

Left ventricular structure and urinary metabolomics in young adults: The African-PREDICT study

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Preface

The article format as approved by the North-West University was chosen for this dissertation. This dissertation consists of four chapters which include a background and motivation, literature overview, research methodology, a manuscript and a concluding chapter. The manuscript will be submitted to the *Journal of Molecular and Cellular Cardiology*. All figures used in this dissertation were personally sketched (unless referenced otherwise) with the use of Wikimedia, available from <https://commons.wikimedia.org/wiki/Category:Images> and Servier Medical Art, available from <https://smart.servier.com/>

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Contributions of the authors

The following researchers contributed to the manuscript for publication:

Mrs D Erasmus Responsible for applying for ethical clearance from the Health Research Ethics Committee of the North-West University, also for compiling background and motivation, literature review, design and planning of the research article, statistical analyses, interpretation of results and inscription of all sections forming this dissertation

Prof R Kruger Supervisor of the dissertation. Responsible for intellectual and technical input, evaluation of statistical analyses, design and planning the research article and dissertation.

Prof CMC Mels Co-supervisor of the dissertation. Responsible for intellectual and technical input, evaluation of statistical analyses, design and planning the research article and dissertation. Provided discipline-specific input in the statistical analyses of the metabolomics.

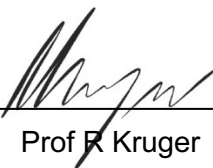
Prof AE Schutte Principal investigator of the African-PREDICT study and co-supervisor of the dissertation. Responsible for intellectual and technical input, evaluation of statistical analyses, design and planning the research article and dissertation.

Prof R Louw Provided discipline-specific input in the interpretation and elucidation of the metabolomic markers and scientific writing of the methods used to perform the metabolic analyses.

Prof C Delles Provided intellectual and technical input in the design and planning of the research article.

The following statement from the co-authors confirms their individual involvement in this study and gives their permission that the relevant research article may form part of this dissertation.

Hereby, I declare that I approved the abovementioned dissertation and that my role in this study (as stated above) is representative of my contribution towards the research article and supervised Master's study. I also give my consent that this research article may be published as part of the dissertation of Dalene Erasmus.



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Summary

Motivation

African populations are more prone to the development of left ventricular structure abnormalities and dysfunction, however limited information exists on potential metabolic pathways contributing to early left ventricular structural changes. Consequently, the importance of identifying possible metabolomic markers in association with left ventricular mass in the youth is vital for future prediction and prevention of hypertension-related organ damage.

To the best of our knowledge, no studies have been done to determine the relationship of left ventricular mass with urinary metabolomics in 20-30 year-old black and white populations from South Africa.

Aim

We aimed to investigate the metabolomic profiles and identify possible metabolites associated with left ventricular mass index in young black and white South African adults.

Methodology

This cross-sectional study formed part of the larger African prospective study on early detection and identification of cardiovascular disease and hypertension (African-PREDICT). We included 20-30 year-old normotensive black (N=80) and white (N=80) participants from the African-PREDICT study, with complete data on urinary metabolomics and echocardiography. This sub-study was approved by the Health Research Ethics Committee of the North-West University (NWU-00029-18-S1) and adhered to all applicable requirements according to the revised Declaration of Helsinki for investigation on human participants.

Questionnaires included a General Health and Demographic Questionnaire and a 24-hour dietary recall questionnaire. Anthropometric measurements included body height and weight to calculate body mass index, as well as waist circumference. Body surface area was additionally calculated. Twenty-four-hour ambulatory blood pressure (ABPM) was determined with a 24-hour ABPM and electrocardiogram (ECG) apparatus (CardXplore, Meditech, Budapest, Hungary). An appropriately sized cuff was used on the non-dominant arm to measure blood pressure in 30-minute intervals during the day, and hourly during night-time. A standard transthoracic echocardiogram was performed by a clinical technologist using the General Electric Vivid E9

device (GE Vingmed Ultrasound A/S, Horten, Norway) to determine left ventricular dimensions for calculating left ventricular mass. Basic biochemical analyses included serum total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, high sensitivity C-reactive protein, total serum protein, creatinine and sodium fluoride plasma glucose. Cotinine, total glutathione and creatine kinase-MB (muscle/brain) analyses were also included. Urinary metabolites were measured using nuclear magnetic resonance spectroscopy, liquid chromatography tandem mass spectrometry and gas chromatography time-of-flight mass spectrometry. We performed univariate statistical analysis, which included independent t-tests (adjusted for multiple comparisons), effect size ($d \geq 0.3$) and single regression analysis to identify the most prominent urinary metabolites. Multivariate adjusted analyses were performed to test for independent associations of left ventricular mass index with identified metabolites.

Results

When comparing the black and white groups, the black group had lower body weight ($p < 0.001$) and protein intake ($p = 0.014$). Left ventricular mass index was similar between black and white participants ($p = 0.97$). Our statistical analyses identified five from a total of 192 metabolites which differed between the groups and associated with left ventricular mass index. In the black group the five metabolites were identified to be more abundant ($p < 0.05$) and these metabolites associated inversely with left ventricular mass index (only in the black group) in multivariable-adjusted regression analyses: hydroxyproline ($\beta = -0.24$; $p = 0.012$), methionine ($\beta = -0.21$; $p = 0.024$), glycine ($\beta = -0.22$; $p = 0.031$), serine ($\beta = -0.19$; $p = 0.047$) and trimethylamine ($\beta = -0.26$; $p = 0.007$).

Conclusion

We found inverse associations between left ventricular mass index and glycine, hydroxyproline, serine, methionine and trimethylamine, only in young black adults. We propose that the biosynthesis of glycine, hydroxyproline, serine and methionine are possibly up-regulated under restricted dietary conditions in the black group. The biosynthesis of these amino acids may be up-regulated to maintain homeostatic collagen synthesis and stability, glutathione synthesis and energy storage to aid in preventing a premature increase in left ventricular mass index.

Key words: collagen synthesis, glutathione, glycine, serine, hydroxyproline, black.

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Chapter layout

The layout of the dissertation is as follows:

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List of abbreviations

β	Beta
γ-glutamyl-AA	Gamma-glutamyl-amino acids
°C	Degrees Celsius
°	Degrees
ABPM	Ambulatory blood pressure measurement
ADP	Adenosine diphosphate
African-PREDICT	African Prospective study on the Early Detection and Identification of Cardiovascular Disease and Hypertension
AGAT	L-arginine:glycine amidinotransferase
AU	Arbitrary Units
ATP	Adenosine triphosphate
BHMT	Betaine-homocysteine methyltransferase
BMI	Body mass index
CK-MB	Creatine kinase muscle and brain
CK	Creatine kinase
CVD	Cardiovascular disease
DMG	Dimethylglycine
ECG	Electrocardiogram
FMO3	Flavin-containing monooxygenases
GAMT	Guanidinoacetate N-methyltransferase
GGT	Gamma-glutamyl transferase
GSH	Glutathione
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
IVSd	End-diastole linear measurement of the interventricular septum
LDL	Low density lipoprotein
LVM	Left ventricular mass
LVMi	Left ventricular mass index
LVIDd	Left ventricular internal diameter at end-diastole
n	Number of participants
NRF	National Research Foundation
PWTd	Inferolateral (posterior) left ventricular wall thickness at end-diastole

ROS	Reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SAMRC	South African Medical Research Council
SARCHI	South African Research Chairs Initiative
SASCO	South African Standard Classification of Occupations
THF	Tetrahydrofolate
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
TSP	Tetradecylacetic acid
cm	Centimetres
g	Grams
g/l	Grams per litre
Hz	Hertz
K	Cluster
kCal	Kilocalorie
kg	Kilogram
kJ	Kilojoules
l/min	Litres per minute
m	Metre
mm	Millimetre
mg	Milligrams
min	Minute
ml	Millilitre
mmHg	Millimetres of mercury
mmol/l	Millimole per litre
mg/dl	Milligrams per decilitre
mg/l	Milligrams per litre
mol/l	Micromoles per litre
mg/ml	Milligrams per millilitres
ml/min	Millilitres per minute
µl	Microlitre
µm	Micrometre

ng/ml	Nanograms per millilitre
ppm	Parts per million
U/l	Units per litre
V	Volt

Chapter 1

*Background, literature review, motivation, aim, objectives
and hypotheses*

1. Background and motivation

Left ventricular mass (LVM) is an independent risk marker for the prediction of cardiovascular events, such as heart failure, coronary heart disease and stroke, in children and adults (1-6). Various studies described African populations as having a higher risk of increased LVM and consequently left ventricular hypertrophy when compared to white populations (5, 7-10). Therefore, even the slightest echocardiographic abnormality, such as increased LVM, merits evaluating mechanistic pathways to identify prevention strategies at younger ages (7, 11).

In a study done on 6-8-year-old black and white boys from South Africa, arterial stiffness associated inversely with β -alanine and 1-methylhistidine, and positively with L-proline, thereby demonstrating a potential early compromise in cardioprotective mechanisms in the black boys (12). These early changes in cardioprotective mechanisms shows the importance of research on children and young adults to discover possible biomarkers associated with early cardiovascular changes, including changes in cardiac structure. Hypothesis-generating techniques, including omics, are often implemented in the search for potential biomarkers related to cardiovascular risk and events.

The use of metabolomic techniques in relation to cardiovascular disease only commenced at the beginning of this decade and focused mainly on population groups with advanced cardiovascular disease (13-16). In this regard, previous studies linked metabolomic markers, such as amino acids, organic acids and acylcarnitines with echocardiographic abnormalities. These studies included different stages of heart failure, heart failure with preserved ejection fraction, heart failure with reduced ejection fraction and left ventricular diastolic dysfunction (14, 15, 17).

Although black populations are more prone to the development of left ventricular structure abnormalities and dysfunction (5, 7-9), the relation of metabolic pathways and left ventricular structure is still poorly understood. Consequently, the identification of early changes that are already occurring at the metabolomic level in association with LVM in young and healthy adults, may lead to therapeutic and lifestyle interventions. This may ultimately lead to the detection and prevention of cardiovascular disease.

To the best of our knowledge, no studies have been done to determine the relationship of LVM with urinary metabolites in young healthy populations. Therefore, we will focus on 20-30 year-old black and white populations from South Africa to investigate this further.

1.1 Metabolomics

Omics studies present an integrated view of the molecules that make up cells, tissues or organisms (18). Omics technologies can be divided into genomics, transcriptomics, proteomics and metabolomics (18) (**Figure 1**). Metabolomics is the unbiased analysis of metabolites, which are the products of metabolism, in a biological specimen (18-20). Furthermore, metabolomics portray the events at a level downstream of gene expression which is closer to the actual phenotype than either proteomics or genomics (21).

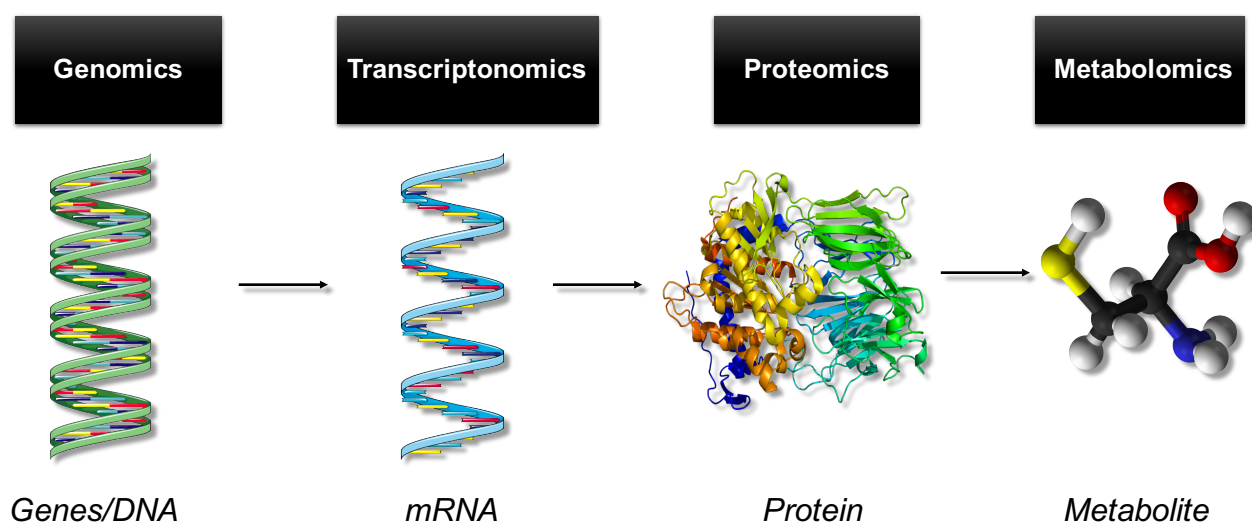


Figure 1: Omics technologies inter-relationship

Metabolomic studies provide insight into the current state and regulation of physiological and pathophysiological processes via the comprehensive investigation of all the metabolites in a system (22). Metabolic profiles can be influenced by a number of factors, including diet, age, ethnicity, gender, lifestyle, gut microbial populations, diseases and medication usage (23, 24). Therefore, the study of metabolomics provides a direct overview of a person's health at a certain point in time (25). Furthermore, by exploring metabolomics, metabolomic profiles associated with pathologies, including cardiovascular diseases, can be identified (26). This approach may lead to biomarker discovery for consequent improved cardiovascular disease diagnosis and ultimately the potential prevention thereof (19). The study of metabolomics may also contribute to inform precision medicine (20) and lead to new therapeutics to target specific needs of a patient, based on their own genetic, biomarker, phenotypic and psychosocial state (27, 28).

1.1.1 Analytical platforms

State of the art analytical methods are used to measure and analyse metabolites. The two main technological approaches include nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) (29, 30). Nuclear magnetic resonance is based on energy absorption and re-emission of an atom nuclei, where certain nuclei possess the property of magnetic spin which can adopt different energy levels when put in a magnetic field (31). Nuclear magnetic resonance provides information on the chemical structure (28) and absolute quantification of metabolites (30). Furthermore, NMR is advantageous since it is non-destructive, requires minimal separation techniques with no ionization of metabolites; however, definite identification and quantification are limited to abundant metabolites (30).

Mass spectrometry methods are dependent on spectral data in the form of the mass-to-charge ratio and relative intensity of the measured sample (28) and provide a higher analytical sensitivity than NMR (30). In spectrometry, biological compounds are ionized to generate different peak signals of each compound to therefore identify each molecule based on their unique peak patterns (23, 28). Mass spectrometry methods are often coupled to chemical separation techniques which are required to separate the different metabolites (23, 28). Separation techniques include gas and liquid chromatography columns (GC and LC), which are based on the interaction of metabolites with the adsorbent substances in the columns (32). Liquid chromatography mass spectrometry (LC-MS) is an extremely sensitive and selective analytical platform (19). Gas chromatography is a more effective separation technique than LC, with moderate shifts in retention time during an analytical run (33). The samples is also volatile to effortlessly introduce samples into the mass spectrometer (33).

However, some samples are not volatile and cause overlap between numerous co-eluting metabolites, making it difficult to quantify each metabolite. Nevertheless, this problem can be resolved with the use of time-of-flight (TOF) instruments which can capture more points across the peak signals in a chromatography run than through conventional methods, such as GS-MS (33, 34). Time-of-flight makes use of an electrical field where samples are accelerated to the same potential (35). This method is extremely sensitive since ions proceed to the detector through a “flight” tube where the time that the ions take to reach the detector correlates with the mass of the ion (35). Therefore, TOF coupled to MS provides a greater accuracy and mass resolution, sensitivity and profiling over a broad molecular weight range of samples (35).

Two different analytical approaches can be followed to measure metabolites, namely a targeted or untargeted approach (19, 36), where NMR and MS methods can be used to measure metabolites (30). These analytical platforms are briefly explained to illustrate the untargeted and targeted approaches. This study will make use of NMR, LC-MS/MS and GC-TOF-MS methods, which will be described in more detail in the methodology chapter (Chapter 2), under section 2.8.

1.1.2 Targeted and untargeted approaches

A targeted metabolomics approach is the quantitative measurement of one or a set of metabolites of known identity which are related to specific molecular pathways (19, 30, 36, 37). Analytical platforms, such as tandem mass spectrometry (MS/MS), GC-MS (38) and liquid chromatography tandem mass spectrometry (LC-MS/MS) (39) are often implemented to measure and analyse targeted metabolites, such as amino acids and acylcarnitines.

An untargeted metabolomics approach involves the non-quantitative analysis of all measurable metabolites in a sample, where more metabolites can be detected than by taking a targeted approach (37, 40). Gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) and NMR analyses are often used to measure non-targeted metabolites (41).

In the following sections, cardiac structure and function related to metabolic studies and pathways will be discussed.

1.3 Cardiac structure and function

Since this study focus on the associations between LVM and urinary metabolomics, it is important to provide a more detailed background on cardiac structure and function. The heart consists of three layers, namely the inner endocardium, middle myocardium and the outer epicardium (**Figure 2**) (42). The myocardium, which is the largest layer of the heart wall, comprises mainly of cardiac fibroblasts (43, 44), cardiomyocytes, endothelial cells, smooth muscle cells and connective tissue (43, 45, 46). Fibroblasts are responsible for the production of structural proteins that form the myocardial extracellular matrix (ECM) (43). The cardiac ECM is an interconnected system that provides structure and support to cardiac cells (47, 48). The ECM proteins include myocytes, fibronectin, laminin, elastin, fibrillin, proteoglycans and glycoproteins (43, 47), where the most abundant ECM proteins belong to the collagen family (48). Cardiomyocytes, along with the fibrillar collagen matrix, perform a key role in the contraction of the myocardium (49), with proline and glycine responsible for the biosynthesis of collagen (50). Alterations in the

homeostasis of the myocardial ECM have been associated with cardiac dysfunction, especially in black populations (44, 49, 51-54).

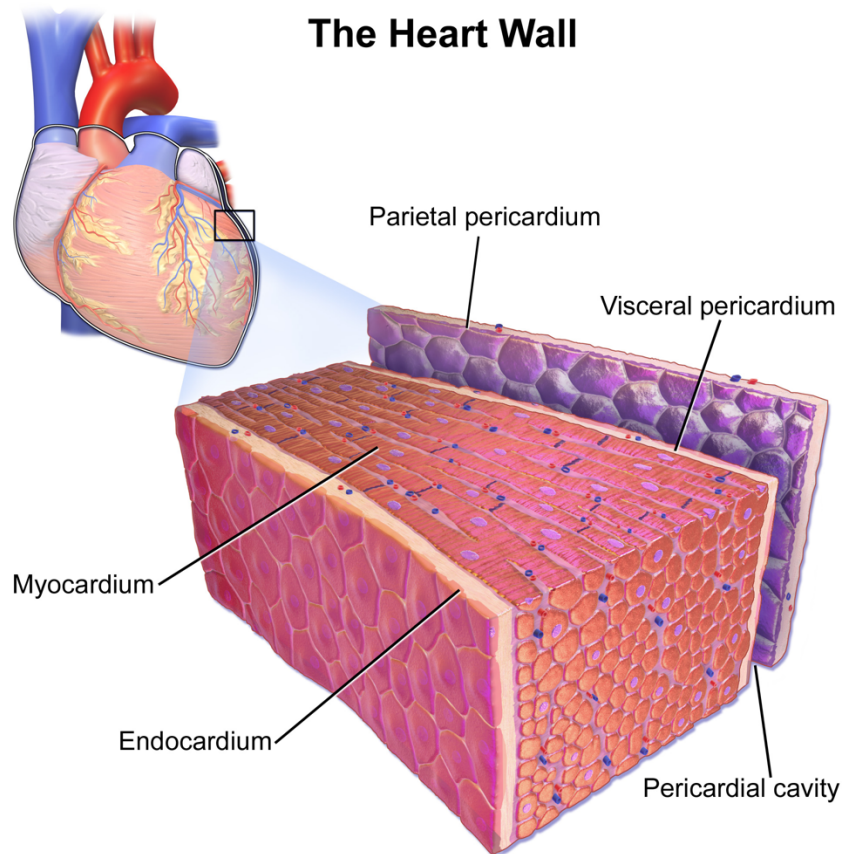


Figure 2: The layers of the heart wall

1.3.1 Left ventricular structure and function

The heart consists of four chambers, namely the right and left atria and ventricles (45). The left ventricle consists of thick myocardial walls with a central cavity, being anteriorly on the left side of the heart (42). The left ventricle (LV) is seen as the pressure pump of the heart, having three times the mass and twice the thickness of the right ventricle (42, 45). Furthermore, the LV is responsible for moving a certain volume of blood with every contraction into the systemic arterial vasculature to all the organs (55). Black populations have significantly greater LV geometry including higher relative wall thickness (10) and LVM than whites (56). Men also have higher LVM than women (57). Changes in the relationship between left ventricular myocardial contractility,

along with geometric and structural components, may reflect change in contractility and/or ventricular remodelling (55).

1.3.2 Cardiac remodelling

Appropriate turnover of the myocardial ECM is necessary to maintain the normal structure and morphogenesis of the heart (54, 58). An imbalance in cardiac ECM turnover differentiates pathological from physiological cardiac remodelling (44, 58). Cardiac remodelling is initially an adaptive response to changes in the hemodynamic state, but can result in irreversible pathological conditions (47, 54, 59). Cardiac remodelling can either be as a result of physiological stimuli, such as aerobic exercise, or as a result of a pathological stimulus, such as hypertension (59, 60). Maladaptive cardiac remodelling is a response to pathological events, such as myocardial injury or sustained increased cardiac load (54, 61-63). Therefore, maladaptive remodelling results in a change of the ECM and consequently a change in size, mass, geometry and functional properties of the heart and LV (47, 61-63) (**Figure 3**).

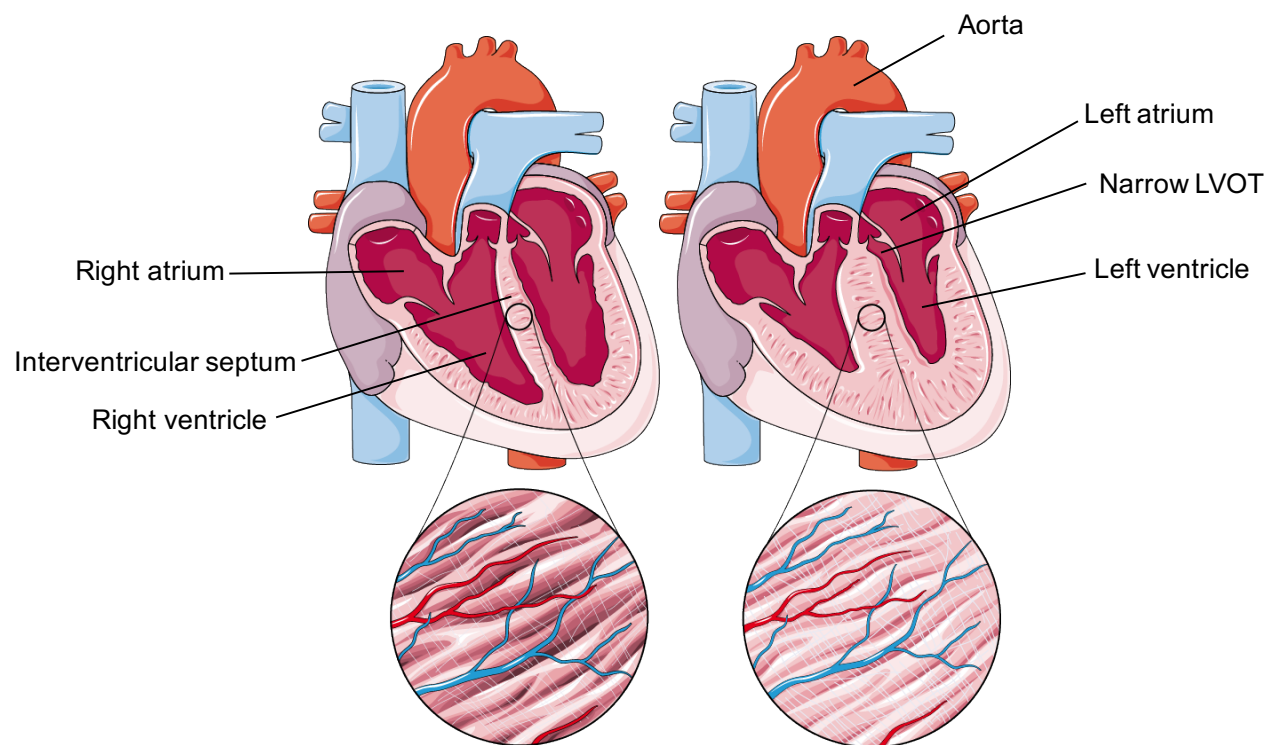


Figure 3: Normal heart (left) compared to hypertrophic heart (right)
Abbreviations: LVOT – left ventricular outflow tract.

Cardiac hypertrophy can be described as an increase in LVM attributable to an increased size of differentiated cardiomyocytes, caused by a physiological (for example in athletes) or pathological response as seen in hypertensive heart disease or heart failure (59, 64). Initially, cardiac hypertrophy is beneficial to normalise wall stress as a response to homeostatic remodelling, but afterward exerts detrimental effects on the heart (64), such as increased left ventricular dimensions, myocardial dysfunction, fibrosis and ultimately heart failure (65). An increased cardiac load will at first automatically increase end-diastolic sarcomere length to increase ventricular contractility (59). If the load further increases, it will act on the neurohormonal autonomic response to activate the sympathetic system (66) to increase cardiac output through augmenting heart rate and relaxation and contraction time of the ventricle (59). Neurohormonal compensatory mechanisms also include the activation of the renin-angiotensin-aldosterone system, resulting in vasoconstriction, increased blood volume and water and salt retention (66). Natriuretic peptides concentrations are also increased and released by the heart in response to myocardial stretch, causing natriuresis and vasodilation to counteract the effect of angiotensin II, aldosterone and renal-tubule sodium reabsorption (66, 67). If increased load persists, changes in the LV chambers' geometry will arise with an increased LVM (55, 59, 68). Physiological or pathological growth indicates changes in heart wall thickness and volume, which can be explained as concentric or eccentric remodelling (60).

1.3.3 Concentric remodelling

Concentric remodelling is described as an increased relative wall thickness with a normal LVM (**Figure 4**) (69, 70).

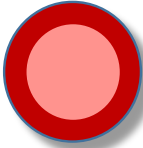
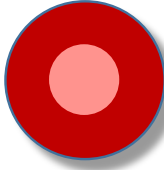
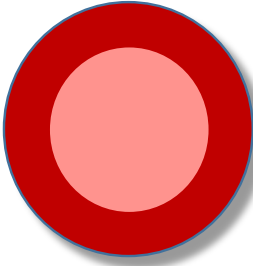
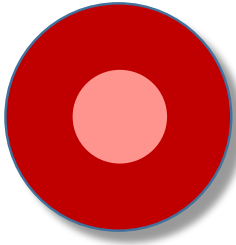
	Normal RWT 0.42	Increased RWT >0.42
LVMi (g/m²) Women ≤95 Men ≤115	Normal 	Concentric remodelling 
LVMi (g/m²) Women >95 Men >115	Eccentric Hypertrophy 	Concentric Hypertrophy 

Figure 4: A schematic representation of cardiac remodelling in the left ventricle adapted from Konstam (69)

Abbreviations: LVMi – left ventricular mass index, RWT – relative wall thickness.

Concentric remodelling as a result of pathological pressure overload (increased cardiac afterload), is attributable to an increase in systolic wall stress due to hypertension or aortic stenosis (61, 71, 72). An increase in LV wall stress as a result of pressure load, leads to hypertrophic growth of cardiomyocytes, which leads to an increase in protein synthesis and stability, along with derangement in energy metabolism (59, 63). Pressure overload causes the cross-sectional area of cardiomyocytes to widen (61) with the synthesis of sarcomeres in parallel that cause a greater increase in LV wall thickness (relative wall thickness) (73) than in cavity size (72), with a normal LVM (63). An increase in cardiomyocyte cell death occurs, causing a decrease in contractile mass and consequently cardiac contractility (63). If concentric remodelling is not treated, it will lead to concentric hypertrophy with an increase in LVM, increased relative wall thickness and decreased LV cavity (72) (63), leading to high intraventricular pressure needed to open the aortic valve (74). Concentric remodelling also decreases LV compliance and LV diastolic filling, leading to LV diastolic dysfunction and consequently heart failure (75). Furthermore,

concentric hypertrophy can also progress to systolic dysfunction, with a decreased ejection fraction, leading to heart failure (75).

1.3.4 Eccentric remodelling

Eccentric remodelling is defined as an increase in LVM with a normal relative wall thickness (69, 70). Maladaptive eccentric remodelling is associated with disorders that cause volume overload (increased preload) and an increase in diastolic wall stress, such as mitral and aortic regurgitation (61, 71). During volume overload, cardiomyocytes lengthen (61) with an increase in LVM and LV dilation (72), as well as an increase in sarcomeres in series which is associated with cardiomyocyte slippage (73). The latter results in eccentric hypertrophy with an increased LVM and a small relative wall thickness (63, 73) (**Figure 4**). Maladaptive LV remodelling in patients with end-stage aortic stenosis undergoing transcatheter aortic valve replacement demonstrated independent associations with long-chain acylcarnitines (76). Less is known about the relation between LVM and metabolomic markers.

1.4 Left ventricular mass (LVM): Factors influencing left ventricular mass

Left ventricular mass is an early, independent marker and predictor for cardiovascular disease (77-80). Numerous factors can influence LVM, including body size, sex, ethnicity, age, blood pressure, smoking, exercise (61, 77, 81) excessive alcohol use (82) and diabetes (63, 80).

1.4.1 Body size and sex

Left ventricular mass increases with increasing body size (83). Left ventricular mass is indexed (LVMI) for body surface area (g/m^2) (61, 77) with different cut-off values for men and women due to differences in body size (women= 43 to 95 g/m^2 and men= 49 to 115 g/m^2) (**Figure 5**) (77). Studies in children from different cohorts showed body size to be the strongest indicator of increased LVM in children (84-86). A study on children showed boys have higher LVM relative to girls (86). In adults, men have shown to have increased LVM and LV volume compared to women due to an increased body height (61, 77). Left ventricular mass is indexed for body surface area to adjust for the effects of body size on the left ventricle (61).

1.4.2 Age

Left ventricular remodelling is a normal phenomenon in advancing age (87). Alterations in cardiac structure, including concentric remodelling described as a greater mass-to-volume ratio, can

progress with advancing age (88-91). Age associated cardiac remodelling may be as a result of increasing blood pressure load on the heart (92) or due to increased body mass index with advancing age (93). Studies found age-associated increases in LVM in women, but not in men (94), likely attributable to increased body mass index in aging women (94). Autopsy findings demonstrated progressive LV myocyte loss, and cardiomyocyte hypertrophy with decreased LVM in men (95).

1.4.3 Ethnicity and genetic factors

Black populations are mostly reported to have an increased LVM when compared to white populations (61, 77, 96). Black populations are also more vulnerable to early onset hypertension, left ventricular hypertrophy and especially concentric hypertrophy (63). Some studies suggest that a genetic predisposition to increased LVM may be possible (63, 81, 97). As such, gene encoding proteins for the structure of the LV, blood pressure control and cell signal transduction may contribute in the development of left ventricular hypertrophy (63, 81, 97). Single nucleotide polymorphisms within an intron of peroxisome proliferator-activated receptor alpha genes have also been associated with increased LVM in response to both physiological and pathological events (97, 98).

1.4.4 Blood pressure

Sustained high blood pressure causes an increase in LV wall stress, which over time may lead to an increase in LV wall thickness and mass to normalize or counterbalance LV wall stress (63, 81, 99). Studies have shown an increase in LVM in children with an increased risk for hypertension (1, 100-102). Furthermore, left ventricular hypertrophy was significantly higher in children with hypertension and pre-hypertension compared to the normotensive subjects (1). It was also found that high blood pressure increases left ventricular stiffness and consequently left ventricular hypertrophy in adults (61, 77, 80). Another study on normotensive offspring of hypertensive Nigerians (aged 15-25 years) demonstrated higher LVMi in offspring of hypertensive parents compared with offspring of normotensive parents (85). The authors from the latter study suggest that normotensive offspring of hypertensive Nigerians have earlier alterations in LVM and LV structure and present a higher need for early dietary and lifestyle alterations to prevent cardiovascular events (85). A study on young hypertensive men (aged 20-35-years-old) had lower levels of glycine, lysine and cysteine which may contribute to increased inflammation, a comprised glutathione system and impaired protein formation (103). Another study on patients with essential

hypertension had increased levels of arginine and decreased levels of methionine, alanine and pyruvates compared to healthy subjects (104). This again highlights the importance of detecting possible metabolites to associate with LVM.

1.4.5 Lifestyle

Associations exist between high systolic blood pressure, people with a history of hypertension and smoking, leading to increased LVM (105). Smoking is one of the known preventable risk factors of cardiovascular disease and associates with increased LVM and abnormal LV geometry (106). A study done on current smokers, showed that increased duration of smoking was associated with increased LVM and worse LV diastolic function (107). Smoking leads to an increase in blood pressure over time and consequently an increase in LVM (108). Excessive alcohol consumption over a long period is also known for its toxic effects on the myocardium and is associated with high blood pressure (109) and increased LVM (81, 82).

Dietary factors, such as high sodium intake have been associated with increased LVM (63, 81, 110). High dietary sodium intake may lead to an increase in blood pressure, intravascular volume and consequently cardiac hypertrophy (63). Studies on dietary sodium intake showed that a reduction in salt intake resulted in a reduction in left ventricular hypertrophy (63). Furthermore, a diet low in animal fat and high in vegetables, fruit and monounsaturated fatty acids is associated with low LVM (111-113).

In addition to dietary factors, obesity also associates with increased LVM (61, 77), independent of high blood pressure (96). When obesity is characterised by central fat distribution, it causes an increase in metabolic demand and consequently elevated systemic blood volume and cardiac output, contributing to possible cardiac strain (63). Furthermore, obesity is associated with increased dietary salt intake and therefore increased water retention, which results in the dilation of the LV and thus eccentric hypertrophy (63). A study done on 5-18 year-old normotensive, pre-hypertensive and hypertensive subjects showed increased left ventricular hypertrophy in children and adolescents with overweight or obesity (1).

Isotonic exercise, which involves the movement of the large muscles causes adaptive hypertrophy by elevating venous return and volume load (61, 114). The latter does not affect relative wall thickness, but leads to an increase in ventricular cavity size with a proportional change in wall thickness (61, 114). Static exercise, such as weight lifting which involves little or no movement

while contracting muscle fibres, leads to pressure overload on the heart and consequently a small increase in the ventricle cavity with increased relative wall thickness (61, 114).

1.5 Metabolomics and cardiovascular disease

Numerous studies have shown associations between cardiovascular events or diseases and altered metabolic pathways:

- A study conducted on women (aged 18-84 years) called The TwinsUK Registry study, showed inverse associations between arterial stiffness and amino acids, such as methionine, glutamine, glycine, serine and trans-4-hydroxyproline (115).
- In a study done on black and white boys from South Africa (6-8 years-old), arterial stiffness was associated with 1-methylhistidine, L-proline and β -alanine in black boys only (116).
- Branched-chain amino acids have also been linked with myocardial infarction, coronary artery disease (13), heart failure and cardiovascular mortality (30, 117).
- Elderly patients with coronary artery disease presented with increased serum levels of medium-chain and long-chain acylcarnitines (117).
- Another study linked a decrease in choline-containing compounds with myocardial ischemia (118) and metabolites, including trimethylamine N-oxide, choline and betaine to predict the development of cardiovascular diseases (119).

Less is known about the associations between LVM and urinary metabolomics in young and healthy populations.

In this MHSc study, various statistical analyses were used to narrow down 192 metabolites to five metabolites that differed between the black and white groups and associated with LVMI (Chapter 3, section 3.2.7). The following metabolites were identified: glycine, 4-hydroxyproline, methionine, serine and trimethylamine. Therefore, the following sections will focus on metabolites relevant to this study, and their roles in maintaining a healthy LVM.

Glycine, 4-hydroxyproline (120) and serine (121) are non-essential amino acids that can be synthesized in the body. Glycine can be synthesized from serine and choline by the liver or kidneys (122, 123) and is involved in glutathione (124) and collagen (50) synthesis. Serine plays a crucial role in the formation of glycine, cysteine, taurine and phospholipids (121). Furthermore, 4-hydroxyproline is synthesized in the lumen of the endoplasmic reticulum through the hydroxylation of proline and is a major metabolite in collagen proteins (50, 125). In contrast,

methionine is an essential amino acid and cannot be synthesized in sufficient quantities by the body and needs to be provided through dietary sources, such as animal protein diets (126) to meet the requirements necessary to maintain growth, development and health (120).

More details on the metabolic processes where these metabolites are involved, will be described in the subsequent sections:

1.5.1 Energy metabolism

Cardiac remodelling, attributable to a pathological stimulus, leads to a decrease in cardiac contractility and metabolic energy production (59, 60, 62). Energy production is essential for the adequate functioning of the myocardium (127). Four key metabolic systems serve as energy providers for the heart, namely the tricarboxylic acid cycle (Krebs cycle), mitochondrial oxidative phosphorylation, mitochondrial oxidation of free fatty acids and the creatine pathway (127, 128). The creatine pathway serves as the most vital energy reserve of the heart (128, 129). Metabolites, including glycine, arginine and methionine are involved in the synthesis of creatine (130, 131), where creatine can either be derived from synthesis in the body or from dietary sources, including meat and fish (131).

Glycine and arginine, through the enzymatic reaction of L-arginine:glycine amidinotransferase (AGAT), produce guanidinoacetate (in the kidneys with S-adenosylmethionine as the methyl group donor), which is methylated through the enzyme N-guanidinoacetate methyltransferase (GAMT) (in the liver) to produce creatine (130, 132) (**Figure 5**).

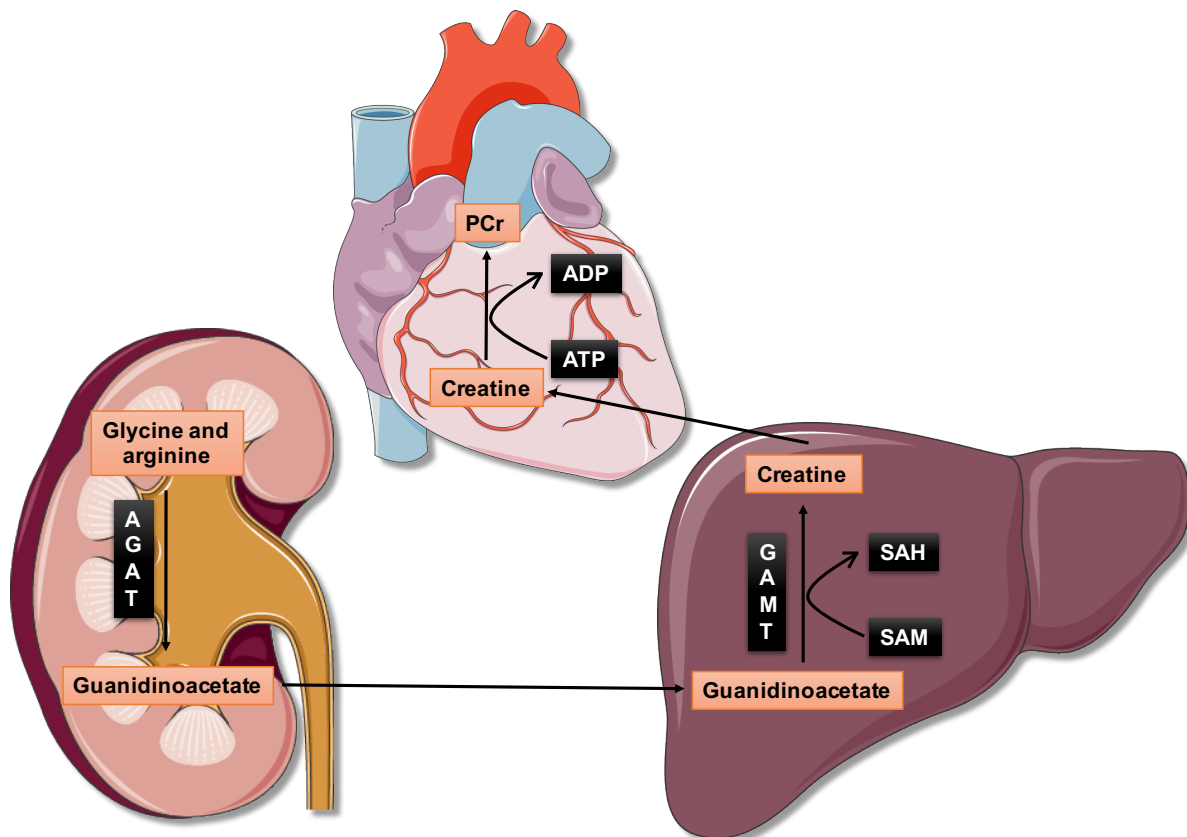


Figure 5: Creatine synthesis in the kidneys and liver

Abbreviations: AGAT – L-arginine:glycine amidinotransferase, GAMT – N-guanidinoacetate methyltransferase, SAM – S-adenosylmethionine, SAH – S-adenosylhomocysteine, ATP – adenosine triphosphate, ADP – adenosine diphosphate, PCr – phosphocreatine.

Creatine is then transported through the blood to the heart where it generates phosphocreatine through the enzyme, creatine kinase, with the conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) (130, 132). Phosphocreatine can either renew ATP or catch any available cellular energy to store in the ATP pool (131). Creatine kinase is responsible for catalysing the reversible phosphorylation of ATP to creatine to facilitate the storage of energy in the form of phosphocreatine in muscle cells (133). Therefore, creatine kinase is crucial in maintaining ATP homeostasis. Furthermore, glycolysis is one of the most important fuel substrates in the body (134), where glucose is transformed into pyruvic acid to take part in the Krebs cycle to produce ATP (135). The creatine system is therefore an important energy reserve in the heart to maintain normal cardiac function and prevent cardiac remodelling.

1.5.2 Oxidative stress

In a normal and healthy heart, a balance exists between reactive oxygen/nitrogen species and antioxidant systems (62). Glutathione is one of the most potent intracellular anti-oxidants (124, 136, 137) and is vital for vascular and cardiac function and a decrease in glutathione would result in oxidative stress in the cardiomyocytes (136). Methionine (138) and serine (as precursors for the transsulfuration pathway) (139) along with glycine (124, 136, 137) are involved in the intracellular biosynthesis of glutathione (**Figure 6**).

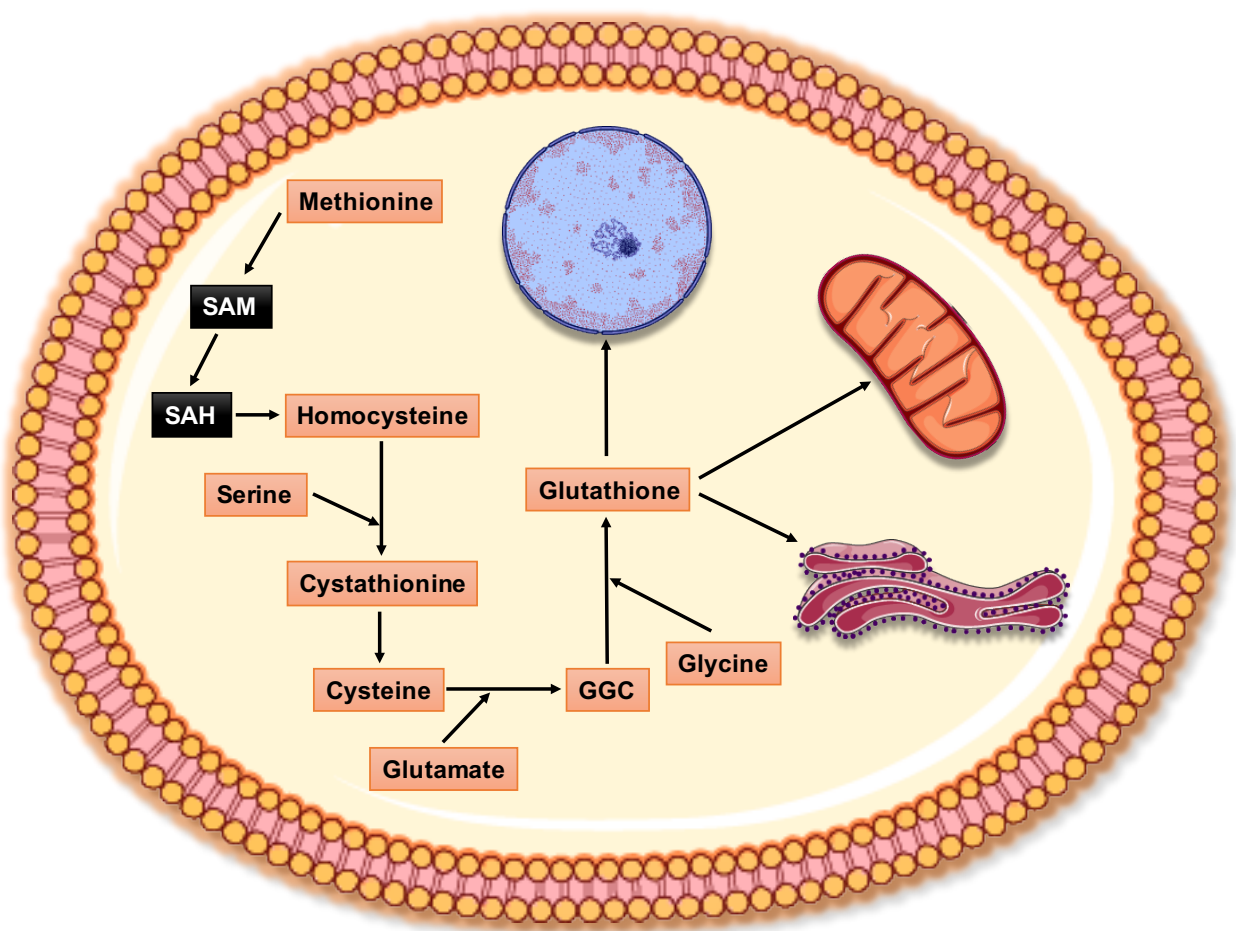


Figure 6: Intracellular glutathione synthesis

Abbreviations: SAM – S-adenosylmethionine, SAH – S-adenosylhomocysteine, GGC – gamma-glutamyl-cysteine.

Methionine is adenylated to generate S-adenosylmethionine (SAM) which is methylated to form S-adenosylhomocysteine (SAH). S-adenosylhomocysteine then produces homocysteine which enters the transsulfuration pathway and is converted to cystathionine. Cystathionine, along with serine, produces cysteine which condenses with glutamate to form gamma-glutamyl-cysteine (GGC). Finally, gamma-glutamyl-cysteine condenses with glycine to form glutathione. Glutathione is then freely distributed through the cytosol of the cells and can also be compartmentalised in the endoplasmic reticulum, nucleus and mitochondria.

A study investigating cardiac glutathione showed that patients undergoing coronary artery disease, aortic stenosis or terminal cardiomyopathy exhibited a deficiency in glutathione and blood glutathione was proposed as a possible biomarker in asymptomatic patients with structural cardiac changes (136). Oxidative stress occurs as the capacity of antioxidant systems becomes insufficient to handle the increased production of reactive oxygen species (ROS) produced from enzyme systems, such as xanthine- and NADPH oxidase, as well as mitochondrial dysfunction. Oxidative stress may in turn lead to DNA damage, cell dysfunction, fibroblast proliferation, protein oxidation, increased apoptosis and altered signalling pathways (140-142). During an increase in ROS beyond the level of antioxidant systems (oxidative stress), various effects are exerted in the cardiac muscle. This includes affecting excitation-contraction coupling and contributing to LV remodelling through the activation of mitogen-activated protein kinase pathways, leading to left ventricular hypertrophy, cell death and fibrosis (126). Furthermore, oxidative stress activates matrix metalloproteinase which decreases the synthesis of collagens in cardiac fibroblast, and consequently regulates myocardial ECM quantity and quality (143, 144). Therefore, oxidative stress seems to play a pivotal role in the process of cardiac remodelling.

1.5.3 Collagen stability

Cardiac collagens perform important roles to support myocytes and myofibrils necessary to maintain the structure and tensile strength of cardiac muscle (48, 49). The cardiac collagen matrix consists mostly of collagen types I and III (145, 146) and is made up of triple helix strands (50). Following the normal pathway of intracellular protein synthesis, collagens are formed by fibroblasts from amino acids, mostly glycine and proline (**Figure 7**) (50, 147). First, glycine and proline residues form procollagen which is processed by the rough endoplasmic reticulum and Golgi apparatus to form collagen (147, 148). During the conversion of procollagen to collagen, the following reactions take place in the rough endoplasmic reticulum: a) hydroxylation of proline residues to transform proline into hydroxyproline, b) hydroxylation of lysine residues to convert

lysine into hydroxylysine (50, 147), c) glycosylation and d) the initiation of intra-chain disulfide bonds between the N- and C-terminal polypeptides (147). These reactions give rise to the conformational changes in polypeptide chains to produce a triple helix structure (50, 147). Procollagens are then moved to the Golgi apparatus to be secreted by the fibroblasts into the extracellular space (147). After exocytosis, extracellular enzymes cleave the N- and C-terminal amino acid sequence to produce collagens (147, 148). The triple helical structure is then coiled after post-translational modifications, which include the conversion of collagen by lysyl oxidase to hydroxylysine, to ensure stable cross-links and therefore provide strong tensile strength to the collagen structure (50, 147). The triple-stranded helix consists of a repeating sequence of X-Y-Glycine-X-Y-Glycine, where every third residue is glycine and X and Y an amino acid (149, 150). The X amino acid is usually proline and the Y amino acid is hydroxyproline (149, 150), with the most common triple helix strand as proline-hydroxyproline-glycine (50).

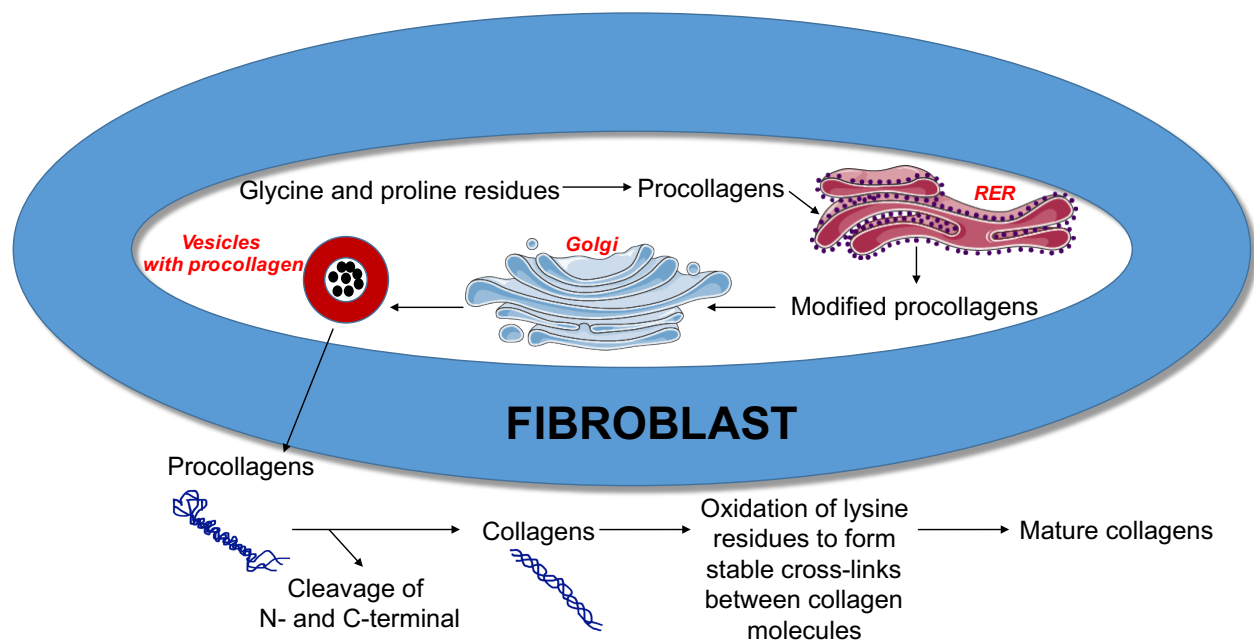


Figure 7: Summary of collagen biosynthesis in fibroblasts adapted from Li (147)

Abbreviations: RER – rough endoplasmic reticulum, Golgi – golgi apparatus.

Glycine and hydroxyproline are known for their fundamental role in collagen synthesis and stability (50, 124). Studies showed that hydroxyproline is essential in the stability of collagen and therefore important in maintaining the normal structure and strength of the heart and blood vessels (50, 147). Collagen provides the heart muscle and blood vessels with tensile strength and mechanical

support (150, 151). Decreased hydroxyproline availability and consequent collagen deficiency, may therefore lead to increased risk for vascular damage (50). Therefore, collagen deficiency may lead to fragile blood vessels, myocyte slippage, ventricular dilation and ultimately cardiac remodelling (151, 152).

1.5.4 Trimethylamine

Trimethylamine (TMA) is a metabolite produced by gut microbiota from dietary sources, such as betaine, choline and carnitine (153, 154). After TMA is absorbed through the intestinal epithelium, it is oxidized to form trimethylamine N-oxide (TMAO) via the enzyme hepatic flavin monooxygenase (FMO3) in the liver (154, 155) and is then secreted by the kidneys (156). Trimethylamine N-oxide has been linked to numerous cardiovascular disorders, including atherosclerosis (153, 157, 158) and coronary artery disease, as well as increased cardiovascular risk (159).

1.6 Motivation and aim

Even though studies have shown that African populations are more prone to the development of left ventricular structure abnormalities and dysfunction, the relation of metabolic pathways and left ventricular structure is still poorly understood. The unique data from a healthy cohort, which includes young black and white adults, provides the opportunity to review possible early actions that are already occurring on the metabolic level, and how these metabolites associate with LVM. This may lead to a better understanding of early factors contributing to increased LVM which may lead to the identification of potential targeted therapeutic and lifestyle interventions. For this reason, this study will also consist of hypothesis-generating work. To the best of our knowledge, no studies have been done to determine the relationship of LVM with metabolic patterns in a young and healthy population.

We aimed to investigate the metabolomic profiles and identify possible metabolites associated with LVM index in young black and white South African adults.

1.7 Objectives

In a study sample of 20-30 year-old black and white adults, we aimed to

- i.) statistically identify the most prominent urinary metabolites,
- ii.) compare identified urinary metabolites between groups,
- iii.) compare LVM between groups, and

- iv.) explore independent associations between LVMi and the identified urinary metabolites in black and white adults, respectively.

1.8 Hypotheses

From our first objective, we hypothesised that:

- The most prominent urinary metabolites will be statistically identified in black and white South Africans.

From our second objective, we hypothesised that:

- Multiple urinary metabolites will differ between black and white South Africans.

From our third objective, we hypothesised that:

- Black South Africans will have higher LVM than white South Africans.

From our fourth objective, we hypothesised that:

- Adverse associations will exist between LVM and urinary metabolites in both black and white South Africans.

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Chapter 2

Methodology

2.1 Introduction

Left ventricular mass (LVM) is an independent predictor of cardiovascular events, such as coronary heart disease, incident ischemic stroke and incident heart failure (1-3). Numerous factors contribute to increased LVM, including obesity, smoking, diabetes, gender, increased age and high blood pressure (1, 2, 4).

In this chapter, the specific methodology for the biochemical analysis, cardiovascular and anthropometric measurements, as well as questionnaires are outlined.

2.2 Population criteria and sample size

This MHSc Study is a cross-sectional sub-study of the African Prospective on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT African-PREDICT study started in 2013 and included 1202 healthy participants at baseline. This longitudinal study was designed to identify the pattern of hypertension development over a period of 10 years. The inclusion criteria were as follows: self-reported black or white ethnicity, an equal distribution of apparently healthy men and women (between 20-30 years old) with no chronic illnesses and a brachial blood pressure of <140/90 mmHg. Research shows that African populations have higher blood pressure (5) and are more prone to higher LVM (6). It is therefore important to compare LVM, as well as metabolomic profiles in black and white individuals and to explore associations between left ventricular mass index (LVMI) and urinary metabolites in the groups.

The aim of the African-PREDICT study is to focus on the development of cardiovascular disease and therefore the participants needed to be healthy. Both men and women were used to determine whether differences occur according to gender. The exclusion criteria was as follows: hypertensive (>140/90 mmHg), self-reported Indian, Asian, mixed origin ethnicity, not a permanent resident of Potchefstroom or its surrounding areas, previously diagnosed with type 1 or 2 diabetes mellitus, glucose levels of >5.6 mmol/l, HIV infected or other infectious illness, fever (ear temperature of >37.5°C), recent surgery or trauma, phobia for needles, using medication for chronic disease, diagnosed with chronic diseases in the past, self-reported previous history of stroke, angina pectoris and myocardial infarction, as well as micro-albuminuria of >30 mg/ml or proteinuria in spot morning urine sample. Pregnant and/or lactating women were also excluded due to the known influence of hormonal and gestational variances in haemodynamic and blood glucose regulation.

Since African populations have increased blood pressure and LVM, white participants were used as a comparison group. Participants were recruited from Potchefstroom and the surrounding areas in the North-West province, South Africa (**Figure 1**).

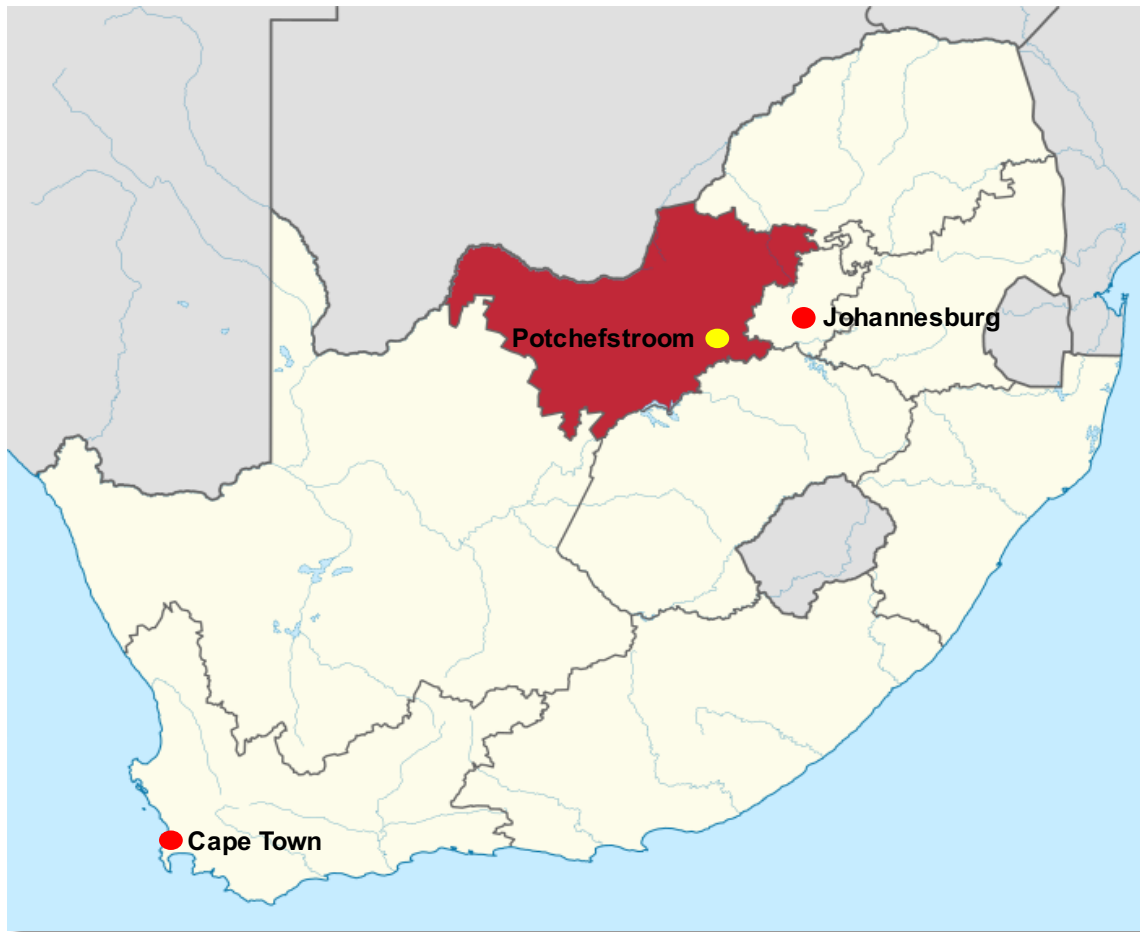


Figure 1: Map indicating Potchefstroom in the North-West Province in South Africa

The inclusion criteria of this study are in line with the main African-PREDICT study. However, due to the expensive nature of omics analyses, existing data on 160 participants (80 black and 80 white participants) with complete urinary metabolomics and echocardiographic data from the first 426 participants who enrolled in 2013-2014 will be used (**Figure 2**).

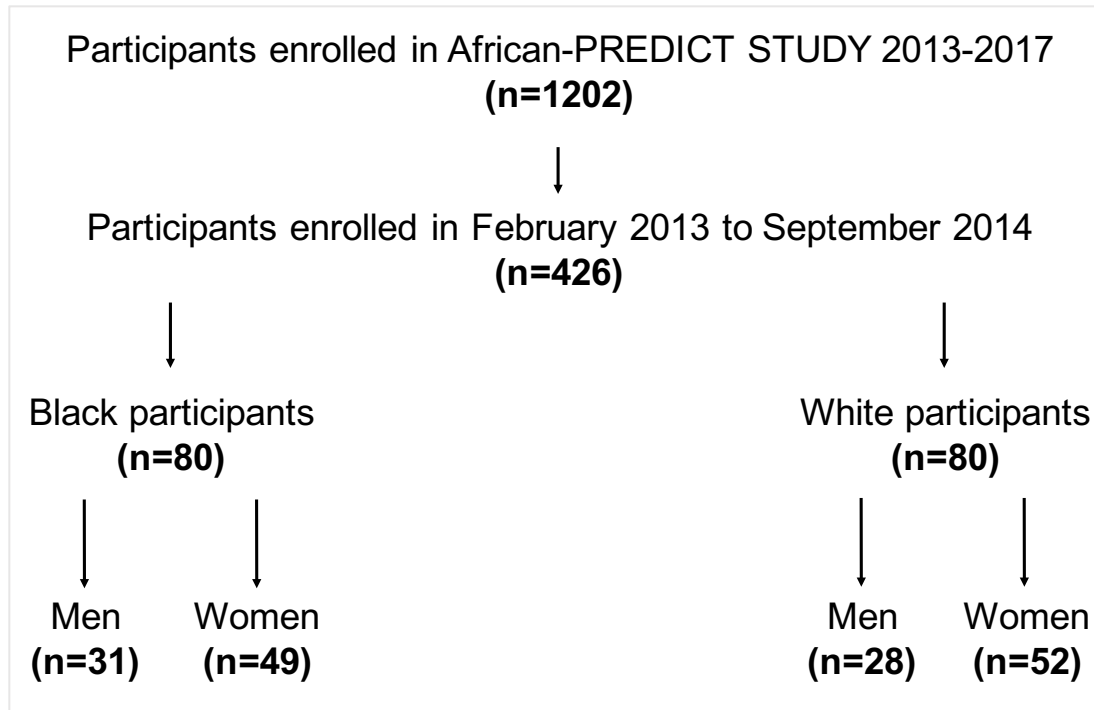


Figure 2: Study population of this MHSc study

This cross-sectional study was approved by the Health Research Ethics Committee of the North-West University (NWU-00029-18-S1) and adhered to all applicable requirements according to the revised Declaration of Helsinki for investigation on human participants

2.3 Basic procedures

The participants of this study were recruited from Potchefstroom in the North-West Province and surrounding areas. Recruitment took place through active contact with a field worker, access through their workplace or through advertisements by radio. Recruited participants were invited to the screening phase of the study to determine their eligibility to take part in the African-PREDICT study. To complete the screening phase, participants were required to complete and sign an informed consent form. The participants not eligible for the study, due to any of the exclusion criteria or health abnormalities, received detailed health feedback and where necessary, a referral letter for further testing or treatment from the research nurse. A private feedback session was also provided to the participants who met the inclusion criteria, after which they received an information leaflet with all the details regarding the research phase of the study and an informed consent form. Participants interested in participating in the research phase

handed in an informed consent form, and were asked to arrive at 08h00 hours at the research facility, where they were shown around the research facility to familiarise themselves with each measurement station. Transport was arranged for participants not able to arrange any themselves. All study measurements were performed in a temperature-controlled rooms. The research phase of the study included biological sampling (fasting blood and urine samples) along with various anthropometric, cardiovascular and biochemical measurements, as well as the completion of a set of questionnaires. After all measurements were done, a light meal and transport to go home were provided to all the participants. Participants received a six-digit ID number to ensure anonymity when processing data.

2.4 Questionnaires

Participants completed a General Health and Demographic Questionnaire, along with 24-hour dietary recall questionnaires. The General Health and Demographic Questionnaire is a self-administered questionnaire, to detect any current disorders (7), which were completed by the participants before any measurements commenced. The General Health and Demographic Questionnaire provided the following information in regards to the participant: demographic (age and locality), self-reported alcohol and tobacco use, employment details and the use of any medication. This questionnaire was completed on iPads using a web-based program.

Numerous studies have outlined the relationship between dietary factors and cardiovascular disease risk and development (8-10). The 24-hour dietary recall questionnaire was completed by a trained dietician or nutritionist on site, and on two occasions in the following week (including a weekend day). The five-step multiple-pass approach was used in conducting a 24-hour recall (11). Each fieldworker used a standardised dietary collection kit containing example pictures, packages, measurement tools and food models. After all three recalls were collected, it was coded according to the South African Medical Research Council's (SAMRC) Food Composition Tables (12), and the SAMRC's Food Quantities Manual (13) to convert household measures to grams. Protein deficiency was calculated according to the United States Dietary Reference Intakes for protein (14). Therefore, protein intake was calculated as 0.8 grams of protein per kilogram body weight, with dietary protein reference intake as 56 grams per day for men and 46 grams per day for women. Socio-economic score is a reliable indicator and predictor of morbidity and mortality (15). Each participant's socio-economic score was calculated from South African Standard Classification of Occupations (SASCO) skill level, highest level of education and total household

income (16). Each participant was then categorised into one of three categories: low, middle or high socio-economic groups.

2.5 Anthropometric measurements

Increased body size is an independent risk marker for cardiovascular morbidity and mortality (17), as well as increased LVM (18). Trained researchers made use of the International Society for the Advancement of Kinanthropometry (ISAK) (19) guidelines to measure the following of each participant: height (m) by using the SECA 213 Portable Stadiometer (SECA, Hamburg, Germany), weight (kg) by using the SECA 813 Electronic Scales with weighing capacity up to 200kg (SECA, Hamburg, Germany), as well as waist circumference (cm) (Lufkin Steel Anthropometric Tape; W606PM; Lufkin, Apex, USA). All measurements were done in a closed and private room to ensure privacy. These measurements were used to calculate the participant's body mass index (BMI) (Formula 1). High body mass index and obesity were associated with increased risk for cardiovascular morbidity and mortality (20), as well as increased LVM (6, 21).

Formula 1: $BMI = weight\ (kg) / height\ (m)^2$.

Body surface area was calculated with the use of the Mosteller formula (formula 2) (22), to index LVM (reason for this indexation is described under section 2.8).

Formula 2: $BSA = height\ (m) \times weight\ (kg) / 3600^{22}$

2.6 Physical activity

Physical activity is known to benefit cardiovascular health (23) and prevent premature cardiovascular disease development (24). Participants were fitted with an ActiHeart physical activity monitor (CamNtech Ltd., England, UK) to record their heart rate, inter-beat-interval and physical activity, to calculate each participant's energy expenditure over a seven-day period. Since physical activity reduces the risk for cardiovascular disease (24), the importance of obtaining a measure of physical activity and energy expenditure should not be neglected.

2.7 Blood pressure measures

Twenty-four-hour ambulatory blood pressure (ABPM) was determined with a 24-hour ABPM and electrocardiogram (ECG) apparatus, (CardXplore, Meditech, Budapest, Hungary) validated by the British Hypertension Society. An appropriate sized cuff was used on the participant's non-dominant arm, which measured blood pressure in 30-minute intervals during the day (06h00 to

22h00 hours), and hourly during night-time (22h00 to 06h00 hours). Only participants with >70% valid 24hr blood pressure measurements and >20-day time measurements and >7- night time measurements were included in the data analysis. The mean successful inflation rate of this group over a 24-hour period was 84.7%.

For this MHSc cross-sectional sub-study, we used 24-hour ambulatory blood pressure readings, rather than office blood pressure. Evidence propose that home readings of blood pressure is a more reliable predictor for cardiovascular events than measurements taken in an unfamiliar place (25), such as a research clinic.

2.8 Echocardiography

A non-invasive transthoracic echocardiography technique was used to determine cardiac structure and function (**Figure 3** and **4**).

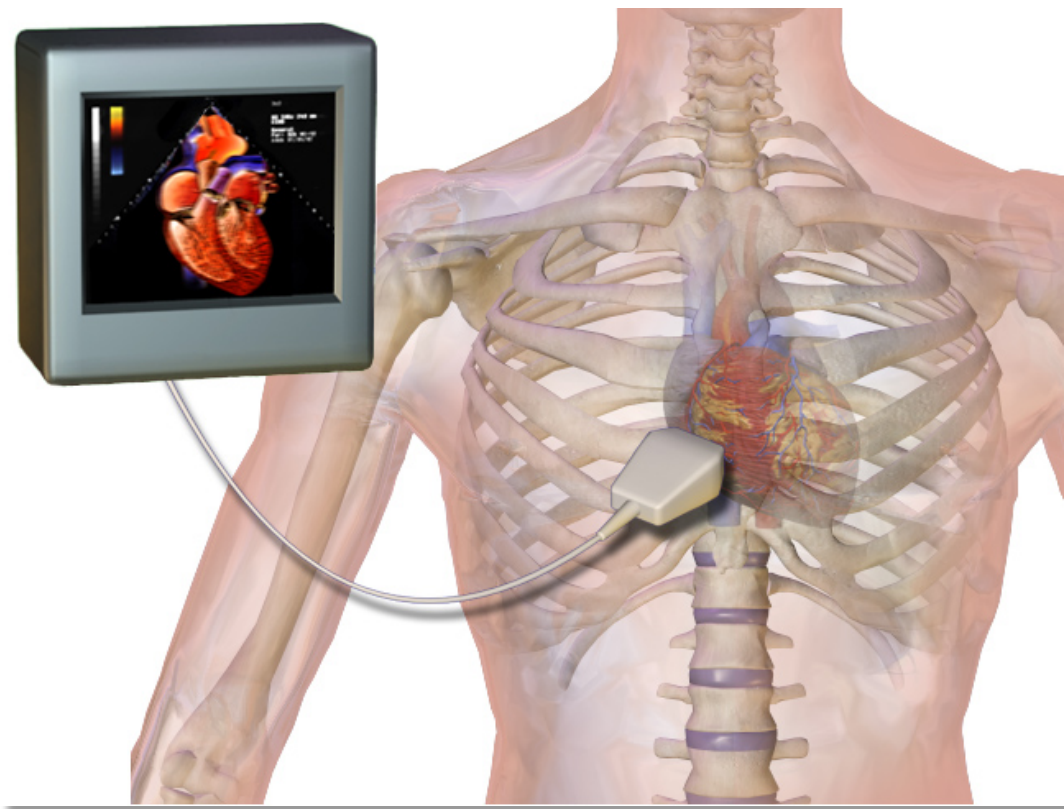


Figure 3: A basic illustration of a non-invasive transthoracic echocardiographic technique

A standard transthoracic echocardiogram was performed by a medical clinical technologist (registered by the Health Professions Council of South Africa) using the General Electric Vivid E9 device (GE Vingmed Ultrasound A/S, Horten, Norway) along with the 2.5 to 3.5 MHz transducer and a single ECG-lead. Standard methods were used to ensure high quality recordings, applying the recommendations as stated by the guidelines of the European Association of Echocardiography and the American Society of Echocardiography (26, 27). A trained ultrasound technician obtained the data, after which it was analysed by a single reader.

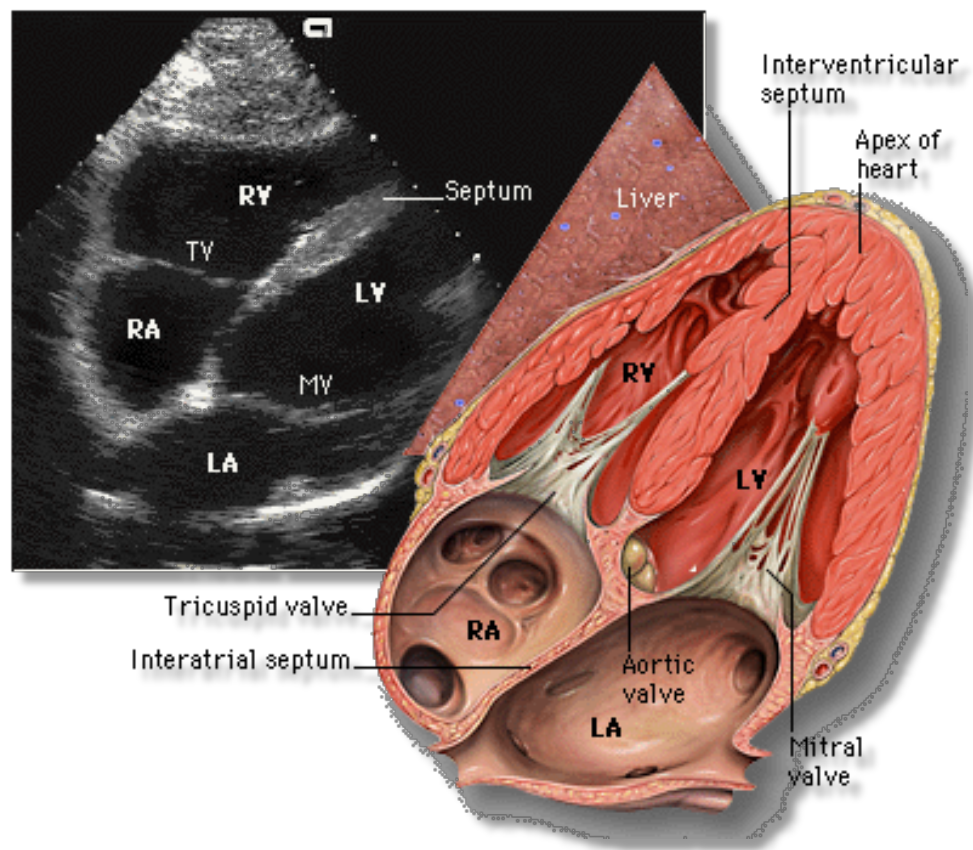


Figure 4: A picture taken by a trained ultrasound technician of the four chambers of the heart
Abbreviations: RV – right ventricle, RA – right atrium, LV – left ventricle, LA – left atrium, TV – tricuspid valve, MV – mitral valve.

The most commonly used method to measure LVM is the linear echocardiographic method (6, 21). We made use of the Devereux and Reichek cube formula (formula 3), which were indexed for body surface area (g/m^2) to calculate left ventricular mass index. Left ventricular mass index

was also used in statistical analyses to correct for height and obesity-related left ventricular hypertrophy (6).

Formula 3: $LVM = 0.8 \times 1.04 \times [(IVSd + LVIDd + PWTd)^3 - LVIDd^3] + 0.6$

The abbreviations used in the formula denotes the following: IVSd is the interventricular septum thickness measured at end-diastole, LVIDd is the LV internal diameter at end-diastole and PWTd is the inferolateral (posterior) LV wall thickness at end-diastole (6, 21).

2.9 Biochemical analysis

Participants were required to fast for eight hours prior to sampling, which included blood sampling (from the antebachial vein with the use of a winged infusion set) and an early-morning spot urine sample, taken by a registered nurse. Fasting reduces variability in biochemical parameters caused by any meals and consequently provide more stable and reliable values (28). Even though some lipid profile components are not affected by meals, such as total cholesterol and high-density lipoprotein, triglycerides in particular are (28). Therefore, fasting conditions provide triglyceride concentrations to be a steadier estimation of the measurement (28).

After biological samples were collected, all samples were taken to an on-site laboratory to be prepared for the bio-freezers (-80°C) in which the samples were stored for later analyses. Basic biochemical measurements included serum total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, high sensitivity C-reactive protein, total serum protein, creatinine and sodium fluoride plasma glucose (Cobas Integra 400 plus, Roche, Basel, Switzerland). Cotinine (Immulate, Siemens, Erlangen, Germany), total glutathione (OxisResearch; CA; USA) and creatine kinase-MB (muscle/brain) analyses (E411, Roche, Basel Switzerland) were also performed.

Urinary metabolites were measured with analytical methods, such as nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography tandem mass spectrometry (LC-MS/MS) and gas chromatography time-of-flight-mass spectrometry (GC-TOF-MS) analyses. For NMR Spectroscopy analysis, 600 µl urine was transferred to a glass vial. The urine was dried under a stream of nitrogen gas at 37°C, after which it was dissolved in 600 µl milliQ water. The total volume was transferred to a micro-centrifuge tube for centrifugation at 10 000g for 10 min at 25°C. Thereafter the 500 µl of the supernatant was transferred to a new tube and to which 50 µl potassium dihydrogen phosphate buffer (pH7.4) and trimethylsilyl-2,2,3,3-tetrahydroxybutyric acid (TSP) in D₂O as internal standard were added. To obtain a homogenous sample, all the

contents of the tube were vortex mixed and then transferred to 5 mm NMR tubes. The NMR analysis was performed on a 500 MHz Bruker, Avance III HD NMR spectrometer equipped with a triple-resonance inverse ^1H [^{15}N , ^{13}C] probe head and x, y, z gradient coils. ^1H spectra were acquired as 128 transients in 32 K data points with a spectral width of 6002 Hz. The temperature of the sample was maintained at 300 K and the water resonance pre-saturated by single-frequency irradiation during a relaxation delay of four seconds with a 90° excitation pulse of eight microseconds. Shimming of the sample was performed automatically on the deuterium signal. The resonance line widths for TSP and metabolites were b1 Hz. Fourier transformation, phase and baseline correction were done automatically. The Bruker Topspin V3.1 software was used to process spectral data and the Bruker AMIX V3.9.12 software was used to distinguish and identify metabolites. The identity of metabolites was confirmed with the Bruker pH7.0 BBO REFCODE spectral library.

Amino acids and acylcarnitines were analysed with a liquid chromatography tandem mass spectrometry (LC-MS/MS) method. A specific volume of each urine sample containing 0.0625 μmoles of creatinine was transferred to a glass vial and 1 ml deuterated isotope mixture used as internal standard was added. The deuterated isotope mixture contained valine (70.5 ppm), isoleucine (22.0 ppm), phenylalanine (57 ppm), lysine (57.5 ppm), free L-carnitine (73.5 ppm), acetylcarnitine (137 ppm), octanoylcarnitine (77.5 ppm) and octadecanoylcarnitine (59 ppm) isotopes. The samples were then evaporated to dryness under nitrogen gas (N_2) at 37°C . 200 μl 3 N butanolic hydrochloric acid was added to the dried residue, and the samples were incubated at 50°C for 60 minutes. The butylated samples were again evaporated to dryness with N_2 at 37°C . The dried residue was reconstituted in 100 μl (50:50) (v/v) containing 0.1% formic acid. The samples were analysed on an Agilent 6410 LC-MS/MS system with 1200 series liquid chromatograph front-end. Separation was done with a C18 Zorbax SB-Aq (150 mm $\times$ 2.1 mm $\times$ 3.5 μm) column from Agilent. The column was kept at 30°C during the entire run. Injection volume was 1 μl sample. The chromatographic gradient started at 95% solvent A (water with 0.1% formic acid) with a flow rate of 0.2 ml/min and maintained for 1 min, before the gradient was increased to 18% solvent B (acetonitrile with 0.1% formic acid) over a period of four minutes. The gradient was then kept constant for three minutes. Next the gradient was increased linearly to 100% solvent B at 15 minutes. Over this period, the flow was linearly increased to 0.3 mL/min. After maintaining the flow at 0.3 ml/min for five min with 100% solvent B, the flow and gradient were gradually decreased to 0.2 ml/min and 5% solvent B over a three-minute period. A post-run of 10 minutes was allowed to ensure equilibration of the column to give a total run time of 33 minutes

(23 min gradient and 10 minutes post-run) per sample. The electrospray ionisation source gas temperature was kept at 300°C, with a flow rate of 7.5 L/min. Nebulizer pressure was kept at 30 psi and capillary voltage at 3500 V.

Gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) analyses were performed to obtain relative quantities of metabolites. The volume of urine samples used was calculated from the creatinine concentration to compensate for variation in urine concentrations. The calculated volume of urine containing 0.125 µmoles of creatinine and internal standard (100 ppm 3-phenylbutyric acid) was transferred to a glass vial and dried under nitrogen gas for 45 minutes at 37°C. Derivatization (oximation and silylation) of samples to achieve sufficient volatility for gas chromatography was performed. Oximation was performed by adding 50 µl reagent (20 mg methoxyamine in 1 ml pyridine) to the dried samples and followed by incubation for 60 minutes at 60°C. Silylation followed with the addition of 50 µl *N,O*-Bis (trimethylsilyl) trifluoroacetamide (containing 1% trimethylsilyl chloride), after which the samples were vortexed for one minute and incubated for 60 minutes at 60°C. The samples were then analysed on a Leco Pegasus HT GC-TOF-MS system with Agilent 7890A gas chromatography front-end. Separation was done with a Rxi-5Sil MS (30 m × 250 µm × 0.25 µm) column from Restek. Helium was used as carrier gas at a constant flow rate of 1.5 mL/min. The inlet temperature was kept at 250°C for injection. Injection volume was 1 µl sample with 1:10 split. The oven temperature initiated at 70°C for one minute followed by a 7°C/min increase to 120°C. The oven temperature was then increased to 230°C at 10°C/min and finally to 300°C at a rate of 13°C/min, where it was kept for one minute before cooling to the initial temperature. The transfer line was kept constant at 225°C while the source was kept at 200°C. Electron impact ionization (-70 V) was performed to fragment all eluting compounds. An acquisition delay of 230 seconds was allowed where after data were acquired at 20 spectra/s (50–950 m/z). The GC-TOF-MS data were processed with ChromaTOF (Leco) software. A baseline offset of 1 (just above the noise) was used with the “spanning” baseline tracking method to remove the baseline. The software performed auto-smoothing. An average peak width of three seconds was selected to detect peaks with N20 signal-to-noise ratio and five apexing masses. The National Institute of Standards and Technology (2011) and an in-house created library were used to identify the compound peaks through spectral matching.

All the samples were randomly assigned to be analysed in one of three batches on each of the different analytical platforms. Urinary metabolite levels were reported as arbitrary units (AU). The data were examined for within-batch and between-batch effects/drifts using quality control samples. Since no drifts were observed, the data were processed further with standard

metabolomics procedures. Briefly, a zero and quality control coefficient of variance filter was applied. The data were normalised (relatively quantified) by using the appropriate internal standards to eliminate any technical variance.

2.10 Statistical analysis

Statistical analyses were performed with IBM® SPSS® Statistics version 25 software (IBM Corporation; Armonk, New York, USA), G*Power version 3.1.9.3 software (Faul, Erdfelder, Lang, & Buchner, 2007) (29) and Metaboanalyst (www.metaboanalyst.ca) (30).

We tested normality for all variables used in statistical analyses through visual inspection of histograms (QQ-plots). We performed logarithmic transformation for each skewed variable (total cholesterol, high and low density lipoprotein cholesterol, triglycerides, c-reactive protein, γ -glutamyl transferase, creatine kinase). All urinary metabolomics data were transformed with the use of a generalised logarithmic transformation to obtain comparable concentration ranges of metabolites. Normally distributed data was expressed as mean $\pm$ standard deviation, where logarithmic transformed variables were expressed as geometric mean with 5th and 95th percentile boundaries.

Univariate statistical analyses included independent t-test (adjusted for multiple comparisons), effect size ($d \geq 0.03$) and single regression analyses to determine the most prominent metabolites differing between black and white groups and to correlate with LVMi. All metabolomics data were visually inspected to determine any missing values before being uploaded to Metaboanalyst. In Metaboanalyst, the data was uploaded as a peak intensity table and zero value imputation was done by replacing the zero values with the minimum value of each column. We performed comparisons between the black and white groups by using independent T-tests. Adjustment for multiple comparisons was carried out to lower the false discovery rate ($q \leq 0.05$). Furthermore, the G*Power software was used to calculate a predefined effect size in order to determine the most sensitive effect size to flag significant differences between groups ($d = 0.27$) (**Table 1**). Consequently, an effect size of $d \geq 0.3$ was chosen to calculate the difference in metabolite levels between the groups.

Table 1: Calculation of effect size with the use of the G*Power software

F-tests – Linear multiple regression: Fixed model, R^2 deviation from zero		
Analysis: Sensitivity: Compute required effect size		
Input:	α err prob	= 0,05
	Power (1- β err prob)	= 0,95
	Total sample size	= 80
	Number of predictors	= 5
Output:	Noncentrality parameter λ	= 21,3129200
	Critical F	= 2,3382776
	Numerator df	= 5
	Denominator df	= 74
	Effect size f^2	= 0.2664115

Duplicate metabolites retrieved from different metabolomics platforms were evaluated and the ones with the largest effect size were kept in the dataset. We performed single regression analyses to explore the relationship between LVMi and the identified metabolites in the black and white group. Only metabolites which differed statistically after adjustment for multiple comparisons ($q \leq 0.05$), with an effect size $d \geq 0.3$ and which correlated with LVMi in single regression analysis were used in further statistical analysis and are defined as the five metabolites that were consistent to be significant in all analyses.

We performed partial regression analyses (with adjustments for sex and 24-hour systolic blood pressure) to investigate the relationship between LVMi and the identified urinary metabolites. Independent relationships between LVMi and the identified urinary metabolites were done by means of multiple linear regression analyses. We included covariates based on bivariate correlations with LVMi and identified metabolites in the multiple regression models. The following covariates were entered into the models: age, sex, C-reactive protein, plant protein intake, and 24-hour systolic blood pressure. We furthermore performed sensitivity analyses to assess the contribution of creatine kinase in the model, since the creatine pathway serves as the most vital energy storage of the heart (31).

2.11 Student contributions

The data used in this study was collected before I started with my postgraduate studies. I am trained and competent to use the following statistical software programs: IBM® SPSS® Statistics version 25 software (IBM Corporation; Armonk, New York, USA), G*Power version 3.1.9.3 software (Faul, Erdfelder, Lang, & Buchner, 2007) (29), REDCap (Research Electronic Data Capture (32), see <http://project-redcap.org>) and Metaboanalyst (www.metaboanalyst.ca) (30). For

this dissertation I was also responsible for all data handling, cleaning and statistical analyses using data from the African-PREDICT study. I am currently involved in the data-capturing for the first follow-up of the African-PREDICT study, where I am responsible for all data-capturing into REDCap. I am further responsible for sorting biochemical samples from the African-PREDICT study for international shipping. I am also responsible for double checking the data captured on REDCap for the EndoAfrica study (Vascular endothelial dysfunction: The putative interface of emerging cardiovascular risk factors affecting population living with and without HIV in Sub-Saharan Africa). I am also involved in the Exercise; Arterial Modulation and Nutrition in Youth South Africa (ExAMIN Youth SA) study, where I perform pulse wave analysis with the use of a validated, oscillometric Mobil-o-Graph monitor (I.E.M GmbH, Germany) with integrated ARCSolver software. I am further responsible and competent in blood pressure measurements with a validated and automated paediatric blood pressure monitor (Omron HBP-1100-E, (OMRON Healthcare Co., Ltd. Kyoto, Japan)). I am also trained and competent to use a Static Retinal Vessel Analyzer (SVA-T, Imedos Systems UG, Jena, Germany) to measure static retinal vessel diameters. I have also assisted with participant recruitment and data capturing for the ExAMIN Youth study.

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Chapter 3

Research manuscript

Left ventricular mass index and urinary metabolomics in young black and white adults: The African-PREDICT study

Summary of the instructions for the author

This article followed the specific guidelines as set out by the *Journal of Molecular and Cellular Cardiology* below. A full list of details regarding the author's instructions are available at: https://www.elsevier.com/wps/find/journaldescription.cws_home/622889?generatepdf=true

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- The article must adhere to approximately 6 000 words containing six-eight Figures and/or Tables and only six keywords.
- The article must contain the following sections: abstract, keywords, introduction, materials and methods, results, discussion, conclusion, figures and tables with captions. The article must be divided into named and numbered sections with each heading on its own separate line.
- Figures and tables can be placed next to the relevant text or on separate pages at the end of the manuscript. Each figure and table should include a caption with a brief explanation of all symbols and abbreviations.
- References must be consistent and may consist of any style or format. All references used in text must be cited under references and *vice versa*.

Left ventricular mass index and urinary metabolomics in young black and white adults: The African-PREDICT study

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Abstract

Background and aims: Increased left ventricular mass is an independent predictor for cardiovascular events, and shown to be higher in black than white populations. To gain a better understanding of early factors contributing to increased left ventricular mass in young black adults, we investigated metabolomic profiles and identified metabolites associated with left ventricular mass index in healthy black and white adults aged 20-30 years.

Methods and results: We included normotensive black (N=80) and white (N=80) participants from the African-PREDICT study, with complete data on urinary metabolomics and echocardiography. Urinary metabolites were measured using nuclear magnetic resonance spectroscopy, liquid chromatography tandem mass spectrometry and gas chromatography time-of-flight mass spectrometry. Univariate statistical analyses, including independent t-test (adjusted for multiple comparisons), effect size ($d \geq 0.03$) and single regression analyses were used to identify metabolites. When comparing the black and white groups, the black group had lower body weight ($p < 0.001$) and protein intake ($p = 0.014$). Left ventricular mass index was similar between black and white groups ($p = 0.97$). Five from a total of 192 metabolites were identified to be more abundant ($p < 0.05$) and inversely associated with left ventricular mass index in the black group only: hydroxyproline ($\beta = -0.24$; $p = 0.012$), methionine ($\beta = -0.21$; $p = 0.024$), glycine ($\beta = -0.22$; $p = 0.031$), serine ($\beta = -0.19$; $p = 0.047$) and trimethylamine ($\beta = -0.26$; $p = 0.007$).

Conclusion: Higher urinary levels of hydroxyproline, methionine, glycine, serine, and trimethylamine were inversely associated with left ventricular mass index only in the black adults. Since these metabolites are important to maintain homeostasis of collagen synthesis and stability, a healthy redox state and energy reserves in the heart, we propose an altered metabolic pathway to promote the biosynthesis of these metabolites under deficient dietary conditions.

Key words: collagen synthesis, glutathione, glycine, serine, hydroxyproline, black.

3.1 Introduction

Novel approaches, such as metabolomic profiling (metabolomics), can be used to discover new biomarkers related to cardiovascular diseases (1, 2). Black populations, including children with increased risk for hypertension, demonstrate increased left ventricular mass (LVM) (3-5) and are more prone to the development of hypertension mediated organ damage such as left ventricular hypertrophy at young ages (6-8). The gravity of this phenomenon is underlined by the fact that increased LVM is an independent predictor for cardiovascular events (9-11). It is therefore important to investigate this predisposition for increased LVM and to identify early factors related to LVM in young black populations, before the onset of potential pathology.

By following a metabolomics approach, metabolic pathways associated with cardiovascular disease risk may be identified. In turn, these pathways can be targeted with lifestyle and therapeutic interventions. Previous metabolomic studies on patients with increased arterial stiffness (12), myocardial ischemia (13), coronary artery disease (14) and atherosclerosis (15), identified metabolites, such as amino acids (glycine, methionine, serine, trans-4-hydroxyproline and creatine) and acylcarnitines associated with an increased risk for the development of cardiovascular disease.

Less is known about metabolomic profiles in young and healthy populations in relation to left ventricular mass index (LVMI), one of the earliest indicators of cardiovascular deterioration (16). Therefore, the metabolomic profiles of young, normotensive black and white adults were investigated in order to identify a urinary metabolite phenotype and the associations thereof with LVMI.

3.2 Methods

3.2.1 Study population

The baseline phase of the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT) included young (aged 20-30 years) and healthy participants. The inclusion criteria were self-reported black or white ethnicity, men and women with no self-reported chronic illnesses or chronic medication use, clinic normotensive (brachial blood pressure of <140 and <90 mmHg) and HIV uninfected.

This analysis of the African-PREDICT database included 160 participants (80 black and 80 white participants) with complete data for urinary metabolomics and echocardiography.

3.2.2 Organisational procedures

The participants were recruited from the North West province, South Africa. After explaining all the procedures, participants signed a written informed consent form. This study was approved by the Health Research Ethics Committee of the North-West University (NWU-00029-18-S1). This study adhered to all applicable requirements of the revised Declaration of Helsinki for investigation on human participants.

3.2.3 Questionnaires

Participants completed a General Health and Demographic Questionnaire, along with 24-hour dietary recall questionnaires. The 24-hour dietary recall questionnaires were completed by a trained dietician on site, and on two occasions in the following week (including a weekend day). The five-step multiple-pass approach was used in conducting a 24-hour recall (17). Each fieldworker used a standardised dietary collection kit containing example pictures, packages, measurement tools and food models. After all three recalls were collected, it was coded according to the South African Medical Research Council's (SAMRC) Food Composition Tables (18), and the SAMRC's Food Quantities Manual (19) to convert household measures to grams. Each participant's socio-economic score was calculated from the South African Standard Classification of Occupations (SASCO) skill level, highest level of education and total household income (20). Protein deficiency was calculated according to the United States Dietary Reference Intakes for protein (21).

3.2.4 Anthropometric measurements and physical activity monitoring

The International Society for the Advancement of Kinanthropometry guidelines were followed to measure body height (m) with a SECA 213 portable stadiometer (SECA, Hamburg, Germany), body weight (kg) using the SECA 813 electronic scale (SECA, Hamburg, Germany), as well as waist circumference (cm) (Lufkin Steel anthropometric tape; W606PM; Lufkin, Apex, USA). These measurements were used to calculate the participant's body mass index (BMI). Participants were fitted with an ActiHeart physical activity monitor (CamNtech Ltd., England, UK) that was worn for a maximum of seven consecutive days, to measure and calculate total energy expenditure.

3.2.5 Cardiovascular measurements

The 24-hour ambulatory blood pressure was determined with the Card(X)plore device validated by the British Hypertension Society (Meditech, Budapest, Hungary). An appropriately sized cuff

was used on the participant's non-dominant arm, which measured blood pressure in 30-minute intervals during the day, and hourly during night-time. The mean successful inflation rate of the group over a 24-hour period was 84.7%.

A standard transthoracic echocardiography procedure was performed by a clinical technologist using a General Electric Vivid E9 device (GE Vingmed Ultrasound A/S, Horten, Norway) along with a 2.5 to 3.5 MHz transducer and a single ECG-lead. Standard methods were used to ensure high quality recordings, applying the recommendations as stated by the guidelines of the European Association of Echocardiography and the American Society of Echocardiography (22, 23). We used the updated Devereux and Reichek cube formula for determining LVM and subsequently indexed by body surface area (LVMI) (22, 23).

3.2.6 Biochemical analysis

Participants were required to fast for at least eight hours prior to blood and early-morning spot urine sampling. After biological samples were collected, all samples were taken to an on-site laboratory to be prepared, aliquoted into cryovials and stored in bio-freezers (-80°C) until later analyses. Basic biochemical measurements included serum total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, high sensitivity C-reactive protein, total protein, creatinine and sodium fluoride plasma glucose (Cobas Integra 400 plus, Roche, Basel, Switzerland). Cotinine (Immulite, Siemens, Erlangen, Germany), total glutathione (OxisResearch; CA; USA) and creatine kinase-MB (muscle/brain) analyses (E411, Roche, Basel Switzerland) were also determined.

Urinary metabolites were measured with three different analytical platforms, including nuclear magnetic resonance (NMR) spectroscopy (500 MHz Bruker, Advance III HD NMR spectrometer), liquid chromatography tandem mass spectrometry (LC-MS/MS) (Agilent 6410 LC-MS/MS system with 1200 series liquid chromatograph front-end) and gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) (Leco Pegasus HT GC-TOF-MS system with Agilent 7890A gas chromatography front-end) analyses as previously described (24). In short, the volume of urine samples used on the different analytical platforms was calculated from the creatinine value to compensate for variation in urine concentration. All the samples were randomly assigned to be analysed in one of three batches on each of the different analytical platforms. Data generated from the various analytical platforms were analysed with appropriate software and the identity of metabolites were confirmed against spectral libraries, including the Bruker pH7.0 BBO REFCODE

spectral library, the National Institute of Standards and Technology (2011) and an in-house created library.

The data were normalised and relatively quantified by using the appropriate internal standards to eliminate any technical variance. Urinary metabolite levels were reported as arbitrary units (AU). The data were examined for within-batch and between-batch effects/drifts using quality control samples. Since no drifts were observed, the data were processed further with standard metabolomics procedures, including application of a zero and quality control coefficient of variance filters.

3.2.7 Statistical analysis

Statistical analyses were performed with IBM® SPSS® Statistics version 25 software (IBM Corporation; Armonk, New York, USA), G*Power version 3.1.9.3 software (Faul, Erdfelder, Lang, & Buchner, 2007) (25) and Metaboanalyst (www.metaboanalyst.ca) (26). All metabolomics data were visually inspected to determine any missing values before being uploaded to Metaboanalyst. In Metaboanalyst, the data was uploaded as a peak intensity table and zero value imputation was done by replacing the zero values with the minimum value of each column. However, none of our identified metabolites had more than one missing data point. All urinary metabolomics data were transformed by using a generalised logarithmic transformation to obtain comparable concentration ranges of metabolites. Non-metabolic variables were tested for normality by visual inspection (QQ-plots). Normally distributed variables were expressed as arithmetic mean and standard deviation. Non-Gaussian data was logarithmically transformed and presented as geometric means with 5th and 95th percentile boundaries. In order to identify the most prominent metabolites differing between black and white groups and to correlate with LVMi, different statistical approaches shown in **Figure 1** were followed. Thereafter, independent t-tests were performed to compare black and white groups. Adjustment for multiple comparisons was carried out to lower the false discovery rate ($q \leq 0.05$). Furthermore, the G*Power software was used to calculate a predefined effect size in order to determine the most sensitive effect size to flag significant differences between groups ($d = 0.27$). Consequently, an effect size of $d \geq 0.3$ was chosen to calculate the difference in metabolite levels between the groups. Duplicate metabolites retrieved from different metabolomics platforms were evaluated and the ones with the largest effect size were kept in the dataset. Finally, single regression analyses in the black and white groups between LVMi and the identified metabolites were performed. Only metabolites which differed statistically after adjustment for multiple comparisons ($q \leq 0.05$), with an effect size $d \geq 0.3$

and which correlated with LVMI in single regression analysis were used in further statistical analysis and are defined as the five metabolites that were consistent to be significant in all analyses (**Figure 1**). (For details on effect size and adjusted p-values to lower the false discovery rate of urinary metabolites refer to **Supplementary Tables S2** and **S3**).

We performed partial regression analyses (with adjustments for sex and 24-hour systolic blood pressure) between LVMI and urinary metabolites. Multiple linear regression analyses were performed to determine independent relationships between LVMI and the urinary metabolites. We included covariates based on bivariate correlations with LVMI and identified metabolites in the multiple regression models. The following covariates were entered into the models: age, sex, C-reactive protein, plant protein intake, and 24-hour systolic blood pressure.

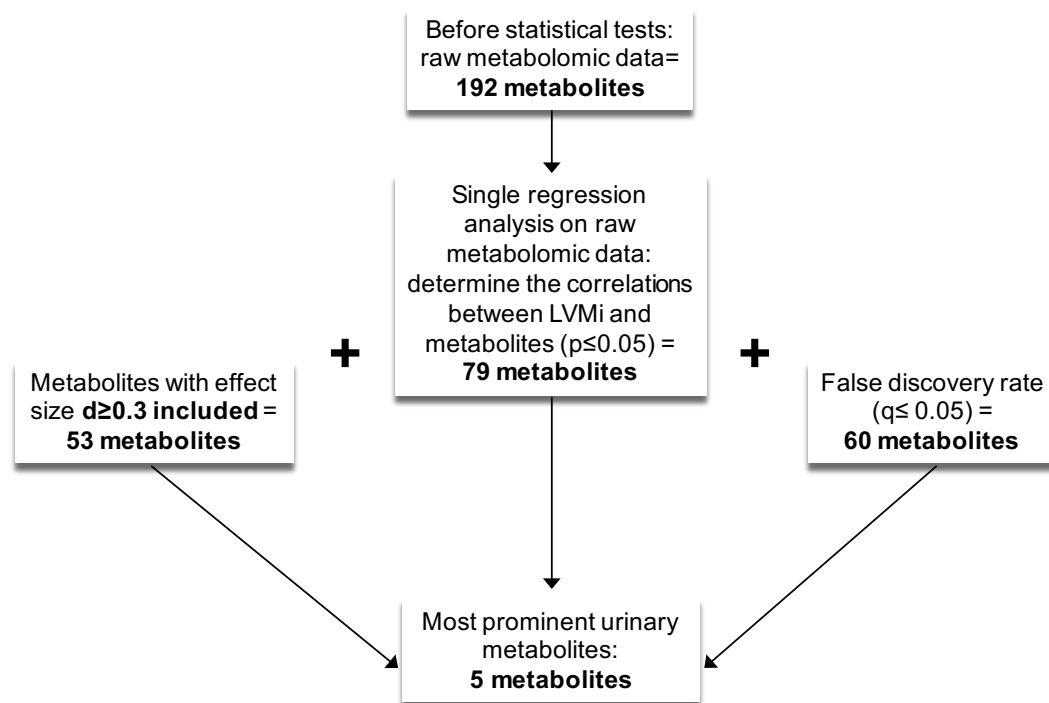


Figure 1: Statistical analyses pathway to identify the most prominent urinary metabolites
Abbreviations: LVMI – left ventricular mass index.

3.3 Results

The general characteristics of the study population are described in **Table 1**. The black group was younger (mean age 23.9 vs 25.8 years; $p<0.001$) and had a lower body weight ($p<0.001$) than the white group, whereas other measures of body composition were similar between the groups.

Left ventricular mass index and 24-hour blood pressure were similar between the two groups (all $p>0.018$). The five identified urinary metabolites, namely hydroxyproline, methionine, serine, glycine and trimethylamine were higher in the black compared to the white group (all $p\leq 0.001$). In addition, a few other metabolites related to the identified metabolic pathways were also compared (**Table 1**), where the black group demonstrated decreased levels of trimethylamine-N-oxide (TMAO) ($p=0.008$) and increased levels of cysteine ($p=0.042$) compared to the white group. The black group indicated an overall favourable lipid and glucose profile (all $p\leq 0.003$) and higher levels of gamma-glutamyl transferase, creatine kinase and total glutathione ($p\leq 0.015$) compared to their white counterparts. The black group demonstrated lower protein and vitamin C intake (all $p<0.001$).

Table 1: Characteristics of black and white groups

	Black (n=80)	White (n=80)	p-value
Age (years)	23.9 ± 3.29	25.8 ± 2.66	<0.001
Sex, male n (%)	31 (38.8)	28 (35.0)	0.63
Body mass index (kg/m ²)	24.3 ± 5.52	25.1 ± 5.68	0.38
Body weight (kg)	64.2 ± 13.3	74.3 ± 19.0	<0.001
Waist circumference (cm)	75.8 ± 10.4	79.5 ± 14.0	0.064
Cardiovascular measurements			
24h Systolic blood pressure (mmHg)	115 ± 9.63	118 ± 8.78	0.72
24h Diastolic blood pressure (mmHg)	69 ± 5.77	70 ± 6.41	0.58
24h Pulse pressure (mmHg)	76 ± 10.8	74 ± 10.7	0.31
24h Mean arterial pressure (mmHg)	88 ± 6.82	89 ± 6.24	0.18
Left ventricular mass index (g/m ²)	69.0 ± 15.8	68.9 ± 15.2	0.97
Urinary metabolites			
Trimethylamine (AU)	0.023 (0.02; 0.03)	0.021 (0.01; 0.03)	0.028*
Glycine (AU)	214 (68.1; 583)	150 (51.3; 402)	0.001*
Serine (AU)	151 (73.1; 292)	119 (60.3; 199)	0.003*
Hydroxyproline (AU)	2.35 (0.75; 8.28)	1.55 (0.70; 3.13)	<0.001*
Methionine (AU)	0.43 (0.16; 0.85)	0.35 (0.22; 0.56)	0.013*
Cysteine (AU)	0.26 (0.10; 0.57)	0.22 (0.10; 0.41)	0.042*
TMAO (AU)	0.0010 (8.6 × 10 ⁻⁴ ; 1.34 × 10 ⁻³)	0.0013 (8.5 × 10 ⁻⁴ ; 1.21 × 10 ⁻²)	0.008*
Biochemical analyses			
Total cholesterol (mmol/l)	3.61 (2.51; 4.96)	4.74 (3.29; 7.22)	<0.001
HDL cholesterol (mmol/l)	1.27 (0.80; 1.82)	1.44 (0.83; 2.42)	0.002
LDL cholesterol (mmol/l)	2.13 (1.21; 3.51)	2.95 (1.67; 5.18)	<0.001
Triglycerides (mmol/l)	0.79 (0.39; 1.58)	0.97 (0.44; 2.18)	0.003
Glucose (mmol/l)	3.86 ± 0.95	4.78 ± 0.76	<0.001
C-reactive protein (mg/l)	1.31 (0.19; 9.36)	1.09 (0.10; 8.58)	0.39
Total protein (g/l)	73.6 ± 5.78	70.7 ± 4.69	0.001
γ-glutamyl transferase (U/l)	26.4 (12.4; 99.8)	16.3 (7.5; 55.9)	<0.001
Creatine kinase (ng/ml)	1.52 (0.62; 4.05)	1.23 (0.56; 2.71)	0.015
Cotinine (ng/ml)	5.19 (1; 365)	2.85 (1; 253)	0.096
Total glutathione (μM)	1264 ± 276	956 ± 245	<0.001

Lifestyle

Self-reported smoking, n (%)	24 (30.0)	13 (16.3)	0.039
Self-reported alcohol use, n (%)	59 (74.8)	53 (66.3)	0.20
Total energy expenditure (kCal)	2120 ± 380	2328 ± 502	0.007
Socio-economic score	16.7 ± 5.04	25.5 ± 5.03	<0.001

Dietary intake

Total energy intake (kJ)	7187 ± 2896	9007 ± 2822	<0.001
Total protein intake (g)	59.3 ± 24.5	84.0 ± 37.0	<0.001
Plant protein intake (g)	21.1 ± 10.1	17.5 ± 7.3	0.012
Animal protein intake (g)	29.2 ± 18.2	55.9 ± 31.5	<0.001
Protein deficiency, n (%) [†]	38 (47.5)	23 (28.8)	0.014
Vitamin C intake (mg)	42.8 (60.0)	98.9 (96.2)	<0.001

Values are arithmetic mean and standard deviation, geometric mean with 5th and 95th percentiles, frequency and percentage. *False discovery rate adjusted p-values.

Abbreviations: AU – arbitrary units, HDL – high-density lipoprotein, LDL – low-density lipoprotein, n – number of participants. [†]Dietary protein reference intake: 0.8 g/kg body weight; men= 56g protein per day; women= 46g protein per day.

In both single and partial regression analyses (**Figure 2** and **Table 2**, respectively), LVMI correlated inversely with hydroxyproline, methionine, serine, glycine and trimethylamine in the black group only (all $p \leq 0.034$). In multivariable-adjusted regression analysis (**Table 3**), previous associations were confirmed. Briefly, LVMI associated inversely with trimethylamine ($\beta = -0.26$; $p = 0.007$), hydroxyproline ($\beta = -0.24$; $p = 0.012$), methionine ($\beta = -0.21$; $p = 0.024$), glycine ($\beta = -0.22$; $p = 0.031$) and serine ($\beta = -0.19$; $p = 0.047$) in the black group only. Significant correlations in the white group between LVMI and the urinary metabolite, methionine, lost significance after adjusting for covariates in partial- and multiple regression analysis (**Table 2** and **3**).

We performed sensitivity analyses by also including creatine kinase in the multivariable adjusted model. This was done since the creatine pathway serves as the most vital energy storage of the heart (27). In forward stepwise regression analysis, creatine kinase did not contribute significantly to the associations between LVMI and the urinary metabolites ($\beta = -0.046$; $p = 0.69$). We confirmed all previous associations between LVMI and trimethylamine ($\beta = -0.27$; $p = 0.006$), hydroxyproline ($\beta = -0.24$; $p = 0.012$), methionine ($\beta = -0.22$; $p = 0.022$), serine ($\beta = -0.20$; $p = 0.034$) and glycine ($\beta = -0.20$; $p = 0.036$).

Table 2: Partially adjusted linear regression analyses between left ventricular mass index and urinary metabolites in black and white groups

	<i>Left ventricular mass index (g/m²)</i>	
	Black (n=80)	White (n=80)
Trimethylamine (AU)	r= -0.31; p=0.006	r= -0.04; p=0.76
Glycine (AU)	r= -0.24; p=0.034	r= 0.07; p=0.52
Serine (AU)	r= -0.24; p=0.033	r= 0.07; p=0.56
Hydroxyproline (AU)	r= -0.29; p=0.012	r= 0.11; p=0.36
Methionine (AU)	r= -0.26; p=0.020	r= -0.10; p=0.37

Adjusted for 24-hour systolic blood pressure and sex.

Abbreviations: AU – arbitrary units, n – number of participants.

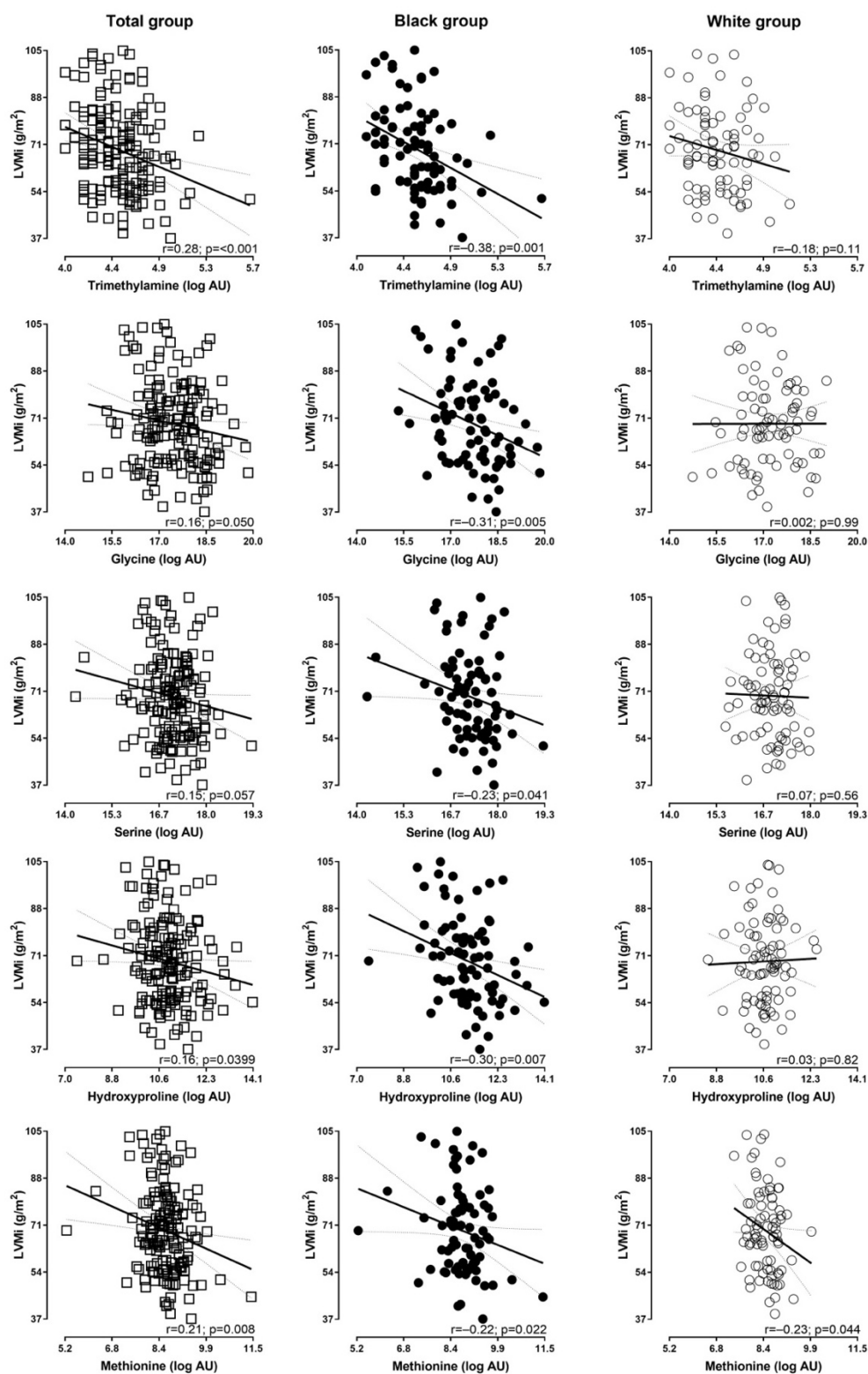


Figure 2: Single linear regression analyses between left ventricular mass index and urinary metabolites

Table 3: Multiple regression analysis of left ventricular mass index with identified metabolites in the black and white group

<i>Left ventricular mass index (g/m²)</i>						
	<i>Black (n=80)</i>			<i>White (n=80)</i>		
	<i>Adj R²</i>	<i>Std β (±95 CI)</i>	<i>p</i>	<i>Adj R²</i>	<i>Std β (±95 CI)</i>	<i>p</i>
Trimethylamine (AU)	0.34	−0.26 (−0.44; −0.06)	0.010	0.25	−0.02 (−0.25; 0.20)	0.83
Sex (women/men)		0.48 (0.20; 0.76)	0.001		0.34 (0.09; 0.64)	0.010
Age (years)		−0.42 (−0.23; 0.15)	0.67		0.09 (−0.14; 0.34)	0.41
Total plant protein (g)		0.10 (−0.10; 0.29)	0.35		0.10 (−0.13; 0.38)	0.32
C-reactive protein (mg/l)		0.01 (−0.23; 0.26)	0.91		−0.09 (−0.29; 0.12)	0.42
24-h systolic blood pressure (mmHg)		0.02 (−0.20; 0.23)	0.90		0.11 (−0.17; 0.39)	0.43
Glycine (AU)	0.33	−0.22 (−0.44; −0.02)	0.031	0.25	0.01 (−0.22; 0.24)	0.94
Sex (women/men)		0.47 (0.21; 0.72)	<0.001		0.42 (0.16; 0.67)	0.002
Age (years)		−0.05 (−0.23; 0.13)	0.59		0.07 (−0.17; 0.34)	0.51
Total plant protein (g)		0.11 (−0.10; 0.30)	0.32		0.12 (−0.12; 0.40)	0.26
C-reactive protein (mg/l)		−0.03 (−0.28; 0.21)	0.76		−0.08 (−0.27; 0.12)	0.46
24-h systolic blood pressure (mmHg)		−0.01 (−0.23; 0.20)	0.91		0.13 (−0.14; 0.40)	0.35
Serine (AU)	0.32	−0.19 (−0.35; −0.002)	0.047	0.25	−0.01 (−0.30; 0.29)	0.97
Sex (women/men)		0.53 (0.28; 0.79)	<0.001		0.42 (0.16; 0.67)	0.002
Age (years)		−0.05 (−0.23; 0.14)	0.64		0.08 (−0.18; 0.35)	0.50
Total plant protein (g)		0.09 (−0.12; 0.27)	0.44		0.12 (−0.11; 0.41)	0.25
C-reactive protein (mg/l)		0.02 (−0.22; 0.26)	0.88		−0.08 (−0.27; 0.13)	0.46
24-h systolic blood pressure (mmHg)		−0.01 (−0.22; 0.21)	0.97		0.12 (−0.15; 0.40)	0.36
Hydroxyproline (AU)	0.34	−0.24 (−0.39; −0.05)	0.012	0.26	−0.06 (−0.19; 0.36)	0.53
Sex (women/men)		0.50 (0.25; 0.75)	<0.001		0.43 (0.17; 0.68)	0.002
Age (years)		−0.07 (−0.25; 0.11)	0.45		0.06 (−0.16; 0.31)	0.53
Total plant protein (g)		0.09 (−0.11; 0.27)	0.40		0.12 (−0.12; 0.38)	0.30
C-reactive protein (mg/l)		−0.01 (−0.25; 0.23)	0.91		−0.09 (−0.28; 0.12)	0.43
24-h systolic blood pressure (mmHg)		0.004 (−0.21; 0.21)	0.97		0.13 (−0.14; 0.40)	0.33
Methionine (AU)	0.33	−0.21 (−0.34; −0.02)	0.024	0.27	−0.12 (−0.48; 0.14)	0.27
Sex (women/men)		0.52 (0.27; 0.77)	<0.001		0.40 (0.14; 0.65)	0.003
Age (years)		−0.06 (−0.24; 0.12)	0.52		0.12 (−0.12; 0.37)	0.31
Total plant protein (g)		0.09 (−0.11; 0.28)	0.41		0.13 (−0.09; 0.40)	0.20
C-reactive protein (mg/l)		0.01 (−0.23; 0.25)	0.94		−0.06 (−0.26; 0.14)	0.57
24-h systolic blood pressure (mmHg)		0.03 (−0.18; 0.24)	0.75		0.12 (−0.16; 0.38)	0.42

Abbreviations: Adj – adjusted, Std β – standardised beta, AU – arbitrary units, n – number of participants.

3.4 Discussion

In this study, urinary metabolites between young, healthy black and white adults were compared and relationships between LVMI and the most prominent urinary metabolites were explored. We identified five prominent urinary metabolites from 192 metabolites that were measured with three different analytical methods/ platforms. These five metabolites include glycine, hydroxyproline, serine, methionine and trimethylamine. In the black adults, these metabolites were all higher and associated inversely with LVMI, but not in the white group.

The black adults had higher levels of non-essential amino acids, including serine, glycine and hydroxyproline which may be explained by the increased plant protein intake and lower animal protein intake in the black group compared to the white group (28). However, almost half (48%) of the black participants in our study were protein deficient, compared to 29% of whites as determined by the Dietary Reference Intakes for protein guidelines of to the United States. The human body is able to change the preference in substrate use according to the change in substrate availability (29). It is therefore possible that the increased plant protein intake in the black group may lead to some non-essential amino acids to be used as substitutes for essential amino acids. This may be to compensate for the lower abundance in other nutrients due to a deficient protein intake, especially animal protein intake. However, information in this regard is limited and more research are necessary to confirm this finding.

There is controversy on whether a high animal protein diet is associated with cardiovascular disease, with some studies indicating an increased risk (30, 31), and other recent studies a lower risk (32-34). Since almost half of the black adults demonstrated protein deficiency with an inverse association between LVMI and the identified amino acids, an altered metabolism seems plausible to contribute to cardioprotective processes (**Figure 3**). We suggest an up-regulation in the biosynthesis of glycine, serine, hydroxyproline and methionine to maintain the homeostasis of collagen synthesis and stability (35, 36), a healthy redox state (37-39) and energy reserves in the heart (40) to prevent premature increase in LVMI.

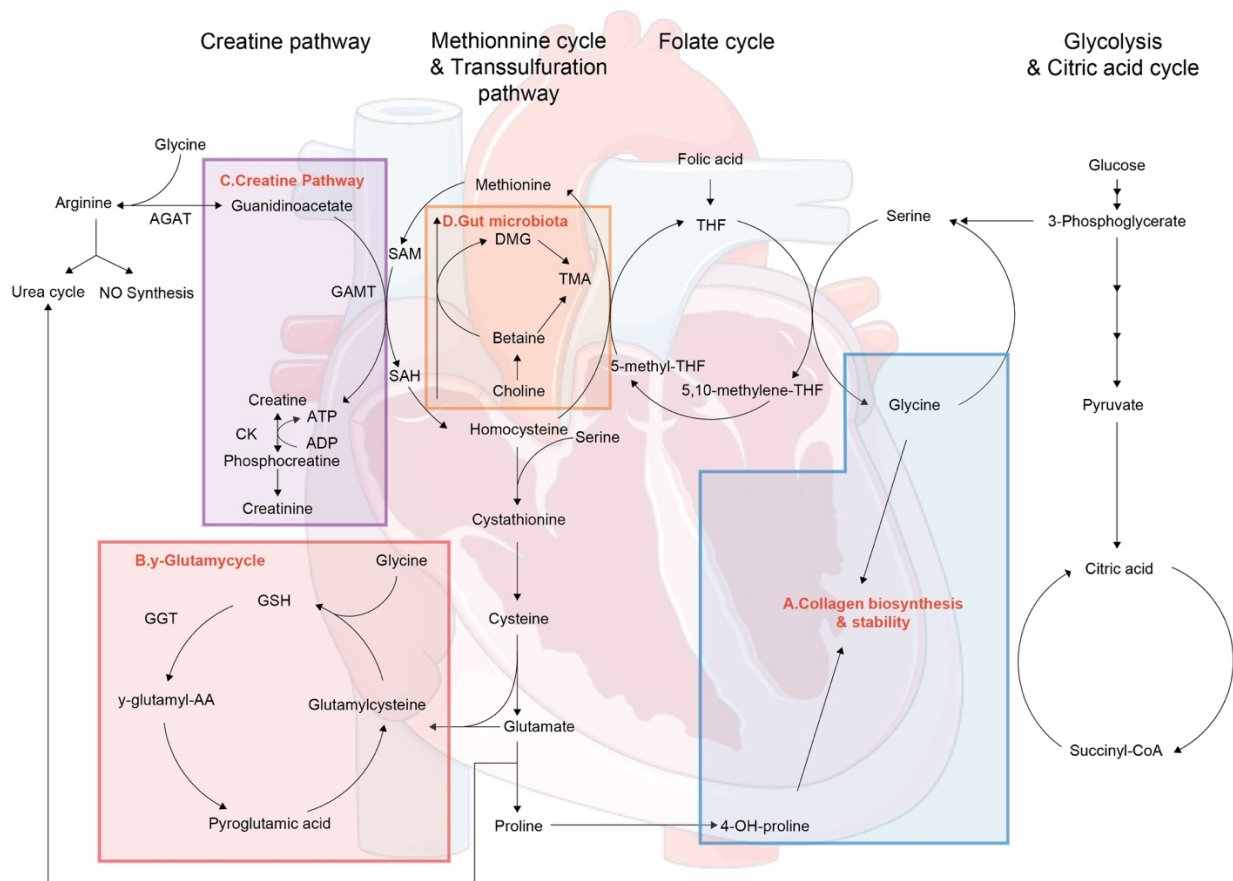


Figure 3: Proposed altered metabolic pathway in the black group

The letters denote the following: A – collagen biosynthesis and stability, B – gamma-glutamyl cycle, C – creatine pathway, D – Trimethylamine synthesis through gut microbiota. Window A: In the glycolysis pathway, glucose is metabolised to pyruvate which can enter the citric acid cycle, with 3-phosphoglycerate as intermediate. 3-Phosphoglycerate can actively be used to synthesise serine which is linked to the folate cycle as a one-carbon donor. During this reaction, serine is converted to glycine. The folate cycle is coupled to the methionine cycle and transsulfuration pathway leading to the formation of glutamate, from which proline can be synthesized where 4-hydroxyproline is formed through the hydroxylation of proline in the presence of vitamin C. Collagen synthesis and stability is highly dependent on the availability of both glycine and 4-hydroxyproline. Window B: Serine, methionine (as precursors for cysteine in the transsulfuration pathway) and glycine are involved in the biosynthesis of the potent intracellular anti-oxidant, glutathione. GSH synthesis begins when homocysteine is converted to cystathionine. First, methionine undergoes adenylation to form SAM, which donates its methyl group to form SAH to generate homocysteine. Homocysteine can then either enter the transsulfuration pathway, or be converted back to methionine. If homocysteine enters the transsulfuration pathway in the liver, it is converted to cystathionine, which condenses with serine to form cysteine. Glutamate and cysteine form gamma-glutamyl-cysteine, where gamma-glutamyl-cysteine condenses with glycine to form GSH. During GSH degradation, GGT acts on GSH to form gamma-glutamyl-amino acid to generate pyroglutamic acid. GGT cleaves extracellular GSH to increase its components, namely glycine, cysteine and glutamate, to regulate intracellular glutathione homeostasis (41). Window C: Glycine, arginine and methionine are required for creatine synthesis. Glycine and arginine, in the presence of AGAT, generates guanidinoacetate. The conversion of SAM to SAH transfers a methyl group to guanidinoacetate. Guanidinoacetate is then methylated through the enzyme GAMT, to produce creatine which generates phosphocreatine through the enzyme CK with the conversion of ATP to ADP. Phosphocreatine is then spontaneously converted to creatinine. Window D: TMA is produced by the gut microbiota from dietary sources, including choline. Choline is the precursor for betaine

and its metabolite, DMG. Betaine donates a methyl group to BHMT to re-methylate homocysteine back to methionine and consequently produce DMG and methionine. 5-methyl-THF can also donate a methyl group to re-methylate homocysteine back to methionine (folate cycle).

Abbreviations: ATP – adenosine triphosphate, ADP – adenosine diphosphate, CK – creatine kinase, GSH – glutathione, GGT – gamma-glutamyl transferase, SAM – S-adenosylmethionine, SAH – S-adenosylhomocysteine, THF – tetrahydrofolate, γ -glutamyl-AA – gamma-glutamyl-amino acids, AGAT – L-arginine:glycine amidinotransferase, GAMT – guanidinoacetate N-methyltransferase, DMG – dimethylglycine, BHMT – betaine-homocysteine methyltransferase, TMA – trimethylamine.

Collagen biosynthesis and stability

Collagen stability (**Window A, Figure 3**) is essential to maintain cardiac structure, where collagen deficiency may lead to cardiomyocyte slippage and cardiac instability (42, 43). This may in turn result in ventricular dilation, cardiac remodelling (43) and consequently increased LVMI. We propose that one of the early mechanisms related to the known predisposition for increased left ventricular hypertrophy in black populations (44) may be associated with an increased need for collagen synthesis. The more abundant levels of glycine and hydroxyproline, which inversely related to LVMI, may suggest an up-regulation in the biosynthesis thereof as a cardioprotective mechanism to maintain cardiac stability. The inverse associations of LVMI with glycine and hydroxyproline may suggest that if concentrations of these metabolites are low, it may result in a loss of collagen's cardioprotective role in cardiac stability and strength. The proposed increased need for collagen synthesis is unknown, but may be exacerbated by dietary factors, such a low protein and vitamin C intake in the black group. In experimental work, rats fed on a low protein diet resulted in impaired collagen synthesis (45). Additionally, in a study on vegetarians, lower expression of genes associated with collagen synthesis were noted (46). Regarding the role of vitamin C in collagen synthesis and stability, it was demonstrated that vitamin C stimulates fibroblast collagen synthesis, facilitates intracellular hydroxylation of proline and lysine residues (47) and converts collagen gene subtypes into mature subtypes that form stable fibrils to export intracellular collagen to form the extracellular matrix (48). Since LVMI was similar when comparing the black and white groups, we propose collagen synthesis and stability to be maintained at this stage, but over time LVMI may increase in the black adults if lower dietary intake of essential dietary components, such as proteins and vitamin C persist.

Glutathione synthesis

We found more abundant levels of glycine, serine and methionine in the black group, which is involved in glutathione synthesis (**Window B, Figure 3**) that counteracts the effects of reactive oxygen species (ROS) (49). These metabolites were also inversely associated with LVMI in the

black group which may reflect an increased need for the potent antioxidant, glutathione. We also found increased levels of glutathione and gamma-glutamyl transferase in our young black population. In previous work from our group, we indicated increased total glutathione levels and ROS in black participants when compared to their white counterparts (49-52). This suggests that with an increase in ROS, glutathione also increases to counteract the harmful effects of ROS (49). The higher gamma-glutamyl transferase activity in the black group may also indicate the increased need to regulate intracellular glutathione synthesis through the up-regulation in glycine and cysteine recycling (**Window B, Figure 3**). We therefore hypothesize that the black group may require increased cardioprotection against oxidative stress and consequently glutathione synthesis to prevent or delay ventricular remodelling (53).

Energy storage

We observed higher creatine kinase-MB levels in the black compared to the white group. With creatine kinase's involvement in maintaining adenosine triphosphate homeostasis (54), thus vital energy reserve of the heart (29) (**Window C, Figure 3**), possible disruptions in energy production can contribute to cardiomyocyte hypertrophy and increased LVM (58). However, we failed to establish a direct link between creatine kinase and LVMi or any of the urinary metabolites.

Trimethylamine synthesis

Left ventricular mass index associated inversely with trimethylamine (TMA) in the black group only. Trimethylamine, produced by the gut microbiota (**Window D, Figure 3**), is converted to trimethylamine-N-oxide (TMAO), which has been linked with cardiovascular disease risk and diseases (54, 55). We suggest that the inverse association between LVMi and TMA, may be due to decreased flavin-containing monooxygenases (FMO3) which catalyses the conversion of TMA to TMAO in the liver, leading to an increased urinary TMA excretion (56). Dietary components, such as indoles, found in cruciferous vegetables (56), including cabbages and broccoli (57), can decrease FMO3 and consequently decrease TMAO levels. Since TMAO was lower in the black group, the inverse association between LVMi and TMA may be explained by the increased urinary TMA in the black group, indicating a decrease in TMAO production and its detrimental cardiovascular effects (55, 58, 59).

To summarise, the black group potentially demonstrates an up-regulation in the biosynthesis of glycine, methionine, hydroxyproline and serine levels, which may contribute to uphold the cardioprotective processes in maintaining a normal LVMi. Although based on cross-sectional

associations, we suggest that this up-regulation may lead to a threshold where these metabolites can no longer be up-regulated to sustain the demand of cardioprotection. In the long term, this can result in an increased LVMI in the black group as reported in older black adults compared to their white counterparts (44), when alternative metabolic adjustments become insufficient.

This study is one of the first to determine the relationship between LVMI with urinary metabolomics in young black and white adults. We made use of three different state-of-the-art analytical methods, including NMR, LC-MS/MS and GC-TOF-MS spectrometry. The strict inclusion criteria of this study with a young and healthy black and white cohort is likely the reason for similarities of blood pressure and LVMI between the ethnic groups with no apparent hypertension-induced organ damage. Nevertheless, the findings suggest that compensatory changes are already occurring on metabolic level to prevent an early increase in LVMI in the black group. Even though this is considered to be hypothesis-generating, the importance of contributing to new hypotheses for future studies to test, by either confirming or opposing these findings, should not be underestimated.

3.5 Conclusion

In conclusion, we found that LVMI relates inversely and independently with glycine, hydroxyproline, serine, methionine and trimethylamine in young black adults, but not in whites. The elevated levels of these urinary metabolites in black adults may be due to the biosynthesis of these amino acids that are possibly up-regulated in response to the potential dietary deficient state in the black group. These amino acids are known to perform important cardioprotective roles by up-regulating collagen synthesis and stability, glutathione synthesis and energy storage to aid in preventing a premature increase in LVMI.

Conflict of interest

The authors report that they have no conflict of interest.

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3.6 Supplementary material

Table S1: Comparison between t-test derived p-values and false discovery rate adjusted p-values

Metabolite name	p-value	FDR
NMR		
3-Hydroxyisovaleric acid (AU)	<0.001	<0.001
1-Methylnicotinamide (AU)	<0.001	<0.001
Dimethylglycine (AU)	<0.001	<0.001
Trigonelline (AU)	<0.001	<0.001
Glycine (AU)	<0.001	<0.001
Alanine (AU)	<0.001	<0.001
Lysine/Arginine/Acetic acid (AU)	<0.001	<0.001
Pyruvic acid (AU)	<0.001	<0.001
Methylmalonic acid (AU)	<0.001	<0.001
Formic acid (AU)	<0.001	<0.001
Xanthine (AU)	<0.001	<0.001
Hippuric acid (AU)	<0.001	0,0012
Phenylacetylglycine (AU)	<0.001	0,0014
Glutamine (AU)	<0.001	0,0015
Acetic acid (AU)	0,001	0,0047
TMAO (AU)	0,002	0,0079
Tyrosine (AU)	0,002	0,0080
Indoxylsulfate (AU)	0,003	0,0117
Histidine/Histamine (AU)	0,003	0,0119
Glycolic acid (AU)	0,004	0,0131
Carnitine (AU)	0,004	0,0131
Guanidinoacetic acid (AU)	0,006	0,0187
Trimethylamine (AU)	0,010	0,0284
Histamine (AU)	0,031	0,073
Lactic acid (AU)	0,032	0,074
2-methylglutaric acid (AU)	0,037	0,085
ATP (AU)	0,041	0,093
Indoxylsulfate (AU)	0,049	0,11
Arginine (AU)	0,057	0,11

Citric acid (AU)	0,07	0,14
Dimethylamine (AU)	0,15	0,26
Succinic acid (AU)	0,21	0,33
Methylguanidine (AU)	0,35	0,51
Hypoxanthine (AU)	0,38	0,55
Valine (AU)	0,40	0,57
Taurine (AU)	0,44	0,61
Histidine/Histamine (AU)	0,52	0,68
Creatine (AU)	0,60	0,75
Isoleucine (AU)	0,60	0,75
Indoxylsulfate (AU)	0,61	0,75
Urea (AU)	0,66	0,77
3-Hydroxybutyric acid (AU)	0,70	0,80
4-Hydroxyphenylacetic acid (AU)	0,82	0,88
2-Hydroxyisobutyric acid (AU)	0,87	0,92
Valine (AU)	0,99	1,00

GC-TOF-MS

2,3-Dihydroxybutanoic acid (AU)	<0.001	<0.001
3-Methylglutaconic acid v	<0.001	<0.001
p-Hydroxyphenyl-ethane-diol (AU)	<0.001	0,0012
Methylsuccinate (AU)	<0.001	0,0022
Citric acid (AU)	0,004	0,0127
2-Hydroxyisobutyric acid (AU)	0,007	0,0195
4-Hydroxybenzene acetate (AU)	0,011	0,0300
Succinate (AU)	0,011	0,0300
3-Hydroxyisobutyric acid (AU)	0,014	0,0362
3-Hydroxyisovaleric acid (AU)	0,045	0,10
5-Hydroxyindole (AU)	0,049	0,11
m-Cresol (AU)	0,051	0,11
Homovanillic acid (AU)	0,068	0,13
Maleic acid (AU)	0,08	0,16
Lactic acid (AU)	0,12	0,22
1H-Indole-3-acetic acid (AU)	0,13	0,23
2-Hydroxyglutaric acid (AU)	0,15	0,26

Hippuric acid (AU)	0,16	0,27
Pyroglutamic acid (AU)	0,20	0,33
Analyte 280 (AU)	0,29	0,45
Phosphate (AU)	0,31	0,47
cis-Aconitic acid (AU)	0,32	0,48
Glycerol (AU)	0,45	0,63
Monostearyl glycerol (AU)	0,48	0,64
Lyxofuranose (AU)	0,64	0,77
N-acetylglycine (AU)	0,65	0,77
Pyrocatechol (AU)	0,69	0,80
4-Hydroxybenzoate (AU)	0,76	0,85
Glycolic acid (AU)	0,80	0,88
Carbamate (AU)	0,92	0,97
Oxalic acid (AU)	0,94	0,97
Trigonelline (AU)	1,00	1,00
LCMS		
Hydroxyproline (AU)	<0.001	<0.001
Glycine (AU)	<0.001	0,0019
Serine (AU)	0,001	0,0034
Histidine (AU)	0,001	0,0049
Glutamine (AU)	0,003	0,0102
GABA (AU)	0,004	0,0131
C0-acylcarnitine (AU)	0,004	0,0131
Methionine (AU)	0,004	0,0131
Alanine (AU)	0,006	0,0187
C2-acylcarnitine (AU)	0,009	0,0237
Arginine (AU)	0,012	0,0308
C3-acylcarnitine (AU)	0,016	0,0403
Cysteine (AU)	0,017	0,0423
Asparagine (AU)	0,050	0,11
Pyroglutamic acid (AU)	0,06	0,11
2-aminoadipic acid (AU)	0,12	0,22
Valine (AU)	0,15	0,26
Ornithine (AU)	0,20	0,33

Tyrosine (AU)	0,20	0,33
C12-acylcarntine (AU)	0,22	0,35
Citrulline (AU)	0,31	0,47
Lysine (AU)	0,320	0,48
Phenylalanine (AU)	0,34	0,51
Leucine/Isoleucine (AU)	0,43	0,61
Glutamic acid (AU)	0,46	0,64
C5-acylcarntine (AU)	0,49	0,65
C10-acylcarntine (AU)	0,51	0,67
Aspartic acid (AU)	0,58	0,75
Beta-alanine (AU)	0,60	0,75
Isothreonine (AU)	0,66	0,77
Tryptophan (AU)	0,68	0,80
Proline (AU)	0,70	0,80
Creatine (AU)	0,81	0,88
C6-acylcarntine (AU)	0,94	0,97
Threonine (AU)	0,94	0,97
C8-acylcarntine (AU)	0,97	0,99
C4-acylcarntine (AU)	0,98	1,00

Abbreviations: NMR – nuclear magnetic resonance, GC-TOF-MS – gas chromatography-time of flight-mass spectrometry, LCMS – liquid chromatography tandem mass spectrometry, C – carbon units, TMA – trimethylamine, TMAO – trimethylamine-N-oxide, ATP – adenosine triphosphate, GABA – gamma-aminobutyric acid.

Table S2: Effect size of urinary metabolites with a false discovery rate $q \leq 0.05$

Metabolite	Effect size (d)
NMR	
Erythronic acid (AU)	0.37
Histidine_Histamine (AU)	0.39
Taurine/TMAO (AU)	0.33
Tyrosine (AU)	0.41
Glycolic acid (AU)	0.35
Indoxysulfate (AU)	0.43
TMAO (AU)	0.38
Phenylacetylglycine (AU)	0.50
Acetic acid (AU)	0.41
Xanthine (AU)	0.54
Hippuric acid (1) (AU)	0.57
Glutamine (AU)	0.49
Formic acid (AU)	0.57
Pyruvic acid (AU)	0.64
Methylmalonic acid (AU)	0.61
Glycine (AU)	0.66
1-methylnicotinamide (AU)	0.60
Alanine (AU)	0.65
Trigonelline (AU)	0.71
Dimethylglycine (AU)	0.90
3-Hydroxyisovaleric acid (AU)	0.99
GC-TOF-MS	
Citric acid (AU)	0.40
Methylsuccinate (AU)	0.45
2-Methyl-3-Hydroxybutaric acid (AU)	0.46
3-Methyl-Hexanedioic acid (AU)	0.49
p-Hydroxyphenyl-ethane-diol (AU)	0.47
4-Hydroxyhippuric acid (AU)	0.47
2,3-Dihydroxybutanoic acid (AU)	0.55
2,5-Furandicarboxylic acid (AU)	0.55
3-Methylglutaconic acid (AU)	0.68

3-Methylglutaric acid (AU)	0.68
2-Hydroxyphenylacetate (AU)	0.69
Ethylmalonic acid (AU)	0.67
3-Hydroxyphenylacetate (AU)	0.73
Malonic acid (AU)	0.90

LCMS

Glutamine (AU)	0.53
Glycine (AU)	0.57
Histidine (AU)	0.55
Methionine (AU)	0.53
Serine (AU)	0.58
Hydroxyproline (AU)	0.71

Abbreviations: NMR – nuclear magnetic resonance, GC-TOF-MS – gas chromatography-time of flight-mass spectrometry, LCMS – liquid chromatography tandem mass spectrometry, C – carbon units, TMA – trimethylamine, TMAO – trimethylamine-N-oxide, AU – arbitrary units.

Chapter 4

Summary of main findings

4.1 Introduction

In this chapter, the main findings of this study are summarised and the hypotheses set out in Chapter 1 are reviewed. Recommendations for future research regarding the link between left ventricular mass index (LVMI) and urinary metabolomics are also provided.

4.2 Summary of main findings

Univariate and multivariate statistical analyses were used to identify five from 192 metabolites to differ between the black and white group and to associate with LVMI. The identified metabolites included glycine, serine, hydroxyproline, methionine and trimethylamine and were more abundant in the black group compared to the white group. We found inverse associations between LVMI with the identified metabolites in the black group only. In the following section, we will address the main findings of this study according to the original hypotheses set out for a study population (n=160) including black and white South Africans.

4.2.1 Hypotheses

Hypothesis 1: The most prominent urinary metabolites will be statistically identified in black and white South Africans.

We accept our first hypothesis as through various univariate statistical analyses, five metabolites, namely glycine, serine, hydroxyproline, methionine and trimethylamine were identified to be the most prominent metabolites relating to LVMI. Univariate statistical analyses included independent t-test (adjusted for multiple comparisons), effect size ($d \geq 0.3$) and single regression analyses.

Hypothesis 2: Multiple urinary metabolites will differ between black and white South Africans.

We also accept our second hypothesis, as we identified 5 from 192 metabolites including glycine, serine, hydroxyproline, methionine and trimethylamine, which were all more abundant in the black group compared to the white group. A few other metabolites also differed significantly between the groups, but did not associate with LVMI.

Hypothesis 3: Black South Africans will have higher LVMI than white South Africans.

We reject our third hypothesis, as LVMI were similar between the young and healthy groups. We do however propose compensatory changes already occurring on metabolomic level to aid in preventing an increase in LVM in the black group.

Hypothesis 4: Adverse associations will exist between LVMi and urinary metabolites in both black and white South Africans.

We reject our fourth hypothesis, as it was demonstrated that only in the black group, LVMi associated inversely with glycine, serine, hydroxyproline, methionine and trimethylamine, which may contribute to cardioprotective mechanisms.

4.3 Comparison to relevant literature

Metabolomic studies on young and healthy populations with a known predisposition for cardiovascular disease are sparse. Since we made use of hypothesis-generating work, comparing the findings of this study with results from other studies revealed some results to contradict or confirm previous findings, where other findings of this study are completely novel and contribute to the gaps in the literature.

Black populations are known to have an increased LVM compared to other populations (1, 2). Considering the fact that our study showed similar results for LVMi between the black and white groups, this result contradicts the findings of these studies (1, 2). This may be as a result of the young and healthy inclusion criteria of our study population with no overt or apparent cardiovascular manifestations. Since inverse associations between LVMi and the identified amino acids were only demonstrated in the black group, we propose compensatory changes already arising on metabolomic level to prevent or delay an increase in LVMi in the black group. These inverse associations between LVMi and identified amino acids in the black group have not been established elsewhere and future studies are needed to confirm or oppose these findings.

4.4 Discussion of main findings

We demonstrated that, only in the black group, urinary metabolites glycine, serine, hydroxyproline, methionine and trimethylamine were more abundant compared to those of their white counterparts, with inverse associations between LVMi and these amino acids. The black group also demonstrated low animal protein, vitamin C and energy intake, with almost half of the black group (48%) being protein deficient.

Hydroxyproline, glycine (3, 4) and vitamin C (5) perform pivotal roles in the regulation of collagen synthesis and stability. Studies on low protein diets have shown that low protein intake impairs collagen synthesis (6) and decrease the expression of genes associated with collagen synthesis (7). We therefore propose that the biosynthesis of hydroxyproline and glycine are up-regulated to

maintain the homeostasis of collagen synthesis and stability as a result of low vitamin C and protein intake in the black group.

Glycine (4), serine (8) and methionine (9) are fundamental precursors in the synthesis of glutathione, which were more abundant in the black group compared to the white group. We suggest that the synthesis of these metabolites may be to increase the synthesis of glutathione to prevent an increase in reactive oxygen species, known to be higher in the black populations (10-13).

The black group demonstrated more abundant levels of glycine, methionine, creatine kinase and lower dietary glucose and energy intake. We hypothesise that the black group is in greater need of stored energy in the heart, through the creatine pathway known to be the most vital energy reserve in the heart (14). Furthermore, we suggest that the biosynthesis of glycine, methionine and serine are up-regulated in an attempt to maintain a constant availability of adenosine triphosphate in the heart in a state of lower glucose availability. However, we failed to establish a direct link between creatine kinase and LVMI or the five identified urinary metabolites.

The black group had a higher plant protein intake compared to the white group. We propose that the increased urinary trimethylamine is a result of a decrease in flavin-containing monooxygenases (FMO3) which catalyse the conversion of TMA to TMAO. We therefore hypothesise that the inverse correlation between LVMI and trimethylamine is due to the decrease in the detrimental effects of trimethylamine-N-oxide on the cardiovascular system (15, 16).

4.5 Limitations, strengths, chance and confounding factors

It is important to reflect on factors that may have affected the results of this cross-sectional study.

A cross-sectional study only identifies the health of the population at a certain point in time. Therefore, our study only exhibits the health and associations of the black and white groups, which cannot declare any causality. Participant recruitment was done in all the surrounding areas of Potchefstroom in the North-West Province, including both rural and urban communities. Therefore, this study is not representative of the general South African population. Nevertheless, the African-PREDICT study was thoroughly designed and followed a concise protocol.

This study made use three different state-of-the-art analytical techniques including liquid chromatography tandem mass spectrometry (LC-MS/MS), gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) and nuclear magnetic resonance (NMR) methods. We made

use of hypothesis-generating techniques, but the importance thereof should not be underestimated in contributing to future hypothesis-testing work. Biesecker (17) says, “The paradigm of hypothesis-generating research does not replace or undermine hypothesis-testing modes of research; instead, it complements them and has facilitated discoveries that may not have been possible without hypothesis-generating research.” To the best of our knowledge, this is the first study in South Africa to determine the associations between LVMi and urinary metabolites in young black and white adults.

Despite using uni- and multivariate regression analysis, the possibility of chance with regard to the results of this study should be taken into consideration. However, we limited the possibility of chance by adjusting for multiple comparisons. We included covariates based on bivariate correlations with LVMi and adjusted for the following: age, sex, C-reactive protein, plant protein intake, and 24-hour systolic blood pressure. Therefore, the adjustments made may have caused an over- or underestimation of the associations observed between LVMi and urinary metabolites. Nonetheless, consistent significant associations were found after taking various confounders into consideration. Even though our population size was relatively small ($n=160$), a power analysis done on the G*Power software indicated an alpha error probability of 0.05 and power at 95.00% (**Table 1**). Our sample size were therefore sufficient to generate our hypotheses.

Table 1: Power analysis report

F-tests – Linear multiple regression: Fixed model, R^2 deviation from zero		
Analysis: Post hoc: Compute achieved power		
Input:	Effect size f^2	= 0,2664115
	α err prob	= 0,05
	Total sample size	= 80
	Number of predictors	= 5
Output:	Noncentrality parameter λ	= 21,3129200
	Critical F	= 2,3382776
	Numerator df	= 5
	Denominator df	= 74
	Power (1- β err prob)	= 0,9500000

In regards to metabolomics, a low sample size may lead to lack of precision, where an unnecessarily large sample size may be a waste of resources for the amount of information gain (18). Commonly used statistical analysis techniques include univariate i.e. parameter-by-parameter fashion, such as independent t-tests and analysis of variance (ANOVA), and/ or multivariate techniques including principal component analysis (PCA and partial least square regression (PLS) (19). Evidence suggest that univariate approaches are better to use with small sample sizes (≤ 200 metabolites) than multivariate approaches (20). Since only 192 metabolites were measured in this study, we primarily made use of univariate analyses with multiple comparisons to identify the most prominent metabolites.

4.6 Conclusion

To conclude, we found urinary metabolites glycine, serine, methionine, hydroxyproline and trimethylamine to be more abundant in the black group compared to the white group. In the black group only, inverse associations between LVMi and the above named urinary amino acids were described. We propose the biosynthesis of these amino acids to be up-regulated in the black group to maintain the homeostasis of collagen synthesis and stability, a healthy redox state and energy storage in the heart in a state of protein deficiency, low vitamin C and energy intake to prevent a premature increase in LVMi.

4.7 Recommendations

Since our study is the first to identify the inverse associations between LVMi and urinary amino

acids, future comparative population samples are needed to explore, confirm or oppose these findings. Also, due to the expensive nature of omics, this study only included the metabolomics data of 160 participants and comparatively larger population samples are needed to explore the link between LVMi and urinary metabolites. The long-term effects of decreased animal and overall protein intake on the cardiac structure, especially the left ventricle, should be evaluated in longitudinal analyses. Future longitudinal studies are needed to explore if associations between LVM and urinary metabolites change over a period of time. Future studies are needed to focus on metabolites involved in specific pathways, such as collagen turnover, glutathione and creatine synthesis, and to explore associations of cardiac structure measures with these pathways.

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Appendix A: Ethics approval certificate for African-PREDICT and this current study



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**Faculty of Health Sciences Ethics
Office for Research, Training
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31 March 2018

Dear Prof Schutte

FEEDBACK ON HREC ANNUAL MONITORING REPORT: NWU-00001-12-A1

We would like to thank you for submitting the annual monitoring report for your project entitled, "**African-PREDICT study (African Prospective study for the Early Detection and Identification of Cardiovascular disease and hyperTension)**", to the Health Research Ethics Committee (HREC) in a timely manner. Please find below the decision of the HREC regarding the continuation of your project.

Classification	Mark with X	Comment	
Clarification			
Completion (Final report)			
Suspended			
Continuation	X	Date of next monitoring report:	31 March 2019
Termination			

Should you have any further queries, please feel free to contact Ms Leanie van Ronge at your earliest convenience (E-mail: Ethics-HRECMonitoring@nwu.ac.za; Tel: 018 299 2197). We wish you well in your future endeavours.

Yours sincerely

Prof Minrie Greeff
Head of Health Sciences Ethics
Office for Research, Training and Support

Dr Wayne Towers
Chairperson: HREC

Current details: (23239522) G:\My Drive\9.1.5.5 HREC Monitoring\9.1.5.5 R.o.P\NWU-00001-12-A1\9.1.5.5.4_Cont_NWU-00001-12-A1_31-03-2018.docm
31 March 2018

File reference: 9.1.5.5.4



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**Health Sciences Ethics Office for Research,
Training and Support**

Health Research Ethics Committee (HREC)
Tel: 018-285 2291
Email: Wayne.Towers@nwu.ac.za

30 May 2018

Dear Prof Kruger

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

Ethics number: NWU-00029-18-S1

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

Study title: Left ventricular structure and urinary metabolomics in young adults: The African-PREDICT study

Study leader: Prof R Kruger

Student: D Erasmus-25348426

Application type: Single study

Risk level: Minimal (monitoring report required annually)

Expiry date: 31 May 2019

You are kindly informed that after review by the HREC, Faculty of Health Sciences, North-West University, your ethics approval application has been successful and was determined to fulfil all requirements for approval. Your study is approved for a year and may commence from 30 May 2018. 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. A monitoring report should be submitted two months prior to the reporting dates as indicated i.e. annually for minimal risk studies, six-monthly for medium risk studies and three-monthly for high risk studies, to ensure timely renewal of the study. A final report must be provided at completion of the study or the HREC, Faculty of Health Sciences must be notified if the study is temporarily suspended or terminated. The monitoring report template is obtainable from the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-HRECMonitoring@nwu.ac.za. Annually, a number of studies may be randomly selected for an internal audit.

The HREC, Faculty of Health Sciences requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the proposal or other associated documentation must be submitted to the HREC, Faculty of Health Sciences prior to implementing these changes. These requests should be submitted to Ethics-HRECAppl@nwu.ac.za with a cover letter with a specific subject title indicating, "Amendment request: NWU-XXXXX-XX-XX". The letter should include the title of the approved study, the names of the researchers involved, the nature of the amendment/s being made (indicating what changes have been made as well as where they have been made), which documents have been attached and any further explanation to clarify the amendment request being submitted. The amendments made should be indicated in **yellow highlight** in the amended documents. The *e-mail*, to which you attach the documents that you send, should have a *specific subject line* indicating that it is an amendment request e.g. "Amendment request: NWU-XXXXX-XX-XX". This e-mail should indicate the nature of the amendment. This submission will be handled via the expedited process.

Any adverse/unexpected/unforeseen events or incidents must be reported on either an adverse event report form or incident report form to Ethics-HRECIncident-SAE@nwu.ac.za. The *e-mail*, to which you attach the documents that you send, should have a specific subject line indicating that it is a notification of a serious adverse event or incident in a specific project e.g. "SAE/Incident notification: NWU-XXXXX-XX-XX". Please note that the HREC, Faculty of Health Sciences has the prerogative and authority to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.

The HREC, Faculty of Health Sciences complies with the South African National Health Act 61 (2003), the Regulations on Research with Human Participants (2014), the Ethics in Health Research: Principles, Structures and Processes (2015), the Belmont Report and the Declaration of Helsinki (2013).

We wish you the best as you conduct your research. If you have any questions or need further assistance, please contact the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-HRECAppl@nwu.ac.za.

Yours sincerely



Prof Wayne Towers
HREC Chairperson



Prof Minrie Greeff
Ethics Office Head

Current details: (23239522) G:\My Drive\9. Research and Postgraduate Education\9.1.5 Ethics\NWU-00029-18-S1\9.1.5.4.1_AL_NWU-00029-18-S1_30-05-2018.docm
30 May 2018

File reference: 9.1.5.4.1

Appendix B: Confirmation of language editing of the dissertation

This serves to confirm that I, Isabella Johanna Swart, registered with and accredited as professional translator by the South African Translators' Institute, registration number 1001128, language edited the following dissertation (excluding 19 pages of References).

Left ventricular mass index and urinary metabolomics in young black and white adults: The African-PREDICT study

by

Dalene Erasmus

A handwritten signature in black ink, appearing to read 'J. Swart' with a stylized, cursive flourish.

Dr Isabel J Swart

Date: 3 November 2018

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Appendix C: Turn-it-in originality report

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Appendix D: Solemn declaration and permission to submit



Higher Degrees Administration

SOLEMN DECLARATION AND PERMISSION TO SUBMIT

1. Solemn declaration by student

I, **Dalene Erasmus**

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

Left ventricular structure and urinary metabolomics in young adults: The African-PREDICT study

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

Master of Health Sciences in Cardiovascular Physiology

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

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

Signature of Student

University Number

25348426

Signed on this 13 day of November of 2018

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

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

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

Signature of Supervisor/Promoter

Prof Ruan Kruger
Digitally signed by Prof Ruan Kruger
Date: 2018.11.13 16:40:18 +02'00'

Date

13 November 2018