

Metabolomics of hypertension in South Africans: The SABPA Study

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Nothing happens in isolation

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Abstract

There has been growing concern in recent years about the alarmingly high prevalence and severity of hypertension and other cardiovascular diseases in individuals from newly (or recently) westernised countries such as South Africa. This is especially true for the Black ethnic group where higher average blood pressure values are seen, compared to their Caucasian counterparts. There is already an established connection between urbanisation and increased prevalence of lifestyle diseases in developed countries such as the USA. However, despite the global effort of clinicians and scientists investigating the aetiology of hypertension, regarding its involvement in cardio-metabolic disease no definitive biological mechanism has been elucidated, especially in the Black ethnic group of South Africa. This study thus aimed to investigate hypertension in Black and Caucasian South Africans in a holistic manner, utilising a metabolomics-based approach together with clinical data and targeted biochemical measured markers. Two metabolomics platforms were used to ensure wider metabolome coverage, namely gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). Study participants were divided into gender and ethnic groups and each group was further divided into quintiles according to average 24-hour ambulatory systolic blood pressure values. Only data from quintile 1 (normotensives) and quintile 5 (extreme hypertensives) were used in statistical analyses to ensure optimal separation between blood pressure groups. In the hypertensive Black males perturbations in several systems involved in ethanol metabolism were evident, being driven by a shifted global NADH/NAD⁺ ratio. Alterations in the bile acid metabolism of the hypertensive Black females were seen, while a more classical pre-diabetic insulin resistant state was observed in the hypertensive Caucasian females. In the hypertensive Caucasian males, disruptions in fatty acid metabolism and liver damage was evident, along with perturbations in detoxification systems. Obesity and perturbations in gut flora metabolism were evident in most of the hypertensive groups. Results from this study serve to demonstrate the power of applied metabolomics in the field of cardiovascular research, as novel metabolic pathways not previously associated with the pathogenesis of hypertension were found to be perturbed in hypertensives compared to their normotensive counterparts.

Key words

Cardiovascular disease; Cardio-metabolic disease; Hypertension; Metabolomics; South Africa

List of Abbreviations & Symbols

°C	degrees celsius
μ	micro
·OH	hydroxyl radical
¹ O ₂	singlet oxygen
4-HNE	4-hydroxy nonenal
ACE	angiotensin converting enzyme
ACTH	adrenocorticotrophic hormone
ADMA	asymmetric dimethyl-L-arginine
AGAT	arginine:glycine amidinotransferase
AGE	advanced glycation end product
AIDS	acquired immunodeficiency syndrome
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AMDIS	automated mass spectral deconvolution and identification software
amu	atomic mass units
AST	aspartate aminotransferase
AT-1	angiotensin-II receptor type 1
BCFA	branched chain fatty acid
BMI	body mass index
BSA	body surface area
BSTFA	O-bis(trimethylsilyl) trifluoroacetamide
CMD	cardio-metabolic disease
CRP	C-reactive protein
CV	coefficient of variance
CVD	cardiovascular disease
DBP	diastolic blood pressure
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
eV	electron volt
FRAP	ferric reducing antioxidant power

<i>g</i>	g-force (9.80665 m/s ²)
GABA	gamma-aminobutyric acid
GC-MS	gas chromatography-mass spectrometry
GGT	gamma-glutamyl transferase
GSH	reduced glutathione
H ₂ O ₂	hydrogen peroxide
HCl	hydrochloric acid
HDL	high density lipoprotein
HIV	human immunodeficiency virus
HMDB	human metabolome database
HPA	hypothalamic–pituitary–adrenal
HR	heart rate
ICAM-1	intercellular adhesion molecule 1
ID	identity document
IL	interleukin
IU	international units
LCFA	long chain fatty acid
LC-MS	liquid chromatography-mass spectrometry
QTOF	quadrupole-time-of-flight
TOF	time-of-flight
LDL	low density lipoprotein
LDL _{ox}	oxidised low density lipoprotein
m/z	mass-to-charge ratio
MCFA	medium chain fatty acid
MET-IDEA	metabolomics ion-based data extraction algorithm
min	minute
MOX	methoxyamine hydrochloride
MSTUS	mass spectrometry total useful signal
n	number
NAD ⁺	oxidised nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NAFLD	non-alcoholic fatty liver disease
NCD	non-communicable disease
NF-κB	nuclear factor kappaB

NHANES	national health and nutrition examination survey
NIST	national institute of standards and technology
NMN	β -nicotinamide mononucleotide
NMNAT	β -nicotinamide mononucleotide adenylyltransferase
NO	nitric oxide
O ₂ ⁻	superoxide anion
ONOO ⁻	peroxynitrite
PAI1	plasminogen activator inhibitor 1
PCA	principle component analysis
PLP	pyridoxal-5'-phosphate
PPAR γ	peroxisome proliferator-activated receptor gamma
PUFA	polyunsaturated fatty acid
Q	quintile
QC	quality control
RAAS	renin-angiotensin-aldosterone-system
RAGE	receptor for advanced glycation end products
RNS	reactive nitrogen species
ROS	reactive oxygen species
SABPA	sympathetic activity and ambulatory blood pressure in africans
SAH	s-adenosyl-L-homocysteine
SAM	s-adenosyl-L-methionine
SBP	systolic blood pressure
SD	standard deviation
SIRT1	sirtuin (silent mating type information regulation 2 homolog) 1 (<i>S. cerevisiae</i>)
SNP	single nucleotide polymorphism
SNS	sympathetic nervous system
T2D	type 2 diabetes
TCA	tricarboxylic acid
TH	tyrosine hydroxylase
TMCS	trimethylchlorosilane
TNF- α	tumour necrosis factor-alpha
VIP	variable importance in projection
VLDL	very low density lipoprotein
WC	waist circumference
WHO	world health organisation

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Chapter 1: Preface

1.1. Introduction

Growing concern surfaced in recent years about the cardiovascular health of individuals in newly (or recently) westernised countries such as South Africa. The problem of lifestyle related diseases, which include hypertension and Type 2 Diabetes (T2D), has been widely researched and reported on in countries such as the United States where obesity and physical inactivity, together with unhealthy diets, have been steadily increasing (Ford et al. 2014). If the situation in these countries can be seen as an example of what is to happen in recently urbanised countries, there is a need for both pro-active prevention as well as screening and early diagnosis in these populations.

Many reports exist on the high prevalence of hypertension in developing countries such as South Africa, with authorities battling to raise awareness of this complicated and often underdiagnosed risk factor (Crush et al. 2011; Lloyd-Sherlock et al. 2014). This is especially troublesome in populations of African descent where the incidence and severity of hypertension is already higher than in other ethnic groups. In various studies conducted in developed countries, researchers consistently report higher blood pressure values in African American participants (Huan et al. 2012; Fox et al. 2011). With the advent of urbanisation, the change in diet and physical activity, as well as an increase in psychosocial stressors, might exacerbate metabolic changes and already higher baseline blood pressure to dangerous levels. South Africa is a country with a variety of different populations and cultures in various stages of urbanisation from rural traditional regions to highly urbanised metropolitan centres.

1.2. Motivation for the study

Despite the global effort of clinicians and scientists investigating the aetiology of hypertension, regarding its involvement in cardio-metabolic disease no definitive biological answer has been obtained. However, as a result of landmark epidemiological studies, such as the Framingham study, various mechanisms responsible for elevations in blood pressure have been reported recently. Further confounding the search for the aetiology of hypertension is the fact that it can be seen as a disease state on its own, but is more likely interconnected with other risk factors of cardio-metabolic disease clouding the issue of cause-and-effect relationships. As blood

pressure is one of the most significant risk factors of cardio-metabolic syndrome, investigating the cause of the hypertension in the Black ethnic group is of high importance in elucidating the underlying cause of their unhealthy phenotypes compared to age-matched Caucasians.

Bearing this in mind, studies investigating the global central- and secondary metabolism as a whole are needed to get a systemic view of metabolic perturbations that cause hypertension, or arise because of hypertension. This could possibly lead to the clarification of novel metabolic mechanisms and pathways involved in hypertension. Analytical techniques that measure all small molecular end-products of metabolism present in a selected sample type, complementing the normal clinical and biochemical testing reported thus far in literature, are thus best suited for this type of investigations.

In this regard, the field of applied metabolomics is the technique of choice because it can be used to achieve rich metabolome information with minimum sample preparation required. Metabolomics techniques aim to measure the complete set of small molecule end-products of metabolism (metabolites) in a given biological tissue or fluid to explain the cause of often subtle differences between control and experimental groups following perturbation. Being a high-throughput and non-invasive technique with untargeted methods in data acquisition and data processing constantly being improved upon, it is a powerful scientific tool still in its infancy.

Metabolomics has however been around long enough for scientists to realise its potential for unlocking the answers to major biological scientific questions. Metabolomics methods have been used extensively in plant science, but has seen increasing popularity in clinical research using samples from cell cultures and laboratory animals. Metabolomics methods also offer a new paradigm for disease biomarker discovery, as they are not driven by any other prior biological hypotheses but by data, thus enabling investigators to detect events that could not have been anticipated from any biological reasoning. However, metabolomics investigations of human subjects are fundamentally more difficult as potentially countless variables and confounding factors are present. Thus, using metabolomics methods in conjunction with clinical measurements and biochemical marker testing would lead to information-rich datasets for more accurate biological interpretation. Also, experimental groups should be as homogenous as possible, thereby decreasing the risk of interference from confounding factors. Therefore, it was decided in the present study to investigate hypertension in ethnic- and gender groups separately to negate the influence that these two variables would inevitably have on

blood pressure, leaving experimental groups sufficiently homogenous for this metabolomics investigation to elucidate possible subtle metabolic disturbances, which could potentially have been masked if investigating all participants together in one group. This also enables the comparison of the groups with each other in terms of the results obtained from the metabolomics methods.

There is a lack of metabolomics-driven studies on the aetiology of the high incidence of hypertension in an urban-dwelling Black ethnic group in South Africa. Keeping this **problem statement** in mind, this study will aim to fill the knowledge gap in this much specialised and as yet under-utilised field of medical science, i.e. applied metabolomics in cardiovascular research in black and white Africans (hereafter referred to as Blacks and Caucasians). A more detailed breakdown of research aims and objectives will follow in Section 2.6.

By design metabolomics is a hypothesis-generating tool in science. Using information obtained from the current study, future investigations into hypertension in South Africans could possibly be guided into the development of therapeutic strategies targeting metabolic pathways not previously associated with this disease.

1.3. Structure of thesis

This thesis consists of eight chapters and three appendices and includes two peer-reviewed publications.

Chapter 2 details the current status of knowledge on the aetiology and pathophysiology of cardio-metabolic disease and its risk factors (including hypertension). The chapter also highlights the intertwined nature of the disease with no single factor contributing in isolation. Chapter 3 deals with the analytical approach followed in this study including a GC-MS metabolomics assay, two LC-MS assays, as well as data handling and statistical methods used. A peer-reviewed paper resulting from the study is presented in Chapter 4 and deals with the metabolomics investigation specifically in the Black male group. Chapter 5-7 consist of results obtained from the metabolomics approach followed in the remaining study groups, namely the Black females (Chapter 5), Caucasian males (Chapter 6) and Caucasian females (Chapter 7). In Chapter 8 results from the four main groups are compared and discussed, ending the thesis with concluding remarks and future research prospects potentially arising from the present study.

Additional information presented as appendices include the following: Firstly, Appendix A is a breakdown of the full SABPA study. As the present study is only part of the main SABPA study and only deals with baseline values and samples, the protocol given in this Appendix will focus only on information relevant to the present study. Secondly, Appendix B is a peer-reviewed publication investigating a genetic mutation associated with hypertension as investigated in the SABPA study cohort and referred to throughout the thesis. Lastly, a table consisting of all features significant in the separation between hypertensives (Q5) and normotensives (Q1) in all four study groups is presented in Appendix C.

1.4. Reference List

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Chapter 2: Literature Review

Hypertension is a disease with multiple causes, but hypertension arising from an unknown single causative factor (essential hypertension) is often associated with lifestyle-related cardiovascular risk factors, such as those included in cardio-metabolic disease, which will form the basis of this chapter.

2.1. Cardio-metabolic disease

2.1.1. Definition

Cardio-metabolic disease (CMD) is a broad term used to describe a collection of lifestyle-related metabolic abnormalities (such as glucose handling problems, dyslipidaemia, high blood pressure and central obesity) co-existing with a pro-thrombotic and pro-inflammatory state that results in a higher risk of developing atherosclerosis and other cardiovascular disease (CVD), as well as Type 2 Diabetes (T2D) (Vasudevan & Ballantyne 2005). It can thus be seen as a disorder involving many biological systems and global metabolic pathways of energy transport and utilisation. This pathological state that includes hypertension as risk factor can also be seen as a syndrome, owing to the additive clustering of its components. However in the current text the term ‘disease’ will be used to avoid confusion with ‘Metabolic Syndrome’, which has a specific definition and set criteria that does not include some of the factors that will be discussed in this text. The risk for coronary heart disease, stroke and myocardial infarction is also much higher in individuals presenting with this cluster of risk factors than those who do not (Isomaa et al. 2001). The risk factors contributing to this disease state often occur together and include lifestyle, anthropometric (Malan et al. 2008) and genetic factors (Govindarajan et al. 2005). Patients with CMD are phenotypically diverse and prominent differences exist between males and females in terms of the importance of one risk factor above another, thus no single risk factor can be taken alone as the most important in the aetiology of this disease state. There is also significant interplay and clustering between combinations of these factors (Betteridge 2004; Grundy 2007). Consequently, the addition of any one of the risk factors to the cluster will increase the overall risk.

It is therefore difficult to elucidate causal relationships in CMD. For example, in some cases it is difficult to determine if having other cardio-metabolic risk factors increases blood pressure

(and therefore the risk of developing hypertension as a disease on its own) or if an already established high blood pressure (as a result of another unrelated or unknown cause and together with one or more other CMD risk factors) increases the chances of developing CMD. Similar relationships exist between other risk factors of CMD (Chen et al. 2011; Li et al. 2013; Wahba & Mak 2007). It must be stated that having CMD does not confer absolute risk for CVD since there are various other diseases and factors, including age, that are not considered in the diagnosis of CMD. However, patients with the risk factors for CMD are twice as likely to develop CVD in the next five to ten years of their lives (Alberti et al. 2009). Diagnosing CMD, which is a serious and growing health problem in the developing world (Hamer & Malan 2012; Hamer et al. 2015; Mozumdar & Liguori 2011), is thus a very important first step in assessing the individual risk for CVD. For years, non-communicable diseases were seen as diseases of the rich but with the advent of urbanisation developing countries are experiencing this group of diseases more than ever. Common to all forms of non-communicable diseases are the risk factors, which will be discussed in the following sections.

Research into the aetiology and pathology has increased dramatically since the emergence of this disease. Although CMD is a topic of recent scientific interest and numerous research studies, its most practical use is as a reminder to physicians (and patients) that the presence of one risk factor should be a warning that other risk factors are also likely to be present. While the results from numerous studies have led to a better understanding of the disease and the implementation of various treatment strategies, the downstream cardiovascular effects of this clustering of risk factors remain among the leading causes of morbidity and mortality worldwide (WHO 2010). This fact highlights the need for further in-depth research into molecular mechanisms governing CMD.

2.1.2. Risk factors and pathophysiology

2.1.2.1. Lifestyle risk factors

Urban-dwelling lifestyle

Boutayeb & Boutayeb reported in 2005 that there has been an alarming switch in the leading cause of death worldwide from communicable (infectious) diseases to non-communicable diseases (NCD's) such as CVD. In a westernised environment there is a seemingly unavoidable shift in the lifestyles of the general population toward behaviours favouring the development of NCD's. Data published in the WHO global status report on NCD's in 2010 showed that the

leading risk factor globally is raised blood pressure (to which 13 % of global deaths are attributed), followed by tobacco use (9 %), hyperglycaemia (6 %), physical inactivity (6 %), and overweight/obesity (5 %). According to this report the risk for CMD (and ultimately, CVD) boils down to four modifiable lifestyle factors: smoking, physical inactivity, unhealthy diet and the harmful use of alcohol. The resulting metabolic consequences of these four factors are hypertension, overweight/obesity, hyperglycaemia (inclusive of insulin resistance) and dyslipidaemia. It is further stated that not only are NCD's the leading cause of death globally, but also that nearly 80 % of NCD deaths occur in low- and middle-income countries. These are developing countries recently urbanised or in the process of urbanisation such as sub-Saharan Africa, a region with one of the fastest rates of urbanisation. CMD can be seen as a disease of urbanisation, occurring more frequently in developing and developed countries (Lloyd-Sherlock et al. 2014).

In a study by Njelekela et al. (2003) it was found that differences in dietary habits as it relates to urbanisation contributed to the risk profile of people in Tanzania where the prevalence of various cardio-metabolic risk factors were lowest in rural areas. Among these differences would be an increased salt intake. This factor has been continually seen in urbanised diets and salt sensitivity is also considered to be the hallmark of hypertension in Black individuals (Richardson et al. 2013). According to Hendriks et al. (2012) some of the cardio-metabolic risk factors such as hypertension are seen more in urban areas than rural areas, a finding shared with Addo et al. (2007). This trend is observed not only in other parts of the developing world, but is also very much present in South Africa (Malan et al. 1992; van Rooyen et al. 2002; Malan et al. 2008). In a study by Steyn et al. (1997) it was seen that those who spent larger parts of their lives in urban settings were more likely to have unhealthier lifestyles compared with their less urbanised counterparts. Years ago, hypertension in rural areas was relatively rare; however, recent findings indicate that the prevalence of this risk factor is even increasing in rural areas suggesting that urbanisation not only consists of changes in geographical location, but also includes the effect of progression of time on populations of the same region (Vaidya et al. 2012).

South Africa is a multi-ethnic society with a large range of cultures and lifestyles at different stages of urbanisation. However, CVD is a major cause of morbidity and mortality in all these groups. Indeed, Dalal et al. (2011) reviewed the available literature on the occurrence of non-communicable diseases in Sub-Saharan Africa and found high prevalence of all forms of CVD in this setting.

Furthermore, hypertension rates were similar in males and females but males were more likely to be smokers and females were more frequently classified as obese. Motala et al. (2011) investigated the prevalence of cardio-metabolic risk factors in a rural Zulu community in Kwa-Zulu Natal. They found a higher prevalence of risk profiles than in studies from other African countries and that gender difference in risk profiles exist. In women, obesity conferred greater risk than blood pressure, which in turn was the greatest risk predictor in men. A high waist circumference (WC) is also associated with the prevalence of CMD in participants from the SABPA urban-dwelling cohort from South Africa (Hoebel et al. 2014). Although in African cultures a fuller figure with a large waistline has traditionally been seen as a status symbol, the rapid rate of urbanisation (and the accompanying increased cardiovascular risk) carries with it insecurities and disruption in socio-economic relationships and could contribute to cognitive distress or a perception of own poorer well-being (Botha et al. 2012).

The incidence and prevalence of CMD reflects global changes in behaviour and lifestyle, which in turn favour the development of obesity and cardio-metabolic disorders.

Other factors along with age, which contribute to the risk for CMD, are genetic factors such as gender and ethnicity. A detailed discussion of each risk factor, as well as connections to other risk factors will follow.

Overweight and Obesity

Although each population differs in the aetiology of the disease state, many researchers believe the occurrence of central (visceral) obesity to be a good starting point (Nesto, 2005; Després 2006; Fezeu et al. 2007) associated with modern lifestyles. The worldwide prevalence of overweight and obesity has increased dramatically over the last few decades and correlates with the pattern of westernisation seen in developing and developed countries. According to Hossain et al. (2007) obesity rates have tripled in the past twenty years in newly westernised (developing) countries involving overconsumption of cheap, energy dense food and a decline in physical activity. Obesity is thus an established problem in developed countries such as America, but an emerging problem in developing countries, suggesting a connection of unhealthy diet and lifestyle with the phenomenon of urbanisation (Malan et al. 2008).

To understand the contribution that overweight/obesity has to the risk of CMD one has to first understand that there are various deposits of adipose tissue in the body divided into specific localised depots with differences in structural organisation, cell size, and biological function.

These depots range from subcutaneous to visceral adipose tissue according to depth; and distribution patterns include abdominal (upper body) and femoral-gluteal (lower body) patterns. The cardiovascular risk of overweight and obesity are more related to body fat distribution rather than total body fat (Després 2012). Abdominal fat distribution is seen more in males and femoral-gluteal is seen more in females (Ross et al. 1994; Demerath et al. 2007).

The adipose depots most associated with an increased risk of developing CMD are visceral and ectopic fat deposits (Bjørndal et al. 2011). Research suggests adipose tissue accumulation in the relevant depots are not merely energy storage in the form of adipocytes, but that adipose tissue is a metabolically active organ (Kershaw & Flier 2004; Galic et al. 2010) with other cell types present such as pre-adipocytes, lymphocytes, macrophages, fibroblasts and vascular cells. Visceral fat depots (consisting of white adipose tissue) are the most metabolically active and thus the depots that confer the highest risk for developing CMD (Ibrahim 2010). This depot is also most frequently connected with or implicated in other risk factors for CMD (Einstein et al. 2005).

Adipocytes release a range of signalling molecules (known as adipokines) with important regulatory functions. Leptin was the first adipokine discovered (Zhang et al. 1994) and is an important modulator of maintenance of energy homeostasis in central and peripheral tissues and serves to initiate a negative feedback loop to suppress appetite and further energy intake. However, leptin levels correlate with total body fat and higher circulating levels of leptin have been observed in obese or overweight subjects (Simonds & Crowley 2013). Leptin also has other functions such as stimulating the production of nitric oxide (NO), which is a vasodilator and important regulator of blood pressure. This seemingly contradictory function of leptin has gained much attention in research and it seems that the vasodilatory function of leptin via NO is attenuated in the obese individual, suggesting a resistance to the effects of leptin, similar to the insulin resistance seen in diabetic individuals (Schinzari et al. 2013).

Adiponectin, also produced by fat cells, is normally present in high amounts in the circulation of healthy humans but levels decrease in individuals with obesity, showing inverse correlation with visceral adipose tissue mass in adults. In a study by Koh and colleagues in 2010 it was seen that adiponectin levels were inversely correlated with metabolic syndrome in non-diabetic patients. When controlling for body mass index (BMI) and fat mass, individuals with higher visceral adipose tissue have lower adiponectin levels than those with less visceral adipose tissue (Bacha et al. 2004). Results from studies in mice show that long term caloric restriction increased adiponectin and insulin sensitivity (Combs et al. 2003). Adiponectin improves the

ability of insulin to suppress hepatic glucose output and adiponectin knockout mice show severe insulin resistance when given a high fat diet (Kubota et al. 2002).

Adiponectin has anti-inflammatory properties (in contrast to most other adipokines) such as decreasing the expression of tumour necrosis factor- α (TNF- α) and inhibition of macrophage to foam cell progression. Plasminogen activator inhibitor 1 (PAI1), an inhibitor of fibrinolysis, is an adipokine that is up regulated in visceral adipose depots in obesity, suggestive of a mechanistic link between obesity and thrombotic disorders (Ouchi et al. 2011; Correia & Haynes 2006). Adipose tissue also produces other pro-inflammatory signalling molecules such as interleukin-6 and -8, as well as TNF- α , and ongoing research has resulted in the continued discovery of even more adipokines released by adipose tissue (Mattu & Randeve 2013).

Furthermore visceral adipose tissue has increased sensitivity to the lipolytic effects of catecholamines and glucocorticoids, which promote the release of free fatty acids, some oxidised derivatives of which can stimulate aldosterone production (Goodfriend et al. 2004). Adipocytes may also produce aldosterone, thereby contributing directly to the systemic aldosterone levels (Calhoun & Sharma 2010) and activating the renin-angiotensin-aldosterone system (RAAS). This may cause increased peripheral vascular resistance and after-load on the heart contributing to hypertension (Castro et al. 2003; Redon et al. 2009). In addition, increased free fatty acid delivery to the liver (via the portal vein situated close to visceral fat depots) can stimulate hepatic very low-density lipoprotein (VLDL) and triglyceride production, leading to dyslipidaemia. Visceral adipose depots also increase hepatic insulin resistance, probably by means of increased fatty acid influx into the liver, suppressing insulin action. This in turn stimulates gluconeogenesis and raises hepatic glucose output (Sun & Lazar 2013). In addition, free fatty acid flux from visceral adipose tissue could be harmful to the liver, inducing insulin resistance.

Further evidence for the role of obesity in the development of CMD comes from caloric restriction studies in which long term reduction of energy intake results in reduction of risk scores for CMD. In a study by Barzilai et al. in 1998 it was found that caloric restriction increased insulin sensitivity by decreasing visceral fat in adult rodents compared with ad libitum feeding. In a review by Anderson & Weindruch (2009) it was stated that altered mitochondrial energy metabolism, enhanced sensitivity of insulin signalling and increased circulating levels of adiponectin are all associated with the positive outcomes of caloric restriction interventions.

An interesting set of phenotypes worth noting is that of the “normal weight but metabolically obese”, and the so-called “obese but metabolically healthy” groups. These phenotypes are seemingly contradictory to the notion that increased fat storage *in itself* promotes the risk for developing CVD. Indeed, many individuals present with normal (or even underweight) BMI, but still exhibit many risk factors of CMD. A plausible reason for this phenomenon can simply be the inadequacy of using BMI as the only measure of adiposity as many individuals with normal range BMI’s have high fat percentage, which is normally seen as an indicator of internal fat storage (visceral fat). In a 2010 study by Romero-Corral et al. the normal weight obesity phenotype was investigated. The authors found that higher fat percentage is associated with higher risk, even in normal range BMI subjects. Predictive models of cardiovascular risk also performed similarly when using fat percentage or WC, suggesting that WC is a better clinical measure of metabolic obesity than BMI as accurate body fat percentage measurement is not always possible in the clinical setting. WC is a better predictor of cardiovascular risk than BMI although differences exist between genders and also between different ethnicities (Botha et al. 2013). For example, Black men and women have lower ratios of intra-peritoneal fat to subcutaneous abdominal fat than their Caucasian counterparts (Grundy et al. 2013). Camhi et al. (2011) share this where it was seen that Black individuals have lower levels of visceral fat compared to Caucasians. However, it has been stated that there is heterogeneity in intra-peritoneal fat depot size for any given level of obesity (Grundy et al. 2013).

The lack of ethnic-specific cut-points for central obesity in prospective studies was addressed in a previous publication on the SABPA study cohort and an ethnic specific WC cut-point model for Black Africans was proposed (Botha et al. 2013). The model was validated by utilising diagnostic tests and non-linear analyses. Furthermore support for the validated ethnic-specific WC cut point model (Black men, ≥ 90 cm; -women, ≥ 98 cm) was associated with cognitive emotional distress and sub-clinical atherosclerosis (Botha et al. 2012). Gender differences in fat distribution also exist, where it has been stated in literature that visceral fat accounts for up to 10–20 % of total fat in men and only 5–8 % in women (Wajchenberg 2000).

It is well recognised that individuals with the same (or similar) BMI’s can present with very diverse metabolic features such as glucose tolerance, lipid profile and blood pressure. In a recent meta-analysis by Kramer et al. (2013) several longitudinal and cross-sectional studies were examined to determine the effect of metabolic status on all-cause mortality and cardiovascular events in individuals with BMI’s ranging from normal to overweight to obese. The authors concluded that there is no “healthy” pattern of overweight, that excess weight is

associated in the short term with the development of subclinical disease and that this leads to CVD in the long term (Kramer et al. 2013).

It is thus clear that body fat percentage and distribution are important predictors of the risk for cardiovascular events but cannot completely explain total risk. Thus a multitude of other factors (metabolic- or otherwise) must be taken into account when assessing metabolic health.

Smoking

Other lifestyle factors associated with increased risk for CMD include tobacco smoke and high intake of alcoholic beverages. Tobacco smoking is the most important avoidable established cause of morbidity and mortality and mere exposure to tobacco smoke is now a proven cause of cancer, cardiovascular, respiratory and other diseases (U.S. Department of Health and Human Services 2014). Even exposure to second hand smoke is implicated in the development of several forms of CVD (Dunbar et al. 2013). Smoking rates are associated with income, educational achievement and ethnicity (Ames et al. 2010; Patterson et al. 2004; Wetter et al. 2005), where individuals with lower socio-economic standing tend to smoke more. While there is a decline in tobacco use in most developed countries, an increase is seen in developing countries. The increasing prevalence of female smokers is of particular concern in many countries (Thun et al. 2012) including South Africa (Peer et al. 2013). While the smoking prevalence currently seen in females is lower than that of males they are projected to rise in many low- and middle-income countries (Hitchman & Fong 2011).

In a recent report by Thun et al. in 2013, examining the smoking trends and smoking-related mortality in the United States over the last fifty years, the authors reported large persistent increases in the risks of smoking-related deaths among female cigarette smokers related to an increase in the number of female smokers. They concluded that, in relative terms, the risks for females now equal those for males. Smoking prevalence also differs between ethnic groups. In a recent case-control study by Sitas et al. in 2013 the authors examined 481 640 South African notifications of death at ages 35–74 years between 1999 and 2007. Observational findings included that the highest smoking-attributed mortality rates in both males and females were in the mixed race (Coloured) group. This group also exhibited the longest constant trend of high smoking rates. Furthermore the lowest smoking-attributed mortality rates were seen in the Black group.

During smoking blood pressure and heart rate increase. Even exposure to second-hand smoke can have deleterious effects on microvasculature and it has been demonstrated that asymmetrical dimethyl arginine (ADMA) levels are elevated, even after cessation of exposure (Argatcha et al. 2008). ADMA is created during protein methylation and is formed from S-adenosyl methionine (SAM), an intermediate in the homocysteine pathway. Interestingly, homocysteine has been implicated as a marker for various forms of CVD such as atherosclerosis, and ADMA has been suggested as possible link between elevated homocysteine levels and endothelial dysfunction (Stühlinger & Stanger 2005). In a meta-analysis by Christen and colleagues in 2000 it was found that homocysteine levels were elevated in 30-90 % of patients with atherosclerotic vascular disease compared to controls. The production and release of ADMA (which is an endogenous inhibitor of NO synthase) by endothelial cells is also elevated by LDL and LDL_{ox}, suggesting a role for ADMA in endothelial dysfunction and atherosclerosis (Böger et al. 2000; Reimann et al. 2013).

Although lower smoking rates were reported historically in black populations of South Africa, the prevalence seems to be increasing in young South African Black urban males, as reported by Peer et al. (2013). In this paper the authors aimed to elucidate differences in several modifiable risk factors such as tobacco and alcohol use, diet and physical activity between urban-rural and male-female groups of young Black South Africans. Data from two studies between 1998 and 2003 were pooled. Main findings included that in males the prevalence of smoking and problem-drinking were high and increased with age. Smoking rates, along with several other risk factors for CMD, were also higher in urban youth compared to their rural counterparts, further highlighting the importance of urbanisation in the development of CMD.

Alcohol use

Consumption of alcoholic beverages in more than recommended quantities hold elevated risks of many diseases such as CVD, alcohol-related liver diseases, etc. There are several established health risks of chronic heavy alcohol use. Alcoholic beverages are high in calories but low in nutrients and alcoholics frequently suffer from malnutrition and anaemia, which can be multifactorial (Lewis et al. 2007). Indeed, Kopczyńska et al. (2003) examined homocysteine, folic acid and vitamin B12 concentrations in 71 male alcoholics. Serum homocysteine concentration was significantly higher and serum folic acid concentration was lower in alcoholic men than in the control group.

Ethanol metabolism can result in a sharp increase in Reactive Oxygen Species (ROS) production and increased alcohol use has been implicated in the development of a systemic oxidative stress status (Albano 2006; Rendón-Ramírez et al. 2013), which *in itself* has been implicated in cancer formation. In a recent review by Ambade & Mandrekar in 2012 the authors indeed investigated whether oxidative stress and inflammation are key players in the development of alcoholic liver disease. They found that oxidative stress in pro-inflammatory signalling and macrophage activation during liver injury cause a positive feedback mechanism in alcoholic liver disease. Furthermore, in alcoholic neuropathy chronic alcohol use leads to nerve damage (Chopra & Tiwari 2011).

Alcohol use has also been associated with various cardiovascular risk factors such as high blood pressure and hypertension (Klatsky & Gunderson 2008; Puddey & Beilin 2006) increasing the risk of stroke (Hillbom et al. 2011). The connection between alcohol use and raised blood pressure has indeed been well established and was first suggested in a study by Lian in 1915. In the last century various epidemiologic studies have found an association between alcohol use and hypertension (Klatsky 1996). The specific biological mechanisms through which alcohol interacts with the cardiovascular and other systems to raise blood pressure are not fully understood, but several mechanisms have been proposed that may play a role. These include increases in sympathetic output, stimulation of the RAAS, increased oxidative stress and endothelial dysfunction (Husain et al. 2014; Marchi et al. 2014). Alcohol decreases the sensitivity of the body's blood pressure sensors and thereby increases sympathetic outflow, resulting in increased cardiac load. In a paper by Wakabayashi in 2007 the author reported a hyperpulsatile or widened pulse pressure in heavy drinkers compared with their age-matched counterparts. The greater increase in blood pressure may support the proposed mechanism of alcohol-induced hypertension concerning increased sympathetic outflow. Ethanol is also a central nervous system depressant and a diuretic, triggering higher metabolic demands (Hastedt et al. 2013).

Previous studies have also demonstrated that alcohol consumption up-regulates the hypothalamus-pituitary-adrenal (HPA) axis. A study by Lee et al. in 2011 found an association between alcohol-mediated increases in brain catecholamines and the stimulation of the HPA axis. Alcohol use can also stimulate the RAAS, increasing the release of renin and aldosterone, either of which may cause systemic arterial vasoconstriction (Husain et al. 2014). Further mechanisms proposed include increased cortisol levels, increased vascular reactivity due to deregulated calcium levels, as well as endothelial dysfunction because of NO destruction by

ROS (Puddey et al. 2001; Slattery et al. 2015). Alcohol intake has of course also been widely associated with ROS, free radical damage and oxidative stress. Alcohol metabolism leads to increased ROS production via enzymatic and non-enzymatic pathways, possibly also involving lipid peroxidation leading to liver damage (Wu & Cederbaum 2003; Albano 2006).

In a recent meta-analysis of hypertension in low- and middle-income countries South Africa rates as one of the countries with the highest hypertension prevalence rates with alcohol abuse being one of the most significant predictors for hypertension in Blacks (Lloyd-Sherlock et al. 2014).

Long-term alcohol use can contribute to the development of hypertension although most of the deleterious effects of alcohol are only largely seen with higher consumption (Shield et al. 2013). The risk for various forms of cardiovascular pathologies resulting from alcohol use forms a “J-curve”, with lowest risk seen in moderate alcohol use and intermediate risk seen in abstainers (Costanzo et al. 2010). This suggests that alcohol may have beneficial properties at lower consumption although this relationship is complex and varies by age, gender and ethnicity (Roerecke & Rehm 2012; Kerr et al. 2011). Moderate alcohol intake has indeed been associated with protection against coronary heart disease (Klatsky 2010). A possible mechanism of this protection is alcohol-induced higher HDL levels. In a paper by e Silva et al. in 2000 the authors tested the hypotheses that alcohol intake can increase levels of HDL. They found that the raise in HDL levels correlated with the amount of alcohol taken and that this increase was possibly caused by an increase in the transport rate of Apolipoprotein AI and II as measured in vivo by turnover of these proteins.

In contrast a study by Fuchs et al. (2001) showed that there seems to be no protective effect of low to moderate alcohol consumption in the Black South African male, where the risk of incident hypertension over 3 years was higher in all alcohol consumption groups. In a 2009 systematic review and meta-analysis by Taylor et al. it was concluded that the risk for hypertension increases linearly with alcohol consumption. Regarding alcohol consumption in South Africans Hamer et al. (2011) found that Black men showed a higher consumption of alcohol than their Caucasian counterparts and that this was connected to depressed heart variability and sub-clinical vascular disease prevalence in this group (Hamer et al. 2011; Malan et al. 2013). This difference was also completely neutralised after adjusting for conventional and behavioural risk factors suggesting that the high prevalence of CMD among Blacks could be due to various modifiable risk factors including a poor diet rich in salt and saturated fats, minimum physical exercise, increased tobacco smoke and chronic consumption of alcohol.

2.1.2.2. Other factors

Ethnicity and gender

Ethnic- and gender differences in risk factors for CMD are seen frequently in literature. For example, compared with Caucasians Asians tend to have a higher prevalence of CVD at lower BMI, which may be due to the tendency of Asians to have abdominal obesity (the so-called apple shape) and increased incidence of diabetes (Prasad et al. 2011). The following results emerged in an analysis by Ervin and colleagues in 2009 on the prevalence of metabolic syndrome and its risk factors on a subset of participants (3 423 male and female Caucasian, Mexican-American and African-American adults) from the National Health and Nutrition Examination Survey (NHANES): abdominal (visceral) obesity and hypertension were the most frequently occurring risk factors in all groups, while Mexican-American females had a higher prevalence of low HDL cholesterol than either of the other two race and ethnic groups. The prevalence of metabolic syndrome increased with each succeeding age group for both sexes. Women also tend to be more obese than men while men tend to be more hypertensive (Ervin et al. 2009).

There is clearly a range of ethnic- and gender differences in terms of prevalence and severity, of the risk factors for CMD. Hoebel and colleagues found in 2011 that Black men had the highest incidence of metabolic syndrome when excluding diabetics. Obesity prevalence was highest among Black women regardless of socio-economic status but microalbuminuria, commonly used as predictor of renal- and endothelial dysfunction, is seen more frequently in Blacks than Caucasians (Lindhorst et al. 2007).

Hormonal influences

Sex hormones also play an important role in the risk for CMD, with testosterone being frequently associated with higher blood pressure in literature (Huisman et al. 2006; Ziemens et al. 2013). In a study by Iliescu et al. in 2007 it was found that androgens increased blood pressure via an oxidative stress mechanism in a rat model of hypertension. The role of oestrogens in blood pressure control in postmenopausal women have historically been controversial with some studies reporting beneficial characteristics and others detrimental, however blood pressure does rise and oestrogen levels decrease with increasing age, becoming most prominent after menopause (Canoletta & Cagnacci 2014). However it was found recently that oestrogens, particularly estradiol, could be linked to increased risk for stroke and

myocardial infarction in postmenopausal women (Scarabin-Carré et al. 2012). Low testosterone levels are frequently accompanied by elevated estradiol levels. In a study by Malan et al. (2012) it was found that increased levels of estradiol might play a role in the development of subclinical kidney damage in men, as well as atherosclerosis in men presenting with low testosterone levels. It is thus evident that the role of sex hormones in blood pressure control is very complicated, being influenced by multiple factors. Other hormones such as cortisol have also been associated with elevated blood pressure. Cortisol is a stress hormone released in response to stress. This hormone influences various biological functions such as increasing blood glucose levels, suppressing the immune system and sensitising the body to the effects of catecholamines. Chronically elevated levels of cortisol have been associated with increased CVD risk (Manenschijn et al. 2013). It is also well known that this hormone increases with age (Woods et al. 2006) and is involved in elevated abdominal fat storage, especially in the postmenopausal woman and that this is linked to the decline of oestrogen during menopause.

Psychological stress influences

Apart from other physiological risk factors chronic exposure to emotional and psychological stress also raises cortisol levels and is also a risk factor for CVD and this is mostly mediated by elevated blood pressure response to stress (Meyburgh et al. 2012). This exaggerated response is particularly true for individuals of African descent (Mashele et al. 2010; Mashele et al. 2014). Chronic stress can lead to perturbations in the regulation of the HPA axis as evidenced by increased circulating levels of adrenocorticotrophic hormone (ACTH), cortisol and corticosterone (Grippe 2009; De Kock 2012). Meyburgh et al. (2012) found higher HPA axis activity in hypertensive Black Africans after exposure to controlled laboratory stressors. The participants showed elevated fasting cortisol levels favouring enhanced α -adrenergic vasoconstriction, predictive of sub-clinical atherosclerosis.

Genetic influences

Many genetic differences between ethnic groups exist that have an influence in the control of blood pressure and several genetic factors have been investigated for contribution to elevated blood pressure. Several genome-wide association studies in Caucasians have been reported, although this type of studies has not identified replicable results in individuals of African descent (Franceschini et al. 2013). Further Non et al. (2012) found that ethnic differences in

blood pressure might be better explained by differences in education than by genetic ancestry. However, one specific single nucleotide polymorphism (SNP) in the tyrosine hydroxylase (TH) gene was shown to have a significant influence on blood pressure. In a population-based study conducted by Nielsen et al. in 2010 it was found that the TH C-824T SNP influences blood pressure in the general Danish population, i.e., blood pressure was significantly lower in participants with the wild-type gene. This C-824T SNP present in the promoter region of the gene causes overexpression of the enzyme and, thus, higher levels of circulating catecholamines in the body (as TH catalyses the rate-limiting step in the catecholamine synthesis pathway, all downstream reactions are affected). This leads to over-stimulation of the sympathetic nervous system (SNS), increasing blood pressure (to be discussed in detail later in the chapter).

Thus, in the course of the investigation into the mechanisms of hypertension in the SABPA study cohort, the participants were screened for this mutation in order to investigate the possibility that this SNP is specifically contributing to the higher prevalence of hypertension in the Black group (Appendix B). Following logistic regression model building, no significant contribution of the SNP to higher blood pressure could be found in Black Africans. However, the sub-study evaluated all 409 participants together, dividing groups only according to the continuous variable of systolic blood pressure (van Deventer et al. 2013).

Dyslipidaemia

Dyslipidaemia is the abnormal concentrations of lipids and lipoproteins in the blood. It is characterised by higher plasma levels of less-dense lipoproteins and triglycerides and lower levels of HDL cholesterol. Oxysterols are oxidative derivatives of cholesterol and elevated levels are present in various risk factors of CMD, such as dyslipidaemia. These molecules are highly cytotoxic, suggesting an involvement of oxidative stress in many factors of the dyslipidaemic state (Mauldin et al. 2008). Various South African studies on Black population groups have historically reported lower prevalence of dyslipidaemia (Mollentze et al. 1995; Oelofse et al. 1996; Steyn et al. 1997). However, results from the Transition in Health during Urbanisation of South Africans (THUSA) study showed increased serum lipid levels in urban-dwelling Black South Africans (van Rooyen et al. 2000), suggesting an increase in the prevalence of dyslipidaemia in parallel with urbanisation. However, these findings could not

be reproduced in the SABPA cohort that presented with normal lipid levels albeit lower HDL levels in Blacks (De Kock et al. 2015).

As repeatedly seen in literature there is a strong involvement of both oxidative stress and/or hypertensive mechanisms in all of the risk factors contributing to CMD. Figure 2.1 shows a visual representation of the interplay between various factors and mechanisms associated with CMD. A detailed discussion on the possible mechanisms of interaction between oxidative stress and hypertension, as well as the contribution of each, will be included later in the chapter. Given the fact that CVD has become one of the most important contributors of global burden of disease, especially in developing countries, understanding the molecular mechanisms of its pathogenesis is of critical importance. Several possible molecular mechanisms for the aetiology of CMD (as risk factor for CVD) were suggested and extensively researched in the scientific community. These include activation of the RAAS, constant elevated sympathetic tone and insulin resistance mediated by various metabolic perturbations. A detailed discussion on these proposed mechanisms will follow.

2.2. Molecular mechanisms involved in CMD

2.2.1. Insulin resistance in CMD

The hallmark of CMD is the presence of insulin resistance, i.e. a decreased sensitivity or responsiveness of peripheral tissues to the metabolic action of insulin (Wilcox 2005). Insulin resistance per se is not T2D but rather a distinct condition possibly leading to end stage disease such as diabetes and atherosclerosis. In terms of pathophysiology insulin resistance involves primarily liver, adipose- and muscle tissue (Wilcox 2005). In response to the development of early insulin resistance the pancreas can often be stimulated to over-produce insulin, overcoming the insulin resistant state temporarily (Forbes & Cooper 2013). Unfortunately the compensatory hyperinsulinaemia may have deleterious effects on some tissues that are still insulin sensitive. The relationship between differential insulin sensitivity, hyperinsulinaemia and metabolic/clinical effects are complex and in many cases still being elucidated. For example, the hypertriglyceridaemia associated with insulin resistance appears to result from at least two defects: increased lipolysis and subsequent delivery of fatty acids to the liver due to insulin resistance in fat cells; and increased production of triglycerides in the liver (Vatner et al. 2015).

Obesity is closely associated with the development of insulin resistance in tissues such as skeletal muscle and the liver (Galic et al. 2010) though insulin resistance has a strong genetic component and not all insulin resistant individuals are overweight. Insulin resistance, compensatory hyperinsulinaemia, and elevated blood glucose are associated with atherosclerotic CVD. A rise in plasma insulin levels has been connected to increased SNS activity (Young et al. 2010). High SNS activity in turn stimulates renin production, raising blood pressure by means of increased renal sodium and water re-absorption and leading to volume expansion. However, aerobic exercise has been shown to improve insulin sensitivity and lower blood pressure among sedentary non-diabetic hypertensive subjects (Castro et al. 2003).

Evidence also suggests that insulin resistance is an inflammatory condition. Studies show correlations between markers of inflammation, such as IL-6 and C-reactive protein (CRP), and fasting insulin concentrations, suggesting that inflammation and insulin resistance might be causally related conditions (Hak et al. 1999; Pradhan et al. 2003) in the pathogenesis of CVD. Insulin has many beneficial properties including up-regulation of endothelial NO synthase. This in turn produces more NO, which is the mechanism whereby insulin increases blood flow to peripheral microcirculation. Insulin also inhibits platelet aggregation and suppresses the production of many inflammatory signalling molecules such as monocyte chemo attractant protein-1 and NF- κ B (Dandona et al. 2004) suggesting that insulin has anti-inflammatory properties. Insulin further enhances sensitisation of the SNS thereby increasing cardiac output to deliver glucose to peripheral tissues for utilisation (Deedwania 2011).

A high percentage of hypertensive patients are also insulin resistant independent of BMI and body fat distribution (Manrique et al. 2005). Impaired microvascular dilation has been associated with sub-optimal glucose uptake in skeletal muscle (resulting in insulin resistance) and also increased blood pressure through increased peripheral resistance, thus increasing blood pressure (Karaca et al. 2014).

2.2.2. The sympathetic nervous system (SNS) in CMD

The SNS is part of the autonomic nervous system along with the para-sympathetic nervous system, which serves to promote homeostasis of the body at rest. However, in times of emergency or perceived immediate danger the SNS serves to activate the fight-or-flight response by releasing various catecholamines, such as norepinephrine and epinephrine, into the

circulation (Parati & Esler 2012). This metabolic response readies the body for action by increasing blood flow to peripheral tissues decreasing gastrointestinal blood flow, dilating the pupils and increasing heart rate and cardiac output, thus increasing blood pressure. Many components of CMD are characterised by pronounced adrenergic hyperactivity.

Moreover, the sympatho-excitatory effect on hypertension and obesity are additive. Indeed, with every added risk factor in the cardio-metabolic risk cluster the urinary excretion of catecholamines increases (Lee et al. 2001). Chronic sympatho-excitation may be driven by high leptin levels derived from adipose tissue as studies showed that acute administration of leptin increase renal and lumbar sympathetic nerve activation in rats (Hilzendeger et al. 2012; Muntzel et al. 2012; Tanida et al. 2000). Constant activation of the SNS also increases renin secretion further contributing to hypertension.

2.2.3. The renin-angiotensin-aldosterone system (RAAS) in CMD

The RAAS is an enzyme cascade system in the body that functions as the most important regulator of long-term blood pressure and extracellular volume. This system mostly acts on the kidneys, influencing sodium and water retention and filtration rate. Changes in blood pressure are sensed by macula densa cells in the distal convoluted tubule. This can be as a result of changes in sodium concentration, pressure, volume or renal perfusion (Otte & Spier 2009). When, for example, a low sodium concentration is sensed, the kidneys release the enzyme renin into circulation, which cleaves angiotensinogen (released by the liver). The product, angiotensin I, is converted to the vasoactive peptide angiotensin II by the angiotensin-converting enzyme (ACE) found in the lungs. ACE also degrades bradykinin, needed for synthesis of NO, a potent vasodilator. Angiotensin II then binds to angiotensin-II receptor type 1 (AT-1) expressed on vascular endothelial cell surfaces, thereby reducing the synthesis of NO.

Reduced bioavailability of NO, together with the stimulation of the AT-1 receptors on smooth muscle cells, cause vasoconstriction. Stimulation of AT-1 receptors also causes adrenal glands to produce aldosterone. This hormone causes cells in the kidney to retain sodium and water, in exchange for potassium. The sodium and water retention, together with vasoconstriction causes blood pressure to rise. Other consequences of AT-1 stimulation include fibrosis, ROS formation, inflammation and activation of the SNS. In a recent paper by Hamer et al. in 2011 it was seen that elevated release of renin during a laboratory stressor test (in the form of a Stroop colour-word conflict chart) was associated with carotid intima-media thickness, a

marker of sub-clinical atherosclerosis. They further stated that this might be a potential mechanism of the increased burden of CVD in urban-dwelling Blacks.

The role of insulin on the RAAS was also implicated in the development of hypertension. Insulin enhances sodium re-absorption in the kidney via increased expression of sodium transporters, which results in a decrease in sodium excretion (Song et al. 2006). This effect could possibly contribute to the development of hypertension in hyperinsulinaemic individuals and these further highlights the deleterious effect of a raised blood insulin level on all insulin tissues and systems, which are still insulin sensitive.

In studies using animal models it has been reported that over-expression of Angiotensinogen (restricted to adipose tissue) can increase total body fat percentage (Guberman et al. 2013). Such animals also become hypertensive (Manrique et al. 2009) suggesting a role for components of the RAAS locally in adipose tissue growth enhancement, and systemically through loss of adequate blood pressure regulation. In a recent study by Kalupahana et al. in 2012 the authors investigated whether over-expression of angiotensinogen in adipose tissue can lead to systemic insulin resistance by means of inflammatory responses. It was found that adipose RAAS over-activation could cause glucose intolerance and systemic insulin resistance, possibly through reduced skeletal muscle glucose uptake. Increased levels of aldosterone are also frequently reported in insulin resistant overweight or obese individuals.

Aldosterone increases blood pressure through enhancement of sodium retention and also through mineralocorticoid receptor-mediated actions (Cooper et al. 2007). Furthermore, aldosterone can also activate NADPH oxidase to trigger oxidative stress (Johar et al. 2006).

2.2.4. Inflammation in CMD

The inflammatory response correlates with multiple metabolic markers including obesity, dyslipidaemia, hypertension and insulin resistance. Obesity causes changes in the cellular composition of the adipose tissue and infiltration of macrophages can occur, resulting in systemic inflammation and insulin resistance. Chronic inflammation is an important factor in the development of CMD and downstream morbidities such as diabetes and CVD. It is characterised by elevated levels of CRP. CRP, in conjunction with elevated TNF- α , interleukin-6 and interleukin-1B, has been demonstrated in patients with CMD and insulin resistance (Hallenbeck 2002; Hansson 2005; Indulekha et al. 2011; Park et al. 2008). T2D has also been

associated with a pro-inflammatory state in patients with obesity (Wellen & Hotamisligil 2005).

Elevated oxidative stress levels also lead to advanced glycation end products (AGE's) which induce inflammatory cytokines and pro-coagulants. These molecules promote plaque instability by accumulating at vulnerable sites along the vessel walls (Farahmand et al. 2003). It is well established that CVD has an inflammation component, but in a recent review by Després (2012) it was suggested that the inflammatory signals generated by visceral fat deposits could contribute to the progressive risk of CVD. The author reviewed various aspects of adiposity including distribution, morphology and function and stated that abdominal obesity (as determined by elevated WC) is the most frequent cause of the CMD state leading to CVD. He further stated that cardio-metabolic syndrome is rarely found in non-obese, active individuals, but rather in sedentary overweight/obese subjects. Major findings include the connection between most of the risk factors of CVD and visceral obesity.

Caloric overload (as is the case in Western diets) favours adipocyte hypertrophy (enlargement) over hyperplasia (increased cell division) (Laforest et al. 2015). The hypertrophic cells become inflamed and start to produce various inflammation markers which then spill over into the circulation and a systemic pro-inflammatory state follows. Evidence for the argument that visceral adipose tissue contribute to a systemic inflammatory state is seen in intervention studies where a diet and exercise induced negative energy balance has been shown to reduce CRP levels (Borel et al. 2012). Emanuela et al. (2012) also constructed a model based on the inflammatory activation in adipose tissue by increased TNF- α , interleukin-6 and lowered levels of adiponectin. These signalling molecules induce endothelial dysfunction, which in turn causes impaired NO production and elevated oxidative stress. Indeed, Matsuzawa-Nagata et al. (2008) demonstrated in a mouse model of high fat diet-induced obesity that the production of ROS precedes the development of insulin resistance. Inflammation of the endothelium is a process implicated in the early stages of atherosclerotic plaque formation and occurs in conjunction with a pro-thrombotic state.

2.2.5. Endothelial dysfunction in CMD

The endothelium is a cell layer situated between the wall of blood vessels and the blood stream that regulates local vasomotor tone and vascular wall proliferation processes by synthesizing and releasing various vasoactive factors known as endothelium-derived factors (Zhou et al.

2012). In normal conditions it responds accordingly by releasing substances that regulate angiogenesis, inflammation and haemostasis, as well as vascular tone and permeability (Félétou & Vanhoutte 2006). Specific endothelial functions include control of vascular tone, modulation of vascular structure by regulation of angiogenesis and proliferation, maintenance of a selective permeability barrier, regulation of lipid oxidation and mediation of immune responses (Bauer & Sotníková 2010; Stanger et al. 2001).

Endothelial dysfunction has been implicated in a number of cardiovascular perturbations and is generally considered an early event in the development of atherosclerosis (Endemann & Schiffrin 2004). It is defined as an impaired response to vasodilatory agents and is associated with a pro-inflammatory and pro-thrombotic state (Endemann & Schiffrin 2004). The most important vasodilatory agent released by the endothelium is NO. Impaired bioavailability of NO, which is due to inhibition of NO production and/or increased inactivation of NO by ROS, is considered the hallmark of endothelial dysfunction. It is well established that overweight/obesity is connected with endothelial dysfunction (Lobato et al. 2012; Kobayasi et al. 2010; Lteif et al. 2005). Indeed, Romero-Corral et al. (2010) investigated whether weight gain will influence endothelial function. In this case-control study one group of normal weight, healthy young volunteers was assigned to gain approximately 4 kg, while the control group gained no extra weight. Endothelial function was measured at baseline after fat gain (8 weeks), and again after fat loss (16 weeks), in the experimental group; and at baseline and 8 weeks in the control group. It was found that endothelial function was attenuated with even modest weight gain, and that increased visceral fat storage is a better predictor of endothelial function than subcutaneous fat. The authors postulated that mechanisms linking visceral adiposity to increased cardiovascular risk include higher levels of adipokines and pro-inflammatory molecules.

In a recent review by Lobato et al. (2012) mechanisms of endothelial dysfunction as it relates to obesity and hypertension were investigated. Endothelial dysfunction via reduced NO availability was caused by uncoupled endothelial NO synthase (eNOS) and increased NADPH oxidase activity, a finding shared by Uys et al. in 2014. When NOS is uncoupled electrons flowing from the reductase domain to the heme groups are diverted to molecular oxygen instead of to L-arginine, resulting in the formation of the superoxide anion (Sullivan & Pollock 2006).

A recently proposed topic of interest is the phenomenon of endoplasmic reticulum (ER) stress as a mechanism of endothelial dysfunction. The ER is the main site for synthesis and folding

of secreted and membrane-bound proteins. Stressors such as ROS can interfere with ER function, leading to accumulation of misfolded or unfolded proteins. This situation results in ER stress. When this happens the unfolded protein response is initiated to cope with the ER stress by first halting protein translation and increasing the expression of protein chaperones to aid in correct protein folding. However, if the response is not sufficient to cope with the cause of the ER stress the apoptotic pathway is activated (Adamopoulos et al. 2014).

Along with an oxidative environment high concentrations of glucose can also induce ER stress via the formation of advanced glycation end products (AGE's). These AGE's are highly reactive molecules that result from the irreversible reaction of glucose with, among other molecules, amino groups in proteins (Baumann 2012). Increased AGE levels have been implicated in hyperinsulinaemic and pro-inflammatory states, highlighting its importance in cardiovascular research (Basta et al. 2004). AGE act on receptors known as receptors of AGE (RAGE) situated in vascular tissue, which in turn can modulate several pathways in the cardiovascular system. AGE's are therefore not only markers, but also mediators of cardiovascular risk (Baumann 2012) and the AGE-RAGE axis seems to correlate with vascular damage processes.

2.3. Hypertension and oxidative stress in CMD – the common soil theory

2.3.1. Hypertension in CMD

High blood pressure is a leading cause of the global disease burden as investigated by Lim et al. in 2013 and a major independent risk factor for CVD. Hypertension is a multifactorial disease caused by dynamic interactions between genetic, physiological and environmental features (Helvacı et al. 2013; Yannoutsos et al. 2014). Interestingly, many of the possible pathogenic mechanisms involved in oxidative stress have also been implicated in the pathophysiology of essential hypertension, such as constant activation of the SNS, up-regulation of the RAAS, abnormal G protein-coupled receptor signalling, and chronic low-grade systemic inflammation (also associated with obesity) (Montezano & Touyz 2012).

Excessive weight gain and the progression to obesity is one of the key causes of hypertension and the hypertensive effect of overweight is potentiated by three main mechanisms. Firstly visceral adipose tissue physically compresses the kidneys, causing sodium and water retention. In a review and meta-analysis by Wang et al. (2008) it was proved that overweight and obesity are implicated in kidney disease and thus can contribute to hypertension. Secondly, chronic

activation of the RAAS (as discussed in Section 2.2.3) by adipocyte-derived signalling molecules can also contribute to chronic blood pressure elevation. In this regard chronic activation of mineralocorticoid receptors (partially independent of aldosterone) is of importance. Lastly, chronic excitation of sympathetic nerves and consequent hyperactivity of the SNS has been implicated in the pathophysiology of hypertension due to increased visceral fat mass. This is mainly potentiated by the action of the adipokine leptin on the central nervous system. Sustained obesity also causes target organ damage, such as cardiac hypertrophy and kidney damage (possibly via an inflammation-related mechanism), further augmenting hypertension and possibly making it resistant to treatment.

A high blood pressure is a very common component of CMD and close quantitative relationships exist between blood pressure values and other risk factors of the disease, regardless of what type of blood pressure measurement is reported (Mancia et al. 2007). Interestingly, the reported risk factors for high blood pressure mirror those listed for CMD, although high blood pressure itself is listed as an independent risk factor for CMD and other CVD's. As previously stated, the significant interplay between these sets of factors cannot be ignored and should always be considered in unison for risk assessment. Various authors report several mechanisms involved in the relationship between hypertension and the rest of the risk factors of CMD, including constant over-activation of the RAAS and SNS, an increase in oxidative stress levels, constant low-grade inflammation, impaired insulin-induced vasodilation and abnormal sodium handling in the kidneys (Betteridge 2004; Deedwania 2011; Ihm et al. 2012).

In a recent report by Peer et al. in 2013 the authors aimed to investigate the prevalence of hypertension in a black urban community in Cape Town and to determine the change in the hypertension rates between 1990 and 2008/09. In addition to already high prevalence rates in 1990, they found that the incidence of hypertension in this group increased significantly. They also concluded that hypertension is rarely seen in isolation, given the significantly higher prevalence of overweight/obesity, diabetes and dyslipidaemia among individuals with hypertension.

Although various studies and meta-analyses have been published on the subject, the enigma over the reasons for the higher prevalence and lower rate of control of hypertension among Black Africans remains to be deciphered.

2.3.2. Oxidative stress in CMD

Oxidative molecules such as ROS and reactive nitrogen species (RNS) are normal by-products of cell metabolism produced by a variety of enzymatic- and non-enzymatic pathways and also act as signal transduction molecules, participating in normal signalling cascades. Sources of ROS include NADPH-oxidases, mitochondrial enzymes, xanthine oxidases, and uncoupled NO- synthase. Forms of ROS include hydroxyl radical ($\cdot\text{OH}$), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), peroxynitrite (ONOO^-) and singlet oxygen ($^1\text{O}_2$). ONOO^- is formed when ROS reacts with the vasodilator, NO. In times of metabolic perturbation, an overproduction of oxidative molecules, together with inability of the body to compensate by increasing antioxidant defence, leads to a state of systemic or localized oxidative stress (Rodrigo et al. 2011). Oxidative free radicals are highly reactive and can bind to most cellular components and macromolecules, turning these molecules into free radicals themselves, thereby perpetuating a cycle of further free radical formation. ROS-mediated cell death can stimulate abnormal cardiac remodelling which leads to diabetic cardiomyopathy (Boudina & Abel 2007).

A common occurrence in most of the risk factors for CMD is an increase in oxidative stress status. It was indeed stated in a review by Ceriello & Motz in 2004 that clinical manifestations of risk factors, as well as the eventual CMD states, were accompanied by the presence of oxidative stress and that this systemic oxidative state is a possible pathogenic mechanism linking obesity and insulin resistance with CMD and eventually end-stage CVD and T2D. The link between oxidative stress and insulin resistance becomes evident in studies where exposure of cultured endothelial cells to glucose results in increased ROS production (Inoguchi et al. 2000).

Many animal studies rely on chemically induced disease states to investigate diseases of lifestyle. Streptozotocin is a naturally occurring chemical that is toxic to insulin producing cells of the pancreas and is often used in animal models to induce both types of diabetes, depending on the dose used. In a study by Ohkuwa et al. in 1995, it was seen that free radical production and blood glucose levels in the rats treated with streptozotocin were directly proportional to the amount of streptozotocin injected. Although the toxin itself also generates some ROS, many human studies have found that an overabundance of ROS is mechanistically associated with the multifactorial etiology of insulin resistance. Several in vitro studies and clinical studies have demonstrated that treatment with antioxidants can improve insulin sensitivity in insulin-resistant individuals. In a study by Vincent et al. (2009) it was found that antioxidant supplementation lowered measures of insulin resistance in overweight young adults by means

of lowered oxidative stress. Similarly, in a study by Udupa et al. in 2012 it was shown that supplementation with alpha lipoic acid, omega 3 fatty acids and vitamin E significantly reduced markers of oxidative stress and insulin resistance.

Oxidative stress is not only involved in insulin resistance and dyslipidaemia, but also in elevated blood pressure or hypertension, which is in itself a factor contributing to CMD risk. Oxidative stress has been shown to amplify blood pressure elevation in the presence of other pro-hypertensive factors, although hypertension also can contribute to the oxidative stress state (Montezano & Touyz 2012). It is not yet clear whether increased oxidative stress is a cause or consequence of high blood pressure as evidence for both scenarios exist in literature. In a study by Rajagopalan et al. (1996) it was seen that angiotensin-II mediated hypertension in rats, increased vascular ROS production via NADPH oxidase activation. Conversely, mice deficient in ROS generating enzymes have lower blood pressure and infusion of angiotensin-II fails to induce hypertension (Haque & Majid 2011; Grote et al. 2006). Possibly, a positive feedback mechanism is involved whereby damage from increased oxidative stress raises blood pressure, which in turn increases ROS production via various proposed mechanisms.

ROS can also act as signal transduction molecules whereby they activate various pro-inflammatory cytokines such as interleukin-8 and Intercellular Adhesion Molecule 1 (ICAM-1), mediating trans-endothelial migration of neutrophils as part of the inflammatory response (Roebuck 1999). Superoxide production in the mitochondrial oxidative phosphorylation complex I is governed by the NADH/NAD⁺ ratio. In normal circumstances when the ratio is low, superoxide production is also low, but in times of raised NADH/NAD⁺ ratio, superoxide production increases. This leads to a vicious cycle where oxidative damage decreases complex I efficiency even more, further stimulating excess superoxide production (Hirst 2013).

A paper by Chen et al. in 2008 discussed the role of ROS in endothelial dysfunction with particular focus on TNF- α . Since endothelial dysfunction and oxidative stress have been implicated in a myriad of diseases related to the cardiovascular system, the authors investigated a possible molecular mechanism that could explain the connection of both causes. They stated that TNF- α , an inflammatory cytokine, induces oxidative stress by elevating ROS production in endothelial cells, thus causing damage to these cells. This further perpetuates the interplay between different risk factors and metabolic perturbations leading to CMD, as TNF- α is also produced by adipose tissue and up-regulated by various pro-inflammatory states, such as hyperglycaemia.

Another detrimental action of ROS is the oxidation of the circulating LDL to form LDL_{ox}, which may play a key role in the pathogenesis of atherosclerosis by its uptake into macrophages, forming foam cells in the process, an initial step in atherosclerotic plaque formation (Oguntibeju et al. 2009). LDL_{ox} can also influence a range of other processes connected with increased CMD risk, including producing chemotactic proteins, increasing the expression of colony-stimulating factors, causing increased expression of adhesion molecules by endothelium cells and inhibiting vasodilation (Maiolino et al. 2013).

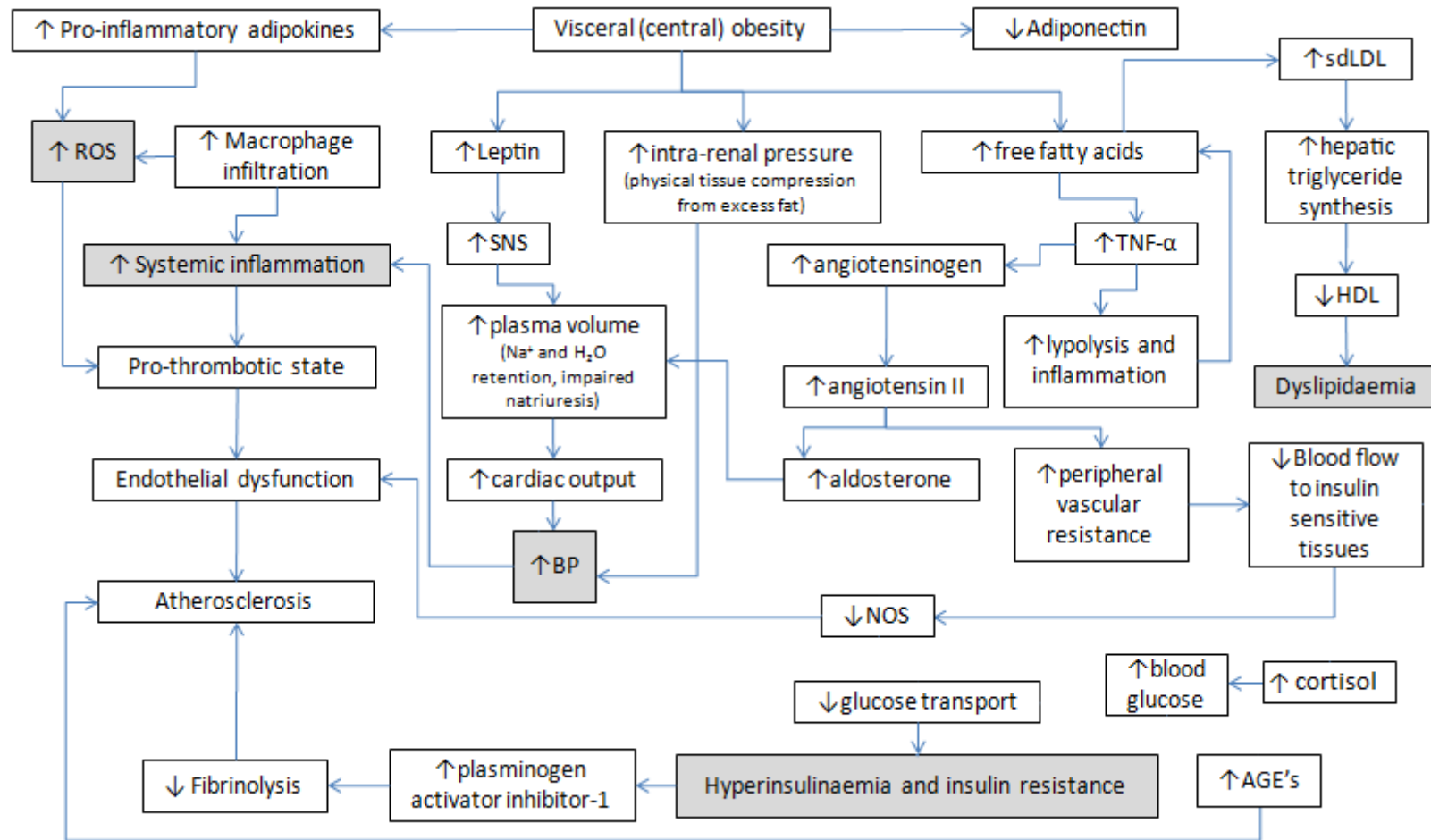


Figure 2.1: Visual representation of the significant interplay between various metabolic perturbations of CMD when looking at only one risk factor, i.e. Obesity. Grey blocks denote connections with other risk factors and oxidative stress involvement (adapted from Castro et al. 2003.). sdLDL, small dense low density lipoprotein; ROS, reactive oxygen species; HDL, high density lipoprotein; TNF- α , tumour necrosis factor alpha; SNS, sympathetic nervous system; BP, blood pressure; NOS, nitric oxide synthase; AGE's, advanced glycation end products. $\uparrow$ = increase; $\downarrow$ = decrease.

2.4. Cardio-metabolic disease and urbanisation

The burden of CMD, in particular the increased incidence of hypertension is evident in urban-dwelling Black South Africans, not surprisingly given the worldwide prevalence already discussed. South Africa is undergoing a demographic transition from rural traditional cultures to Western urban lifestyles (Malan et al. 2006, 2008; van Rooyen et al. 2002). The percentage of Blacks (the largest population group in SA) in urban areas has increased dramatically in the last twenty years. With this transition come increases in the risk for non-communicable diseases such as CMD (Vorster 2002), with elevated blood pressure one of the most important risk factors consistently seen in this population group. But is the increased prevalence of hypertension in the Black population compared to other ethnic groups simply a by-product of an urban lifestyle, or differences in genetics causing impaired biological responses to stress, or are there some lifestyle-related underlying metabolic perturbations in these individuals, increasing the severity of risk factors associated with CMD? The statistics reveal a lack of prevention, early detection and effective management of cardiovascular risk factors in SA and other developing countries (Yusuf et al. 2014).

Members of the scientific community generally agree that maintaining a healthy lifestyle by eating a healthy diet rich in fruit and vegetables, engaging in frequent sessions of moderate to high intensity physical exercise, not smoking and moderate intake of alcohol, would be beneficial in lowering the risk level for CMD in the long term. Optimal management of cardio-metabolic risk will require efforts on government, clinical, community and individual levels, monitoring risk factors and adapting interventions where applicable. Weight loss and lifestyle intervention studies have generally proven to be less effective on the long term though, possibly as a result of participants reverting back to 'pre-intervention' unhealthy lifestyle patterns (Pagoto & Appelhans 2013).

This is why this easily preventable disease is still a matter of vigorous and ongoing research by various groups around the world. Proper education on the importance of sustained healthy lifestyle habits is therefore important for future planned research on prevention strategies. According to Okafor (2012) a final set of cardio-metabolic risk factors for the Black South African population is difficult to define as there seem to be a lack of data applicable to this group. Studies on Blacks generally use definitions based on data from Western populations of developed countries and thus there is a substantial need for research on CMD among Blacks. Keeping this in mind, we therefore planned the current study to involve methods of research that would provide high-throughput, reliable measurements of as much of the metabolism as

possible to attempt to elucidate the mechanisms involved in the global perturbations seen in CMD. A metabolomics platform is optimally suited to this specific need and was thus preferred as methodological platform in this study.

2.5. Metabolomics in cardiovascular research

As stated in Chapter 1 the use of metabolomics methodologies is a powerful tool when applied to biological sciences. Indeed, the number of scientific publications employing metabolomics methods in biological investigations has shown a steep upwards trend in recent years (Duarte et al. 2014; Fillet & Frédérick 2015) along with ongoing advancement and changes in analytical aspects of this technique (Raterink et al. 2014; Sévin et al. 2015). Research papers include reports on elucidating underlying metabolic mechanisms in disease risk, diagnosis, progression and therapeutic intervention. Studies range from simple experimental vs. control group investigations to large-scale longitudinal clinical and epidemiological cohorts.

Metabolomics has also been used in studies investigating genetic aspects of CVD. In a paper by Then et al. (2013) the authors investigated metabolic alterations in carriers of a common genetic polymorphism in the transcription factor 7-like 2 gene associated with T2D. They found a genotype-mediated link to early metabolic abnormalities prior to the development of disturbed glucose tolerance. In a paper by Xie et al. in 2012 the authors reviewed metabolomics investigations in obesity. Main sites of metabolic perturbation were central energy metabolism pathways such as glycolysis and the Tricarboxylic Acid (TCA) cycle as evidenced by metabolites from classes such as organic acids, lipids and amino acids.

Recent publications on metabolomics investigations into various aspects of metabolic syndrome also exist (Scherer et al. 2015; Menni et al. 2015). In an article by Akira and colleagues in 2012 the authors investigated essential hypertension in spontaneously hypertensive rats by utilising a metabolomics approach. They found alterations in intestinal microflora populations; also the occurrence of metabolic acidosis in the urine of the hypertensive rats when compared with normal Wistar-Kyoto rats. In a recent review by Djekic et al. (2015) the authors summarise the role metabolomics played in atherosclerosis research. Main findings include alteration in pathways including amino acid metabolism (with increases in branched chain amino acids being implicated), decreases in TCA cycle intermediates and pyruvate, and elevated ketone bodies. However, the authors concluded that the current evidence in this field is limited.

Although papers were published recently on metabolomics applied to hypertension research in Black ethnic groups (Zheng et al. 2013) there is a distinct lack of reports focussing entirely on investigating this disease in the high risk Black ethnic group in South Africa.

2.6. Research aims and objectives

Some questions arise after reviewing literature on hypertension and its pathogenesis. Firstly, it is clear from literature that hypertension is a major burden in the Black population group of South Africa. However, there seems to be a distinct lack of research on the exact mechanism of this disease on specifically Black South Africans. Is there an as yet unknown metabolic contribution to increased blood pressure in this population group, or can the hypertension incidence be explained in part by all of the mechanisms discussed in this chapter? Furthermore, the brevity of metabolomics-driven studies in the investigation of CVD is also apparent.

2.6.1. Aim of this study

Therefore, the aim of this study was to investigate hypertension in South African Blacks and Caucasians (males and females separately, as discussed in Chapter 1) by utilising metabolomics-driven methodologies.

2.6.2. Specific objectives of this study

To accomplish the aim of this study the following objectives were set (a visual representation of the strategy and objectives of this study can be seen in Figure 2.2):

- Dividing blood pressure data from participants in the main ethnic- and gender groups into quintiles (representing 20 % of the data spread of the variable of interest, namely 24-hour ambulatory systolic blood pressure) separately. This will result in the maximization of subtle metabolic differences caused by increased blood pressure, leading to a more robust differentiation of the hypertensive experimental groups (quintile 5 – Q5) from the normotensive control groups (quintile 1 – Q1) by the metabolomics methodology used;
- Utilising two different metabolomics techniques – gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) – for broader metabolome coverage;

- Processing data obtained from clinical, biochemical- and metabolomics analyses, comparing Q5 with Q1 within each of the four main groups, constructing an all-inclusive single data matrix for each of the four groups separately;
- Performing feature selection and multivariate statistical analyses in the form of principle component analysis (PCA) in each of the four data matrices resulting in a list of variables important in the separation of Q5 and Q1 for each of the four groups;
- Biological interpretation and pathway analyses can then be performed, resulting in four metabolic maps representing the main metabolic perturbations seen in the four groups; and
- Finally comparing the metabolic perturbations to highlight possible similarities and/or differences between main study groups.

2.7. Study approach

This study forms part of the SABPA prospective cohort study. For a detailed description and protocol of the SABPA study the reader is referred to Appendix A. Briefly: The SABPA study was conceptualised to investigate the influence of a hyperactive nervous system on lifestyle diseases (such as CMD) in urban-dwelling Black and Caucasian South Africans. An urban lifestyle has been associated with increased incidence of CMD and downstream CVD events, as well as increased prevalence of risk factors for these diseases such as obesity, hyperglycaemia and hypertension. The aim of the SABPA study was therefore to aid in the successful treatment and eventual prevention of these lifestyle diseases in Africans.

The aim in the present study, as discussed above, is therefore relevant given the fact that raised blood pressure is seen as one of the most important predictors of future cardio-metabolic perturbations and downstream CVD events (Edwards et al. 2014). By investigating the metabolomes from these experimental groups, we aim to elucidate possible molecular mechanisms contributing to the deleterious effect of hypertension in these urban-dwelling individuals.

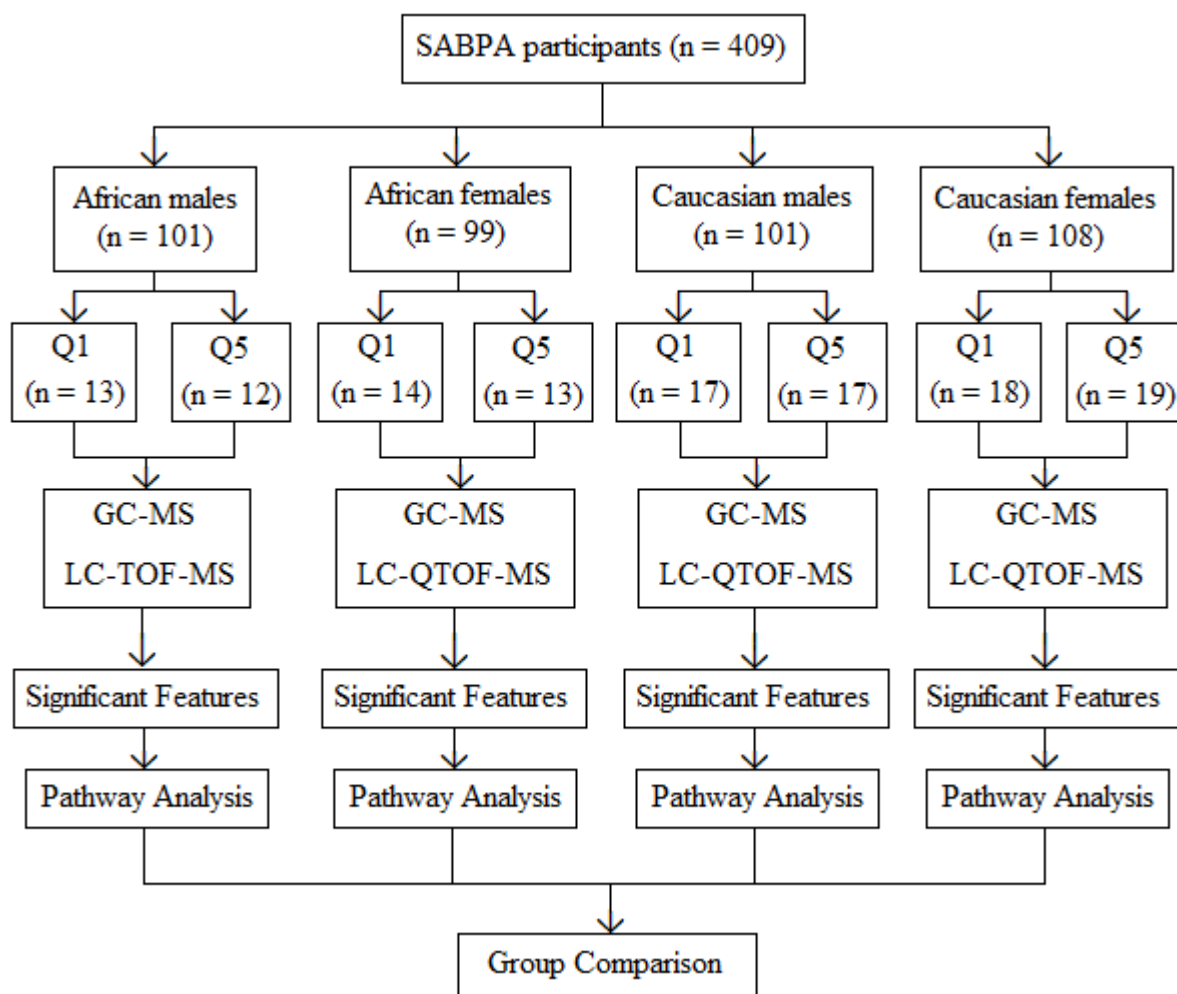


Figure 2.2: Flow diagram of analytical approach followed in this study. Participants divided into quintiles according to 24-hour ambulatory systolic blood pressure from lower normotensives (Q1) to extreme hypertensives (Q5). Significant features refer to metabolites from the metabolomics methods that differed significantly between Q5 and Q1, as well as variables that showed significant differences in Q5 compared to Q1 from clinical and anthropometric markers. These features were pooled for subsequent pathway analyses and biological interpretations on each group separately, after which the metabolic perturbations observed in the separate groups were compared and discussed in the final group comparison.

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Chapter 3: Analytical approach

3.1. Introduction

This study forms part of the Sympathetic Activity and ambulatory Blood Pressure in Africans (SABPA) study. For a detailed description of the main study the reader is referred to Appendix A. A metabolomics experiment is a powerful tool for biological investigations. However, there are numerous aspects of this technique to consider (as described in the flow diagram in Figure 3.1). After careful consideration of all these steps, urine was chosen as sample type as it is an easily obtainable and non-invasive sample available in large quantities.

In addition, urine represents the endpoint of metabolism and thus reflects the characteristics of the circulation as well as its excreted products. Urine also has substantially less protein content and less pre-treatment steps are needed when used in a metabolomics approach. As for the analytical considerations both gas chromatography (GC) and liquid chromatography (LC) separation techniques were used in conjunction with the very sensitive mass spectrometry (MS) detection, enabling the detection of a substantially larger portion of the metabolome. No single method exists that can detect and quantify the whole metabolome at once. This is because the metabolome comprises of metabolite classes of vastly different physicochemical properties and differences of concentrations ranging orders of magnitude. Thus, a dual method metabolomics approach is best suited.

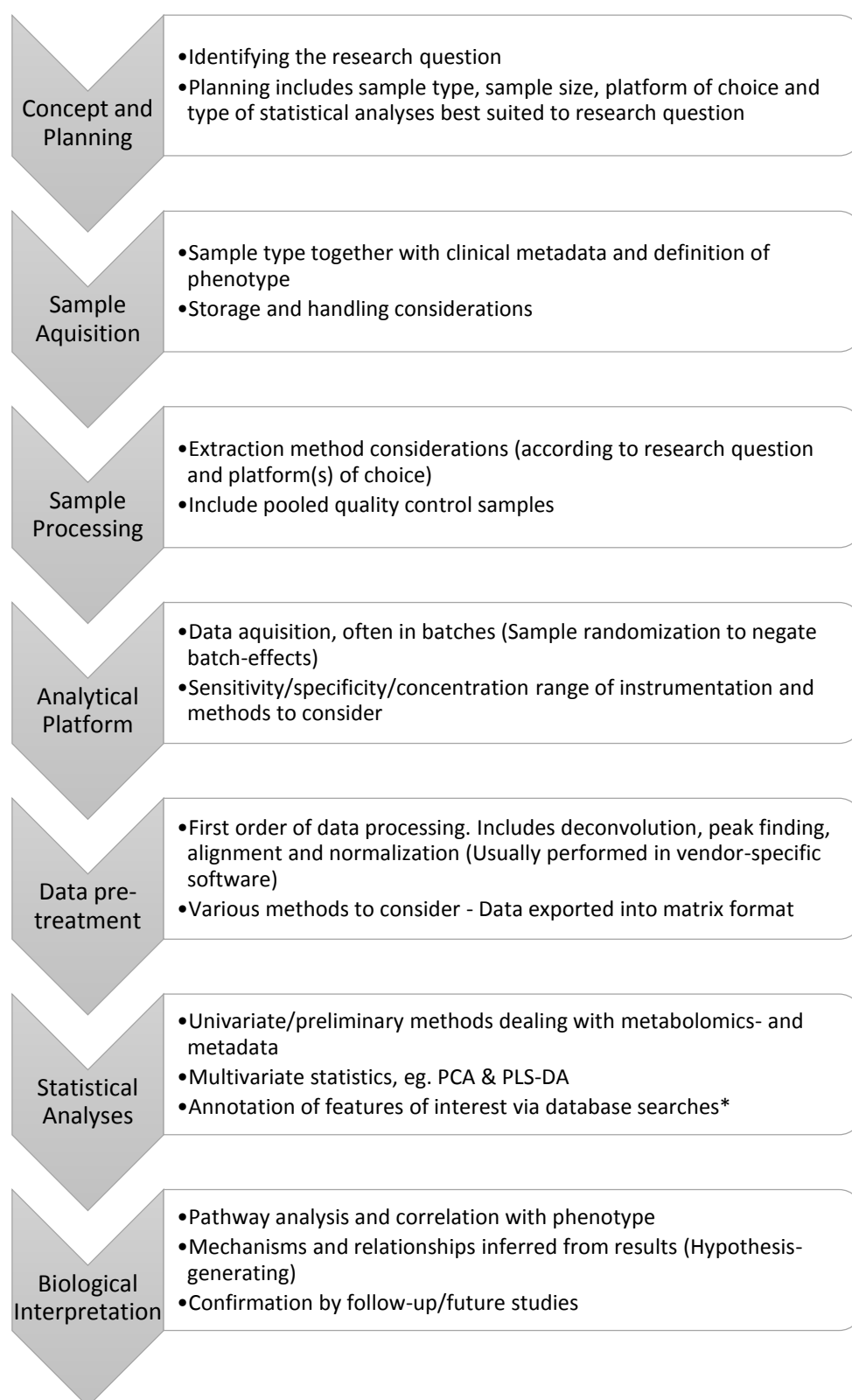


Figure 3.1: Workflow of a typical metabolomics experiment. *This represents the greatest bottleneck/rate-limiting step in the metabolomics field thus far. Identification of features only possible with subsequent MS-MS confirmation.

The study complied with all applicable institutional guidelines and terms of the Declaration of Helsinki of 1975 (as revised in 2004) for investigation of human participants and was approved by the Ethics Review Board of the North-West University, Potchefstroom Campus (00036-07-S6) (Malan et al. 2014). The nature, benefits, and risks of the study were explained to the participants in their mother tongue. Written informed consent was obtained from all participants before being included in the study.

3.2. Sample collection and preparation

First-void, fasting urine samples were used in this study as set out in the main study protocol (Appendix A). Samples were transported on ice to the laboratory where it was aliquotted into 1.5 mL polypropylene tubes and stored at -70 °C until use. A pooled quality control (QC) sample was prepared by adding aliquots from all the participants together. This QC sample was added on all batches to ensure repeatability. Samples were randomized and processed in batch-format adding a blank sample and QC sample to each batch.

3.3. Metabolic profiling methods

3.3.1. GC-MS Organic acid analysis

Organic acid extraction

In this study alternative GC-MS metabolomics sample preparation and derivatisation methods were investigated on test samples but yielded non-reproducible and unsatisfactory results (data not shown). These alternative methods investigated included simple urine deproteination, followed by evaporation and re-suspension in organic solvents. Test samples were then derivatised in a microwave-assisted derivatisation technique with an alternative derivatisation reagent, namely methoxyamine hydrochloride (MOX) in pyridine in an effort to shorten derivatisation time to mere minutes. However, further investigations into these non-reproducible methods were discarded; instead a standard, widely used organic acid extraction technique was followed for urine samples in this study (Chalmers & Lawson 1982). Firstly, aliquots of urine from each participant (along with an aliquot of the pooled QC sample) were thawed at room temperature, mixed and kept on ice during preparation. Batches were prepared by randomisation of participant numbers and adding a blank (pure hexane) and QC sample to each batch. The volume of urine to be used in the extraction was calculated for each sample

based on the previously determined creatinine concentration according to standard guidelines as can be seen in Table 3.1.

Table 3.1: Guidelines of starting volume of urine to be used in organic acid extraction method

Creatinine concentration (mmol/L)	Volume of urine used (mL)
> 8.8	0.5
0.44 – 8.8	1
0.18 – 0.44	2
< 0.18	3

The correct volume of urine for each sample was added to clean, numbered silanised glass tubes (Kimax) after which the internal standard (3-phenylbutyric acid - Aldrich, cat # 11680-7) was added to a final concentration of 180 mmol/mol creatinine. The samples were then acidified with approximately 5-6 drops of 5 N HCl, ensuring that the pH level was less than two. The liquid-liquid extraction started by addition of 6 mL of ethylacetate, followed by shaking of the mixture on a rotary wheel for 20 minutes. After centrifugation at $\approx 2\,000 \times g$ for 3 minutes, the upper organic phase was transferred to a clean silanised glass tube, numbered accordingly. To the water phase, 3 mL of diethyl ether was added and the mixture was shaken on the rotary wheel for a further 10 minutes. After a subsequent centrifugation step at $\approx 2\,000 \times g$ for 3 minutes, the upper organic phase was added to the organic phase from the first extraction step. Approximately 100 mg sodium sulphate was added to the organic phase mixture and the tube vortexed for 30 seconds to remove any residual water. After a final centrifugation step at $\approx 2\,000 \times g$ for 3 minutes the organic phase mixture was transferred to a clean silanised glass tube, numbered accordingly and evaporated to dryness under a constant stream of nitrogen at 37 °C. To the dried organic acids, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and pyridine were added in a ratio of 5:1:1. The exact volumes of these reagents to be added to each sample was calculated based on the creatinine value. Firstly, the creatinine value (mmol/L) was converted to mg %; the amount of derivatisation reagents as follows: $\text{mg \% creatinine} \times 3 = \text{BSTFA (mL)}$ and $\text{mg \% creatinine} \times 0.6 = \text{TMCS (mL)}$. The samples were derivatised at 70 °C for 45 minutes in a temperature-controlled sand bath, after which the derivatised mixtures were transferred to 2 mL glass vials with tapered glass inserts and sealed with new septa until analysis.

GC-MS analysis of urinary organic acids

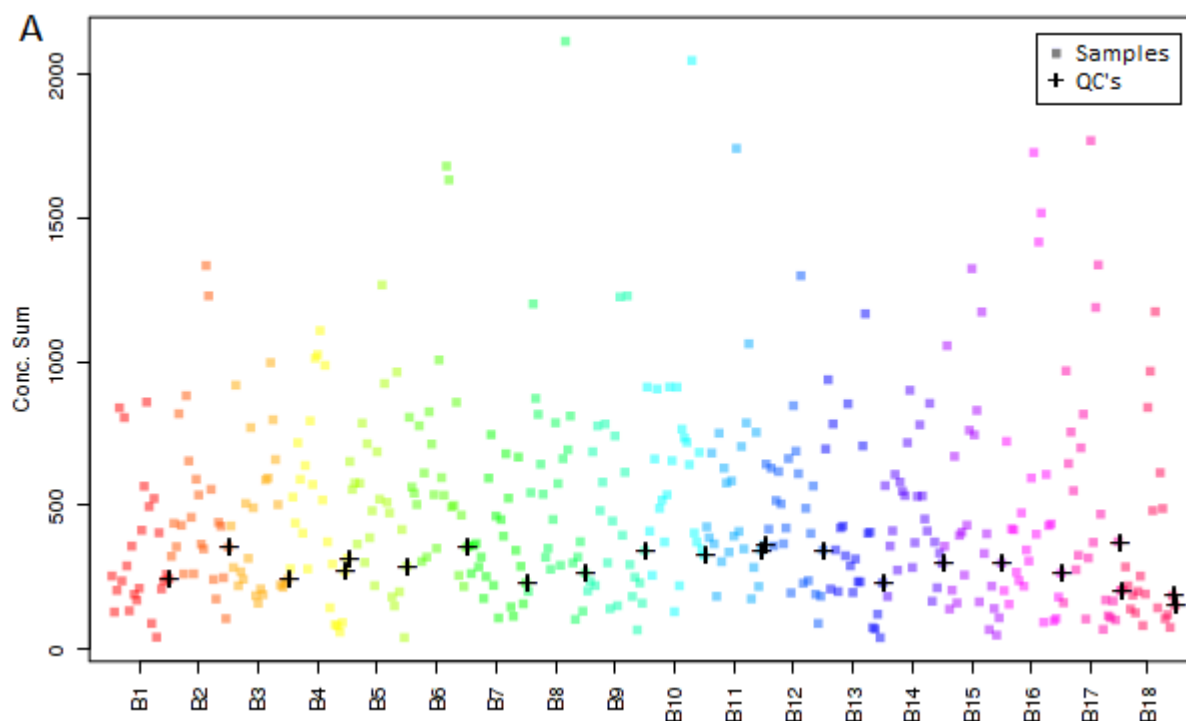
The GC-MS system consisted of a 7890A GC system, 7683B series injector and 5975B inert XL mass analyser and Chemstation Software (Revision E.02.00) from Agilent Technologies, Santa Clara, CA, USA. Chromatography was performed with a DB-1MS column (0.25 μ m, 250 μ m x 30 m) from J&W Scientific (Part#: 122-0132). A volume of 1 μ l sample was injected in split mode (5:1) at a temperature of 280 °C. The temperature program started at 60 °C for 2 minutes, increasing at 4 °C/min to 120 °C, and then at 6 °C/min to 285 °C where the temperature was kept for 2 min. The carrier gas was helium (17.73 psi) and electron impact ionisation was applied at 70 eV. MS acquisition was performed in scan mode (50–600 amu) for the organic acid analysis. The MS source and quadrupole temperatures were 230 and 150 °C, respectively.

GC-MS data

GC-MS data files were analysed in Automated Mass Spectral Deconvolution and Identification Software (AMDIS) (Stein 1999) in batch mode with the analytical settings as follows: minimum factor = 60 % and type of analysis = “Use of an internal standard for RI”. The deconvolution settings were as follows: component width = 20, adjacent peak subtraction of 1, and resolution, peak shape and sensitivity set at low. To limit the detection of false positives, the masses 73 and 147 were ignored as model ions. The National Institute of Standards and Technology (NIST08), and an in-house created organic acid library (Reinecke et al. 2012), were used to identify detected compounds. The pooled QC sample reports generated in AMDIS were inspected in order to choose a single QC sample with the most detected compounds. This QC sample was selected as a reference file in METabolomics Ion-based Data Extraction Algorithm (MET-IDEA) (Broeckling et al. 2006) because it best represented the entire batch. After data extraction, the data matrices were also inspected for deconvolution errors such as over-deconvolution. The correlation analysis function in MET-IDEA was used to identify variables that corresponded linearly across all samples. These compounds were then inspected manually to rule out metabolite correlation and the replicate variables removed. As a rule, the higher number model ion was retained as it is more compound-specific in comparison to lower ions. Feature identification was performed with the NIST08 library, which is a database of fingerprints used to identify the chemical compounds obtained from chromatographic separation.

Data pre-processing and normalisation

The GC-MS data were normalised with the internal standard and afterwards inspected for between-batch effects and carry-over. Although no major batch-effect was observed, it was decided to perform a between-batch correction to improve the quality of data before unreliably measured compounds were removed. The between-batch correction method of van der Kloet et al. (2009) was performed. A visual representation on the reproducibility of the GC-MS method over all the test batches with randomised samples and QC aliquots dispersed throughout can be seen in Figure 3.2, with Figure 3.2A showing scattered data points before between-batch correction and Figure 3.2B showing data points after between-batch correction. This serves to highlight the importance of data pre-processing steps.



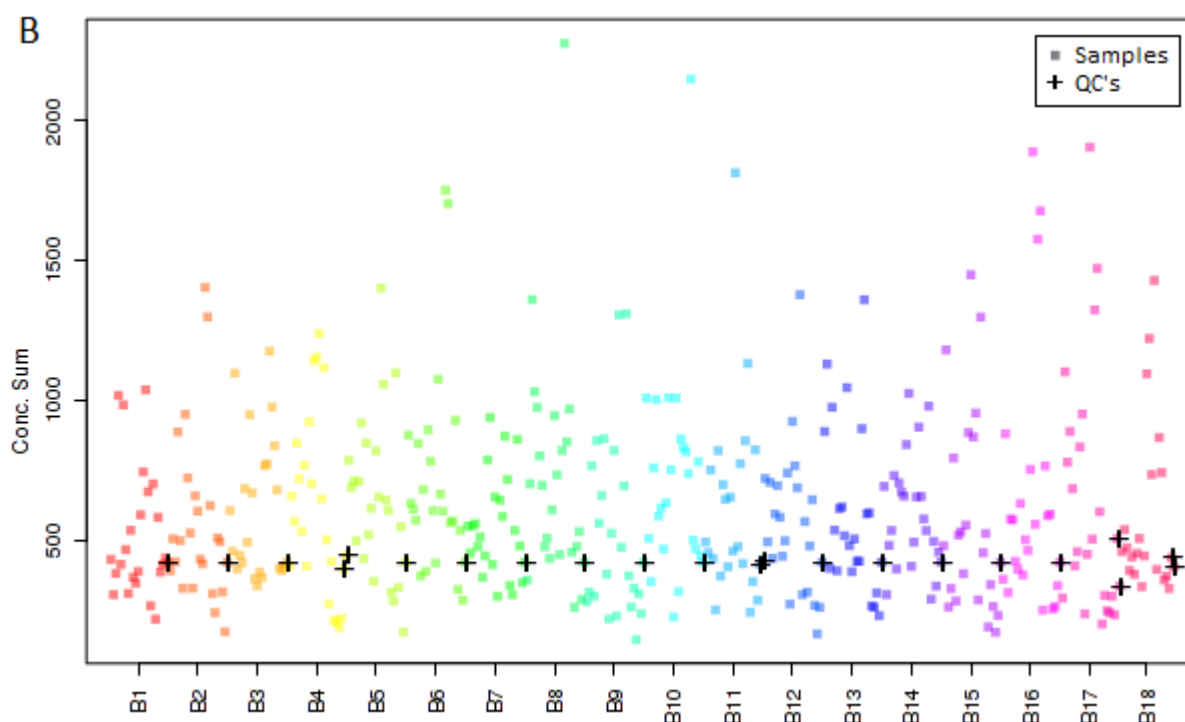


Figure 3.2: Scatterplots showing GC-MS batches in run order. A = before normalisation, B = after normalisation. Y-axis represents total concentration, while X-axis represents the order of batches over time. After normalisation, QC samples show linearity over time.

A QC coefficient of variance (CV) filter was applied to the data and compounds with CV > 50 % across all the QC's were removed. Heat maps were inspected and “outlier” cases were removed according to the following algorithm: The sum of all metabolite values were calculated per case and subsequently the median and standard deviation (Figure 3.3). Cases with standard deviation of more than two from the median were removed. The calculation cycles continued until no case was more than two standard deviations from the median.

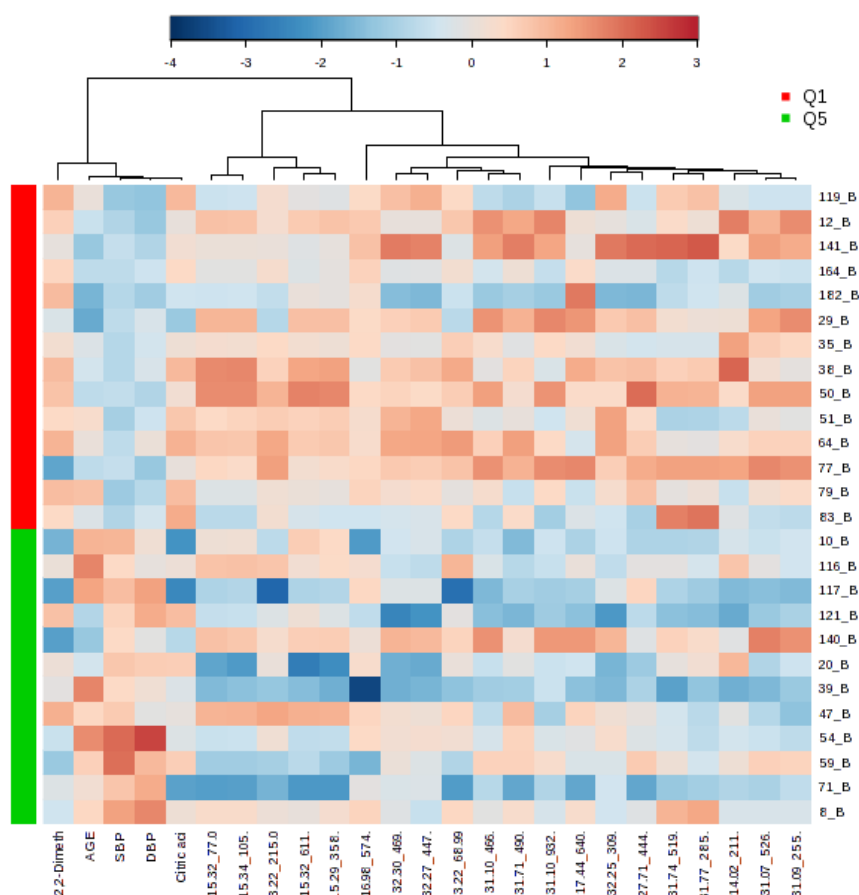


Figure 3.3: Example of a heat map from the Black female group showing selected variables as visual representation of data. This technique is useful when analysing data for outlier cases, as cases with abnormally high (red) or low (blue) can easily be identified.

3.3.2. LC-TOF-MS Metabolic profiling method

Sample preparation

To ensure as little as possible metabolite loss, minimum sample preparation steps were carried out for the LC-TOF-MS method. Briefly: Aliquots of urine/QC samples were thawed on ice and thoroughly vortexed. This was followed by centrifugation (at 4 °C) for 20 minutes at 15 000 x *g* to remove crystalline deposits. Thereafter 100 µL of the supernatant was diluted with an equal volume of chromatography-grade water containing 0.1 % formic acid and the internal standard (norleucine from Fluka, cat # 74560) to a final internal standard concentration of 50 µg/mL. A blank sample was included in every batch to monitor contaminating compounds in the solvents, artefacts from the preparation procedures and carry-over during chromatography.

Analysis of urine samples

An Agilent 1200 Rapid resolution LC instrument coupled to a 4GHz Agilent 6210 Time-of-flight (TOF) mass spectrometer was used. Chromatography was performed with a ZORBAX SB-Aq reverse phase column (3.5 μ m, 2.1 x 150 mm; Part#: 830990-914) fitted with a ZORBAX SB-Aq guard column (5 μ m, 2.1 x 12.5 mm; Part#: 821125-933) also from Agilent.

The chromatographic separation was started with 100 % solvent A (water and 0.1 % formic acid) for the first 5 minutes before the gradient was increased to reach 35 % solvent B (acetonitrile and 0.1 % formic acid) at 30 minutes. The gradient was increased linearly from 35 % solvent B at 30 minutes to 70 % solvent B at 45 minutes. From 45 minutes, the gradient was rapidly increased and reached 100 % solvent B at 50 minutes where it was kept constant for 5 minutes. The gradient was returned to the initial conditions within three minutes.

A 5 minute post-run was allowed to ensure equilibration of the column. A constant flow rate of 0.2 mL/minute and a column temperature of 30 °C were used. The dual ESI source was set for positive ionisation. A gas temperature of 280 °C, gas flow of 8 L/min and nebuliser pressure of 30 psi were used. The TOF scan window was set from 50 to 1,000 m/z. A reference solution containing masses 121.050873 [M+H]⁺ and 922.009798 [M+H]⁺ were constantly infused as accurate mass reference. Samples were kept at 4 °C in the auto-sampler. An injection volume of 10 μ L was used for all samples.

LC-TOF-MS data

LC-MS data was extracted using Agilent's Mass Hunter Qualitative data analysis software (B.05.00) and Mass Profiler Professional (B.02.02). The molecular feature extraction and find-by-ion algorithms were used according to Agilent's specifications. METLIN (<http://metlin.scripps.edu>) and the human metabolome database (HMDB: www.hmdb.ca) were used to annotate compounds using the accurate mass obtained. Retention times were not used in the search and a mass difference of 5 ppm were allowed between the theoretical (database) and observed masses. Isotope and adduct patterns were used to generate formulas for the relevant masses after which the accurate masses [M] and [M+H]⁺ were used for identification.

3.3.3. LC-QTOF-MS Metabolic profiling method

Sample preparation

For the LC-QTOF-MS method a deproteination step was added to the sample preparation steps, ensuring that samples were free of interfering proteins. Briefly: Urine/QC samples were thawed on ice and mixed thoroughly. To ensure uniform metabolite levels urine samples were diluted to match the sample with the lowest creatinine concentration and chromatography grade water was added up to 100 μ L. Norleucine (Fluka, cat # 74560) was added as internal standard for a final concentration of 100 ppm. The samples were deproteinated with 300 μ L ice cold acetonitrile. The samples were then vortexed and allowed to incubate on ice for 10 min. This was followed by the centrifugation of the samples (at 4 °C) for 10 minutes at 13 000 x *g*. Thereafter, the supernatant was transferred to a clean 1.5 mL polypropylene tube and evaporated to dryness at 37 °C under vacuum in a Speedvac centrifuge on high speed to remove any remaining acetonitrile. The samples were re-suspended in 100 μ L of solvent A (chromatography grade water containing 0.1 % formic acid), mixed and transferred to a clean glass vial fitted with a new septum. A blank sample was included in every batch to monitor contaminating compounds in the solvents, artefacts from the preparation procedures and carry-over during chromatography.

Analysis of urine samples

An Agilent 1200 Rapid resolution LC coupled to a 6510 Quadrupole-Time-Of-Flight (Q-TOF) mass analyser. Chromatography was performed with a ZORBAX SB-Aq reverse phase column (3.5 μ m, 2.1 x 150 mm; Part#: 830990-914) fitted with a ZORBAX SB-Aq guard column (5 μ m, 2.1 x 12.5 mm; Part#: 821125-933), also from Agilent.

The chromatographic separation was started with 100 % solvent A (water and 0.1 % formic acid) for the first 5 minutes before the gradient was increased to reach 35 % solvent B (acetonitrile and 0.1 % formic acid) at 30 minutes. The gradient was increased linearly from 35 % solvent B at 30 minutes to 70 % solvent B at 45 minutes. From 45 minutes, the gradient was rapidly increased and reached 100 % solvent B at 50 minutes where it was kept constant for 5 minutes. The gradient was returned to the initial conditions within three minutes.

A 5 minute post-run was allowed to ensure equilibration of the column. A constant flow rate of 0.2 mL/minute and a column temperature of 30 °C were used. The dual ESI source was set for positive ionization. A gas temperature of 280 °C, gas flow of 8 L/min and nebulizer pressure

of 30 psi were used. The TOF scan window was set from 50 to 1,000 m/z. A reference solution containing masses 121.050873 [M+H]⁺ and 922.009798 [M+H]⁺ were constantly infused as accurate mass reference. Samples were kept at 4 °C in the auto-sampler. An injection volume of 10 µL was used for all samples. The all ions MS/MS data acquisition mode was used, which is a new mode in Agilent Masshunter software that allows for simultaneous detection of precursor- and fragment ions. This is done by alternating between low- and high energy scans during data acquisition. This serves to ease identification of analytes by essentially doubling the available data points, i.e. precursor ions are produced by low energy scans and fragment ions are produced by high energy scans.

LC-QTOF-MS data

Raw Agilent LC-QTOF-MS data files were imported into Progenesis QI software (version 1, NonLinear Dynamics). Peak detection, deconvolution, data alignment and normalization were carried out in an automated workflow using default settings. Structure data files (SDF files) from HMDB (www.hmdb.ca), LipidMaps (www.lipidmaps.org), ChEBI (www.ebi.ac.uk/chebi/) and DrugBank (www.drugbank.ca) were used to annotate the features using accurate mass as well as in silico fragmentation matching.

3.3.4. LC-MS data pre-processing and normalisation

LC-MS data were filtered by removing variables that were not completely present in at least one of the two groups. Missing values in the remaining features were replaced by half of the lowest value in the dataset. The data were normalised where necessary using the MS total useful signal (MSTUS) normalization method (Warrack et al. 2009) and inspected for batch effects before further data processing. The between-batch correction method described by Wagner et al. (2007) was used to correct a clear between-batch effect seen in the LC-TOF-MS data. No batch correction method was performed for the LC-QTOF-MS data. A visual representation on the reproducibility of the LC-TOF-MS method over the test batches with randomised samples and QC aliquots dispersed throughout can be seen in Figure 3.4, with Figure 3.4A showing scattered data points before normalization and Figure 3.4B showing data points after between-batch correction.

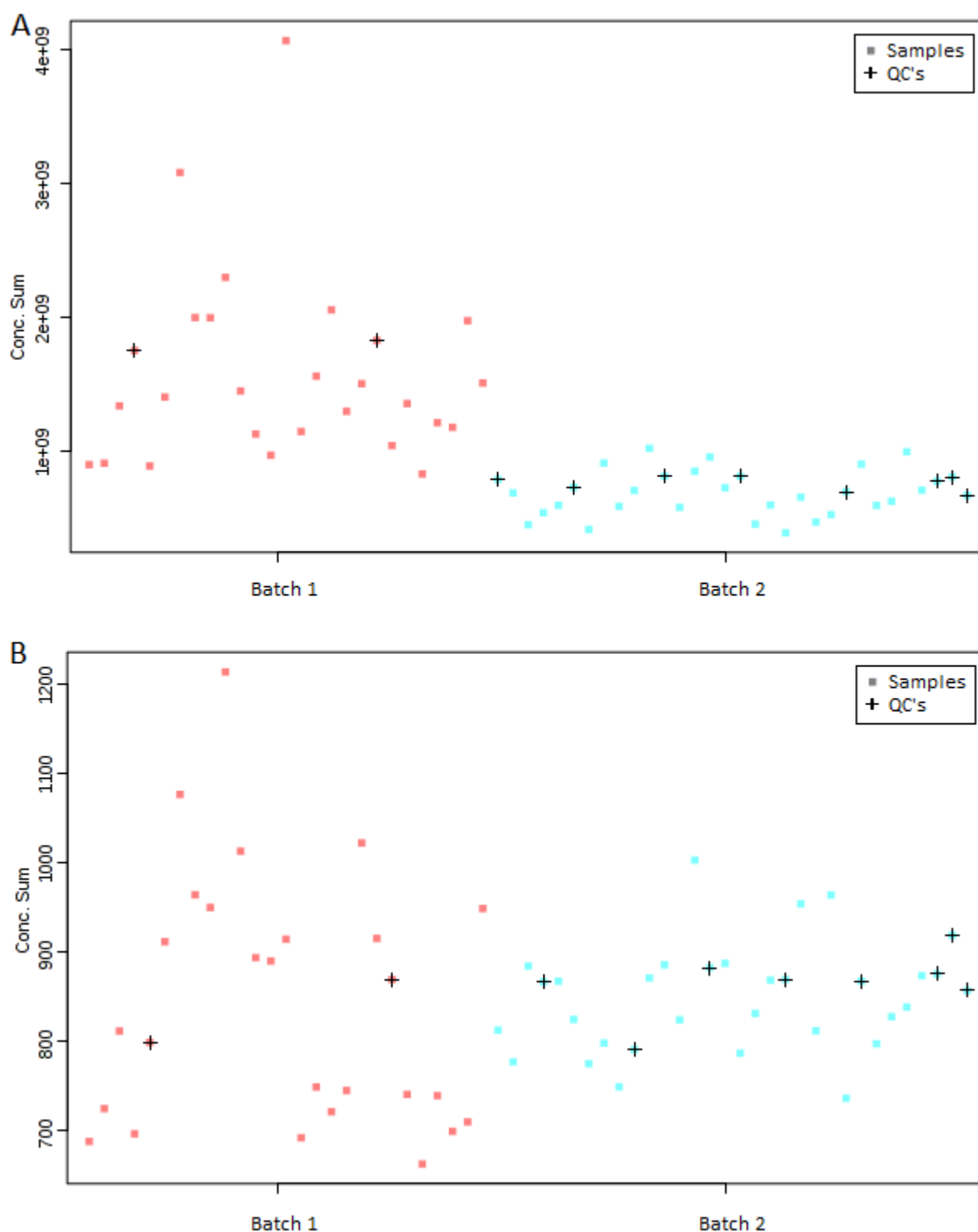
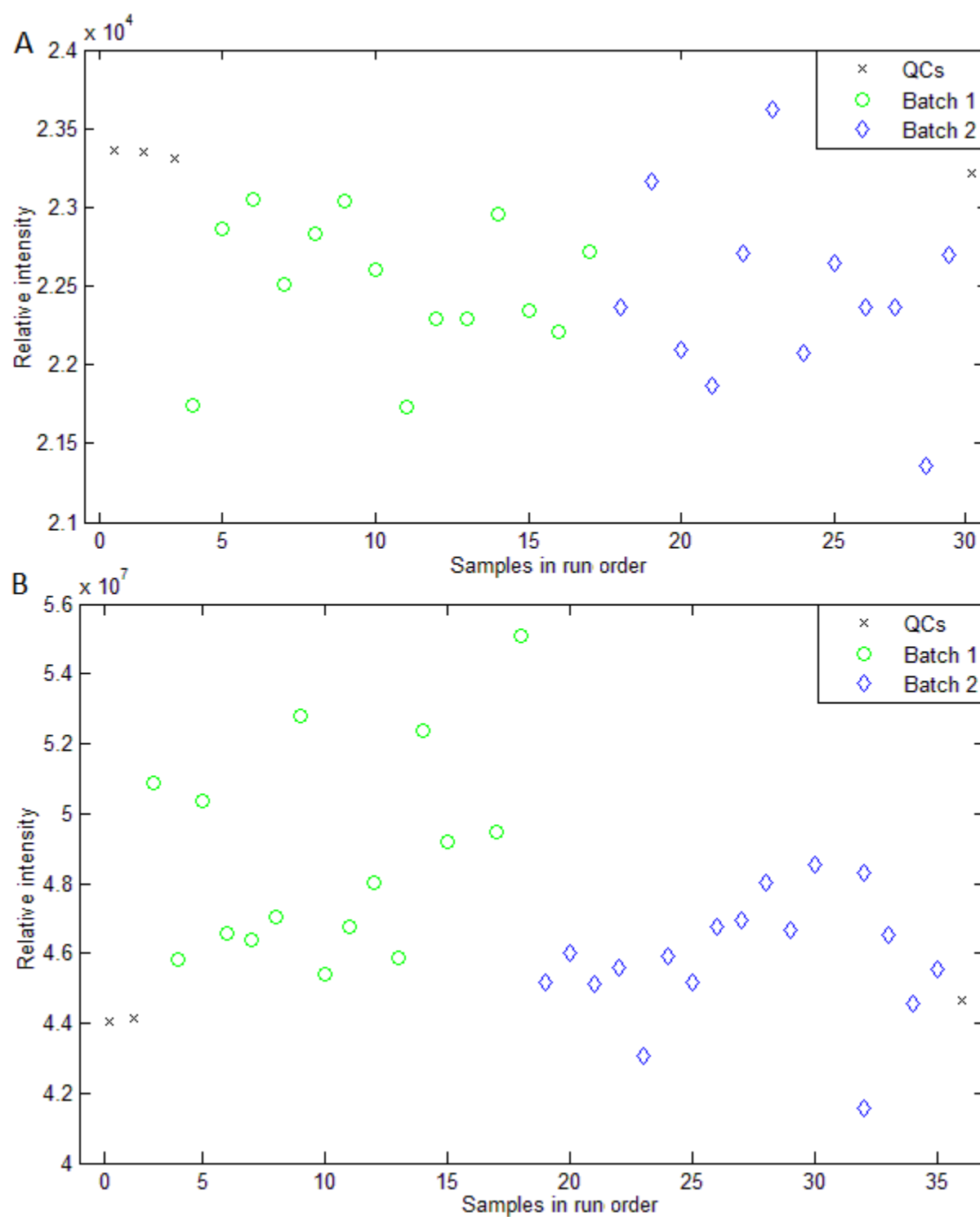


Figure 3.4: Scatterplots showing LC-TOF-MS batches in run order. A = before normalisation, B = after normalisation. After normalisation, batch effects (if present) are neutralised.

No batch effect was observed for the LC-QTOF-MS method in the three main groups. Each group run was randomised, starting and ending with QC samples. With the selected visualisation technique, the QCs were in a straight line, indicating that no major batch effect

was present (Figure 3.5). Data quality plots for the three remaining groups can be seen in Figure 3.5, with Figure 3.5A showing data points for the Black females, Figure 3.5B for the Caucasian males and finally Figure 3.5C showing data points for the Caucasian females.



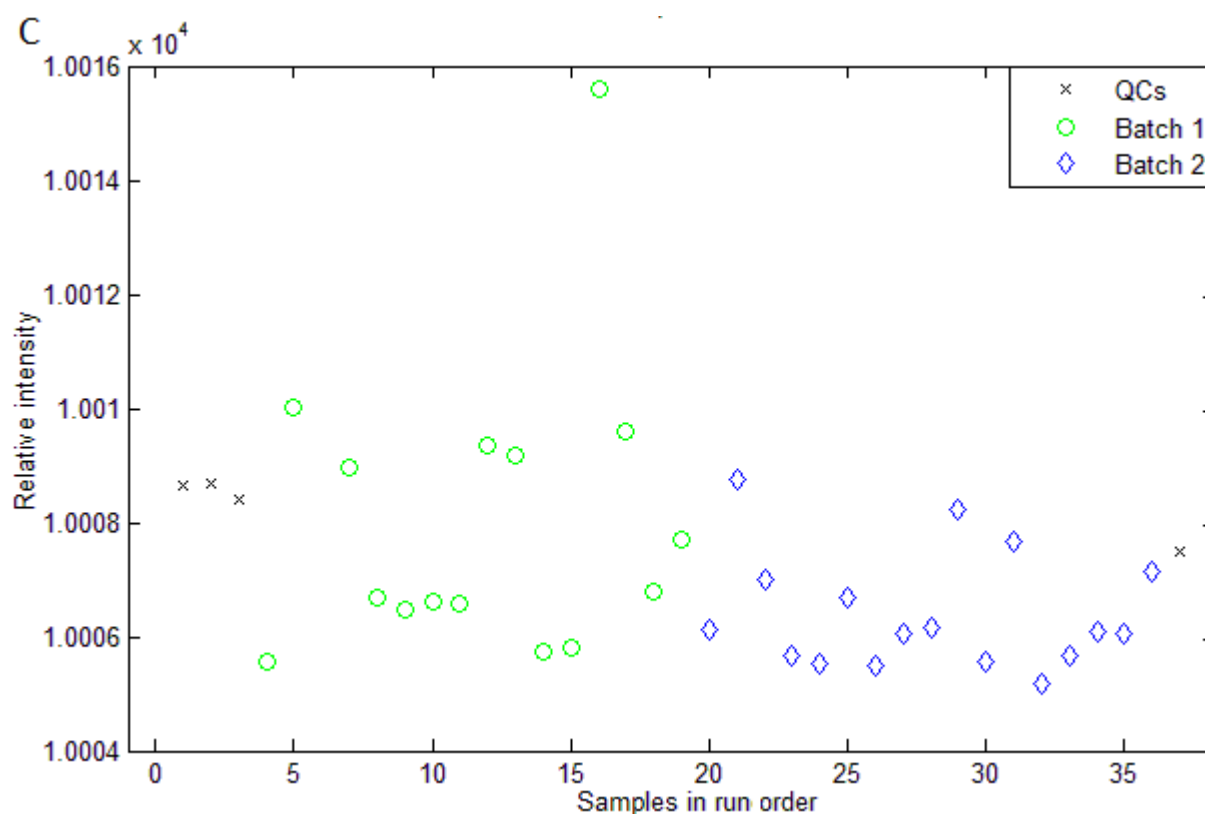


Figure 3.5: Data quality plots for the LC-QTOF-MS method. A = Black females; B = Caucasian males and C = Caucasian females. Y-axis refers to relative intensity for LC-MS method and X-axis refers to the order of batches over time. No normalisation was necessary for these groups, as total relative intensity of QC samples run at the start and end of batches correspond.

A QC CV filter was applied as mentioned above. The identity of important annotated features was verified. Two assumptions were tested, namely (1) the mass represent the intact molecular ion of the compound of interest or (2) it represents a fragment ion of the compounds of interest. With the first assumption, a putative name was given where possible and all lower masses that co-eluted and apexed at roughly the same time were used as possible fragment ions of the compound that could confirm its identity. With the second assumption, all higher masses that co-eluted and apexed at the same time as the mass of interest were used as possible molecular ions from which the fragment ion originated; fragmentation spectra were then investigated for this mass. LC-MS data are presented in this thesis as relative intensity, not absolute concentrations, and are included in results tables where comparing Q5 with Q1.

3.4. Data analyses

Student's t-test and Principle Component Analysis (PCA) were performed in MetaboAnalyst (www.metaboanalyst.ca). Data matrices from GC-MS and LC-MS methods were pre-processed separately according to standardised method specific procedures as described above. Thereafter, a single working data matrix was constructed from the LC-MS, GC-MS, clinical- and biochemical datasets for each of the four main groups separately. The data were transformed using the generalized log algorithm (glog transformation) before univariate and multivariate statistics were done in order to minimize influences from differences in concentration ranges of the metabolites and to allow for parametric statistical methods to be used. In short: The Student's t-test was used to test the equality of group means for each variable in the final data matrix. Data from the mutation analysis (as described in detail in Appendix B) were used in its glog transformed state as continuous variable in the final data matrix for each group. The variables from the final combined data matrix that differed significantly ($P < 0.05$) between the two blood pressure groups in each main group were then used for final PCA analyses to visualise group separation.

With the final data matrices compiled and metabolomics VIP's annotated where possible, biological interpretation and pathway analyses could be performed for each main group, namely the two Caucasian gender groups and the two black gender groups, respectively. The results obtained from the pathway analyses will be discussed in subsequent chapters.

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Chapter 4: Manuscript - Use of metabolomics to elucidate the metabolic perturbation associated with hypertension in a black South African male cohort: the SABPA study.

Co-Author consent form

To whom it may concern,

We, the co-authors of the research paper titled “Use of metabolomics to elucidate the metabolic perturbation associated with hypertension in a black South African male cohort: the SABPA study. 2015. *Journal of the American Society of Hypertension*, 9(2):104-114.” hereby give permission that this paper, as presented in Chapter 4, may be included as part of the thesis submitted for the degree *Philosophiae Doctor*.



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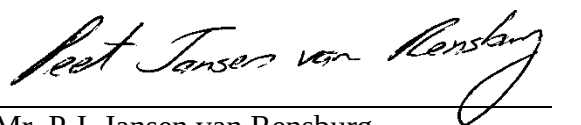
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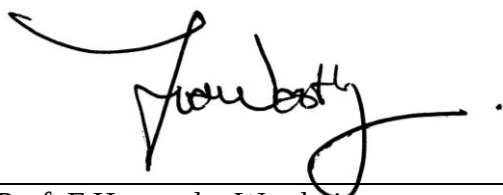
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Research Article

Use of metabolomics to elucidate the metabolic perturbation associated with hypertension in a black South African male cohort: the SABPA study

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Abstract

There is concern about the increasing burden of essential hypertension in urban–dwelling black South Africans, especially males. Several studies have investigated urbanization and hypertension in South Africans, but in–depth metabolomics studies on these urbanized hypertensives are still lacking. We aimed to investigate hypertension via two metabolomics methods in order to explore underlying biological mechanisms, demonstrating the effectiveness of these methods in cardiovascular research. A comprehensive characterization of a group ($n = 25$) of black male South Africans was performed using urinary gas chromatography–mass spectrometry and liquid chromatography–mass spectrometry metabolic profiling in conjunction with 24–hour ambulatory blood pressure readings and anthropometric, clinical, and biochemical markers. Average 24–hour blood pressure readings served as the grouping variable, and test subjects were divided into quintiles. Statistical analyses were performed on Quintile 1 (normotensive subjects) and Quintile 5 (extreme hypertensive subjects). After feature selection was performed, several metabolites and cardiometabolic risk markers, including abdominal obesity and markers of liver damage, inflammation, and oxidative stress were significantly perturbed in Quintile 5 (hypertensives) compared with Quintile 1 ($P < .05$). Pathway analysis revealed perturbations in several systems involved in ethanol metabolism via shifted global NADH/NAD⁺ ratio. Although alcohol abuse has been established as a risk factor for hypertension, this study illustrated a metabolic perturbation associated with alcohol abuse, contributing to the development of hypertension—possibly by altering bioenergetics through a shift in the NADH/NAD⁺ ratio. Following this finding, future intervention studies on alcohol moderation, as well as further enhancement of metabolomics methods in cardiovascular research are highly recommended. *J Am Soc Hypertens* 2015;■(■):1–11. © 2015 American Society of Hypertension. All rights reserved.

Keywords: Alcohol abuse; cardiometabolic disease; hypertension; metabolomics.

Introduction

Cardiometabolic disease (including essential hypertension) is an emerging problem among urban–dwelling black Africans in South Africa.^{1–3} Peltzer reported that of the

estimated 5.5 million people in South Africa with hypertension, three million were black males,⁴ which is an alarming statistic. Black adults in an urban setting seem to be most prone to significant increases in cardiovascular disease.^{5,6} Walker already predicted, in 1972, that increasing

Funding grants for this study were obtained from the North–West University (Potchefstroom campus); North–West Department of Education; the National Research Foundation; Medical Research Council; ROCHE Diagnostics, South Africa, the Biotechnology Partnerships and Development (BIOPAD), now called the Technology Innovation Agency (TIA), and the Metabolic Syndrome Institute, France. The funding organizations played no role in the design and conduct of the study; collection,

management, analysis and interpretation of the data; preparation, review, or approval of the manuscript.

Conflict of interest: none.

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urbanization and a rise in socio-economic status in developing populations would increase their proneness to obesity, hypertension, diabetes, and stroke.⁷ Unfortunately, with urbanization comes changes in lifestyle, such as poor diet, physical inactivity, and increases in tobacco smoking and alcohol consumption that favor the development of non-communicable diseases. Tracking the influences of these and many other risk factors of hypertension on the body holistically is a challenge, since present clinical markers are measured singularly. However, the field of metabolomics aims to provide a global profile of all small-molecule metabolites in cells and biologic fluids, free of observational biases inherent to more focused studies of metabolism. This may have major advantages over traditional cardiovascular disease studies, as it is not restricted to known patho-physiologic pathways or single biomarker measurements that reflect only a specific aspect of the disease process of interest.⁸ Numerous metabolomics studies have contributed to the acquisition of patho-physiologic knowledge of cardiovascular disease, as well as the search for novel biomarkers of this disease.^{9–12} Also, the high-throughput nature of metabolomics makes it ideal to perform early screening for diseases or to follow drug efficacy. Metabolomics, in conjunction with known risk factors, can therefore theoretically be used to detect early metabolic signs of hypertension before major systemic and end-organ damage occur, in order to implement successful lifestyle intervention methods. In individuals presenting with hypertension (or other cardiovascular diseases), it is likely that more than one metabolic pathway and process is affected, which in turn will contribute to the overall disease state. Understanding how metabolites relate with each other and with established risk factors will not only be important in assessing their value as potential biomarkers, but will likely shed more light on our understanding of the vast metabolic web of interactions in global pathways and may also lead to novel connections between metabolic pathways.¹³

Knowledge about preventable diseases is important to promote a healthy lifestyle, especially in a recently urbanized and multi-cultural setting like South Africa. The objective of this study was to investigate the systemic perturbations involved in the pathogenesis of essential hypertension in the black South African male in an attempt to explore the main contributing factors or mechanisms. To achieve this goal, a metabolomics approach was used, employing two popular hyphenated methods which cover a wide variety of metabolite classes. Urine was the chosen sample type of this study, as it constitutes the best matrix for systemic investigation of end-products of metabolism.

Methods

Ethics Statement

The study complied with all applicable institutional guidelines and terms of the Declaration of Helsinki of

1975 (as revised in 2004) for investigation of human participants and was approved by the Ethics Review Board of the North-West University, Potchefstroom Campus (00,036–07–S6).¹⁴ The nature, benefits, and risks of the study were explained to the participants in their mother tongue. Written informed consent was obtained from all participants before being included in the study.

Test Subjects

This study was performed on a subset of samples ($n = 25$) from the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) cross-sectional study conducted between February 2008 and May 2009.¹⁴ To fit the requirements of the SABPA main study, urbanized participants with similar demographic statistics were needed. It was therefore decided during study design to perform the main study on African and Caucasian male and female educators from schools in the same education district, thus minimizing socio-economic variability between participants. Participants for the SABPA study were recruited from the Dr Kenneth Kaunda Education district in the North-West Province, South Africa, and consisted of 409 educators (roughly equally divided into African and Caucasian males and females), aged 25–65 years. Exclusion criteria for the SABPA study included psychotropic substance users, tympanum temperature $>37.5^{\circ}\text{C}$, and vaccination or blood donation within 3 months prior to participation. For the present sub-study, additional exclusion criteria were used: since hypertension is one of the most common cardiovascular risk factors in the black South African males,^{5,15,16} it was decided to only include African men ($n = 101$) in this metabolomics study. Furthermore, participants that were HIV-positive ($n = 13$) and/or used anti-hypertensive drugs ($n = 17$) were excluded from any further analyses. The remaining participants ($n = 71$) were ranked according to their ambulatory 24-hour systolic blood pressure (SBP) before they were divided into quintiles (Q1 to Q5). Only the participants in Q1 (lowest SBP, $n = 13$) and the participants in Q5 (highest SBP, $n = 12$) were further used in this metabolomics study as illustrated in Figure 1. The reason for only using Q1 and Q5 is that metabolomics investigations are most successfully conducted when the two groups are clearly distinguished from one another.¹⁷ According to the 2013 Guidelines of the European Society of Hypertension/European Society of Cardiology for a 24-hour period,¹⁸ all the participants in Q1 can be classified as normotensive while the participants in Q5 are all hypertensive (SBP ≥ 130 mm Hg and/or DBP ≥ 80 mm Hg).

Sample Collection and Preparation

Ambulatory blood pressure measurement (ABPM) over a 24-hour period was done, as it reflects a more accurate

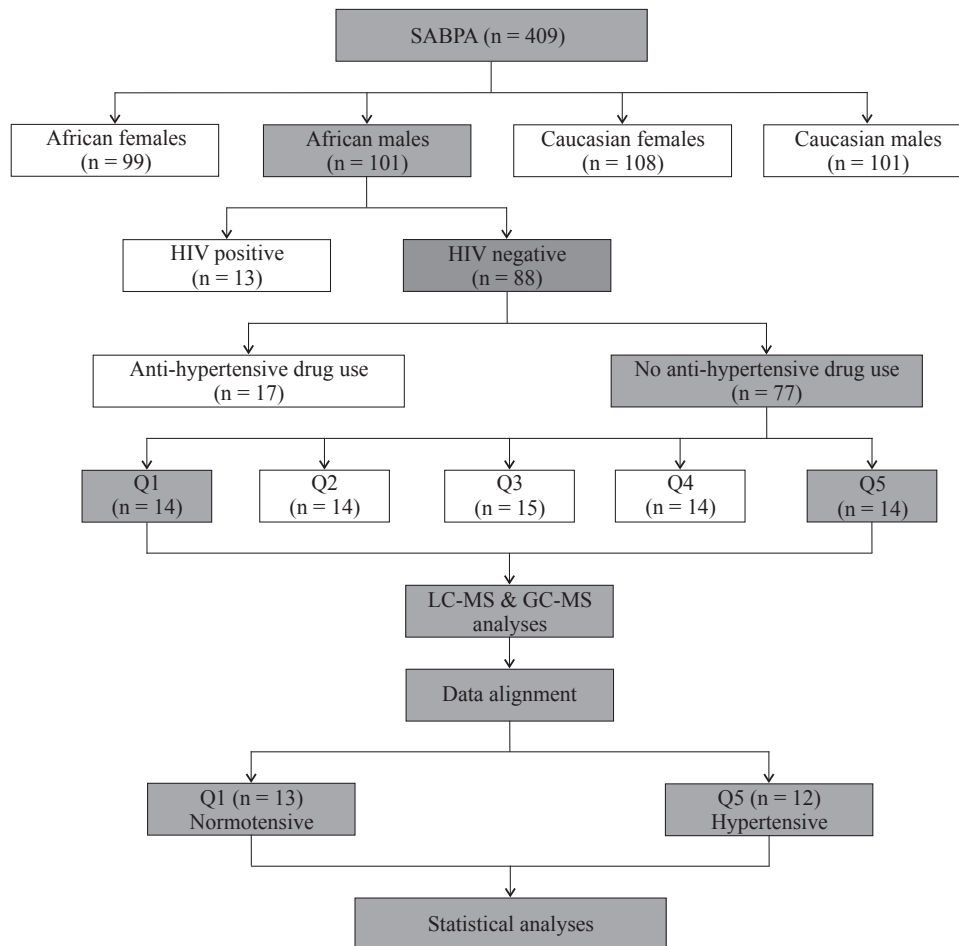


Figure 1. Flow diagram and rationale of participant selection. GC–MS, gas chromatography–mass spectrometry; LC–MS, liquid chromatography–mass spectrometry; Q, quintile; SAPBA, Sympathetic Activity and Ambulatory Blood Pressure in Africans.

blood pressure reading than clinic blood pressure measurements,¹⁹ thus bypassing the possible effect of white coat hypertension. ABPM was conducted during a normal working day for each participant. At ~07h00, participants were fitted with a physical activity meter and an ABPM apparatus (CE120 Cardiotens; Meditech, Budapest, Hungary), applying suitable cuff sizes to the non-dominant arm. The ABPM software was programmed to measure blood pressure at 30-minute intervals during the day (08h00–22h00) and every hour during night time (22h00–06h00). Successful mean inflation rate for the ABPM period was 82.7% ($\pm 3.8\%$) in African men. Participants were asked to continue with normal daily activities and record any abnormalities such as visual disturbances, headache, nausea, fainting, palpitations, and also physical activity and emotional stress on their ambulatory diary cards. The data were analyzed using the CardioVisions 1.19 Personal Edition software (Meditech). At 16h30, the participants arrived at the Metabolic Unit Research Facility of the North–West University, where they were familiarized with the experimental setup. Participants filled in

questionnaires, received a standardized dinner, and were advised to go to bed at 22h00. At 06h00 the next morning, the ABPM apparatus was removed and an 8-hour fasting mid-stream urine sample was obtained, which was kept on ice until aliquotted. Several anthropometric measurements were taken. With participants in a semi-recumbent position, a registered nurse then obtained a fasting blood sample with a sterile winged infusion set from the brachial vein branches.

Anthropometric Measurements

Body height (stature), weight, and waist circumference were measured with calibrated instruments in triplicate (Invicta Stadiometer, IP 1465, London, UK; Precision Health Scale, A&D Company, Tokyo, Japan; Holtain unstretchable flexible 7 mm wide metal tape, Crosswell, Wales) while participants were in their underwear. The body mass index (BMI) and body surface area (BSA) were calculated for each participant. All measurements were done by Level II anthropometrists according to standard procedures.²⁰

Biochemical Measurements

Blood and urine biochemical analyses were performed as described elsewhere.^{21–25} These included quantification of total serum cholesterol, serum high-density lipoprotein (HDL) cholesterol, serum glucose, HbA1C%, high sensitivity C-reactive protein (hsCRP), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), cotinine, reactive oxygen species (ROS), ferric reducing antioxidant power (FRAP), and total glutathione.

Metabolic Profiling

A metabolomics approach was used to analyze urine samples from the participants for metabolic profiling. The exact methods and protocols used from sample preparation to metabolite identification are described in detail in the [Online Supplementary Information file](#).

Statistical Analyses

Student *t*-tests were performed in Matlab (Mathworks), and Principle Component Analysis (PCA) was performed in MetaboAnalyst (www.metaboanalyst.ca). Data matrices from gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) methods were pre-processed separately according to standardized method specific procedures. Thereafter, a single working data matrix was constructed from the GC–MS, LC–MS, clinical, and biochemical datasets. The data were normalized using the generalized log algorithm (glog transformation) before univariate and multivariate statistics were done. In short, the Student *t*-test was used to test the equality of group means for each variable in the final data matrix. Proportions and prevalence were computed with χ^2 statistics. The variables that differed significantly ($P < .05$) between the two blood pressure groups were then used in a PCA to visualize group separation. This was done to determine if the selected physiologic and biochemical markers contained any co-variance structure (or relationships). Variables that have no biological relation and differ only by chance between groups will often not have any covariance structure, leading to the overlap of experimental groups in the PCA score plot.

Results

Characteristics of the Participants

Table 1 lists the general characteristics (before log-transformation) of the normotensive and hypertensive participants. Our test groups were chosen based on averaged 24-hour ABPM obtained from the ambulatory blood pressure monitor worn by the participants during a 24-hour period in a normal work week. The data spread of this

continuous variable was divided into quintiles, of which only the lowest and highest 20% were chosen as control and experimental groups, respectively. All variables in **Table 1** relate to differences between these two groups only. Average systolic and diastolic blood pressure values of the two groups can be seen in **Table 1**. The hypertensive group demonstrated higher mean BMI, BSA, waist circumference, total cholesterol, cholesterol/HDL ratio, and 24-hour heart rate; as well as means indicative of a pre-diabetic (HbA1C >5.7%) and low-grade inflammatory (CRP >3 mg/L) state when compared with the normotensive group. Furthermore, the hypertensive group had significantly higher oxidative stress levels, as evidenced by higher reactive oxygen species (ROS) values, than the normotensive group. Several liver enzymes such as ALP, ALT, and ASP were elevated in the hypertensive group. The liver enzyme GGT showed the biggest difference ($P < .05$) between the groups, being significantly elevated in the hypertensive group. No differences in the history of alcohol abuse or self-reported alcohol use were revealed in the hypertensive versus the normotensive groups. Self-reported data contradicted the increased GGT levels of the hypertensives, and therefore underscores its use as a marker of liver damage eminent of alcohol abuse.^{26–28}

Metabolic Profiling Results

After performing the Student *t*-test on the final (log-transformed) dataset, 38 variables differed significantly ($P < .05$) between the two blood pressure groups. These included clinical and biochemical parameters, as well as metabolites/variables from the metabolic profiling data obtained with the GC–MS and LC–MS analyses ([Online Supplementary Information file](#)). PCA was used to demonstrate that the 38 variables that differed significantly ($P < .05$) between the two blood pressure groups could clearly separate the two groups. Indeed, PCA showed clear separation of the two groups when all 38 selected features were used ([Online Supplementary Information file](#)). From this list of 38 variables, the metabolites that differed significantly between the two groups are shown in **Figure 2** as box-and-whisker plots. Because some of the LC–MS features could not be identified, emphasis was only given to the compounds that could be identified, as well as their involvement in the underlying metabolic perturbations of cardiometabolic disease.

Several clinical markers as well as blood pressure markers were important in the separation of the normotensive and hypertensive groups. Interestingly, the hypertensive group demonstrated higher physical activity than the normotensive group ($P = .001$). This may be due to the fact that the overweight/obese body needs to expend more energy for everyday movement compared with individuals with normal BMI. Also, a higher baseline

Table 1Baseline characteristics of the study group (means $\pm$ standard deviation)

Characteristics	Quintile 1 (Normotensive)	Quintile 5 (Hypertensive)	P Values
N	13	12	
Age, y	38.7 $\pm$ 7.7	45.9 $\pm$ 7.4	.03
Anthropometric measurements			
Stature, m	1.71 $\pm$ 0.06	1.71 $\pm$ 0.05	.94
Body weight, kg	65.5 $\pm$ 8.8	89.1 $\pm$ 13.5	<.01
Body mass index, kg/m ²	22.3 $\pm$ 2.6	30.6 $\pm$ 5.8	<.01
Waist circumference, cm	79.0 $\pm$ 8.4	104.2 $\pm$ 12.5	<.01
Body surface area, m ²	1.76 $\pm$ 0.13	2.05 $\pm$ 0.15	<.01
Cardiovascular measurements			
24-hour SBP, mm Hg	115.9 $\pm$ 4.3	161.8 $\pm$ 13.0	<.01
24-hour DBP, mm Hg	73.7 $\pm$ 4.1	102.0 $\pm$ 9.7	<.01
24-hour HR, beats/min	75.7 $\pm$ 5.7	84.6 $\pm$ 13.1	.04
Biochemical analyses			
Total serum cholesterol, mmol/L	4.30 $\pm$ 0.90	5.08 $\pm$ 1.03	.05
Serum HDL cholesterol, mmol/L	1.22 $\pm$ 0.37	1.00 $\pm$ 0.36	.14
Total cholesterol: HDL ratio	3.71 $\pm$ 0.95	5.75 $\pm$ 2.67	.02
Serum glucose, mmol/L	4.97 $\pm$ 0.52	6.86 $\pm$ 2.76	.02
HbA1c, %	5.68 $\pm$ 0.32	6.28 $\pm$ 1.16	.09
C-reactive protein, mg/L	2.24 $\pm$ 1.83	4.60 $\pm$ 2.84	.02
Alkaline phosphatase (ALP), U/L	81.8 $\pm$ 16.2	112.3 $\pm$ 21.8	<.01
Alanine aminotransferase (ALT), U/L	12.0 $\pm$ 4.8	26.1 $\pm$ 21.8	.03
Aspartate aminotransferase (AST), U/L	20.1 $\pm$ 5.7	34.4 $\pm$ 31.0	.12
γ -Glutamyltransferase (GGT), U/L	43.9 $\pm$ 18.5	118.4 $\pm$ 100.1	.02
History of alcohol use, n (%)	3 (23.08)	1 (8.33)	.32
Self-reported current alcohol use, n (%)	2 (15.38)	5 (41.67)	.14
Cotinine, ng/mL	6.85 $\pm$ 17.3	27.6 $\pm$ 43.7	.13
ROS, units*	71.5 $\pm$ 12.6	94.3 $\pm$ 16.3	<.01
FRAP, μ M	404.4 $\pm$ 31.0	418.8 $\pm$ 61.7	.46
Total glutathione, μ M	953 $\pm$ 162	919 $\pm$ 253	.69
Other measurements			
Physical activity, kcal/24-h	2209 $\pm$ 335	3146 $\pm$ 949	<.01
Tympanic temperature, °C	36.5 $\pm$ 0.4	36.7 $\pm$ 0.2	.11

DBP, diastolic blood pressure; FRAP, ferric reducing antioxidant power; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; HR, heart rate; n, number of participants; ROS, reactive oxygen species; SBP, systolic blood pressure.

* 1 unit = 1.0 mg/L H₂O₂.

sympathetic tone can contribute to higher energy expenditure,²⁹ which was evident in the male group of the main study.³⁰ Several variables from the metabolomics data set were important in separation of the groups. Firstly, organic acids such as lactic acid, 2-OH-isovaleric acid, 4-OH-phenyllactic acid, and fumaric acid were elevated in the hypertensive group, whereas tricarballic acid and indole carboxylic acid glucuronide were decreased in this group. All of the LC-MS metabolites that were significant in separation showed significantly lower levels in the hypertensive group compared with the normotensive group.

Discussion

In this study, we used metabolomics methods on urine samples from a group of hypertensive black South African males, compared with their normotensive counterparts, in an effort to elucidate the underlying metabolic perturbation involved in hypertension and cardiometabolic disease. We demonstrated that several general cardiometabolic risk factors, including abdominal obesity, were significantly disturbed in the hypertensive group (Q5) compared with the normotensive group (Q1). Furthermore, more specific

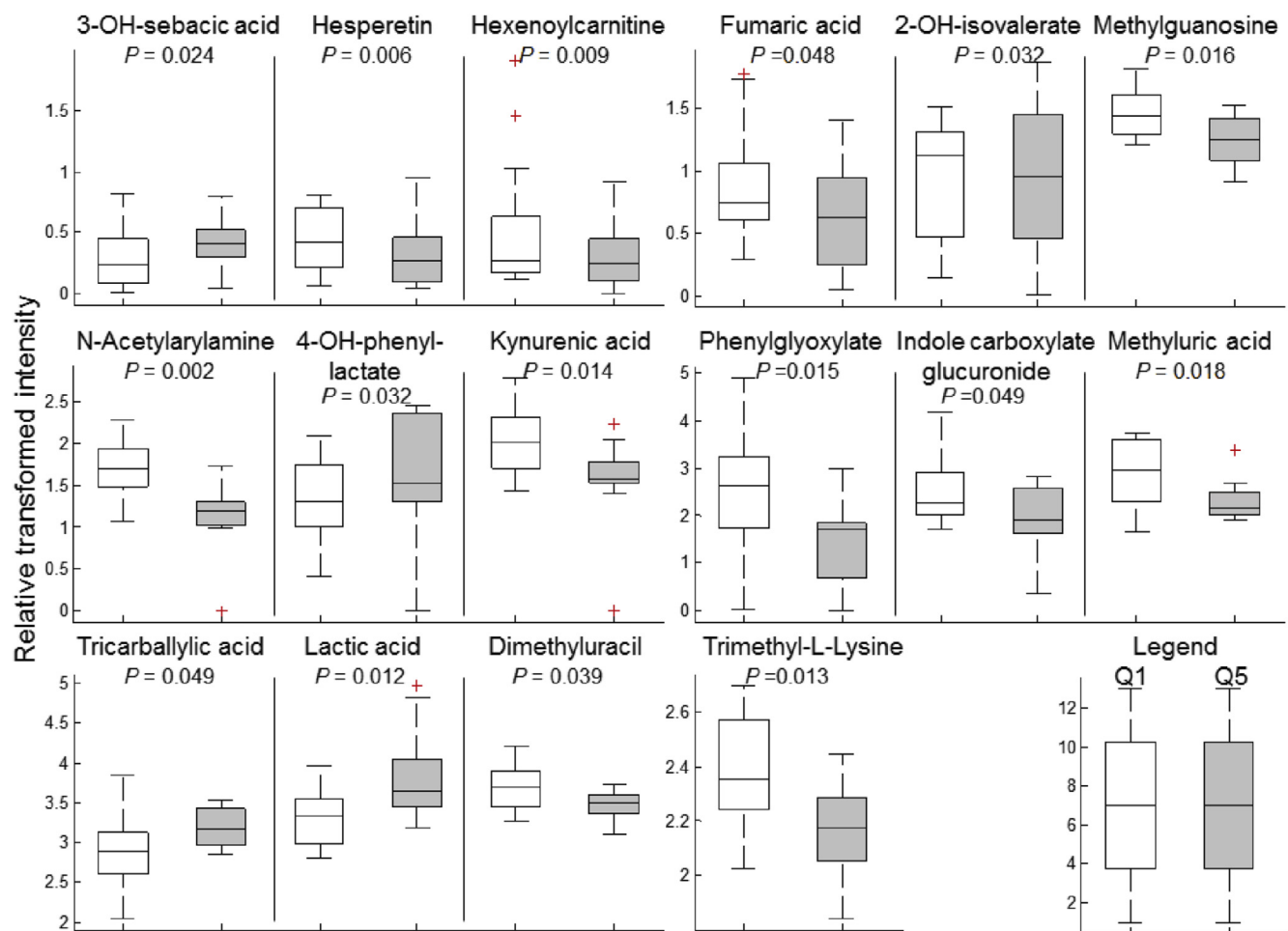


Figure 2. Box plots showing relative intensities of log-transformed data variables important in separation of the two blood pressure groups. The horizontal line within the box represents the median, with the box representing the 25th and 75th percentiles, while the whiskers represent $1.5 \times$ interquartile range (outliers shown as +). Q, quintile.

markers associated with liver damage, inflammation, and oxidative stress in the hypertensive group prompted suspicion into the possible cumulative deleterious roles of lifestyle habits such as smoking and alcohol use that result in elevated inflammation and oxidative stress levels. However, the cotinine level, which was used as a marker of current smoking status, did not differ between the two blood pressure groups. Bearing this in mind, the metabolite profiling using GC-MS and LC-MS platforms then confirmed chronic alcohol use/abuse as a possible major contributing factor in the pathophysiology of hypertension in this test group.

Clinical and Biochemical Markers

Since GGT, which was highly elevated in the hypertensive group, can also be used as a marker for alcohol abuse,³¹ it suggests that there might be increased alcohol use/abuse in the hypertensive group compared with their normotensive counterparts. The elevated levels of ALT

and ALP found in the hypertensives would also be consistent with increased alcohol abuse and could be an indication of liver damage. Alcohol is metabolized by three enzyme systems, namely alcohol dehydrogenase in the cytosol, the microsomal ethanol-oxidizing system involving the Cytochrome P450 2E1 (CYP2E1) enzyme of the cytochrome P450 enzyme system, and the peroxisomal oxidation of ethanol by the catalase system.³² Catabolism of the ethanol to acetaldehyde by the CYP2E1 enzyme generates ROS such as superoxide anion radicals and hydrogen peroxide. In the presence of metal catalysts, these formed reactive species can be converted to even more powerful oxidants like the hydroxyl radical. Ethanol-induced oxidative stress seems to play a major role in the mechanism where ethanol induces liver injury.³³ In this study, highly increased ROS levels were detected in the hypertensive group compared with the normotensive group. However, although oxidative stress levels were elevated, antioxidant capacity (FRAP) in the hypertensive group was not negatively influenced by the underlying

metabolic perturbations, suggesting that antioxidant systems were possibly up-regulated in response to the oxidative damage. Furthermore, oxidative stress in this black hypertensive group may stimulate the release of tumor necrosis factor α (TNF- α) from Kupffer cells.³⁴ This can activate inflammatory responses, such as evidenced by the low grade inflammatory state of the hypertensive group. Also, chronic inflammation is an important factor in the development of diabetes and cardiovascular disease^{35–38} where type 2 diabetes has been associated with a pro-inflammatory state in patients with obesity.³⁹ Finally, markers commonly associated with cardiometabolic symptoms were seen in the form of elevated serum glucose level, CRP, and total cholesterol:HDL,⁴⁰ which support our own findings.

Metabolic Perturbation

The metabolomic profiling revealed a systemic metabolic perturbation associated with higher ethanol intake/abuse as illustrated in Figure 3. First, GC–MS analyses revealed that lactic, fumaric, 4-hydroxyphenyllactic, and 2-hydroxyisovaleric acid were elevated in the hypertensives compared with the normotensives. These four organic acids have previously been reported to be elevated in pediatric patients with mitochondrial dysfunction.⁴¹ Therefore, the increase of these metabolites in the hypertensive group could indicate the involvement of altered mitochondrial metabolism. The main catabolic mechanism for ethanol is by cytosolic alcohol dehydrogenase to form acetaldehyde. In the process, oxidized nicotinamide adenine dinucleotide (NAD⁺) is converted to its reduced form (NADH). Acetaldehyde is further metabolized in the mitochondrion by alcohol dehydrogenase to form acetate. Again NAD⁺ is converted to NADH. The formed acetate can now be converted to acetyl-CoA by AceCS1. Chronic alcohol metabolism thus results in increased NADH levels with reduced free NAD⁺ available.^{42–44} But since the tricarboxylic acid (TCA) cycle is regulated by the NADH/NAD⁺ ratio, the cycle will be inhibited if the NADH/NAD⁺ ratio increases, resulting in lowered TCA-cycle turnover.

In the TCA cycle, malic acid is converted to oxaloacetate by malate dehydrogenase, consuming NAD⁺ and producing NADH. With low NAD⁺ available, malic acid is then converted to fumaric acid by fumarase, explaining the elevated fumaric acid in the hypertensives. Also, the conversion of pyruvate to lactate is favored, re-supplying the NAD⁺ pool to continue the glycolysis process.⁴⁵ This also explains the elevated lactic acid in the hypertensives. During inhibition of the TCA cycle, protein catabolism is activated to supply the TCA cycle with intermediates that can be used to feed biosynthetic pathways to produce lipids, proteins, and nucleic acids via reductive carboxylation.⁴⁶ Tyrosine is catabolized to 4-hydroxyphenylpyruvic

acid before being converted to homogentisate (and will later enter the TCA cycle via fumaric acid). However, due to an increased NADH/NAD⁺ ratio, the conversion of 4-hydroxyphenylpyruvic acid to 4-hydroxyphenyllactic acid is increased as observed in the hypertensives. Valine released during the increased protein catabolism is catabolized to 2-ketoisovaleric acid before being converted to isobutyryl-CoA via the conversion of NAD⁺ to NADH. Lowered NAD⁺ in the hypertensive group resulted in an increased conversion of 2-ketoisovaleric acid to 2-hydroxyisovaleric acid with higher levels of 2-hydroxyisovaleric acid detected in the hypertensive group.

From the LC–MS data, lowered methyluric acid was also observed. Since methyluric acid is formed from methylxanthine during the conversion of NAD⁺ to NADH, lowered methyluric acid may be caused by an increased NADH/NAD⁺ ratio. This altered ratio may be responsible for the majority of the metabolic perturbations observed in the hypertensive group compared with the normotensives. No data currently suggests that this altered mitochondrial metabolism is caused by genetic differences between the hypertensive and normotensive group. Therefore, increased alcohol use/abuse is the most likely cause of this increased NADH/NAD⁺ ratio.

Chronic alcohol metabolism and the resulting alcoholic liver disease are also linked to lowered S-adenosylmethionine (SAM) levels.^{47,48} Since SAM is responsible for DNA methylation, lowered SAM will result in hypomethylation of DNA and decreased urinary methylguanosine,⁴⁷ as observed in the hypertensives. SAM is also responsible for the methylation of proteins. Trimethyl-L-lysine (TML) is formed when proteins are catabolized and ultimately used for glutathione synthesis. Lowered SAM will cause lowered protein methylation and result in lower urinary TML, as observed in the hypertensive group. Chronic alcohol metabolism is also linked to lowered pyridoxal-5-phosphate (PLP) levels⁴⁹ and may be responsible for the lowered 3-indole carboxylic acid glucuronide detected in the hypertensives. Furthermore chronic alcohol metabolism is linked to lowered GSH levels^{50–52} Although GSH was not significantly lower in the hypertensive group, the lowered kynurenic acid detected in the hypertensives could be due to slightly lower GSH levels available for detoxification via glucuronidation.

Although the main metabolic perturbation seems to be due to an altered NADH/NAD⁺ ratio, other metabolites were also important in separation of the hypertensive and normotensive groups. These included lowered 3-hydroxysebacic acid and hexenoylcarnitine that can be linked to alterations in beta-oxidation. Dimethyluracil could indicate abnormal purine metabolism,⁵³ while hesperetin and tricarballic acid are dietary metabolites. The reasons why the levels of these metabolites are altered in the hypertensives, compared to the normotensive group, are not clear from the current data.

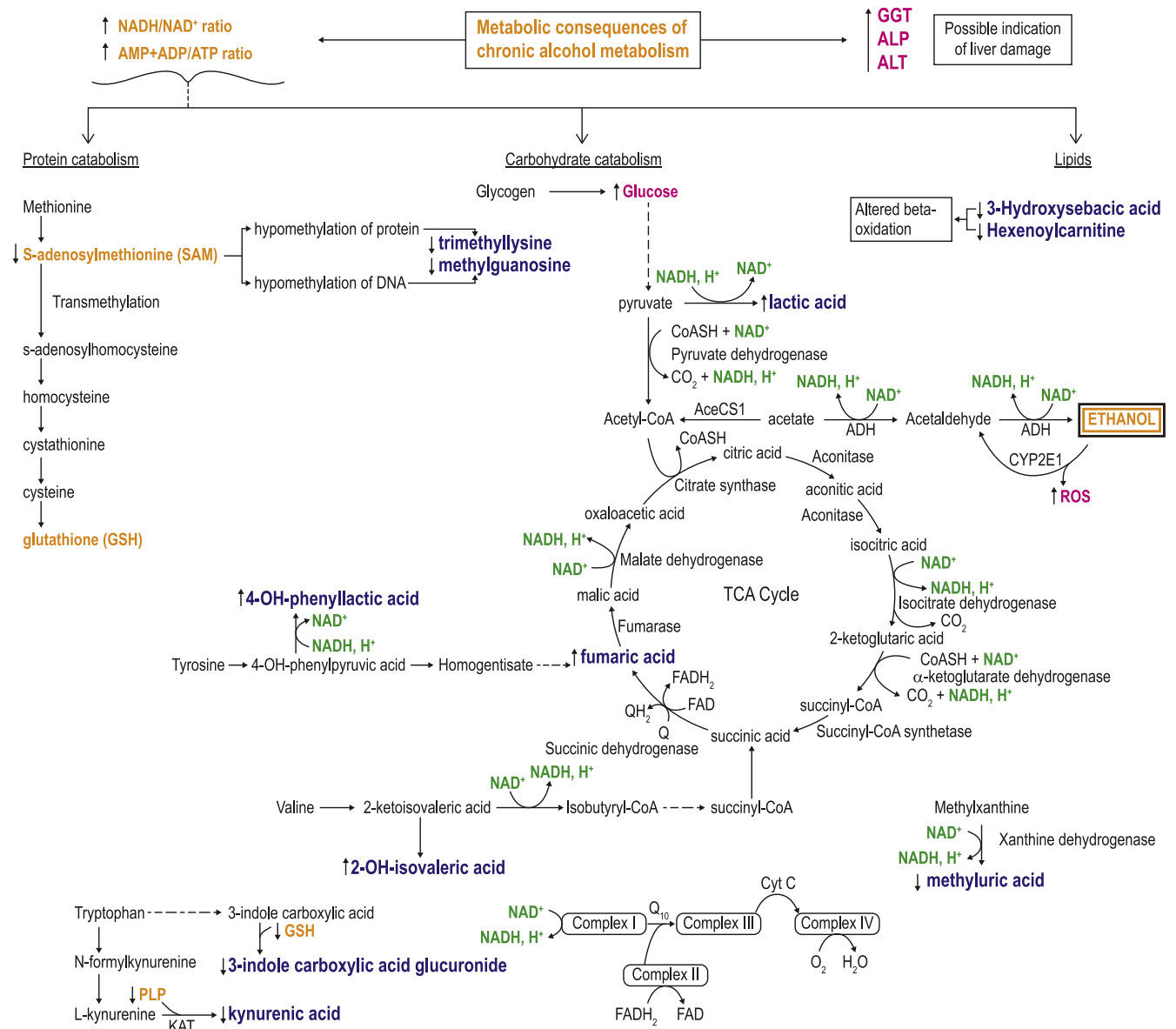


Figure 3. A schematic representation of global metabolic perturbations observed in the hypertensive group compared with the normotensive group. ↓ = decreased in the hypertensive group relative to the normotensive group; ↑ = increased in the hypertensive group relative to the normotensive group. Variable Importance in Projection (VIPs) measured with the metabolomics approach are indicated in blue; biochemical marker VIPs are marked in pink; and markers/alterations linked to chronic alcohol intake (from the literature) are marked in orange. NADH and NAD⁺ are marked in green to highlight their importance in the metabolic perturbation.

Chronic alcohol abuse

The metabolic profiling data from this study indicate that chronic alcohol abuse is most likely responsible for the main metabolic perturbation in this hypertensive black South African male cohort, compared to their normotensive counterparts. The exact mechanism how alcohol abuse leads to cardiovascular disease remains unclear, but oxidative stress might be involved. The connection of oxidative stress to various risk factors of cardiovascular disease has been reported.^{54–56}

Hamer et al identified alcohol consumption as a possible risk factor, among others, for hypertension in urban black South African males.⁵⁷ Alcohol abuse is seen as the most common form of drug abuse, and dose-dependent relationships exist between alcohol consumption and the incidence of diabetes, hypertension, cardiovascular disease, and stroke.⁵⁸ However, a demanding individualistic urban environment might also contribute to chronic stress where an ethnic group with a collectivistic orientation may not be able to exert control.³⁰ Behavioral adjustment to psychosocial stress was demonstrated in black African males who

revealed apparent abuse of alcohol. Hamer et al demonstrated the odds of early structural vascular changes based on high GGT levels were 3.1 (95% confidence interval, 0.6–15.5) in a black African male cohort, independent of other confounders. This was not found in black females or white gender groups.⁵⁷ It is thus possible that alcohol abuse in the black African male is the first line of defense to cope with taxing situations.³⁰ These findings support results from the present study, suggesting perturbations in various metabolic processes and systems, as a result of chronic alcohol use/abuse along with other risk factors like obesity and low-grade inflammation.

Strengths of this study include wide coverage of the urine metabolome; by using both GC–MS and LC–MS methods as at present, no single metabolomics method exists that can cover all hydrophilic and hydrophobic classes of metabolites with the sensitivity offered by mass spectrophotometric hyphenated methods, such as those used in this study. More robust and reliable identification of metabolites from overlapping classes measured by both methods is then also possible. Although our eventual experimental and control groups were small, a large portion of measured and identified compounds related to a shift in a very specific biologic ratio and not many vague, unrelated metabolic pathways. The NADH/NAD⁺ ratio is highly regulated and very important in various enzyme systems in the body, and it stands to reason that when this ratio is disturbed, many systems will be affected.

Limitations of this study include small experimental and control groups. Unfortunately with metabolomics methods measuring thousands of metabolites in the nanomolar range, it is imperative to have clearly defined groups so as to maximize the separation ability of these methods. Thus only participants from Quintile 1 and Quintile 5 were used in this study, resulting in smaller experimental groups. Furthermore, measurement of other long-term alcohol consumption markers was not included in the main study. We would therefore strongly recommend assessment of alcohol consumption and its connection with possible pathology of hypertension in prospective studies. The use of GGT as routine test in clinical practice for consumption of alcohol is advised as it was supported by the metabolomics findings. However, the exact mechanism on alcohol abuse, metabolism, and possible cardiovascular disease risk must be scrutinized for future therapeutic interventions. Therefore metabolomics analyses, despite its costs, might be justified.

Conclusions

Changes in lifestyle and diet as seen in urbanization bring about a range of metabolic shifts favoring the development of hypertension and eventual cardiovascular disease states. The aim of this study was to use a metabolomics approach to investigate the metabolic

perturbation associated with hypertension in urban-dwelling black males in South Africa. Data from various clinical and biochemical parameters that were measured were consistent with increased alcohol abuse in the hypertensive group, compared with the normotensives. Our metabolite profiling methods, in conjunction with clinical markers, were able to clearly separate the two blood pressure groups based on secondary metabolites of ethanol consumption. We therefore achieved our goal by demonstrating the powerful disruptive effect of alcohol abuse on the metabolism of the hypertensive individual. Thus, chronic alcohol abuse, along with an elevated waist circumference (indicating central obesity), bring about various symptoms of dysmetabolic state including high blood pressure, increasing the risk of eventual cardiovascular disease. The mechanism underlying this metabolic perturbation can possibly be attributed to an elevated oxidative stress status, as many of the systems involved in ethanol metabolism also produce elevated oxidative end-products, bringing about a range of secondary consequences. Bearing this in mind, further in-depth investigation into the pathology of ethanol metabolism is highly recommended. Furthermore, intervention studies on moderation of alcohol intake in the hypertensive African male would be warranted, as we have demonstrated the important role this modifiable lifestyle habit has in the development and maintenance of hypertension in this group. To our knowledge, this is the first study to use a metabolomics approach to study cardiometabolic disease in black South African men. The significance of our findings provides convincing evidence to substantiate the recent and continued interest in metabolomics as a useful tool in cardiovascular disease research.

Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jash.2014.11.007>.

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Chapter 5: Metabolomics of hypertension in Black females

5.1. Introduction

Chronic elevated blood pressure is one of the most important risk factors for cardiovascular disease (CVD) in urban-dwelling communities in sub-Saharan Africa and is set to become one of the most important contributors to the growing burden of CVD in this group of countries (Hendriks et al. 2012). In fact, it may be a more important contributor than other well-known risk factors of CVD like tobacco use, obesity and lipid disorders (He & MacGregor 2007). A systematic review of studies reporting on hypertension prevalence in the sub-Sahara Africa region indeed linked hypertension rates to level of urbanisation, and also reported on the higher level of hypertension awareness among black African women compared to black males (Addo et al. 2007). Sanderson et al. reported in 2007 that, in South Africa, the risk of death from hypertension had increased by a substantial amount in less than ten years. Black South Africans suffering from hypertension are also more likely to develop secondary complications, such as kidney disease, leading to congestive heart failure, as opposed to Caucasians.

In the previous chapter a very specific metabolic perturbation was reported for the Black males. This metabolic shift seemed to centre on chronic alcohol intake, possibly leading to dysregulation in the NAD^+/NADH ratio. To investigate possible causes or contributing factors of hypertension in the Black females, the same GC-MS organic acid analysis (Section 3.3.1) method was used as for the Black males (Chapter 4). However, an improved LC method was used, utilising an LC-QTOF-MS platform, together with Progenesis software for LC data processing as described in Section 3.3.3. Participants were assigned to quintiles (Q) according to the average 24-hour ambulatory systolic blood pressure measurements. Again, only Q1 (lowest 20 % blood pressure values) and Q5 (highest 20 % blood pressure values) were used in statistical analyses.

This chapter will deal with the results obtained from the metabolomics methods along with clinical- and biochemical markers for the Black female group, comparing Q5 with Q1.

5.2. Methods

In Figure 5.1 the classification and experimental strategy for the chosen Black female participants is shown. From the total group of Black females ($n = 99$) participants who were

HIV-positive and/or using anti-hypertensive medication were excluded. The remaining 60 participants were grouped into quintiles according to 24-hour ambulatory systolic blood pressure (SBP) values. For the purposes of the metabolomics methods only samples from participants in Q1 (lowest 20 % SBP) and Q5 (highest 20 % SBP) were further processed. After data alignment from the two metabolomics methods, Q1 consisted of 14 participants and Q5 consisted of 12 participants on which further statistical analyses were done.

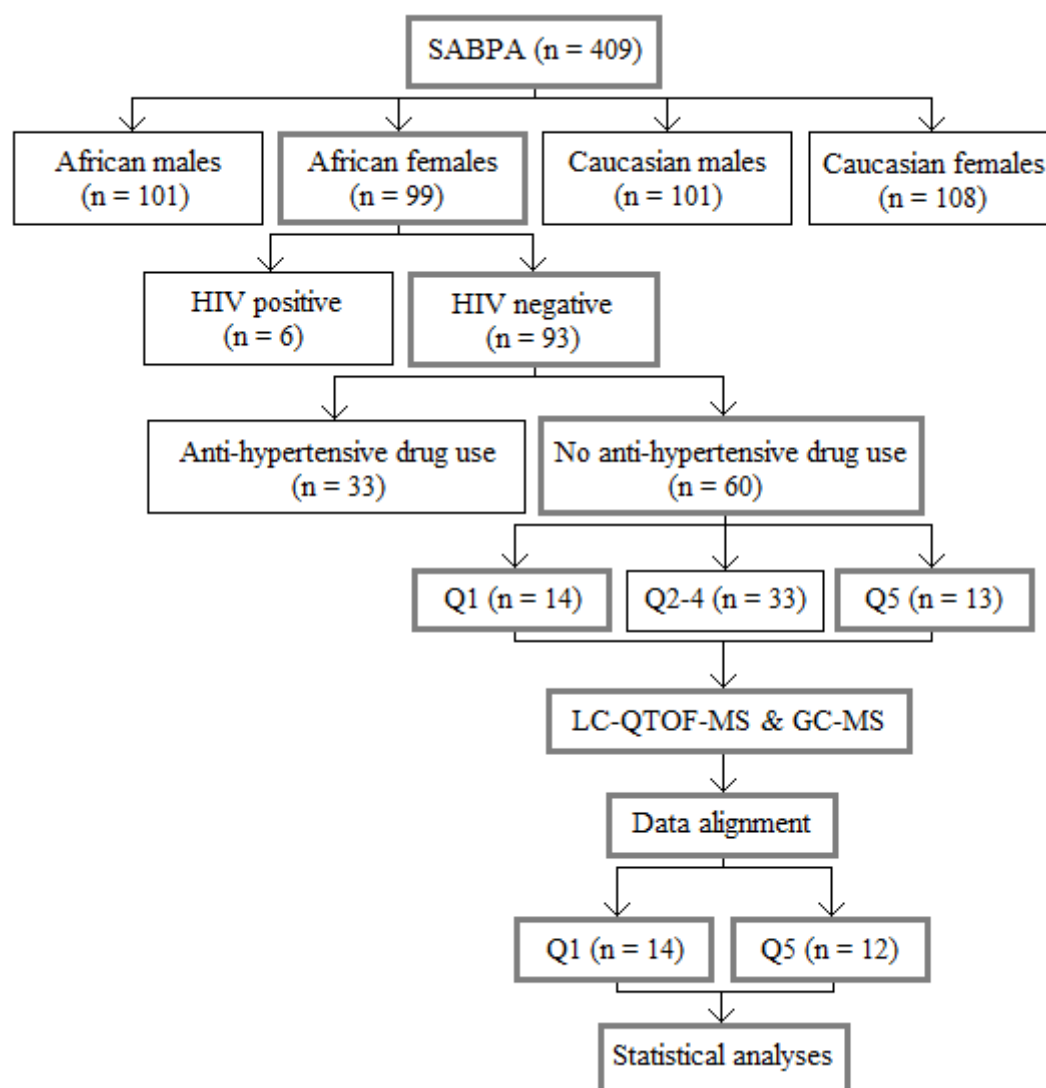


Figure 5.1: Flow diagram of strategy of participant selection in the Black female group. GC-MS, gas chromatography-mass spectrometry; LC-QTOF-MS, liquid chromatography-quadrupole-time-of-flight-mass spectrometry; Q, quintile; SABPA, Sympathetic activity and Ambulatory Blood Pressure in Africans.

For a detailed description on the clinical- and biochemical targeted measurements done, the reader is referred to Appendix A. Briefly: Body height, mass and waist circumference (WC)

were measured, after which BMI and BSA were calculated. Blood and urine biochemical analyses included quantification of total serum cholesterol, serum high-density lipoprotein (HDL) cholesterol, serum glucose, HbA1c%, high sensitivity C-reactive protein (hsCRP), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), cotinine, reactive oxygen species (ROS), ferric reducing antioxidant power (FRAP), and total glutathione.

For a detailed description on metabolomics methods followed in this study, the reader is referred to Chapter 3. Briefly: The two metabolomics analyses were performed on eight-hour overnight fasting first void urine samples. Statistical analyses (Section 3.4) consisted of t-tests and principle component analysis (PCA) on normalised, glog transformed data, after which biological interpretation was done.

5.3. Results

Table 5.1 lists results from general clinical, anthropometric and biochemical targeted measured markers in Black females before log-transformation.

Table 5.1: Baseline characteristics of the Black females study group (means $\pm$ standard deviation)

	Quintile 1 (Normotensive)	Quintile 5 (Hypertensive)	P- values
n	14	13	-
Age, years	43 $\pm$ 4.6	50 $\pm$ 7.6	0.007
<i>Anthropometric measurements</i>			
Stature, m	1.58 $\pm$ 0.05	1.59 $\pm$ 0.07	0.533
Body mass, kg	76.98 $\pm$ 17.07	87.86 $\pm$ 17.22	0.112
Body mass index, kg/m ²	30.87 $\pm$ 6.37	34.54 $\pm$ 5.54	0.124
Waist circumference, cm	84.96 $\pm$ 11.90	99.72 $\pm$ 16.91	0.014
Body surface area, m ²	1.83 $\pm$ 0.22	1.97 $\pm$ 0.21	0.108
<i>Cardiovascular measurements</i>			
24-hour SBP, mmHg	110.87 $\pm$ 3.46	149.62 $\pm$ 14.94	0.000
24-hour DBP, mmHg	69.07 $\pm$ 4.23	86.85 $\pm$ 10.82	0.000
24-hour HR, beats/min	79.50 $\pm$ 7.57	80.38 $\pm$ 13.64	0.835
<i>Biochemical analyses</i>			
Total serum cholesterol, mmol/L	4.50 $\pm$ 1.20	3.82 $\pm$ 0.92	0.115
Serum HDL cholesterol, mmol/L	1.21 $\pm$ 0.28	1.23 $\pm$ 0.24	0.826
Total cholesterol : HDL ratio	3.85 $\pm$ 1.12	3.21 $\pm$ 1.01	0.132

Serum glucose, mmol/L	4.83 ± 0.38	4.75 ± 0.79	0.733
HbA1c, %	5.49 ± 0.37	5.72 ± 0.29	0.076
C-reactive protein, mg/L	8.55 ± 6.18	11.04 ± 11.56	0.488
Alkaline phosphatase (ALP), U/L	97.52 ± 25.54	111.51 ± 63.60	0.454
Alanine aminotransferase (ALT), U/L	10.54 ± 5.01	10.73 ± 6.86	0.935
Aspartate aminotransferase (AST), U/L	18.12 ± 4.98	18.95 ± 7.53	0.737
γ-Glutamyltransferase (GGT), U/L	38.35 ± 24.49	45.28 ± 24.85	0.472
Self-reported current alcohol use, n (%)	1 (7 %)	2 (15 %)	-
Cotinine, ng/mL	7.07 ± 21.58	12.53 ± 33.77	0.618
ROS, Units*	104.57 ± 31.20	110.95 ± 36.81	0.636
FRAP, μM	334.12 ± 77.58	362.35 ± 60.81	0.305
Total glutathione, μM	900.54 ± 94.87	864.99 ± 191.45	0.542

Other measurements

Physical activity, kcal/24h	2507.79 ± 534.5	3027.72 ± 1196.56	0.152
Tympanic temperature, °C	36.62 ± 0.48	36.72 ± 0.29	0.580

*Tyrosine Hydroxylase SNP***

CC n (%)	2 (14 %)	3 (25 %)	-
CT n (%)	7 (50 %)	5 (42 %)	-
TT n (%)	5 (36 %)	4 (33 %)	-

Note: n, number of participants; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; ROS, reactive oxygen species; FRAP, ferric reducing antioxidant power; *1 unit = 1.0 mg/L H₂O₂; significant P-values shown in bold. **only data from this variable was tested in its glog-transformed state for significant difference in Q5 when compared to Q1.

Baseline clinical- and biochemical results

Firstly, an apparent observation from Table 5.1 is that there were not many clinical and biochemical targeted markers that showed any significant difference between the blood pressure groups in these women. A few of the markers, however, did show marked increases in the hypertensive group, compared to the normotensive group. Firstly, age was significantly higher in the Q5 group than in the Q1 group for these women ($P = 0.007$). As mentioned previously age has been reported as being an independent risk factor for hypertension and CVD in women, particularly of African descent (Rose et al. 2013). Women have been shown to present with steeper increases in blood pressure with increase in age as compared to males. This may additionally be supported by a decline in estradiol in menopausal states (Dubey et al. 2002; Chen et al. 2014). Furthermore, the only anthropometric measure that could possibly explain the higher blood pressure was WC ($P = 0.014$). This is in accordance with literature where it has been reported that WC was a very good predictor of risk of CVD (Goh et al. 2014; Zhu et al. 2005). Indeed, Murphy et al. (2014) found WC to be a useful primary screening tool

for cardio-metabolic risk in a rural Black population. Furthermore, Ware et al. 2014 found that both WC and waist-to-hip ratio significantly predicted all cardio-metabolic risk factors in a five year follow-up study in women. Apart from age and WC, none of the other clinical or biochemical markers differed significantly in the hypertensives compared to their normotensive counterparts. However, according to the guidelines of the American Diabetic Association a tendency for a mean pre-diabetic state ($\text{HbA1c\%} \geq 5.7 \%$) was evident in the hypertensives. Also after performing t-tests on glog transformed TH SNP data, no significant difference was observed in the prevalence of this mutation in Q5 relative to Q1.

With not much in lieu of clinical and targeted markers explaining the higher blood pressure in Black females, the usefulness of global metabolite profiling and metabolomics methods becomes apparent.

GC- and LC metabolomics results

After data clean up the GC-MS dataset consisted of a total of 64 features and the LC-MS dataset contained 165 features, 48 of which differed significantly between Q1 and Q5 according to t-tests. A single working data matrix was constructed, used in all subsequent statistical analyses, with the clinical, biochemical and metabolomics data. The data were normalised by glog transformation and then inspected for any natural separation using preliminary PCA. As can be seen in Figure 5.2 no natural separation exists between the normotensive and hypertensive groups although the data used did include cardiovascular measurements, such as blood pressure. Thus, there appeared to be confounding variables, possibly masking the contribution of important features to the separation of Q1 and Q5. To highlight the contribution of the variables involved in the rise in blood pressure in Q5 feature selection was performed.

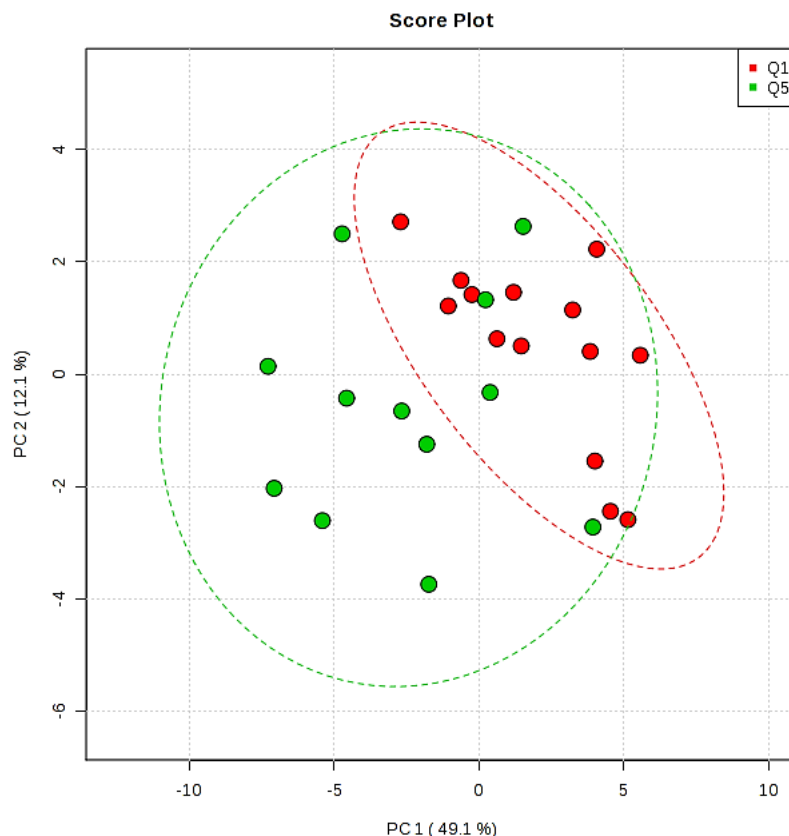


Figure 5.2: Preliminary PCA plot of Black female blood pressure groups with all variables included. Red circles indicate cases from quintile (Q) 1 ($n = 14$) and green circles indicate cases from Q5 ($n = 12$).

Of the 64 organic acids measured only two differed significantly between the two blood pressure groups (listed in Table 5.2), namely 2,2-dimethyl succinic acid and citric acid. In both cases the highest mean was in the normotensive group. It is therefore clear that only one metabolomics-related method is not suitable for elucidation of the variables important in separation of the Black female test groups in this study. The lack of variance between the test groups in terms of classical markers associated with high blood pressure and risk for CVD, such as anthropometric variables and markers of glucose and lipid metabolism, also raises a question about the true causative factors of hypertension in the Black females.

Table 5.2: GC organic acid profiling method VIP's for Black females

Organic acid	Means (mg/g creatinine) $\pm$ SD		P-value	Direction of change*
	Q1	Q5		
2,2-Dimethyl Succinic acid	6.45 $\pm$ 3.28	3.49 $\pm$ 3.33	0.029	-
Citric acid	107.38 $\pm$ 29.08	82.71 $\pm$ 31.55	0.045	-

Note: Means = Mean relative abundance; SD = standard deviation. *In Q5 relative to Q1.

In the final log-transformed LC data matrix a total of 48 metabolites differed significantly between the two blood pressure groups. These metabolites were then subjected to online database searches (both simple compound and fragment searches) for possible identification/annotation of the compounds. Non-biologicals and xenobiotics were removed from the list as far as possible. Table 5.3 lists the resulting significant features, 11 of which could be annotated and four of which also had confirmatory fragments.

Table 5.3: LC metabolomics method VIP's for Black females.

Compound	Putative Annotation	Metabolite Class	Confirmatory Masses	Relative Intensity $\pm$ SD		Direction of Change*
				Q1	Q5	
2.56_62.9892m/z	Adrenic acid	ω -6 LCFA	62.99, 189.12	203 $\pm$ 52	286 $\pm$ 146	+
3.22_192.0530n	Citric acid	TCA cycle intermediates	215.02, 157.04, 69	61886 $\pm$ 20610	44455 $\pm$ 24401	-
15.28_179.0633n	Hippuric acid	Products: polyphenol metabolism	180.07	1257591 $\pm$ 461109	927554 $\pm$ 452122	-
15.32_611.1405n	5-methyltetrahydropteroyl tri-L-glutamate	Substrate of EC 2.1.1.14	576.13	96662 $\pm$ 27512	71190 $\pm$ 31119	-
31.10_466.2525n	Androsterone glucuronide	Products: hormone metabolism	431.25	21158 $\pm$ 13933	11454 $\pm$ 10013	-
31.71_557.3315m/z	-	Sterol lipids	513.27	2593 $\pm$ 2564	884 $\pm$ 773	-
31.76_279.2239m/z	Pinolenic acid	ω -3 LCFA	285.25	8465 $\pm$ 8649	3161 $\pm$ 3133	-
32.25_917.5764m/z	-	Glycerophospholipids	519.29	1620 $\pm$ 3386	298 $\pm$ 451	-
32.27_447.2978n	Glycocholic acid	Bile acids	448.31, 355.26	30338 $\pm$ 26415	9973 $\pm$ 10386	-
6.44_196.9685m/z	Oxidised dithiothreitol	Co-factor in vitamin K cycle	-	631 $\pm$ 142	735 $\pm$ 114	+
25.63_535.1846m/z	Estradiol glucuronide	Products: hormone metabolism	-	2700 $\pm$ 1135	1844 $\pm$ 1363	-
31.09_255.2077m/z	-	Products: hormone metabolism	-	6167 $\pm$ 3922	3228 $\pm$ 2892	-
31.09_61.0394m/z	Urea	Major nitrogenous end-product	-	834 $\pm$ 410	573 $\pm$ 366	-
34.52_473.2531m/z	Cholic acid	Primary bile acids	-	757 $\pm$ 658	3544 $\pm$ 352	-

Note: Compound = Retention time plus detected mass (where n = neutral mass and m/z = mass-to-charge ratio); Q1 = normotensives, Q5 = hypertensives. Relative intensity refers to abundance and is not an absolute concentration (See Section 3.3.4); All compounds listed differed significantly between Q1 and Q5 (P-values < 0.05). *in Q5 relative to Q1. LCFA, long chain fatty acids; TCA, tricarboxylic acid

Regarding the LC features from Table 5.3 only two showed higher values in the hypertensives, namely adrenic acid and oxidised dithiothreitol. Adrenic acid is a long chain fatty acid in the ω -6 polyunsaturated fatty acid class. Oxidised dithiothreitol is part of the ubiquinone biosynthesis pathway and also a substrate for vitamin-K-epoxide reductase, which is part of the vitamin K cycle. All the other features had lower levels in the hypertensives and were mostly part of the lipid class of metabolites, such as pinolenic acid, glycocholic and cholic acid, and two products of hormone metabolism (estradiol glucuronide and androsterone glucuronide). Citric acid and hippuric acid, both organic acids, were also lower in hypertensives. Furthermore urea excretion was also lower in hypertensives than normotensives. Lastly 5-methyltetrahydropteroyltri-L-glutamate, which is a product of gut bacteria and also a hippuric acid, showed lower levels in the hypertensive group than their normotensive counterparts. The feature selection process resulted in a total of 17 variables important in separation of Black females Q1 and Q5 and consisted of age, WC, SBP, DBP, 2,2-dimethylsuccinic acid, citric acid and the 11 LC metabolites from Table 5.3. The resulting PCA plot (Figure 5.3), using these variables only, showed separation of the two groups.

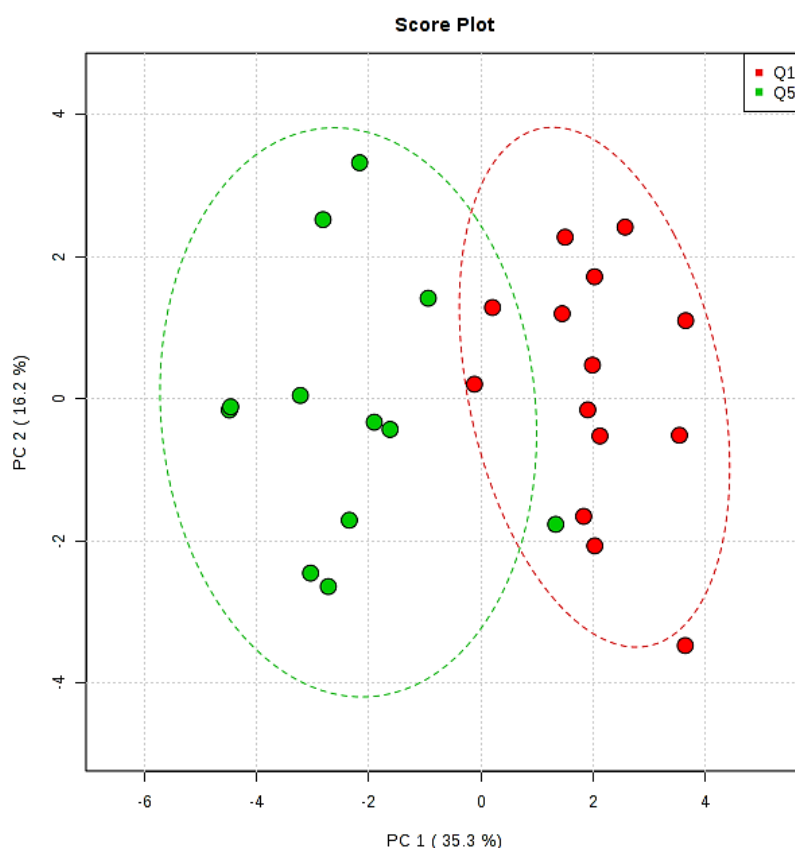


Figure 5.3: Final PCA plot of Black female blood pressure groups showing separation of Q1 and Q5. Red circles indicate cases from quintile (Q) 1 (n = 14) and green circles indicate cases from Q5 (n = 12).

5.4. Discussion

In the Black female group of this study very few variables differed significantly between normotensives and hypertensives although this group presented with higher average WC values and a mean pre-diabetic state compared to their normotensive counterparts. This was noted in literature where some research groups reported on the suitability of WC as a predictor of future CVD risk (Arnold et al. 2014). As a consequence of an urban-dwelling environment of the Black population in South Africa (and other parts of Sub-Saharan Africa) there may be a marked change in diet from a diet rich in plant-derived nutrients and fibre content to a diet rich in refined sugars, high in animal-derived fats and low in plant-derived complex fibre and nutrients (Pieters & Vorster 2008). This change in diet, together with a lack of physical exercise also observed in urban-dwelling populations, can lead to overweight and obesity.

Changes in diet during urbanisation can also lead to a constant state of mild metabolic acidosis as a result of high dietary acid load, worsening with age due to a decline in kidney function (Adeva & Souto 2011). This increasing prevalence of metabolic acidosis can lead to insulin resistance and dietary acid load may be an important variable in predicting CVD risk (Souto et al. 2011). Compensation for the acidic state by kidneys leads to lowered urine pH and lowered urinary citric acid excretion, and increased calcium elimination, together with nitrogen and phosphate wasting (Adeva & Souto 2011). In the Black female group lower citric acid was indeed observed in the hypertensives compared to the normotensives. Urinary citric acid excretion is heavily influenced by the acid-base status of the body and lower levels of this metabolite have been associated with insulin resistance in humans and in obese animal models (Cupisti et al. 2007). This may support the inclination to a pre-diabetic state in the hypertensives and their future risk for full-blown development of insulin resistance or T2D. In contrast, elevated 2,2-dimethyl succinic acid has been identified in children with inborn errors of metabolism (Giordano et al. 1990). The reason why this metabolite showed lower levels in Q5 compared to Q1 in the Black females remains unclear from the present study.

Citric acid is an intermediate metabolite of the TCA cycle in mitochondria. Mitochondrial stress/dysfunction has been reported in literature in individuals with the metabolic syndrome and other cardiovascular and cardio-metabolic diseases, leading to increased oxidative stress, a well-supported mechanism involved in the pathogenesis of CVD and metabolic syndrome (Selvaraju et al. 2012). However, no significant increase in ROS was seen in the hypertensive group compared to the normotensive group.

Bile acids have long been thought of purely as detergent molecules, which help to solubilise ingested fats obtained from the diet. However, recent research suggest a more comprehensive biological importance of these molecules, including endocrine functions (Houten et al. 2006) whereby bile acids has been shown to bind various receptors such as farsenoid x receptor, whose activation also affects lipid and glucose metabolism.

Bile acids are produced in the liver hepatocytes as the primary bile acids, cholic acid and chenodeoxycholic acid in the multi-step process involving cytochrome P450-mediated oxidation of cholesterol and represent the major output pathway of cholesterol (Pandak et al. 2002). This pathway is up-regulated at the rate-limiting step, catalysed by the enzyme cholesterol-7- α hydroxylase, by cholesterol and down-regulated in a negative feedback loop by cholic acid. After synthesis, the bile acids are then conjugated to glycine or taurine, making them more water-soluble. These bile acids (sometimes referred to as bile salts) are then stored in the gallbladder during non-digestive states and secreted into the intestines during digestion. In the lower small intestine and colon, gut bacteria remove conjugated glycine/taurine and partial hydroxylation takes place, forming the secondary bile acids deoxycholic acid and lithocholic acid. These and other forms of bile acids are then reabsorbed into the hepatic portal circulation and delivered back into the liver via the portal vein. This process is referred to as enterohepatic circulation (Hofmann 2011) and can occur multiple times during a single digestive phase.

Both primary bile acids, cholic acid and glycocholic acid (the glycine conjugate of cholic acid), showed lower levels in the hypertensive Black females, suggesting either impaired synthesis or increased elimination (and thus lower levels in the enterohepatic circulation). Indeed, lower bile acid levels, especially cholic acid, would impair negative regulation of the bile acid synthesis pathway and thus speed up production of more bile acids, leading to increased metabolism of cholesterol. While higher total cholesterol levels are usually seen in hypertensive individuals, in the Black female group the levels of total cholesterol actually show a downwards trend in the hypertensives though not statistically significant ($P = 0.115$). It has also been reported that an urban-dwelling population may experience more chronic psychosocial- or environmental stress, resulting in a possible down-regulation of steroid hormones (Hamer & Malan 2010).

Bile acids are crucial for the absorption of ingested fats. Bile acids form part of mixed micelles, important vessels for absorption of fats and fat-soluble vitamins, like vitamin K (phylloquinone, menaquinone). Lower levels of bile acids can therefore lead to inadequate

absorption of these vitamins. Lower vitamin K metabolism has indeed been implicated in the pathology of CVD (van den Heuvel et al. 2014; Vermeer 2012). Vitamin K is recycled in the body via the vitamin K cycle (Stafford 2005). This cycle starts with vitamin K quinone obtained from the diet, which is reduced to vitamin K quinol by vitamin K quinone reductase; then vitamin K γ -glutamyl carboxylase enzyme catalyses the conversion of vitamin K quinol to vitamin K epoxide via simultaneous conversion of prothrombin precursor glutamic acid to prothrombin carboxyglutamic acid. Then vitamin K epoxide reductase (with oxidised dithiothreitol as co-factor) converts vitamin K epoxide back to vitamin K quinone. In the hypertensive group, there was an elevated level of oxidised dithiothreitol present. This can be due to the lowered metabolism of vitamin K quinone, leading to the elevated level of co-factors used in preceding reactions in the vitamin K cycle. Indeed, as discussed above, the lowered absorption of fat-soluble vitamins (because of lower bile acids) can possibly lead to impaired vitamin K cycle turnover. This raises the question if the absorption of other fat-soluble vitamins (A and E) could possibly also be impaired as a result of the above mentioned perturbation. Although levels of these vitamins were not measured in the current study, it has been found in literature that plasma levels of vitamins A and E are associated with reduced adiposity, greater weight loss and an improved cardio-metabolic profile in overweight and obese individuals after a therapeutic intervention program (Guerendiain et al. 2015).

The hypertensive Black females also showed lower levels of hormone metabolites, such as estradiol glucuronide and androsterone glucuronide. This can be because of lower levels of bile acids available to absorb dietary fats and cholesterol, which is the starting point of all other steroid hormone synthesis pathways. It has also been widely reported that an age-related decline in steroid hormones occur, as can be inferred from the significant higher age of our hypertensive Black female group. Also, as both of these metabolites are glucuronic acid conjugates, this brings into question the possibility of negatively impacted detoxification in these hypertensives as glucuronic acid conjugation is part of the phase II detoxification system. Glucuronidation is also used for easier transportation of hormones in the circulation. It is possible that a lower level of bio-available glucuronic acid is present in these hypertensives as a result of a fatigued detoxification system. Furthermore, it may imply a possible down-regulation in the estradiol receptor system, inducing vasoconstrictive responsiveness (Komesaroff et al. 1999) which can worsen blood pressure increases.

Oestrogen has many endocrine roles in the body and also participates in glucose homeostasis via regulation of genes that are involved in insulin sensitivity and glucose uptake. Oestrogen

can also bind to various receptors such as oestrogen receptor- β , an anti-lipogenic and anti-adipogenic receptor, which is also a negative regulator of peroxisome proliferator-activated receptor (PPAR) γ (Mauvais-Jarvis 2011). PPAR γ regulates fatty acid storage and glucose metabolism by generating adipose tissue.

Interestingly the hypertensive Black females showed higher levels of adrenic acid, an ω -6 polyunsaturated fatty acid (PUFA), which has been reported as a pro-thrombotic agent and an inhibitor of prostacyclin. Prostacyclin is a vasodilator, also an inhibitor of platelet activation, but also has anti-mitogenic and anti-inflammatory properties. Conversely, the levels of pinolenic acid (an ω -3 PUFA) are decreased in the hypertensive group. Although pinolenic acid is only found in pine nuts, it is an isomer of α -linolenic acid, an essential fatty acid obtained from the diet, which inhibits lipogenesis. It is also an isomer of γ -linolenic acid. The exact isomer of this metabolite could not be identified within the current study. Although it is unclear whether the levels of these two fatty acids can be attributed to diet in these women it has been reported in literature that higher dietary intake of ω -3 PUFA to ω -6 PUFA has significant beneficial impact in a number of different disease states, whereas a high ω -6 PUFA/ ω -3 PUFA is associated with fatty liver and other chronic diseases (Xin et al. 2008). Indeed, ω -3 polyunsaturated fatty acids are able to limit triglyceride deposition in the liver (Schmitz & Ecker 2008).

Lower urinary urea was observed in the hypertensives. Being a major metabolite of protein intake the long-term involvement of diet is possible. Studies have also found an inverse relationship of urinary urea levels to blood pressure (Cirillo et al. 2002; Bispo et al. 2013), although the mechanism or cause for this inverse relationship is not stated. However, conditions associated with water balance and kidney damage (such as elevated blood pressure) can result in changes in urea excretion. Mitochondrial dysfunction has also repeatedly been implicated in the pathologies of non-communicable diseases (Kim et al. 2008; Liang 2011; Sharma et al. 2013; Ward et al. 2013). The lower urinary level of citric acid (a TCA cycle intermediate) in the hypertensive group also substantiates this. Lower urinary citric acid may also be part of the compensatory function of the kidneys when a high dietary acid load is encountered, as discussed earlier in the chapter. All of the features discussed in this section can be seen in Figure 5.4.

Lastly, two metabolites connected with the metabolism of gut microbiota, hippuric acid and 5-methyltetrahydropteroyltri-L-glutamate, showed lower levels in the hypertensive group compared to the normotensive group. It should be noted that hippuric acid is also a major

metabolite of the preservative sodium benzoate. However, the test participants all received a standardised diet during the study and fasting urine samples were used. It has been reported that Western-type diets can interfere with host-gut microbiota interactions and alter the metabolism of these microbial communities in the lower intestinal tract, with studies reporting on the connection with perturbations in normal gut flora to inflammation, insulin resistance, T2D and atherosclerosis (Cani & Delzenne 2009). Interestingly, a study by Samsel & Seneff in 2013 found a connection between residues from a herbicide left on foodstuffs generally consumed in a westernised diet and inhibition of the cytochrome P450 system, while simultaneously disrupting the biosynthesis of several amino acids by gut bacteria. In the present study the level of 5-methyltetrahydropteroyltri-L-glutamate, which is a substrate for methionine synthase and thus involved in methionine biosynthesis by gut bacteria, was indeed found to be lower in hypertensive Black females. Several studies have also found lower hippuric acid levels in models of various components of CVD, such as insulin resistance, obesity and hypertension (Harris et al. 2012).

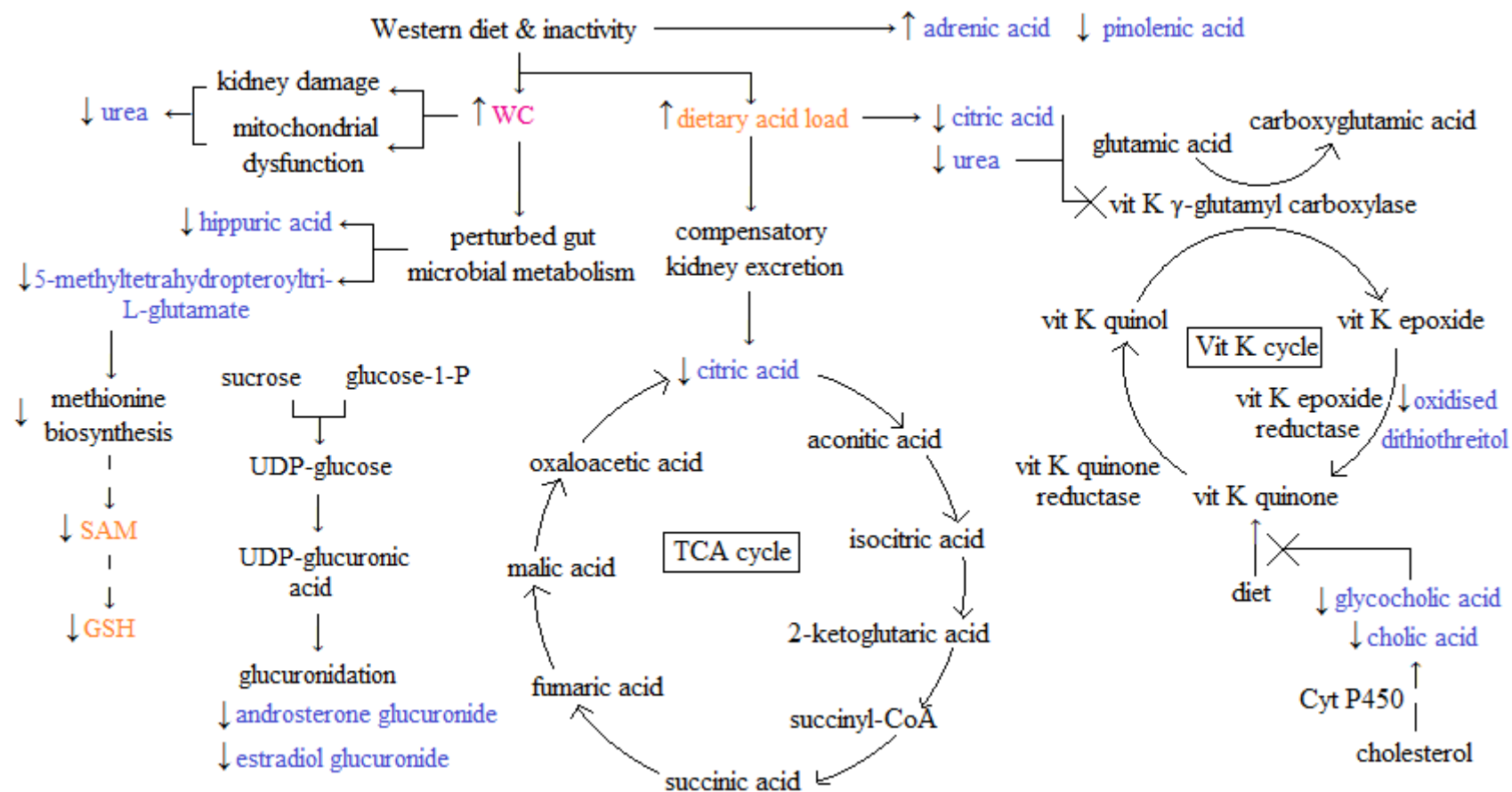


Figure 5.4: A schematic representation of global metabolic perturbations observed in the hypertensive group compared with the normotensive group in Black females. ↓ = decreased in the hypertensive group relative to the normotensive group; ↑ = increased in the hypertensive group relative to the normotensive group. Variable Importance in Projection (VIPs) measured with the metabolomics approach are indicated in blue; biochemical marker VIPs are marked in pink; and markers/alterations linked to cardio-metabolic disease (from literature) are marked in orange.

5.5. Conclusions

In the Black female group of this study the main urine metabolic markers that distinguish hypertensives from normotensives appear to be diet-related where urban-dwelling Black females consume a diet high in acid load, causing various metabolic perturbations in central metabolism and placing pressure on detoxification systems. This is supported by a number of conclusions that can be made from the metabolic data. Firstly, lower bile acids and consequences thereof were apparent in the hypertensive group. Bile acids are important modulators of cholesterol metabolism and also have various endocrine functions. As a result, inadequate absorption of fat-soluble vitamins, such as vitamin K takes place resulting in elevated levels of intermediates in the vitamin K cycle, as well as higher CVD risk connected to lower vitamin K in the body.

Furthermore, steroid hormone levels were lower in hypertensives. Lower levels of steroid hormones have been associated with hypertension, but this may be associated with the higher age of the hypertensive group compared to the normotensives. Perturbations in gut microbiota metabolism, which can be a result of unhealthy diet is also present in the hypertensives. This observation presents with interesting therapeutic possibilities which should be further investigated as it has been reported that prebiotic/probiotic supplementation is associated with alleviation of CVD symptoms and risk (Aron-Wisnewsky et al. 2013; Cani & Delzenne 2009), although longitudinal research in this field is warranted.

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Chapter 6: Metabolomics of hypertension in Caucasian males

6.1. Introduction

In the previous two chapters a metabolomics-based approach was described to investigate hypertension in Black gender groups. Hypertension in Caucasians has been researched extensively and factors involved in its pathogenesis can generally be elucidated by clinical and biochemical measured markers. However, data on the underlying mechanistic aspects causing the clinical manifestations of the disease are still lacking.

To investigate possible mechanistic aspects or contributing factors of hypertension in the Caucasian males a GC-MS organic acid analysis method was used as described in Section 3.3.1, in conjunction with an improved LC-QTOF-MS method, utilizing Progenesis software for LC data processing as described in Section 3.3.3. Participants were assigned to quintiles (Q) according to the average 24-hour ambulatory systolic blood pressure measurements. Again, only Q1 (lowest 20 % blood pressure values) and Q5 (highest 20 % blood pressure values) were used in statistical analyses.

This chapter will describe the outcome of the metabolomics investigation in the Caucasian male group, comparing the metabolic- and other clinical data of cases in Q5 with Q1.

6.2. Methods

The classification and experimental strategy for the chosen Caucasian male participants appears in Figure 6.1. Participants who were HIV-positive and/or using anti-hypertensive medication were excluded from the total group of Caucasian males ($n = 101$). The remaining 87 participants were grouped into quintiles according to 24-hour ambulatory systolic blood pressure (SBP) values. For the purpose of the metabolomics methods only samples from participants in Q1 (lowest 20 % SBP) and Q5 (highest 20 % SBP) were further processed. After data alignment from the two metabolomics methods Q1 consisted of 16 participants and Q5 consisted of 17 participants on which further statistical analyses were done.

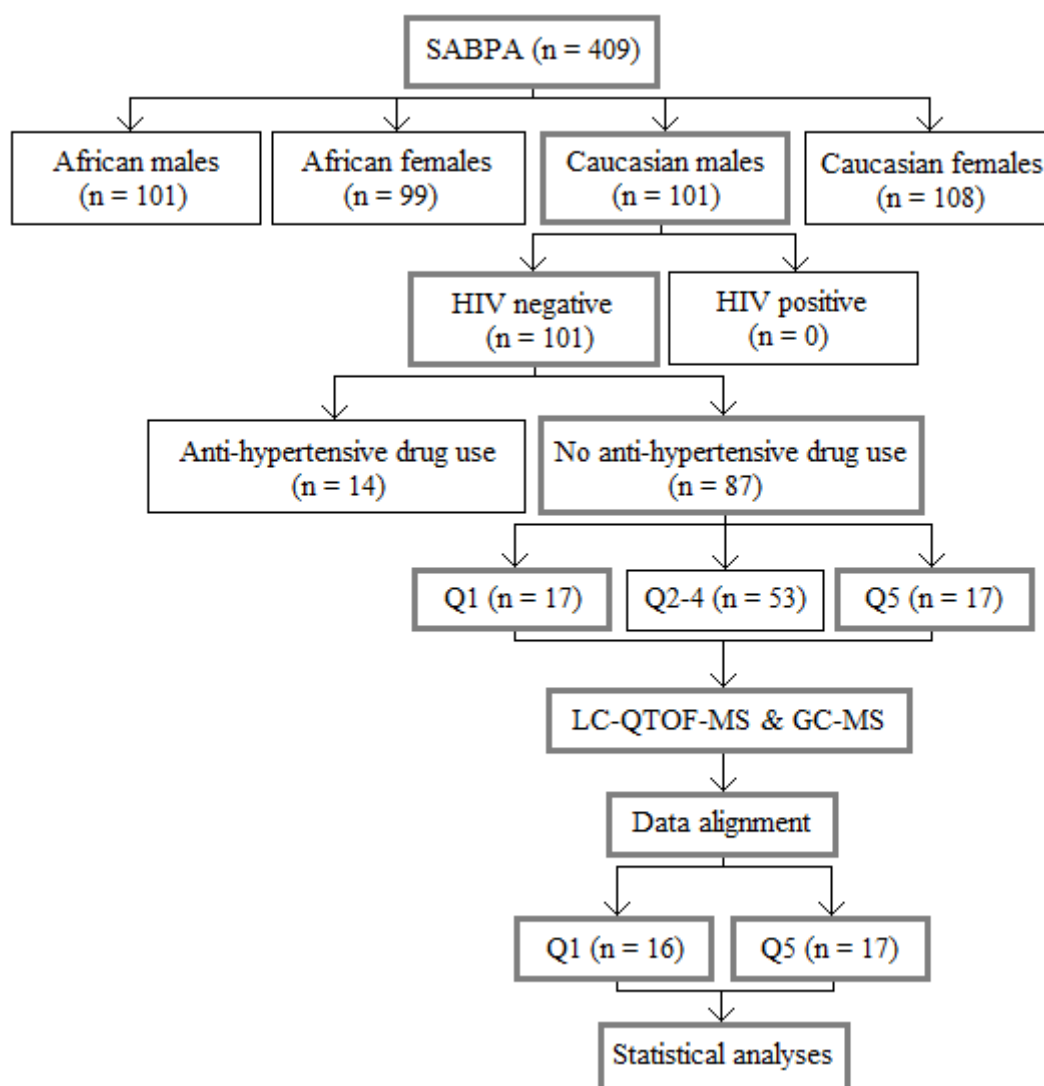


Figure 6.1: Flow diagram of strategy of participant selection in the Caucasian male group. GC-MS, gas chromatography-mass spectrometry; LC-QTOF-MS, liquid chromatography-quadrupole-time-of-flight-mass spectrometry; Q, quintile; SABPA, Sympathetic activity and Ambulatory Blood Pressure in Africans.

For a detailed description on the clinical aspect of this study, i.e. information on clinical and biochemical targeted measurements and markers collected, the reader is referred to Appendix A. Briefly: Body height, mass and WC were measured, after which BMI and BSA were calculated. Blood and urine biochemical analyses included quantification of total serum cholesterol, serum high-density lipoprotein (HDL) cholesterol, serum glucose, HbA1c%, high sensitivity C-reactive protein (hsCRP), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), cotinine, reactive oxygen species (ROS), ferric reducing antioxidant power (FRAP), and total glutathione.

For a detailed description on metabolomics methods followed in this study, the reader is referred to Chapter 3. Briefly: The two metabolomics analyses were performed on fasting first void urine samples. Statistical analyses (Section 3.4) consisted of t-tests and PCA analyses on normalised, glog transformed data, after which biological interpretation was done.

6.3. Results

Table 6.1 lists results from general clinical, anthropometric and biochemical targeted measured markers in Caucasian males before log-transformation.

Table 6.1: Baseline characteristics of the Caucasian males study group (means $\pm$ standard deviation)

	Quintile 1 (Normotensive)	Quintile 5 (Hypertensive)	P- values
n	17	17	-
Age, years	43 $\pm$ 11.9	44 $\pm$ 13.5	0.883
<i>Anthropometric measurements</i>			
Stature, m	1.82 $\pm$ 0.07	1.81 $\pm$ 0.07	0.541
Body mass, kg	87.34 $\pm$ 12.32	104.35 $\pm$ 23.81	0.013
Body mass index, kg/m ²	26.26 $\pm$ 3.48	31.92 $\pm$ 7.29	0.007
Waist circumference, cm	93.54 $\pm$ 12.25	107.12 $\pm$ 17.16	0.012
Body surface area, m ²	2.10 $\pm$ 0.17	2.28 $\pm$ 0.27	0.030
<i>Cardiovascular measurements</i>			
24-hour SBP, mmHg	116.94 $\pm$ 3.13	145.82 $\pm$ 7.69	0.000
24-hour DBP, mmHg	73.35 $\pm$ 5.80	88.06 $\pm$ 7.39	0.000
24-hour HR, beats/min	68.06 $\pm$ 11.69	74.47 $\pm$ 11.56	0.118
<i>Biochemical analyses</i>			
Total serum cholesterol, mmol/L	5.45 $\pm$ 1.15	5.69 $\pm$ 1.38	0.577
Serum HDL cholesterol, mmol/L	1.06 $\pm$ 0.22	0.93 $\pm$ 0.36	0.219
Total cholesterol : HDL ratio	5.30 $\pm$ 1.27	6.63 $\pm$ 1.77	0.017
Serum glucose, mmol/L	5.66 $\pm$ 0.38	6.31 $\pm$ 1.38	0.068
HbA1c, %	5.43 $\pm$ 0.27	5.96 $\pm$ 0.81	0.016
C-reactive protein, mg/L	2.09 $\pm$ 2.31	3.38 $\pm$ 2.48	0.126
Alkaline phosphatase (ALP), U/L	66.68 $\pm$ 13.60	72.77 $\pm$ 18.57	0.283
Alanine aminotransferase (ALT), U/L	18.21 $\pm$ 5.70	31.12 $\pm$ 13.35	0.001
Aspartate aminotransferase (AST), U/L	20.52 $\pm$ 4.88	25.50 $\pm$ 6.49	0.016
γ -Glutamyltransferase (GGT), U/L	25.24 $\pm$ 16.95	44.35 $\pm$ 34.67	0.049
Self-reported current alcohol use, n (%)	6 (35 %)	5 (29 %)	-
Cotinine, ng/mL	0.06 $\pm$ 0.24	41.24 $\pm$ 108.03	0.126
ROS, Units*	80.32 $\pm$ 17.33	81.61 $\pm$ 17.90	0.832

FRAP, μM	555.63 ± 92.70	513.28 ± 72.69	0.169
Total glutathione, μM	762.62 ± 153.35	864.46 ± 250.42	0.162
<i>Other measurements</i>			
Physical activity, kcal/24h	3046.79 ± 465.86	3817.07 ± 1055.84	0.012
Tympanic temperature, $^{\circ}\text{C}$	36.42 ± 0.28	36.48 ± 0.32	0.536
<i>Tyrosine Hydroxylase SNP**</i>			
CC n (%)	8 (50 %)	4 (24 %)	-
CT n (%)	7 (44 %)	10 (59 %)	-
TT n (%)	1 (6 %)	3 (18 %)	-

Note: n, number of participants; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; ROS, reactive oxygen species; FRAP, ferric reducing antioxidant power; *1 unit = 1.0 mg/L H_2O_2 ; significant P-values shown in bold. **only data from this variable was tested in its glog-transformed state for significant difference in Q5 when compared to Q1.

Baseline clinical- and biochemical results

In the Caucasian hypertensive males, an overall trend of increases in cardio-metabolic risk markers such as obesity, low-grade inflammation, pre-diabetic state and disturbed liver enzymes were evident compared to their normotensive counterparts. Also, after performing t-tests on glog transformed TH SNP data, no significant difference was observed in the prevalence of this mutation in Q5 relative to Q1. Lastly, the hypertensive Caucasian males showed increased total energy expenditure ($P = 0.012$) compared to the normotensives.

GC- and LC metabolomics results

After data clean up the GC-MS dataset consisted of a total of 64 features (none of which differed significantly between the hypertensives and normotensives) and the LC-MS dataset contained 321 features, 122 of which differed significantly between Q1 and Q5 according to t-tests. All clinical, biochemical and metabolomics variables were compounded into a single working data matrix, which was used in all subsequent statistical analyses. The data were normalised by glog transformation and then inspected for any natural separation using preliminary PCA. As can be seen in Figure 6.2 no sufficient natural separation exists between the normotensive and hypertensive groups although the data used did include cardiovascular measurements, such as blood pressure. Thus, as with previous groups, there appeared to be confounding variables, possibly masking the contribution of important features to the

separation of Q1 and Q5. To highlight the contribution of the variables involved in the rise in blood pressure in Q5, feature selection was performed.

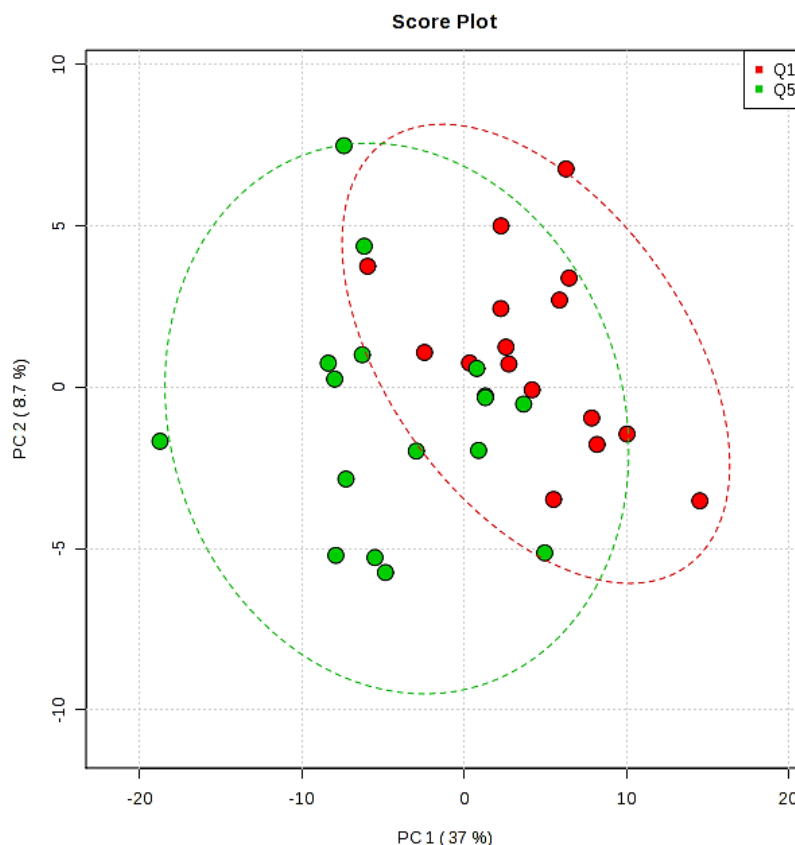


Figure 6.2: Preliminary PCA plot of Caucasian male blood pressure groups with all variables included. Red circles indicate cases from quintile (Q) 1 ($n = 16$) and green circles indicate cases from Q5 ($n = 17$).

As the organic acid profiling method showed no significantly different metabolites between Q1 and Q5 in the Caucasian male group, only the LC-MS metabolites were considered in the feature selection process. This resulted in 31 LC-MS metabolites as shown in Table 6.2 being used in the subsequent PCA analysis, which is visually represented in Figure 6.3.

Table 6.2: LC metabolomics method VIP's for Caucasian males.

Compound	Putative Annotation	Metabolite Class	Confirmatory Masses	Relative Intensity $\pm$ SD		Direction of Change*
				Q1	Q5	
1.73_201.0010n	Selenocysteine seleninic acid	Amino acid metabolite	242.99	123190 $\pm$ 31449	99421 $\pm$ 23026	-
2.14_208.0472m/z	1-methylhistidine/ 3-methylhistidine	Amino acid metabolite	187.1, 82.05, 170.09	8211 $\pm$ 1554	6989 $\pm$ 1337	-
3.26_625.0996m/z	-	Flavonoids	601.11	4273 $\pm$ 993	3207 $\pm$ 928	-
3.30_553.1712m/z	-		443.12	3557 $\pm$ 1699	1980 $\pm$ 909	
3.31_610.1230n	-		435.13	7844.92 $\pm$ 1727.24	5591 $\pm$ 1136	
3.36_356.0423n	Beta-Nicotinamide mononucleotide	Nicotinic acid nucleotides	357.05	41794 $\pm$ 9368	28380 $\pm$ 8895	-
17.15_264.1108n	Alpha-N-Phenylacetyl-L-glutamine	Amino acid metabolite	265.12	2740106 $\pm$ 642694	2176148 $\pm$ 897232	-
18.41_317.1823n	L-suberoylcarnitine	Acylcarnitines	318.19	65434 $\pm$ 15587	95154 $\pm$ 43014	+
18.42_85.0291m/z	Suberic acid	Dicarboxylic acid	55.05	17181 $\pm$ 4604	21653 $\pm$ 8057	+
26.63_85.0280m/z	L-palmitoylcarnitine	Acylcarnitines	60.08	30780 $\pm$ 10330	42078 $\pm$ 17650	+
29.98_427.2179m/z	Hydrocortisone succinate	Xenobiotic metabolite	251.13, 445.23, 209.12	3074 $\pm$ 829	4175 $\pm$ 2666	+
30.97_625.3439n	Glycochenodeoxycholic acid 3-glucuronide	Products: bile acid conjugation	626.34	12225 $\pm$ 4068	16830 $\pm$ 5010	+
31.76_314.2406n	Di-OH Octadecenoic acid	Hydroxylated LCFA	279.23	11770 $\pm$ 4558	15284 $\pm$ 5687	+
1.93_986.5053m/z	-	Phosphatidylinositol bisphosphates	-	4887 $\pm$ 964	4199 $\pm$ 1012	-
2.17_156.0755m/z	Histidine	Amino acids	-	12339 $\pm$ 7506	8210 $\pm$ 5784	-
2.26_76.0396m/z	Glycine		-	907 $\pm$ 325	689 $\pm$ 208	-
2.33_129.0657m/z	Glutamine		-	13714 $\pm$ 6383	9349 $\pm$ 3126	-
13.51_290.1588m/z	3-Methylglutarylcarnitine	Acylcarnitines	-	45127 $\pm$ 8441	55652 $\pm$ 16846	+

2.23_146.0781m/z	OH-butyric acids, OH-isobutyric acids, Isobutyrylglycine		-	7634 ± 5664	4447 ± 5373	-
2.33_176.0651m/z	Guanidinosuccinic acid	Alpha amino acids and derivatives	-	5061 ± 2713	3537 ± 1049	-
3.07_263.1924m/z	-	Unsaturated fatty acids	-	4718 ± 3635	2717 ± 2122	-
3.67_100.0766m/z	-	Aminopentanoic acids	-	9210 ± 4646	13797 ± 7013	+
19.97_616.2223m/z	Chenodeoxycholic acid disulfate	Bile acid metabolites	-	3467 ± 760	4119 ± 837	+
20.71_171.1013m/z	3-Methylsuberic acid	Dicarboxylic acid	-	2970 ± 1135	4991 ± 3027	+
21.32_258.1521n	L-Propionylcarnitine	Acylcarnitines	-	19351 ± 10464	24817 ± 10650	+
21.79_578.3328m/z	-	Glycerophospholipids/ Steroid derivatives	-	10190 ± 19865	2737 ± 1758	-
25.91_432.3439m/z	-	Vit D3 derivatives/ Sterol derivatives	-	6035 ± 8244	36667 ± 54507	+
30.34_596.3490n	L-Phosphaditic acid	Diacyl-glycerophospholipids	-	13914 ± 4032	18329 ± 6733	+
30.43_618.3739m/z	-	Monoacylglycerophosphocholines/ Monoacylglycerophosphoglycerols	-	2643 ± 1038	5644 ± 9077	+
31.45_342.2614m/z	Methyl-hexadecanedioic acid	Methylated LCFA	-	6283 ± 2480	9033 ± 3463	+
40.06_378.2973m/z	Pristanoylglycine, N-Hexadecyl-L-hydroxyproline, N-palmitoyl valine	N-acyl amides	-	2306 ± 775	1864 ± 396	-

Note: Compound = Retention time plus detected mass (where n = neutral mass and m/z = mass-to-charge ratio); Relative intensity refers to abundance and is not an absolute concentration (See Section 3.3.4); Q1 = normotensives, Q5 = hypertensives. All compounds listed differed significantly between Q1 and Q5 (P-values < 0.05).*in Q5 relative to Q1. LCFA, long chain fatty acid.

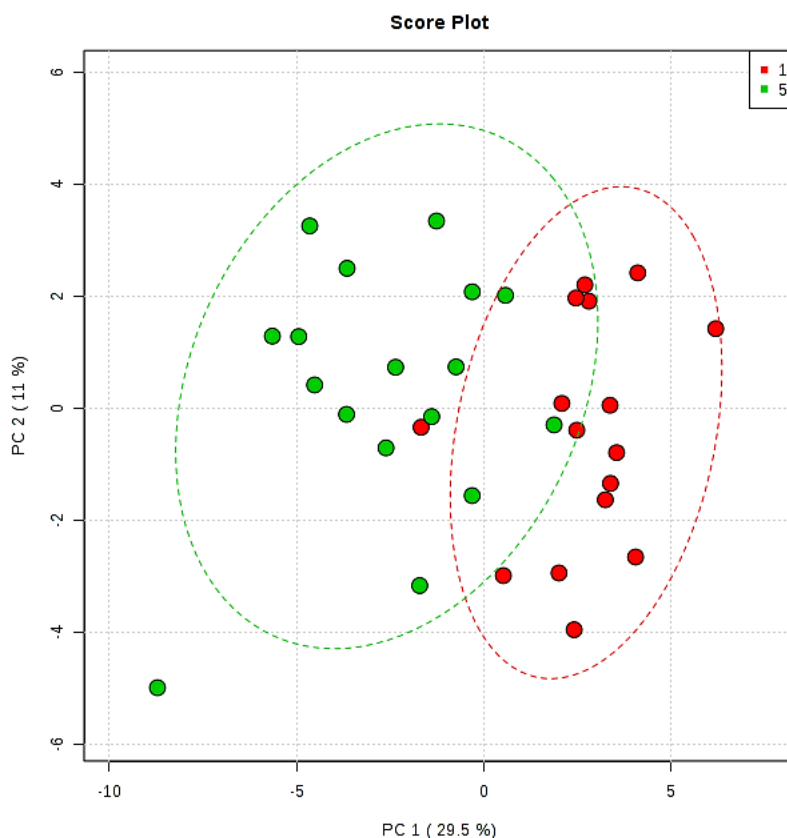


Figure 6.3: Final PCA plot of Caucasian male blood pressure groups, showing increased separation of Q1 and Q5, compared to Figure 6.2. Red circles indicate cases from quintile (Q) 1 (n = 16) and green circles indicate cases from Q5 (n = 17).

From Table 6.2 it is immediately apparent that there is a strong representation of amino acids, fatty acids of various chain lengths and acylcarnitines in the VIP list. In certain cases, the online database Metlin failed to identify a compound accurately based on the given mass-to-charge ratio although all possible identities for the compound in question belonged to the same class of biological compounds. In these cases the metabolite class is given in Table 6.2. Metabolites with specific identifications according to Metlin will be discussed first, followed by the metabolites with general allocations. Firstly, the amino acids histidine (as well as 1-methylhistidine/ 3-methylhistidine, which are both markers of muscle protein turnover), glycine and glutamine (together with α -N-phenylacetyl glutamine) were all lower in hypertensive subjects than in normotensives. Several carnitine conjugates, including 3-methylglutaryl carnitine, L-suberoylcarnitine, L-palmitoylcarnitine and L-propionylcarnitine were all elevated in the hypertensives. Furthermore, various lipid species were elevated in hypertensive subjects. These included suberic acid, 3-methyl suberic acid, dihydroxy octadecenoic acid and L-phosphatidic acid. Also, a metabolite annotated as methyl-

hexadecenoic acid was elevated in the hypertensive group compared to the normotensive group, but this could be a falsely annotated metabolite. Other metabolites showed lower levels in the hypertensives, including guanidinosuccinic acid, selenocysteine seleninic acid and β -nicotinamide mononucleotide. Interestingly, both chenodeoxycholic acid disulfate and glycochenodeoxycholic acid-3-glucuronide (which are bile acid metabolites) were elevated in the hypertensives. Lastly, a metabolite annotated as hydrocortisone succinate was elevated in the hypertensive group compared to the normotensive group. The higher hydrocortisone succinate that was seen in the hypertensives compared to the normotensives could be a falsely annotated LC feature, as this is a metabolite of cortisone treatment. Regarding the VIP's which could only be annotated into general metabolite classes (and thus were interpreted with caution) several lipid species, including phosphatidylinositol biphosphates, unsaturated fatty acids, flavonoids, glycerophospholipids and acyl amides were decreased in the hypertensive group. In contrast, vitamin D3/sterol derivatives and monoacylglycerophosphocholines/monoacylglycerophosphoglycerols were increased in the hypertensives. Lastly, aminopentanoic acids were increased in the Q5 group, compared to the Q1 group.

6.4. Discussion

In the Caucasian hypertensive males, markers of mild liver damage and long-term glucose handling problems were evident whereby no short-term elevated blood glucose levels were seen, but significantly elevated HbA1c % (an average of three-month blood glucose levels) was seen in the hypertensives compared to the normotensives. This, taken together with a profile of obesity, a picture of obesity-related pathogenic cardio-metabolic shifts is evident. Interestingly, an age-related hypertensive state was not seen in the hypertensive men. This is in accordance with literature, where males are at greater life-long risk for hypertension and cardiovascular disease, compared to age-matched, pre-menopausal females. Indeed, it is well-known that male gender is an independent risk factor for hypertension (Reckelhoff 2001; Mancia et al. 2013). Presently the ratio of total cholesterol to HDL and not the individual components were higher in the hypertensives, accompanied by no differences in oxidative stress levels compared to the normotensives. Also, no difference was seen in antioxidant capacity between the two blood pressure groups. At first glance it would seem that the contributing factors of hypertension in the Caucasian male group centres on liver damage and not oxidative damage, which is widely reported to be involved in hypertension (Rodrigo et al. 2011). Using the information obtained from the clinical-, anthropometric- and biochemical

targeted markers as a platform for the biological interpretation of important metabolites from the metabolomics methods, a distinct metabolic shift associated with amino acid and lipid metabolism was observed in the Caucasian male group. Figure 6.4 represents a visual summary of metabolic changes seen in this group.

Firstly, the amino acids histidine, glycine and glutamine showed lower levels in the urine of hypertensive males compared to normotensive males. Histidine is an aromatic, glucogenic amino acid and is considered an essential amino acid. Histidine has been shown to have anti-oxidative and anti-inflammatory effects and is also involved in the regulation of the acid-base status of the body. Furthermore, lower circulating levels of histidine have been associated with chronic kidney disease and CVD in patients, compared to controls, via various proposed mechanisms including attenuated nitric oxide synthesis and increases in sympathetic nervous system activity (Watanabe et al. 2008; Toba et al. 2010). Indeed, Tuttle et al. (2012) found that there was an inverse relationship between dietary intake of histidine and blood pressure. Watanabe et al. (2008) also found a positive correlation between histidine deficiency and inflammation. Histidine supplementation may improve nutritional status in malnourished individuals. Thus, it would seem that the lower histidine levels in our hypertensive Caucasian males as compared to the normotensives could possibly be associated with the lower marker of protein intake. Furthermore, histidine supplementation may improve nitrogen balance without increasing blood urea concentration.

Glycine is a conditionally non-essential amino acid that has been found to lend protection in pathological instances such as metabolic syndrome. El Hafidi et al. (2006) demonstrated that glycine can lower blood pressure in a rat metabolic syndrome model. It seems that glycine lowers blood pressure by increasing nitric oxide availability, and up-regulating the synthesis of structural proteins like elastin, which plays a role in vessel elasticity, and thus blood pressure. On the other hand glycine itself can be synthesised from serine by the enzyme serine hydroxymethyltransferase in a reaction requiring pyridoxal-5-phosphate (PLP) as a co-factor. Levels of PLP, the active form of vitamin B6, have been shown to be lower in alcoholics (see discussion in Chapter 4). Even though the Caucasian male Q5 group did not show a specific alcohol-related metabolic perturbation, markers of liver damage was increased in hypertensives compared to normotensives (liver damage is frequently reported in literature on alcoholics). If the level of PLP is low, glycine synthesis could be impaired as well, possibly resulting in lower glycine levels (as seen in the Caucasian male Q5 group). Also, low levels of PLP are linked to hypertension development. In a study by Lin et al. (2008), the authors investigated whether

low PLP, along with homocysteine and a specific genetic mutation had any effect on the risk of high blood pressure. The main finding was that low PLP was a significant and independent risk factor of hypertension.

Lower levels of glycine conjugates, such as isobutyryl-glycine were also seen in the hypertensives, further reflecting the possible metabolic consequences of lower glycine levels in these males as glycine is a major contributor in the phase II detoxification pathway (Liska 1998).

L-Carnitine is used not only as transporter for long chain fatty acids to cross the mitochondrial membranes and undergo β -oxidation, but also in conjugation and elimination of otherwise insoluble compounds for excretion in the urine. It therefore makes sense that adequate levels of carnitine stores in the body would be beneficial, for not only enhancing fatty acid oxidation in the mitochondria thereby lowering circulating levels of fatty acids and thus being anti-atherogenic in nature, but also for eliminating potentially toxic compounds via conjugation. In the hypertensive Caucasian males higher levels of carnitine conjugates were seen including 3-methylglutaryl carnitine, L-suberoyl carnitine, L-palmitoyl carnitine and L-propionyl carnitine. As these men presented with mean obesity levels a higher circulating pool of triglycerides and fatty acids might be present, although these were not clinically measured in the study. This pool would need to be eliminated, thus potentially putting more pressure on the pool of available free carnitine than in the normotensives and increasing the urinary acyl carnitine metabolites. Lower levels of 1-methylhistidine/ 3-methylhistidine (markers of muscle protein turnover) were seen in the hypertensives. As the exact isomer could not be identified with certainty in this study, both isomers are reported. 1-Methylhistidine is a marker of dietary protein intake, resulting from the metabolism of the dipeptide anserine found in meats and 3-methylhistidine is indicative of the rate of muscle protein breakdown. As most of the carnitine pool is supplemented by dietary intake of meat and dairy, with carnitine synthesised from dietary lysine, the lower methylhistidine isomer seen in Q5 correlates with a lower bioavailable carnitine pool leading to impaired fatty acid oxidation and higher circulating fatty acids, further adding pressure on the carnitine pool via increased conjugation reactions. In addition, glycine is a by-product of carnitine synthesis from dietary lysine and this metabolite was also lower in the hypertensives. High circulating lipid species can also contribute to liver damage and the pathogenesis of non-alcoholic fatty liver disease (NAFLD), as evidenced by the higher levels of liver enzymes in the hypertensive group as compared to the normotensive group.

A generally lower level of flavonoids was measured in the hypertensives. Flavonoids are polyphenolic plant metabolites that have been widely reported to have beneficial influence in human health, particularly cardio-metabolic well-being. Indeed, several population studies have found an inverse relationship between flavonoid intake and risk of cardiovascular disease (Feliciano et al. 2015). Westernised diets are deficient in flavonoid-rich food, such as fresh fruit and vegetables (Martin et al. 2013), and this is reflected in the lower urinary excretion of the flavonoid class of metabolites in the hypertensive Caucasian male group. Flavonoids can improve endothelial function, have strong antioxidant- and anti-inflammatory properties and are also involved in various signalling pathways (Funakoshi-Tago et al. 2015; Kim et al. 2015) in many tissue types, including adipose tissue. There are various proposed mechanisms of the beneficial actions of flavonoids, including flavonoid-mediated improvement in adipocyte functionality and fatty acid oxidation (Galleano et al. 2012). As fatty acid oxidation occurs in mitochondria, this neatly ties in with the proposed mitochondrial dysfunction connected to insulin resistance in obese subjects in literature. However, it is still uncertain whether mitochondrial dysfunction results from or causes insulin resistance (Martin & McGee 2014).

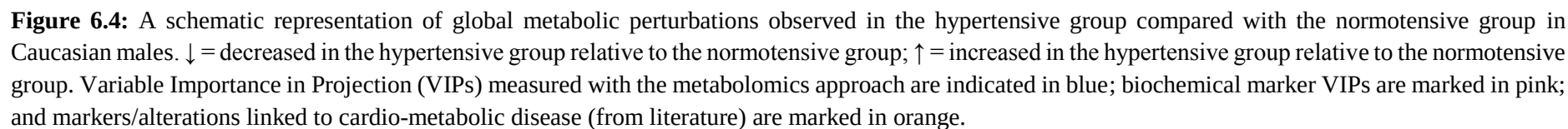
Some of the metabolites which are known to be uremic toxins, like guanidinosuccinic acid and phenylacetyl glutamine showed lower excretion in the urine of the hypertensive Caucasian males. Guanidinosuccinic acid increases after protein intake, its source being arginine. A lower level of dietary protein intake is reflected in the metabolic profile of the hypertensives, as seen in metabolites such as the methyl histidine isomer, which fits with the lower guanidinosuccinic acid. Glutamine and phenylacetylglutamine levels were also lower in the hypertensives. Glutamine is an amino acid synthesised from glutamate and higher levels have been shown to increase production of gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system. GABA serves to regulate muscle tone and therefore has an anti-hypertensive effect (Yang et al. 2012). It stands to reason that lower precursor substrates for GABA synthesis would be connected to higher blood pressure.

Higher levels of methylated metabolites were seen in the hypertensive group compared to the normotensive group, including 3-methylglutaryl carnitine, 3-methylsuberic acid and methyl hexadecenoic acid. It has been shown that the westernised diet (generally followed by urbanised individuals) is high in methyl-consuming compounds which can exacerbate oxidative stress status and lead to subsequent tissue injury (Zhou et al. 2011). These compounds are metabolised in methylation reactions utilising SAM as methyl donor, which is in turn converted to S-adenosylhomocysteine (SAH). SAH is rapidly hydrolysed into homocysteine

and adenosine. Remethylation of homocysteine completes the cycle via folate or vitamin B12-dependent pathways. Adequate supply of methyl donors is crucial to the function of the cycle, and in times of high demand for methyl donors the cycle can be interrupted leading to higher levels of homocysteine. Also, this leads to depletion of the pool of bio-available methyl groups and subsequent perturbed methylation status (Zhou et al. 2011). Both the occurrence of high levels of homocysteine and low levels of SAM have been connected to increased cardiovascular disease risk and metabolic syndrome-like pathological states (Sengwayo et al. 2013; Barve et al. 2011).

A lower level of β -nicotinamide mononucleotide (NMN) was seen in the hypertensives compared to the normotensives. This metabolite is an intermediate in the synthesis pathway of the widely used co-factor NAD^+ . This pathway starts with dietary tryptophan in ATP-consuming reactions. NMN is converted to NAD^+ by members of the nicotinamide/nicotinic acid mononucleotide adenylyltransferase (NMNAT) enzyme family. Decreased levels of NAD^+ (and thus the NAD^+/NADH ratio) have been implicated in various pathophysiological states, including hypertension and cardio-metabolic disease, where mitochondrial dysfunction is a key mechanism. Indeed, in a recent review by Massudi et al. (2012) the role of NAD^+ in various cellular functions is highlighted and the authors suggest that up-regulated NAD^+ synthesis can be a therapeutic target for degenerative disorders. Low NMN levels can have wide-reaching effects as circulating levels of tryptophan, a precursor of both NAD^+ and serotonin (a neurotransmitter involved in the regulation of appetite) has been shown to be low in obese subjects (Gebler et al. 2015). Also SIRT1, a master regulator of energy metabolism and the main energy-sensing molecule in the body, is dependent upon NAD^+ levels for proper function and is also activated by high levels of NMN (Imai & Yoshino 2013). Being involved in energy metabolism and situated in the mitochondrion, SIRT1 levels are also widely reported in studies investigating mechanisms in the pathogenesis of cardio-metabolic and cardiovascular diseases. Indeed, deficiency in (or inhibition of) the sirtuins has been implicated in the risk for metabolic syndrome and other cardiovascular diseases, and mechanisms of up-regulating their production and biological function has been proposed as therapeutic targets against these lifestyle diseases (Herranz et al. 2010; Wang et al. 2012). Therefore, as the level of the co-factor NAD^+ is insufficient for proper function of the sirtuins, systemic metabolic perturbations follows in central energy metabolism pathways leading to (and possibly simultaneously resulting from) obesity-related mitochondrial dysfunction (Morris 2013).

Lastly, a lower level of selenocysteine seleninic acid was seen in the hypertensive group. Selenocysteine is an amino acid present in several peroxidase enzymes with a structure similar to cysteine except for the replacement of the sulphur atom with that of selenium. Selenium acts as a co-factor for the reduction of antioxidant enzymes, such as in glutathione peroxidase reactions. The seleninic acid derivative of selenocysteine is produced as an intermediate in the main reduction reaction catalysed by glutathione peroxidase. Thus it stands to reason that, in states of mitochondrial dysfunction and high levels of oxidative stress such as in obesity, the antioxidant system that includes the glutathione peroxidase enzymes would be impaired, leading to reduced levels of its intermediate metabolites.



6.5. Conclusions

In the Caucasian hypertensive males urine metabolomics revealed higher lipid levels and evidence of a perturbation in the accompanying metabolism of this class of metabolites, as levels of acylcarnitines and carnitine conjugates were also elevated. Also, mild liver damage was seen in the hypertensives with levels of liver enzymes being significantly higher than in the normotensive group. Although the mean levels of liver enzymes from the two blood pressure groups did not fall outside the normal clinical ranges, the levels of these markers were significantly elevated in the hypertensive group compared to the normotensive group, which can potentially be a sign of early liver damage. This corroborates the widely reported high-fat diet induced occurrence of NAFLD. In the urbanised individual a diet deficient in micronutrients and abundant in saturated fats and simple sugars is seen, giving rise to the overfed but undernourished state, as evidenced by the lower markers of dietary protein intake seen in these men.

Also, low levels of several amino acids and their derivatives were seen in the hypertensive group, leading to possible dysfunction of several metabolic pathways in which these amino acids feature such as anti-oxidant and anti-inflammatory pathways. It is concluded from the data presented here that the hypertensive males' detoxification systems are compromised or under pressure, and an interesting point of note is that the damaged liver, failing detoxification systems and associated metabolic derangements, form part of a vicious circle metabolically being involved in both the cause and consequence of elevated blood pressure.

This means that the liver damage is both a cause and consequence of the failing detoxification system. Likewise the high blood pressure is also both cause and consequence of chronic oxidative stress and inflammatory states. The overall endpoint of this metabolic perturbation is a cardio-metabolic disease state possibly resulting from (as well as possibly resulting in) dysregulation in amino acid and lipid metabolism. This could have secondary consequences including disruption in mitochondrial function (and dysregulation of central energy homeostasis as a result of inhibition of the sirtuins) and upregulation of the SNS.

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Chapter 7: Metabolomics of hypertension in Caucasian females

7.1. Introduction

In the previous chapter an interesting metabolic shift was seen in the hypertensive Caucasian males with emphasis on perturbed lipid and amino acid metabolism affecting detoxification systems.

To investigate possible causes or contributing factors of hypertension in the Caucasian females a GC-MS organic acid analysis method was used as described in Section 3.3.1, in conjunction with an improved LC-QTOF-MS method utilising Progenesis software for LC data processing as described in Section 3.3.3. Participants were assigned to quintiles (Q) according to the average 24-hour ambulatory systolic blood pressure measurements. Only Q1 (lowest 20 % blood pressure values) and Q5 (highest 20 % blood pressure values) were used in statistical analyses.

This chapter will deal with the results obtained from the metabolomics methods along with clinical- and biochemical markers for the Caucasian female group, comparing Q5 with Q1.

7.2. Methods

In Figure 7.1 the experimental approach and strategy for the chosen Caucasian female participants is shown. From the total group of Caucasian females ($n = 108$), participants that were HIV-positive and/or using anti-hypertensive medication were excluded. The remaining 95 participants were grouped into quintiles according to 24-hour ambulatory systolic blood pressure (SBP) values. For the purposes of the metabolomics methods only samples from participants in Q1 (lowest 20 % SBP) and Q5 (highest 20 % SBP) were further processed. After data alignment from the two metabolomics methods Q1 consisted of 17 participants and Q5 consisted of 16 participants on which further statistical analyses were done.

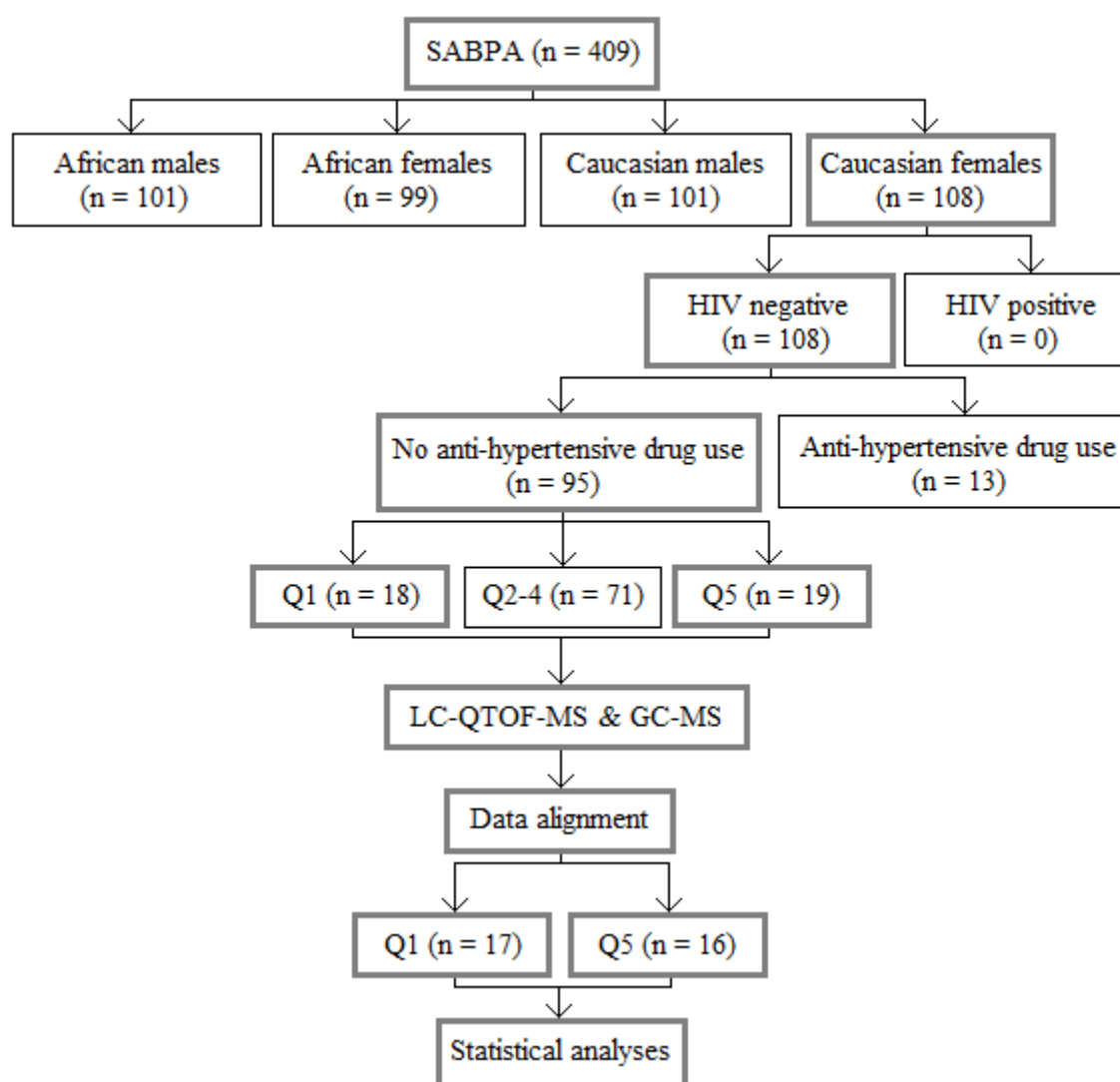


Figure 7.1: Flow diagram of strategy of participant selection in the Caucasian female group. GC-MS, gas chromatography-mass spectrometry; LC-QTOF-MS, liquid chromatography-quadrupole-time-of-flight-mass spectrometry; Q, quintile; SABPA, Sympathetic activity and Ambulatory Blood Pressure in Africans.

For a detailed description on the clinical- and biochemical targeted measurements done in the SABPA study the reader is referred to Appendix A. In brief: Body height, mass and WC were measured, after which BMI and BSA were calculated. Blood and urine biochemical analyses included quantification of total serum cholesterol, serum high-density lipoprotein (HDL) cholesterol, serum glucose, HbA1c%, high sensitivity C-reactive protein (hsCRP), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), cotinine, reactive oxygen species (ROS), ferric reducing antioxidant power (FRAP) and total glutathione.

For a detailed description on metabolomics methods followed in this study the reader is referred to Chapter 3. Briefly: The two metabolomics analyses were performed on fasting first void urine samples. Statistical analyses (Section 3.4) consisted of t-tests and PCA analyses on normalised, glog transformed data, after which biological interpretation was done.

7.3. Results

Table 7.1 lists results from general clinical, anthropometric and biochemical targeted measured markers in Caucasian females before log-transformation.

Table 7.1: Baseline characteristics of the Caucasian females study group (means $\pm$ standard deviation)

	Quintile 1 (Normotensive)	Quintile 5 (Hypertensive)	P- values
n	18	19	-
Age in years	41 $\pm$ 10.1	48 $\pm$ 8.8	0.038
<i>Anthropometric measurements</i>			
Stature, m	1.67 $\pm$ 0.07	1.65 $\pm$ 0.05	0.294
Body mass, kg	62.52 $\pm$ 10.39	85.86 $\pm$ 23.85	0.001
Body mass index, kg/m ²	22.26 $\pm$ 2.92	31.34 $\pm$ 8.56	0.000
Waist circumference, cm	75.01 $\pm$ 8.72	90.87 $\pm$ 15.68	0.001
Body surface area, m ²	1.70 $\pm$ 0.16	1.97 $\pm$ 0.27	0.001
<i>Cardiovascular measurements</i>			
24-hour SBP, mmHg	107.17 $\pm$ 2.38	133.42 $\pm$ 6.08	0.000
24-hour DBP, mmHg	67.22 $\pm$ 3.69	79.47 $\pm$ 5.82	0.000
24-hour HR, beats/min	72.28 $\pm$ 8.07	78.21 $\pm$ 10.44	0.062
<i>Biochemical analyses</i>			
Total serum cholesterol, mmol/L	4.93 $\pm$ 1.49	5.55 $\pm$ 1.39	0.199
Serum HDL cholesterol, mmol/L	1.43 $\pm$ 0.38	1.33 $\pm$ 0.48	0.516
Total cholesterol : HDL ratio	3.49 $\pm$ 0.75	4.52 $\pm$ 1.47	0.012
Serum glucose, mmol/L	5.17 $\pm$ 0.47	5.60 $\pm$ 0.49	0.011
HbA1c, %	5.24 $\pm$ 0.23	5.63 $\pm$ 0.28	0.000
C-reactive protein, mg/L	1.44 $\pm$ 0.97	6.07 $\pm$ 7.39	0.013
Alkaline phosphatase (ALP), U/L	44.41 $\pm$ 10.86	67.33 $\pm$ 16.16	0.000
Alanine aminotransferase (ALT), U/L	10.16 $\pm$ 2.65	20.36 $\pm$ 13.27	0.003
Aspartate aminotransferase (AST), U/L	14.78 $\pm$ 3.22	21.20 $\pm$ 6.86	0.001
γ -Glutamyltransferase (GGT), U/L	13.28 $\pm$ 15.91	21.32 $\pm$ 13.62	0.107
Self-reported current alcohol use, n (%)	9 (50 %)	8 (47 %)	-
Cotinine, ng/mL	10.44 $\pm$ 42.84	19.74 $\pm$ 59.59	0.591
ROS, Units*	86.94 $\pm$ 26.55	112.56 $\pm$ 37.72	0.023

FRAP, μM	395.87 ± 129.10	373.74 ± 110.92	0.579
Total glutathione, μM	836.76 ± 105.24	800.86 ± 155.47	0.419
<i>Other measurements</i>			
Physical activity, kcal/24h	2110.21 ± 316.22	3058.65 ± 756.80	0.000
Tympanic temperature, $^{\circ}\text{C}$	36.47 ± 0.34	36.58 ± 0.28	0.280
<i>Tyrosine Hydroxylase SNP**</i>			
CC n (%)	7 (41 %)	5 (31 %)	-
CT n (%)	8 (47 %)	8 (50 %)	-
TT n (%)	2 (12 %)	3 (19 %)	-

Note: n, number of participants; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; ROS, reactive oxygen species; FRAP, ferric reducing antioxidant power; *1 unit = 1.0 mg/L H_2O_2 ; significant P-values shown in bold. **only data from this variable was tested in its log-transformed state for significant difference in Q5 when compared to Q1.

Baseline clinical- and biochemical results

In the Caucasian female group, a considerable number of clinical- and biochemical variables differed significantly between the two blood pressure groups, as can be seen in Table 7.1. Firstly, hypertensive Caucasian women were older, had higher average body mass and thus BMI & BSA, as well as higher average WC ($P < 0.05$). As mentioned previously both age and WC are good predictors and risk factors for higher blood pressure, especially in Black women. This seems to hold true for their Caucasian counterparts in this study. Furthermore, neither total cholesterol nor HDL cholesterol differed between the two blood pressure groups. However, the total cholesterol/HDL cholesterol ratio was significantly higher in the hypertensives suggesting that a greater portion of their total cholesterol consists of LDL cholesterol, which is an unfavourable situation that is in itself a general risk factor for atherosclerosis and other CVD's.

Hypertensive Caucasian females also showed higher levels of serum glucose and HbA1c% levels than the normotensives, suggesting perturbed glucose handling in these individuals. Although still within the clinically acceptable range, the higher glucose and HbA1c% seen in the hypertensives as compared to the normotensives could indicate a tendency toward insulin resistance. This is supported by the higher levels of C - reactive protein (CRP), a marker of systemic inflammation indicative of chronic low-grade inflammation (Mancia et al. 2013). Higher average ROS was also observed in the hypertensives, suggesting marked elevations in oxidative stress, although no differences in antioxidant defence was noted between these two groups. Furthermore, several liver enzymes were elevated in the hypertensives (including ALP,

ALT and AST). However, GGT, a general marker of liver damage, was not significantly elevated in the hypertensive group compared to the normotensives. Interestingly the hypertensive (and more overweight) group showed higher total energy expenditure levels than the normotensives. This has been noted in literature with several studies reporting on the higher energy expenditure in overweight/obese women (Das et al. 2004; Gepner et al. 2015). Indeed, DeLany et al. (2013) found that high body mass in obese individuals leads to a high total energy expenditure where net energy cost of walking was higher in obese subjects. Also, after performing t-tests on glog transformed TH SNP data, no significant difference was observed in the prevalence of this mutation in Q5 relative to Q1 although the hypertensives did show a higher percentage of the TT allele, together with a lower percentage of the CC allele.

GC- and LC metabolomics results

After data clean up the GC-MS dataset consisted of 64 features (three of which differed significantly between the two blood pressure groups) and the LC-MS dataset contained 62 features, 27 of which differed significantly between Q1 and Q5 according to t-tests. A single working data matrix for the Caucasian females was used in all subsequent statistical analyses and was constructed with the clinical, biochemical and metabolomics data. The data were normalised by glog transformation and then inspected for any natural separation using preliminary PCA. As can be seen in Figure 7.2 no natural separation exists between the normotensive and hypertensive groups although the data used did include cardiovascular measurements such as blood pressure. Thus, there appeared to be confounding variables possibly masking the contribution of important features to the separation of Q1 and Q5. To highlight the contribution of the variables involved in the rise in blood pressure in Q5, feature selection was performed.

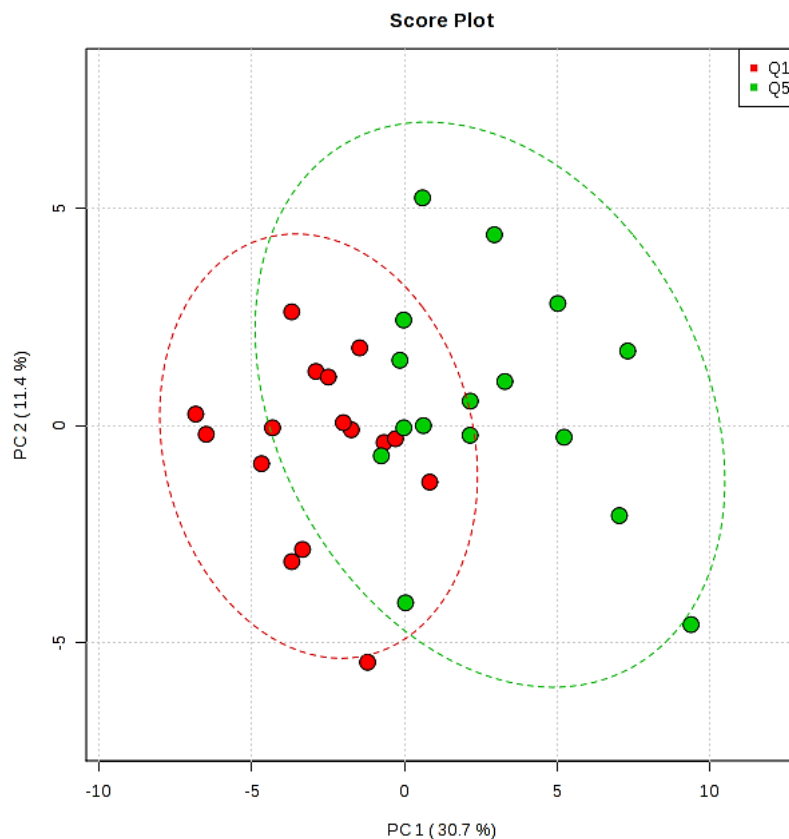


Figure 7.2: Preliminary PCA plot of Caucasian female blood pressure groups with all variables included. Red circles indicate cases from quintile (Q) 1 (n = 17) and green circles indicate cases from Q5 (n = 16).

After feature selection 3 organic acids from the GC-MS metabolic profiling method and 11 LC-MS metabolites differed significantly between Q1 and Q5 and were selected to include in the subsequent PCA analysis. Firstly, the three selected organic acids can be seen in Table 7.2. All three organic acid metabolites showed higher average values in the hypertensive group ($P < 0.05$). Both pimelic acid and suberic acid are dicarboxylic acids, while succinic acid is an intermediate in the TCA cycle.

Table 7.2: GC organic acid profiling method VIP's for Caucasian females

Organic acid	Means (mg/g creatinine) $\pm$ SD		P-value	Direction of change*
	Q1	Q5		
Pimelic acid	1.83 ± 1.20	3.44 ± 2.76	0.029	+
Suberic acid	1.91 ± 1.39	3.66 ± 3.21	0.041	+
Succinic acid	0.21 ± 0.14	0.36 ± 0.26	0.035	+

Note: Means = Mean relative abundance; SD = standard deviation. *In Q5 relative to Q1.

The feature selection process resulted in 11 LC metabolites that can be seen in Table 7.3. Of these most showed higher levels in Q1, suggesting lowered or inhibited production and/or excretion in hypertensives. Firstly 4-hydroxy-estradiol, one of the products of oestrogen metabolism, was lower in hypertensives. This metabolite is formed by cytochrome P450 (CYP1B1) catalysed hydroxylation of estradiol forming 2-hydroxy-estradiol and 4-hydroxy-estradiol, the former being the major route (Hayes et al. 1996; Itoh et al. 2010). Citric acid, an intermediate in the TCA cycle, was also higher in Q1 compared to Q5. Furthermore 3-(2-hydroxyphenyl) propionic acid, a metabolite derived from gut microbiota metabolism, showed higher levels in the normotensive group. Androsterone and androsterone glucuronide (a conjugation metabolite of androsterone) were also higher in normotensives, as well as S-adenosyl-4-methylthio-2-oxobutanoate, a by-product of biotin synthesis by gut microbiota. The following metabolites showed higher levels in the hypertensive group: N1-acetylspermidine, an intermediate in polyamine metabolism; epinephrine, an intermediate in catecholamine biosynthesis and involved in the sympathetic nervous system activity; 4-hydroxynonenal glutathione, a lipid peroxidation product and also a marker of oxidative stress conjugated to glutathione for excretion, and (+)-gamma-hydroxy-L-homoarginine, which is possibly involved in nitric oxide synthesis. Furthermore the level of trimethoxy resveratrol was higher in the hypertensive group compared to the normotensives. This is in contrast to literature where the cardioprotective characteristics of resveratrol as a plant-derived polyphenol has been extensively reported (Pervaiz & Holme 2009).

Table 7.3: LC metabolomics method VIP's for Caucasian females.

Compound	Putative Annotation	Metabolite Class	Confirmatory Masses	Relative Intensity $\pm$ SD		Direction of Change*
				Q1	Q5	
2.16_288.1235m/z	4-OH-Estradiol	Hormones	175.08, 148.08	10344 $\pm$ 2898	8197 $\pm$ 2290	-
3.25_87.0446m/z	Citric acid	TCA cycle intermediates	215.02	1691 $\pm$ 297	1467 $\pm$ 369	-
3.69_188.0995m/z	N1-Acetylspermidine	Polyamines	100.08	10457 $\pm$ 4285	15635 $\pm$ 8877	+
22.79_131.0525m/z	3-(2-OHphenyl) propionic acid	Phenylpropanoic acids	103.05	23891 $\pm$ 13867	14323 $\pm$ 10346	-
26.27_270.1467n	trismethoxy resveratrol	Plant-derived phenols	288.16	10650 $\pm$ 3694	15961 $\pm$ 7294	+
26.91_464.2024m/z	4-OH-nonenal glutathione	Lipid peroxidation product	288.16, 486.18	18556 $\pm$ 6439	26714 $\pm$ 12187	+
30.70_272.2123n	Androsterone	Hormones	199.02	49948 $\pm$ 33328	28188 $\pm$ 11673	-
30.71_466.2532n	Androsterone glucuronide	Products: hormone metabolism	484.29	24032 $\pm$ 13275	16423 $\pm$ 4162	-
4.54_184.0967m/z	Epinephrine	Catecholamines	-	2464 $\pm$ 499	2889 $\pm$ 679	+
18.92_362.0914m/z	S-adenosyl-4-methylthio-2-oxobutanoate	By-product of biotin biosynthesis	-	4792 $\pm$ 3605	2738 $\pm$ 1980	-
26.28_205.1286m/z	(+)-gamma-hydroxy-L-homoarginine	Products: amino acid metabolism	-	2585 $\pm$ 2202	13641 $\pm$ 37473	+

Note: Compound = Retention time plus detected mass (where n = neutral mass and m/z = mass-to-charge ratio); Q1 = normotensives, Q5 = hypertensives. Relative intensity refers to abundance and is not an absolute concentration (See Section 3.3.4); All compounds listed differed significantly between Q1 and Q5 (P-values < 0.05).*in Q5 relative to Q1.

The feature selection process for the Caucasian females resulted in a total of 30 variables important in separation of Caucasian females Q1 and Q5 and consisted of all clinical and biochemical variables that differed significantly between the two groups, as well as the three GC organic acid metabolites and the 11 LC metabolites from Table 7.3. These VIP's were used in a PCA analysis and separation of the two blood pressure groups is evident (Figure 7.3).

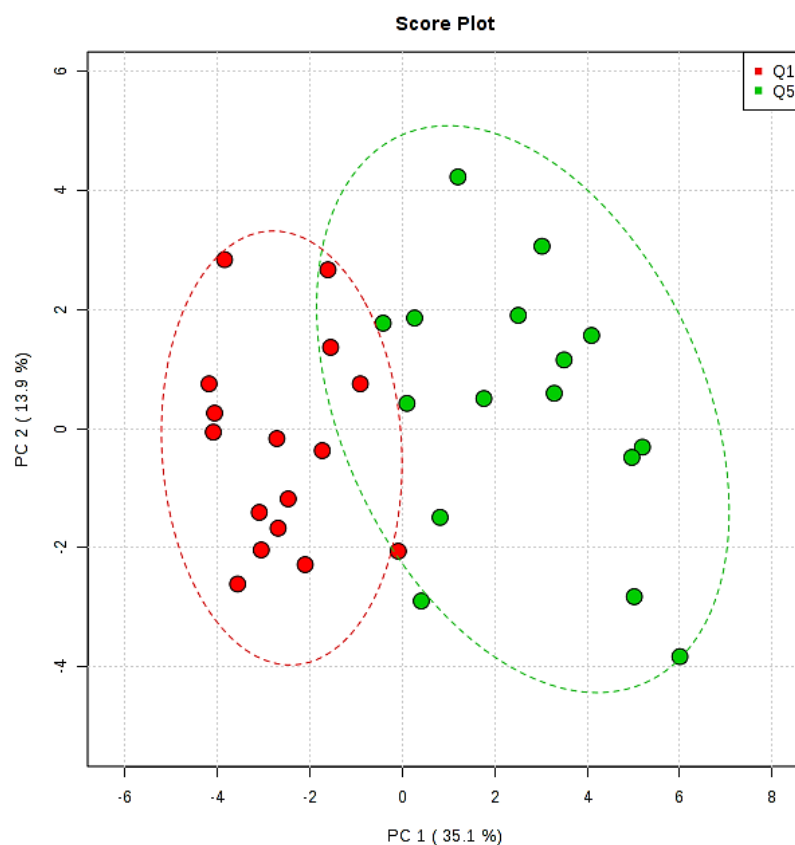


Figure 7.3: Final PCA plot of Caucasian female blood pressure groups, showing separation of Q1 and Q5. Red circles indicate cases from quintile (Q) 1 (n = 17) and green circles indicate cases from Q5 (n = 16).

7.4. Discussion

In the Caucasian female group of this study a substantial amount of clinical- and biochemical variables differed significantly between Q1 and Q5 blood pressure groups and this included many of the known consensus diagnostic criteria of the metabolic syndrome. Firstly, hypertensives were older than normotensives. This difference is widely reported in literature (Barnes et al. 2014; Chen et al. 2014) although Santos Machado et al. (2014) found that Caucasian ethnicity in women was associated more with lower cumulative incidence of

hypertension over time than women of other ethnicities. The hypertensives also showed disturbed obesity and lipid profiles, as well as low-grade inflammation. A visual representation of the discussion above can be seen in Figure 7.4.

In the hypertensive group there was a marked elevation in epinephrine, indicative of a stimulation of the sympathetic nervous system (SNS). This ties in with the lower estradiol level in the hypertensives, as clinical studies have shown that oestrogen can inhibit the SNS. Indeed, in a study by Hinojosa-Laborde et al. (2008) it was seen that oestrogen loss resulted in higher contribution of the SNS to hypertension in salt-sensitive rats on a high salt diet. In contrast oestrogen replacement attenuated this contribution. In addition, the level of visceral fat has been connected to elevated SNS activity (Brooks et al. 2015), tying the higher obesity level in the hypertensives to a possible up-regulation of the SNS, which can contribute to hypertension development.

Oestrogen can also act as an endocrine molecule via regulation of genes involved in glucose metabolism (Mauvais-Jarvis 2011). It is thus conceivable that older females presenting with lower oestrogen levels will tend towards an insulin-resistant and lipogenic phenotype, which would seem to be the case in the hypertensive Caucasian females. Oestrogen can also participate in blood pressure regulation by promoting the release of both prostacyclin and nitric oxide (NO) (Paulin et al. 2012), thereby slowing down the atherogenic process (Miller & Mulvagh 2007). This ties in with the increased level of oxidative stress seen in the hypertensives compared to the normotensives as elevated ROS levels lead to a decrease in NO levels.

NO is produced from L-arginine by nitric oxide synthase (NOS). Homoarginine (synthesized by arginine:glycine amidinotransferase (AGAT), an enzyme with high promiscuity), can replace arginine in the NOS reaction forming as an intermediate the metabolite gamma-OH-homoarginine, which was elevated in the hypertensives. Interestingly, oestrogen decreases AGAT activity. This ties in with the lowered hormone metabolite seen in hypertensives which could be implicated in the elevated activity of AGAT, producing higher levels of homoarginine, which compete with arginine in the NOS reaction to produce NO. However, as this is a less desirable substrate for the enzyme, impaired completion of the pathway takes place resulting in elevated levels of the gamma-OH-homoarginine intermediate product (as observed in the hypertensive Caucasian female group compared to their normotensive counterparts) and less NO being produced.

Higher oxidative stress levels, as seen in the hypertensives, can lead to lipid peroxidation, the products of which include 4-OH-nonenal (4-HNE). This metabolite (conjugated to glutathione) was higher in the hypertensive group, suggesting increased LDL-ox levels in the circulation of the hypertensives, which is atherogenic and can contribute to plaque formation. This ties in with the increased LDL fraction of total cholesterol (and not LDL per se, but increased LDL-ox), supported by increased levels of ROS in the hypertensives, a marker of systemic oxidative stress. Increased lipid peroxidation enhances formation of atherosclerotic plaque, and thus chronic inflammation, as seen in the elevated CRP levels of hypertensives. Higher levels of lipid peroxidation end-products have also been reported to lead to insulin resistance (Pillon et al. 2012). Thus, it seems that oxidative stress can contribute to insulin resistance but that insulin resistance also causes oxidative stress.

A possible tendency towards an insulin resistant state, as evidenced by the elevated HbA1c% levels in the hypertensives, could lead to a state of protein and lipid breakdown as glucose is not properly taken up by cells for oxidation. However, the liver takes up glucose independent of insulin levels, forming fatty acids in the process, contributing to elevated levels of free fatty acids in circulation. These circulating fatty acids could however also be as a result of high dietary fat intake. In an article by Rhee et al. in 2011 the authors found that shorter chain fatty acids were associated with type 2 diabetes, whereas longer chain fatty acids in circulation were less associated with the disease. These fatty acids can also contribute to the liver damage seen in the hypertensives. Studies have also shown that increasing circulating free fatty acids in humans rapidly induces insulin resistance (Roden et al. 1996; Belfort et al. 2005) leading to inflammation in adipocytes as evidenced by higher CRP in the hypertensives.

As stated above a state of insulin resistance promotes protein-breakdown releasing amino acids for gluconeogenesis. Metabolism of glucogenic amino acids, such as branched chain amino acids, deliver substrates to the TCA cycle in mitochondria as evidenced by increased levels of succinic acid in the hypertensives. However, this does not mean that there is a higher TCA cycle turnover as there is a discrepancy in the levels of TCA cycle intermediates with citric acid being lower in the hypertensives and succinic acid being higher.

In the hypertensive Caucasian females two markers of gut microbial metabolism were lower than in normotensives, namely 3-(2-Hydroxyphenyl) propionic acid and S-adenosyl-4-methylthio-2-oxobutanoate. Propionic acid (and associated metabolites) is produced by gut microbiota after digestion of complex carbohydrates such as fibre that cannot be digested by humans, and lowered levels of this metabolite can possibly point to an altered gut microbiome

composition or impaired gut microbiome metabolism because of poor diet. A range of microbial end-products have been shown to be beneficial to their human hosts and it has been shown that microbial diversity is highly sensitive to diet and lifestyle changes (David et al. 2014). It has been reported that poor diet (such as the Western type diet) can lead to impaired gut microbial diversity and metabolism (Cani 2013) with lowered levels of secondary metabolites available for uptake by the gut endothelium. This is supported by the lower level of S-adenosyl-4-methylthio-2-oxobutanoate seen in the hypertensives. This metabolite is a by-product of microbial biotin biosynthesis, a B vitamin that enhances insulin secretion, and the lowered bioavailable biotin from gut metabolism ties in with the lowered actions of insulin (being an anabolic hormone) resulting in a metabolic shift towards catabolism. This is frequently seen in studies that report high circulating amino acids and fatty acids in obesity and T2D.

Biotin is essential for re-methylation of homocysteine to methionine in the methionine salvage pathway and elevated levels of homocysteine have been implicated in hypertension and cardiovascular disease (Christen et al. 2000). The conversion of methionine to homocysteine is thus possibly favoured over its conversion to s-adenosylmethionine (SAM) in the hypertensives. A major role of SAM is in polyamine biosynthesis. Polyamines (putrescine, spermidine and spermine) are ubiquitous molecules that play important roles in cell growth and viability, also possessing anti-inflammatory and antioxidant properties, but perhaps the most noteworthy role of these molecules is the maturation and maintenance of intestinal mucosal barriers.

Thus low SAM availability for polyamine synthesis, coupled with the catabolic effect of pro-inflammatory signals on polyamines, result in stimulated back-conversion of these molecules, forming oxidative products such as aldehydes and peroxides in the process. Back-conversion reactions in the polyamine pathway result in accumulation of N1-acetylspermidine, the levels of which was elevated in the hypertensive Caucasian female group. As stated earlier polyamines are important in the defence against inflammation and also high dietary acid load. In cases of obesity and poor diet species of polyamine-producing gut bacteria are selected against, thereby lowering the integrity of the intestinal mucosa and lowering the defence against inflammation. This lowered acid tolerance can be seen in the lower citric acid excreted by the hypertensives, as also observed in the hypertensive Black female group.

Dysbiosis in gut bacteria could also affect bioavailability of dietary sources of methyl groups and can lead to lower bioavailable SAM (Shenderov et al. 2012) leading to reduced levels of

glutathione being produced and thus lowered antioxidant capacity to deal with the oxidative stress associated with insulin resistance as seen in the hypertensives.

Lastly, cis/trans trimethoxy resveratrol (as best annotated by the Metlin database) showed higher levels in the hypertensive group. This is in contrast to literature where the health and cardio-protective benefits of resveratrol have been widely reported (Novelle et al. 2015; Petrovski & Das 2010). However, trans-trimethoxy resveratrol has been used as an internal standard by research groups (Urpi-Sarda et al. 2007; Yashiro et al. 2012) and thus is not a normal biological metabolite. However, Bolca et al. (2013) did report on the diversity of different metabotypes of gut microbiomes concerning uptake and metabolism of dietary phenols. Thus, as seen in overweight/obese individuals, there is a decline in gut microbial diversity that leads to impaired utilisation of dietary phenols and that can possibly lead to increased levels of beneficial compounds being lost in urine output.

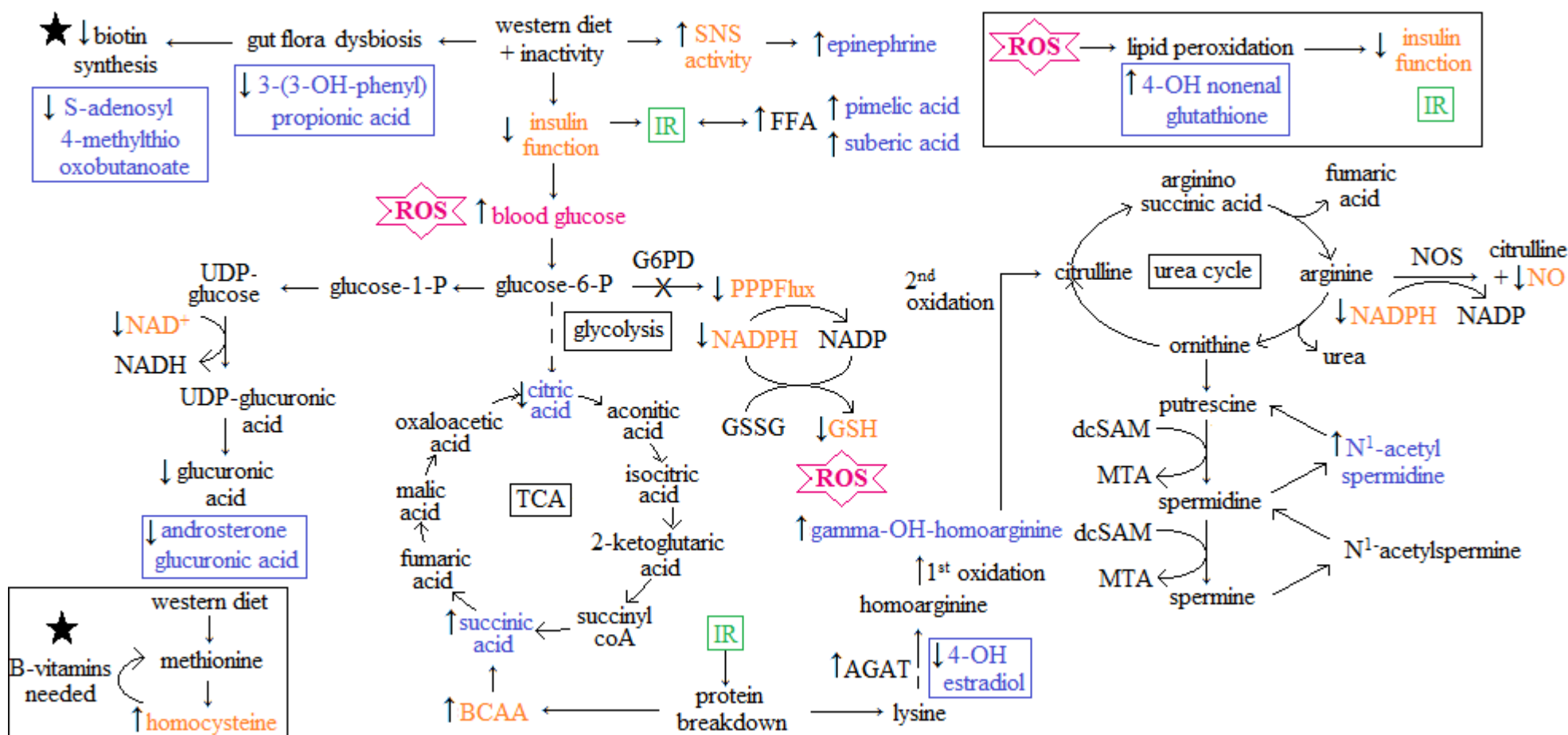


Figure 7.4: A schematic representation of global metabolic perturbations observed in the hypertensive group compared with the normotensive group in Caucasian females. ↓ = decreased in the hypertensive group relative to the normotensive group; ↑ = increased in the hypertensive group relative to the normotensive group. Variable Importance in Projection (VIPs) measured with the metabolomics approach are indicated in blue; biochemical marker VIPs are marked in pink; and markers/alterations linked to cardio-metabolic disease (from literature) are marked in orange, the common theme is insulin resistance and oxidative stress, which are marked in green blocks and pink stars, respectively. SNS, sympathetic nervous system; ROS, reactive oxygen species; IR, insulin resistance; NO, nitric oxide; NOS, nitric oxide synthase; BCAA, branched chain amino acids; FFA, free fatty acids.

7.5. Conclusions

Historically Caucasians of European heritage have been urbanised for a longer period than Black Africans. This reflects in the almost classic presentation of metabolic syndrome-like symptoms in the Caucasian females. The biggest contribution to hypertension in the Caucasian female group concerned perturbations in glucose metabolism and resulting insulin resistance-like profile, together with liver damage and impaired lipid profiles, also involving chronic low-grade inflammation and elevated levels of oxidative stress. However, after metabolic investigation the picture appeared to be more complicated than mere clinical and biochemical perturbations with involvement evident from dysbiosis in the intestinal flora. The contribution of a range of metabolites derived from the gut microbiota to the pathology of hypertension in the Caucasian females of this study serves to highlight the power of metabolomics for gleaning useful information from complex datasets, rather than relying solely on limited information derived from clinical manifestations and targeted biochemical markers.

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Chapter 8: Group comparison, conclusions and future prospects

8.1. Introduction

In the preceding chapters Black- and Caucasian hypertensive gender groups were investigated separately. In this chapter the possible mechanism(s) in the pathogenesis of hypertension as a disease in its own right, as well as a risk factor for other cardio-metabolic disease states, as observed in the four study groups, will be compared and discussed.

South Africa is a developing country undergoing changes from a more traditional collectivistic lifestyle to a more urban individualistic setting. Urbanisation brings about unfavourable changes in diet and lifestyle leading to a higher prevalence of non-communicable diseases. Among these is the so-called cardio-metabolic syndrome and its related pathologies, of which hypertension is a major independent risk factor. Hypertension is especially prevalent and more severe in individuals of African descent, placing an immense financial and socio-economic burden on this population group.

Although there has been extensive cardiovascular research done on this ethnic group internationally, there is a lack of reports investigating the pathogenesis of hypertension in South Africans in a holistic manner. Therefore, in this study, the main aim was to attempt to address and identify metabolic perturbations associated with hypertension in a well-defined study population. We used metabolic profiling methods in a hypothesis-generating manner, which is a first in South Africa, especially investigating metabolic signatures separately in gender- and ethnic groups.

As gender and ethnicity have proved to be confounding factors in various disease states, it was decided to treat each gender and ethnic group as a separate experimental entity using 24-hour ambulatory systolic blood pressure as dependent variable for each ethnic- and gender group. This resulted in four main study groups, subdivided into normotensive (Q1) and hypertensive (Q5) groups, on which urinary metabolic profiling and pathway analyses were done. Specific objectives further included the use of metabolomics methodology that would cover as much as possible of the metabolome in the chosen sample-type of this study. In fact, both a standardised and widely used GC-MS organic acid extraction method and two previously validated LC-MS metabolic profiling methods were utilised, thus fulfilling the requirement of the specific objective. Furthermore, data processing and statistical analyses relevant to the study design

were utilised, inclusive of univariate statistics, such as student's t-tests and multivariate statistics, such as principle component analyses.

Data from the metabolomics methods was pooled with clinical data for each main group separately and biological interpretation of significant features followed. As main findings for each main group are discussed in detail in Chapters 4-7, only a brief discussion of the main findings and possible biological interpretations will follow for each group.

8.2. Brief discussion on main findings

8.2.1. Black males

From the data, a metabolic perturbation associated with alcohol abuse was concluded in this group. It is noteworthy that data obtained from the health questionnaires filled in by the participants did not reflect greater alcohol use in the hypertensives. This indicates the limited added value of data from questionnaires on alcohol use in these types of studies. Chronic alcohol abuse, along with indications of central/abdominal obesity, were the main contributors to high blood pressure, bringing about various disruptions in central energy metabolism in the form of a perturbed NAD^+/NADH ratio. Also, the mechanism underlying this metabolic perturbation can possibly be attributed to an elevated oxidative stress status as many of the systems involved in ethanol metabolism produce elevated oxidative end-products, as well as depleting crucial co-factors, bringing about a range of secondary consequences. Bearing this in mind further in-depth investigation into the pathology of ethanol metabolism is essential. In addition, the urine metabolomics investigation on this group demonstrates the profound effect that modifiable lifestyle factors have on systemic metabolic function.

8.2.2. Black females

In the Black females the main contributors of hypertension, which were mostly observed from the metabolomics data of this study, appeared to be diet-related. Firstly, metabolic effects linked to a high dietary fat- and acid load were evident with compensatory pathways resulting in kidney damage and hypocitraturia. This translates into decreased TCA cycle turnover affecting mitochondrial biogenesis and central energy metabolism. The hypertensives were also more obese than normotensives but without significant changes in any other clinical markers present. Perturbations in bile acid metabolism were also evident, with lowered bile

acid levels leading to inadequate absorption of fat-soluble vitamins, as well as disruptions in bile acid-mediated endocrine functions. Finally, a perturbation in gut microbial metabolism is proposed in this study, as several metabolites specifically produced by gut flora were significantly lower in hypertensives. This has wide-reaching effects including putatively lower antioxidant capacity. Therefore, in this group an unfavourable diet and possible inactive lifestyle may result in kidney damage and lower antioxidant capacity, paving the way for increased cardiovascular risk.

8.2.3. Caucasian males

In this group, the main finding was that of a NAFLD-like syndrome with liver damage evident and high levels of several lipid metabolites detected in urine of hypertensives. Accompanying the lipid metabolites was an increase in carnitine conjugates as these lipid metabolites need to be removed from the system. This could lead to decreased bioavailable free carnitine for transport of fatty acids into the mitochondrion, resulting in a vicious circle of accumulating lipids, which is in itself a risk factor (and early symptom) of atherosclerosis. These hypertensive individuals also presented with liver damage, possibly as result of the damaging effects of chronic excess fatty acids in the liver. A diet deficient in micronutrients and abundant in saturated fats and simple sugars is indeed possibly a causing effect, giving rise to the overfed, but undernourished state.

The lower levels of some (semi)essential amino acids could be evidence of this perturbed diet, leading to perturbations in metabolic pathways in which these amino acids play crucial roles. This could result in an attenuated antioxidant capacity and lower levels of anti-inflammatory metabolites. Furthermore, detoxification systems in these men also appear to be affected negatively with depletion in methyl-donors evident. Lastly, metabolites consistent with a lower function of the nicotinic acid metabolic pathway were observed, having major downstream consequences such as lower SIRT activity. This in turn may lead to perturbations in many central energy pathways, including insulin signalling. In conclusion: Perturbations in mitochondrial β -oxidation, along with malfunctions in the major energy-regulating system of the body, were observed and associated with elevated blood pressure in this group with implications in insulin sensitivity and an overstimulation of the sympathetic nervous system also present.

8.2.4. Caucasian females

There were significant differences in this main group in many of the clinical and biochemical markers normally associated with a rise in blood pressure. The main metabolic perturbations seen in these women involved oxidative stress-generating reactions and indications of impaired glucose metabolism – classic symptoms related to hypertension. Results from this metabolomics investigation support the findings of increased gluconeogenesis, together with increased activity of the SNS and lowered antioxidant capacity. Lower metabolites from gut flora metabolism were also observed, supporting the view that diet has a profound effect on gut microbial metabolism. Overall, a rather classical picture of hypertension presented in this group consisting of insulin resistance and the accompanying rise in oxidative stress.

8.3. Group comparison

In this section, the resulting (vastly different) metabolic profiles obtained from the metabolomics methods (interpreted along with clinical markers) for hypertensives in each main group will be compared. It is important to note that, given the significant impact that variables such as gender and ethnicity will inevitably have on blood pressure, multivariate analyses, in the form of PCA, were not performed on Q5 vs. Q1 data from pooled gender and ethnic groups. This would have opposed the objectives of the study, as results from pooled PCA analyses would amplify the confounding influence from major variables, such as gender and ethnicity, while masking the effects of possible variables of interest, as noted above. For this reason, only conclusions drawn from clinical markers, as well as the eventual metabolic- and biological interpretation for each main group, will be compared.

From the clinical characteristics given for each main group in Chapters 5-7 the first important observation made was that of the lack of clinical markers that differed significantly in the hypertensive Black females, compared to their normotensive counterparts. This suggests a metabolic perturbation in these women that could possibly not be explained by measuring known markers associated with elevated blood pressure alone, potentially leading to under-diagnosis of cardio-metabolic disease in these women. However, the metabolic profiling data provided a novel avenue of information by highlighting pathways previously not connected to hypertension per se in this specific population group.

Another important observation to make is that of the difference in age between the blood pressure groups in both the Black gender groups and Caucasian females, although this

difference does not exist in the Caucasian male groups ($P = 0.883$). It is widely accepted that age is an independent risk factor for hypertension in females, with various studies reporting a positive correlation between age and blood pressure, especially in post-menopausal women (Awotidebe et al. 2014, Modena 2014, Zhou et al. 2015). It is therefore interesting that the Black males Q5 group was also significantly older than the normotensive group, suggesting that hypertension is a symptom that may accumulate risk over time. No difference was observed in stature for the blood pressure groups in any of the main groups. However BMI and BSA did differ between the Caucasian blood pressure groups, as well as in the Black male group. However, although there was no difference observed in body mass and BMI between Q1 and Q5 in the Black females group, the Black females did show higher mean obesity levels than their Caucasian counterparts, with BMI being significantly higher in Black female Q1 than Caucasian female Q1 groups. This is in accordance with literature, suggesting that Black females present with higher apparently healthy baseline values for widely accepted anthropometric risk factors of metabolic syndrome (Botha et al. 2013; Hoebel et al. 2014). Black South African women in fact present with less abdominal adipose tissue for the same WC than their Caucasian counterparts and show lower BMI at a set percentage of body fat (Rush et al. 2007).

This supports the expressed need for race/ethnicity-specific cut-off values for WC, especially in this population group as discussed by Hoebel et al. (2014). In the Black male group, hypertensives showed a significantly higher heart rate than normotensives. There was also evidence for perturbed lipid- and central energy metabolism in the main groups collectively. Liver damage was prominent in the male groups with the Black males' liver damage probably resulting from chronic alcohol intake, and the Caucasian males' liver damage resulting from a NAFLD-like state. Of the targeted clinical markers measured, there was no difference observed in serum cotinine, FRAP or glutathione levels in any group. The absence of significant decreases or compensatory increases in FRAP and glutathione as antioxidants are noteworthy, however these are only two markers of numerous possible enzyme systems and antioxidants in the body.

Each group's metabolic profile highlights different aspects of hypertension as a disease on its own, as well as its interconnected nature, being a risk factor for the metabolic syndrome and end-organ damage. Each group showed various aspects of proposed mechanisms contributing to the pathogenesis of hypertension and metabolic syndrome, like oxidative stress and insulin resistance, and each group showed effects associated with a possible poor diet and sedentary

lifestyle and the profound impact this can have on the central- and secondary metabolism pathways. The fact that each gender and ethnic group showed such marked differences in the metabolic perturbations regarding the pathogenesis of hypertension also highlights the impact of confounding factors in such studies, and the importance of taking adequate measures to ensure homogeneity of populations in such studies.

Although all the different groups had very different metabolic perturbations and likely effects on the pathogenesis of hypertension as end stage disease and also as a symptom of other CVD's, certain elements were observed in most of the groups. Firstly, evidence of perturbed antioxidant systems was observed starting with lower bioavailable SAM in the hypertensives (Q5) of most of the main groups. This could be due to constraints on the methylation pathways due to overload of methyl-consumers in the diet. Lower antioxidant capacity is linked to higher oxidative stress levels, a major implicating factor in the pathogenesis of hypertension. Oxidative stress is indeed such an important and well-known factor of hypertension that it is part of the “common soil” theory of the metabolic syndrome as discussed in Section 2.3.

Essentially excessive increased delivery of fatty acids to muscle will flood the mitochondrion with fuel, leading to build-up of bioactive lipid metabolites. This will activate serine kinase pathways and interfere with insulin regulation pathways. Considering this a striking similarity in all four main groups is the fact that hypertensives are more overweight than their normotensive counterparts are. In fact increased waist circumferences, as seen in the hypertensive groups, indicate central- or abdominal obesity, a factor that has also been widely associated with hypertension and hypertension-related pathologies. Perturbations in lipid metabolism are also rife, connecting with the incidence of obesity in the hypertensives and causing organ damage.

An interesting point to mention is the contribution of the human gut flora to the host's metabolic function. For instance, gut microbial metabolism and breakdown of dietary fibre produce secondary metabolites, which are essential in human metabolism. Unfavourable dietary input is indeed detrimental to gut microbial communities leading to damaged intestinal mucosal walls and inflammation, not to mention decreased production of essential secondary metabolites. A recurring central theme in most of our hypertensive study groups is that of gut microbial dysbiosis resulting from poor diet (and excessive alcohol use in the Black males). In most of the groups there were at least some metabolites that could be traced back to gut microbial metabolism. This is an interesting avenue of science only relatively recently better investigated – that the almost 100 trillion bacterial cells in the human gut can have such an

immense impact on our metabolic wellbeing. This makes sense given the fact that much of what we eat is metabolised first by our microbial colonies. Although there are apparent shifts in the microbial community profiles in obese and type 2 diabetic patients (taxonomic differences have been reported), the contribution of the microbiome to host metabolism is not yet completely understood.

Finally: Regarding the single genetic variant investigated in this study in the form of the tyrosine hydroxylase C-824T single nucleotide polymorphism (SNP), the following conclusion was made: This SNP (used in its log-transformed state as continuous input variable) did not cause significant separation between blood pressure groups in any of the main groups and was consequently not used in any of the final PCA analyses; thus this SNP apparently does not contribute to the development of hypertension in our study group, even when investigated in more extreme blood pressure subgroups.

8.4. Final conclusion and future prospects

Together with biochemical and clinical data from targeted measurements, a metabolomic approach, consisting of both GC- and LC-MS methods, was followed to investigate the characteristics and mechanisms of hypertension in a well-defined South African cohort. This resulted in a wealth of information and new insights into the metabolic perturbations that could be associated with a rise in blood pressure. Results clearly highlight known and novel metabolic involvements, as either result or possible cause of hypertension in these groups. Novel findings include the involvement of bile acid metabolism in hypertension in Black women as well as the involvement of altered gut microbial metabolism, which is an emerging field of interest in cardiovascular research. Also the lack of significant changes in clinical markers between normotensive and hypertensive Black women is noteworthy. The involvement of central energy metabolism in the Black male group is also noteworthy, possibly being driven by an altered NAD^+/NADH ratio. As this ratio is an important factor in central metabolism, many pathways could potentially be affected. In the Caucasian men possible perturbations in detoxification systems were inferred from the results. This too can have far-reaching effects in metabolism, especially considering various lifestyle-related factors of which diet is probably the most influential factor. Indeed, obesity resulting from an urban-dwelling lifestyle seemed to confer the most important influence with Q5 groups in all four main experimental groups showing higher anthropometric values. An urban lifestyle is associated with higher levels of

psychosocial stress (Malan et al. 2008), which is evidenced in the hypertensive groups via enhancement of neuroendocrine systems. This includes hyperactivity of the HPA axis and the SNS leading to a vast array of downstream metabolic perturbations, which could possibly lead to, but also be mediated, by T2D and CVD. Also chronic stress-induced elevation in blood pressure leads to downstream endothelial damage and a pro-inflammatory state. This neatly demonstrates the fact that hypertension is a multi-factorial disease.

Even though each main group showed a very different metabolic picture, metabolomics highlight the fact that many different metabolic systems and pathways are perturbed or affected by the pathogenesis of, in this case, hypertension (which is in itself a symptom of the metabolic syndrome of which all the groups showed other symptoms as well). Even though we used systolic blood pressure as grouping variable, the metabolic pictures of these groups brought out the fact that one cannot simply take one single symptom of this disease on its own, as there was a strong involvement in all of the groups of inflammation, insulin resistance and dyslipidaemia – all risk factors of metabolic syndrome. Therefore, using metabolomics-based approaches applied in cardiovascular research, there is no bias toward a single metabolic pathway or system as in the classical “tunnel vision” view of measuring a single clinical marker, but a holistic view of entire metabolism. Indeed, although the four groups had different metabolic pathways that came to the forefront of the respective discussions, they all intertwined on a certain level and reveal similar perturbations associated with known symptomatic states and risk factors of cardiovascular disease, i.e. metabolic syndrome criteria/symptoms. Literature on metabolomics applied to cardiovascular research has boomed in the last few years. This is not surprising given the enormous power of the method to generate information-rich datasets. The future of metabolomics, however, will rest on the reliable and easy identification of metabolites of interest for biological interpretation. This study highlights the importance of the evolution of metabolomics from being a purely analytical technique to being applied in the biological sciences for better understanding of the underlying metabolic processes and perturbations in various disease states. This also lays the basis for future studies to expand upon the insights gained in the present study, as metabolomics is a mostly hypothesis-generating technique.

Bearing this in mind a few hypotheses can be formulated given the conclusions made from the biological pathway analyses of the four groups. Firstly, it is clear that diet has a major influence on metabolism. This is a logical statement as most of the metabolic pathways in the body rely on dietary elements for proper functioning. Therefore, a hypothesis could be put forward stating

that any change in a “westernised” diet toward a diet with increased micronutrients and fibre and decreased salt intake, for example, would directly lead to a change in the prevalence and severity of risk factors related to cardio-metabolic disease. Secondly, reducing the impact of unfavourable modifiable lifestyle choices, such as alcohol intake and physical inactivity, by means of lifestyle intervention plans would also lead to a reduction in factors associated with hypertension. As little is known at present about the exact mechanism of the involvement of the gut flora in human metabolism, planning and executing a metabolomics study on cultured gut flora extracts could furthermore potentially lead to a wealth of new information provided that correct grouping, according to the dependent variable in question, is followed, be it hypertension, obesity or related disease states. Future studies utilising more than one metabolomics method and platform would also benefit from cross-validation of metabolites of interest. Although different analytical methods have differences in sample preparation steps and different instrument platforms have vastly different sensitivity and detection ranges, obtaining the same metabolite from both methods would strengthen confidence in its correct identification. In addition, this study also supports the hypothesis that genetic elements could exist between gender and ethnic groups, which could influence the development of metabolic syndrome.

From the conclusions made in previous sections it is clear that, at least in the African ethnic group of South Africa, great progress in the fight against high blood pressure would be made with future studies focussed on reducing alcohol abuse and obesity rates in this population group.

Critical assessment of the study

This study aimed to investigate mechanistic metabolic aspects of the pathogenesis of hypertension in four groups. Main shortcomings and points of concern include small sample sizes as the starting number of participants ($n = 409$) had to be sub-divided into groups with ever-decreasing numbers as metabolomics experiments must rely on robust grouping owing to its most sensitive analytical capabilities. Taking this into account, inclusion criteria for future investigations must be stricter, implying thorough critical planning stages keeping in mind the analytical platform to be utilised.

Bearing in mind that metabolomics is a young member of the “omics” world of science, which is constantly evolving and being improved upon, studies with large numbers of potential

samples should be completed in sufficient time to ensure that relevant analytical procedures are followed that are not out-dated at the time of completion. With the present study as an example of a large timeframe between analyses of some of the groups, better technology and data handling software were already being made available. As each group was investigated separately, and comparisons only drawn after biological interpretation, this negates the potential negative impact of this particular drawback; however, it is worth noting.

Regarding the metabolites that could not be annotated, or perhaps not accurately annotated (as is possibly the case with the resveratrol metabolite in the Caucasian females), this is one case where the biggest pitfall of metabolomics technology is evident: The uncertainty when annotating compounds from databases after performing searches on the m/z ratio values obtained from the LC-MS. As there is an ongoing global effort to expand the quality and scope of information in these databases, it can be expected that some metabolites still need to be measured, verified and entered into the database of interest. Also, (given that metabolomics is essentially the science of isomers), the metabolite in question can very well be an isomer of *cis/trans*methoxy resveratrol although biologically different with unrelated functions/reactions. The greatest bottleneck of metabolomics studies presently is the lack of ease in correctly identifying metabolites of interest. Confident identification of a metabolite indeed relies on subsequent MS/MS analysis of pure standards, unobtainable for every metabolite of interest in real-world situations. As data on a metabolite of interest consists of mass-to-charge ratio and retention time for precursor ions and, in some cases fragmentation ion patterns, theoretically performing an online database search for said ion could potentially result in several isomers being listed with identifications ranging from common biological intermediates to metabolites from xenobiotics, with the final annotation based on biological relevance only. It is immediately evident in the current study that several isomers exist for the majority of metabolites obtained from the LC method, which could possibly lead to subsequent misinterpretation based on incorrect isomer information.

In this study the levels of urinary metabolites were interpreted as if being in direct relationship with the levels of metabolites in circulation or in specific organs, as urine constitutes the global endpoint of metabolism. The problem remains however to what extent urinary levels of metabolites reflect the true levels in the cell and circulation. This is not a problem confined to the current study, but urinary metabolomics in general and there is no simple solution to this problem as of yet.

Finally in this study accurate data on dietary intake were not available for participants; therefore none of the conclusions made relating to dietary impact could be independently tested. Also, taking into account the significant role that amino acid metabolism played in the metabolic perturbation seen in the Caucasian males, anthropometric measurements in future studies should include a measure of muscle mass in relation to fat mass, as BMI alone can be influenced by water content and does not give any information on muscle mass. Adding data on muscle mass to clinical information in future studies, a more detailed view of protein metabolism may be possible, especially if analysed together with untargeted amino acid metabolic profiling methods. Nonetheless, although numerous limitations were encountered during this study, the aim and all of the specific objectives set for this study were met.

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Appendix A: SABPA prospective cohort study (2008/9)

A.1. Introduction

The Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study, of which the present study forms part, is a prospective cohort study investigating the impact of a hyperactive nervous system on lifestyle diseases in an urban-dwelling South African cohort (Malan et al. 2014). This well-controlled study is the first of its kind in South Africa and involves collaborators from multiple disciplines, both nationally and internationally. The SABPA study abides by the institutional guidelines and terms of the Declaration of Helsinki 1975 (revised 2008), and is approved by the Ethics Review Board of the North-West University (0003607S6).

A brief description of the main study will follow, focussing on information relevant to the current study only, as only baseline values were used. For details and a comprehensive characterisation of the entire study protocol and cohort profile the reader is referred to Malan et al. (2014).

A.2. Background (Motivation for the SABPA study)

An urban-dwelling lifestyle in Africans has been implicated as aetiological factor in the prevalence rates of lifestyle diseases such as cardiovascular disease, as evidenced by higher obesity levels, high blood pressure and the experiencing of more psychosocial stress (van Rooyen et al. 2000; Malan et al. 2008). Coping disability in Africans is associated with higher vascular activity (Malan et al. 2006; 2008; 2013; 2014) and this in turn can cause further metabolic and psychological perturbations if not controlled or properly treated, a finding shared by Nykliček and colleagues (2005).

The SABPA study is thus a multi-disciplinary project where the connection between autonomic nervous system activity, in particular the sympathetic nervous system, and the cardiovascular system has been investigated by means of measuring markers of sympathetic nervous system activity at baseline and in response to controlled laboratory stress testing.

Aims of the SABPA study include aiding in the eventual prevention and successful treatment of lifestyle diseases in Africans where the prevalence of these diseases is increasing. In order to achieve this aim specific goals of the study were formulated, namely investigating the link

between increased sympathetic nervous system activity and lifestyle diseases via measuring biological markers involved in many biological pathways and systems. These include renin-angiotensin-aldosterone system, stress hormone and catecholamines profile, oxidative stress profile, thrombotic- and inflammatory markers, cardio-metabolic syndrome markers and markers of end-organ damage. To obtain accurate blood pressure measurements and eliminating the white-coat effect (Obrist 1981) a 24-hour ambulatory blood pressure recording will be done via the Cardiotens ambulatory blood pressure apparatus which will measure blood pressure oscillometrically every 30 minutes during the day (08h00 – 22h00) and every hour during the night (22h00 – 06h00) (Kohara et al. 1995). To investigate the above-mentioned effect in a homogenous urban-dwelling subject group primary and secondary school teachers were chosen. This group, consisting of individuals with similar socio-economic status, have to deal with changing curricula and constant disciplinary problems whilst also dealing with the burden of modern urban living.

Power analyses were done for this cohort using previous studies. Ambulatory autonomic dysfunction (Kohara et al. 1995) and cortisol data (Rogauch et al. 2006; Sato et al. 2012) were used to obtain relevant effect sizes based on differences in biological profiles and genotyping hypothalamic-pituitary-adrenal (HPA) axis or emotional stress variation. Resulting sample sizes of 50 – 416 would enable explanation of biological differences and detection of single nucleotide polymorphisms (SNPs) with a statistical power of 0.8 and a significance level of 0.05.

A.3. SABPA study design

The SABPA prospective cohort study underwent extensive planning and was performed in a well-controlled setting. This study required participants to stay overnight in the Metabolic Unit at the Potchefstroom campus of the North-West University. This building is well ventilated with four bedrooms and two bathrooms, a sitting room and fully equipped kitchen. Each participant (4 per day) had their own bedroom but bathrooms were shared by two participants. The study protocol spanned two days with day one from Mondays to Thursdays and day two from Tuesdays to Fridays. Holidays and weekends were not used for data collection. To avoid the impact of seasonal changes, the study was broken down to accommodate half of the participants (Africans) in February-May of 2008 and the other half (Caucasians) in the same months of the next year. This study was conducted by highly trained researchers and personnel,

consisting of both national and international experts from the health, natural and social sciences. Instruments and apparatus were routinely maintained and calibrated, and standardised protocols were used.

A.4. SABPA participants

In October and November 2007 (Phase I) and November 2008 (Phase II) roughly 200 Caucasian and 200 African male and female teachers, aged 25-65 years, were recruited from primary- and secondary schools in the Dr Kenneth Kaunda education district in the North-West Province of South Africa. A total of 2 170 teachers from 43 schools were invited to participate. The purpose and scope of the study was communicated to potential participants and volunteering individuals were screened for eligibility to comply with inclusion criteria. They signed informed consent forms. After the screening process, 409 participants were enrolled for the study (200 Africans: 99 male and 101 female; and 209 Caucasians: 101 male and 108 female).

Inclusion- and exclusion criteria were formulated in terms of the specific study design and also in line with the set aims and objectives. Inclusion criteria were 200 Caucasian (100 male and 100 female) and 200 African (100 male and 100 female), apparently healthy educators aged 25-65 years old, recruited in the Dr Kenneth Kaunda education district in the North-West Province of South Africa. Exclusion criteria consisted of pregnancy and lactation, users of α - and β -blockers, and psychotropic substance abuse. Furthermore blood donors and individuals vaccinated in the previous three months were excluded, as well as individuals with tympanum temperatures above 37.5 °C.

In the SABPA study the guidelines of the European Society of Hypertension were followed with regards to ambulatory blood pressure values and participants with SBP $\geq$ 130 mm Hg and/or DBP $\geq$ 80 mm Hg were considered to be hypertensive (Mancia et al. 2013).

A.5. Samples Collected

Anthropometric measurements were done on day two with participants wearing minimal clothing and included height (stature), weight, waist circumference, hip circumference and neck circumference (to the nearest cm or kg). Measurements were done with calibrated instruments (Precision Health Scale, A & D Company, Tokyo, Japan; Invicta Stadiometer, IP

1465, Invicta, London, UK; Holtain unstretchable flexible 7 mm wide metal tape). Measurements were taken in triplicate using standard methods (International Society for the Advancement of Kinanthropometry 2001). Ambulatory (24-hour) measurements included: The Actical (Mini Mitter, Bend OR, Montréal, Québec), an omnidirectional accelerometer monitor worn around the waist, which determined physical activity for 24 hours taking the metabolic rate into account. Ambulatory blood pressure and 2-lead ECG measurements (according to a pre-set programme for 20 seconds at five minute intervals for assessing silent ischaemic events) were obtained with the Cardiotens CE120® apparatus (Meditech, Budapest, Hungary), validated by the British Hypertension Society in 2003. Along with anthropometric and cardiovascular measurements, biological samples were collected, consisting of fasting blood and 8 hour overnight urine samples. Resting blood samples were collected at baseline (resting values). Overnight fasting urine samples were obtained and kept on ice until being frozen at -80 °C. Blood samples consisted of 2 x 10 ml and 1 x 5 ml serum separation tubes; 1 x 1 ml NaF plasma tube; 1 x 5 ml citrate plasma tube, and 1 x 10 ml and 1 x 5 ml EDTA plasma tubes taken at baseline and handled according to specified protocols.

A.6. SABPA analyses done

Anthropometric measurements were used to calculate body mass index (BMI) and body surface area (BSA). HIV status was determined on resting serum samples according to the protocol of the National Department of Health of South Africa. Briefly, a rapid antibody test in plasma was performed with the First response kit (PMC Medical, Daman, India) and confirmed with the Pareekshak test (BHAT Bio-Tech, Bangalore, India). Analyses for fasting blood glucose, total and high-density lipoprotein (HDL) cholesterol, triglycerides, creatinine, ultra-high-sensitivity C-reactive protein (hs-CRP) and gamma glutamyl transferase were done on a sequential multiple analyser computer (Konelab 20i; Thermo Scientific, Vantaa, Finland). Estimated creatinine clearance was calculated by using the Cockcroft-Gault formula. The percentage of glycated haemoglobin (HbA1c) was determined by means of the turbidimetric inhibition immunoassay on whole blood using the Roche Integra 400 (Roche, Basel, Switzerland). Serum cotinine levels were determined with a homogeneous immunoassay (Automated Modular Roche, Switzerland). Reactive oxygen species (ROS), ferric reducing ability of plasma (FRAP) and total glutathione (GSH) were determined with high-throughput spectrophotometric kinetic assays. The data from the Cardiotens were analysed using the CardioVisions 1.15.2 Personal Edition software (Meditech®, Budapest, Hungary). The

Mosteller formula was used to calculate body surface area (BSA) (m^2) = $([height (cm) \times weight (kg)]/3600)$. Leucine/isoleucine and valine levels were determined with an electrospray ionisation tandem mass spectrometry method (Chace et al. 1997).

A.7. Brief SABPA study protocol

On the morning of day one (07:00-09:00) at the respective schools, the participants' consent forms were checked, after which the four participants of the day were fitted with the Cardiotens 24-hour ambulatory blood pressure and ECG measuring apparatus, applying suitable cuffs on the non-dominant arm, as well as accelerometers. Participants were instructed to continue with their normal daily routine at school. The participants also received 24-hour ambulatory diary cards on which they could indicate any symptoms such as visual disturbances, headache, nausea, fainting, palpitations, physical activity and emotional stress. At about 16:30 the participants of the day were transported to the metabolic unit on campus. After welcoming and introduction to the experimental setup, participants filled in demographic, health and Berlin sleep questionnaires and received pre-counselling on HIV/AIDS. Participants also received participant sheets, on which all measurement details (clinical measures for immediate feedback on day two) were indicated by the researchers at the respective stations. The number on the form corresponded to the participant number and each form stayed with each participant throughout the sampling process. Participants were then allowed to freshen up before signing indemnity forms, completing personal information and IDs, and receiving a dinner standardised according to carbohydrate, fat and protein content. After dinner, the psychosocial battery of questionnaires was completed and participants received coffee/tea and cookies. Participants then socialized and were advised to go to bed at 22:00 to ensure valid daytime and night time cardiovascular measurements from the Cardiotens apparatus. If less than 75 % of the measurements for a particular subject were successful, the measurement was repeated the next day (Mancia et al. 2013). Participants fasted overnight.

On the morning of day two at about 05:45 the Cardiotens apparatus was removed and an overnight eight hour fasting urine sample was collected. Thereafter anthropometric measurements were taken by level II anthropometrists. The measurements included length (stature), weight, and neck-, waist- and hip circumference. Two participants then underwent blood sampling, while the other two participants had other clinical measures taken, such as arterial stiffness and sonar indices. For the two participants at the cardiovascular test station,

the ECG electrodes were applied and the first round of resting sampling took place, namely a Riva-Rocci-Korotkoff blood pressure reading (taken in duplicate), ECG readings taken for six cardiac cycles and blood sampling done. Venous blood (48 mL) was collected with a winged infusion set. The infusion set was left in situ with anti-clotting solution (0.5 ml of Heparin Sodium-Fresenius 5000 IU/ml in 50 ml normal saline solution) added to the infusion set to avoid blood clotting. All resting sampling (blood pressure readings and blood sampling) was done after the participants were in a semi-recumbent position for at least 30 minutes. The four participants of the day then received feedback on their health and post counselling for HIV/AIDS, as well as monetary compensation for successful completion of the Stroop stressor test (calculated on a sliding scale), after which they were allowed to shower and have breakfast. The participants were then transported back to the school and received feedback reports within one week.

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Appendix B: Manuscript – The Contribution of the C-824T Tyrosine Hydroxylase Polymorphism to the Prevalence of Hypertension in a South African Cohort: The SABPA Study.

Co-Author consent form

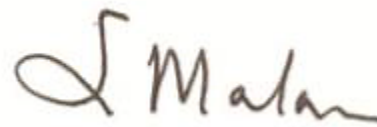
To whom it may concern,

We, the co-authors of the research paper titled “The Contribution of the C-824T Tyrosine Hydroxylase Polymorphism to the Prevalence of Hypertension in a South African Cohort: The SABPA Study. (2013) *Clinical and Experimental Hypertension*, 35(8), 614-619.” hereby give permission that this paper may be included as part of the thesis submitted for the degree *Philosophiae Doctor*.



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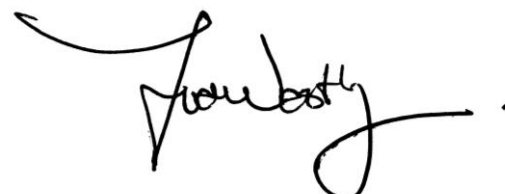
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The Contribution of the C-824T Tyrosine Hydroxylase Polymorphism to the Prevalence of Hypertension in a South African Cohort: The SABPA Study

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Abstract

The C-824T single nucleotide polymorphism in the promoter region of the tyrosine hydroxylase gene has been associated with hypertension. It is well documented that African South Africans exhibit a higher prevalence of hypertension than Caucasians. However, the possible role of this mutation on 24-hour ambulatory blood pressure (ABBP) has not been investigated in African South Africans. Blood samples of 409 South Africans were screened for the mutation. Ambulatory blood pressure and lifestyle factors were also measured. Africans had higher incidence of hypertension and higher occurrence of the mutation. However, the contribution of the tyrosine hydroxylase C-824T single nucleotide polymorphism to hypertension could not be confirmed in our cohort.

Keywords: tyrosine hydroxylase, mutation analysis, blood pressure, hypertension, South African

INTRODUCTION

High blood pressure (BP) can be caused by a magnitude of factors, some of which are universally accepted to be risk factors for the development of hypertension (HT) (1). In South Africa, the prevalence of HT is significantly higher in Africans than in other ethnic groups (2). In a study by Connor et al., logistic regression revealed that Caucasians were 44% less likely to be hypertensive than Africans (3), even after adjusting for age and gender (odds ratio 0.66, 95% CI: 0.59–0.74). A possible contributing factor is constant sympathetic nervous system overactivity perpetuated by the action of the catecholamines on target receptors in the body (2).

Tyrosine hydroxylase (TH), also known as tyrosine 3-monooxygenase (EC 1.14.16.2), is the cytosolic enzyme which catalyzes the first (rate-limiting) reaction in the catecholamine biosynthesis pathway (4). This highly specific enzyme is a homotetrameric mixed-function oxidase that uses molecular oxygen (O₂) and tyrosine as substrates and tetrahydrobiopterin as cofactor to form dihydroxyphenylalanine (DOPA), a precursor for dopamine, norepinephrine, and epinephrine. In times of stress or

emergencies, stimulation of the adrenergic nerves increases TH activity, thus speeding up the formation of catecholamines to function in response to these stressors. Several clinically significant polymorphisms in the TH gene have been described, some of which have pathological implications (5,6). Rao et al. identified the C-824T single nucleotide polymorphism (SNP) as a common base variation (7). They concluded that human catecholamine secretory traits are heritable, showing pleiotropy with autonomic activity and BP. This outcome is also consistent with findings from Goldstein, where the results indicated that decreased baroreflex-cardiac sensitivity, increased sympathetic outflow, and pressor hyper-responsiveness tend to occur together in some patients with essential HT (8).

The C-824T SNP in the promoter region of the TH gene causes overexpression of the enzyme and, thus, higher levels of catecholamines in the body (7). In a population-based study conducted by Nielsen et al., it was found that the TH C-824T SNP does influence BP in the general Danish population, i.e., BP was significantly lower in participants with the wild-type gene (9).

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Participants with the mutated (T/T) genotype had a 45% increased risk of hypertension (odds ratio 1.45, 95% CI: 1.12–1.88) compared to participants with the wild-type (C/C) genotype.

The aim of this report was to investigate the possible role of the TH C-824T SNP in predicting HT in a South African study cohort. Considering the association of this variation with high BP, we hypothesized that a high incidence of this variation exists and that, together with environmental stress caused by urbanization (10), it could contribute to HT in Africans.

MATERIALS AND METHODS

Study Population

This study forms part of the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study (February 2008–May 2009). The study included 409 teachers, 200 Africans (101 males and 99 females) and 209 Caucasians (101 males and 108 females), aged 20–63 years. After screening for the list of exclusion criteria ($>37^{\circ}\text{C}$ ear temperature, psychotropic substance dependence or abuse, blood donors, and individuals vaccinated in the preceding 3 months), participants were recruited from schools in the Dr. Kenneth Kaunda Education District in the North-West Province, ensuring homogeneity of the study population. This study was executed in two phases, with the African group in the first phase (February–May 2008) and the Caucasian group in the second phase (February–May 2009). This eliminated the effect of seasonal variation in the study. The study was approved by the Ethics Committee of the North-West University (number 00036-07-S6) and informed consent was obtained from all the participants after every aspect of the study was conveyed to them. Participants filled in health and demographic questionnaires, where smoking status, alcohol consumption, and other confounding factors were indicated. Anthropometric measurements, such as body height (stature), weight, and waist circumference, were measured according to standardized procedures (11). Subsequently, the body mass index (BMI) and body surface area (BSA) (12) were calculated for each participant. Participants were considered hypertensive with 24-hour ambulatory blood pressure (ABPM) equal to or higher than 125 mm Hg systolic and/or 80 mm Hg diastolic measurements (13). Participants were considered to be smokers with blood cotinine levels greater than 15 $\mu\text{g/L}$ (14).

Cardiovascular Measurements

ABPM measurements were conducted during a normal working day for each participant. At $\sim 07:00$ AM, participants were fitted with a physical activity meter and an ambulatory blood pressure measurement (ABPM) apparatus (CE120 Cardiotens; Meditech, Budapest, Hungary) applying suitable cuff sizes to the nondominant arm. The ABPM software

was programmed to measure BP at 30-minute intervals during the day (8:00 AM–10:00 PM) and every hour during nighttime (10:00 PM–6:00 AM). Mean successful inflation rates were calculated (72.6% for the Africans and 84.6% for the Caucasians). Participants continued with their daily activities. Each participant received an ABPM diary card where symptoms associated with BP measurement (including dizziness, palpitations, visual problems, etc.) could be indicated. At 4:30 PM, the participants arrived at the Metabolic Unit Research Facility of the North-West University where they were familiarized with the experimental setup. They later received a standardized dinner and were advised to go to bed at 10:00 PM. The next morning at 6:00 AM, the ABPM apparatus was removed. Participants then filled in questionnaires, and anthropometric measurements were taken. With participants in a semi-recumbent position, a registered nurse then obtained a fasting blood sample with a sterile winged infusion set from the brachial vein branches. EDTA whole blood was stored at -80°C until use.

Biochemical Analyses

EDTA whole blood obtained from participants was used to screen for the presence of the C-824T SNP in the TH proximal promoter. A polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method was developed to screen participants for this SNP. A 1013-bp region overlapping the C-824T polymorphism in the TH gene (GenBank NG_008128.1) was amplified by PCR using the PCR Blood Direct reagent (KAPA Biosystems, Boston, MA, USA) and the following primer set: forward 5'-ccaggggctattgtgaaga-3' ($T_m = 60.4^{\circ}\text{C}$) and reverse 5'-caggccgtgtgtctttaga-3' ($T_m = 62.5^{\circ}\text{C}$). The PCR reaction consisted of 10 μL of the Blood Direct reagent, forward and reverse primers (20 pmoles of each), and 2 μL of EDTA whole blood in a final volume of 25 μL . A Thermo Hybaid® Multiblock System 0.2G thermocycler was used for amplification. The PCR conditions were as follows: initial denaturation step at 95°C for 10 minutes, followed by 30 cycles of amplification (94°C for 30 s, 61°C for 30 s, and 72°C for 30 s). PCR was concluded by an elongation step for 10 minutes at 72°C ; thereafter the products were kept at 4°C . The RFLP analysis consisted of 2 μL Buffer R (supplied with the restriction enzyme), 7.8 μL PCR grade water, 0.2 μL *Tru1* enzyme with a recognition site of 5'-T↓TAA-3' (10 Units/ μL ; Fermentas Life Sciences; Burlington, Ontario, Canada), and 10 μL PCR product containing the amplicon. No clean-up step was used to purify the amplicon prior to the restriction analysis. The PCR mixture was transferred directly from the PCR tube to the new restriction analysis tube with a micropipette. The reaction mixture was incubated for 4 hours at 65°C in the Thermo Hybaid® Multiblock System 0.2G thermocycler. Products of the restriction analysis were analyzed by separation with agarose gel electrophoresis. A 2% (w/v) agarose gel was prepared with tris–acetate–EDTA (TAE) buffer

(consisting of 40 mM tris base, 40 mM acetic acid, 1 mM EDTA). A loading dye containing bromophenol blue (0.05% w/v) and glycerol (30%) was used to assist with the loading of the products onto the agarose gel. The voltage applied varied according to the size of the gel, with an average of 10 V/cm. The fragments were visualized using a UV fluorescence transilluminator at 254 nm with the use of ethidium bromide (EtBr, 0.5 µg/mL gel). The results were photographed with a ChemiGenius Bio imaging system from Syngene, using GeneSnap version 6.05 (2004) as bio imaging software. The presence of a homozygous C-824T polymorphism results in complete recognition and thus two fragments of 345 and 668 bp, whereas the presence of wild-type allele results in no digestion and one fragment of 1013 bp. Digestion products from heterozygous carriers would thus consist of three fragments (345, 668, and 1013 bp).

Statistical Analyses

Statistical analyses were performed using PASW Statistics software for Windows, version 18 (SPSS Inc., Chicago, IL, USA). Normality of data was tested and preliminary analyses were conducted to describe socio-demographic- and cardiovascular-related variables presented as mean $\pm$ standard deviation (SD) for continuous data and percentages for categorical data. Differences between ethnic groups for each confounding variable were assessed using unpaired *t*-tests for continuous variables, whereas the χ^2 test was used to test for dependence between categorical variables. Power calculations based on the largest standard deviation of 24-hour systolic BP have shown that 162 participants per group in this type of study are more than sufficient to show significant differences in genetic profiles (15). Hardy–Weinberg equilibrium was assessed for genetic data by the classic χ^2 test. A single two-way ANCOVA tested interaction on main effects (ethnicity $\times$ HT) for genetic and cardiovascular data. A logistic regression model with HT status as dependant variable was constructed for each ethnic group separately. Input variables consisted of body surface area, physical activity (kcal/h), γ -glutamyl transferase (γ GT, U/L, as a measure of liver damage from alcohol usage, among other causes), as well as use of anti-HT medication, antidiabetic medication, and the TH SNP for both logistic regression models. The sensitivity and specificity, as well as the statistical power, of the regression models were tested. Throughout this study, statistical significance was concluded for values of nominal $P < .05$, and BP differences of ≥ 5 mm Hg were considered to be clinically significant.

RESULTS

The T-allele frequency in the total study sample was 0.52, and the genotypes were in Hardy–Weinberg equilibrium ($\chi^2 = 0.05$, $P = .82$). In the Caucasian group, the T-allele frequency was 0.41 ($\chi^2 = 0.81$, $P = .37$) and in

the African group the T-allele frequency was 0.63 ($\chi^2 = 0.03$, $P = .86$). Table 1 summarizes the characteristics for the SABPA study group stratified according to the ethnicity. No difference was observed in age and gender between these two ethnic groups. The hypertension incidence among Africans was 68.7% and 49.0% for the Caucasians, while 74.1% of all males were hypertensive in comparison to 43.1% of all females. The percentage distribution of C/C, C/T, and T/T genotypes were 13.3%, 47.2%, and 39.5%, respectively, for the Africans and 33.2%, 51.4%, and 15.4% for the Caucasians. The interaction between ethnicity, hypertension status, and genotype was not significant. This is evident from Table 2 where similar distributions are observed, especially for the Caucasians. Also, a two-way ANCOVA showed no significant interaction between the effects of ethnicity and genotype for either SBP or DBP.

Hence, from this result, it seems that genotype alone does not necessarily influence the occurrence of HT, in either of the ethnic groups. In Table 3, *t*-tests demonstrated significant differences between hypertensives ($n = 236$) and normotensives ($n = 167$) for several risk factors where hypertensives had higher BSA (m^2) and physical activity (kcal/h) measurements than normotensives. The hypertensives also had higher γ GT levels and were older than the normotensives. A χ^2 test showed no significant interaction between smoking status and HT status. Subsequently, we investigated the effect of the TH C-824T SNP on HT incidence, accounting for a number of covariates via logistic regression model for each ethnic group. Results from the two logistic regression ethnic models are given in Table 4. Sensitivity and specificity calculations were done on the prediction results from the logistic regression analysis. The logistic regression model for Caucasians conferred 69.6% sensitivity and 77.4% specificity, whereas the logistic regression model for the Africans conferred 86.6% sensitivity and 28.3% specificity. The genotype did not predict HT in the African or the Caucasian group.

DISCUSSION

In South Africa, higher BP and HT are becoming a public health crisis and are more prevalent in Africans than in Caucasians (2). Many studies on sub-Saharan African populations indicated a multifactorial cause of this rise in BP, including environmental factors such as urbanization (16,17) and genetic factors (18,19). We aimed to investigate whether the difference in the occurrence of HT between the Caucasian and African South Africans can be partly explained by ethnic differences in the prevalence of the C-824T SNP.

Our main findings showed various differences in risk factors of HT between the two ethnic groups, with the African group showing higher mean BP values than the Caucasian group. The TH C-824T SNP genotype distributions also differed, where a

Table 1. Baseline characteristics of participants in the SABPA study ($n = 409$)

Variable	African ($n = 200$)	Caucasian ($n = 209$)	<i>P</i> value
Demographic factors			
Age, y	44.28 $\pm$ 8.01	44.92 $\pm$ 10.86	.494
Males, <i>n</i> (%)	101 (50.5)	101 (48.3)	.693
Females, <i>n</i> (%)	99 (49.5)	108 (51.7)	
Anthropometric factors			
Body mass index, kg/m ²	30.13 $\pm$ 7.01	27.60 $\pm$ 5.94	.000
Body surface area, m ^{2a}	1.92 $\pm$ 0.23	2.00 $\pm$ 0.28	.001
Waist circumference, cm	93.57 $\pm$ 15.48	92.98 $\pm$ 16.14	.707
Lifestyle factors			
Smokers, <i>n</i> (%)	52 (26)	25 (12)	.000
Nonsmokers, <i>n</i> (%)	148 (74)	184 (88)	
Serum γ GT, U/L ^a	66.33 $\pm$ 82.39	26.91 $\pm$ 33.91	.000
Physical activity, kcal/h ^a	2680.87 $\pm$ 793.69	3112.65 $\pm$ 1596.54	.001
Metabolic factors			
Serum Cholesterol, mmol/L	4.60 $\pm$ 1.19	5.54 $\pm$ 1.28	.000
Hemodynamic factors			
24 h SBP, mm Hg	133.19 $\pm$ 16.18	124.06 $\pm$ 12.04	.000
24 h DBP, mm Hg	83.46 $\pm$ 10.70	76.62 $\pm$ 8.04	.000
24 h heart rate, bpm	79.80 $\pm$ 10.73	73.63 $\pm$ 10.17	.000
Hypertensive, <i>n</i> (%)	137 (68.5)	102 (48.8)	.000
Normotensive, <i>n</i> (%)	63 (31.5)	107 (51.2)	
Medication ^a			
On antihypertensive meds, <i>n</i> (%)	43 (21.5)	18 (8.6)	.000
Not on antihypertensive meds, <i>n</i> (%)	157 (78.5)	191 (91.4)	
On antidiabetic meds, <i>n</i> (%)	10 (5)	2 (1)	.015
Not on antidiabetic meds, <i>n</i> (%)	190 (95)	207 (99)	
Genotypes ^a			
CC (Wild type), <i>n</i> (%)	26 (13.3)	69 (33.2)	.000
CT, <i>n</i> (%)	92 (47.2)	107 (51.4)	
TT, <i>n</i> (%)	77 (39.5)	32 (15.4)	

Values presented are the mean $\pm$ 1 standard deviation for continuous variables and percentages for categorical variables. Abbreviations: γ GT – γ -glutamyl transferase; SBP – 24-h mean ambulatory systolic blood pressure; DBP – 24-h mean ambulatory diastolic blood pressure. Hypertension: 125/80 mm Hg. Smoking: blood cotinine values >15 μ g/L.

^aInput variables of logistic regression models for each ethnicity.

Table 2. Distributions of genotypes across HT status for each ethnic group ($n = 403$)

Ethnicity	Normotensive ($n = 167$)			Hypertensive ($n = 236$)		
	C/C	C/T	T/T	C/C	C/T	T/T
African	13.1	41	45.9	13.4	50	36.6
Caucasian	37.7	50.9	11.3	28.4	52	19.6

Values presented are the percentages of each genotype in each ethnic group. Hypertension $\geq 125/80$ mm Hg.

Table 3. Common risk factors for HT measured in the total sample ($n = 403$)

Variable	Hypertensive ($n = 236$)	Normotensive ($n = 167$)	<i>P</i> value
Body surface area, m ²	2.04 $\pm$ 0.26	1.85 $\pm$ 0.22	.000
Physical activity, kcal/h	3049.96 $\pm$ 869.54	2692.80 $\pm$ 1688.75	.006
Smoking, <i>n</i> (%)	51 (67.1)	25 (32.9)	.120
Alcohol intake (γ GT), U/L	59.87 $\pm$ 80.22	26.49 $\pm$ 23.61	.000
Age, y	45.72 $\pm$ 9.07	43.05 $\pm$ 10.04	.005

Values presented for continuous variables are the mean $\pm$ 1 standard deviation. Values presented for the categorical variable are the percentage of smokers. A χ^2 test of dependence between the HT status and smoking status showed no significant interaction. Hypertension: 125/80 mm Hg; Smoking: blood cotinine levels >15 μ g/L.

Abbreviation: γ GT – γ -glutamyl transferase.

larger portion of the African group had the T-allele (associated with HT) than the Caucasian group, which had a higher percentage of the wild-type (C/C) genotype. Since this polymorphism is predicted

to cause higher levels of catecholamines, it has been implicated in the prevalence of HT.

The C-824T SNP in the promoter region of the TH gene putatively results in overexpression of the enzyme

Table 4. Prevalence and odds ratio's for prediction of HT in TH C-824T genotypes

Genotype	African			Caucasian		
	OR	95% CI	P value	OR	95% CI	P value
C/C	1 ^a	–	–	1 ^a	–	–
C/T	0.706	0.254–1.964	.505	0.813	0.391–1.690	.579
T/T	0.950	0.340–2.657	.923	0.393	0.137–1.125	.082

Abbreviations: OR – odds ratio; CI – confidence intervals.

^aReference group. R^2 African = 0.206, R^2 Caucasian = 0.371.

(7). In their cohort Nielsen et al. found a ~45% (CI 1.08–1.97) increase in the risk of HT in the T/T genotype group compared to the C/C genotype group. However, the contributions of some risk factors to hypertension, as well as the effect of ethnicity, could not be ignored in our cohort and had to be accounted for. Thus, adjustments for other risk factors identified were carried out in our logistic regression models. From the logistic regression models, it is clear that, for the *SABPA study cohort*, genotype is not important to predict HT status, thus the SNP is not a significant risk factor for HT. As seen from poor effect sizes (<0.05) when testing the statistical power in our cohort, it is probable that larger numbers in each group would be needed for significant separation. However, similar results to ours were obtained by Chen et al. in the Chinese Han population (20). They concluded that there is no association between the TH C-824T SNP and essential HT susceptibility in the Hunan Han population ($n = 721$). In our study, data from a total of 409 (200 Africans and 209 Caucasians) participants were used to predict HT status while correcting for the possible influence of several risk factors for HT. The aim was to unmask the possible influence of the SNP on BP and thus HT status. The greatest risk factors for HT were ethnicity and gender, but by constructing two separate regression models, these effects became nonsignificant. Thus, after correcting for other risk factors of HT we conclude that the TH C-824T SNP on its own or in conjunction with other risk factors measured in our study does not convey greater risk of HT in our study cohort. However, it is possible that the negligible effect size of the SNP can become significant when the contributions of all other risk factors (not measured in our study) of HT (environmental and genetic) are taken into account. Also, as this SNP is implicated in the catecholamine biosynthesis pathway the possibility does arise that when resting BPs are used in conjunction with BPs taken after activation of the sympathetic nervous system, the difference in BP for carriers of the T-allele from baseline to stressor exposure could be larger than for participants with the wild-type (C/C) genotype. van Lill et al. recently revealed attenuated baroreceptor sensitivity in African males at rest and during stressor exposure (21). This finding supports

the need for an investigation pertaining to the above-mentioned reactivity hypothesis.

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Appendix C: Table containing all features/variables that differed significantly ($P < 0.05$) between the hypertensive group (Q5) and normotensive group (Q1), for all four main study groups.

Black males	Black females	Caucasian males	Caucasian females
<i>Anthropometric, Clinical & Biochemical</i>			
Age	Age	Body mass	Age
Body mass	WC	BMI	Body mass
BMI	SBP	WC	BMI
WC	DBP	BSA	WC
BSA		SBP	BSA
SBP		DBP	SBP
DBP		Total cholesterol:HDL ratio	DBP
HR		HbA1c	Total cholesterol:HDL ratio
Total serum cholesterol		ALT	Serum Glucose
Total cholesterol:HDL ratio		AST	HbA1c
Serum Glucose		GGT	CRP
CRP		Physical activity	ALP
ALP			ALT
ALT			AST
GGT			ROS
ROS			Physical activity
Physical activity			

<i>GC-MS</i>			
Lactic acid	2,2-Dimethyl Succinic acid		Pimelic acid
2-OH-isovaleric acid	Citric acid		Suberic acid
4-OH-phenyl-lactic acid			Succinic acid
Fumaric acid			
Tricarballic acid			
Indole carboxylic acid glucuronide			
<i>LC-MS</i>			
'80.95363@1.7025'	1.67_126.9688m/z	0.80_81.9469m/z	2.17_175.0758m/z (4-OH-Estradiol)
'C18 H33 N O4'	1.69_172.9399m/z	1.63_273.0098m/z	3.25_87.0446m/z (Citric acid)
'C9 H11 N O6 S'	1.71_520.8544m/z	1.63_118.9906m/z	3.30_488.1320m/z
'Methylguanosine'	1.78_455.8746m/z	1.64_166.0563n	3.68_100.0770m/z (N1-acetylspermidine)
'181.95493@1.7806154'	1.82_319.9202m/z	1.64_58.9604m/z	4.54_184.0967m/z (Epinephrine)
'C12 H18 O2'	2.56_62.9892m/z (Adrenic acid)	1.68_373.9607m/z	17.47_884.2920n
'Dimethyluracil**'	3.12_520.9850m/z	1.73_201.0010n (selenocysteine seleninic acid)	17.82_928.4056m/z
'Kynurenic acid'	3.22_215.0166m/z (Citric acid)	1.75_235.9837n	18.02_507.6859m/z
'Trimethyl-L-lysine'	3.22_68.9984m/z (Citric acid)	1.76_175.9506n	18.92_362.0914m/z (S-adenosyl-4-methylthio-2-oxobutanoate)

'Hydroxysebacic acid'	6.44_196.9685m/z (Oxidized dithiothreitol)	1.77_152.8968n	21.07_302.1945m/z
'Methyluric acid'	7.52_132.9569m/z	1.78_478.8373m/z	22.79_131.0525m/z (3-(2-Hydroxyphenyl)propionic acid)
'N-Acetylarlyamine*'	12.16_228.0315m/z	1.78_472.9305m/z	24.45_567.2729m/z
'C13 H4 N2'	14.02_211.0754m/z	1.81_334.8592m/z	25.58_361.3031n
'Hesperetin'	15.23_235.0816m/z	1.81_480.8053m/z	25.77_603.3040m/z
'C15 H20 O4'	15.25_53.0375m/z	1.81_610.7739m/z	25.96_542.2643n
'Hexenoylcarnitine'	15.28_179.0633n (Hippuric acid)	1.81_115.9675n	26.27_270.1467n (Trismethoxy resveratrol)
'Indole carboxylic acid glucuronide'	15.28_286.1071n	1.87_600.6962m/z	26.28_205.1286m/z (gamma-hydroxy-L-homoarginine)
'Phenylglyoxylic acid'	15.29_358.1093n	1.89_277.8552n	26.35_389.1982m/z
'Testosterone*****'	15.32_77.0394m/z	1.92_397.8238n	26.91_288.1583m/z (4-OH-nonenal glutathione)
'C14 H15 N O8'	15.32_611.1405n (5-methyltetrahydropteroyltri-L-glutamate)	1.92_578.7094m/z	27.72_594.3071n
	15.34_105.0345m/z	1.93_840.5977m/z	27.73_345.1905m/z
	15.37_435.0357m/z	1.93_818.6064m/z	27.77_589.2943m/z
	15.90_64.9771m/z	1.93_986.5053m/z (Phosphatidylinositol biphosphates)	29.79_444.1700n
	16.96_157.0011m/z	1.94_931.5561n	30.70_272.2123n (Androsterone)
	16.97_810.1950m/z	1.96_282.8445m/z	30.71_466.2532n (Androsterone glucuronide)
	16.98_574.1928m/z	1.96_560.7021m/z	30.72_526.2822n

16.98_284.0922m/z	2.00_602.7303m/z	30.93_507.0578n
17.44_640.2331n	2.08_151.9527m/z	31.46_342.2592m/z
25.63_535.1846m/z (Estradiol glucuronide)	2.12_686.9372m/z	
27.71_444.3041m/z	2.14_208.0472m/z (3-Methyl histidine)	
31.07_526.2795n	2.16_82.0531n	
31.09_255.2077m/z (LCFA)	2.16_187.0951n	
31.09_61.0394m/z (Urea)	2.17_156.0755m/z (Histidine)	
31.10_466.2525n (Androsterone glucuronide)	2.23_146.0781m/z (Isobutyrylglycine)	
31.10_932.5069n	2.26_76.0396m/z (Glycine)	
31.71_557.3315m/z (Sterol lipids)	2.33_129.0657m/z (Glutamine)	
31.71_490.2760n	2.33_176.0651m/z (Guanidinosuccinic acid)	
31.74_519.2865m/z	3.07_263.1924m/z (Unsaturated fatty acids)	
31.77_285.2515m/z (Pinolenic acid)	3.26_625.0996m/z (Flavonoids)	
32.25_309.2557m/z	3.28_277.0942m/z	
32.25_917.5764m/z (Glycerophospholipids)	3.30_553.1712m/z (Flavonoids)	
32.27_447.2978n (Glycocholic acid)	3.30_641.0623m/z	
32.27_234.6212m/z	3.31_610.1230n (Flavonoids)	

32.30_469.2821n	3.36_356.0423n (Beta-nicotinamide mononucleotide)
34.52_473.2531m/z (Cholic acid)	3.43_623.1027m/z
36.25_118.9923m/z	3.67_100.0766m/z (aminopentanoic acids)
39.43_89.0594m/z	3.81_217.1341m/z
40.54_446.8886m/z	6.12_202.1149m/z
	12.81_158.0768m/z
	13.51_290.1588m/z (3-Methylglutaryl carnitine)
	13.65_326.0919m/z
	13.70_230.0678n
	14.02_151.1729m/z
	14.26_153.0571n
	15.27_635.2795m/z
	15.39_601.1136m/z
	15.86_67.0523m/z
	16.57_707.5768m/z
	17.08_303.0719m/z
	17.08_236.1124n
	17.08_528.2211n
	17.11_443.1222m/z
	17.11_435.1266m/z

17.15_264.1108n (Alpha-N-phenylacetyl-L-glutamine)
17.15_544.1856n
17.16_523.1848m/z
17.16_344.1284m/z
17.29_243.1280m/z
17.31_316.1764m/z
17.48_884.2944n
17.54_221.1015m/z
17.65_412.1341n
17.65_281.0993m/z
17.66_162.0420m/z
17.84_928.4070m/z
18.03_507.6857m/z
18.22_682.3759m/z
18.41_317.1823n (L-suberoylcarnitine)
18.42_85.0291m/z (Suberic acid)
18.42_244.1327n
18.43_157.0837m/z
19.86_459.1324m/z
19.97_616.2223m/z (chenodeoxycholic acid disulfate)
20.10_934.3564m/z

20.71_171.1013m/z (3-Methylsuberic acid)
20.89_385.1474m/z
21.32_258.1521n (L-propionylcarnitine)
21.45_344.2022m/z
21.79_578.3328m/z (Glycerophospholipids)
21.88_388.2270m/z
22.39_274.1942m/z
22.85_346.2188m/z
25.91_432.3439m/z (Sterol derivatives)
25.92_489.2364m/z
26.36_330.2145n
26.63_85.0280m/z (L-palmitoylcarnitine)
26.65_182.0930n
26.95_746.3750n
28.37_652.3562n
28.47_630.3318n
28.47_649.9890m/z
28.49_634.3687m/z
29.46_682.3726m/z
29.75_466.1556n
29.76_624.3245m/z

29.94_874.5698n
29.95_484.1630n
29.96_616.3626m/z
29.98_427.2179m/z (hydrocortisone succinate)
30.04_596.3713m/z
30.34_596.3490n (L-phosphaditic acid)
30.35_614.3618n
30.36_402.3083n
30.43_618.3739m/z (Monoacylglycerophospholipids)
30.97_625.3439n (Glycochenodeoxycholic acid 3-glucuronide)
31.16_628.3759n
31.45_342.2614m/z (Methyl-hexadecanedioic acid)
31.75_490.2722n
31.76_314.2406n (Dihydroxy octadecenoic acid)
32.22_431.2994n
32.23_432.3025m/z
40.06_378.2973m/z (N-acylamines)

Note: BMI, body mass index; BSA, body surface area; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; CRP, C-reactive protein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase; ROS, reactive oxygen species.