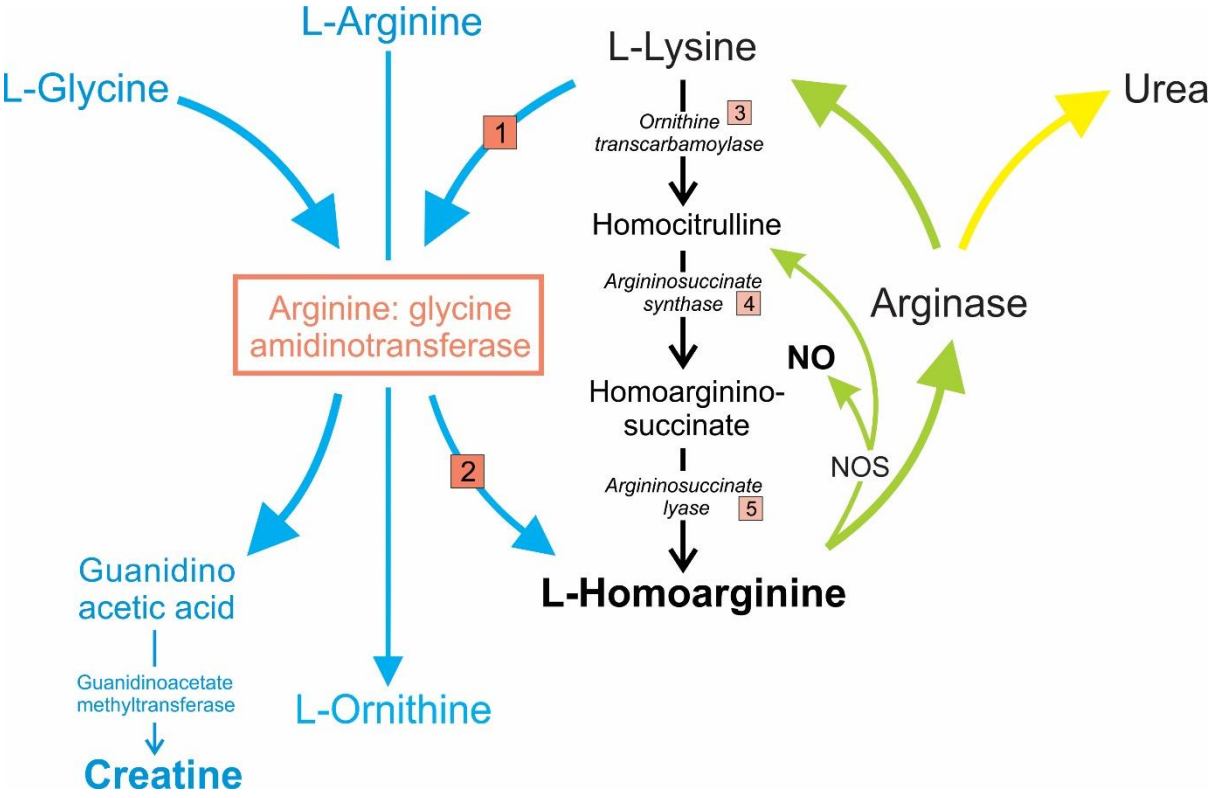


**Figure 5. Metabolic pathways for L-homoarginine metabolism.**

The first suggested pathway is the process whereby arginine: glycine amidinotransferase utilises L-lysine (1) instead of L-glycine in the synthesis of L-homoarginine (2). Another possible pathway for L-homoarginine synthesis is the enzymatic reaction in the urea cycle. The enzyme ornithine transcarbamolyase catalyses L-lysine (3) to form homocitrulline. Homocitrulline is converted to homoargininosuccinate via argininosuccinate synthase (4) and subsequently converted to L-homoarginine by argininosuccinate lyase (5) [104, 112, 113, 115].

Some animal studies have suggested the cardioprotective effects of L-homoarginine [118, 119]; however, the precise mechanisms of the hypothesised beneficial effects still remain to be elucidated. Taking into consideration the structural similarities between L-arginine and L-homoarginine, it has been speculated that at least some of the cardiovascular protective effects that L-homoarginine portrays may be mediated by its interference with the classical L-arginine-NO pathway [116]. L-homoarginine either directly or indirectly interferes with the L-arginine-NO pathway by serving as a weak NOS substrate for NO production, or, alternatively, by increasing NO production from L-arginine via the inhibition of arginase [120]. However, the physiological relevance of L-homoarginine acting as a direct NOS substrate as well as the L-homoarginine-mediated inhibition of arginase causes speculation, as the circulating levels of L-arginine are approximately 30 times higher than the circulating levels of L-homoarginine [121]. However, L-homoarginine impacts the metabolism of NO, endothelial function and the inhibition of platelet aggregation, which are all important in the maintenance of cardiovascular health [121].

**Dimethylarginines**

Although several substrates, metabolites and end products of NO metabolism are known to enhance the synthesis and bioavailability of NO as previously outlined, it has been well documented that dimethylarginines such as ADMA and SDMA are well-known NO synthesis inhibitors [122, 123]. Both dimethylarginines have been reported to hinder the synthesis of NO [122, 123]. These dimethylarginines are synthesised by methylating enzymes such as protein arginine methyltransferase (PRMT) [122, 124]. The PRMT therefore methylates protein to release ADMA and SDMA [122, 124].

Asymmetric dimethylarginine competes with L-arginine to bind with NOS and consequently decreases the synthesis of NO [123, 124]. Asymmetric dimethylarginine metabolism involves the hydrolytic degradation to L-citrulline and dimethylamine that is subsequently catalysed by the enzyme dimethylamine dimethylaminohydrolase (DDAH) [125]. As noted above, ADMA is a potent endogenous NOS inhibitor and may cause endothelial dysfunction [126, 127]. It has been established that ADMA is an emerging risk factor in several cardiovascular-related diseases, including hypertension [128].

On the other hand, SDMA does not directly inhibit eNOS, as seen with ADMA, but is known to interfere with L-arginine uptake [129, 130]. Symmetric dimethylarginine therefore inhibits the transporters responsible for mediating the intracellular uptake of L-arginine [130] and therefore inhibits the absorption of renal tubular L-arginine [131]. Symmetric dimethylarginine has the ability not to only reduce cellular availability of L-arginine, but it also competes with L-arginine for cellular uptake. In addition, it converts eNOS to its uncoupled state, thus inducing monocyte activation accompanied by an increase in the production of reactive oxygen species (ROS) as seen by the presence of oxidative stress [132-134]. When ROS levels increase, it may react with NO, and subsequently lessens the bioavailability of NO. This ultimately results in impaired vasodilation [135, 136].

The biological evidence of ADMA as an endogenous inhibitor of NOS has been well described; however, less attention was focused on SDMA in this regard [137-139]. Moreover, the precise role of NO synthesis inhibitors in the onset of CVD is controversial. Several studies have indicated a positive association between elevated ADMA levels and BP as seen by higher plasma ADMA levels in hypertensive patients when compared to normotensive healthy subjects [127, 140]. Conversely, another study failed to establish an association altogether [43].

## 4. Pathophysiological effects of a reduced nitric oxide bioavailability

### 4.1. Endothelial dysfunction and hypertension

Endothelial dysfunction is described by a reduced NO bioavailability, which may, in part, be mediated by the presence of oxidative stress [141-143]. However, whether the reduction in the bioavailability of NO is the result or the cause of endothelial dysfunction is still not fully understood.

Endothelial dysfunction is one of the main underlying mechanisms in the development of hypertension [1], and is characterised by a loss in homeostatic balance between vasodilation and vasoconstriction, in favour of the latter [141]. The endothelium therefore takes on a pathophysiological state, releasing several vasoconstricting, pro-inflammatory and pro-thrombotic factors [18]. Therefore, the pathophysiological effects of endothelial dysfunction extend further than the cardiovascular system as it is also of particular importance to kidney function [144]. However, for the purpose of this thesis, cardiovascular function will be the main focus.

Since BP represents the net effect of vasoconstriction and vasodilation, hypertension–an elevation in BP–can either reflect defective vasodilation, enhanced vasoconstriction, or both [145]. It has been shown that animal models of hypertension have impaired endothelial function [146]. In hypertensives, vasodilation in the forearm, coronary and renal arteries was impaired and endothelial dysfunction was found to increase the risk of CVD [128]. Possible mechanisms for the development of endothelial dysfunction in hypertension are proposed from alterations in the L-arginine-NO pathway or an increased amount of the endogenous NOS inhibitor ADMA which causes NO inactivation [147, 148].

Deficient L-arginine substrate availability has been proposed not to only decrease NO bioavailability, but also further diminish endothelial function [149]. This, in turn, not only results in the development of hypertension but also resultant CVD (**Figure 6**) [150-152]. Endothelial dysfunction, in addition to hypertension, is the most prevalent and poorly controlled risk factor in individuals with CVD [153].

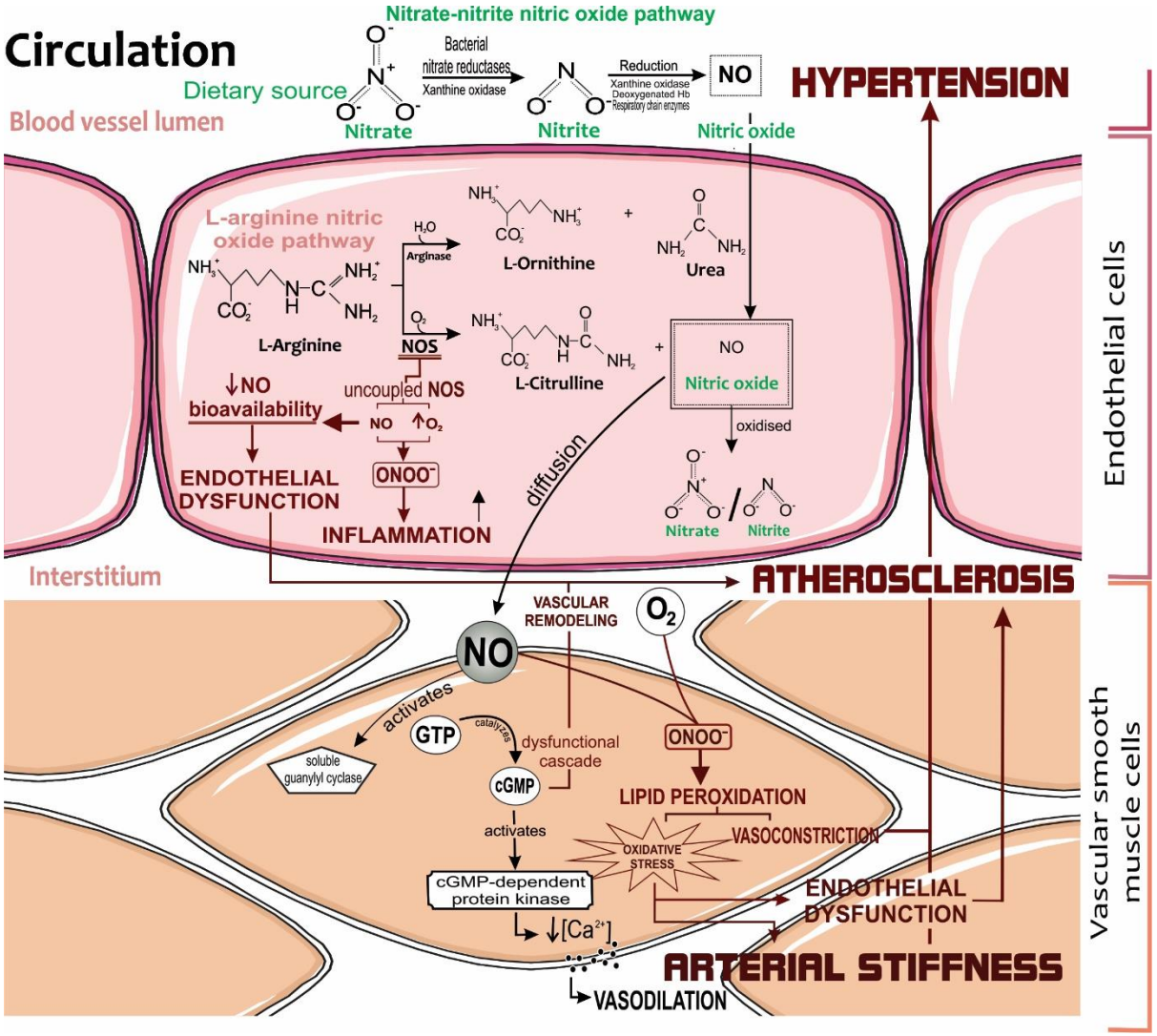


**Figure 6**. **The pathophysiological effects of a diminished nitric oxide bioavailability.**

It remains unclear whether endothelial dysfunction is the cause or consequence of hypertension. Endothelial function is impaired as BP increases [154]. In addition, the degree of dysfunction relates to the magnitude of BP elevation, suggesting endothelial dysfunction to be the result of hypertension [155, 156]. However, Taddei *et al*. showed offspring of hypertensive parents had a reduced response to acetylcholine linked to a defect in the NO pathway, illustrating that endothelial dysfunction precedes hypertension [157].

The assessment of endothelial function is important. Apart from using biomarkers, a well-described non-invasive technique is the use of flow-mediated dilation (FMD) [158]. Flow-mediated dilation therefore provides a measure of *in vivo* endothelium-dependent NO bioavailability [159]. Therefore, endothelial dysfunction is depicted by a diminished FMD response. Although FMD is clinically validated by numerous trials, it is limited by the need for highly-trained and experienced clinical technicians with the expense of the equipment as well

as the influence of certain physiological (exercise, food intake and caffeine ingestion) and environmental (temperature variation) effects of various stimuli [160].

Another non-invasive technique is the urinary albumin-to-creatinine ratio (uACR) which is proposed to evaluate urinary albumin excretion in a spot urine sample [161]. Several studies have reported a significant inverse correlation between uACR and FMD [162, 163]. The presence of urinary albumin has been reported to show associations with impaired endothelial vasodilation and thus represents a reflection of systemic endothelial dysfunction [164]. An increased uACR represents glomerular capillary leakage [165, 166]. Elevated urinary albumin excretion is associated with increased risk of CVD in apparently healthy individuals [165]. The pathophysiological mechanism underlying this association is not fully understood. Clausen *et al*. proposed that in addition to higher BP indices, healthy individuals with elevated urinary albumin excretion may also be characterised by a higher risk of subclinical atherosclerotic development [167, 168]. An elevation in the excretion of urinary albumin is said to associate with an impaired capacity for the artery to dilate. This indicates an impaired response to both endogenous and exogenous NO, which could be a result of structural alterations in the arterial wall [167].

### 4.2. Arterial stiffness

Arterial stiffness is described as a reduction in the expansion and contraction capability of the artery in response to a change in pressure [169]. Arterial stiffness is known to run parallel with several other cardiovascular-related diseases, as seen in the presence of atherosclerosis [170].

Several histological changes occur due to an increase in arterial stiffness. With an increase in arteriole pressure, a rise in transmural pressure is inevitable. With increasing age, the elastic lamella is subjected to a disruption and fragmentation with an alteration in the artery's scaffolding protein (collagen and elastin) ratio [171]. A reduced elastin production will result in increased levels of collagen deposits (**Figure 7**) [169]. This results in the stretching and stiffening of large artery elastic lamellae. The presence of arterial stiffness can be considered an inevitable consequence of the ageing process; however, the magnitude to which arterial stiffness develops could be relevant to the presence and extent of various cardiovascular complications.

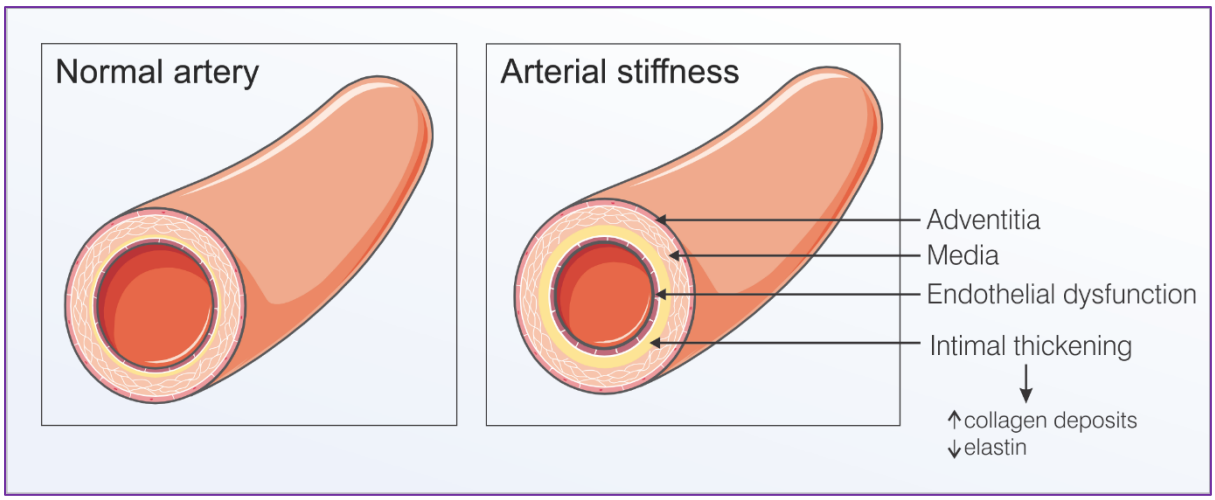


**Figure 7. Structural alterations in arterial stiffness.**

Walls of larger arteries lose elasticity over time. This process results in a loss of elastin fibres and an accumulation of collagen fibres in the arterial wall resulting in arterial stiffness [169].

Clinical implications of arterial stiffness include an increase in BP (both systolic and diastolic), pulse pressure and mean arterial pressure in conjunction with the obvious increase in arterial wall thickness [170, 172, 173]. The major consequence of arterial stiffening is the increase in afterload pressures in the aorta. This increase in afterload, which is attributed to reduced arterial compliance and quicker return of the reflected pulse wave following the narrowing of sections along the arterial tree, generates greater pressure demands from the cardiac muscle to the aortic valve [172, 174]. Due to larger elastic arteries being the central supply to smaller arteries of the periphery, a change in vessel diameter results in the pulsatile wave being reflected back towards the aorta and with an increase in arterial stiffness, the speed at which the reflected wave travels increases.

Clinical evaluations of the cardiovascular system tend to rely on the measurements of different arterial pressures. The most common measure is the measurement of brachial BP; however, central aortic stiffness, measured by pulse wave velocity (PWV), is considered a biomarker for adverse cardiovascular events (i.e. heart failure, renal disease and mortality) [170, 172]. The use of PWV to indicate arterial stiffness is of clinical relevance due to the measurement being easy to perform, reliable and its usefulness as a predictor of CVD morbidity and mortality [175]. These attributes make PWV an easy evaluation of cardiovascular risk [176].

According the Reference Values for Arterial Stiffness' Collaborations, the PWV across the carotid-to-femoral arteries for individuals younger than 30 years of age is optimal at 6.1 m/s, normal at 6.6 m/s and high normal at 6.8 m/s [175]. It was further shown that PWV increases with age and BP [175]. However, specific ethnic reference values have not yet been established. Age, height and mean BP are deemed independent predictors for the

measurement of PWV in children, which resulted in generating curves of PWV percentiles [177]. Moreover, an increase in PWV increases the peripheral vascular resistance which could result from sympathetic over-activity or endothelial dysfunction [171].

Asymmetric dimethylarginine was reported to associate with PWV in an ethnically diverse cohort [178]. Previous findings concluded that the infusion of ADMA resulted in vasoconstriction and elevates peripheral vascular resistance [179, 180]. Kapil *et al*. established a role of dietary inorganic nitrate in preventing arterial stiffness [181]. Moreover, to the best of our knowledge, no results have been reported with regards to the relationship between arterial stiffness and L-homoarginine.

### 4.3. Atherosclerosis

Endothelial dysfunction is a precursor of atherosclerosis, an inflammatory condition that primarily affects large blood vessels such as the aorta, and carotid arteries [182]. Fatty deposits accumulate on the arterial surface and progress to form plaques (**Figure 8**) [183]. Plaque build-up occludes the artery and limits blood flow [183, 184]. Atherosclerosis is therefore a well-described cardiovascular risk factor [148] where impaired endothelial dependent-vasodilation due to an impaired L-arginine-NO pathway is also likely to occur [150, 185].

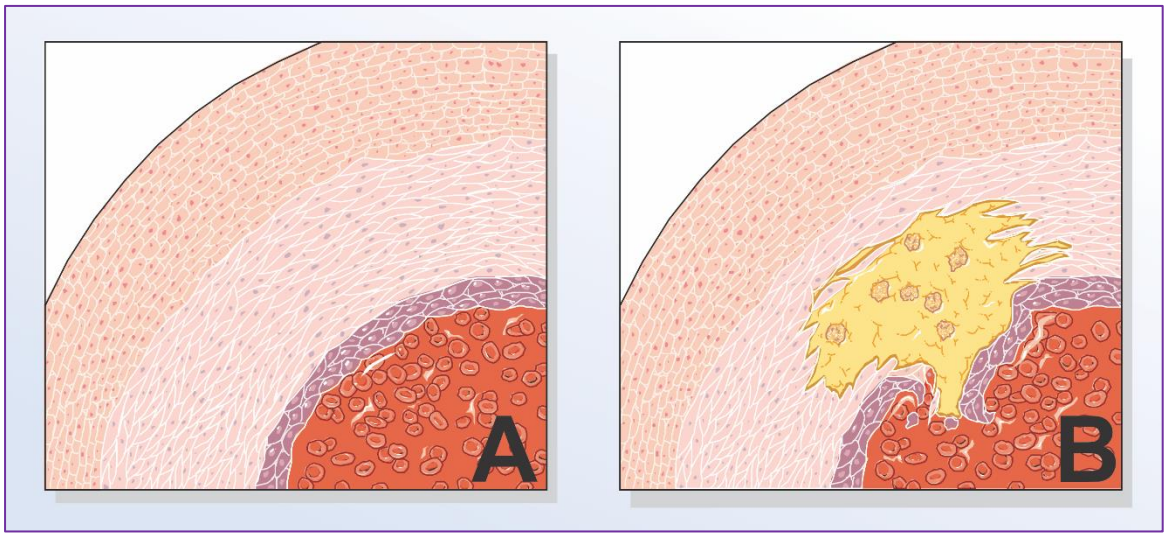


**Figure 8. Atherosclerotic development.**

A healthy artery (A) and an artery affected by atherosclerosis (B).

Since the carotid artery is an elastic artery, an increase in carotid intima media thickness (cIMT) is most often observed [186]. Several studies have noted an increase in intima media thickness (IMT) each year in individuals with known CVD [187]. Therefore, an increase in IMT

can be regarded as a surrogate marker of generalised atherosclerosis [188]. Individuals with atherosclerotic arteries can remain asymptomatic for several decades. Hence, the non-invasive assessment of IMT could pose great value in screening asymptomatic individuals who are at increased risk.

It has also been perceived that CVD is initiated early in life possibly due to inherited and environmental risk factors that may trigger endothelial dysfunction during childhood. Studies have shown repeatedly that childhood BP correlated significantly with cIMT [189, 190], thus providing the appropriate evidence that premature arterial abrasions are already prevalent in asymptomatic individuals [189]. An increase in IMT is an early marker for the development of atherosclerotic plaque which is accompanied by arterial stiffness [191].

Several factors are known to associate with cIMT. These include the presence of oxidative stress and inflammation [192-195]. It has been reported that a reduced NO synthesis capacity as seen in white men with lower plasma NO-related markers and higher NOS inhibitors may possibly affect systemic and endothelial function as well as contribute to the development of atherosclerosis [196].

## 5. Risk factors associated with pathophysiological changes in the endothelium

Modifiable lifestyle risk factors such as tobacco and alcohol use, contribute to the development of CVD, through the development of hypertension, arterial stiffening and atherosclerosis [184, 185, 197]. Similarly, non-modifiable risk factors such as increased age [150, 197], ethnicity [198] and possible genetic factors are known to also result in hypertension [199]. Both modifiable and non-modifiable risk factors have an effect on the early onset of CVD, which may possibly affect NO and related pathways.

### 5.1 Age and sex

Age-related endothelial dysfunction may justify the heightened CVD development risk seen in elderly individuals [150, 197]. With age, several structural and functional alterations in the vascular system occur over time [200]. In addition to disease states such as hypertension, in the elderly, endothelium-dependent vasodilation is impaired [185, 201] and ADMA levels are heightened [202]. It is also known that plasma and urinary nitrate levels are higher in younger children and lessen with age [203]. The specific means for this age-related decrease seen in both plasma and urinary nitrate levels is uncertain, and speculation is made that younger individuals have heightened basal NOS activity [203].

Elli *et al.* reported no significant sex-related differences between plasma and urinary nitrate levels [203]. Sex is a noteworthy CVD risk factor, due to the different physiological effects of sex hormones on cardiovascular function. It has been reported that both high and low testosterone levels associate with cardiovascular risk [204]. In a study reported in a tri-ethnic cohort (African-American, Mexican American and non-Hispanic white), males presented with higher BP when compared to females, independent of ethnicity [205]. This trend of the male sex presenting with higher BP than women was also evident in normotensive populations [206].

### 5.2 Ethnicity

The prevalence of hypertension has been long documented as a common occurrence in black South Africans [207]. Nearly a century ago, the main cause of morbidity and mortality in Sub-Saharan African countries was infectious diseases and malnutrition [207]. Many years later, the disease prevalence in Africa changed [208] and chronic diseases became apparent in low-income countries, and of greater importance with increasing age [208]. Urban black communities in Sub-Saharan Africa began to show signs of CVD development such as hypertension when compared to rural communities [209, 210]. It is now well documented that hypertension associates with end-organ damage [178, 199, 209, 211], renal failure [198] and events such as a stroke, and presents higher in black Africans when compared to their white counterparts [178, 209, 211]. Although it is apparent that black communities experience inequalities, low access to healthcare and treatment [212], a possible explanation for the disparity in disease prevalence in black and white populations may occur due to salt sensitivity and an abnormal haemodynamic reactivity [20]. Salt sensitivity is more prevalent in black populations [213] characterised by increased peripheral resistance [211], in which a decrease in NO-dependent vasodilation is also evident [213, 214].

Since endothelial dysfunction is predominant in black compared to white individuals [178, 199, 209, 211], black populations are more susceptible to CVD development [197]. In a South African study comparing black and white populations, de Kock *et al.* confirmed the black populations presented with lower levels of cortisol, increased uACR and decreased eGFR, also indicating the renal disparities among this population [215].

The role of dimethylarginines in ethnically diverse populations reveal differing results [178, 197]. It has been reported that ADMA levels are higher in white populations showing an association with CVD development [178, 216, 217]. Yet, in contrast, two other studies reported black populations to have higher ADMA levels, indicating CVD risk is higher in the black

population [178, 197]. Moreover, it was shown that L-arginine levels are significantly lower in black men with a reported higher BP [211].

Additionally, it has been shown that PWV associates with ADMA in black Africans, thus suggesting ADMA may regulate arterial stiffness [178]. It has also been reported that black populations have higher PWV when compared to white populations [218]. Therefore, since endothelial dysfunction is prevalent in black populations, it may play a pivotal role in the early onset of arterial stiffening and atherosclerotic development [41, 219, 220]. In support of this, even apparently healthy normotensive black Africans demonstrate signs of endothelial dysfunction as shown by an interaction of L-arginine with cardiovascular risk factors [211].

### 5.3 Lifestyle risk factors

Lifestyle factors such as tobacco use and alcohol consumption significantly influence the association between L-arginine, ADMA and SDMA [211]. In populations who are known smokers, endothelial dysfunction is evident as the endothelium falls prey to the harmful toxic compounds found in cigarettes [184]. Nicotine and carbon monoxide can cause alterations of the endothelium [221] and it is also noteworthy to mention that free radical components in cigarette smoke have shown to cause endothelial damage in model systems [222]. Smoking is also known to accelerate the ageing process through the enhanced production of ROS, which, in turn, results in impaired endothelium-dependent vasodilation [185].

Tobacco use is a cardiovascular risk factor due to its association with peripheral artery disease [223]. In the general population, smokers present with lower levels of high density-lipoprotein cholesterol (HDL-C) and higher levels of low density-lipoprotein cholesterol (LDL-C) and triglycerides [224]. Nicotine–a stimulant and potent parasympathomimetic alkaloid–elevates cardiac output and heart rate which ultimately results in an increase in BP [225, 226]. It has also been reported that more men with a lower socio-economic score tend to smoke [225].

Another modifiable cardiovascular risk factor is the excessive consumption of alcohol. Alcohol consumption accounts for an elevation in cardiovascular morbidity and mortality [227]. The risk for hypertension development is low when alcohol consumption is low to moderate; however, when alcohol consumption is heightened, the risk for CVD increases [228].

Benefits of moderate alcohol consumption have been reported [227]. Moderate consumption increases eNOS activity, thus aiding in NO synthesis, decreasing fibrinolytic activity and increasing both pro-thrombotic and anti-inflammatory activity [227]. However, chronic alcohol consumption outweighs any benefits and reportedly lowers the glycoprotein von Willebrand

factor that is involved in homeostasis [227]. Additionally, alcohol abuse lowers anti-oxidant capacity (lowers glutathione production) and increases hydrogen peroxide ($H_2O_2$) formation, thus resulting in oxidative stress [229]. Chronic alcohol consumers tend to have increased acetyl Coenzyme-A conversion to triglycerides, which is further secreted in the circulation as unwanted LDL-C [224]. The excess triglycerides are responsible for the accumulation of fat in the liver. This, in turn, causes serum gamma glutamyltransferase (GGT) to increase [230, 231]. Gamma glutamyltransferase is an enzyme located on the surface of the cellular membrane, and is involved in the cleavage of glutathione (antioxidant) and cellular defence against oxidative stress [232]. Therefore, an elevated level of GGT is a known predictive CVD biomarker [230, 231].

## 6. Problem statement and motivation

Cardiovascular disease has become a health concern across the globe. It seems that CVD has translated from a wealthy man's disease to one for the poor, where poorer populations are now considered vulnerable to CVD development and fall victim in both developed and developing countries [8]. The pathophysiological link between the role of endothelial dysfunction and premature endothelial alterations associating with cardiovascular risk factors is of importance. Endothelial dysfunction, characterised by a diminished NO bioavailability, is now perceived as an initial, reversible precursor to the development of CVD [233-235]. Several hypotheses reported that the deficiency of NO in early life is a central stimulus for the development and progression of hypertension in adulthood [236-238].

Many traditional risk factors are known to track into adulthood which are known to cause endothelial dysfunction with NO deficiency in children [239]. Evidence linking these risk factors are significant in black populations where elevated levels of dimethylarginines (ADMA and SDMA) along with diminished levels of NOS substrates (L-homoarginine and L-arginine) have been reported [240, 241]. Therefore, the availability of NO can be explored by determining the association between factors that are involved in its synthesis and bioactivity, as well as markers related to cardiovascular structure and function.

There have been numerous studies stating the impact of an impaired NO bioavailability on cardiovascular health. However, most studies have reported findings on older populations, and individuals with diseased states [178, 196, 211, 242]. There are limited studies exploring the NO profile in South African populations and the influence of NO-related markers on the development of endothelial dysfunction in a generally healthy population, which in the most part, forms the motivation for this study. Since markers of NO synthesis pose great value as

promising risk predictors, it is necessary to investigate these biomarkers in young healthy populations and how these associate with measures of cardiovascular structure and function.

Having an insight on the interactions of large artery structure and function with biomarkers related to the synthesis and bioavailability of NO may hinder the progression of CVD, and therefore reduce the possible onset of resultant cardiovascular compromise. Black South Africans are in fact more susceptible to developing cardiovascular complications. However, the precise role of an unfavourable NO profile amongst this population still warrants exploring.

## 7. Aims, objectives and hypotheses

The central aim of this thesis is to investigate the relationships between measures of arterial structure and function and markers of NO metabolism in children and young adults from South Africa.

**Manuscript 1**

In black and white boys (aged 6-8 years) and men (aged 20-30 years), the objectives were as follows:

i. To compare blood pressure and carotid wall thickness (BP and cIMT), along with urinary biomarkers related to NO bioavailability (nitrate and $U_{NOx}R$);
ii. To determine the associations of BP and cIMT with urinary biomarkers related to NO bioavailability (nitrate and $U_{NOx}R$).

Hypotheses

- Urinary nitrate and $U_{NOx}R$ will present lower in black boys and men, than their white counterparts.
- Blood pressure and cIMT will associate inversely with nitrate and $U_{NOx}R$ in both groups.

**Manuscript 2**

In black and white men and women (aged 20-30 years), the objectives were as follows:

i. To compare central systolic blood pressure (cSBP) and arterial stiffness (PWV), along with NO substrates and metabolites in plasma (arginine, homoarginine, ADMA and SDMA) and urine (arginine, homoarginine, ADMA, SDMA, ornithine/citrulline, nitrate and $U_{NOx}R$);

ii. To determine whether central BP (cSBP) and arterial stiffness (PWV) are associated with NO substrates and metabolites in plasma (arginine, homoarginine, ADMA and SDMA) and urine (arginine, homoarginine, ADMA and SDMA, ornithine/citrulline, nitrate and $U_{NOx}R$).

Hypotheses

- Central BP (cSBP) and arterial stiffness (PWV) will present higher in the black populations compared to their white counterparts;
- In plasma and urine, ADMA and SDMA will present higher in the black group while NO substrates and metabolites (arginine, homoarginine, ornithine/citrulline, nitrate and $U_{NOx}R$) will present lower in the black group.
- Central BP (cSBP) and arterial stiffness (PWV) will associate inversely with NO substrates and metabolites (arginine, homoarginine, ornithine/citrulline, nitrate and $U_{NOx}R$) and positively with NO synthesis inhibitors (ADMA and SDMA) in both groups.

**Manuscript 3**

In black and white men and women (aged 20-30 years), the objectives were as follows:

i. To compare a marker of endothelial function (uACR) and NO substrates and metabolites in plasma (arginine, homoarginine, ADMA, SDMA) and urine (arginine, homoarginine, ornithine/citrulline, ADMA, SDMA, nitrate and $U_{NOx}R$);
ii. To determine whether endothelial function (uACR) is associated with NO substrates and metabolites in plasma (arginine, homoarginine, ADMA, SDMA) and urine (arginine, homoarginine, ornithine/citrulline, ADMA, SDMA, nitrates and $U_{NOx}R$).

Hypotheses

- uACR will be higher in black adults when compared to their white counterparts;
- In plasma and urine, NO substrates and metabolites (arginine, homoarginine, ornithine/citrulline, nitrates and $U_{NOx}R$) will present lower, while NO synthesis inhibitors (ADMA and SDMA) higher in the black compared to white group.
- uACR will inversely associate with NO substrates and metabolites (arginine, homoarginine, ornithine/citrulline, nitrates and $U_{NOx}R$) and positively with NO synthesis inhibitors (ADMA and SDMA) in the black group only.

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# Chapter II

## Methodology

## 1. Study design

For this PhD thesis, data from both the Arterial Stiffness in Offspring Study (ASOS) and the African Prospective study on the Early Detection and Identification of Cardiovascular Disease and Hypertension (African-PREDICT) was used.

The ASOS study was a cross-sectional study conducted from April 2015 to October 2015. This study included 40 black and 41 white boys (between the ages of 6-8 years). Both black and white boys were recruited from urban schools in Potchefstroom, North West province, South Africa (**Figure 1**) to ensure comparability in terms of socio-economic background


**Figure 1. Geographical location of Potchefstroom, North West province, South Africa.**

The prospective African-PREDICT study is an ongoing longitudinal study that screened black and white participants (aged 20-30 years) from Potchefstroom and the surrounding areas of the North West province (**Figure 1**). The aim of the prospective African-PREDICT study is to identify premature cardiovascular disease (CVD) development in young South Africans.

In this cross-sectional baseline study, 80 black and white ASOS participants and 1110 black and white African-PREDICT participants were included, after the exclusion of participants with

nitrite outliers and those with missing data of interest. This chapter will therefore outline the specific methodology and justifications as to the anthropometric, cardiovascular and biochemical analyses used in compiling the prevailing manuscript chapters.

## 2. Inclusion and recruitment processes

### 2.1 The ASOS study

Based on the aims of the ASOS study which were to investigate the links between arterial function and stiffness profiles, body composition phenotypes and urinary biomarkers in black and white boys, the following exclusion criteria were applied: children aged <6 and >8 years; girls (to exclude hormonal influences of unknown pubescence); obese children (with a body mass index z-score greater than 95th percentile, as indicated by the World Health Organization) and those using any chronic medication, or with self-reported type 1 diabetes mellitus, renal disease or cancer.

Upon obtaining permission, schools within the Potchefstroom area were contacted and a meeting with the school principals was requested. Some schools only consisted of either black or white children, whereas others included both ethnic groups; however, all schools were in the urban setting of Potchefstroom, and only children of black or white ethnicity were recruited. The Principal Investigator of the study further presented and discussed the purpose of the project, along with the specific details of each measurement. Teachers who were not directly involved with the particular children were appointed as potential mediators. Invitation letters were distributed to the parents whose children fitted the inclusion criteria inviting them to an information session. A Microsoft® PowerPoint presentation was presented to the parents outlining and explaining the purpose and objectives of the ASOS study, as well as the intended measurements. At the end of the information session, information sheets were distributed to the parents. Parents were given a two-week period in which to decide if they were willing to allow their child to participate. Parents and their children who agreed to participate were informed on which date they would take part upon the Principal Investigator scheduling and liaising with the schools.

Research stations were arranged in secured private rooms that were provided by the schools, and measurements on at least 2–4 children were performed daily in preselected time slots. Children were given general health questionnaires, consent/assent and permission forms as well as cooler boxes with a urine specimen collection kit at their schools to take home on the day prior to their scheduled date of participation. Both the parents and their respective children were instructed to complete informed consent/assent and permission forms before basic

health measures were conducted. Spot urine samples on the morning of participation were collected in the privacy of their own homes. Urine samples were returned to the schools, along with the questionnaires (completed by the parents) and consent/assent and permission forms, where a research assistant collected them. Urine samples were taken to the laboratory for further handling and storage. When children arrived at the research stations, they were introduced to the research environment after which all the procedures were thoroughly explained to them.

## 2.2 The African-PREDICT study

To broaden the knowledge on the processes and markers involved in the premature development of CVD, the African-PREDICT study included black and white apparently healthy (self-reported) individuals (aged 20-30 years). Considering it has been shown that black South Africans are at higher risk of hypertension [1], black participants will be compared to their white counterparts with equal sex distribution in an attempt to balance the socio-economic score [2].

Recruitment, screening and assessment of normotensive, apparently healthy volunteer participants was conducted at the Hypertension Research and Training clinic, located within the North-West University, Potchefstroom campus in addition to external locations (the workplace). Recruitment of participants took place via contact with external field workers, through media and radio advertisements and access through the workplace until the full baseline sample was reached.

To determine their eligibility, recruited participants were invited to a screening phase of the study. A private feedback session was provided to participants who met the inclusion criteria, after which they received an information leaflet with all the relevant details pertaining to the research phase of the study and an informed consent form. Participants were required to complete the informed consent form before participation. Those participants who were not eligible to participate in the study (due to exclusion criteria or health abnormalities) received a detailed health feedback report, and, if necessary, a referral letter for further testing or treatment.

Due to CVD development being an endpoint of the African-PREDICT study, all hypertensive participants (office blood pressures ≥140/90 mmHg), individuals with any self-reported diseases or risk factors that may influence cardiovascular health (human immunodeficiency virus (HIV), diabetes mellitus, liver disease, cancer, tuberculosis or renal disease) as well as

the use of chronic medication were excluded from the study. Pregnant and/or lactating women were also excluded due to known influences of hormones on cardiovascular health [3, 4].

Interested participants who met the inclusion criteria were enrolled in the African-PREDICT study in the years 2013–2017 (*n*=1202). Participants who enrolled were instructed to arrive at the Hypertension Research and Training Clinic at 08h00 on the morning of participation, after which they were given a tour of the research facility to familiarise themselves with each measurement. Transport arrangements were made for those participants who did not have their own transport. Participants then received a six-digit identification number to ensure anonymity when processing data. The research phase of the study included biological sampling (fasting blood and urine samples), along with various anthropometric, cardiovascular and biochemical measurements, as well as the completion of a set of questionnaires. All measurements were performed in a private temperature-controlled room. Upon completion of all measurements, a light meal was provided to those who participated.

## 3. Research methodology

Since this study made use of existing data (**Table 1**) and no additional measurements were performed, all protocols and procedures used in compiling the manuscripts of this thesis are in line with the ASOS and African-PREDICT studies [2] and conformed to the ethical guidelines of the Declaration of Helsinki [5] (revised in 2008) for investigation of human participants. This cross-sectional PhD study was also approved as a single study by the Health Research Ethics Committee of the North-West University (NWU-00051-18-S1) (**Appendix C**).

**TABLE 1: Summary of the research measures used in this cross-sectional study.**

| **The ASOS study** (aged 6-8 years) | **The African-PREDICT study** (aged 20-30 years) |
|---|---|
| **Anthropometry** | |
| Height and weight | Height and weight |
| Waist circumference | Waist circumference |
| Body mass index | Body mass index |
| | Waist-to-hip ratio |
| **Cardiovascular measures** | |
| Brachial blood pressure | Brachial blood pressure |
| Carotid intima media thickness | Carotid intima media thickness |
| | 24-hour Ambulatory blood pressure |
| | Central blood pressure |
| | Pulse wave velocity |
| **Biological sampling** | |
| Spot urine samples | Spot urine samples |
| | Fasted blood sample |

### 3.1 Questionnaires

Both the ASOS and the African-PREDICT participants completed a general health questionnaire. The general health questionnaire is seen as a self-administered screening tool used and designed to detect current disorders [6]. Prior to participation, each participant completed a questionnaire. Parents of the participating children were expected to complete the questionnaire on behalf of the child, and these questionnaires were collected by the research assistant on the day of participation. The following information was gathered from the questionnaire: demographic (age and locality), self-reported alcohol and tobacco use, employment information as well as the use of medication. Hardcopies of the questionnaires were completed by the parents of the ASOS children participants, while African-PREDICT participants completed their questionnaires on Apple iPads (Hon Hai Precision Industry Co., Ltd.) using a web-based program which took participants approximately 15 minutes to complete.

### 3.2 Anthropometric measures

All anthropometric measures were performed according to the set guidelines determined by the International Society for the Advancement of Kinanthropometry (ISAK) [7] in order to describe the body composition of the study population.

Comprehensive lists of all anthropometric measures and devices used in the ASOS and African-PREDICT studies (**Figure 2**) are outlined below (**Table 2**).

**TABLE 2: Summary of the anthropometric measures obtained in the ASOS and African-PREDICT studies.**

| | **The ASOS study** (aged 6-8 years) | **The African-PREDICT study** (aged 20-30 years) |
|---|---|---|
| **Measure** | **Device used** | |
| Height (cm) | Seca 213 stadiometer, Birmingham, United Kingdom | Seca 213 portable stadiometer; Seca, Hamburg, Germany |
| Weight (kg) | Seca 813 digital scale, Birmingham, United Kingdom | Seca 813 electronic scales; Seca, Hamburg, Germany |
| Waist and hip circumference (cm) | Lufkin® executive thinline 2mm steel tape (Apex tool group B.V.; AK Emmen, Netherlands | Lufkin steel anthropometric tape; w606pm; Lufkin; Apex; USA |

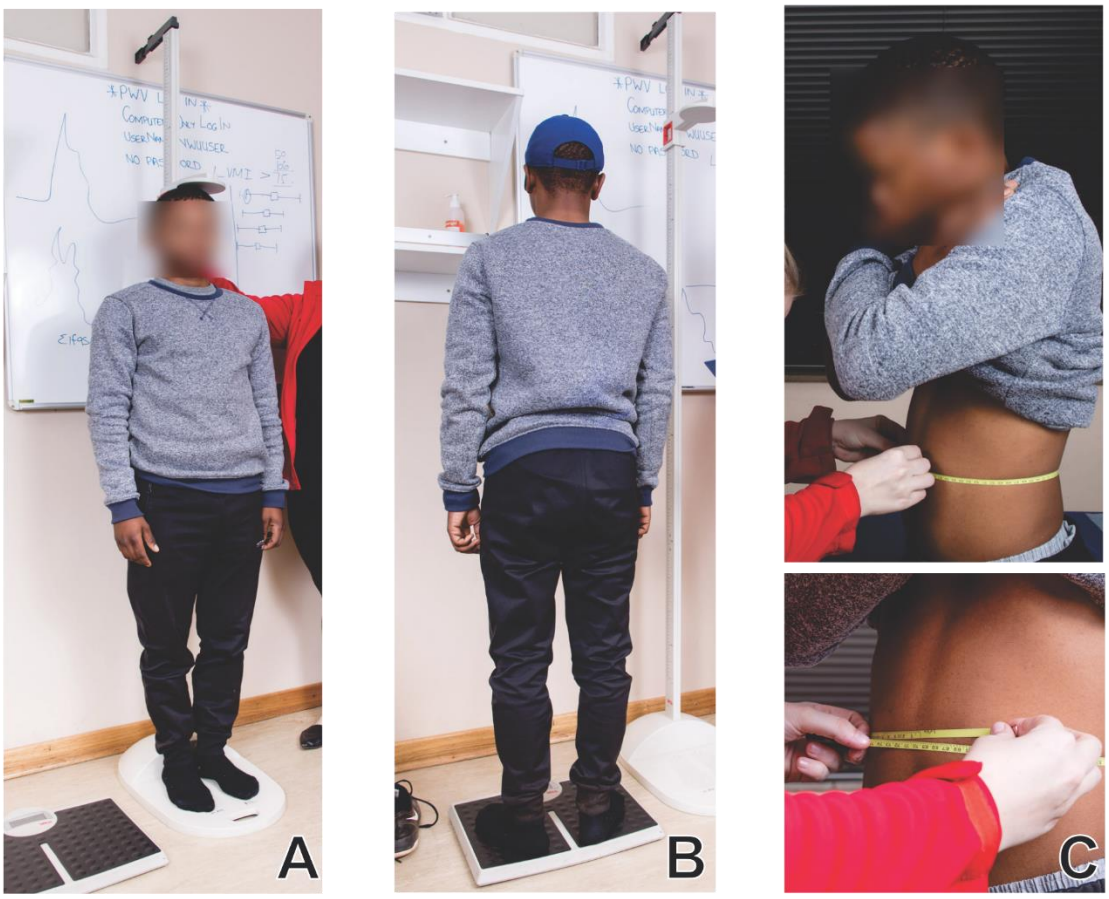


**Figure 2. Anthropometric measurements preformed for the African-PREDICT study**. Each participant's body height (A), body weight (B) and waist circumference (C) were measured [2].

In addition to the basic anthropometry, waist-to-hip ratio (waist circumference (cm) / hip circumference (cm)) was calculated in African-PREDICT study and body mass index (BMI) was calculated (weight (kg) / height ($m^2$)) for both studies and used as an indirect measure of adiposity [8, 9]. As BMI of children tends to change throughout childhood and different growth patterns occur between boys and girls [10, 11], BMI z-scores were utilised for the assessment of body composition in the ASOS study. International growth references developed from the World Health Organization Multicentre Growth Reference Study were used to compare the children's BMI z-scores. Thresholds of the child growth reference were used in order to determine whether the child's BMI z-scores are above or below the defined thresholds [12, 13].

### 3.3 Cardiovascular measures

Various cardiovascular measures were used to prepare the research manuscripts. These included brachial and ambulatory blood pressure (BP), arterial stiffness indices and carotid intima media thickness (cIMT), which are discussed in detail below.

**Blood pressure**

Brachial office BP was measured in both the ASOS and the African-PREDICT studies while participants remained in a rested seating position. The ASOS study made use of the Omron HEM-759-E (750IT) device (Omron Healthcare, Tokoyo, Japan), while the African-PREDICT study used the Dinamap® ProCare 100 Vital Signs Monitor (GE Medical Systems, Milwaukee, USA). The Omron device is validated for BP measurement in children [14]. Three consecutive measurements were obtained and the mean value of the lowest two measurements was calculated. The mean arterial pressure (MAP) was subsequently calculated by using the following formula: diastolic BP (DBP) + (0.4 x pulse pressure) [15].

Central systolic BP (cSBP) of the African-PREDICT participants was obtained in duplicate using the SphygmoCor® XCEL device (AtCor Medical Pty. Ltd., Sydney, Australia) (**Figure 3**) with the correctly sized brachial BP cuff on the upper right arm. In the event the measures differed by more than 3mmHg, additional measures were performed.

**Figure 3. Central systolic blood pressure measure in the African-PREDICT study [2].**

Since evidence suggests that BP readings taken in the familiarity of your home environment more accurately predict cardiovascular events when compared to measures taken in an unfamiliar setting [16], African-PREDICT participants were equipped with an ambulatory BP monitor apparatus (CardioXplore, Meditech, Budapest, Hungary, British Hypertension Society (BHS) validated). The monitoring apparatus was fitted at roughly the same time each day. This device takes predetermined recordings at 30-minute intervals during the day (from 06h00 to 22h00) and every hour throughout the night (from 22h00 to 06h00), of which a 24-hour ambulatory BP (ABPM) was obtained. Systolic BP (SBP), DBP and heart rate were captured from each measurement.

Additionally, with successful ABPM, the African-PREDICT participants were classified as normotensive or masked hypertensive. In order to be stratified into the normotensive category, brachial office BP needed to be below 140/90 mmHg, together with a normal ABPM (24-hour

readings indicative of <130/80 mmHg, daytime readings ≥135/85 mmHg and night-time readings <120/70 mmHg). Similarly, in order to be stratified into the masked hypertensive category, participants presented with a normal brachial office BP below 140/90 mmHg but an elevated ABPM (24-hour readings indicative of <130/80 mmHg, daytime readings ≥135/85 mmHg and night-time readings <120/70 mmHg) [17].

**Arterial stiffness**

Pulse wave velocity (PWV) is used as an index of arterial stiffness [18]. Carotid-femoral PWV (**Figure 4**) is deemed the golden standard for the non-invasive measurement of arterial stiffening [19], and therefore represents the propagation of pressure waves which can be measured regionally across any point within the arterial tree [19].

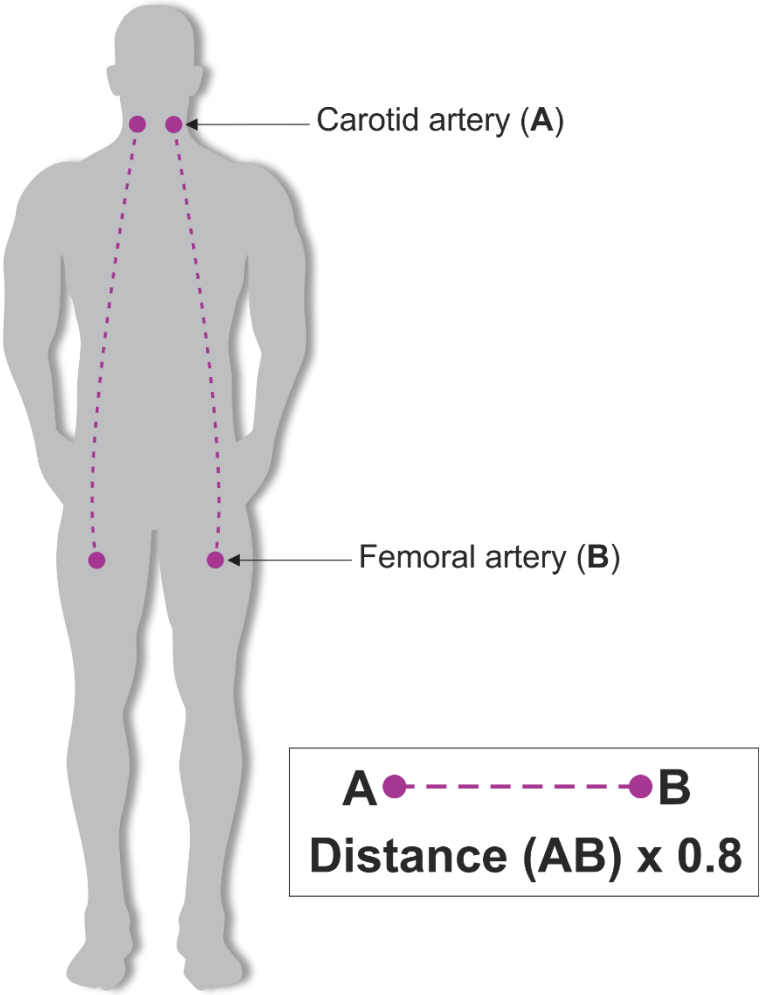


**Figure 4. Illustration of carotid femoral pulse wave velocity.**

Carotid femoral PWV is a measure of arterial stiffness and determined by the time taken for the arterial pulse to propagate from the carotid artery down to the femoral artery.

The SphygmoCor® XCEL device (AtCor Medical Pty. Ltd., Sydney, Australia) was used to obtain the carotid-femoral PWV (**Figure 5**) of the African-PREDICT participants. Prior to measurement, participants were required to remain in the supine position in a relaxed state for the duration of five minutes. The augmentation index was estimated by performing pulse wave analysis with the use of the general transfer function. Thereafter, to identify the participant's strongest pulse point, the carotid artery was then located by means of palpation. An infantometer (SECA 207 infantometer, SECA, Hamburg, Germany) was used to measure the pulsated sites of which 80% of these distances were used as the pulse wave travelled distance. The carotid pulse of each participant was measured using a tonometer simultaneously as the femoral pulse was measured using a correctly sized femoral cuff placed on the upper right thigh. The PWV of each participant was therefore measured along the descending thoracic abdominal aorta with the use of the foot-to-foot method.

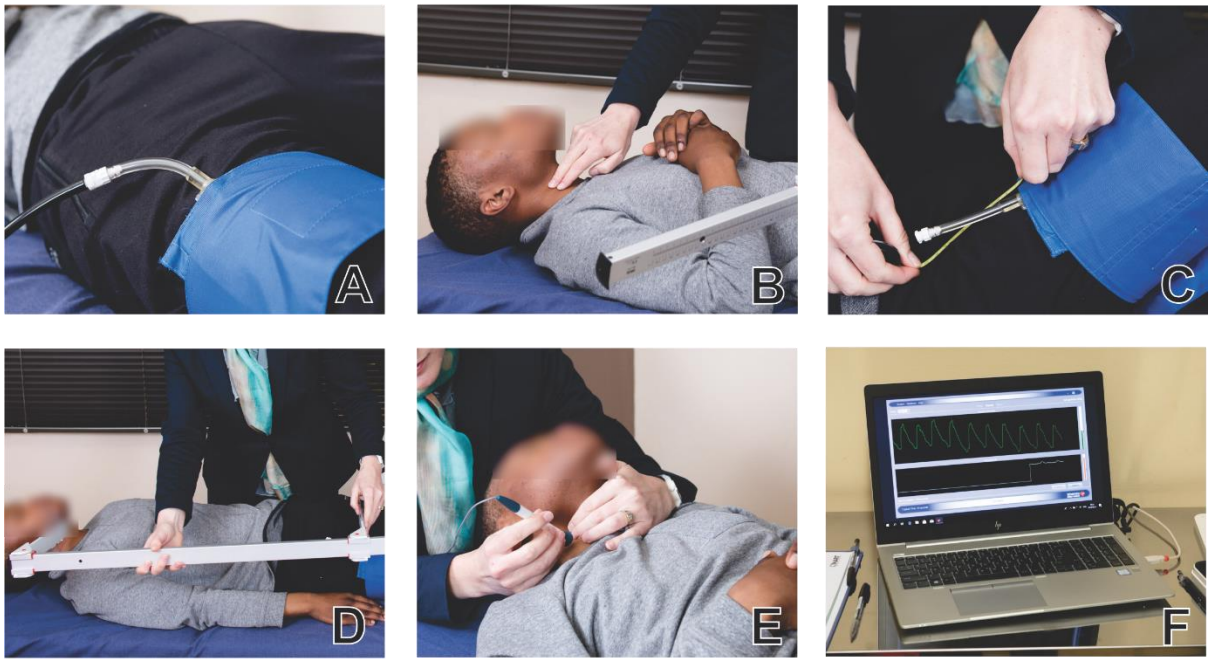


**Figure 5. Pulse wave analysis measurement for the African-PREDICT study.**

Participants were fitted with the correctly sized BP cuff the right upper thigh (A). The participant's strongest pulse point was located by means of palpation (B) whereby each pulsated site was measured (C, D). The carotid pulse of each participant was then measured using a tonometer (E), while the femoral pulse was measured simultaneously by the femoral cuff. The participant's PWV was measured along the descending thoracic-abdominal aorta with the use of the foot-to-foot method (SphygmoCor® XCEL device (AtCor Medical Pty. Ltd., Sydney, Australia)) (F) [2].

All PWV measurements were taken in duplicate. Additional measures were performed if the participants' PWV measure differed by more than 0.5m/s [20]. In the event that more than two measurements were obtained, the two closest measures were captured.

**Carotid intima media thickness**

The assessment of a participants' cIMT was used as a non-invasive measure of alterations to the arterial wall. With a simple reproducible ultrasound technique, this is considered a well-described method for assessing atherosclerotic development and predicting cardiovascular risk [21-23].

Participants were asked to remain in a supine position. Each participants' cIMT was assessed on the left and right common carotid artery, as well as the internal carotid according to the Mannheim Consensus (**Figure 6**). This was achieved by B-mode ultrasonography (General Electric Vivid E9, GE Vingmed Ultrasound A/S, Horten, Norway) along with the 2.5 to 3.5 MHz transducer in the African-PREDICT study, the high resolution SonoSite Micromaxx ultrasound system (SonoSite Micromaxx, Bothell, WA) and a 6 to 13 MHz linear array probe in the ASOS study.

**Figure 6. General Electric Vivid E9 device used for measuring carotid intima media thickness in the African-PREDICT study [2].**

Digitised images were imported in the Artery Measurement System software for analyses (Gustavvson, Sweden) [24].

### 3.4 Biochemical analyses

In both the ASOS and African-PREDICT studies, all biochemical measures and analyses were performed independently by a trained researcher. It was requested that participants fast for approximately eight hours, preferably overnight prior to the day of participation.

Upon arrival at the Hypertension Research and Training Clinic, North-West University, all African-PREDICT participants were required to provide an early morning spot urine and blood sample. Fasting blood collection was taken by a registered nurse (**Figure 7**) with the use of a sterile winged butterfly infusion set and syringe. Venous blood samples were collected from the brachial vein branches. This is an invasive procedure, yet carries minimal risk for the participant.

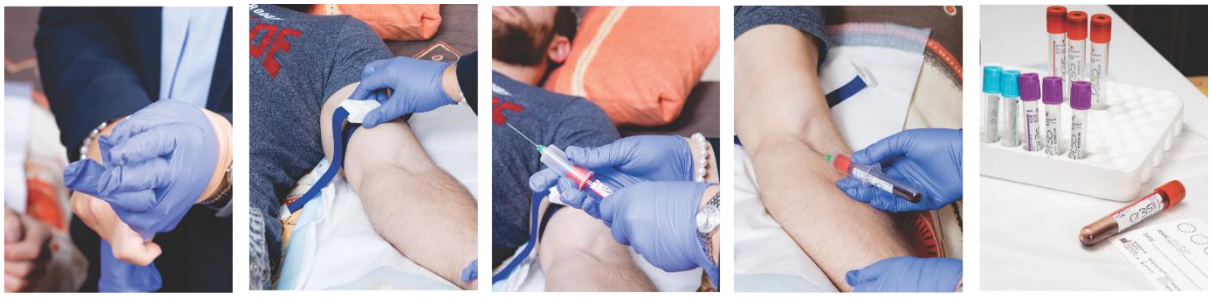

**Figure 7. Blood sampling performed by a registered nurse of the African-PREDICT study [2].**

After the biological samples were collected, all samples were taken to an on-site laboratory to be prepared and stored at –80°C until further analyses (**Figure 8**). Basic biochemical measures included urinary albumin, creatinine (Cobras Integra® 400plus, Roche, Basel, Switzerland) and the urinary albumin-to-creatinine ratio (uACR) was determined. Lipid profiles (triglycerides, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and total cholesterol), high sensitivity C-reactive protein, creatinine, albumin and gamma glutamyltransferase (GGT) were also determined in serum (Cobas Integra 400 plus Roche, Basel Switzerland). Plasma glucose was determined from blood processed in sodium fluoride tubes (Cobas Integra 400 plus Roche, Basel Switzerland). Cotinine levels were determined in serum with Chemiluminescence method on the Immulite (Siemens, Erlangen, Germany). Plasma creatine kinase (CK) was determined with electrochemiluminescence method on the E411 (Roche, Basel Switzerland).

**Figure 8. Biological samples stored in biological freezers until analysed [2].**

With the use of the Chronic Kidney Disease Epidemiology (CKD-EPI) formula [25, 26], estimated glomerular filtration rate (eGFR) was calculated. Reactive oxygen species were measured as serum peroxides (reported in units, where 1 mg $H_2O_2$/L is equivalent to one unit) using a high-throughput spectrophotometric assay and analysed on a Synergy HT microplate reader (BioTek, Winooski, VT, USA). The intra- and inter-assay variability for all African-PREDICT biochemical components was below 3.5%.

All measurements of the ASOS study were non-invasive, hence no blood samples were obtained from the child participants. Urine specimen collection kits were provided to each participant on the day prior to their participation. Collection kits were clearly marked and a cooler box with a gel ice pack including instructions on how to collect the urine sample was also provided. A mid-stream first early morning urine sample was required before any fluids were consumed. This urine collection sample was taken in the privacy of the child's home to ensure comfortability. Parents were asked to assist their children where necessary. Parents were asked to keep the urine sample bottle inside the cooler box with the provided frozen ice pack in order to maintain urine samples at low temperatures. Urine samples of the children were prepared according to standard procedures and stored at –80°C until analysed (**Figure 8**). Upon analyses, urinary albumin and creatinine concentrations were determined using the Cobas Integra® 400 plus (Roche Diagnostics Mannheim, Germany). The intra- and inter-assay variability for all ASOS biochemical components were below 2.5%.

Nitric oxide (NO)-related markers were evaluated as main independent variables in this study. With the use of gas chromatography-mass spectrometry (GC-MS) and previously reported validated protocols [27], the concentration of all urinary NO-related markers in both the ASOS and the African-PREDICT studies was determined at the Institute of Toxicology, Core Unit Proteomics, Hannover Medical School, Germany under the supervision of Professor Dimitrios

Tsikas. Internal standards were made available with the use of commercially available stable isotope labelled analogues of the NO substrates, metabolites and inhibitors. Additionally, nitrate, nitrite, malondialdehyde (MDA) and creatinine were measured simultaneously using $^{15}$N-nitrate, $^{15}$N-nitrite, [1,3-$^{2}H_2$] MDA and [*methylo*-$^{2}H_3$] creatinine as the internal standards, respectively, and derivatisation with pentafluorobenzyl bromide [27, 28]. Nitric oxide-related substrates (arginine and homoarginine), metabolites (citrulline/ornithine) and NO synthesis inhibitors (asymmetric (ADMA) and symmetric dimethylarginine (SDMA)) were measured using self-made trideuteromethyl esters followed by *N*-acylation using pentafluoropropionic anhydride [29, 30]. Symmetric dimethylarginine was analysed using the commercially available [$N^{G}$,$N'^{G}$-dimethylo-$^{2}H_6$-] SDMA as the internal standard and a single derivatisation step with pentafluoropropionic anhydride.

Equipped with an autosampler AS 1310 (ThermoFisher (Dreieich, Germany)), all GC-MS analyses were performed on a single-stage quadrupole mass spectrometer model ISQ directly interfaced with a Trace 1310 series gas chromatograph. The gas chromatograph was kitted with a fused-silica capillary column Optima 17 (0.25 mm I.D., 0.25µm film thickness) of 15 metres in length (Macherey-Nagel (Düren, Germany)). With the use of the following oven temperature regime as detailed below and the addition of helium as a carrier gas (1 mL/min), gas chromatograph separation was achieved: The oven temperature for nitrate, nitrite, MDA and creatinine was maintained at 70°C for the duration of one minute, following an increase to the temperature of 250°C at a rate of 30°C/minute followed by an increase to 320°C at a rate of 70°C/minute and held at 320°C for the duration of one minute. Interface, injector and ion-source temperatures were kept at 260°C, 200°C and 250°C, respectively. For negative-ion chemical ionisation (NICI), methane (1.0 mL/min) was used as the reagent gas. For NO substrates, metabolites and inhibitors, the oven temperature was maintained at 40°C for the duration of half a minute, following an increase to 210°C at a rate of 15°C/minute and lastly increased at a rate of 35°C/minute to 320°C which was held for the duration of one minute. Interface, injector and ion-source temperatures were kept at 300°C, 280°C and 250°C, respectively. Methane (2.4 mL/min) was used as the reagent gas for both the NICI and positive-ion chemical ionisation (PICI). The electron multiplier voltage, electron energy and electron current was set to 2025V, 70eV and 50µA respectively. The concentration of urinary NO-related markers and MDA was reported as µM and/or µmol analyte per mmol creatinine (µmol/mmol). The urinary (U) nitrate-to-nitrite molar ratio ($U_{NOx}R$) was additionally calculated ($[nitrate]_U/[nitrite]_U$) [31].

In the African-PREDICT study only, plasma NO-related markers were determined at the Department of Clinical Pharmacology and Toxicology, University Medical Center Hamburg-

Eppendorf under the supervision of Professor Edzard Schwedhelm. With the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS), plasma arginine, ADMA and SDMA were measured. Plasma (50 µL) was mixed with 50 µL of internal standard of (13)C-arginine and d(7)-ADMA followed by protein precipitation with methanol containing 1% ammonium acetate (300 µL). The supernatant (100 µL) was mixed with 300 µL of acetonitrile with 1% formic acid after centrifugation, and the mixture was injected onto a silica column monitored by a mass spectrometer. The analytical cycle time was five minutes. The method was linear from 5.7 to 489.7 µM for arginine, 0.34 to 5.65 µM for ADMA and 0.06 to 5.15 µM for SDMA, with an accuracy of 99% - 120%. Total coefficients of variation for all analytes ranged from 2.7% - 7.7% for three concentration levels [32]. Additionally, the LC–MS/MS method, together with a stable-isotope dilution, was utilised to determine the arginine homologue, plasma homoarginine. The method of using LC–MS/MS electrospray ionisation in the positive mode (ESI+) provides a high sample throughput of 25-l aliquots of plasma with a four-minute analysis time. Specific transitions for homoarginine and l-[13C6]-homoarginine were m/z → 245 m/z → m/z 211 and m/z 251 → m/z 217, respectively. The mean intra- and inter-assay critical values were 7.4 ± 4.5% (±SD) for 0.1–50 mol/L and 7.5 ± 2.0% for 2 and 5 mol/L, respectively [33].

## 4. Statistical analyses

### 4.1. Power analyses

Due to the small sample size of the ASOS study, a *priori* power analyses (**Figure 9**) was performed using G*Power v3.1.9.4 software [34]. The preselected power was 80% and the prespecified significance level was estimated at α=0.05. The priori analysis calculated that an *n* value or population of 72 (i.e. 36 black boys in one group and 36 white in the other) is sufficient for the hypothesis of this study.

t tests - Means: Difference between two independent means (two groups)

| | | | |
|---|---|---|---|
| Analysis: | Post hoc: Compute achieved power | | |
| Input: | Tail(s) | = | One |
| | Effect size d | = | 0.6 |
| | α err prob | = | 0.05 |
| | Sample size group 1 | = | 36 |
| | Sample size group 2 | = | 36 |
| Output: | Noncentrality parameter δ | = | 2.5455844 |
| | Critical t | = | 1.6669145 |
| | Df | = | 70 |
| | Power (1-β err prob) | = | 0.8094855 |

**Figure 9. Sample size calculation of the ASOS study [34].**

A power analysis was additionally performed. This study, embedded in the ASOS study, should detect an effect size of 0.2125507 (**Figure 10**) given a sample size of 39 (smallest group) and a significance of 0.05 for multiple linear regression with NO-related markers as the main independent variables with a maximum of two covariates. For the African-PREDICT study an effect size of 0.0313953 (**Figure 11**) was detected, given a sample size of 252 (smallest group) with a power of 80% and a significance level set at 0.05, for multiple linear regression with NO-related markers as the main independent variables with a maximum of nine covariates.

t tests - Linear multiple regression: Fixed model, single regression coefficient

| | | | |
|---|---|---|---|
| Analysis: | Sensitivity: Compute required effect size | | |
| Input: | Tail(s) | = | Two |
| | α err prob | = | 0.05 |
| | Power (1-β err prob) | = | 0.80 |
| | Total sample size | = | 39 |
| | Number of predictors | = | 2 |
| Output: | Noncentrality parameter δ | = | 2.8791452 |
| | Critical t | = | 2.0280940 |
| | Df | = | 36 |
| | Effect size $f^2$ | = | 0.2125507 |

**Figure 10. Power analysis of the ASOS study for 39 participants [34].**

t tests – Linear multiple regression: Fixed model, single regression coefficient

| | | | |
|---|---|---|---|
| Analysis: | Sensitivity: Compute required effect size | | |
| Input: | Tail(s) | = | Two |
| | α err prob | = | 0.05 |
| | Power (1−β err prob) | = | 0.80 |
| | Total sample size | = | 252 |
| | Number of predictors | = | 9 |
| Output: | Noncentrality parameter δ | = | 2.8127608 |
| | Critical t | = | 1.9698151 |
| | Df | = | 242 |
| | Effect size $f^2$ | = | 0.0313953 |

**Figure 11. Power analysis of the African-PREDICT study for 252 participants [34].**

### 4.2. Statistical considerations

Statistical analyses were performed with IBM® SPSS® Statistics version 26 software (IBM Corporation; Armonk, New York, USA). G*Power version 3.1.9.4 software (Faul, Erdfelder, Lang, & Buchner, 2007) [34] and GraphPad Prism version 5.03 for Microsoft® Windows (GraphPad Software, San Diego, California, USA) was used to analyse and plot results. A detailed description of the exact statistics used in each manuscript is outlined in each prevailing chapter.

## 5. Skills development and candidate contribution to data collection and analyses

I (Ms. A. Craig) am trained and competent to use the following statistical software programs: IBM® SPSS® Statistics software (IBM Corporation; Armonk, New York, USA), G*Power software [34] and GraphPad Prism for Microsoft® Windows (GraphPad Software, San Diego, California, USA).

*The ASOS study*

The ASOS data used in this study was collected before I enrolled as a student at the Hypertension in Africa Research Team (HART) and commenced my postgraduate studies. However, my contribution to this study was my ability to utilise my training in the REDCap (Research Electronic Data Capture [35], see http://project-redcap.org) software program to capture, clean and quality control NO-related data, processed after the conclusion of the study.

*The African-PREDICT study*

I was involved in the initial screening phase of the African-PREDICT study in 2016 and 2017. With reference to the African-PREDICT screening, I was responsible for urine analysis, cholesterol and glucose testing as well as BP measures and blood grouping. I was actively involved in various measurements of the follow-up phase of the African-PREDICT study in which I was responsible for pulse wave analysis using the SphygmoCor® XCEL device (AtCor Medical Pty. Ltd., Sydney, Australia), BP measures using the Dinamap® ProCare 100 Vital Signs Monitor (GE Medical Systems, Milwaukee, USA) and accurately performing a 12-lead ECG (Norav Medical Ltd, PC 1200, v5.030, Israel).

I was responsible for arranging the international biological sample shipment of 650 plasma and 1202 urinary African-PREDICT samples for the analyses of specific NO-related data. My responsibilities included sorting biological samples for international courier shipment, liaising with the respective recipients (plasma samples at the University Medical Centre, Hamburg-Eppendorf, Germany; urinary samples at the Hannover Medical School, Hannover, Germany) and compiling/arranging the required documentation between North-West University and the respective recipient institutes (ethics application for the transporting of biological samples (NWU), material agreement transfer and applying for an export permit at the Department of Health).

*Other studies*

I was also actively involved in the Exercise; Arterial Modulation and Nutrition in Youth South Africa (ExAMIN Youth SA) study in children, in which I was responsible and competent in the measurements of pulse wave analysis with the use of the validated, oscillometric Mobil-o-Graph monitor (I.E.M. GmbH, Germany). I was given administrative duties to which I oversaw specific data handling (consent/assent, general health questionnaire, food survey). Upon the conclusion of the initial screening phase of the ExAMIN Youth SA study in April 2019, I was responsible for compiling health feedback reports, which were then redistributed to the child participants.

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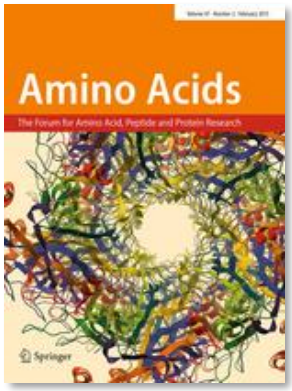
# Chapter III

## Research manuscript

This manuscript followed the specific guidelines as set out by the journal *Amino Acids*. A full list of details regarding the author's instructions are available at: https://www.springer.com/journal/726/submission-guidelines

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# Nitric oxide-related markers link inversely to blood pressure in black boys and men: The ASOS and African-PREDICT studies

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**Abstract and keywords**

**Objectives:** Nitric oxide plays an important role in maintaining endothelial function while increased oxidative stress may lead to nitric oxide inactivation and cardiovascular disease. If nitric oxide biosynthesis/bioavailability is already suppressed early in life, it may potentially predispose an individual to the early development of cardiovascular disease. We therefore aimed to identify differences in nitric oxide-related markers (urinary nitrate, nitrite and the nitrate-to-nitrite ratio ($U_{NOx}R$)) between young black and white individuals, and whether these markers associate with blood pressure and carotid intima media thickness.

**Methods:** We included black and white healthy boys (*n*=80; aged 6-8 years) and men (*n*=510; aged 20-30 years) and measured blood pressure and carotid intima media thickness, along with urinary biochemical markers including nitrate and nitrite.

**Results:** The black boys and men had lower nitrate and $U_{NOx}R$ (all $p \leq 0.003$) than their white counterparts. In single and multiple regression analyses, we found an inverse association of diastolic blood pressure in the black boys (adj. $R^2=0.27$; $\beta=-0.32$; $p=0.030$), and systolic blood pressure in black men (adj. $R^2=0.07$; $\beta=-0.13$; $p=0.036$) with nitrate. Carotid intima media thickness associated inversely with $U_{NOx}R$ in the black men (adj. $R^2=0.02$; $\beta=-0.14$; $p=0.023$), but not in the boys.

**Conclusion:** Lower urinary nitrate in black boys and young men associated negatively with blood pressure, suggesting potentially lower nitric oxide bioavailability in young black individuals may contribute to hypertension development in later life.

**Keywords:** Carotid intima media thickness, ethnicity, nitrate, nitrite, nitric oxide, urinary nitrate-to-nitrite ratio

## Introduction

Hypertension is one of the most common cardiovascular risk factors in populations of African descent [1-3]. Raised blood pressure (BP) tends to develop early in life, and is known to track into adulthood [4], ultimately leading to cardiovascular morbidity and mortality [5]. Decreased synthesis and bioavailability of nitric oxide (NO), an important endogenous regulator of vascular tone, in early life has been implicated in the development and progression of hypertension and renal disease in adulthood [6-8].

Since NO is essential in the regulation of normal endothelial function, factors influencing its availability are repeatedly explored [9]. The inorganic anions nitrite and nitrate were previously thought to be inert end products of endogenous NO metabolism [10, 11]. However, it has been shown that nitrite and nitrate may be recycled to form NO, representing an alternative source for NO synthesis apart from the classic L-arginine-NO synthase pathway [10, 11]. Furthermore, the availability of NO is suppressed by factors that enhance its inactivation, such as increased oxidative stress [12].

Nitric oxide is oxidised to nitrite (by autoxidation) and to nitrate (by oxyhaemoglobin), both being excreted in urine [13]. Urinary nitrate excretion is lower in adults with essential hypertension, suggesting impaired whole-body NO production [14]. Enhanced excretion of urinary nitrite may contribute to NO-related dysfunction in the renal and cardiovascular systems [15]. It has been proposed that the urinary nitrate-to-nitrite ratio ($U_{NOx}R$) might be a more suitable measure of nitrite reabsorption or excretion [15]. It was previously reported that black boys participating in the Arterial Stiffness in Offspring Study (ASOS) presented with lower $U_{NOx}R$ compared to white boys, which could be a result of diminished renal nitrite reabsorption and loss of circulating nitrite, a major reservoir of NO [15]. This may reflect the early onset of endothelial dysfunction in young black asymptomatic individuals [15]. Whether this is also evident in young adults warrants investigation.

Apart from studies indicating that disease states influence NO synthesis and bioavailability [16-18], less is known about the NO profile amongst individuals with no apparent cardiovascular disease (CVD), such as healthy children and young adults. It is also unclear whether BP or carotid intima media thickness (cIMT), a measure to determine thickening of the arterial wall [19], will also associate with urinary nitrate and $U_{NOx}R$ at young ages. We therefore compared urinary nitrate and $U_{NOx}R$ in two healthy bi-ethnic cohorts consisting of children and young adults.

## Methodology

We included data of black (*n*=39) and white (*n*=41) boys (aged 6-8 years) from the ASOS study and black (*n*=252) and white (*n*=258) men (aged 20-30 years) from the African Prospective study on Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT) [20], with complete datasets for urinary nitrate and nitrite.

The exclusion criteria for the ASOS study were children aged younger than 6 years and older than 8 years; girls (to exclude hormonal influences of unknown pubescence onset); obese children (whose body mass index z-score were greater than the 95$^{th}$ percentile, as indicated by the World Health Organisation) and those using any chronic medication, or with self-reported type 1 diabetes mellitus, renal disease or cancer. The study population and protocol for the African-PREDICT study has been described elsewhere [20]. Briefly, participants with office BP >140/90 mmHg, or with any self-reported diseases or risk factors that may influence cardiovascular health, internal ear temperature >37.5°C, human immunodeficiency virus, diabetes mellitus, liver disease, cancer, tuberculosis or renal disease as well as the use of chronic medication were excluded from the African-PREDICT study. Pregnant and lactating women were also excluded due to known influences of hormones on cardiovascular health. Since the ASOS study only included boys, for our main analyses the African-PREDICT study were also done in men only.

## Anthropometric measures

All anthropometric procedures were performed according to specific guidelines set out by the International Society for the Advancement of Kinanthropometry (ISAK) [20, 21]. Body mass index (BMI) (weight (kg) / square height ($m^2$)) of each participant was then calculated (SECA portable 213 stadiometer; SECA 813 electronic scale; Hamburg, Germany). Body mass index z-scores were used for the assessment of body composition in children. Thresholds derived from a child growth reference was used to classify the BMI z-scores of children according to their age and sex [22].

## Cardiovascular measures

In the ASOS study, brachial BP was measured in triplicate on the upper left arm at heart level with a paediatric validated Omron HEM-759-E (750IT) device (Omron Healthcare, Tokyo, Japan) [23]. With the use of a Dinamap® ProCare 100 Vital Signs Monitor, brachial BP of the African-PREDICT participants was measured on the left arm, thereafter on the right arm in duplicate followed by a repeated measure on the left upper arm (GE Medical Systems, Milwaukee, USA). In this study, the left BP measurement was used in the analyses. Systolic

blood pressure (SBP) and diastolic blood pressure (DBP) were captured from each measurement. The mean arterial pressure (MAP) was subsequently calculated from brachial BP recordings. Mean arterial pressure was calculated using the formula: (DBP) + (0.4*pulse pressure) [24].

In the ASOS and African-PREDICT studies, cIMT was measured using B-mode ultrasonography (ASOS: SonoSite MicroMaxx, Bothell, WA; African-PREDICT: General Electric Vivid E9 device; GE Vingmed Ultrasound A/S, Horten, Norway) according to the Mannheim Consensus [25]. The cIMT was calculated from at least two optimal angles on both the left and the right side of the neck. The Artery Measurement System (Gustavvson, Sweden) software was used to quantify cIMT and the derived luminal diameter of the common carotid arteries. A maximal 10 mm segment with good image quality of the near and far wall was chosen for offline analysis. The far wall measurement of cIMT was used in both studies.

**Biochemical analyses**

A first voided mid-stream urine sample was acquired from each ASOS participant in the privacy of their own home. Participants were provided with sealable cups and containers to collect first urine samples on the day of participation. The African-PREDICT participants were required to provide an early morning spot urine sample at the Hypertension Clinic of the North-West University. Urine samples were prepared according to standard procedures.

In the ASOS and African-PREDICT studies, nitrite, nitrate, malondialdehyde (MDA) and creatinine were measured simultaneously as described elsewhere [26]. All nitrite, nitrate and MDA data were normalised to creatinine excretion, and the urinary (u) nitrate-to-nitrite molar ratio ($U_{NOx}R$) was calculated ($[nitrate]_u/[nitrite]_u$) [15]. In the African-PREDICT study, serum peroxides, as an indicator of reactive oxygen species (ROS), were determined using a high-throughput spectrophotometric assay and analysed on a Synergy HT microplate reader (BioTek, Winooski, VT, USA). ROS is reported in units, where 1 mg $H_2O_2$/L is equivalent to one unit [27].

**Statistical analyses**

For statistical analyses, IBM®, SPSS® version 25 (IBM Corporation, Armonk, New York) was used. We tested for interactions of ethnicity on the association of BP or cIMT with NO metabolites (nitrate and $U_{NOx}R$). Variables were tested for normality using the Kolmogorov-Smirnov test and QQ-plots. Non-Gaussian variables were logarithmically transformed. Data

was expressed as mean ± standard deviation if normally distributed and as geometric mean with 5$^{th}$ and 95$^{th}$ percentile boundaries for logarithmically transformed variables.

For comparisons between ethnic groups, independent T-tests were used. The correlations of BP or cIMT with urinary nitrate and $U_{NOx}R$ were performed using Pearson correlations. Standard multiple regression analyses were conducted where either BP or cIMT were included as dependent variables, and tested separately for their association with urinary nitrate and $U_{NOx}R$. Covariates considered for entry in the multiple regression models included age and BMI. When cIMT was the dependent variable it was additionally adjusted for MAP.

**Results**

We found significant interactions of ethnicity on the associations of BP and cIMT with urinary nitrate and $U_{NOx}R$ as presented in **Table 1**. We therefore stratified our groups according to black and white ethnicity.

The characteristics of the study populations stratified by age and ethnicity are presented in **Table 2**. In boys, body composition was comparable between the groups. Diastolic BP, MAP and cIMT of the black boys were higher than their white counterparts (all $p \leq 0.006$). The black boys also presented with lower nitrate and $U_{NOx}R$ (all $p \leq 0.003$). In young adults, the black men presented with lower body composition measures (all $p \leq 0.001$), and lower nitrate concentration and $U_{NOx}R$ (all $p \leq 0.003$) compared to the white men. All cardiovascular measures were comparable between the groups.

In **Figure 1**, urinary nitrate and $U_{NOx}R$ were compared between boys and men, stratified by ethnicity. By doing so, men of both ethnicities had significantly lower mean levels of urinary nitrate and $U_{NOx}R$ than boys (all $p \leq 0.023$).

In single regression analysis presented in **Table 3**, DBP (r= –0.349; $p$=0.030) and MAP (r= –0.337; $p$=0.036) associated inversely with nitrate in the black boys. In the men, SBP (r= –0.118; $p$=0.004) and MAP (r= –0.134; $p$=0.033) associated inversely with nitrate in the black men and cIMT positively with nitrate concentration in the white men (r= 0.131; $p$= 0.035). Carotid intima media thickness also correlated inversely with the $U_{NOx}R$ in the black men only (r= –0.142; $p$=0.024).

In multiple regression analysis (**Table 4**), we found a consistent negative association between DBP and urinary nitrate in the black boys (adj. $R^2$= 0.274; β= –0.323; $p$= 0.030) only. Systolic BP associated inversely with urinary nitrate only in the black men (adj. $R^2$= 0.065; β= –0.132;

*p*= 0.036). Carotid intima media thickness also correlated inversely with the $U_{NOx}R$ in the black men (adj. $R^2$= 0.022; β= –0.143; *p*= 0.023) only. A positive association between cIMT and nitrate remained in the white men (adj. $R^2$= 0.027; β= 0.128; *p*= 0.039).

**Sensitivity analyses**

To investigate whether the reported associations of BP and/or cIMT with NO-related markers are dependent on oxidative stress, we included urinary MDA as a marker of *in vivo* lipid peroxidation in our multiple regression model (**Supplementary Table 1**). In healthy adults (aged, 60-70 years), a strong positive correlation was found between urinary nitrite and MDA (*r*=0.819, *p*<0.0001) [28]. In the present study, we found positive correlations between urinary nitrite and MDA in black (*r*=0.425, *p*<0.001) and white (*r*=0.265, *p*<0.0001) men, in black boys (*r*=0.441, *p*=0.006), but not in white boys (*r*=-0.025, *p*=0.876). The significant associations previously reported in black (SBP and nitrate: adj. $R^2$= 0.064; β= –0.127; *p*= 0.044, cIMT and $U_{NOx}R$: $R^2$= 0.005; β= –0.140; *p*= 0.040) and white (cIMT and nitrate: adj. $R^2$= 0.040; β= 0.127; *p*= 0.039) men remained robust. In black boys the association between DBP and nitrate (adj. $R^2$= 0.252; β= –0.330; *p*= 0.033) also remained significant.

We alternatively included another marker of oxidative stress, i.e., serum peroxides as a measure of ROS in the models for young men. All the significant associations previously reported in Table 4 lost significance when ROS was included (SBP and nitrate: adj. $R^2$= 0.026; β= –0.135; *p*= 0.26, cIMT and $U_{NOx}R$: adj. $R^2$= –0.013; β= –0.148; *p*= 0.21 and cIMT and nitrate: adj. $R^2$= –0.009; β= 0.148; *p*= 0.23).

Due to the inclusion criteria of the ASOS study, we decided to only include men from the African-PREDICT study for comparison. However, as the African-PREDICT study also captured data from women, we additionally compared the characteristics of black and white women (**Supplementary Table 2**). Black women presented with higher body composition measures (all *p*≤0.007) compared to the white women. Systolic BP, DBP and MAP of the black women presented higher than their white counterparts (all *p*<0.001). The black women also presented with lower nitrate and $U_{NOx}R$ (all *p*<0.001) levels, than white women. In addition, we also performed a multiple regression analyses (**Supplementary Table 3**) however, after full adjustments for potential confounders no significant associations were found.

## Discussion

Our study compared urinary nitrate and $U_{NOx}R$ in two healthy cohorts consisting of black and white boys (aged 6-8 years; ASOS) and men (aged 20-30 years; African-PREDICT), and we determined whether these NO-related biomarkers associate with BP and cIMT in these young populations. Urinary nitrate and $U_{NOx}R$ levels were lower in black boys and men, when compared to their white counterparts. We found that BP associated independently and negatively with urinary nitrate in both black boys and men, but not in the white groups. We also found cIMT to associate inversely with $U_{NOx}R$ in the black men and positively with urinary nitrate in the white men.

In urine samples of black and white boys and men, black men in our study (independent of age) had significantly lower urinary nitrate and $U_{NOx}R$ levels, yet had similar nitrite, when compared to their white counterparts. Similarly, in women, we found the same trend of lower nitrate and $U_{NOx}R$ levels in black compared to white women. The lower nitrate and $U_{NOx}R$ levels in the black groups may indicate a reduced capacity in NO metabolism via the alternative renal pathway of nitrite reabsorption and supports previous findings reporting lower L-arginine (primary substrate for NO production) in black men, compared to whites, which may affect NO synthesis [29]. The origin of these differences in NO metabolism in these bi-ethnic populations is unknown. Furthermore, the lower $U_{NOx}R$ values of the black group of the present study could indicate a lower reabsorption or higher excretion of nitrite, presumably due to genetic differences in the renal carbonic anhydrase isoforms and anion transporters [15, 30]. Lower nitrate excretion and lower $U_{NOx}R$ indicate diminished carbonic anhydrase dependent nitrite reabsorption and ultimately lower NO bioavailability via this alternative renal pathway of NO production. Ethnic differences in CVD development, including renal physiology, of black and white populations have been long reported [31-33]. For instance, black South Africans are prone to lower fractional sodium excretion, thus increasing sodium retention which further promotes hypertension development [32, 33]. The underlying mechanisms surrounding ethnic disparities seem to be multifactorial, and it is unknown what the contribution of socio-economic status, genetic and environmental factors are that may explain the differences in NO metabolism and risk for hypertension when comparing these population groups [31-34].

We also investigated how BP or carotid wall thickness is associated with these markers of NO metabolism. We found an inverse relationship between BP and urinary nitrate levels in both of the black age groups. This result confirms the findings of others who found that an increase in BP is, at least in part, induced by a decrease in circulating NO metabolites [35, 36]. Our finding may suggest potential diminished NO bioavailability via an alternative renal pathway

of NO production in the black groups and may further decline and contribute to hypertension development in later life. Lower NO bioavailability is associated with reduced vasodilation and favours vasoconstriction thus increasing BP [37, 38]. In adults, elevated SBP predicts future CVD [39], but in children, DBP seems to be a better measure of cardiovascular risk [40, 41]. The inverse associations between BP and markers of NO bioavailability observed in this present study may therefore suggest that the level of NO expressed precedes raised BP in later life. Although the black group displayed lower nitrate and $U_{NOx}R$ levels when compared to the white group, boys showed higher nitrate and $U_{NOx}R$ levels when compared to men. It was already reported that in children, urinary nitrate levels are high and would decrease with age [42], as confirmed by the findings of the present study. The mechanism for an age-related decrease in urinary nitrate levels may be due to younger individuals having a heightened basal NO synthase activity [42, 43].

We further showed an independent inverse association between cIMT and $U_{NOx}R$ in black men. Such an association has not been reported previously. Urinary nitrate-to-nitrite ratio is a suitable measure of renal reabsorption of urinary nitrite in humans [30]. Hence, the inverse relationship between cIMT and $U_{NOx}R$ found in our study, indicates increased nitrite excretion or poorer renal reabsorption of nitrite, which is in agreement with an inverse association observed between IMT and plasma nitrite in individuals with endothelial dysfunction [44]. In addition to the endothelial protective effects of NO, it has been demonstrated that NO has anti-atherogenic properties [45]. These results suggest that although cIMT was similar between young healthy black and white men, the lower $U_{NOx}R$ levels in black men and the independent negative association with cIMT suggest that lower systemic nitrite levels may have implications on systemic endothelial function which may contribute to the potential development of atherosclerosis [9]. Additionally, we found a positive association between cIMT and nitrate in the white men, however, after full adjustment for potential confounders this association lost significance.

The last prominent finding from our study is that black men presented with higher urinary MDA markers of whole-body lipid peroxidation [46] and systemic ROS, respectively. Elevated oxidative stress is known to contribute to hypertension and play a pivotal role in the progression of CVD ([47, 48]. We determined oxidative stress markers could potentially mediate the associations reported between BP or cIMT with urinary nitrate and $U_{NOx}R$. In our exploratory analysis, the negative associations between DBP (in black boys), SBP and urinary nitrate as well as between cIMT and $U_{NOx}R$ in black men remained significant when urinary MDA was added to the model. However, the absence of associations of BP or cIMT with the NO-related markers after correcting for ROS in the men indicates a physiological interaction

between reactive molecules and NO within this young group of men. With serum peroxides being the main component measured in the ROS assay, it is rather non-specific compared to MDA, a marker of lipid peroxidation indicating whole-body lipid radical formation. Therefore, these two markers may reflect differing degrees of oxidative stress. Whether the presence of increased oxidative stress affects the bioavailability of NO within this group warrants further investigation.

Our study must be interpreted within the context of its strengths, limitations and recommendations. Our study was well planned and executed under strict conditions. Our study populations included participants from the North West province of South Africa, and are not representative of the population as a whole. Our study is limited by its cross-sectional design; hence we were unable to investigate precise mechanisms and causal relationships. The studies in boys (ASOS) and men (African-PREDICT) were two independent studies. Future studies on NO bioavailability should include girls and consider diet, as it may influence nitrate and nitrite levels and BP. To expand on the present findings, future studies should include flow-mediated dilation as a measurement of endothelial function as well as to investigate serum systemic ROS in children as marker of oxidative stress.

In conclusion, black boys and men presented with lower nitrates and $U_{NOx}R$ compared to white boys and men. In addition, BP associated inversely with nitrate in black boys and men, whereas cIMT associated inversely with $U_{NOx}R$ in black men only. Our findings suggest that lower NO biosynthesis and bioavailability may increase the susceptibility of the black population for premature hypertension development in later life.

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**Conflict of interest**

The authors report that they have no conflict of interest.

**Ethical Statement**

Participants were fully informed about the objectives of both studies (written informed consent and assent were obtained from all participants included in the study). All procedures performed in study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Health Research Ethics Committee of the North-West University; ASOS study: NWU-00007-15-A1; African-PREDICT study: NWU-00001-12-A1) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards [49].

**TABLE 1: Interaction terms of ethnicity on the relationship of blood pressure and carotid intima media thickness with creatinine-corrected urinary nitrate (μM/mM) and nitrate-to-nitrite ratio.**

| | Boys | Men |
|---|---|---|
| | ***p* value** | ***p* value** |
| **Systolic blood pressure** | | |
| Nitrate (μM/mM) | 0.006 | 0.002 |
| Urinary nitrate-to-nitrite ratio | 0.002 | 0.002 |
| **Diastolic blood pressure** | | |
| Nitrate (μM/mM) | 0.017 | 0.003 |
| Urinary nitrate-to-nitrite ratio | 0.006 | 0.002 |
| **Mean arterial pressure** | | |
| Nitrate (μM/mM) | 0.003 | 0.003 |
| Urinary nitrate-to-nitrite ratio | 0.001 | 0.002 |
| **Intima media thickness** | | |
| Nitrate (μM/mM) | 0.010 | 0.002 |
| Urinary nitrate-to-nitrite ratio | 0.008 | 0.003 |

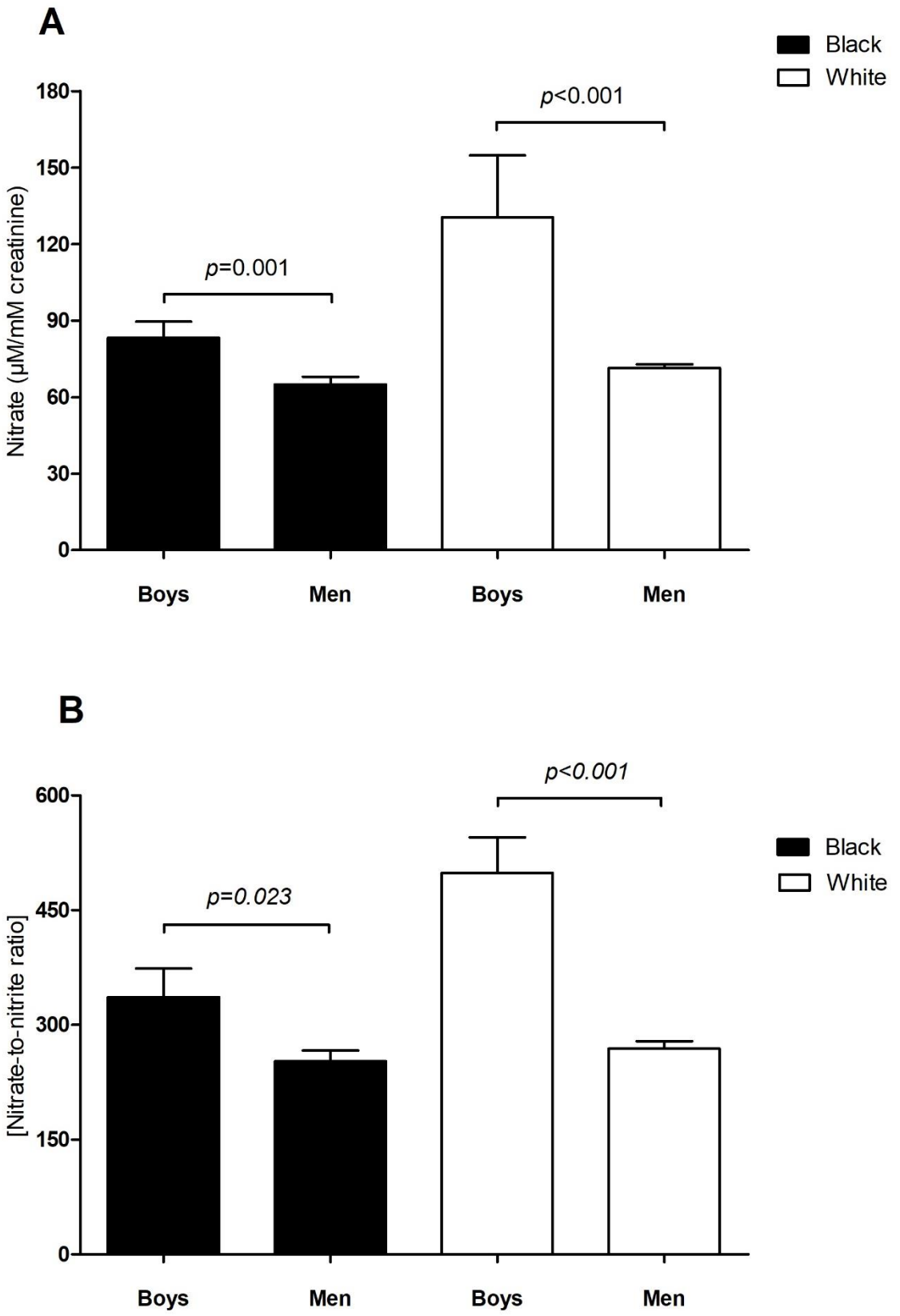


**Figure 1: Comparing the mean values of (A) creatinine-corrected urinary nitrate levels and (B) urinary nitrate-to-nitrite ratio in boys and men.**

**TABLE 2: General characteristics and markers related to nitric oxide and oxidative stress of two male study populations stratified according to age and ethnicity.**

| | Boys | | | Men | | |
|---|---|---|---|---|---|---|
| | **Black** (*n*= 39) | **White** (*n*= 41) | ***p* value** | **Black** (*n*= 252) | **White** (*n*= 258) | ***p* value** |
| Age (years) | 7.83 ± 0.71 | 7.68 ± 0.97 | 0.42 | 24.29 ± 3.06 | 24.78 ± 3.03 | 0.072 |
| **Body composition** | | | | | | |
| Waist circumference (cm) | 57.14 ± 5.62 | 58.03 ± 5.76 | 0.48 | 76.47 ± 9.34 | 88.73 ± 11.76 | **<0.001** |
| Body Height (mm) | 125.86 ± 6.37 | 125.99 ± 7.14 | 0.93 | 169.96 ± 6.65 | 179.03 ± 6.20 | **<0.001** |
| Body mass index (kg/m²) | 16.51 ± 2.07 | 16.03 ± 1.68 | 0.25 | 22.23 ± 3.99 | 26.56 ± 4.58 | **<0.001** |
| **Cardiovascular measures** | | | | | | |
| Systolic blood pressure (mmHg) | 105 ± 11 | 102 ± 7 | 0.16 | 123 ± 12 | 124 ± 10 | 0.38 |
| Diastolic blood pressure (mmHg) | 69 ± 9 | 63 ± 8 | **0.001** | 81 ± 9 | 80 ± 8 | 0.085 |
| Mean arterial pressure (mmHg) | 83.72 ± 9.21 | 78.71 ± 6.61 | **0.006** | 97 ± 9 | 97 ± 8 | 0.82 |
| Carotid intima-media thickness (mm)* | 0.47 ± 0.07 | 0.43 ± 0.06 | **0.003** | 0.45 ± 0.07 | 0.44 ± 0.07 | 0.18 |
| **Nitric oxide- related markers** | | | | | | |
| Nitrite (µM/mM creatinine) | 0.28 (0.11 – 1.04) | 0.25 (0.14 – 0.55) | 0.37 | 0.29 (0.10 – 0.90) | 0.27 (0.12 – 0.85) | 0.35 |
| Nitrate (µM/mM creatinine) | 76.7 (50.2 – 194.1) | 105 (45.1 – 322) | **0.003** | 58.9 (31.3 – 128) | 65.7 (36.2 – 126) | **0.003** |
| Urinary nitrate-to-nitrite ratio | 271 (78.3 – 645) | 415 (929 – 1074) | **0.001** | 205 (64.3 – 576) | 241 (85.7 – 529) | **0.002** |
| **Oxidative stress related markers** | | | | | | |
| Malondialdehyde (µM/mM creatinine) | 0.21 (0.10 – 0.80) | 0.17 (0.10 – 0.53) | 0.105 | 0.16 (0.07 – 0.41) | 0.12 (0.06 – 0.29) | **<0.001** |
| Reactive oxygen species (mg/L $H_2O_2$) | - | - | - | 157.60 ± 47.52 | 138.28 ± 38.77 | **0.008** |

Values are arithmetic mean ± standard deviation or geometric mean (5th and 95th percentiles) for logarithmically transformed variables. * Carotid intima media thickness was adjusted for mean arterial pressure, † Samples measured in urine (µM/mM creatinine), ‡ *Samples measured in whole blood (µM).* Abbreviations: *n* – number of participants. Bold values denote statistical significance (p<0.05).

**TABLE 3: Pearson correlations between anthropometric and cardiovascular measures and markers related to nitric oxide synthesis in males stratified by age and ethnicity in the ASOS and African-PREDICT studies.**

| | Nitrate (µM/mM creatinine) | | | | Urinary nitrate-to-nitrite ratio | | | |
|---|---|---|---|---|---|---|---|---|
| | Boys | | Men | | Boys | | Men | |
| | **Black** (n= 39) | **White** (n= 41) | **Black** (n= 252) | **White** (n= 258) | **Black** (n= 39) | **White** (n= 41) | **Black** (n= 252) | **White** (n= 258) |
| Age (years) | r = –0.209<br>*p* = 0.201 | r = –0.281<br>*p* = 0.075 | r = –0.027<br>*p* = 0.675 | r = 0.061<br>*p* = 0.333 | r = –0.202<br>*p* = 0.218 | r = –0.093<br>*p* = 0.564 | r = 0.058<br>*p* = 0.358 | r = 0.095<br>*p* = 0.128 |
| Waist circumference (cm) | r = –0.118<br>*p* = 0473 | r = –0.223<br>*p* = 0.161 | **r = –0.224**<br>***p* <0.001** | r = 0.033<br>*p* = 0.603 | r = 0.087<br>*p* = 0.600 | r = –0.023<br>*p* = 0.885 | r = –0.018<br>*p* = 0.775 | r = 0.002<br>*p* = 0.975 |
| Body height (mm) | r = –0.097<br>*p* = 0.557 | r = –0.366<br>*p* = 0.019 | r = –0.118<br>*p* = 0.061 | r = 0.060<br>*p* = 0.344 | r = –0.018<br>*p* = 0.913 | r = –0.149<br>*p* = 0.353 | **r = 0.129**<br>***p* = 0.039** | r = 0.079<br>*p* = 0.208 |
| Body mass index (kg/m²) | r = –0.116<br>*p* = 0.313 | r = –0.006<br>*p* = 0.969 | **r = –0.231**<br>***p* <0.001** | r = 0.023<br>*p* = 0.714 | r = 0.037<br>*p* = 0.823 | r = –0.049<br>*p* = 0.762 | r = 0.054<br>*p* = 0.390 | r = –0.032<br>*p* = 0.604 |
| Systolic blood pressure (mmHg) | r = –0.273<br>*p* = 0.093 | r = –0.083<br>*p* = 0.606 | **r = –0.118**<br>***p* = 0.004** | r = 0.093<br>*p* = 0.137 | r = –0.133<br>*p* = 0.420 | r = –0.077<br>*p* = 0.634 | r = 0.006<br>*p* = 0.920 | r = –0.028<br>*p* = 0.655 |
| Diastolic blood pressure (mmHg) | **r = –0.349**<br>***p* = 0.030** | r = 0.026<br>*p* = 0.872 | r = –0.066<br>*p* = 0.298 | r = 0.027<br>*p* = 0.666 | r = –0.171<br>*p* = 0.298 | r = –0.101<br>*p* = 0.951 | r = 0.036<br>*p* = 0.571 | r = –0.002<br>*p* = 0.973 |
| Mean arterial pressure (mmHg) | **r = –0.337**<br>***p* = 0.036** | r = –0.017<br>*p* = 0.916 | **r = –0.134**<br>***p* = 0.033** | r = 0.061<br>*p* = 0.327 | r = –0.166<br>*p* = 0.312 | r = –0.040<br>*p* = 0.805 | r = 0.012<br>*p* = 0.853 | r = –0.036<br>*p* = 0.560 |
| Carotid intima media thickness (mm) | r = –0.107<br>*p* = 0.516 | r = –0.093<br>*p* = 0.563 | r = –0.002<br>*p* = 0.978 | **r = 0.131**<br>***p* = 0.035** | r = –0.174<br>*p* = 0.290 | r = –0.145<br>*p* = 0.366 | **r = –0.142**<br>***p* = 0.024** | r = 0.095<br>*p* = 0.129 |
| Malondialdehyde (µM/mM creatinine) | r = 0.060<br>*p* = 0.718 | r = 0.194<br>*p* = 0.225 | r = 0.106<br>*p* = 0.092 | r = 0.008<br>*p* = 0.903 | r = –0.269<br>*p* = 0.097 | r = –0.080<br>*p* = 0.621 | **r = –0.377**<br>***p* <0.001** | **r = –0.277**<br>***p* <0.001** |
| Reactive oxygen species (mg/L $H_2O_2$) | - | - | r = 0.065<br>*p* = 0.581 | **r = 0.243**<br>***p* = 0.038** | - | - | r = –0.033<br>*p* = 0.781 | r = 0.197<br>*p* = 0.095 |

† Samples measured in urine (µM/mM creatinine), ‡ Samples measured in whole blood (µM). Abbreviations: n – number of participants. Bold values denote statistical significance (p<0.05).

**TABLE 4: Multiple regression analyses with cardiovascular measures as dependent variables in males stratified by age and ethnicity in the ASOS and African-PREDICT studies.**

| | Boys † | | | Men ‡ | | |
|---|---|---|---|---|---|---|
| | **Adjusted $R^2$** | **Std β (95 % CI)** | ***p* value** | **Adjusted $R^2$** | **Std β (95 % CI)** | ***p* value** |
| | | | **Nitrate (µM/mM creatinine)** | | | |
| **Systolic blood pressure (mm/Hg)** | | | | | | |
| Black | 0.235 | –0.189 (–0.683; 0.151) | 0.20 | **0.065** | **–0.132 (–0.265; –0.009)** | **0.036** |
| White | 0.058 | –0.057 (–0.278; 0.195) | 0.73 | 0.086 | 0.081 (–0.036; 0.193) | 0.18 |
| **Diastolic blood pressure (mm/Hg)** | | | | | | |
| Black | **0.274** | **–0.323 (–0.726; –0.040)** | **0.030** | 0.078 | –0.046 (–0.173; 0.079) | 0.46 |
| White | –0.009 | 0.024 (–0.261; 0.302) | 0.88 | 0.111 | 0.008 (–0.106; 0.122) | 0.89 |
| **Mean arterial pressure (mm/Hg)** | | | | | | |
| Black | 0.287 | –0.281 (–0.740; 0.007) | 0.054 | 0.052 | –0.104 (–0.235; 0.021) | 0.10 |
| White | 0.035 | –0.007 (–0.257; 0.247) | 0.97 | 0.083 | 0.047 (–0.070; 0.163) | 0.43 |
| **Carotid intima media thickness (mm)*** | | | | | | |
| Black | 0.002 | –0.017 (–0.417; 0.379) | 0.93 | 0.001 | 0.010 (–0.114; 0.132) | 0.88 |
| White | –0.002 | –0.114 (–0.414; 0.204) | 0.50 | **0.027** | **0.128 (0.007; 0.265)** | **0.039** |
| | | | **Urinary nitrate-to-nitrite ratio** | | | |
| **Systolic blood pressure (mm/Hg)** | | | | | | |
| Black | 0.057 | –0.052 (–0.307; 0.220) | 0.74 | 0.049 | –0.004 (–0.126; 0.117) | 0.94 |
| White | 0.220 | –0.145 (–0.539; 0.186) | 0.33 | 0.081 | –0.029 (–0.150; 0.091) | 0.63 |
| **Diastolic blood pressure (mm/Hg)** | | | | | | |
| Black | –0.009 | 0.001 (–0.312; 0.314) | 0.99 | 0.076 | 0.016 (–0.102; 0.134) | 0.79 |
| White | 0.225 | –0.237 (–0.547; 0.062) | 0.12 | 0.111 | –0.016 (–0.137; 0.103) | 0.78 |
| **Mean arterial pressure (mm/Hg)** | | | | | | |
| Black | 0.036 | –0.021 (–0.299; 0.261) | 0.89 | 0.042 | –0.006 (–0.126; 0.115) | 0.93 |
| White | 0.252 | –0.211 (–0.567; 0.091) | 0.15 | 0.082 | –0.043 (–0.167; 0.078) | 0.48 |
| **Carotid intima media thickness (mm)*** | | | | | | |
| Black | 0.006 | –0.142 (–0.492; 0.191) | 0.38 | **0.022** | **–0.143 (–0.246; –0.018)** | **0.023** |
| White | 0.026 | –0.155 (–0.479; 0.182) | 0.37 | 0.017 | 0.081 (–0.046; 0.227) | 0.19 |

Variables included in the models were: age and body mass index. * Carotid intima media thickness was additionally adjusted for mean arterial pressure, † Black (n= 39) and white (n= 41), ‡ Black (n= 252) and white (n= 258). Abbreviations: n – number of participants. Bold values denote statistical significance ($p<0.05$).

**SUPPLEMENTARY TABLE 1: Multiple regression analyses of cardiovascular measures with nitrate and nitrate-to-nitrite ratio in male study population additionally adjusted for malondialdehyde.**

| | Boys † | | | Men ‡ | | |
|---|---|---|---|---|---|---|
| | **Adjusted $R^2$** | **Std β (95 % CI)** | ***p* value** | **Adjusted $R^2$** | **Std β (95 % CI)** | ***p* value** |
| | **Nitrate (µm/mm)** | | | | | |
| **Systolic blood pressure (mm/Hg)** | | | | | | |
| Black | 0.216 | –0.184 (–0.678; 0.164) | 0.22 | 0.064 | –0.127 (–0.244; –0.003) | **0.044** |
| White | 0.041 | –0.040 (–0.273; 0.214) | 0.81 | 0.086 | 0.081 (–0.033; 0.180) | 0.18 |
| **Diastolic blood pressure (mm/Hg)** | | | | | | |
| Black | 0.252 | –0.330 (–0.749; –0.034) | **0.033** | 0.075 | –0.043 (–0.179; 0.087) | 0.50 |
| White | –0.036 | 0.022 (–0.270; 0.306) | 0.90 | 0.108 | 0.008 (–0.112; 0.128) | 0.89 |
| **Mean arterial pressure (mm/Hg)** | | | | | | |
| Black | –0.006 | –0.148 (–0.458; 0.181 | 0.38 | 0.051 | –0.100 (–0.231; 0.025) | 0.12 |
| White | –0.046 | –0.080 (–0.483; 0.301) | 0.64 | 0.079 | 0.047 (–0.070; 0.163) | 0.43 |
| **Intima media thickness (mm)*** | | | | | | |
| Black | 0.017 | –0.064 (–0.448; 0.307) | 0.71 | 0.002 | 0.004 (–0.129; 0.137) | 0.95 |
| White | –0.034 | –0.129 (–0.440; 0.201) | 0.45 | 0.040 | 0.127 (0.008; 0.285) | **0.039** |
| | **Urinary nitrate-to-nitrite ratio** | | | | | |
| **Systolic blood pressure (mm/Hg)** | | | | | | |
| Black | 0.223 | –0.204 (–0.617; 0.124) | 0.19 | 0.050 | –0.032 (–0.151; 0.091) | 0.63 |
| White | 0.044 | –0.063 (–0.321; 0.215) | 0.69 | 0.081 | –0.048 (–0.161; 0.071) | 0.44 |
| **Diastolic blood pressure (mm/Hg)** | | | | | | |
| Black | 0.210 | –0. 276 (–0.620; 0.054) | 0.10 | 0.074 | 0.001 (–0.132; 0.134) | 0.99 |
| White | –0.037 | –0.003 (–0.325; 0.319) | 0.99 | 0.108 | –0.011 (–0.142; 0.118) | 0.85 |
| **Mean arterial pressure (mm/Hg)** | | | | | | |
| Black | 0.014 | –0.208 (–0.447; 0.111) | 0.23 | 0.042 | –0.030 (–0.158; 0.099) | 0.65 |
| White | –0.046 | 0.079 (–0.329; 0.535) | 0.63 | 0.079 | –0.044 (–0.172; 0.082) | 0.49 |
| **Intima media thickness (mm)*** | | | | | | |
| Black | 0.027 | –0.118 (–0.448; 0.223) | 0.50 | 0.005 | –0.140 (–0.270; –0.006) | **0.040** |
| White | –0.033 | –0.129 (–0.490; 0.216) | 0.44 | 0.037 | 0.122 (–0.005; 0.296) | 0.058 |

Variables included in the models were: age, body mass index and malondialdehyde. *Intima media thickness was additionally adjusted for mean arterial pressure, † Black (*n*= 39) and white (*n*= 41), ‡ Black (*n*= 252) and white (*n*= 258). Abbreviations: n – number of participants. Bold values denote statistical significance (p<0.05).

**SUPPLEMENTARY TABLE 2: General characteristics and markers related to nitric oxide and oxidative stress of a women study population (African-PREDICT) stratified according to ethnicity.**

| | Women | | |
|---|---|---|---|
| | **Black** (*n*= 265) | **White** (*n*= 285) | ***p* value** |
| Age (years) | 24.54 ± 3.31 | 24.46 ± 3.10 | 0.77 |
| **Body composition** | | | |
| Body height (cm) | 159.09 ± 6.11 | 166.68 ± 6.36 | **<0.001** |
| Body mass index (kg/m²) | 26.62 ± 5.92 | 24.48 ± 5.33 | **<0.001** |
| Waist circumference (cm) | 79.08 ± 12.25 | 76.50 ± 11.74 | **0.007** |
| **Cardiovascular measures** | | | |
| Systolic blood pressure (mmHg) | 114 ± 10 | 110 ± 10 | **<0.001** |
| Diastolic blood pressure (mmHg) | 78 ± 8 | 75 ± 7 | **<0.001** |
| Mean arterial pressure (mmHg) | 92 ± 7 | 89 ± 8 | **<0.001** |
| Intima-media thickness (mm) * | 0.43 ± 0.007 | 0.43 ± 0.06 | 0.92 |
| **Urinary nitric oxide-related markers** | | | |
| Nitrite (µM/mM creatinine) | 0.29 (0.09 – 0.87) | 0.29 (0.10- -0.95) | 0.93 |
| Nitrate (µM/mM creatinine) | 60.4 (34.9 – 124) | 75.2 (37 – 168) | **<0.001** |
| Nitrate-to-nitrite-ratio | 209 (68.9 – 578) | 259 (88.2 – 717) | **<0.001** |
| **Oxidative stress related markers** | | | |
| Malondialdehyde (µM/mM creatinine) | 0.18 (0.08 – 0.67) | 0.13 (0.06 – 0.44) | **<0.001** |
| Reactive oxygen species (mg/L $H_2O_2$) | 219.15 ± 69.84 | 205.58 ± 102.34 | **0.049** |

Values are arithmetic mean ± standard deviation or geometric mean (5th and 95th percentiles) for logarithmically transformed variables. * Intima media thickness was adjusted for mean arterial pressure. Abbreviations: n – number of participants. Bold values denote statistical significance (p<0.05).

**SUPPLEMENTARY TABLE 3: Multiple regression analyses with cardiovascular measures as dependent variables in women stratified by ethnicity (African-PREDICT study).**

| | Women | | | | | |
|---|---|---|---|---|---|---|
| | Nitrate (µM/mM) | | | $U_{NOx}R$ | | |
| | **Adjusted $R^2$** | **Std β (95 % CI)** | ***p* value** | **Adjusted $R^2$** | **Std β (95 % CI)** | ***p* value** |
| **Systolic blood pressure (mm/Hg)** | | | | | | |
| Black (*n*= 265) | 0.065 | –0.056 (–0.234; 0.128) | 0.56 | 0.091 | –0.171 (–0.294; 0.014) | 0.075 |
| White (*n*= 285) | 0.175 | –0.121 (–0.236; 0.041) | 0.17 | 0.178 | –0.133 (–0.262; 0.033) | 0.13 |
| **Diastolic blood pressure (mm/Hg)** | | | | | | |
| Black (*n*= 265) | 0.001 | –0.021 (–0.235; 0.191) | 0.84 | 0.007 | –0.078 (–0.257; 0.112) | 0.44 |
| White (*n*= 285) | 0.105 | –0.128 (–0.252; 0.042) | 0.16 | 0.108 | –0.140 (–0.279; 0.034) | 0.12 |
| **Mean arterial pressure (mm/Hg)** | | | | | | |
| Black (*n*= 265) | 0.040 | –0.061 (–0.281; 0.148) | 0.54 | 0.054 | –0.132 (–0.310; 0.058) | 0.18 |
| White (*n*= 285) | 0.134 | –0.146 (–0.299; 0.028) | 0.10 | 0.137 | –0.156 (–0.328; 0.020) | 0.082 |
| **Intima media thickness (mm)*** | | | | | | |
| Black (*n*= 265) | 0.019 | –0.079 (–0.319; 0.138) | 0.43 | 0.020 | 0.086 (–0.113; 0.285) | 0.39 |
| White (*n*= 285) | 0.008 | –0.036 (–0.204; 0.140) | 0.71 | 0.008 | 0.030 (–0.155; 0.213) | 0.76 |

Variables included in the models were: age, body mass index, reactive oxygen species and oral contraceptives. * Intima media thickness was additionally adjusted for mean arterial pressure. Abbreviations: n – number of participants, $U_{NOx}R$ – urinary nitrate-to-nitrite ratio**.** Bold values denote statistical significance ($p<0.05$).

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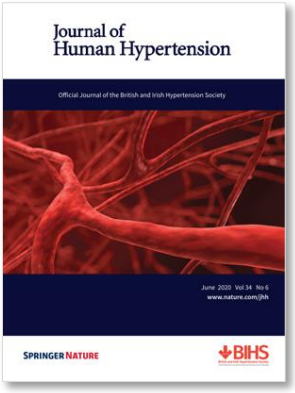
# Chapter IV

## Research Manuscript

This manuscript followed the specific guidelines as set out by the *Journal of Human Hypertension as* specified below. A full list of details regarding the author's instructions are available at: https://www.nature.com/documents/jhh-gta.pdf.




Formatting changes were made to maintain uniformity throughout the thesis, including text font, line spacing, margins, page numbers and reference style.

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# Central systolic blood pressure relates inversely to nitric oxide synthesis in young black adults: The African-PREDICT study

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**Short title:** Central pressure relates inversely to nitric oxide bioavailability

**Figures:** 1

**Tables:** 2

**Supplementary files:** 4

**Abstract**

**Background and aims:** Lower nitric oxide (NO) bioavailabilty associates with hypertension in patients and elderly populations. With hypertension known to develop earlier in black populations, we compared both plasma and urinary NO-related markers and their associations with central systolic blood pressure (cSBP) and arterial stiffness in healthy young black and white adults.

**Methods:** We included healthy black and white men and women (*n*=1110; 20-30 years) and measured cSBP and pulse wave velocity (PWV), along with both plasma and urinary arginine, homoarginine, asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), as well as urinary ornithine/citrulline, nitrite and nitrate. Additionally the urinary nitrate-to-nitrite ratio ($U_{NOx}R$) was calculated.

**Results:** The black men and women had higher cSBP and higher plasma arginine and ADMA, but lower urinary nitrate and $U_{NOx}R$ (all $p \leq 0.003$) than their white counterparts. In single and forward stepwise multiple regression analyses, we found an inverse association of cSBP (adj. $R^2 = 0.124$; $\beta = -0.134$; $p = 0.006$) and plasma homoarginine in black men. Central SBP associated inversely with $U_{NOx}R$ in black women only (adj. $R^2 = 0.171$; $\beta = -0.130$; $p = 0.029$). In the white women, cSBP associated positively with urinary ADMA (adj. $R^2 = 0.372$; $\beta = 0.162$; $p = 0.015$). PWV associated inversely with plasma ADMA (adj. $R^2 = 0.253$; $\beta = -0.163$; $p = 0.024$) in the white women only.

**Conclusion:** The lower NO synthesis and the higher cSBP in our black cohort support the notion of a potential increased risk for future large artery stiffness and hypertension development in later life.

**Keywords:** Central blood pressure, ethnicity, nitric oxide, homoarginine, urinary nitrate-to-nitrite ratio, asymmetric dimethylarginine, hypertension

## Introduction

High blood pressure (BP) is one of the main contributing factors in the development of cardiovascular disease (CVD), with high BP being particularly prevalent in black populations [1]. Left undiagnosed and untreated, hypertension increases the rate of morbidity and mortality [2]. A number of factors are responsible for the development of hypertension. Among these is the lower bioavailability of the potent endogenous vasodilator, nitric oxide (NO) [3].

Nitric oxide is synthesised via the oxidation of the NO synthase (NOS) substrates, L-arginine and L-homoarginine, or alternatively via the recycling of inorganic anions, nitrate and nitrite [4,5]. The bioavailability of NO encompasses the production and reaction of endothelial NO and a reduced bioavailability is associated with CVD progression [6]. Several factors are known to decrease the production of NO. These include lower NOS activity resulting from elevated synthesis of endogenous NOS inhibitors, lower availability of the NOS substrates L-arginine and L-homoarginine and loss of nitrite in the renal nitrate-nitrite-NO cycle [7,8]. Measuring of NO-related biomarkers in plasma and urine allows for the estimate of the status of the L-arginine-NO pathway in health and disease. The concentration of the major NO metabolites, nitrate and nitrite, in circulation and in urine is indicative of whole-body NO bioavailability, respectively [9].

Blood pressure associates inversely with the concentration of NO metabolites and positively with the concentration of endogenous NOS inhibitors in hypertensive individuals [10,11]. Within a South African setting, reports indicated that the black population present with lower plasma arginine but surprisingly also with lower levels of the endogenous NOS inhibitor, asymmetric dimethylarginine (ADMA), when compared to white populations [12,13]. Yet, some reports have failed to establish a difference between NOS inhibitors in black and white groups altogether [14]. Owing to the inhibitory effect of ADMA on endothelial NOS activity, ADMA has also been implicated in the progression of arterial stiffness as reported by a positive association between plasma ADMA concentration and pulse wave velocity (PWV) in black men [14]. These studies support the notion that the bioavailability of NO may have substantial influences on endothelial function.

There is solid knowledge of NO bioavailability and the associations with biomarkers of the L-arginine-NO pathway in diseased and elderly populations [13,15,16], while less is known about its status in young healthy populations. The lack of knowledge surrounding the role of both NO metabolites and NOS inhibitors in central blood pressure and arterial stiffness in healthy young individuals and considering central systolic BP (cSBP) as an important prognostic hemodynamic parameter [17], spurred our investigation. In this hypothesis-generating study,

we determined the status of the L-arginine-NO pathway in plasma and urine of samples of young black and white adults from South Africa, compared NO-related biomarkers and determined their associations with cSBP and PWV.

**Methodology**

The African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT), screened and assessed 1202 apparently healthy volunteers (aged 20-30 years). In this cross-sectional study we included data from 1110 participants stratified according to ethnicity and sex, included black (*n*=261) and white (*n*=271) men and black (*n*=283) and white (*n*=295) women, after the exclusion of participants with missing urinary data (*n*=7) and those with urinary nitrite outliers (*n*=85). Nitrite outliers included those participants with an uncommonly high nitrite concentration when compared to nitrate, possibly due to asymptomatic bacteriuria and bacterial nitrate reductase catalysed reduction of nitrate to nitrite.

The study population and protocol for the African-PREDICT study has been described elsewhere [18]. Briefly, participants with office BP >140/90 mmHg during screening, or with any self-reported diseases or risk factors that may influence cardiovascular health, internal ear temperature >37.5°C, human immunodeficiency virus (HIV), diabetes mellitus, liver disease, cancer, tuberculosis or renal disease as well as the use of chronic medication were excluded from the African-PREDICT study. Pregnant and lactating women were also excluded due to known influences of hormones on cardiovascular health.

Participants were fully informed about the objectives of the study and written informed consent was acquired from each participant. The African-PREDICT study was conducted in line with the ethical principles of the Declaration of Helsinki [19] and was approved by the Health Research Ethics Committee of the North-West University.

**Anthropometric measures**

All anthropometric procedures were performed according to specific guidelines set out by the International Society for the Advancement of Kinanthropometry (ISAK) [18,20]. Waist circumference (cm) was obtained using a standard protocol (Lufkin Steel Anthropometric Tape; W606PM; Lufkin; Apex; USA). Body mass index (BMI) [weight (kg) / square height ($m^2$)] of each participant was calculated (SECA portable 213 stadiometer; SECA 813 electronic scale; Hamburg, Germany).

**Cardiovascular measures**

With the use of a Dinamap® ProCare 100 Vital Signs Monitor, office BP was measured on the left arm, thereafter on the right arm in duplicate followed by a repeated measure on the left upper arm (GE Medical Systems, Milwaukee, USA). In this study, the left BP measurement was used in the analyses. Systolic BP (SBP) and diastolic BP (DBP) were captured from each measurement. Mean arterial pressure (MAP) was calculated using the following formula, (DBP) / (0.4*pulse pressure) [21]. Participants were also fitted with a 24-h ambulatory BP monitor and ECG apparatus (CardioXplore®, CE0120; Meditech, Budapest, Hungary). Masked hypertension was defined as a normal clinic BP (<140 and <90 mmHg), but elevated 24-h SBP and/or DBP (≥130/80 mmHg).

The SphygmoCor XCEL device (AtCor Medical Pty. Ltd., Sydney, New South Wales, Australia) was used to measure carotid-femoral PWV [22]. With the participants in supine position the right carotid artery was located by means of palpation to identify the strongest pulse point. The carotid pulse was measured using a tonometer while the femoral pulse was measured by a femoral cuff placed around the thigh of the participant. The transit-distance method was used and 80% of the distance calculated and entered after which the carotid-femoral PWV was measured along the descending thoracic abdominal aorta using the foot-to-foot velocity method. Duplicate measurements were taken and the mean value was used in subsequent analyses. Any measurements not considered of sufficient quality were repeated based on an operator index and additional quality indices reflecting the degree of variation above acceptable limits [23]. By using a brachial cuff, a central arterial waveform was produced and using pulse wave analysis of the peripheral arterial waveform [24], cSBP readings were derived.

**Biochemical analyses**

Participants were required to fast for at least 8 hours before they were required to provide an early morning spot urine sample, and blood samples were taken by a registered research nurse at the Hypertension Clinic of the North-West University.

Basic serum analyses included lipids (triglycerides, high density lipoprotein cholesterol, low density lipoprotein cholesterol and total cholesterol), gamma glutamyltransferase, creatinine and high sensitivity C-reactive protein (Cobas Integra 400 plus Roche, Basel Switzerland). Serum cotinine levels were determined with a chemiluminescence method on the Immulite (Siemens, Erlangen, Germany). Sodium fluoride plasma glucose was determined (Cobas Integra 400 plus Roche, Basel Switzerland). The intra-assay variability and inter-assay

variability of all variables were below 10%. Furthermore, the Chronic Kidney Disease Epidemiology (CKD-EPI) creatinine formula was utilised to calculate the estimated glomerular filtration rate (eGFR) [25].

Plasma NO-related markers (arginine, homoarginine, ADMA and symmetric dimethylarginine (SDMA)) of the first 561 participants were quantified by liquid chromatography tandem mass spectrometry [26,27]. Urinary arginine, homoarginine, ornithine/citrulline, nitrite, nitrate, malondialdehyde (MDA) and creatinine of 1110 participants were measured by gas chromatography-mass spectrometry [28]. All urinary measures were normalised to creatinine excretion. The urinary (U) nitrate-to-nitrite molar ratio ($U_{NOx}R$) was calculated ($[nitrate]_U/[nitrite]_U$) [29].

**Statistical analyses**

For statistical analyses, IBM®, SPSS® version 25 (IBM Corporation, Armonk, New York) was used. We tested for interactions of sex and ethnicity on the association of cSBP or PWV with NO-related markers. Variables were tested for normality using the Kolmogorov-Smirnov test and QQ-plots. Non-Gaussian variables were log transformed. Data was expressed as mean ± standard deviation if normally distributed and as geometric mean with $5^{th}$ and $95^{th}$ percentile boundaries for log transformed variables.

For comparisons between ethnic groups, independent T-tests were used. Proportions were determined with cross-tabs with significant differences indicated by Chi-square tests and presented as number and percentage. Associations of cSBP or PWV with plasma and urinary concentrations of the NO-related biomarkers were tested using Pearson correlations. Forward stepwise multiple regression analyses were conducted where either cSBP or PWV were included as dependent variables, and tested separately for their association with plasma and urinary NO-related markers. Covariates in the multiple regression models included age, BMI, low-density lipoprotein cholesterol, C-reactive protein, MDA, eGFR, masked hypertension and smoking. When PWV was the dependent variable it was additionally adjusted for mean arterial pressure.

**Results**

We found interactions of sex and ethnicity on the associations of cSBP or PWV with both plasma and urinary NO-related markers (**Supplementary Table S1**). We therefore stratified our groups according to male and female sex, as well as to black and white ethnicity.

The general characteristics of the study population are presented in **Table 1**. Both black men and women had higher cSBP when compared to their white counterparts (all $p \leq 0.001$). A comparison of plasma and urinary NO-related markers (**Figure 1 and Supplementary Table S2**) showed that plasma ADMA and plasma arginine levels were higher in the black group (all $p \leq 0.001$), independent of sex. Urinary nitrate excretion and $U_{NOx}R$ were lower in the black group compared to the white group (all $p \leq 0.001$). Urinary SDMA excretion was lower in the black women compared to the white women ($p \leq 0.004$).

We performed Pearson correlations (**Supplementary Table S3**) which showed cSBP associated inversely with plasma homoarginine (r= –0.187; $p$= 0.040) in black men and positively with urinary ADMA (r= 0.176; $p$= 0.002) in white women. Additionally, PWV associated inversely with urinary ADMA (r= –0.181; $p$= 0.003) in the black women. In partial regression analyses (**Supplementary Table S4**) (adjusted for age and BMI), cSBP associated inversely with plasma homoarginine ($r$= –0.295; $p$= 0.001) and $U_{NOx}R$ ($r$= –0.127; $p$= 0.034) in black men and women, respectively. Additionally, PWV associated inversely with plasma ADMA ($r$= –0.164; $p$= 0.045) in white women only.

Plasma homoarginine levels were comparable between the ethnic groups (both $p \geq 0.96$) (**Supplementary Table S2**). In forward stepwise multiple regression analysis, we found consistent inverse associations between cSBP (adj. $R^2$= 0.124; β= –0.134; $p$= 0.001) and plasma homoarginine in black men (**Table 2**). cSBP also correlated inversely with the $U_{NOx}R$ in the black women (adj. $R^2$= 0.171; β= –0.130; $p$= 0.029). In white women only, cSBP associated positively with urinary ADMA excretion (adj. $R^2$= 0.372; β= 0.162; $p$= 0.015), whereas PWV associated inversely with plasma ADMA concentration (adj. $R^2$= 0.253; β= –0.163; $p$= 0.024).

## Discussion

We determined the status of the L-arginine-NO pathway in healthy young black and white men and women in plasma and urine and investigated whether cSBP and large artery stiffness are associated with members of this pathway. In black men and women, urinary nitrate and $U_{NOx}R$ levels were lower and plasma ADMA and arginine levels were higher compared to their white counterparts. Central SBP associated independently and inversely with plasma homoarginine concentration and $U_{NOx}R$ in black men and women, respectively, but not in the white groups. In white women, we found cSBP to associate positively with urinary ADMA excretion, and PWV to associate inversely with plasma ADMA concentration.

The lower urinary nitrate excretion and the lower $U_{NOx}R$ levels seen in black men and women suggest a reduced whole-body NO synthesis in combination with loss of circulating nitrite, the major NO reservoir, presumably via an impaired renal carbonic anhydrase dependent reabsorption of nitrite [29]. Black men and women also presented with higher plasma levels of ADMA, an endogenous NOS inhibitor and cardiovascular risk factor, along with higher plasma levels of arginine, the natural NOS substrate. Our results confirm the findings of previous studies reporting higher plasma arginine levels in black populations [13,30] as well as higher plasma ADMA levels in black normotensive African men [31]. Previous studies also reported higher levels of NO metabolites and NOS inhibitors which were attributed to an inhibition of L-arginine transport, that may also decrease NO synthesis [32]. It was additionally speculated that higher plasma L-arginine levels initiate a counter-regulatory response aimed at compensating inhibition of NOS activity by higher plasma ADMA levels [33]. Although only reported in hypertensives, we confirm the findings of higher plasma arginine together with higher plasma ADMA concentrations [32,33]. It is therefore noteworthy that we report such findings in a young healthy black population, a population that is prone to early hypertension development [1]. The reduced NO bioavailability seen in our black cohort is associated with higher cSBP, when compared to whites, and suggests a potential for increased risk for future hypertension development in the blacks.

We also investigated in this young cohort whether cSBP and arterial stiffness associate with NO-related biomarkers. We found an inverse relationship between cSBP and plasma homoarginine concentration in black men only. Homoarginine is a homologue of L-arginine and may also serve as a substrate for NOS [34]. The higher cSBP of black men and the independent inverse association with plasma homoarginine concentration suggests that NO synthesis from homoarginine may be more relevant in the black group. Central BP measurements including cSBP have shown stronger associations to hypertensive end-organ damage [35,36]. Central pressure is also known to reflect central arterial stiffness [37]. The inverse associations between cSBP and plasma homoarginine concentration in black men [14], may suggest an early onset of hypertension and arterial stiffening amongst young black asymptomatic men [38].

The urinary nitrate-to-nitrite ratio is a reliable measure of renal carbonic anhydrase dependent reabsorption of urinary nitrite and may reflect NO bioavailability [29,39]. A previous study on South African children reported lower $U_{NOx}R$ in black boys compared to white boys [29], presumably as a result of diminished renal nitrite reabsorption and loss of circulating nitrite, seemingly due to genetic differences in the renal carbonic anhydrase isoforms and anion transporters [29,39]. The inverse relationship between cSBP and $U_{NOx}R$ found in the black

women of our study has not been described previously. Lower $U_{NOx}R$ values may indicate diminished carbonic anhydrase-dependent nitrite reabsorption and may ultimately result in diminished NO bioavailability in the kidney and presumably in the circulation [29]. Taken together, these results suggest that lower renal and systemic nitrite levels may have implications in NO bioavailability in the cardiovascular system in the black group and may further decline and contribute to hypertension development in later life.

From our study, cSBP associated positively with urinary ADMA levels in white women. Since renal excretion of ADMA is one of the main pathways of its elimination and plasma ADMA concentrations increase as renal function declines, ADMA is considered a uremic toxin [40]. Interestingly, it has been proposed that lower levels of urinary ADMA is a more consistent predictor of all-cause mortality when compared to higher plasma ADMA levels in renal transplant recipients [41]. Black women presented with lower and white women with higher levels of urinary symmetric dimethylarginine (SDMA), which is a combined measure of renal function and extent of coronary artery disease [42], yet with poorly understood biological activities. ADMA is an endogenous inhibitor of NOS [43,44]. Hence, it is inevitable that ADMA associates with BP in normal human physiology, as well as in the rat [45]. Urinary ADMA excretion is an interesting, yet largely understudied component of ADMA homeostasis. In patients with coronary artery disease, low urinary ADMA concentrations are associated with impaired cardiac function and predict cardiovascular as well as all-cause mortality [46]. The positive association between cSBP and urinary ADMA excretion in our white women is not fully understood, however, the white women of this study population presented with a more beneficial NO profile (lower NOS inhibitors) compared to their black counterparts, taken together with a combination of a lower cSBP, may aid to explain the finding of the positive association we observed.

We found that plasma ADMA concentration associated inversely with PWV in white women. Studies regarding the association between plasma ADMA levels and indices of vascular damage are limited and controversial [37,47]. The Cardiovascular Risk in Young Finns Study also found that plasma ADMA levels associated inversely with PWV in young healthy individuals [48]. It is known that ADMA acts as a biological regulator of vascular tone and may produce adverse cardiovascular events in humans at elevated concentrations [49]. The white women of our study presented with lower plasma ADMA levels compared to black women. Physiologically, lower plasma ADMA seems to have an alternative role in young healthy individuals. It is possible that this inverse association is not observed in individuals with CVD or in the elderly where plasma ADMA levels are heightened [50]. Based on our findings, in a

physiological setting, plasma ADMA seems to play a role in the regulation of vascular tone in young healthy individuals.

This explorative study must be interpreted within the context of its strengths and limitations. This study was well planned and executed under strict conditions. Populations included participants from the North West province of South Africa are not representative of the population as a whole. This study is limited by its cross-sectional design; hence we were unable to investigate precise mechanisms and causal relationships. This study also lacked the use of flow-mediated dilation to assess endothelial function. More-over, due to the hypothesis-generating nature of this study, our findings of cSBP and arterial stiffness indices relating to NO-related biomarkers should be investigated in future studies. Such studies should also consider dietary behaviour which may influence BP.

In conclusion, black men and women presented with lower urinary nitrate excretion, lower $U_{NOx}R$, higher plasma arginine and ADMA levels compared to white men and women. cSBP associated inversely with plasma homoarginine in black men. Central SBP associated inversely with $U_{NOx}R$ in black women only. Our findings suggest that lower NO bioavailability occurs already in healthy young black South Africans. We, therefore, hypothesize that diminished NO synthesis may increase the susceptibility of the black population for premature arterial stiffening and hypertension development.

**Conflict of interest**

The authors report that they have no conflict of interest.

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recommendations expressed in this material are those of the authors, and therefore, the NRF does not accept any liability in this regard.

**Author contributions**

AC performed the literature search, data cleaning, statistical analyses, interpretation of data and writing of the draft manuscript. AES is the principal investigator of the African-PREDICT study, and together with CMCM and RK were responsible for the research planning and design, acquisition of data, interpretation of data and revising article critically for intellectual content. Additionally, DT, RHB and ES revised the article for intellectual content. All authors approved the final version.

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**TABLE 1: General characteristics of the study population stratified according to sex and ethnicity.**

| | Men | | | Women | | |
|---|---|---|---|---|---|---|
| | **Black** (*n*= 261) | **White** (*n*= 271) | ***p* value** | **Black** (*n*= 283) | **White** (*n*= 295) | ***p* value** |
| Age (years) | 24.29 ± 3.06 | 24.78 ± 3.03 | 0.072 | 24.54 ± 3.31 | 24.46 ± 3.10 | 0.77 |
| **Body composition** | | | | | | |
| Waist circumference (cm) | 76.47 ± 9.34 | 88.73 ± 11.76 | **<0.001** | 79.08 ± 12.25 | 76.50 ± 11.74 | **0.007** |
| Body mass index (kg/m²) | 22.23 ± 3.99 | 26.56 ± 4.58 | **<0.001** | 26.62 ± 5.92 | 24.48 ± 5.33 | **<0.001** |
| Overweight/Obese (*n* %) | 62 (24%) | 170 (63%) | **<0.001** | 176 (62%) | 109 (37%) | **<0.001** |
| **Cardiovascular measures** | | | | | | |
| Office systolic blood pressure (mmHg) | 124 ± 12 | 124 ± 10 | 0.43 | 114 ± 10 | 110 ± 10 | **<0.001** |
| Office diastolic blood pressure (mmHg) | 81 ± 9 | 80 ± 8 | 0.058 | 78 ± 8 | 75 ± 7 | **<0.001** |
| Office mean arterial pressure (mmHg) | 98 ± 9 | 97 ± 8 | 0.57 | 92 ± 8 | 90 ± 8 | **<0.001** |
| Ambulatory 24hr systolic blood pressure (mmHg) | 120 ± 8 | 124 ± 8 | **<0.001** | 113 ± 9 | 112 ± 9 | 0.92 |
| Ambulatory 24hr diastolic blood pressure (mmHg) | 70 ± 7 | 70 ± 6 | 0.77 | 68 ± 6 | 68 ± 6 | 0.11 |
| Central systolic blood pressure (mmHg) | 112 ± 9 | 109 ± 9 | **<0.001** | 109 ± 9 | 103 ± 9 | **<0.001** |
| Pulse wave velocity (m/s) * | 6.81 ± 0.92 | 6.67 ± 0.77 | 0.091 | 5.95 ± 0.79 | 6.01 ± 0.84 | 0.43 |
| Masked hypertension (*n* %) | 37 (14%) | 61 (23%) | **<0.001** | 29 (10%) | 21 (7%) | 0.81 |
| **Biochemical measures** | | | | | | |
| High density-lipoproteins (mmol/L) | 1.26 ± 0.34 | 1.13 ± 0.30 | **<0.001** | 1.26 ± 0.34 | 1.52 ± 0.41 | **<0.001** |
| Low-density lipoproteins (mmol/L) | 2.25 (1.31 – 3.85) | 3.24 (1.95 – 5.15) | **<0.001** | 2.39 (1.25 – 4.05) | 2.80 (1.71 – 4.43) | **<0.001** |
| Triglycerides (mmol/L) | 0.78 (0.41 – 1.63) | 1.14 (0.54 – 3.93) | **<0.001** | 0.68 (0.35 – 1.34) | 0.87 (0.41 – 2.12) | **<0.001** |
| Total cholesterol (mmol/L) | 3.65 (2.67 – 5.33) | 4.76 (3.29 – 6.68) | **<0.001** | 3.78 (2.67 – 5.46) | 4.50 (3.18 – 6.43) | **<0.001** |
| eGFR (ml/min/1.73m²) | 117.80 ± 14.33 | 103.37 ± 16.31 | **<0.001** | 117.94 ± 14.99 | 108.19 ± 15.68 | **<0.001** |
| C-reactive protein (mg/L) | 0.65 (0.09 – 6.20) | 0.89 (0.10 – 8.09) | **0.030** | 1.86 (0.18 – 13.6) | 1.09 (0.10 – 11.3) | **<0.001** |
| Glucose (mmol/L) | 4.29 (2.88 – 5.57) | 4.96 (4.14 – 5.79) | **<0.001** | 4.40 (3.00 – 5.56) | 4.51 (3.54 – 5.58) | 0.44 |
| Malondialdehyde (µM/mM creatinine) | 0.16 (0.07 – 0.41) | 0.12 (0.06 – 0.29) | **<0.001** | 0.18 (0.08 – 0.67) | 0.13 (0.06 – 0.44) | **<0.001** |
| Gamma-glutamyl transferase (U/L) | 28.28 (13.1 – 89.4) | 25.48 (10.9 – 64.9) | 0.084 | 22.79 (10.1 – 67.6) | 14.60 (7.40 – 39.3) | **<0.001** |
| Cotinine (ng/ml) | 9.27 (1.00 – 445) | 5.23 (1.00 – 367) | **0.023** | 1.98 (1.00 – 182) | 2.21 (1.00 – 258) | 0.45 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Self-reported** | | | | | | |
| Smoking (*n* %) | 123 (47%) | 80 (30%) | **<0.001** | 31 (11%) | 51 (17%) | **0.029** |
| Alcohol drinking (*n* %) | 169 (65%) | 165 (61%) | 0.58 | 146 (52%) | 160 (54%) | 0.63 |
| Contraceptive use (*n* %) | - | - | - | 113 (40%) | 113 (38%) | 0.73 |

Values are arithmetic mean ± standard deviation or geometric mean (5th and 95th percentiles) for logarithmically transformed variables. * Pulse wave velocity was adjusted for mean arterial pressure. Abbreviations: *n* – number of participants, eGFR – estimated glomerular filtration rate. Bold values denote statistical significance ($p<0.05$).

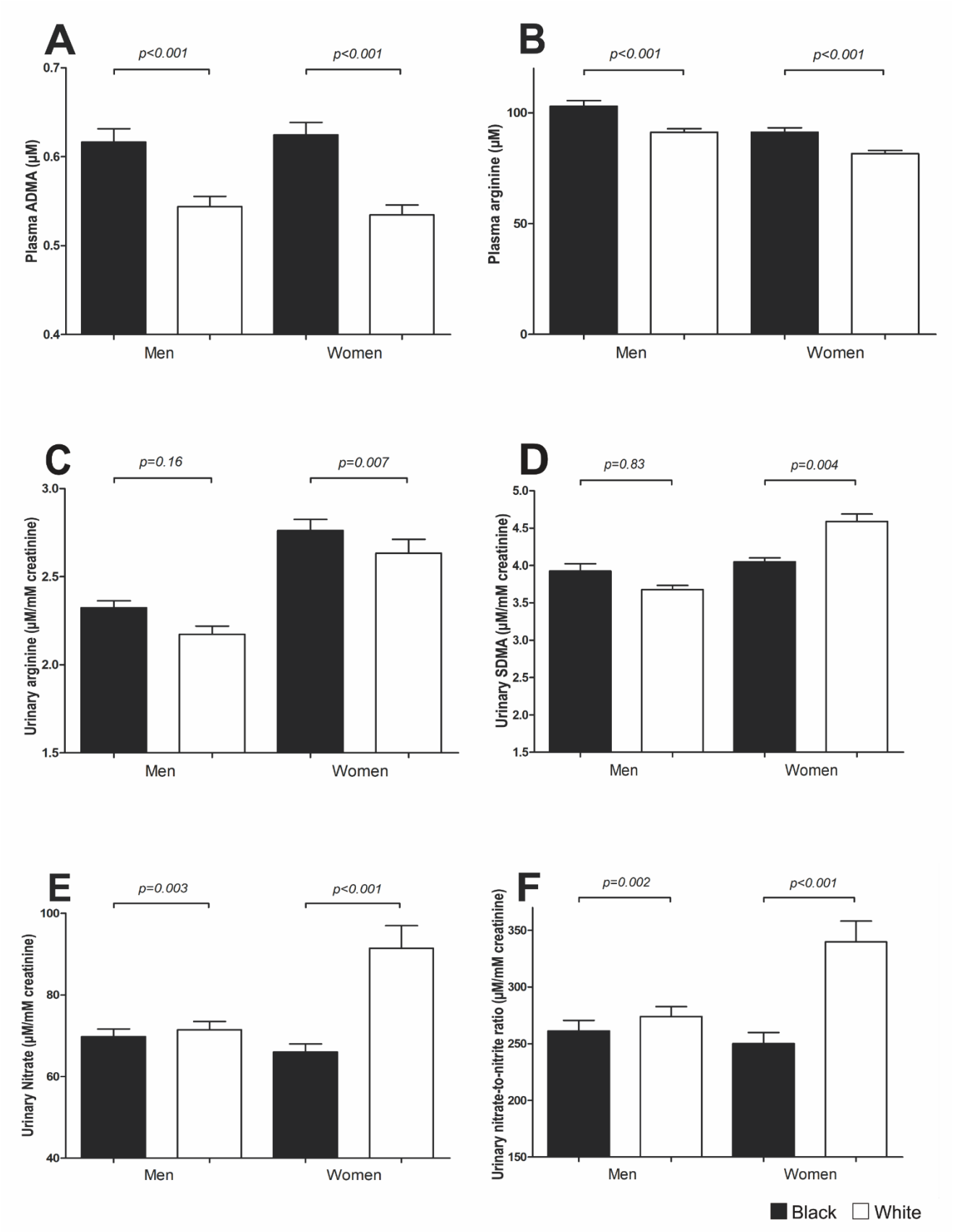


**Figure 1: Comparing the mean values of (A) plasma ADMA, (B) plasma arginine, (C) creatinine-corrected urinary arginine, (D) creatinine-corrected urinary SDMA, (E) creatinine-corrected urinary nitrate levels and (F) urinary nitrate-to-nitrite ratio in men and women.**

**TABLE 2: Forward stepwise multiple regression analyses with cardiovascular measures as dependent variables, with the population stratified by sex and ethnicity.**

| | Men | | | Women | | |
|---|---|---|---|---|---|---|
| | **Adjusted $R^2$** | **Std β (95 % CI)** | ***p* value** | **Adjusted $R^2$** | **Std β (95 % CI)** | ***p* value** |
| | | | **Plasma** | | | |
| | | | **Homoarginine (μM) ǂ** | | | |
| **Central systolic blood pressure (mmHg)** | | | | | | |
| Black | 0.124 | –0.134 (–0.496; –0.121) | **0.001** | - | - | - |
| White | - | - | - | - | - | - |
| | | | **Asymmetric dimethylarginine (μM) ǂ** | | | |
| **Pulse wave velocity (m/s) *** | | | | | | |
| Black | - | - | - | - | - | - |
| White | - | - | - | 0.253 | –0.163 (–0.297; –0.021) | **0.024** |
| | | | **Urine** | | | |
| | | | **Asymmetric dimethylarginine (μM/mM creatinine) †** | | | |
| **Central systolic blood pressure (mmHg)** | | | | | | |
| Black | - | - | - | - | - | - |
| White | - | - | - | 0.372 | 0.162 (0.032; 0.296) | **0.015** |
| | | | **Urinary nitrate-to-nitrite ratio †** | | | |
| **Central systolic blood pressure (mmHg)** | | | | | | |
| Black | - | - | - | 0.171 | –0.130 (–0.315; –0.017) | **0.029** |
| White | - | - | - | - | - | - |

Variables included in the models were: age, BMI, low-density lipoproteins, C-reactive protein, malondialdehyde, eGFR, masked hypertension and smoking. * Pulse wave velocity was additionally adjusted for mean arterial pressure. ǂ Samples measured in plasma (black men *n*=115; white men *n*=128; black women *n*=164; white women *n*=154). Abbreviations: *n* – number of participants, eGFR – estimated glomerular filtration rate. Bold values denote statistical significance ($p<0.05$).

**SUPPLEMENTARY TABLE 1: Interaction terms of ethnicity and sex on the relationship of cardiovascular and arterial stiffness measures with plasma (μM) and creatinine-corrected urinary nitric oxide metabolites (μM/mM).**

| | Central systolic blood pressure | | Central pulse pressure | | Pulse wave velocity | |
|---|---|---|---|---|---|---|
| | **Ethnicity** | **Sex** | **Ethnicity** | **Sex** | **Ethnicity** | **Sex** |
| | ***p* value** | ***p* value** | ***p* value** | ***p* value** | ***p* value** | ***p* value** |
| **Plasma samples** | | | | | | |
| Arginine (μM) | **<0.001** | **<0.001** | **0.005** | **<0.001** | 0.24 | **<0.001** |
| Homoarginine (μM) | **0.012** | **<0.001** | **0.007** | **<0.001** | 0.59 | **<0.001** |
| ADMA (μM) | **<0.001** | **<0.001** | 0.076 | **<0.001** | 0.31 | **<0.001** |
| SDMA (μM) | **<0.001** | **<0.001** | **0.003** | **<0.001** | 0.38 | **<0.001** |
| **Urine samples** | | | | | | |
| Arginine (μM/mM creatinine) | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **0.031** | **<0.001** |
| Homoarginine (μM/mM creatinine) | **<0.001** | **<0.001** | 0.40 | **<0.001** | **0.030** | **<0.001** |
| ADMA (μM/mM creatinine) | **<0.001** | **<0.001** | 0.77 | **<0.001** | **0.023** | **<0.001** |
| SDMA (μM/mM creatinine) | **<0.001** | **<0.001** | 0.99 | **<0.001** | **0.028** | **<0.001** |
| Ornithine/Citrulline (μM/mM creatinine) | **<0.001** | **<0.001** | 0.37 | **<0.001** | 0.11 | **<0.001** |
| Nitrate (μM/mM creatinine) | **<0.001** | **<0.001** | 0.62 | **<0.001** | 0.069 | **<0.001** |
| Nitrite (μM/mM creatinine) | **<0.001** | **<0.001** | 0.17 | **<0.001** | 0.46 | **<0.001** |
| Urinary nitrate-to-nitrite ratio | **<0.001** | **<0.001** | 0.45 | **<0.001** | 0.11 | **<0.001** |

Bold values denote statistical significance ($p<0.05$).

**SUPPLEMENTARY TABLE 2: Plasma and urinary nitric oxide-related markers of the study population stratified according to sex and ethnicity.**

| | Men | | | Women | | |
|---|---|---|---|---|---|---|
| | **Black** (*n*= 261) | **White** (*n*= 271) | ***p* value** | **Black** (*n*= 283) | **White** (*n*= 295) | ***p* value** |
| **Plasma nitric oxide-related markers †** | | | | | | |
| Arginine (µM) | 99.77 (68.9 – 168) | 89.31 (64.6 – 127) | **<0.001** | 88.13 (57.6 – 145) | 79.54 (53.7 – 115) | **<0.001** |
| Homoarginine (µM) | 2.02 (0.98 – 3.67) | 2.04 (1.10 – 3.60) | 0.93 | 2.04 (0.94 – 4.74) | 2.07 (1.00 – 4.16) | 0.96 |
| ADMA (µM) | 0.60 (0.30 – 0.79) | 0.53 (0.33 – 0.77) | **<0.001** | 0.60 (0.38 – 0.95) | 0.52 (0.39 – 0.93) | **<0.001** |
| SDMA (µM) | 0.56 ± 0.14 | 0.59 ± 0.13 | 0.10 | 0.51 ± 0.14 | 0.54 ± 0.13 | 0.082 |
| **Urinary nitric oxide-related markers** | | | | | | |
| Arginine (µM/mM creatinine) | 2.15 (1.37 – 3.74) | 2.07 (1.26 – 3.63) | 0.16 | 2.59 (1.38 – 4.48) | 2.39 (1.37 – 4.48) | **0.007** |
| Homoarginine (µM/mM creatinine) | 0.12 (0.04 – 0.35) | 0.13 (0.004 – 0.56) | 0.13 | 0.18 (0.05 – 0.73) | 0.17 (0.005 – 0.71) | 0.30 |
| ADMA (µM/mM creatinine) | 3.33 (2.06 – 4.96) | 3.23 (1.89 – 5.26) | 0.081 | 4.12 (2.42 – 6.36) | 3.96 (2.16 – 6.51) | 0.13 |
| SDMA (µM/mM creatinine) | 3.41 (2.23 – 5.19) | 3.47 (2.19 – 5.30) | 0.83 | 3.86 (2.50 – 5.44) | 4.14 (2.40 – 6.05) | **0.004** |
| Ornithine/Citrulline (µM/mM creatinine) | 3.42 (2.18 – 5.48) | 3.28 (1.97 – 5.21) | 0.10 | 3.54 (2.19 – 5.52) | 3.38 (1.76 – 5.76) | 0.078 |
| Nitrite (µM/mM creatinine) | 0.29 (0.10 – 0.90) | 0.27 (0.12 – 0.85) | 0.39 | 0.29 (0.09 – 0.87) | 0.29 (0.10- -0.95) | 0.91 |
| Nitrate (µM/mM creatinine) | 58.9 (31.3 – 128) | 65.7 (36.2 – 126) | **0.003** | 60.4 (34.9 – 124) | 75.2 (37 – 168) | **<0.001** |
| Urinary nitrate-to-nitrite ratio | 205 (64.3 – 576) | 241 (85.7 – 529) | **0.002** | 209 (68.9 – 578) | 259 (88.2 – 717) | **<0.001** |

Values are arithmetic mean ± standard deviation or geometric mean (5th and 95th percentiles) for logarithmically transformed variables. † Samples measured in plasma (black men *n*=115; white men *n*=128; black women *n*=164; white women *n*=154). Abbreviations: *n* – number of participants. Bold values denote statistical significance (p<0.05).

**SUPPLEMENTARY TABLE 3: Pearson correlations between anthropometric and cardiovascular measures and markers related to nitric oxide synthesis in the study population stratified by sex and ethnicity.**

| | Plasma | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Homoarginine (µM) | | | | Asymmetric dimethylarginine (µM) | | | |
| | Men | | Women | | Men | | Women | |
| | Black | White | Black | White | Black | White | Black | White |
| | (*n*=261) | (*n*=271) | (*n*=283) | (*n*=295) | (*n*=261) | (*n*=271) | (*n*=283) | (*n*=295) |
| Age (years) | r=0.011 | **r=0.209** | r=0.023 | r=–0.002 | **r=–0.267** | r=0.035 | r=0.023 | r=0.072 |
| | *p*=0.901 | ***p*=0.017** | *p*=0.762 | *p*=0.983 | ***p*=0.003** | *p*=0.690 | *p*=0.759 | *p*=0.358 |
| Waist circumference (cm) | **r=0.355** | r=0.074 | r=0.044 | r=0.096 | r=0.044 | **r=0.214** | **r=0.156** | r=0.055 |
| | ***p*<0.001** | *p*=0.405 | *p*=0.553 | *p*=0.220 | *p*=0.633 | ***p*=0.015** | ***p*=0.036** | *p*=0.485 |
| Body mass index (kg/m²) | **r=0.371** | r=0.076 | r=0.013 | r=0.124 | r=0.074 | **r=0.183** | r=0.144 | r=0.065 |
| | ***p*<0.001** | *p*=0.393 | *p*=0.860 | *p*=0.113 | *p*=0.422 | ***p*=0.038** | *p*=0.054 | *p*=0.405 |
| Central systolic blood pressure (mmHg) | **r=–0.187** | r=–0.034 | r=0.006 | r=0.088 | r=–0.066 | r=0.005 | r=–0.010 | r=0.143 |
| | ***p*=0.040** | *p*=0.704 | *p*=0.932 | *p*=0.263 | *p*=0.474 | *p*=0.957 | *p*=0.893 | *p*=0.069 |
| Pulse wave velocity (m/s) | r=–0.022 | r=0.062 | r=0.081 | r=0.090 | r=–0.172 | r=0.082 | r=–0.048 | r=–0.088 |
| | *p*=0.818 | *p*=0.492 | *p*=0.315 | *p*=0.270 | *p*=0.066 | *p*=0.367 | *p*=0.555 | *p*=0.284 |
| High-density lipoproteins (mmol/L) | r=–0.021 | r=–0.069 | r=–0.011 | **r=0.167** | r=–0.033 | r=–0.046 | r=–0.107 | r=0.029 |
| | *p*=0.826 | *p*=0.442 | *p*=0.884 | ***p*=0.038** | *p*=0.730 | *p*=0.607 | *p*=0.172 | *p*=0.720 |
| Low-density lipoproteins (mmol/L) | **r=0.204** | r=0.080 | **r=0.197** | r=0.035 | r=0.042 | r=–0.029 | r=–0.097 | r=–0.071 |
| | ***p*=0.025** | *p*=0.365 | ***p*=0.008** | *p*=0.655 | *p*=0.648 | *p*=0.744 | *p*=0.197 | *p*=0.365 |
| Triglycerides (mmol/L) | r=–0.055 | r=–0.059 | **r=0.264** | **r=0.192** | r=–0.077 | r=0.097 | r=0.010 | r=–0.126 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *p*=0.564 | *p*=0.509 | ***p*=0.001** | ***p*=0.017** | *p*=0.415 | *p*=0.280 | *p*=0.899 | *p*=0.120 |
| Total cholesterol (mmol/L) | **r=0.211** | r=0.070 | **r=0.185** | r=0.157 | r=–0.003 | r=–0.003 | r=–0.152 | r=–0.072 |
| | ***p*=0.025** | *p*=0.437 | ***p*=0.018** | *p*=0.051 | *p*=0.974 | *p*=0.974 | *p*=0.052 | *p*=0.372 |
| | r=–0.047 | r=–0.161 | r=0.028 | r=0.148 | **r=0.320** | r=0.078 | r=0.090 | r=0.091 |
| eGFR (ml/min/1.73m²) | *p*=0.611 | *p*=0.069 | *p*=0.713 | *p*=0.059 | ***p*<0.001** | *p*=0.380 | *p*=0.233 | *p*=0.220 |
| | r=0.122 | –0.027r= | r=–0.091 | r=0.041 | r=–0.191 | r=–0.114 | **r=–0.170** | r=0.032 |
| Glucose (mmol/L) | *p*=0.194 | *p*=0.763 | *p*=0.250 | *p*=0.611 | *p*=0.041 | *p*=0.203 | ***p*=0.030** | *p*=0.691 |
| | **r=0.191** | r=0.052 | r=–0.035 | r=0.024 | r=–0.015 | r=0.092 | r=0.127 | **r=–0.159** |
| C-reactive protein (mg/L) | ***p*=0.037** | *p*=0.557 | *p*=0.636 | *p*=0.757 | *p*=0.874 | *p*=0.298 | *p*=0.088 | ***p*=0.041** |
| | r=–0.041 | r=0.067 | **r=0.157** | r=–0.039 | r=–0.110 | r=–0.079 | r=–0.023 | r=0.029 |
| Malondialdehyde (µM/mM creatinine) | *p*=0.656 | *p*=0.455 | ***p*=0.038** | *p*=0.624 | *p*=0.235 | *p*=0.373 | *p*=0.768 | *p*=0.711 |
| | r=0.077 | r=–0.067 | r=0.090 | r=0.007 | r=–0.114 | r=–0.009 | r=0.054 | r=–0.044 |
| Gamma-glutamyl transferase (U/L) | *p*=0.401 | *p*=0.451 | *p*=0.231 | *p*=0.927 | *p*=0.213 | *p*=0.920 | *p*=0.469 | *p*=0.577 |
| | **r=–0.333** | **r=–0.210** | r=–0.016 | r=–0.008 | r=0.103 | r=0.108 | r=0.011 | r=0.011 |
| Cotinine (ng/ml) | ***p*<0.001** | ***p*=0.016** | *p*=0.838 | *p*=0.922 | *p*=0.268 | *p*=0.222 | *p*=0.886 | *p*=0.885 |
| | **r=–0.281** | **r=–0.198** | r=0.081 | r=–0.115 | r=0.160 | r=0.164 | r=0.056 | r=–0.016 |
| Smoking (yes/no) | ***p*=0.002** | ***p*=0.024** | *p*=0.281 | *p*=0.141 | *p*=0.080 | *p*=0.063 | *p*=0.452 | *p*=0.841 |
| | **r=–0.190** | **r=0.182** | r=0.004 | r=0.024 | r=0.081 | r=0.103 | r=0.106 | r=–0.050 |
| Alcohol drinking (yes/no) | ***p*=0.038** | ***p*=0.038** | *p*=0.953 | *p*=0.757 | *p*=0.380 | *p*=0.244 | *p*=0.160 | *p*=0.524 |

| | Urine | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Asymmetric dimethylarginine (μM/mM creatinine)** | | | | **Urinary nitrate-to-nitrite ratio** | | | |
| | **Men** | | **Women** | | **Men** | | **Women** | |
| | **Black** (*n*=261) | **White** (*n*=271) | **Black** (*n*=283) | **White** (*n*=295) | **Black** (*n*=261) | **White** (*n*=271) | **Black** (*n*=283) | **White** (*n*=295) |
| Age (years) | r=–0.106 | **r=–0.128** | r=–0.109 | r=–0.028 | r=0.027 | r=0.046 | r=–0.083 | r=0.098 |
| | *p*=0.071 | ***p*=0.032** | *p*=0.057 | *p*=0.625 | *p*=0.644 | *p*=0.440 | *p*=0.145 | *p*=0.085 |
| Waist circumference (cm) | r=0.040 | r=0.099 | **r=0.138** | **r=0.184** | r=0.053 | r=0.051 | r=0.036 | r=0.008 |
| | *p*=0.501 | *p*=0.098 | ***p*=0.015** | ***p*=0.001** | *p*=0.365 | *p*=0.395 | *p*=0.528 | *p*=0.883 |
| Body mass index (kg/m²) | r=0.047 | r=0.083 | **r=0.190** | **r=0.221** | r=0.097 | r=0.016 | r=0.010 | r=0.041 |
| | *p*=0.424 | *p*=0.281 | ***p*=0.001** | ***p*<0.001** | *p*=0.102 | *p*=0.784 | *p*=0.862 | *p*=0.466 |
| Central systolic blood pressure (mmHg) | r=0.034 | r=–0.065 | r=–0.018 | **r=0.176** | r=0.038 | r=–0.002 | r=–0.094 | r=–0.044 |
| | *p*=0.565 | *p*=0.283 | *p*=0.752 | ***p*=0.002** | *p*=0.520 | *p*=0.978 | *p*=0.100 | *p*=0.444 |
| Pulse wave velocity (m/s) | r=–0.040 | r=0.057 | **r=–0.181** | r=–0.025 | r=0.023 | r=0.001 | r=–0.002 | r=–0.015 |
| | *p*=0.518 | *p*=0.357 | ***p*=0.003** | *p*=0.674 | *p*=0.714 | *p*=0.987 | *p*=0.980 | *p*=0.797 |
| High-density lipoproteins (mmol/L) | r=0.079 | **r=–0.190** | **r=–0.158** | r=–0.086 | r=–0.059 | r=–0.096 | r=0.097 | r=–0.022 |
| | *p*=0.316 | ***p*=0.015** | ***p*=0.012** | *p*=0.210 | *p*=0.453 | *p*=0.222 | *p*=0.125 | *p*=0.755 |
| Low-density lipoproteins (mmol/L) | r=–0.050 | r=–0.050 | r=–0.107 | r=0.025 | r=–0.059 | r=–0.062 | r=–0.094 | r=–0.122 |
| | *p*=0.524 | *p*=0.519 | *p*=0.086 | *p*=0.717 | *p*=0.456 | *p*=0.431 | *p*=0.133 | *p*=0.072 |
| Triglycerides (mmol/L) | r=0.003 | r=0.124 | r=–0.099 | r=–0.006 | r=0.001 | r=–0.092 | r=0.035 | r=–0.108 |
| | *p*=0.968 | *p*=0.115 | *p*=0.116 | *p*=0.930 | *p*=0.988 | *p*=0.242 | *p*=0.581 | *p*=0.116 |
| Total cholesterol (mmol/L) | r=0.005 | r=–0.093 | **r=–0.195** | r=–0.026 | r=–0.016 | r=–0.085 | **r=–0.147** | **r=–0.152** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *p*=0.944 | *p*=0.237 | ***p*=0.002** | *p*=0.707 | *p*=0.835 | *p*=0.278 | ***p*=0.020** | ***p*=0.027** |
| Glucose (mmol/L) | r=–0.153 | r=0.022 | r=0.004 | r=–0.039 | **r=–0.155** | r=–0.046 | r=–0.007 | r=0.035 |
| | *p*=0.063 | *p*=0.780 | *p*=0.947 | *p*=0.576 | ***p*=0.040** | *p*=0.555 | *p*=0.908 | *p*=0.600 |
| eGFR (ml/min/1.73m²) | **r=0.272** | **r=0.522** | **r=0.341** | **r=0.479** | r=0.042 | r=–0.071 | r=0.031 | r=–0.019 |
| | ***p*<0.001** | ***p*<0.001** | ***p*<0.001** | ***p*<0.001** | *p*=0.609 | *p*=0.369 | *p*=0.638 | *p*=0.786 |
| C-reactive protein (mg/L) | r=0.059 | r=0.045 | **r=0.142** | r=0.014 | r=0.112 | r=–0.004 | r=0.064 | r=–0.032 |
| | *p*=0.435 | *p*=0.565 | ***p*=0.019** | *p*=0.830 | *p*=0.137 | *p*=0.955 | *p*=0.294 | *p*=0.631 |
| Malondialdehyde (µM/mM creatinine) | r=–0.076 | r=0.069 | **r=–0.189** | **r=–0.304** | **r=–0.386** | **r=–0.171** | **r=–0.295** | **r=–0.233** |
| | *p*=0.198 | *p*=0.251 | ***p*=0.001** | ***p*<0.001** | ***p*<0.001** | ***p*=0.004** | ***p*<0.001** | ***p*<0.001** |
| Gamma-glutamyl transferase (U/L) | r=0.026 | r=–0.027 | **r=–0.122** | r=0.069 | r=0.028 | r=–0.016 | r=–0.067 | r=–0.050 |
| | *p*=0.734 | *p*=0.730 | ***p*=0.044** | *p*=0.303 | *p*=0.712 | *p*=0.834 | *p*=0.269 | *p*=0.462 |
| Cotinine (ng/ml) | r=0.014 | **r=0.191** | r=0.014 | r=–0.046 | r=0.034 | r=–0.027 | r=0.023 | r=–0.014 |
| | *p*=0.844 | ***p*=0.009** | *p*=0.810 | *p*=0.480 | *p*=0.628 | *p*=0.716 | *p*=0.699 | *p*=0.827 |
| Smoking (yes/no) | r=0.071 | **r=0.132** | r=0.006 | r=0.028 | r=0.034 | r=–0.106 | **r=0.138** | r=0.000 |
| | *p*=0.230 | ***p*=0.027** | *p*=0.918 | *p*=0.619 | *p*=0.565 | *p*=0.076 | ***p*=0.016** | *p*=0.995 |
| Alcohol drinking (yes/no) | r=–0.033 | r=–0.010 | r=0.054 | r=–0.053 | r=0.035 | r=–0.029 | r=0.007 | r=–0.086 |
| | *p*=0.572 | *p*=0.872 | *p*=0.344 | *p*=0.348 | *p*=0.561 | *p*=0.630 | *p*=0.907 | *p*=0.131 |

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**SUPPLEMENTARY TABLE 4: Partial correlations between anthropometric and cardiovascular measures and markers related to nitric oxide synthesis in the study population stratified by sex and ethnicity.**

| | Plasma | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Homoarginine (µM) ∓ | | | | Asymmetric dimethylarginine (µM) ∓ | | | |
| | Men | | Women | | Men | | Women | |
| | Black | White | Black | White | Black | White | Black | White |
| | (*n*=261) | (*n*=271) | (*n*=283) | (*n*=295) | (*n*=261) | (*n*=271) | (*n*=283) | (*n*=295) |
| Central systolic blood pressure (mmHg) | **r=–0.295** | r=–0.095 | r=–0.031 | r=0.056 | r=–0.053 | r=–0.034 | r=–0.060 | r=0.153 |
| | ***p*=0.001** | *p*=0.296 | *p*=0.695 | *p*=0.500 | *p*=0.580 | *p*=0.706 | *p*=0.499 | *p*=0.061 |
| Pulse wave velocity (m/s)* | r=0.055 | r=0.011 | r=0.096 | r=0.125 | r=–0.107 | r=0.090 | r=0.003 | **r=–0.164** |
| | *p*=0.565 | *p*=0.902 | *p*=0.238 | *p*=0.130 | *p*=0.262 | *p*=0.324 | *p*=0.973 | ***p*=0.045** |
| High-density lipoproteins (mmol/L) | r=0.121 | r=–0.103 | r=–0.008 | **r=0.221** | r=0.022 | r=–0.004 | r=–0.084 | r=0.038 |
| | *p*=0.202 | *p*=0.251 | *p*=0.918 | ***p*=0.006** | *p*=0.817 | *p*=0.966 | *p*=0.290 | *p*=0.639 |
| Low-density lipoproteins (mmol/L) | r=0.129 | r=0.039 | **r=0.172** | r=0.012 | r=0.093 | r=–0.066 | r=–0.107 | r=–0.082 |
| | *p*=0.176 | *p*=0.670 | ***p*=0.029** | *p*=0.878 | *p*=0.319 | *p*=0.458 | *p*=0.156 | *p*=0.298 |
| Triglycerides (mmol/L) | r=–0.155 | r=–0.094 | **r=0.264** | **r=0.167** | r=–0.053 | r=0.047 | r=–0.021 | r=–0.145 |
| | *p*=0.103 | *p*=0.300 | ***p*=0.001** | ***p*=0.040** | *p*=0.579 | *p*=0.606 | *p*=0.793 | *p*=0.074 |
| Total cholesterol (mmol/L) | r=0.174 | r=0.005 | **r=0.184** | r=0.149 | r=0.065 | r=–0.047 | **r=–0.170** | r=–0.087 |
| | *p*=0.067 | *p*=0.957 | ***p*=0.019** | *p*=0.067 | *p*=0.499 | *p*=0.602 | ***p*=0.031** | *p*=0.286 |
| Glucose (mmol/l) | r=0.037 | r=–0.027 | r=–0.100 | r=0.033 | r=–0.164 | r=–0.106 | **r=–0.199** | r=0.026 |
| | *p*=0.696 | *p*=0.769 | *p*=0.207 | *p*=0.690 | *p*=0.083 | *p*=0.240 | ***p*=0.011** | *p*=0.747 |
| eGFR (ml/min/1.73m$^2$) | r=–0.001 | r=–0.129 | r=0.036 | r=0.136 | **r=0.276** | r=0.042 | r=0.102 | r=0.105 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *p*=0.991 | *p*=0.152 | *p*=0.635 | *p*=0.085 | ***p*=0.003** | *p*=0.640 | *p*=0.179 | *p*=0.186 |
| C-reactive protein (mg/L) | r=0.061 | r=0.069 | r=–0.069 | r=–0.021 | r=–0.002 | r=–0.018 | r=0.053 | **r=–0.180** |
| | *p*=0.525 | *p*=0.442 | *p*=0.362 | *p*=0.792 | *p*=0.984 | *p*=0.844 | *p*=0.479 | ***p*=0.026** |
| Malondialdehyde (μM/mM creatinine) | r=–0.008 | r=0.044 | **r=0.174** | r=–0.039 | r=–0.100 | r=–0.085 | r=–0.024 | r=0.025 |
| | *p*=0.937 | *p*=0.625 | ***p*=0.030** | *p*=0.625 | *p*=0.286 | *p*=0.347 | *p*=0.751 | *p*=0.754 |
| Gamma-glutamyl transferase (U/L) | r=–0.068 | r=–0.117 | r=0.082 | r=–0.032 | r=–0.093 | r=–0.119 | r=0.015 | r=–0.070 |
| | *p*=0.475 | *p*=0.193 | *p*=0.278 | *p*=0.682 | *p*=0.316 | *p*=0.183 | *p*=0.846 | *p*=0.373 |
| Cotinine (ng/ml) | **r=–0.281** | **r=–0.187** | r=–0.018 | r=–0.013 | r=0.098 | r=0.092 | r=0.006 | r=0.006 |
| | ***p*=0.003** | ***p*=0.036** | *p*=0.812 | *p*=0.871 | *p*=0.296 | *p*=0.303 | *p*=0.933 | *p*=0.942 |
| Smoking (yes/no) | **r=–0.225** | **r=–0.183** | r=0.085 | **r=–0.205** | r=0.137 | r=0.144 | r=0.068 | r=–0.024 |
| | ***p*=0.017** | ***p*=0.040** | *p*=0.261 | ***p*=0.011** | *p*=0.138 | *p*=0.104 | *p*=0.366 | *p*=0.760 |
| Alcohol drinking (yes/no) | r=–0.185 | r=0.154 | r=0.004 | r=0.022 | r=0.094 | r=0.076 | r=0.099 | r=–0.056 |
| | *p*=0.052 | *p*=0.086 | *p*=0.963 | *p*=0.782 | *p*=0.314 | *p*=0.395 | *p*=0.195 | *p*=0.474 |

Variables included in the models were: age and body mass index. * Pulse wave velocity was adjusted for mean arterial pressure. ǂ Samples measured in plasma (black men *n*=115; white men *n*=128; black women *n*=164; white women *n*=154). Abbreviations: *n* – number of participants, eGFR – estimated glomerular filtration rate. Bold values denote statistical significance (p<0.05)

| | Urine | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Asymmetric dimethylarginine (µM/mM creatinine) †** | | | | **Urinary nitrate-to-nitrite ratio †** | | | |
| | **Men** | | **Women** | | **Men** | | **Women** | |
| | **Black** (*n*=261) | **White** (*n*=271) | **Black** (*n*=283) | **White** (*n*=295) | **Black** (*n*=261) | **White** (*n*=271) | **Black** (*n*=283) | **White** (*n*=295) |
| Central systolic blood pressure (mmHg) | r=0.040 | r=–0.079 | r=–0.069 | r=0.096 | r=0.028 | r=–0.026 | **r=–0.127** | r=–0.064 |
| | *p*=0.493 | *p*=0.193 | *p*=0.232 | *p*=0.093 | *p*=0.640 | *p*=0.671 | ***p*=0.034** | *p*=0.261 |
| Pulse wave velocity (m/s) * | r=–0.025 | r=0.063 | r=–0.059 | r=0.000 | r=–0.017 | r=–0.007 | r=0.068 | r=–0.014 |
| | *p*=0.721 | *p*=0.427 | *p*=0.322 | *p*=0.998 | *p*=0.809 | *p*=0.931 | *p*=0.257 | *p*=0.831 |
| High-density lipoproteins (mmol/L) | r=0.093 | **r=–0.156** | r=–0.111 | r=–0.023 | r=–0.052 | r=–0.142 | r=0.117 | r=–0.058 |
| | *p*=0.236 | ***p*=0.048** | *p*=0.081 | *p*=0.742 | *p*=0.514 | *p*=0.072 | *p*=0.066 | *p*=0.401 |
| Low-density lipoproteins (mmol/L) | r=–0.042 | r=–0.048 | r=–0.102 | r=–0.023 | r=–0.082 | r=–0.077 | **r=–0.145** | r=–0.121 |
| | *p*=0.595 | *p*=0.543 | *p*=0.105 | *p*=0.738 | *p*=0.299 | *p*=0.327 | ***p*=0.026** | *p*=0.074 |
| Triglycerides (mmol/L) | r=0.026 | r=0.110 | **r=–0.140** | r=–0.074 | r=–0.018 | r=–0.087 | r=0.045 | r=–0.103 |
| | *p*=0.746 | *p*=0.166 | ***p*=0.027** | *p*=0.287 | *p*=0.819 | *p*=0.274 | *p*=0.481 | *p*=0.136 |
| Total cholesterol (mmol/L) | r=0.040 | r=–0.087 | **r=–0.199** | r=–0.059 | r=–0.039 | r=–0.109 | **r=–0.129** | **r=–0.173** |
| | *p*=0.608 | *p*=0.269 | ***p*=0.002** | *p*=0.399 | *p*=0.619 | *p*=0.166 | ***p*=0.043** | ***p*=0.012** |
| Glucose (mmol/l) | r=–0.134 | r=0.028 | r=0.002 | r=–0.060 | r=0.021 | r=–0.075 | r=0.059 | r=–0.022 |
| | *p*=0.107 | *p*=0.720 | *p*=0.974 | *p*=0.387 | *p*=0.799 | *p*=0.340 | *p*=0.371 | *p*=0.753 |
| eGFR (ml/min/1.73m$^2$) | **r=0.302** | **r=0.517** | **r=0.387** | **r=0.537** | r=–0.150 | r=–0.048 | r=–0.034 | r=0.055 |
| | ***p*=0.001** | ***p*<0.001** | ***p*=<0.001** | ***p*<0.001** | *p*=0.048 | *p*=0.544 | *p*=0.576 | *p*=0.419 |
| C-reactive protein (mg/L) | r=0.061 | r=–0.033 | r=0.078 | r=–0.079 | r=0.098 | r=–0.028 | r=0.050 | r=–0.031 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *p*=0.418 | *p*=0.673 | *p*=0.200 | *p*=0.243 | *p*=0.196 | *p*=0.721 | *p*=0.416 | *p*=0.647 |
| Malondialdehyde (μM/mM creatinine) | r=–0.068 | r=0.066 | **r=–0.126** | **r=–0.190** | **r=–0.386** | **r=–0.279** | **r=–0.283** | **r=–0.160** |
| | *p*=0.248 | *p*=0.268 | ***p*=0.035** | ***p*=0.001** | ***p*<0.001** | ***p*<0.001** | ***p*<0.001** | ***p*=0.006** |
| | r=0.035 | r=–0.076 | **r=–0.148** | r=–0.001 | r=0.005 | r=–0.045 | r=–0.084 | **r=–0.229** |
| Gamma-glutamyl transferase (U/L) | *p*=0.648 | *p*=0.333 | ***p*=0.020** | *p*=0.983 | *p*=0.944 | *p*=0.567 | *p*=0.172 | ***p*=0.001** |
| | r=0.014 | **r=0.224** | r=0.021 | r=–0.056 | r=0.048 | r=–0.027 | r=0.029 | r=–0.018 |
| Cotinine (ng/ml) | *p*=0.840 | ***p*=0.002** | *p*=0.726 | *p*=0.391 | *p*=0.493 | *p*=0.713 | *p*=0.625 | *p*=0.779 |
| | r=0.063 | **r=0.149** | r=0.011 | r=0.001 | r=0.045 | r=–0.110 | r=0.139 | r=–0.001 |
| Smoking (yes/no) | *p*=0.288 | ***p*=0.015** | *p*=0.842 | *p*=0.988 | *p*=0.448 | *p*=0.067 | *p*=0.015 | *p*=0.981 |
| | r=–0.028 | r=–0.149 | r=0.032 | r=–0.058 | r=0.040 | r=–0.042 | r=–0.005 | r=–0.093 |
| Alcohol drinking (yes/no) | *p*=0.635 | *p*=0.933 | *p*=0.575 | *p*=0.310 | *p*=0.505 | *p*=0.487 | *p*=0.924 | *p*=0.102 |

Variables included in the models were: age and body mass index. * Pulse wave velocity was adjusted for mean arterial pressure. ₸ Samples measured in plasma (black men *n*=115; white men *n*=128; black women *n*=164; white women *n*=154). Abbreviations: *n* – number of participants, eGFR – estimated glomerular filtration rate. Bold values denote statistical significance (p<0.05)

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# Chapter V

## Research Manuscript

This manuscript followed the specific guidelines as set out by the journal of *Hypertension Research as* specified below. A full list of details regarding the author's instructions are available at: https://www.nature.com/nature-research/for-authors.


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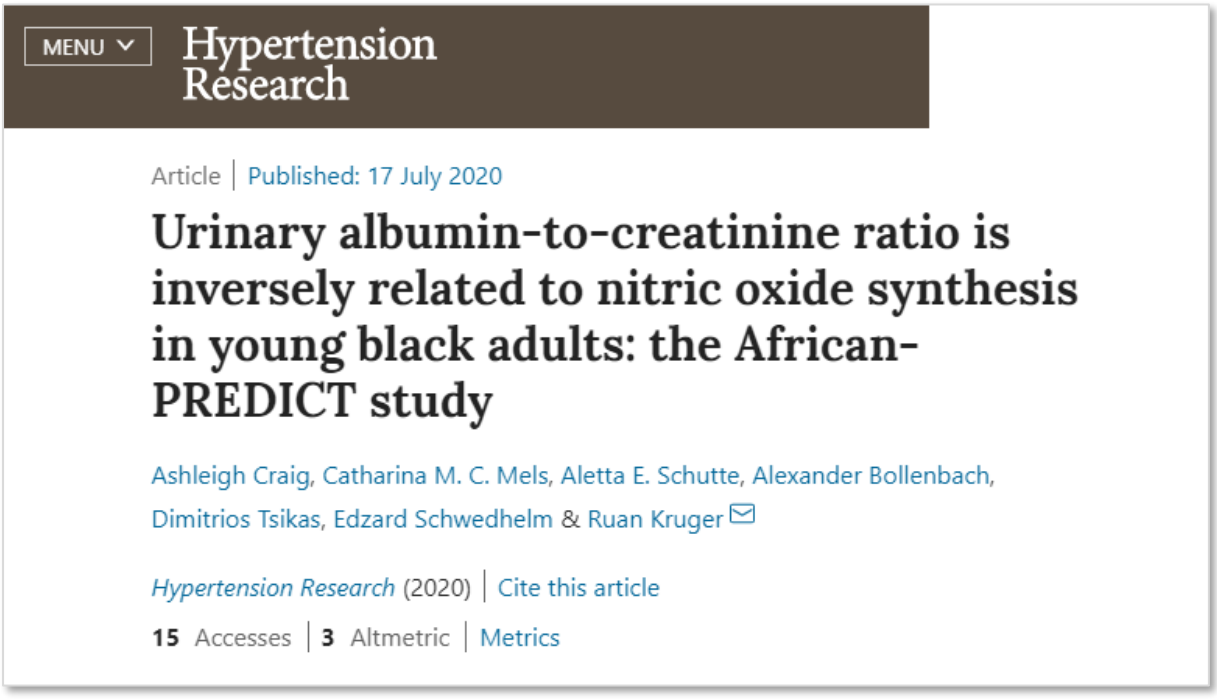
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# Urinary albumin-to-creatinine ratio is inversely related to nitric oxide synthesis in young black adults: The African-PREDICT study

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**Abstract**

Hypertension is common in black populations and is known to be associated with low nitric oxide (NO) bioavailability. We compared plasma and urinary NO-related markers and plasma creatine kinase (CK) levels between young healthy black and white adults along with the associations of these markers with the urinary albumin-to-creatinine ratio (uACR), which is a surrogate marker of endothelial and kidney function. We included 1105 participants (20-30 years). We measured the uACR, plasma CK, plasma and urinary arginine, homoarginine, asymmetric (ADMA) and symmetric dimethylarginine (SDMA), urinary ornithine/citrulline, nitrate and nitrite, and malondialdehyde (MDA). Additionally, the urinary nitrate-to-nitrite ratio ($U_{NOx}R$) was calculated and used as a measure of circulating NO bioavailability. The uACR was comparable between the groups, yet the black group had lower urinary nitrate (by –15%) and $U_{NOx}R$ values (by –18%) (both $p \leq 0.001$), higher plasma (by +9.6%) and urinary (by +5.9%) arginine (both $p \leq 0.004$), higher plasma (by +13%) and urinary (by +3.7%) ADMA (both $p \leq 0.033$), and higher CK (by +9.5%) and MDA (by +19%) (both $p<0.001$) compared to white adults. Plasma and urinary homoarginine were similar between the groups. In the multiple regression analysis, we confirmed the inverse associations of the uACR with both plasma (adj. $R^2=0.066$; $\beta=–0.209$; $p=0.005$) and urinary (adj. $R^2=0.066$; $\beta=–0.149$; $p=0.010$) homoarginine and with the $U_{NOx}R$ (adj. $R^2=0.060$; $\beta=–0.122$; $p=0.031$) in the black group only. The overall less favourable NO profile and higher CK and MDA levels in the black cohort along with the adverse associations with the uACR may reflect the vulnerability of this cohort to the early development of hypertension.

**Keywords:** Urinary albumin-to-creatinine ratio, nitric oxide, homoarginine, urinary nitrate-to-nitrite ratio, hypertension

## Introduction

Increased blood pressure (BP) is a main contributing factor to cardiovascular outcomes [1], yet the origins of the early phases of disease development remain unclear. Noticeably, a cardiovascular risk factor such as hypertension – which is common in black populations [2] – may be partly due to underlying endothelial dysfunction [3, 4]. Endothelial dysfunction is measured by several non-invasive methods, including flow-mediated dilation, which has been widely used in the last few decades [5,6]. However, more recently, the urinary albumin-to-creatinine ratio (uACR) has been introduced as a surrogate marker of both vascular endothelial and renal function [7-9]. Moreover, an elevated uACR has also been recognized to predict hypertension [10] and mortality [11] in the general population.

One of the important mediators of endothelial function is nitric oxide (NO). Nitric oxide is synthesized from L-arginine and L-homoarginine, which serve as substrates in a reaction catalysed by NO synthase (NOS) [12]. Alternatively, NO can be formed via the reduction of the inorganic anions nitrate and nitrite, which are the main metabolites of NO [13]. Asymmetric (ADMA) and symmetric dimethylarginine (SDMA) are inhibitors of NOS activity and arginine transport [14]. Impairment of the synthesis or bioavailability of NO results in a pro-constrictive phenotype [15] and has been reported to be more common in black populations [16] and hypertensive patients [10]. L-homoarginine is synthesized by L-arginine:glycine amidinotransferase (AGAT) [17], an enzyme that is also responsible for the synthesis of the energy-related metabolite creatine [18]. Creatine is the substrate of creatine kinase (CK), and its synthesis requires larger quantities of available L-arginine than NO synthesis [18, 19]. This may suggest that the CK system may be sensitive to a reduction in L-arginine bioavailability. Nitric oxide synthesis from L-arginine is therefore regulated not only by L-arginine availability but also by competition for substrates between NO and creatine synthesis.

Studies comparing the uACR and CK levels in black and white populations have provided consistent findings of increased uACR [20-22] and CK levels [23, 24] in black populations. However, reports on NO profiles in black and white populations have been inconsistent [20, 25, 26]. One study reported a greater NO synthesis capacity in a black population with higher levels of arginine [20], while another study observed reduced NO production and lower levels of arginine in black men [25]. Additionally, a study presenting the first evaluation of the urinary nitrate-to-nitrite ratio ($U_{NOx}R$) suggested that the $U_{NOx}R$ was a better measure of carbonic acid anhydrase-dependent nitrite reabsorption and reported a lower $U_{NOx}R$ in black boys compared to that in age-matched white boys, potentially suggesting an association of reduced NO synthesis with black ethnicity [27]. There have also been inconsistent findings for the

endogenous NOS inhibitor ADMA, which is considered a marker of endothelial dysfunction in adults [14,28]. Circulating ADMA was found to be lower in black populations [20, 29], higher in black Africans [26] or similar [25] when compared to that in white individuals. Asymmetric dimethylarginine and SDMA are produced by the catalytic dimethylation by protein-arginine methyltransferase (PRMT) of proteinic L-arginine residues. Asymmetric dimethylarginine and SDMA circulate in the blood and are excreted in the urine, and ADMA is also metabolized by dimethylarginine dimethylaminohydrolase (DDAH) [30].

Due to the varying findings for the NO profile in black and white populations, a deeper look into NO profiles among these populations is warranted. Furthermore, no prior studies have examined both the plasma and urinary NO profiles of young and apparently healthy individuals in bi-ethnic populations. Thus, in young black and white adults, we compared NO-related markers derived from urine and plasma along with the respective plasma CK levels and determined their relationship with the uACR as a measure of endothelial and kidney function.

**Methodology**

The African Prospective Study on the Early Detection and Identification of Cardiovascular Disease and Hypertension (African-PREDICT) screened and assessed 1202 apparently healthy volunteers (aged 20-30 years). In this cross-sectional study, we included data from 1105 participants who were stratified according ethnicity, i.e., black (*n*=539) and white (*n*=566) men and women, after the exclusion of participants with nitrite outliers and those with missing NO-related and uACR data (*n*=97).

The population and the protocol for the African-PREDICT study have been described elsewhere [31]. Briefly, participants with an office BP >140/90 mmHg during screening or with any self-reported diseases or risk factors that may influence cardiovascular health, an internal ear temperature >37.5°C, human immunodeficiency virus (HIV), diabetes mellitus, liver disease, cancer, tuberculosis or renal disease as well as the use of chronic medication were excluded from the African-PREDICT study. Pregnant and lactating women were also excluded due to the known influences of hormones on cardiovascular health.

Participants were fully informed about the objectives of the study, and written informed consent was acquired from each participant. The African-PREDICT study was conducted according to the ethical principles of the Declaration of Helsinki [32] and was approved by the Health Research Ethics Committee of North-West University.

**Anthropometric measures**

All anthropometric procedures were performed according to specific guidelines set out by the International Society for the Advancement of Kinanthropometry (ISAK) [31, 33]. Waist circumference (cm) was obtained using a standard protocol (Lufkin Steel Anthropometric Tape; W606PM; Lufkin; Apex; USA). The body mass index (BMI) (weight (kg)/square height ($m^2$)) (SECA portable 213 stadiometer; SECA 813 electronic scale; Hamburg, Germany) and waist-to-hip ratio (waist circumference (cm)/hip circumference (cm)) of each participant were then calculated.

**Cardiovascular measures**

With a Dinamap® ProCare 100 Vital Signs Monitor, the office BP was measured in the left arm and then in the right arm in duplicate, which was followed by a repeated measurement in the left upper arm (GE Medical Systems, Milwaukee, USA). In this study, the mean left BP measurement was used in the analyses. The systolic BP (SBP) and diastolic BP (DBP) were determined from each measurement. With a SphygmoCor XCEL device (AtCor Medical Pty. Ltd., Sydney, New South Wales, Australia), supine central SBP (cSBP) readings were derived using pulse wave analysis [34].

**Biochemical analyses**

Participants were required to fast for at least 8 hours before they provided an early morning spot urine sample. Blood samples were obtained by a registered research nurse at the Hypertension Clinic of North-West University.

Basic serum analyses included lipids (total cholesterol, high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C) and triglycerides), gamma glutamyltransferase, creatinine and high sensitivity C-reactive protein (Cobas Integra 400 plus Roche, Basel Switzerland). Serum cotinine levels were determined with a chemiluminescence method with an Immulite system (Siemens, Erlangen, Germany). CK was determined with the electrochemiluminescence method by using an E411 (Roche, Basel Switzerland). Sodium fluoride plasma glucose (Siemens, Erlangen, Germany) and EDTA whole blood glycated haemoglobin were also determined (Cobas Integra 400 plus Roche, Basel Switzerland). The intra-assay variability and inter-assay variability of all variables were below 10%. Urinary albumin (mg/L) and creatinine (mmol/L) were determined (Cobras Integra® 400plus, Roche, Basel, Switzerland), and the uACR was calculated. Furthermore, the Chronic Kidney Disease

Epidemiology (CKD-EPI) formula was utilized to calculate the estimated glomerular filtration rate (eGFR) from the serum creatinine values [35].

The plasma NO-related markers (arginine, homoarginine, ADMA and SDMA) of the first 561 participants were quantified by liquid chromatography-tandem mass spectrometry [36, 37]. Urinary NO-related markers (arginine, homoarginine, ADMA, SDMA, ornithine/citrulline, nitrite, and nitrate) as well as malondialdehyde (MDA), which is a biomarker of oxidative stress, and creatinine were measured simultaneously by gas chromatography-mass spectrometry [38]. All urinary NO-related data were normalized based on creatinine excretion and are expressed as µM analyte to mM creatinine (µM/mM). The $U_{NOx}R$ values were calculated by dividing the urinary nitrate concentration by the urinary nitrite concentration [27].

**Statistical analysis**

For the statistical analyses, IBM®, SPSS® version 26 (IBM Corporation, Armonk, New York) was used. We tested the interactions of ethnicity with the association with the uACR with both plasma and urinary NO-related markers. Variables were tested for normality using the Kolmogorov-Smirnov test and QQ plots. Non-Gaussian variables were log transformed. Data were expressed as the mean ± standard deviation if they were normally distributed and as the geometric mean with the $5^{th}$ and $95^{th}$ percentile boundaries for log transformed variables.

For comparisons between ethnic groups, independent T-tests were used. The proportions were determined with cross-tabs, with significant differences indicated by Chi-square tests and presented as numbers and percentages. The correlations of the uACR with plasma and urinary NO-related markers were tested using Pearson correlations. Standard multiple regression analyses were conducted with the uACR as a dependent variable, which was tested separately for its association with plasma and urinary NO-related markers. The covariates included in the multiple regression models included age, sex, waist-to-hip ratio, total cholesterol, eGFR, office systolic blood pressure, MDA, smoking, glycated haemoglobin and CK levels.

**Results**

The general characteristics of the study population are presented in **Table 1**. The black group had lower values for body composition measures, such as waist circumference, body height and BMI (all $p \leq 0.002$), than the white group, while body weight was comparable between the groups ($p=0.36$). Diastolic BP, cSBP, and the MDA and CK levels were higher in the black

group (all $p \leq 0.001$) than in the white group. Additionally, the mean uACR was similar ($p=0.34$) between the groups.

When comparing NO-related markers (**Table 2**), the black group presented with lower urinary nitrate (by –15%) and $U_{NOx}R$ values (by –18%) (both $p \leq 0.001$) and higher plasma (by +13%) and urinary (by +3.7%) ADMA values (both $p \leq 0.033$). However, both the plasma (by –5.2%) and urinary (by –4.1%) SDMA values (both $p \leq 0.022$) were lower in the black group than in the white group. No differences were observed for plasma and urinary homoarginine (both $p \geq 0.81$). An interaction of ethnicity with the associations of the uACR with both plasma (all $p \leq 0.002$) and urinary (all $p \leq 0.001$) NO-related markers was observed (**Supplementary Table 1**).

We performed single regression analyses (**Supplementary Table 2**) that showed that the uACR was associated inversely with the plasma ($r=–0.176$; $p=0.006$) and urinary ($r=–0.100$; $p=0.049$) homoarginine and $U_{NOx}R$ ($r=–0.169$; $p=0.001$) values in the black group. Additionally, the uACR was positively associated with urinary nitrate ($r=0.139$; $p=0.008$) in the white group.

In the partial regression analyses (**Supplementary Table 3**) (adjusted for age, sex and BMI), we found inverse correlations of the uACR with the plasma homoarginine ($r=–0.178$; $p=0.006$) and urinary homoarginine ($r=–0.139$; $p=0.007$) and $U_{NOx}R$ ($r=–0.172$; $p=0.001$) values in the black group only. uACR was positively associated with urinary nitrate in the white group ($r=0.124$, $p=0.018$). Noticeably, CK levels were inversely associated with urinary homoarginine ($r=–0.413$; $p=0.012$) in the black group only. The oxidative stress biomarker MDA was inversely associated with the $U_{NOx}R$ in the black ($r=–0.319$; $p<0.001$) and white groups ($r=–0.204$; $p<0.001$).

In the multivariable-adjusted regression analysis (**Figure 1**), we observed consistent inverse associations between the uACR and both plasma (adj. $R^2=0.066$; $\beta=–0.209$; $p=0.005$) and urinary (adj. $R^2=0.066$; $\beta=–0.149$; $p=0.010$) homoarginine as well as between the uACR and the $U_{NOx}R$ (adj. $R^2=0.060$; $\beta=–0.122$; $p=0.031$).

## Discussion

In the present exploratory study involving a healthy cohort of both black and white patients, we compared plasma and urinary NO-related markers along with the CK levels and determined the associations with the uACR, which is an early marker of endothelial dysfunction [39]. The NO-related markers included the NOS substrates arginine and homoarginine [12, 17], the NOS inhibitors ADMA and SDMA [14, 40], urinary nitrate, which is

a major NO metabolite reflecting whole-body NO synthesis [41], and urinary nitrite, which is a measure of the loss of circulating NO bioactivity [27]. We also measured urinary MDA, which is a measure of kidney-related oxidative stress [42]. We observed an overall less favourable NO profile and elevated oxidative stress in the black cohort. Although the uACR did not differ between the black and white groups, the uACR was found to be independently and inversely associated with the $U_{NOx}R$, plasma homoarginine and urinary homoarginine in the black cohort only. Additionally, the plasma CK levels were inversely associated with urinary homoarginine in the black group only.

The results of our study are in line with those of a previous bi-ethnic study on black and white children, which found that black boys presented lower (by –35%) $U_{NOx}R$ levels than white boys [27]. The reduced urinary nitrate and $U_{NOx}R$ (by –18%) levels in the young black adults in the present study illustrate that whole-body NO synthesis is reduced in black individuals, with the difference between black and white individuals appearing to decrease with age. This may be attributable to genetic differences in the enzymes involved in the L-arginine-NO pathway, including NOS, AGAT, PRMT and DDAH, as well as in the renal carbonic anhydrases and anion transporters involved in nitrite excretion and reabsorption from the primary urine, as previously reported [27, 43]. In addition to reduced urinary nitrate and $U_{NOx}R$ values, the black group also displayed increased plasma and urinary levels of arginine and ADMA, which are an endogenous NOS substrate and inhibitor, respectively. Studies have reported increased arginine [20, 44] and ADMA levels [26] in black adults; however, other studies have reported findings of both increased arginine levels and increased ADMA levels in the same study population [28, 45]. The metabolic fate of arginine is complex and involves the arginase-catalysed hydrolysis of arginine to generate ornithine, thus potentially decreasing arginine concentrations and local NO synthesis [46]. Animal studies have suggested that the expression of arginase may be elevated in hypertensive rats [47, 48]. However, the bi-ethnic differences in the arginine and ornithine/citrulline concentrations observed in our study were relatively small, especially with respect to the differences in whole-body NO synthesis. Therefore, differences in arginase expression and activity are probably unlikely to have affected NO synthesis from arginine in our study. The ethnic disparities in regard to the NO profile of our bi-ethnic cohort seem to be multifactorial and include genetic and environmental factors, which, however, are unknown. Since decreased NO synthesis is associated with hypertension [49], the decrease in NO synthesis found in the black group in this study could be responsible for the increased susceptibility of the population to the development of hypertension later in life.

In the first instance, the uACR is an indicator of kidney function, primarily that of the glomerulus [8]. However, the uACR is also generally accepted to be an indicator of cardiovascular disease (CVD) [9] and early endothelial dysfunction [39]. In our study, we found inverse associations of the uACR with the $U_{NOx}R$ and homoarginine (both in plasma and in urine) in the black group only, although both groups presented with relatively similar $U_{NOx}R$ values. Such associations have not been previously described in a black population and may suggest the considerable contribution of the kidney to these ethnic differences. Indeed, the black group presented with higher (by +10%) eGFR values compared to the white group of the cohort. The decreased $U_{NOx}R$ values measured in the black young adults in this study may indicate a relative increase in the loss of circulating nitrite, which is an important reservoir of NO bioactivity, due to attenuated renal carbonic anhydrase activity in the proximal tubule of the nephron. Interestingly, the attenuation of renal carbonic anhydrase activity is more pronounced in childhood [27] than in adulthood in black individuals.

The inverse association of plasma CK with urinary homoarginine ($r$=–0.413; $p$=0.012) but not with plasma homoarginine in the black group only may suggest the considerable contribution of kidney function to this association. By comparing the homoarginine level with the ADMA plasma and SDMA urine concentrations, the urinary homoarginine content was found to be relatively low (**Table 2**). This suggests the occurrence of considerable tubular reabsorption of homoarginine. CK expressed in tubular cells of the kidney provides the adenosine triphosphate (ATP) necessary for $Na^+/K^+$-ATPase activity, which drives several tubular transport mechanisms, including sodium ($Na^+$) reabsorption in Henle's loop and the distal tubule [50, 51]. In a small cohort of African and European young men, plasma CK was found to be inversely associated with $Na^+$ excretion [51]. Increased CK activity has been observed in people from the sub-Saharan African region and has been discussed as a factor contributing to hypertension in individuals of this ethnicity [23, 24].

Our large study confirms the increased (by +10%) CK levels in black individuals. The increased urinary MDA excretion rate (by +19%) measured in the black group may be an indication that the kidneys of the young black adults of this study may have also been subjected to higher oxidative stress than the kidneys of their white counterparts. Elevated oxidative stress may in itself reduce NO bioavailability by increasing NO inactivation, as seen in Africans [52] and Europeans [53]. Not only does an increase in oxidative stress damage the endothelium by impairing endothelium-dependent vascular relaxation (diminished NO), it also increases vascular contractile activity, which may result in hypertension [54]. These findings further reiterate that an increase in oxidative stress (MDA) [20], combined with decreased renal and systemic nitrite levels, may reduce NO bioavailability in the black population, which

may contribute to structural changes in blood vessels and ultimately result in the early onset of hypertension.

The inverse association of plasma CK with urinary homoarginine may indicate that there is a mutual interaction between CK and AGAT in the mitochondria and cytoplasm of kidney cells. This is expected because AGAT not only produces homoarginine but also guanidino acetic acid (GAA). Guanidino acetic acid is further converted to creatine, which, in turn, is the substrate of CK and an inhibitor of the AGAT-catalyzed synthesis of homoarginine and GAA [17, 55]. Notably, homoarginine has been shown to be associated with many human diseases. In particular, low circulating and excretory levels of homoarginine are associated with all-cause mortality [17, 56]. However, the biological activities of L-homoarginine remain almost entirely unknown. The kidney has been reported to be a major contributor to circulating and urinary homoarginine [57-59], underlining the major importance of renal AGAT activity in humans. In the present study, we did not find differences in the plasma and urinary homoarginine levels between black and white young adults. In the healthy young adults in this study, both plasma and urinary homoarginine levels were closely comparable to those reported for healthy humans [56]. However, the disparate associations of urinary homoarginine with CK and the uACR indicate the occurrence of ethnic differences in these biomarkers, which presumably are related to kidney physiology.

This explorative study must be interpreted within the context of its strengths and limitations. The study was well planned and executed under strict conditions. As our population included participants from the North West province of South Africa, it is not representative of the population as a whole. The study is limited by its cross-sectional design; hence, we were unable to investigate the precise mechanisms and causal relationships. The participants in our study did not observe strict abstinence from a nitrate-rich diet. Dietary nitrate may have contributed to some degree to urinary nitrate but not to other biomarkers, including ADMA and homoarginine. Forthcoming studies on the potential involvement of L-arginine-NO and related pathways need to include a nitrate-poor dietary protocol.

To conclude, young black asymptomatic adults partially differ from their white counterparts with respect to various biomarkers and some of the associations between these biomarkers. Reduced urinary nitrate excretion indicates decreased whole-body NO synthesis, and a reduced $U_{NOx}R$ indicates renal carbonic anhydrase dependent loss of circulating nitrite, which is a major reservoir of NO; increased plasma ADMA levels are indicative of the elevated inhibition of NOS activity. These occurrences and the inverse associations, notably those with the uACR, which is a biomarker of early endothelial dysfunction, plasma CK, urinary MDA,

which is a biomarker of kidney-associated oxidative stress, and urinary homoarginine, which is an endogenous substance with largely unknown biological activities, were observed only in the black cohort in the study, suggesting that there were ethnicity-dependent differences in several overlapping L-arginine-involving pathways, i.e., pathways involving L-arginine-NO, AGAT, arginase, CK, and oxidative stress. Our study suggests that these differences originate in the cardiovascular and renal systems. Young black individuals seem to show increased susceptibility to the development of premature hypertension later in life.

**Acknowledgments**

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**Conflict of interest**

The authors report that they have no conflict of interest.

**TABLE 1: General characteristics of young adults stratified according to ethnicity.**

| | **Black** (*n*= 539) | **White** (*n*= 566) | ***p* value** |
|---|---|---|---|
| Age (years) | 24.4 ± 3.14 | 24.6 ± 3.05 | 0.41 |
| Sex men (*n* %) | 261 (48%) | 271 (47%) | 0.86 |
| **Body composition** | | | |
| Waist circumference (cm) | 77.6 ± 11.1 | 82.7 ± 13.7 | **<0.001** |
| Body height (cm) | 164.45 ± 8.48 | 172.49 ± 8.73 | **<0.001** |
| Body weight (kg) | 71.24 ± 17.97 | 72.20 ± 17.06 | 0.36 |
| Body mass index (kg/m$^2$) | 24.6 ± 5.77 | 25.6 ± 5.42 | **0.002** |
| **Cardiovascular measures** | | | |
| Systolic blood pressure (mmHg) | 118 ± 12 | 117 ± 12 | 0.052 |
| Diastolic blood pressure (mmHg) | 80 ± 9 | 78 ± 8 | **<0.001** |
| Central systolic blood pressure (mmHg) | 110 ± 9 | 106 ± 9 | **<0.001** |
| **Biochemical measures** | | | |
| Urinary albumin-to-creatinine ratio | 0.48 (0.15 – 2.00) | 0.47 (0.17 – 2.01) | 0.34 |
| Total cholesterol (mmol/L) | 3.73 (2.68 – 5.40) | 4.61 (3.19 – 6.50) | **<0.001** |
| High density-lipoproteins (mmol/L) | 1.26 ± 0.336 | 1.36 ± 0.416 | **<0.001** |
| Low-density lipoproteins (mmol/L) | 2.33 (1.30 – 3.98) | 2.98 (1.80 – 4.81) | **<0.001** |
| Triglycerides (mmol/L) | 0.72 (0.37 – 1.41) | 0.98 (0.44 – 2.36) | **<0.001** |
| C-reactive protein (mg/L) | 1.27 (0.13 – 12.9) | 0.99 (0.10 – 10.3) | **0.014** |
| eGFR (mL/min/1.73m$^2$) | 118 ± 14.9 | 106 ± 16.0 | **<0.001** |
| Glucose (mmol/L) | 4.35 (2.95 – 5.56) | 4.70 (3.71 – 5.64) | **0.001** |
| Glycated haemoglobin (%) | 5.44 ± 0.30 | 5.20 ± 0.28 | **<0.001** |
| Malondialdehyde (µM/mM creatinine) | 0.16 (0.07 – 0.51) | 0.13 (0.06 – 0.32) | **<0.001** |
| Gamma-glutamyl transferase (U/L) | 24.9 (10.4 – 75.9) | 18.7 (7.84 – 51.0) | **<0.001** |
| Cotinine (ng/mL) | 3.77 (1.00 – 343) | 3.25 (1.00 – 313) | 0.31 |
| Creatine kinase (ng/mL) * | 1.59 (0.64 – 4.58) | 1.35 (0.57 – 3.80) | **<0.001** |
| **Lifestyle risk factors** | | | |
| Smoke (*n* %) | 154 (29%) | 131 (23%) | **0.040** |
| Alcohol (*n* %) | 146 (27%) | 160 (28%) | 0.35 |

Values are arithmetic mean ± standard deviation or geometric mean (5$^{th}$ and 95$^{th}$ percentiles) for logarithmically transformed variables. * Creatine kinase was adjusted for age, sex and active energy expenditure. Abbreviations: *n* – number of participants, eGFR – estimated glomerular filtration rate. Bold values denote statistical significance (p<0.05).

**TABLE 2: Plasma and urinary nitric oxide-related markers of the study population stratified according to ethnicity.**

| | **Black** (*n*= 539) | **White** (*n*= 566) | ***p* value** |
|---|---|---|---|
| **Plasma nitric oxide-related markers** | | | |
| Arginine (µM) | 92.7 (60.3 – 149) | 83.8 (56.7 – 117) | **<0.001** |
| Homoarginine (µM) | 2.05 (0.97 – 4.33) | 2.05 (1.06 – 3.77) | 0.99 |
| ADMA (µM) | 0.60 (0.39 – 0.92) | 0.52 (0.31 – 0.78) | **<0.001** |
| SDMA (µM) | 0.533 ± 0.142 | 0.562 ± 0.129 | **0.012** |
| **Urinary nitric oxide-related markers** | | | |
| Arginine (µM/mM creatinine) | 2.37 (1.38 – 4.18) | 2.23 (1.31 – 3.95) | **0.004** |
| Homoarginine (µM/mM creatinine) | 0.15 (0.04 – 0.52) | 0.15 (0.05 – 0.63) | 0.81 |
| ADMA (µM/mM creatinine) | 3.82 (2.27 – 6.01) | 3.68 (2.15 – 5.94) | **0.033** |
| SDMA (µM/mM creatinine) | 3.73 (2.44 – 5.36) | 3.89 (2.59 – 5.76) | **0.022** |
| Ornithine/Citrulline (µM/mM creatinine) | 3.48 (2.19 – 5.49) | 3.57 (1.91 – 5.51) | **0.017** |
| Nitrate (µM/mM creatinine) | 59.6 (32.7 – 127) | 70.5 (37.8 – 146) | **<0.001** |
| Nitrite (µM/mM creatinine) | 0.29 (0.10 – 0.90) | 0.28 (0.11 – 0.95) | 0.51 |
| Urinary nitrate-to-nitrite ratio | 204 (64.49 – 573) | 248 (88.19 – 601) | **<0.001** |

Values are arithmetic mean ± standard deviation or geometric mean (5th and 95th percentiles) for logarithmically transformed variables. † Samples measured in plasma (black *n*=279; white *n*=282). Abbreviations: *n* – number of participants. Bold values denote statistical significance (p<0.05).

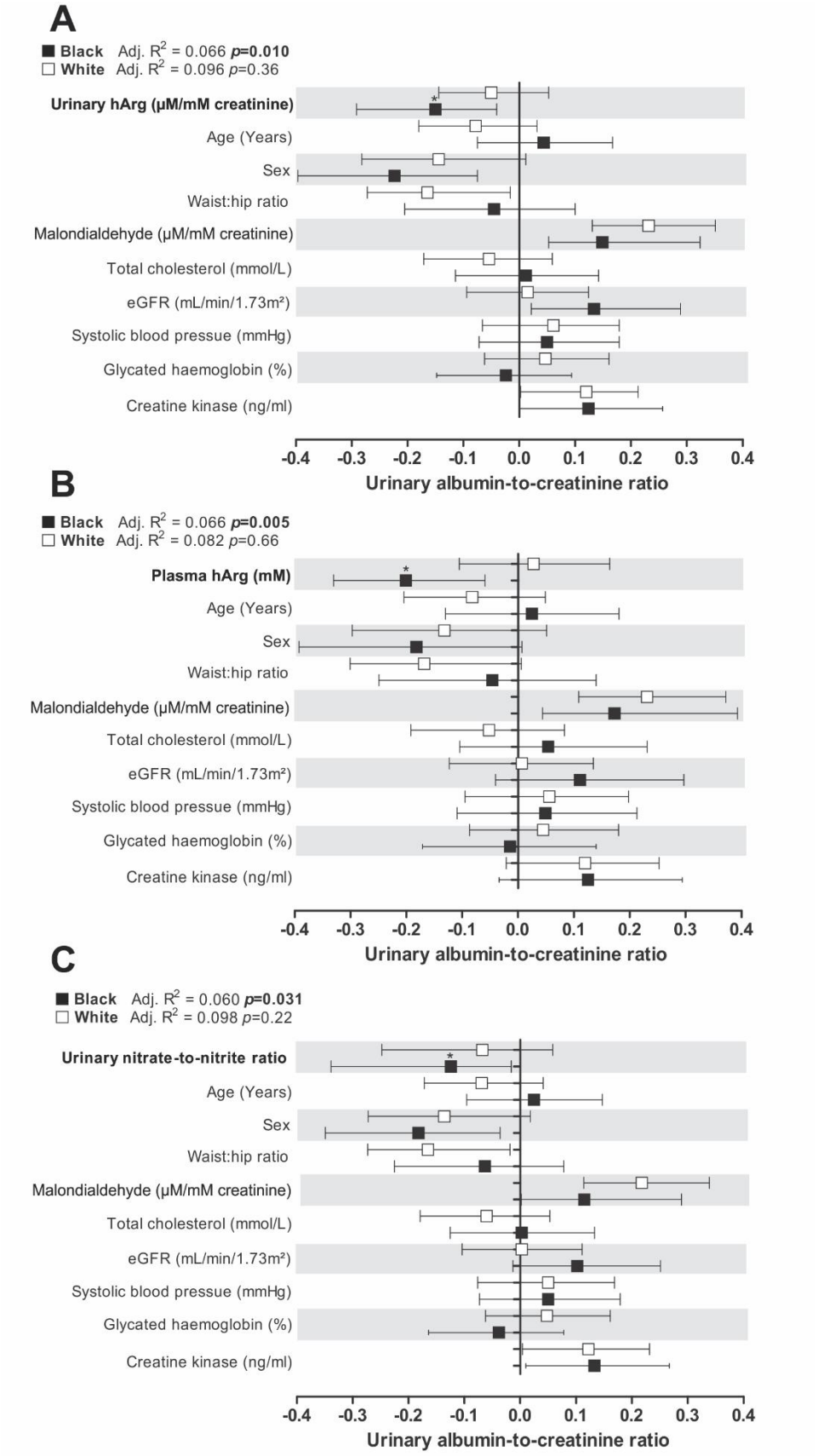


**Figure 1: Multiple regression anaylses of uACR with (A) creatinine-corrected urinary homoarginine, (B) plasma homoarginine, and (C) urinary nitrate-to-nitrite ratio levels in the population stratified by ethnicity.**

**SUPPLEMENTARY TABLE 1: Interaction terms of ethnicity on the relationship of the urinary albumin-to-creatinine ratio (uACR) with plasma and creatinine-corrected urinary nitric oxide-related metabolites.**

| **Interaction of ethnicity on urinary albumin-to-creatinine ratio** | |
|---|---|
| **Biomarker** | ***p* value** |
| **Plasma nitric oxide-related markers** | |
| Arginine (µM) | **<0.001** |
| Homoarginine (µM) | **<0.001** |
| ADMA (µM) | **0.002** |
| SDMA (µM) | **0.001** |
| **Urinary nitric oxide-related markers** | |
| Arginine (µM/mM creatinine) | **<0.001** |
| Homoarginine (µM/mM creatinine) | **0.001** |
| ADMA (µM/mM creatinine) | **<0.001** |
| SDMA (µM/mM creatinine) | **<0.001** |
| Ornithine/Citrulline (µM/mM creatinine) | **<0.001** |
| Nitrate (µM/mM creatinine) | **<0.001** |
| Nitrite (µM/mM creatinine) | **<0.001** |
| $U_{NOx}R$ | **<0.001** |

Bold values denote statistical significance ($p<0.05$).

**SUPPLEMENTARY TABLE 2: Pearson correlations between anthropometric and cardiovascular measures and markers related to nitric oxide synthesis in the study population stratified by ethnicity.**

| | **Homoarginine** | | | | **Urinary nitrate-to-nitrite ratio** | | **Nitrate** | |
|---|---|---|---|---|---|---|---|---|
| | **Plasma (μM)** | | **Urine (μM/mM creatinine)** | | **Urine (μM/mM creatinine)** | | **Urine (μM/mM creatinine)** | |
| | **Black** (*n*=539) | **White** (*n*=566) | **Black** (*n*=539) | **White** (*n*=566) | **Black** (*n*=539) | **White** (*n*=566) | **Black** (*n*=539) | **White** (*n*=566) |
| Age (years) | r=0.019<br>*p*=0.749 | r=0.078<br>*p*=0.189 | r=0.062<br>*p*=0.151 | r=0.004<br>*p*=0.921 | r=–0.042<br>*p*=0.336 | **r=0.137**<br>***p*=0.001** | r=0.021<br>*p*=0.629 | **r=0.103**<br>***p*=0.014** |
| Waist circumference (cm) | **r=0.156**<br>***p*=0.009** | r=0.066<br>*p*=0.269 | **r=0.260**<br>***p*<0.001** | r=0.019<br>*p*=0.644 | r=0.020<br>*p*=0.642 | r=–0.053<br>*p*=0.210 | r=–0.079<br>*p*=0.067 | r=–0.040<br>*p*=0.338 |
| Waist-to-hip ratio | r=0.057<br>*p*=0.341 | r=0.042<br>*p*=0.479 | r=–0.043<br>*p*=0.333 | r=–0.051<br>*p*=0.227 | r=0.021<br>*p*=0.631 | r=–0.066<br>*p*=0.119 | r=–0.050<br>*p*=0.255 | **r=–0.098**<br>***p*=0.020** |
| Body mass index (kg/m²) | **r=0.120**<br>***p*=0.046** | r=0.087<br>*p*=0.146 | **r=0.311**<br>***p*<0.001** | **r=0.097**<br>***p*=0.021** | r=0.012<br>*p*=0.776 | r=–0.022<br>*p*=0.594 | r=–0.058<br>*p*=0.182 | r=0.008<br>*p*=0.848 |
| Systolic blood pressure (mm/Hg) | r=–0.023<br>*p*=0.701 | r=–0.004<br>*p*=0.949 | **r=–0.144**<br>***p*=0.001** | r=–0.015<br>*p*=0.716 | r=–0.056<br>*p*=0.192 | **r=–0.089**<br>***p*=0.034** | **r=–0.110**<br>***p*=0.011** | **r=–0.093**<br>***p*=0.027** |
| Diastolic blood pressure (mm/Hg) | r=–0.019<br>*p*=0.753 | r=0.016<br>*p*=0.785 | r=–0.078<br>*p*=0.070 | r=0.027<br>*p*=0.524 | r=–0.019<br>*p*=0.657 | r=–0.070<br>*p*=0.097 | r=–0.041<br>*p*=0.346 | r=–0.078<br>*p*=0.064 |
| Central systolic blood pressure (mm/Hg) | r=–0.064 | r=0.039 | r=–0.021 | r=0.026 | r=–0.026 | r=–0.039 | r=–0.023 | r=–0.068 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *p*=0.285 | *p*=0.513 | *p*=0.621 | *p*=0.542 | *p*=0.541 | *p*=0.353 | *p*=0.599 | *p*=0.108 |
| Urinary albumin-to-creatinine ratio | **r=–0.176** | r=0.021 | **r=–0.100** | r=–0.018 | **r=–0.169** | r=–0.097 | r=0.062 | **r=0.139** |
| | ***p*=0.006** | *p*=0.739 | ***p*=0.049** | *p*=0.738 | ***p*=0.001** | *p*=0.065 | *p*=0.227 | ***p*=0.008** |
| High-density lipoproteins (mmol/L) | r=–0.016 | r=0.083 | r=–0.031 | r=0.019 | r=0.034 | r=–0.004 | **r=0.097** | r=–0.036 |
| | *p*=0.793 | *p*=0.163 | *p*=0.526 | *p*=0.719 | *p*=0.489 | *p*=0.944 | ***p*=0.049** | *p*=0.487 |
| Low-density lipoproteins (mmol/L) | **r=0.190** | r=0.066 | r=–0.008 | r=–0.065 | **r=–0.120** | **r=–0.120** | r=–0.037 | r=–0.032 |
| | ***p*=0.001** | *p*=0.269 | *p*=0.875 | *p*=0.207 | ***p*=0.017** | ***p*=0.021** | *p*=0.464 | *p*=0.543 |
| Triglycerides (mmol/L) | r=0.115 | r=0.075 | r=–0.077 | r=–0.091 | r=0.029 | **r=–0.117** | r=–0.081 | r=–0.002 |
| | *p*=0.055 | *p*=0.212 | *p*=0.119 | *p*=0.079 | *p*=0.556 | ***p*=0.024** | *p*=0.101 | *p*=0.971 |
| Total cholesterol (mmol/L) | **r=0.195** | r=0.114 | r=0.000 | r=–0.089 | **r=–0.098** | **r=–0.133** | r=0.006 | r=–0.026 |
| | ***p*=0.001** | *p*=0.056 | *p*=1.000 | *p*=0.085 | ***p*=0.047** | ***p*=0.010** | *p*=0.898 | *p*=0.615 |
| eGFR (mL/min/1.73m²) | r=–0.004 | r=0.005 | **r=0.142** | **r=0.182** | r=–0.050 | r=–0.079 | **r=0.127** | **r=0.147** |
| | *p*=0.941 | *p*=0.934 | ***p*=0.004** | ***p*<0.001** | *p*=0.310 | *p*=0.125 | ***p*=0.010** | ***p*=0.004** |
| Glucose (mmol/L) | r=0.000 | r=0.015 | r=0.054 | r=–0.024 | r=0.029 | r=–0.044 | r=–0.022 | r=–0.058 |
| | *p*=0.995 | *p*=0.797 | *p*=0.293 | *p*=0.648 | *p*=0.577 | *p*=0.396 | *p*=0.675 | *p*=0.264 |
| Glycated haemoglobin (%) | r=0.105 | r=0.101 | **r=0.113** | r=0.013 | r=0.008 | r=0.018 | r=0.018 | **r=0.131** |
| | *p*=0.084 | *p*=0.095 | ***p*=0.009** | *p*=0.766 | *p*=0.851 | *p*=0.678 | *p*=676 | ***p*=0.002** |
| C-reactive protein (mg/L) | r=0.061 | r=0.073 | **r=0.261** | r=0.010 | r=0.013 | r=0.005 | r=0.028 | r=0.052 |
| | *p*=0.308 | *p*=0.223 | **p<0.001** | *p*=0.844 | *p*=0.785 | *p*=0.918 | *p*=0.568 | *p*=0.312 |
| Malondialdehyde (µM/mM creatinine) | r=0.087 | r=0.027 | r=–0.027 | r=0.023 | **r=–0.319** | **r=–0.204** | r=0.047 | r=0.048 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *p*=0.153 | *p*=0.661 | *p*=0.533 | *p*=0.592 | ***p*<0.001** | ***p*<0.001** | *p*=0.275 | *p*=0.252 |
| Gamma-glutamyl transferase (U/L) | r=0.080 | r=–0.019 | r=0.006 | r=–0.070 | r=–0.023 | **r=–0.142** | r=–0.068 | r=–0.080 |
| | *p*=0.181 | *p*=0.754 | *p*=0.901 | *p*=0.176 | *p*=0.638 | ***p*=0.006** | *p*=0.164 | *p*=0.122 |
| | **r=–0.170** | **r=–0.131** | **r=–0.119** | **r=–0.186** | r=0.067 | r=–0.035 | **r=0.131** | r=0.075 |
| Cotinine (ng/mL) | ***p*=0.005** | ***p*=0.028** | ***p*=0.010** | ***p*<0.001** | *p*=0.153 | *p*=0.479 | ***p*=0.005** | *p*=0.126 |
| | r=0.001 | r=–0.010 | **r=–0.170** | r=–0.076 | r=0.015 | r=0.017 | r=0.016 | r=–0.005 |
| Creatine kinase (ng/mL) | *p*=0.988 | *p*=0.878 | ***p*=0.001** | *p*=0.154 | *p*=0.773 | *p*=0.749 | *p*=0.754 | *p*=0.930 |
| | r=–0.093 | **r=–0.185** | **r=–0.198** | **r=–0.144** | r=0.088 | r=–0.063 | **r=0.149** | r=0.036 |
| Smoke | *p*=0.121 | ***p*=0.002** | ***p*<0.001** | ***p*=0.001** | *p*=0.042 | *p*=0.132 | ***p*=0.001** | *p*=0.388 |
| | r=–0.111 | r=0.111 | r=–0.052 | r=0.038 | r=0.012 | r=–0.044 | r=0.066 | r=0.012 |
| Alcohol | *p*=0.066 | *p*=0.063 | *p*=0.225 | *p*=0.363 | *p*=0.782 | *p*=0.296 | *p*=0.131 | *p*=0.777 |

Abbreviations: *n* – number of participants, eGFR – estimated glomerular filtration rate. Bold values denote statistical significance (p<0.05).

**SUPPLEMENTARY TABLE 3: Partial correlations between anthropometric and cardiovascular measures and markers related to nitric oxide synthesis in the study population stratified by ethnicity.**

| | Homoarginine | | | | Urinary nitrate-to-nitrite ratio | | Nitrate | |
|---|---|---|---|---|---|---|---|---|
| | Plasma (µM) | | Urine (µM/mM creatinine) | | Urine (µM/mM creatinine) | | Urine (µM/mM creatinine) | |
| | **Black** (*n*=539) | **White** (*n*=566) | **Black** (*n*=539) | **White** (*n*=566) | **Black** (*n*=539) | **White** (*n*=566) | **Black** (*n*=539) | **White** (*n*=566) |
| Urinary albumin-to-creatinine ratio | **r=–0.178** | r=0.028 | **r=–0.139** | r=–0.041 | **r=–0.172** | r=–0.102 | r=0.056 | **r=0.124** |
| | ***p*=0.006** | *p*=0.658 | ***p*=0.007** | *p*=0.436 | ***p*=0.001** | *p*=0.053 | *p*=0.278 | ***p*=0.018** |
| High-density lipoproteins (mmol/L) | r=0.012 | r=0.101 | r=0.019 | r=–0.023 | r=0.043 | r=–0.073 | r=0.077 | **r=–0.144** |
| | *p*=0.843 | *p*=0.093 | *p*=0.701 | *p*=0.652 | *p*=0.379 | *p*=0.161 | *p*=0.117 | ***p*=0.005** |
| Low-density lipoproteins (mmol/L) | **r=0.176** | r=0.053 | r=–0.076 | r=–0.060 | **r=–0.120** | **r=–0.114** | r=–0.035 | r=–0.006 |
| | ***p*=0.003** | *p*=0.377 | *p*=0.137 | *p*=0.247 | ***p*=0.019** | ***p*=0.028** | *p*=0.490 | *p*=0.912 |
| Triglycerides (mmol/L) | r=0.099 | r=0.062 | r=–0.087 | r=–0.095 | r=0.033 | **r=–0.103** | r=–0.064 | r=0.034 |
| | *p*=0.100 | *p*=0.303 | *p*=0.076 | *p*=0.066 | *p*=0.498 | ***p*=0.047** | *p*=0.194 | *p*=0.519 |
| Total cholesterol (mmol/L) | **r=0.188** | r=0.096 | r=–0.054 | r=–0.094 | r=–0.093 | **r=–0.152** | r=0.005 | r=–0.030 |
| | ***p*=0.002** | *p*=0.112 | *p*=0.277 | *p*=0.070 | *p*=0.058 | ***p*=0.003** | *p*=0.916 | *p*=0.558 |
| eGFR (mL/min/1.73m²) | r=0.004 | r=0.004 | **r=0.181** | **r=0.149** | r=–0.066 | r=–0.058 | **r=0.139** | **r=0.152** |
| | *p*=0.948 | *p*=0.954 | ***p*<0.001** | ***p*=0.004** | *p*=0.182 | *p*=0.267 | ***p*=0.005** | ***p*=0.003** |
| Glucose (mmol/L) | r=–0.023 | r=0.013 | r=–0.006 | r=–0.013 | r=0.037 | r=–0.040 | r=–0.018 | r=–0.046 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *p*=0.702 | *p*=0.825 | *p*=0.910 | *p*=0.801 | *p*=0.474 | *p*=0.446 | *p*=0.724 | *p*=0.383 |
| Glycated haemoglobin (%) | r=0.079 | r=0.075 | r=0.037 | r=–0.023 | r=0.007 | r=–0.007 | r=0.031 | **r=0.101** |
| | *p*=0.176 | *p*=0.218 | *p*=0.389 | *p*=0.590 | *p*=0.866 | *p*=0.873 | *p*=0.471 | ***p*=0.016** |
| C-reactive protein (mg/L) | r=–0.001 | r=0.041 | r=0.086 | r=–0.075 | r=0.010 | r=0.029 | r=0.060 | r=0.042 |
| | *p*=0.990 | *p*=0.493 | *p*=0.080 | *p*=0.146 | *p*=0.841 | *p*=0.579 | *p*=0.226 | *p*=0.420 |
| Malondialdehyde (µM/mM creatinine) | r=0.091 | r=0.021 | r=–0.031 | r=0.009 | **r=–0.324** | **r=–0.213** | r=0.046 | r=0.036 |
| | *p*=0.136 | *p*=0.729 | *p*=0.471 | *p*=0.827 | ***p*<0.001** | ***p*>0.001** | *p*=0.292 | *p*=0.389 |
| Gamma-glutamyl transferase (U/L) | r=0.059 | r=–0.049 | r=0.002 | r=–0.056 | r=–0.024 | **r=–0.139** | r=–0.046 | r=–0.027 |
| | *p*=0.330 | *p*=0.412 | *p*=0.960 | *p*=0.284 | *p*=0.624 | ***p*=0.009** | *p*=0.355 | *p*=0.606 |
| Cotinine (ng/ mL) | **r=–0.116** | **r=–0.129** | r=–0.015 | **r=–0.166** | r=0.073 | r=–0.016 | **r=0.149** | **r=0.116** |
| | ***p*=0.006** | ***p*=0.031** | *p*=0.753 | ***p*=0.001** | *p*=0.118 | *p*=0.751 | ***p*=0.001** | ***p*=0.019** |
| Creatine kinase (ng/ mL) | r=0.017 | r=–0.006 | **r=–0.413** | r=–0.022 | r=0.019 | r=0.047 | r=0.039 | r=0.229 |
| | *p*=0.815 | *p*=0.929 | ***p*=0.012** | *p*=0.686 | *p*=0.719 | *p*=0.385 | *p*=0.445 | *p*=0.349 |
| Smoke | r=–0.077 | **r=–0.192** | r=–0.077 | **r=–0.140** | **r=0.096** | r=–0.047 | **r=0.179** | r=0.063 |
| | *p*=0.204 | ***p*=0.001** | *p*=0.073 | ***p*=0.001** | ***p*=0.026** | *p*=0.262 | ***p*<0.001** | *p*=0.136 |
| Alcohol | r=–0.106 | r=0.102 | r=–0.013 | r=0.042 | r=0.011 | r=–0.051 | r=0.070 | r=0.013 |
| | *p*=0.081 | *p*=0.088 | *p*=0.759 | *p*=0.317 | *p*=0.801 | *p*=0.227 | *p*=0.108 | *p*=0.764 |

Variables included in the models were: age, sex and body mass index. † Samples measured in urine († Samples measured in urine (black *n*=539; white *n*=566), ₸ Samples measured in whole blood (black *n*=279; white *n*=282). Abbreviations: *n* – number of participants, eGFR – estimated glomerular filtration rate. Bold values denote statistical significance (p<0.05).

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# Chapter VI

## Summary of the main findings

## 1. Introduction

In this chapter, the main findings of the study are summarised. The results of each manuscript will be compared to relevant literature and briefly discussed. Additionally, recommendations on future studies exploring nitric oxide (NO)-related markers and cardiovascular structure and function will be made.

## 2. Summary of main findings

The central aim of this study was to investigate the relationships between measures of arterial structure and function and markers of NO metabolism in children and young adults from South Africa. The associations of plasma and urinary NO-related markers with blood pressure (BP) indices and large artery structure and function were investigated in Chapters III, IV and V respectively.

### *2.1 Nitric oxide related markers link inversely to blood pressure in black boys and men: The ASOS and African-PREDICT studies.*

NO bioavailability as indicated by the NO-related markers (urinary nitrate, and the nitrate-to-nitrite ratio ($U_{NOx}R$)) between young black and white boys (aged 6-8 years) and men (aged 20-30 years) was explored. It was also determined whether these markers associated with BP and carotid intima media thickness (cIMT). This study initially hypothesised the following (as previously outlined in Chapter I):

i. Urinary nitrate and $U_{NOx}R$ will present lower in black boys and men, than their white counterparts and,
ii. Blood pressure and cIMT will associate inversely with nitrate and $U_{NOx}R$ in both groups.

In this cross-sectional analysis, both urinary nitrate and $U_{NOx}R$ levels were lower in black boys and men when compared to their white counterparts, and the first hypothesis is therefore accepted. An inverse association of diastolic blood pressure (DBP) in black boys, and systolic blood pressure (SBP) in black men with urinary nitrate was found. Additionally, cIMT associated inversely with $U_{NOx}R$ in black men only. Due to the inverse associations in the black groups and no associations presenting in the white groups, the second hypothesis is partially accepted.

## *2.2 Central systolic blood pressure relates inversely to nitric oxide synthesis in young black adults: The African-PREDICT study.*

Both plasma and urinary NO-related markers in healthy young black and white men and women (aged 20-30 years) were compared and additionally the NO-related associations with central systolic blood pressure (cSBP) and arterial stiffness were explored. In Chapter I, this study hypothesised that:

i. Central BP (cSBP) and arterial stiffness (pulse wave velocity (PWV)) will present higher in the black populations compared to their white counterparts;
ii. In plasma and urine, asymmetric (ADMA) and symmetric dimethylarginine (SDMA) will present higher in the black group while NO substrates and metabolites (arginine, homoarginine, ornithine/citrulline, nitrate and $U_{NOx}R$) will present lower in the black group and;
iii. Central BP (cSBP) and arterial stiffness (PWV) will associate inversely with NO substrates and metabolites (arginine, homoarginine, ornithine/citrulline, nitrate and urinary nitrate-to-nitrite ratio) and positively with NO synthesis inhibitors (ADMA and SDMA) in both groups.

Higher cSBP in black men and women compared to the white groups, but similar PWV between the groups was observed, and therefore the first hypothesis was partially accepted. When a comparison of NO-related markers between black and white men and women (**Figure 1**) was made, plasma ADMA presented higher and urinary nitrate and $U_{NOx}R$ levels lower in both black men and women. The second hypothesis was accepted in part due to black women having higher plasma and urinary arginine levels and lower urinary SDMA and black men having higher plasma arginine when compared to their respective counterparts. An inverse association between cSBP and plasma homoarginine in black men, between cSBP and $U_{NOx}R$ in black women and a positive association between cSBP and urinary ADMA in white women was found. In addition, PWV was found to associate inversely with plasma ADMA in white women. The third hypothesis was partially accepted due to the inverse association this study reported between PWV and plasma ADMA, a potent NOS inhibitor.

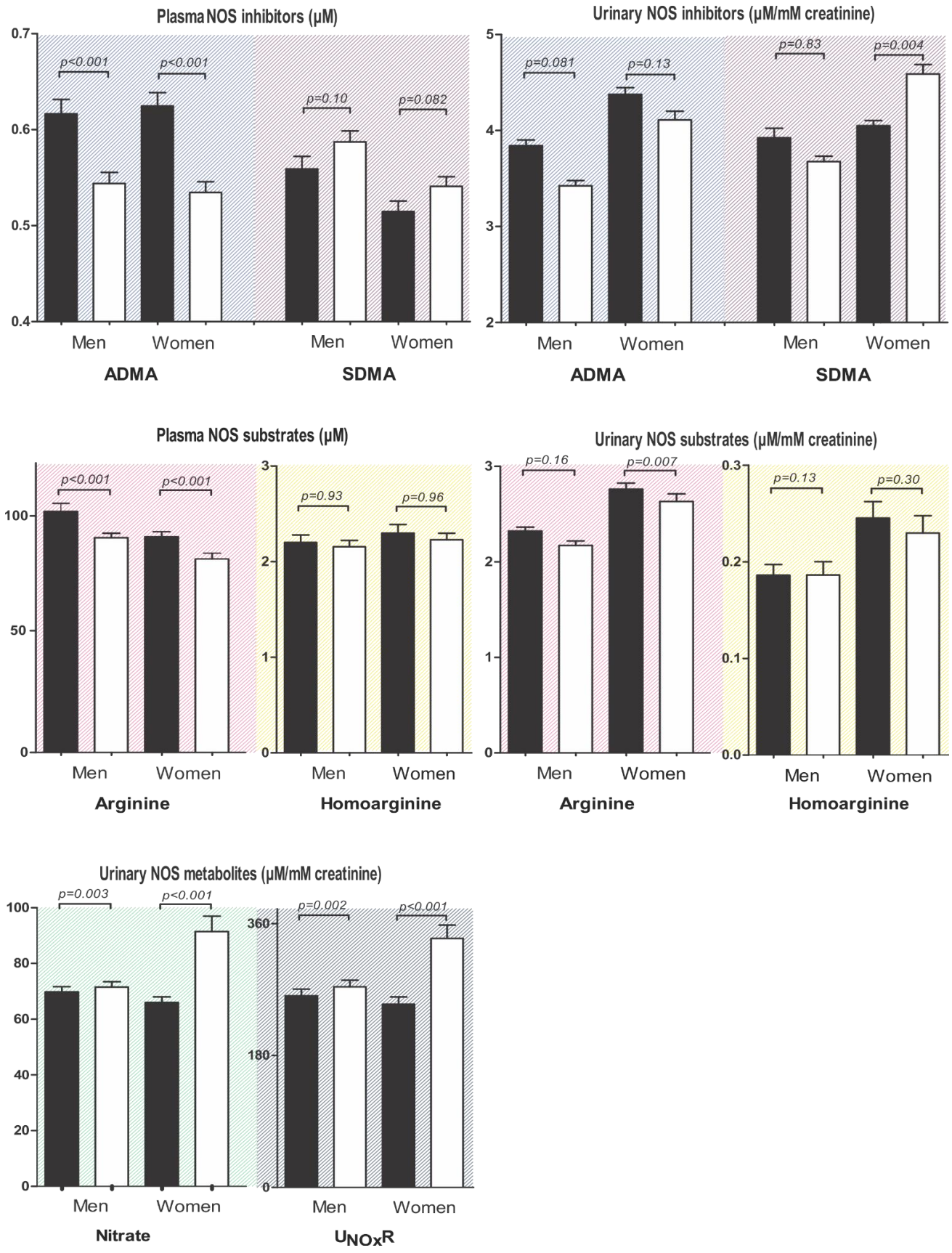


**Figure 1. A comparison of NO-related markers in a biethnic adult cohort comprising of men and women.**

*2.3 Urinary albumin-to-creatinine ratio is inversely related to nitric oxide synthesis in young black adults: The African-PREDICT study.*

We compared NO-related markers in plasma and urine and plasma creatine kinase (CK) levels in healthy black and white young adults (aged 20-30 years). We also determined the associations of NO-related markers with urinary albumin-to-creatinine ratio (uACR), a known marker of systemic endothelial function. As previously outlined in Chapter I, the following hypotheses were deduced:

i. uACR will be higher in black adults when compared to their white counterparts;
ii. In plasma and urine, NO substrates and metabolites (arginine, homoarginine, and urinary ornithine/citrulline, nitrates and $U_{NOx}R$) will present lower, while NO synthesis inhibitors (ADMA and SDMA) higher in the black compared to the white group and;
iii. uACR will inversely associate with NO substrates and metabolites (arginine, homoarginine, ornithine/citrulline, nitrates and $U_{NOx}R$) and positively with NO synthesis inhibitors (ADMA and SDMA) in the black group.

The uACR was similar in the black and white adult groups and therefore the first hypothesis was rejected. When viewing the comparison of NO-related markers between the groups (**Figure 2**), plasma and urinary ADMA presented higher and urinary nitrate, $U_{NOx}R$ and citrulline/ornithine presented lower in the black group. The second hypothesis was partially accepted due to the black group having higher plasma and urinary arginine and lower plasma and urinary SDMA levels when compared to the white group. This study further confirmed consistent inverse associations of uACR with both plasma and urinary homoarginine as well as between uACR and $U_{NOx}R$ in the black group only. The third hypothesis was accepted in part due to the lack of positive associations between NO synthesis inhibitors (ADMA and SDMA) and uACR in the black group.

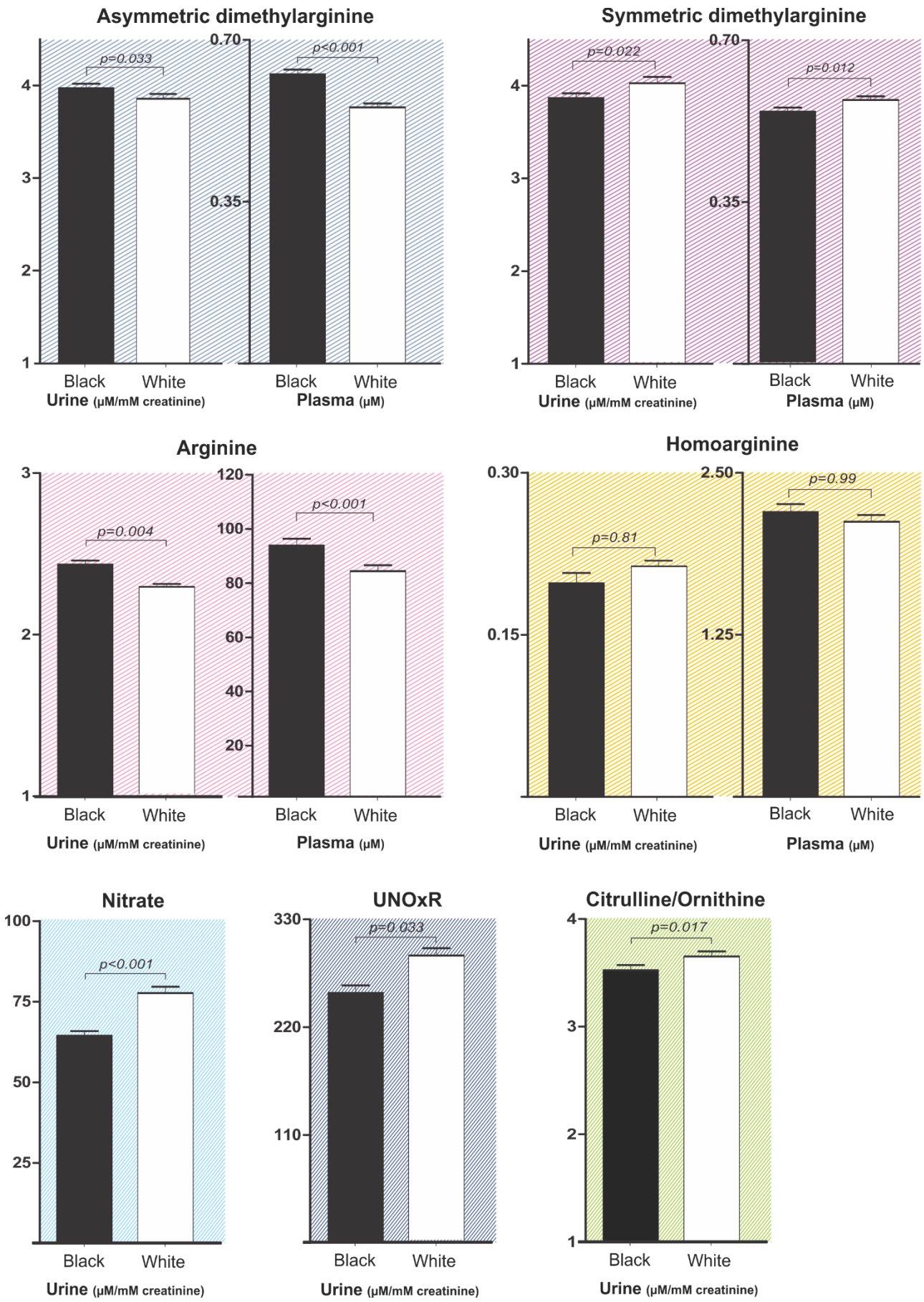


**Figure 2: A comparison of NO-related markers in a biethnic adult cohort.**

## 3. Discussion and comparison of main findings to the relevant literature

Evidence of studies exploring the plasma and urinary NO profiles in young apparently healthy populations is sparse. Upon comparing the findings of this hypothesis-generating study to previous findings, it has revealed some of the findings of this study confirm while some of the findings of this study contradict those previously reported. Some of the findings of this study were also reported to be novel and contributed to the limited body of knowledge surrounding the NO profile and associations with cardiovascular structure and function in black and white South Africans.

### *3.1 Nitric oxide-related markers link inversely to blood pressure in black boys and men: The ASOS and African-PREDICT studies.*

This study demonstrated that, only in black boys and men, urinary nitrate (boys: by –27%; men by –10%) and $U_{NOx}R$ (boys: by –35%; men by –15%) levels presented lower when compared to their white counterparts, with the difference seeming to decrease with age. The finding of lower urinary nitrate and $U_{NOx}R$ levels in the black groups not only confirms previous findings of black boys participating in the Arterial Stiffness in Offspring Study (ASOS) [1] who presented with lower $U_{NOx}R$ levels, but also, further supports previous findings by Glyn *et al.* who reported lower NO production in black men who present with lower NOS substrate arginine (primary NOS substrate in the L-arginine-NO pathway) [2]. Additionally, this study confirmed the findings from Johnsen *et al*. who reported that urinary nitrate levels in children are high and seem to decrease with age [3]. In the present study, children (aged 6-8 years) presented with higher urinary nitrate levels when compared to men (aged 20-30 years) (**Figure 3**). The precise mechanism for this age-related decrease in urinary nitrate levels as seen in adults when compared to children may be due to younger individuals having a heightened basal NOS activity [3, 4].

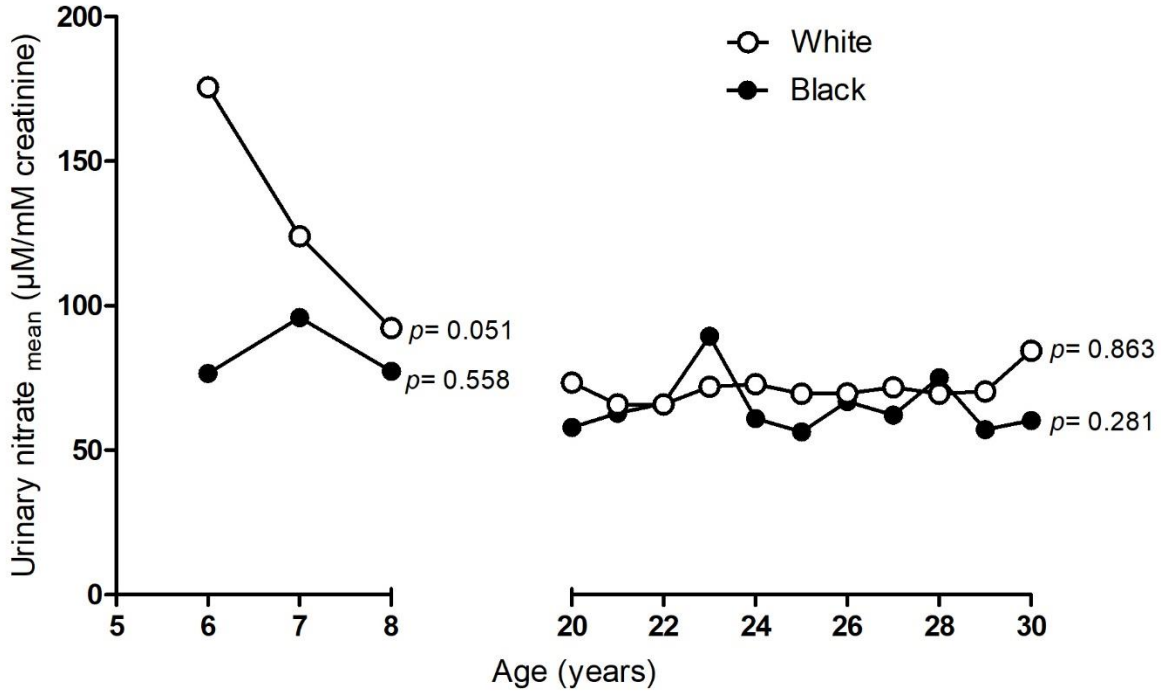


**Figure 3: Age plotted against urinary nitrate levels in boys (aged 6-8 years) and men (aged 20-30 years).**

Since urinary nitrate and urinary nitrite are co-dependent of each other, $U_{NOx}R$ as described by Tsikas *et al*. is suggested to be a better estimate of renal nitrite reabsorption in the kidney [1]. It was suspected that the black boys and men of this study would have a reduced capacity in NO metabolism via this alternative renal pathway (nitrite-to-nitrate-NO pathway) of nitrite reabsorption. It has been previously reported in two independent studies that the ethnic disparities of lower $U_{NOx}R$ levels (lower reabsorption of urinary nitrite) found in black populations is suggested to result from genetic differences in the renal carbonic anhydrase isoforms and anion transporters [1, 5]. However, it was not possible to investigate the precise mechanisms or genetic variations contributing to the ethnic differences observed due to the nature of this hypothesis-generating study.

Inverse associations with DBP and urinary nitrate in black boys, and with SBP and urinary nitrate in black men were reported. The findings of this are in line with two previous independent studies [6, 7], that indicated an increase in BP is partly induced by a decrease in circulating NO metabolites. As NO is seen as a potent vasodilator, a reduction in its bioactivity results in attenuated vasodilatory effects, favouring vasoconstriction and thus increasing BP [8, 9]. It has been proposed that the measurement of adolescent BP may predict the progress and frequency in which hypertension develops in adults [10]. In adults, an elevated SBP is

considered a risk for the development of CVD [11] while in children, DBP is more often considered a better measure of cardiovascular risk prediction [12, 13]. The inverse associations between BP and markers of NO bioavailability observed in this present study may suggest that the lower level of NO expressed precedes raised BP in later life.

Another finding of this study is the reported inverse association between cIMT and $U_{NOx}R$ in black men. Such an association has not yet been reported. Since $U_{NOx}R$ is considered a suitable measure of renal nitrite reabsorption [5], we can confirm the findings of Kleinbongard *et al*. who reported an inverse association between IMT and plasma nitrite in individuals with endothelial dysfunction [14]. As previously outlined in Chapter I, a reduced NO bioavailability is, on its own, a hallmark for endothelial dysfunction occurring early in atherosclerosis. The black men of the present study presented with lower $U_{NOx}R$ levels, ultimately reducing the bioavailability of NO. The reported result of this study may suggest that the lower $U_{NOx}R$ levels indicate lower renal systemic nitrite levels which may ultimately impair systemic endothelial function.

The last finding from this study is that black men had higher urinary malondialdehyde (MDA) (by +25%), and systemic reactive oxygen species (ROS) (by +12%), when compared to age-matched white men. This study can confirm the findings of the SABPA study, reported by Mels *et al*. who showed black men to have higher ROS levels when compared to their white counterparts [15]. However, to the best of the knowledge of the researchers involved in this study, no prior studies have compared the urinary MDA levels in a biethnic cohort. As MDA is a marker of whole-body lipid peroxidation, this study can confirm the findings from an independent study who reported higher levels of the lipid peroxidation molecule, thiobarbituric acids reactive substances (TBARS) in black South Africans [16].

As part of an explorative analyses in this study, the negative associations between DBP reported in black boys, SBP reported in black men and urinary nitrate, as well as cIMT and $U_{NOx}R$ in black men, remained significant when MDA was added into the multivariate analyses. However, when ROS was added as a possible covariate in the multivariate regression model, the reported associations of BP or cIMT with NO-related markers lost significance. A possible explanation could be the fact that serum peroxides as measured by ROS and lipid peroxidation as measured by MDA are two markers that may reflect differing degrees of oxidative stress. This may, in part, be due to serum peroxides being rather non-specific and MDA indicating whole-body lipid radical formation.

*3.2 Central systolic blood pressure relates inversely to nitric oxide synthesis in young black adults: The African-PREDICT study.*

In a comparison between black and white men and women (aged 20-30 years), black men and women presented with lower urinary nitrate (men: by –10%; women: by –20%) and $U_{NOx}R$ (men: by –15%; women: by –19%) levels, higher plasma ADMA (men: by +12%; women: by +13%) and arginine (men: by +10%; women: by +9.7%), when compared to their white counterparts. This study can confirm the findings of two previous studies reporting significantly lower urinary nitrate and $U_{NOx}R$ levels in black males when compared to white males [1, 17]. To the best of the knowledge of the researchers involved in this study, no prior studies have explored the urinary nitrate levels in healthy black and white men and women, although Cubeddu *et al*. reported findings of lower urinary nitrate levels in salt-sensitive adults [18]. It is noteworthy to mention that salt sensitivity is an important contributor to hypertension, where both salt sensitivity and hypertension are found more prominent in black individuals when compared to white individuals [19]. However, salt-sensitivity was outside the scope of this thesis.

In the black men and women of this study–a population with known increased risk for hypertension [20]–it was expected that higher levels of the NOS inhibitor, ADMA would be reported. It was unexpected that higher levels of the NOS substrate, arginine, in the black population, would also be reported. This study therefore confirms the findings of higher plasma ADMA in black Africans [21] and higher plasma arginine in black populations [22, 23]. Although found in hypertensives when compared to their normotensive counterparts, this study also confirmed the findings of an independent study who reported higher plasma ADMA and arginine in the same study population [24]. The intriguing finding of both an elevation in arginine and ADMA (within the limits of the normal range) was firstly attributed to the inhibition in L-arginine transport that may limit the synthesis of NO [25], and secondly, may be a compensatory mechanism aimed to counter balance the production of both a NOS substrate and inhibitor [24]. As proposed by Perticone *et al*., the phenomenon of high plasma arginine, as well as high plasma ADMA, may be due to the direct relationship linking plasma arginine and plasma ADMA [24]. This study therefore confirms a positive association between plasma arginine and plasma ADMA in white women (**Figure 4**) and suspects that this direct relationship between a NOS substrate and inhibitor could aid the explanation of this reported finding in the white women group.

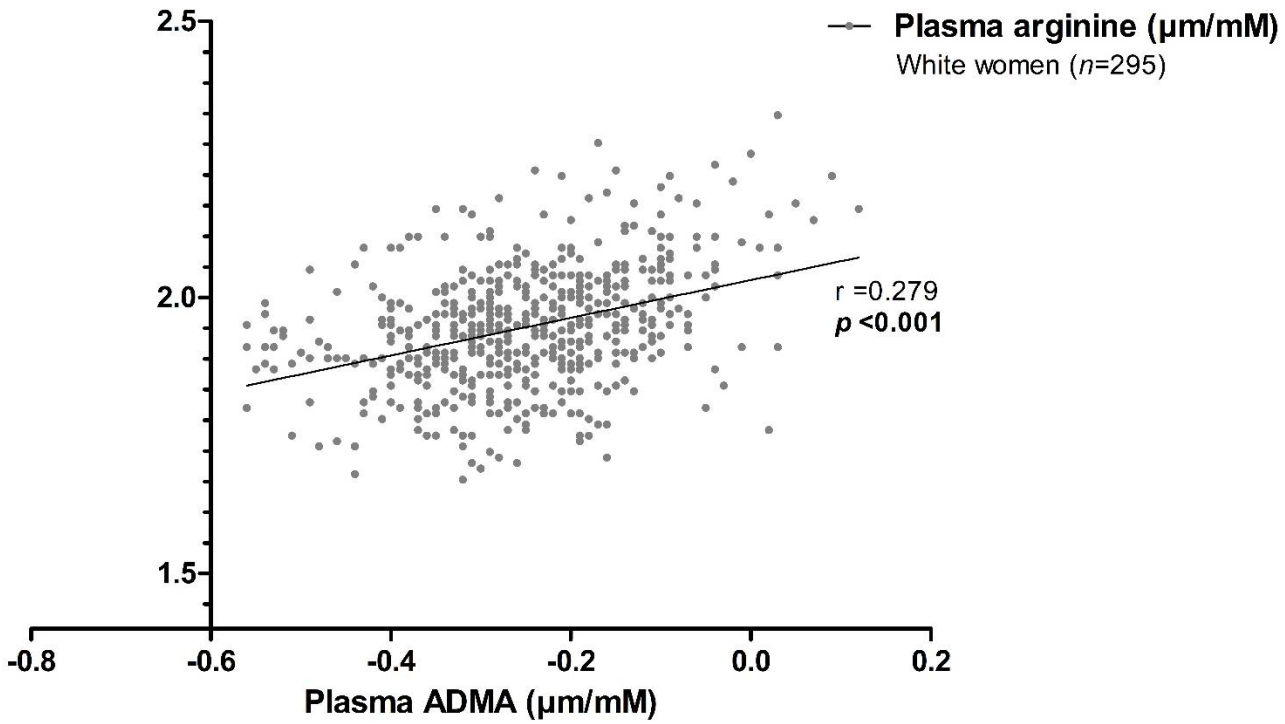


**Figure 4: Scatter plot showing a significant positive association between log plasma arginine and log plasma ADMA in white women (aged 20-30 years) participating in the African-PREDICT study.**

This study found inverse associations between cSBP and plasma homoarginine in black men and between cSBP and $U_{NOx}R$ in black women. Additionally, in white women, cSBP associated positively with urinary ADMA and PWV associated inversely with plasma ADMA.

The plasma homoarginine levels in the black men and women of the present study were comparable with their respective counterparts. Although the precise role of L-homoarginine is not evidently clear, as previously outlined in Chapter I, L-homoarginine is a homologue to L-arginine and considered a weak alternative NOS substrate [26], which may increase the NO bioavailability. The results of this study contradict previous findings of higher plasma homoarginine levels in black men and women when compared to white men and women participating in the Dallas Heart Study [27]. With regards to the cardiovascular measures of this study, black men and women presented with higher cSBP when compared to white men and women, while PWV was similar between the groups (**Figure 5**). This study therefore confirms the findings from a previous African-PREDICT study that showed cSBP to present higher in the black group as well as similar PWV measures between black and white groups [28].

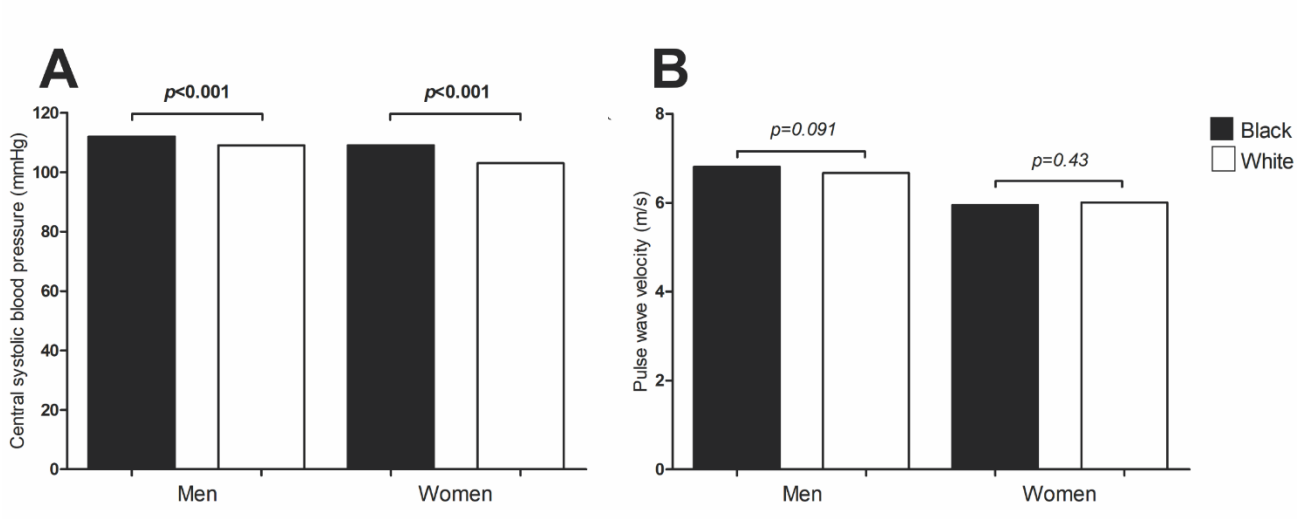


**Figure 5: Unadjusted mean values of (A) cSBP and (B) PWV in men and women (aged 20-30 years).**

Although the association between cSBP and plasma homoarginine found in the black men has not been previously reported, this study can deduce that the higher cSBP of the black men, and the independent inverse association with plasma homoarginine in the black men, may suggest that NO synthesis from L-homoarginine may be more relevant in the black population.

This study found it difficult to reflect on the association of cSBP associating positively with urinary ADMA levels in white women, as urinary ADMA was comparable between the black and white groups. Considering that ADMA is a potent endogenous inhibitor of NOS, this study expected cSBP to associate positively with ADMA, yet the expectation was in the black group and not in the white group as was reported. Additionally, this study expected an association with plasma ADMA and not urinary ADMA as was found. A recent article outlined lower urinary ADMA being a more consistent predictor of all-cause mortality when compared to plasma ADMA as previously reported [29]. This study thus concluded that although ADMA levels were comparable between the groups, SDMA–a structural isomer of ADMA and combined measure of renal function [30]–was significantly lower in black women and higher in white women. This study confirms the findings of comparable ADMA levels in black and white children and adults and lower SDMA levels in black adults as presented by Bollenbach *et al*. [31]. Thus, due to the fact that the white women of the present study presented with a more favourable NO profile as indicated by lower NO synthesis inhibitors when compared to their black counterparts, in combination with lower cSBP, this may possibly explain the positive association which was reported.

The final finding of this study is the inverse association between PWV and plasma ADMA in white women. Studies surrounding the association between ADMA and macrovascular damage are scarce and limited. Several reported studies have failed to report an association between PWV and ADMA altogether. However, to the best of the knowledge of the researchers involved in this study, only one reported study has shown an inverse association between PWV and plasma ADMA in young healthy individuals [32]. This study therefore confirms the findings of the Cardiovascular Risk in Young Finns study who reported that in a physiological setting, ADMA may have an alternative role in healthy individuals [32]. Asymmetric dimethylarginine, when elevated, is said to actively regulate vascular tone, thus producing adverse cardiovascular effects. According to the findings of Päivä *et al*. and the Cardiovascular Risk in Young Finns study, high levels of ADMA result in an increase in vascular tone. However, in individuals within a normal range, ADMA's cellular level may be altered to maintain the appropriate vascular resistance [32]. Therefore, as the white women of this study presented apparently healthy with ADMA levels within normal range, this could aid in explaining the inverse association which was reported.

### *3.3 Urinary albumin-to-creatinine ratio is inversely related to nitric oxide synthesis in young black adults: The African-PREDICT study.*

In the final manuscript comprising of black and white young healthy adults (aged 20-30 years), this study observed an overall less favourable NO profile in the black group with elevated levels of plasma CK and oxidative stress, when compared to the white group.

Firstly, the findings of this study are in line with multiple studies indicating NO synthesis may be reduced in black individuals [1, 2, 17, 21]. Secondly, this study further confirms that NO synthesis via the alternative renal pathway of nitrite reabsorption may be lowered in black individuals ultimately impairing NO bioavailability [1, 17]. Since the black group of this present study presented with lower urinary nitrate, lower $U_{NOx}R$, higher plasma and urinary ADMA and arginine, this may be due to genetic differences in the enzymatic processes involved in the classical L-arginine-NO pathway including NOS, arginine: glycine amidinotransferase (AGAT), protein-arginine methyltransferase (PRMT) and dimethylarginine dimethylaminohydrolase (DDAH), as well as in the renal carbonic anhydrases and anion transporters involved in nitrite excretion and reabsorption as previously reported [1, 5]. Although genetic determinations were outside the scope of this thesis.

The black group of this study also presented with elevated levels of kidney-associated oxidative stress (MDA) and plasma CK levels, when compared to the white group. This study

confirms the findings of higher MDA levels in black individuals, when compared to white individuals [17], which further highlights the ethnic differences between the black and white groups. It is also a common reported finding of black individuals presenting with higher levels of CK, and this study therefore confirms the findings of several studies reporting higher CK levels in black populations [33-35].

Additionally, this study found an inverse association between plasma CK levels and urinary homoarginine in the black group only. Although this association has not been reported elsewhere, it was indeed anticipated in the field of renal physiology. Arginine: glycine amidinotransferase is the enzyme responsible for the synthesis of homoarginine and guanidino acetic acid (GAA) as detailed in Chapter I. In short, GAA is converted to creatine, which is the substrate for CK, and a well-known inhibitor of AGAT-catalysed synthesis of homoarginine and GAA [36, 37]. Therefore, this inverse association of plasma CK with urinary homoarginine in the black group of this study suggests a considerable influence of kidney function to this association and may further indicate a mutual interaction between CK and AGAT in the mitochondria and cytoplasm of kidney cells.

This study further reported an inverse association with uACR and both plasma and urinary homoarginine and between uACR and $U_{NOx}R$ in the black group. The inverse associations of NO-related markers with uACR in the black group, along with the lower $U_{NOx}R$ levels indicate a relatively higher loss of circulating nitrite, an important NO reservoir, possibly due to an attenuated carbonic anhydrase activity. Additionally, although the eGFR values in both the groups were well within normal range, further reiterating a healthy population group, the black group presented with significantly higher estimated glomerular filtration rate (eGFR) compared to the white group of the cohort. This study confirms the findings of higher eGFR in black versus white populations [28], which may again suggest a considerable contribution of the kidney to these ethnic differences observed in this study.

## 4. Strengths, limitations, chance and confounding

There are some factors that may have confounded the findings of this study which need to be critically reflected upon.

Both the ASOS study and the larger African-PREDICT study were well planned and executed under strict conditions. Both studies recruited participants from the North West province of South Africa; however, these results cannot be representative of the entire South African population. The inclusion criteria of both the ASOS and African-PREDICT studies included

participants who were apparently healthy; however, underlying infections or diseases were not certain. While the impact of diet on NO-related associations was not included in the scope of this study, dietary data of the participants of the African-PREDICT study were collected and allows for further research on the dietary components of the NO profile in black and white populations.

Due to the cross-sectional design of this study in combination with the use of data from both studies, causality cannot be inferred from the results obtained in these populations. Even though this study reported significant results as outlined in Chapters III, IV and V, it cannot exclude any unknown interactions that may have played a role in these findings. The correlations in this study should therefore be confirmed in future studies.

The potential covariates used as adjustments in each multivariate regression analysis were outlined in each manuscript chapter. These potential confounding factors could have under- or overestimated the associations between NO-related markers and cardiovascular structure and function. Regardless, this study provides some of the first findings on a comparison between both a plasma and urinary NO profile in black and white healthy South Africans. Moreover, as discussed in Chapter II, the sample size of our cohorts (ASOS *n*=80; African-PREDICT *n*=1110) proved to be statistically significant for testing our intended hypotheses.

## 5. Conclusion

This study was the first to indicate independent relationships between NO-related markers measured in plasma and urine with arterial structure and function in a healthy biethnic cohort comprising of both children and young adults.

The results of this study persistently found that the black cohort (children and adults) presented with a less favourable NO profile when compared to their age-matched white counterparts. This potentially lower NO synthesis and bioavailability found in the black cohort may contribute to the early onset of hypertension. Additionally, the associations reported between NO-related markers with BP (DBP in black boys; SBP in black men and cSBP in black men and women), a marker of cardiovascular structure (cIMT in black men), and with a marker of endothelial function (uACR in black adults) further supports the susceptibility of black South Africans to the risk of early onset of endothelial dysfunction, arterial stiffness and potential hypertension related complications in later life.

## 6. Recommendations

Vascular endothelial function remains an understudied topic in South Africa. The following are recommendations for future studies that aspire to tackle the limited knowledge surrounding NO.

- Participants of the ASOS and African-PREDICT studies were recruited solely from the North West province of South Africa and therefore larger study populations are required to confirm the relevance of the results in the entire South Africa or the Sub-Saharan African setting. Additionally, multi-country and multi-centred studies are also required to understand the development of CVD in a broader population.
- Although the dietary components of the African-PREDICT participants were collected, the dietary aspects of NO were neither a focus nor included under the ethical approval of this study. The participants did not abstain from a nitrate-rich diet. It is indeed clear that dietary nitrate contributes to some degree of urinary nitrates. Therefore, forthcoming studies on the potential involvement of the L-arginine-NO and related pathways should consider a nitrate-poor dietary protocol compared to a control group.
- Intervention studies are needed to confirm causality in cardiovascular deterioration by evaluating the changes in the NO profile of South African populations in response to dietary administration of L-arginine and nitrates.
- Future studies exploring markers of cardiovascular risk and pathophysiological traits should include genetic methods (mendelian randomisation or casual inference) as an alternative way to show causality.
- Additional methods to assess endothelial function, such as the use of flow-mediated dilation, should be used to confirm the reported findings and explore its correlation to urinary and plasma biomarkers of NO bioavailability.

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# Appendices

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**Health Research Ethics Committee (HREC)**

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22 August 2017

Prof AE Schutte
Physiology-HART

Dear Schutte

## APPROVAL OF YOUR AMENDMENT REQUEST APPLICATION BY THE HEALTH RESEARCH ETHICS COMMITTEE (HREC) OF THE FACULTY OF HEALTH SCIENCES

**Ethics number: NWU-00001-12-A1**

Kindly use the ethics reference number provided above in all correspondence or documents submitted to the Health Research Ethics Committee (HREC) secretariat.

**Study title: The African-PREDICT study**

**Study leader/supervisor: Prof AE Schutte**

The Health Research Ethics Committee (HREC) has reviewed your amendment request to the single study entitled, "The African Prospective Study on the Early Detection and Identification of Cardiovascular Disease and Hypertension (The African-PREDICT Study)," via the expedited process. The request was to make changes to the exclusion criteria of the study, the addition of a video explaining procedures to be undertaken during the process of obtaining consent, addition of an additional feedback procedure regarding baseline analyses at follow-up data collection, change to the remuneration amount, and changes to the questionnaires to be completed during the follow-up phases. The HREC approves this amendment request.

Please note that this approval is only for the amendment request and does not impact on the approval status of the overall project.

Please inform us immediately if there are any other amendments required to your study. If there are any queries, please let us know at your earliest convenience.

Yours sincerely

Prof Wayne Towers
HREC Chairperson

Prof Minrie Greeff
Ethics Office Head

Current details: (13210572) C:\Users\13210572\Documents\HREC\HREC - Applications\2017 Applications\Applications 07-17 August 2017\NWU-00001-12-A1(AE Schutte)\NWU-00001-12-A1(AE Schutte)-AL\NWU-00001-12-A1(AE Schutte)-AL.docm
14 August 2017

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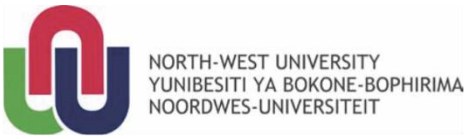


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**Ethics Committee**
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**ETHICS APPROVAL OF PROJECT**

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

**Project title:** THE ARTERIAL STIFFNESS IN OFFSPRING STUDY (ASOS)

**Project Leader:** Dr R Kruger

**Ethics number:** NWU-00007-15-A1
(Institution – Project Number – Year – Status)

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

**Approval date**: 2015-03-16 **Expiry date**: 2018-10-31

Special conditions of the approval (if any):

- Any changes to the approved application must be submitted to the Health Research Ethics Committee for approval before implementation.

General conditions:

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

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

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

Yours sincerely

Linda du Plessis

Digitally signed by Linda du Plessis
DN: cn=Linda du Plessis, o=NWU, Vaal Triangle Campus, ou=Vice-Rector: Academic, email=linda.duplessis@nwu.ac.za, c=US
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**ETHICS APPROVAL LETTER OF STUDY**

Based on approval by the North West University Health Research Ethics Committee (NWU-HREC) on 25/06/2018, the NWU Health Research Ethics Committee hereby approves your study as indicated below. This implies that the North-West University Research Ethics Regulatory Committee (NWU-RERC) grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

**Study title: Nitric oxide bioavailability and cardiovascular function in children and young adults**
**Study Leader/Supervisor (Principal Investigator)/Researcher: Prof R Kruger**
**Student: A Craig**

**Ethics number**:

| N | W | U | - | 0 | 0 | 0 | 5 | 1 | - | 1 | 8 | - | A | 1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Institution | | | | Study Number | | | | | | Year | | | Status | |

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

**Application Type: Single study**
**Commencement date: 2018/06/25**
**Expiry date: 2019/06/30**

**Risk:** **Adult: Minimal**
**Children: Not greater than minimal risk**

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

**Special in process conditions of the research for approval (if applicable): None**

**General conditions:**
*While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:*

- *The study leader/supervisor (principle investigator)/researcher must report in the prescribed format to the NWU-HREC:*
  - *annually (or as otherwise requested) on the monitoring of the study, whereby a letter of continuation will be provided, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the study*

*leader/researcher must apply for approval of these amendments at the NWU-HREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*

- *Annually a number of studies may be randomly selected for an external audit.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility the NWU-RERC and NWU-HREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-HREC or that information has been false or misrepresented;*
    - *submission of the annual (or otherwise stipulated) monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and / or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-HREC can be contacted for further information or any report templates via Ethics-HRECApply@nwu.ac.za or 018 299 1206.*

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

Yours sincerely

Prof Wayne Towers
Chair NWU Health Research Ethics Committee

Current details: (22351930) M:\DSS1\8533\Monitoring and Reporting Cluster\Ethics\Certificates\Templates\Research Ethics Approval Letters\9.1.5.4.2 HREC Ethical Approval Letter.docm
3 December 2018

File reference: 9.1.5.4.2

JOINT MEETING
**ESH-ISH 2020**
**Glasgow**

European Society of Hypertension | International Society of Hypertension | BIHS

**May 29 - June 1, 2020**
**Scottish Exhibition Campus**
Glasgow, United Kingdom
www.hypertension2020.org

Rome, February 6, 2020

Dear Dr. ASHLEIGH,

On behalf of the Scientific Programme Committee, we are delighted to inform you that your abstract **"CENTRAL SYSTOLIC BLOOD PRESSURE RELATES INVERSELY TO NITRIC OXIDE SYNTHESIS IN YOUNG BLACK ADULTS: THE AFRICAN-PREDICT STUDY"** (ID 1541) has been accepted for a **POSTER PRESENTATION** at the Joint Meeting of the European Society of Hypertension (ESH) and the International Society of Hypertension (ISH) in Glasgow, UK, from May 29, 2020 to June 1, 2020.
Posters may be upgraded to Best Poster (E-Poster) or Oral presentation for ISH New Investigator Presentation session or ISH NIC Austin Doyle Award Session, based on the final score received.
**Further information regarding the session, date and time will follow shortly.**

We would be most grateful if you would confirm your participation by e-mailing the ESH-ISH Scientific Secretariat (Mrs Marta Vittori - hypertension2020.abs@aimgroup.eu) **by February 10, 2020** and pay the relevant registration fee via the webpage https://www.hypertension2020.org/registration/.

*Please provide the following information upon confirmation of your accepted abstract:*
*1) years post highest degree obtained*
*2) your membership status of the ISH/ESH/BIHS, i.e. Research Fellow or Emerging Leader or Professional member"*

**Please note that AIM Italy will submit to AIFA (Italian Ministry of Health) a Pre-request of authorisation, including the list of all presenting authors and ESH-ISH 2020 scientific programme, by March 15, 2020.**
**Please kindly note that, from March 15, 2020, confirmed posters cannot be withdrawn, since the scientific programme is final and cannot be further modified.**

**PUBLICATION OF ABSTRACTS**
The abstract book will be available online before the congress, by the end of May 2020.
**Registration is compulsory in order to publish the abstract. Therefore, any presentation without a paid registration will be removed from the abstract book.**
**Please note:**

- Registration is compulsory in order to present an abstract at the meeting.
- Each presenting author may present up to three abstracts with one paid registration.
- A special registration fee is available for authors with one accepted abstract aged younger than 35 years (a copy of your ID card is required).

We looking forward to welcoming you to Glasgow.
Your sincerely
Marta Vittori - *ESH-ISH 2020 Scientific Secretariat*

**ORGANISING AND SCIENTIFIC SECRETARIAT**
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Via Flaminia 1068 - 00189 Rome ITALY - Ph +39.06.33053.1 - Fax +39.06.2332.5630 - hypertension2020@aimgroup.eu - www.hypertension2020.org

## APPENDIX E – CONFIDENTIALITY AGREEMENT FOR LANGUAGE EDITING

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24 July 2020

To Whom It May Concern

**CONFIDENTIALITY AGREEMENT**

I, Mrs L.K. Gouws, herewith agree to keep all information regarding the PhD research study of Ms. A. Craig entitled "**Nitric oxide bioavailability and cardiovascular function in children and young adults**" in utmost confidence, and undertake not to disclose any information or have discussions with any person other than the researcher or promoter of the research, Professor R. Kruger, about the content or results thereof.

I further undertake not to divulge any information to any person or institution for the next five (5) years, regarding the particulars or details thereof, unless consent is given to me in writing by the abovementioned researcher or promoter.

Signed at PORT ELIZABETH on this 24TH day of JULY 20.20

L.K. Gouws.

Signature of L.K. Gouws

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## CONFIRMATION OF PROOFREADING

**Candidate:**
A. Craig

**Research study for the Doctor of Philosophy in Science with Physiology thesis entitled:**

NITRIC OXIDE BIOAVAILABILITY AND CARDIOVASCULAR FUNCTION IN CHILDREN AND YOUNG ADULTS

This serves to confirm that I, Lisa Kirsten Gouws,
have provided proofreading services to the candidate,
ASHLEIGH CRAIG,
in preparation for the submission of the aforementioned research thesis.

Yours faithfully,

LK Gouws

Lisa K. Gouws (Mrs)

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Chapter 1

Introduction

Cardiovascular disease (CVD) is one of four leading non-communicable diseases (NCDs) reported globally and is the primary cause of mortality and morbidity [1]. The burden of NCDs in Sub-Saharan Africa is increasing [2, 3] and will account for a projected mortality rate of 46% in the Sub-Saharan region by the year 2020 [4]. The increased incidence of NCD is linked to rapid urbanisation, which is accompanied by a change in lifestyle [3, 5]. Lifestyle risk factors that are associated with disease development include obesity, physical inactivity, obesity, tobacco and tobacco use [6, 7].

The prevalence of CVD in developing countries, such as South Africa, is twice as high in comparison to developed countries [8]. This is seen together with a high prevalence of hypertension amongst children and adults [9, 10], and a relatively younger age of CVD-related deaths [3]. Therefore, the identification of early predictors for the development of cardiovascular compromise in both children and young adults is warranted.

Nitric oxide (NO) plays a pivitol regulatory role in maintaining vascular homeostasis [11]. A decrease in the synthesis or bioavailability of NO is firstly, associated with endothelial dysfunction and secondly, implicated in several adverse diseases including hypertension and atherosclerosis. The disruption in vasoactive substances such as NO leads to endothelial dysfunction, which in turn, leads to structural and functional changes of the vasculature [12]. Therefore, a healthy endothelium is vital in cardiovascular protection and healthy ageing.

Nitric oxide synthesis is regulated via the availability of particular substrates (L-arginine, L-homoarginine), metabolites (L-ornithine/L-citrulline, nitrates and nitrites) and the influence of NO synthase (NOS) inhibitors (asymmetric (ADMA) and symmetric dimethylarginine (SDMA). However, the impact of NO-related markers on CVD in the context of the South African population is limited and controversial. Therefore, we aimed to investigate associations of NO-related markers with markers of cardiovascular structure and function in black and white South African children and young adults.

The endothelium

More than a century ago, physician Rudolf Virchow, once considered the "Pope of Medicine" spotted a cellular layer within a capillary vessel and referred to it as a simple membrane with flattened nuclei [13]. Half a century later, Swiss anatomist Wilhelm His invented the word endothelium [13] and later, the endothelium was redefined as the inner cellular lining of blood vessels [13, 14].



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# SOLEMN DECLARATION AND PERMISSION TO SUBMIT

## 1. Solemn declaration by student

I, **Miss Ashleigh Craig**

declare herewith that the thesis/dissertation/mini-dissertation/article entitled (**exactly as registered/approved title**),

Nitric oxide bioavailability and cardiovascular function in children and young adults

which I herewith submit to the North-West University is in compliance/partial compliance with the requirements set for the degree:

Doctor of Philosophy in Sciences (Physiology)

is my own work, has been text-edited in accordance with the requirements and has not already been submitted to any other university.

**LATE SUBMISSION: If a thesis/dissertation/mini-dissertation/article of a student is submitted after the deadline for submission, the period available for examination is limited. No guarantee can therefore be given that (should the examiner reports be positive) the degree will be conferred at the next applicable graduation ceremony. It may also imply that the student would have to re-register for the following academic year.**

Signature of Student: Ashleigh Craig — Digitally signed by Ashleigh Craig Date: 2020.06.30 18:22:28 +02'00'

University Number: 27751023

Signed on this 15 day of July of 20 20

## 2. Permission to submit and solemn declaration by supervisor/promoter

The undersigned declares that the thesis/dissertation/mini-dissertation complies with the specifications set out by the NWU and that:

- the student is hereby granted permission to submit his/her mini-dissertation/ dissertation/thesis:
  (●) Yes ( ) No
- that the student's work has been checked by me for plagiarism (by making use of TurnItIn software for example) and a satisfactory report has been obtained:
  (●) Yes ( ) No

Signature of Supervisor/Promoter: Prof Ruan Kruger — Digitally signed by Prof Ruan Kruger Date: 2020.07.30 14:09:02 +02'00'

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