

CHAPTER 1 : INTRODUCTION

1.1 Cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity globally. In 2004, approximately 30% of deaths worldwide were attributed to CVD ('Cardiovascular Diseases' <http://www.who.int/mediacentre/factsheets/fs317/en/index.html> [accessed 19 November 2010]).

In the United States it is estimated that more than eighty one million adults (more than one in three) have one or more types of CVD (American Heart Association, 2010). Middle- and lower income countries account for up to 80% of CVD deaths (Wu and Meininger, 2000). In the year 2000 in South Africa, non-communicable diseases, including CVD, claimed 37% of adult mortalities (Bradshaw *et al*, 2003).

Cardiovascular disease is a broad term for an array of disorders affecting not only the vasculature but the heart itself. It includes disorders such as congestive heart failure, aneurysm, angina, atherosclerosis, arteriosclerosis, cardiac myopathies, acute myocardial infarction, peripheral vascular disease and hypertension. Consequences of these disorders are not exclusive to the cardiovascular system but proliferate into other disorders such as kidney failure and stroke. As discussed in the INTERHEART study, smoking, diabetes, abdominal waste-to-hip ratio, apolipoprotein B-to-A ratio and hypertension are some of the risk factors for acute myocardial infarction (Steyn *et al*, 2005). Of greater importance, to this study, is the critical modifiable risk factor, hypertension.

1.2 Hypertension

Hypertension or high blood pressure is the leading cause of stroke and one of the most important modifiable risk factors for CVD (American Heart Association, 2010; Mensah, 2008). Hypertension has been implicated in various other cardiovascular complications such as coronary artery disease, left ventricular hypertrophy, heart failure and stroke (Gokce, 2004). Two thirds of stroke cases, the second most common cause of death worldwide, occur in developing regions such as sub-Saharan Africa (Connor and Bryer, 2006). In South Africa, stroke is the fourth most common cause of death claiming 6% of all deaths annually. Of concern is recent hospital based study data showing age specific stroke in younger adults between the ages 35 to 54 years (Connor and Bryer, 2006) in whom hypertension appears to be a major contributing cause. Hypertension is a major health care problem affecting nearly 50 million people in the US (Boger and Ron, 2005).

Previously hypertension was rarely diagnosed amongst rural Africans but with urbanisation hypertension is more commonly diagnosed, particularly in sub-Saharan Africans (Opie and Seedat, 2005). In South Africa more than 6 million people, 15 years and older (approximately one in four) live with hypertension (Steyn, 2007). This burdens the economy in excess of R200 million per year with the reality that not all treatments are successful.

The 2010 Heart Disease and Stroke Statistics data showed that people with African ancestry have higher prevalence of hypertension and stroke rates, 23.3% and 2.7% respectively in whites compared with 31.8% and 3.6% respectively in blacks (American Heart Association). People with African ancestry tend to develop hypertension earlier in life and have higher average blood

pressures than any other racial grouping. Consequently this equates to a 4.2 times higher risk of developing kidney disease (American Heart Association, 2010).

Known risk factors for CVD can be divided into those which are modifiable and those which are non-modifiable (Table 1.1). Aggressive intervention can reduce the risk and thus the development or progression of CVD before mortalities and morbidities occur. Hypertension is a modifiable risk factor and underlies many cardiovascular disorders (WHO, 2011). However, even when the contribution of these risks is taken into account, they do not explain the high prevalence and incidence of CVD. Our knowledge of cardiovascular disease, whether it be mechanism or understanding, is still incomplete and other approaches are needed to explain the excess CVD in populations.

Hypertension has few modifiable mechanisms one of which is the ability to manipulate endogenous vaso constrictors and dilators. One such substance is nitric oxide (NO), a potent vasodilator, which has been closely reviewed with the likes of arginine (Wu and Morris 1998; Gokce, 2004; Cylwik *et al*, 2005; Huynh and Chin-Dusting, 2006a).

Table 1.1 Modifiable and non-modifiable risk factors for cardiovascular disease

Risk factors for cardiovascular disease	
Modifiable	Non-modifiable
<p>Hypertension $\geq 140/90$ mmHg</p> <p>Lipid Levels \uparrow Cholesterol</p> <p> \uparrow Triglycerides</p> <p> \uparrowLDL /\downarrowHDL</p> <p>Tobacco use</p> <p>Physical inactivity</p> <p>Obesity</p> <p>Diabetes</p> <p>Stress</p> <p>Anxiety</p> <p>Depression</p> <p>Alcohol use</p>	<p>Family history</p> <p>Gender A risk in men when compared with premenopausal women</p> <p>Ethnicity African and Asian ancestry develop CVD more than any other racial group</p>

Adapted from *American Heart Association and World Health Federation, 2010*

1.3 Nitric oxide

Nitric oxide is an endogenous gas involved in an array of physiological processes such as leucocyte activation, smooth muscle cell proliferation, extra cellular matrix deposition, anti-coagulation, platelet aggregation, immunological reactions and cell to cell interactions (Chin-Dusting *et al*, 2007, Boger and Ron, 2005). Nitric oxide deficiency is a major risk factor for stroke, renal insufficiency and end stage renal failure. To this study, the key attributes of NO pertain to the relationships between NO and the cardiovascular system. Nitric oxide regulates cardiac contractility and is a mediator of vascular tone and integrity (Durante *et al*, 2007). Nitric oxide also plays a vital role in the prevention of atherosclerosis by suppressing abnormal vascular smooth muscle cell proliferation (Napoli *et al*, 2006). Nitric oxide may be produced by any of the nitric oxide synthases one of which is the endothelial nitric oxide synthase (eNOS) and, as the name implies, is expressed in the endothelium of blood vessels. Defective endothelial cells or impaired NO production has adverse implications on the cardiovascular system and therefore may be one plausible etiology or contributing factor of hypertension (BP systolic ≥ 140 mmHg and/or diastolic ≥ 90 mmHg).

Endothelial derived NO regulates arterial tone through the activation of a cascading second messenger system. The release of NO activates guanylyl cyclase to generate cyclic 3'5'-guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) in smooth muscle cells. Elevated cGMP in turn causes vascular smooth muscle relaxation (Wu and Meininger, 2000; Gokce, 2004). Nitric oxide is not restricted to working through second messenger systems but

may also initiate vasodilatory effects by directly activating calcium-dependant potassium channels in vascular smooth muscle cell membranes (Yang and Kaye, 2006).

Inactivation of NO for reasons such as excess generation of reactive oxygen species, decreased bioavailability of L-arginine or defects in intracellular transduction have all been implicated in the pathophysiology of hypertension (Gokce, 2004). In some instances decreased bioavailability of NO has been ascribed to the etiology of endothelial dysfunction, a state in which normal endothelial regulated vascular tone is disturbed (Yang and Kaye, 2006). West *et al* in 2005 also showed that administration of L-arginine is beneficial in the treatment and management of peripheral vascular disease and associated stroke risk factors. Indeed, both peripheral vascular disease and stroke are conditions in which NO plays a crucial role in preventing the onset and countering disease effects. Aberrations in the L-arginine/NO pathway are considered to be one of the common mechanisms through which several cardiovascular risk factors mediate their deleterious effects on the vascular wall.

1.3.1 Nitric oxide synthesis

Nitric oxide is synthesized by the nitric oxide synthase (NOS) family of enzymes (Armengou *et al*, 2003). Three isoforms of NOS are present namely: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Huynh and Chin-Dustling, 2006). Both nNOS and eNOS are regulated by calcium, calmodulin and by post translational specializations of these enzymes. Inducible NOS is regulated by cytokine stimulation and produces more NO than the eNOS and iNOS isoforms (Napoli *et al*, 2006). Tetrahydrobiopterin (BH₄), nicotinamide-adenine-dinucleotide phosphate, flavin adenine dinucleotide and flavin mononucleotide are bound to the

NOS enzymes as part of their functional groups (Huynh and Chin-Dustling, 2006). The NOS family relies solely upon arginine as substrate. The NOS enzymes oxidize the substrate L-arginine to form NO and citrulline.

1.4 Arginine

There are two isomers of arginine, namely the D and L isomers. L-arginine is the isomeric form that is naturally available and considered the active form in the human body. For the duration of this study ‘arginine’ refers to the L-form of the amino acid isomer in all instances. Arginine was first discovered by Schultze in 1886 and is considered to be a nutritionally semi-essential amino acid (Cylwik *et al*, 2005; Murray *et al*, 1990). Essential amino acids must be supplied in the diet as the body cannot synthesize adequate amounts to support growth in children or to maintain regular physiological functions in adults. Arginine is an endogenous amino acid but is not produced at sufficient rates to support growth of children (Murray *et al*, 1990). Arginine can be found in considerably high amounts in sea food, nuts, seeds, algae, meat, rice protein concentrate and soy protein isolate (Collins *et al*, 2007). A recent report indicates that 25% of people living in the United States consume suboptimal levels of arginine per day and that preterm infants (10-12% of newborns) exhibit arginine deficiency resulting in multi-organ failure or hyperammonemia (Wu *et al*, 2008).

Arginine is involved in many physiological and nutritional processes such as the transport, storage and excretion of nitrogen, the urea cycle as an intermediate in the elimination of urea, in the ammonia detoxification and the formation of creatinine, as a structural amino acid in proteins and as an amino acid at the catalytic centre of enzymes (Milner, 1985; Cylwik *et al*, 2005; Wu *et*

al, 2008). Furthermore, with particular reference to the adrenal and pituitary glands, arginine serves as a stimulator of some endocrinal secretions such as insulin, growth hormone, glucagon, prolactin and catecholamines (Appleton, 2002). As an inhibitor of the angiotensin-converting-enzyme, arginine possesses anti-hypertensive properties. These anti-hypertensive properties are achieved by altering circulating angiotensin II. Decreased angiotensin II limits the re-absorption of fluid in the proximal tubules of the kidney resulting in a lower plasma volume and finally a reduction in blood pressure is achieved (Wu and Meininger, 2000).

Most importantly, arginine is the precursor to the endothelial derived vasodilator, NO (Chin-Dusting *et al*, 2007). The importance of arginine in CVD is mainly derived from studies demonstrating that arginine supplementation: decreases blood pressure (Siani *et al*, 2000), improves status of heart failure patients (Rector *et al*, 1996) and small vessel coronary endothelial function (Lerman *et al*, 1998), improves haemodynamic responses (Cooke, 1997; Napoli, 2006), and inhibits atherogenesis (West *et al*, 2005).

Considering the role of arginine as the NO precursor and the subsequent beneficial effects on the cardiovascular system, there is sufficient rationale that investigations should not only surround arginine but include factors affecting arginine, precursors of arginine and arginine metabolites. Normal plasma arginine concentrations range between 80 – 120 μmolL^{-1} (Morris Jr., 2007), but what are the concentrations in patients with cardiovascular disease? Do arginine concentrations differ in patients with CVD and if so, what are the factors that dictate this change? Is arginine limiting per se or are aberrations down or upstream of the arginine metabolic pathway contributing to hypertension or CVD's?

1.4.1 Metabolism of arginine

Free arginine within the body is derived from one of three sources: diet, protein turnover and endogenous synthesis (Morris Jr., 2006). Arginine synthesis can occur in a variety of sites such as the intestines, kidneys and the liver but the majority of synthesis, in adults, occurs through an intestinal-renal axis (Chin-Dusting *et al*, 2007; Wu *et al*, 2008; Wu and Morris, 1998). The kidneys are responsible for approximately 60% of arginine synthesis. Enterocytes of the small intestine synthesize citrulline from glutamate, glutamine and proline. In infants synthesized citrulline is converted to arginine locally within enterocytes. However in adults, citrulline synthesized by the enterocytes is transported to the kidneys and then converted to arginine (Wu and Morris, 1998; Wu *et al*, 2008). In the proximal tubules of the kidney, arginosuccinate synthase (ASS) and arginosuccinate lyase (ASL) are responsible for the conversion of citrulline to arginine. Good renal health is therefore paramount to ensure adequate arginine synthesis which in turn will promote cardiovascular health.

The intestinal-renal axis for the synthesis of arginine, also referred to as the inter-organ pathway, is thus named because all enzymes required for arginine synthesis are not present at either one of these sites (Wu and Morris, 1998). The highest rates of arginine synthesis do occur within the hepatic urea cycle, a cycle that needs to be continuously replenished in order to sustain further urea synthesis (Wu and Morris, 1998). However, net production of arginine from the liver is small compared with the inter-organ pathway. This is due to high arginase activity and the constricting channeling of intermediates within the urea cycle (Huynh and Chin-Dusting, 2006; Wu and Morris, 1998). There is, however, a disparity in the literature as to whether arginine

produced in the urea cycle, albeit small quantities, is used for NO production (Wettstein *et al*, 1994; Wu and Morris, 1998).

The present study is focused on determining arginine concentrations in the plasma which plays a role in maintaining the integrity of blood vessels (Wu and Morris, 1998). Arginine directly and as the NO precursor has been shown to positively influence the integrity of blood vessels thus improving endothelial function and lowering or normalizing blood pressures (Gokce, 2004). In addition to the vascular effects, arginine reduces endothelin-1 and angiotensin II, both of which are vasoconstrictive agents.

Despite the numerous mechanisms put forward, the exact pathway by which arginine regulates vascular tone still remains unclear. This disparity is evident in the ‘arginine paradox’ which shows that although intracellular arginine may be at saturated concentrations for utilization by the NOS enzymes, increasing extracellular arginine can further enhance NO synthesis by providing more substrate (Yang and Kaye, 2006; Wu and Morris, 1998; Huynh and Chindusting, 2006). Perhaps vasomotor regulation by arginine involves an array of interconnected pathways or involves other amino acids as mediators (Figure 1)?

Figure 1. Arginine metabolism and pathways

1.5 Amino acids

Amino acids are the monomer units of peptides, polypeptides and finally protein. Although there are approximately 300 different amino acids that exist in nature, the hydrolysis of protein only yields 20 amino acids (Table 1.2) (Brosnan, 2003). Amino acids have various metabolic functions and are constituents of many biomedical compounds that are important for normal physiological function. A number of diseases are attributed to abnormal intra and intercellular amino acid transport. These diseases are often characterized by increased or decreased concentrations of specific amino acids in the plasma or urine (Murray *et al*, 1998).

Given that single amino acid concentrations may vary with disease, gluconeogenesis, protein synthesis, muscle anabolism and catabolism, it would be more accurate and beneficial to evaluate an amino acid profile rather than the concentration of a single amino acid. An example of such a profile is the Fischer's ratio which evaluates the ratio between branch chained amino acid and aromatic amino acid concentrations (Leucine + Valine + Isoleucine)/Tyrosine + Phenylalanine). Fischer's ratio has commonly been used to diagnose and monitor pharmacological treatment efficacy of hepatic encephalopathy (Kimura *et al*, 2009). Such ratio monitoring or amino acid profiling provides valuable insight into the progression, regression and possible etiology of a given disease. In addition to evaluating amino acid concentrations and their ratios, it may also prove beneficial to evaluate associations between various amino acids and between amino acids and other metabolites.

The use of an amino acid profile or a specified group of amino acids to monitor disease diagnosis, progression or regression is called an AminoIndex (Kimura *et al*, 2009). Published

applications of AminoIndex are still limited particularly with the specific target area of diagnosis (Kimura *et al*, 2009). According to research at the Metametrix Clinical Laboratory (www.metametrix.com), conditions associated with plasma amino acid changes are numerous and include hypertension and congestive heart failure.

1.6 Summary

The importance of arginine in CVD is derived mainly from studies showing that arginine supplementation improves outcome in CVD. However, factors affecting arginine concentrations have not been fully elucidated. There appears to be a paucity of data on amino acid profiling for South African patients with CVD, the leading cause of death in all age groups, and the relationships between various amino acids, and amino acids and other cardiovascular variables, and related metabolites. In particular, data is limiting concerning the concentrations of the sole NOS substrate, arginine, and its relationship with CVD in the local South African population. Furthermore, literature illuminates the paucity of population-related data accounting for variation of amino acid concentrations in categories such as sex, age and geographic region (Cynober, 2002). Vital questions such as the concentrations and associations of amino acids related to the cardiovascular system still remain unanswered. Finally, a further reason for this study is the absence of data of amino acid profiles in CVD, particularly in hypertension, in the local South African population.

1.7 Aims

The aim of my study is to determine the concentrations of plasma and urinary amino acids in hypertension using a local South African cohort. This study will also determine the relationships between either individual, structurally related or biochemical pathway intermediate related amino acids, and arginine concentrations, and whether such concentrations are related, or may be determinants, of blood pressure.

Table 1.2 Essential and non-essential amino acids obtained from the hydrolysis of protein

Amino Acids in Protein	
Essential	Non-essential
Leucine **	Arginine *
Isoleucine **	Asparagine
Methionine	Aspartic acid
Phenylalanine	Cysteine
Tryptophan	Glutamic acid
Valine **	Glutamine
Lysine	Glycine
Threonine	Alanine
Histidine	Serine
Tyrosine	Proline

** Branch chain * Semi - essential

CHAPTER 2 : MATERIALS AND METHODS

This study is designed to compare amino acid profiles, concentrations and associations of plasma and urinary amino acids, in subjects of African ancestry. Approval for the study was obtained from the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg (Ethics number M070228).

2.1 Patients and control subjects

Participants were apparently healthy individuals, not receiving treatment for hypertension, who were recruited from clinics at the Chris Hani-Baragwanath Hospital, the inner city and laboratory staff at the National Health Laboratory Services at the Charlotte Maxeke (Johannesburg) Hospital. Participants were from the same geographical area, ie. from the townships around Johannesburg and had similar lower socio-economic status. All study participants were informed verbally about the rationale for and the protocol of the study and were then each requested to provide written informed consent to participate. All participants were compensated for their transport costs incurred.

Participants were excluded if they had angina or a previous myocardial infarction, impaired renal function, liver damage or suspected abuse of alcohol (raised γ -GT level above normal laboratory values), obvious infection from wounds or illness, diabetes mellitus (type 1), or if they had been taking any investigational drug or participated in any other trial during the previous 12 weeks or had been using non-steroidal anti-inflammatory drug in the previous 7 days.

2.2 Blood pressure measurements

Twenty four hour ambulatory blood pressures were recorded using oscillometric monitors (Spacelabs, model: 90207) with all participants wearing the blood pressure cuff on the non-dominant arm. The ambulatory blood pressure monitors (ABPM) were programmed to take measurements at 20 minute intervals from 06:00 – 18:00 and at 30 minute intervals from 18:00 – 06:00.

Upon completion of the 24hour ABPM demographic data, including age, gender, ancestry, alcohol and tobacco use, current medications and a medical history was obtained from participants. If subjects were found, after the 24 hour ambulatory blood pressure monitoring, to be newly diagnosed for hypertension, they were referred for follow-up at the hypertension clinic.

2.3 Blood and urine sampling.

Upon obtaining consent, and fitting the ABPM monitor, participants were requested not to eat from 10pm to obtain fasting early morning blood and urine samples the following morning. Two 5 ml blood samples were drawn into EDTA vacutainers (Beckton-Dickinson) from either the left or right arm depending on patient preference and/or vascular convenience. Participants were then asked to provide a spot urine sample collected in a standard plastic urine sample container.

Both the urine and blood samples were stored on ice and transported to the Wits Percy Fox Laboratory where blood samples were separated by centrifugation using a Beckman TJ-6 Centrifuge at 2000 rpm and eight degrees Celsius for 10 minutes. Plasma extract was then

aliquotted into 2 x 1ml eppendorf capped tubes and stored, alongside the corresponding urine sample, at -70 degrees Celcius.

2.4 Sample preparation and laboratory analysis

Plasma and urine samples were thawed and centrifuged through a 10kDa centricon unit (Z648078 Sigma, Microcon centrifugal filter unit YM-10 membrane). The resultant 50 micro liter filtrate was analyzed by a developed method (AD Cromarty, Pharmacology, Basic Medical Sciences, University of Pretoria) utilizing high performance liquid chromatography (HPLC) with mass spectrometry (MS).

2.5 HPLC-MS.

Concentrations of amino acids were determined by HPLC/MS (Chaimbault et al. 2000, Piraud et al. 2003). Quantification of amino acids was performed on an Agilent HPLC system with a Phenomenex Prodigy C18 column (4.6x100mm; 3µm particle size) equilibrated with 25mM heptafluorobutyric acid in water containing 5% acetonitrile and 0.1% formic acid (Mobile phase A) at 45°C. Separation was achieved with a gradient of 25mM heptafluorobutyric acid in acetonitrile containing 5% water and 0.1% formic acid.

The gradient was as follows: 0-3 minutes: 100% A; 3-9.5 minutes: 30% B; 9.5-13.5 minutes: 32% B; 13.5-14 minutes 60% B with a 4 minute re-equilibration step. Flow rate set at 700µl/min with a 10µl injection volume. Eluting peaks were identified by retention time and multiple reaction monitoring using a MSD Sciex 4000QTrap mass spectrometer in positive mode at 5200V.

2.6 Data analysis

All data were recorded in Microsoft Excel 2007 and imported into Statistica (Statsoft Version 8.0) for statistical analysis. Results were reported as mean \pm SD or as median [range]. A student's t-test or Mann-Whitney test was used to determine differences between groups for normally and non-normally distributed data respectively. A Chi-Squared test was used to compare frequency of dichotomized data. Pearson's correlation co-efficient was used to determine association between variables with multiple regression analyses to determine variables predicting blood pressure. A p-value of less than 0.05 was considered to be of statistical significance.

CHAPTER 3 : RESULTS

3.1. Patient demographics

The study population were all of African ancestry and mostly male. When subjects were dichotomized into those with mean daytime ambulatory blood pressures less than or equal to SBP/DBP 140/90 mm Hg and greater than SBP/DBP 140/90 mm Hg, there were no differences between normotensive and hypertensive patients with respect to age, gender, body mass index, smoking habits and use of alcohol (Table 3.1).

In all patients (Table 3.1), no correlation between age and blood pressures was noted in this study (e.g. daytime SBP $r=0.0693$; $p=0.51$, daytime DBP $r=0.0284$; $p=0.79$). The correlation of body mass index (BMI) SBP approached significance (mean night time SBP $r=0.1913$; $p=0.06$) and DBP did not correlate. The correlation of BMI with blood pressure was significant in males for 24h ($r=0.2432$; $p=0.03$), daytime ($r= 0.2675$; $p=0.02$) and night time ($r= 0.2260$; $p=0.05$) but not subjects with a BMI $<30\text{kg/m}^2$.

In all subjects, smoking and alcohol consumption did not correlate with blood pressure but did correlate with each other ($r=0.5791$; $p<0.0001$). In subjects of BMI $<30\text{kg/m}^2$, these parameters correlated inversely with BMI ($r= -0.286$; $p=0.02$ for alcohol and $r= -0.2492$; $p=0.04$ and smoking respectively). The association was stronger in males in this latter group for alcohol consumption ($r=-0.3912$; $p=0.003$) but the correlation with smoking remaining unchanged.

3.2. Arginine and related amino acids

In all subjects, the amino acids using the y⁺-transporter, arginine and lysine, were not statistically elevated in subjects with a mean daytime systolic >140mm Hg and a mean diastolic (>90 mm) Hg ambulatory blood pressure. Other amino acids related to arginine metabolism, were also not different in subjects with elevated blood pressures (Table 3.2.).

3.2.1. Correlations of amino acids with blood pressure and demographic parameters

Arginine concentrations decreased with age and this correlation remained significant while the correlation increased when arginine concentrations were corrected for total amino acid nitrogen, as with non-obese subjects (BMI<30kg/m²). Corrected arginine concentrations also decreased with increasing BMI in all subjects and in males but not in those with non-obese subjects and non-obese males. Plasma arginine correlated with smoking ($r^2=0.3740$; $p=0.0058$).

Plasma arginine correlated only with mean nocturnal SBP and DBP in males, non-obese subjects and in non-obese males (Table 3.3) However, when corrected for total amino acid nitrogen, the association with blood pressures were no longer significant (mean night DBP $r^2=0.14$; $p=0.20$) (Table 3.3) suggesting a concentration effect.

In contrast, plasma lysine concentrations did not correlate with age but did correlate positively with BMI although only in non-obese males. Lysine also correlated with mean ambulatory blood pressure, with most significant correlations observed with nocturnal pressures (Table 3.4). After correcting for amino acid nitrogen, the association of blood pressure with lysine remained significant (Table 3.4.).

Arginine concentrations correlated positively with most amino acids using the same transporter, other urea cycle amino acids and the inhibitor ADMA, but not with total homocystine and ornithine. When corrected for amino acid nitrogen, the correlation was weaker but remained significant. The correlation with proline was particularly strong ($r^2 > 0.9$; $p < 0.0001$ for all groups) and remained highly significant after correcting for amino acid nitrogen ($r^2 > 0.7$; $p < 0.0001$ for all groups).

Lysine concentrations correlated with other amino acids but not with tHcys, ornithine, ADMA and cystine. Unlike arginine, the correlations were no longer significant after correcting for amino acid nitrogen (Table 3.4).

3.2.2 Urinary amino acids.

No differences in the concentrations of urinary amino acids between subjects with normal and those with raised SBP and DBP were noted (Table 3.5). Concentrations ranged considerably and were sometimes undetectable. Indeed ornithine concentrations were not detectable in approximately half of the subjects.

3.3 Multiple linear analyses.

To determine factors associated with blood pressure, multiple linear regression analysis was performed on variables which included age, BMI, use of cigarettes and alcohol and amino acids linked to arginine metabolism, as covariates in the models (Table 3.6.). BMI and age were further dichotomized by the median and inserted into multiple linear regression analysis. However, this did not have any effect on the results.

Body mass index and plasma lysine were independent predictors of mean systolic and mean systolic and diastolic blood pressures respectively with citrulline predicting night time diastolic pressure. When plasma amino acids were corrected for amino acid nitrogen, BMI was a significant determinant of systolic blood pressure with lysine and gender being independent predictors of all measures of blood pressures. Citrulline predicted both nocturnal systolic and diastolic pressure as well 24h diastolic pressure (Table 3.6.).

When urinary amino acids were included in the model, except for mean daytime diastolic pressure, gender was an important determinant of blood pressure with use of alcohol inversely determining systolic pressures and cigarette use inversely associated with night time diastolic pressure. Arginine excretion independently determined both daytime systolic and diastolic blood pressure, with citrulline determining both 24h and daytime diastolic pressures. Urinary lysine and orotic acid were independent determinants of daytime diastolic pressure but this association was not strong (Table 3.6.).

All the major covariates were found to significantly predict blood pressures (Table 3.6) were then modeled to determine whether plasma amino acid concentrations or urinary amino acid excretion independently determined blood pressure (Table 3.7). Gender in this study consisting mostly of males, was a determinant of all measures of blood pressure. Urinary arginine excretion independently determined both 24h and daytime systolic and diastolic blood pressures but not night time pressures. Urinary citrulline also determined all blood pressures with lower citrulline excretion associated with higher blood pressure measurements. Serum lysine determined only mean nighttime DBP and this association was not strong.

The ratio of the concentrations of urinary arginine (as μmol arginine/ mmol creatinine) to plasma arginine (μmol arginine/ mg N) did not correlate with any of the measured blood pressure measurements with a similar finding for the equivalent citrulline concentrations (data not shown).

Table 3.1. Subject demographic data.

Criteria for hypertension were mean daytime ABP, SBP \geq 140mm Hg and DBP \geq 90mm Hg.

Parameter	All Subjects (n=97)	Normotensives (n=56)	Hypertensives (n=41)	Normotensive vs hypertensive (p)
Age (years)	46.4 \pm 9.4	45.9 \pm 10.3	47.6 \pm 7.9	0.38
Gender (f/m; % male)	20/77 (79.4%)	14/42 (75.0%)	6/35 (85.4%)	0.21
BMI (kg/m ²)	27.9 \pm 5.3	24.4 \pm 5.2	28.7 \pm 5.3	0.26
BMI>27.5	39 (40.2%)	24 (42.9%)	26 (65.4%)	0.53*
Smoking (incl. ex-)	51 (52.5%)	30 (53.6%)	21 (51.2%)	0.82*
Non-	46 (47.4%)	26 (46.4%)	20 (48.8%)	
Ex-	15 (15.4%)	8 (14.3%)	7 (17.1%)	
Present	36 (37.1%)	22 (39.3%)	14 (34.1%)	
Alcohol	44 (45.4%)	25 (48.1%)	19 (48.3%)	0.87*
ABP (mean SBP/DBP; mm Hg):				
24h	136 \pm 18/88 \pm 12	125 \pm 12/81 \pm 8	152 \pm 13/99 \pm 8.7	<0.0001/<0.0001
Day	140 \pm 19/93 \pm 13	128 \pm 12/85 \pm 8	157 \pm 13/105 \pm 9	<0.0001/<0.0001
Night	132 \pm 17/84 \pm 12	123 \pm 12/78 \pm 9	146 \pm 14/93 \pm 10	<0.0001/<0.0001

ABP: Ambulatory blood pressure; BMI: Body mass index; SBP: Systolic blood pressure; DBP: diastolic blood pressure.

*Chi-square test

Table 3.2. Subject plasma arginine and related amino acid concentrations.

Determined by HPLC-MS in subjects with daytime ambulatory blood pressure SBP/DBP \leq 140/90mm Hg or SBP/DBP $>$ 140/90mm Hg. N=97 unless otherwise stated.

Amino acid	Significance & relationship to arginine	All Subjects (n=97) (μ mol/L)	Normotensive (n=56) (μ mol/L)	Hypertensive (n=41) (μ mol/L)	P
Arginine	Common y+ transporter	60.2 \pm 21.6 [30.6 – 138.4]	59.6 \pm 21.6 [30.6 – 131.1]	62.9 \pm 22.4 [32.4 – 138.4]	0.43
Lysine	Common y+ transporter	79.1 \pm 19.7 [44.2 – 136.5]	77.7 \pm 18.9 [44.5 – 117.1]	81.5 \pm 22.0 [44.2 – 136.5]	0.48
Histidine	Common y+ transporter	41.0 \pm 7.4 [27.2 - 68.3]	42.0 \pm 7.3 [28.1 – 63.0]	40.3 \pm 7.9 [27.2 – 68.3]	0.17
Total homocysteine (n = 53)	risk factor y+ transporter inhibitor	11.3 \pm 6.4 [3.1 – 31.1]	10.7 \pm 4.8 [3.1 - 25.9; n=30]	12.0 \pm 8.1 [4.2 - 31.1; n=23]	0.84
Citrulline	Intermediate Urea cycle	13.1 \pm 5.0 [1.0 – 28.5]	12.8 \pm 4.6 [1.0 – 28.5]	13.8 \pm 5.6 [1.0 – 27.9]	0.32
Ornithine	Intermediate Urea cycle	13.5 \pm 10.7 [0 – 40.5]	13.4 \pm 11.5 [0 – 40.5]	13.1 \pm 10.3 [0 – 35.7]	0.82
ADMA	Arginine analogue and NO synthase inhibitor	0.8 \pm 0.6 [0 – 2.8]	0.8 \pm 0.6 [0-2.8]	0.7 \pm 0.6 [0-2.0]	0.60
Proline	Arginine precursor	73.6 \pm 23.8 [35.8 – 163.2]	73.2 \pm 25.3 [35.8 \pm 163.2]	75.7 \pm 23.0 [52.6 \pm 159.5]	0.54
Cystine (n=29)	Disulphide – uses y+transporter	31.8 \pm 21.0 [2.0 – 104.3]	37.2 \pm 27.3 [2.0 – 104.3]	28.1 \pm 14.5 [4.4 – 43.4]	0.52

HPLC-MS: High performance liquid chromatography and mass spectrometry; SBP: Systolic blood pressure; DBP: diastolic blood pressure; ADMA: asymmetric dimethyl-arginine

Table 3.3. Associations (Pearson correlation coefficient: r) of plasma arginine, blood pressure and other amino acids.

#p<0.10; *: p<0.05; **p<0.005

	All subjects (n=97)		Males (n=69)		BMI<30kg/m2 (n=65)		Males BMI<30kg/m2 (n=53)	
	(μmol/L)	(μmol/mg amino acid N)	(μmol/L)	(μmol/mg amino acid N)	(μmol/L)	(μmol/mg amino acid N)	(μmol/L)	(μmol/mg amino acid N)
Age (years)	-0.2338*	-0.2999*	-0.23#	-0.2626*	-0.2972*	-0.4021**	-0.2960^	-0.2587*
BMI (kg/m²)	-0.07	-0.2306*	-0.07	-0.2471*	0.03	0.12	0.73	-0.12
24h ABPM								
SBP (mm Hg)	0.15	0.08	0.15	0.06	0.24#	0.14	0.22	0.11
DBP (mm Hg)	0.20#	0.14	0.17	0.10	0.2733*	0.20	0.22	0.14
Daytime								
SBP (mm Hg)	0.11	0.06	0.10	0.02	0.18	0.12	0.15	0.07
DBP (mm Hg)	0.16	0.11	0.14	0.07	0.20	0.17	0.19	0.11
Night time								
SBP (mm Hg)	0.18	0.07	0.20	0.08	0.2824*	0.13	0.2800*	0.14
DBP (mm Hg)	0.2153*	0.14	0.19	0.11	0.2951*	0.20	0.24#	0.15
Amino acids								
Lysine	0.6301***	0.2662*	0.6785***	0.3735**	0.6847***	0.3325*	0.7139***	0.3960**
Histidine	0.6474***	0.2670*	0.6333***	0.2782*	0.6820***	0.2883*	0.6659***	0.2922*
tHcys	0.14 (n=47)	0.16 (n=47)	-0.20 (n=42)	0.18 (n=42)	0.18 (n=35)	0.21 (n=35)	0.22 (n=34)	0.22 (n=34)
Proline	0.9352***	0.6923***	0.9428***	0.7215***	0.9539***	0.7113***	0.9548***	0.7302***
Citrulline	0.3239**	0.2404*	0.4137***	0.3641**	0.3415*	0.24#	0.4300**	0.3712*
Ornithine	0.10	0.01	0.2384*	0.16	0.19	0.12	0.25#	0.18
ADMA	0.3000*	0.3123**	0.2684*	0.2737*	0.2718*	0.2855*	0.24#	0.26#
Cystine	0.5503**	0.4528*	0.6190**	0.44#	0.5909*	0.5301*	0.6538*	0.48# (n=13)

Amino acid N shows that the data was corrected for total amino acid nitrogen to reduce the concentration effect. SBP: Systolic blood pressure; DBP: diastolic blood pressure; ABP: ambulatory blood pressure; tHcys: total homocysteine; ADMA: asymmetric dimethyl-arginine

Table 3.4. Associations (Pearson correlation coefficient: r) of plasma lysine, blood pressure and other amino acids.

#p<0.10; *: p<0.05; **p<0.005; ***p<0.0005.

	All subjects (n=87)		Males (n=69)		BMI<30kg/m2 (n=65)		Males BMI<30kg/m2 (n=53)	
	($\mu\text{mol/L}$)	($\mu\text{mol/L/mg}$ amino acid N)	($\mu\text{mol/L}$)	($\mu\text{mol/L/mg}$ amino acid N)	($\mu\text{mol/L}$)	($\mu\text{mol/L/mg}$ amino acid N)	($\mu\text{mol/L}$)	($\mu\text{mol/L/mg}$ amino acid N)
Age (years)	-0.13	-0.16	-0.21	-0.16	-0.18	-0.14	-0.16	-0.16
BMI (kg/m²)	0.12	-0.06	0.16	0.12	0.23	0.18	0.3236*	0.2792*
24h ABP								
SBP (mm Hg)	0.2656*	0.3187**	0.2593*	0.2774*	0.3780**	0.3212*	0.3542*	0.27#
DBP (mm Hg)	0.3051**	0.3430**	0.2899*	0.3014*	0.3862**	0.3331*	0.3577*	0.2938*
Daytime								
SBP (mm Hg)	0.2224*	0.2991**	0.20#	0.2403*	0.2936*	0.2938*	0.2733*	0.23#
DBP (mm Hg)	0.2407*	0.2816*	0.2512*	0.2491*	0.2751*	0.2532*	0.3033*	0.24#
Night time								
SBP (mm Hg)	0.3086**	0.3271**	0.3300*	0.3253*	0.4588***	0.3402*	0.4504**	0.3256*
DBP (mm Hg)	0.3383**	0.3656**	0.3439**	0.3527**	0.4443***	0.3696**	0.4212**	0.3551*
Amino acids								
Arginine	0.6301***	0.14	0.6785***	0.17	0.6847***	0.16	0.7139***	0.17
Histidine	0.5543***	-0.03	0.5236***	-0.06#	0.5687***	-0.05	0.5514***	-0.04
tHcys	-0.02 (n=47)	0.09 (n=47)	-0.10 (n=42)	0.08 (n=41)	-0.06 (n=35)	0.08 (n=35)	-0.13 (n=34)	0.07
Proline	0.6358***	0.12	0.6555***	0.13	0.6786***	0.14	0.6933***	0.14
Citrulline	0.2362*	0.03	0.3111*	0.12	0.23#	-0.03	0.3091*	0.08
Ornithine	0.19#	0.09	0.21#	0.06	0.22#	0.07	0.22	0.03
ADMA	0.10	-0.03	0.15	0.03	0.16	0.09	0.22	0.20
Cystine	0.12 (n=25)	-0.20 (n=24)	0.09 (n=20)	-0.36 (n=20)	0.10 (n=17)	-0.29 (n=16)	0.01 (n=13)	-0.54#(n=13)

SBP: Systolic blood pressure; DBP: diastolic blood pressure; ABP: ambulatory blood pressure; tHcys: total homocysteine; ADMA: asymmetric dimethyl-arginine

Table 3.5. Subject urinary arginine and related amino acid concentrations.

Determined by HPLC-MS in subjects with daytime ambulatory blood pressure SBP/DBP \leq 140/90mm Hg or SBP/DBP $>$ 140/90mm Hg. N=60 unless otherwise stated.

Amino acid	All Subjects (n=60) ($\mu\text{mol/L}$)		Normotensive (n=56) ($\mu\text{mol/L}$)		Hypertensive (n=41) ($\mu\text{mol/L}$)		Significance p
	($\mu\text{mol/L}$)	Corrected ($\mu\text{mol}/\text{mmol}$ creatinine)	($\mu\text{mol/L}$)	Corrected ($\mu\text{mol}/\text{mmol}$ creatinine)	($\mu\text{mol/L}$)	Corrected ($\mu\text{mol}/\text{mmol}$ creatinine)	NT/HT
Arginine	0.160 \pm 0.100 [0.003 - 0.430]	6.0 \pm 03.5 [0.3- 20.6]	0.156 \pm 0.097 [0.003 – 0.430]	6.1 \pm 3.8 [0.3 – 20.7]	0.171 \pm 0.110 [0.042 – 0.430]	6.0 \pm 2.7 [2.2 – 11.6]	0.76/1.0
Lysine	0.605 \pm 0.911 [0 - 4.586]	29.4 \pm 51.9 [0.0 – 285.2]	0.652 \pm 1.059 [0 – 4.586]	32.9 \pm 60.5 [0 – 285.2]	0.484 \pm 0.310 [0.132 – 1.332]	20.5 \pm 14.3 [3.7 – 53.8]	0.17/0.47
Histidine	2.164 \pm 1.772 [0.125 - 10.384]	97.7 \pm 97.1 [12.0 – 524.1]	2.106 \pm 1.956 [0.125 – 10.383]	97.8 \pm 108.6 [12.0 – 524.2]	2.313 \pm 1.226 [0.558 – 4.614]	97.6 \pm 61.3 [16.7 – 214.7]	0.21/0.32
Citrulline	0.195 \pm 0.106 [0.0 - 0.500]	7.7 \pm 3.7 [0.0 – 24.5]	0.194 \pm 0.100 [0.033 – 0.500]	7.9 \pm 3.9 [3.2 – 24.5]	0.199 \pm 0.125 [0 – 0.479]	7.0 \pm 3.3 [0 – 13.4]	0.89/0.49
Ornithine	0.034 \pm 0.049 [0.0 – 0.199]	1.7 \pm 3.5 [0.0 – 18.4]	0.039 \pm 0.054 [0 – 0.199]	2.1 \pm 4.0 [0 – 18.4]	0.024 \pm 0.035 [0 – 0.088]	0.7 \pm 1.1 [0 – 3.1]	0.49/0.36
ADMA	0.810 \pm 0.397 [0.075-1.644]	3.0 \pm 0.9 [0.8 – 5.2]	0.782 \pm 0.405 [0.075 – 1.643]	3.0 \pm 0.9 [0.8 – 5.2]	0.882 \pm 0.382 [0.322 – 0.152]	3.2 \pm 0.8 [1.6 – 4.5]	0.37/0.36
Proline	0.088 \pm 0.084 [0.004 – 0.453]	3.6 \pm 3.5 [0.5 – 19.6]	0.091 \pm 0.090 [0.004 – 0.453]	3.8 \pm 4.0 [0.5 – 19.6]	0.08 \pm 0.070 0.009 – 0.252	2.8 \pm 2.1 [0.6 – 7.5]	0.82/0.41
Cystine	0.728 \pm 2.125 [0.0 – 9.535]	29.2 \pm 83.1 0.0 – 359.1	0.927 \pm 2.476 [0 – 9.535]	37.5 \pm 96.9 [0 – 359.1]	0.225 \pm 0.418 [0.015 – 1.612]	8.1 \pm 14.1 [0.8 – 44.9]	0.09/0.09
Orotic acid	2.720 \pm 1.901 [0.0 – 11.494]	121.6 \pm 104.9 [0.0 – 580.3]	2.666 \pm 2.150 [0 – 11.494]	122.8 \pm 118.8 [0 – 580.3]	2.856 \pm 1.074 [1.073 – 4.674]	118.7 \pm 59.2 [32.2 – 246.7]	0.16/0.45
Creatinine	25.3 \pm 8.5 [9.6 – 45.3]	- -	24.784 \pm 9.060 [9.554 – 45.268]	- -	26.706 \pm 7.105 [15.362 – 8.598]	- -	0.35/ -

SBP: Systolic blood pressure; DBP: diastolic blood pressure; ABP: ambulatory blood pressure; tHcys: total homocysteine; ADMA: asymmetric dimethyl-arginine; HPLC-MS: high performance liquid chromatography and mass spectrometry.

Table 3.6. Multiple linear analysis of determinants of blood pressure using subject demographics and amino acid concentrations as covariates as shown below the Table. All intercepts were significant ($p < 0.0001$) unless stated otherwise. Adjusted # $p < 0.15$; $p > 0.05$; * $p < 0.05$; ** $p < 0.005$. Values as parameter estimates \pm std error.

	24h SBP	24h DBP	Daytime SBP	Daytime DBP	Night time SBP	Night time DBP
Plasma amino acids						
Model significance (p)	p=0.0155	p=0.0358	p=0.0265	p=0.0562	p=0.0057	p=0.0062
Intercept	95.3157 \pm 13.7425	75.8976 \pm 5.8975	98.4177 \pm 15.0664	80.6774 \pm 6.0925	89.3718 \pm 12.8934	75.0714 \pm 6.2631
BMI (kg/m ²)	0.9067 \pm 0.4258*	ns	0.9712 \pm 0.4668*	ns	0.9197 \pm 0.3995*	ns
Lysine (μ mol/L)	0.1905 \pm 0.1063#	0.1500 \pm 0.0702*	0.1805 \pm 0.1165#	0.1451 \pm 0.0748#	0.2099 \pm 0.0997*	0.2062 \pm 0.0703**
Citrulline (μ mol/L)	ns	ns	ns	ns	ns	-0.5949 \pm 0.2922*
Plasma amino acids (/ mg amino acid N)						
Model significance (p)	p=0.0005	p=0.0093	p=0.0006	p=0.0172	p=0.0005	p=0.0013
Intercept	63.7168 \pm 17.0053	65.5020 \pm 10.0569	62.3730 \pm 18.7797**	52.6647 \pm 12.6423	72.4789 \pm 17.1178	62.1633 \pm 9.8807
BMI (kg/m ²)	0.9567 \pm 0.3948*	ns	1.0415 \pm 0.4360*	0.4303 \pm 0.2935#	0.9153 \pm 0.3699*	ns
Gender (F=0;M=1)	13.43837 \pm 4.9568*	5.7314 \pm 3.4280#	14.9468 \pm 5.4740*	6.4714 \pm 3.6851#	12.7875 \pm 4.6548*	5.7691 \pm 3.3679#
Lysine (μ mol/mg N)	5.52910 \pm 2.0641*	3.7239 \pm 1.4322*	5.8434 \pm 2.2795*	3.6136 \pm 1.5345*	4.9113 \pm 1.9352*	4.0414 \pm 1.4072**
Citrulline (μ mol/mg N)	ns	-5.3085 \pm 3.5224#	ns	ns	-7.1792 \pm 4.7696#	-8.0553 \pm 3.4607*
Urinary amino acids (as μmol/mmol creatinine)						
Model significance (p)	p=0.0029	p=0.0021	p=0.0009	p=0.0009	p=0.0013	p=0.0013
Intercept	124.7836 \pm 6.5829	84.4877 \pm 4.4296	138.9320 \pm 8.1204	89.9111 \pm 4.3263	122.5758 \pm 6.5401	78.3295 \pm 4.2996
Gender (F=0;M=1)	16.9679 \pm 4.9381**	7.0142 \pm 3.3967*	12.4032 \pm 5.0010**	ns	17.7472 \pm 4.9060**	11.0638 \pm 4.0603**
Risk alcohol	-6.4489 \pm 3.7569#	ns	-6.2021 \pm 3.7205#	ns	-7.4429 \pm 3.7325#	ns
Risk smoking	ns	ns	ns	ns	ns	-2.4290 \pm 1.5346#
Arginine	ns	1.6418 \pm 0.9238#	3.9679 \pm 1.3595*	3.1638 \pm 0.9466*	ns	ns
Citrulline	ns	-2.2176 \pm 0.8854*	-4.1180 \pm 1.2984#	-2.9890 \pm 0.8808*	ns	ns
Lysine	ns	ns	ns	-0.1301 \pm 0.0550#	ns	ns
Orotic acid	ns	ns	ns	0.0407 \pm 0.0239#	ns	ns
Proline	ns	ns	ns	ns	ns	-0.7998 \pm 0.4036*

Plasma amino acids: age, BMI (body mass index), gender, alcohol and smoking, plasma arginine, lysine, proline, ADMA (asymmetric dimethyl-arginine), ornithine and citrulline

Corrected amino acids: age, BMI, gender, alcohol and smoking, plasma arginine, lysine, proline, ADMA, ornithine and citrulline

Urinary amino acids: age, BMI, gender, alcohol and smoking, urinary (corrected for urinary creatinine) arginine, lysine, proline, ADMA, ornithine, citrulline, histidine, orotic acid and creatinine

Table 3.7. Multiple regression analysis of using all the significant determinants of blood pressure (derived from Table 3.6), as covariates as shown below the Table. All intercepts were significant ($p < 0.0001$) unless stated otherwise. # $0.15 > p > 0.05$; * $p < 0.05$; ** $p < 0.005$. Values as adjusted parameter (beta coefficients) estimates \pm std error.

	24h SBP	24h DBP	Daytime SBP	Daytime DBP	Night time SBP	Night time DBP
Model significance (p)	p=0.0013	p=0.007	p=0.0003	p=0.0003	p=0.0029	p=0.0019
Intercept	124.9691 \pm 6.2287	85.5320 \pm 4.2856	129.9709 \pm 6.3225	90.6246 \pm 4.4664	118.6202 \pm 6.1400	63.0401 \pm 11.0349
Gender (F=0;M=1)	12.8645 \pm 4.9628**	6.2482 \pm 3.4146#	12.2926 \pm 5.0376*	5.8161 \pm 3.5587#	14.3805 \pm 4.9963*	9.9737 \pm 3.6735*
Urinary arginine (μ mol/mmol creatinine)	2.3280 \pm 1.2853#	1.5729 \pm 0.8844#	3.5015 \pm 1.3047*	2.2164 \pm 0.9217*	ns	ns
Citrulline (μ mol/mmol creatinine)	-2.9086 \pm 1.2167*	-2.3905 \pm 0.8371*	-4.0927 \pm 1.2350**	-3.0048 \pm 0.8724**	-0.8741 \pm 0.4805#	-0.8372 \pm 0.3576*
Plasma lysine (μ mol/mg nitrogen)	ns	ns	ns	ns	ns	2.5337 \pm 1.6654#

Model covariates (see Table 3.6): BMI, gender, alcohol and smoking, plasma lysine, citrulline and urinary concentrations of arginine, lysine, proline, citrulline (corrected for urinary creatinine). SBP: Systolic blood pressure; DBP: diastolic blood pressure;

CHAPTER 4 : DISCUSSION AND CONCLUSIONS

Known risk factors for cardiovascular disease include age, increased BMI, smoking and alcohol, the presence of diabetes mellitus, high blood pressure as well as increased biochemical markers such as cholesterol. However, such risk factors do not fully account for cardiovascular morbidity and mortality especially in patients of African ancestry, a group with excess risk for CVD (Chobanian *et al*, 2003). Thus, alternative risk factors need to be found to identify such subjects who may be particularly at risk. The only amino acid to be identified as a cardiovascular risk factor is total homocysteine concentrations where the actual mechanism is still not clear (Brattström and Wilcken, 2000). To date, no study has determined the associations between physiological amino acid concentrations and blood pressure as we have done in this study. Although altered amino acid concentrations may indicate changes in amino acid metabolism, associations between amino acids and blood pressures may provide insight into disturbances in metabolic pathways or transport, suggesting pathophysiological changes which may accompany hypertension. This study was set up to determine whether indeed such associations occur in African subjects who live in the urban townships surrounding Johannesburg and who are at increased risk for CVD.

4.1 Methodology.

As study participants were of similar lower socio-economic status (participants were from the surrounding townships and informal settlements), measures of such status or a recall of food intake were not recorded. As the association of 24 hour blood pressure monitoring with target organ damage is better than clinic pressures, subjects were screened using 24 hour ambulatory blood pressure monitoring to determine blood pressure, which excluded patients with white

coat hypertension (O'Brien, 2007). Other studies (Brunini *et al.* 2004, Perticone *et al.* 2005) which determined the associations between arginine concentrations and blood pressures have only used clinic pressures and did not excluded white coat hypertension. In my study, blood and urine samples were taken after a 16 hour fast and were thus standardised. Although 24 hour urine collections would be preferable, these are seldom complete collections and metabolite degradation may occur. For convenience, spot urine samples were taken which in many cases correlate reasonably with the 24 hour collections (Shahbazian and Hosseini-Asl, 2008). In reviewing the data, it was noted that all amino acids were elevated in certain subjects, suggesting a concentration effect. As haematocrit was not measured, the plasma amino acids were corrected to total amino acid nitrogen to account for this concentration effect. Urinary amino acid concentrations were corrected for urinary creatinine concentration.

4.2 Arginine and blood pressure.

Arginine is the sole precursor of the potent arterial vasodilator, NO and if this were an important determinant of blood pressure, one would anticipate an inverse association between plasma arginine concentrations and blood pressure, i.e. less arginine would produce a blood pressure elevation. In support of this, blood pressures decreased in patients with CVD, following oral supplementation of significant amounts of arginine (1-12g/day) (Siani *et al.*, 2000; Palloshi, 2004; Rector *et al.*, 1996; West *et al.*, 2005). However, other studies (Adams *et al.*, 1995; Adams *et al.*, 1997; Chin-Dusting *et al.*, 2006a; Chin-Dusting, 1996b; Lerman 1998) with limited numbers of patients were unable to show such changes to be significant. If plasma arginine concentrations were unchanged or indeed were elevated, this would not exclude arginine not being available for NO production, since reduced arginine uptake into cells or

increased urinary arginine excretion may still limit NO production, manifesting as increased blood pressure.

The finding from my study, showing neither plasma nor urinary arginine concentrations to be different in subjects with hypertension in this cohort of Black South Africans, is in contrast to other studies from Caucasian subjects (Brunini *et al.* 2004, Perticone *et al.* 2005). Indeed, none of the concentrations of the amino acids, either using the same biochemical pathway (the urea cycle), or those using the same y+ amino acid transport system, were changed in subjects with elevated blood pressures compared to those with normal blood pressures. Brunini *et al.* (2004) collected plasma samples of thirty six normotensive controls and thirty four patients with hypertension while these patients were still on therapy. Therefore, whether the results of this study (Brunini *et al.*, 2004) represent an impact of treatment or the presence of hypertension is uncertain. Perticone *et al.* (2005) found plasma arginine concentrations to be increased in 36 never-treated hypertensives as compared to only 8 control participants. The latter study may reflect a false positive outcome since the study sample of control participants was very small.

Overall my study showed that plasma arginine concentrations were not an important determinant of blood pressure since the associations of arginine concentrations with blood pressure became insignificant once concentrations were corrected for amino acid nitrogen. In contrast, increased urinary excretion of arginine may be an important determinant of daytime blood pressures, since this remained significant after covariates, including plasma amino acids, were taken into account.

The possibility that arginine may not be available for NO production as a result of reduced arginine transport was investigated by measuring lysine and histidine concentrations, amino acids which use the same membrane transporter (Deves, 1998). As for the arginine concentrations, both lysine and histidine plasma concentrations were unchanged in subjects diagnosed with hypertension. Both histidine and lysine correlated significantly with arginine concentrations, even after correcting for amino acid nitrogen. My study also showed correlations between arginine and its precursor, proline (0.7302), citrulline, an intermediate in the urea cycle and a product of NO production, but did not correlate with ornithine. These associations were significant in males, participants with a body mass index less than 30 kg/m² and males with low BMI.

An understanding of arginine relatives and other biological cycle intermediates suggest plausible explanations to such correlations in the absence of changes in concentrations of these amino acids in participants with elevated blood pressure. Arginine synthesis from proline and citrulline may be up-regulated to provide precursor for NO production. Arginine, lysine and histidine share the same y⁺ amino acid membrane transporter and thus may be expected to correlate. However, it should be noted that arginine can be synthesized from other sources, proline and citrulline, whereas lysine, an essential amino acid, is available only from the diet or protein turn-over. Increased formation of NO would result in elevated citrulline which was not observed in the present study. I did not investigate nitrite/nitrate metabolites to measure NO synthesis as described by Lundberg *et al*, 2008 and Ignarro *et al*, 1981 which may have suggested increased degradation in hypertension. The strong association of arginine with

proline even after correction for amino acid nitrogen suggests that this association may be more important than the association with ornithine and citrulline (figure 1).

My study further demonstrated significant associations between lysine and 24 hour, day and night time blood pressure, which remained significant after correcting for total amino acid nitrogen as well as covariates including gender and BMI. The stronger association of lysine than arginine may be explained by competitive cationic amino acid transport were limiting NO production. Lysine, but not arginine, is an essential amino acid and lysine concentrations would be determined by diet and re-absorption from nephrons and transport. Arginine can be recycled from citrulline following NO production (Hecker *et al*, 1990) in endothelial cells, and thus concentrations would also be determined by *de novo* biosynthesis. Plasma lysine concentrations may be sensitive to limited or impaired cellular transport and thus explains the ability of plasma lysine concentrations to replace plasma arginine concentrations as an independent predictor of BP in multiple linear regression analysis. Indeed higher lysine was an independent predictor of blood pressure with BMI predicting mean systolic, not diastolic pressures.

With all significant variables as determinants of blood pressure, besides gender, multiple linear analysis showed increased urinary arginine, and decreased urinary citrulline to independently predict blood pressure. Thus, increased arginine loss in the urine and low urinary citrulline concentrations may imply less arginine is available for NO synthesis if arginine limits NO production in subjects with elevated blood pressure. However, the ratio of urinary to plasma arginine and citrulline concentrations did not correlate with blood pressure (Hecker *et al*, 1990) and such hypotheses would need to be confirmed by isotopic studies.

The results of the present study need to be interpreted in the context of the study limitations. My study was conducted in a study sample with a high proportion of men (80%) and the findings may reflect a gender-specific effect. My study was also performed in a convenient sample and hence further studies are required in a larger study group of randomly recruited participants. Future studies should include intracellular arginine measurements to assess and compare the y+ transporter functionality in association with blood pressure. Moreover, such studies should consider nitrite/nitrate measurements to the assessment of the NOS enzymes.

My approach of measuring and determining the relationships of amino acid concentrations in CVD, particularly hypertension, is novel and certainly is the first time this has been measured in African subjects. This study also showed that neither plasma nor urinary amino acid concentrations are altered in patients with hypertension when compared with those in normotension. With particular reference to the amino acid arginine, this study, in contrast to other studies (Brunini *et al.* 2004, Perticone *et al.* 2005), was not elevated in patients with hypertension. The novel association of plasma lysine with blood pressure should be further explored to be incorporated as a potential marker for cardiovascular disease. The present study showing urinary excretion of arginine to independently predict blood pressure in this cohort of subjects of African descent may be important and needs to be confirmed in subsequent studies.

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