


**Marinobufagenin and markers of early cardiovascular  
risk in a young black and white population:  
The African-PREDICT study**

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

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**Co-promoter:** Prof. W Smith

**Graduation:** October 2019

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## Acknowledgements

Hereby, I would like to acknowledge and express my gratitude towards the following people for their contributions and support throughout the successful completion of this thesis:

- \* **Professor AE Schutte**, firstly for her invaluable guidance, support and advice given throughout the completion of this thesis. Secondly, for her patience, mentorship and commitment to help me develop vital skills as a future researcher. And lastly, I would like to acknowledge what an incredible inspiration Professor AE Schutte has been to me, not only as a researcher but also in my personal journey.
- \* **Associate Professor W Smith**, not only for his knowledge and insightful input to better this thesis, but also for his continuous guidance and encouragement throughout these years.
- \* **Associate Professor R Kruger**, for his helpful input and critical advice.
- \* **Doctor OV Fedorova, Doctor W Wei and Doctor A Bagrov** for the analyses of the biomarker marinobufagenin (MBG) at the Laboratory of Cardiovascular Science, National Institute on Ageing. Also, for the valuable knowledge and helpful interpretation of the findings for each research article.
- \* The **participants of the African-PREDICT study** for the voluntary participation that made this study possible.
- \* **Members of the Hypertension in Africa Research Team (HART), African-PREDICT collaborators and postgraduate students** for their contributions to the collection of data used in this research study.
- \* The financial assistance of the **National Research Foundation (NRF) and the NRF in collaboration with the German Academic Exchange Service (DAAD)** that enabled me to complete this research study. *“This work is based on the research supported in part by the National Research Foundation of South Africa (Grant Number: 111862).”*
- \* **Servier Medical Art** was used to create all illustrations for this thesis.
- \* **My family and friends** for their support.
- \* **Mother and grandmother** for their endless love and encouragement.
- \* **My fiancé Marco**, for his love and support.

**Above all I would like to thank my Heavenly Father for His absolute grace and love.**

## **Preface**

This thesis entitled “*Marinobufagenin and markers of early cardiovascular risk in a young black and white population: The African-PREDICT study*” is presented in an article-format, in accordance with the guidelines of the North-West University. The thesis consists of eight chapters outlined below. All research articles included in this thesis were submitted and/or published in international peer-reviewed journals.

### **Chapter layout of this thesis:**

Chapter 1: Literature overview, motivation, aims, objectives and hypotheses

Chapter 2: Study design, protocol and methodology

Chapter 3: Research article 1 (Published in the *Journal of Hypertension*)

Chapter 4: Research article 2 (Published in the *European Journal of Preventive Cardiology*)

Chapter 5: Research article 3 (Published in *Nutritional Neuroscience*)

Chapter 6: Research article 4 (To be submitted for publication)

Chapter 7: Review article (Published in *Current Hypertension Reports*)

Chapter 8: Final remarks and recommendations for future studies

The text and referencing style of each research article in this thesis, Chapters 3 to 7, are presented in the format as set out in the author instructions of the abovementioned respective journals.

- \* In order to improve the legibility of this thesis for examination purposes, I deviated from the respective journal author instructions by inserting tables and figures in between the text of all chapters. Also, paragraphs throughout this thesis have been justified.

## **Author contributions**

The following contributions of each researcher were instrumental to the success of this thesis:

### **Ms M Strauss**

Ms Strauss conducted the initial literature search to propose the concept and design of each research article presented in this thesis. She was responsible for writing the PhD proposal and also conducting an in-depth literature review. She completed the ethics application for this study as part of the larger African-PREDICT study. She helped with the collection of data for the African-PREDICT study as postgraduate student and performed biochemical analyses of urine samples. As the first author of the research articles, the PhD candidate performed all statistical analyses, interpreted the results of all analyses and drafted the thesis, including each manuscript for publication.

### **Professor AE Schutte**

In Professor Schutte's role as promoter and Principal Investigator of the African-PREDICT study, she supervised and guided all stages of this study. She contributed to the statistical analyses, interpretation of results, intellectual input and critical evaluation of all research articles and the final thesis.

### **Associate Professor W Smith**

In Professor Smith's role as co-promoter, he provided guidance on the statistical analyses and interpretation of results. He also provided intellectual input and critically revised each article and the final thesis.

### **Associate Professor R Kruger**

As a co-author for the second article presented as Chapter 4, Professor Kruger contributed to the interpretation of statistical analyses, provided intellectual input and critically revised the article.


**Doctor OV Fedorova, Doctor W Wei and Doctor A Bagrov**

Doctors Bagrov and Fedorova developed the competitive immunoassay to measure urinary marinobufagenin (MBG) in the Laboratory of Cardiovascular Science, National Institute on Aging, Baltimore, USA. Doctors Wei and Fedorova performed biochemical analyses to measure urinary MBG from the African-PREDICT study's 24-hour urine samples. Doctor Fedorova contributed to the interpretation of statistical analyses, provided intellectual input and critically revised all research articles. Doctor Bagrov provided intellectual input and critically revised the first and fourth research articles presented as Chapter 3 and Chapter 6.

## Statement by the authors


The following is a statement from the co-authors verifying their individual roles in the study and giving their permission that the research articles may form part of this thesis:

*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. I also give my consent that these manuscripts may be published as part of the PhD thesis of Ms Michél Strauss.*



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Professor AE  
Schutte



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A/Professor W  
Smith



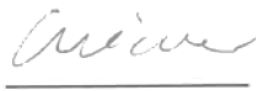
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A/Professor R Kruger



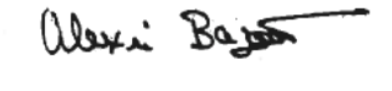
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Doctor OV  
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Doctor W Wei



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Doctor A Bagrov

## Publications and conference presentations during PhD

### Publications

Strauss M, Smith W, Wei W, Bagrov AY, Fedorova OV, Schutte AE. Large artery stiffness is associated with marinobufagenin in young adults: The African-PREDICT study. *Journal of Hypertension*. 2018; 36: 2333-2339.

Strauss M, Smith W, Kruger R, Wei W, Fedorova OV, Schutte AE. Marinobufagenin and left ventricular mass in young adults: The African-PREDICT study. *European Journal of Preventive Cardiology*. 2018; 25: 1587-1595.

Strauss M, Smith W, Kruger R, van der Westhuizen B, Schutte AE. Large artery stiffness is associated with salt intake in young healthy black but not white adults: The African-PREDICT study. *European Journal of Nutrition*. 2018;57: 2649-2656. **(Appendix A)**

Strauss M, Smith W, Wei W, Fedorova OV, Schutte AE. Autonomic activity and its relationship with the endogenous cardiotonic steroid marinobufagenin: The African-PREDICT study. *Nutritional Neuroscience*. 2019; 7: 1-11. DOI: 10.1080/1028415X.2018.1564985.

Strauss M, Smith W, Fedorova OV, Schutte AE. The Na<sup>+</sup>K<sup>+</sup>-ATPase inhibitor Marinobufagenin, and early cardiovascular risk in humans: A review of recent evidence. *Current Hypertension Reports*. 2019; 21: 38. DOI: 10.1007/s11906-019-0942-y.

Schutte AE, Strauss M. Salt is bad for you: but how it affects your body is still frontier science. *The Conversation*. 11 March 2019. <https://theconversation.com/salt-is-bad-for-you-but-how-it-affects-your-body-is-still-frontier-science-112895>. **(Appendix B)**

## Conference presentations

Strauss M, Smith W, Kruger R, van der Westhuizen B, Schutte AE. Large artery stiffness is associated with salt intake in young healthy black but not white adults: The African-PREDICT study. *Dr Kenneth Kaunda Research Day: Department of Health*. Klerksdorp, North West Province, South Africa. 26-27 July 2018. Poster presentation.

Strauss M, Smith W, Kruger R, van der Westhuizen B, Schutte AE. Large artery stiffness is associated with salt intake in young healthy black but not white adults: The African-PREDICT study. *The 3rd Biennial Congress of the South African Stroke and South African Hypertension Societies*. Stellenbosch, Western Cape, South Africa. 3-5 August 2018. Oral presentation, and winner of the best oral presentation by a postgraduate student.



## **Summary**

### **Marinobufagenin and markers of early cardiovascular risk in a young black and white population: The African-PREDICT study**

#### **Motivation**

There have been many arguments about the harmful effect of increased or low salt intake and its concurrent role in cardiovascular health. While an overwhelming amount of research has focused on the relationship between salt and blood pressure, more attention has been brought to novel markers associated with increased salt consumption and their role in cardiovascular pathophysiology. One such biomarker is the steroidal hormone and  $\text{Na}^+/\text{K}^+$ -ATPase inhibitor, marinobufagenin (MBG), released in response to the sodium induced angiotensin-aldosterone-sympatho-excitatory pathway. In vitro and animal model studies have shown MBG infusion to promote microvascular hyperpermeability, cardiovascular fibrosis and cardiac hypertrophy. These findings were concomitant with increased plasma MBG and urinary MBG excretion in animals. Studies in humans however are far more limited, with the majority of studies investigating relationships between cardiovascular risk markers and MBG (urinary and plasma MBG) being performed in populations with reported pathologies. Information on relationships between markers of cardiovascular risk and MBG in young healthy adults is scarce. If we are able to determine associations between established markers of cardiovascular risk and MBG at an early age and prior to cardiovascular pathologies, especially in a population consuming excessive amounts of salt, this would reinforce the need to implement sodium reduction strategies in an effort to reduce cardiovascular risk.

Furthermore, there is a lack of research pertaining to MBG and the possible diverse physiological or pathophysiological roles thereof in men and women or black and white ethnic groups, respectively. There are various studies indicating increased salt-sensitivity in women as well as black populations. It is therefore also possible that these specific groups may be more sensitive to the cardiovascular effects of MBG and may be at a greater risk.

## **Aim**

The central aim of this study was to investigate the role of 24hr urinary MBG as a potential early marker in the development of cardiovascular disease. This was done by exploring the relationships of 24hr urinary MBG with established markers of early cardiovascular risk in young healthy adults. This study specifically investigated these relationships within respective sex (men and women) and ethnic groups (black and white) so to bring forth new evidence and add to a body of literature where information in these respective populations are scant. Taking into consideration the young age, as well as the peak cardiovascular health of the participants of this study, new evidence of possible relationships between 24hr urinary MBG and early markers of cardiovascular risk may highlight the adverse role of MBG on the cardiovascular system prior to the onset of disease.

## **Methodology**

The original research study included in this thesis made use of the data of the first 711 consecutively enrolled participants from the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT) (42% men and 51% black) with complete 24hr urinary data. The African-PREDICT study enrolled black and white, men and women (between the ages of 20-30 years), from diverse communities and socio-economic backgrounds from in and around the Potchefstroom area (North-West Province, South Africa). All individuals who took part in the study voluntarily underwent health screening prior to inclusion into the African-PREDICT study. The following criteria were used to determine eligibility for inclusion into the study: participants were normotensive (office blood pressure <140/90 mmHg); HIV uninfected; had not been previously diagnosed with any chronic illnesses (self reported); and were not using chronic medication. Also, none of the women who participated in the study were pregnant or lactating at the time that measurements were performed. All participants gave written informed consent. Individuals who met the inclusion criteria were invited back to take part in the advanced measurements of the African-PREDICT study.

Simultaneous ambulatory blood pressure monitoring (Card(X)plore device Meditech, Budapest, Hungary, British Hypertension Society (BHS)) and 24hr electrocardiography (ECG) (Cardio Visions 1.15.2 Personal Edition software, Meditech, Budapest, Hungary) were done to determine 24hr blood pressure (including night-time systolic blood pressure dipping status) and heart rate variability. Data from the 24hr ECG time and frequency

domains included low frequency heart rate variability (LF HRV), high frequency heart rate variability (HF HRV), LF/HF heart rate variability ratio, and the standard deviation of normal-to-normal intervals (SDNN).

Large artery stiffness was measured as carotid-femoral pulse wave velocity (cfPWV)(Sphygmocor® XCEL device, AtCor Medical Pty. Ltd., Sydney, Australia), and microvascular function determined as the peak retinal artery dilation in response to a light flicker provocation (Dynamic Retinal Vessel Analyzer (DVA), Imedos Systems, Jena, Germany). The central retinal artery (CRAE) and central retinal vein equivalent (CRVE) were also determined using static retinal images.

Indices of left ventricular structure (left ventricular mass index (LVMI)) and function (stroke volume index (SVI), cardiac output index (COi), left atrial to aortic ratio (LA:Ao), mitral valve E to A ratio (E:A), ratio of mitral peak velocity of early filling (E) to early diastolic mitral annular velocity (e') (E:e')) were determined by means of transthoracic echocardiography (General Electric Vivid E9 device, GE Vingmed Ultrasound A/S, Horten, Norway).

The current recommendation of the World Health Organization refers to 24hr urine sampling as the golden standard for determining the average population wide salt intake. In this study, 24hr urinary biomarkers included MBG, sodium, potassium, creatinine and albumin. Estimated salt intake was calculated from 24hr urinary sodium excretion. Estimated glomerular filtration rate (eGFR) and albuminuria were used as indices of renal function. Additional biochemical analyses were performed using serum samples to measure aldosterone (RIA Aldosterone Kit, Beckman Coulter, Immunotech, Radiova, Czech Republic), interleukin-6 (IL-6) (high sensitivity Quantikine ELISA kit), C-reactive protein (CRP), creatinine, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), total cholesterol (TC), triglycerides, gamma glutamyltransferase (GGT), glucose (Cobas Integra 400plus, Roche, Basel Switzerland) and cotinine (chemiluminescence method on the Immulite, Siemens, Erlangen, Germany).

### *Statistical analyses*

For each manuscript we tested for an interaction of sex and ethnicity on the relationships between MBG and cardiovascular risk markers. Group stratifications for further statistical analyses were done accordingly. The Kolmogorov-Smirnov test was conducted to test for the normal distribution of data. Normally distributed data were shown as the mean and standard deviation, whereas non-Gaussian distributed data were logarithmically transformed and presented as geometric means with the 5th and 95th percentiles. Independent T-tests, analyses of covariance and chi-square tests were used to compare the respective means and proportions. We performed Pearson, partial and multivariate regression analyses to demonstrate relationships between 24hr urinary MBG and markers of early cardiovascular risk. A more detailed outline of the group stratifications and statistical analyses are provided in the respective chapters.

## **Results**

### *Basic characteristics*

Based on interaction testing, we performed statistical analyses and group comparisons in men and women. Sensitivity analysis for ethnicity was also performed, although our results seemed to be more sex-specific than ethnic-specific. The mean estimated salt intake for this population was 7.69 g/day, with men consuming approximately 8.32 g/day (2.42; 21.2 g/day, 5<sup>th</sup> and 95<sup>th</sup> percentiles; N=296) and women 7.27 g/day (2.70; 19.2 g/day, 5<sup>th</sup> and 95<sup>th</sup> percentiles; N=415) ( $p<0.003$ ). Expectedly, men also had increased mean 24hr urinary MBG excretion (4.13 nmol/day) compared to women (2.69 nmol/day) ( $p<0.001$ ). Mean 24hr urinary MBG excretion was higher in white compared to black men (4.59 vs. 3.70 nmol/day,  $p<0.001$ ), but similar for white and black women (2.83 vs. 2.56 nmol/day,  $p=0.11$ ). While salt intake did not differ between black and white men, black women (7.83 g/day) consumed more salt than their white counterparts (6.68 g/day) ( $p=0.007$ ).

Men from this study demonstrated higher 24hr blood pressure, cfPWV and LF HRV ( $p<0.001$ ) compared to women, while women had elevated 24hr heart rate and HF HRV ( $p<0.001$ ). When comparing the characteristics of participants within the lowest quartile of MBG excretion to those in the highest quartile, adults with excessive MBG excretion (quartile four) had higher 24hr systolic blood pressure, LVMI, EDVi and SVi (all  $p<0.050$ ).

We also compared the cardiovascular, retinal microvascular and biochemical profiles of dippers (night-time blood pressure dipping >10%) and non-dippers (night-time blood pressure dipping <10%). Night-time systolic blood pressure was significantly higher in non-dippers ( $113 \pm 9.01$  mmHg) compared to dippers ( $105 \pm 8.99$  mmHg) ( $p < 0.001$ ), with non-dippers exhibiting a mean night-time systolic blood pressure dipping percentage of only 5.9%. Non-dippers had narrower CRAE compared to dippers, although CRVE and retinal microvascular reactivity to a stressor did not differ. There was also no difference in the estimated salt intake or 24hr urinary MBG excretion between the two groups.

### *Regression analyses*

We firstly reiterated results from previous studies that included older or hypertensive adults, by demonstrating a strong correlation between estimated salt intake and 24hr urinary MBG ( $r = 0.49$ ;  $p < 0.001$ ) in the young healthy men and women from the African-PREDICT study.

Secondly, we found that only in women, cfPWV increased significantly across increasing quartiles of 24hr urinary MBG excretion independent of mean arterial pressure and estimated salt intake ( $p = 0.001$ ). The positive association persisted after performing multiple regression analyses adjusting for several covariates (Adj.  $R^2 = 0.23$ ; std.  $\beta = 0.15$ ;  $p = 0.002$ ). In addition, LVMI associated positively with MBG excretion only in women after partial ( $r = 0.38$ ;  $p < 0.001$ ) and multi-variable regression analyses (Adj.  $R^2 = 0.06$ ; std.  $\beta = 0.127$ ;  $p = 0.015$ ).

In the total group, we found significant relationships of MBG with systolic blood pressure, indices of early target organ damage and SVi in unadjusted analyses. MBG correlated positively with systolic blood pressure ( $r = 0.20$ ;  $p < 0.001$ ), LVMI ( $r = 0.21$ ;  $p < 0.001$ ), EDVi ( $r = 0.20$ ;  $p < 0.001$ ), SVi ( $r = 0.10$ ;  $p = 0.008$ ) and negatively with eGFR ( $r = -0.08$ ;  $p = 0.031$ ). However, after adjusting for age, sex and ethnicity, the negative relationship between MBG and eGFR lost significance. After performing multivariate adjusted regression analyses the relationships of MBG with LVMI, EDVi and SVi became borderline significant. Still, LVMI was positively associated with MBG excretion in the highest MBG excretion quartile (Adj.  $R^2 = 0.20$ ; std.  $\beta = 0.15$ ;  $p = 0.043$ ;  $N = 165$ ).

Thirdly, we indicated that both estimated salt intake and plasma aldosterone contribute positively to multiple regression models with MBG excretion as the main dependent variable in men and women (both  $p < 0.001$ ). However, contrasting associations of MBG with indices of autonomic activity were evident between women and

men, with MBG excretion associating positively with LF HRV in women (Adj.  $R^2=0.33$ ;  $\beta=0.11$ ;  $p=0.030$ ) and negatively with HF HRV in men ( $R^2=0.36$ ;  $\beta=0.12$ ;  $p=0.034$ ).

Lastly, we demonstrated in young healthy adults with ambulatory blood pressure <140/90 mmHg, who exhibit a non-dipping night-time blood pressure pattern, that MBG excretion was associated with reduced peak retinal artery dilation (Adj.  $R^2=0.34$ ;  $\beta=-0.26$ ;  $p<0.001$ ). To a lesser extent, estimated salt intake was also inversely associated with peak retinal artery dilation (Adj.  $R^2=0.30$ ;  $\beta=-0.14$ ;  $p=0.051$ ), but this relationship was significantly confounded by MBG excretion (Adj.  $R^2=0.33$ ;  $\beta=-0.015$ ;  $p=0.86$ ).

#### *Sensitivity analyses for ethnicity*

Taking into consideration the known positive relationship between MBG and salt-sensitivity, and the literature linking black ethnicity with increased salt-sensitivity, we performed additional sensitivity analyses for the interaction of ethnicity on the relationships of MBG with cardiovascular risk factors. While we saw clear sex differences in the relationships of MBG with cfPWV and LVMI, we found no interaction of ethnicity on the latter relationships.

We did, however, find that the relationships between MBG and indices of autonomic activity were only evident in black women and men, but not their white counterparts. MBG excretion associated positively with LF HRV in women (Adj.  $R^2=0.38$ ;  $\beta=0.13$ ;  $p=0.036$ ) and negatively with HF HRV in men (Adj.  $R^2=0.40$ ;  $\beta=0.18$ ;  $p=0.045$ ).

#### **Discussion and Conclusion**

For the first time in a young human cohort, this study demonstrated significant associations between markers of increased cardiovascular risk and elevated 24hr urinary MBG excretion. Additionally, our results support previous findings, only demonstrated in animals, of the possible involvement of the sodium-induced angiotensinergic-sympatho-excitatory pathway in MBG stimulation.

In young healthy adults, we found positive associations between known predictors of increased cardiovascular risk (large artery stiffness, increased left ventricular mass and reduced retinal microvascular function in non-dippers) and 24hr urinary MBG excretion. Our results suggest that MBG may play a harmful role in the early development of cardiovascular disease, especially in populations consuming a habitual high salt diet. Although

we found no association between MBG and kidney function it is possible that MBG may have an adverse effect on the kidneys at a later stage in life.

Notably, our results were predominantly evident in women who are reportedly more salt-sensitive compared to men. We therefore propose that women are likely more sensitive to the cardiovascular effect of MBG and may be at greater risk when consuming increased amounts of salt. However, the lack of relationships between MBG and markers of early cardiovascular risk in black adults was unforeseen. Especially since black ethnicity is associated with salt-sensitivity.

This study, furthermore, demonstrated positive relationships of estimated salt intake and aldosterone with 24hr urinary MBG excretion in men and women. However, only in women did we find a positive association between indices of sympathetic activity and 24hr urinary MBG excretion. We were able to replicate previous findings from animals in a human cohort, namely that MBG was associated with components of the proposed sodium induced angiotensinergic-sympatho-excitatory pathway.

Despite the cross-sectional study design being a main limitation and prohibiting conclusions on causality, this study provides valuable insights into the possible early pathophysiological role of MBG in humans. In addition, taking into consideration the results of this study and the comprehensive literature linking salt intake and MBG, the study's main findings support the implementation of sodium reduction strategies in South Africa.

**Key words:** autonomic activity; arterial stiffness; left ventricular mass; microvasculature; marinobufagenin; salt; women; salt; microvasculature.

## Table of contents

Acknowledgements .....	ii
Preface.....	iii
Author contributions .....	iv
Statement by the authors .....	vi
Publications and conference presentations during PhD.....	vii
Summary.....	ix
List of tables and figures .....	xx
List of abbreviations .....	xxv

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## Chapter 1: Literature overview, motivation, aims, objectives and hypotheses

1. Introduction .....	2
2. Salt and blood pressure .....	3
2.1 The physiology behind salt intake and the concurrent pressor response .....	4
3. Marinobufagenin .....	5
3.1 Marinobufagenin and salt-sensitivity.....	8
3.2 Marinobufagenin and the endothelium .....	9
3.3 Marinobufagenin and the vasculature.....	11
3.3.1 Marinobufagenin and the microvasculature .....	11
3.3.2 Marinobufagenin and the macrovasculature.....	11
3.4 Marinobufagenin and subclinical target organ damage .....	13
3.4.1 Renal function.....	13
3.4.2 Cardiovascular structure and function .....	14
4. Potential confounding role of sex and ethnicity on the relationship between MBG and indices of early cardiovascular risk .....	15
4.1 Sex .....	15
4.2 Ethnicity .....	16



4.3	Lifestyle behaviours.....	17
5.	Motivation .....	18
6.	Aims, objectives and hypotheses.....	18
6.1	Marinobufagenin and large artery function .....	18
6.2	Marinobufagenin and its association with subclinical target organ damage .....	18
6.3	Autonomic activity, aldosterone and marinobufagenin .....	19
6.4	Marinobufagenin and microvascular function in non-dipping adults .....	19
	References.....	20

---

## **Chapter 2: Study design, protocol and methodology**

1.	The African-PREDICT study.....	36
2.	Organisational procedures.....	37
3.	Methodology used this PhD study .....	39
3.1.	Questionnaire data .....	39
3.2.	Anthropometric measurements.....	39
3.3.	Cardiovascular measurements.....	40
3.4.	Biological sampling and biochemical analyses.....	46
3.5.	PhD candidate's contributions to the African-PREDICT study.....	48
4.	Statistical analyses .....	49
4.1.	Power analyses .....	49
4.2.	Statistical considerations .....	50
	References.....	52

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## **Chapter 3: Research article 1**

	Large artery stiffness is associated with marinobufagenin in young adults: The African-PREDICT study .....	58
--	--	----

---

## **Chapter 4: Research article 2**

Marinobufagenin and left ventricular mass in young adults: The African-PREDICT study.....	85
---	----

---

## **Chapter 5: Research article 3**

Autonomic activity and its relationship with the endogenous cardiogenic steroid marinobufagenin: The African-PREDICT study.....	119
---	-----

---

## **Chapter 6: Research article 4**

Microvascular function in non-dippers: potential involvement of the salt-sensitive biomarker, marinobufagenin. The African-PREDICT study .....	148
--	-----

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## **Chapter 7: Review article**

The Na <sup>+</sup> K <sup>+</sup> -ATPase inhibitor Marinobufagenin, and early cardiovascular risk in humans: A review of recent evidence.....	172
---	-----

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## **Chapter 8: Final remarks and recommendations for future studies**

Introduction .....	202
Aims, objectives and hypotheses.....	202
1. Marinobufagenin and large artery function .....	202
2. Marinobufagenin and its association with subclinical target organ damage .....	203
3. Autonomic activity, aldosterone and marinobufagenin.....	203
4. Marinobufagenin and microvascular function in non-dipping adults .....	204
Strengths and limitations of this study.....	204
Recommendations and perspectives for future studies .....	205
Conclusion .....	207

## Appendices

Appendix A: Published article: *Large artery stiffness is associated with salt intake in young healthy black but not white adults: The African-PREDICT study*. European Journal of Nutrition.

Appendix B: Published article: *Salt is bad for you: but how it affects your body is still frontier science*. The Conversation.

Appendix C: Health Research Ethics Committee approval of the African-PREDICT study

Appendix D: Health Research Ethics Committee approval for this PhD study

Appendix E: African-PREDICT study informed consent form

Appendix F: Language editing

Appendix G: Turnitin report

## List of tables and figures

### Chapter 1: Literature overview, motivation, aims, objectives and hypotheses

- Figure 1:** Proposed brain ouabain-angiotensinergic-sympatho-excitatory pathway to increase MBG secretion in response to salt intake.
- Figure 2:** Natriuretic function of marinobufagenin.
- Figure 3:** Vasoconstrictive mechanism associate with vascular Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibition by marinobufagenin.
- Figure 4:** Caspase-3 mediated β-catenin translocation to the endothelial nucleus.
- Figure 5:** Signalling pathway whereby marinobufagenin promotes vascular fibrosis.
- Figure 6:** Proposed cardiac effects of marinobufagenin.
- 

### Chapter 2: Study design, protocol and methodology

- Table 1:** Eligibility criteria for inclusion into the African-PREDICT study
- Table 2:** Variables considered for this sub-study of the African-PREDICT study
- Figure 1:** Geographic location of the Hypertension Research and Training Clinic at the North-West University located in Potchefstroom, North-West Province, South Africa.
- Figure 2:** Monochrome retinal image centred on the optic disc.
- Figure 3:** Vessel selection for dynamic retinal vessel analyses.
- Figure 4:** Temporal response curve.
- Figure 5:** Power analysis for this PhD study for 711 participants.
- Figure 6:** Power analysis for this PhD study for 145 participants.
-

### Chapter 3: Research article 1

#### *Large artery stiffness is associated with marinobufagenin in young adults: The African-PREDICT study*

- Table 1:** Cross-immunoreactivity of 4G4 monoclonal anti-MBG antibody with the components of the contraceptive treatments
- Table 2:** Interaction of ethnicity on the relationship between MBG excretion and arterial stiffness in the total group, men and women
- Table 3:** Basic characteristics of young men and women
- Table 4:** Single and partial regression analyses with carotid-femoral pulse wave velocity as dependent variable
- Table 5:** Multiple regression analyses with carotid-femoral pulse wave velocity as dependent variable
- Figure 1:** Displacement of binding 4G4 anti-MBG monoclonal antibody to MBG-thyroglobulin conjugate by MBG, progesterone, drospirenone, ethinyl estradiol, and levonorgestrel in dissociation-enhanced fluoroimmunoassay (DELFLIA) competitive reverse phase immunoassay.
- Figure 2:** Arterial stiffness according to increasing quartiles of MBG excretion within men (■) and women (●).
- Figure 3:** Pearson correlation between 24hr urinary MBG excretion and estimated NaCl intake.

**Supplemental Digital Content 1, Figure:** Arterial stiffness according to increasing quartiles of MBG excretion in women who use (■) and do not use hormonal contraceptives (■).

**Supplemental Digital Content 2, Table:** Multiple regression analyses in women

**Supplemental Digital Content 3, Table:** Sensitivity analyses for aldosterone

---

## Chapter 4: Research article 2

### *Marinobufagenin and left ventricular mass in young adults: The African-PREDICT study*

**Table 1:** Comparison of general participant characteristics across quartiles of MBG excretion ( $N=707$ )

**Figure 1:** Pearson correlations between indices of subclinical target organ damage and MBG excretion in the total group.

**Figure 2:** Left ventricular mass index of young adults across increasing quartiles of MBG excretion. (A) Single and partially adjusted regression analyses (□ Q1-Q3 and ■ Q4). (B) Multivariate adjusted regression analyses adjusted for age, sex, ethnicity, 24hr SBP, eGFR, HDL-C, CRP, GGT and glucose.

**Supplementary Table 1:** Multiple regression analyses with MBG excretion as the main independent variable

**Supplementary Table 2:** Pearson and partial correlations across increasing quartiles of MBG excretion

**Supplementary Table 3:** Forward stepwise multiple regression analyses in the highest MBG excretion quartile with LVMI as dependant variable ( $N=165$ )

**Supplementary Table 4:** Sensitivity analyses in the highest MBG excretion quartile for Estimated NaCl intake with LVMI as dependant variable ( $N=165$ )

**Supplementary Table 5:** Interaction of sex and ethnicity on the relationship between MBG excretion and indices subclinical target organ damage

**Supplementary Table 6:** Pearson, partial and multiple regression analyses with MBG excretion as the main independent variable in men and women

## Chapter 5: Research article 3

### *Autonomic activity and its relationship with the endogenous cardiotonic steroid marinobufagenin: The African-PREDICT study*

- Table 1:** Characteristics of men and women from the African-PREDICT study
- Table 2:** Pearson and partial correlations between indices of autonomic activity and MBG excretion
- Table 3:** Fully adjusted multiple regression models with MBG excretion as dependent variable in men and women
- Table 4:** Fully adjusted multiple regression models with MBG excretion as dependent variable in black and white men and women
- Figure 1:** Unadjusted, partially adjusted and fully adjusted  $\beta$ -values of heart rate variability estimates (LF HRV ((a) men; (b) women) or HF HRV ((c) men; (d) women)) as part of regression analyses with MBG excretion as dependent variable.
- Figure 2:** Suggested ouabain-angiotensinergic-sympatho-excitatory-MBG pathway.
- Supplementary Figure 1:** Unadjusted, partially adjusted and fully adjusted  $\beta$ -values of heart rate variability estimates ((A) LF HRV; (B) HF HRV) in the total group, as part of regression analyses with MBG excretion as dependent variable.
- Supplementary Table 1:** Pearson and partial correlations between autonomic activity and MBG excretion in the total group
- Supplementary Table 2:** Pearson and partial correlations between indices of autonomic activity and MBG excretion in black and white men and women
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## Chapter 6: Research article 4

### *Microvascular function in non-dippers: potential involvement of the salt-sensitive biomarker, marinobufagenin. The African-PREDICT study*

- Table 1:** Basic characteristics of dippers and non-dippers
- Table 2:** Correlations of MBG excretion and peak artery dilation with night-time dipping percentage
- Table 3:** Pearson and partial correlations
- Table 4:** Multiple regression analyses in non-dippers
- Figure 1:** Retinal arterial responses to a light flicker provocation in individuals with (A) normal retinal arterial dilation and (B) suppressed retinal arterial dilation.
- Figure 2:** Unadjusted (●) and adjusted (●) relationship between peak artery dilation and MBG excretion in (A) non-dippers and (B) dippers.

### **Supplementary Table 1:** Multiple regression analyses in dippers

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## Chapter 7: Review article

### *The Na<sup>+</sup>K<sup>+</sup>-ATPase inhibitor Marinobufagenin, and early cardiovascular risk in humans: A review of recent evidence*

- Table 1:** 24hr Urinary marinobufagenin in human cohorts without reported kidney or heart disease
- Figure 1:** Mechanisms whereby MBG have been implicated increasing cardiovascular risk.
- Figure 2:** Summary of the evidence linking MBG to an increased cardiovascular risk.



## List of abbreviations

<b>ABPM</b>	Ambulatory blood pressure monitoring
<b>ACE</b>	Angiotensin converting enzyme
<b>African-PREDICT</b>	African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension
<b>AHA</b>	American Heart Association
<b>ATII</b>	Angiotensin II
<b>AVR</b>	Arterio-to-venous ratio
<b>BHS</b>	British Hypertension Society
<b>BMI</b>	Body mass index
<b>BSA</b>	Body surface area
<b>CARDIA</b>	Coronary Artery Risk Development in Young Adults study
<b>CEN</b>	Centre of Excellence for Nutrition
<b>CKD-EPI</b>	Chronic Kidney Disease Epidemiology
<b>COi</b>	Cardiac output index
<b>CVD</b>	Cardiovascular disease
<b>cfPWV</b>	Carotid-femoral pulse wave velocity
<b>CRAE</b>	Central retinal artery equivalent
<b>CRP</b>	C-reactive protein
<b>CRVE</b>	Central retinal vein equivalent
<b>cSBP</b>	Central systolic blood pressure
<b>DBP</b>	Diastolic blood pressure

<b>DELFLIA</b>	Dissociation-Enhanced Lanthanide Fluorescent Immunoassay
<b>DVA</b>	Dynamic retinal vessel analyses
<b>E</b>	Peak transmitral flow velocity of early ventricular filling
<b>e'</b>	Diastolic mitral annular velocity
<b>E/A ratio</b>	Peak transmitral flow velocity of early to late ventricular filling
<b>E/e' ratio</b>	Ratio of mitral peak velocity of early filling to early diastolic mitral annular velocity
<b>ECG</b>	Electrocardiogram
<b>ECM</b>	Extracellular matrix
<b>EDV</b>	End diastolic volume
<b>eGFR</b>	Estimated glomerular filtration rate
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>ESC</b>	European Society of Cardiology
<b>ESH</b>	European Society of Hypertension
<b>ESV</b>	End systolic volume
<b>FE<sub>Na</sub></b>	Fractional sodium excretion
<b>Fli-1</b>	Friend leukemia integration factor 1
<b>FMD</b>	Flow mediated dilation
<b>FS</b>	Fractional shortening
<b>GGT</b>	gamma glutamyltransferase
<b>HART</b>	Hypertension in Africa Research Team
<b>HDL-C</b>	High density lipoprotein cholesterol
<b>HF HRV</b>	High frequency heart rate variability

<b>HRV</b>	Heart rate variability
<b>HyperGEN</b>	Hypertension Genetic Epidemiologic Network study
<b>ICAM-1</b>	Intercellular adhesion molecule-1
<b>IDACO</b>	International Database on Ambulatory blood pressure in relation to Cardiovascular Outcomes
<b>IL-6</b>	Interleukin-6
<b>LA:Ao</b>	Left atrium to aortic root ratio
<b>LDL-C</b>	Low density lipoprotein cholesterol
<b>LF HRV</b>	Low frequency heart rate variability
<b>LV</b>	Left ventricular
<b>LVMi</b>	Left ventricular mass index
<b>MAP</b>	Mean arterial pressure
<b>MBG</b>	Marinobufagenin
<b>MCP-1</b>	Monocyte chemoattractant protein-1
<b>MMP</b>	Matrix Metalloproteinase
<b>MSNA</b>	Muscle sympathetic nerve activity
<b>MU</b>	Measuring unit
<b>NaCl</b>	Sodium chloride
<b>Na<sup>+</sup>/K<sup>+</sup>-ATPase</b>	Sodium potassium adenosine triphosphate
<b>NCD</b>	Non-communicable diseases
<b>NUTRICODE</b>	Global Burden of Diseases Nutrition and Chronic Diseases Expert Group
<b>PAHO</b>	Pan American Health Organization
<b>PhasREC</b>	Physical activity, Sport and Recreation

<b>PKC</b>	Protein kinase C
<b>PWA</b>	Pulse wave analysis
<b>PWV</b>	Pulse wave velocity
<b>SBP</b>	Systolic blood pressure
<b>SDNN</b>	Standard deviation of normal NN interval
<b>SES</b>	Socio-economic status
<b>SVi</b>	Stroke volume index
<b>TC</b>	Total cholesterol
<b>TPR</b>	Total peripheral resistance
<b>TOD</b>	Target organ damage
<b>UN</b>	United Nations
<b>VCAM-1</b>	Vascular cellular adhesion molecule-1
<b>VSMC</b>	Vascular smooth muscle cells
<b>WHO</b>	World Health Organisation
<b>WHR</b>	Waist to height ratio

# Chapter 1

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Literature overview, motivation, aims, objectives  
and hypotheses

## 1. Introduction

Global mortality rates due to non-communicable diseases (NCDs) have steadily increased from 1990 (27 million deaths) to 2015 (39.8 million death), constituting 73.1% of all deaths.<sup>1, 2</sup> Data from the 2013 Global Burden of Disease Study indicated that more than 17.3 million deaths were attributed to cardiovascular disease (CVD),<sup>3, 4</sup> accounting for approximately one third of all mortalities.<sup>1</sup> This number increased to 17.9 million in 2015, establishing CVD as the leading cause of NCD related deaths.<sup>2</sup> The *Global Status Report on Non-communicable Diseases 2010*, was one of the first detailed reports that highlighted the growing global burden of NCD.<sup>5</sup> The World Health Organisation (WHO) called for an effort to devote resources and generate sustainable strategies to monitor and reduce NCDs, including CVD.<sup>5, 6</sup> In accordance, the 66<sup>th</sup> United Nations (UN) General Assembly, 19 September 2011, focused specifically on the global challenges surrounding the control and prevention of NCDs.<sup>7</sup> In line with the agenda of the WHO,<sup>5, 8</sup> the UN recognised the profound need to implement effective multi-sectoral policies, which intervene and create awareness with regard to behavioural risk factors (sedentary lifestyle, high blood pressure, obesity, salt intake, tobacco and alcohol use) contributing to the prevalence of NCDs.<sup>7</sup> Indeed, a reduction in these modifiable risk factors is projected to decrease the number of NCD attributed premature deaths.<sup>9-11</sup> Amongst several suggested interventions the UN acknowledged the responsibility of the food industry to lower the sodium content of certain foods, so to reduce population salt intake.<sup>7</sup> This objective was again highlighted as a necessary commitment at the recent 73rd United Nations (UN) General Assembly on the prevention and control of non-communicable diseases, 27 September 2018.<sup>12</sup>

The salt consumption recorded by the Global Burden of Diseases Nutrition and Chronic Diseases Expert Group (NUTRICODE) in 2010 (10.06 g/day),<sup>13</sup> from 21 global regions, exceeded the daily recommendation of both the WHO (5 g/day)<sup>14</sup> and the American Heart Association (3.75 g/day).<sup>15</sup> Alarmingly, Mozaffarian *et al.* reported that 1.65 million CVD related deaths were attributed to excessive sodium intake greater than 2 g/day or alternatively 5 grams of salt per day.<sup>16</sup> Recently, Swanepoel *et al.* indicated that the median salt intake of different South African populations from 2013 to 2015 was 7.2 g/day,<sup>17</sup> which coincides with the recorded salt intake from 2010.<sup>13</sup> From thenceforth, in 2016, the South African National Department of Health implemented a new sodium legislation in an effort to regulate non-discretionary salt intake from a range of staple foods.<sup>18</sup> The implementation of the sodium legislation constitutes a proactive approach to ameliorate the rising national burden of CVD.<sup>18, 19</sup> It

was estimated that a moderate reduction of 0.85 gram salt/day, per person, could prevent approximately 7400 CVD related deaths per annum in South Africa.<sup>19, 20</sup> In the light of the national statistics indicating that 44% of the premature mortalities due to NCD are attributed to CVD,<sup>21</sup> the sodium regulation strategies mentioned are a step in the right direction.

## 2. Salt and blood pressure

A recent comprehensive Medline search by Rexhaj and colleagues, using the terms: “sodium”, “salt” “hypertension” and “high blood pressure”, revealed over 20,000 articles published from 1966 up until 2017.<sup>22</sup> The association between salt and blood pressure has thus been a hot topic for researchers, bringing forth different arguments and views on the deleterious role of sodium consumption for many years - that still remain relevant today.

A large study that included 102,216 participants from the Prospective Urban Rural Epidemiology (PURE) study, enrolling adults (aged 35-70) from 667 communities in 18 countries, indicated a significant positive association between sodium excretion and blood pressure.<sup>23</sup> They demonstrated that an increment of 1g in estimate sodium excretion resulted in an 1.46 mmHg increase in systolic blood pressure.<sup>23</sup> This positive association between estimate sodium excretion and systolic blood pressure was confirmed in another sub-study from PURE published two years later in 2016.<sup>24</sup> The latest study, however, published in 2018 by Mente *et al.* reported that a positive relationship observed between sodium and blood pressure was only evident within the highest sodium intake tertile.<sup>25</sup> The PURE study made use of the Kawasaki formula in order to calculate the estimate 24hr sodium excretion from spot urine samples, as opposed to the preferred method using 24hr urinary collections.<sup>23</sup> In support, a meta-analysis published by Mozaffarian *et al.* evaluated the dose-response relationship between sodium reduction and blood pressure, based on 107 intervention studies, with cumulatively 6970 subjects.<sup>16</sup> They indicated a strong linear relationship, in which instance a 2.3 g/day reduction in sodium intake was associated with a 3.82 mmHg attenuation in blood pressure.<sup>16</sup> These associations between sodium and blood pressure have also been confirmed in other research studies,<sup>26-30</sup> with several review papers discussing this relationship and the concurrent physiological mechanisms in detail.<sup>22, 31-34</sup>

## 2.1 The physiology behind salt intake and the concurrent pressor response

Blood pressure defined as the amount of force exerted by blood against the vessel wall area, is a function of the cardiac output and total vascular resistance.<sup>35</sup> Therefore, circulatory changes including alterations in the total peripheral resistance and cardiac function determine blood pressure.<sup>35, 36</sup> Whilst blood pressure regulation can be influenced by numerous factors, the kidneys have been identified as playing a prominent role.<sup>36</sup> In 1966, Guyton and colleagues first discovered what they had called the infinite feedback gain kidney-fluid mechanism.<sup>36</sup> This mechanism describes the physiological inter-regulation of salt and water intake and output, with blood pressure – reflective of the renal function capacity to adapt in an effort to maintain an arterial pressure equilibrium point.<sup>36</sup> Recently, however, Kitada *et al.* have challenged this well-known mechanism by proposing natriuretic-ureotelic regulation of extracellular water homeostasis in response to a high salt diet.<sup>37</sup> They propose that a high salt intake promotes urea osmolyte production and excretion, thereby resulting in osmotic-driven Na<sup>+</sup> excretion together with water conservation by tubular reabsorption.<sup>37</sup> Still, more research is needed to substantiate this regulatory mechanism.

Known factors controlling renal function and blood pressure include the renin-angiotensin-aldosterone-system (RAAS) and vasopressin (antidiuretic hormone), which have been thoroughly described and are considered textbook knowledge.<sup>35</sup> In brief, sodium intake induces volume expansion whereby arterial pressure is increased.<sup>35</sup> High concentrations of NaCl detected by macula densa cells as well as an increase in arterial pressure, inhibits renin release from juxtaglomerular cells and thereby angiotensin II formation.<sup>35</sup> The concurrent attenuation of angiotensin II and aldosterone<sup>35</sup> promotes vasorelaxation, natriuresis and diuresis in order to restore normal blood pressure.<sup>35</sup> Excessive salt intake, however, together with a dysregulation in the kidney-fluid mechanism, could result in a shift in the renal function curve, resulting in a new blood pressure equilibrium.<sup>35, 36</sup> This occurs as renal sodium excretion is compromised and sodium induced volume expansion increases cardiac output and therefore blood pressure,<sup>35, 36</sup> a phenomenon described as volume overload hypertension.<sup>38</sup> Secondary to the initial increase in cardiac output and decrease in total peripheral resistance (TPR), TPR increases after prolonged excessive sodium intake - as the aforementioned compensating mechanism is diminished. At this second stage during which TPR increases, volume overload hypertension has already developed.<sup>35</sup>



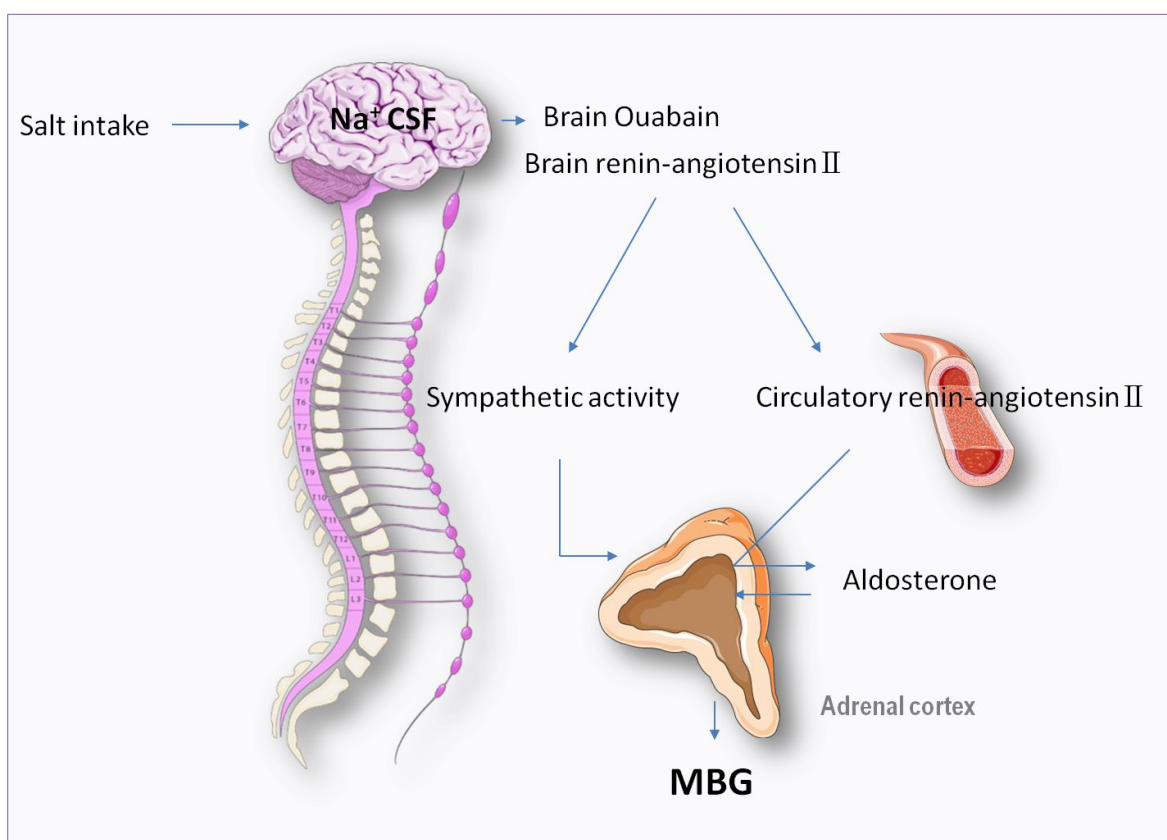
Although the detrimental effect of excessive sodium consumption in the development of CVD, attributed to a pressor response is well known,<sup>23, 24, 26, 27, 32, 33</sup> animal<sup>39</sup> and human<sup>40-43</sup> studies suggest a blood pressure independent role of sodium in the pathogenesis of cardiovascular dysfunction preceding CVD. Evidently, studies reported an attenuation in the micro<sup>42, 43</sup>- and macrovascular<sup>40, 41</sup> function of healthy individuals subjected to a high salt diet, in the absence of a pressor response. These results support the possibility of an alternative pressure-independent role of sodium, promoting early cardiovascular alterations prior to the onset of CVD. Importantly, an increase in sodium strongly relates to an increase in the novel endogenous biomarker, marinobufagenin (MBG).<sup>44-46</sup> A detailed discussion with regard to MBG and the pathophysiological implications thereof follow subsequently.

### 3. Marinobufagenin

Native to the southern parts of Texas, western Mexico and central Brazil<sup>47</sup> the *Bufo marinus* toad was imported to Queensland, Australia from Hawaii, in 1935, in an effort to control sugar cane pests. The *Bufo marinus* toad has since become an ecological invader raising concern amongst Australian ecologists<sup>48</sup> due to the toxicity of the bufotoxins on wildlife.<sup>47, 49</sup> Several variations of these bufotoxin compounds have been isolated and identified.<sup>49</sup> Lichtstein *et al.* demonstrated the acclimation ability whereby bufadienolide concentrations secreted by the skin of amphibians are altered according to environmental salinity.<sup>50</sup> One such bufadienolide namely MBG, was discovered as a digitalis-like factor produced through the skin of the *Bufo marinus* toad, to regulate the water and electrolyte homeostasis via the Na<sup>+</sup>/K<sup>+</sup>-ATPase pump.<sup>49-53</sup>

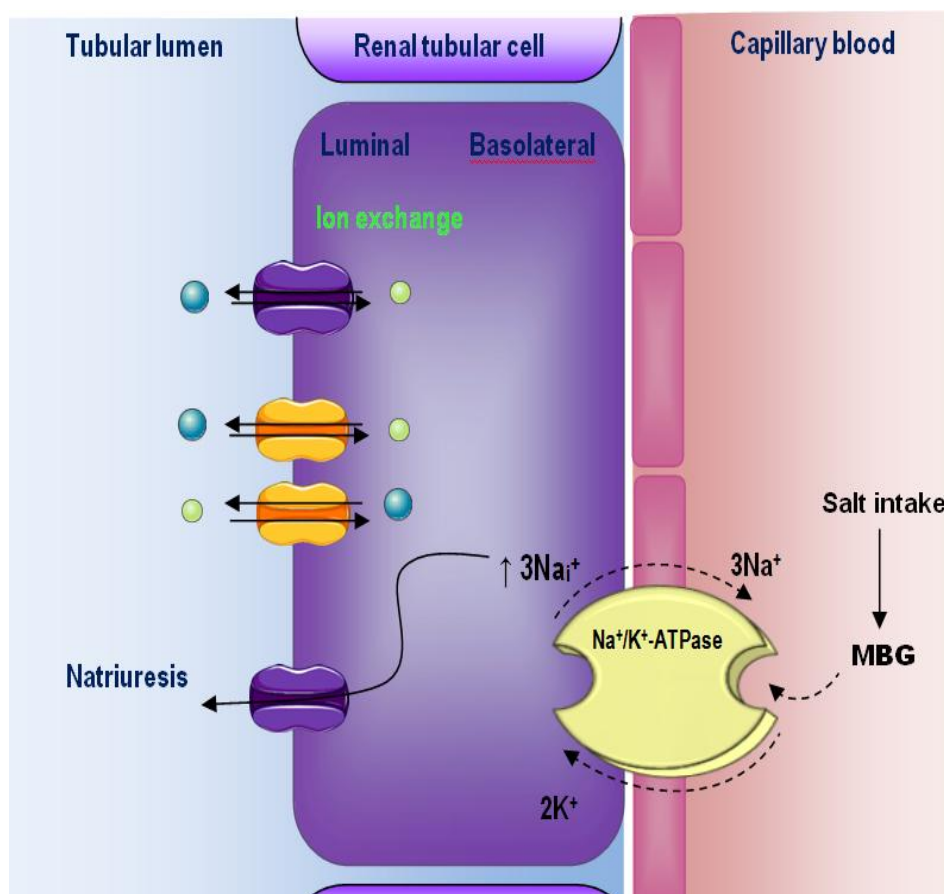
MBG, an Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibitor, has since been identified as a mammalian endogenous steroid,<sup>44, 54-56</sup> synthesized and secreted from the adrenal cortex<sup>55</sup> as a result of sodium-induced volume expansion.<sup>45, 57, 58</sup> Indeed, sodium loading in salt-sensitive animals proved to significantly increase both plasma and urinary MBG excretion.<sup>45, 58</sup> Sodium intervention studies in humans, however, have indicated contrasting results. While Fedorova *et al.* demonstrated increased plasma MBG in the absence of significant changes in urinary MBG excretion,<sup>56</sup> after a high salt intervention, Jablonski *et al.* reported the converse.<sup>59</sup> Discrepancies in the findings of these studies may include differences in the study population, study design and the habitual diet of participants prior to the study inclusion.

Fedorova *et al.* were the first to demonstrate the biosynthesis of MBG via the acidic bile acid pathway, involving the regulatory CYP27A1 enzyme.<sup>55</sup> They demonstrated a significant increase in adrenocortical CYP27A1 expression in response to sodium loading in Dahl salt-sensitive rats.<sup>55</sup> Sodium loading was suggested to increase MBG secretion via the brain ouabain-angiotensinergic-sympatho-excitatory pathway in rats (**Figure 1**).<sup>60</sup> Still, there are no studies in humans supporting the role of angiotensin II, aldosterone or sympathetic activity in MBG secretion, despite MBG being proven to be strongly related to salt intake in studies where its role in blood pressure has been investigated.<sup>44, 56</sup>

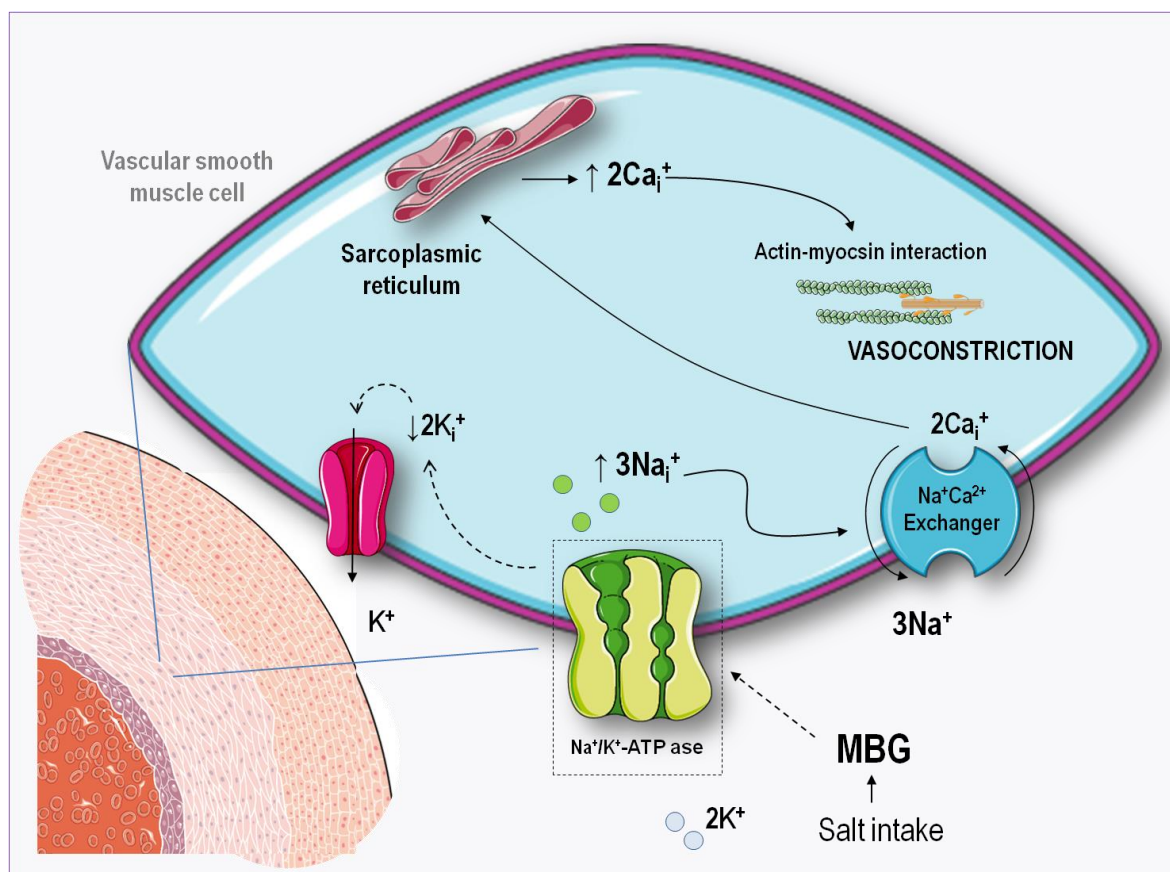


**Figure 1:** Proposed brain ouabain-angiotensinergic-sympatho-excitatory pathway to increase MBG secretion in response to salt intake. Solid lines indicate stimulatory pathways. CSF, cerebral spinal fluid; MBG, marinobufagenin.

The sodium-induced pressor effect of MBG is largely attributed to its interaction with the renal and vascular  $\alpha 1$ - $\text{Na}^+/\text{K}^+$ -ATPase isoform,<sup>45, 46, 53, 58, 61</sup> a key regulator of intracellular sodium concentration.<sup>62, 63</sup> The natriuretic nature of MBG is ascribed to its ability to promote natriuresis by inhibiting renal  $\text{Na}^+/\text{K}^+$ -ATPase as a compensatory mechanism to lower blood pressure in response to a high salt diet (**Figure 2**).<sup>44, 46</sup> However, a blunted natriuretic response to MBG could result in excessive MBG levels exerting an adverse response whereby blood pressure is elevated.<sup>45, 58</sup> Inhibition of the vascular  $\text{Na}^+/\text{K}^+$ -ATPase pump increases intracellular sodium concentrations, thereby altering the transmembrane electrochemical gradient, which indirectly inverts the function of the  $\text{Na}^+/\text{Ca}^{2+}$ -exchanger.<sup>62</sup> Ultimately, high intracellular sodium concentrations cause an accumulation of calcium ions inside the vascular smooth muscle cell (VSMC), increasing vasomotor tone,<sup>31, 64</sup> and thereby blood pressure (**Figure 3**).



**Figure 2:** Natriuretic function of marinobufagenin. Dashed lines indicate the physiological natriuretic function whereby MBG inhibits the  $\text{Na}^+/\text{K}^+$ -ATPase pump and concurrently normal ion transport. This results in increased intracellular  $\text{Na}^+$  and ultimately natriuresis. MBG, marinobufagenin.



**Figure 3:** Vasoconstrictive mechanism associate with vascular Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibition by marinobufagenin.

Dashed lines indicated inhibition and solid lines indicate stimulation of ion transport. MBG, marinobufagenin.

### 3.1 Marinobufagenin and salt-sensitivity

Fedorova *et al.*, however, have indicated that certain individuals may be more sensitive to MBG compared to others.<sup>66</sup> In support, the differential physiological and pathophysiological effects of MBG in Dahl salt-sensitive versus normotensive rats were previously demonstrated.<sup>65</sup> With sodium loading, urinary MBG excretion increased significantly in both salt-sensitive versus normotensive rats - although natriuretic and pressor responses were notably different between the two groups.<sup>65</sup> In normotensive rats, sodium excretion was two-fold higher compared to the sodium excretion of salt-sensitive rats, paralleled with a 24% inhibition compared to the 14% inhibition in renal Na<sup>+</sup>/K<sup>+</sup>-ATPase pump activity.<sup>65</sup> In contrast, only salt-sensitive rats exhibited a significant pressor response, with SBP increasing by 18mmHg. Additionally, vascular Na<sup>+</sup>/K<sup>+</sup>-ATPase pump activity was inhibited by 22% in these animals, with no significant inhibition indicated in normotensive rats.<sup>65</sup> While these results support that MBG may play an adverse role on the cardiovascular of salt-sensitive animals, no

research has been done to investigate the link between MBG, salt-sensitivity and early cardiovascular risk in humans. Considering the abovementioned, it is possible that black populations,<sup>66-69</sup> women<sup>70-72</sup> and individuals who exhibit non-dipping night-time blood pressure patterns (non-dippers)<sup>73</sup> - all associated with a salt-sensitive phenotype – may be at greater risk for the harmful role of MBG on their cardiovascular system. Indeed, non-dippers (night-time blood pressure dipping < 10%),<sup>74</sup> were shown to have an increased cardiovascular risk despite being normotensive,<sup>75, 76</sup> reinforcing the need to investigate alternative mechanisms increasing their cardiovascular risk.

Although speculative, salt-sensitivity and sensitivity to MBG may play a deleterious role in the microvascular dysfunction of these individuals - preceding macrovascular changes and hypertension. In fact, microvascular dysfunction in response to a high salt diet has been observed in healthy adults without concomitant changes in blood pressure.<sup>77</sup> Furthermore, de Jongh *et al.* have also reported an inverse association between salt-sensitivity and microvascular function in normotensive and hypertensive adults.<sup>78</sup> It is possible, however, that the observed microvascular dysfunction in salt-sensitive individuals (including non-dippers) is not a direct result of increased salt *per se*, but a rather a result of the adverse vascular effects of MBG associated with increased salt intake. A more detailed description of the harmful role of MBG on the endothelium and microvasculature will follow subsequently.

Apart from the known hemodynamic pressor response to MBG, it has been shown to promote oxidative stress,<sup>79</sup> microvascular endothelial dysfunction,<sup>80, 81</sup> apoptosis<sup>80</sup> and fibrosis.<sup>82-84</sup> As mentioned previously, animal<sup>39</sup> and human<sup>40, 41, 43</sup> studies suggest a blood pressure independent role of sodium in vascular dysfunction. Therefore, it lends to speculation on whether MBG might contribute to a sodium-related, pressure independent mechanism promoting early cardiovascular dysfunction prior to the onset of CVD.

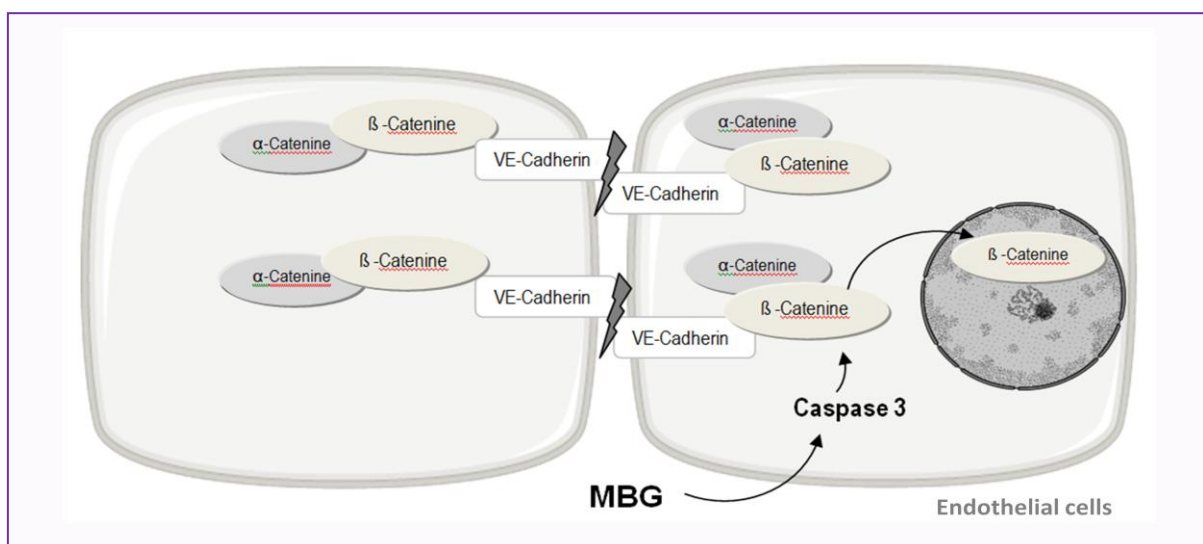
### **3.2 Marinobufagenin and the endothelium**

The endothelium forms one of the three distinct layers of the vessel wall structure.<sup>85, 86</sup> Endothelial cells play an important role in maintaining a homeostatic environment upon exposure to various physical and chemical stimuli,<sup>86, 87</sup> while also regulating vascular permeability.<sup>88, 89</sup> Stimulation of the endothelium activates several cascading pathways by which a range of circulating factors regulating vasomotor tone, inflammatory processes

and general homeostasis are released.<sup>86, 87</sup> Although studies demonstrate that a high salt diet impairs endothelial function<sup>40, 41</sup> the exact mechanism remains speculative.

The endothelial monolayer regulates the passage of several circulating proteins and cells from the plasma into surrounding structures.<sup>90</sup> Permeability of the endothelial barrier is greatly determined by the durability of the cellular adhesion junctions.<sup>90</sup> Interaction of the transmembrane protein VE-cadherin with the endothelial adhesion protein  $\beta$ -catenin is essential in maintaining the cell to cell junctional strength of the endothelial adhesive structure.<sup>90, 91</sup> Dissociation of the VE-cadherin- $\beta$ -catenin complex compromises the structural integrity of the endothelial cell barrier resulting in hyperpermeability.<sup>90</sup> Caspase-3 dependent cleavage of adhesive protein  $\beta$ -catenin decreases its affinity to VE-cadherin, resulting in the displacement of  $\beta$ -catenin to the endothelial cell nucleus (**Figure 4**).<sup>88, 92</sup> Evidently, MBG was shown to significantly increase caspase-3 activity and disrupt  $\beta$ -catenin-endothelial cell junctions.<sup>80</sup>

Interestingly, similar  $\beta$ -catenin fluorescence patterns were evident in endothelial cells treated with either 100nM MBG<sup>80</sup> or active caspase-3.<sup>92</sup> Thus MBG-induced translocation of  $\beta$ -catenin from the endothelial cell membrane to the cellular nucleus might be mediated by caspase-3 dependent uncoupling of the VE-cadherin- $\beta$ -catenin complex. Accordingly, MBG treatment of both rat lung endothelial monolayers<sup>80</sup> as well as human brain microvascular endothelial cells<sup>93</sup> significantly increase endothelial permeability.



**Figure 4:** Caspase-3 mediated  $\beta$ -catenin translocation to the endothelial nucleus. **Adapted from Tharakan et al.**<sup>92</sup> Solid lines indicate stimulatory pathways. MBG, marinobufagenin.

### 3.3 Marinobufagenin and the vasculature

#### 3.3.1 Marinobufagenin and the microvasculature

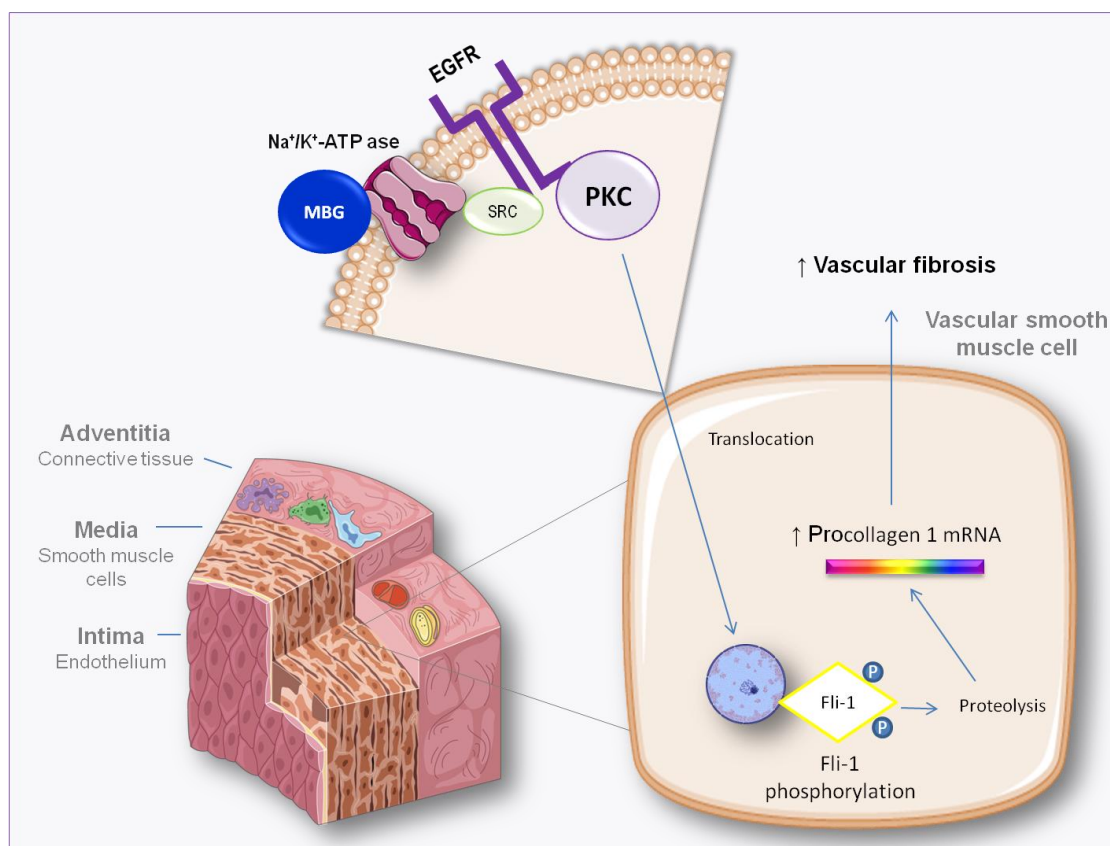
The adverse precursory role of endothelial dysfunction on microvascular alterations have been previously described.<sup>94</sup> Considering, the known effect of MBG on microvascular endothelial hyperpermeability,<sup>90</sup> as discussed in detail, it is possible that MBG may promote microvascular dysfunction. Microvascular alterations (both structural and functional) play a crucial role the development and progression of target organ damage, especially in the heart, kidney and brain that have high perfusion rates.<sup>94</sup> This includes amongst others: microvascular rarefaction, decreased vasodilation and altered wall-to-lumen ratio of arterioles. Although high salt intake has been shown to impair skin microvascular function of healthy adults,<sup>42, 43</sup> there are no studies investigating the relationship between MBG excretion microvascular function in young healthy adults.

The non-invasive assessment of microvascular function has become more achievable with advances in methods such as dynamic retinal microvascular imaging. The retinal microvasculature has been proposed to reflect the state of the systemic microvasculature,<sup>95</sup> and alterations have been associated with several cardiovascular risk factors.<sup>96-98</sup> Retinal artery and vein dilation in response to a light flicker provocation is used as the method for assessing retinal microvascular function, with suppressed dilation reflecting impaired functionality.<sup>98</sup> Attenuated retinal artery dilation has been associated with diabetes mellitus,<sup>99</sup> hypertension<sup>99</sup> coronary artery disease<sup>100</sup> and cardiac stress in black adults.<sup>101</sup>

#### 3.3.2 Marinobufagenin and the macrovasculature

Vascular remodelling involves the downstream activation of intra- and intercellular signalling pathways altering the function of endothelial and VSMC.<sup>85, 102</sup> In addition to the effect of MBG on the endothelium, it was also demonstrated to promote VSMC proliferation<sup>103</sup> and fibrosis.<sup>39, 84</sup> MBG mediated aortic collagen deposition in normotensive Wistar rats, subjected to a high salt diet, was observed in the absence of a pressor response.<sup>39</sup> Elkaher *et al.* suggests a mechanism whereby MBG increases procollagen-1 expression via a pathway involving the phosphorylation of Fli-1 by protein kinase C (PKC) (**Figure 5**).<sup>83</sup> Fli-1 has been implicated in the down regulation of collagen synthesis.<sup>83</sup> These findings were supported by Fedorova *et al.* who demonstrated the profibrotic effect, together with the down-regulation of Fli-1 in rat aortic explants incubated

for 24hr in 100 nmol/L MBG.<sup>84</sup> An overproduction of collagen reduces the elastic capacity of blood vessels, thereby promoting arterial stiffness.<sup>104</sup>



**Figure 5:** Signalling pathway whereby marinobufagenin promotes vascular fibrosis. EGFR, endothelial growth factor receptor; Fli-1, friend leukaemia factor 1; MBG, marinobufagenin; PKC, protein kinase C.

Indeed, human studies have indicated a positive relationship between salt intake<sup>105</sup> as well as urinary MBG<sup>59</sup> with carotid femoral pulse wave velocity (cfPWV), which is accepted as the golden standard for measuring arterial stiffness.<sup>104</sup> Avolio *et al.* demonstrated the relationship between salt intake and arterial stiffness in an urban Australian population by comparing the carotid, femoral and radial PWVs of 57 participants on a low salt diet (27 men and 30 women) with that of 51 participants following an habitual diet.<sup>105</sup> They found that participants on a low salt diet between the ages of 29 to 44 exhibited lower aortic, femoral and radial PWV, whilst older participants exhibited lower aortic and femoral PWV (aged 45 to 66) compared to their respective control groups.<sup>105</sup> Furthermore, Jablonski *et al.* demonstrated a significant reduction in aortic PWV, associated with reduced 24hr urinary MBG excretion, following a 10 week low sodium intervention in 11 hypertensive subjects (8 men and 3 women).<sup>59</sup>



Although vascular remodelling is commonly associated with an increase in arterial stiffness, Lemaré *et al.* explains that arterial distensibility can actually increase during the onset phases of remodelling as a result of extracellular matrix (ECM) degradation and reorganisation.<sup>85</sup> The activation of matrix metalloproteinases (MMP's) influence the vessel wall compliance in order to withstand and dampen the effect of tensile forces brought on by hemodynamic changes.<sup>85</sup> Over an extended period of time, however, the resynthesis of ECM proteins override the initial compensatory mechanism, thereby promoting arterial stiffness.<sup>85</sup> This supports previous findings in young black women (aged 20-30), where the MBG/Na<sup>+</sup> excretion ratio related positively to stroke volume and negatively with total peripheral resistance, suggesting that the compensatory homeostatic mechanisms are still intact.<sup>106</sup> This compensatory mechanism, however, could deteriorate over time resulting in a more adverse cardiovascular environment.

### **3.4 Marinobufagenin and subclinical target organ damage**

#### **3.4.1 Renal function**

As mentioned earlier, MBG is known to play a role in promoting or impairing natriuresis by means of its interaction with the enzymatic activity of the Na<sup>+</sup>/K<sup>+</sup>-ATPase pump in salt-sensitive and salt-resistant phenotypes.<sup>44-46, 56, 58</sup> However, the effect of MBG on the renovasculature might not be limited to the natriuretic function via the Na<sup>+</sup>/K<sup>+</sup>-ATPase pump, since MBG promotes endothelial cell hyperpermeability by disrupting endothelial cell junctions,<sup>80, 93</sup> as mentioned earlier. Relationships between MBG and estimates of renal damage and dysfunction (albuminuria, estimate glomerular filtration rate (eGFR) and fractional sodium excretion (FE<sub>Na</sub>)) could possibly provide novel insights with regard to the pathophysiological role of MBG in the kidneys, contributing to renal and CVD development.

Endothelial damage to the glomerular barrier results in the leakage of molecules such as albumin into the urinary tract.<sup>107</sup> Indeed, urinary albumin excretion is an accepted marker of renal damage<sup>108</sup> and endothelial dysfunction<sup>109, 110</sup> associated with an increase in salt intake.<sup>29, 111</sup> Urinary albumin is considered normal between the ranges of 5-10 mg/day in young adults,<sup>108</sup> with higher levels shown to predict all cause<sup>112-115</sup> and cardiovascular mortality.<sup>113, 115</sup> Urinary albumin levels greater than 30 mg/day reflect structural alteration in the glomerular capillary wall.<sup>108</sup> Ultimately, structural damage will influence the kidney function.<sup>116</sup> eGFR has been

identified and accepted as the preferred measure of kidney function.<sup>108, 117-119</sup> Values for the eGFR of healthy adults (<40 years) is considered normal between 120-130 mL/min per 1.73 m<sup>2</sup><sup>108</sup> with an eGFR < 60 mL/min per 1.73 m<sup>2</sup> defined as the cut-off value for chronic kidney disease.<sup>119</sup> A decrease in eGFR is associated with an increased risk of CVD<sup>119</sup> as well as all cause and cardiovascular mortality.<sup>120</sup>

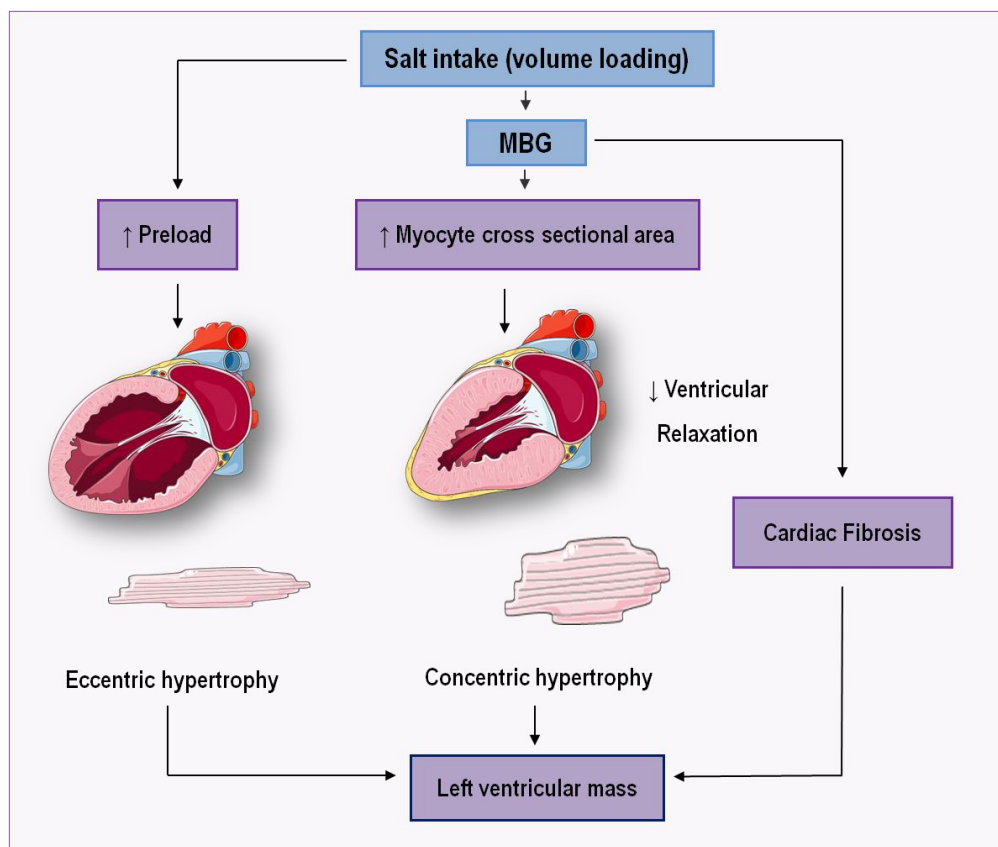
Fractional sodium excretion is defined as the percentage of sodium filtered by the glomerular apparatus of the kidneys.<sup>121</sup> The FE<sub>Na</sub> is used to differentiate between pre-renal and intrinsic kidney injury.<sup>122</sup> Pre-renal injury refers to a condition of renal hypoperfusion during which blood supply to the kidneys is diminished.<sup>123</sup> Concurrently, sodium and water reabsorption is increased resulting in FE<sub>Na</sub> < 1%.<sup>122, 123</sup> However, due to the intact intrinsic function of the kidneys, pre-renal injury is reversible in the case where blood flow and vascular hemodynamics are restored.<sup>123</sup> Therefore, increased sodium intake associated with volume loading may increase FE<sub>Na</sub>.<sup>124</sup> In contrast, intrinsic renal injury refers to a condition whereby tubular damage restricts appropriate sodium reabsorption resulting in FE<sub>Na</sub> > 3%.<sup>122</sup> MBG has been positively related to FE<sub>Na</sub>,<sup>44</sup> indicative of renal tubular damage. Indeed, MBG has been shown to promote renal tubular fibrosis.<sup>125</sup> Thus, these findings support a possible deleterious role of MBG in the kidneys, contributing to a more adverse internal environment.

### **3.4.2 Cardiac structure and function**

Both left ventricular mass (LVM) and indices of left ventricular function are predictors of future cardiovascular risk that provide valuable prognostic information.<sup>126, 127</sup> Left ventricular hypertrophy (LVH), defined as an increase in the LVM, is classified as either eccentric or concentric based on the occurrence of ventricular dilation, ventricular wall thickening or both.<sup>128</sup> The progression of eccentric and concentric LVH is characterised by distinct mechanisms whereby volume or pressure overload, respectively, cause myocardial strain. In both instances of LVH, remodelling adversely affects cardiac function over time. Intriguingly, elevated MBG is observed in patients with heart failure,<sup>79</sup> and adversely associates with functional and morphological cardiac changes in animal studies.<sup>79, 81, 82</sup>

Consistent with the notion that MBG is increased during a volume overloaded state, Kennedy *et al.* observed elevated levels of MBG in 245 patients with heart failure (aged 58 ± 13 years).<sup>79</sup> This is the only study to the best of my knowledge with reference to MBG and cardiac structure or function in a human cohort. However, in

support, Sprague-Dawley rats receiving MBG infusion were shown to exhibit a greater end diastolic pressure<sup>81</sup> along with an increase in their pressure-volume relationship,<sup>79</sup> also indicative of an increased volume load. Further effects of MBG infusion on cardiac structure of rats include increased cardiac myocyte cross-sectional wall area,<sup>82</sup> fibrosis<sup>81, 82</sup> and cardiac weight (**Figure 6**).<sup>81, 82</sup>



**Figure 6:** Proposed cardiac effects of marinobufagenin. Solid lines indicate stimulatory pathways. MBG, marinobufagenin.

#### 4. Potential confounding role of sex, ethnicity and lifestyle behaviours on the relationship between MBG and indices of early cardiovascular risk

##### 4.1 Sex

Although we have previously demonstrated a significant positive correlation between MBG and sodium excretion in black and white, men and women,<sup>106</sup> distinct contrasting relationships of systolic blood pressure and MBG have been noted between men and women. While, MBG was shown to be positively associated with systolic blood pressure in middle-aged white men (N=20) (9 grams salt per day),<sup>56</sup> a negative association was observed

in middle-aged white women (N=28) (16.32 grams salt/day)<sup>44</sup> on an induced high salt diet. Although we were unable to replicate these results in the young white men (8.91 grams salt/day) from the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT), young white women (6.68 grams salt/day) displayed a similar negative trend in the relationship between systolic blood pressure and MBG,<sup>106</sup> previously described by Anderson *et al.*<sup>44</sup> In addition, significant positive associations of central and 24hr systolic blood pressure with the MBG/Na<sup>+</sup> excretion ratio, a proposed estimate of sodium excretion resistance to MBG, have been observed in black women.<sup>106</sup>

While there is little information on the relationship between MBG and sex hormones, women are reportedly more salt-sensitive than men.<sup>70-72</sup> As previously mentioned, Bagrov *et al.* have demonstrated increased vascular Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibition by MBG in salt-sensitive animals compared to normotensive controls.<sup>65</sup> Therefore, although increased salt intake has been shown to promote urinary MBG excretion in both men and women,<sup>44, 59</sup> it is possible that women may be more sensitive to the cardiovascular effects of MBG compared to men. Despite insufficient literature on MBG and sex hormones, the reported salt-sensitivity in women substantiates the need to investigate relationships between MBG and markers of early cardiovascular risk in different sex groups.

## 4.2 Ethnicity

Anderson *et al.* indicated 24hr urinary MBG to be higher in middle-aged white individuals (N=395) when compared to their black counterparts (N=125).<sup>129</sup> The authors, however, did not report the population salt intake, which can be regarded as a limitation of this study. Their results were not confirmed in a young, apparently healthy black (N=145) and white cohort (N=186) from the African-PREDICT study.<sup>106</sup> Nonetheless, we previously demonstrated contrasting relationships between measures of systolic blood pressure and MBG in black and white women,<sup>106</sup> suggesting ethnic differences in the role of MBG.

Moreover, various studies have demonstrated ethnic differences in the salt-sensitivity,<sup>66-69</sup> RAAS system,<sup>130</sup> inflammatory profile,<sup>131</sup> endothelial,<sup>132, 133</sup> micro-<sup>133-137</sup> and macrovascular<sup>133-135, 138, 139</sup> function of black and white populations, possibly implicating different underlying physiological mechanisms in the pathogenesis of CVD. These studies indicated impaired endothelial,<sup>132, 133</sup> micro-<sup>133-136</sup> and macrovascular<sup>133-135, 138, 139</sup> function in black individuals. Additionally, several large population-based studies including the Dallas Heart Study,<sup>140</sup> Coronary

Artery Risk Development in Young Adults study (CARDIA)<sup>141</sup> and the Hypertension Genetic Epidemiologic Network study (HyperGEN),<sup>142</sup> found black individuals to have a greater LVM when compared to white individuals. Black populations might also be more susceptible to the development of CVD at a younger age due to the early onset of a more adverse cardiovascular profile.<sup>139, 143</sup> Black children and adults have been shown to exhibit a greater total peripheral resistance<sup>139, 144, 145</sup> and PWV<sup>143, 145</sup> together with a lower arterial compliance<sup>139, 144, 145</sup> when compared to white populations. In terms of microvascular function, black ethnicity has been associated with retinal arterial narrowing in a young apparently healthy population indicative of an increased cardiovascular risk.<sup>146</sup>

### 4.3 Lifestyle behaviours

Modifiable lifestyle risk factors are known contributors of CVD, and are essential targets for reducing cardiovascular morbidity and mortality rates.<sup>10</sup> While this literature review has summarized the limited literature on salt intake and MBG in humans, there is no literature to our knowledge investigating relationships of MBG with other lifestyle behaviours. This is specifically surprising for relationships between MBG and obesity, since salt intake is known to be associated with increased obesity.<sup>147</sup> Bagrov *et al.*, however, summarised several findings that described the potential implications of cardiotonic steroids, as Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibitors, on alcohol addiction, diabetes mellitus and mood disorders in animal studies.<sup>148</sup>

Bagrov *et al.* firstly, demonstrated that the administration of ethanol in rats resulted in an increase in urinary MBG excretion, and that immunisation against MBG promoted alcohol seeking behaviour in the same animals.<sup>149</sup> Secondly, in rats with induced type 1 and type 2 diabetes mellitus, it was found that urinary MBG excretion was significantly increased together with the inhibition of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity. This response was augmented especially in those animals with type 1 diabetes mellitus.<sup>150, 151</sup> The role of cardiotonic steroids in mood disorders have also been proposed to alter Na<sup>+</sup>/K<sup>+</sup>-ATPase and thereby neural activity and transmission.<sup>152, 153</sup>

## 5. Motivation

In support of the aforementioned sodium legislation and global actions to reduce salt consumption, as well as previous work on the association between MBG and blood pressure, this study focuses on the relationships between MBG and markers of cardiovascular risk, beyond blood pressure alone. Investigation into these relationships is necessary in order to gain a better understanding with regards to the possible role of MBG contributing to the development of CVD in humans, adding to the body of evidence based on cell cultures and animal models. This study will furthermore bring forth new evidence with regard to associations of MBG and markers of early cardiovascular risk within young black and white adults.

## 6. Aims, Objectives and Hypotheses

The central aim of this study is to evaluate the role of 24hr urinary MBG as a potential early marker in the development of CVD. This will be done by exploring the relationship of 24hr urinary MBG with established markers of early cardiovascular risk in adults aged 20-30 years without overt CVD.

An overarching objective, encompassing all the study objectives below, is to determine the interaction of sex and ethnicity on the relationship between 24hr urinary MBG and markers of early cardiovascular risk.

### 6.1 Marinobufagenin and large artery function

#### *Objective*

- To determine whether large artery stiffness (cfPWV) is related to higher levels of MBG.

#### *Hypothesis*

- Large artery stiffness (cfPWV) associates positively with MBG.

### 6.2 Marinobufagenin and its association with subclinical target organ damage

#### *Objectives*

- To establish whether indices of left ventricular structure (left ventricular mass index (LVMI)) and function (stroke index (SVi), cardiac index (COi), left atrial to aortic root ratio (LA:Ao), mitral valve E to A ratio (E:A),

ratio of mitral peak velocity of early filling (E) to early diastolic mitral annular velocity (e') (E:e') relate adversely to MBG.

- To determine the relationship between estimates of renal function and MBG.

### ***Hypotheses***

- Positive associations exist between LVMi, SVi, COi, LA:Ao, E:e' and MBG.
- A negative association exists between the E:A ratio and MBG.
- eGFR is negatively associated with MBG, whereas 24hr urinary albumin is positively associated with MBG.

## **6.3 Autonomic activity, aldosterone and marinobufagenin**

### ***Objectives***

- To explore whether relationships exist between indices of autonomic activity and MBG.
- To determine the relationship between aldosterone and MBG.

### ***Hypotheses***

- Aldosterone associates positively with MBG.
- Low frequency heart rate variability (an index of sympathetic tone, with a parasympathetic component<sup>154, 155</sup>) is positively related to MBG.
- High frequency heart rate variability (an index of parasympathetic tone, with a sympathetic component<sup>154, 155</sup>) is negatively associated with MBG.

## **6.4 Marinobufagenin and microvascular function in non-dipping adults**

### ***Objective***

- To determine whether MBG is related to impaired microvascular function (retinal peak artery dilation in response to light flicker stimulation) in adults with non-dipping versus dipping night-time blood pressure.

### ***Hypothesis***

- MBG is negatively related to microvascular function (retinal peak artery dilation) in non-dipping adults, but not in adults with a normal night-time blood pressure dipping status.

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# Chapter 2

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Study design, protocol and methodology

## 1. The African-PREDICT study

The African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT) is one of few longitudinal studies in Africa designed to track and monitor the development of cardiovascular pathology in young black and white adults. The African-PREDICT study was initiated by the principal investigator Professor Alta Schutte and enforced by the Hypertension in Africa Research Team (HART) at the North-West University of Potchefstroom, in collaboration with several national and international researchers and institutions. The study's coordinating office and the Hypertension Research and Training Clinic, where measurements were performed, is located on the North-West University Potchefstroom Campus in the North-West province of South Africa.<sup>1</sup>

The study aims to create and follow up on the detailed cardiovascular profile of 1202 young (aged 20-30) apparently healthy black and white, men and women every five years over a 10-year time period. The baseline phase for the study was completed between 2013 and 2017, with the first follow-up measurements starting in 2018. Participants were screened to be healthy upon initial inclusion into the study which allows researchers to monitor changes in the cardiovascular profile of participants. The study employs a multidisciplinary approach to perform several advanced cardiovascular and biochemical measurements in an effort to:

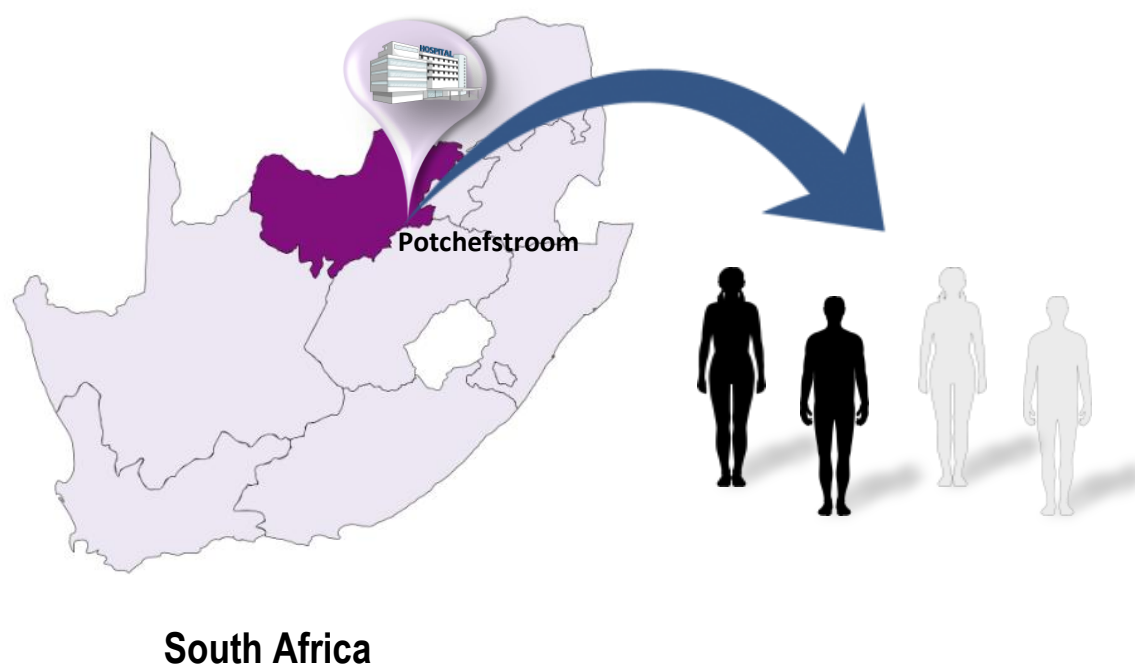
- (i) yield new knowledge with regard to existing and novel mechanisms and biomarkers involved in the pathogenesis of cardiovascular disease (CVD);
- (ii) identify early predictors of cardiovascular risk that will help introduce and support the implementation of successful prevention strategies, in order to reduce cardiovascular morbidity and mortality and;
- (iii) provide a more in-depth insight into the understanding of the divergent aetiologies in the cardiovascular pathophysiology of black and white ethnic groups.<sup>1</sup>

The African-PREDICT study was endorsed by the Provincial and National Department of Health, and approved by the Health Research Ethics Committee of the North-West University (NWU-00001-12-A1) (**Appendix C**). All protocols and procedures conformed to Institutional guidelines and the Declaration of Helsinki. The African-PREDICT study is also registered at ClinicalTrials.gov (Identifier: NCT03292094).<sup>1</sup>



## 2. Organisational procedures

Community members living in proximity of the Potchefstroom area were invited to participate in the research study at the Hypertension Research and Training Clinic on a voluntary basis (**Figure 1**). Participation in the African-PREDICT study consisted of two phases namely the (i) initial screening phase and; (ii) advanced research phase. All participants gave written informed consent at each instance, so to comply with institutional, national and international ethical regulations and standards.



**Figure 1:** Geographic location of the Hypertension Research and Training Clinic at the North-West University located in Potchefstroom, North-West Province, South Africa.

Participant screening took place in order to determine eligibility, as specified by the inclusion and exclusion criteria, for the study (**Table 1**). Screening procedures included a General Health and Demographic Questionnaire, office blood pressure, blood glucose (finger prick), anthropometric measurements and HIV testing. Participants who met the inclusion criteria were thereafter informed about all procedures of the advanced research phase and invited to take part in these measurements. Of the 1886 individuals who underwent the initial screening 1202 participants were included into the African-PREDICT study for advanced measurements. All procedures and measurements, which are described below, were performed by trained members, postgraduate

students and collaborators (The Centre for Human Metabolomics; Physical activity, Sport and Recreation (PhasREC) and the Centre of Excellence for Nutrition (CEN)) of the Hypertension in Africa Research Team (HART). Individuals who were recruited for participation were stratified according to their socio-economic status, sex and ethnicity in order to account for its known confounding effects on hypertension development.<sup>1</sup>

**Table 1: Eligibility criteria for inclusion into the African-PREDICT study**

Inclusion criteria	Justification
<ol style="list-style-type: none"> <li>1. Black or white ethnicity</li> <li>2. Men or women</li> <li>3. Aged 20-30 years</li> <li>4. Apparently healthy</li> <li>5. Normotensive or pre-hypertensive with the systolic blood pressure (SBP)/diastolic blood pressure (DBP) being less than 140/90mmHg, based on an average of four repeated office blood pressure measurements during screening</li> </ol>	<p>HART as well as others have shown that black individuals tend have the highest blood pressure and a more adverse cardiovascular profile. Therefore, the cardiovascular and biochemical profiles of black participants were compared with those of white participants, as indicated in the aims of the African-PREDICT study. (Inclusion criterion 1).</p> <p>The study included men and women in order to determine whether sex differences occur. (Inclusion criterion 2)</p> <p>The aim of the African-PREDICT study entails to follow the progression of cardiovascular dysfunction over time. The inclusion of young normotensive individuals (20-30 years) who were apparently healthy at the stage of enrolment, allow researchers to monitor and track changes in cardiovascular and biochemical markers in the pathogenesis of CVD. (Inclusion criterion 3,4 &amp; 5)</p>
Exclusion criteria	Justification
<ol style="list-style-type: none"> <li>1. Not being a permanent resident of the surrounding Potchefstroom area (i.e. intention to move)</li> <li>2. Fever on the research day (internal ear temperature &gt; 37.5°C)</li> <li>3. Elevated fasting glucose (&gt; 5.6mmol/L) and confirmed glycated haemoglobin (HbA1c) <math>\geq</math> 6.5%</li> <li>4. Previously diagnosed chronic diseases including: type 1 or 2 diabetes mellitus, renal or liver disease, tuberculosis, cancer, history of angina pectoris, stroke or myocardial infarction. Recent surgery or trauma (within the past three months). The use of</li> </ol>	<p>Due to the longitudinal study design of African-PREDICT it was important for researchers to have a measure of certainty that participants could be followed-up over the required period (Exclusion criterion 1).</p> <p>Known risk factors, diseases or medication that could influence cardiovascular health had to be taken into consideration, due to the specific aim of the African-PREDICT study to follow up on the progression of cardiovascular dysfunction in an initially healthy population. (Exclusion criterion 2-6).</p>

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<p>chronic medication, i.e. antihypertensive, anti-inflammatory, anti-diabetic or anti-retroviral</p> <p>5. Proteinuria or microalbuminuria &gt; 30 mg/ml in spot morning urine</p> <p>6. HIV infected</p> <p>7. Pregnant or lactating women</p>	<p>Hormones have been shown to influence the cardiovascular health of pregnant and lactating women; thus these women were excluded (Exclusion criterion 7).</p>
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### 3. Methodology used in this PhD study

This study made use of existing data collected as part of the African-PREDICT study, and no additional measurements were performed. Thus, all protocols and procedures for this PhD study were in coherence with those of the African-PREDICT study which are subsequently described,<sup>1</sup> and was approved as a single study by the Health Research Ethics Committee of the North-West University (NWU-00027-17-S1). (**Appendix D**) The study included the data of the first 711 participants with a complete 24hr urinary profile and data available for MBG, with data collected between February 2013 and September 2016. This study included the data of 296 men (N=145 black men; N=151 white men) and 415 women (N=217 black women; N=198 white women).

#### 3.1 Questionnaire data

Participants completed a general health and demographic questionnaire with the assistance of a trained research assistant, postgraduate student or research nurse. Information obtained from the questionnaire included the participant's age, sex, ethnicity, socio-economic status, self-reported alcohol use, smoking, family history and medication (e.g. hormonal contraceptive) use.

#### 3.2 Anthropometric measurements

Anthropometric measurements were performed in accordance with the guidelines set by the International Society for the Advancement of Kinanthropometry.<sup>2</sup> The body height (m) (SECA 213 Portable Stadiometer, SECA, Hamburg, Germany), weight (kg) (SECA 813 Electronic Scales, SECA, Hamburg, Germany) and waist circumference (cm) (Lufkin Steel Anthropometric Tape; W606PM; Lufkin, Apex, USA) were measured. The body mass index (BMI) (weight (kg) / height (m<sup>2</sup>)) and waist to height ratio (WHtR) was subsequently calculated for each participant.

### 3.3 Cardiovascular measurements

#### *Office blood pressure*

The Dinamap Procare 100 Vital Signs Monitor (GE Medical Systems, Milwaukee, USA) was used to measure office brachial blood pressure bilaterally and in duplicate. Participants were seated with their arm rested at heart level. The first blood pressure measurement was taken in the left arm and thereafter the right. A waiting period of five minutes followed. Thereafter, the blood pressure of the right arm was measured for a second time followed by the last blood pressure measurement in the left arm.

#### *Ambulatory blood pressure monitoring*

We made use of the Card(X)plore device (Meditech, Budapest, Hungary) to measure 24hr ambulatory blood pressure (ABPM) in 30-minute intervals from 06:00-22:00, and hourly from 22:00-06:00. Participants were fitted with an appropriately sized brachial blood pressure cuff to the non-dominant arm and given instructions on ensuring successful inflations during the 24hr time period. Participants also received an ABPM diary card which was completed during the duration of the measurement in order to report events that might influence ABPM readings. Cardio Visions 1.15.2 Personal Edition software (Meditech, Budapest, Hungary) was used to analyse 24hr blood pressure data including inflation rate and dipping status. The criteria defined by the European Society of Hypertension (ESH) for successful ABPM measurements were as follow: (i) at least 20 successful daytime and seven night-time measures; (ii) or 70% of the total measurements being valid.<sup>3</sup> Although not all participants met these criteria, 24hr ABPM data included for research purposes in this study met the most recently published recommendations for the necessary amount of ABPM inflations, based on data from the International Database on Ambulatory blood pressures in relation to Cardiovascular Outcomes (IDACO).<sup>4</sup> Investigators concluded that eight or more daytime and four or more night-time ABPM recordings were sufficient for analyses in population based studies.<sup>4</sup> Additionally, we stratified participants into dippers and non-dippers for the last manuscript (Chapter 5). Non-dippers were defined as individuals with a systolic night-time blood pressure dipping less than 10% from their daytime systolic blood pressure.

We made use of the Cardio Visions 1.15.2 Personal Edition software (Meditech, Budapest, Hungary) to analyse continuous 24hr electrocardiography (ECG) recordings to obtain heart rate variability data (HRV) data from both

the time (standard deviation of NN intervals (SDNN)) and frequency domains. Power spectral density analyses were used to determine the low frequency heart rate variability (LF HRV) (0.04-0.15 Hz), high frequency heart rate variability (HF HRV) (0.15-0.40 Hz) and LF/HF ratio.

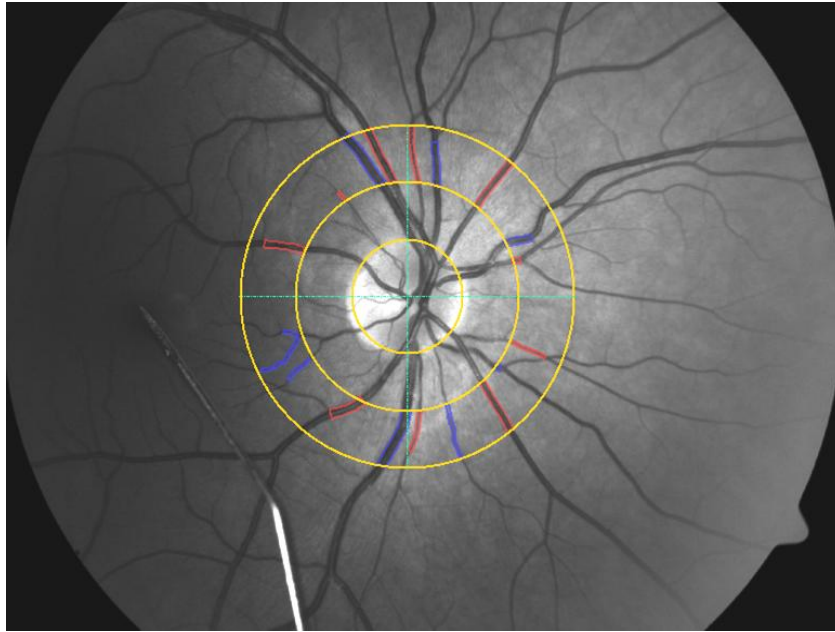
#### *Pulse wave analyses and pulse wave velocity*

The Sphygmocor® XCEL device (AtCor Medical Pty. Ltd., Sydney, Australia) was used as a non-invasive instrument to determine the central SBP (cSBP) and carotid-femoral pulse wave velocity (cfPWV). In a private room, participants laid in a relaxed supine position for approximately five minutes before the start of the measurement. A brachial blood pressure cuff was placed on the upper arm and a femoral cuff on the thigh of each participant. Using the pulse wave analyses (PWA) function the cSBP was derived from arterial pulsations recorded at the location of the brachial cuff using the generalised transfer function. In addition, supine brachial SBP and DBP was measured, from which the mean arterial pressure (MAP) was derived ( $bDBP + 1/3(bSBP - bDBP)$ ). For the subsequent cfPWV measurement, the strongest carotid pulse point was located by means of palpation, and the distance thereof measured from the upper femoral cuff using an infantometer (SECA 207 Infantometer, SECA, Hamburg, Germany). To determine the cfPWV travel distance, 80% of the direct distance measured between the arterial points (carotid to cuff, and femoral to cuff) was calculated.<sup>5</sup> This calculation ( $\text{common carotid artery} - \text{common femoral artery} * 0.8$ ) is recommended by experts, who agree that it most accurately reflects the estimate body surface distance.<sup>5</sup> The cfPWV was quantified using the cuff/tonometer sync mode, during which the carotid arterial pulse wave was determined by means of appliation tonometry, and the femoral pulse wave determined by the inflation of the femoral cuff. Both the PWA and cfPWV measurements were taken in duplicate. Additional measurements were made if the cSBP differed by more than 3 mmHg, or cfPWV differed by more than 0.5 m/s.<sup>5</sup> Where three or four measurements were taken, the closest two measurements were captured, and the average of the two values was calculated for the purpose of statistical analyses.

*Retinal microvascular calibres and retinal vessel response to a light flicker provocation*

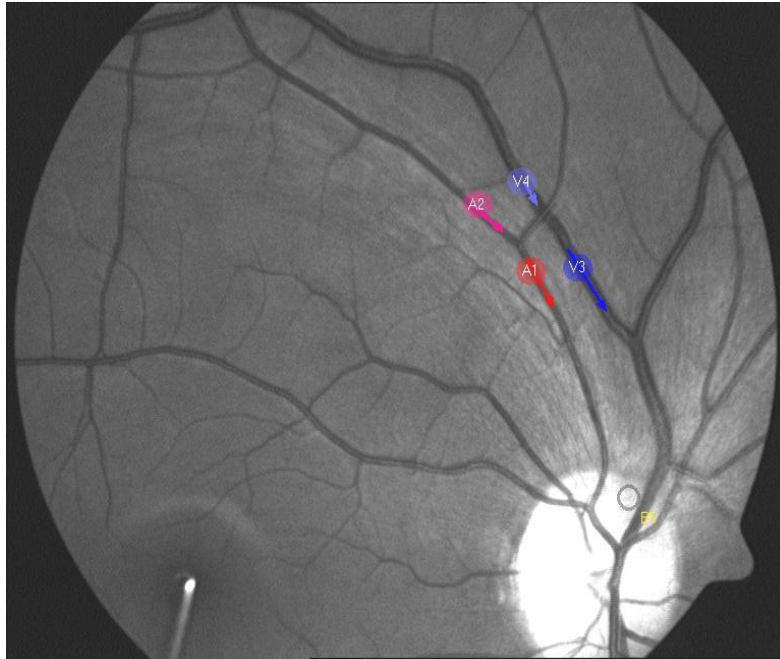
The Dynamic Retinal Vessel Analyzer (DVA) (Imedos Systems, Jena, Germany) which is fitted with a Zeiss Fundus Camera (FF-450 Plus), was used to perform retinal photography and functional assessment of the retinal microvascular response to a light flicker provocation. Participants refrained from eating, drinking, smoking or exercising one hour before the measurement. Any history of epilepsy or glaucoma was noted and these participants were not examined. The intraocular pressure measured by a research nurse to determine whether participants could partake in this measurement. All procedures and conditions were thoroughly explained to the participant before the measurement commenced. One drop of Tropicamide (1% Alcon) (Alcon laboratories, Braynston, South Africa) was administered into the right eye of the participant, 30 minutes prior to the measurement, to induce mydriatic conditions.

Monochrome as well as colour retinal images centred on the optic disc were captured with the camera angle set at 50° (Visualis 2.81 software). The best quality image was selected for analysis (preferably the monochrome image) (VesselMap2 software, Imedos Systems, Jena, Germany). All vessels located within a 0.5–1.0 optic disc diameter from the outer margin of the optic disk were selected as arteries or veins (**Figure 2**). Individual diameter data were extracted from Visualis software, and retinal vessel calibres namely the central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE) were computed separately using revised formulas.<sup>6</sup> In contrast to the Parr-Hubbard formula, the revised Knudtson formulas restrict vessel selection to the six largest arteries and veins. This significantly reduces the effect of several other vessel diameters on the calculation of CRAE and CRVE. In addition, the revised formulas are independent of image scale. The CRAE and CRVE were measured in measuring units (MU) where 1MU is equivalent to 1 $\mu$ M.

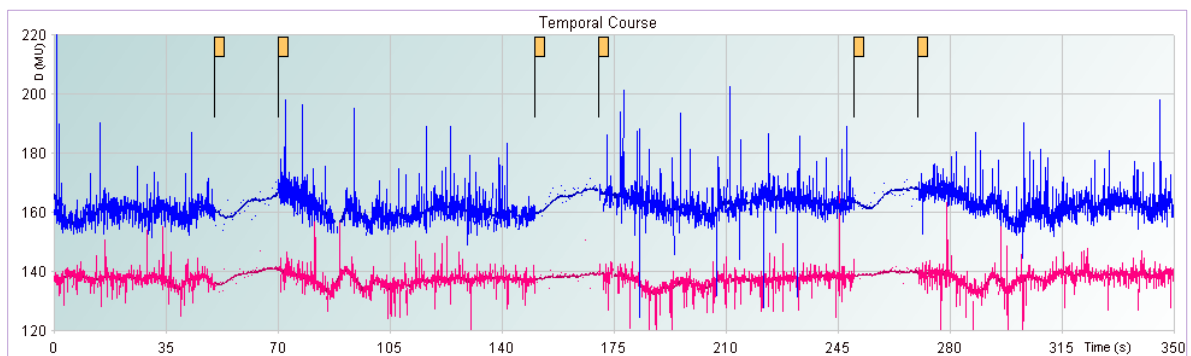


**Figure 2:** Monochrome retinal image centred on the optic disc. The outer two yellow circles demonstrate the 0.5-1.0 outer margin wherein vessels are selected. Vessels selected in red demonstrate arteries and vessels selected in blue demonstrate veins.

For dynamic retinal vessel response to a light flicker provocation, the Fundus camera was set at a 30° angle with the participant focusing on the tip of the fixation rod. Two to six artery and vein segments, 0.5-2.0 optic disc diameters from the outer margin of the optic disc, were selected from the upper or lower temporal quadrant of the fundus image, using RVA version 4.50 software (**Figure 3**). For the flicker assessment (3 flicker cycles), the baseline phase lasted for 50 second followed by a 20 second flicker period and an 80 second recovery/2<sup>nd</sup> baseline period. In total the measurement lasted for 350 seconds (**Figure 4**). From thenceforth, data were exported to Excel sheets where the peak artery dilation, peak artery constriction and peak vein dilation were determined.<sup>7</sup> The median vessel lengths for the retinal arteries and veins for this study were (1130) MU and (1235) MU respectively, with 1MU being equivalent to 1µM.



**Figure 3:** Vessel selection for dynamic retinal vessel analyses. Vessels selected in red demonstrate arteries and vessels selected in blue demonstrate veins.



**Figure 4:** Temporal response curve. Blue and red lines respectively reflect venous and arterial diameter changes over the examination time period. Firstly a 50 Second baseline examination, then three 20 second flicker cycles (area between orange flags) with 80 second intermitted recovery periods (VesselMap2 software, Imedos Systems, Jena, Germany).



*Transthoracic echocardiography*

Standard transthoracic echocardiography was performed using the General Electric Vivid E9 device (GE Vingmed Ultrasound A/S, Horten, Norway) along with a 2.5-3.5 MHz transducer and single electrocardiogram (ECG) lead for timing purposes. With the head of the examining table slightly elevated, participants were asked to lay in a partial left decubitus position for these measurements. A clinical technologist employed standardised procedures<sup>8,9</sup> to ensure that high quality recordings were obtained. EchoPAC software (version 10.8.1) was used to analyse data and determine measures of left ventricular (LV) structure and function.

LV mass index (LVMI), stroke volume index (SVI) and cardiac output index (COI) were calculated using standard formulae, normalised for body surface area and or height. LV end-systolic (ESV) and end-diastolic volumes (EDV) were calculated using the Teichholz's formula. Furthermore, the estimate ESV and EDV, derived from two-dimensional imaging according to the biplane method, were used to calculate the LV ejection fraction.

In order to assess LV filling, a pulse wave Doppler was performed in the apical four-chamber view. A 1-3 mm sample volume was placed in parallel alignment to inflow, between the tips of the mitral valve leaflets. The parameters obtained indicative of LV diastolic function included: (i) the peak transmitral flow velocities of early (E) and late (A) ventricular filling; (ii) the E:A ratio and (iii) the mitral E-wave deceleration time. Additionally, Tissue Doppler imaging was performed to calculate the myocardial tissue velocity (diastolic mitral annular velocity (e')) in relation to the transmitral blood flow velocity on both the lateral and anterior planes. The E:e' ratio distinguishes between normal LV filling pressures (<8 mmHg) and elevated filling pressures (>12 mmHg).

### 3.4 Biological sampling and biochemical analyses

As indicated in the in the African-PREDICT Research Informed Consent Form (**Appendix E**), participants were requested to fast overnight. Early morning biological sampling entailed the collection of blood and spot urine samples by a trained research nurse in a private room.

On the morning of participation all study participants received a five-litre plastic bottle in order to collect 24hr urinary samples. Participants were asked to discard the first passed urine of the day and collect all subsequent urinary voids thereafter. Bottles containing the 24hr urine samples were retrieved from participants the following day. 24hr urine samples were aliquoted into four 1.5ml cryovials and stored without added preservatives. Completeness of 24hr urine samples were verified using a volume cut-off point of <300 ml.<sup>10, 11</sup> Participants with incomplete or invalid 24hr urine samples were omitted from this study. Estimate sodium intake of this study cohort was derived from the 24hr urinary sodium excretion.

$$\text{Estimate NaCl (g/day)} = \frac{(24\text{hr Urinary Na (mmol/L)} * \text{Urinary volume (L)}) * 58.44}{1000}$$

In line with the Informed Consent Form, 24hr urine samples were also sent to an expert international laboratory at the National Institute on Aging (Laboratory of Cardiovascular Science, National Institute of Health, Baltimore, Maryland, United States of America) for analysis of 24hr urinary MBG.

Blood sample collection was performed by a trained nurse using a sterile winged infusion set and syringes from the antebraichial vein. Samples collected in red top serum tubes and sodium fluoride plasma tubes were taken from the research clinic to the onsite laboratory. Trained postgraduate students, under the supervision of the laboratory manager (Prof. Carina Mels) were responsible for the preparation of biological samples in a temperature-controlled laboratory. Biological samples (plasma, serum, whole blood and urine) were aliquoted into cryovials and stored in on-site biofreezers at -80°C (building F12 on the Potchefstroom Campus or at CEN). Samples prepared for serum analyses were left at room temperature for 30 minutes to clot and then centrifuged for 30 minutes at 1602g at room temperature (Hettich Universal 320; Andreas Hettich GmbH & Co., Tuttlingen, Germany) before being aliquoted into cryovials (16 x 500µl, 50 x 200 µl aliquots). Sodium fluoride plasma tubes were placed on ice and centrifuged for 10 minutes at 2307 g (Hettich Universal 32R, Andreas Hettich GmbH &

Co., Tuttlingen, Germany) (2 x 500µl aliquots). All biochemical analyses were done by a qualified biochemist according to standardised procedures, using calibrated instruments.

C-reactive protein (CRP), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), total cholesterol (TC), triglycerides, sodium, creatinine and gamma glutamyltransferase (GGT) were measured from serum sample analyses, and glucose was measured using sodium fluoride plasma analysis. 24hr and spot urine sodium, potassium, albumin and creatinine were also measured. These analyses were done with the Cobas Integra 400plus (Roche, Basel Switzerland). Estimate glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology (CKD-EPI) creatinine formula.<sup>12, 13</sup> We also calculated fractional sodium excretion ( $FE_{Na}$ )  $((\text{plasma creatinine} \times \text{urinary sodium}) / (\text{plasma sodium} \times \text{urinary creatinine}))$ .<sup>14</sup> From serum, cotinine was determined using the chemiluminescence method on the Immulite (Siemens, Erlangen, Germany). The high sensitivity Quantikine ELISA kit was used to measure serum interleukin-6 (IL-6). Aldosterone was quantified using the RIA Aldosterone Kit (Beckman Coulter, Immunotech, Radiova, Czech Republic). 24hr urinary MBG was determined with the use of a solid-phase Dissociation-Enhanced Lanthanide Fluorescent Immunoassay (DELFI A), based on a 4G4 anti-MBG mouse monoclonal antibody, previously reported in detail by Fedorova *et al.*<sup>15</sup>

Bio-waste disposal from all biochemical analyses were in accordance with the North-West University's policy and existing legislation. Urine samples shipped to the USA for MBG analysis were analysed and afterwards destroyed by Dr. Fedorova as per the Material Transfer Agreement.

### 3.5 PhD candidate's contributions to the African-PREDICT study

Despite the use of existing data for this study, I have contributed to the data collection of the African-PREDICT study since 2015. As a BSc Honours (2015), MSc (2016) and PhD student (2017-2019), as well as research assistant of HART (2016), I have been actively involved in various phases of the African-PREDICT study including the initial participant screening, advanced cardiovascular measurements and the participant follow-up strategy.

Firstly, I have worked together with a trained research nurse and research assistant to screen community members for inclusion into the study. As mentioned earlier, these screening tests involved general health and demographic questionnaires, dipstick urine sample, office blood pressure, blood glucose, lipids and anthropometric measurements. Secondly, with the advanced measurements I have gained several skills in performing PWV and PWA measurements (working with the Sphygmocor® XCEL device), standard 12 lead ECG measurements (Norav Medical Ltd, PC 1200, v5.030, Israel) and connecting 24hr blood pressure devices (CardXplore devices). Working with these devices, I was responsible for explaining the appropriate procedures, equipping each participant with the respective apparatuses and performing the measurements. I was also closely involved with processing, capturing and cleaning the data from these devices. In addition, I performed all statistical data analyses. Although echocardiography and dynamic retinal vessel analysis were performed by specialist scientists, I have personally observed these measurements in order to gain the necessary knowledge with regard to the methodology of the study. I assisted with the analyses of the static retinal images for data capturing purposes.

In the laboratory I analysed 24hr and spot urine samples for sodium, potassium, albumin and creatinine using the Cobas Integra 400plus device. I was responsible for sorting, packing and sending 24hr urine sample for analyses of 24hr urinary MBG at the Laboratory of Cardiovascular Science, National Institute on Aging, NIH, Baltimore, Maryland, United States of America. Lastly, as part of the follow up strategy I contributed by calling, emailing and sending letters to participants enquiring about their health, contact details and willingness to remain part of the African-PREDICT study for the follow up measurements. This formed part of my activities as research assistant. I signed a confidentiality agreement ensuring that I would not disclose any of the participants' personal information.

## 4. Statistical analyses

### 4.1 Power analyses

The power analysis of the African-PREDICT study indicated a sample size of 1200, with the scope of this study being a longitudinal investigation. For this PhD study a post hoc statistical power analysis was done for the main objective, which included cross-sectional analyses.

This study, embedded in the African-PREDICT study, should be able to detect an effect size of 0.0110 with a power of 80% using 711 participants, with the sample size and significance level set at 0.05 for a multiple linear regression, with MBG as the main independent variable with a maximum of 10 covariates (**Figure 5**). With the stratification of participants into four groups according to sex and ethnicity after interaction testing, the study should be able to detect an effect size of 0.055 with a power of 80% given a sample size of 145 (smallest group) and significance level set at 0.05, for a multiple linear regression with MBG as the main independent variable with a maximum of 10 covariates (**Figure 6**). Power analyses were performed using the G\*Power 3 statistical analysis program.<sup>16</sup>

Test family: t test	
Statistical test: Linear multiple regression: Fixed model, single regression coefficient	
Type of power analysis: Sensitivity: Compute required effect size – given $\alpha$ , power, and sample size	
<b>Input Parameters:</b>	<b>Output Parameters:</b>
Tail(s): two	Noncentrality parameter $\delta$ : 2.8054327
A err probability: 0.05	Critical t: 1.9633587
Power: 0.80	Df: 700
Total sample size: 711	Effect size $f^2$ : 0.0110696
Number of predictors: 10	

**Figure 5:** Power analysis for this PhD study for 711 participants

Test family: t test	
Statistical test: Linear multiple regression: Fixed model, single regression coefficient	
Type of power analysis: Sensitivity: Compute required effect size – given $\alpha$ , power, and sample size	
<b>Input Parameters:</b>	<b>Output Parameter:</b>
Tail(s): two	Noncentrality parameter $\delta$ : 2.8218605
A err probability: 0.05	Critical t: 1.9778258
Power: 0.80	Df: 134
Total sample size: 145	Effect size $f^2$ : 0.0549165
Number of predictors: 10	

**Figure 6:** Power analysis for this PhD study for 145 participants

## 4.2 Statistical considerations

Statistica Version 13 was used to perform statistical analyses. Interaction testing was done to determine whether an interaction of sex or ethnicity existed on the relationship between MBG and markers of early cardiovascular risk in each manuscript. The Kolmogorov-Smirnov test was conducted to test for the normal distribution of data. Normally distributed data were shown as the mean and standard deviation, whereas non-Gaussian distributed data were logarithmically transformed presented as geometric means with the 5th and 95th percentiles. Group comparisons were made using independent t-tests and one-way analysis of variance. Group comparisons were performed using a Mann-Whitney U test or Kruskal-Wallis test for variables that did not follow a normal distribution. Chi-square tests were conducted to compare proportions between groups. The relationship between early markers of cardiovascular risk and MBG was explored using Pearson-, partial- and multiple regression analysis. A more in-depth description with regard to the statistical analyses is discussed within the respective manuscript Chapters 3 to 6.

**Table 2: Variables considered for this sub-study of the African-PREDICT study**

Demographic data and health behaviours	Age (20-30 years), ethnicity (black and white), sex (male and female), socio-economic status (low/ middle/ high), self-reported alcohol, smoking and contraceptive use
Body composition	Anthropometric measures: weight, height, waist circumference, WHtR and BMI
Blood pressure measurements	24hr ABPM, cSBP, MAP and office blood pressure (SBP and DBP)
Autonomic activity	HF HRV, LF HRV, LF:HF ratio and SDNN
Markers of vascular function	cfPWV
Parameters of retinal microvascular calibres and dilation	CRAE, CRVE, AVR, peak artery dilation, peak artery constriction and peak vein dilation
Estimates of renal function	FE <sub>Na</sub> , CKD-EPI, 24hr urinary albumin and creatinine
Markers of left ventricular structure and function	LVMi, SVi, COi, E:A, E:e', LA:Ao, EDV, ESV
Other biochemical parameters serving as potential confounders	GGT, cotinine, glucose, HDL-C, LDL-C, total cholesterol, triglycerides, CRP, IL-6, aldosterone.

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# Chapter 3

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Research article 1:

Large artery stiffness is associated with marinobufagenin  
in young adults: The African-PREDICT study

Journal of Hypertension			
			
<b>Impact factor</b>	4.099		
<b>Publisher</b>	Wolters Kluwer Health		
<b>Citation</b>	<p>Strauss M, Smith W, Wei W, Bagrov AY, Fedorova OV, Schutte AE. Large artery stiffness is associated with marinobufagenin in young adults: The African-PREDICT study. <i>Journal of Hypertension</i>. 2018;36:2333-2339. DOI: 10.1097/HJH.0000000000001866.</p> 		
<b>Aims &amp; Scope</b>	<p>The <i>Journal of Hypertension</i> publishes papers reporting original clinical and experimental research which are of a high standard and which contribute to the advancement of knowledge in the field of hypertension.</p>		
Author Instructions			
<b>Language</b>	Not specified	<b>Font</b>	Not specified
<b>Spacing</b>	Double Spacing	<b>Margins</b>	3 cm
<b>Page numbers</b>	<p>Number consecutively starting on title page Number in top right hand corner</p>		
<b>Alignment</b>	Not justified		
<b>Manuscript</b>	<p>Manuscripts should include the following sections, each starting on a separate page: Title page, Abstract and Keywords, Text (Introduction, Methods, Results &amp; Discussion),</p>		

	Acknowledgements, References, Tables and Captions.
<b>Title page</b>	<ul style="list-style-type: none"> <li>• Full title (20 words)</li> <li>• Running head (40 characters, including spaces)</li> <li>• Full first name, middle initial(s) and last (family name- in capital letters) name of each author should appear</li> <li>• Affiliations of all the authors connected using a,b,c</li> <li>• Sources of funding</li> <li>• Conflict of interest statement</li> <li>• Corresponding author information</li> <li>• Word count (including references, but not tables and legends)</li> <li>• Number of tables</li> <li>• Number of figures</li> <li>• Number of supplementary digital content files</li> </ul>
<b>Abstract</b>	Structured abstract (250 words) – Objectives, Methods, Results & Conclusion
<b>Keywords</b>	3–10 keywords
<b>Text</b>	Introduction, Methods, Results & Discussion
<b>Acknowledgements</b>	Include acknowledgements
<b>Conflict of interest</b>	Include statement on title page
<b>Funding</b>	Include funding on title page
<b>Ethical considerations</b>	<ul style="list-style-type: none"> <li>• All work must be conducted in accordance with the Declaration of Helsinki.</li> <li>• Include (1) a statement that consent was obtained from participants and (2) of ethical approval.</li> </ul>
<b>References</b>	<ul style="list-style-type: none"> <li>• Number consecutively in the order in which they first appear in the text.</li> <li>• Assigned Arabic numerals in brackets, e.g. [17].</li> <li>• Include the names of all authors and any Study Group named in the primary author list when six or fewer; when seven or more, list only the first six names and add et al.</li> <li>• Journal names should be abbreviated as MEDLINE</li> </ul> <p><i>Articles in journals</i></p> <p>Zhou M-S, Schulman IH, Raij L. Vascular inflammation, insulin resistance, and endothelial dysfunction in salt-sensitive hypertension: role of nuclear factor kappa B activation. <i>J Hypertens</i> 2010; <b>28</b>:527–535</p>
<b>Tables</b>	<ul style="list-style-type: none"> <li>• Each table should be typed on a separate page in double spacing</li> <li>• Each table should be assigned an Arabic numeral and a brief title</li> </ul>
<b>Figures</b>	<ul style="list-style-type: none"> <li>• Cite figures consecutively in your manuscript.</li> </ul>

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	<ul style="list-style-type: none"><li>• Number figures in the figure legend in the order in which they are discussed</li></ul>
<b>Legends for illustrations</b>	<ul style="list-style-type: none"><li>• Captions should be typed in double spacing, beginning on a separate page.</li><li>• Each one should have an Arabic numeral corresponding to the illustration to which it refers.</li></ul>
<b>Supplemental Digital Content</b>	<ul style="list-style-type: none"><li>• Cited consecutively in the text of the submitted manuscript.</li><li>• Should include the type of material submitted (Audio, Figure, Table, etc.), be clearly labelled as "Supplemental Digital Content," include the sequential list number, and provide a description of the supplemental content.</li></ul>

*\*Formatting changes were made to maintain uniformity throughout this thesis, including text font, line spacing, margins, page numbers, tables and figures.*

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**Large artery stiffness is associated with marinobufagenin in young adults:****The African-PREDICT study**

**Short title:** Marinobufagenin and arterial stiffness

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**Conflicts of interest:** None declared

**Sources of funding:** The research funded in this manuscript is part of an ongoing research project financially supported by the South African Medical Research Council (SAMRC) with funds from National Treasury under its Economic Competitiveness and Support Package; the South African Research Chairs Initiative (SARChI) of the Department of Science and Technology and National Research Foundation (NRF) of South Africa; GlaxoSmithKline R&D, the UK Medical Research Council and with funds from the UK Government's Newton Fund; as well as corporate social investment grants from Pfizer (SA), Boehringer Ingelheim (SA), Novartis (SA), the Medi Clinic Hospital Group (SA) and in kind contributions of Roche Diagnostics (SA). Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors, and therefore, the NRF does not accept any liability in regard. This study was supported in part by the Intramural Research Program, National Institute on Aging, National Institutes of Health, USA.

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**Word count:** 3348

**Tables:** 5 tables;

**Figures:** 3 figures

**Supplementary online content files:** 3

## Abstract

**Objectives:** The cardiotonic steroid, marinobufagenin (MBG), has been shown to play a physiological natriuretic role in response to salt intake. However, recent studies in clinical and animal models demonstrated possible links between elevated levels of endogenous MBG and increased arterial stiffness. Large artery stiffness is a known predictor of future cardiovascular disease. We, therefore, investigated whether large artery stiffness relates to 24hr urinary MBG excretion in young apparently healthy black and white adults.

**Methods:** This study included data of 711 participants (black 51%, men 42%, mean age  $24.8 \pm 3.02$  years). We measured the carotid-femoral pulse wave velocity (cfPWV), 24hr urinary MBG and sodium excretion.

**Results:** In single, partial and multivariable adjusted regression analyses we found a persistent positive association between cfPWV and MBG excretion in women (Adj.R<sup>2</sup>=0.23; std.  $\beta$ =0.15;  $p$ =0.002), but not men (Adj.R<sup>2</sup>=0.17; std.  $\beta$ =0.06;  $p$ =0.31). Multiple regression models were adjusted for ethnicity, age, waist-to-height ratio, mean arterial pressure, high density lipoprotein cholesterol, C-reactive protein,  $\gamma$ -glutamyl transferase and glucose.

**Conclusion:** In conclusion, already at a young age heightened endogenous MBG levels may contribute to large artery stiffness in women via pressure-independent mechanisms, increasing their risk for future cardiovascular disease.

**Key words:** Healthy, marinobufagenin, pulse wave velocity, salt, sodium, young adult



## Introduction

The pathophysiological role of sodium in the aetiology of cardiovascular disease remains obscure. The mammalian bufadienolide marinobufagenin (MBG), a steroidal  $\text{Na}^+/\text{K}^+$ -ATPase inhibitor with natriuretic properties, has been identified as an endogenous biomarker released from the adrenal cortex in response to high sodium intake [1]. Several studies have implicated MBG in blood pressure regulation via its inhibitory function on  $\text{Na}^+/\text{K}^+$ -ATPase [2-4]. However, increasing evidence from experimental animal models [5,6] and in vitro [7] studies have suggested alternate pathways through which MBG may adversely contribute to cardiovascular disease development beyond blood pressure.

Evidently, MBG was shown to exhibit profibrotic properties by promoting collagen deposition in rat aortic explants [7]- possibly contributing to large artery stiffness [7,8]. Indeed, MBG has been associated with arterial stiffness, an established predictor of cardiovascular risk and mortality [9-11], in a small pre-hypertensive population (n=11, mean age  $60 \pm 2$  years) [7].

However, to increase our understanding on the functioning of MBG, it is important to explore whether large artery stiffness relates to MBG, particularly in a young apparently healthy population previously reported to consume excessive amounts of salt [12]. Should our study already indicate an association between MBG and arterial stiffness in young healthy adults, it would underline specific population targeted approaches to reduce sodium intake and avoid or delay arterial stiffening at young ages.

Due to known reports on the sex specific effects of MBG [2,4,13], we investigated these relationships in men and women, aged 20-30 years. Further taking into consideration the steroidal nature of MBG [1] we tested for cross-immunoreactivity of the anti-MBG monoclonal antibody used in this study with hormonal contraceptives.

This is the first study to the best of our knowledge to explore the relationship between large artery stiffness and 24hr urinary MBG excretion in a young healthy black and white population.

## Methods

### *Study design and participant recruitment*

The African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT) assesses and monitors the lifestyle, biochemical and cardiovascular profiles of young apparently healthy black and white individuals from communities in close proximity to the Potchefstroom area, in the North West province of South Africa. Participant recruitment for this study took place on a voluntary basis, and community members were screened before inclusion into the study. Eligibility for participation was determined by the following inclusion criteria: normotensive based on office blood pressure (<140/90 mmHg); HIV uninfected; microalbuminuria less than 30 mg/ml; not pregnant or lactating and no previously diagnosed chronic illness (self-reported). In addition, none of the participants included into the study made use of medication for hypertension or other chronic diseases.

For the purpose of this study we analysed the cross-sectional data of the first 711 (black 51%, men 42%) consecutively enrolled participants from February 2013 to October 2016, with complete 24hr urinary data.

The African-PREDICT study was approved by the Health Research Ethics Committee of the North-West University. The study is registered at Clinical Trials.gov (Nr. NCT03292094). All procedures conformed to the relevant principles outlined by institutional guidelines and the Declaration of Helsinki. All procedures were thoroughly explained, and each participant provided written informed consent prior to initial screening and participation in the advanced measurements of this study.

### *Questionnaire and Anthropometric data*

Detailed information on demographics and lifestyle habits were obtained through general health questionnaires completed by each participant. Questionnaire data included age, sex, ethnicity, socio-economic status, family history, self-reported smoking, alcohol and hormonal contraceptive use.

Anthropometric measurements were performed in triplicate, according to the guidelines set by the International Society for the Advancement of Kinanthropometry [14]. The body weight (kg) (SECA 813 Electronic Scales), height (m) (SECA 213 Portable Stadiometer) (SECA, Hamburg, Germany) and waist circumference (cm) (Lufkin

Steel Anthropometric Tape; W606PM; Lufkin, Apex, USA) were measured, and the body mass index (weight (kg) / height (m<sup>2</sup>)) calculated.

#### *Cardiovascular measurements*

##### *Arterial stiffness*

We used the Sphygmocor® XCEL device (AtCor Medical Pty. Ltd., Sydney, Australia) to measure carotid-femoral pulse wave velocity (cfPWV) non-invasively while participants rested in a supine position. Both the femoral and carotid artery waveforms were captured simultaneously by means of a femoral cuff placed on the upper right thigh, and carotid artery applanation tonometry. In order to determine the cfPWV travel distance, 80% of the distance measured between the arterial points (carotid to cuff measured using an infantometer, and femoral to cuff via a tape measure) was calculated [15]. cfPWV was automatically calculated as distance/pulse transit time. In addition, we determined supine brachial systolic and diastolic blood pressure. Mean arterial pressure (MAP) was subsequently calculated using supine brachial blood pressures ( $bDBP + 1/3(bSBP - bDBP)$ ). cfPWV as well as blood pressure measurements were performed in duplicate, and repeated if PWV differed by more than 3 m/s.

##### *Ambulatory blood pressure monitoring*

Each participant was fitted with a validated CardioXplore apparatus (Meditech, Budapest, Hungary, British Hypertension Society) on their non-dominant arm so to obtain 24hr ambulatory blood pressure measurements. Blood pressure measurements were recorded over 30 minute intervals during the day (06:00-22:00), and hourly at night (22:00-06:00). 24hr blood pressure data was considered successful with (a) at least 70% of the total 24hr blood pressure readings being valid; or (b)  $\geq 20$  valid daytime and seven night-time measurements [16].

*Biological sampling and biochemical analyses*

Participants refrained from eating or drinking at least 8 hours before measurements took place. Early morning biological sampling included blood and spot urine collection, by a trained research nurse. 24hr urine was collected from participants starting on the morning of participation. Participants were requested to discard the first passed urine after which all subsequent urinary voids were collected. 24hr urine samples were considered complete if the total urinary volume  $\geq 300\text{mL}$  [17].

24hr urinary potassium, sodium, creatinine and albumin were measured using the Cobas Integra 400plus (Roche, Basel Switzerland). 24hr urinary MBG was analysed using a solid-phase Dissociation-Enhanced Lanthanide Fluorescent Immunoassay, based on a 4G4 anti-MBG mouse monoclonal antibody, described in detail by Fedorova *et al.* [18]. For our competitive reverse phase immunoassays we use a highly specific anti-MBG monoclonal antibody (mAb; clone 4G4) with very low immunoreactivity to several substances, including aldosterone. We also tested for immunoreactivity with contraceptive hormones [18]. The typical contraceptives reported by women in the present study include Nur-Isterate (progesterone), Yasmin (drospirenone and ethinyl estradiol), Triphasil (ethinyl estradiol and levonorgestrel), Minerva (progesterone with estrogens), and Ginette (progesterone with estrogens). MBG, progesterone, drospirenone, ethinyl estradiol and levonorgestrel (MilliporeSigma, St Louis, MO, USA) were diluted in the assay buffer and tested in our 4G4 immunoassay to compete with immobilised antigen (MBG-thyroglobulin) for a limited number of binding sites on 4G4 anti-MBG mAb. Furthermore, we used the C18 column for sample extraction. This column extracts a panel of steroids and other hydrophobic substances from the sample because of the nature of its silica-based sorbent [19].

We analysed serum low density lipoprotein cholesterol, high density lipoprotein cholesterol (HDL-C), triglycerides, C-reactive protein (CRP), glucose and gamma glutamyltransferase (GGT) using the Cobas Integra 400plus (Roche, Basel Switzerland). The chemiluminescence method on the Immulite (Siemens, Erlangen, Germany) was used to measure serum cotinine. We measured serum aldosterone using the RIA Aldosterone Kit (Beckman Coulter, Immunotech, Radiova, Czech Republic).

### *Statistical analyses*

All statistical analyses were performed with Statistica version 13.2 (Dell Inc., Tulsa, Oklahoma, USA) and figures drawn with GraphPad Prism version 5.0 (GraphPad Software Inc., California, USA). Data following a normal distribution was presented as the arithmetic mean  $\pm$  standard deviation. Variables following a non-Gaussian distribution were logarithmically transformed, and the central tendency and spread presented as the geometric mean; 5<sup>th</sup> and 95<sup>th</sup> percentile intervals. Independent t-tests were done to compare the anthropometric, cardiovascular, 24 urinary, biochemical and lifestyle profiles of men and women, and Chi-square tests for categorical data (socio-economic status, smoking, alcohol and hormonal contraceptive use). We performed analyses of covariance to determine significant differences in cfPWV across increasing quartiles of MBG excretion, while adjusting for age, waist to height ratio (WHtR) and MAP. Pearson-, partial- and multiple regression analyses were done in each group, with cfPWV as dependent variable, to explore the relationship with MBG excretion. While several covariates were considered for inclusion as possible independent variables, we ultimately included: ethnicity, age, WHtR, MAP, high density lipoprotein cholesterol (HDL-C), C-reactive protein (CRP),  $\gamma$ -glutamyl transferase (GGT) and glucose based on the strongest bivariate associations, with MBG excretion and cfPWV.

## **Results**

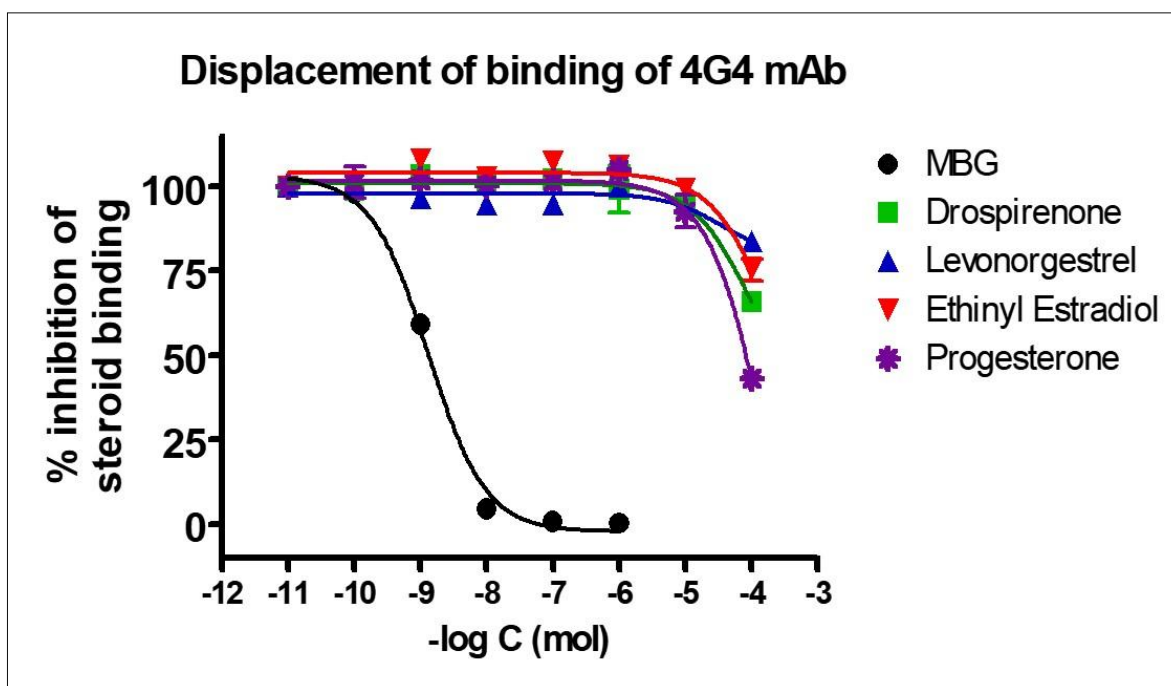
### *Cross-immunoreactivity of 4G4 anti-MBG mAb with contraceptive hormones*

We found low cross-immunoreactivity of 4G4 anti-MBG mAb with contraceptive hormones (less than 0.001-0.01 %; **Table 1, Figure 1**). This insured that MBG was reliably measured in the non-extracted urine samples in the presence of other steroids and hormones.

**Table 1: Cross-immunoreactivity of 4G4 monoclonal anti-MBG antibody with the components of the contraceptive treatments**

Cross-reactants	Cross-reactivity (%)
MBG	100
Progesterone	0.0007 (<0.001)
Drospirenone	0.0014
Ethinyl estradiol	0.0007 (<0.001)
Levonorgestrel	0.0034

MBG, Marinobufagenin



**Figure 1:** Displacement of binding 4G4 anti-MBG monoclonal antibody to MBG-thyroglobulin conjugate by MBG, progesterone, drospirenone, ethinyl estradiol, and levonorgestrel in dissociation-enhanced fluoroimmunoassay (DELFLIA) competitive reverse phase immunoassay.

*Participant characteristics*

Due to known reports on salt-sensitivity in black populations [20,21], we tested for an interaction of ethnicity on the relationships between MBG excretion and cfPWV, but found no interaction (**Table 2**).

**Table 2: Interaction of ethnicity on the relationship between MBG excretion and arterial stiffness in the total group, men and women**

	MBG excretion (nmol/day)		
	Total group N=711	Men N=296	Women N=415
cfPWV (m/s)*	$p=0.60$	$p=0.90$	$p=0.35$

cfPWV, Carotid femoral pulse wave velocity

\*Adjusted for mean arterial pressure

The general characteristics of this study population (mean age  $24.8 \pm 3.02$  years) are outlined in **Table 3**. Clear sex differences were evident in the cardiovascular profiles, with men demonstrating higher 24hr SBP, 24hr DBP, MAP and cfPWV compared to women (all  $p < 0.001$ ).

Even though 24hr urinary volume output was similar for men and women, men had higher MBG excretion ( $p < 0.001$ ) and  $\text{Na}^+$  excretion ( $p = 0.003$ ). In entirety, 78.3% of this study population was on a high salt habitual diet, consuming more than 5g of salt per day with a collective mean intake of 7.69 g/day and a  $\text{Na}^+/\text{K}^+$  ratio  $> 3$ .

Table 3: Basic characteristics of young men and women

	Men N=296	Women N=415	P
Ethnicity, Black, N (%)	145 (49.0)	217 (52.3)	0.39
Age (years)	24.9 ± 2.95	24.8 ± 3.08	0.70
Socio economic status, N (%)			0.27
<i>Low</i>	113 (38.2)	158 (38.0)	
<i>Middle</i>	65 (22.0)	111 (26.8)	
<i>High</i>	118 (39.8)	146 (35.2)	
<b>Anthropometric measurements</b>			
Height (m)	1.75 ± 7.65	1.63 ± 0.07	<0.001
Weight (kg)	76.2 ± 18.6	69.3 ± 16.6	<0.001
BMI (kg/m <sup>2</sup> )	24.8 ± 5.32	26.0 ± 6.15	0.008
WC (cm)	83.4 ± 13.1	78.7 ± 13.0	<0.001
Waist to height ratio	0.48 ± 0.07	0.48 ± 0.08	0.42
<b>Cardiovascular profile</b>			
24hr SBP (mmHg)	121 ± 8.26	113 ± 8.44	<0.001
24hr DBP (mmHg)	69.8 ± 5.99	68.1 ± 5.36	<0.001
MAP (mmHg)	92.2 ± 7.61	88.7 ± 7.91	<0.001
CfPWV (m/s)*	6.71 ± 0.81	5.95 ± 0.79	<0.001
<b>24hr Urinary profile</b>			
Volume (L/24hr)	1.42 ± 0.75	1.38 ± 0.83	0.51
MBG conc. (nmol/L)	3.29 (1.26; 7.23)	2.26 (0.70; 5.79)	<0.001
MBG exc. (nmol/day)	4.13 (1.46; 10.2)	2.69 (0.92; 7.92)	<0.001
MBG/Na <sup>+</sup> ratio	0.03 (0.01; 0.08)	0.02 (0.01; 0.06)	<0.001
Na <sup>+</sup> exc. (mmol/day)	141 (41.1; 360)	123 (45.8; 326)	0.003
NaCl intake (g/day)	8.32 (2.42; 21.2)	7.27 (2.70; 19.2)	0.003
K <sup>+</sup> exc. (mmol/day)	41.6 (12.6, 104)	39.3 (15.9; 101)	0.35
Na:K ratio	3.41 (1.36; 7.14)	3.22 (1.40; 6.78)	0.15
Albumin (mg/L)	3.55 (2.21; 8.13)	4.15 (2.32; 11.1)	<0.001



Creatinine (mmol/L)	9.85 (4.55; 20.2)	7.72 (3.08; 16.3)	<0.001
<b>Biochemical profile</b>			
Glucose (mmol/L)	4.82 ± 0.78	4.64 ± 0.66	0.002
HDL-C (mmol/L)	1.15 (0.77; 1.74)	1.33 (0.81; 2.13)	<0.001
LDL-C (mmol/L)	2.75 (1.54; 4.57)	2.61 (1.50; 4.25)	0.070
Trig (mmol/L)	0.90 (0.45; 2.05)	0.76 (0.39; 1.67)	<0.001
CRP (mg/L)	0.73 (0.10; 5.94)	1.38 (0.13; 12.3)	<0.001
Interleukin-6 (pg/ml)	0.92 (0.34; 3.29)	1.13 (0.35; 3.98)	<0.001
Aldosterone (pg/ml)	63.7 (17.5; 203)	73.1 (17.1; 425)	0.055
<b>Lifestyle measures</b>			
Smoking, N (%)	101 (34.2)	57 (13.7)	<0.001
Cotinine >10 (ng/ml)	85 (35.3)	54 (16.3)	<0.001
Alcohol intake, N (%)	181 (61.8)	207 (50.4)	0.003
GGT (U/L)	26.0 (12.8; 66.2)	18.1 (7.80; 54.0)	<0.001
Hormonal contraception, N (%)	—	163 (40.3)	

Arithmetic mean ± standard deviation; geometric mean (5<sup>th</sup> percentile; 95<sup>th</sup> percentile intervals).

\*Adjusted for MAP

cfPWV, Carotid femoral pulse wave velocity; CRP, C-reactive protein; DBP, Diastolic blood pressure; GGT,  $\gamma$ -glutamyl transferase; HDL-C, High density lipoprotein cholesterol; K<sup>+</sup>, Potassium; MAP, Mean arterial pressure; MBG, Marinobufagenin; Na<sup>+</sup>, Sodium; SBP, Systolic blood pressure; Trig, Triglycerides; WC, waist circumference.

### Regression analyses

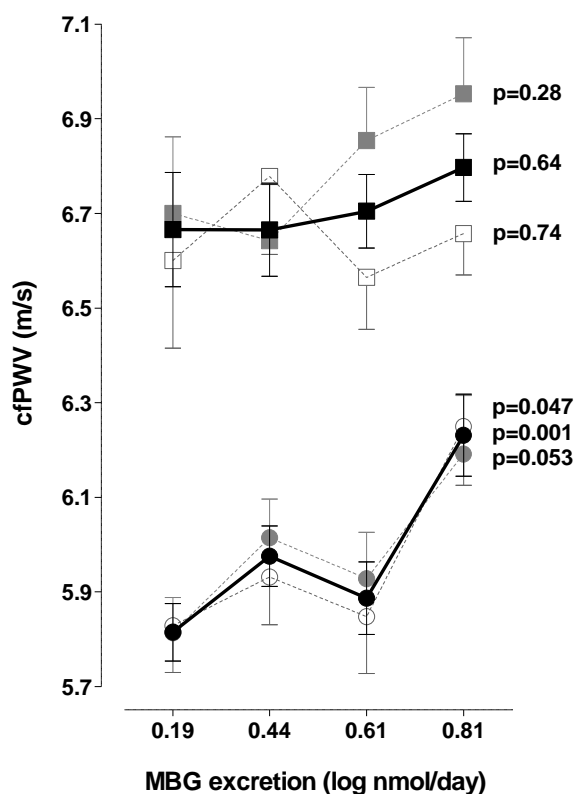
Single and partial regression analyses are presented in **Table 4**, with multiple regression analyses depicted in **Table 5**. We found that cfPWV associated positively across quartiles of MBG excretion in women ( $p=0.001$ ), adjusting for age, WHtR and MAP (**Figure 2**). Multivariable adjusted regression analyses underlined this positive association of cfPWV with MBG excretion women (Adj.R<sup>2</sup>=0.23; std.  $\beta$ =0.15;  $p=0.002$ ).

**Table 4: Single and partial regression analyses with carotid-femoral pulse wave velocity as dependent variable**

	<i>MBG excretion (nmol/day)</i>	
	<b>Women</b>	<b>Men</b>
Age (years)	$r=-0.11$ ; $p=0.03$	$r=-0.03$ ; $p=0.64$
Waist to height ratio	$r=-0.06$ ; $p=0.20$	$r=0.13$ ; $p=0.024$
<b>Cardiovascular measures</b>		
cfPWV (m/s)*	<b><math>r=0.17</math>; <math>p=0.003</math></b>	$r=0.04$ ; $p=0.46$
<i>Adjusted for ethnicity, age and waist to height ratio</i>		
cfPWV (m/s)*	<b><math>r=0.19</math>; <math>p&lt;0.001</math></b>	$r=0.07$ ; $p=0.26$

\*Adjusted for mean arterial pressure

cfPWV, Carotid femoral pulse wave velocity; MBG, Marinobufagenin.



**Figure 2: Arterial stiffness according to increasing quartiles of MBG excretion within men (■) and women (●).**

Black men (■); white men (□); black women (●) and white women (○).

Adjusted for age, waist to height ratio and mean arterial pressure.

**Table 5: Multiple regression analyses with carotid-femoral pulse wave velocity as dependent variable**

	<i>Carotid-femoral Pulse Wave Velocity (m/s)</i>			
	<b>Women</b>		<b>Men</b>	
	<b><math>\beta</math> (SE)</b>	<b><i>P</i></b>	<b><math>\beta</math> (SE)</b>	<b><i>P</i></b>
Adj. R <sup>2</sup>	0.23		0.23	
MBG (nmol/day)	0.150 (0.048)	0.002	0.062 (0.061)	0.31
Age (years)	0.259 (0.049)	<0.001	0.225 (0.062)	<0.001
MAP (mmHg)	0.441 (0.052)	<0.001	0.367 (0.064)	<0.001
Waist to height ratio	-0.232 (0.058)	<0.001	-0.158 (0.080)	0.048
Ethnicity (black/white)	0.063 (0.055)	0.23	0.011 (0.075)	0.88
CRP (mg/L)	-0.059 (0.055)	0.28	0.070 (0.065)	0.28
GGT (U/L)	0.047 (0.053)	0.38	0.096 (0.064)	0.14
HDL-C (mmol/L)	-0.043 (0.051)	0.40	-0.050 (0.063)	0.43
Glucose (mmol/L)	0.034 (0.049)	0.48	0.007 (0.069)	0.92
<i>Sensitivity Analyses for Hormonal Contraceptive use*</i>				
Adj. R <sup>2</sup>	0.21			
MBG (nmol/day)	0.139 (0.050)	0.005	—	
Hormonal Contraceptives (yes/no)	0.090 (0.049)	0.067	—	
<i>Sensitivity Analyses for Estimate NaCl intake*</i>				
Adj. R <sup>2</sup>	0.23		0.16	
MBG (nmol/day)	0.142 (0.056)	0.012	0.033 (0.075)	0.66
NaCl intake (g/day)	0.021 (0.056)	0.70	0.045 (0.073)	0.74

\*Multiple regression models for sensitivity analyses are additionally adjusted for age, ethnicity, MAP, waist to height ratio, HDL, CRP, GGT and glucose

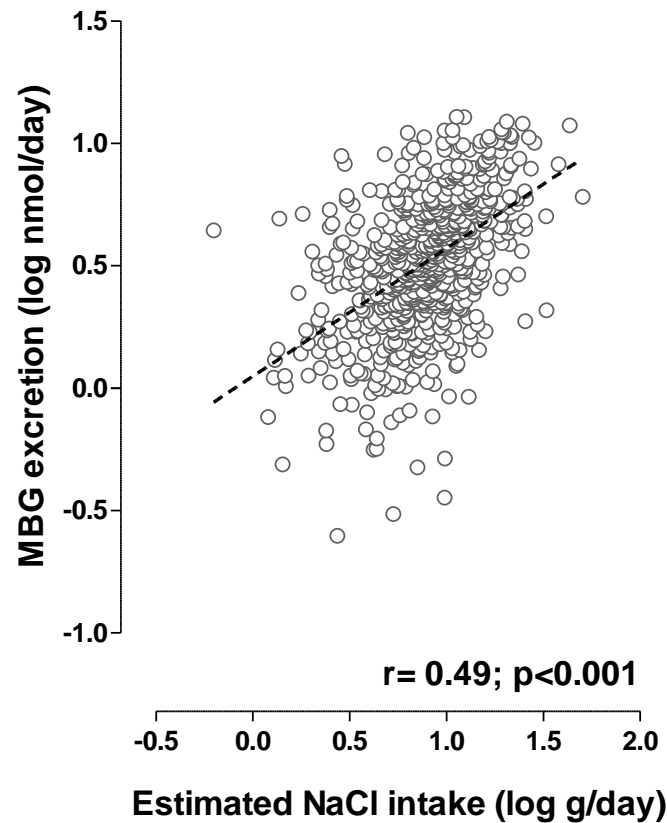
cfPWV, Carotid femoral pulse wave velocity; CRP, C-reactive protein; GGT,  $\gamma$ -glutamyl transferase; HDL-C, High density lipoprotein cholesterol; MAP, Mean arterial pressure; MBG, Marinobufagenin.

### *Sensitivity analyses*

Taking into account our sex specific results as well as the steroidal nature of endogenous MBG, we performed a sensitivity analyses to determine the possible confounding role of hormonal contraceptive use on the relationship between cfPWV and MBG in women (**Table 5**). This association between cfPWV and MBG remained significant (Adj.R<sup>2</sup>=0.21; std.  $\beta$ =0.14;  $p$ =0.005). Noteworthy however, was the borderline significant association between cfPWV and hormonal contraceptive use (Adj.R<sup>2</sup>=0.21; std.  $\beta$ =0.090;  $p$ =0.067). In addition we performed separate analyses in women who either use or do not use hormonal contraceptives. When plotting cfPWV across quartiles of MBG excretion, we found a significant trend in women not using hormonal contraceptives ( $p$ =0.005), and no relationship in those who do use contraceptives ( $p$ =0.27) (see Figure, Supplemental Digital Content 1, demonstrating cfPWV across quartiles of MBG excretion in women who made use of hormonal contraceptives and women who do not). In multiple regression analyses, our results were confirmed in women not using hormonal contraceptives (N=217; Adj.R<sup>2</sup>=0.19; std.  $\beta$ =0.18;  $p$ =0.005) (see Table, Supplemental Digital Content 2, which illustrates multiple regression analyses in women who made use of hormonal contraceptives and women who do not). In women who used contraceptives, the association was lost (N=140; Adj.R<sup>2</sup>=0.22; std.  $\beta$ =0.06;  $p$ =0.50).

We additionally performed a sensitivity analyses for aldosterone, which is known to influence sodium excretion, to determine whether the relationship between MBG excretion and cfPWV in women is robust (see Table, Supplemental Digital Content 3, demonstrating the sensitivity analyses for aldosterone). We found that the relationship of MBG excretion with cfPWV was not confounded by aldosterone (Adj.R<sup>2</sup>=0.23; std.  $\beta$ =0.16;  $p$ =0.001), and that aldosterone did not associate with cfPWV (Adj.R<sup>2</sup>=0.23; std.  $\beta$ =-0.013;  $p$ =0.81).

Several studies report the known relationship between NaCl intake and MBG excretion, also confirmed in our population ( $r$ =0.49;  $p$ <0.001) (**Figure 3**). We, therefore, performed additionally sensitivity analyses to determine if the associations of cfPWV with MBG excretion in women were confounded by NaCl intake (**Table 5**). In doing so, we found that the association of cfPWV with MBG excretion was independent of NaCl intake (Adj.R<sup>2</sup>=0.23; std.  $\beta$ =0.14;  $p$ =0.012), confirming the robustness of our results.



**Figure 3:** Pearson correlation between 24hr urinary MBG excretion and estimated NaCl intake.

## Discussion

We investigated whether large arterial stiffness is related to MBG excretion in a young healthy population. This study showed for the first time a positive and independent association between large artery stiffness and MBG excretion in women. In addition, our findings suggest that MBG may contribute to sodium and pressure-independent vascular changes at an early age, in the absence of detected cardiovascular disease.

Intriguingly, it has been previously proposed that large artery stiffness may play a precursory role in the development of hypertension as opposed to being a mere complication thereof [22]. Arterial stiffness characterises the reduced capability of the vessel wall to expand in response to hemodynamic changes [23]. The relationship between arterial stiffness and MBG in this young population may be attributed to MBG-mediated alterations in the composition of the scaffolding proteins, specifically collagen [7], essential in determining the mechanical properties of the arterial wall. Indeed, MBG has been shown to stimulate collagen-I synthesis via the

Na<sup>+</sup>/K<sup>+</sup>-ATPase-PKC $\delta$ -Fli-1 signalling pathway [6,7], and has been associated with arterial stiffness in an aged pre-hypertensive population (n=11) [8]. Importantly, the relevance of our results are underlined in a recently published study demonstrating that arterial stiffness is a strong independent predictor of elevated blood pressure and hypertension in young normotensive adults (aged 38 $\pm$ 5 years) [24].

Our findings additionally suggest that women may be more sensitive to the vascular effects of MBG despite having lower urinary MBG excretion compared to men. Although evidence from human studies explaining our sex specific result is limited, Goel *et al.* demonstrated increased PKC $\beta$ 2 expression in female rat aorta, previously shown to sensitise Na<sup>+</sup>/K<sup>+</sup>-ATPase to MBG [25]. Also, our study supports the role of sex hormones on the relationship between arterial stiffness and MBG, indicated by the sensitivity analyses performed for hormonal contraceptive use in women. The clear finding of an association only in women not using hormonal contraceptives, support the notion that sex hormones may interact with MBG as a steroidal hormone and its association with arterial stiffness, and should be accounted for in future analyses. In spite of the fact that our results add to a restricted body of literature with regards to MBG and sex, we did not specifically explore the relationship between MBG and sex hormones. Therefore, we cannot discuss causality as a more in-depth investigation into the relationship between MBG and sex hormones is imperative.

A strength of this study includes the measurement of arterial stiffness as cfPWV, accepted as the golden standard [26]. Due to the cross-sectional nature of our analyses the findings of this study should be interpreted within the appropriate context. Also, although urinary MBG was used to reflect plasma MBG levels, the measurement of plasma MBG for future studies would shed further light on the relationship with arterial stiffness.

In conclusion, in a young healthy population, natriuretic MBG may contribute to large artery stiffness early in life via a pressure independent manner, only in women, despite men exhibiting significantly higher estimated 24-h sodium excretion, MBG and higher arterial stiffness. This paper highlights the possible harmful implications of elevated MBG, shown to increase with a high salt diet, on large artery stiffness, thereby potentially increasing the risk for future CVD.

**Acknowledgements**

The authors of this study are grateful towards all individuals participating voluntarily in the study. The dedication of the support and research staff as well as students at the Hypertension Research and Training Clinic at the North-West University is also duly acknowledged.

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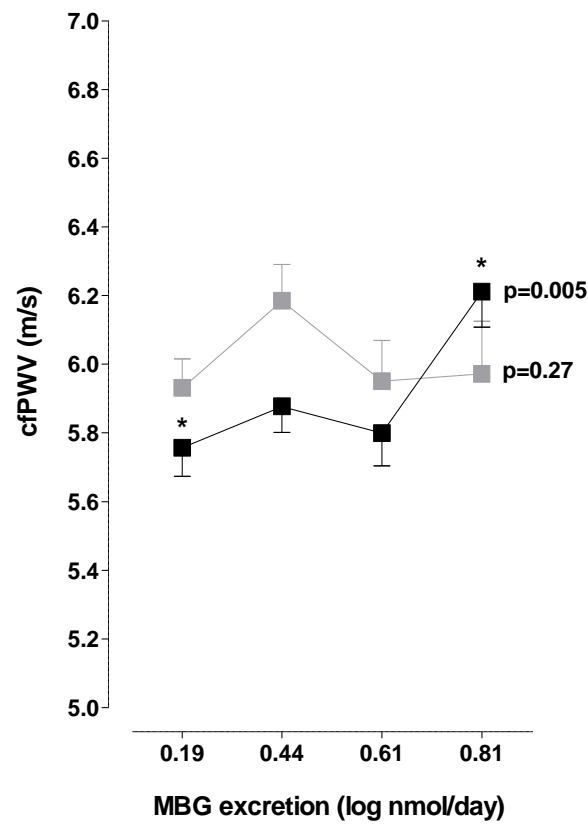
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## Supplemental digital content



**Supplemental Digital Content 1, Figure:** Arterial stiffness according to increasing quartiles of MBG excretion in women who use (■) and do not use hormonal contraceptives (■).

Adjusted for age, waist to height ratio and mean arterial pressure.

\*Interquartile difference  $p=0.021$

Supplemental Digital Content 2, Table: Multiple regression analyses in women

<i>Carotid-femoral Pulse Wave Velocity (m/s)</i>				
	Women not using hormonal contraceptives		Women using hormonal contraceptive	
	N=217		N=140	
	$\beta$ (SE)	<i>P</i>	$\beta$ (SE)	<i>P</i>
Adj. R <sup>2</sup>	0.19		0.22	
MBG (nmol/day)	0.179 (0.063)	0.005	0.055 (0.082)	0.50
Age (years)	0.230 (0.066)	<0.001	0.278 (0.079)	<0.001
MAP (mmHg)	0.4381 (0.070)	<0.001	0.432 (0.082)	<0.001
Waist to height ratio	-0.185 (0.084)	0.023	-0.232 (0.010)	0.010
Ethnicity (black/white)	0.073 (0.070)	0.30	0.021 (0.104)	0.84
CRP (mg/L)	-0.076 (0.079)	0.33	-0.090 (0.083)	0.28
GGT (U/L)	-0.003 (0.072)	0.96	0.066 (0.089)	0.46
HDL-C (mmol/L)	0.021 (0.065)	0.75	-0.135 (0.092)	0.15
Glucose (mmol/L)	0.179 (0.063)	0.60	0.023 (0.080)	0.78

cfPWV, Carotid femoral pulse wave velocity; CRP, C-reactive protein; GGT,  $\gamma$ -glutamyl transferase; HDL-C, High density lipoprotein cholesterol; MAP, Mean arterial pressure; MBG, Marinobufagenin.

Supplemental Digital Content 3, Table: Sensitivity analyses for aldosterone

<i>Carotid-femoral pulse wave velocity (m/s)</i>					
<b>Adjusted R<sup>2</sup></b>	<b>MBG included in model</b>		<b>MBG not included into model</b>		
	<b>0.23</b>		<b>0.21</b>		
	<b><math>\beta</math></b>	<b><i>P</i></b>	<b><math>\beta</math></b>	<b><i>P</i></b>	
MBG exc. (nmol/day)	0.16	<b>0.001</b>	—		
Aldosterone (pg/ml)	-0.013	0.81	-0.013	0.80	
Ethnicity (black/white)	0.063	0.29	0.090	0.13	
Age (years)	0.267	<0.001	0.236	<0.001	
Waist:height ratio	-0.231	<0.001	-0.217	<0.001	
MAP (mmHg)	0.436	<0.001	0.446	<0.001	
HDL (mmol/L)	-0.039	0.46	-0.071	0.18	
CRP (mg/L)	-0.058	0.28	-0.099	0.069	
Glucose (mmol/L)	0.040	0.42	0.050	0.32	
GGT (U/L)	0.047	0.38	0.079	0.15	

CRP, C-reactive protein; HDL-C, High density lipoprotein cholesterol; MAP, Mean arterial pressure; MBG, Marinobufagenin; GGT,  $\gamma$ -glutamyl transferase.

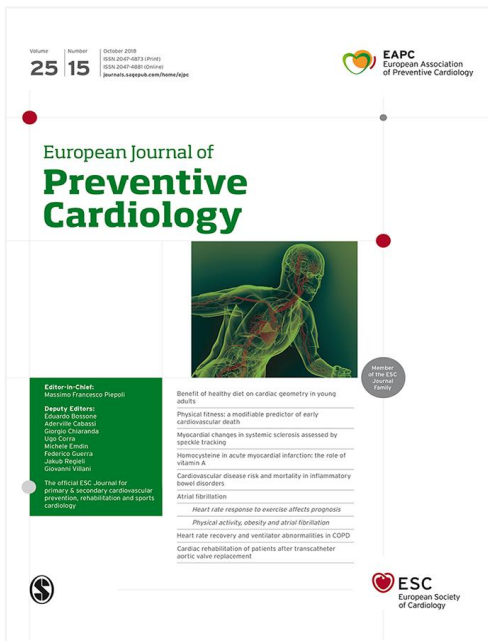
Adjusted for Aldosterone, age, ethnicity, waist:height ratio, mean arterial pressure, HDL-C, CRP, GGT and Glucose

# Chapter 4

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Research article 2:

Marinobufagenin and left ventricular mass in young  
adults: The African-PREDICT study

European Journal of Preventive Cardiology			
<p>European Journal of</p> <h1>Preventive Cardiology</h1>			
Impact factor	4.542		
Publisher	SAGE		
Citation	<p>Strauss M, Smith W, Kruger R, Wei W, Fedorova OV, Schutte AE. Marinobufagenin and left ventricular mass in young adults: The African-PREDICT study. <i>Eur J Prev Cardiol.</i> 2018;25:1587-1595.</p> 		
Aims & Scope	<p>A fully refereed journal embracing all the scientific, clinical and public health disciplines that address the causes and prevention of cardiovascular disease, as well as cardiovascular rehabilitation and exercise physiology.</p>		
Author Instructions			
Language	Not specified	Font	Not specified
Spacing	Double Spacing	Margins	Not specified
Word count	5000 words	Tables & figures	Maximum of 6
References	± 40	Alignment	Not justified

<b>Page numbers</b>	Number consecutively starting on the title page
<b>Manuscript</b>	Manuscripts should include the following sections (1) Title page, (2) Abstract and up to six Keywords, (3) Introduction, (4) Methods, (5) Results, (6) Discussion, (7) Acknowledgements, (8) Funding, (9) Conflict of interest, (10) Authors' Contributions, (11) References, (12) Figure legends, (14) Tables, (15) Figures
<b>Title page</b>	<ul style="list-style-type: none"> <li>• Full title (20 words)</li> <li>• Full first name, middle initial(s) and last (family name) name of each author</li> <li>• Affiliations of all the authors connected using a,b,c</li> <li>• Sources of funding</li> <li>• Conflict of interest statement</li> <li>• Corresponding author information</li> <li>• Word count (including references, tables and legends)</li> </ul>
<b>Abstract</b>	Structured or unstructured abstract (250 words) – Aims, Methods, Results & Conclusion
<b>Keywords</b>	3–10 keywords
<b>Text</b>	Introduction, Methods, Results & Discussion
<b>Author contributions</b>	Provide the initials and contributions of each author
<b>Acknowledgements</b>	Include acknowledgements
<b>Conflict of interest</b>	Include statement on title page
<b>Funding</b>	Include funding on title page
<b>Ethical considerations</b>	<ul style="list-style-type: none"> <li>• All work must be conducted in accordance with the Declaration of Helsinki.</li> <li>• Include (1) a statement that consent from participants and (2) of ethical approval.</li> </ul>
<b>References</b>	<ul style="list-style-type: none"> <li>• Number consecutively in the order in which they first appear in the text.</li> <li>• SAGE Vancouver reference style</li> </ul>
<b>Tables</b>	<ul style="list-style-type: none"> <li>• Each table should be typed on a separate page in double spacing</li> <li>• Each table should be assigned an Arabic numeral and a brief title</li> </ul>
<b>Figures</b>	<ul style="list-style-type: none"> <li>• Cite figures consecutively in your manuscript.</li> <li>• Number figures in the figure legend in the order in which they are discussed</li> </ul>
<b>Legends for illustrations</b>	<ul style="list-style-type: none"> <li>• Captions should be typed in double spacing, beginning on a separate page.</li> </ul>
<b>Supplemental Digital Content</b>	<ul style="list-style-type: none"> <li>• Cited consecutively in the text of the submitted manuscript.</li> </ul>

*\*Formatting changes were made to maintain uniformity throughout this thesis, including text font, line spacing, margins, page numbers, tables and figures.*



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**Marinobufagenin and left ventricular mass in young adults:****The African-PREDICT study**

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**Sources of support:** The research funded in this manuscript is part of an ongoing research project financially supported by the South African Medical Research Council (SAMRC) with funds from National Treasury under its Economic Competitiveness and Support Package; the South African Research Chairs Initiative (SARChI) of the Department of Science and Technology and National Research Foundation (NRF) of South Africa; GlaxoSmithKline R&D, the UK Medical Research Council and with funds from the UK Government's Newton Fund; as well as corporate social investment grants from Pfizer (SA), Boehringer Ingelheim (SA), Novartis (SA), the Medi Clinic Hospital Group (SA) and in kind contributions of Roche Diagnostics (SA). Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors, and therefore, the NRF does not accept any liability in regard. This study was supported in part by the Intramural Research Program, National Institute on Aging, National Institutes of Health, USA.

**Disclaimers:** The authors wish to disclose the unrestricted educational grants from Boehringer Ingelheim, Novartis, the Medi Clinic Hospital Group, in kind contributions of Roche Diagnostics (South Africa) and support of patient transport minibus from Pfizer (South Africa). Contributions from GlaxoSmithKline R&D is acknowledged within its partnership with the UK Government's Newton Fund.

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**Word count:** 5036

## Abstract

**Background:** The endogenous steroidal inhibitor of Na/K-ATPase and natriuretic hormone, Marinobufagenin (MBG), plays a physiological role in ionic homeostasis. Animal models suggest that elevated MBG adversely associates with cardiac and renal, structural and functional alterations. It remains uncertain whether MBG relates to the early stages of target organ damage (TOD) development, especially in young adults without cardiovascular disease. We therefore explored whether elevated 24hr urinary MBG excretion related to indices of subclinical TOD in young healthy adults.

**Design:** This cross-sectional study included 711 participants from the African-PREDICT study (black 51%, men 42%,  $24.8 \pm 3.02$  years).

**Methods:** We assessed cardiac geometry and function by two dimensional echocardiography and pulse wave Doppler imaging. 24hr urinary MBG and Na<sup>+</sup> excretion were measured, and estimated glomerular filtration rate determined.

**Results:** Across MBG excretion quartiles, left ventricular mass (LVMi)( $p<0.001$ ), end diastolic volume (EDVi)( $p<0.001$ ), stroke volume (SVi)( $p=0.004$ ) and Na<sup>+</sup> excretion ( $p<0.001$ ) were higher within the fourth compared to the first quartile. Partial regression analyses indicated LVMi ( $r=0.08$ ,  $p=0.043$ ), EDVi ( $r=0.10$ ,  $p=0.010$ ) and SVi ( $r=0.09$ ,  $p=0.022$ ) to be positively related to MBG excretion. In multivariate-adjusted regression analysis, LVMi associated positively with MBG excretion only in the highest MBG excretion quartile (Adj. $R^2=0.20$ ;  $\beta=0.15$ ;  $p=0.043$ ). This relationship between LVMi and MBG excretion was evident in women (Adj. $R^2=0.06$ ;  $\beta=0.127$ ;  $p=0.015$ ), but not men (Adj. $R^2=0.06$ ;  $\beta=0.007$ ;  $p=0.92$ ).

**Conclusions:** LVMi positively and independently associates with MBG excretion in young healthy adults with excessively high MBG excretion. Women may be more sensitive to the effects of MBG on early structural cardiac changes.

**Word count:** 250

**Key words:** apparently healthy, left ventricular mass index, marinobufagenin, young adults

## Introduction

Mammalian cardiotoxic steroids include the endogenous bufadienolide marinobufagenin (MBG), which is synthesized by the adrenal cortex<sup>1</sup> in response to sodium-induced plasma volume expansion.<sup>2</sup> In accordance, we have previously indicated a strong correlation between estimated salt intake and MBG in young adults.<sup>3</sup> Apart from MBG's primary natriuretic function via the classic ionic pathway whereby renal  $\alpha 1$ -Na<sup>+</sup>/K<sup>+</sup>-ATPase activity is inhibited, MBG additionally induces pro-fibrotic signalling through the Na<sup>+</sup>/K<sup>+</sup>-ATPase in cardiovascular tissue.<sup>4, 5</sup> This effect of MBG on the cellular signalling function of Na<sup>+</sup>/K<sup>+</sup>-ATPase is proposed to play an important role in disease development.<sup>4</sup>

Increasing evidence demonstrates the deleterious role of MBG particularly on cardiac<sup>5-8</sup> and renal<sup>9, 10</sup> structure and function, implicating MBG in the pathogenesis of several conditions such as preeclampsia,<sup>11, 12</sup> uremic cardiomyopathy<sup>5, 6</sup> and heart failure.<sup>7, 13</sup> Accordingly, elevated levels of MBG were shown to promote cardiac fibrosis<sup>5, 6</sup> and hypertrophy<sup>5</sup> while also being associated with impaired left ventricular relaxation.<sup>5, 8</sup> Apart from cardiac effects, MBG promotes renal fibrosis<sup>9</sup> and was shown to be elevated in patients with chronic kidney disease.<sup>14</sup>

Studies pertaining to MBG and the effect thereof on target organ damage (TOD) were performed in animals<sup>5, 9</sup> or human cohorts with present pathology such as heart failure (aged  $58 \pm 13$ ),<sup>7</sup> chronic kidney disease (aged  $52.8 \pm 3.7$ )<sup>14</sup> and renal artery stenosis (aged  $70.5 \pm 1.3$ ).<sup>10</sup> Thus, it is not yet clear whether MBG might be related to the initial stages of TOD development in young healthy adults, especially those previously shown to consume high amounts of salt,<sup>15</sup> with resultant elevated MBG levels.<sup>3</sup> It is of particular interest to demonstrate a possible relationship in this young population without overt cardiovascular disease (CVD), which should contribute to our understanding of the early pathophysiological role of MBG in CVD development. We therefore explored whether elevated levels of 24hr urinary MBG excretion are related to indices of subclinical TOD in a young apparently healthy adult population aged 20-30 years.

Contingent upon a relationship between MBG and indices of subclinical TOD in this young population, our study would support further investigation into MBG as a potential marker of early cardiovascular risk. Moreover,

evidence of a relationship between MBG and indices of subclinical TOD in this population consuming a habitual high salt diet,<sup>15</sup> would endorse the implementation of global preventative salt reduction legislations.

## Methods

### *Study design and participant recruitment*

This cross-sectional study included data of the first 711 consecutively enrolled participants (black 51%, men 42%) from the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT), with complete 24hr urinary data.

The study recruited young apparently healthy black and white adults (aged 20-30) from the North West Province of South Africa. Participants were recruited between 2012 and 2017. Community members, in proximity to the Potchefstroom area, were invited on a voluntary basis to participate in the initial health screening prior to inclusion into the study. The inclusion criteria were microalbuminuria < 30 mg/ml and HIV uninfected. Participants were included based on office blood pressure <140/90 mmHg, which is in line with the 2013 Guidelines set by the European Society of Hypertension and European Society of Cardiology.<sup>16</sup> In addition, none of the participants were previously diagnosed with any chronic illness, or were using antihypertensive or chronic disease medication (self-reported). None of the women participating in this study were pregnant or lactating.

Eligible participants were invited back for further measurements at the Hypertension Research Clinic on the North-West University campus. Participants arrived at 08:00 where they were introduced to the research environment. Organisational procedures were explained prior to the commencement of measurements.

The African-PREDICT study protocol was approved by the Health Research Ethics Committee of the North-West University, and is registered at Clinical Trials.gov (Nr. NCT03292094). All procedures complied with institutional guidelines and the Declaration of Helsinki. Written informed consent was provided by each participant before participation in the study. Reporting of the study conforms to STROBE statement along with references to the STROBE statement and the broader EQUATOR guidelines.<sup>17</sup>

*Cardiovascular measurements*

A single registered medical clinical technologist performed two dimensional echocardiography using the General Electric Vivid E9 device (GE Vingmed Ultrasound A/S, Horten, Norway), and a 2.5 to 3.5 MHz transducer and a single ECG-lead. Echocardiographic imaging was performed in accordance with standardized procedures outlined by the American Society of Echocardiography and the European Association of Cardiovascular Imaging.<sup>18-20</sup> Measures including the left ventricular mass (LVM) and LV volumes were determined using the biplane method, and defined by indexation to BSA or height. LVM was normalized for BSA (LVMi),<sup>20</sup> stroke volume for height to the power 2.04 (SVi) and cardiac output for height to the power 1.83.<sup>21</sup> End systolic (ESV) and end diastolic volume (EDV) were calculated using the Teichholz formula,<sup>22</sup> and EDV subsequently indexed for height (EDVi). In addition, we determined indices of systolic function including fractional shortening (FS) and LV ejection fraction (EF)  $((EDV-ESV)/EDV)$ .

LV filling was assessed by means of pulse wave Doppler imaging performed in the apical 4-chamber view. Sample volumes were placed 1-3 mm between the tips of the mitral valve leaflets in parallel alignment to inflow. The peak velocities of early (E) and late (A) diastolic filling, the E/A ratio and the left atrial to aortic root ratio (LA: Ao) were measured as parameters of diastolic function. Additionally, we determined myocardial tissue movement using tissue Doppler imaging (TDI) in order to calculate the ratio between the transmitral peak E velocity and the early diastolic mitral annular velocity ( $E/\dot{e}$ ).

*Biological sampling and biochemical analyses*

Participants were requested not to eat or drink at least 8 hours prior to measurements. A trained research nurse collected early morning blood samples at approximately the same time every morning and before cardiovascular measurements commenced. Following blood sample collection using red top serum tubes and sodium fluoride plasma tubes, samples were immediately taken from the Hypertension Research Clinic to the on-site temperature controlled laboratory. Samples prepared for serum were allowed to clot for 30 minutes at room temperature and were subsequently centrifuged for 30 minutes at 1602g at room temperature (Hettich Universal 320; Andreas Hettich GmbH & Co., Tuttlingen, Germany), and aliquoted into cryovials. Sodium fluoride plasma tubes were placed on ice, and centrifuged for 10 minutes at 2307 g (Hettich Universal 32R, Andreas Hettich

GmbH & Co., Tuttlingen, Germany). Aliquoted samples were stored in the on-site biofreezers controlled at  $-80^{\circ}\text{C}$ , until analysis.

Serum C-reactive protein (CRP), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), triglycerides,  $\gamma$ -glutamyl transferase (GGT), creatinine and  $\text{Na}^+$ , and plasma glucose were analysed using the Cobas Integra 400plus (Roche, Basel Switzerland). In addition, serum interleukin-6 (IL-6) (High sensitivity Quantikine ELISA kit R&D systems, Minneapolis, USA) and cotinine (Chemiluminescence method on the Immulite, Siemens, Erlangen, Germany) were measured.

The 24hr urine sampling for this study, from which we measured  $\text{Na}^+$ ,  $\text{K}^+$ , albumin and creatinine (Cobas Integra 400plus, Roche, Basel Switzerland), has previously been described<sup>3</sup> and follow standard protocols by the Pan American Health Organisation/ World Health Organisation (PAHO/WHO).<sup>23</sup> 24hr urinary MBG was additionally analysed with a solid-phase Dissociation-Enhanced Lanthanide Fluorescent Immunoassay based on a mouse monoclonal 4G4 anti-MBG antibody.<sup>24</sup> We determined eGFR using the CKD-EPI equation,<sup>25</sup> and determined fractional sodium excretion (FENa)<sup>26</sup> using the following formula:

$$\text{FENa} = (\text{urinary Na} \cdot \text{plasma creatinine}) / (\text{plasma Na} \cdot \text{urinary creatinine}) \cdot 100$$

*Additional methods can be seen in the online supplement.*

#### *Statistical analyses*

We used Statistica version 13.2 (Dell Inc., Tulsa, Oklahoma, USA) for data analyses. Non-Gaussian distributed variables were logarithmically transformed. We performed analyses of covariance to determine differences in cardiac structure, function and renal function across increasing quartiles of MBG excretion while adjusting for age, sex and ethnicity. Pearson, partial and multiple regression analyses were done to explore relationships of subclinical TOD with MBG excretion. We additionally performed multiple regression analyses within respective MBG excretion quartiles, where measures of TOD (RWT, LVMI, EDVi, SVi, COi, LA:Ao, E:A, E:é, FENa, eGFR or 24hr urinary albumin) were included into separate models as dependent variables. While we considered several covariates for inclusion as possible independent variables, sex, age, waist:height (WHtR), 24hr SBP, HDL-C, CRP, GGT and glucose were ultimately included in the final models based on the strongest bivariate

associations with indices of TOD and MBG excretion. Multiple regression models in which case the dependent variables were normalized for BSA or height, were not adjusted for WHtR. Also, multiple regression models with echocardiographic parameters as dependent variables were additionally adjusted for eGFR. Interaction testing was performed for sex and ethnicity, and Pearson, partial and multiple regression analyses were repeated accordingly. Missing data for dependent variables included LVMi (N=2), relative wall thickness (N=2), EDVi (N=2), SVi (N=3), COi (N=4), EF (N=2), FS (N=3), LA:Ao (N=2), E:A (N=5), E:é (N=7), FENa (N=37), eGFR (N=22), albumin (N=18). Pearson and partial analyses were performed using pairwise deletion of data, while casewise deletion was used for multiple regression analyses.

## Results

### *Participant characteristics*

**Table 1** presents the descriptive characteristics of this young adult population (aged  $24.8 \pm 3.02$  years) by increasing quartiles of MBG excretion. We found that EDVi ( $p=0.003$ ), SVi ( $p=0.003$ ) and 24hr SBP ( $p=0.001$ ) increased along increasing quartiles of MBG. Differences between quartiles 1 and 4 for EDVi ( $p<0.001$ ), SVi ( $p=0.004$ ) and 24hr SBP ( $p<0.001$ ) were also noted after adjustment for age, sex and ethnicity. Similarly, LVMi was higher within the fourth quartile when compared to the first of MBG excretion ( $p<0.001$ ). As expected, individuals with increased MBG had significantly higher 24hr urinary volume, Na<sup>+</sup>, K<sup>+</sup> and creatinine excretion.

### *Regression analyses*

Unadjusted analyses indicated significant relationships between indices of subclinical TOD and MBG excretion (**Figure 1 and Figure 2**). Once we adjusted for age, sex and ethnicity the relationships with LVMi ( $r=0.08$ ,  $p=0.043$ ), EDVi ( $r=0.10$ ,  $p=0.010$ ) and SVi ( $r=0.09$ ,  $p=0.022$ ) remained significant, but not eGFR ( $r=0.004$ ,  $p=0.92$ ). When we applied full adjustments in multivariate adjusted analyses in the total group, these relationships became borderline significant, namely with LVMi (Adj.  $R^2=0.23$ ; std.  $\beta=0.066$ ;  $p=0.084$ ), EDVi (Adj.  $R^2=0.22$ ; std.  $\beta=0.068$ ;  $p=0.079$ ) and SVi (Adj.  $R^2=0.07$ ; std.  $\beta=0.072$ ;  $p=0.089$ ) (**Supplementary Table 1**).

Table 1: Comparison of general participant characteristics across quartiles of MBG excretion (N=707)

	<i>MBG excretion quartiles</i>				<i>P for trend</i>	<i>P Q1 vs Q4</i>
	<i>Q1 (N=178)</i>	<i>Q2 (N=178)</i>	<i>Q3 (N=176)</i>	<i>Q4 (N=179)</i>		
Limits of quartiles (nmol/day)	0.250 – 2.175	2.176 – 3.282	3.283 – 5.021	5.022 – 12.81	<0.001	<0.001
Age (years)	25.1 ± 0.23	24.6 ± 0.23	24.8 ± 0.23	24.7 ± 0.23	0.34	0.88
Black race, N (%)	102 (57.3)	102 (57.3)	90 (51.1)	68 (38.0)	<0.001	<0.001
Men, N (%)	37 (20.8)	57 (32.0)	90 (51.1)	112 (62.6)	<0.001	<0.001
Low SES, N (%)	65 (36.5)	78 (43.8)	70 (39.8)	58 (32.4)	0.33	0.74
<b><i>Anthropometric measurements</i></b>						
Height (m)	1.64 ± 0.66	1.66 ± 0.66	1.69 ± 0.66	1.72 ± 0.66	<0.001	<0.001
Body mass index (kg/m <sup>2</sup> )	26.1 ± 0.44	25.0 ± 0.44	24.9 ± 0.44	26.1 ± 0.44	0.076	0.99
Waist to height ratio	0.49 ± 0.01	0.47 ± 0.01	0.47 ± 0.01	0.49 ± 0.01	0.067	0.99
<b><i>Adjusted for age, sex, ethnicity</i></b>						
<b><i>Cardiovascular profile</i></b>						
24hr SBP (mmHg)	116 ± 0.63	115 ± 0.62	117 ± 0.63	118 ± 0.64	0.001	<0.001



	<i>MBG excretion quartiles</i>				<i>P for</i>	<i>P</i>
	<i>Q1 (N=178)</i>	<i>Q2 (N=178)</i>	<i>Q3 (N=176)</i>	<i>Q4 (N=179)</i>	<i>trend</i>	<i>Q1 vs Q4</i>
24hr DBP (mmHg)	68.6 ± 0.43	68.1 ± 0.42	69.2 ± 0.42	69.2 ± 0.43	0.24	0.58
LVMi (g/m <sup>2</sup> )	70.4 (68.2; 72.6)	72.0 (69.9; 74.3)	72.6 (70.4; 74.9)	74.1 (71.9; 76.5)	0.16	<0.001
Relative wall thickness (cm)	0.36 (0.35; 0.37)	0.36 (0.35; 0.38)	0.36 (0.35; 0.37)	0.36 (0.35; 0.37)	0.70	0.99
EDVi (ml/min)	59.0 (57.2; 60.8)	58.9 (57.2; 60.7)	58.9 (57.1; 60.6)	63.1 (61.2; 65.0)	0.003	<0.001
SVi (ml.min <sup>2.04</sup> )	22.7 (21.9; 23.5)	22.9 (22.2; 23.7)	22.6 (21.8; 23.3)	24.4 (23.6; 25.3)	0.003	0.004
COi (L/min <sup>1.83</sup> )	1.66 (1.60; 1.73)	1.66 (1.60; 1.72)	1.64 (1.58; 1.70)	1.76 (1.70; 1.83)	0.030	0.99
Ejection fraction (%)	65.9 ± 0.47	66.5 ± 0.46	66.2 ± 0.46	67.1 ± 0.47	0.39	0.99
Fractional shortening (%)	36.4 ± 0.37	36.9 ± 0.36	36.6 ± 0.36	37.4 ± 0.37	0.35	0.99
LA:Ao	1.10 ± 0.01	1.09 ± 0.01	1.08 ± 0.01	1.09 ± 0.01	0.84	0.99
E:A	2.07 (2.00; 2.15)	2.02 (1.96; 2.10)	2.04 (1.97; 2.12)	2.07 (2.00; 2.15)	0.78	0.99
E:é	6.33 (6.16; 6.51)	6.28 (6.12; 6.45)	6.25 (6.09; 6.42)	6.37 (6.20; 6.55)	0.78	0.98
<b>24hr Urinary profile and renal function</b>						
Volume (L/24hr)	0.99 ± 0.05	1.22 ± 0.05	1.46 ± 0.05	1.89 ± 0.05	<0.001	<0.001

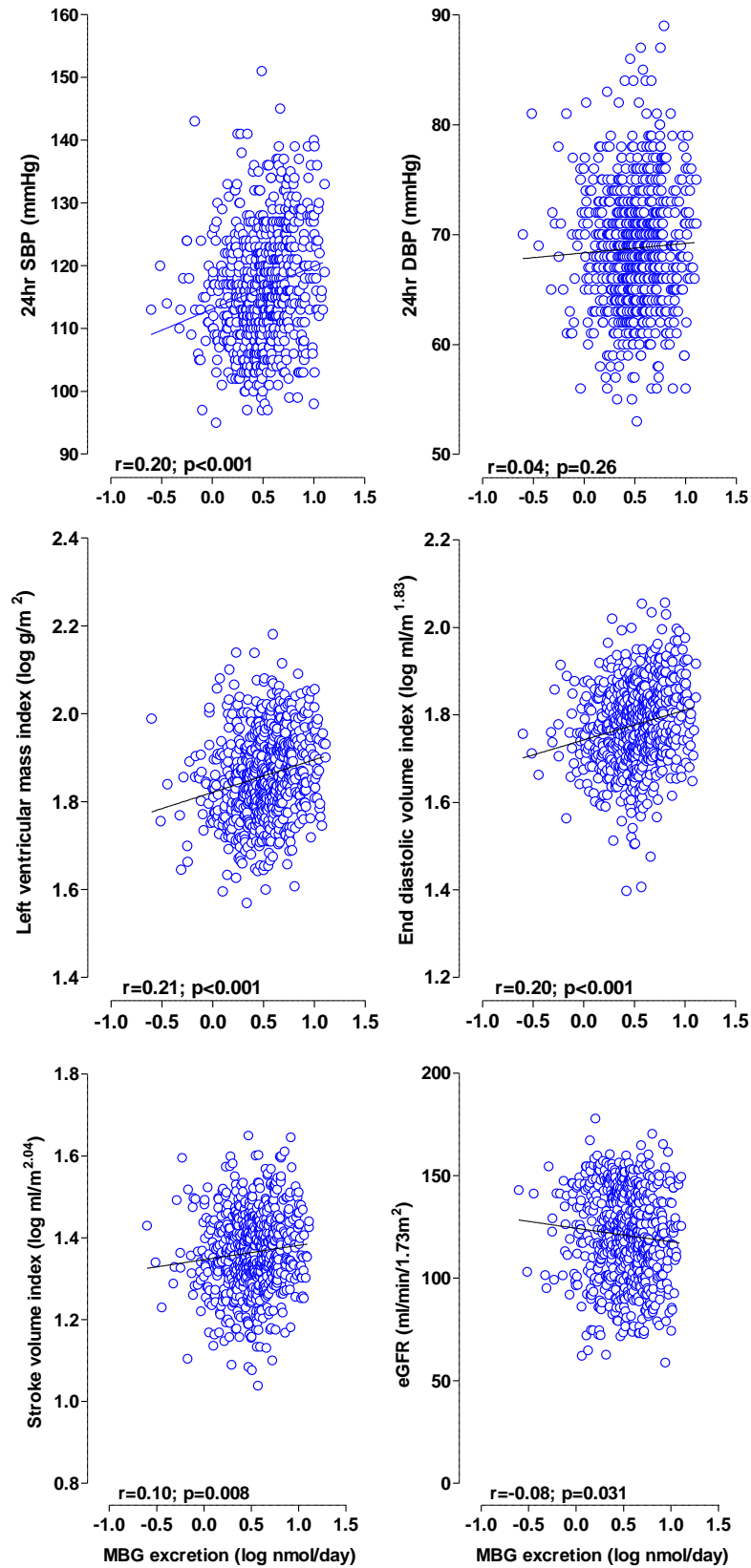
	<i>MBG excretion quartiles</i>				<i>P for</i>	<i>P</i>
	<i>Q1 (N=178)</i>	<i>Q2 (N=178)</i>	<i>Q3 (N=176)</i>	<i>Q4 (N=179)</i>	<i>trend</i>	<i>Q1 vs Q4</i>
MBG concentration (nmol/L)	1.56 (1.45; 1.68)	2.48 (2.31; 2.66)	3.10 (2.89; 3.33)	4.06 (3.77; 4.37)	<0.001	<0.001
MBG excretion (nmol/day)	1.41 (1.36; 1.46)	2.73 (2.63; 2.84)	4.04 (3.89; 4.19)	6.92 (6.66; 7.19)	<0.001	<0.001
Na <sup>+</sup> excretion (mmol/day)	87.7 (80.8; 95.3)	116 (107; 126)	139 (132.9; 151)	201 (185; 218)	<0.001	<0.001
MBG:Na <sup>+</sup> ratio	0.02 (0.01; 0.02)	0.02 (0.02; 0.03)	0.03 (0.03; 0.03)	0.03 (0.03; 0.04)	<0.001	<0.001
NaCl intake (g/day)	5.17 (4.76; 5.61)	6.85 (6.33; 7.41)	8.22 (7.60; 8.90)	11.8 (10.9; 12.8)	<0.001	<0.001
K <sup>+</sup> excretion (mmol/day)	27.2 (25.1; 29.5)	33.7 (31.2; 36.4)	43.1 (39.9; 46.6)	65.7 (60.8; 71.0)	<0.001	<0.001
Na:K ratio	3.34 (3.09; 3.61)	3.48 (3.23; 3.75)	3.25 (3.02; 3.50)	3.15 (2.93; 3.40)	0.33	0.99
FENa (%)	0.55 (0.49; 0.61)	0.70 (0.63; 0.77)	0.80 (0.72; 0.88)	1.06 (0.96; 1.18)	<0.001	<0.001
eGFR (ml/min/1.73m <sup>2</sup> )	120 ± 1.26	122 ± 1.21	121 ± 1.22	120 ± 1.24	0.76	0.010
Albumin (mg/L)	3.92 (3.68; 4.19)	3.67 (3.45; 3.91)	3.89 (3.65; 4.14)	4.07 (3.82; 4.34)	0.17	0.99
Creatinine (mmol/L)	7.88 (7.30; 8.51)	8.19 (7.60; 8.82)	8.56 (7.95; 9.22)	9.54 (8.84; 10.3)	0.006	<0.001
<b>Biochemical profile</b>						
Glucose (mmol/L)	4.76 ± 0.05	4.68 ± 0.05	4.71 ± 0.05	4.71 ± 0.05	0.79	0.99

	<i>MBG excretion quartiles</i>				<i>P for</i>	<i>P</i>
	<i>Q1 (N=178)</i>	<i>Q2 (N=178)</i>	<i>Q3 (N=176)</i>	<i>Q4 (N=179)</i>	<i>trend</i>	<i>Q1 vs Q4</i>
HDL-C ( mmol/L)	1.31 (1.25; 1.37)	1.26 (1.21; 1.31)	1.23 (1.18; 1.28)	1.22 (1.17; 1.27)	0.11	<0.001
LDL-C (mmol/L)	2.71 (2.57; 2.86)	2.67 (2.54; 2.81)	2.58 (2.45; 2.71)	2.70 (2.57; 2.84)	0.52	0.99
C-Reactive protein (mg/L)	1.37 (1.11; 1.70)	1.02 (0.83; 1.25)	1.00 (0.81; 1.23)	0.92 (0.74; 1.13)	0.055	<0.001
IL-6 (pg/ml)	1.06 (0.96; 1.18)	1.02 (0.92; 1.14)	1.05 (0.94; 1.16)	1.01 (0.91; 1.12)	0.91	0.041
<b><i>Lifestyle measures</i></b>						
Cotinine >10 (ng/ml)	209 ± 26.9	198 ± 23.7	228 ± 27.5	168 ± 23.9	0.43	0.99
γ-glutamyl transferase (U/L)	19.3 (17.8; 21.0)	20.4 (18.8; 22.1)	23.3 (21.5; 25.2)	21.2 (19.5; 23.0)	0.018	0.033

Data presented as arithmetic mean ± SEM or geometric mean (5<sup>th</sup> and 95<sup>th</sup> percentile intervals).

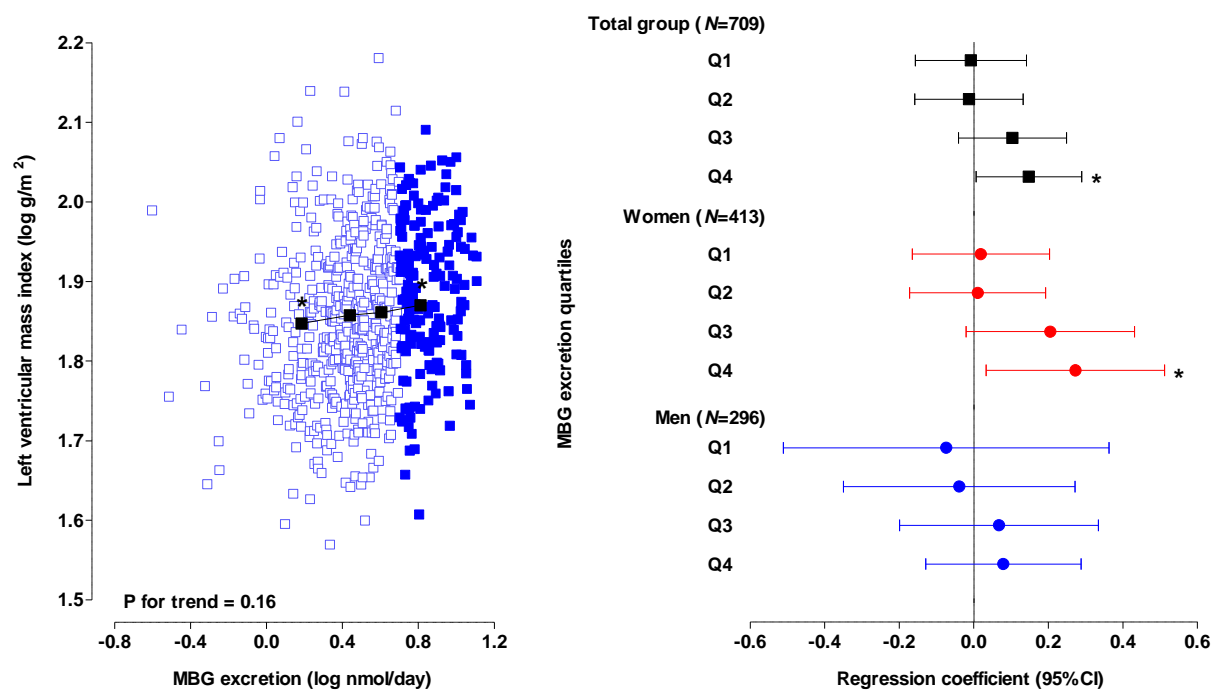
Cardiovascular, renal, biochemical and lifestyle measure were adjusted for age, sex and ethnicity.

SES, Socio-economic status in total group; COi, Cardiac index; DBP, Diastolic blood pressure; E:A, Mitral peak velocity of early filling (E) to late diastolic filling (A); EDVi, End diastolic volume index; E:e', Mitral peak velocity of early filling (E) to early diastolic mitral annular velocity (e'); EF, Ejection fraction; eGFR, Estimate glomerular filtration rate; FENa, Fractional sodium excretion; FS, Fractional shortening; HDL-C, High density lipoprotein cholesterol; K<sup>+</sup>, Potassium; LA:Ao, Left atrial to aortic ratio; LVMI, Left ventricular mass index; MBG, Marinobufagenin; Na<sup>+</sup>, Sodium; SBP, Systolic blood pressure; SVi, Stroke index.



**Figure 1:** Pearson correlations between indices of subclinical target organ damage and MBG excretion in the total group.

However, since we noted marked differences in LVMI, EDVi, SVi and eGFR between the lowest and highest quartiles of MBG excretion in **Table 1**, we additionally investigated these relationships more thoroughly within respective quartiles. When performing single or partially adjusted linear regression analyses, we found no correlations of LVMI, EDVi, SVi, or eGFR with MBG excretion in either quartile (**Supplementary Table 2**). However, in multivariate adjusted regression analyses we found that LVMI demonstrated a positive tendency across increasing quartiles, and associated positively with MBG excretion in the highest MBG excretion quartile only (Adj.  $R^2=0.20$ ; std.  $\beta=0.15$ ;  $p=0.043$ ;  $N=165$ ) (**Figure 2, Supplementary Table 3**).



**Figure 2:** Left ventricular mass index of young adults across increasing quartiles of MBG excretion. (A) Single and partially adjusted regression analyses ( $\square$  Q1-Q3 and  $\blacksquare$  Q4). (B) Multivariate adjusted regression analyses adjusted for age, sex, ethnicity, 24hr SBP, eGFR, HDL-C, CRP, GGT and glucose.

\* Comparing Q1 and Q 4 adjusting for age sex and ethnicity;  $p<0.05$ .

\* Indicates  $p<0.05$

### *Sensitivity analyses*

Evident in this study was the high salt intake of the population, with more than 78 % (N=557) of the young adults consuming more than the recommended 5 grams of salt per day . We have previously demonstrated a positive relationship between estimated NaCl intake and MBG excretion,<sup>3</sup> also confirmed in this population ( $r=0.39$ ;  $p<0.001$ ). We therefore performed a sensitivity analyses to determine whether the association found in the highest quartile between LVMI and MBG excretion was independent of NaCl intake (**Supplementary Table 4**). Forward stepwise multiple regression analysis indicated that this relationship was not confounded by NaCl as LVMI remained significantly related to MBG excretion (Adj.  $R^2=0.21$ ; std.  $\beta=0.15$ ;  $p=0.041$ ), and NaCl did not enter the model.

### *Interaction of sex and ethnicity*

Interaction testing for sex and ethnicity on the relationships between indices of subclinical TOD and MBG excretion or MBG excretion quartiles is presented in **Supplementary Table 5**. We found a significant interaction with sex only on the relationship of MBG excretion with SVI, COi and FENa, while no interaction existed with ethnicity. Therefore, Pearson, partial and multiple regression analyses were repeated in men and women respectively (**Supplementary Table 6**).

Notably, the relationship between LVMI and MBG excretion was observed only in women (N=374) (Adj.  $R^2=0.06$ ; std.  $\beta=0.127$ ;  $p=0.015$ ), while the relationship of EDVi (Adj.  $R^2=0.09$ ;  $\beta=0.128$ ;  $p=0.043$ ), and COi (Adj.  $R^2=0.18$ ; std.  $\beta=0.119$ ;  $p=0.048$ ) with MBG excretion were predominant in men (N=252).

## **Discussion**

To our knowledge, our study is the first to investigate whether elevated levels of urinary MBG excretion relate to indices of subclinical TOD in young adults, free of detected cardiovascular or other chronic diseases. We found a positive, blood pressure independent, association of LVMI, the most sensitive estimate of subclinical cardiac TOD, with 24hr urinary MBG excretion in young individuals with excessively high levels of MBG excretion.

Although our study cannot confer causality, previous work by Elkareh *et al.*,<sup>5, 6</sup> and Kennedy *et al.*<sup>7, 8</sup> demonstrated an increase in the cardiac weight<sup>5</sup> of rats subjected to MBG infusion, along with concurrent cardiac

histological changes.<sup>5-8</sup> Accordingly, the observed marked increases in collagen-1,<sup>5,6</sup> fibronectin<sup>8</sup> and  $\alpha$  smooth muscle cell actin<sup>5</sup> were observed in the cardiac tissue of these animals. Further evidence indicates that immunization against MBG attenuates cardiac fibrosis.<sup>5,8</sup> With no evidence from human studies, we could therefore speculate that sustained exposure to high levels of MBG may induce similar histological changes in human cardiac tissue.

Notably, the relationship between LVMI and MBG excretion was evident only in women, despite men consuming more salt,<sup>15</sup> and having a higher MBG excretion.<sup>3</sup> Although speculative, our findings suggest that women, exhibiting lower urinary MBG excretion, may be more sensitive to the cardiac effects of MBG. Indeed, sex specific analyses of ion channel distribution in human hearts indicated  $\alpha 1\text{-Na}^+/\text{K}^+\text{-ATPase}$  to be expressed more abundantly in women compared to men.<sup>27</sup> With further limited evidence in human studies, observations from animal studies may be considered explanatory to our findings. Goel *et al.* previously demonstrated increased protein kinase C  $\beta 2$  expression in female rats,<sup>28</sup> which promotes  $\alpha 1\text{-Na}^+/\text{K}^+\text{-ATPase}$  sensitivity to MBG.<sup>29</sup> In support, the female sex hormone estradiol was also shown to increase cardiac  $\alpha 1\text{-Na}^+/\text{K}^+\text{-ATPase}$  activity in rats.<sup>30</sup> Conversely, in men, the relationships of EDVi and COi with excessively high levels of MBG excretion support a volume overload profile associated with MBG. These apparent sex differences warrant a more in-depth investigation into the relationship between MBG and sex hormones.

Where studies also indicated impaired LV relaxation during MBG exposure in rats,<sup>5,8</sup> we found no relationships between MBG excretion and indices of LV function. This suggests that the adverse effect of MBG on cardiac structure precedes that of function in young adults. Furthermore, we found no relationship between reduced kidney function and MBG excretion, possibly due to the young age of our population. However, MBG might alter kidney function at a later stage in this population consuming high amounts of salt.

Our findings should be interpreted in context of the strengths and limitations of this study. This young study population provides a unique opportunity to gain valuable insight with regards to the possible role of MBG at an early age prior the onset of CVD. However, our cohort may not be representative of the general South African population, since we screened young adults to be apparently healthy upon enrolment. Nonetheless, our findings of a positive association between LVMI and MBG in young healthy adults with excessively high levels MBG

excretion may be of particular importance, as these individuals are presumably at their peak cardiovascular health. Importantly, Levy *et al.* reported LVMi to be a predictor of future CVD development, - mortality and all cause mortality.<sup>31</sup> It is possible that the adverse effects of elevated MBG on LVMi might be aggravated during the long term exposure to modifiable risk factors and advancing age, thereby increasing the risk for future CVD. Due to the novelty of our findings in a young cohort with elevated MBG, our work is of a hypothesis generating nature, and we encourage replication of our findings in other populations.

Taking into consideration the aforementioned, and the levels of MBG excretion in those consuming high amounts of salt, our study supports global initiatives on sodium reduction by organisations such as the European Society of Cardiology,<sup>32</sup> United Nations<sup>33</sup> and World Health Organisation.<sup>34</sup>

In conclusion, this is the first study demonstrating that LVMi is positively and independently associated with MBG in young apparently healthy adults with excessively high MBG excretion. These findings suggest that elevated levels of MBG, particularly in women, may increase the risk for the development of future CVD.

### **Author contributions**

MS, WS, RK, OVF and AES contributed to the concept and design. MS, WS, RK, WW, OVF and AES contributed to the acquisition, analyses and interpretation of data. MS drafted the manuscript. WS, RK, WW, OVF and AES critically revised the manuscript. “All authors gave final approval and agree to be accountable for all aspects of work ensuring integrity and accuracy.”

### **Acknowledgements**

The authors acknowledge the voluntary participation of all participants in the African-PREDICT study. We would like to thank the researchers, support staff and postgraduate students assisting at the Hypertension Research and Training Clinic at the North-West University. The Authors are grateful to Dr. Alexei Y. Bagrov for the discussion of the results.



**Funding**

The research funded in this manuscript is part of an ongoing research project financially supported by the South African Medical Research Council (SAMRC) with funds from National Treasury under its Economic Competitiveness and Support Package; the South African Research Chairs Initiative (SARChI) of the Department of Science and Technology and National Research Foundation (NRF) of South Africa; GlaxoSmithKline R&D, the UK Medical Research Council and with funds from the UK Government's Newton Fund; as well as corporate social investment grants from Pfizer (SA), Boehringer Ingelheim (SA), Novartis (SA), the Medi Clinic Hospital Group (SA) and in kind contributions of Roche Diagnostics (SA). Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors, and therefore, the NRF does not accept any liability in regard. This study was supported in part by the Intramural Research Program, National Institute on Aging, National Institutes of Health, USA.

**Declaration of conflict of interest**

The authors wish to disclose the unrestricted educational grants from Boehringer Ingelheim, Novartis, the Medi Clinic Hospital Group, in kind contributions of Roche Diagnostics (South Africa) and support of patient transport minibus from Pfizer (South Africa). Contributions from GlaxoSmithKline R&D is acknowledged within its partnership with the UK Government's Newton Fund.

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## Supplemental digital content

### Methods

#### *Questionnaire data*

General health and demographic questionnaires were completed by participants in order to obtain detailed information with regards to certain demographics (age, ethnicity, sex, socio-economic status).

#### *Anthropometric measurements*

We measured body height (SECA 213 Portable Stadiometer), weight (SECA 813 Electronic Scales) and waist circumference (Lufkin Steel Anthropometric Tape; W606PM; Lufkin, Apex, USA) in triplicate, in accordance with the guidelines of International Society for the Advancement of Kinanthropometry.<sup>1</sup> Body mass index (weight (kg) / height (m<sup>2</sup>)) and body surface area (BSA)(Mosteller formula)<sup>2</sup> were calculated.

#### *Ambulatory blood pressure measurement*

24hr Ambulatory blood pressure measurements (ABPM) were done using validated CardioXplore devices (CardioXplore, MediTech, Budapest, Hungary), with appropriately sized brachial cuffs fitted to each participant on their non-dominant arm. We programmed devices to record daytime systolic (SBP) and diastolic (DBP) blood pressure during 30 minute intervals (6 am to 10 pm) and hourly at night (10 pm to 6 am). Quality control for ABPM was insured by only including data from participants with more than a total of 70% successful recordings, or 20 daytime and 7 night-time measurements.<sup>3</sup>

**Supplementary Table 1: Multiple regression analyses with MBG excretion as the main independent variable.**

Dependent variable	MBG excretion (nmol/day)		
	Total group (N=626)		
	Adj. R <sup>2</sup>	$\beta$ (SE)	P
<b>Cardiac structure and function</b>			
LVMi (g/m <sup>2</sup> )	0.23	0.066 (0.038)	0.084
Relative wall thickness (cm)	0.06	0.004 (0.025)	0.93
EDVi (ml/m)	0.22	0.068 (0.038)	0.079
SVi (ml/m <sup>2.04</sup> )	0.07	0.072 (0.042)	0.089
COi (ml/m <sup>1.83</sup> )	0.14	0.052 (0.040)	0.20
EF (%)	0.07	0.039 (0.042)	0.36
FS (%)	0.05	0.038 (0.042)	0.37
LA:Ao	0.03	-0.031 (0.043)	0.47
E:A	0.08	-0.026 (0.042)	0.53
E:e'	0.08	-0.006 (0.042)	0.89
<b>Renal function</b>			
Fractional sodium excretion (%)	0.11	0.355 (0.042)	<0.001
eGFR (ml/min/1.73m <sup>2</sup> )	0.51	-0.022 (0.030)	0.48
Albumin (mg/L)	0.03	0.034 (0.043)	0.43

Adjusted for age, sex, ethnicity, 24hr SBP, HDL-C, CRP, GGT and Glucose

Variables not indexed for BSA or height were additionally adjusted for WtHR

Cardiac structure and function markers are additionally adjusted for eGFR.

COi, Cardiac index; E:A, mitral peak velocity of early filling (E) to late diastolic filling (A); EDVi, End diastolic volume index; E:e', of mitral peak velocity of early filling (E) to early diastolic mitral annular velocity (e'); EF, Ejection fraction; eGFR, Estimate glomerular filtration rate; FS, Fractional shortening; LA:Ao, left atrial to aortic ratio; LVMi, Left ventricular mass index; MBG, Marinobufagenin; SVi, Stroke index

**Supplementary Table 2: Pearson and partial correlations across increasing quartiles of MBG excretion.**

Dependent variable	MBG excretion (nmol/day)			
	Q1 (N=177)	Q2 (N=178)	Q3 (N=175)	Q4 (N=177)
<b>Cardiac structure and function</b>				
LVMi (g/m <sup>2</sup> )	$r=-0.007; p=0.92$	$r=0.004; p=0.96$	$r=0.19; p=0.012$	$r=0.073; p=0.34$
RWT (cm)	$r=-0.04; p=0.64$	$r=0.09; p=0.23$	$r=0.03; p=0.74$	$r=0.01; p=0.90$
EDVi (ml/m)	$r=0.06; p=0.43$	$r=-0.06; p=0.45$	$r=0.10; p=0.17$	$r=0.007; p=0.93$
SVi (ml/m <sup>2.04</sup> )	$r=0.004; p=0.96$	$r=-0.06; p=0.40$	$r=0.03; p=0.73$	$r=-0.02; p=0.78$
COi (ml/m <sup>1.83</sup> )	$r=-0.01; p=0.86$	$r=-0.09; p=0.20$	$r=-0.02; p=0.79$	$r=-0.04; p=0.63$
EF (%)	$r=-0.04; p=0.56$	$r=0.06; p=0.43$	$r=-0.09; p=0.23$	$r=-0.06; p=0.44$
FS (%)	$r=-0.04; p=0.59$	$r=0.05; p=0.48$	$r=-0.09; p=0.21$	$r=-0.06; p=0.42$
LA:ao	$r=0.06; p=0.45$	$r=0.15; p=0.049$	$r=0.02; p=0.83$	$r=0.08; p=0.30$
E:A	$r=-0.12; p=0.13$	$r=0.16; p=0.031$	$r=-0.08; p=0.32$	$r=0.05; p=0.51$
E:e'	$r=-0.12; p=0.11$	$r=-0.03; p=0.65$	$r=0.03; p=0.70$	$r=-0.06; p=0.47$
<b>Renal function</b>				
FENa (%)	$r=-0.01; p=0.85$	$r=0.08; p=0.27$	$r=0.14; p=0.072$	$r=0.16; p=0.033$
eGFR (ml/min/1.73m <sup>2</sup> )	$r=-0.04; p=0.64$	$r=-0.08; p=0.31$	$r=-0.01; p=0.87$	$r=0.12; p=0.13$
Albumin (mg/L)	$r=-0.04; p=0.56$	$r=0.002; p=0.98$	$r=0.15; p=0.043$	$r=0.11; p=0.15$
<i>Adjusted for age sex and ethnicity</i>				
	Q1 (N=177)	Q2 (N=178)	Q3 (N=175)	Q4 (N=177)
<b>Cardiac structure and function</b>				
LVMi (g/m <sup>2</sup> )	$r=-0.05; p=0.47$	$r=-0.07; p=0.36$	$r=0.133; p=0.099$	$r=0.12; p=0.13$
RWT (cm)	$r=-0.04; p=0.57$	$r=0.07; p=0.35$	$r=0.03; p=0.70$	$r=0.03; p=0.73$
EDVi (ml/m)	$r=0.04; p=0.62$	$r=-0.10; p=0.19$	$r=0.04; p=0.63$	$r=0.03; p=0.69$
SVi (ml/m <sup>2.04</sup> )	$r=0.007; p=0.93$	$r=-0.06; p=0.40$	$r=0.01; p=0.87$	$r=-0.02; p=0.78$
COi (ml/m <sup>1.83</sup> )	$r=0.009; p=0.91$	$r=-0.07; p=0.39$	$r=0.01; p=0.85$	$r=-0.04; p=0.64$
EF (%)	$r=-0.03; p=0.73$	$r=0.09; p=0.22$	$r=-0.04; p=0.56$	$r=-0.09; p=0.26$
FS (%)	$r=-.02; p=0.75$	$r=0.08; p=0.28$	$r=-0.05; p=0.48$	$r=-0.09; p=0.24$
LA:Ao	$r=0.07; p=0.37$	$r=0.16; p=0.040$	$r=-0.01; p=0.90$	$r=0.09; p=0.23$



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E:A	$r=-0.12; p=0.13$	$r=0.16; p=0.037$	$r=-0.09; p=0.26$	$r=0.02; p=0.77$
E:e'	$r=-0.11; p=0.14$	$r=-0.006; p=0.93$	$r=0.08; p=0.33$	$r=-0.06; p=0.42$

**Renal function**

FENa (%)	$r=-0.009; p=0.91$	$r=0.11; p=0.15$	$r=0.12; p=0.11$	$r=0.15; p=0.046$
eGFR (ml/min/1.73m <sup>2</sup> )	$r=-0.03; p=0.71$	$r=-0.06; p=0.44$	$r=0.06; p=0.45$	$r=0.05; p=0.53$
Albumin (mg/L)	$r=-0.03; p=0.71$	$r=0.06; p=0.48$	$r=0.19; p=0.012$	$r=0.09; p=0.23$

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COi, Cardiac index; E:A, mitral peak velocity of early filling (E) to late diastolic filling (A); EDVi, End diastolic volume index; E:e', of mitral peak velocity of early filling (E) to early diastolic mitral annular velocity (e'); EF, Ejection fraction; eGFR, Estimate glomerular filtration rate; FS, Fractional shortening; LA:Ao, left atrial to aortic ratio; LVMi, Left ventricular mass index; RWT, Relative wall thickness; SVi, Stroke index

**Supplementary Table 3: Forward stepwise multiple regression analyses in the highest MBG excretion quartile with LVMI as dependant variable (N=165).**

Independent variable	Left ventricular mass index (g/m <sup>2</sup> )	
	Adj. R <sup>2</sup>	
	0.20	
	$\beta$ (SE)	P
MBG excretion (nmol/L)	0.147 (0.071)	0.041
Sex	0.275 (0.074)	<0.001
Ethnicity	-0.116 (0.077)	0.13
Age (Years)	0.191 (0.076)	0.013
HDL-C (mmol/L)	-0.139 (0.073)	0.058
C-reactive protein (mg/L)	-0.197 (0.071)	0.006
Glucose (mmol/L)	0.142 (0.079)	0.075
24 SBP (mmHg)	—	—
$\gamma$ -glutamyl transferase (U/L)	—	—
eGFR (ml/min/1.73m <sup>2</sup> )	—	—

eGFR, Estimate glomerular filtration rate; HDL-C, High density lipoprotein cholesterol; SBP, Systolic blood pressure

**Supplementary Table 4: Sensitivity analyses in the highest MBG excretion quartile for Estimated NaCl intake with LVMi as dependant variable (N=165).**

	<i>Left ventricular mass index (g/m<sup>2</sup>)</i>	
	<b>Adj. R<sup>2</sup></b>	
	<b>0.21</b>	
<b>Independent variable</b>	<b>β (SE)</b>	<b>P</b>
MBG excretion (nmol/L)	0.147 (0.071)	0.041
Sex	0.275 (0.074)	<0.001
Age (Years)	0.191 (0.076)	0.013
C-reactive protein (mg/L)	-0.197 (0.072)	0.007
HDL-C (mmol/L)	-0.139 (0.073)	0.058
Glucose (mmol/L)	0.142 (0.079)	0.075
Ethnicity	-0.116 (0.077)	0.13
NaCl (g/day)	—	—
24 SBP (mmHg)	—	—
eGFR (ml/min/1.73m <sup>2</sup> )	—	—
γ-glutamyl transferase (U/L)	—	—

eGFR, Estimate glomerular filtration rate; HDL-C, High density lipoprotein cholesterol;  
NaCl, Salt intake; SBP, Systolic blood pressure

**Supplementary Table 5: Interaction of sex and ethnicity on the relationship between MBG excretion and indices subclinical target organ damage.**

Dependent variable	MBG excretion (nmol/day)		MBG excretion quartiles	
	Sex	Ethnicity	Sex	Ethnicity
LVMi (g/m <sup>2</sup> )	$p= 0.28$	$p= 0.41$	$p=0.25$	$p=0.95$
Relative wall thickness (cm)	$p= 0.08$	$p= 0.36$	$p=0.098$	$p=0.91$
EDVi (ml/m)	<b><math>p= 0.050</math></b>	$p= 0.40$	$p=0.078$	$p=0.35$
SVi (ml/m <sup>2.04</sup> )	$p= 0.093$	$p= 0.73$	$p=0.20$	$p=0.52$
COi (ml/m <sup>1.83</sup> )	<b><math>p= 0.009</math></b>	$p= 0.50$	<b><math>p=0.044</math></b>	$p=0.28$
EF (%)	$p= 0.72$	$p= 0.45$	$p=0.44$	$p=0.67$
FS (%)	$p= 0.78$	$p= 0.36$	$p=0.53$	$p=0.54$
LA:Ao	$p= 0.12$	$p= 0.47$	$p=0.23$	$p=0.86$
E:A	$p= 0.63$	$p= 0.080$	$p=0.92$	$p=0.29$
E:e'	$p= 0.29$	$p= 0.22$	$p=0.69$	$p=0.66$
FENa (%)	<b><math>p=0.023</math></b>	$p= 0.54$	$p=0.098$	$p=0.71$
eGFR (ml/min/1.73m <sup>2</sup> )	$p= 0.99$	$p= 0.36$	$p=0.96$	$p=0.54$
Albumin(mg/L)	$p= 0.38$	$p= 0.13$	$p=0.54$	$p=0.098$

COi, Cardiac index; E:A, mitral peak velocity of early filling (E) to late diastolic filling (A); EDVi, End diastolic volume index; E:e', of mitral peak velocity of early filling (E) to early diastolic mitral annular velocity (e'); EF, Ejection fraction; eGFR, Estimate glomerular filtration rate; FS, Fractional shortening; LA:Ao, left atrial to aortic ratio; LVMi, Left ventricular mass index; MBG, Marinobufagenin; SVi, Stroke index

**Supplementary Table 6: Pearson, partial and multiple regression analyses with MBG excretion as the main independent variable in men and women.**

	MBG excretion (nmol/day)		
	Men (N=252)		
	Pearson correlations	Partial correlations	Multiple regression
<b>Cardiac structure and function</b>			
LVMi (g/m <sup>2</sup> )	<i>r</i> =0.02; <i>p</i> =0.80	<i>r</i> =0.02; <i>p</i> =0.78	R <sup>2</sup> =0.06; β= 0.007; <i>p</i> =0.92
Relative wall thickness (cm)	<i>r</i> =-0.11; <i>p</i> =0.058	<i>r</i> =-0.10; <i>p</i> =0.096	R <sup>2</sup> <0.001; β=-0.099; <i>p</i> =0.14
EDVi (ml/m)	<b><i>r</i>=0.19; <i>p</i>=0.001</b>	<b><i>r</i>=0.15; <i>p</i>=0.011</b>	<b>R<sup>2</sup>=0.09; β=0.128; <i>p</i>=0.043</b>
SVi (ml/m <sup>2.04</sup> )	<b><i>r</i>=0.16; <i>p</i>=0.007</b>	<b><i>r</i>=0.14; <i>p</i>=0.014</b>	R <sup>2</sup> =0.05; β=0.119; <i>p</i> =0.066
COi (ml/m <sup>1.83</sup> )	<b><i>r</i>=0.18; <i>p</i>=0.002</b>	<b><i>r</i>=0.14; <i>p</i>=0.019</b>	<b>R<sup>2</sup>=0.18; β=0.119; <i>p</i>=0.048</b>
EF (%)	<i>r</i> =0.02; <i>p</i> =0.76	<i>r</i> =0.04; <i>p</i> =0.48	R <sup>2</sup> =0.05; β=0.008; <i>p</i> =0.90
FS (%)	<i>r</i> =0.02; <i>p</i> =0.68	<i>r</i> =0.05; <i>p</i> =0.42	R <sup>2</sup> =0.05; β=0.016; <i>p</i> =0.80
LA:Ao	<i>r</i> =0.08; <i>p</i> =0.18	<i>r</i> =0.07; <i>p</i> =0.23	R <sup>2</sup> <0.001; β=0.059; <i>p</i> =0.38
E:A	<i>r</i> =0.004; <i>p</i> =0.95	<i>r</i> =0.04; <i>p</i> =0.46	R <sup>2</sup> =0.16; β=0.046; <i>p</i> =0.45
E:e'	<i>r</i> =0.005; <i>p</i> =0.93	<i>r</i> =0.04; <i>p</i> =0.53	R <sup>2</sup> =0.12; β=0.043; <i>p</i> =0.49
<b>Renal function</b>			
FENa (%)	<b><i>r</i>=0.38; <i>p</i>&lt;0.001</b>	<b><i>r</i>=0.38; <i>p</i>&lt;0.001</b>	<b>R<sup>2</sup>=0.06; β=0.127; <i>p</i>&lt;0.001</b>
eGFR (ml/min/1.73m <sup>2</sup> )	<i>r</i> =-0.05; <i>p</i> =0.38	<i>r</i> =0.11; <i>p</i> =0.074	R <sup>2</sup> =0.11; β=0.006; <i>p</i> =0.54
Albumin(mg/L)	<i>r</i> =0.09; <i>p</i> =0.14	<i>r</i> =0.09; <i>p</i> =0.13	R <sup>2</sup> =0.14; β=0.044; <i>p</i> =0.17
	Women (N=374)		
	Pearson correlations	Partial correlations	Multiple regression
	<b>Cardiac structure and function</b>		
LVMi (g/m <sup>2</sup> )	<b><i>r</i>=0.11; <i>p</i>=0.025</b>	<b><i>r</i>=0.12; <i>p</i>=0.015</b>	<b>R<sup>2</sup>=0.06; β=0.127; <i>p</i>=0.015</b>
Relative wall thickness (cm)	<i>r</i> =0.02; <i>p</i> =0.70	<i>r</i> =0.04; <i>p</i> =0.46	R <sup>2</sup> =0.11; β=0.006; <i>p</i> =0.22
EDVi (ml/m)	<i>r</i> =0.06; <i>p</i> =0.24	<i>r</i> =0.07; <i>p</i> =0.18	R <sup>2</sup> =0.14; β=0.044; <i>p</i> =0.37
SVi (ml/m <sup>2.04</sup> )	<i>r</i> =0.04; <i>p</i> =0.38	<i>r</i> =0.05; <i>p</i> =0.29	R <sup>2</sup> =0.06; β=0.042; <i>p</i> =0.42
COi (ml/m <sup>1.83</sup> )	<i>r</i> =-0.005; <i>p</i> =0.92	<i>r</i> =-0.004; <i>p</i> =0.93	R <sup>2</sup> =0.09; β=0.007; <i>p</i> =0.89

EF (%)	$r=0.05; p=0.29$	$r=0.05; p=0.34$	$R^2=0.01; \beta=0.038; p=0.48$
FS (%)	$r=0.05; p=0.31$	$r=0.04; p=0.37$	$R^2=0.01; \beta=0.033; p=0.54$
LA:Ao	$r=-0.04; p=0.39$	$r=-0.03; p=0.51$	$R^2=0.05; \beta=-0.071; p=0.83$
E:A	$r=-0.04; p=0.47$	$r=-0.04; p=0.37$	$R^2=0.04; \beta=-0.074; p=0.17$
E:e'	$r=-0.08; p=0.093$	$r=-0.06; p=0.23$	$R^2=0.06; \beta=-0.042; p=0.42$

**Renal function**

FENa (%)	<b><math>r=0.25; p&lt;0.001</math></b>	<b><math>r=0.26; p&lt;0.001</math></b>	<b><math>R^2=0.09; \beta=0.298; p&lt;0.001</math></b>
eGFR (ml/min/1.73m <sup>2</sup> )	$r=-0.06; p=0.21$	$r=0.05; p=0.32$	$R^2=0.46; \beta=-0.044; p=0.27$
Albumin(mg/L)	$r=0.01; p=0.78$	$r=0.02; p=0.76$	$R^2=0.014; \beta=-0.003; p=0.95$

Partial regression analyses adjusted for age and ethnicity

Multiple regression analyses adjusted for age, ethnicity, 24hr SBP, HDL-C, CRP, GGT and Glucose.

Variables not indexed for BSA or height were additionally adjusted for WtHR

Cardiac structure and function markers are additionally adjusted for eGFR.

CO<sub>i</sub>, Cardiac index; E:A, mitral peak velocity of early filling (E) to late diastolic filling (A); EDV<sub>i</sub>, End diastolic volume index; E:e', of mitral peak velocity of early filling (E) to early diastolic mitral annular velocity (e'); EF, Ejection fraction; eGFR, Estimate glomerular filtration rate; FS, Fractional shortening; LA:Ao, left atrial to aortic ratio; LVMI, Left ventricular mass index; MBG, Marinobufagenin; SV<sub>i</sub>, Stroke index

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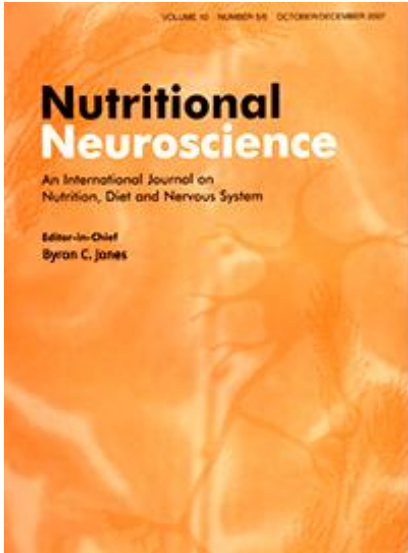
# Chapter 5

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Research article 3:

Autonomic activity and its relationship with the  
endogenous cardiotonic steroid marinobufagenin: The  
African-PREDICT study



Nutritional Neuroscience			
			
<b>Impact factor</b>	3.313		
<b>Publisher</b>	Taylor & Francis Productions		
<b>Citation</b>	Strauss M, Smith W, Wei W, Fedorova OV, Schutte AE. Autonomic activity and its relationship with the endogenous cardiogenic steroid marinobufagenin: the African-PREDICT study. <i>Nutritional Neuroscience</i> . 2019:1-11. DOI:10.1080/1028415X.2018.1564985		
<b>Aims &amp; Scope</b>	<p>An international, interdisciplinary broad-based journal for reporting both basic and clinical research in the field of nutrition that relates to the central and peripheral nervous system. Studies may include the role of different components of normal diet, dietary supplements, and food additives on neurochemistry, neurobiology, and behavioral biology of all vertebrate and invertebrate organisms. Ideally this journal will serve as a forum for neuroscientists, nutritionists, neurologists, psychiatrists, and those interested in preventive medicine.</p> <div style="text-align: center;">  </div>		
Author Instructions			
<b>Language</b>	Not specified	<b>Font</b>	Times New Roman 12pt
<b>Spacing</b>	Double Spacing	<b>Margins</b>	2.5
<b>Word count</b>	± 6000 words	<b>Tables&amp; figures</b>	6 to 8
<b>References</b>	± 25	<b>Alignment</b>	Not justified

<b>Manuscript</b>	Manuscripts should include the following sections, each starting on a separate page: Title page, Abstract and Keywords, Text, References, Tables and Captions.
<b>Title page</b>	<ul style="list-style-type: none"> <li>• Full title (Bold)</li> <li>• Full first name and last (family name) name of each author should appear</li> <li>• Affiliations of all the authors connected using a,b,c</li> <li>• Word count</li> </ul>
<b>Abstract</b>	Structured abstract (250 words) – Aims, Methods, Results & Conclusion
<b>Keywords</b>	3–10 keywords
<b>Text</b>	Introduction, Methods, Results & Discussion
<b>Acknowledgements</b>	Include acknowledgements
<b>Conflict of interest</b>	Include statement declaring conflict of interest
<b>Funding</b>	Include funding
<b>Ethical considerations</b>	<ul style="list-style-type: none"> <li>• All work must be conducted in accordance with the Declaration of Helsinki.</li> <li>• Include (1) a statement that consent was obtained from participants and (2) of ethical approval.</li> </ul>
<b>References</b>	<ul style="list-style-type: none"> <li>• Number consecutively in the order in which they first appear in the text.</li> <li>• Include the names of all authors and any Study Group named in the primary author list when six or fewer; when seven or more, list only the first six names and add et al.</li> <li>• Journal names should be abbreviated as MEDLINE</li> </ul> <p><i>Articles in journals</i></p> <p>Zhou M-S, Schulman IH, Raji L. Vascular inflammation, insulin resistance, and endothelial dysfunction in salt-sensitive hypertension: role of nuclear factor kappa B activation. <i>J Hypertens</i> 2010; <b>28</b>:527–535</p>
<b>Tables</b>	<ul style="list-style-type: none"> <li>• Each table should be typed on a separate page in double spacing</li> <li>• Each table should be assigned an Arabic numeral and a brief title</li> </ul>
<b>Figures</b>	<ul style="list-style-type: none"> <li>• Cite figures consecutively in your manuscript.</li> </ul>
<b>Legends for illustrations</b>	<ul style="list-style-type: none"> <li>• Captions should be typed in double spacing, beginning on a separate page.</li> <li>• Each one should have an Arabic numeral corresponding to the illustration to which it refers.</li> </ul>
<b>Supplemental Digital Content</b>	<ul style="list-style-type: none"> <li>• Cited consecutively in the text of the submitted manuscript.</li> </ul>

*\*Formatting changes were made to maintain uniformity throughout this thesis, including text font, line spacing, margins, page numbers, tables and figures.*

**Autonomic activity and its relationship with the endogenous cardiotonic steroid  
marinobufagenin: The African-PREDICT study**

**Short title:** Autonomic activity and marinobufagenin

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**Word count:** 5874

**Acknowledgements**

The authors of this study are grateful towards all individuals participating voluntarily in the study. The dedication of the support and research staff as well as students at the Hypertension Research and Training Clinic at the North-West University is also duly acknowledged.

**Abstract**

**Aim:** Marinobufagenin (MBG), a cardiotoxic steroid and a natriuretic hormone, is elevated in response to high salt diet consumption. In animal models salt intake stimulates adrenocortical MBG secretion via increased angiotensin II, sympathetic activity and aldosterone. No evidence in humans exists to suggest the involvement of the angiotensinergic-sympatho-excitatory pathway in MBG production. We investigated whether MBG is related to indices of autonomic activity in men and women.

**Methods:** This cross-sectional study included 680 black and white, men and women from the African-PREDICT study (aged 20-30 years). Continuous 24hr ECG recordings were used to obtain low and high frequency (LF, HF) heart rate variability (HRV). We measured 24hr urinary MBG excretion and serum aldosterone.

**Results:** We found a positive association of MBG excretion with estimated salt intake ( $p < 0.001$ ) and aldosterone ( $p < 0.001$ ) in women and men. In women only, a positive relationship was evident between MBG excretion and LF HRV in multivariate adjusted regression analyses (Adj.  $R^2=0.33$ ;  $\beta=0.11$ ;  $p=0.030$ ). In men, MBG excretion associated positively with HF HRV in similar regression analyses (Adj.  $R^2=0.36$ ;  $\beta=0.12$ ;  $p=0.034$ ). Sex-specific results were corroborated only in blacks, namely, a positive association of MBG excretion with LF HRV in black women (Adj.  $R^2=0.38$ ;  $\beta=0.13$ ;  $p=0.036$ ), and negative association with HF HRV in black men (Adj.  $R^2=0.40$ ;  $\beta=0.18$ ;  $p=0.045$ ). No relationships were evident in white women ( $p=0.58$ ) or men ( $p=0.27$ ).

**Conclusion:** Our findings in this human cohort support suggested mechanisms whereby MBG is elevated as a result of increased salt intake, including autonomic activity, previously demonstrated in Dahl salt-sensitive hypertension.

**Key words:** autonomic activity, human, marinobufagenin, salt intake, women

## Introduction

It is well known that excessive salt intake is associated with increased cardiovascular risk.<sup>1</sup> In response to a high salt intake, endogenous cardiogenic steroids are released – with reports indicating clear adverse effects of these steroids.<sup>2,3</sup> This includes, amongst others, marinobufagenin (MBG), a circulating bioactive steroid, synthesized in the adrenal cortex by means of CYP27A1 enzymatic activity.<sup>4</sup> We have previously confirmed this significant positive relationship between salt intake and MBG in this study cohort.<sup>5</sup> While MBG has been linked to several pathological states,<sup>3</sup> we have recently demonstrated in healthy young adults that MBG excretion is associated with increased blood pressure,<sup>6</sup> arterial stiffness<sup>5</sup> and left ventricular mass.<sup>7</sup>

Fedorova *et al* demonstrated that salt intake stimulates adrenocortical MBG secretion in Dahl salt-sensitive rats.<sup>2,8</sup> Acute salt loading promoted short-term increases in pituitary endogenous ouabain and angiotensin II (AT II), along with sustained increases in adrenocortical AT II, plasma norepinephrine and MBG. It was proposed that MBG production was increased in response to salt loading via increased brain ouabain, and concurrent renin-angiotensin-aldosterone-system and sympathetic activity.<sup>8</sup> Intrahippocampal administration of a low-dose ouabain mimics the effects of salt loading and stimulates marinobufagenin production in Dahl salt-sensitive rats.<sup>9</sup> Administration of anti-ouabain antibody inhibited the abovementioned brain ouabain-angiotensinergic-sympatho-excitatory pathway and resulted in lower circulating MBG and urinary MBG excretion.<sup>8</sup> In addition, the administration of losartan decreased AT II, norepinephrine and MBG excretion but not endogenous ouabain.<sup>8</sup>

Although MBG levels are elevated in young adults on a habitual high salt diet,<sup>5-7</sup> no evidence in humans exists to suggest the involvement of the angiotensinergic-sympatho-excitatory pathway. We, therefore, investigated whether MBG is related to indices of autonomic activity in young healthy men and women on a habitual sodium chloride intake, without detected cardiovascular disease. Heart rate variability (HRV) is one of several methods used as an indirect measure of autonomic activity.<sup>10</sup> Whilst the heart has an intrinsic pacemaker,<sup>11</sup> HRV is mainly controlled by the complex inter-regulation of the sympathetic and parasympathetic branches of the autonomic nervous system. Based on the results from Fedorova *et al.*,<sup>8</sup> we hypothesize that MBG excretion will be positively associated with indices of sympathetic autonomic activity. We will also explore the relationship between MBG

excretion and aldosterone, taking into account the possible role thereof as a stimulatory factor in MBG synthesis and excretion.

We previously indicated that men had higher levels of MBG excretion than women, but that women may paradoxically be more sensitive to the effects thereof.<sup>5,6</sup> Therefore, we investigated the association between autonomic activity and MBG excretion separately in men and women. This may provide useful insights into the unparalleled observation of higher sensitization to MBG despite lower levels, in accordance with reported increased salt sensitivity in women.

## **Methods**

This cross-sectional study included the first consecutive 680 participants with complete 24hr urinary data, from the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT). Protocols of the African-PREDICT study conform to Institutional guidelines and the declaration of Helsinki, and were approved by the Institutional Health Research Ethics Committee. The study is registered at ClinicalTrials.gov (Nr. NCT03292094).

### *Participant recruitment*

A fieldwork team recruited black and white adults between the ages of 20 to 30 from communities in and around Potchefstroom in the North West province of South Africa. Recruited volunteers were screened to determine eligibility for inclusion into the African-PREDICT study. Inclusion criteria were office blood pressure <140/90 mmHg,<sup>12</sup> HIV uninfected, no previous diagnosis or medication use for chronic illnesses (self-reported), not pregnant or lactating. Eligible participants were invited to the Hypertension Research Clinic. All participants gave written informed consent before health screening and study measurements. General health and demographic questionnaires were completed by participants to obtain data on demographics (age, sex, ethnicity and socio-economic status) and lifestyle habits (e.g. contraceptive use).

### *Anthropometry*

We measured height (SECA 213 Portable Stadiometer) (SECA, Hamburg, Germany), weight (SECA 813 Electronic Scales) and waist circumference (Lufkin Steel Anthropometric Tape; W606PM; Lufkin, Apex, USA) using standard methods, as reported earlier,<sup>7</sup> and calculated body mass index (BMI)(kg/m<sup>2</sup>).

### *Cardiovascular measurements*

Participants were fitted with a 24hr ambulatory blood pressure (ABPM) and electrocardiogram (ECG) apparatus (Card(X)plore, Meditech, Budapest, Hungary). An appropriately size brachial blood pressure cuff was attached to the non-dominant arm of participants with the apparatus attached to their waist. Daytime blood pressure recordings were taken in 30 minute intervals (6 AM – 10 PM) and night-time blood pressure every hour (10 PM – 6 AM). Continuous 24hr ECG recordings were analyzed using Cardio Visions 1.15.2 Personal Edition software (Meditech, Budapest, Hungary) to obtain HRV data and provide information on the time- and frequency domains. Although the time domain (including the standard deviation of normal to normal interval (SDNN)) provides information on changes in total HRV, this technique is limited in providing information on specific components contributing to the variance.<sup>11,13,14</sup> The frequency domain, however, reflects different components of the autonomic nervous system with the low frequency (LF) HRV band being an index of sympathetic tone, with a parasympathetic component, and the high frequency (HF) HRV band a reflector of parasympathetic tone, with a sympathetic component.<sup>13</sup> Power spectral density analyses were used to calculate the LF HRV (0.04-0.15 Hz) and HF HRV (0.15-0.40 Hz), expressed in normalized units,<sup>14</sup> as well as the LF/HF ratio.

### *Biological sampling and biochemical analyses*

Participants fasted from 10 PM the evening prior to study measurements. A registered nurse took early morning blood samples using a sterile winged infusion set and syringes, in a private room. Thorough instructions were provided to each participant on how to collect 24hr urine after they discarded the first passed urine of the day.

24hr urinary MBG was analyzed using a solid-phase Dissociation-Enhanced Lanthanide Fluorescent Immunoassay (DELFLIA), based on a 4G4 anti-MBG mouse monoclonal antibody,<sup>15</sup> which demonstrates a low cross-immunoreactivity with contraceptive hormones<sup>5</sup> and aldosterone.<sup>15</sup> We previously indicated that MBG from nonextracted urine samples, in the presence of other hormones and steroids, is measured reliably.<sup>5</sup> When comparing MBG levels from extracted urine samples on C18 columns versus nonextracted urine samples, the highly specific 4G4 anti-MBG mouse monoclonal antibody consistently detected similar levels of MBG in both samples.

The Cobas Integra 400plus (Roche, Basel Switzerland) was used to determine 24hr urinary sodium and potassium, as well as serum concentrations of high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), triglycerides, C-reactive protein (CRP),  $\gamma$ -glutamyltransferase (GGT) and glucose. We measured serum cotinine using the chemiluminescence method on the Immulite (Siemens, Erlangen, Germany), and serum aldosterone using the RIA Aldosterone Kit (Beckman Coulter, Immunotech, Radiova, Czech Republic)

Estimated salt intake for this study was calculated as:

$$\text{Estimate NaCl (g/day)} = \frac{(24\text{hr Urinary Na (mmol/L)} * \text{Urinary volume (L)}) * 58.44}{1000}$$

### *Statistical Analyses*

We made use of Statistica version 13.2 (TIBCO Software Inc., Tulsa, Oklahoma, USA) to perform statistical analyses and GraphPad Prism version 5.0 (GraphPad Software Inc., California, USA) to draw figures. Skewed data were logarithmically transformed, and presented as the geometric mean; 5<sup>th</sup> and 95<sup>th</sup> percentile intervals. We performed independent t-tests to compare the basic characteristics (continuous data) of men and women and Chi-square tests for categorical data. Pearson, partial and multiple regression analyses were done in the total group, men and women to investigate the relationship between indirect indices of autonomic activity and MBG excretion. We considered several covariates for inclusion as possible independent variables based on the strongest bivariate associations with HRV variables and MBG excretion. In order to explore the potential



influence of covariates on the relationship of LF HRV and HF HRV with MBG excretion we assessed the unadjusted, partially adjusted and fully adjusted  $\beta$ -values as part of regression analyses with MBG excretion as dependent variable. Pearson, partial and multiple regression analyses were repeated in subgroup analyses of black and white, men and women. We performed sensitivity analyses for hormonal contraceptive use in black and white women.

## Results

**Table 1** outlines the basic characteristics of the young men (aged  $24.9 \pm 2.98$  years) and women (aged  $24.8 \pm 3.08$  years) from this study. The groups had equal distribution of black and white ethnicity ( $p=0.42$ ). Men had higher blood pressure, LF HRV, LF/HF and SDNN ( $p<0.001$ ), while women demonstrated elevated 24hr HR and HF HRV ( $p<0.001$ ). In agreement with what we have previously published, men compared to women had a greater estimated salt intake ( $p=0.004$ ), and in accordance, demonstrated higher levels of MBG excretion ( $p<0.001$ ).<sup>5</sup> Additionally, in this study population 77% (N=303) of women and 81% (N=229) of men consumed more than 5 grams of salt per day. The women and men from this study respectively consumed approximately 7.24 (95% C.I. 7.99; 9.11) and 8.29 (95% C.I. 9.17; 10.5) grams of salt per day, which are higher than the World Health Organizations recommended amount for adults (5g salt per day).<sup>16</sup> While estimated salt intake did not differ between black and white adults in the total group ( $p=0.21$ ) or in men ( $p=0.34$ ), black women consumed significantly more salt compared to white women ( $p=0.007$ ).

### *Regression analyses*

We firstly performed single, partial and multiple regression analyses in the total group which indicated a significant contribution of sex on the relationship between MBG excretion and HRV autonomic activity parameters (**Supplementary Table 1 & Supplementary Figure 1**). Subsequent single, partial and multiple regression analyses were thus performed according to sex stratification (**Table 2**).

Table 1: Characteristics of men and women from the African-PREDICT study

	Men N=284	Women N=396	P
Ethnicity, Black, N (%)	136 (40.2)	202 (59.8)	0.42
Age (years)	24.9 ± 2.98	24.8 ± 3.08	0.59
Socio economic status, N (%)			0.28
<i>Low</i>	105 (37.0)	149 (37.6)	
<i>Middle</i>	61 (21.5)	103 (26.0)	
<i>High</i>	118 (41.5)	144 (36.4)	
<b>Anthropometric measurements</b>			
BMI (kg/m <sup>2</sup> )	24.9 ± 5.32	25.8 ± 5.93	0.047
WC (cm)	83.7 ± 13.2	78.3 ± 12.7	<0.001
<b>Cardiovascular profile</b>			
24hr SBP (mmHg)	121 ± 8.18	113 ± 8.12	<0.001
24hr DBP (mmHg)	69.7 ± 5.98	68.0 ± 5.27	<0.001
24hr Heart rate (bpm)	69.5 ± 9.22	78.7 ± 9.76	<0.001
24hr LF HRV (n.u.)	64.9 ( 46.0; 83.0)	57.9 (37.0; 78.0)	<0.001
24hr HF HRV (n.u.)	29.7 (16.0; 49.0)	35.9 (20.0; 60.0)	<0.001
24hr LF/HF HRV	2.17 (0.90; 5.10)	1.60 (0.60; 3.70)	<0.001
24hr HRV SDNN (ms)	167 (107; 262)	131 (85.0; 206)	<0.001
<b>24hr Urinary profile</b>			
Volume (L/24hr)	1.42 ± 0.76	1.36 ± 0.79	0.35
MBG conc. (nmol/L)	3.26 (1.26; 7.16)	2.26 (0.72; 5.75)	<0.001
MBG exc. (nmol/day)	4.11 (1.59; 10.2)	2.69 (0.93; 8.01)	<0.001
Na <sup>+</sup> exc. (mmol/day)	141 (41.1; 360)	123 (45.8; 326)	0.004
NaCl intake (g/day)	8.29 (2.42; 21.2)	7.24 (2.70; 19.2)	0.004
<i>Black</i>	7.98 (2.18; 23.7)	7.83 (2.94; 20.5)†	
<i>White</i>	8.57 (3.22; 17.2)	6.68 (2.52; 15.4)†	
K <sup>+</sup> exc. (mmol/day)	41.8 (13.2; 103)	39.3 (15.7; 103)	0.18
Na <sup>+</sup> :K <sup>+</sup> ratio	3.41 (1.36; 7.14)	3.22 (1.40; 6.78)	0.17

**Biochemical profile**

Aldosterone (pg/ml)	64.1 (16.8; 210)	74.7 (17.1; 423)	0.034
Glucose (mmol/L)	4.85 ± 0.75	4.67 ± 0.63	0.001
HDL-C (mmol/L)	1.15 (0.75; 1.73)	1.34 (0.81; 2.14)	<0.001
LDL-C (mmol/L)	2.76 (1.54; 4.71)	2.62 (1.50; 4.23)	0.054
Triglycerides (mmol/L)	0.90 (0.45; 2.06)	0.76 (0.39; 1.67)	<0.001
C-reactive protein (mg/L)	0.72 (0.10; 6.37)	1.34 (0.12; 12.0)	<0.001

**Lifestyle measures**

Smoking, N (%)	96 (33.9)	54 (13.6)	<0.001
Cotinine >10 (ng/ml)	82 (35.5)	52 (16.6)	<0.001
GGT (U/L)	26.2 (12.8; 66.2)	18.0 (7.80; 54.6)	<0.001
Hormonal contraception, N (%)	—	155 (40.2)	

Arithmetic mean ± standard deviation; geometric mean (5<sup>th</sup> percentile; 95<sup>th</sup> percentile intervals).

BMI, Body mass index; DBP, Diastolic blood pressure; GGT,  $\gamma$ -glutamyl transferase; HDL-C, High density lipoprotein cholesterol; K<sup>+</sup>, Potassium; LDL-C, Low density lipoprotein cholesterol; MBG, Marinobufagenin; Na<sup>+</sup>, Sodium; NaCl, Estimated salt intake; SBP, Systolic blood pressure; SDNN, Standard deviation of normal R-R intervals; WC, waist circumference; 24hr LF HRV, Normalized low frequency power heart rate variability ; 24hr HF HRV, Normalized high frequency power heart rate variability, 24hr LF/HF HRV, Ratio of low-to high frequency power

†  $P=0.007$

In women only, a positive relationship was evident between MBG excretion and LF HRV in single ( $r=0.11$ ;  $p=0.033$ ), partial ( $r=0.10$ ;  $p=0.055$ ) (**Table 2**) and multivariate adjusted regression analyses (Adj.  $R^2=0.33$ ;  $\beta=0.11$ ;  $p=0.031$ ) (**Table 3 & Figure 1**). Partially adjusted  $\beta$ -values of LF HRV in regression analyses with MBG excretion as dependent variable (**Figure 1**), indicated that the inclusion of estimated salt intake (Adj.  $R^2=0.23$ ;  $p=0.006$ ) or aldosterone (Adj.  $R^2=0.005$ ;  $p=0.12$ ) significantly adjusted the relationship between MBG excretion and LF HRV.

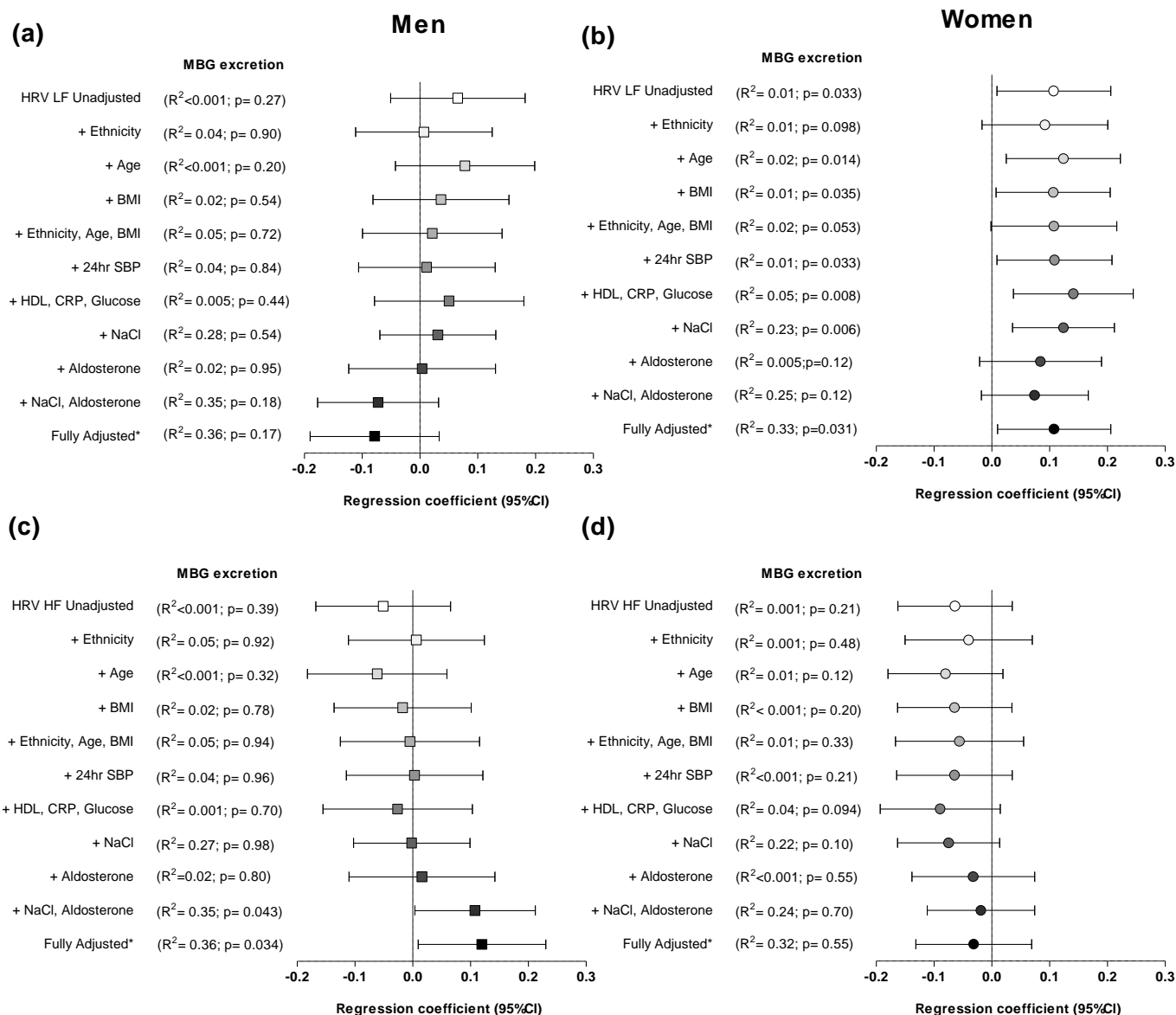
**Table 2: Pearson and partial correlations between indices of autonomic activity and MBG excretion**

	<i>MBG excretion (nmol/day)</i>	
	<b>Men N=284</b>	<b>Women N=396</b>
24hr Heart rate (bpm)	r<0.001; p=0.99	r=-0.06; p=0.20
24hr LF HRV (n.u.)	r=0.07; p=0.27	<b>r=0.11; p=0.033</b>
24hr HF HRV (n.u.)	r=-0.05; p=0.39	r=-0.06; p=0.21
24hr LF/HF HRV	r=0.06; p=0.31	r=0.08; p=0.13
SDNN	r=-0.02;p=0.70	r=0.004;p=0.94
<i>Adjusted for age, ethnicity and body mass index</i>		
24hr Heart rate (bpm)	r=-0.04; p=0.51	r=-0.06; p=0.26
24hr LF HRV (n.u.)	r=0.021; p=0.73	<b>r=0.10; p=0.055</b>
24hr HF HRV (n.u.)	r=-0.01; p=0.93	r=-0.05; p=0.34
24hr LF/HF HRV	r=0.01; p=0.81	r=0.06; p=0.21
SDNN	r<-0.001;p=0.99	r=-0.02;p=0.68

24hr LF HRV, Normalized low frequency power heart rate variability; 24hr HF HRV, Normalized high frequency power heart rate variability, 24hr LF/HF HRV, Ratio of low-to high frequency power; SDNN, Standard deviation of normal R-R intervals

Although we found no correlation between MBG excretion and autonomic parameters in single or partial regression analyses in men (**Table 2**), MBG excretion associated positively with HF HRV in fully adjusted multiple regression analyses ( $R^2=0.36$ ;  $\beta=0.12$ ;  $p=0.034$ ) (**Table 3**). Notably, the relationship of MBG excretion with HF HRV is altered by the parallel inclusion of estimated salt intake and aldosterone, as indicated by partially adjusted and fully adjusted  $\beta$ -values of HF HRV (**Figure 1**).

As expected we found a significant positive association of MBG excretion with estimated salt intake ( $p<0.001$ ) and aldosterone ( $p<0.001$ ) in women and men.



**Figure 1:** Unadjusted, partially adjusted and fully adjusted  $\beta$ -values of heart rate variability estimates (LF HRV ((a) men; (b) women) or HF HRV ((c) men; (d) women)) as part of regression analyses with MBG excretion as dependent variable.

\*In fully adjusted multiple regression models HRV estimates were adjusted for age, ethnicity, body mass index (BMI), 24hr systolic blood pressure (24hr SBP), high density lipoprotein cholesterol (HDL-C), C- reactive protein (CRP), glucose, estimated NaCl intake, aldosterone

**Table 3: Fully adjusted multiple regression models with MBG excretion as dependent variable in men and women**

<i>MBG excretion (nmol/day)</i>				
	<b>Men</b>		<b>Women</b>	
	<b>N=243</b>		<b>N=349</b>	
	<b>R<sup>2</sup></b>		<b>R<sup>2</sup></b>	
	0.36		0.33	
	<b>β (S.E.)</b>	<b>P</b>	<b>β (S.E.)</b>	<b>P</b>
<b>LF HRV(n.u.)</b>	-0.078 (0.057)	0.17	<b>0.108 (0.050)</b>	<b>0.031</b>
Ethnicity (black/ white)	<b>0.164 (0.067)</b>	<b>0.016</b>	0.045 (0.056)	0.42
Age (years)	-0.024 (0.056)	0.67	<b>-0.106 (0.046)</b>	<b>0.023</b>
BMI (kg/m <sup>2</sup> )	-0.023 (0.074)	0.75	0.004 (0.061)	0.95
24hr SBP (mmHg)	-0.025 (0.061)	0.68	0.070 (0.054)	0.20
NaCl intake (g/day)	<b>0.567 (0.055)</b>	<b>&lt;0.001</b>	<b>0.542 (0.046)</b>	<b>&lt;0.001</b>
HDL-C (mmol/l)	-0.068 (0.057)	0.23	<b>-0.210 (0.049)</b>	<b>&lt;0.001</b>
CRP (mg/l)	-0.064 (0.059)	0.28	<b>-0.213 (0.051)</b>	<b>&lt;0.001</b>
Glucose (mmol/l)	-0.085 (0.062)	0.17	-0.084 (0.047)	0.073
Aldosterone (pg/ml)	<b>0.203 (0.059)</b>	<b>&lt;0.001</b>	<b>0.165 (0.049)</b>	<b>&lt;0.001</b>
<i>MBG excretion (nmol/day)</i>				
	<b>R<sup>2</sup></b>		<b>R<sup>2</sup></b>	
	0.36		0.32	
	<b>β (S.E.)</b>	<b>P</b>	<b>β (S.E.)</b>	<b>P</b>
<b>HF HRV (n.u.)</b>	<b>0.120 (0.056)</b>	<b>0.034</b>	-0.031 (0.051)	0.55
Ethnicity (black/ white)	<b>0.173 (0.068)</b>	<b>0.011</b>	0.076 (0.057)	0.18
Age (years)	-0.014 (0.056)	0.80	<b>-0.095 (0.047)</b>	<b>0.045</b>
BMI (kg/m <sup>2</sup> )	-0.022 (0.075)	0.76	0.011 (0.062)	0.88
24hr SBP (mmHg)	-0.021 (0.061)	0.74	0.076 (0.055)	0.17
NaCl intake (g/day)	<b>0.566 (0.055)</b>	<b>&lt;0.001</b>	<b>0.546 (0.046)</b>	<b>&lt;0.001</b>
HDL-C (mmol/l)	-0.068 (0.059)	0.24	<b>-0.197 (0.049)</b>	<b>&lt;0.001</b>

CRP (mg/l)	-0.063 (0.059)	0.29	<b>-0.209 (0.052)</b>	<b>&lt;0.001</b>
Glucose (mmol/l)	-0.086 (0.062)	0.16	<b>-0.093 (0.047)</b>	<b>0.049</b>
Aldosterone (pg/ml)	<b>0.207 (0.059)</b>	<b>&lt;0.001</b>	<b>0.163 (0.050)</b>	<b>0.001</b>

BMI, Body mass index; CRP, C-reactive protein; HDL-C, High density lipoprotein cholesterol; 24hr LF HRV, Normalized low frequency power heart rate variability; 24hr HF HRV, Normalized high frequency power heart rate variability, NaCl intake, Estimated salt intake; SBP, Systolic blood pressure.

### *Ethnicity*

Due to known salt-sensitivity in black populations,<sup>17</sup> we additionally performed subgroup analyses in black and white men and women. Our sex-specific results were corroborated only in black women and men (**Supplementary Table 2 & Table 4**). We found a significant positive association of MBG excretion with LF HRV in black women ( $R^2=0.38$ ;  $\beta=0.13$ ;  $p=0.036$ ), and HF HRV in black men ( $R^2=0.40$ ;  $\beta=0.18$ ;  $p=0.045$ ), but not in their white counterparts (all  $p>0.26$ ).

### *Sensitivity analysis for contraceptive use*

We have previously reported on the need to perform sensitivity analysis for hormonal contraceptive use when reporting associations of MBG with cardiovascular measures in women.<sup>5</sup> Forty percent of the young women in this study made use of hormonal contraceptives (41.8 % of black and 38.5 % of white women). Hormonal injection was more common in black women (black women N=55 (28.4%); white women N=6 (3.13%)) compared to oral contraceptive use in white women (white women N=68 (35.4%); black women N=23 (12%)). These differences in hormonal contraceptive use methods between ethnic groups are in line with a recently published study in the South African Medical Journal.<sup>18</sup> Taking this into account we performed sensitivity analyses for hormonal contraceptive use in black and white women respectively (**Table 4**). In black women the relationship between MBG and LF HRV remained robust ( $R^2=0.36$ ;  $\beta=0.14$ ;  $p=0.040$ ), and there was no relationship between MBG excretion and hormonal contraceptive use ( $R^2=0.36$ ;  $\beta=0.040$ ;  $p=0.55$ ). Interestingly, although there was no relationship between MBG and LF HRV in white women ( $R^2=0.38$ ;  $\beta=0.17$ ;  $p=0.79$ ), MBG excretion negatively associated with hormonal contraceptive use ( $R^2=0.38$ ;  $\beta=0.25$ ;  $p<0.001$ ).

**Table 4: Fully adjusted multiple regression models with MBG excretion as dependent variable in black and white men and women**

	<i>MBG excretion (nmol/day)</i>							
	<i>Black</i>				<i>White</i>			
	<i>Men</i>		<i>Women</i>		<i>Men</i>		<i>Women</i>	
	<i>N=102</i>		<i>N=168</i>		<i>N=141</i>		<i>N=181</i>	
	<i>R<sup>2</sup></i>		<i>R<sup>2</sup></i>		<i>R<sup>2</sup></i>		<i>R<sup>2</sup></i>	
	0.39		0.38		0.25		0.32	
	<i>β (S.E.)</i>	<i>P</i>	<i>β (S.E.)</i>	<i>P</i>	<i>β (S.E.)</i>	<i>P</i>	<i>β (S.E.)</i>	<i>P</i>
LF HRV (n.u.)	-0.158 (0.086)	0.071	<b>0.134 (0.063)</b>	<b>0.036</b>	-0.005 (0.076)	0.94	0.036 (0.064)	0.58
Age (years)	-0.002 (0.087)	0.98	<b>-0.154 (0.066)</b>	<b>0.021</b>	-0.047 (0.077)	0.54	-0.063 (0.064)	0.33
BMI (kg/m <sup>2</sup> )	-0.009 (0.095)	0.93	-0.018 (0.084)	0.83	-0.024 (0.099)	0.81	0.016 (0.088)	0.85
24hr SBP (mmHg)	-0.027 (0.089)	0.76	0.138 (0.070)	0.058	-0.013 (0.084)	0.88	0.027 (0.084)	0.75
NaCl intake (g/day)	<b>0.618 (0.084)</b>	<b>&lt;0.001</b>	<b>0.609 (0.066)</b>	<b>&lt;0.001</b>	<b>0.517 (0.077)</b>	<b>&lt;0.001</b>	<b>0.502 (0.063)</b>	<b>&lt;0.001</b>
HDL-C (mmol/l)	-0.142 (0.085)	0.10	-0.087 (0.064)	0.18	0.012 (0.079)	0.88	<b>-0.261 (0.067)</b>	<b>&lt;0.001</b>
CRP (mg/l)	-0.076 (0.086)	0.38	-0.102 (0.074)	0.17	-0.072 (0.089)	0.42	<b>-0.282 (0.071)</b>	<b>&lt;0.001</b>
Glucose (mmol/l)	-0.019 (0.085)	0.82	<b>-0.150 (0.065)</b>	<b>0.023</b>	<b>-0.160 (0.075)</b>	<b>0.034</b>	0.019 (0.064)	0.77
Aldosterone (pg/ml)	<b>0.260 (0.087)</b>	<b>0.004</b>	<b>0.192 (0.065)</b>	<b>0.004</b>	0.148 (0.077)	0.056	<b>0.138 (0.064)</b>	<b>0.031</b>
<b>Sensitivity analysis for hormonal contraceptive use</b>								
	<i>MBG excretion (nmol/day)</i>							
	<i>R<sup>2</sup></i>				<i>R<sup>2</sup></i>			
	<b>0.36</b>				<b>0.38</b>			
	<i>β (S.E.)</i>	<i>P</i>	<i>β (S.E.)</i>	<i>P</i>	<i>β (S.E.)</i>	<i>P</i>	<i>β (S.E.)</i>	<i>P</i>
LF HRV (n.u.)	—		<b>0.138 (0.067)</b>	<b>0.040</b>	—		0.017 (0.062)	0.79
Contraceptive use (no/yes)	—		0.040 (0.067)	0.55	—		<b>-0.252 (0.066)</b>	<b>&lt;0.001</b>



Table 4: Fully adjusted multiple regression models with MBG excretion as dependent variable in black and white men and women (continue)

	<i>MBG excretion (nmol/day)</i>							
	<i>Black</i>				<i>White</i>			
	<i>Men</i>		<i>Women</i>		<i>Men</i>		<i>Women</i>	
	<i>N=102</i>		<i>N=168</i>		<i>N=141</i>		<i>N=181</i>	
<i>R<sup>2</sup></i>		<i>R<sup>2</sup></i>		<i>R<sup>2</sup></i>		<i>R<sup>2</sup></i>		
0.40		0.37		0.24		0.32		
	<i>β (S.E.)</i>	<i>P</i>	<i>β (S.E.)</i>	<i>P</i>	<i>β (S.E.)</i>	<i>P</i>	<i>β (S.E.)</i>	<i>P</i>
<b>HF HRV (n.u.)</b>	<b>0.175 (0.086)</b>	<b>0.045</b>	-0.101 (0.063)	0.11	0.085 (0.076)	0.27	0.024 (0.064)	0.71
Age (years)	0.002 (0.086)	0.98	<b>-0.145 (0.067)</b>	<b>0.031</b>	-0.028 (0.077)	0.71	-0.057 (0.064)	0.38
BMI (kg/m <sup>2</sup> )	-0.001 (0.095)	0.99	-0.011 (0.085)	0.90	-0.035 (0.100)	0.72	0.022 (0.088)	0.80
24hr SBP (mmHg)	-0.027 (0.089)	0.76	0.140 (0.073)	0.056	0.002 (0.086)	0.98	0.037 (0.083)	0.66
NaCl intake (g/day)	<b>0.630 (0.084)</b>	<b>&lt;0.001</b>	<b>0.607 (0.066)</b>	<b>&lt;0.001</b>	<b>0.502 (0.077)</b>	<b>&lt;0.001</b>	<b>0.507 (0.063)</b>	<b>&lt;0.001</b>
HDL-C (mmol/l)	-0.147 (0.085)	0.087	-0.067 (0.065)	0.30	0.020 (0.080)	0.81	<b>-0.255 (0.067)</b>	<b>&lt;0.001</b>
CRP (mg/l)	-0.067 (0.085)	0.43	-0.088 (0.075)	0.24	-0.072 (0.090)	0.43	<b>-0.287 (0.070)</b>	<b>&lt;0.001</b>
Glucose (mmol/l)	-0.021 (0.085)	0.80	<b>-0.163 (0.065)</b>	<b>0.014</b>	<b>-0.167 (0.075)</b>	<b>0.028</b>	0.017 (0.065)	0.79
Aldosterone (pg/ml)	<b>0.261 (0.086)</b>	<b>0.003</b>	<b>0.188 (0.066)</b>	<b>0.005</b>	0.152 (0.078)	0.052	<b>0.141 (0.063)</b>	<b>0.028</b>

BMI, Body mass index; CRP, C-reactive protein; HDL-C, High density lipoprotein cholesterol; 24hr LF HRV, Normalized low frequency power heart rate variability; 24hr HF HRV, Normalized high frequency power heart rate variability, NaCl intake, Estimated salt intake; SBP, Systolic blood pressure.

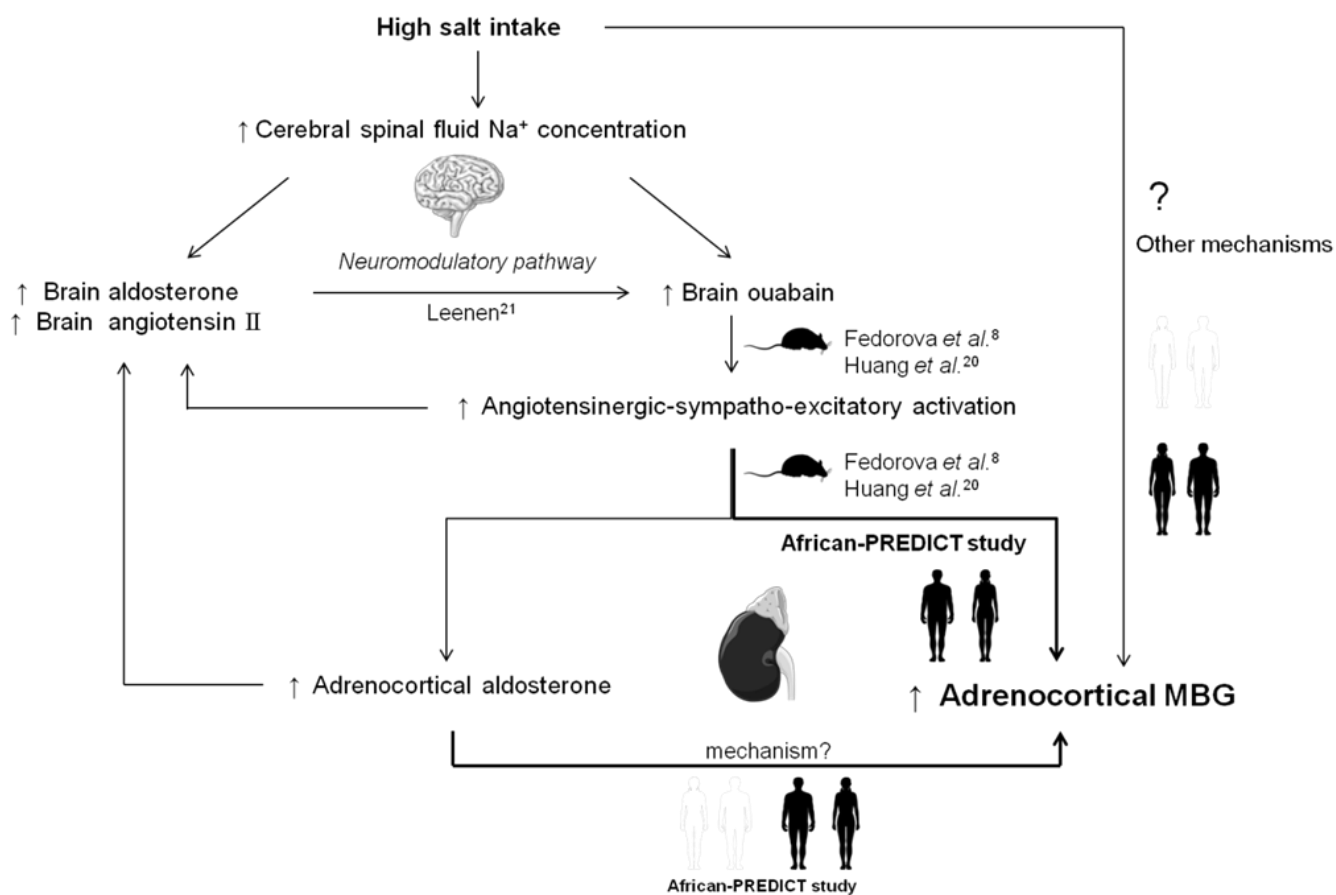
## Discussion

### *Main findings*

For the first time in a human cohort, our study indicated a significant positive association between the endogenous cardiotoxic steroidal inhibitor of Na<sup>+</sup>/K<sup>+</sup>-ATPase, MBG, and autonomic activity in young healthy men and women, supporting a previously demonstrated pathway in animal models.<sup>8</sup> In addition, our results were

especially evident in black men and women, highlighting ethnic specific differences with regards to possible autonomic pathways whereby MBG excretion may be mediated.

Although the relationship between salt intake and MBG excretion is well established in humans,<sup>7</sup> including this study population,<sup>5</sup> the relevant stimulatory mechanisms whereby MBG is released have only been described in animal models.<sup>2,8</sup> Salt loading was shown to increase brain ouabain and concurrently AT II and sympathetic activity,<sup>8,19,20</sup> thereby promoting MBG excretion via this ouabain-angiotensinergic-sympatho-excitatory pathway (**Figure 2**).<sup>8</sup> Brain aldosterone has been also been suggested to stimulate the ouabain-angiotensinergic-sympatho-excitatory pathway via the neuromodulatory pathway.<sup>21</sup>



**Figure 2:** Suggested ouabain-angiotensinergic-sympatho-excitatory-MBG pathway.

We found that salt intake as well as aldosterone positively and independently associated with MBG excretion in both men and women, which is in accordance with previous studies.<sup>22,23</sup> Tomaschitz *et al.* found significantly elevated MBG levels in patients with primary aldosteronism, and demonstrated positive relationships between

plasma aldosterone and MBG.<sup>22</sup> However, in women only, an independent positive relationship between MBG excretion and an indirect measure of sympathetic activity, LF HRV,<sup>24,25</sup> was found. This relationship was strengthened when including estimated salt intake in the regression model, supporting the suggested mechanism whereby MBG excretion is stimulated via increased autonomic activity in response to salt intake. Intriguingly, we have previously demonstrated positive associations of MBG excretion with blood pressure,<sup>6</sup> arterial stiffness<sup>5</sup> and left ventricular mass,<sup>7</sup> predominantly in women. In support, other studies have found the effect of salt intake to be more pronounced in women,<sup>26-28</sup> and that women demonstrate a 15% greater reactivity of aldosterone levels in response to AT II infusion compared to men.<sup>28</sup>

MBG may additionally play a role in the autonomic feedback regulation via its effect on Na<sup>+</sup>/K<sup>+</sup>-ATPase,<sup>29</sup> implicated in cardiac sympathetic regulation.<sup>30</sup> There is some indication in the previous publications on a high sensitivity of human Na<sup>+</sup>/K<sup>+</sup>-ATPase to MBG. Accordingly, human skeletal muscle  $\alpha$ -2 Na<sup>+</sup>/K<sup>+</sup>-ATPase exhibited a high affinity to MBG.<sup>31</sup> In addition, human  $\alpha$ 1-Na<sup>+</sup>/K<sup>+</sup>-ATPase with a high affinity for MBG is expressed in the brain and nerve endings, although it is not the primary isoform.<sup>29,32</sup> The effect of MBG on human neuronal Na<sup>+</sup>/K<sup>+</sup>-ATPase, and specifically  $\alpha$ -3 subunit, abundant in the nerve endings,<sup>32</sup> would merit future detailed investigations. This feedback mechanism may also exaggerate sympathetic activation in women. Our results in the women of this study population,<sup>5-7</sup> along with the findings of other studies,<sup>26-28</sup> may suggest that women are likely at a greater risk to the harmful effects of salt in the development of cardiovascular disease- where MBG may play an important role.<sup>5-7</sup>

Conversely, in men a significant positive relationship between MBG excretion and a measure of parasympathetic activity,<sup>14</sup> HF HRV, was only evident after parallel adjustment for both estimated salt intake and aldosterone. Although we anticipated relationships of salt intake and aldosterone with MBG excretion, the positive association between MBG excretion and HF HRV (potentially reflecting parasympathetic activity) was unexpected, as only indices of sympathetic activity have previously been implicated in increased MBG excretion.<sup>8</sup>

The apparent sex-specific relationship of MBG excretion with LF HRV in women, and HF HRV in men warrants further investigation into sympathetic and parasympathetic modulation of MBG excretion. Although 24hr LF HRV and HF HRV have been thought to reflect sympathetic and parasympathetic activity, respectively,<sup>24</sup> this

assumption is controversial.<sup>13,33,34</sup> While vagal activity has been recognized as a major component of HF HRV by the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology,<sup>14</sup> the physiological interpretation of LF HRV has been debated.<sup>34</sup> Some have argued that that HRV does not reflect sympathetic activation based on the dissociation between HRV and norepinephrine spillover,<sup>33</sup> peripheral muscle sympathetic nerve activity (MSNA)<sup>35</sup> and expected increased sympathetic activity with exercise.<sup>36</sup> However, other studies demonstrate contrasting results. Furlan *et al.* indicated a significant increase in LF HRV (n.u.) along with heart rate, MSNA, plasma epinephrine, norepinephrine, and a decrease in HF HRV during a tilt test in healthy adults.<sup>25</sup> In support, Sandrone *et al.* have found that beta blockers reduce LF HRV after myocardial infarction.<sup>37</sup> It has also been observed that the circadian variation of LF HRV corresponds with expected decreased sympathetic activity at night and early morning sympathetic surge.<sup>24</sup> We, therefore, recommend that our findings be repeated using more direct measures of sympathetic activity such as microneurography.<sup>38</sup>

Apart from the sex differences, the relationship between MBG excretion and autonomic activity was particularly evident in black women and men, who are known to be more salt sensitive,<sup>17</sup> and have increased autonomic activity.<sup>39</sup> While the young age of these participants might still be protective to prevent MBG over production, exaggerated sympathetic drive and aldosterone sensitivity<sup>40</sup> associated with black ethnicity could increase their cardiovascular risk over time.

#### *Strengths and limitations*

Our young study population allows us to investigate the possible relationship of MBG excretion with indices of autonomic activity in adults who are at their peak cardiovascular health, while also consuming a high salt diet (77% of the women and 81% of the men had salt intake > 5 g/day).<sup>16</sup> This is the first study to investigate this relationship in a human cohort that supports mechanisms previously only demonstrated in animal model studies. The cross-sectional design limits the discussion of cause and effect, and therefore the observational results should be interpreted accordingly. For this study a single 24hr urine sample was collected for each participant as opposed to the multiple samples for the estimation of salt intake. The World Health Organization, however, have indicated that the use of a single 24hr urine sample, as the method for estimating population sodium intake, is

sufficient.<sup>41</sup> The impact of age and cardiovascular diseases on the association of MBG and autonomic activity would merit further investigations.

### *Conclusion*

Our study demonstrated a positive association between MBG excretion and autonomic activity in young healthy men and women, and particularly in black adults. Our findings in this human cohort support suggested mechanisms whereby MBG is elevated as a result of increased salt intake, including autonomic activity, previously demonstrated in Dahl salt-sensitive hypertension.

### **Sources of funding**

The research funded in this manuscript is part of an ongoing research project financially supported by the South African Medical Research Council (SAMRC) with funds from National Treasury under its Economic Competitiveness and Support Package; the South African Research Chairs Initiative (SARChI) of the Department of Science and Technology and National Research Foundation (NRF) of South Africa (Grant numbers: UID 86895; 111862); the SAMRC with funds received from the South African National Department of Health; GlaxoSmithKline R&D (Africa Non-Communicable Disease Open Lab grant), the UK Medical Research Council and with funds from the UK Government's Newton Fund; as well as corporate social investment grants from Pfizer (SA), Boehringer Ingelheim (SA), Novartis (SA), the Medi Clinic Hospital Group (SA) and in kind contributions of Roche Diagnostics (SA). The study is also supported by the National Institute on Aging, NIH Intramural Research Program (USA). Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors, and therefore, the NRF does not accept any liability in regard.

### **Conflict of interest**

None

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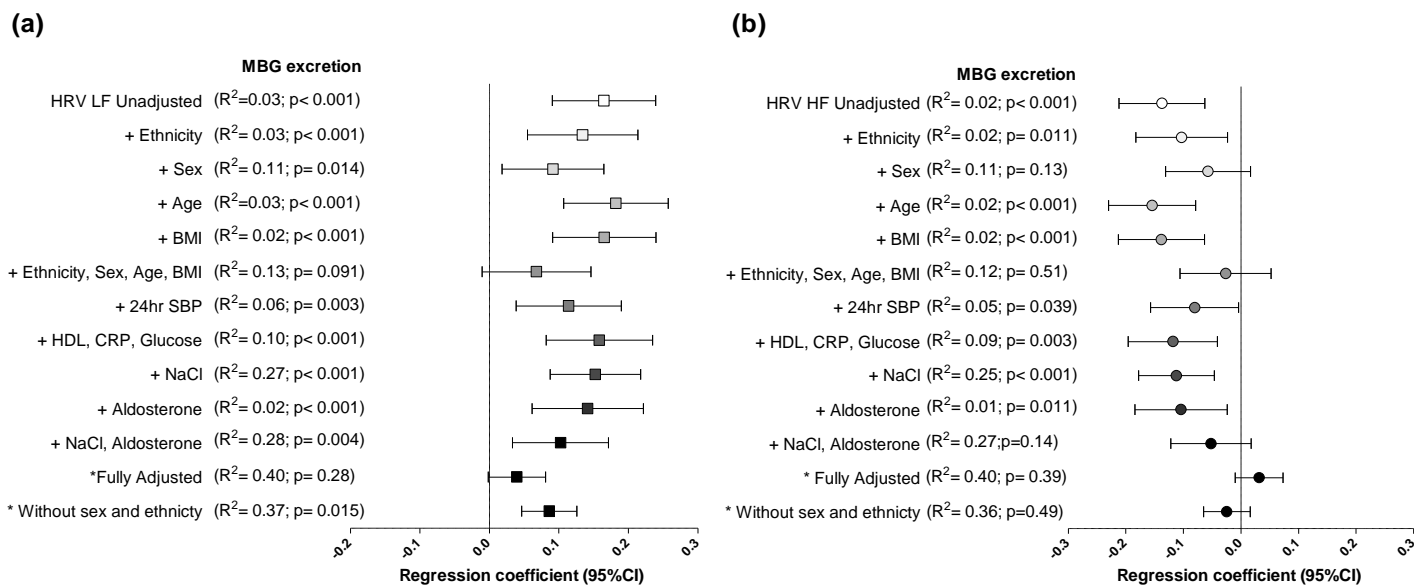
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## Supplemental digital content



**Supplementary Figure 1:** Unadjusted, partially adjusted and fully adjusted  $\beta$ -values of heart rate variability estimates ((A) LF HRV; (B) HF HRV) in the total group, as part of regression analyses with MBG excretion as dependent variable.

**Supplementary Table 1: Pearson and partial correlations between autonomic activity and MBG excretion in the total group**

	<i>MBG excretion (nmol/day)</i>		
	<b>Unadjusted N=679</b>	<b>Adjusted for age, ethnicity and BMI N=679</b>	<b>Additional adjustment for sex N=679</b>
24hr Heart rate (bpm)	<b>r=-0.17; p&lt;0.001</b>	<b>r=-0.18; p&lt;0.001</b>	r=-0.04; p=0.33
24hr LF HRV (n.u.)	<b>r=0.16; p&lt;0.001</b>	<b>r=0.14; p=0.001</b>	r=0.07; p=0.13
24hr HF HRV (n.u.)	<b>r=-0.14; p&lt;0.001</b>	<b>r=-0.11; p=0.010</b>	r=-0.03; p=0.54
24hr LF/HF HRV	<b>r=0.15; p&lt;0.001</b>	<b>r=0.12; p=0.005</b>	r=0.04; p=0.37
24hr HRV SDNN (ms)	<b>r=0.12; p=0.002</b>	<b>r=0.11; p=0.011</b>	r=-0.02; p=0.66

BMI, Body mass index; 24hr LF HRV, Normalized low frequency power heart rate variability ; 24hr HF HRV, Normalized high frequency power heart rate variability, 24hr LF/HF HRV, Ratio of low-to high frequency power; SDNN, Standard deviation of normal R-R intervals

**Supplementary Table 2: Pearson and partial correlations between indices of autonomic activity and MBG excretion in black and white men and women**

	<i>MBG excretion (nmol/day)</i>			
	Men		Women	
	Black N=136	White N=148	Black N=201	White N=194
24hr LF HRV (n.u.)	r=-0.03; p=0.74	r=0.05; p=0.52	r=0.13; p=0.069	r=0.03; p=0.66
24hr HF HRV (n.u.)	r=-0.01; p=0.93	r=0.02; p=0.80	r=-0.11; p=0.11	r=0.03; p=0.69
24hr LF HRV/HF	r=-0.01; p=0.93	r=0.02; p=0.83	r=0.12; p=0.093	r=-0.02; p=0.83
SDNN	r=0.02; p=0.79	r=-0.06; p=0.48	r=-0.03; p=0.70	r=-0.003; p=0.97
	Adjusted for age and BMI			
24hr LF HRV (n.u.)	r=-0.02; p=0.84	r=0.06; p=0.44	<b>r=0.14; p=0.043</b>	r=0.05; p=0.51
24hr HF HRV (n.u.)	r=-0.01; p=0.89	r=0.01; p=0.88	r=-0.12; p=0.079	r=0.01; p=0.88
24hr LF HRV/HF	r=-0.002; p=0.99	r=0.03; p=0.76	r=0.13; p=0.062	r<0.001; p=0.99
SDNN	r=0.04; p=0.69	r=-0.05; p=0.55	r=-0.02; p=0.75	r=-0.02; p=0.79

BMI, Body mass index; 24hr LF HRV, Normalized low frequency power heart rate variability ; 24hr HF HRV, Normalized high frequency power heart rate variability, 24hr LF HRV/HF, Ratio of low-to high frequency power; SDNN, Standard deviation of normal R-R intervals


# Chapter 6

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Research article 4:

Microvascular function in non-dippers: potential involvement  
of the salt-sensitivity biomarker, marinobufagenin.

The African-PREDICT study

Clinical Nutrition			
			
<b>Impact factor</b>	5.496		
<b>Publisher</b>	Elsevier		
<b>Citation</b>	Manuscript prepared for submission to <i>Clinical Nutrition</i>		
<b>Aims &amp; Scope</b>	<i>Clinical Nutrition</i> , the official journal of The European Society for Clinical Nutrition and Metabolism, an international journal providing essential scientific information on nutritional and metabolic care and the relationship between nutrition and disease both in the setting of basic science and clinical practice.		
Author Instructions			
<b>Language</b>	Not specified	<b>Font</b>	Not specified
<b>Spacing</b>	Double Spacing	<b>Alignment</b>	Not specified
<b>Page numbers</b>	Number consecutively starting on title page.		
<b>Line numbering</b>	Lines should be numbered consecutively starting on the title page.		
<b>Manuscript</b>	Manuscripts should include the following sections, each starting on a separate page: Title Page, Abstract, Introduction, Materials and Methods (including statistical considerations and ethical statement), Results, Discussion, Acknowledgements, Statement of Authorship, Conflict of Interest Statement and Funding sources, References; Figure and Table Legends.		
<b>Title page</b>	<ul style="list-style-type: none"> <li>• Full title</li> <li>• Full first name and family name of each author should appear</li> <li>• Affiliations of all the authors connected using a,b,c</li> <li>• Corresponding author information</li> </ul>		
<b>Abstract</b>	Structures abstract should include: Background & aims, Methods, Results and Conclusion		
<b>Keywords</b>	A maximum of 6 keywords		
<b>Text</b>	Introduction should be limited to 1.5 pages and the Discussion to 4 pages.		
<b>Acknowledgements</b>	Include acknowledgements section.		
<b>Conflict of interest</b>	Include disclosure statement.		

<b>Funding</b>	Include funding section.
<b>Authorship</b>	Include authorship statement.
<b>Ethical considerations</b>	<ul style="list-style-type: none"> <li>• All work must be conducted in accordance with the Declaration of Helsinki.</li> <li>• Include (1) a statement that consent was obtained from participants and (2) of ethical approval.</li> </ul>
<b>References</b>	<ul style="list-style-type: none"> <li>• References can be in any style or format as long as the style is consistent.</li> </ul>
<b>Tables</b>	<ul style="list-style-type: none"> <li>• Manuscript should include at least 2 tables.</li> <li>• Each table should be typed on a separate page in double spacing.</li> <li>• Tables can be placed either next to the relevant text or at the end of the manuscript on a separate page.</li> </ul>
<b>Figures</b>	<ul style="list-style-type: none"> <li>• Manuscript should include at least 2 figures.</li> <li>• Please ensure the figures are included in the single file and placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file. The corresponding caption should be placed directly below the figure.</li> </ul>
<b>Supplemental Digital Content</b>	<ul style="list-style-type: none"> <li>• Supplements should be cited consecutively in the text of the submitted manuscript.</li> </ul>

*\*Formatting changes were made to maintain uniformity throughout this thesis, including text font, line spacing, margins, page numbers, tables and figures.*

**Microvascular function in non-dippers: potential involvement of the salt-sensitivity biomarker,  
marinobufagenin.**

**The African-PREDICT study**

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## Abstract

**Background & Aims:** Suppressed night-time blood pressure dipping is associated with salt-sensitivity and may increase the hemodynamic load on the microvasculature. The mechanism remains unknown whereby salt-sensitivity may increase the cardiovascular risk of non-dippers. Marinobufagenin, a novel steroidal biomarker, is associated with salt-sensitivity and other cardiovascular risk factors independent of blood pressure. We investigated whether microvascular function in non-dippers is associated with marinobufagenin.

**Methods:** We included 220 dippers and 154 non-dippers (aged 20-30 years) from the African-PREDICT study, with complete 24hr urinary marinobufagenin and sodium data. We determined dipping status using 24hr blood pressure monitoring, and defined night-time non-dipping <10%. We measured microvascular reactivity as retinal artery dilation in response to light flicker provocation.

**Results:** Young healthy non-dippers and dippers presented with similar peak retinal artery dilation, urinary sodium and MBG excretion ( $p>0.05$ ). However, only in non-dippers did peak retinal artery dilation relate negatively to marinobufagenin excretion after single ( $r=-0.20$ ;  $p=0.012$ ), partial ( $r=-0.23$ ;  $p=0.004$ ) and multivariate adjusted regression analyses (Adj.  $R^2=0.34$ ;  $\beta=-0.26$ ;  $p<0.001$ ). We also noted a relationship between peak artery dilation and estimated salt intake (Adj.  $R^2=0.30$ ;  $\beta=-0.14$ ;  $p=0.051$ ), but it was lost upon inclusion of marinobufagenin (Adj.  $R^2=0.33$ ;  $\beta=-0.015$ ;  $p=0.86$ ). No relationship between microvascular reactivity and marinobufagenin was evident in dippers ( $p=0.77$ ).

**Conclusion:** Marinobufagenin, representing salt-sensitivity, may be involved in early microvascular functional changes in young non-dippers, and thus contribute to the development of hypertension and cardiovascular disease later in life.

**Key words:** blood pressure, cardiotonic steroids, dietary salt, human, marinobufagenin, non-dipping, retinal microvascular function, salt-sensitivity, vasorelaxation, young

## Introduction

Night-time blood pressure dipping forms part of the normal circadian rhythm where blood pressure is elevated during the daytime and lowered with more than 10% during the night.<sup>1</sup> This physiological rhythm, however, is impaired in individuals who are classified as non-dippers (BP dipping < 10%).<sup>1</sup> The non-dipping phenotype is associated with salt-sensitivity<sup>2,3</sup> which has recently been recognized by the American Heart Association (AHA) as a cardiovascular risk factor independent of blood pressure.<sup>4</sup> It is reported that approximately 30 to 50% of hypertensive<sup>5</sup> and 1 in 4 normotensive individuals<sup>6</sup> are salt-sensitive. Salt-sensitivity is associated with increased mortality not only in hypertensive adults but also in normotensive adults.<sup>7</sup> Both the AHA<sup>4</sup> and Weinberger *et al.*<sup>7</sup> noted that continued research is needed to investigate possible mechanisms whereby salt-sensitivity increases cardiovascular risk beyond blood pressure, especially in normotensive individuals. However, the accurate classification of salt-sensitivity remains difficult due to the known variability of blood pressure regardless of multiple measurements, and requires highly controlled salt intervention.<sup>4</sup> In addition to the aforementioned, these studies are labour intensive, time consuming and costly. The identification of surrogate markers of salt-sensitivity may be useful, especially in population-based studies that do not have the resources to adhere to the strict protocols for accurate salt-sensitivity classification.

The precursory role of microvascular dysfunction in the development of hypertension and cardiovascular disease has been recognised.<sup>8</sup> Although there is little information on dipping status and microvascular function, salt-sensitivity was indeed associated with impaired microvascular function.<sup>9</sup> Past examinations of the microvasculature were limited and invasive, however, technological advancements have made it possible to now gain valuable information via methods including retinal microvascular imaging<sup>10</sup> – reflective of the systemic microvasculature state.<sup>11</sup> Structural changes in the retinal microvascular calibres including small artery narrowing and vein widening have been consistently associated with increased blood pressure and inflammation, respectively.<sup>12-14</sup> In addition, retinal microvascular responses to a light flicker provocation (function changes) may be indicative of microvascular endothelial function,<sup>11</sup> as reduced retinal artery dilation was related to hypertension,<sup>15</sup> diabetes mellitus,<sup>15</sup> obesity<sup>16</sup> and coronary artery disease.<sup>17</sup>

The novel steroidal biomarker, cardiotonic steroid marinobufagenin (MBG), shown to markedly increase with increased salt intake,<sup>18,19</sup> strongly associates with salt-sensitivity.<sup>20-23</sup> In vitro investigation of the adverse role of MBG on the microvasculature indicated elevated MBG to promote endothelial damage of human brain microvascular endothelial cells.<sup>24</sup> Studies investigating relationships between MBG and microvascular function in vivo, especially in healthy adults are scarce. We demonstrated that MBG associates with increased large artery stiffness and left ventricular mass in young normotensive adults consuming excessive amounts of salt, independent of blood pressure.<sup>18,25</sup> It is, therefore, possible that MBG may play a harmful role in early microvascular function, independent of blood pressure, in individuals who are reportedly more salt-sensitive - including those with a non dipping night-time blood pressure profile. We therefore investigated the relationship of microvascular function with MBG excretion in young normotensive non-dippers, when compared to dippers.

## Methods

This study forms part of the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT), and included the data of the first 374 consecutively enrolled participants with complete 24hr urinary, 24hr blood pressure and dynamic retinal vessel analyses data. The African-PREDICT study aims to investigate novel markers of early cardiovascular risk, while identifying potential strategies for the prevention of adverse cardiovascular outcomes. All procedures for the African-PREDICT study adhered to institutional guidelines and the Declaration of Helsinki, and were approved by the Health Research Ethics Committee of the North-West University (NWU-00001-12-A1). The study is registered at Clinical Trials.gov (Nr. NCT03292094).

The African-PREDICT study enrolled young apparently healthy black and white, men and women (between the ages of 20 to 30 years), from communities near Potchefstroom, in the North West Province of South Africa. Participant recruitment and data collection took place from 2012-2017. Community members took part in screening to determine eligibility for inclusion into the study based on the following criteria: office blood pressure less than 140/90 mmHg,<sup>26</sup> microalbuminuria less than 30 mg/ml, HIV uninfected, no self-reported previous diagnosis of chronic illnesses and did not make use of antihypertensive or chronic disease medication. Women included into the study were not pregnant or lactating at the time of participation.

Participants who met these inclusion criteria were invited back for additional measurements at the Hypertension Research Clinic on the North-West University campus. Participation in the study was voluntary, and all participants completed written informed consent prior to the screening and study measurements.

#### *Questionnaire and anthropometric data*

General health and demographic questionnaires were completed by each participant to collect data on ethnicity, sex, age, self-reported alcohol use and smoking.

The body height (m) (SECA 213 Portable Stadiometer) (SECA, Hamburg, Germany), weight (kg) (SECA 813 Electronic Scales) and waist circumference (cm) (Lufkin Steel Anthropometric Tape; W606PM; Lufkin, Apex, USA) of participants were measured, and body mass index (BMI) (weight (kg)/height (m<sup>2</sup>))<sup>27</sup> and waist to height ratio (WHtR) calculated.

### **Cardiovascular measurements**

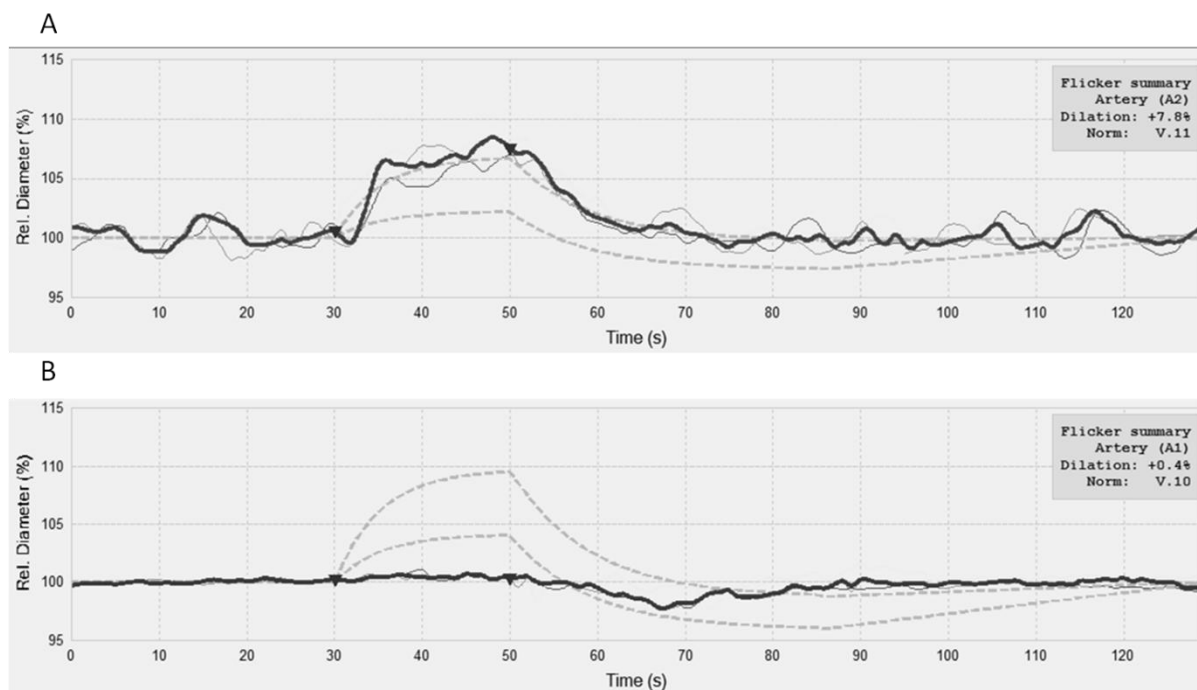
#### *Ambulatory blood pressure*

Ambulatory blood pressure (ABPM) was used to identify dipper status of participants. Each participant was fitted with a Card(X)plore device (Meditech, Budapest, Hungary) following the European Society of Hypertension practice guidelines.<sup>1</sup> We programmed ABPM devices to measure daytime blood pressure in 30 minute intervals (06:00-22:00) and night-time blood pressure every hour (22:00-06:00). Participants included into this study had more than 70 % successful ABPM recordings, or 20 daytime and 5 night-time measurements.<sup>28</sup> Non-dipping was defined as night-time systolic blood pressure dipping <10%.<sup>1</sup>

#### *Microvascular reactivity*

Participants refrained from eating at least one hour before the retinal microvascular measurements were performed. A registered nurse measured the intraocular pressure (Tonopen, Avia, Reichert Technologies) prior to retinal microvascular measurements, and those with an intraocular pressure exceeding 24mmHg, did not participate in further retinal microvascular assessments. . Mydriasis was induced by administering a drop of Tropicamide (1% Alcon) to the right eye 15 to 30 minutes before the measurement commenced.

Microvascular reactivity in response to light flicker provocation was measured non-invasively using the Dynamic Retinal Vessel Analyzer (Imedos, Jena, Germany), fitted with a Zeiss Fundus camera FF-450 set at a 30° angle. The dynamic retinal vessel analyses were performed using the standard flicker protocol of the Imedos Systems. Using RVA version 4.50 software, segments of both the artery and vein branches, between 0.5-2.0 optic disc diameter from the optic disc, were selected for analysis. The first light flicker stimulus was applied for 20s after a 50s baseline phase. The 20s flicker stimulus was repeated for three cycles, each interrupted by a 80s recovery period. The quality of each measurement was assessed as previously described.<sup>29</sup> Raw data were exported to Excel sheets with built in macros. The maximum retinal artery and vein dilation in response to FLIP was calculated as a percentage of baseline previously described by Kotliar et al.<sup>16</sup> **Figure 1** demonstrates the expected retinal artery dilation in response to FLIP (**Figure 1 A**), in comparison to suppressed retinal artery dilation (**Figure 1 B**).



**Figure 1:** Retinal arterial responses to a light flicker provocation in individuals with (A) normal retinal arterial dilation and (B) suppressed retinal arterial dilation.

*Microvascular calibres*

The Dynamic Retinal Vessel Analyzer was also used to capture retinal images so determine the central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE), as previously described.<sup>30</sup> Retinal images were captured with the Fundus camera angled at 50° and individual diameter data extracted from Visualis software. Vessels located between 0.5 -1.5 optic disc diameters from the optic disc were selected as either arteries or veins, and the CRAE and CRVE were subsequently calculated using the revised formulas.<sup>31</sup>

**Biological sampling and biochemical analyses**

All participants were requested to fast from 22:00 the night before the study measurements. Early morning blood samples were collected at approximately the same time every morning by trained research nurse. We have previously published a detailed description on the handling of the biological samples.<sup>25</sup> The 24hr urine sampling protocol for this study, followed standard protocols of the Pan American Health Organisation/ World Health Organisation (PAHO/WHO).<sup>32</sup>

24hr Urinary MBG was analysed using a solid-phase Dissociation-Enhanced Lanthanide Fluorescent Immunoassay, based on a 4G4 anti-MBG mouse monoclonal antibody, described in detail by Fedorova *et al.*<sup>33</sup> We measured 24hr urinary sodium and potassium, serum low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), triglycerides, C-reactive protein (CRP), glucose and gamma glutamyltransferase (GGT) using the Cobas Integra 400plus (Roche, Basel Switzerland). Serum cotinine was determined using the chemiluminescence method (Immulite, Siemens, Erlangen, Germany) and IL-6 using the high sensitivity Quantikine ELISA kit (R & D Systems, Minneapolis, United States). Estimated salt intake was calculated from 24hr urinary sodium using the equation:<sup>34</sup>

$$\text{Estimate NaCl (g/day)} = \frac{(24\text{hr Urinary Na (mmol/L)} * \text{Urinary volume (L)}) * 58.44}{1000}$$

## Statistical analyses

All statistical analyses were performed with Statistica version 13 (TIBCO Software Inc., Tulsa, Oklahoma, USA). Normally distributed data was presented as the arithmetic mean and standard deviation, with non-Gaussian distributed data presented as the geometric mean, 5<sup>th</sup> and 95<sup>th</sup> percentiles. We performed independent *t* tests to compare continuous data, and the chi-square test for categorical data, between dippers and non-dippers. Pearson, partial and multiple regression analyses were conducted to investigate the relationships of peak artery dilation with MBG excretion and estimated salt intake respectively, in dippers and non-dippers. Covariates included into multiple regression models were included based on the strongest bivariate associations with peak artery dilation, MBG excretion or estimated salt intake. The model included waist to height ratio (WHtR), 24hr systolic blood pressure, IL-6, LDL-C, cotinine, and glucose. Multiple regression analyses with peak artery dilation as dependent variable, additionally included artery segment diameter as a covariate.

## Results

**Table 1** demonstrates the basic characteristic of this young adult population according to their nocturnal dipping status. Of those exhibiting a normal night-time blood pressure dipping pattern the proportion of black (35%) compared to white adults (65%) was significantly lower. Although, 24hr systolic and diastolic blood pressure did not differ between dippers and non-dippers, non-dippers had lower daytime blood pressure ( $p=0.055$ ) and expectantly higher night-time blood pressure ( $p<0.001$ ). The CRAE was narrower in non-dippers ( $p=0.050$ ). We observed no differences in the microvascular reactivity between dippers and non-dippers. Also, there were no differences in the 24hr urinary volume, sodium, potassium or MBG excretion.

### *Regression analyses*

We firstly performed Pearson correlations of night-time dipping with MBG and peak artery dilation. Only in non-dippers did we find a borderline negative correlation between night-time dipping and MBG excretion ( $r=-0.15$ ;  $p=0.064$ ) – but not between night-time dipping and peak artery dilation ( $r=-0.036$ ;  $p=0.66$ ) (**Table 2**). Also, we found a negative correlation between peak artery dilation and MBG excretion only in non-dippers ( $r=-0.20$ ;  $p=0.012$ )

(Figure 2 A), which remained significant after partial adjustment for age, sex, ethnicity and WHtR ( $r=-0.23$ ;  $p=0.004$ ) (Table 3).

**Table 1: Basic characteristics of dippers and non-dippers**

	<b>Dippers N=220</b>	<b>Non-dippers N=154</b>	<b>P</b>
Sex, men, N (%)	83 (37.7)	68 (44.2)	0.21
Ethnicity, black, N (%)	77 (35.0)	76 (49.7)	0.005
<b>Cardiovascular Profile</b>			
24hr SBP (mmHg)	116 ± 9.68	117 ± 8.79	0.14
<i>Day</i>	122 ± 10.2	120 ± 8.88	0.055
<i>Night</i>	105 ± 8.99	113 ± 9.01	<0.001
24hr DBP (mmHg)	68.6 ± 5.93	69.2 ± 5.90	0.36
<i>Day</i>	74.0 ± 6.31	72.5 ± 6.13	0.025
<i>Night</i>	58.0 ± 5.92	62.7 ± 6.68	<0.001
Night-time Dipping (%)	14.1 ± 2.57	5.92 ± 2.66	<0.001
24hr Heart rate (bpm)	74.4 ± 10.4	74.9 ± 10.9	0.62
<i>Day</i>	79.0 ± 11.2	79.3 ± 11.1	0.76
<i>Night</i>	65.8 ± 10.5	67.1 ± 11.4	0.24
<b>Retinal microvascular calibres</b>			
CRAE (MU)	160 ± 11.6	158 ± 12.3	0.050
CRVE (MU)	249 ± 17.7	248 ± 16.8	0.46
<b>Microvascular reactivity to an acute stressor</b>			
Retinal peak artery dilation (%)	4.30 ± 2.30	4.45 ± 2.26	0.53
<b>24 hr Urinary profile</b>			
Volume (L/24hr)	1.43 ± 0.81	1.39 ± 0.78	0.63
MBG excretion (nmol/day)	3.28 (1.04; 9.37)	3.40 (1.03; 10.1)	0.60
Na <sup>+</sup> excretion (nmol/day)	124 (47.9; 291)	128 (54.2; 326)	0.46
K <sup>+</sup> excretion (mmol/day)	40.5 (14.3; 99.2)	42.7 (16.7; 104)	0.40
Na <sup>+</sup> /K <sup>+</sup> ratio	3.11 (1.45; 6.65)	3.13 (1.17; 6.04)	0.94



Salt intake (g/day)	7.33 (2.82; 17.2)	7.66 (3.19; 19.2)	0.46
<b>Biochemical profile</b>			
Aldosterone (pg/ml)	77.6 (20.7; 405)	79.8 (20.4; 336)	0.75
Glucose (mmol/L)	4.69 ± 0.68	4.64 ± 0.76	0.47
HDL-C (mmol/L)	1.32 (0.82; 2.24)	1.31 (0.82; 2.11)	0.90
LDL-C (mmol/L)	2.72 (1.60; 4.37)	2.59 (1.39; 4.32)	0.17
Triglycerides (mmol/L)	0.87 (0.42; 2.05)	0.87 (0.41; 1.90)	0.95
C-reactive protein (mg/L)	1.00 (0.12; 10.9)	1.00 (0.10; 9.37)	0.97
Interleukin-6 (pg/ml)	0.88 (0.29; 3.03)	0.93 (0.30; 3.21)	0.44
<b>Lifestyle</b>			
Smoking, N (%)	52 (23.6)	27 (17.5)	0.15
Cotinine, N (%) >10 (ng/ml)	59 (27.1)	29 (19.5)	0.094
Alcohol, N (%)	139 (63.2)	88 (58.7)	0.38
GGT (U/L)	19.9 (8.10; 53.3)	22.0 (8.80; 65.4)	0.014

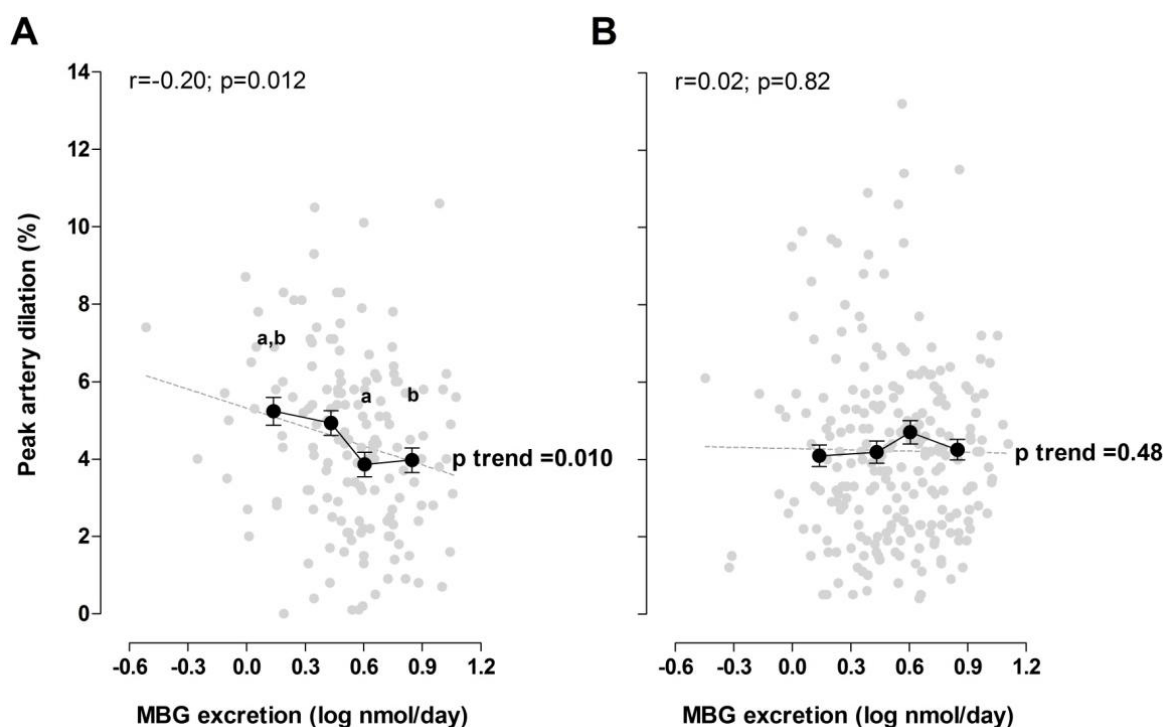
Arithmetic mean ± standard deviation; geometric mean (5<sup>th</sup> percentile; 95<sup>th</sup> percentile intervals).

CRAE, Central retinal artery equivalent; CRVE, Central retinal vein equivalent; DBP, Diastolic blood pressure; GGT,  $\gamma$ -glutamyl transferase; HDL-C, High density lipoprotein cholesterol; K<sup>+</sup>, Potassium; LDL-C, Low density lipoprotein cholesterol; MBG, Marinobufagenin; Na<sup>+</sup>, Sodium; SBP, Systolic blood pressure

**Table 2: Correlations of MBG excretion and peak artery dilation with night-time dipping percentage**

	Night-time dipping (%)	
	Dippers	Non-dippers
	N=220	N=154
Peak artery dilation (%)	r=-0.003; p= 0.97	r=-0.036; p=0.66
MBG excretion (nmol/day)	r=0.024; p= 0.72	r=-0.15; p= 0.064

MBG, Marinobufagenin



**Figure 2:** Unadjusted (●) and adjusted (●) relationship between peak artery dilation and MBG excretion in (A) non-dippers and (B) dippers.

\*Adjusted for age, sex, ethnicity and waist-to-height ratio

a, b  $P=0.025$

**Table 3: Pearson and partial correlations**

	MBG excretion (nmol/day)		Salt intake (g/day)	
	Dippers N=220	Non-dippers N=154	Dippers N=220	Non-dippers N=154
Peak artery dilation (%)	$r=0.02$ ; $p=0.82$	<b><math>r=-0.20</math>; <math>p=0.012</math></b>	$r=-0.01$ ; $p=0.85$	$r=-0.11$ ; $p=0.16$
Adjusted for sex, age, ethnicity and waist-to-height ratio				
Peak artery dilation (%)	$r=0.03$ ; $p=0.71$	<b><math>r=-0.23</math>; <math>p=0.004</math></b>	$r=-0.05$ ; $p=0.45$	$r=-0.14$ ; $p=0.091$

MBG, Marinobufagenin. Bold values denotes  $p<0.05$ .

We determined whether estimated salt intake associates with peak artery dilation, and found a weak relationship after partial adjustments ( $r=-0.14$ ,  $p=0.091$ ). When we performed multivariate-adjusted regression analyses in non-dippers (**Table 4**), the borderline significant association between peak artery dilation and estimated salt

intake persisted (Adj.  $R^2=0.30$ ;  $\beta=-0.14$ ;  $p=0.051$ ). However, this association was altered after adding MBG excretion into the multiple regression model (Adj.  $R^2=0.33$ ;  $\beta=-0.015$ ;  $p=0.86$ ). Conversely, the negative association between peak artery dilation and MBG excretion remained robust (Adj.  $R^2=0.34$ ;  $\beta=-0.26$ ;  $p<0.001$ ) before and after including estimated salt intake into the model (Adj.  $R^2=0.33$ ;  $\beta=-0.25$ ;  $p=0.006$ ). No relationships were evident between peak artery dilation and MBG excretion (Adj.  $R^2=0.21$ ;  $\beta=0.02$ ;  $p=0.77$ ) or estimated salt intake (Adj.  $R^2=0.21$ ;  $\beta=-0.06$ ;  $p=0.32$ ) in dippers (**Table 3 & Supplementary Table 1**).

**Table 4: Multiple regression analyses in non-dippers**

	<i>Retinal peak artery dilation (%)</i>					
	Salt model		MBG model		Salt & MBG model	
	N=144		N=145		N=144	
	Adj. $R^2$		Adj. $R^2$		Adj. $R^2$	
	$\beta$ (S.E.)	<i>P</i>	$\beta$ (S.E.)	<i>P</i>	$\beta$ (S.E.)	<i>P</i>
MBG excretion (nmol/day)	N/A		<b>-0.260 (0.075)</b>	<b>&lt;0.001</b>	<b>-0.250 (0.090)</b>	<b>0.006</b>
Salt intake (g/day)	-0.144 (0.073)	0.051	N/A		-0.015 (0.085)	0.86
Age (years)	0.070 (0.076)	0.36	0.066 (0.073)	0.37	0.064 (0.074)	0.39
Sex (women/men)	-0.074 (0.092)	0.42	0.003 (0.093)	0.98	0.002 (0.094)	0.98
Ethnicity (black/white)	<b>-0.362 (0.085)</b>	<b>&lt;0.001</b>	<b>-0.348 (0.083)</b>	<b>&lt;0.001</b>	<b>-0.348 (0.083)</b>	<b>&lt;0.001</b>
WHtR	<b>0.196 (0.095)</b>	<b>0.041</b>	<b>0.194 (0.092)</b>	<b>0.036</b>	<b>0.196 (0.093)</b>	<b>0.037</b>
24 hr SBP (mmHg)	0.092 (0.101)	0.37	0.110 (0.098)	0.27	0.107 (0.099)	0.28
Interleukin-6 (pg/ml)	<b>-0.192 (0.081)</b>	<b>0.019</b>	<b>-0.184 (0.079)</b>	<b>0.020</b>	<b>-0.186 (0.079)</b>	<b>0.021</b>
Cotinine (ng/ml)	<b>0.173 (0.077)</b>	<b>0.027</b>	<b>0.158 (0.075)</b>	<b>0.037</b>	<b>0.158 (0.076)</b>	<b>0.039</b>
LDL-C (mmol/L)	<b>-0.225 (0.080)</b>	<b>0.006</b>	<b>-0.228 (0.077)</b>	<b>0.004</b>	<b>-0.229 (0.078)</b>	<b>0.004</b>
Glucose (mmol/L)	-0.126 (0.083)	0.13	-0.153 (0.080)	0.059	-0.150 (0.082)	0.070

LDL-C, Low density lipoprotein cholesterol; MBG, Marinobufagenin; SBP, Systolic blood pressure; WHtR, Waist to height ratio

## Discussion

In young adults with suppressed night-time blood pressure dipping, we found that their acute microvascular dilatory responses were independently and negatively associated with a biomarker of salt-sensitivity, namely MBG. This was not found in those with normal dipping.

Night-time blood pressure dipping forms part of the normal physiological circadian rhythm that plays an important role in lowering unnecessary cardiovascular hemodynamic load while sleeping. The lack of a decrease in blood pressure from day to night-time, increases cardiovascular load and concurrently cardiovascular risk. Indeed, Hermida *et al.* showed that non-dippers with a normal 24hr blood pressure (<135/90 mmHg) demonstrated similar hazard ratios of total cardiovascular events compared to hypertensive dippers.<sup>35</sup> Also, non-dipping blood pressure is associated with increased mortality, even in those with normotensive blood pressures.<sup>36</sup> Accordingly, the non-dippers in our study population had a narrower retinal artery equivalent, which itself is associated with increased risk of hypertension<sup>13</sup> and cardiovascular mortality.<sup>37</sup> This brings forth the question as to the physiological mechanisms promoting early cardiovascular risk independent of blood pressure in these individuals.

Non-dipping is associated with salt-sensitivity,<sup>2,3</sup> a recognised cardiovascular risk factor.<sup>4</sup> An endogenous inhibitor of Na<sup>+</sup>K<sup>+</sup>-ATPase, the cardiotoxic steroid MBG, is strongly associated with salt-sensitivity.<sup>20-22</sup> The interaction of MBG with Na<sup>+</sup>K<sup>+</sup>-ATPase via either the inhibitory or signalling pathway,<sup>38</sup> has been shown to promote vasoconstriction<sup>39</sup> and vascular fibrosis<sup>40,41</sup> respectively, ultimately impairing vasorelaxation. In support, Fedorova *et al.* have demonstrated impaired sodium nitroprusside induced vasorelaxation of rat aortic explants pretreated with MBG.<sup>40</sup> Notably, the effect of MBG on vascular Na<sup>+</sup>K<sup>+</sup>-ATPase can be potentiated by the mechanisms related to the salt-sensitivity.<sup>42</sup> Our findings – albeit based on cross-sectional association studies – suggest that MBG may play an adverse role in altering microvascular function in non-dipping normotensive adults, thereby increasing their cardiovascular risk independent of blood pressure. Salt-sensitivity is not characterized by an altered salt balance, but rather abnormal sodium handling and the concurrent hypertensive responses in these individuals.<sup>4</sup> Therefore, although salt intake and MBG did not differ between dippers and non-dippers, the negative relationship observed between microvascular function and MBG only in non-dippers

suggest differential sodium handling and MBG functionality. The inverse association observed between estimated salt intake and microvascular reactivity, confounded by MBG excretion, suggests that the salt-sensitive phenotype associated with non-dipping may likely be resultant of MBG. In support, Fedorova *et al.* previously demonstrated distinct patterns of renal and vascular Na<sup>+</sup>K<sup>+</sup>-ATPase inhibition in normotensive and salt-sensitive rats – despite similar increases in MBG in response to salt loading. Normotensive rats exhibited greater inhibition of renal Na<sup>+</sup>K<sup>+</sup>-ATPase to promote natriuresis, while vascular Na<sup>+</sup>K<sup>+</sup>-ATPase was only inhibited in salt-sensitive rats.<sup>42</sup>

Microvasculature functionality is crucial in terms of regulating the exposure of capillaries to alterations in pulsatile pressure.<sup>8</sup> The question of whether microvascular dysfunction precedes macrovascular dysfunction, or vice versa, remains subjective.<sup>8</sup> In our study, however, it was evident that an association between MBG excretion and attenuated microvascular reactivity was prominent in these young adults. Relationships between MBG and macrovascular reactivity at a later stage remains possible as MBG is associated with large artery stiffness.<sup>18</sup>

This study is limited by its cross-sectional design, and therefore the results should be interpreted within the appropriate context. Also, while MBG is strongly associated with salt intake in normotensive rats<sup>41</sup> and humans,<sup>18</sup> and Dahl salt-sensitive hypertension,<sup>20-22</sup> more studies are needed to establish MBG as a marker of salt-sensitivity in humans. Strengths of this study include the use of gold standard measurements and high quality data from a unique healthy population sample of black and white adults in Africa. The young age of this study population allowed researchers to identify early associations of MBG with established risk factors prior to the onset of cardiovascular disease that might be exaggerated over time and contribute to cardiovascular disease development.

We conclude that MBG is associated with reduced retinal microvascular artery dilation in young healthy normotensive adults, exhibiting a non-dipping blood pressure pattern. Salt intake, with resultant elevation in circulating MBG, may have profound effects in those with salt-sensitivity and non-dipping night-time pressures. It is possible that MBG may play a pathophysiological role contributing to increased cardiovascular risk, independent of blood pressure, observed in those with impaired night-time blood pressure dipping.

**Acknowledgements:** The authors of this study are grateful towards all individuals participating voluntarily in the study. The dedication of the support and research staff as well as students at the Hypertension Research and Training Clinic at the North-West University is also duly acknowledged.

**Statement of Authorship:** MS, WS, OVF and AES contributed to the concept and design of the study. MS, WS, WW, AYB, OVF and AES contributed to the acquisition, analyses and interpretation of data. MS drafted the manuscript. WS, WW, AYB, OVF and AES critically revised the manuscript.

**Disclosures:** None

**Sources of funding:** The research funded in this manuscript is part of an ongoing research project financially supported by the South African Medical Research Council (SAMRC) with funds from National Treasury under its Economic Competitiveness and Support Package; the South African Research Chairs Initiative (SARChI) of the Department of Science and Technology and National Research Foundation (NRF) of South Africa (Grant numbers: UID86895; 111862); the SAMRC with funds received from the South African National Department of Health; GlaxoSmithKline R&D (Africa Non-Communicable Disease Open Lab grant), the UK Medical Research Council and with funds from the UK Government's Newton Fund; as well as corporate social investment grants from Pfizer (SA), Boehringer Ingelheim (SA), Novartis (SA), the Medi Clinic Hospital Group (SA) and in kind contributions of Roche Diagnostics (SA). The study is also supported by the National Institute on Aging, NIH Intramural Research Program (USA). Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors, and therefore, the NRF does not accept any liability in regard.

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## Supplemental digital content

Supplementary Table 1: Multiple regression analyses in dippers

	<i>Retinal peak artery dilation (%)</i>					
	N=214					
	Salt model		MBG model		Salt & MBG model	
	Adj. R <sup>2</sup>		Adj. R <sup>2</sup>		Adj. R <sup>2</sup>	
	0.21		0.21		0.21	
	$\beta$ (S.E.)	<i>P</i>	$\beta$ (S.E.)	<i>P</i>	$\beta$ (S.E.)	<i>P</i>
MBG excretion (nmol/day)	N/A		0.020 (0.067)	0.77	0.070 (0.077)	0.37
Salt intake (g/day)	-0.064 (0.064)	0.32	N/A		-0.095 (0.073)	0.19
Sex (women/men)	0.133 (0.075)	0.076	0.112 (0.77)	0.15	0.115 (0.077)	0.14
Ethnicity (black/white)	<b>-0.446 (0.073)</b>	<b>&lt;0.001</b>	<b>-0.451 (0.074)</b>	<b>&lt;0.001</b>	<b>-0.461 (0.074)</b>	<b>&lt;0.001</b>
Age (years)	-0.019 (0.065)	0.77	-0.014 (0.066)	0.83	-0.009 (0.066)	0.89
WHtR	0.119 (0.077)	0.12	0.122 (0.076)	0.11	0.115 (0.077)	0.14
24hr SBP (mmHg)	0.072 (0.079)	0.36	0.069 (0.079)	0.38	0.073 (0.079)	0.36
Interleukin-6 (pg/ml)	0.004 (0.69)	0.95	-0.002 (0.069)	0.98	0.003 (0.06)	0.97
Cotinine (ng/ml)	-0.084 (0.066)	0.20	-0.070 (0.065)	0.28	-0.084 (0.066)	0.20
LDL-C (mmol/L)	0.039 (0.067)	0.56	0.043 (0.067)	0.52	0.041 (0.067)	0.54
Glucose (mmol/L)	-0.010 (0.071)	0.89	0.008 (0.071)	0.91	0.016 (0.071)	0.81


LDL-C, Low density lipoprotein cholesterol; MBG, Marinobufagenin; SBP, Systolic blood pressure; WHtR, Waist to height ratio

# Chapter 7

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Review article:

The Na<sup>+</sup>K<sup>+</sup>-ATPase inhibitor Marinobufagenin, and early cardiovascular risk in humans: A review of recent evidence

Current Hypertension Repots			
			
<b>Impact factor</b>	3.234		
<b>Publisher</b>	Springer US		
<b>Citation</b>	Strauss M, Smith W, Fedorova OV, Schutte AE. The Na <sup>+</sup> K <sup>+</sup> -ATPase inhibitor Marinobufagenin, and early cardiovascular risk in humans: A review of recent evidence. <i>Current Hypertension Reports</i> . 2019; 21: 38. DOI: 10.1007/s11906-019-0942-y.		
<b>Aims &amp; Scope</b>	<i>Current Hypertension Reports</i> provides in-depth review articles contributed by international experts on the most significant developments in the field. By presenting clear, insightful, balanced reviews that emphasize recently published papers of major importance, the journal elucidates current and emerging approaches to the diagnosis, treatment, management, and prevention of conditions associated with hypertension.		
Author Instructions			
<b>Font</b>	Normal plain font	<b>Spacing</b>	Double Spacing
<b>References</b>	± 100	<b>Words</b>	± 6000 excluding abstract and references
<b>Page numbers</b>	Make use of the autonomic page numbering tool.		
<b>Title page</b>	<ul style="list-style-type: none"> <li>• Full title</li> <li>• Names and degrees of each author should appear</li> <li>• Affiliations of all the authors</li> <li>• Corresponding author information</li> <li>• Auther ORCID IDs</li> </ul>		
<b>Abstract</b>	Structured abstract (150 words) – Purpose of review, Recent findings & Summary		
<b>Keywords</b>	Up to 6 keywords on title page		
<b>Text</b>	Relevant headings including clear Introduction and Conclusion sections		
<b>Acknowledgements</b>	Include acknowledgements on title page		
<b>Conflict of interest</b>	Include statement		

<b>Funding</b>	Include funding on title page
<b>References</b>	<ul style="list-style-type: none"> <li>• Number consecutively in the order in which they first appear in the text.</li> <li>• Assigned Arabic numerals in brackets, e.g. [17].</li> <li>• Bullet important (●) or very important (●●) recent references (<b>within past 3 years</b>).</li> </ul> <p><i>Articles in journals</i></p> <p>O'Neil S, Rose SL, Cheever AJ, Ballinger, JR, Smith CK, Barber RW, et al. Finding an optimal method for imagining lymphatic vessels. Eur J Nucl Med 2015;31:555-63. Doi:10.1007/s0059-003-1399-3.</p>
<b>Tables</b>	<ul style="list-style-type: none"> <li>• 2 Tables</li> </ul>
<b>Figures</b>	<ul style="list-style-type: none"> <li>• 1 Figure</li> <li>• Figure legends should accompany figure and be cited in text.</li> </ul>

*\*Formatting changes were made to maintain uniformity throughout this thesis, including text font, line spacing, margins, page numbers, tables and figures.*

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**The Na<sup>+</sup>K<sup>+</sup>-ATPase inhibitor Marinobufagenin, and early cardiovascular risk in humans: A review  
of recent evidence**

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**Sources of funding:** The South African Medical Research Council (SAMRC), the South African Research  
Chairs Initiative (SARChI) of the Department of Science and Technology and National Research Foundation  
(NRF) of South Africa (UID 86895 and 111862); Any opinion, findings, and conclusions or recommendations  
expressed in this material are those of the authors, and therefore, the NRF does not accept any liability in regard.  
This research was supported in part by the Intramural research Program of the NIH, National Institute on Aging,  
Baltimore, Maryland, USA.

**Key words:** early cardiovascular risk, humans, marinobufagenin, women, salt-sensitivity



**Abstract**

**Purpose of Review:** This review synthesizes recent findings in humans pertaining to the relationships between marinobufagenin (MBG), a steroidal Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibitor and salt-sensitivity biomarker, with early cardiovascular risk markers.

**Recent findings:** 24hr Urinary MBG strongly associates with habitual salt intake in young healthy adults (aged 20-30 years). Furthermore, in young healthy adults free of detected cardiovascular disease, MBG associates with increased large artery stiffness and left ventricular mass independent of blood pressure. These findings in human studies corroborate mechanistic data from rat studies whereby stimulation of MBG by a high salt intake or MBG infusion increased vascular fibrosis and cardiac hypertrophy.

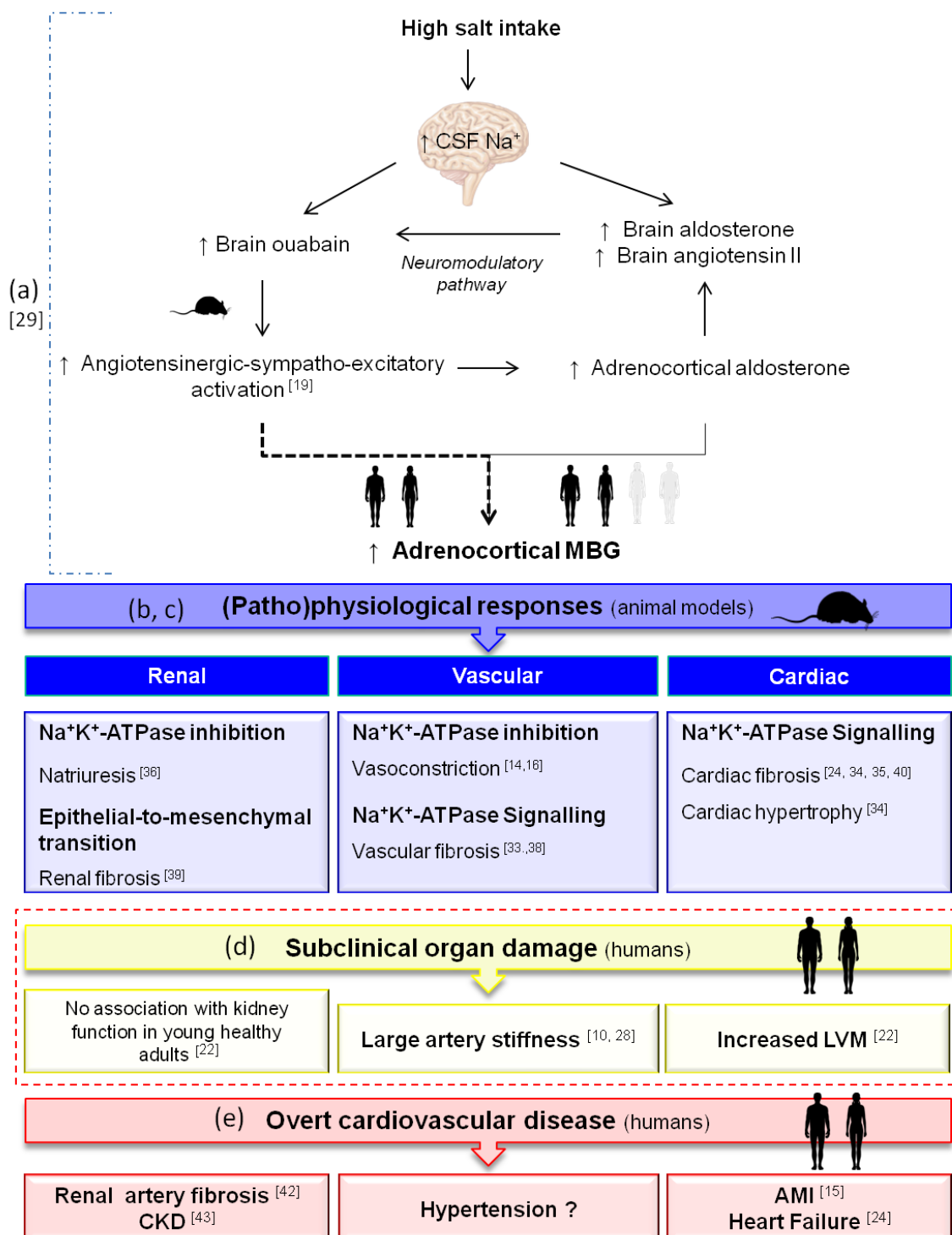
**Summary:** 24hr Urinary MBG may be a potential biomarker of early cardiovascular risk. Adverse associations between MBG –which increases with salt consumption- and early cardiovascular risk markers, support the global efforts to reduce population-wide salt intake in an effort to prevent and control the burden of non-communicable diseases.

## Background and introduction

There is currently little doubt that high salt intake significantly increases the risk for hypertension and cardiovascular disease [1]. At present, the global mean salt intake is twice the amount recommended by the World Health Organization (namely 10g salt/day [2] vs <5g salt/day) [3]. Thus, with 1.65 million cardiovascular related deaths in 2010 [4] and 2.3 million all-cause related deaths in 2016 [5] being attributed to a high salt diet, reducing excessive dietary salt intake remains a key priority. Global organizations including the World Health Organization [6], the United Nations [7, 8] and the Resolve to Save Lives initiative [9] have taken steps to commit to the initiation and implementation of sodium reduction strategies, in an effort to reduce the growing burden of non-communicable diseases.

In light of the aforementioned, the importance of understanding underlying mechanisms whereby salt intake increases cardiovascular risk is vital. This review specifically focuses on the cardiotonic steroid and Na/K-ATPase inhibitor, marinobufagenin (MBG), as a possible novel biomarker directly relating to salt intake and implicated in increased cardiovascular risk [10].

The purpose of this review is to highlight recent evidence from human studies that support previous animal studies demonstrating a link between elevated MBG and cardiovascular risk. As portrayed in **Figure 1**, the subsequent sections will review research on the physiological functions of MBG and how it relates to measures of cardiovascular risk in humans.



**Figure 1:** Summary of the evidence linking MBG to an increased cardiovascular risk. (a) Salt intake stimulates MBG synthesis and secretion via the angiotensinergic-sympatho-excitatory pathway. (b) Under normal physiological conditions MBG acts as a natriuretic hormone to stimulate natriuresis as a compensation for increased salt intake. (c) Excessive MBG production promotes pathophysiological responses including vasoconstriction, vascular, renal and cardiac fibrosis. (d) Evidence from human studies demonstrate that

elevated MBG associates with measures of subclinical target organ damage that may promote the development of cardiovascular disease. (e) Elevated MBG has already been observed in several overt cardiovascular diseases. AMI, Acute myocardial infarction; CKD, Chronic kidney disease; CSF, Cerebral spinal fluid; MBG, Marinobufagenin.

### **Endogenous marinobufagenin**

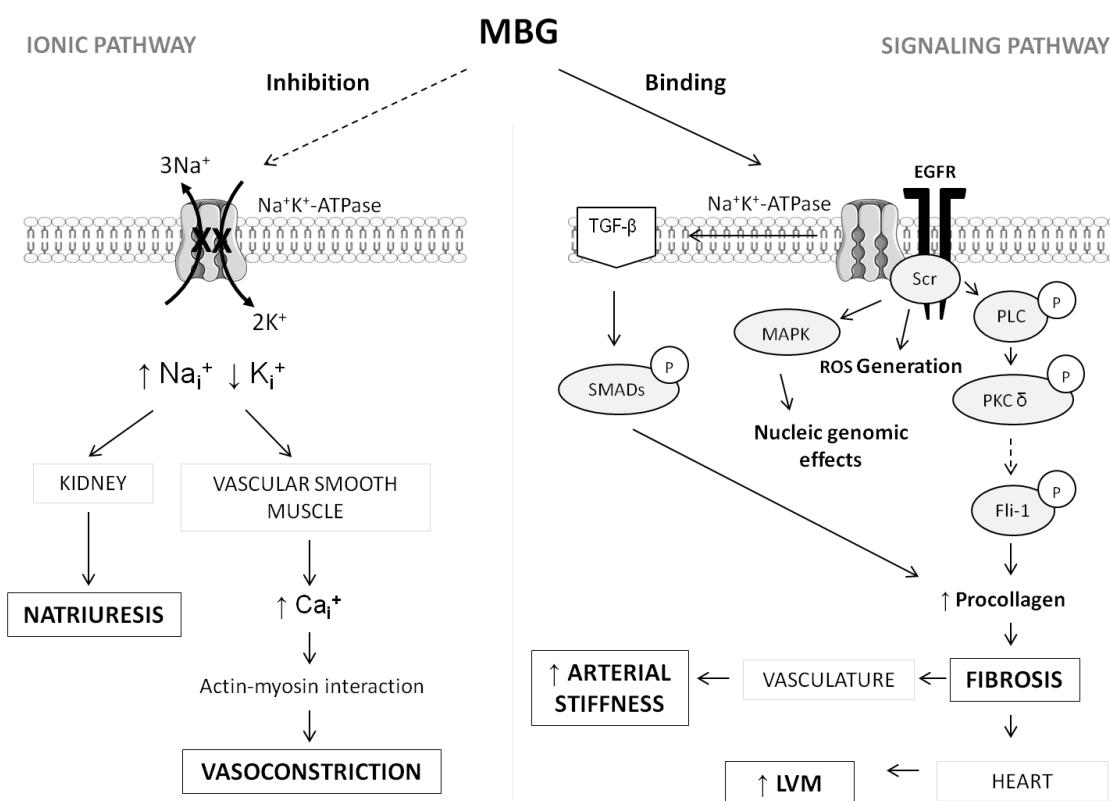
Bufadienolides were firstly recognized as playing a regulatory role in the salt acclimation of amphibians via its inhibitory function on skin  $\text{Na}^+/\text{K}^+$ -ATPase [11, 12]. Later it was found that one of the compounds of the venom of the *Bufo marinus* toad, MBG, is structurally and functionally similar to digitalis-like steroid from mammalian plasma and urine that inhibited the  $\text{Na}^+/\text{K}^+$ -ATPase and exhibited vasoconstrictive properties [13-16]. This digitalis-like steroid was identified as MBG [15, 16], and shown to be stimulated by sodium-induced volume loading [17]. Fedorova *et al.* have previously demonstrated MBG synthesis in adrenal cortical and placental cells under control of the bile acid CYP27A1 enzyme, but recognize that MBG synthesis may not be limited to these areas [18]. It was also demonstrated, that mammalian MBG production is stimulated by angiotensin II (ANGII) in the animal model of salt-sensitive hypertension and in the adrenocortical cell culture [19].

Mammalian MBG has since been extracted from the plasma and urine of humans [10, 20-24], and we have recently demonstrated the reliability of the non-invasive measurement of 24hr urinary MBG in the presence of other steroidal hormones, using a solid-phase Dissociation-Enhanced Lanthanide Fluorescent Immunoassay, based on a 4G4 anti-MBG mouse monoclonal antibody [10]. In the clinical study with the salt-loaded subjects [21], and in the animal model of salt-sensitive hypertension with an enhanced MBG production, urine MBG exceeded the concomitant changes in plasma MBG [25-27], though the MBG changes in both biological fluids exhibited a similar profile. Notably, one of the studies reported plasma MBG increases in the absence of MBG excretion changes on a high salt diet [20], while the other study demonstrated exclusively urine MBG changes in the presence of high salt intake [28]. This discrepancy may be due to the differences in the experimental designs (sequence of the dietary interventions, amount of sodium chloride in the diet, habitual style life prior to the study, etc.), and is discussed in detail in "Marinobufagenin and blood pressure" section.

Several studies have shown that elevated MBG associates with increased salt intake in animals [25, 26] and humans [10, 21, 28]. The suggested stimulatory pathway was described in detail by Fedorova *et al.* [19] Briefly, an increase in salt intake promotes increased angiotensin II, aldosterone and sympathetic activity, which in turn stimulates adrenocortical MBG synthesis and secretion [19]. In support, we have recently shown in a human cohort, that increased autonomic activity and aldosterone are associated with increased MBG excretion [29].

### Physiological function

In a review by Bagrov *et al.* the (patho)physiological interaction of MBG with the Na<sup>+</sup>/K<sup>+</sup>-ATPase pump was thoroughly described [30], clearly distinguishing between the two pathways through which MBG acts on the Na<sup>+</sup>/K<sup>+</sup>-ATPase pump [30] (**Figure 2**).



**Figure 2:** Mechanisms whereby MBG have been implicated increasing cardiovascular risk. Ca<sup>+</sup>, Calcium; Fli-1, Friend leukemia integration factor-1; K<sup>+</sup>, Potassium; LVM, Left ventricular mass; MBG, Marinobufagenin; MAPK, Mitogen activated protein kinase; Na<sup>+</sup>, Sodium; PKC, Protein kinase C; PLC, Phospholipase C; ROS, Reactive oxygen species; TGF- $\beta$ , Transforming growth factor beta; P, phosphorylated form of the protein.

The first, identified as the classic “ionic pathway”, involves the inhibition of membrane bound  $\text{Na}^+/\text{K}^+$ -ATPase and concurrently altered transmembrane ion transport. It is via the inhibition of the renal  $\text{Na}^+/\text{K}^+$ -ATPase pump that MBG was described to promote natriuresis as part of its normal physiological function in response to sodium-induced volume loading [25, 26]. This acts as a compensatory mechanism to lower blood pressure. However, MBG has also been shown to promote vasoconstriction via the inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase in the vascular smooth muscle cells [14, 16] - the proposed result of excessive MBG production. Vascular  $\text{Na}^+/\text{K}^+$ -ATPase inhibition increases intracellular sodium concentrations, and concurrently reverses the function of the vascular  $\text{Na}^+/\text{Ca}^{2+}$ -exchanger. This reversed functionality results in an influx of calcium ions into the vascular smooth muscle cells that further stimulates calcium-induced calcium release from the sarcoplasmic reticulum. The elevated intracellular calcium concentration increases the vascular actin-myosin interactions thereby promoting vasoconstriction (**Figure 2**) [30, 31].

In contrast, the second pathway namely the “signaling pathway”, involves the binding of MBG to the  $\text{Na}^+/\text{K}^+$ -ATPase, which activates several downstream signaling cascades [30]. These include the activation of mitogen activated protein kinases, reactive oxygen species and the promotion of fibrosis [30, 32]. It is via these signaling pathways through which MBG has been shown to promote vascular [32, 33] and cardiac fibrosis [34, 35], which will be described in more detail further on. The aforementioned, also demonstrated in **Figure 2**, indicates the mechanistic pathways through which excessive MBG production could promote cardiovascular disease, overriding the normal physiological function thereof.

### **Marinobufagenin and cardiovascular risk**

Investigations into the mechanistic pathways whereby MBG contributes to cardiovascular disease have been predominantly performed in rats. Increased plasma MBG, either brought about by salt loading or osmotic pump infusion, was shown to promote pressor responses [19, 25, 26, 36], microvascular alterations [37], vascular [33, 38], renal [39] and cardiac fibrosis [24, 34, 35, 40].

Indeed, human studies focusing on diseased populations have also observed elevated plasma levels of MBG in patients with primary aldosteronism [41], heart failure [24], renal artery stenosis [42] and chronic kidney disease [43, 44] and elevated urinary MBG in patients with acute myocardial infarction [15]. However, it remains unclear

whether elevated urinary levels of MBG, specifically 24hr MBG excretion – due to excessive dietary salt intake – in young healthy populations would already confer increased cardiovascular risk prior to the onset of disease. If so, MBG may be considered a biomarker of early cardiovascular risk.

We recently investigated in young healthy adults whether urinary MBG is associated with blood pressure and measures of early cardiovascular risk, including large artery stiffness [10], and increased left ventricular mass (LVM) [22] – thereby evaluating MBG's potential as a biomarker of early cardiovascular risk.

### *Marinobufagenin and blood pressure*

When taking into consideration the well-known relationship between salt, and especially salt-sensitivity, and blood pressure [45], it is not surprising that elevated MBG is associated with pressor responses [21, 23, 28] (**Table 1**). Indeed, MBG was shown to exhibit both natriuretic as well as vasoconstrictive properties via the inhibition of renal and vascular Na<sup>+</sup>/K<sup>+</sup>-ATPase [16, 26]. Although the relationship between MBG and blood pressure was originally largely confirmed in rats [19, 25, 26, 36, 46], recent investigations have been performed in humans [20, 21, 23, 28], with one study including young adults with clinic blood pressures <140/90 mmHg [23].

The first human study investigating the relationship between MBG and blood pressure included 28 normotensive white women (aged  $53 \pm 1.6$  years) who underwent 12 days of dietary sodium intervention (a 6 day low sodium diet of approximately 2.86 g/day, followed by a 6 day high sodium diet of 16.32 g salt per day day) [21]. They demonstrated a 35% increase in 24hr urinary MBG, from approximately 1.83 nmol/day to 2.45 nmol/day, when comparing sodium interventions (**Table 1**). Only during the high salt diet, did systolic blood pressure (SBP) inversely correlate with MBG excretion, possibly reflecting the natriuretic function of MBG in this cohort of the normotensive subjects [21]. This study was performed over a short time period that might reflect the short-term homeostatic mechanism whereby increased natriuresis may lower blood pressure as a protective mechanism to excessive salt intake in the healthy subjects. Nonetheless we also demonstrated non-significant inverse relationship between the MBG/Na<sup>+</sup> ratio (but not MBG) and SBP measures in young normotensive white women (aged  $25.6 \pm 2.78$  years) from the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT) [47], consuming approximately 6.68 grams of salt

per day (n=112) [23]. The MBG/Na<sup>+</sup> ratio was used as an indication of Na<sup>+</sup> excretion resistance to elevated levels of urinary MBG [23], and may be reflective of the natriuretic functionality of MBG.

In an intervention study performed by Jablonski *et al.* in 11 men and women, with SBP ranging from 130-159 mmHg and DBP <99 mmHg, they also found that MBG was significantly attenuated ( $2.04 \pm 0.16$  nmol/day) during 5 weeks of low salt intake (mean 3.75 g/day), compared to MBG levels ( $2.45 \pm 0.17$  nmol/day) during a high salt intake (mean 9 g/day). However, they found that MBG related positively with SBP only during the high salt diet intervention [28]. In contrast to the short term dietary intervention performed by Anderson *et al.* [21], the study performed by Jablonski and colleagues may be reflective of a long term homeostatic mechanisms where the natriuretic function of MBG to high salt intake may be overridden by the vasoconstrictive properties of MBG [28]. Indeed, the authors alluded to the vasoconstrictive characteristic of MBG as a possible explanation for the observed positive association between MBG and blood pressure in their cohort [28]. Additionally, possible kidney dysfunction in these prehypertensive and hypertensive participants may overbalance and diminish the natriuretic function of MBG, which would cause an additional stimulation of MBG production and will initiate and feed a vicious circle of salt-sensitivity.

We also demonstrated a significant positive association between the MBG/Na<sup>+</sup> ratio and central SBP in young black women (n=74) (aged  $24.3 \pm 3.64$  years), that was in contrast to our finding of a borderline negative association between MBG/Na<sup>+</sup> and SBP in white women from the African-PREDICT study [23]. There was no relationship between the MBG/Na<sup>+</sup> ratio and SBP in either black or white men [23].

In contrast to the abovementioned studies, Fedorova *et al.* found that while both men (n=20) and women (n=19) (aged  $53 \pm 11$  years) displayed increases in SBP with salt loading, neither plasma nor urinary MBG levels changed significantly [20]. In the total group however only plasma MBG was significantly lower during low compared to high salt intake. While men and women were included into the study based on no reported history of hypertension, it was evident that some of the participants did indeed have hypertension with the mean SBP being  $139 \pm 13.3$  mmHg and DBP  $86.3 \pm 7.4$  mmHg [20]. Participants from this study were firstly examined at baseline so to take into account their habitual salt diet, after which they participated in a double blind cross-over study. Participants were placed on a strict diet containing only 3g of salt per day, for the entire study period of



eight weeks. Additionally, participants received 6 g salt or placebo capsules that were randomly taken for two periods of four weeks each. Thus, each participant consumed a four week high salt diet (9g salt per day) and a four week low salt diet (3g salt per day). Mean baseline 24hr urinary MBG in men and women were 1.30 nmol/day and 1.06 nmol/day (on a habitual diet), and after four weeks of a high salt diet 1.19 nmol/day and 0.97 nmol/day, respectively [20] – noticeably lower compared to normotensive adults from another study [10]. 24hr Urinary MBG was 1.13 nmol/day and 0.98 nmol/day for men and women after 4 weeks of low sodium intervention. Notably, baseline 24hr urinary sodium as well as MBG was higher in comparison to urinary sodium and MBG after the high salt intervention. This may suggest that participants consumed a habitual high salt diet in excess of the high sodium intervention of 9g of salt per day. Fedorova *et al.* found that the change in the plasma MBG levels were related to the changes in SBP from a high to a low salt diet, although no relationships was evident between urinary MBG and SBP [20].

From the abovementioned studies it seems that the reported relationships between urinary MBG and blood pressure in humans are inconsistent. While animal studies have provided compelling evidence on the functionality of MBG and the effect to increase blood pressure, more evidence in human studies are needed. Importantly, the differences in the study designs, population characteristics and sample sizes of these studies cannot be overlooked when bearing in mind the discrepancies (**Table 1**). Still, some intriguing observations on the contrasting relationships between MBG and blood pressure in specific groups, including white and black women, support the potential divergent properties of MBG on blood pressure in humans.

Table 1: 24hr Urinary marinobufagenin in human cohorts without reported kidney or heart disease

	Population description Mean urinary MBG excretion	Population salt intake	Study Results (Observed associations with MBG)
Anderson <i>et al.</i> [21]	<b>Women (n=28):</b>		
	· 1.86 nmol/day	Low salt diet (2.86 g/day)	
	· 2.45 nmol/day	High salt diet (16.32 g/day)	MBG associated inversely with SBP - natriuretic response
Strauss <i>et al.</i> [10, 22, 23]	<i>The African-PREDICT study</i>		
	<b>White women (n=112):</b>		
	· 2.52 nmol/day [23]	Habitual salt intake (mean 6.68 g/day)	Trend of a negative association with SBP [23]
	<b>Black women (n=74):</b>		
	· 2.82 nmol/day [23]	Habitual salt intake (mean 6.65 g/day)	MBG associated positively with central SBP [23]
	<b>White men (n=77)</b>		
	· 4.69 nmol/day [23]	Habitual salt intake (mean 8.91 g/day)	No association with blood pressure [23]
	<b>Black men (n=68)</b>		
	· 3.99 nmol/day [23]	Habitual salt intake (mean 8.52 g/day)	No association with blood pressure [23]
	<b>Black and white women (n=415):</b>		
	· 2.69 nmol/day [10]	Habitual salt intake (mean 7.27 g/day)	MBG positively associated with arterial stiffness [10]
	<b>Black and white men (n=296):</b>		
	· 4.13 nmol/day [10]	Habitual salt intake (mean 8.32 g/day)	No association with large artery stiffness
	<b>Black and white men and women within the highest quartile of MBG excretion (n=179) from the total study population of n=711.</b>		
	· 6.92 nmol/day [22]	Habitual salt intake (mean 11.8 g/day) [22]	MBG associates positively with increased LVMi [22]

Jablonski <i>et al.</i> [28]	<b>Men and Women (n=11):</b>		
	· 2.04 nmol/day	Low salt diet (3.75 g/day)	
	· 2.45 nmol/day	Habitual salt diet (9 g/day)	MBG positively associated with SBP  MBG positively associated with arterial stiffness
Fedorova <i>et al.</i> [20]	<b>Men (n=20):</b>		
	· 1.30 nmol/day	Habitual high salt intake (mean > 9 g/day)	No relationships of urinary MBG and systolic blood pressure were evident during either condition.
	· 1.19 nmol/day	High salt diet (9 g/day)	
	· 1.13 nmol/day	Low salt diet (3 g/day)	
	<b>Women (n=19):</b>		
	· 1.06 nmol/day	Habitual high salt intake (mean ≥ 9 g/day)	No relationships of urinary MBG and systolic blood pressure were evident during either condition.
	· 0.97 nmol/day	High salt diet (9 g/day)	
	· 0.98 nmol/day	Low salt diet (3 g/day)	

LVMi; Left ventricular mass index; MBG, Marinobufagenin; SBP, Systolic blood pressure

### *Marinobufagenin and arterial stiffness*

Several studies have established large artery stiffness as a predictor of increased cardiovascular risk and mortality [48] in young [49], middle aged and older populations [50-53] beyond blood pressure. Salt intake was shown to be associated with arterial stiffness – not only in hypertensive [54, 55] but also young healthy adults [56]. Arterial stiffness measured as the pulse wave velocity (PWV) within the carotid to femoral (cf) section of the arterial tree is currently considered as the gold standard measurement of large artery stiffness [57]. The first human study investigating the relationship between arterial stiffness and MBG was performed by Jablonski *et al.*, and included 11 participants, namely men (n=8) and women (n=3), aged  $62 \pm 2$  years, with high or hypertensive blood pressures [28]. They demonstrated a positive correlation between cfPWV and MBG excretion [28]. In support, in young healthy women (aged  $24.8 \pm 3.08$  years; N=415) consuming a habitual high salt diet (mean 7.27g/day) we have recently found that cfPWV associated positively with MBG excretion, independent of salt

intake [10]. When performing sensitivity analyses for salt intake on the relationship between MBG and arterial stiffness, salt intake remained non-significant [10] – possibly implying that relationships observed between salt intake and arterial stiffness [54-56] may be via MBG as opposed to salt in itself. This relationship was also independent of mean arterial pressure [10]. Since arterial stiffness may precede the development of hypertension [58], this relationship in young normotensive healthy adults, and the possible blood pressure independent effect of MBG on large artery stiffness, is highlighted [10].

Although there are no human studies demonstrating mechanistic links between MBG and arterial stiffness, MBG was shown to promote vascular fibrosis in rat aortic explants [33] and increase in collagen production in the cultured rat vascular smooth muscle cells [32] - which indicate a pressure-independent effect of MBG on vascular fibrosis. Both Fedorova [33] and Elkareh *et al.* [35], have described the MBG-dependent signaling pathway in the promotion of collagen deposition - initiated by MBG binding to Na<sup>+</sup>K<sup>+</sup>ATPase [33, 35]. Findings from these studies indicated a significant down-regulation of transcription factor Friend leukemia integration-1 (Fli-1), in response to MBG, and concurrently increased collagen-1 synthesis [33, 35]. The scaffolding protein collagen reduces the arterial wall elasticity, thereby adversely influences large artery function [59]. In support, recently published findings from Grigorova *et al.* demonstrated that increased dietary sodium resulted in concurrent increased MBG excretion, aortic collagen expression and arterial stiffness via TGF- $\beta$  in normotensive rats [32]. Contrarily, sodium reduction and concurrent attenuation of MBG excretion resulted in decreased aortic collagen abundance and restored large artery elasticity [32]. If this applies to humans, it may further strengthen current strategies to reduce salt intake.

All together, the positive findings from the two studies investigating the relationship between MBG and arterial stiffness in humans support the role of MBG in the development of arterial stiffness. However, whether the exact mechanisms shown in rats, whereby MBG may promote arterial stiffness as a result of vascular fibrosis, holds true in humans, remains to be investigated.

### *Marinobufagenin and structural cardiac alterations*

Left ventricular mass (LVM) determined by echocardiography is a predictor of increased cardiovascular risk and mortality [60]. Findings from the Coronary Artery Risk Development in Young Adults study (CARDIA) indicated that 24hr urinary sodium associated positively with LVM in young adults ( $30.1 \pm 3.6$  years), although this relationship was confounded by obesity [61]. We therefore speculated that higher levels of MBG as a result of increased salt intake, would also be associated with increased LVM [22]. Indeed, our research group found a significant positive association between MBG and LVM index in young adults with excessively high MBG levels [22]. In accordance with international guidelines, the LVM index takes into account intra-individual body composition, and was normalized for body surface area [62]. The association between LVM index and MBG excretion was therefore independent of obesity. In addition, the latter relationship was also independent blood pressure, suggesting alternate mechanisms whereby MBG promotes cardiac hypertrophy [22]. Although there are no human studies investigating histological cardiac changes in response to MBG, others have demonstrated increased cardiac myocyte hypertrophy and fibrosis in response to MBG infusion in Sprague-Dawley rats. These observations were parallel with an increased cardiac mass in these animals [34, 35].

It is, therefore, likely that excessively high levels of MBG may cause corresponding histological changes in the cardiac tissue of humans – thereby increasing the cardiac mass. Although our findings suggest that the structural cardiac changes associated with elevated MBG may precede cardiac dysfunction at an early age, it is possible that cardiac functionality may be adversely altered at a later stage.

### **Marinobufagenin and ethnicity**

It is well known that black ethnicity is associated with increased salt-sensitivity and abnormal sodium handling [63-65]. It would therefore be important to investigate whether MBG – a marker of salt sensitivity – is elevated in black populations. Contradictory to expectations, Anderson *et al.* indicated that white adults ( $n=40$ ) had higher concentrations of 24hr urinary MBG (mean  $2.7 \pm 0.2$  pmol) compared to black adults ( $n=40$ ) (mean  $2.1 \pm 0.2$  pmol) who participated in the Baltimore Longitudinal Study on Aging [66]. A limitation of this study, however, was that the researchers did not report the salt intake of participants [66], which highly correlates with 24hr urinary

MBG [10]. It is therefore not possible to accurately interpret findings on the observed ethnic differences in 24hr urinary MBG concentrations.

We have also investigated whether there are ethnic differences in 24hr urinary MBG excretion between young healthy black and white adults from the African-PREDICT study while also reporting their estimated salt intake based on 24hr urinary sodium [23]. We found no significant difference in estimated salt intake or the 24hr urinary MBG excretion when comparing black and white men and black and white women [23]. Also, unexpectedly, no interaction of ethnicity was evident on the relationship of MBG with arterial stiffness [10] or left ventricular mass [22]. The absences of these interactions were unforeseen, especially with salt intake shown to be associated with large artery stiffness in black but not white adults [55, 56]. As previously described in detail, we did however observed a difference in the relationship of the MBG/Na<sup>+</sup> ratio and SBP between black and white women. While the MBG/Na<sup>+</sup> ratio associated positively with central SBP in black women, a tendency for a negative association was evident in white women [23]. Future studies may look at relationships between the MBG/Na<sup>+</sup> ratio and cardiovascular risk markers between ethnic groups, especially if the ratio is used as an indication of Na<sup>+</sup> excretion resistance to elevated urinary MBG [23] – taking into account the differential sodium handling between black and white populations [63].

These findings bring rise to the question with regards to salt-sensitivity, MBG-sensitivity and black ethnicity. Does salt-sensitivity associated with black ethnicity [64], automatically imply increased sensitivity to the cardiovascular effects of MBG? Our results suggest that while increased salt intake may increase cardiovascular risk in blacks, they may not at this young age be as susceptible to the adverse effects of elevated MBG.

Nonetheless, a phenomenon of increased autonomic activity during stress [67-69] and cardiovascular sensitivity to sympathetic outflow [70] as observed in black adults may at a later stage exaggerate MBG production resulting in excessive MBG levels to increase their cardiovascular risk at an older age. This suggestion is supported by our recent findings of increased autonomic activity being positively associated with MBG excretion only in black men and women, but not their white counterparts [29].

Still there is limited research on MBG in ethnic groups, and at this stage the young age and healthy status of the African-PREDICT participants may mask the influence of ethnicity on MBG levels. More in-depth research is needed to further investigate ethnic differences and the cardiovascular effects of MBG.

### **Marinobufagenin and sex**

While reports on the relationship between MBG and blood pressure have been inconsistent in different sex groups [20, 21, 23, 28], the relationship between MBG and cardiovascular risk factors including increased arterial stiffness and left ventricular mass seem more prominent in young women [10, 22]. We have previously suggested that women may likely be more sensitive to the cardiovascular effects of MBG, despite having lower salt intake and lower MBG levels than men [22]. In support of this suggestion, women have been shown to be more salt-sensitive compared to men when consuming similar amounts of salt [71-73], and exhibit greater increases in aldosterone levels, in response to ANGII infusion [73].

Importantly, the possible role of sex hormones cannot be disregarded. While there is no human study to our knowledge investigating the direct relationship between MBG and sex hormones, we have demonstrated the possible confounding effect thereof. While exploring the association between MBG and arterial stiffness in women we performed a sensitivity analyses for hormonal contraceptive use, and repeated subgroup analyses in women who made use of hormonal contraceptives (N=140) and those who did not (N=217) [10]. Our finding of a positive association between MBG and arterial stiffness remained significant only in women who did not make use of hormonal contraceptives [10]. These findings suggest an interaction between the steroidal hormone, MBG, and other sex hormones, which exhibit regular cyclic changes, that require further research.

Understanding the underlying mechanisms of MBG and salt-sensitive hypertension, and particularly the role of sex, is challenging since studies investigating the relationships and relevant mechanisms of MBG with salt sensitive hypertension [26], arterial stiffness [74], cardiac hypertrophy [34, 40, 75], cardiac [34, 40], vascular [33] and renal fibrosis [39] have all been performed in male rats except the studies on the model of preeclampsia [76, 77]. Therefore, none of these studies investigated or compared the mechanisms whereby MBG promotes cardiovascular dysfunction in female rats. In the one study including both male and female rats, the SBP, plasma MBG (and its regulatory enzyme CYP27A1), were significantly increased after 4 weeks of sodium loading in both

sexes [18]. However, consistent with reports of lower 24hr urinary MBG in women [10], female Dahl salt-sensitive rats had lower levels of plasma MBG and CYP27A1 mRNA expression at the baseline and after 4 weeks of a high salt diet compared to male rats, despite consuming similar amounts of salt [18].

Taking into consideration the abovementioned, it is unclear why the adverse relationship between MBG and early markers of cardiovascular risk is predominantly seen in women, despite their lower MBG. One possible mechanism includes the sensitization of the  $\alpha 1$ -Na<sup>+</sup>/K<sup>+</sup>-ATPase to MBG. Indeed, elevated levels of protein kinase C  $\beta 2$  expression have been found in female rats [78], previously shown to sensitize  $\alpha 1$ -Na<sup>+</sup>/K<sup>+</sup>-ATPase to MBG [27].

In view of the recent findings in women it would seem that women may be more sensitive to the cardiovascular effects of steroidal MBG compared to men. The female's childbearing function demands disparate requirements to salt handling compared to men, which may be one of the explanations of the above difference. Normal pregnancy is accompanied by plasma volume expansion involving retention of sodium ions and fluid [79-81], which concurrently increases the levels of MBG as a natriuretic factor to control the water/salt balance. It was found that in women with normal pregnancies plasma MBG increased up to 2-fold compared to non-pregnant age-matched controls [82] with a further dramatic elevation (up to 8-fold) in preeclampsia [82, 83]. In the rat model of preeclampsia, BP increase was achieved by addition of 1.8% NaCl to the drinking water [46, 77] or by a combination of high NaCl (0.9% in the water) and deoxycorticosterone acetate treatments for the duration of their pregnancy [76]. In rats, 24hr urinary MBG and BP were higher in pregnant and non-pregnant animals on a high salt intake in comparison to normal pregnancies and non-pregnant controls [46, 76, 77]. Similarly to the humans, even normal pregnancies exhibited significantly higher MBG levels than non-pregnant controls [46, 76, 77]. The exaggerated production of MBG in preeclampsia contributes to BP increase via direct vasoconstriction [46, 76, 77, 82-84], and to the pathologies associated with the Fli-1-dependent fibrotic changes in the umbilical arteries [83] and in placenta [85] (**Figure 2**). The latter would affect fetal blood supply and placentation. MBG impairs the proliferation, migration and invasion of the cultured first trimester human cytotrophoblast cells. This is done through the activation of Jnk, P38 and Src leading to augmented apoptosis [86, 87], which provides a mechanistic insight on the impaired placentation. Still, normal pregnancy is accompanied by an increase in MBG due to the association of normal pregnancy with salt and water retention [79-81]. It is possible that the sensitivity



of Na<sup>+</sup>/K<sup>+</sup>-ATPase to MBG inhibition in normal pregnancies predominantly promotes the normal physiological natriuretic function of MBG [77]. The rat model of preeclampsia is accompanied by increased salt intake, which indicates that the water/salt balance is vulnerable in pregnancy. This outlines the necessity of dietary salt control during pregnancy in order to ensure balanced functioning of the renal and cardiovascular systems. Still, there are no clear answers when it comes to the role of sex, especially female sex, on the functionality of MBG. Thus, the multifaceted role of MBG in non-pregnancy, pregnancy and preeclampsia merit future investigations.

### **Future directions and Conclusion**

24hr Urinary MBG may serve as a potential biomarker of early cardiovascular risk in young adults who consume a habitual high salt diet. This review highlights recent findings on the associations between MBG – which markedly increases with increased salt intake – and established cardiovascular risk factors in young healthy adults, including large artery stiffness and increased left ventricular mass. These important new findings on the potential harmful role of MBG in adults with no detected cardiovascular disease, add to a body of literature indicating elevated levels of MBG in older populations with reported pathology. These results also support mechanistic studies in rats demonstrating the pathophysiological mechanisms promoted by increased MBG, including vasoconstriction, vascular and cardiac fibrosis as demonstrated in **Figure 2**. Evidently, sodium reduction may be pivotal in reducing the cardiovascular risk associated with elevated MBG.

The most recent body of work investigating MBG and early cardiovascular risk in young healthy adults forms part of the African-PREDICT study [47]. The study enrolled young black and white men and women (20-30 years of age) with no prior history of cardiovascular disease, and who were screened to be healthy and clinic normotensive upon inclusion into the study. The African-PREDICT study is the first longitudinal study that will measure and track the MBG levels of healthy adults over a time period, providing a unique insight into the possible prognostic value of MBG [47].

Establishing MBG as an early biomarker of increased cardiovascular risk, furthermore, will support the efforts of several international legislations to lower salt intake of populations.

**Ethical considerations**

This manuscript does not contain patient data.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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# Chapter 8

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Final remarks and recommendations for future  
studies

## Introduction

Chapter 7 included a review article that summarised and compared the key findings from this PhD thesis with existing literature pertaining to the endogenous biomarker marinobufagenin (MBG). Therefore, this conclusive chapter addresses the aims, objectives and hypotheses for this thesis outlined in Chapter 1. Additionally, final recommendations and conclusions are made for future human studies investigating associations between 24hr urinary MBG excretion and markers of early cardiovascular risk.

## Aims, objectives and hypotheses

The central aim of this study was to evaluate the role of 24hr urinary MBG as a potential early marker in the development of cardiovascular disease. This was to be done by exploring the relationship of 24hr urinary MBG with established markers of early cardiovascular risk.

An overarching objective, encompassing all the study objectives, was to determine the interaction of sex and ethnicity on the relationship between 24hr urinary MBG and markers of early cardiovascular risk. Notably, we observed clear sex differences in these associations. It was apparent that women may be more sensitive to MBG, as positive associations found with increased sympathetic activity, large artery stiffness and left ventricular mass were not evident in men.

### 1. Marinobufagenin and large artery function

**Hypothesis 1:** *Large artery stiffness (cfPWV) associates positively with 24hr urinary MBG excretion.*

In research article one, Chapter 3, we demonstrated that large artery stiffness is positively and independently associated with 24hr urinary MBG excretion only in women. Taking into consideration our sex specific results I partially accept the abovementioned hypothesis as it is applicable to women, but not to men.

## 2. Marinobufagenin and its association with subclinical target organ damage

**Hypothesis 2:** *Positive associations exist between left ventricular mass index (LVMI), stroke volume index (SVI), cardiac output index (COi), left atrial to aortic root ratio (LA:Ao), ratio of mitral peak velocity of early filling (E) to early diastolic mitral annular velocity (e') (E:e') and 24hr urinary MBG excretion.*

**Hypothesis 3:** *A negative association exists between the mitral peak velocity of early filling (E) to late diastolic filling (A) ratio (E:A) and 24hr urinary MBG excretion.*

**Hypothesis 4:** *eGFR is negatively associated with 24hr urinary MBG excretion, whereas 24hr urinary albumin is positively associated with 24hr urinary MBG excretion.*

Research article two presented as Chapter 4, focused on the relationship of 24hr urinary MBG excretion with target organ damage - which included indices of cardiac structure and function, as well as renal function. Indeed, 24hr urinary MBG excretion positively associated with LVMI in those with the highest levels of MBG. I, therefore, partially accept the second hypothesis in relation to cardiac structure. No associations, however, were evident between 24hr urinary MBG excretion and indices of cardiac or renal function after multiple regression analyses. Thus, hypothesis two, pertaining to relationships between MBG and indices of cardiac function, as well as hypotheses three and four are rejected.

## 3. Autonomic activity, aldosterone and marinobufagenin

**Hypothesis 5:** *Aldosterone associates positively with 24hr urinary MBG excretion.*

**Hypothesis 6:** *Low frequency heart rate variability (an index of sympathetic tone, with a parasympathetic component<sup>1, 2</sup>) is positively related to 24hr urinary MBG excretion.*

**Hypothesis 7:** *High frequency heart rate variability (an index of parasympathetic tone, with a sympathetic component<sup>1, 2</sup>) is negatively associated with 24hr urinary MBG excretion.*

Based on our findings in Chapter 5, research article three, I accept hypothesis five and partially accept hypothesis six. As expected, aldosterone associated positively with MBG excretion in black and white, men and women. However, an index of sympathetic activity (low frequency heart rate variability) was only associated with elevated MBG excretion in women. In contrast, an unforeseen positive association was found between

parasympathetic activity (high frequency heart rate variability) and MBG excretion in men. Therefore, I reject hypothesis seven.

#### **4. Marinobufagenin and microvascular function in non-dipping adults**

**Hypothesis 8:** *24Hr urinary MBG excretion is negatively related to microvascular function (retinal peak artery dilation) in non-dipping adults, but not in adults with a normal nighttime blood pressure dipping status.*

For the first time, in research article four, we indicated a negative and blood pressure independent association between 24hr urinary MBG excretion and microvascular function in non-dippers, who are reportedly more salt-sensitive, possibly contributing to an increased cardiovascular risk in these individuals. This association was absent in adults exhibiting a normal circadian blood pressure pattern. The eighth hypothesis is therefore accepted.

#### **Strengths and limitations of this study**

The main limitation for this study was the use of cross-sectional data. Due to the cross-sectional nature of the study design, findings cannot be interpreted as the pathophysiological sequence of events, but merely reflect associations between markers of cardiovascular risk and MBG excretion. Nonetheless, our observations in an apparently healthy human cohort<sup>3, 4</sup> for the first time support findings from animal studies investigating mechanistic pathways through which MBG acts on the cardiovascular system.<sup>5-8</sup> Therefore, our results add to the limited knowledge with regard to the possible deleterious role of MBG in the development of cardiovascular disease in human cohorts. In future, the African-PREDICT study will also provide the first longitudinal data on 24hr urinary MBG in humans that could highlight the prognostic value thereof.<sup>9</sup> This data will be especially useful to determine whether 24hr urinary MBG would be related to diminished cardiac and renal function over time, as these associations were absent at the baseline phase of the study where participants are screened to be healthy.

Participants enrolled in the African-PREDICT study were recruited only from communities in the North-West province of South Africa. Therefore, our results might not portray the true demographic stance of the South African youth in entirety. Still, our study provides some of the first findings on associations of 24hr urinary MBG



excretion with early cardiovascular risk markers in apparently healthy adults. Moreover, the sample size of our cohort (N=711) far exceeded that of other human studies performed by Anderson *et al.* (N=28),<sup>10</sup> Bagrov *et al.* (N=12),<sup>11</sup> Fedorova *et al.* (N=39),<sup>12</sup> Jablonski *et al.* (N=11),<sup>13</sup> Kennedy *et al.* (N=245 heart failure patients and N=13 controls),<sup>14</sup> Piecha *et al.* (N=68 haemodialysis patients),<sup>15</sup> Tian *et al.* (N= 60 patients with hypertension or angina; N=49 patients with renal artery stenosis and N=26 healthy controls)<sup>16</sup> and Tomaschitz *et al.* (N=20 hypertensive and N=20 primary aldosteronism patients).<sup>17</sup>

Another limitation of this study was the calculation of estimated salt intake of participants based on a single 24hr urine sample, considering the intra-individual diurnal variation.<sup>18</sup> Nonetheless, the use of a single urine sample is accepted for the calculation of estimated salt intake in larger population based studies by the World Health Organization.<sup>19, 20</sup> Urine samples for the African-PREDICT study were obtain in accordance with guidelines from the Pan American Health Organization and World Health Organization.<sup>4, 19</sup>

Our study did not collect data on pregnancy history (e.g. preeclampsia), or the number of children women had that were included into our study. This additional data may have provided more detail on the observed associations between MBG and early cardiovascular risk markers predominantly observed in women.

### **Recommendations and perspectives for future studies**

- To date there are a limited amount of studies focussing on sex and MBG. However, based on the sex specific results from the first three research article in this study, we propose that more in-depth research be done with regard to sex hormones and the interaction thereof on the functioning of MBG. This includes the role of hormonal contraceptives. It would also be interesting to compare the associations of 24hr urinary MBG with early cardiovascular risk factors in pre and post menopausal women.
- Anderson *et al.*,<sup>10</sup> Fedorova *et al.*<sup>12</sup> and Jablonski *et al.*<sup>13</sup> have previously published findings on urinary MBG and blood pressure in response to salt intervention in hypertensive adults. However, it is possible that homeostatic mechanisms essential in sodium regulation may already be altered in these individuals. We suggest that sodium intervention be performed in young healthy men and women, in a controlled metabolic research unit where daytime and night-time urine samples are taken. With the majority of adults

already consuming a habitual high salt diet, comparative data on 24hr urinary MBG and its association with early cardiovascular risk factors after salt reduction would be informative.

- The longitudinal study design of the African-PREDICT study will allow researchers to monitor and follow up on the 24hr urinary MBG levels of individuals who were recruited before the implementation of the national sodium reduction legislation in 2016. Data on the changes in the urinary MBG levels of these adults from before (at baseline) and after the sodium legislation implementation (five-year follow-up) will provide valuable information on habitual sodium reduction and its relationship with urinary MBG.
- The investigation of relationships between additional components of the renin-angiotensin-aldosterone system and MBG in humans would provide additional data on the stimulatory pathway implicated in MBG secretion in response to salt intake.
- While there has been a lot of focus on investigating the relationship between MBG and sodium, there are few studies that have focused on MBG and potassium. Since sodium and potassium work hand in hand in many physiological systems, investigations into the relationship between MBG and potassium may provide new insights into the possible protective or harmful role of MBG in individuals who consume a low potassium diet.
- Future research may look at the relationships between MBG and other modifiable risk factors in humans including alcohol consumption, smoking, obesity and fasting glucose. Bagrov *et al.* have indicated that alcohol administration<sup>21</sup> and induced type 1 and type 2 diabetes mellitus<sup>22, 23</sup> in animals, significantly increase MBG excretion. It would, therefore, be of interest to see whether this holds true in humans as well.

## Conclusion

This study aimed to investigate the role of 24hr urinary MBG excretion as a potential early marker in the development of cardiovascular disease. This was done by exploring the relationship of 24hr urinary MBG excretion with established markers of early cardiovascular risk. Our study for the first time demonstrated relationships between autonomic activity and MBG in a human cohort, supporting proposed stimulatory pathways indicated in rats. In addition, our results confirmed previous observations from animal studies where we found that elevated 24hr urinary MBG excretion adversely associated with arterial stiffness and LVMI. Our findings in young healthy adults brings forth new evidence that support the need to provide more in-depth investigations into 24hr urinary MBG as a possible predictive marker of increased cardiovascular risk. Moreover, the positive association between 24hr urinary MBG and salt intake, also highlights the importance of implementing population wide sodium reduction strategies (**Appendix B**).

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# Appendix A

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Large artery stiffness is associated with salt intake in  
young healthy black but not white adults: The  
African-PREDICT study

**Citation:** Strauss M, Smith W, Kruger R, van der Westhuizen B, Schutte AE. Large artery stiffness is associated with salt intake in young healthy black but not white adults: The African-PREDICT study. *Eur J Nutr.* 2018; 57:2649-2656. The final publication is available at <https://doi.org/10.1007/s00394-018-1791-1>.

*Large artery stiffness is associated with salt intake in young healthy black but not white adults: the African-PREDICT study*

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**European Journal of Nutrition**

ISSN 1436-6207  
Volume 57  
Number 7

Eur J Nutr (2018) 57:2649-2656  
DOI 10.1007/s00394-018-1791-1



 Springer



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**Large artery stiffness is associated with salt intake in young healthy black but not white adults:  
the African-PREDICT study**

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**Acknowledgements:** The authors of this study are grateful towards all individuals participating voluntarily in the study. The dedication of the support and research staff as well as students at the Hypertension Research and Training Clinic at the North-West University is also duly acknowledged.

**Sources of funding:** The research funded in this manuscript is part of an ongoing research project financially supported by the South African Medical Research Council (SAMRC) with funds from National Treasury under its Economic Competitiveness and Support Package; the South African Research Chairs Initiative (SARChI) of the Department of Science and Technology and National Research Foundation (NRF) of South Africa; the Strategic Health Innovation Partnerships (SHIP) Unit of the SAMRC with funds received from the South African National Department of Health; GlaxoSmithKline R&D, the UK Medical Research Council and with funds from the UK Government's Newton Fund; as well as corporate social investment grants from Pfizer (SA), Boehringer Ingelheim (SA), Novartis (SA), the Medi Clinic Hospital Group (SA) and in kind contributions of Roche Diagnostics (SA). Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors, and therefore, the NRF does not accept any liability in regard.

**Abstract**

**Purpose:** There is global consensus on the benefits of reducing excessive salt intake. Indeed, lower salt intake associates with reduced arterial stiffness, a well established predictor of cardiovascular risk, in older populations. Whether high habitual salt intake in healthy normotensive youth may already contribute to increased arterial stiffness, is unknown. We therefore determined whether estimated salt intake is associated with large artery stiffness in young healthy black and white adults.

**Methods:** We included 693 black and white adults (51% black; 42% men), aged 20 – 30 years. Participants were normotensive based on clinic blood pressure, and no previous diagnosed chronic illnesses. We measured carotid femoral pulse wave velocity (cfPWV) and determined estimated salt intake based on 24hr urinary sodium excretion.

**Results:** We found estimated salt consumption of > 5 g/day in 47% of our population, whereas 21% consumed > 10 g/day. In multivariable-adjusted regression analyses a positive association existed between estimated salt intake and cfPWV in the total group (Adj.R<sup>2</sup>=0.32; std.  $\beta$ =0.10;  $p$ =0.007), and black adults (Adj.R<sup>2</sup>=0.37; std.  $\beta$ =0.12;  $p$ =0.029). This was independent of age, sex, mean arterial pressure and other covariates. No association was evident in white individuals ( $p$ =0.19).

**Conclusion:** Excessive salt intake is positively associated with large artery stiffness – independent of blood pressure – in young adults, especially in black individuals. Our results suggest a potential contributory role of salt consumption towards early vascular ageing.

**Key words:** Arterial stiffness, black, estimated salt intake, healthy, young

## Introduction

Salt intake, as a modifiable risk factor, has long been a focus point in cardiovascular epidemiology. Studies demonstrated discrepancies on the adverse cardiovascular effects of an excessively low[1] or high[2] sodium intake, sparking a great debate amongst healthcare professionals and scientists. Nonetheless, convincing evidence demonstrates a decrease in cardiovascular risk when reducing sodium intake in populations consuming high amounts of salt, as opposed to achieving a general low sodium intake in entire populations [3]. Therefore, the existing recommendations outlined by the World Health Organization of 5 grams of salt or 2 grams of sodium per day is widely accepted [4]. However, the reported global salt intake levels far exceed the latter [2,5]. Evidently in 2010, excessive sodium intake reportedly attributed to approximately 1.65 million cardiovascular disease related deaths [2].

The mechanism whereby salt intake promotes hypertension by means of volume overloading has been widely described [6]. However, recently more attention was placed on the effect of salt intake on arterial stiffness as a possible contributor [7]. While it was previously thought that arterial stiffness was promoted by increased pulsatile wall stress, as a result of elevated systolic blood pressure, the precursory role thereof has alternatively been proposed [8]. Indeed, arterial stiffness was shown to be an independent predictor of increased blood pressure and hypertension in young healthy adults [9]. Moreover, arterial stiffness was identified as an independent predictor of cardiovascular morbidity and all cause mortality [10]. A recent meta-analysis assessing the relationship between salt intake and arterial stiffness, based on data from 11 randomized controlled trials, concluded that sodium reduction was associated with a decrease in arterial stiffness. The majority of these studies however, were performed in older populations with reported pathology [7]. With high intake of salt already occurring from a young age, it is plausible that excessive salt may already contribute to arterial stiffening prior to hypertension development in the young. There are no studies, to the best of our knowledge, investigating the relationship between salt intake and arterial stiffness in young healthy adults.

This study therefore determined whether estimated salt intake is associated with large artery stiffness in a young black and white population. We will specifically investigate this relationship in black versus white adults, due to the known reports on salt-sensitivity in black populations [11,12]. Should our study indicate a relationship

between estimated salt intake and arterial stiffness, it lends further credence to the implementation of global sodium reduction strategies affecting the larger population.

## **Methods**

This study cohort included young normotensive black and white adults (aged 20-30 years) consecutively enrolled in the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT). The African-PREDICT study was approved by the Health Research Ethics Committee of the North-West University (NWU-00001-12-A1), and is registered at Clinical Trails.gov (Nr. NCT03292094).

### *Participant recruitment*

Community members living in close proximity to the Potchefstroom area, in the North West province of South Africa, were invited to participate in the initial health screening phase of the African-PREDICT study. Eligible volunteers, who underwent screening based on the inclusion criteria, were invited back for further measurements. The inclusion criteria was defined as being normotensive based on office blood pressure (<140/90 mmHg) with no previously diagnosed chronic illness (self-reported). Participants who made use of medication for hypertension or other chronic diseases were excluded. In addition, we included participants that were non-diabetic; HIV uninfected and had microalbuminuria < 30 mg/ml. None of the women who were enrolled in the study were pregnant or lactating.

Of the initial 1886 participants recruited for eligibility screening (between 2013 and 2017), 1262 fulfilled the inclusion criteria and gave consent for inclusion in the African-PREDICT study. Main reasons for exclusion included: incorrect age (n=36), not being of black or white ethnicity (N=22), increased blood pressure (N=47), chronic disease and/or made use of chronic medication (N=52), pregnant or lactating (N=9), and other e.g. refused HIV testing, were not South African residents, or withdrew from the study (N=458). From the 1262 participants who took part in the baseline data collection, only 1202 participants' data were ultimately included into the African-PREDICT database (an ultimate inclusion rate of 64%). Participant exclusion after baseline measurement included: incomplete 24hr blood pressure monitoring (N=9), detected chronic illness (N=15), no biological sampling possible (N=13), withdrawal (N=7) and other reasons (N=16). For the present study, we

included data of the first 693 consecutively enrolled participants for whom 24hr urinary biochemical analyses has been performed at the time of this study, and who had complete 24hr urinary data.

#### *Organizational procedures*

Participants arrived at the Hypertension Research Clinic on the North-West University campus at 08:00 where they were familiarized with the research environment. All organizational procedures were then thoroughly explained, and participants who voluntarily proceeded with the study gave written informed consent. All procedures abided by the Declaration of Helsinki and institutional guidelines.

#### *Questionnaire and anthropometric data*

Each participant completed a detailed General Health and Demographic Questionnaire pertaining to information on demographics and lifestyle habits. Questionnaires included data on ethnicity, sex, age, socio-economic status (SES), family history, self-reported alcohol use and smoking.

Body height (m) (SECA 213 Portable Stadiometer) (SECA, Hamburg, Germany), weight (kg) (SECA 813 Electronic Scales) and waist circumference (cm) (Lufkin Steel Anthropometric Tape; W606PM; Lufkin, Apex, USA) were measured in triplicate, in accordance to International Society for the Advancement of Kinanthropometry guidelines [13]. We subsequently calculated the body mass index (weight (kg)/height (m<sup>2</sup>)) and waist-to-height ratio (WHtR) (waist circumference (cm)/height (m)).

#### *Arterial stiffness*

We measured carotid-femoral pulse wave velocity (cfPWV) using the Sphygmocor® XCEL device (AtCor Medical Pty. Ltd., Sydney, Australia). The carotid and femoral artery waveforms were captured simultaneously with participants resting in the supine position, by means of carotid artery applanation tonometry and a femoral cuff. The cfPWV travel distance was calculated as 80% of the distance measured between the carotid pulse point and femoral cuff. Measurements were performed in duplicate and repeated if the cfPWV differed more than 3 m.s<sup>-1</sup> [14].

We additionally determined supine brachial systolic (SBP) and diastolic blood pressure (DBP), and calculated the mean arterial pressure (MAP) accordingly ( $bDBP + 1/3(bSBP - bDBP)$ ).

#### *Ambulatory blood pressure*

We measured 24hr ambulatory blood pressure using the validated CardioXplore apparatus (Meditech, Budapest, Hungary, British Hypertension Society). An appropriately sized brachial blood pressure cuff was fitted to the non-dominant arm of each participant. Blood pressure measurements were recorded over 30 minute intervals during the day (06:00-22:00), and hourly at night (22:00-06:00). Data for 24hr blood pressure was considered successful when (a) at least 70% of the total 24hr blood pressure readings being valid; or (b) there were at least 20 valid daytime and seven nighttime measurements [15].

#### *Biological sampling and biochemical analyses*

Participants were requested to fast at least 8 hours prior to early morning biological sampling which included blood and spot urine collection. Participants also received instructions on the protocol for 24hr urine collection, starting on the morning of participation, where they discarded the first passed urine of the day. All subsequent urinary voids were collected in a container provided by the clinic. 24hr urine samples were considered complete if the total urinary volume  $\geq 300\text{mL}$  [16].

We measured 24hr urinary potassium and sodium using the Cobas Integra 400plus (Roche, Basel Switzerland). The device was also used to analyze serum high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol, triglycerides, C-reactive protein (CRP),  $\gamma$ -glutamyltransferase (GGT) and glucose. Serum cotinine was determined using the chemiluminescence method on the Immulite (Siemens, Erlangen, Germany).

Estimated salt intake was calculated using the following equation:

$$\text{Estimate NaCl (g/day)} = \frac{(24\text{hr Urinary Na (mmol/L)} * \text{Urinary volume (L)}) * 58.44}{1000}$$

### Statistical Analyses

We performed all statistical analyses using Statistica 13.3 (Dell Inc., Tulsa, Oklahoma, USA). Data was reported as the arithmetic mean  $\pm$  standard deviation when following a normal distribution. Non-Gaussian distributed data was logarithmically transformed and presented as the geometric mean (5<sup>th</sup> and 95<sup>th</sup> percentile intervals). We performed independent t-tests to compare ethnic differences in the anthropometric, cardiovascular, 24 urinary sodium and potassium, biochemical and lifestyle profiles of black and white participants. Chi-square tests were performed for categorical data (SES, smoking, alcohol consumption). Analyses of covariance were done in the total group and within black and white ethnic groups, to determine significant differences in cfPWV across increasing quartiles of estimated salt intake, adjusting for age, WHtR and MAP. We performed single, partial and multiple regression analyses in each group to explore the relationship between cfPWV and estimated salt intake. Several covariates were considered for inclusion as possible confounders. Sex, age, WHtR, MAP, HDL-C, CRP, GGT, glucose and cotinine were ultimately included into multiple regression models based on the strongest bivariate associations with estimated salt intake and cfPWV. We additionally adjusted for ethnicity in the total group.

### Results

We performed interaction testing for sex but found no interaction ( $p=0.47$ ) on the relationship between cfPWV and estimated salt intake. This was confirmed when testing for main effects of ethnicity\*estimated salt intake on cfPWV ( $p=0.71$ ).

The general characteristics of this young bi-ethnic population (mean age  $24.8 \pm 3.01$  years) are presented in **Table 1**. Notable there was a difference in the SES of black and white participants ( $p<0.001$ ), with 58% of black participants from a low SES compared to 18% of white participants. With regards to cardiovascular differences, white adults had higher 24hr SBP ( $p=0.009$ ) and cfPWV ( $p=0.006$ ) compared to their black counterparts. Although 24hr urinary volume output and estimated salt intake was similar for both ethnicities, 24hr urinary potassium excretion was significantly lower in black adults ( $p<0.001$ ). Alarming, within this population 47% of participants consumed  $> 5$  -10 grams salt/day, 21% between  $< 10$ -15 grams salt/day and 12% consumed more than 15 grams salt/day.

Table 1: Basic characteristics

	Total N=693	Black N=350	White N=343	Black vs White P
Sex, Men, N (%)	291 (42.0)	140 (40.0)	151 (44.0)	0.28
Age (years)	24.8 ± 3.01	24.5 ± 3.12	25.2 ± 2.85	0.001
Socio economic status, N (%)				<0.001
<i>Low</i>	262 (37.8)	202 (57.7)	60 (17.5)	
<i>Middle</i>	170 (24.5)	88 (25.2)	82 (23.9)	
<i>High</i>	261 (37.7)	60 (17.1)	201 (58.6)	
<b>Anthropometric measurements</b>				
Height (m)	1.68 ± 0.09	1.64 ± 0.08	1.72 ± 0.08	<0.001
Weight (kg)	72.4 ± 17.7	68.0 ± 15.7	76.8 ± 18.6	<0.001
BMI (kg.m <sup>-2</sup> )	25.6 ± 5.84	25.4 ± 6.18	25.7 ± 5.47	0.51
Waist to height ratio	0.48 ± 0.08	0.48 ± 0.08	0.48 ± 0.08	0.60
<b>Cardiovascular profile</b>				
24hr SBP (mmHg)	116 ± 9.32	115 ± 8.73	117 ± 9.81	0.009
24hr DBP (mmHg)	68.8 ± 5.63	68.7 ± 5.52	68.9 ± 5.73	0.61
Clinic supine MAP (mmHg)	90.2 ± 7.98	92.4 ± 7.68	87.9 ± 7.65	<0.001
CfPWV (m.s <sup>-1</sup> )*	6.71 ± 0.81	6.19 ± 0.88	6.37 ± 0.88	0.006
<b>24hr Urinary profile</b>				
Volume (L/24hr)	1.38 ± 0.73	1.37 ± 0.70	1.39 ± 0.07	0.67
Na <sup>+</sup> exc. (mmol/day)	129 (42.8; 331)	134 (42.3; 361)	124 (43.6; 275)	0.096
Salt intake (g/day)	7.64 (2.53; 19.5)	7.92 (2.49; 21.3)	7.35 (2.57; 16.2)	0.096
K <sup>+</sup> exc. (mmol/day)	40.1 (14.3; 101)	35.4 (12.7; 103)	45.4 (17.8; 101)	<0.001
Na <sup>+</sup> :K <sup>+</sup> ratio	3.30 (1.38; 6.89)	3.92 (1.87; 7.59)	2.76 (1.15; 6.03)	<0.001
<b>Biochemical profile</b>				
Glucose (mmol/L)	4.72 ± 0.72	4.45 ± 0.82	4.95 ± 0.51	<0.001
HDL-C (mmol/L)	1.25 (0.79; 2.02)	1.21 (0.79; 1.87)	1.29 (0.79; 2.24)	0.005
LDL-C (mmol/L)	2.67 (1.53; 4.36)	2.38 (1.30; 3.93)	2.97 (1.83; 4.71)	<0.001



LDL:HDL ratio	2.11 (0.98;4.34)	1.94 (0.89; 3.89)	2.29 (1.08; 4.71)	<0.001
CRP (mg/L)	1.06 (0.11; 10.5)	1.18 (0.12; 12.3)	0.95 (0.10; 8.58)	0.047
<b>Lifestyle measures</b>				
Smoking, N (%)	153 (22.1)	77 (22.1)	76 (22.2)	0.98
Cotinine >10 (ng/ml)	137 (24.4)	67 (25.5)	70 (23.4)	0.57
Alcohol intake, N (%)	379 (55.3)	181 (26.4)	198 (57.7)	0.19
GGT (U/L)	21.1 (8.80; 59.7)	24.3 (10.5; 67.7)	18.3 (7.80; 52.7)	<0.001

Arithmetic mean  $\pm$  standard deviation; geometric mean (5<sup>th</sup> percentile; 95<sup>th</sup> percentile intervals).

\*Adjusted for MAP

cfPWV, Carotid femoral pulse wave velocity; CRP, C-reactive protein; DBP, Diastolic blood pressure; GGT,  $\gamma$ -glutamyl transferase; HDL-C, High density lipoprotein cholesterol; K<sup>+</sup>, Potassium; MAP, Mean arterial pressure; Na<sup>+</sup>, Sodium; SBP, Systolic blood pressure.

### Regression analyses

In single regression analyses we found a positive correlation between estimated salt intake and cfPWV in the total group ( $r=0.08$ ;  $p=0.023$ ) and the white group ( $r=0.12$ ;  $p=0.027$ ) (**Table 2**). After partial adjustments for age, WHtR and MAP we found that cfPWV was greater across increasing quartiles of estimated salt intake in the total ( $p=0.014$ ) and black groups ( $p=0.035$ ) (**Table 2, Figure 1**). Forward stepwise multiple regression analyses (**Table 3**) confirmed this positive association between estimated salt intake and cfPWV in the total group (Adj.R<sup>2</sup>=0.32; std.  $\beta=0.10$ ;  $p=0.007$ ), supported by the positive association particularly in the black group (Adj.R<sup>2</sup>=0.37; std.  $\beta=0.12$ ;  $p=0.029$ ). In white participants, estimated salt intake did not enter the forward stepwise multiple regression model significantly. Due to the lower potassium levels in black adults we additionally performed Pearson and partial analyses between the Na<sup>+</sup>:K<sup>+</sup> ratio and cfPWV. We found no correlation between the Na<sup>+</sup>:K<sup>+</sup> ratio and cfPWV in black or white adults, before ( $r=0.06$ ;  $p=0.31$ ) ( $r=-0.02$ ;  $p=0.70$ ) or after adjustments( $r=0.05$ ;  $p=0.34$ ) ( $r=-0.03$ ;  $p=0.54$ ).

**Table 2: Pearson and partial correlations**

	<b>Salt intake (g/day)</b>		
	<i>Total group</i> N=693	<i>Black</i> N=350	<i>White</i> N=343
Age (years)	$r=-0.02$ ; $p=0.62$	$r=-0.07$ ; $p=0.21$	$r=0.06$ ; $p=0.25$
WHtR	<b><math>r=0.10</math>; <math>p=0.008</math></b>	<b><math>r=0.11</math>; <math>p=0.046</math></b>	$r=0.09$ ; $p=0.091$
cfPWV (m.s <sup>-1</sup> )*	<b><math>r=0.08</math>; <math>p=0.023</math></b>	$r=0.07$ ; $p=0.20$	<b><math>r=0.12</math>; <math>p=0.027</math></b>
<i>Adjusted for sex, age and waist to height ratio</i>			
cfPWV (m.s <sup>-1</sup> )*	<b><math>r=0.08</math>; <math>p=0.036</math></b>	<b><math>r=0.12</math>; <math>p=0.033</math></b>	$r=0.05$ ; $p=0.32$

\*Adjusted for mean arterial pressure. Bold values indicate  $p<0.05$ .

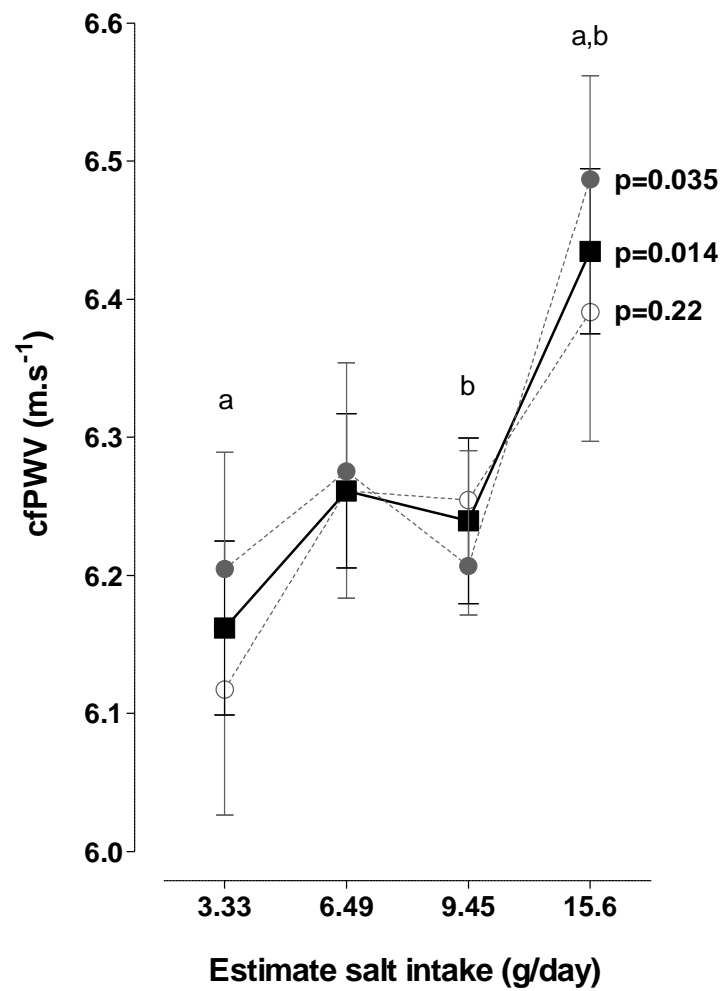
A partial regression analysis in the total group was additionally adjusted for ethnicity.

**Table 3: Multiple regression analyses with cfPWV as dependant variable and Salt intake as main independent variable**

	<b>Pulse Wave Velocity (m.s<sup>-1</sup>)</b>					
	<b>Total (N=538)</b>		<b>Black (N=250)</b>		<b>White (N=288)</b>	
	<b><math>\beta</math> (SE)</b>	<b>P</b>	<b><math>\beta</math> (SE)</b>	<b>P</b>	<b><math>\beta</math> (SE)</b>	<b>P</b>
Adj. R <sup>2</sup>	0.32		0.37		0.28	
Salt (g/day)	0.099 (0.037)	0.007	0.115 (0.052)	0.029	0.246 (0.188)	0.19
MAP (mmHg)	0.382 (0.039)	<0.001	0.396 (0.053)	<0.001	0.353 (0.055)	<0.001
Sex (women/men)	0.267 (0.039)	<0.001	0.220 (0.059)	<0.001	0.242 (0.061)	<0.001
Age (years)	0.198 (0.037)	<0.001	0.234 (0.053)	<0.001	0.192 (0.052)	<0.001
WHtR	-0.0180 (0.038)	<0.001	-0.254 (0.059)	<0.001	-0.154 (0.059)	0.010
Cotinine (ng/ml)	0.092 (0.037)	0.014	0.099 (0.054)	0.070	—	—
Ethnicity(black/white)	0.066 (0.037)	0.079	—	—	—	—
HDL-C (mmol/L)	—	—	—	—	-0.138 (0.062)	0.038

Covariates included age, sex, ethnicity, WHtR, MAP, estimated salt intake, HDL-C, CRP, GGT, glucose, cotinine

CRP, C-reactive protein; GGT,  $\gamma$ -glutamyl transferase; HDL-C, High density lipoprotein cholesterol; MAP, Mean arterial pressure



**Fig 1** PWV across increasing quartiles of estimated salt intake in the total group (■), black (●) and white (○) participants

<sup>a,b</sup> Interquartile differences in the total group ( $p < 0.05$ )

Adjusted for age, waist-to-height ratio and MAP

## Discussion

In this study we found a positive and independent association between estimated salt intake and large artery stiffness in a young healthy population on a habitual high salt diet. This finding was particularly apparent in the young black participants.

It is widely acknowledged that black populations tend to be more salt-sensitive, possibly due to a genetic predisposition [11,12,17], and demonstrate early vascular aging [18-20]. Our results suggest that black individuals might also be more susceptible to the adverse effect of excessive salt intake on large artery stiffness at an early age. To our knowledge there are few studies reporting the relationship between salt intake and arterial stiffness, especially in a young healthy bi-ethnic adult population.

The first long term observational study conducted in 1986 by Avolio *et al.* compared the arterial stiffness of two small urban Australian population groups, matched for age and blood pressure [21]. Individuals following the low salt diet did so for a period ranging between 5 months to 8 years. Notably, large artery stiffness of individuals consuming a low salt diet was 21.8% lower in adults between the ages of 29-44 (N=26), and 22.7% lower in adults between the ages of 45-66 (N=15) compared to those consuming a habitual diet high salt diet [21]. The average salt intake of those following a low salt diet was 41 mmol/24hr and 22 mmol/24hr (translating to 2.4 grams and 1.3 grams salt per day), respectively, determined from the mean molar Na/K ratio using spot urine samples. Although this study did not measure sodium excretion of control subjects on an habitual diet, they reported that the estimated sodium excretion of the Australian population ranged between 130 to 200 mmol/24hr (translating to 7.6 - 11.7 grams salt per day) [21]. Notable limitations of the study published by Avolio *et al.* were the methods used to determine estimated salt intake, namely, sodium questionnaires and morning spot urine tests. In addition, participants in this study were also classified as being normotensive with blood pressure being <160/95 mmHg.

The abovementioned observation of an increased PWV with higher sodium intake over time was supported in a more recently published study by Jung *et al.*, where baPWV and 24hr urinary sodium excretion of participants were measured 3 times over an eight year period [22]. They reported a positive association between high sodium intake and brachial ankle PWV, in Korean adults (older than 40 years). Once again a limitation of this long term

observational study was the use of questionnaire data as opposed to 24hr urinary data to determine estimated salt intake [22].

Although short term intervention studies have demonstrated similar results to ours, the population size was smaller and the participants were notably older compared to our study population (aged 20-30). In support of our results in black individuals, reduced salt intake was reported to attenuate cfPWV in a group of older hypertensive black adults (N=69; mean age  $50 \pm 9$ ) but not their white counterparts (N=71; mean age  $52 \pm 12$ ) after 6 weeks of sodium intervention [23]. Similarly, Todd *et al.* found no relationship between salt intake and arterial stiffness in healthy middle-aged white adults after a 4 week sodium intervention (N=23; mean age 43.7 years) [24]. This lack of an association in white adults is in agreement with the findings of our study, where we found no relationship between estimated salt intake and arterial stiffness in young white adults, despite 29% consuming >10 grams of salt per day.

Contrary to the aforementioned study by He *et al.*, Redelinguys *et al.* reported no association between estimated salt intake and arterial stiffness in black adults (N= 552; mean age  $45.1 \pm 18.4$  years) [25] in their cross-sectional study. It is worth mentioning that the 24hr urinary sodium excretion of their population (105 mmol/day) was markedly lower compared to the black participants from our study (134 mmol/day), as well as that from the study performed by He *et al.* (132 mmol/day). In addition, an important limitation and potential confounder of their study was the inclusion of participants who made use of antihypertensive medication, including diuretics (approximately 21.3% of the total population) [25]. Other cross sectional studies in Portuguese (N=426) [26] and Chinese (N=341) [27] adult cohorts have reported positive associations between estimated salt intake and arterial stiffness. Still some important differences should be highlighted. While the study performed by Polonia *et al.* included 82 young normotensive adults (mean age  $22 \pm 3$  years), all analyses were performed in the total group which included 245 hypertensive adults (mean age  $49 \pm 18$ ), including 38 individuals with familial history of stroke (mean age  $60 \pm 20$ ) [26]. Also, Sun *et al.* included 341 older hypertensive Chinese adults in their study.

Taking into consideration all of the above, our study firstly reports this ethnic difference in the manner in which salt associates with arterial stiffness in young healthy black and white adults. Although speculative, one

mechanism that could be considered for our results in black adults, might include the activation of the extracellular matrix protein metalloproteinase-9 (MMP-9), which is associated with an increase in sodium intake, estimated by three self-reported instruments [28], as well as arterial stiffness [29]. Indeed MMP-9 levels were shown to be elevated in black individuals [30]. MMP activity degrades the elastic scaffolding protein elastin within the arterial wall, thereby compromising the extracellular matrix composition, promoting arterial stiffness [29,31]. Intriguingly, in the white adults of our study, lipids may play a more prominent role in contributing to increased arterial stiffness. Notably we observed a significant negative association between arterial stiffness and HDL-C in white adults only. These apparent ethnic differences suggest that alternate contributing factors may play a role in increasing arterial stiffness in black and white adults early in life.

### ***Strengths and limitations***

This is the first study performed in a young healthy bi-ethnic population, allowing us to investigate the relationship between salt intake and large artery stiffness in both black and white adults at an early age prior the onset of cardiovascular disease. The sample size of our study (N=693) also exceeded most previous studies evaluating this relationship, namely Avolio *et al.* (N=57) [21], Todd *et al.* (N=23) [24], He *et al.* (N=169) [23], Redelinghuys *et al.* (N=552) [25] and that of the entire population sample accounted for in the meta-analyses performed by D'Elia (N=431) [7]. Limitations of our study include that participants were not randomly selected for inclusion into the study. We additionally acknowledge that the use of repeated 24hr collection is recommended to determine estimated salt intake, in order to account for day to day variability. Although the cross-sectional design of our study prohibits us from discussing causality, the positive association between a habitual high salt diet and arterial stiffness in this population can be meaningfully interpreted. Moreover, with African-PREDICT being a longitudinal study, future studies reporting on the follow-up data will allow us to further establish the harmful role of salt on the progression of arterial stiffness, especially according to black and white ethnic groups.

In conclusion, excessive salt intake is positively and independently associated with arterial stiffness, particularly in young black adults, early in life. Our findings suggest that excessive salt intake may promote early vascular aging and accordingly, our findings endorse the implementation of South Africa's sodium reduction legislation

(Proclamation No. R. 214, 2013) as a precursory measure to reduce the risk of early cardiovascular disease development.

### **Perspectives**

Our study indicated for the first time a positive and independent association between estimated salt intake and large artery stiffness in a young healthy population consuming a habitual high salt diet. Our findings were particularly apparent in the young black adults. This paper highlights the possible harmful implications of a habitual high salt diet, on large artery function, possibly increasing the risk for future cardiovascular disease. Furthermore, we demonstrate ethnic differences in the possible effect of excessive salt intake on large artery stiffness. Although is important to interpret the results of this cross-sectional study within context, our results support the general consensus of several experts to reduce the salt intake of populations consuming excessive amounts of salt.

### **Ethical Standards**

All organizational procedures were then thoroughly explained, and participants who voluntarily proceeded with the study gave written informed consent. All procedures abided by the Declaration of Helsinki and institutional guidelines.

### **Conflicts of interest**

The authors declare that they have no conflict of interest.

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# Appendix B

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Salt is bad for you: but how it affects your body is  
still frontier science

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# THE CONVERSATION

**Citation:** Schutte AE, Strauss M. Salt is bad for you: but how it affects your body is still frontier science. *The Conversation*. 11 March 2019. <https://theconversation.com/salt-is-bad-for-you-but-how-it-affects-your-body-is-still-frontier-science-112895>.

## **Salt is bad for you: but how it affects your body is still frontier science**

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## **Funding**

Alta Schutte receives funding from the South African Medical Research Council. She is also a SARChI Research Chair funded by the Department of Science and Technology. She is the President of the International Society of Hypertension.

Michél Strauss receives funding from the NRF in collaboration with the German Academic Exchange Service (DAAD). The NRF/DAAD In-country Doctoral Scholarship (UID 111862)

**Key words:** Public health; heart disease; Hypertension; salt; South Africa; high blood pressure; salt intake; sodium

Research has [shown](#) that excess salt intake is harmful to people's health. It can lead to high blood pressure and increase the risk of heart disease and stroke.

Globally salt reduction programmes are gaining [traction](#), whether through awareness campaigns or through governmental interventions.

This has been true in South Africa too. Three years ago it became the first country to implement mandatory [salt targets](#) for staple foods such as bread and soups. This is in line with World Health Organisation (WHO) [recommendations](#) to reduce salt intake by 30% by 2025. In South Africa a [further reduction](#) in sodium targets is set to come into effect later this year.

The approach South Africa took was to target the non-discretionary intake of salt – that's salt already added as an ingredient to food. It's view was that this would be the most cost effective approach to prevent hypertension – a key driver of heart disease and stroke.

Research [estimates](#) that reducing the amount of salt people eat could prevent an estimated 23,000 cardiovascular diseases and 5,600 deaths every year in South Africa. And that the new laws to reduce salt intake could save the country [US\\$ 51.25 million](#) on health care for cardiovascular diseases.

It's still early days on whether the new laws are having the desired effect on health – that will take a few more years to feed through. But the policy is certainly working in reducing salt in [staple](#) foods such as bread.

As part of a [WHO study on global ageing](#) we reviewed South Africans's salt intake before the new laws were implemented, and are now repeating this exercise to determine whether salt intake has reduced.

South Africa has also made concerted efforts to promote [public awareness](#) on excessive salt intake and cardiovascular health. Research [suggests](#) that this has been effective in changing people's behaviour such as adding salt to food when cooking and at the table during meals.

But, in the medium to longer term, will these interventions produce the health outcomes the government has forecast? The key indicators would be lower blood pressure, fewer cardiovascular events such as hearts attacks and strokes.

The answer lies partly in policies being adjusted to take into account new scientific findings about how salt affects the body. This is a frontier that's being explored by scientists around the world. It's also the subject of [our research](#).

Our research challenge some assumptions that have been held for decades about how salt affects the body. Our findings – together with those of other international researchers – suggest that the mechanisms surrounding salt and cardiovascular health may be more complex than originally thought. This in turns suggests that there's significant scope to fine tune policies to improve prevention and treatment of common disorder like hypertension.

We highlight, for example, that salt reduction may significantly reduce the harmful effects of hormones associated with high salt intake. Blood pressure, heart structure and blood vessels can all be affected. This provides further evidence of the important of policies that target salt intake.

### **What we know now**

Compelling [evidence](#) over many years has strongly linked high [salt intake](#) with raised blood pressure and cardiovascular events like heart attacks. But emerging research has started to raise questions about the physiological mechanisms for the link between salt intake and raised blood pressure.

A common [understanding](#) – prevalent in medical text books for decades – has been that a high salt intake results in thirst. The higher water intake consequently leads to increased blood volume which raises blood pressure, and ultimately both water and salt are excreted by the kidneys and blood pressure is maintained.

But German researcher, Jens Titze, recently found salt to be stored in the [skin](#). Researchers have further shown that a high salt intake is accompanied by [minimal water loss](#). These surprising findings were met with scepticism by the global health sciences community, but underscores that a much better understanding of blood pressure mechanisms is required.

US investigators, Alexei Bagrov and Olga Fedorova, identified another [player](#) in how salt affects cardiovascular health. [Marinobufagenin](#) is a steroid hormone that has similar properties to active substances found in the venom of the [Bufo marinus toad](#). The hormone's function is to [manage salt balance](#) and is thus produced in response to high salt intake.

But very high levels of the steroid hormone, in response to excessive salt intake, resulted in [increased blood pressure](#), affected heart structure and [increased](#) the stiffness of the blood vessel walls in animals.

We [recently](#) set out to test this for the first time in healthy young humans. We [confirmed](#) a strong positive [association](#) between increased salt intake and an increase in the steroid hormone.

We [found](#) that high salt intake was associated with the stiffness of the aorta, even in very young people. We then [tested](#) whether this was due to the steroid hormone or salt itself. When we included both in our statistics, we found that the culprit was the steroid hormone, and not necessarily salt.

Not only was the steroid hormone associated with [aortic stiffness](#), but also with increased [blood pressure](#), and [left ventricular mass](#) in young healthy adults who consumed a mean of 11.8 grams (more than two teaspoons) of salt a day. The WHO [recommends](#) an intake of less than 5 grams (one teaspoon) of salt per day.

## **Conclusion**

Our findings support South Africa's second phase implementation of reducing salt in staple foods to further lower the daily intake of salt.

The evidence also strongly supports the importance of continued public awareness campaigns to reduce excessive salt intake to protect cardiovascular health.



# Appendix C

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Health Research Ethics Committee approval of the

African-PREDICT study



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2012/07/31

**ETHICS APPROVAL OF PROJECT**

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

<b>Project title :</b> African Prospective study for the Early Detection and Identification of Cardiovascular disease and hyperTension. (African-PREDICT study)																															
<b>Project Leader:</b> Prof A Schutte																															
<b>Ethics number:</b>	<table border="1"> <tr> <td>N</td><td>W</td><td>U</td><td>-</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>-</td><td>1</td><td>2</td><td>-</td><td>A</td><td>1</td> </tr> <tr> <td colspan="3">Institution</td> <td colspan="5">Project Number</td> <td colspan="2">Year</td> <td colspan="5">Status</td> </tr> </table>	N	W	U	-	0	0	0	0	1	-	1	2	-	A	1	Institution			Project Number					Year		Status				
N	W	U	-	0	0	0	0	1	-	1	2	-	A	1																	
Institution			Project Number					Year		Status																					
Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation																															
<b>Approval date:</b> 2012/04/12							<b>Expiry date:</b> 2017/04/11																								

Special conditions of the approval (if any): None

**General conditions:**

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

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

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

Yours sincerely

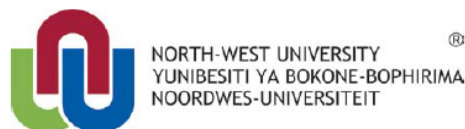
Prof Amanda Lourens  
(chair NWU Ethics Committee)

# Appendix D

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Health Research Ethics Committee approval for this

PhD study



Private Bag X6001, Potchefstroom,  
South Africa, 2520

Tel: (018) 299-4900  
Faks: (018) 299-4910  
Web: <http://www.nwu.ac.za>

**Institutional Research Ethics Regulatory Committee**

Tel: +27 18 299 4849  
Email : [Ethics@nwu.ac.za](mailto:Ethics@nwu.ac.za)

## ETHICS APPROVAL CERTIFICATE OF STUDY

Based on approval by Health Research Ethics Committee (HREC) on 02/05/2017 after being reviewed at the meeting held on 19/04/2017, the North-West University Institutional Research Ethics Regulatory Committee (NWU-IRERC) hereby approves your study as indicated below. This implies that the NWU-IRERC grants its permission that provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

<b>Study title:</b> Marinobufagenin and markers of cardiovascular risk in a young black and white population: The African-PREDICT study														
<b>Study Leader/Supervisor:</b> Prof AE Schutte														
<b>Student:</b> M Strauss														
<b>Ethics number:</b>														
N	W	U	-	0	0	0	2	7	-	1	7	-	A	1
Institution			Study Number					Year		Status				
Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation														
<b>Application Type:</b> Single study														
<b>Commencement date:</b> 2017-05-02														
<b>Risk:</b> <span style="border: 1px solid black; padding: 2px;">Minimal</span>														
<b>Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation.</b>														

### Special conditions of the approval (if applicable):

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

### General conditions:

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

- The study leader (principle investigator) must report in the prescribed format to the NWU-IRERC via HREC:
  - annually (or as otherwise requested) on the monitoring of the study, and upon completion of the study
  - without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.
- Annually a number of studies may be randomly selected for an external audit.
- The approval applies strictly to the proposal as stipulated in the application form. Would any changes to the proposal be deemed necessary during the course of the study, the study leader must apply for approval of these amendments at the HREC, prior to implementation. Would there be deviated from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.
- The date of approval indicates the first date that the study may be started.
- In the interest of ethical responsibility the NWU-IRERC and HREC retains the right to:
  - request access to any information or data at any time during the course or after completion of the study;
  - to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.
  - withdraw or postpone approval if:
    - any unethical principles or practices of the study are revealed or suspected,
    - it becomes apparent that any relevant information was withheld from the HREC or that information has been false or misrepresented,
    - the required amendments, annual (or otherwise stipulated) report and reporting of adverse events or incidents was not done in a timely manner and accurately,
    - new institutional rules, national legislation or international conventions deem it necessary.
- HREC can be contacted for further information or any report templates via [Ethics-HRECAppl@nwu.ac.za](mailto:Ethics-HRECAppl@nwu.ac.za) or 018 299 1206.

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

Yours sincerely

Prof LA  
Du Plessis

Digitally signed by  
Prof LA Du Plessis  
Date: 2017.05.11  
11:13:04 +02'00'

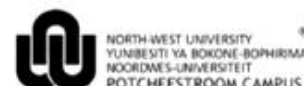
Prof Linda du Plessis  
Chair NWU Institutional Research Ethics Regulatory Committee (IRERC)

# Appendix E

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African-PREDICT study informed consent form

Informed Consent Form – Research Version Aug 2016



### INFORMED CONSENT FORM FOR THE African-PREDICT STUDY (RESEARCH PHASE):

**TITLE OF THE RESEARCH PROJECT:** African Prospective study on the Early Detection and Identification of Cardiovascular disease and HyperTension (African-PREDICT)

**ETHICS REFERENCE NUMBER:** NWU-00001-12-A1

**PRINCIPAL INVESTIGATOR:** Prof. Alta Schutte (PhD Physiology)

*Prof. Schutte and the research team have the expertise and interest in Cardiovascular Physiology, namely to understand the biological processes in humans when high blood pressure and heart disease develop.*

**ADDRESS:** NORTH-WEST UNIVERSITY (Potchefstroom Campus); Hypertension in Africa Research Team (HART); Hypertension Research and Training Clinic Building F11, Office 101.

**CONTACT NUMBERS:** 018 299 2444 / 018 285 2466 / 018 299 2780

You are invited to take part in the African-PREDICT research study. Please take some time to read the information presented here, which will explain the details of this study. Please ask the researcher or person explaining the research to you any questions about any part of this study that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research is about and how you might be involved. Also, your participation is **entirely voluntary** and you are free to say no to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part now.

This study has been approved by the Health Research Ethics Committee of the Faculty of Health Sciences of the North-West University (NWU-00001-12-A1) and will be conducted according to the ethical guidelines and principles of Ethics in Health Research: Principles, Processes and Structures (DoH, 2015) and other international ethical guidelines applicable to this study. It might be necessary for the research ethics committee members or other relevant people to inspect the research records.



#### What is this research study all about?

You will know already from taking part in the screening phase of the study that heart disease and especially high blood pressure (or hypertension) is a big problem in South Africa. Also, many people are unaware of it, as it has no symptoms. High blood pressure is a very important risk factor which may result in heart disease, kidney disease and stroke. *(When blood stops flowing to the heart, this can cause a heart attack and part of the heart dies. A stroke is when there is a problem with the blood supply to the brain and a part of the brain is damaged.)* That is why many people in South Africa suffer from these diseases resulting in death.

Since heart disease is mostly seen in older people, the purpose of this study is to include and focus on young healthy people to understand how high blood pressure and heart disease develop. It is believed that our lifestyle (e.g. what we eat, drink, and do) may have an impact on whether we will develop high blood pressure and heart disease. Also, it is not well known whether there are perhaps certain measurements (e.g. in your blood or urine) that may predict whether you will develop heart disease when you are older.

The aim of this study is therefore to determine how high blood pressure and heart disease develop in a group of 1200 healthy young South Africans living in and around Potchefstroom, by tracking everyone over 5-20 years. It is therefore of great importance that we take detailed measurements of your lifestyle, and your current health (e.g. heart, blood vessels, eyes, blood and urine). These measurements will be made at the beginning of the study, but it will be most important to repeat these measurements in following visits every 5 years, to see how these health measurements have changed. We expect that some participants will remain healthy with normal blood pressures, and other will develop high blood pressure. Only by tracking the changes in blood pressures and other detailed measurements will be able to understand the influences of e.g. lifestyle on changes in blood pressure.

If your results show that a certain measurement predicts that high blood pressure will develop later in life, this information could help doctors and nurses to prevent more people in the local community having strokes and heart attacks in the future.



#### Why have you been invited to participate?

Your screening tests show that you are healthy and suitable to take part in this study. You are also in the most important age group of 20 to 30 years. As we would like to follow you over time it is ideal that you have indicated that you intend to stay in or around or visit Potchefstroom for the next 5 years at least.

It will also be very important for us to be able to keep in touch with you. We kindly ask that you **tell us immediately about any changes of your contact details** (address, telephone number, email address etc.).

Once we have performed all of the measurements as described below, we will have a much better understanding of your health status. If we are not able to obtain important measurements (such as 24-hour blood pressure measurements, or if we are unable to obtain a blood sample, or if we detect a serious health abnormality), you will most likely not be able to take further part in the research project. Once we have completed the measurements, we will discuss your results with you and the way forward.



#### What will be expected of you?

The research team will make an appointment with you, and if necessary, transport will be provided to bring you to the Hypertension Clinic (Building F12) on the Potchefstroom Campus of the North-West University. Such an appointment will be made for early in the morning, as the measurements will start at approximately 08:00, and in total will take about 5 hours to complete.

To make sure that your results are valid and useful, it is important to take note of the following:

1. **The evening before – Do not eat or drink anything except water after 10pm** or before you come to the clinic in the morning.
2. **On the study day, please wear comfortable clothing** such as trousers and a top that can be easily removed for the tests (please avoid wearing skirts, dresses or tights as we will need to access your bare foot and put a blood pressure cuff around your thigh over your trousers).
3. **Please bring with you:**
  - All medication you currently are taking
  - Your ID document & clinic card/book

## Informed Consent Form – Research Version Aug 2016

- Some good quality sunglasses to protect your eyes after the measurements
4. Let us know if transport should be arranged for you.

If you are happy to participate, we will ask you to sign this consent form stating that you are volunteering to participate in this study and that you understand all the procedures that will be performed. You are free to contact us with any questions should there be any uncertainty about any of the information provided. Then we will take the measures listed in the table below. Tests will be done in the Hypertension Clinic and we will provide you with a meal during the day. You will not be able to bath or shower for 24 hours after your clinic appointment due to the equipment you will be wearing when you leave the clinic.

**WHAT TESTS WILL BE DONE?**

- **Body composition:** we will measure your height, weight, waist, hip and neck circumference in a private room, while you are wearing your underwear. In another room, while you are clothed and lying down on a bed, we will also measure your body fat percentage by using a device that connects with sensors on your hand and on your foot. This is a completely painless procedure. (the measurements should take about 20 minutes to complete)
- **Biological samples:** early in the morning while you are lying down on a bed, a research nurse will take a blood sample from a vein in your arm by using standard clinical procedures.(10-20 min) We will also ask you to provide a urine sample in the morning, in a private restroom. At the end of the day, we will kindly request that you collect your urine over the next 24 hours (we will give you the containers and detailed instructions for this). These urine and blood samples will be used to test for genetic and a detailed range of biochemical markers (biomarkers) related to high blood pressure, heart disease and diabetes, such as glucose, cholesterol and markers of inflammation. You are more likely to have high blood pressure if one of your parents or a close family member has high blood pressure. This is because high blood pressure can be caused by differences in our genes. Our genes are like a very complicated "manual" in each of our cells that tells the body how to work properly. When there are changes in the genes, it changes the "manual" and the body then does not work as well as it should for example causing high blood pressure. We share our genes with our family because half of the gene "manual" comes from your mother and half from your father. Therefore, if they have high blood pressure due to differences in their genes then it is likely that you will get the same changes in your genes and develop high blood pressure. We would like to find out what these differences are in order to better understand how they cause high blood pressure so that we can find ways to stop it happening.  
Take note that some of your samples may be stored for many years in freezers before we will analyse the samples. We may also need to ship some of your to other local or international expert laboratories for analyses.
- **Blood pressure:** while you are sitting down in a private room, we will measure blood pressure twice on both arms, by placing a cuff around your upper arm. (20 min) Another blood pressure measurement will also be done by placing a small blood pressure cuff around your finger, and upper arm, while you are lying on a bed. We will then test your blood pressure responses when you do a colour word reading test and when you place your hand in cold water for 1 minute. (30 min) At the end of the measurement day, we will fit a portable blood pressure monitor to you which will assess your blood pressure over the next 24 hours, thus over a day and when you are sleeping at night. It is important that the device is not removed during this time to ensure a reliable measurement.
- **Blood vessel & heart health:** in a private room we will again ask you to lie down comfortably on a bed. We will first test your blood pressure at your upper arm, with a device that will also measure the blood pressure at your heart. We will then test how stiff your blood vessels are by using a small pen-like device rested on your neck to register the pulse in your neck on a computer. At the same time another blood pressure cuff will be placed around your thigh. (15 min) Afterwards, in a



semi-dark room we will use a sonar device (usually used during pregnancy) to take some sonar pictures and video clips of the blood vessels in your neck and of your heart on the bare chest. We will provide a blanket or gown for cover. (20 min)

- **ECG (Electrocardiography test) for heart health:** while you are lying down on a bed in a private room, we will test the natural electrical activity of your heart by placing several stickers with sensors on your chest. We will take care to ensure your privacy. (10 min)
- **Eye Pressure:** a research nurse will put some eye drops in both eyes and then she will measure the pressure in your eyes with a device that rests lightly on your eye. (10 min) This test will inform us whether you have a condition called glaucoma, which means that the pressure within your eyes are quite high. If so, we will advise you and refer you for necessary treatment. If the pressure is normal, we will continue with the next eye test as described below.
- **Testing the small vessels of the eye:** a research nurse will put an eye drop in one eye, and a researcher will ask you to look into a special camera, named a fundoscope. This is the same device used by ophthalmologists (eye doctors). This camera will shine a light into your eye and we will take some pictures of the small blood vessels at the back of your eye (there will be a camera-like flash). We will also check how well your small blood vessels respond to light flickering, by doing a light-flicker test with this special camera. (20-30 min)
- **Physical activity:** at the end of the measurement day, a researcher will place a small monitor on your chest that will record your activity and movement levels for 7 days. No pain or discomfort is associated with this device, and you are kindly requested not to remove the device before the 7 day measurements were completed.
- **HIV test:** As this test was done during the screening phase, we will not test again for HIV. However, with each follow-up visit every 5 years, we would like to perform this test again.
- **Questionnaires:** during the course of the morning, you will be asked to complete several questionnaires with the help of a researcher. These include a general health questionnaire (with questions about your age, family history of disease, education, occupation, lifestyle habits, 15 min), Berlin sleep questionnaire (asking questions about how well you sleep, 5 min), physical activity questionnaire (to report on how active your lifestyle is, 5 min), dietary questionnaire (with the help of a dietician you will be asked what you ate during the past day (30 min). Within the next week the dietician will contact you again on two occasions to complete the questionnaire again. This should give us the best reflection on your eating habits). Finally, a trained psychologist will help you to complete a number of questionnaires on your personal well-being (including questions on stress and how well you cope with stress, 30-45 min).



#### Will you gain anything from taking part in this research?

- You will receive direct feedback during each advanced measurement on your health status. All of these advanced clinic tests are provided to you at no cost (worth ±R3 000).
- Should any abnormalities be detected, we will refer you to doctors, clinics or hospitals for further tests or treatment and the test results may assist your doctor in making decisions about further treatment.
- Apart from this personal benefit, your research data will help biomedical health researchers to gain a better understanding on how high blood pressure and heart disease develops, and may help us to develop better programmes to prevent or treat these diseases in our community and elsewhere. The data may also be used to advise the Ministry of Health on changes to the health system that may benefit the broader South Africa.



#### Are there risks involved in you taking part in this research and what will be done to prevent them?

To help you with a better understanding of the potential risks, and what we are doing to prevent these, please refer to the table below:

## Informed Consent Form – Research Version Aug 2016

Risks	Precautions
<ul style="list-style-type: none"> <li>• Taking a blood sample at a vein in the upper arm, may cause some pain and discomfort;</li> <li>• Applying an eye drop may cause a slight burning sensation;</li> <li>• Performing the eye pressure test is slightly uncomfortable;</li> <li>• Performing a light flicker test may also be slightly uncomfortable.</li> <li>• After the eye measurement some discomfort may be experienced (similar to a visit to an eye doctor) while waiting for the pupil to constrict.</li> <li>• Placing the hand in an ice water bucket for 1 minute may cause some pain in your hand.</li> <li>• You may experience some discomfort when having to undress for the body measurements or heart sonar measurements.</li>   <li>• When you complete the psychological questionnaires you may feel uncomfortable when giving personal information, such as feeling depressed or stressed.</li> <li>• All health measurements may cause some anxiety when you are worried about the results of the tests.</li> <li>• If a health abnormality is identified, others may become aware of this private information, e.g. diabetes.</li> </ul>	<ul style="list-style-type: none"> <li>• A trained registered research nurse perform all blood sampling and regularly undergo training on clinical measurements.</li>   <li>• She also performs the eye pressure test and apply the eye drop. To ensure correct procedures and minimum participant discomfort she has undergone training at an eye doctor to ensure that she use the safest techniques to make the measurement quickly and correctly. The light flicker test may cause discomfort but the researcher is highly experienced and ensures that the measurement is done quickly and accurately. It does not cause any long term harm and is comparable to standard eye doctor measures. Afterwards, when the pupil is dilated, the eye is sensitive to light. Therefore an eye patch is provided and all lights of the clinic turned off when these assessments start (at the end of the day's measurements). You are also encouraged to bring sunglasses for when you leave the clinic. We also provide transport to you after we are finished as you are not encouraged to drive if your eye has not yet returned to normal.</li> <li>• Placing the hand in ice water causes some pain due to the very cold water. The time is only for 1 minute to reduce discomfort to a minimum, and a small electric blanket or hot water bottle is provided afterwards to heat up the hand and ensure comfort.</li> <li>• All measurements are done in private temperature controlled rooms. For sensitive measurements a female scientist is trained to perform measurements to ensure especially comfort of female participants. All staff are also trained in these aspects to be highly professional and discreet and to ensure maximum comfort and to avoid any embarrassment. For heart sonars, an expert clinical technologist has vast experience in performing the sonars in a semi-dark room and also provides a blanket should you require this.</li> <li>• For psychological questionnaires a psychologist is well trained to complete the questionnaires in a private area. All necessary aspects are adhered to to make sure it is done in a professional and comfortable manner. If any abnormality is detected, the psychologist informs the research nurse, who will then privately discuss the results with you.</li> <li>• For other health measurements, such blood pressure, the results may be stressful. We will therefore provide you with the information privately and if we note something abnormal, we will ensure that you are referred appropriately for further tests or treatment.</li> <li>• If any health abnormalities was identified, you will meet individually with the research nurse in a private room for a feedback session. She will explain your results to you and provide you with a letter of referral for further testing or treatment. This will also be placed in a</li> </ul>

## Informed Consent Form – Research Version Aug 2016

<ul style="list-style-type: none"> <li>As measurements take place during the working week you may suffer from a loss of income, or may get into trouble for not being at work due to time spent in the project.</li> </ul>	<p>sealed envelope.</p> <ul style="list-style-type: none"> <li>If you will lose wages due to your participation in the study, you need to inform the research nurse, who will make sure that communication is taken up with your employer. We will normally discuss your participation with your employer beforehand to make sure there won't be any loss in income. Once your employer agrees that you can attend the study during normal working hours without having to take leave or lose any wages, you can join the study.</li> </ul>
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There are more gains for you in joining this study than there are risks.



#### **How will we protect your confidentiality and who will see your findings?**

Anonymity of your findings will be protected by all of the researchers involved. A number, and not your name, will be assigned to your research results, and all scientists using your data will only note this number, and not your name. Your privacy will be respected by making sure that all the measurements are taken in private rooms and performed by well-trained scientists. Your results will be kept confidential by storing hard copies of your documentation in a locked cupboard within the Hypertension Clinic, and only the Principal Investigator, Head of the Hypertension Clinic and Data Manager having direct access. Electronic files with data are stored and handled by the Data Manager in a password protected online database using the University web-network (with firewall and security features), as well as some backup files on external password protected harddrives. Only the researchers, their postgraduate students and local and international collaborators will be able to look at your findings – however, all findings will be anonymised using your unique participant number. As this is a long term project, your data will be stored for 20 years or longer.



#### **What will happen with the findings or samples?**

As indicated above, your research results are safely stored on electronic files, with some results on hard copies, and in the form of blood or urine samples in biofreezers. We will store your data and your blood and urine samples for at least 30 years. Over time the research team will make sure that all of this information is analysed in the utmost detail to create new knowledge on how high blood pressure, heart disease, and related diseases develop over time. It is important to store the data and samples for a long period, as new scientific discoveries on markers of high blood pressure will be made by other scientists or ourselves in the future. It will then allow us to test if these markers are also useful in your (the South African) samples, and whether these can be used throughout South Africa in the future.

Some of your biological samples (from urine and blood) will be analysed immediately, but others will be stored for many years before analyses are performed. Please note that we will perform the biochemical analyses in our laboratories on the Potchefstroom Campus. But we may need to ship some of your samples to other laboratories in South Africa or internationally, when we do not have the funds, skills or the equipment to perform the analyses locally. Samples will be shipped using courier services approved for handling biological samples, to ensure the safekeeping and protection of the samples during transit. We will also ensure that the appropriate approvals from the South African Department of Health (export permit) and the Health Research Ethics Committee are obtained prior to shipping the samples.

Apart from your samples, your anonymised data may also be shared with other national or international collaborators. It is therefore possible that your anonymised results will be reported as stand alone data as part of the African-PREDICT study, or your data may be pooled into other datasets from the province, country or

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**Informed Consent Form – Research Version Aug 2016**

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globally in further research studies on high blood pressure and related health status. Your data will therefore be used to analyse your original state of blood pressure and health – in South Africa and in comparison to other local and international populations – and to analyse how your health status changes over time.

If we were to share your anonymised data or samples with external groups, the external groups will sign confidentiality and data or material transfer agreements with us. This process is overseen by the Legal Services of the North-West University. This will ensure that your information is adequately handled and protected, and that your data is only used for the intended purpose as described in the agreement.

It is also possible that your data may be useful for other purposes apart from the aim of the present study. When the data is to be used for such purposes, new applications will be submitted to the Health Research Ethics Committee, where the Committee will stand in on your behalf.

Findings from the study will be published in scientific journals, and discussed locally and internationally with scientific experts and the Department of Health.

**How will you know about the results of this research?**

During the course of the day you will receive direct feedback from each research station on your health status and findings. As described earlier, if any abnormalities are detected, a detailed report within a referral letter will be compiled by the research nurse and you will be directed to the appropriate healthcare provider. If at any stage (also after you have visited the clinic) you wish to know any of your research results, you are welcome to contact the researchers at the Hypertension Clinic.

The research team also intends to publish the research findings of the larger study in scientific literature, but also in local media, and perhaps also national media. This will not include you as an individual, but the collective findings of all the research participants. Furthermore, as this is a longitudinal study, the research team may provide you with further results of the study when you return to the clinic during follow-up measurements. As the research team will contact you annually to ensure that your contact details are still correct, we will inform you if any important research findings became apparent that you need to take note of.

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

No, you will not be paid to take part in the study, but the research team will provide you with a R50 gift voucher as a token of appreciation for your participation. We hope that the results of the measurements will be useful to you to understand your own health status.

We will provide transport to all participants, and a meal will be served during the course of the morning after you have given a blood sample.

There will thus be no costs involved for you, if you do take part in this study.

To cover all of the research expenses, this study is funded by several local and international funding bodies, including the Department of Science and Technology (National Research Foundation), Medical Research Council of South Africa and the Medical Research Council of the United Kingdom, as well as scientific grants from industry (GlaxoSmithKline, Pfizer, Boehringer-Ingelheim, Medi-Clinic Hospital Group).

**Note\* What happens after the study day?**

At the end of the study, you may have one eye covered so it is advisable not to drive until you see that your eye has recovered, due to a possible loss of depth perception. You will know that the eye is fully recovered when the black part of the treated eye (pupil) has been reduced to a similar size as the pupil of the untreated eye. In the week following the study day, we will make **three short appointments** with you to collect the blood

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**Informed Consent Form – Research Version Aug 2016**

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pressure monitor, your urine collection and the activity monitor and to do two more short interviews (20-30 minutes) about your diet. We will give you a diary sheet so you can keep track of these appointments and they will be arranged to suit your schedule.

**Is there anything else that you should know or do?**

- You can contact Sr. Adele Burger (or Prof. Alta Schutte) at 018 285 2261/2446 if you have any further questions or have any problems.
- You can also contact the Health Research Ethics Committee via Mrs Carolien van Zyl at 018 299 1206 or [carolien.vanzyl@nwu.ac.za](mailto:carolien.vanzyl@nwu.ac.za) if you have any concerns that were not answered about the research or if you have complaints about the research.
- You will receive a copy of this information and consent form for your own purposes.

Address: Building F11, Potchefstroom Campus, North-West University, Potchefstroom 2520  
Tel: 018-285 2261 (Office hours Mon-Fri) Fax: 018-285 2260; Email: [adele.burger@nwu.ac.za](mailto:adele.burger@nwu.ac.za)

## Informed Consent Form – Research Version Aug 2016

**Declaration by participant**

By signing below, I ..... agree to take part in the research study titled: The African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT).

I declare that:

- I have read this information/it was explained to me by a trusted person in a language with which I am fluent and comfortable.
- The research was clearly explained to me.
- I have had a chance to ask questions to both the person getting the consent from me, as well as the researcher and all my questions have been answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be handled in a negative way if I do so.
- I may be asked to leave the study before it has finished, if the researcher feels it is in the best interest, or if I do not follow the study plan, as agreed to.

I agree that my blood or urine samples may be sent to laboratories in South Africa or in other countries for analyses (with my personal details removed, and only identifiable by an anonymous number).	<b>Yes</b>	<b>No</b>
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Signed at (*place*) ..... on (*date*) ..... 20....

\_\_\_\_\_  
Signature of participant

\_\_\_\_\_  
Signature of witness

**Declaration by person obtaining consent**

I (*name*) ..... declare that:

- I clearly and in detail explained the information in this document to .....
- I did not use an interpreter.
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above.
- I gave him/her time to discuss it with others if he/she wished to do so.

Signed at (*place*) ..... on (*date*) ..... 20....

\_\_\_\_\_  
Signature of person obtaining consent

\_\_\_\_\_  
Signature of witness

**Declaration by researcher**

I, *Aletta E.Schutte*, declare that:

- I explained the information in this document to the Head of the Hypertension Clinic, Head of Screening, and research assistants.
- I did not use an interpreter.
- I encouraged them to ask questions and took adequate time to answer them.

And that I was available should they want to ask any further questions.

- The informed consent was obtained by an independent person.
- I am satisfied that she adequately understands all aspects of the research, as described above.
- I am satisfied that she had time to discuss it with others if she wished to do so.

Signed at (*place*) ..... on (*date*) ..... 20....

\_\_\_\_\_  
**Signature of researcher**

\_\_\_\_\_  
**Signature of witness**

# Appendix F

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Language editing



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 a thesis (for the qualification Doctor of Philosophy in Physiology), written by Ms M Strauss from the North-West University (student number 23423714).

Title of the thesis: Marinobufagenin and makers of early cardiovascular risk in a young black and white population: The African-PREDICT study



9 April 2019

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C Vorster

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Date

# Appendix G

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Turnitin report

4/10/2019

Turnitin

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