Marinobufagenin and its relationship with systolic blood pressure in a young black and white population: The African-PREDICT study

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Preface

This study, "*Marinobufagenin and its relationship with systolic blood pressure in a young black and white population: The African-PREDICT study"*, forms part of the dissertation in fulfillment of the requirements for the degree *Magister Scientiae* in Physiology at the Potchefstroom Campus of the North-West University. This dissertation consists of 5 chapters presented in an article format as advised and approved by the North-West University.

The chapter outlay of this dissertation is as follows:

Chapter 1: Background and Motivation for the study.

Chapter 2: Literature study, Aim, Objectives and Hypotheses.

Chapter 3: Study Protocol and Methodology.

Chapter 4: The Manuscript for Publication.

Chapter 5: Final Remarks and Recommendations for Future Studies.

The manuscript is prepared for submission to the peer-reviewed journal *Hypertension,* which requires United States English. The referencing style of Chapters 1, 2, 3 and 5 are also prepared according to the author instructions of *Hypertension.*

* To improve legibility for examination purposes I deviated from the author instructions of the *Hypertension* journal on: the justification of paragraphs throughout the dissertation; order of assembly of manuscript; format and text size of tables and the insertion of tables and figures in between the text of the results section of the manuscript.

Author contributions

Ms. M Strauss

Responsible for conducting the initial literature search, writing of the research proposal, completing the ethics application for this study, writing the literature study, performing and interpreting statistical analyses together with the writing of the manuscript. The African-PREDICT study is a multi-disciplinary research study where the candidate also contributed to the success of various methodological aspects including: the initial screening for eligibility of participants prior to enrolment in the study; electrocardiography measurements; the fitting of ambulatory blood pressure apparatuses for the measurement of 24hr blood pressures; the fitting of ActiHeart monitors; and lastly the entry of data into the African-PREDICT database.

Dr. W Smith

Study co-supervisor. Dr. Smith co-supervised and provided expertise in the writing of the proposal, ethics application, literature study and manuscript. Dr. Smith contributed to the interpretation of the data. Dr. Smith provided guidance and support with regards to the statistical analyses and the initial planning and design of the manuscript. Dr. Smith also contributed to the intellectual input of the manuscript.

Prof. AE Schutte

Study supervisor and principal investigator of the African-PREDICT study. Prof. Schutte worked with Dr. Fedorova in the initial conception of this sub-study with regards to MBG. Prof. Schutte supervised the writing of the initial proposal, ethics application, literature study and manuscript. Prof. Schutte contributed to the intellectual input of this dissertation. Prof. Schutte contributed to the collecting and

interpretation of the data. Prof. Schutte furthermore provided guidance with regards to the statistical analysis; initial planning and design of the manuscript.

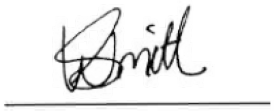
Dr. Olga Fedorova, Dr. Wen Wei and **Dr. Alexei Bagrov**

Dr. Fedorova participated in the initiation of this sub-study. Dr. Fedorova along with Prof. Schutte discussed the possible mechanistic role of the novel endogenous cardiotonic steroid, MBG, in the development of salt-sensitive hypertension and cardiovascular disease (CVD) in black populations, when compared to their white counterparts. Drs. Bagrov and Fedorova developed a competitive immunoassay, based on a monoclonal anti-MBG antibody, also developed in their laboratory. Drs. Wen and Fedorova measured MBG in the biological samples from African-PREDICT study. Dr. Fedorova participated in the database analysis. Drs. Fedorova and Bagrov contributed to the interpretation of data and provided intellectual input with regards to MBG, in the manuscript presented as Chapter 4.

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

Hereby, I declare that I approved the aforementioned manuscripts and that my role in this study, as stated above, is representative of my actual contribution.

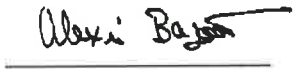
Prof. AE Schutte



Dr. W Smith

Dr. OV Fedorova

Dr. W Wei



Dr. AY Bagrov

Summary

Marinobufagenin and its relationship with systolic blood pressure in a young black and white population: The African-PREDICT study

Motivation

Hypertension remains one of the foremost causes of cardiovascular morbidity and mortality in sub-Saharan Africa. Importantly, the prevalence of hypertension within black and white populations has been ascribed to distinct pathophysiological mechanisms. Numerous studies have shown that black individuals are predisposed to hypertension in part due to their genetic susceptibility to be salt-sensitive. Hence, the scope of research investigating possible underlying mechanisms of salt-sensitivity remains a subject of growing interest.

There is emerging evidence indicating an association between salt-sensitivity and the novel biomarker, MBG. This endogenous sodium-pump ligand's role in blood pressure regulation is attributed to its ability to inhibit both renal as well as cardiovascular α1-Na^+/K^+-ATPase subunits. Studies have demonstrated a vasoconstrictive response to MBG in Dahl salt-sensitive rats — with attenuated pressure-natriuresis — as opposed to the expected homeostatic natriuretic response. Accordingly, black populations portray a similar salt-sensitive phenotype with an impaired pressure-natriuresis profile. Thus, we calculated the 24hr urinary MBG/Na^+ excretion ratio as a proposed estimate of Na^+ excretion resistance to higher levels of urinary MBG.

A better understanding with regards to both ethnic and sex differences in MBG/Na^+ and its association with measures of cardiovascular function, could provide new insight into the possible role of MBG in the salt-sensitive hypertension phenotype.

Aim

The aim of this study was to compare the MBG and 24hr urinary sodium profiles between black and white, men and women. Furthermore, we aimed to investigate the association of the MBG/Na^+ excretion ratio with systolic blood pressure (SBP) and hemodynamic parameters in this young bi-ethnic population.

Methods

This cross-sectional study is affiliated with the African Prospective study on the Early Detection and Identification of Cardiovascular Disease and Hypertension (African-PREDICT), and was reviewed and approved by the Health Research Ethics Committee (HREC) of the North-West University (NWU-00022-16-A1).

The overarching aim of the African-PREDICT study partly entails the early identification of novel biomarkers involved in the development of CVD especially in young black South Africans. We investigated the data of the first consecutive 331 participants (42.9% black, 43.8% men) with complete 24hr urinary data.

We obtained basic anthropometric measurements including height, weight and waist circumference, after which the body mass index (kg/m^2) as well as the waist:height ratio were calculated. Cardiovascular measurements included central systolic blood pressure (cSBP), 24hr SBP and beat-to-beat hemodynamic measurements including heart rate, stroke volume and total peripheral resistance (TPR). Participants were asked to collect 24hr urine samples in which the 24hr urinary sodium, potassium and

MBG concentrations were determined. Furthermore, we used blood samples to determine the high density lipoprotein cholesterol (HDL-C), total cholesterol, and γ-glutamyltransferase (GGT), glycated haemoglobin (HbA1c) and aldosterone levels.

After performing interaction testing participants were stratified by sex and ethnicity. Accordingly we used T-tests and Chi-square tests to compare means and proportions between groups. Subsequent single, partial and multiple regression analyses were performed to explore the relationship between MBG and the MBG/Na^+ excretion ratio with SBP and other hemodynamic variables. *P*-values ≤0.05 were considered significant.

Results

Interaction testing performed in the entire cohort, indicated an interaction of sex on the relationship between cSBP and MBG/Na^+ excretion ratio (p=0.027), while there was an interaction of ethnicity on the associations between cSBP and 24hr SBP with MBG/Na^+ in women (p=0.010 and p=0.012). Black men and women displayed a higher cSBP and TPR with a lower stroke volume compared to whites, whereas white men had higher 24hr SBP measures. We observed no apparent ethnic differences in either MBG excretion or MBG/Na^+ in men or women, although men had a significantly higher salt intake of 8.58 g/day and MBG excretion when compared to women.

Black women portrayed a significant positive trend in cSBP (p=0.003) as well as nighttime systolic ABPM (p=0.013) across increasing MBG/Na^+ quartiles. Furthermore, in black women only single and multiple regression analyses indicated a positive association of central SBP (R^2=0.26; *ß*=0.28; p=0.039), 24hr SBP (R^2=0.46; *ß*=0.30; p=0.011) and stroke volume (R^2=0.26; *ß*=0.29; p=0.036) with

MBG/Na^+, whereas a negative association was found between MBG/Na^+ and TPR (R^2=0.21; *ß*=−0.33; *p*=0.018). Conversely, in white women a negative association existed between MBG/Na^+ and nighttime SBP (*r*=−0.20; *p*=0.038), which became non-significant after adjusting for multiple covariates (R^2=0.36; *ß*=−0.13; *p*=0.12). There were no significant trends or associations in young black and white men with regards to the MBG/Na^+ excretion ratio.

Conclusion

Compared with white women, black women might be more vulnerable to early cardiovascular risk brought on by an apparent resistance to sodium excretion, based on MBG/Na^+ and its association with an increase in cSBP, 24hr SBP and stroke volume. Yet, clear contrasting associations in young white women supports the normal physiological natriuretic effect of MBG. Our results suggest that the inter-regulation of MBG and Na^+ may partially contribute to the prevalence of a salt-sensitive hypertension phenotype. The absence of any associations with the MBG/Na^+ excretion ratio in men requires further investigation.

Key words: Black women, Hemodynamics, Marinobufagenin, Salt, Systolic blood pressure, Young

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CHAPTER 5

Figure 1: Comparison of 24hr Urinary MBG excretion of participants from the African-PREDICT study (Total mean age 25 ± 3.15) and other human studies. (a) Anderson *et al*.—apparently healthy population of white women (n=28) (Total mean age 53 ± 1.6 years). (b) Jablonski *et al.*—8 men and 3 women with a resting SBP between 130-159 mmHg (Total mean age 60 ± 2 years). (c) Fedorova *et al.*—apparently healthy older population of white men (n=20) and women (n=19) (Total mean age 53 ± 11).

Figure 2: Multiple regression analyses in women with MBG/Na^+ as the main independent variable.

Figure 3: Hormonal control of the α1-Na^+/K^+-ATPase pump.

List of abbreviations

ABPM	Ambulatory blood pressure monitoring
ACE	Angiotensin converting enzyme
ATP	Adenosine triphosphate
BHS	British Hypertension Society
BMI	Body mass index
CRIBSA	The Cardiovascular Risk in Black South Africans study
CVD	Cardiovascular disease
cSBP	Central systolic blood pressure
DASH	Dietary Approaches to Stop Hypertension
DBP	Diastolic blood pressure
DELFIA	Dissociation-Enhanced Lanthanide Fluorescent Immunoassay
ECG	Electrocardiogram
HDL-C	High density lipoprotein cholesterol
HREC	Health Research Ethics Committee
IC50	Half maximal inhibitory concentration
ISAK	International Society for the Advancement of Kinanthropometry
LDL-C	Low density lipoprotein cholesterol
MBG	Marinobufagenin

Na^+/K^+-ATPase	Sodium/Potassium-ATPase
NCD	Non-communicable diseases
NUTRICODE	Global Burden of Diseases Nutrition and Chronic Diseases Expert Group
PURE	Prospective Urban Rural Epidemiology
PWV	Pulse wave velocity
RAS	Renin-angiotensin-system
RAAS	Renin-angiotensin-aldosterone-system
SBP	Systolic blood pressure
TPR	Total peripheral resistance
VSMC	Vascular smooth muscle cells
WHO	World Health Organization

Chapter 1

Background and Motivation

Background

The World Health Organization's (WHO) global status report on non-communicable diseases 2014, reported an annual 1.7 million deaths globally due to cardiovascular disease (CVD) attributed to the excessive consumption of sodium.[1] Indeed, a high dietary salt intake is associated with an increased risk of hypertension and CVD.[1-3]

It is said that the hominid ancestry adapted to a low sodium environment, and only exhibited blood pressure changes after progressing into an urbanized high sodium environment.[4] Weinberger *et al*. described salt-sensitivity as a fluctuation of more than 10 mmHg in mean arterial blood pressure in response to an intervention of saline infusion or sodium and volume depletion, whereas salt-resistance was described as a < 5 mmHg fluctuation.[5]

Dietary salt habits may help account for the differences in the prevalence rates of hypertension amongst different populations.[6] Studies show that certain populations have a predisposition to hypertension because of their inherited salt-sensitive phenotype.[7] Black populations have been identified as such a population with a more common genetic predisposition for salt-sensitivity.[8] Accordingly, they exhibit significantly higher intracellular sodium levels compared to whites,[9] because of their tendency to reabsorb more sodium.[8,10,11] Bochud *et al*. indicated that blacks reabsorb a greater majority of their sodium load in the proximal tube of the nephron rather than the distal tube, when compared to whites.[8] This results in a lower fractional sodium excretion rate in blacks irrespective of salt-sensitivity.[8,12] Furthermore they showed that sodium reabsorption within certain segments of the nephron is highly heritable, in particular the proximal tubular sodium reabsorption of blacks.[8] In the aforementioned study white participants from Belgium also portrayed

a steeper decrease in proximal sodium reabsorption along with a higher fractional sodium excretion when compared to black South Africans. This may indicate the incapability of black South Africans to reduce sodium reabsorption during sodium-loading, underlying their predisposition to salt-sensitive hypertension.[8]

The traditional lifestyle of tribal South Africans, including amongst others the Khoi-San, changed to a more westernized lifestyle — with a low potassium, high sodium and calorie diet.[13] The South African Demographic and Health Survey confirmed the influence of urbanization and socio-economic status on hypertension, with rural blacks portraying a significantly lower risk for hypertension compared to urban blacks and whites.[14] They also indicated that immoderate salt intake, less exercise and increased stress in urban men were associated with hypertension.[14]

Bochud *et al*. points out that ethnic differences in blood pressure may be due to lifestyle factors or genetics, but that the genetic heritability of proximal reabsorption in blacks might play a predominant role in sodium handling.[8] Even though an association exists between heritability and salt-sensitivity, especially in black populations, the contribution of vascular, hormonal and transport mechanisms may also play a role in sodium handling and therefore hypertension.[7]

Campese *et al*. observed an elevated blood pressure response during sodium loading in black salt-sensitive participants, reflecting an increase in sodium retention together with less efficient sodium handling when compared to whites.[15] Hypertensive as well as normotensive salt-sensitive subjects have been reported to exhibit lower levels of plasma renin activity than salt-resistant subjects at baseline before any saline intervention.[5] Low renin subjects also portrayed a greater blood pressure sensitivity to saline infusion than those with normal or high renin levels.[5]

These findings are similar to studies detecting low renin salt-sensitivity in black participants during sodium loading.[10,11] Plasma renin levels are known to increase as a result of hypovolemia associated with low salt intake, stimulating the renin-angiotensin-system (RAS) in order to restore blood pressure.[16] This was not evident in salt-sensitive participants after attempting to stimulate the RAS by inducing sodium and volume depletion.[5] Plasma renin activity remained low in both hypertensive and normotensive salt-sensitive subjects compared to those who were salt-resistant.[5] Hoosen *et al*. found a low renin activity in urban Zulus compared to their rural counterparts, emphasizing environmental influences.[17] Despite these findings, Weinberger *et al*. emphasizes that renin should not be considered as the sole predictor of blood pressure reactivity to sodium, since salt-resistant individuals may exhibit low renin levels, and salt-sensitive individuals may portray normal renin.[5]

Membrane bound Na^+/K^+-ATPase enzymes are adenosine triphosphate (ATP) energy dependent ion transporters, located in an abundance of mammalian cells, exporting three sodium ions out of the cell and importing two potassium ions providing an electrochemical gradient.[18-21] Renal Na^+/K^+-ATPase is located on the basolateral membrane of tubular epithelial cells of the kidney.[19,21,22] Na^+/K^+-ATPase regulates intracellular Na^+ concentration and plays a crucial role in renal sodium reabsorption,[20,21,23] volume loading,[16] vascular tone[23,24] as well as cardiac contraction[23] through indirectly controlling free calcium concentrations, and cellular membrane potentials.[18,25]

Kawasaki *et al*. found that salt-sensitive subjects retained more sodium than salt-resistant subjects exposed to a high salt diet,[26] resulting in volume expansion.[27] Volume expansion induces the release of natriuretic substances with the capacity to inhibit Na^+/K^+-ATPase, leading to natriuresis and stimulating vascular reactivity.[28]

Endogenous ligands are structurally similar to cardiac glycosides, such as ouabain, and act as natural modulators of Na^+/K^+-ATPase in various tissues.[18] Urinary and plasma marinobufagenin (MBG) has been identified as such an endogenous digitalis receptor ligand[29] synthesized in the mammalian adrenal cortex and placenta.[30] An increased production and urinary excretion of MBG was observed in young normotensive humans, following a change from a low to a high salt diet.[31,32] This was also evident during a high salt diet in Dahl salt-sensitive rats.[33] However, Fedorova *et al*. indicated that a low or high salt intake did not result in elevated MBG in all participants, and they were subsequently identified as non-MBG responders.[32] The relationship between MBG and the salt-sensitive pressor effect seems to be more evident in men.[32] Consistent with these findings, Fedorova *at al*. demonstrated that female Dahl salt-sensitive rats exhibit a lower blood pressure, as well as lower plasma MBG and CYP27A1 mRNA expression compared to males.[30] CYP27A1 initiates the biosynthesis of MBG via the acidic bile acid pathway.[30]

Increased MBG, due to high salt intake, has two main functions: 1) It acts as a compensatory mechanism for impaired pressure natriuresis,[33] inhibiting Na^+/K^+-ATPase on the basolateral membrane of the proximal tubules, thereby blunting sodium reabsorption.[32-34] 2) However, excessive levels of MBG also inhibits vascular smooth muscle Na^+/K^+-ATPase,[32] thereby increasing intracellular sodium, and lowering calcium efflux, via the Na^+/Ca^{2+} exchanger. This increases intracellular calcium resulting in vascular smooth muscle contraction[18,35,36] and increased total vascular peripheral resistance (TPR).[36]

Gates *et al*. found a significant reduction in systolic blood pressure (SBP) and increased large artery compliance within two weeks of an eight week salt-restricted diet study.[37] They propose that the increase in large artery compliance as a result of

extracellular matrix changes is unlikely, due to the short time, and therefore rather suggest a modulation of vascular smooth muscle tone. They speculate that participants were in a relative sodium-loaded state at baseline, and that a salt restricted diet depleted the sodium level resulting in a reduction of MBG.[37] MBG has been demonstrated to also inhibit the Na^+/K^+-ATPase in human mesenteric arteries.[24] Lower levels of MBG, therefore, relieve the inhibition of Na^+/K^+-ATPase, consequently reducing vascular smooth muscle contraction and increasing arterial compliance.[37] This corresponds with findings that a high salt diet causes a significant increase in arterial stiffness as well as blood pressure.[38,39] In contrast, non-MBG responders exhibited virtually no variation in plasma-MBG when shifting from a low salt to a high salt intake, and evoked neither systolic nor diastolic salt-sensitivity.[32]

Summary

It can be hypothesized that both black and white populations in South Africa have elevated MBG levels as a result of westernized dietary habits with excessive sodium content.[40] However, blacks have a genetic predisposition to reabsorb more sodium than whites,[8,41] causing volume expansion,[27] which induces a further increase in MBG[28] synthesis from adrenal cortex cells[30] to inhibit Na^+/K^+-ATPase and increase natriuresis.[32-34] The genetic predisposition of blacks to reabsorb more sodium in the proximal tube,[8] could override the natriuretic activity of MBG.[34] Hence, increasing MBG production in response to the elevated sodium reabsorption[34] may result in excessive MBG exerting a cardiovascular response.[39,42,43] In contrast salt intake in a white population contributes to MBG production which increases sodium excretion as a compensating mechanism.[31] Concurrent natriuresis reduces extracellular volume which consequently decreases blood pressure.[27]

Motivation

Blacks have been identified as genetically predisposed to salt-sensitive hypertension due to increased proximal sodium reabsorption in the nephron compared to their white counterparts.[8] An elevated blood pressure evoked in salt-sensitive participants during sodium loading, reflects an increase in sodium retention and a less efficient sodium handling, in comparison with salt-resistant individuals.[15] The elevated pressor effect seems to be more apparent in blacks, compared to whites, due to their insufficient sodium handling.[8,15]

To the best of my knowledge, evidence exploring the role of MBG in modulating blood pressure of black populations is limited. Therefore, the purpose of this study is to investigate the ethnic differences with regard to the association between urinary MBG, 24hr sodium excretion and SBP. Additionally we will explore the association of SBP with a newly proposed MBG/Na^+ excretion ratio demonstrating the inter-regulation of MBG and sodium. This study will be the first to investigate the potential role of urinary MBG in blood pressure regulation in a healthy black population.

This study will be performed with the purpose of hypothesis generating research to help provide insight for future studies regarding the possible role of MBG in sodium handling and cardiovascular function in a young black and white population.

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Chapter 2

Literature study

Aims, Objectives and Hypotheses

Table of contents

1. Introduction

Non-communicable diseases (NCD) are well established as the leading cause of mortality worldwide, accounting for 65.5% of all deaths.[1] Approximately 17.3 million deaths globally due to NCD in 2013 were ascribed to cardiovascular diseases (CVD),[2] with a significant increase of 41.7% from 1990.[2] Kearney *et al*. highlights the rising global burden of hypertension contributing to CVD, with a projected increase of 60% in hypertension amongst adults from 2000 by 2025.[3] They furthermore indicate that the population burden of hypertension is more formidable in developing countries compared to economically developed countries due to the large number of individuals in these countries that are affected.[3] Twagirumukiza *et al*. reported that there were approximately 74.7 million hypertensive individuals living in Sub-Saharan Africa in 2008, which is projected to increase by an estimated 68% by 2025.[4] Reportedly in 2010, an estimated total of 1.65 million CVD related deaths worldwide attributed to specifically immoderate sodium consumption were recorded.[5] This is in accordance with the World Health Organization's (WHO) 2014 Global Status Report on NCD.[6] Concordantly, the incidence of hypertension and cardiovascular events such as coronary heart disease, myocardial infarction, stroke or death are projected to be significantly minimized with a modest reduction in dietary salt consumption.[7,8]

Salt has played an evolutionary role throughout the history of humanity, and has since become an established commodity[9] being regarded as the main source of dietary sodium consumption.[6] It is said that the hominid ancestry adapted to a low sodium environment, and only exhibited blood pressure changes after progressing into an urbanized environment.[10] Tribal South Africans' traditional lifestyle changed dramatically by adapting to a more western lifestyle.[11] A previous low sodium, high

potassium diet with high levels of exercise and low levels of obesity have radically changed to a high sodium and calorie diet.[11] Hence there is a notable shift in the dietary habits of populations due to the greater availability of processed foods with a high salt content consumed in a westernized diet.[6,12] The inadequate ability of the kidneys to adapt to a westernized diet could evoke hypertension[13] as a result of excessive sodium retention which instigates thirst and water retention to promote volume overload (**Figure 1**).[13,14]

In order to decrease blood pressure and reduce the risk of coronary heart disease or stroke, the WHO's guidelines recommend that daily salt intake be reduced to less than 5g/day (2g/day sodium).[8] According to the Global Burden of Diseases Nutrition and Chronic Diseases Expert Group (NUTRICODE) almost all countries consume more than the recommended daily salt intake (**Figure 2**).[15] Indeed a nutritional investigation by Charlton *et al*. in three different ethnic populations within South Africa revealed that black, white and mixed ancestry populations consume more than 6g of salt per day[16] exceeding the daily recommendations of the WHO.[8] The Cardiovascular Risk in Black South Africans (CRIBSA) Study reported an increase in the prevalence of hypertension in urban blacks in 2008/2009 compared to 1990,[17] possibly ascribed to a high sodium intake[11,18] and less efficient sodium handling.[19]

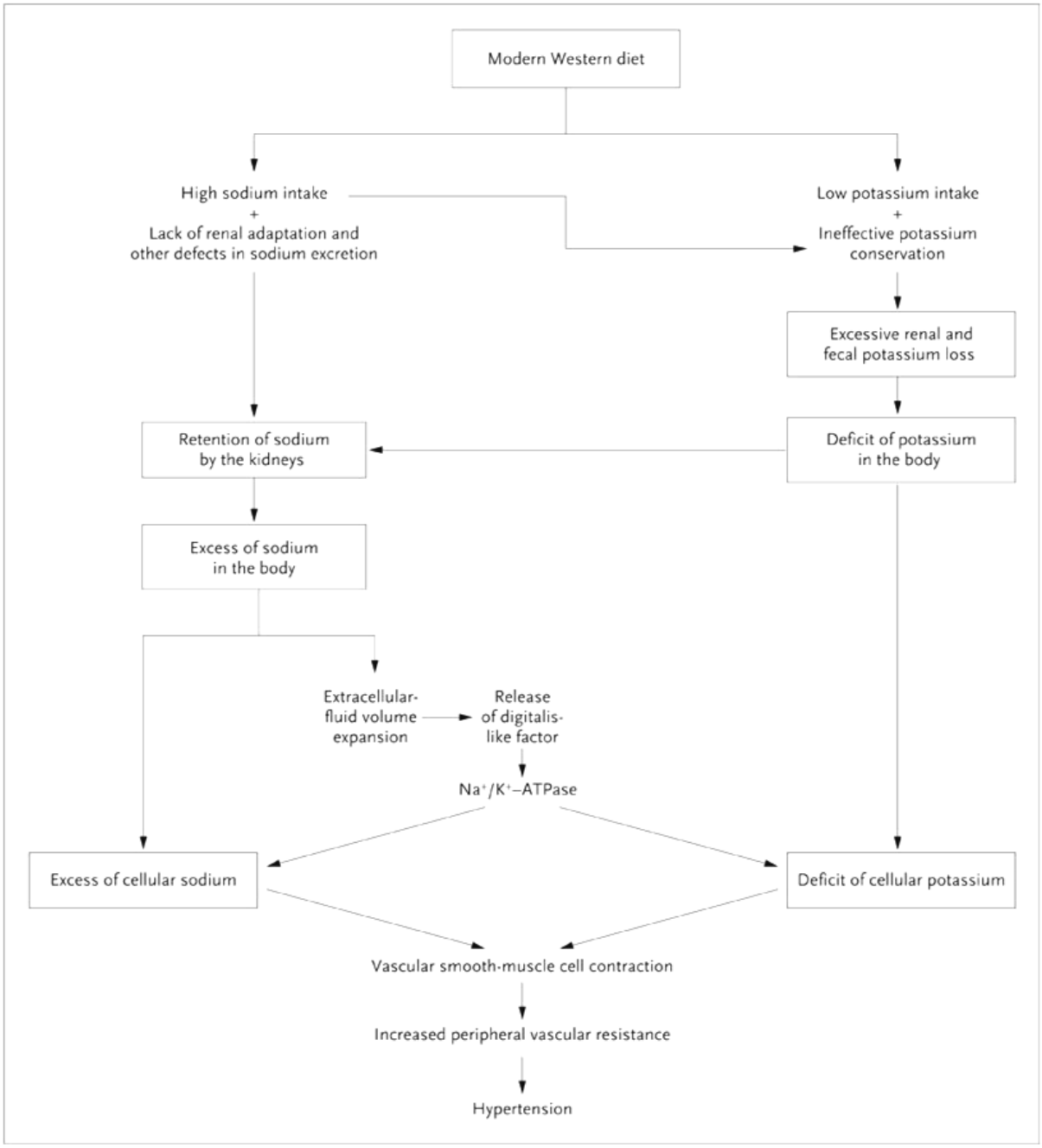


Figure 1: The role of a habitual westernized diet in the development of hypertension.[13]

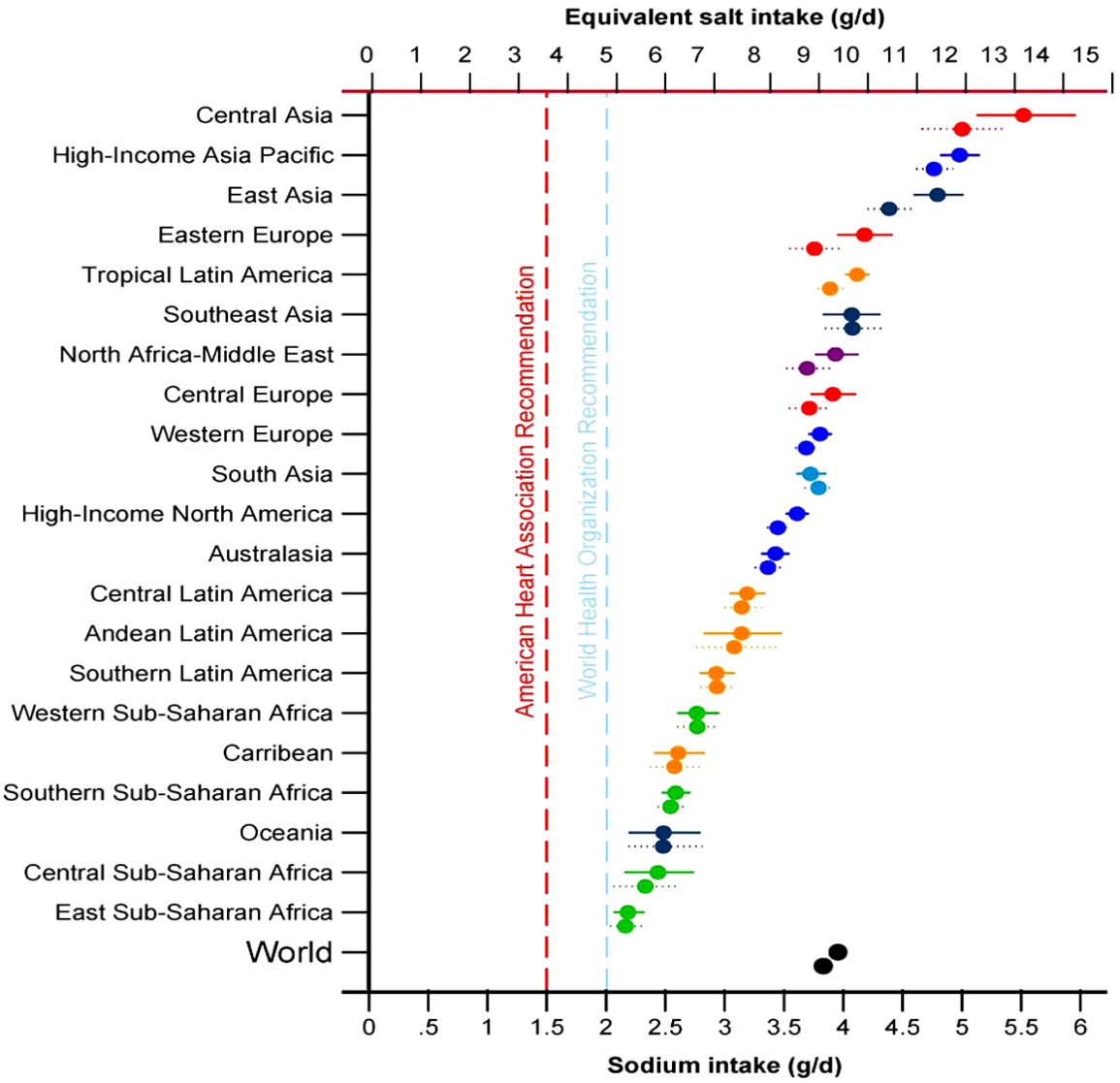


Figure 2: Sodium intake of 21 Global Burden of Disease countries in 1990 (lower symbol) and 2010 (upper symbol).[15]

Nevertheless, there is an on-going controversy regarding salt intake in the existing literature. A large sub-study of the Prospective Urban Rural Epidemiology (PURE) study assessed the relationship between urinary sodium and potassium excretion with cardiovascular events and mortality to investigate the optimal range of salt intake. Both sodium excretion levels lower than 3g/day and higher than 6g/day were associated with an increased risk of incident cardiovascular events and mortality, resulting in a J-shaped association curve. [20] Stolarz-Skrzypek *et al*. concordantly demonstrated that low sodium excretion levels were associated with a higher

prevalence of cardiovascular mortality.[21] However, a meta-analysis conducted as a part of the NUTRICODE, indicated a linear dose–response relationship between sodium intake and blood pressure.[5] Similarly large population studies also indicated a positive association between sodium excretion and blood pressure, and a negative association between potassium excretion and blood pressure.[22] It is important to note that the 24hr urinary sodium excretion from the PURE study conducted by O'Donnelle *et al.* made use of the Kawasaki formula to calculate an estimated 24hr urinary sodium excretion from an early morning spot urine sample.[20] Therefore, the omission of an actual 24hr urinary sample collection is an important limitation of the PURE study,[20] as 24hr urinary collection is regarded as the golden standard for determining sodium excretion.[23,24] Conversely, tertile 24hr urinary sodium excretion analyses conducted by Stolarz-Skrzypek *et al*. reduced the vulnerability of the study's limitations to the high intra-individual variability of urinary sodium excretion in the cohort.[21] Although the topic on sodium and cardiovascular morbidity and mortality is renowned, it is not the purpose of this literature study to comprehensively discuss this matter. Indeed, there is accumulative evidence linking high sodium intake to hypertension and an increased risk of CVD.[12,25]

2. The role of the renin-angiotensin-aldosterone system in response to changes in sodium consumption

The role of the renin-angiotensin-aldosterone system (RAAS) in volume-pressure control is well established.[14,26] The protease, renin, is released from juxtaglomerular cells in the kidney in response to three effectors including 1) volume depletion which causes renal arteriole perfusion to decrease, resulting in stimulation of afferent arteriole baroreceptors; 2) high levels of circulating catecholamines due to

sympathetic activation which stimulates β1-adrenergic receptors located in the glomerular cells; and 3) a reduction in tubular sodium or chloride ion concentrations stimulating macula densa cells.[27,28] Under normal physiological conditions sodium-restricted volume depletion induces renin release and thus results in the conversion of angiotensinogen to angiotensin I and subsequently the conversion of angiotensin I to angiotensin II via the angiotensin converting enzyme (ACE).[27,28] Elevated circulatory levels of angiotensin II exert a potent pressor effect on the vasculature by means of the angiotensin II receptor AT1.[27,28] Subsequent RAAS stimulation mediates an increased blood pressure via sympathetic activation, vasoconstriction, renal sodium reabsorption as well as adrenal aldosterone secretion, which, if stimulated chronically, could ultimately contribute to the development of hypertension.[27,28] The mineralocorticoid, aldosterone, regulates sodium and potassium concentrations.[27] The principle function of aldosterone is to facilitate the transport of Na^+ mainly by increasing luminal Na^+ channel activity.[27] Aldosterone furthermore, facilitates sodium reabsorption by upregulating the sodium-potassium ATPase pump (Na^+/K^+-ATPase pump) which causes extracellular fluid volume expansion resulting in an increase in blood pressure.[27]

As outlined in the abovementioned, there is an ambiguous relationship between salt intake and blood pressure. Conversely, the salt-dependent regulation of the RAAS might not be as apparent. Based on findings from previous studies[29,30] it can be stated that not all individuals respond as markedly to sodium loading; Weinberger *et al*. demonstrated that salt-sensitive individuals exhibit a lower plasma renin activity compared to salt-resistant individuals regardless of sodium loading or sodium and volume depletion.[31] Salt-sensitivity has been described as a fluctuation of more than

10 mmHg in mean arterial blood pressure in response to an intervention of saline infusion or sodium and volume depletion, whereas salt-resistance was described as a < 5 mmHg fluctuation.[31] It is especially noted that white populations tend to be more salt resistant whereas black populations have been identified to be more salt sensitive, in conjunction with lower levels of plasma renin activity.[32] Laragh *et al*. described two opposite sides of the hypertension spectrum as high renin hypertension and low-renin sodium-volume hypertension,[33] where vasoconstriction occurs as a result of sodium retention.[33] Since the RAAS is suppressed during sodium loading,[32] it has been deemed to be not essential in the pathogenesis of sodium induced hypertension.[34] However, although plasma renin activity is suppressed, sodium loading enhances brain RAAS activity which increases sympathetic activation, thereby essentially contributing to the development of hypertension.[34]

3. The role of the kidneys in sodium handling

The kidneys predominantly control long term blood pressure regulation by means of the pressure-natriuresis and diuresis mechanism.[14] Immoderate salt intake increases thirst and thereby water consumption, which results in volume expansion.[14] Hence sodium ions are the primary determinants of extracellular fluid volume.[26,35] The kidneys play a pivotal role in maintaining a relatively constant extracellular fluid volume and composition via excretory and metabolic functions.[35] Changes in the body's sodium concentration and subsequent blood volume alterations most likely account for the notable relationship between sodium intake and blood pressure.[36] The proximal tube is the first segment located along the nephron accounting for approximately 60%-70% of the total sodium reabsorption (**Figure 3**).[35] A further

25%-30% of sodium is reabsorbed along the ascending loop of Henle with a mere 5%-10% of sodium being reabsorbed in the distal portion of the nephron. The kidneys utilize approximately 7%-10% of the available bodily oxygen supply from the circulatory system, of which an estimated two thirds are expended on active sodium reabsorption via the energy dependent Na^+/K^+-ATPase pump.[36] Luminal sodium reabsorption via apical sodium transporters is driven by the negative intracellular electrochemical gradient brought on by the extrusion of sodium via the Na^+/K^+-ATPase pump (**Figure 3**).[36]

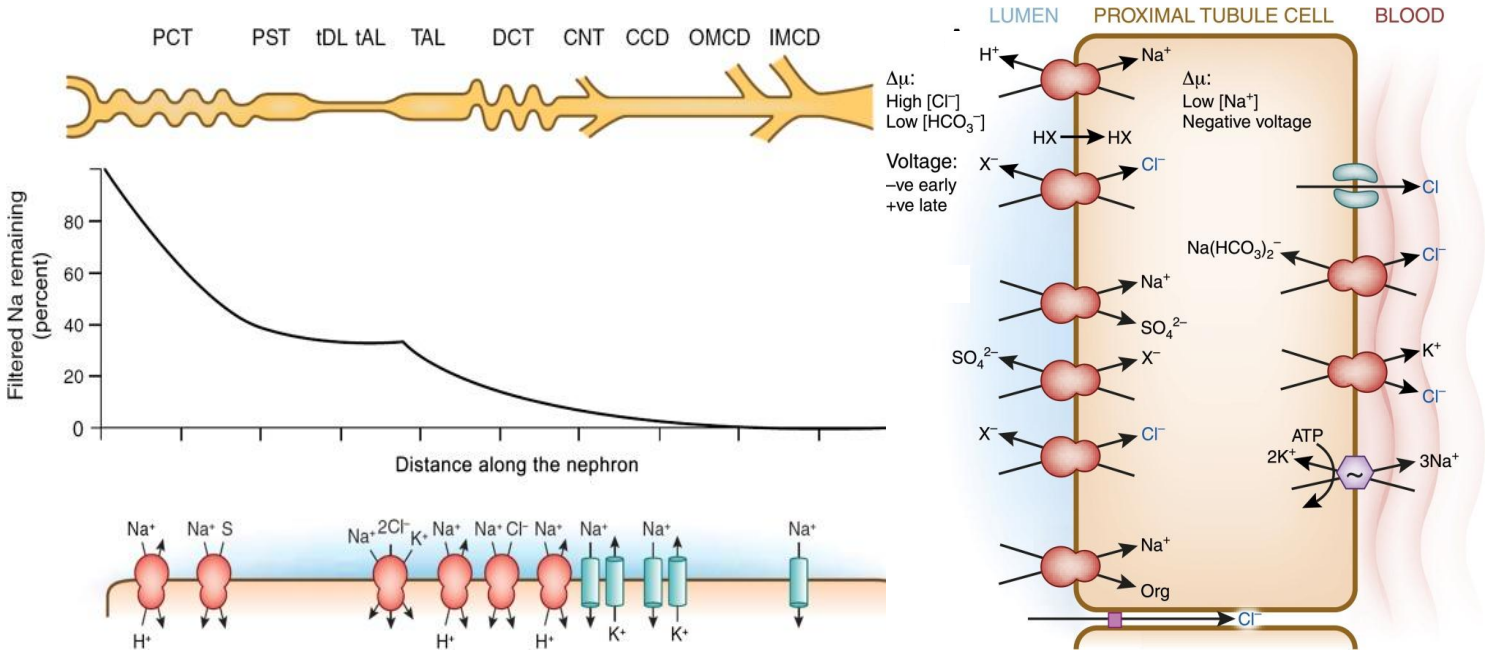


Figure 3: Sodium transport along the nephron.[35,36] PCT, proximal convoluted tubule; PST, proximal straight tubule; tDL, thin descending limb; tAL, thin ascending limb; TAL, thick ascending limb; DCT, distal convoluted tubule; CNT, connecting tubule; CCD, cortical collecting duct; OMCD, outer medullar collecting duct; IMCD, inner medullary collecting duct.

4. The Na^+/K^+-ATPase pump

In 1997 JC Skou was awarded The Nobel Prize in Chemistry *"for the first discovery of an ion-transporting enzyme, Na+, K+ -ATPase"* in the active extrusion of sodium ions from the leg nerve fiber of the *Carcinus Maenas* crab.[37,38] Na^+/K^+-ATPase is a membrane bound protein located in the majority of mammalian cells, regulating intracellular sodium concentrations.[39,40] This functional macromolecule is a transmembrane tetramer composed of alpha and beta subunits.[41] Na^+/K^+-ATPase actively transports three sodium ions across the membrane out of the cell in exchange for two potassium ions into the cell by means of hydrolysis of adenosine triphosphate (ATP).[40] This generates a negative electrochemical gradient across the membrane to help maintain intracellular osmotic and ionic homeostasis.[40] Sodium induced volume expansion[14] stimulates the production of various endogenous digitalis-like factors[42] which have been demonstrated to inhibit Na^+/K^+-ATPase pump activity.[43,44] Inhibition of the Na^+/K^+-ATPase pump by cardiac glycosides such as ouabain[41] or other ouabain-like compounds such as bufadienolides,[45] result in a transient increase and accumulation of intracellular sodium concentrations.[41] A detailed discussion regarding the preceding with respect to the implication thereof on the function of sarcolemmal Na^+/Ca^{2+}-exchangers, will follow in the subsequent section.

5. Marinobufagenin

Bufadienolides of both plant and animal origin have been discovered[45] and have long been recognized as digitalis-like factors, located in the skin and plasma of the *Bufo marinus* toad,[46,47] thought to be responsible for the long-term regulation of water and salt homeostasis in amphibians.[48] Marinobufagenin (MBG), a novel mammalian

Bufadienolide,[49,50] has been identified as an endogenous α1-Na^+/K^+-ATPase pump inhibitor[50-52] stimulated as a result of hypervolemia[42]. MBG is a bioactive steroid synthesized from cholesterol in mammalian adrenal[50] and placental cells via the extra hepatic acidic bile acid pathway.[50] The synthesis of MBG is initiated and controlled by the extra hepatic CYP27A1 enzyme.[50]

Various studies have demonstrated the role of MBG as a Na^+/K^+-ATPase inhibitor in the kidneys,[44,52,53] vasculature[43,54] and heart.[55,56] Wang *et al*. studied the regional expression of Na^+/K^+-ATPase isoforms in rat and human tissue (**Figure 4**).[57] While both α1-Na^+/K^+-ATPase and α3-Na^+/K^+-ATPase were located in brain and cardiac tissue of humans, α1-Na^+/K^+-ATPase was exclusively expressed in the kidneys.[57] Fedorova *et al*. investigated the responsiveness of the various Na^+/K^+-ATPase isoforms to ouabain and MBG. Importantly, they found in rat aorta membrane fractions, that MBG exhibited a greater affinity to the α1-Na^+/K^+-ATPase isoform in comparison with ouabain.[43] Firstly, they isolated a membrane fraction containing predominantly α3-Na^+/K^+-ATPase isoforms, depicting neuronal plasmalemma, followed by another membrane fraction containing α1-Na^+/K^+-ATPase representing the vascular smooth muscle sarcolemma. The half maximal inhibitory concentration (IC50) inhibiting neural plasmalemma Na^+/K^+-ATPase was significantly greater for ouabain, whereas MBG elicited a greater vasoconstrictor response in the vascular smooth muscle sarcolemma.[43]

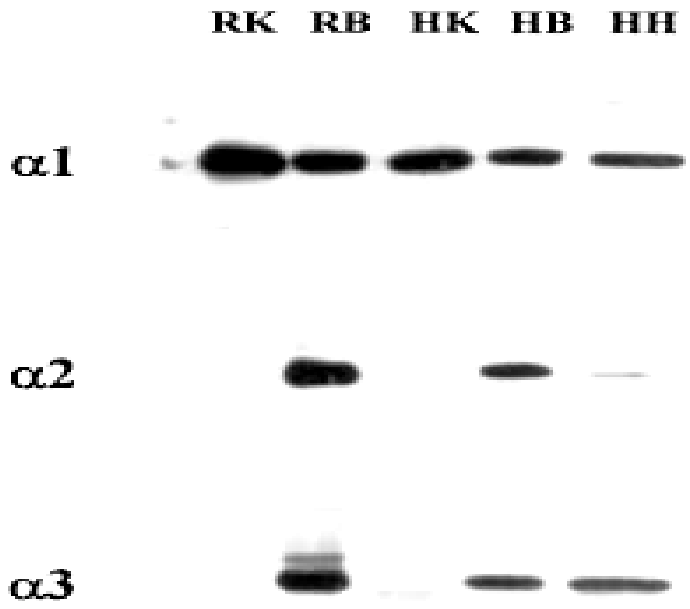


Figure 4: Immunodetection of sarcolemmal Na^+/K^+-ATPase in various rat and human tissues.[57] RK, rat kidney; RB, rat brain; HB, human brain; HK, human kidney; HH, human heart.

There are six sodium influx pathways located in the sarcolemma. However, the Na^+/K^+-ATPase is the one major sodium extrusion pathway regulating intracellular sodium concentrations.[39] The Na^+/Ca^{2+}-exchanger functions at a membrane potential of -40mV in order to transport three sodium ions into the cell in exchange for one calcium ion getting transported to the outside of the cell.[58] Increased intracellular sodium concentrations due to Na^+/K^+-ATPase inhibition,[13,39,58] homologous with the function of MBG,[43,44,52-56] disturb the electrochemical gradient across the cell membrane creating a more positive gradient, thereby dissipating the electrochemical gradient for calcium extrusion through the Na^+/Ca^{2+}-exchanger.[13,39,58,59] This results in an increased intracellular calcium concentration, which in turn provokes calcium induced calcium release from the sarcoplasmic reticulum.[58] Binding of calcium to troponin C causes a conformational change to

relieve the inhibition of actin-myosin cross-bridge bindings caused by the troponin-tropomyosin complex, resulting in cross-bring interaction and contractile shortening (**Figure 5**).[13,58]

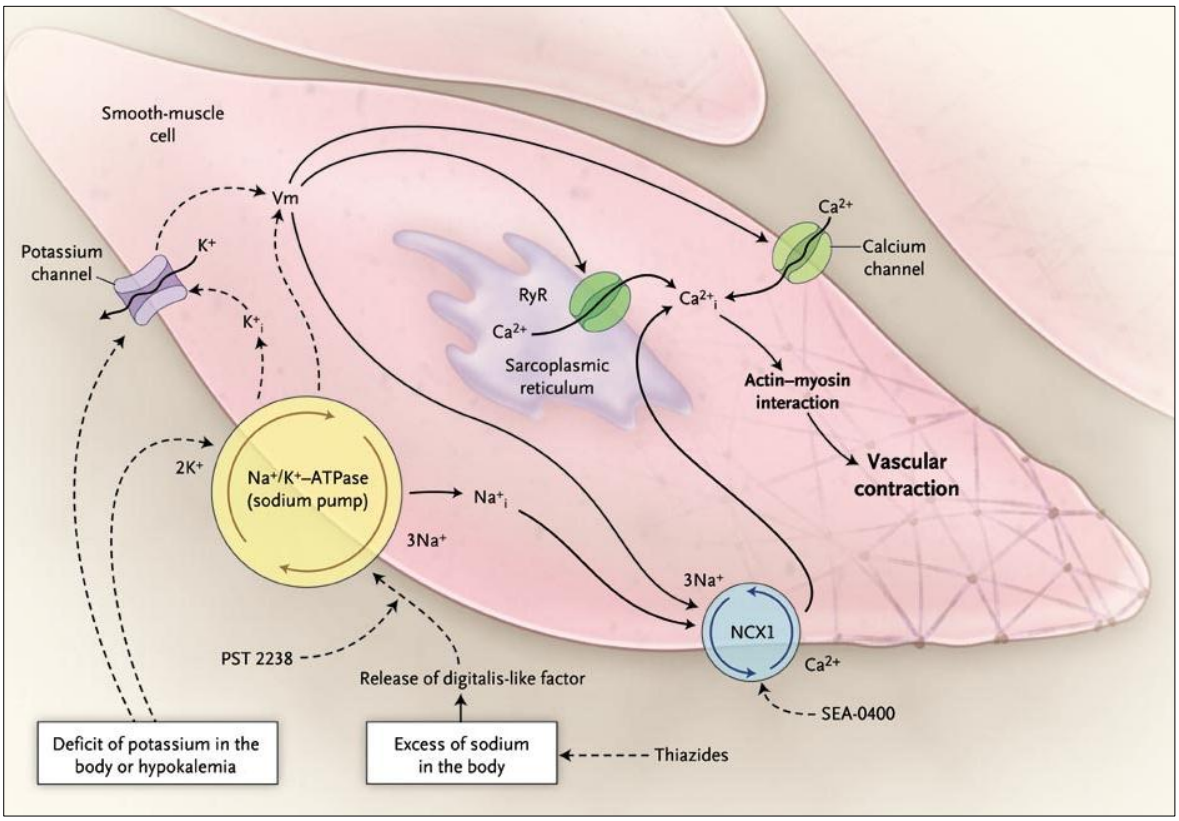


Figure 5: Interdependence of the sodium pump and calcium exchanger in vascular smooth muscle cells.[13] Solid arrows demonstrate stimulation whereas dashed arrows indicate inhibition.

5.1 Marinobufagenin and its association with measures of cardiovascular function

The previously described mechanism might also be applicable to the digitalis-like nature of MBG in the heart.[56] An increase in intracellular calcium causes a positive inotropic effect,[58] resulting in a greater stroke volume and concurrent increase in blood pressure.[34]

Additionally, small changes in the electrochemical sodium gradient provokes an elevation in the calcium-tension curve in vascular smooth muscle cells (VSMC), which increases vasoconstriction and thus the total peripheral resistance (TPR) of the vasculature.[59] Short term intervention studies have positively associated a high sodium diet with an increase in pulse wave velocity (PWV) and blood pressure.[60] A less apparent association between blood pressure and PWV with sodium during sodium loading, prompts a putative relationship between sodium and arterial vascular reactivity independent of blood pressure.[60]

In accordance with the above, a reduction in dietary sodium intake, during a two week period, has been associated with an increase in arterial compliance together with a decrease in 24hr blood pressure.[61] Structural changes in the vascular wall bought on by extracellular matrix remodeling occur over a considerable time period. This emphasizes the unlikely contribution of structural changes during a two week low-sodium diet to the significant improvement of large artery compliance.[61] Gates *et al*. suggest that the increase in large artery compliance during sodium restriction might rather be mediated by lower concentrations of endogenous sodium pump ligand MBG which modulates VSMC tonus.[61] Jablonski *et al*. confirmed this when they demonstrated similar findings during sodium restriction along with reduced urinary MBG levels.[62] Evidently they found that urinary MBG levels are positively associated with blood pressure and aortic PWV.[62] Studies have shown that MBG induces vasoconstriction in human pulmonary[63] and mesenteric[54] arteries by inhibiting α1-Na^+/K^+-ATPase.[43] Lower levels of MBG thus relieve the inhibition of Na^+/K^+-ATPase, consequently reducing vascular smooth muscle contraction and increasing arterial compliance.[61]

The preceding passage emphasizes previous findings and associations of MBG with blood pressure. However, these associations were not explored with regard to the variance in central systolic blood pressure (cSBP) and brachial systolic blood pressure (**Figure 6**).[64] Previous studies have indicated that cSBP, rather than brachial systolic blood pressure, is more strongly related to adverse cardiovascular end organ markers, such as atherosclerosis,[65] left ventricular mass,[65,66] estimated glomerular filtration rate[66] and carotid intima-media thickness.[65,66] In addition, Wang *et al*. found that cSBP was a stronger determent of cardiovascular mortality, as opposed to brachial systolic blood pressure.[66] Thus cSBP might be a more accurate estimate of predicting cardiovascular risk, and its association with MBG should be explored independent of brachial systolic blood pressure.

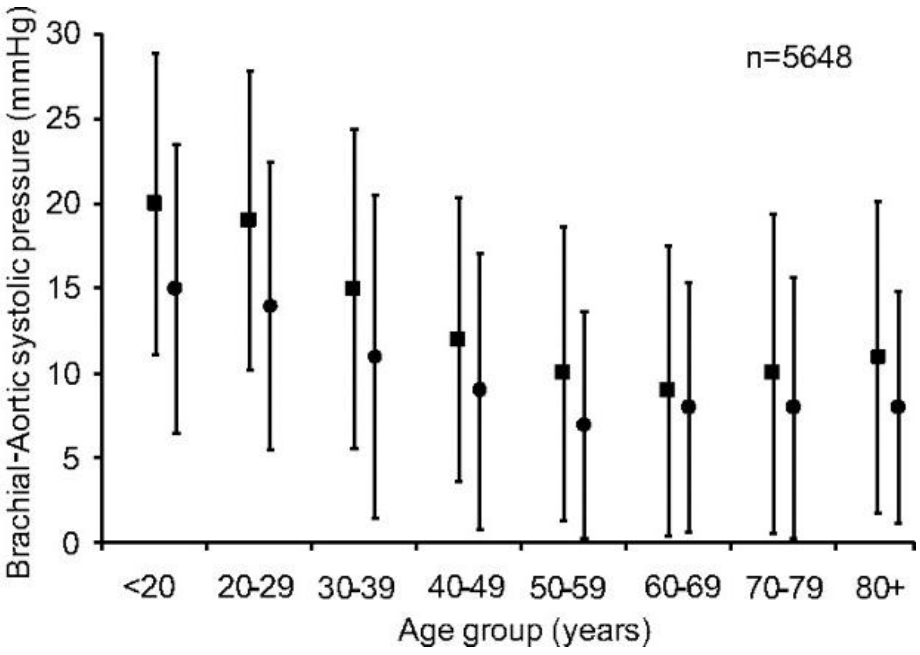


Figure 6: The discrepancies between aortic and brachial systolic blood pressure for healthy men=■ and women=●.[64]

6. Possible confounding factors that may influence marinobufagenin

It should be emphasized that literature relative to MBG and the following confounding factors are restricted, due to the limited data available on MBG and sodium handling in humans. To the best of my knowledge there are only four previous intervention studies with reference to sodium handling and MBG in humans.[52,62,67]

6.1 Ethnicity

Anderson *et al.* conducted a study using end tidal carbon dioxide (CO_2) as a marker of volume expansion and salt-sensitivity to demonstrate ethnic differences in urinary MBG in an older black and white population.[68] It has been demonstrated that a hypoventilatory state (higher end tidal CO_2), in normotensive individuals, associates with a decrease in urinary sodium,[69] an increase in plasma MBG, systolic blood pressure, diastolic blood pressure as well as the inhibition of Na^+/K^+-ATPase.[70] These observations led to the identification of end tidal CO_2 as a marker of salt-sensitivity used by Anderson *et al.*[68] They hypothesized that a higher end tidal CO_2, indicative of impaired renal sodium excretion, would be associated with higher urinary levels of MBG in African Americans compared to whites.[68] Contradictory to this hypothesis, they found that African American individuals excreted less MBG, even though their end tidal CO_2 was higher.[68] This study, however, was performed in an older population (mean age 53). Taking into consideration the overwhelming evidence of possible relationships between MBG and renal sodium regulation with blood pressure, ethnic differences in MBG should be explored further.

The net effect of MBG as a natriuretic or vasoreactive substance depends on the sustained intake of salt; the sensitivity of renal and vascular sodium pumps to

digitalis-like factors; and the potency or sensitivity of these pumps to other natriuretic or vasoactive substances.[67] Fedorova *et al*. demonstrated that Dahl salt-sensitive rats exhibited an exaggerated increase in MBG production and blood pressure along with a blunted natriuretic response, compared to Dahl salt-resistant rats during sodium loading.[44,51] They noted that impairment of Na^+/K^+-ATPase in the basolateral membrane of Dahl salt-sensitive rats might be due to an α1-subunit mutation,[71] resulting in the incapability of MBG to act as a compensating mechanism.[44,51] This mutation of the α1-Na^+/K^+-ATPase subunit associated with salt-sensitivity in Dahl salt-sensitive rats was demonstrated by Herrera *et al*., who indicated that transgenic Dahl salt-sensitive rats bearing the α1-Na^+/K^+-ATPase cDNA of the Dahl salt-resistant rats were less salt-sensitive.[69] Ultimately, the excessive levels of MBG production fail to compensate for the genetic pressure-natriuresis impairment [44] but lead to an increase in vascular resistance and blood pressure due to its effect on the VSMC.[51] This might also be true in black populations with a predisposition to hypertension and insufficient sodium handling due to their inherit propensity to reabsorb more sodium[19,72,73] regardless of salt-sensitivity status.[19,74] This, however, is mere speculation.

One possible genetic factor might be the expression of CYP3A5*1 alelle carrier proteins in the kidney, limited to the proximal nephrotic tube,[75] where blacks have been shown to reabsorb more sodium compared to their white counterparts [19]. Bochud *et al*. demonstrated that CYP3A5*1 carriers of African descent had lower urinary sodium excretion and higher blood pressure compared to non-CYP3A5*1 carriers.[76] Thereupon 43.7% of the black study population were carriers of the CYP3A5*1 allele.[76] The association between CYP3A5*1 and blood pressure has

been confirmed in other studies, giving rise to the speculation that CYP3A5*1 might be implicated in sodium sensitive hypertension in blacks.[77]

White normotensive women present an increased urinary MBG, associated with a decreased SBP, likely due to the natriuretic effect of MBG.[67] Conversely, black populations have been reported to excrete less sodium compared to whites even after exposure to physical and mental stressors (**Figure 7**).[30,78-81] Bochud *et al*. established that proximal sodium reabsorption in black South Africans is highly heritable with the greater majority of their sodium load being reabsorbed in the proximal tube rather than the distal tube, when compared to whites.[19] This results in a lower fractional sodium excretion rate in blacks, irrespective of salt-sensitivity.[19,74,80,81] Based on MBG studies in Dahl salt-sensitive rats mentioned previously,[44,51] one may speculate that the lower fractional sodium excretion observed in blacks might be indicative of the inherit sodium reabsorption overriding the renal function of MBG. A blunted response to MBG in the kidneys (diminished natriuretic and diuretic) is associated with excessive MBG production which will cause an increase in the vascular tone of VSMC,[51,54] and might contribute to a sustained cardiovascular effect causing an elevated blood pressure response (**Figure 1)**.

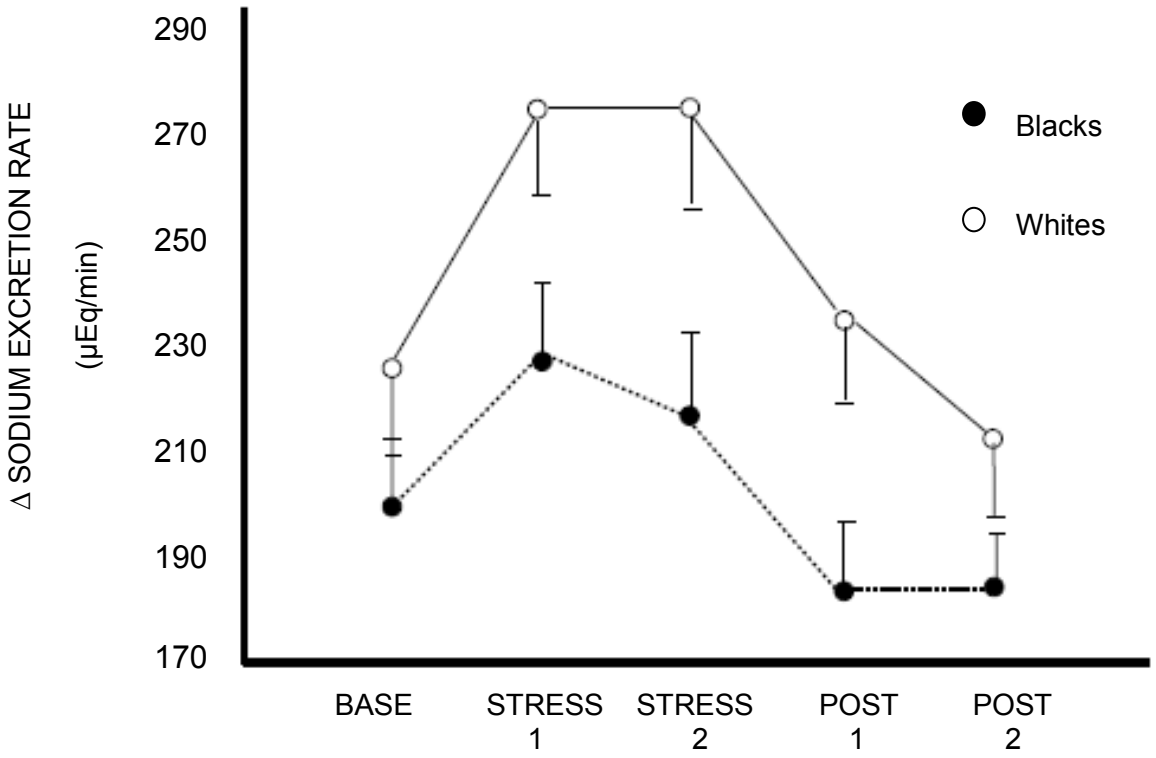


Figure 7: Sodium excretion rates of black and white individuals after exposure to a series of mental stressors, including arithmetic and reaction time tasks.[81]

Complementary to this, renin profiling in ethnic demographics indicate that while white individuals with a high renin phenotype respond to beta-blockers and ACE inhibitors, black individuals respond more often to calcium channel blockers and diuretics to relieve sodium dependent vasoconstriction (**Figure 8**).[33,82] This supports the notion that black individuals are predisposed to the sodium-volume end of the hypertension spectrum,[33,82] and that black and white individuals may exhibit pathophysiological differences contributing to the development and progression of hypertension.[82,83]

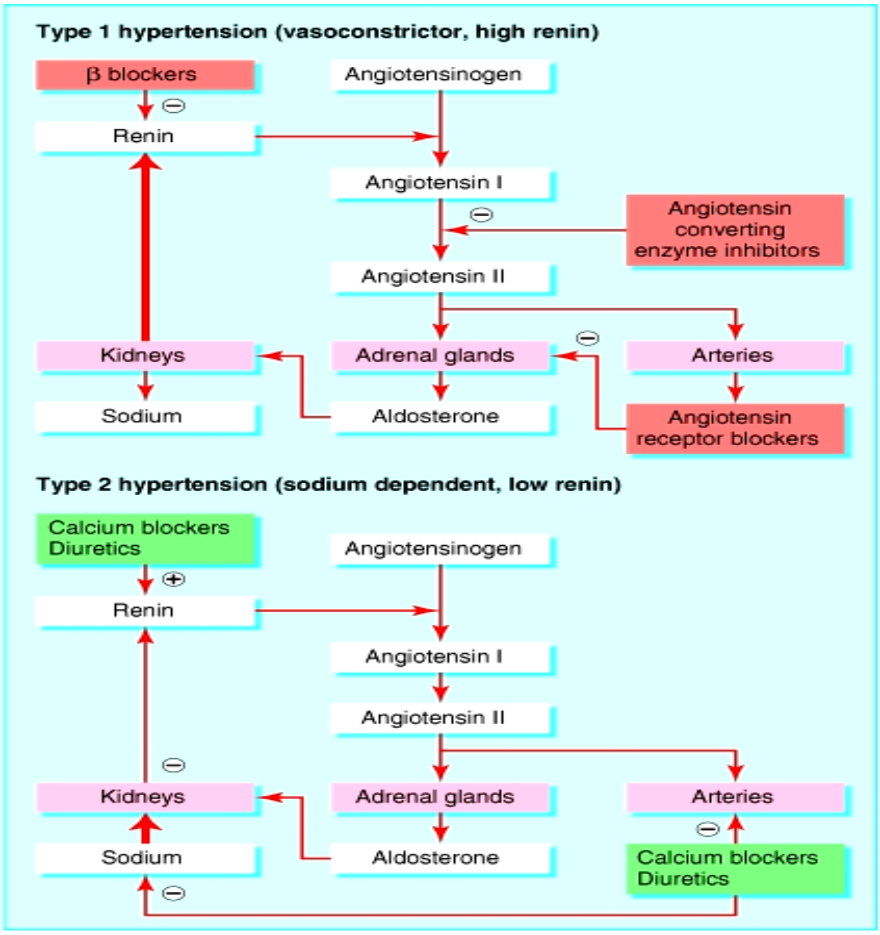


Figure 8: Two types of hypertension: the function of the RAAS.[82] Type 1: High-renin phenotype with exaggerated sodium elimination most common in young white people. Type 2: Low-renin phenotype with suppressed renin secretion via the detection of excessive sodium by the macula densa cells of the kidney, most common in young black people.

In a follow-up study investigating the ethnic differences in blood pressure response to aldosterone, it was determined that aldosterone sensitivity is a significant determinant of blood pressure in black children and young adults — although this is unaccompanied by a proportional increase in their aldosterone levels.[84] Wanzhu *et al.* suggest that black individuals might be more sensitive to aldosterone, in a similar manner to their salt-sensitive predisposition.[82] Moreover, their tendency to retain

sodium may consequently enhance the effect of aldosterone on their blood pressure.[84] They ultimately found that although blacks had lower levels of aldosterone, the positive relationship between aldosterone and blood pressure inversely correlated to low levels of plasma renin activity, indicative of volume expansion (**Figure 9**).[84] Furthermore, aldosterone increases sodium retention which in turn results in volume expansion which stimulates the release of ouabain-like substances (**Figure 10**),[27] such as marinobufagenin.[42]

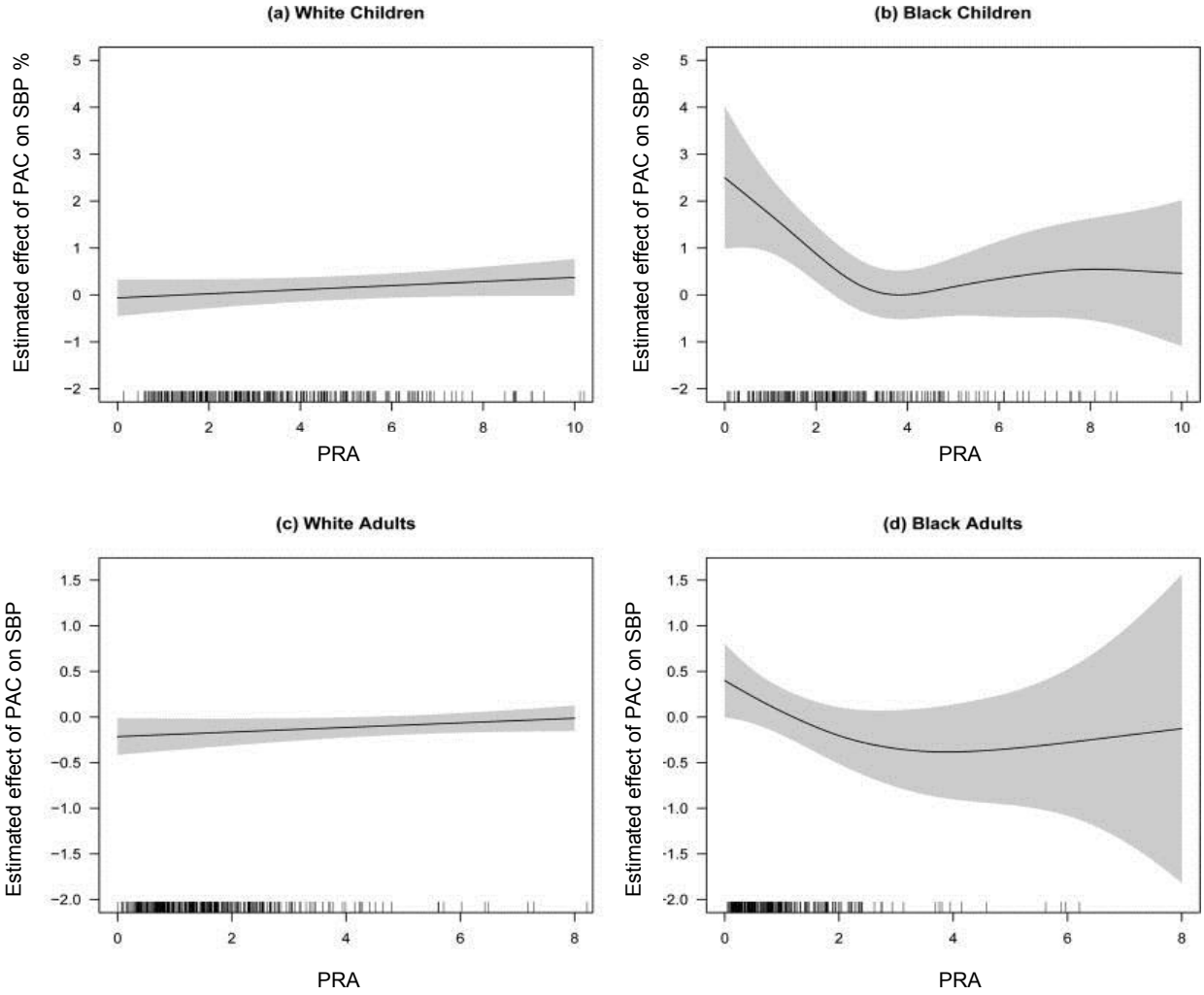


Figure 9: Effect of plasma aldosterone concentrations on systolic blood pressure in black and white children and young adults.[84] PAC, Plasma aldosterone concentration; PRA, Plasma renin activity; SBP, systolic blood pressure.

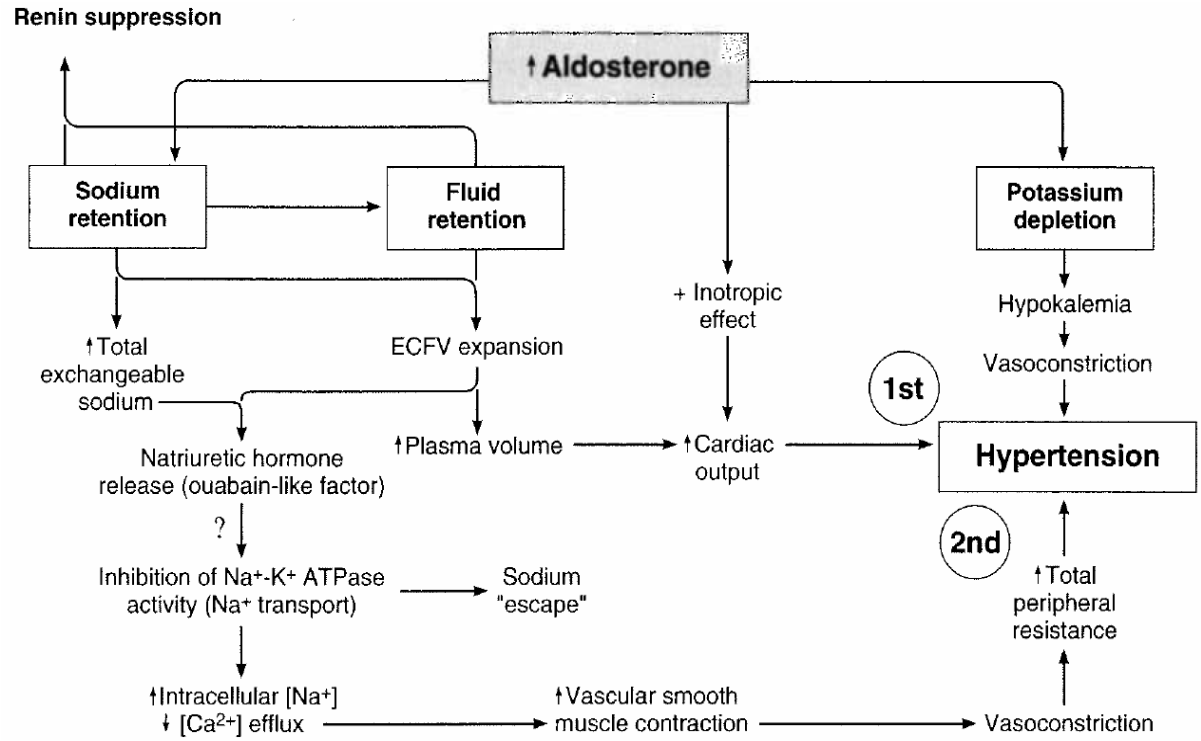


Figure 10: The role of aldosterone in hypertension.[27]

6.2 Sex

The relationship between MBG and the salt-sensitive pressor effect seems to be more evident in men.[52] Consistent with this notion, Fedorova *et al*. demonstrated that female Dahl salt-sensitive rats exhibit lower blood pressure, as well as lower plasma MBG levels and CYP27A1 mRNA expression compared to males,[50] although this has not yet been confirmed in human subjects. Moreover the female sex hormone estradiol stimulates cardiac sarcolemmal Na^+/K^+-ATPase activity believed to play a cardioprotective role.[85] In contrast, Weinberger *et al*. found no blood pressure differences regarding sex in a normotensive subject group when administering sodium interventions.[31] It is essentially important to note that although both sex hormones and MBG are derived from cholesterol, their biosynthesis are dependent on different pathways, initiated by separate enzymes.[50] The biosynthesis of steroidal

MBG entails the side chain cleavage by the CYP27A1 enzyme whereas traditional steroidogenesis involves the cleavage by CYP11A1.[50] Taking into consideration the aforementioned, it is possible that a physiological relationship between MBG and sex hormones might exist, demanding further investigation.

6.3 Smoking and alcohol

As far as could be established, there are no studies indicating any relationship of MBG with either smoking or alcohol consumption. This might be due to the limited amount of population-based studies investigating MBG in humans. Strauss *et al*. studied the effect of ethanol on renal function and concluded that alcohol consumption promoted diuresis without an equivalent electrolyte loss, creating a negative water-electrolyte balance.[86] Indeed, the absence of a relationship between alcohol and sodium excretion was confirmed by De Marchi *et al.* when they demonstrated that alcohol ingestion provoked metabolic acidosis and ketonuria but that there was no difference between sodium excretion during alcohol admission or abstinence.[87] Hence, while both alcohol intake and sodium are associated with an increase in blood pressure, these associations are independent of one and other.[88] A study investigating the renal effect of smoking indicated that although non-smokers displayed a greater renal vasoconstriction compared to smokers, there were no differences in renal sodium excretion in response to nicotine administration.[89] Cigarette smoke has been shown to diminish the Na^+/K^+-ATPase activity in lung cells,[90] although its effect on other Na^+/K^+-ATPase pumps have yet to be established. Since it is not evident that urinary sodium excretion is influenced by either alcohol intake or smoking status, an association with MBG is unforeseen.

6.4 Age

An increase in systolic blood pressure during sodium loading is positively associated with age,[67] while urinary MBG is inversely related to age.[52,67] A mechanistic relationship between MBG and age has yet to be investigated.

6.5 Obesity

Body mass index is not a determinant of proximal sodium reabsorption in either black or white individuals.[19] It has not yet been investigated in detail whether or not a causative link exists between MBG and obesity, although obesity has been associated with impaired Na^+/K^+-ATPase activity and reduced expression in animal and human tissue.[91,92] Anderson and colleagues have, however, noted a negative association between renal excretion of MBG and body weight together with a decrease in natriuresis.[67] Despite these observations, they did not provide data on the plasma MBG concentration, and it is therefore unclear whether a decrease in urinary MBG was accompanied by an increase in plasma MBG.

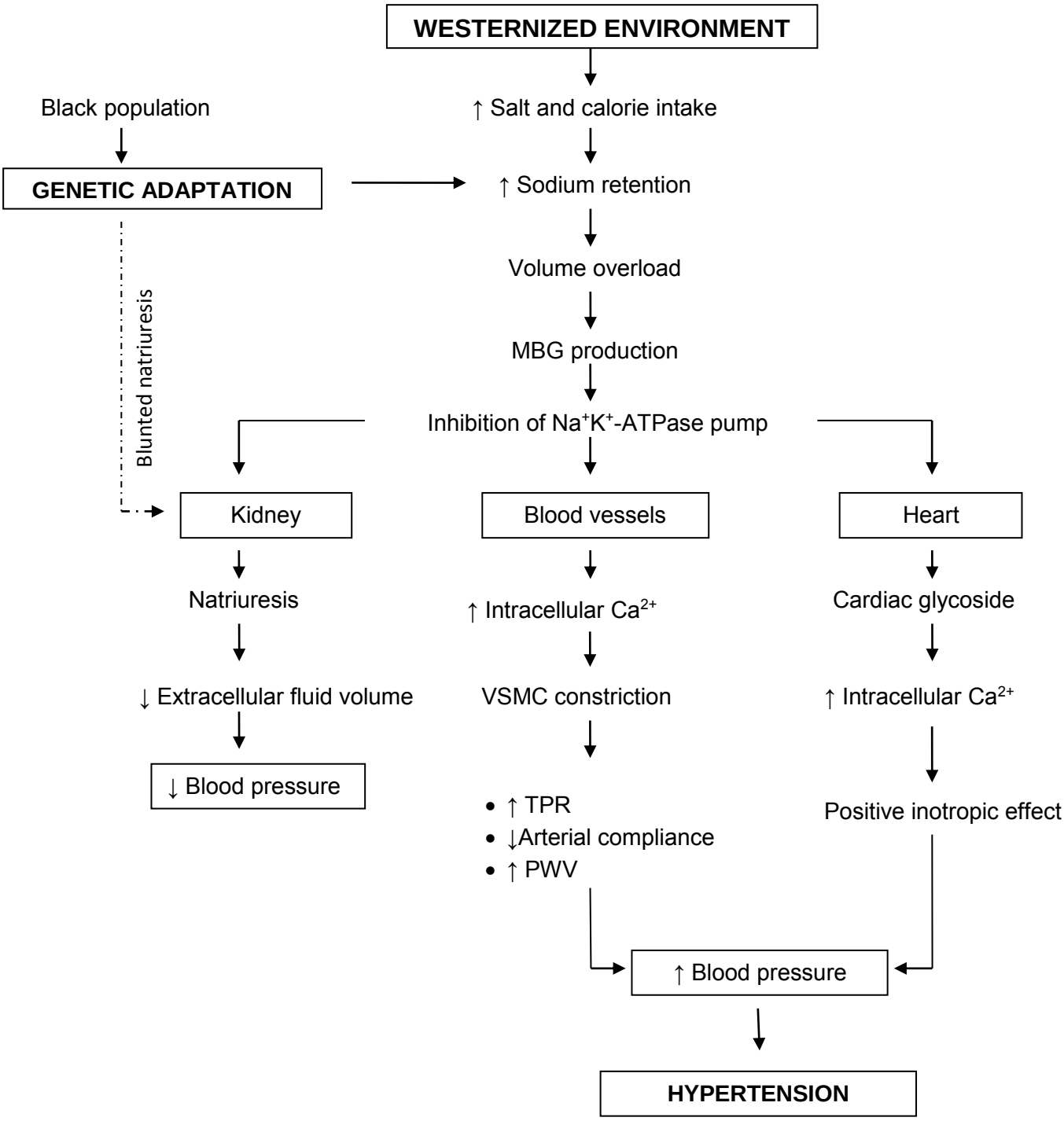
6.6 Diabetes

Djemli-Shipkolye *et al.* demonstrated that depressed Na^+/K^+-ATPase activity in erythrocytes of type 1 diabetes mellitus patients is restored by insulin infusion.[93] Furthermore, patients with diabetes portray an increase in proximal sodium retention compared to healthy subjects.[94,95]

7. Motivation

Despite numerous studies associating salt intake with adverse measures of cardiovascular function[60-62] and events,[12,25] black and white South Africans still consume more than the WHO's recommended daily salt intake.[16] The distinct regulation of sodium handling in these different demographics[19] may contribute to long term blood pressure dysregulation and the development of hypertension. However, knowledge regarding the differences between black and white, men and women, regarding urinary MBG levels and also its associations with 24hr urinary sodium excretion and measures of cardiovascular function, is lacking. We calculated the MBG/Na^+ excretion ratio as a proposed estimate of Na^+ excretion resistance to higher levels of urinary MBG.

7.1 Integration of concepts with reference to marinobufagenin



MBG, Marinobufagenin; PWV, Pulse wave velocity; TPR, Total peripheral resistance; VSMC, Vascular smooth muscle cell. Solid arrows denote stimulation and dashed arrows inhibition.

8. Aim

The aim of this study is to compare the MBG and 24hr urinary sodium profiles between black and white, men and women, and to investigate the association of MBG with systolic blood pressure and hemodynamic parameters in a young bi-ethnic population.

9. Objectives

- To compare MBG and 24hr urinary sodium excretion profiles between black and white groups, as well as between men and women.
- To determine whether the MBG/Na^+ excretion ratio, used as a proposed estimate of Na^+ excretion resistance, could be used as a more sensitive measure as opposed to MBG excretion alone.
- To investigate the associations between MBG and the MBG/Na^+ with:
  a) 24hr urinary sodium excretion, and 24hr urinary volume;
  b) cSBP, 24hr blood pressure, stroke volume; and TPR.

10. Hypotheses

The following hypotheses are made, taking into consideration the literature and the objectives of this study:

- Urinary MBG levels are higher in blacks compared to whites.
- Urinary MBG levels are higher in men compared to women.
- MBG excretion associates positively with sodium excretion and urinary volume.
- MBG excretion as well as the MBG/Na^+ excretion ratio are positively associated with cSBP and 24hr systolic blood pressure, TPR and stroke volume in blacks. Inverse associations are expected in whites.

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Chapter 3

Study Protocol and Methodology

1. Study Design and Participants

The African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT) is an on-going prospective study in the North West province of South Africa. The study will recruit 1200 black and white participants, men and women (20-30 years of age) and perform follow-up measurements for 10-20 years. The African-PREDICT study aims to identify and gain a better understanding of novel early biomarkers as well as predictors involved in the development of cardiovascular diseases. Furthermore, the study aims to substantiate the importance of the markers which could contribute to the successful implementation of cardiovascular risk prevention programs in young black South Africans. The African-PREDICT study was approved by the Health Research Ethics Committee (HREC) of the North-West University (NWU-00001-12-A1). All procedures were in adherence with institutional guidelines and the Declaration of Helsinki.

This study consists of two phases including the initial screening phase, for inclusion into the study, as well as the advanced measurements. Screening for the study began in November 2012 and advanced measurements subsequently followed in February 2013. Black and white individuals of both low- and high socio-economic status, living in close proximity to the Potchefstroom area, were invited to take part in the initial health screening at the Hypertension Research and Training Clinic on the Potchefstroom Campus in building F11. A transitory outline of participant stratification is shown in **Figure 1**.

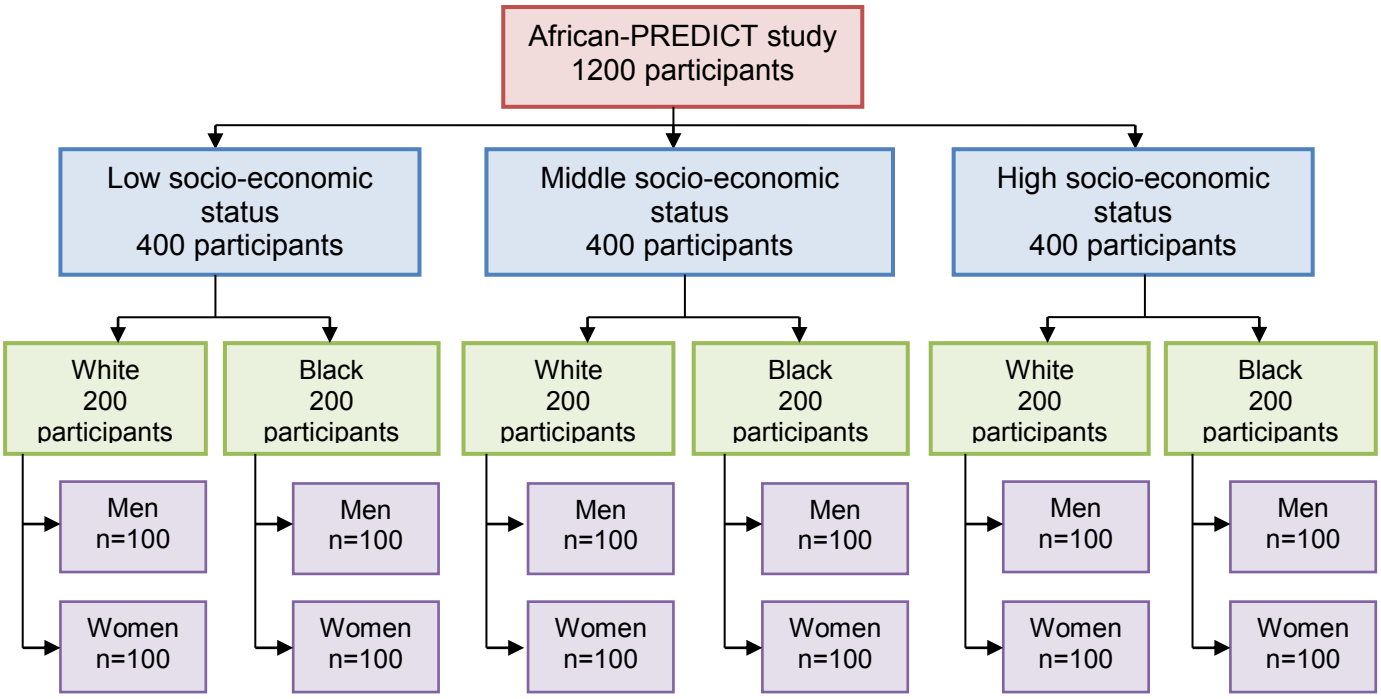

Figure 1: Stratification of participants according to age-group, ethnicity and employment status in the African-PREDICT study.

Organizational Procedures

Participants were transported to the Hypertension Research and Training Clinic and arrived at approximately 08:00 where they were introduced to the organizational procedures and registered to take part in the health screening. Prior to participation all procedures were explained and conveyed to individuals by a trained staff member before giving written informed consent to voluntarily take part in the health screening.

Participants who met the study inclusion criteria (not being hypertensive, not HIV-infected, not using chronic medication for serious illnesses such as tuberculosis or diabetes) (**Table 1**) during the screening visit, were invited to take part in advanced measurements as part of the African-PREDICT study. A professional counselor was present to console and provide the necessary information to individuals who did not

comply with these inclusion criteria. They were also referred to get the appropriate medical care.

Participants who were willing to participate in the African-PREDICT study after the health screening were transported to and from the Hypertension Research and Training Clinic on the Potchefstroom campus, building F12. Information leaflets were distributed to participants prior to taking measurements. Procedures were thoroughly explained to participants beforehand and all participants had the opportunity to ask questions throughout all of the procedures. Participation in the study was voluntary and participants were given the opportunity to withdraw from the study at any time. Participants arrived at the Hypertension Research and Training Clinic at 08:00 where they were familiarized with the research environment and experimental setup. All participants signed a written informed consent form before the commencement of the measurements. All measurements were explained and performed by trained researchers under the supervision of a registered nurse, as head of the Hypertension Research and Training Clinic. Although participants were required to fast prior to early morning measurements, all participants were on a habitual diet. Participants received a light lunch as well as a R50 Checkers retail store voucher at the end of the day as a token of appreciation for their participation and time away from work. It was ensured that the amount was not of such a nature that it would jeopardize the voluntary nature of participation.

For the purpose of this study affiliated with the African-PREDICT study, the data of the first 331 consecutive participants with complete 24hr urinary collections, meeting the inclusion criteria, was analyzed cross-sectionally. The HREC of the North-West University (Potchefstroom Campus) approved this study (NWU-00022-16-S1), and all procedures were performed according to institutional guidelines.

Table 1: Detailed eligibility criteria and concurrent justification for the African-PREDICT study.

Inclusion criteria:	Justification:
1. Black or white ethnicity 2. Aged 20-30 years 3. Men and Women (equally distributed) 4. Apparently healthy 5. Normotensive or pre-hypertensive (systolic blood pressure (SBP) <140 and diastolic blood pressure<90mmHg) based on the average of four blood pressure measures in one day	The African-PREDICT study is a longitudinal study, aimed to include and track young healthy individuals over a period of 10-20 years in order to monitor especially the early phases of cardiovascular disease development. (Inclusion criterion 4) In South Africa, our research group and others have shown that black populations have the highest blood pressure and were therefore used in this study. Black participants were compared with white participants as per the aims of the study. (Inclusion criterion 1). As the aim of the study was to follow the progression of vascular function in young normotensive people, the researchers thought that the 20-30 year old age category was a good place to start. As hypertension was our primary endpoint, it was necessary to exclude those with hypertension (based on office blood pressure), and those on chronic medication or with CVD (Inclusion criterion 2).

	Both men and women were used to determine whether difference occur in gender (Inclusion criterion 3). International hypertension cut-off points were used to determine whether a participant was hypertensive or normotensive (Inclusion criterion 5).
Exclusion criteria: 1. Hypertensive 2. Indian, Asian or mixed origin ethnicity 3. Not permanent resident of Potchefstroom surrounding area (i.e. intend to move to another area) 4. Diagnosed Type 1 or 2 Diabetes Mellitus 5. Elevated glucose >5.6 mmol/L and confirmed glycated haemoglobin (HbA1c) ≥ 6.5% 6. HIV infected 7. Fever (internal ear temperature > 37.5ºC on the research day) 8. Known liver disease, cancer, tuberculosis or renal disease	Justification: As hypertension development was an endpoint in this study, hypertensive participants were excluded from the start of the study (Exclusion criterion 1). From known populations, black individuals have elevated blood pressure and were therefore used in the study, whereas the white population was used as a comparison group. Thus the focus is on ethnic differences between the black and white populations (Exclusion criterion 2). Due to the longitudinal nature of the study researchers made sure that participants could be followed over the required time period (Exclusion criterion 3). Individuals with any known diseases or risk factors that may influence cardiovascular

9. Microalbuminuria > 30 mg/ml in spot morning urine or proteinuria 10. Medication use for chronic disease, i.e. antihypertensive, anti-diabetic, anti-retroviral or anti-inflammatory 11. Pregnant or lactating women 12. Recent surgery or trauma (within the past three months) 13. Previous history of stroke, angina pectoris or myocardial infarction	health were excluded (Exclusion criterion 4-10, 12, 13). Due to the known influences of hormones on cardiovascular health pregnant and lactating women were excluded (Exclusion criterion 11).

2. Materials and Methods

2.1 Questionnaires

The socio-economic statuses as well as self reported smoking and alcohol intake of participants were recorded using General Health Questionnaires.

2.2 Anthropometric Measurements and Physical Activity

An anthropometrist used standard procedures to obtain height (m) determined by the SECA 213 Portable Stadiometer (SECA, Hamburg, Germany), weight (kg) using the SECA 813 Electronic Scales with weighing capacity up to 200kg (SECA, Hamburg, Germany) and waist circumference (cm)(Lufkin Steel Anthropometric Tape; W606PM;Lufkin, Apex, USA). The body mass index (BMI) (weight (kg) / height (m^2)) was calculated. All anthropometric measurements were performed according to

guidelines described by the International Society for the Advancement of Kinanthropometry.[1]

After being fitted with the 24hr ambulatory blood pressure apparatus, participants were fitted with an ActiHeart physical activity monitor (CamNtech Ltd., England, UK) to their chest. The device was used to record heart rate, inter-beat-interval, physical activity and heart rate variability data. Furthermore, the ActiHeart calculated and measured the total activity energy expenditure. The ActiHeart device was worn for a maximum of seven days.

2.3 Cardiovascular Measurements

All cardiovascular measurements were done in temperature controlled private rooms to take into consideration the privacy of the participant. Central systolic blood pressure (cSBP) was determined non-invasively, using the Sphygmocor® XCEL device (AtCor Medical Pty. Ltd., Sydney, Australia). Participants were fitted with a brachial blood pressure cuff, and requested to lie in a supine position for approximately five minutes before the commencement of the measurement. The cSBP was subsequently derived from the recorded brachial pressure pulsations using the pulse wave analysis function.

Beat-to-beat hemodynamic measurements were taken non-invasively using a validated Finometer device (FMS, Finapres Measurement Systems, Amsterdam, Netherlands), computing precise values based on the non-linear three element model.[2] These measurements included heart rate, stroke volume and total peripheral resistance (TPR). Participants lay in the Fowler's position with their arm at heart level, connected to a brachial blood pressure cuff as well as a finger cuff. Following a resting period, the apparatus was calibrated for a two minute period to provide a

subject-level individual adjustment of the finger arterial pressure with the brachial artery pressure, after which cardiovascular measurements were taken over a five minute period.

The 24hr ambulatory blood pressure (ABPM) was measured using a 24hr ABPM and electrocardiogram (ECG) apparatus (CardioXplore, Meditech, Budapest, Hungary, British Hypertension Society (BHS) validated). An appropriate sized cuff was fitted to the participant's non-dominant arm and instructions were given to participants on how to ensure successful inflations across the 24hr time period. The ABPM apparatus was programmed to measure blood pressure in 30 minute intervals during the day (08:00-22:00) and every hour during the night (22:00-06:00), while the ECG recorded measurements every five minutes for 20 seconds. An ambulatory diary card was distributed and completed by participants during the 24hr duration of the measurements. ABPM was repeated if measurements were not successful and when readings did not meet the pre-specified parameter criteria as required by the European Society of Hypertension (ESH). The required criteria were as follow: at least 70% of the measurements being successful or having at least 20 valid daytime together with seven nighttime measures.[3]

2.4 Biological Sampling and Biochemical Analysis

Participants were requested to fast overnight for an 8-10hr period prior to early morning sampling. Early morning spot urine sampling and all blood samples were collected by a registered nurse in a private room. Participants were also asked to collect all urine that they passed during the 24hr time period (day and night) in a large five liter plastic bottle and store it in a cool dark place until a research assistant came to collect it after the 24hr period. Biological samples (serum, plasma, whole

blood and urine) were stored in cryovials and kept in biofreezers at -80°C in an on-site laboratory in building F12 on the Potchefstroom Campus. All biological waste was disposed of in accordance with existing legislation and the University's policy. Biochemical analyses were performed by a qualified biochemist using calibrated instruments, and according to standardized methodology and internationally recognized biochemical procedures. High density lipoprotein cholesterol (HDL-C), total cholesterol (TC), and gamma glutamyltransferase (GGT) were determined in serum, whereas the percentage of glycated haemoglobin (HbA1c) was analyzed in EDTA whole blood. The 24hr urinary sodium and potassium were determined using ion-selective electrodes. All of the aforementioned analyses were performed using the Cobas Integra 400plus (Roche, Basel Switzerland). Aldosterone was determined using the Radioimmunoassay (RIA) Aldosterone Kit (Beckman Coulter, Immunotech, Radiova, Czech Republic). The 24hr urinary MBG concentrations were measured using a solid-phase DELFIA (Dissociation-Enhanced Lanthanide Fluorescent Immunoassay) fluoroimmunoassay based on 4G4 anti-MBG mouse monoclonal antibody, as reported in detail by Fedorova *et al.*[4]. The assay was based on the principle that the immobilized antigen (MBG-glycoside-thyroglobulin), MBG, alternative cross-reactants and other endogenous cardiotonic steroids, within the urine sample, competed for a restricted amount of binding sites on an anti-MBG mAb.[5]

2.5 Statistical Analysis

The dataset of the first consecutive 331 participants, with complete 24hr urinary sodium excretion data was analyzed cross-sectionally. Data analysis was performed using Statistica v13.0 (Statsoft Inc., Tulsa, USA, 2010). The Kolmogorov-Smirnov test was conducted to test whether data followed a normal distribution. Normally

distributed data was presented as mean with the standard deviation. Variables following a non-Gaussian distribution were logarithmically transformed and data represented as geometric means with 5th and 95th percentile.

Interaction testing was performed using multiple regression analysis to determine the potential influence of sex and ethnicity on the relationship between MBG/Na^+ and SBP. Subsequent group divisions were made. Group comparisons for continuous data (urinary MBG, 24hr urinary sodium excretion, 24hr blood pressure, stroke volume, TPR, fasting glucose, lipids (HDL-C, LDL-C or TG), cotinine, GGT) were made using independent t-tests, and in the event that there were more than two groups, the one-way analysis of variance (ANOVA) was used. Chi-square tests were conducted to compare proportions of categorical variables (self-reported smoking and alcohol intake) between groups.

Analyses of covariance were performed to determine differences in SBP regarding MBG/Na^+ quartiles within groups, adjusting for age and waist:height ratio. The relationship between MBG/Na^+ and hemodynamic measurements was determined using Pearson-, partial- and multiple regression analysis with MBG/Na^+ as the main independent variable. Various cardiovascular variables were included, comprising cSBP, 24hr ABPM, stroke volume and TPR as dependent variables in separate models. Several covariates were considered as possible independent variables based on the strongest bivariate associations with the dependent and independent variables. Finally, age, waist:height ratio, GGT, HbA1c, TC:HDL and MBG/Na^+ were included into multiple regression models. The number of covariates was limited to the number of participants in the smallest group (one covariate per 10 participants). Alpha was set at 0.05.

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Chapter 4

Manuscript for Publication

MARINOBUFAGENIN IS RELATED TO ELEVATED CENTRAL AND 24 HR SYSTOLIC BLOOD PRESSURES IN YOUNG BLACK WOMEN: THE AFRICAN-PREDICT STUDY

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Abstract

Marinobufagenin is an endogenous bioactive steroid and α1-Na^+K^+-ATPase inhibitor. Because of its role in sodium handling it has been associated with both antihypertensive and prohypertensive effects. Marinobufagenin is positively associated with blood pressure in Dahl salt-sensitive rats exhibiting a similar hypertensive phenotype to black populations, characterized by low urinary Na^+ excretion. However, clinical studies exploring marinobufagenin's vascular effects in black populations are scant. We determined whether the 24hr marinobufagenin/Na^+ excretion ratio (a proposed estimate of Na^+ excretion resistance to marinobufagenin) is related to systolic blood pressure (SBP) in young black men and women, compared to whites. We included 331 apparently healthy participants (20-30 years) (42.9% black, 43.8% men) on a habitual diet. We obtained 24hr and central SBP, and 24hr urinary Na^+ and marinobufagenin levels. We found no ethnic differences in marinobufagenin, Na^+ or marinobufagenin/Na^+. In black women SBP related positively to marinobufagenin/Na^+ in single and multi-variable adjusted regression models: central SBP (R^2=0.26; *ß*=0.28; *p*=0.039), 24hr SBP (R^2=0.46; *ß*=0.30; *p*=0.011), daytime (R^2=0.38; *ß*=0.28; *p*=0.023) and nighttime SBP (R^2=0.38; *ß*=0.33; *p*=0.009). In contrast, inverse associations of marinobufagenin/Na^+ with nighttime SBP were evident in white women (*r*=−0.20; *p*=0.038) but lost significance after multiple adjustments (R^2=0.36; *ß*=−0.13; *p*=0.12). We found no associations in men. To conclude, we found independent positive associations of SBP with marinobufagenin/Na^+ excretion ratio in black women. This data supports the concept that reduced MBG mediated Na^+ excretion can contribute to adverse hemodynamics.

Key words: Black populations, Marinobufagenin, Sodium handling, Systolic blood pressure, Young.

Introduction

Hypertension contributes to an increased risk of cardiovascular morbidity and mortality.[1] Black populations are particularly predisposed to hypertension in part due to a genetic susceptibility to retain more sodium[2-4] with concurrent volume expansion.[5] Sodium-induced plasma volume expansion stimulates the production of the endogenous steroidal sodium pump ligand, marinobufagenin (MBG),[6] as a compensatory natriuretic mechanism.[7] MBG has been implicated in the well-known relationship between sodium handling and blood pressure (BP) regulation via the inhibition of both renal and cardiovascular α1-Na^+K^+-ATPase.[7-10]

A sustained increase in MBG production, along with an elevated BP and diminished natriuresis, has been noted in Dahl salt-sensitive rats with genetically impaired pressure-natriuresis.[7] A blunted natriuretic response of the kidneys along with excessive levels of plasma MBG, also increases the tone of vascular smooth muscle cells (VSMC),[7,10] thereby increasing the total peripheral vascular resistance (TPR). The aforementioned is consistent with findings that excessive MBG is related to increased BP in middle-aged individuals (n=20)[11] as well as arterial stiffness in older salt-sensitive hypertensive subjects (n=11).[8] Dahl salt-sensitive rats exhibit a similar genetic predisposition to the hypertension phenotype characterized by a low fractional sodium excretion[7,10] observed in black populations.[2-4] Essentially the genetic predisposition of blacks to reabsorb more sodium in the proximal tube[4] might override the compensatory natriuretic activity of MBG.[7] Notably, prior studies conducted in small cohorts of middle-aged white individuals, reported contrasting sex-specific relationships of MBG with systolic blood pressure (SBP).[9,11] They demonstrated that MBG was positively related to SBP in men,[11] whereas a negative association was observed in women (n=28).[9] These sex-specific patterns have yet to be investigated in a young black population.

This study presents the first findings on 24hr urinary MBG, central SBP (cSBP), 24hr BP and hemodynamic parameters in a young black population, in comparison to a homogenous white population (n=331). We hypothesized that due to less efficient sodium handling in blacks, [2-4] higher levels of sodium will continue to drive MBG production with a resultant higher MBG/Na^+ ratio. This prompts an increase in BP due to MBG's vasoconstrictive effect at high levels. To address this hypothesis we measured 24hr urinary sodium and MBG excretion, as well as cSBP and 24hr SBP in young black and white adults. Our investigation of MBG in this young bi-ethnic population sample might provide insight into the role of MBG prior to the onset of pathology. We calculated the MBG/Na^+ excretion ratio as an estimate of Na^+ excretion resistance to higher levels of urinary MBG.

Methods

Protocol and Participants

The protocol of the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT) study was approved by the Health Research Ethics Committee of the North-West University in South Africa. All procedures were in adherence with institutional guidelines and the Declaration of Helsinki.

The African-PREDICT study is an ongoing prospective study that will recruit 1200 black and white, men and women (20-30 years of age) and perform follow-up measurements for 10-20 years. For the purpose of this sub-study, the data of the first 331 consecutively enrolled participants with complete 24hr urinary collections, was analyzed cross-sectionally. Participants were informed about the objectives and procedures of the study before enrolment, after which all participants gave informed consent. Participants were screened for inclusion in the African-PREDICT study, and were included provided that they were normotensive based on clinic BP, HIV uninfected, not previously diagnosed with chronic disease nor using antihypertensive medication or any other medication for chronic disease.

Organizational procedures

Information leaflets were provided and discussed with participants prior to the day on which the study measurements commenced. Participants willing to take part in the African-PREDICT study were transported to the Hypertension Clinic, arriving at approximately 08:00, where they were familiarized with the research environment and experimental setup. All measurements were explained and performed by trained researchers. After informed consent was given general health questionnaires were completed to obtain information on socio-economic status, smoking and alcohol intake.

Anthropometric Measurements

Body height (SECA 213 Portable Stadiometer (SECA, Hamburg, Germany)), weight (SECA 813 Electronic Scales (SECA, Hamburg, Germany)) and waist circumference (Lufkin Steel Tape; W606PM; Lufkin, Apex, USA) were measured according to the guidelines of the International Society for the Advancement of Kinanthropometry by an anthropometrist using calibrated instruments. We calculated body mass index (BMI) (weight (kg) / height (m^2)) and waist:height ratio.

Cardiovascular Measurements

cSBP was measured in duplicate, non-invasively using the Sphygmocor XCEL device (AtCor Medical Pty. Ltd., Sydney, Australia). Participants were fitted with an appropriate sized brachial cuff and requested to lie in a supine position for approximately 5 minutes before commencement of the measurement.

Beat-to-beat hemodynamic measurements were taken non-invasively using a validated Finometer device (FMS, Finapres Measurement Systems, Amsterdam, Netherlands), computing precise values based on the non-linear three element model.[12] These measurements included heart rate, stroke volume and total peripheral resistance (TPR).

Participants lay in the Fowler's position with their arm at heart level, connected to a brachial BP cuff as well as a finger cuff. Following a resting period the apparatus was calibrated for a 2 minute period to provide a subject-level individual adjustment of the finger arterial pressure with the brachial artery pressure, after which continuous hemodynamic measurements were taken over a 5 minute period.

CardioXplore devices (Meditech, Budapest, Hungary, British Hypertension Society (BHS) validated) were used to obtain ambulatory BP (ABPM) data over a 24hr time period. Participants were fitted with an appropriate sized cuff and instructed to be relaxed while measurements were taken. BP readings were recorded in 30 minute intervals during the day (08:00-22:00) and hourly at night (22:00-06:00). An ambulatory diary card was distributed and completed during the duration of the measurements to report any abnormalities. ABPM was repeated if measurements were not successful and readings did not meet the prespecified parameter criteria, with at least 70% of the measurements being successful or having at least 20 valid daytime together with 7 nighttime measures.[13]

Biological Sample Collection and Biochemical Analyses

All participants were on a habitual diet. Participants refrained from eating or drinking, except for water, approximately 8-10hrs prior to biological sampling. Participants were then asked to collect all urine that they passed over a 24hr time period. Biological samples (serum, plasma, whole blood and urine) were stored in cryovials and kept in biofreezers at -80°C. High density lipoprotein cholesterol (HDL-C), total cholesterol (TC), and γ-glutamyltransferase (GGT) were determined in serum whereas the percentage of glycated haemoglobin (HbA1c) was analyzed in EDTA whole blood. 24hr urinary sodium and potassium were determined using ion-selective electrodes. Estimate NaCl intake was subsequently derived from the 24hr urinary sodium excretion. All of the aforementioned analyses were performed using the Cobas Integra 400plus (Roche, Basel, Switzerland). Aldosterone was determined using the

Radioimmunoassay (RIA) Aldosterone Kit (Beckman Coulter, Immunotech, Radiova, Czech Republic). 24hr urinary MBG concentrations were measured using a solid-phase DELFIA (Dissociation-Enhanced Lanthanide Fluorescent Immunoassay) fluoroimmunoassay based on 4G4 anti-MBG mouse monoclonal antibody, as reported in detail by Fedorova *et al.*[10]

Statistical Analyses

Statistical analyses were performed using Statistica v13.0 (Statsoft Inc., Tulsa, USA, 2010). Normally distributed data was presented as mean ± standard deviation. Non-Gaussian distributed data was logarithmically transformed, with the central tendency and spread of these variables represented as geometric means with 5th and 95th percentile intervals. We performed interaction testing using multiple regression analysis to determine the potential influence of sex and ethnicity on the relationship between MBG/Na^+ and SBP. Subsequent group divisions were made; where means and proportions were compared between groups using independent *t*-tests and Chi-square tests, respectively. Analyses of covariance were performed to determine significant differences in BP across MBG/Na^+ quartiles within groups, adjusting for age and waist:height ratio. We determined the relationship between MBG/Na^+ and hemodynamic measurements using Pearson-, partial- and multiple regression analysis with MBG/Na^+ as the main independent variable. We included various cardiovascular variables including cSBP, 24hr ABPM, stroke volume and TPR as dependent variables in separate models. Several covariates were considered as possible independent variables based on the strongest bivariate associations with the dependent and independent variables. We finally included age, waist:height ratio, GGT, HbA1c, TC:HDL and MBG in multiple regression models. $P < 0.05$ was considered as statistically significant.

Results

Participant Characteristics

We found an interaction of sex on the relationship between cSBP and MBG/Na^+ excretion ratio in the total group (N=331; p=0.027). In all women (N=186) we also found an interaction of ethnicity on the associations between either cSBP or 24hr SBP with MBG/Na^+, respectively (p=0.010 and p=0.012). These interactions of ethnicity were absent in black and white men (p=0.49 and p=0.89, respectively). Based on the focus of this paper, participants were therefore subsequently stratified by sex and ethnicity. **Table 1** provides the characteristics of the study population.

Table 1. Basic characteristics of young black and white, men and women

	Men N=145			**Women** N=186		
	Black	White	***P*-value**	Black	White	***P*-value**
N (%)	68 (46.9)	77 (53.1)		74 (39.8)	112 (60.2)	
Age (years)	24.1 ± 3.24	25.7 ± 2.76	0.002	24.3 ± 3.64	25.6 ± 2.78	0.008
Socio economic status, N (%)			<0.001			<0.001
Low	48 (70.6)	9 (11.7)		47 (63.5)	6 (5.4)	
Middle	9 (13.2)	11 (14.3)		21 (28.4)	25 (22.3)	
High	11 (16.2)	57 (74)		6 (8.1)	81 (72.3)	
Self-reported smoking, N (%)	26 (48.2)	15 (21.7)	0.002	9 (14.5)	12 (12)	0.64
Self-reported alcohol intake, N (%)	37 (68.5)	47 (68.1)	0.26	33 (53.2)	64 (64)	0.025
Anthropometric measurements						
Height (cm)	169 ± 5.75	179 ± 6.07	<0.001	159 ± 6.63	168 ± 5.96	<0.001
Weight (kg)	62.1 (47.8; 88.0)	87.4 (63.2; 124)	<0.001	65.8 (46.7; 96.3)	66.9 (52.9; 102)	0.59
Body mass index (kg/m^2)	21.7 (17.1; 30.1)	27.3 (20.2; 40.4)	<0.001	25.9 (18.5; 39.9)	23.7 (18.3; 34.9)	0.004
Waist circumference (cm)	73.9 (63.0; 95.0)	90.3 (70.0; 122)	<0.001	78.3 (63.7; 103)	74.5 (63.3; 103)	0.029
Waist:Height ratio	0.44 (0.38; 0.55)	0.50 (0.40; 0.67)	<0.001	0.49 (0.39; 0.64)	0.44 (0.38; 0.62)	<0.001

	Men N=145			Women N=186		
	Black	White	***P*-value**	Black	White	***P*-value**
Clinic blood pressure						
bSBP (mmHg)	125 ± 11.6	125 ± 8.32	0.72	115 ± 9.32	109 ± 9.85	<0.001
bDBP (mmHg)	81.1 ± 8.25	80.2 ± 6.76	0.45	78.5 ± 7.29	74.9 ± 6.91	<0.001
cSBP (mmHg)	113 ± 9.63	110 ± 7.24	0.013	108 ± 7.56	103 ± 8.67	<0.001
24hr Blood pressure						
SBP (mmHg)	119 ± 8.40	125 ± 7.28	<0.001	113 ± 8.17	113 ± 8.46	0.83
Day	124 ± 8.69	129 ± 7.78	<0.001	117 ± 8.17	117 ± 8.75	0.66
Night	111 ± 10.4	115 ± 9.47	0.014	105 ± 9.53	103 ± 8.93	0.26
DBP (mmHg)	69.1 ± 6.27	70.9 ± 6.09	0.098	68.8 ± 5.07	68.3 ± 5.56	0.53
Day	73.9 ± 6.62	75.7 ± 6.73	0.12	72.8 ± 5.28	73.2 ± 5.84	0.62
Night	59.6 ± 7.39	61.4 ± 7.55	0.16	60.7 ± 6.33	58.6 ± 6.03	0.027
Beat-to-beat cardiovascular measurements						
Heart rate (bpm)	57.7 ± 7.55	62.3 ± 9.94	0.002	69.7 ± 9.08	66.9 ± 10.1	0.063
Stroke volume (mL)	81.4 (52.5; 119)	109 (82.3; 157)	<0.001	75.1 (49.2; 103)	85.8 (62.7; 129)	<0.001
TPR (mmHg/ml/s)	1.34 (0.88; 2.04)	0.88 (0.54; 1.36)	<0.001	1.19 (0.82; 1.80)	1.01 (0.67; 1.56)	<0.001
24hr Urinary profile						
MBG (nmol/L)	3.36 (1.58; 7.49)	3.49 (1.59; 6.94)	0.65	2.53 (0.88; 5.93)	2.28 (0.81; 5.93)	0.16
MBG excretion (nmol/L/24hr)	3.99 (1.92; 10.2)	4.69 (1.87; 10.1)	0.067	2.82 (1.29; 6.45)	2.52 (0.81; 7.86)	0.20
Na^+ (mmol/day)	146 (54.8; 324)	152 (51.7; 287)	0.61	114 (50.5; 270)	114 (47.6; 257)	0.95
MBG/Na^+ excretion ratio	0.03 (0.01; 0.06)	0.03 (0.02; 0.07)	0.16	0.02 (0.01; 0.07)	0.02 (0.01; 0.07)	0.24
NaCl intake (g/day)	8.52 (3.20; 18.6)	8.91 (3.02; 16.8)	0.61	6.65 (2.78; 15.0)	6.68 (2.95; 15.8)	0.95
Volume (ml)	1185 (546;2457)	1342 (557; 3057)	0.12	1117 (550;2057)	1134 (557; 2946)	0.82
K^+ (mmol/L)	28.8 (13.7; 72.4)	36.7 (17.1; 73.3)	0.011	27.5 (11.5; 59.9)	34.7 (12.3; 74.6)	0.004
Na:K ratio	4.26 (2.17; 7.14)	3.10 (1.21; 6.60)	<0.001	3.72 (1.73; 7.62)	2.86 (1.41; 5.68)	<0.001
Albumin (mg/24hr)	4.19 (1.53; 16.2)	3.87 (1.58; 10.4)	0.49	4.72 (1.98; 11.8)	3.98 (1,19; 12.3)	0.11
Biochemical profile						
Total cholesterol (mmol/L)	3.77 (2.80; 5.10)	4.58 (3.29; 5.95)	<0.001	3.84 (2.70; 5.35)	4.68 (3.42; 6.50)	<0.001

	Men N=145			Women N=186		
	Black	White	***P*-value**	Black	White	***P*-value**
HDL-C (mmol/L)	1.33 (0.84; 1.82)	1.09 (0.77; 1.57)	<0.001	1.81 (0.79; 1.88)	1.57 (1.03; 2.48)	<0.001
HbA1c (%)	5.48 ± 0.28	5.28 ± 0.24	<0.001	5.52 ± 0.31	5.29± 0.27	<0.001
γ-glutamyl transferase (U/L)	27.5 (13.0; 76.6)	24.3 (10.8; 65.4)	0.28	24.3 (10.5; 57.4)	14.1 (6.70; 40.7)	<0.001
Aldosterone (pg/ml)	61.3 (16.8; 155)	88.5 (30.8; 308)	0.004	70.1 (28.1; 224)	104 (28.4; 614)	0.003

Mean ± standard deviation; geometric mean (5 percentile; 95 percentile).
bSBP, Brachial systolic blood pressure bDBP, Brachial diastolic blood pressure; cSBP, Central systolic blood pressure; DBP, Diastolic blood pressure; HbA1c, Glycated haemoglobin; HDL-C, High density lipoprotein cholesterol; K^+, Potassium; MBG, Marinobufagenin; Na^+, Sodium; SBP, Systolic blood pressure; TPR, Total peripheral resistance.

All anthropometric measures were higher in white men compared to black men, whereas black women had a larger BMI, waist circumference and waist:height ratio than white women. In terms of the cardiovascular profile, black men and women displayed a higher cSBP and TPR, and lower stroke volume than whites. However, black men displayed lower systolic ABPM measurements, than white men. A collective mean 24hr salt intake of 8.44 g/day was found in all four groups which exceeds the World Health Organization's (WHO) daily recommendation of less than 5g/day.[14] There were no apparent ethnic differences in MBG excretion ($p \geq 0.16$) or MBG/Na^+ ($p \geq 0.067$) in men or women, but both black ($p=0.005$) and white men ($p<0.001$) had a higher salt intake as well as MBG excretion compared to women. Both black men and women had significantly lower aldosterone levels compared to their white counterparts.

Regression Analyses

Single regression analyses for men and women are shown in **Supplementary table 1A** and **Supplementary table 1B**, respectively. The results of the regression analyses with regards to the MBG/Na^+ excretion ratio will mainly be discussed in women as we found no statistically significant associations with cardiovascular variables in either black or white men. 24hr SBP (r=0.24; p=0.038) and daytime SBP (r=0.23; p=0.050) correlated significantly with MBG excretion alone in white men and black women respectively. Only in black women the MBG/Na^+ excretion was positively related to cSBP (r=0.25; p=0.034), daytime (r=0.23; p=0.047) and nighttime (r=0.24; p=0.045) systolic ABPM (**Supplementary table 1A**). In white women there was a negative association with nighttime SBP (r=-0.20; p=0.038).

When comparing SBP according to MBG/Na^+ quartiles (**Figure 1**), black women exhibited a significant positive trend in cSBP (p=0.003) as well as nighttime systolic ABPM (p=0.013) with increasing MBG/Na^+ quartiles. Additionally all the SBP measurements of black women were higher within the highest MBG/Na^+ excretion quartile when compared to white women, as seen in **Figure 1**. We found no significant trends in men. Adjusting for age and waist:height ratio we compared 24hr urinary MBG excretion across increasing quartiles of NaCl intake. It was evident that MBG excretion increased significantly along with an increase in NaCl within black and white, men and women (**Supplementary Figure 1**). Regardless it is clear that there is no correlation between NaCl and SBP with the exception of nighttime ABPM in white men.

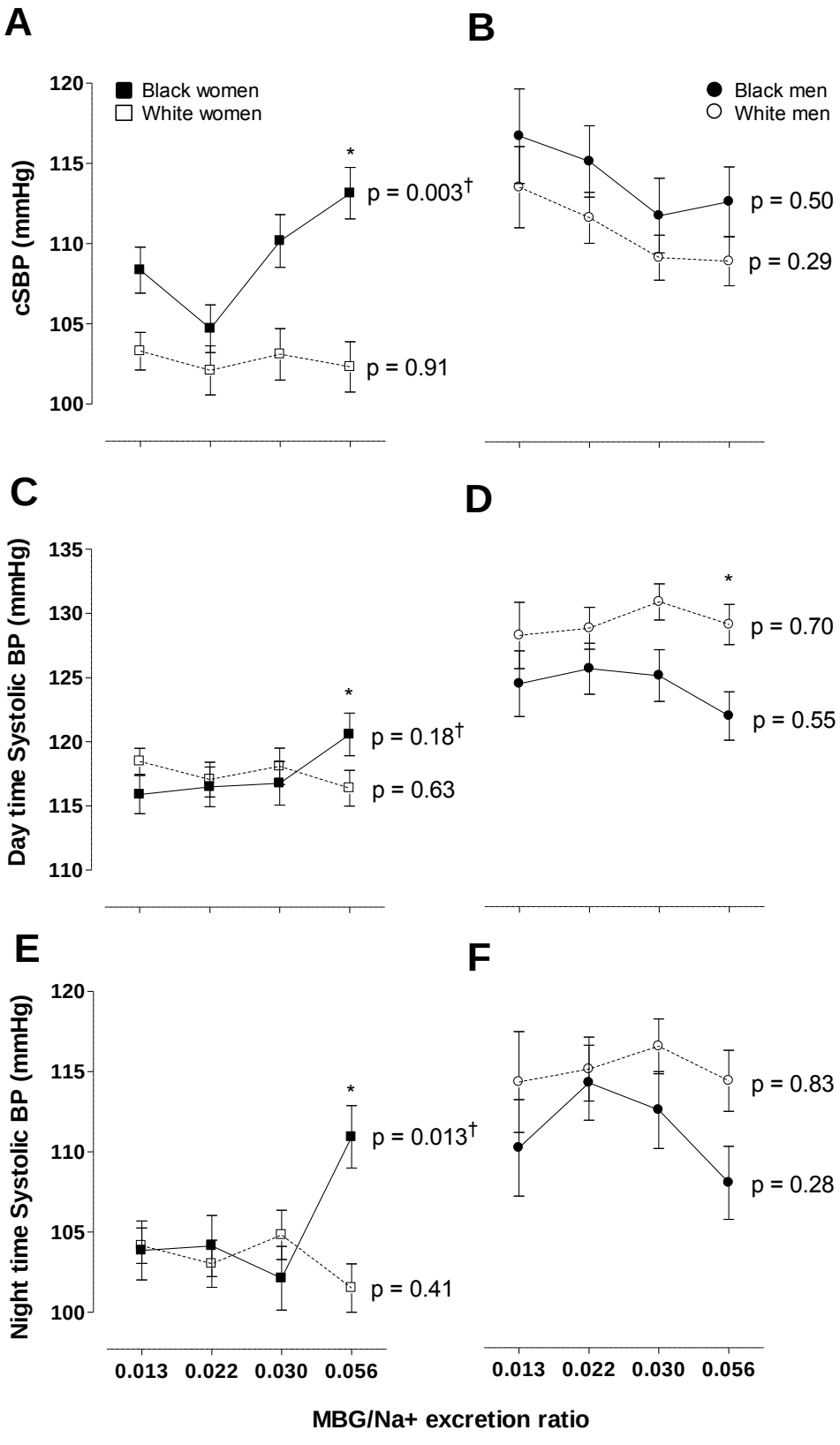

Figure 1: **Ethnic differences in cSBP, daytime systolic ABPM and nighttime systolic ABPM within MBG/Na$^+$ quartiles (adjusted for age and waist:height ratio).**

*Significant differences (P<0.05) between black and white participants within the same MBG/Na$^+$ quartile.
†Significant differences (P< 0.05) between 1st and 4th quartiles within group.

To determine the independent associations between SBP and MBG/Na^+ excretion ratio in women we performed multiple regression analyses adjusting for potential confounders, as shown in **Table 2**. Only in black women we found independent positive associations of cSBP (R^2=0.26; *ß*=0.28; *p*=0.039), 24hr SBP (R^2=0.46; *ß*=0.30; *p*=0.011), daytime (R^2=0.38; *ß*=0.28; *p*=0.023) and nighttime SBP (R^2=0.38; *ß*=0.33; *p*=0.009) with MBG/Na^+ excretion ratio, confirming previous unadjusted associations. In addition, TPR (*ß*=-0.33; *p*=0.018) and stroke volume (*ß*=0.29; *p*=0.036) were significantly explained by MBG/Na^+. In contrast, non-significant inverse associations of all SBP measures with the MBG/Na^+ excretion ratio were evident in white women. Nonetheless independent associations of all hemodynamic measures with MBG/Na^+ were absent in young black and white men (not shown).

Sensitivity Analyses

We performed a sensitivity analysis to investigate whether the associations between cSBP and 24hr SBP with the MBG/Na^+ excretion ratio were confounded by socio-economic status of participants. In doing so, these results confirmed the robustness of our associations, which remained significant in black women (**Supplementary table 2**).

Table 2. Respective multiple regression analyses of blood pressure and hemodynamic variables with MBG/Na^+ excretion ratio as the main independent variable in black and white women

	MBG/Na^+ excretion ratio					
	Black women			White women		
Dependent variable	**Adj. R^2**	**ß (95% CI)**	***P***	**Adj. R^2**	**ß (95% CI)**	***P***
cSBP (mmHg)	0.259	0.279 (0.02; 0.54)	**0.039**	0.251	-0.112 (-0.29; 0.07)	0.23
24hr SBP (mmHg)	0.458	0.298 (0.08; 0.52)	**0.011**	0.412	-0.082 (-0.24; 0.08)	0.31
Day	0.379	0.283 (0.05; 0.52)	**0.023**	0.392	-0.053 (-0.21; 0.11)	0.52
Night	0.381	0.334 (0.09; 0.57)	**0.009**	0.361	-0.134 (-0.30; 0.03)	0.12
24hr DBP (mmHg)	0.192	0.223 (-0.05; 0.49)	0.11	0.117	-0.03 (-0.23; 0.17)	0.75
Day	0.112	0.262 (-0.02; 0.54)	0.075	0.076	-0.030 (-0.23; 0.15)	0.77
Night	0.099	0.222 (-0.07; 0.51)	0.14	0.165	-0.037 (-0.17; 0.21)	0.71
Stroke volume (mL)	0.259	0.286 (0.03; 0.55)	**0.036**	0.171	0.023 (-0.17; 0.21)	0.81
TPR (mmHg/ml/s)	0.214	-0.334 (-0.60; -0.07)	**0.018**	0.164	0.115 (-0.07; 0.30)	0.24

Each dependent variable represents a separate multiple regression analysis.
All models included the following covariates: age; waist:height ratio; γ-glutamyl transferase; glycated haemoglobin; total cholesterol: high density lipoprotein cholesterol ratio; MBG/Na^+ excretion ratio.
cSBP, central systolic blood pressure; DBP, diastolic blood pressure; MBG, marinobufagenin; Na^+, sodium; SBP, systolic blood pressure; TPR, total peripheral resistance.
Bold values indicate statistical significance ($P<0.05$).

Discussion

We found that cSBP, as well as daytime and nighttime SBP, related positively and independently with a proposed estimate (MBG/Na^+) of Na^+ excretion resistance to elevated MBG levels in young black, but not white women. Additionally we found no significant relationship between different measures of SBP and MBG/Na^+ in either black or white men.

To our best knowledge, only a single reference to MBG and ethnicity was published in 2001, demonstrating that an alternative measure of salt-sensitivity was a significant independent predictor of increased urinary MBG excretion in black women.[15] Although higher urinary

MBG was indicative of volume expansion in their study, they did not measure cSBP, stroke volume or vascular resistance to explore possible hemodynamic mechanisms. Our results add to these findings in black women,[15] suggesting that a blunted natriuretic response to MBG could initiate a volume related increase in SBP.

Despite similar daytime and nighttime SBP in young black and white women from our study, the black women had significantly higher cSBP and vascular resistance, and lower stroke volume, albeit within the normal ranges. cSBP is an independent predictor of increased cardiovascular risk and mortality,[16,17] and therefore these differences may reflect the distinct manner in which BP is regulated within black women. Our results are in agreement with previous reports in black and white men,[18] women[19] and children[20], indicating that black populations have a significantly increased vascular resistance together with a lower stroke volume compared to their white counterparts. Nonetheless, we found a positive association between stroke volume and MBG/Na^+ in black women, which may reflect the initiation of sodium-induced volume expansion. Accordingly elevated MBG has been associated with pathophysiological volume expansion.[21] Volume overloading causes the blood vessels to distend as a compensatory homeostatic mechanism, and although speculative may explain the inverse association observed with MBG/Na^+ and TPR in black women. Thus despite our initial hypothesis, a higher TPR in black women compared to white women may not be attributed to MBG/Na^+ at this young age.

Nonetheless the long-term shift to a high pressure environment within blood vessels promotes endothelial cell activation, stimulating the release of neurohormonal and inflammatory markers.[22] Coincidently, early vascular alterations in young black individuals tend to be pressure-related.[23] Indeed, we have shown that the young black women from the present study exhibit an elevated inflammatory profile with higher levels of interleukin-6, C-reactive

protein, tumor necrosis factor-α along and monocyte chemoattractant protein-1, a marker of endothelial activation.[24]

We found clear contrasting results in white women, namely a significant inverse association between nighttime SBP and MBG/Na^+ in single regression. This relationship became weaker after adjusting for various covariates (ß=-0.13, p=0.12). This natriuretic tendency is in accordance with the normal physiological response expected from a renal α1-Na^+K^+-ATPase inhibitor, similarly demonstrated by Anderson et al.[9]

Apart from these findings in women, based on the literature we did expect a strong relationship between sodium, MBG and SBP[7,10,25] in men.[8,11] A previous study in a small cohort of middle-aged men (n=20) found that MBG associated positively with BP.[11] Similar results of a positive correlation (single regression analyses) between 24hr SBP and MBG excretion was evident in the young white men from our study. The absence of associations between MBG/Na^+, SBP and hemodynamic measures in fully adjusted models in our study were surprising since both black and white men had a significantly higher MBG excretion compared to women, likely due to a higher salt intake. The physiological reason as to why these associations with SBP are absent in men is not clear, warranting further investigation as sex differences are observed in human[11] and animal[26] studies.

This is the first known study highlighting ethnic as well as sex differences with associations between MBG/Na^+, SBP, stroke volume and TPR in a large young healthy population. In addition, we suggest using the MBG/Na^+ excretion ratio as a proposed estimate of a blunted natriuretic response in the kidney to excessive 24hr MBG production. We found that the MBG/Na^+ depicted more prominent correlations with SBP and other hemodynamic variables when compared to MBG excretion or Na^+ alone.

Previous short-term intervention studies attributed the BP lowering effect of MBG to its natriuretic activity via the inhibition of renal α1-Na^+/K^+-ATPase,[10,25] and a BP elevating effect due to its vasoconstrictive effect via the inhibition of α1-Na^+/K^+-ATPase in VSMC.[10] Our study however was performed based on habitual dietary salt intake which differs from these studies performed in both Dahl salt-sensitive rats[7,10,25] and humans.[8,9,11,15] As 79% of our participants had a habitual salt intake of more than 5g/day, our results may represent a sustained hemodynamic effect of MBG in young healthy black and white men and women in response to a long-term high sodium diet.[8] Our study extends the limited amount of research conducted in small human cohorts,[8,9,11] by demonstrating associations of MBG/Na^+ in a larger young population, enabling us to explore the role of MBG prior to the onset of pathology.

A strength of our study was the parallel tendencies observed in cSBP, 24hr SBP and supported by stroke volume and TPR hemodynamics, as indicated by three independent devices. As this is a cross-sectional study it limits our ability to examine the physiological sequence of hemodynamic changes as a result of the MBG in a young healthy population. Therefore our results reflect only associations between the MBG/Na^+ excretion ratio and hemodynamic variables and should be interpreted as such. Albeit, the longitudinal design of the African-PREDICT study will allow us to monitor the progression of cardiovascular changes associated with MBG/Na^+ in black and white, men and women. We were not able to take into account salt-sensitivity within each group, since this was not an intervention study. Furthermore we acknowledge that it might be of interest to measure plasma MBG in addition to urinary MBG.

To conclude, in young black women the MBG/Na^+ excretion ratio associated with an increase in cSBP, 24hr SBP and stroke volume which might reflect an increased cardiovascular risk due to abnormal sodium handling. Conversely, the inverse correlation between MBG/Na^+ and

nighttime SBP in young white women supports the concept of the expected natriuretic effect of MBG.

Perspectives

MBG's association with blood pressure has been largely attributed to a salt-sensitive phenotype.[7,10,11] Since black populations are more often than not described to be salt-sensitive,[2-4] even during adolescence,[2] it gives rise to the question as to why significant associations were only observed in black women and not men in this cohort. In this study we showed that MBG/Na^+ excretion ratio associated positively with an increase in cSBP, 24hr SBP and stroke volume in black women, lending support to the notion that MBG may contribute adversely to cardiovascular hemodynamics. Reasons for the dissociating relationship between MBG/Na^+ and various hemodynamic parameters observed in black women when compared to the other groups is unknown. Thus this study suggests ethnic as well as sex differences with regards to the pathophysiological role of MBG, meriting additional in-depth research.

Furthermore, for the first time we show that the use of a ratio comprising urinary MBG and Na^+ excretion, yielded more prominent correlations with SBP and other hemodynamic variables when compared to MBG excretion or Na^+ alone. This could suggest a better sensitivity of this ratio beyond the individual use of MBG to detect vascular alterations as the ratio may be more descriptive of the blunted renal natriuretic response to excessive MBG production. The usefulness of this ratio should be considered in future studies.

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Disclosures

None.

Novelty and Significance

What is New?

- For the first time we introduce the use of the MBG/Na^+ excretion ratio as a proposed estimate of a blunted natriuretic response in the kidney to excessive MBG.
- Investigating MBG in young healthy black individuals compared to their white counterparts we demonstrated clear ethnic and sex differences with regards to the relationship of the MBG/Na^+ excretion ratio with cSBP, 24hr SBP, stroke volume and TPR.

- The MBG/Na^+ excretion ratio associates positively with an increase in cSBP, 24hr SBP and stroke volume, but only in black women, supporting suggestions that MBG may contribute adversely to cardiovascular hemodynamics.

What is Relevant?

Our findings suggest that a blunted natriuretic response to MBG could contribute to an unfavorable hemodynamic milieu in black women, with possible implications for future cardiovascular risk. Conversely, MBG adheres to its normal natriuretic tendency expected from a renal α1-Na^+K^+-ATPase inhibitor in young healthy white women. This study thus supports an alternative mechanism possibly contributing to the prevalence of salt-sensitive hypertension.

Summary

Our results lend support to the possible role of MBG and its inter-regulation with Na^+ as indicated by the findings with the MBG/Na^+ excretion ratio. Despite similar urinary MBG excretion and MBG/Na^+, the role of MBG together with Na^+ already seems deviant within different sex and ethnic groups. In black women, high MBG along with attenuated natriuresis may play an important role in mediating cardiovascular hemodynamics reflecting a salt-sensitive hypertension phenotype. Conversely, it seems apparent that in white women MBG adheres to its normal physiologic natriuretic tendency.

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Online data supplement

MARINOBUFAGENIN IS RELATED TO ELEVATED CENTRAL AND 24 HR SYSTOLIC BLOOD PRESSURES IN YOUNG HEALTHY BLACK WOMEN: THE AFRICAN-PREDICT STUDY

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Supplementary table 1A. Pearson correlations with MBG excretion or MBG/Na^+ excretion ratio in women

	Black		White	
	MBGexc (nmol/L/24hr)	MBG/Na^+	MBGexc (nmol/L/24hr)	MBG/Na^+
Age (years)	r= -0.23; p= 0.049	r= -0.24; p= 0.036	r= -0.11; p= 0.24	r= -0.83; p= 0.38
Waist:Height ratio	r= 0.087; p= 0.46	r= 0.057; p= 0.63	r= -0.11; p= 0.24	r= -0.15; p= 0.11
Blood pressure measurements				
cSBP (mmHg)	r= 0.18; p= 0.13	r= 0.25; p= 0.034	r= -0.14; p= 0.15	r= -0.15; p= 0.11
24hr SBP (mmHg)	r= 0.21; p= 0.067	r= 0.21; p= 0.073	r= -0.10; p= 0.27	r= -0.17; p= 0.067
Day	r= 0.23; p= 0.050	r= 0.23; p= 0.047	r= -0.10; p= 0.27	r= -0.15; p= 0.12
Night	r= 0.13; p= 0.28	r= 0.24; p=0.045	r= -0.11; p= 0.27	r= -0.20; p= 0.038
24hr DBP (mmHg)	r= 0.11; p= 0.35	r= 0.15; p= 0.22	r= -0.17; p= 0.073	r= -0.084; p= 0.38
Day	r= 0.12; p= 0.29	r= 0.23; p= 0.048	r= -0.15; p= 0.11	r= -0.065; p=0.50
Night	r= 0.010; p=0.93	r= 0.11; p= 0.37	r= -0.19; p= 0.05	r= -0.10; p= 0.29
Urinary and biochemical profile				
24hr U-volume (ml)	r= 0.31; p= 0.008	r= -0.098; p= 0.41	r= 0.41; p≤ 0.001	r= 0.001; p= 0.99
NaCl intake (g/day)	r= 0.29; p= 0.011	r= -0.57; p≤ 0.001	r= 0.41; p≤ 0.001	r= -0.38; p≤ 0.001
24hr U-Na (mmol/day)	r= 0.29; p= 0.011	r= -0.57; p≤ 0.001	r= 0.41; p≤ 0.001	r= -0.38; p≤ 0.001
24hr U-K (mmol/L)	r= 0.11; p= 0.35	r= -0.075; p= 0.53	r= 0.032; p= 0.74	r= 0.026; p= 0.79
24hr U Na:K ratio	r= -0.11; p= 0.35	r= -0.44; p≤ 0.001	r= -0.033; p= 0.73	r= -0.42; p≤ 0.001
24hr U-Albumin (mg/24hr)	r= 0.19; p= 0.16	r= -0.22; p= 0.10	r= 0.33; p= 0.001	r= 0.007; p= 0.95
Total cholesterol (mmol/L)	r= -0.13; p= 0.32	r= -0.26; p= 0.047	r= -0.22; p= 0.027	r= -0.19; p= 0.054
TC:HDL-C	r= -0.72; p= 0.61	r= -0.022; p= 0.88	r= 0.20; p= 0.049	r= 0.13; p= 0.22
HbA1c (%)	r= -0.031; p= 0.82	r= -0.34; p= 0.008	r= -0.13; p= 0.18	r= -0.13; p= 0.20
Aldosterone (pg/ml)	r= 0.10; p= 0.42	r= 0.15; p= 0.23	r= 0.034; p= 0.74	r= 0.009; p= 0.93
γ-glutamyl transferase (U/L)	r= 0.17; p= 0.20	r= 0.034; p= 0.79	r= -0.015; p= 0.88	r= -0.19; p= 0.063

cSBP, Central systolic blood pressure; DBP, Diastolic blood pressure; HbA1c, Glycated haemoglobin; K^+, Potassium; MBG, Marinobufagenin; Na^+, Sodium; SBP, Systolic blood pressure; TC:HDL-C, Total cholesterol: High density lipoprotein cholesterol ratio.

Supplementary table 1B. Pearson correlations with MBG excretion or MBG/Na^+ excretion ratio in men

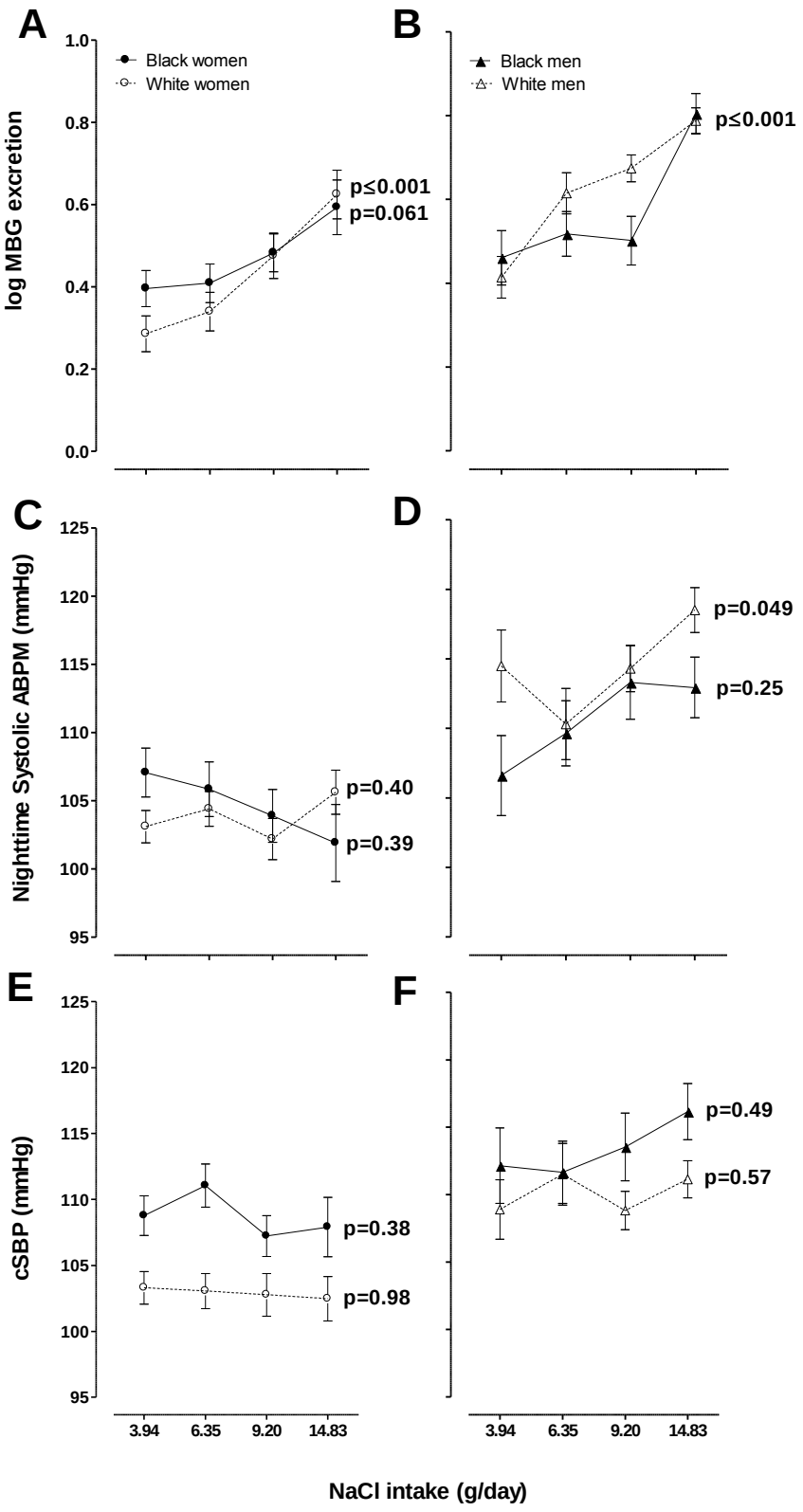
	Black		White	
	MBGexc (nmol/L/24hr)	MBG/Na^+	MBGexc (nmol/L/24hr)	MBG/Na^+
Age (years)	r= -0.12; p= 0.33	r= -0.032; p= 0.79	r= 0.008; p= 0.95	r= -0.081; p= 0.48
Waist:Height ratio	r= 0.22; p= 0.055	r= -0.057; p= 0.62	r= 0.040; p= 0.75	r= -0.13; p=0.30
Blood pressure measurements				
cSBP (mmHg)	r= -0.089; p= 0.47	r= -0.21; p= 0.087	r= -0.085; p= 0.47	r= -0.189; p= 0.10
24hr SBP (mmHg)	r= 0.084; p= 0.50	r= -0.090; p= 0.47	r= 0.24; p= 0.038	r≤ 0.001; p= 1.00
Day	r= 0.017; p= 0.89	r= -0.073; p= 0.56	r= 0.22; p= 0.055	r= 0.016; p= 0.92
Night	r= 0.18; p= 0.14	r= -0.047; p= 0.71	r= 0.17; p=0.13	r= -0.025; p= 0.83
24hr DBP (mmHg)	r= -0.003; p= 0.98	r= 0.025; p= 0.84	r= 0.11; p= 0.36	r= 0.014; p= 0.91
Day	r= -0.090; p= 0.47	r= -0.017; p= 0.89	r= 0.11; p= 0.35	r= 0.011; p= 0.92
Night	r= 0.084; p= 0.50	r= 0.13; p= 0.30	r= 0.07; p= 0.54	r= -0,013; p= 0.91
Urinary and biochemical profile				
24hr U-volume (ml)	r= 0.47; p≤ 0.001	r= -0.20; p= 0.099	r= 0.47; p≤ 0.001	r= -0.34; p= 0.003
NaCl intake (g/day)	r= 0.51; p≤ 0.001	r= -0.43; p≤ 0.001	r= 0.63; p≤ 0.001	r= -0.54; p≤ 0.001
24hr U-Na (mmol/day)	r= 0.51; p≤ 0.001	r= -0.43; p≤ 0.001	r= 0.63; p≤ 0.001	r= -0.55; p≤ 0.001
24hr U-K (mmol/L)	r= 0.04; p= 0.75	r= -0.19; p= 0.12	r= 0.33; p= 0.004	r= 0.34; p= 0.003
24hr U Na:K ratio	r= 0.055; p= 0.66	r= -0.075; p= 0.55	r= -0.13; p= 0.26	r= -0.61; p≤ 0.001
24hr U-Albumin (mg/24hr)	r= 0.54; p≤ 0.001	r= -0.069; p= 0.63	r= 0.38; p= 0.001	r= -0.18; p= 0.13
Total cholesterol (mmol/L)	r= 0.10; p= 0.48	r= 0.066; p= 0.65	r= 0.15; p= 0.23	r= 0.019; p= 0.88
TC:HDL-C	r= 0.19; p= 0.19	r= -0.035; p= 0.81	r= 0.12; p= 0.32	r= -0.058; p= 0.64
HbA1c (%)	r= -0.014; p= 0.92	r= -0.039; p= 0.79	r= -0.067; p= 0.59	r= 0.11; p= 0.39
Aldosterone (pg/ml)	r= 0.35; p= 0.011	r= 0.24; p= 0.088	r= 0.12; p= 0.31	r= 0.11; p= 0.39
γ-glutamyl transferase (U/L)	r= 0.081; p= 0.57	r= 0.37; p= 0.79	r= 0.11; p= 0.38	r= 0.059; p= 0.63

cSBP, Central systolic blood pressure; DBP, Diastolic blood pressure; HbA1c, Glycated haemoglobin; K^+, Potassium; MBG, Marinobufagenin; Na^+, Sodium; SBP, Systolic blood pressure; TC:HDL-C, Total cholesterol: High density lipoprotein cholesterol ratio.

Supplementary table 2. Multiple regression analyses of blood pressure and hemodynamic variables with MBG/Na^+ excretion ratio as the main independent variable in black and white women

	Independent variable MBG/Na^+ excretion ratio					
	Black women			**White women**		
Dependent variable	**Adj. R^2**	**ß**	***P***	**Adj. R^2**	**ß**	***P***
cSBP (mmHg)	0.247	0.286	0.037	0.244	-0.114	0.22
24hr SBP (mmHg)	0.482	0.314	0.007	0.406	-0.083	0.31
Day	0.419	0.303	0.013	0.386	-0.055	0.51
Night	0.367	0.336	0.010	0.355	-0.133	0.12
Stroke volume (mL)	0.256	0.305	0.028	0.171	0.027	0.78
TPR (mmHg/ml/s)	0.207	-0.351	0.015	0.156	0.113	0.25

Each dependent variable represents a separate multiple regression analysis.
All models included the following covariates: socio-economic status; age; waist:height ratio; γ-glutamyl transferase; glycated haemoglobin; total cholesterol: high density lipoprotein cholesterol ratio; MBG/Na^+ excretion ratio.
cSBP, central systolic blood pressure; MBG, marinobufagenin; Na^+, sodium; SBP, systolic blood pressure; TPR, total peripheral resistance.



Supplementary Figure 1. Ethnic differences in MBG excretion according to NaCl intake quartiles in women (A) and men (B). Nighttime SBP (women (C); men (D)) and cSBP (women (E); men (F)) according to NaCl intake quartiles (adjusted for age and waist:height ratio).

Chapter 5

Final Remarks and Recommendations for Future Studies

5.1 Introduction

This chapter interprets and summarizes the main findings of this study, allowing me to accept or reject the initial hypotheses set in Chapter 2. In conjunction, these findings will be compared to the relevant literature on marinobufagenin (MBG). Finally, recommendations will be made for future studies investigating the potential role of 24hr urinary MBG in sodium handling, with possible implications on the cardiovascular hemodynamics of black and white adults.

5.2 Interpretations and Summary of Key Findings

The literature underlines the disparity between the pathophysiology contributing to cardiovascular disease (CVD) in black and white individuals,[1-4] encompassing mechanisms contributing to salt-sensitivity, especially in black populations.[5-9] Studies have proposed a role for an alternate mode of MBG functioning in the salt-sensitive hypertension phenotype which include natriuretic[10,11] and vasoconstrictive[12,13] properties. Thus, this study reviewed the 24hr urinary MBG and sodium profiles between young black and white, men and women. Thereafter, the ratio of 24hr urinary MBG and sodium (MBG/Na^+) was determined, which provides an indication of the amount of MBG molecules required to inhibit the reabsorption of one Na^+ ion via the Na^+/K^{+-}ATPase pump. Therefore, the ratio was used as a proposed estimate of renal sodium excretion resistance. We further investigated the association of the MBG/Na^+ excretion ratio with systolic blood pressure (SBP) within the four sex and ethnic groups. Although literature on MBG in humans is scant, it was possible to derive certain hypotheses based on these studies in combination with observations made in Dahl salt-sensitive rats.[12]

Hypothesis 1: ***Urinary MBG levels are higher in blacks compared to whites.***

We found no ethnic differences with regards to MBG excretion or in the MBG/Na^+ excretion ratio in our sample of young healthy black and white men and women. This is in contrast to the findings of Anderson *et al*. who demonstrated a higher urinary MBG excretion in older white individuals without any previous history of chronic illness.[14] Their initial hypothesis was in line with the hypothesis of this study, based on the literature regarding the relationship between MBG and salt-sensitivity.[12,15] Despite findings from several studies indicating higher sodium retention in black individuals[5-9] even at a younger age,[5] the sodium excretion of black and white men and women where similar. Since this study was performed in young individuals on a habitual diet, it was not possible to account for salt-sensitivity within groups. Previously described in a young adult cohort between the ages of 18 to 23 years, 37.3% of the black participants were characterized as salt-sensitive in comparison to 18.4% of white participants.[16] However, it has been demonstrated by Bochud *et al.* that the fractional sodium excretion of black South African individuals (0.55%) is significantly lower when compared to that of white individuals (0.88%), on a habitual diet, indicative of less efficient sodium handling regardless of salt-sensitivity.[9] Still, it is possible that some of the participants from this study might be salt-resistant, hindering the ability to truly investigate the ethnic differences in the functioning of MBG. Regardless of this, based on the basic characteristics of this young healthy study population, I reject my first hypothesis.

Hypothesis 2: ***Urinary MBG levels are higher in men compared to women.***

The results of this study show that the 24hr urinary MBG excretion of young black and white men is significantly higher when compared to black and white women respectively. Similarly, Fedorova *et al.* demonstrated that in an older white population, with an average age of 53 ± 11 years, the men also had a higher urinary MBG excretion than women.[15] Pearson analyses indicated a strong positive correlation between salt intake and MBG in all four subgroups of black and white, men and women, which persisted after adjusting for age and waist:height ratio. This relationship is expected since MBG is synthesized and released in response to sodium-induced volume expansion.[17] The confirmation of this positive relationship in all four subgroups, therefore, asserts the robustness of our study's results and biochemical analyses. Thus a higher salt consumption by the men in this study is expected to augment their MBG production and excretion. Notably the men and women from the African-PREDICT study had higher concentrations of 24hr urinary MBG excretion in comparison to other studies (**Figure 1**). It was not possible to compare the 24hr urinary MBG excretion from the black and white participants from our study with the 24hr urinary MBG measured in the study performed by Anderson *et al.*,[14] due to incomparable measuring units. Urinary MBG excretion has been shown to be inversely related to age,[18] which might explain the higher urinary MBG concentrations in the young African-PREDICT cohort in comparison to the other studies. It is however more likely that the higher 24hr urinary MBG concentrations are attributed to 79% of the population consuming more than five grams of salt per day in their habitual diet considering the significant positive correlation with sodium intake. In conclusion, it can be confirmed that men presented with higher 24hr urinary MBG concentrations than women, and therefore this hypothesis is accepted.

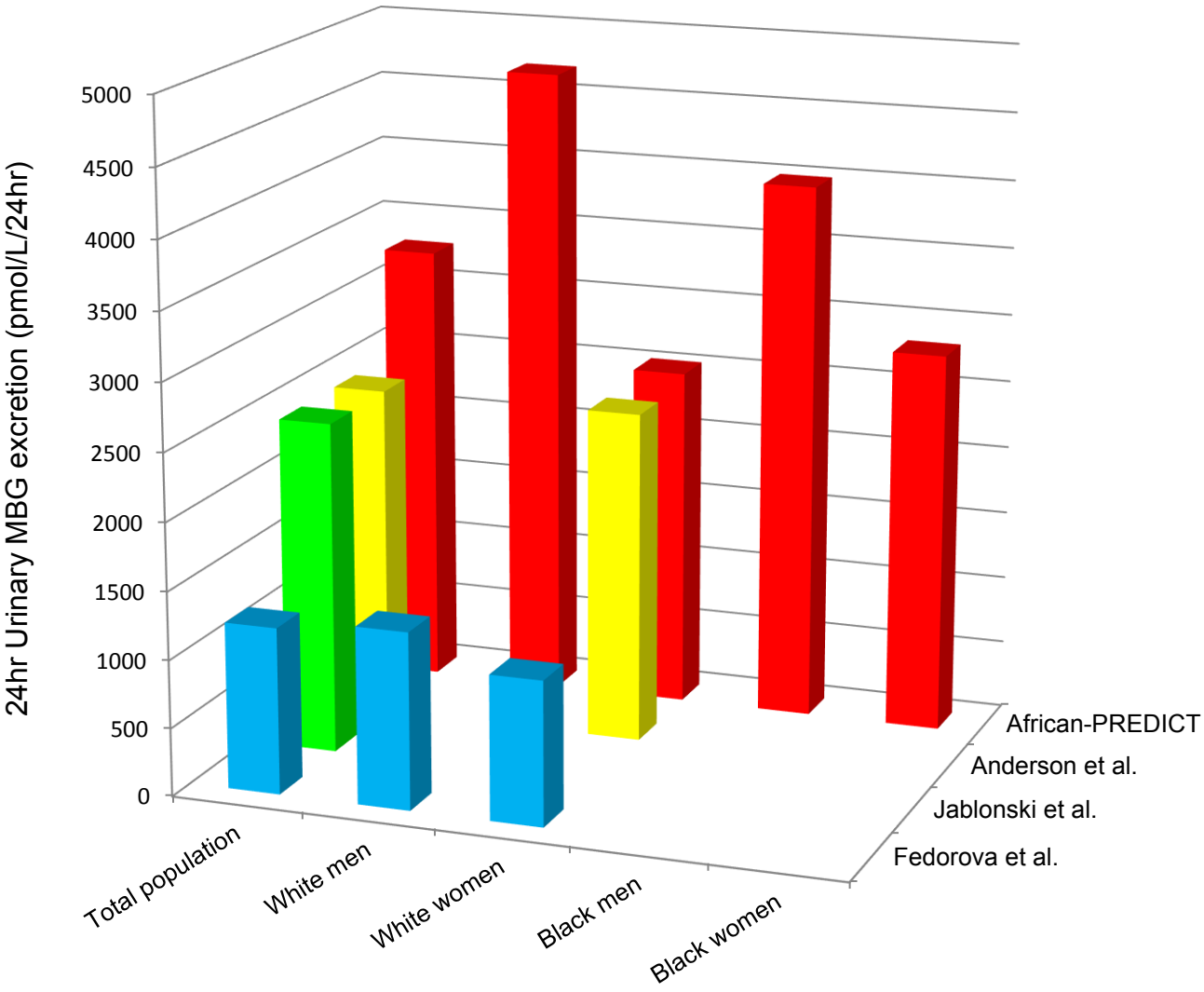


Figure 1: Comparison of 24hr Urinary MBG excretion of participants from the African-PREDICT study (Total mean age 25 ± 3.15) and other human studies. (a) Anderson *et al*.[18]—apparently healthy population of white women (n=28) (Total mean age 53 ± 1.6 years). (b) Jablonski *et al.*[19]—8 men and 3 women with a resting SBP between 130-159 mmHg (Total mean age 60 ± 2 years). (c) Fedorova *et al.*[15]—apparently healthy older population of white men (n=20) and women (n=19) (Total mean age 53 ± 11).

Hypothesis 3: ***MBG excretion associates positively with sodium excretion and urinary volume.***

Using single linear regression analyses a significant positive correlation between MBG excretion and 24hr urinary volume in black and white, men and women was found (Supplementary table 1A and 1B, p. 92 and 93). The 24hr urinary MBG excretion, furthermore, correlated positively with urinary sodium excretion (mmol/day) in all groups, although these correlations disappeared with 24hr urinary sodium concentration measured as mmol/L, in all but the white men. This observation was in accordance with findings by Fedorova *et al.*[15] The strong correlation between MBG excretion and sodium excretion cohered with the expected physiological function of MBG. The 24hr urinary sodium concentration (mmol/L) was calculated using the formula: sodium excretion (mmol/day)/24hr urinary volume (L), adding a volume component. Thus, the strong correlation of MBG excretion with both sodium excretion and volume are clear, but not clearly evident with 24hr urinary sodium concentration. We cannot neglect possible other factors, which cannot be accounted for, influencing the relationship between MBG, natriuresis and diuresis such as antidiuretic hormone (ADH). Based on all the above findings, we therefore, partially accept this hypothesis.

Hypothesis 4: ***MBG excretion as well as the MBG/Na^+ excretion ratio are positively associated with cSBP and 24hr systolic blood pressure, TPR and stroke volume in blacks. Inverse associations are expected in whites.***

We found that in black women only the MBG/Na^+ excretion ratio was positively associated with cSBP, 24hr systolic blood pressure and stroke volume (**Figure 2**). Associations were weaker or non-significant when MBG excretion or 24hr urinary

sodium was entered in models alone. This suggested that the inter-regulation of MBG and sodium might have been important in the functioning of MBG, leading us to speculate whether a larger percentage of black women from this study were salt-sensitive. As was expected, high urinary MBG levels together with a blunted natriuretic response, induced volume expansion, resulting in an augmented pressor response in black women. These results correspond with findings by Anderson *et al*. associating MBG with volume expansion in black women.[14] Nevertheless, the results of this study are in contrast with the literature associating MBG with an increase in TPR as a result of vascular smooth muscle cell α1-Na^+/K^+-ATPase inhibition.[12]

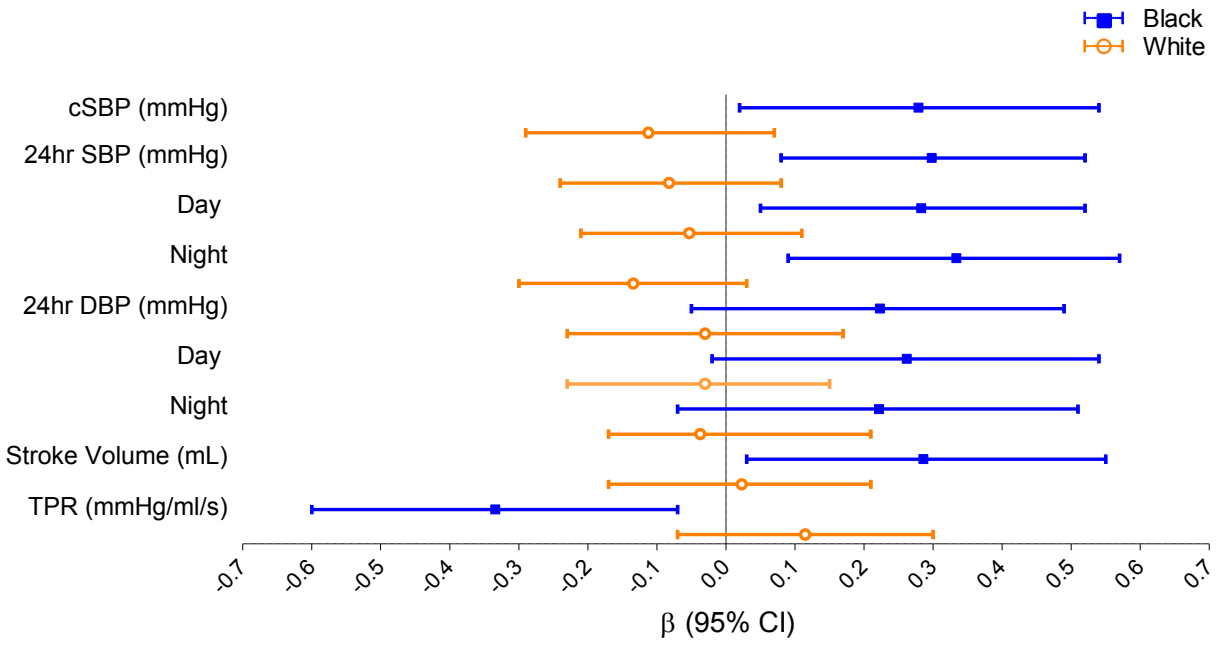
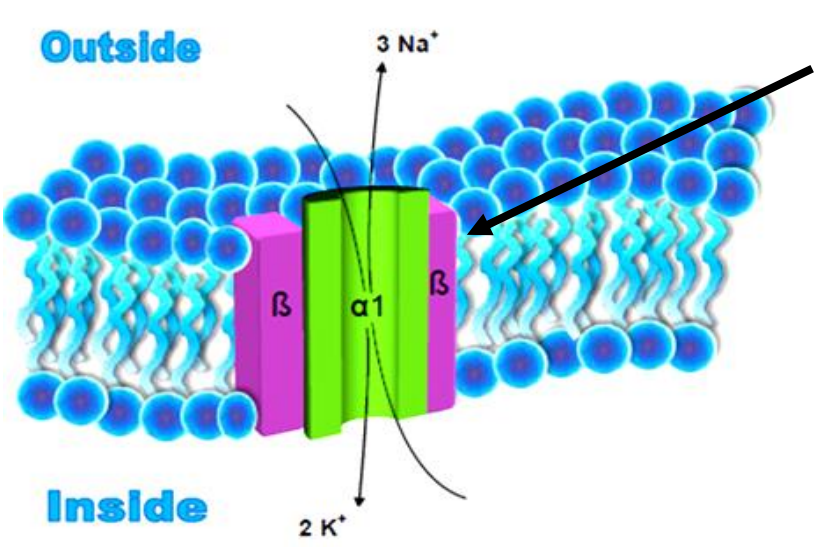


Figure 2: Multiple regression analyses in women with MBG/Na^+ as the main independent variable.

Covariates for each model included age; waist:height ratio; γ-glutamyl transferase; glycated haemoglobin; total cholesterol: high density lipoprotein cholesterol ratio; and MBG/Na^+ excretion ratio.

In the present study a positive association of MBG/Na^+ with stroke volume and SBP together with an inverse association with TPR was observed in black women. This is in coherence with basic cardiovascular ($MAP = CO * TPR$) and hemodynamic principles derived from Ohm's law ($F = \frac{\Delta P}{R}$). In this regard it might suggest that an increase in stroke volume associated with MBG/Na^+, and the concomitant increase in blood flow exerts an increased force within the blood vessels, causing the blood vessels to distend. Additionally, it is possible that the stimulation of stretch sensitive baroreceptors might attenuate the sympathetic outflow to the heart and vasculature, inducing a compensatory decrease in TPR. Blood pressure, however, is not regulated by one pressure control system, but is instead a result of various complex inter-regulated short and long term mechanisms, including amongst others; baroreceptor activity, the renin-angiotensin-aldosterone-system and natriuretic hormones. In fact, the shift to a high pressure environment promotes endothelial cell activation by increasing the shear stress and mechanical stretch, stimulating the release of neurohormonal and inflammatory markers.[20,21] When reviewing the role of MBG in blood pressure and sodium regulation, one cannot disregard the influence of other Na^+/K^+-ATPase regulators including, amongst others, aldosterone,[22,23] ADH,[23,24] progesterone[25] and dopamine[26] (**Figure 3**).



↑ **Na^+/K^+-ATPase activity**

- Aldosterone
- Antidiuretic hormone

Both hormones increase the expression of Na^+/K^+-ATPase

↓ **Na^+/K^+-ATPase activity**

- Progesterone
- Dopamine

Figure 3: Hormonal control of the α1-Na^+/K^+-ATPase pump.

Moreover, in white women a negative relationship, although not significant, between MBG/Na^+ and SBP was found, which is in agreement with the findings of Anderson *et al*., indicative of a natriuretic tendency.[18] Surprisingly, no associations existed in young apparently healthy black and white men. The absence of any associations or trends in both black and white men requires additional research. There are no indices from the existing literature explaining these results. Hence, taking into account the results of this study, I partially accept the last hypothesis: MBG excretion as well as the MBG/Na^+ excretion ratio are positively associated with cSBP and 24hr SBP and stroke volume in blacks with inverse associations expected in whites.

5.3 Limitations, Chance and Confounding factors

While our findings are novel, it is important to reflect on possible limitations and confounding factors that might have influenced this study.

Participants from the African-PREDICT study were recruited from in and around the Potchefstroom area in the North West Province. Therefore, the results of this study might not reflect the true demographic stance across the whole of the young South

African population. Furthermore, 66.9% of the black participants were from a low socio-economic status, whereas 73% of the white participants were from the high socio-economic status group. Some studies have shown that individuals living in rural areas are more vulnerable to an increased risk of cardiovascular morbidity and mortality as a result of unfavorable, modifiable lifestyle and behavioral risk factors such as alcohol consumption, diet, obesity and tobacco use.[27,28] However, other studies have reported an increased burden of hypertension in urban areas, possibly due to a high salt, sedentary and stressful lifestyle.[29,30] Taking into account the latter when entering socio-economic status into multiple regression models, it did not change the robust associations between dependent cardiovascular hemodynamic variables and MBG/Na^+ in black women.

The cross-sectional design of this study limited the ability to examine the physiological sequence of hemodynamic changes as a result of the MBG in a young healthy population. The results, therefore, only reflect associations between the MBG/Na^+ excretion ratio and hemodynamic variables and should be interpreted as such, not implying causality. Furthermore, because no sodium intervention was performed, it was not possible to take into account salt-sensitivity within each group. The sodium intake (g/day) from this study was derived from a single 24hr urinary Na excretion sample (mmol/L). This is also regarded as a limitation since it has been recommended that 24hr urinary sampling be repeated at least 10-15 times in order to decrease the intra-individual variability in estimating sodium intake.[31] We also acknowledge that it might be of interest to measure plasma MBG in addition to urinary MBG, as plasma MBG was shown to be an indicator of salt-sensitivity in older white men.[15]

The likelihood of chance findings should be considered. The influence of this is, however, unlikely as tendencies persisted throughout both single and multiple regression analyses, adjusting for several covariates. Furthermore, similar trends in the relationship of cSBP and 24hr SBP with MBG/Na^+ in black and white women were observed using two independent blood pressure devices, namely the Sphygmocor® XCEL and CardioXplore.

5.4 Recommendations for future studies

- Our study took place as part of the initial phase in the baseline data collection of 1200 participants. The longitudinal study design of the African-PREDICT study provides a unique opportunity to gain valuable information with regard to the association of MBG and cardiovascular hemodynamic changes contributing to the progression of cardiovascular alterations. We recommend that urinary MBG measurements be repeated during the follow-up time period of the African-PREDICT study. Follow-up measurements could help evaluate the predictive value of MBG with particular consideration to the role in sodium handling and deleterious cardiovascular endpoints, such as kidney dysfunction, cardiovascular fibrosis and left ventricular hypertrophy, especially in black women.
- Further investigation should be done with regard to the usefulness of the MBG/Na^+ excretion ratio to confirm our findings as opposed to MBG or Na^+ alone. Succeeding follow-up analyses should determine the predictive value of the MBG/Na^+ excretion ratio, and its relationship with SBP in subclinical end organ damage within stratified sex and ethnic groups.
- We propose that a salt intervention study be performed with the specific purpose of highlighting ethnic and sex differences in MBG and MBG/Na^+. Ideally a study conducted in a sodium controlled metabolic unit, where participant are screened

for salt-sensitivity beforehand, could help in identifying a more accurate role of MBG. This would allow for a more in-depth study of MBG and ethnicity under a controlled sodium environment. Furthermore, an intervention study in which plasma MBG is measured could identify participants within specific groups who might possibly be non-MBG responders.

- An observational study exploring the differences in MBG excretion and its association with cardiovascular hemodynamic variables between a well-defined normotensive and hypertensive population could also be informative. This could allow researchers to investigate MBG as a potential therapeutic target in selective salt-sensitive hypertensive populations.
- We suggest that this study be repeated in a larger randomly selected population representative of the entire South African demographic composition to increase the statistical power within subsequent sex and ethnic group divisions. Additionally, since our study was performed in a young healthy population this study could be repeated in a healthy older population with similar ethnic and sex group divisions. This could help explore the relationship between MBG, cardiovascular aging and renal sodium reabsorption.
- It would be of interest to determine whether the habitual salt intake of individuals with different levels of socio-economic status affects the 24hr urinary MBG excretion, and whether this has an effect on blood pressure regulation. This could be investigated in prospective studies, with a larger sample size, recruiting individuals from different socio-economic backgrounds.
- Elevated MBG has been linked to an increase in oxidative stress (reactive oxygen species) and cardiac fibrosis in animal studies, as well as human cell cultures. Future studies could explore the relationship between MBG, MBG/Na^+

and markers of cardiovascular dysfunction including arterial stiffness, microvascular function and inflammatory and oxidative markers in human participants. This would add to the limited amount of knowledge regarding the potential functionalities of MBG in a human cohort. Furthermore such information could provide an alternative mechanism contributing to adverse vascular alterations contributing to cardiovascular disease.

- It might also be informative if future studies could collect daytime and nighttime urine samples respectively. Luft *et al*. have demonstrated that white normotensive men and women excrete more sodium during the day in comparison to black individuals excreting more sodium during the night.[6] This raises the possibility that MBG excretion might also vary during the daytime and nighttime of black and white, men and women. It follows that disturbances in circadian variations in MBG excretion could be associated with the loss of nocturnal dipping of blood pressure, contributing to an increased risk in CVD. This however needs to be explored.
- The steroidal nature of MBG along with the sex specific results from our study furthermore warrants more in-depth research with regards to the relationship of MBG with sex hormones such as progesterone, estrogen and testosterone amongst others. As the menstrual cycle of the young women from this study is unknown, we recommend that future studies account for the role of sex hormones during this cycle, possibly influencing the function of MBG.

5.5 Conclusion and Perspectives

This study presented the first findings regarding the association of the MBG/Na^+ excretion ratio with cSBP, 24hr SBP, stroke volume and TPR. Furthermore, our findings suggest that the MBG/Na^+ excretion ratio might be associated with the initiation of a volume overload phenotype in young black women. We speculate that the latter could bare implications of a future increased cardiovascular risk due to abnormal sodium handling, providing a possible alternative mechanism contributing to the prevalence of salt-sensitive hypertension. Finally, this study yields evidence with regard to excessive sodium consumption possibly contributing to adverse hemodynamics and increased blood pressure in black women — a population previously shown to have an increased cardiovascular risk.[32]

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Appendices

AUTHOR INSTRUCTIONS

Hypertension

Submitted manuscripts should be original work and may not contain material that has been previously published, with the exception of an abstract. Nor should it be under consideration for publication in another journal. Manuscripts should uphold the requirements of manuscripts submitted to biomedical journals. All manuscripts are examined by journal editors as well as expert reviewers after which a decision will be communicated with regards to the acceptance. Acceptances for the publication of manuscripts are based on the scientific excellence, originality and adherence to the aim and scope of the journal.

Original articles may not exceed a total of 6000 words together with a total of 6 tables or figure. Figures may contain up to a maximum of 6 panels, labeled A, B, C etc. The word count includes all text in the manuscript as calculated by the MS-Word tool accept for supplementary data.

Assembling guidelines:

- Double spaced, 12 point font (including references, figure legends and tables).
- Page margins: 1-inch on all sides.
- Should not use proportional spacing or justified margins.
- Each page should be numbered accept for the title page.
- Figures and tables must be cited in numerical order.
- In text references should be cited in numerical order in the reference section.
- Abbreviations must be defined in the first instance of mention in the text.

Title Page

- Full title of manuscript, in capital letters, limited to 120 characters total.
- Authors' full names and affiliations
- A short title (total characters must not exceed 50, including spaces) to be typeset at the top of the journal page
- Word count of manuscript, including references, figures, legends, word count of abstract, and total number of figures
- The full name, title, and complete address for corresponding author, including street and post office box as well as telephone and fax numbers, and email address

Abstract

- Maximum of 250 words
- Do not use acronyms, abbreviations, subheadings or cite references.
- The abstract should include the rationale for the study, a brief description of methods and presentation of significant results, and a succinct interpretation of the data.
- Provide five to seven key words.

Methods

- Methods have to provide sufficient detail with regards material and protocol in order for experiments to be reproduced.
- Blood pressure methods should be described in detail with regards to the instrument, number of readings, cuff size, arm position and training.
- Statistics should be a subsection under methods and indicate the measures of variance.

Ethics

- Authors should indicate that the study was approved by the institutional ethics committee and that it adheres to the Declaration of Helsinki. Participant characteristics should be described, and that informed consent was given.

Discussion

Should highlight and interpret results, without repeating results.

Perspectives

Less than 250 words, indicating the broad implications, importance and future directions of the study.

References

References should be listed in accordance with the American Medical Association Manual of Style, 10th ed, Baltimore, MD, Williams & Wilkins, 1998. The use of *et al*. in the author listing of references is allowed only when the author list exceeds 15 authors. Author listings with 16 or more authors should list the first 3 authors followed by *et al.*

E.g. Brown JE, Smyth PT. AMA and other styles: how to format references. *J Med Style.* 2007;83:1-15.

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Ethics Office
Tel: 018-285 2291

Email: Wayne.Tovers@nwu.ac.za

23 May 2016

Prof AE Schutte
HART

Dear Prof Schutte

HREC APPROVAL OF YOUR APPLICATION

Ethics number: NWU-00022-16-S1

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

Project title: The endogenous sodium pump ligand, marinabufagenin, and its relationship with 24hr urinary sodium excretion and cardiovascular function in a young black and white population: The African-PREDICT Study

Project leader/supervisor: Prof AE Schutte

Student: M Strauss

Application type: Full Single

Risk level descriptor: Minimal

You are kindly informed that at the meeting held on 12/04/2016 of the HREC, Faculty of Health Sciences, the aforementioned was approved.

The period of approval for this project is from 23/05/2016 to 30/04/2017.

After ethical review:

Translation of the informed consent document to the languages applicable to the study participants should be submitted to the HREC (if applicable).

The HREC requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the protocol or other associated documentation must be submitted to the HREC prior to implementing these changes. Any adverse/unexpected/unforeseen events or incidents must be reported on either an adverse event report form or incident report form.

A progress report should be submitted within one year of approval of this study and before the year has expired, to ensure timely renewal of the study. A final report must be provided at completion of the study or the HREC must be notified if the study is temporarily suspended

or terminated. The progress report template is obtainable from the Ethics Office at Ethics-Monitoring@nwu.ac.za. Annually a number of projects may be randomly selected for an external audit.

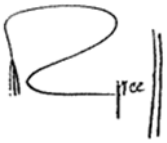
Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.

Please note that for any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the HREC. Ethics approval is required BEFORE approval can be obtained from these authorities.

The HREC complies with the South African National Health Act 61 (2003), the regulations on Research with Human Participants of 2014 of the Department of Health and Principles, the Declaration of Helsinki, 2013, the Belmont Report and the Ethics in Health Research: Principles, Structures and Processes (SANS document).

We wish you the best as you conduct your research. If you have any questions or need further assistance, please contact the Ethics Office at Ethics-HRECApply@nwu.ac.za or 018 299 1206.

Yours sincerely



Dr Wayne Towers

HREC Chairperson

Prof Minrie Greeff

Ethics Office Head

Current details: (13210572) C:\Users\13210572\Documents\HREC\HREC - Applications\2016 Applications\Applications 03 - 12 April 2016\NWU-00022-16-S1 (AE Schutte-M Strauss)\NWU-00022-16-S1 (AE Schutte-M Strauss) - AL\NWU-00022-16-S1 (AE Schutte-M Strauss) - AL.docm
23 May 2016

File reference: 9.1.5.3

DECLARATION

I, C Vorster (ID: 710924 0034 084), Language editor and Translator, and member of the South African Translators' Institute (SATI member number 1003172), herewith declare that I did the language editing of the dissertation of ms M Strauss from the North-West University (student number 23423714).

Title of the dissertation: Marinobufagenin and its relationship with systolic blood pressure in a young black and white population: The African-PREDICT study

C Vorster _____ 11/10/2016 _____

C Vorster *Date*

23423714:M_Strauss_turn-it-in.pdf

ORIGINALITY REPORT

%8	%5	%7	%7
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Strauss, Michél, Wayne Smith, and Aletta E. Schutte. "Inter-arm Blood Pressure Difference and its Relationship with Retinal Microvascular Calibres in Young Individuals: The African-PREDICT Study", Heart Lung and Circulation, 2016. Publication	%3
2	Submitted to North West University Student Paper	%3
3	dspace.nwu.ac.za Internet Source	%1
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