ASSOCIATION OF BRANCHED CHAIN AMINO ACIDS AND AROMATIC AMINO ACIDS WITH CARDIO-METABOLIC RISK FACTORS

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Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements of the degree of Masters of Science in Medicine

Johannesburg, 2016

DECLARATION

I, Lungile Khambule declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signed:  Date: 14/11/2016

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ABSTRACT

Branched chain amino acids (BCAAs), tyrosine and phenylalanine were shown to be associated with insulin resistance and the metabolic syndrome in Caucasian and European populations. However, little is known about the BCAAs, tyrosine and phenylalanine associations with cardio-metabolic risk factors in Asian Indians and black Africans. The aim of this was to describe the associations of BCAAs and aromatic amino acids (AAAs) with cardiovascular risk factors in black Africans and Asian Indians.

Ultra-performance liquid chromatography (UPLC) system interfaced to a triple quadruple mass spectrometer (TQ-MS) was used to chromatographically separate and quantify BCAAs, tyrosine and phenylalanine in plasma samples of 718 participants from the caregivers of the Birth to Twenty Cohort (369 black Africans and 349 Asian Indians). Body mass index (BMI), age and gender independent associations between the BCAA, tyrosine and phenylalanine levels and metabolic syndrome components (fasting glucose, triglycerides, high density lipoprotein –cholesterol (HDL-cholesterol), systolic blood pressure and diastolic blood pressure) were made in the two ethnic groups. Comparisons of the amino acid levels in individuals with the metabolic syndrome and those without and across BMI categories (normal weight, overweight and obese) in both ethnic groups were made.

Phenylalanine levels were significantly associated with systolic blood pressure (OR; 2.44[95% CI; 1.17, 5.11], p=0.018) in black Africans and valine and tyrosine levels were significantly associated with triglycerides (OR; 2.00[95% CI; 1.19, 3.37], p=0.009) and fasting glucose (OR; 2.51 [95% CI; 1.10, 5.7], p=0.027), in Asian Indians, respectively. Furthermore, total amino acids and individual amino acids are higher in overweight and obese Asian Indians compared with normal weight Asian Indians (p<0.05 for all), but this was not the case for

black Africans. . In black Africans, phenylalanine was the only amino acid that was different between normal weight and obese individuals ($p<0.05$). BCAAs, tyrosine and valine levels were elevated in individuals with the metabolic syndrome and those with one or two of the metabolic syndrome components in Asian Indians ($p<0.05$) but not in black Africans.

In conclusion plasma BCAAs, tyrosine and phenylalanine are higher in obese and overweight Asian Indians and are elevated in individuals with one or more metabolic syndrome components in Asian Indians. In black Africans, phenylalanine levels were associated with systolic blood pressure and were elevated in obese individuals.

ACKNOWLEDGEMENTS

Firstly, I would like to give thanks to my God from whom my strength and wisdom come from. I am grateful to my supervisor **Prof. Jaya George** who has worked extra hours to make sure that this dissertation becomes a reality and who has been patient with me throughout this journey. I believe I could not have made it this far without her. It has been very humbling working with her.

I am hugely indebted to my co-supervisor **Mrs. Tracy Snyman** who has trained and supervised me. I was a novice in LCMS systems but now I well informed and trained, thanks to her.

I would also like to extend my gratitude to **Mrs. Genevieve Mezoh** who supported me and helped me shape up my writing skills.

Lastly, I would also like to acknowledge the University of Witwatersrand, the National Health Laboratory Service (NHLS) Research Fund and National Research Foundation (NRF) for financing me and the project.

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CHAPTER 1: INTRODUCTION

There is a rising trend of obesity in Africa with the greatest prevalence in black African women in South Africa (Popkin *et al.*, 2012). This is accompanied by increasing type 2 diabetes mellitus (T2DM) and cardiovascular diseases (CVD). T2DM is set to rise exponentially, with the African region contributing the largest proportion by 2030, followed by the Eastern Mediterranean Middle East region (Shaw *et al.*, 2010). The burden of CVD faced by African countries is expected to double from1990 to 2020 mainly as a result of the epidemiological transition, obesity, population growth and aging (Mensah *et al.*, 2015). The metabolic syndrome is a clustering of risk factors associated with the increased risk of CVD. Hyperglycemia, hypertriglyceridemia, hypertension, low high-density lipoproteins-cholesterol (HDL-C) levels and abdominal obesity are used as criteria for the diagnosis of the metabolic syndrome. The harmonized definition of the metabolic syndrome is when an individual has three or more of the cardiovascular risk factors (Alberti *et al.*, 2009).

Ethnic groups vary in their susceptibility to cardio-metabolic risk. For instance;

- Although, insulin resistance is associated with obesity and is a significant risk factor for T2DM; obesity is more common in Black African than Asian-Indian populations and yet T2DM and CVD are more common in the latter populations. This has been attributed to higher visceral adiposity in Asian Indians (Ferris *et al.*, 2005).
- Black Africans, African Americans and Asian Indians are more insulin resistant then BMI–matched white subject (Bajaj and Banerji, 2004; van der Merwe *et al.*, 2000)
- Black Africans commonly present with hypertension and not hypertriglyceridemia (van Rooyen *et al.*, 2000; Opie and Seedat, 2005; Sumner *et al.*, 2010).

The increase in cardio-metabolic risk is not fully explained by obesity as not all obese individuals have the metabolic syndrome and not all lean individuals are free from the metabolic syndrome (Wildman *et al.*, 2008; Batch *et al.*, 2013)

Data from Johannesburg showed a high prevalence of the metabolic syndrome in both Black Africans (92%) and Asian Indians (46%) in a cohort of healthy adults living in Johannesburg (George *et al.*, 2013). The investigation also showed that although black African women had more visceral adiposity than Asian Indian women; Asian Indian women were more insulin resistant. On the other hand, Asian Indian men have greater amounts of visceral adiposity and were more insulin resistant than African men (George *et al.*, 2014).

Ideally, biomarkers should detect risk long before there is clinical evidence of disease. Several studies have shown that metabolic profiling may help to identify biomarkers that can predict cardio-metabolic risk, years before it is clinically apparent (Todd and Bloom, 2007; Huffman *et al.*, 2009; Newgard *et al.*, 2009; Batch *et al.*, 2013). Individuals with the metabolic syndrome have been shown to present with higher fasting plasma branched chain amino acids (BCAAs) and aromatic amino acids (AAA, except for tryptophan) concentrations when compared to individuals without the metabolic syndrome (Batch *et al.,* 2013). Longitudinal studies on individuals with low insulin sensitivity show BCAAs, tyrosine and phenylalanine as predictors of insulin resistance and new onset-T2DM (Wang *et al.*, 2011; Würtz *et al.*, 2013). Furthermore, BCAAs reduce with improvement in metabolic function. For example, studies have shown that gastric bypass surgery is associated with greater improvement of metabolic state than dietary intervention alone. In addition, obese subjects who had a gastric bypass showed a greater decline in BCAA, C3 and C5 acylcarnitines and phenylalanine and

tyrosine than those with dietary intervention even though weight loss was equal in both (Adams *et al.*, 2007; She *et al.*, 2007).

Leucine, isoleucine and valine are classified as branched chain amino acids (BCAAs) whereas; phenylalanine, tyrosine and tryptophan are classified as aromatic amino acids (AAAs). BCAAs and AAAs are the building blocks of proteins and are therefore largely involved in protein synthesis. In addition, the amino acids, particularly BCAAs have regulatory roles in insulin secretion, protein synthesis and the brain uptake of precursor amino acids (Brosnan and Brosnan, 2006). Tyrosine and phenylalanine function as precursors for the sympathetic nervous system effectors known as catecholamines which prepare the body for fight or flight response (Jensen, 1986).

1.1 STUDY HYPOTHESIS

Our hypothesis is that BCAAs and AAAs are higher in black Africans and Asian Indians with the metabolic syndrome than in those individuals without the metabolic syndrome.

1.2 AIM AND OBJECTIVES

1.2.1 Aim:

Describe the associations of BCAAs and AAAs with cardiovascular risk factors in black Africans and Asian Indians.

1.2.2 Study objectives:

- Describe the association of BCAAs and AAAs with cardio-metabolic variables namely; HDL- cholesterol, triglyceride and blood glucose concentrations as well as blood pressure in black Africans and Asian Indians populations.

- Asses the correlations of metabolic syndrome with fasting plasma BCAAs and AAAs in the two ethnic groups adjusted for confounding factors *i.e.* age, gender and BMI.
- Develop and validate an amino acid analysis method.

CHAPTER 2: LITERATURE REVIEW

The notion that branched chain amino acids (BCAAs) together with tyrosine and phenylalanine are potential biomarkers for cardio-metabolic risk suggests that BCAAs, tyrosine and phenylalanine play a role in cardio-metabolic dysfunction. In attempt to describe the BCAAs, tyrosine and phenylalanine association with cardio-metabolic risk factors, this chapter will describe the chemical and structural properties of the amino acids, followed by their metabolic pathways. Furthermore, this chapter will describe the proposed BCAA/ mammalian target of rapamycin (mTOR) mediated insulin resistance and BCAA induced mitochondrial toxicity. This will be followed by the description of the metabolic syndrome and the ethnic differences observed in individuals with the metabolic syndrome components. Finally, this chapter will discuss the association of BCAAs with cardio-metabolic risk factors.

2.1 CHEMICAL AND STRUCTURAL PROPERTIES OF BCAAs AND AAAs

All amino acids have an alpha carbon bound to an amine group, carboxylic group, hydrogen atom and a side chain. The amine and carboxylic group bind to the alpha carbon forming a positively charged ammonium ion and a negatively charged carboxylate ion respectively. This reaction results in a zwitterion, a term given to molecules which have chemical functional groups that exert equal opposite charges resulting in a neutral overall charge. Amino acids have chiral centers which enables them to display D & L configurations. However, majority of free amino acids exhibit the L-configurations. Figure 2.1 illustrates the general structure of amino acids.

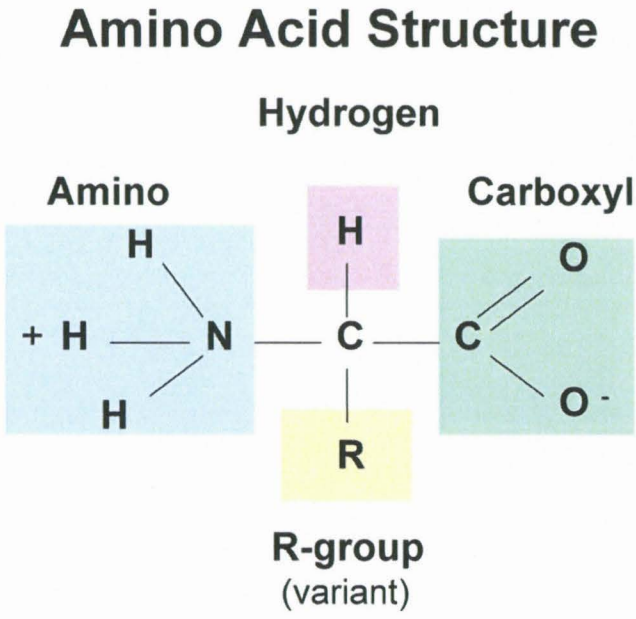

Figure 2.1 The general structure of amino Acids
Taken from Garret and Grisham (2008)

The side chain of the amino acid differentiates one amino acid from another. Amino acids can be classified according to groups that match their chemical or structural properties. For example, leucine, isoleucine and valine all have branched side chains. Therefore, they are classified as branched chain amino acids (BCAAs) (Figure2.2). The side chain of leucine and isoleucine is an isobutyl whereas; valine side chain is an isopropyl.

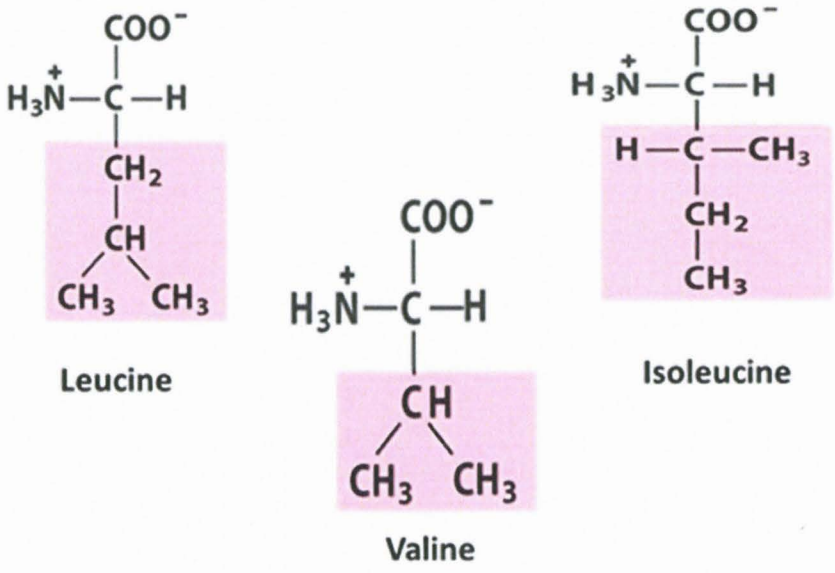

Figure 2.2 The chemical structure of branched chain amino acids
Taken from Garrett and Grisham (2008)

Likewise, tyrosine, phenylalanine and tryptophan all have aromatic rings on their side chains. For this reason they are classified as aromatic amino acids (AAAs) (Figure 2.3). Unlike phenylalanine and the BCAAs which can only be obtained from the diet, tyrosine can be synthesized inside the body from phenylalanine. Thus BCAAs and phenylalanine are essential amino acids whereas; tyrosine is a nonessential amino acid. Phenylalanine side chain has a benzene ring and tyrosine has a hydroxyl benzene ring formally known as phenol. On the other hand, tryptophan side chain has an indole group. For the purpose of this study, tryptophan will not be studied or reviewed because of its insignificant association with the cardiovascular risk factors. Although tryptophan will not be studied in the present study, phenylalanine and tyrosine will be referred to as aromatic amino acids.

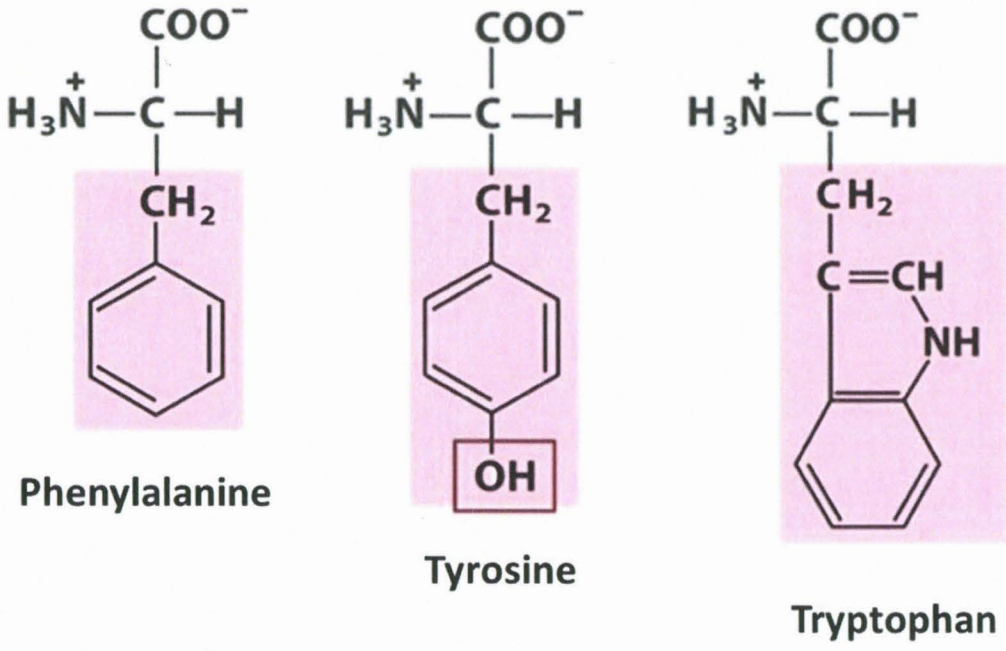

Figure 2.3 The chemical structure of aromatic amino acids
Taken from Garrett and Grisham (2008)

The BCAAs and AAAs are all non-polar (hydrophobic). However tyrosine has a hydroxyl group which makes it slightly polar (Figure 2.3). It is the hydrophobic nature of these amino acids that is responsible for the synthesis of globular proteins, membranous proteins and coiled-coiled structures. In addition to protein synthesis, BCAAs in particular have regulatory roles in lipid metabolism, insulin secretion, protein synthesis and the brain uptake of precursor amino acids (Brosnan and Brosnan, 2006). Tyrosine and phenylalanine function as precursors for the sympathetic nervous system effectors known as catecholamine which prepares the body for fight or flight response.

2.2 METABOLIC PATHWAY OF BCAAs and AAAs

Metabolic pathway impairment in both BCAAs and AAAs result in elevated BCAAs and AAAs in the blood circulation. The normal BCAAs, tyrosine and phenylalanine metabolic pathway will be discussed below

2.2.1 BCAA Metabolic Pathway

Majority of BCAA catabolism occurs in skeletal muscles. There are two main steps involved in BCAA metabolism, the transamination step and the oxidation step (Brosnan and Brosnan, 2006) The transamination step is the only reversible step in BCAA metabolism; the amino group is detached from the amino acid resulting in a formation of a branched-chain α-keto acid (BCKA) (Figure 2.4). The detached amino group is used in glutamate formation in the presence of α-ketoglutarate. This reaction is catalyzed by the enzyme branched-chain amino acid aminotransferase (BCAT) which is present in two isoforms, cytosolic (BCATc) and mitochondrial (BCATm) encoded by *BCT1* and *BCT2* genes, respectively (Naylor and Shows, 1980). The BCKAs formed from L-leucine, L-isoleucine and L-valine are ketoisocaproate, α-keto-β-methylglutarate and α-ketoisovalerate, respectively (Brosnan and Brosnan, 2006).

The second step in BCAA metabolism is the oxidation and decarboxylation of the BCKAs, catalyzed by the branched chain α-keto-acid dehydrogenase complex (BCKDC) which forms the BCAA intermediates isovaleryl-CoA, α-methylbutyryl-CoA and isobutyryl-CoA. The BCAA intermediates go through a series of reactions until the formation of acetoacetate, acetyl-Coenzyme A, propionyl-Co-enzyme-A. The end product of this reaction depends on the BCAA metabolized. For example, leucine (ketoisocaproate) catabolism will produce acetoacetate and acyl-Coenzyme-A, valine (ketoisovalerate) - propionyl-Co-enzyme A and

isoleucine (α-keto-β-methylglutarate)-acetyl-Coenzyme A and propionyl-Coenzyme-A (Figure 2.4). Propionyl-Coenzyme-A can be further degraded into methyl-malonyl-Coenzyme-A then finally into succinyl-Coenzyme-A which is used as a heme precursor in hemoglobin and myoglobin formation. Acyl-Coenzyme A can be converted to acylcarnitines which are toxic to the body when in high amounts (Adams *et al.*, 2009).

The human BCKD complex is made up of three subunits: a branched-chain a-ketoacid decarboxylase/dehydrogenase, a dihydrolipoyl transacylase, and a dihydrolipoamide dehydrogenase, which are called E1, E2, and E3, respectively (Adeva *et al.*, 2012). The E1 and E2 subunits are the active sites used for BCKA decarboxylation and oxidation, whereas, E3 is shared with the mitochondrial pyruvate dehydrogenase complex and the a-ketoglutarate dehydrogenase complex.

The branched chain alpha-ketoacid dehydrogenase kinase (BCKDK) inhibits the enzyme activity of BCKDC through serine phosphorylation of the E1 subunit (Joshi *et al.*, 2006). The activation of BCKDC can also be regulated by the protein phosphatase 1 K (PPM1K) substrate also known as PP2Cm. This substrate dephosphorylates the E1/ Ser293 residue which consequently activate the BCKDC enzyme (Lu *et al.*, 2009). Interestingly, PPM1K loss is associated with T2DM risk factors such as lipid peroxidation, lipid toxicity, oxidative stress and mitochondrial apoptosis (Lu *et al.*, 2007), suggesting that individuals with insulin resistance and T2DM might have a dysfunctional PPMIK substrate.

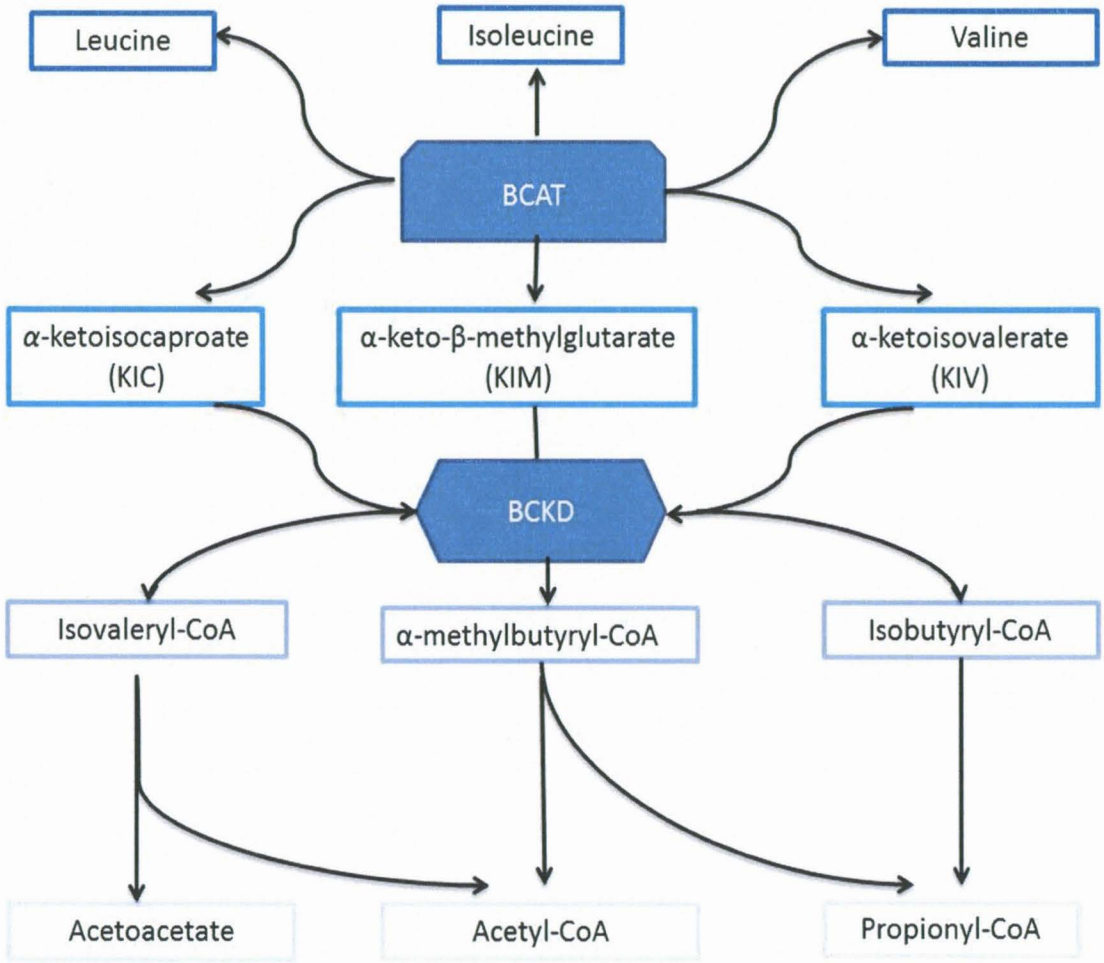

Figure 2.4 Summarized metabolic pathway of branched chain amino acids. Firstly, the branched chain amino acids (leucine, isoleucine and valine) are broken down by the branched-chain amino acid aminotransferase (BCAT), forming branched-chain α-keto acids (BCKAs). The BCKAs formed from L-leucine, L-isoleucine and L-valine is ketoisocaproate (KIC), alpha-keto-b-methylglutarate (KIM) and alpha-ketoisovalerate (KIV), respectively. The BCAA intermediates go through oxidation and decarboxylation catalyzed by the branched chain keto-acid dehydrogenase (BCKD). The BCAA intermediates are broken down further ultimately forming acetoacetate, acetyl-Coenzyme A and propionyl-Coenzyme A.

2.2.2AAAs Metabolic Pathway

AAA metabolism is mostly performed in the liver. Phenylalanine is firstly converted to tyrosine before further catabolism proceeds; the purpose of the transformation is to prepare the amino acid for the forthcoming catabolic reactions. Phenylalanine is hydroxylated in the presence of oxygen molecule, NADPH and the coenzyme tetra-hydrobiopterine. The hydroxylation is catalyzed by the liver specific enzyme called phenylalanine hydroxylase. Patients who have mutations of the *PAH* gene which codes for phenylalanine hydroxylase are diagnosed with phenylketonuria. The lack of the *PAH* gene inhibits the formation of phenylalanine into tyrosine. Consequently, toxic phenylalanine accumulates in plasma and in peripheral tissues which may cause neurological disorders.

The next step following phenylalanine transformation is the reversible transamination reaction catalyzed by the tyrosine specific enzyme called glutamate aminotransferase. The amino group is detached from tyrosine resulting in 4-hydroxy phenylpyruvate formation. Concurrently, the detached amino group is transferred to α-ketoglutarate resulting in glutamate formation. The tyrosine intermediate (4-hydroxy phenylpyruvate) is decarboxylated by the enzyme 4-hydroxyphenylpyruvate dioxygenase in the presence of vitamin C. This reaction results in homogentisic acid formation. Homogentisic acid is broken down in various steps which ultimately result to acetoacetate and fumarate formation (Figure 2.5)

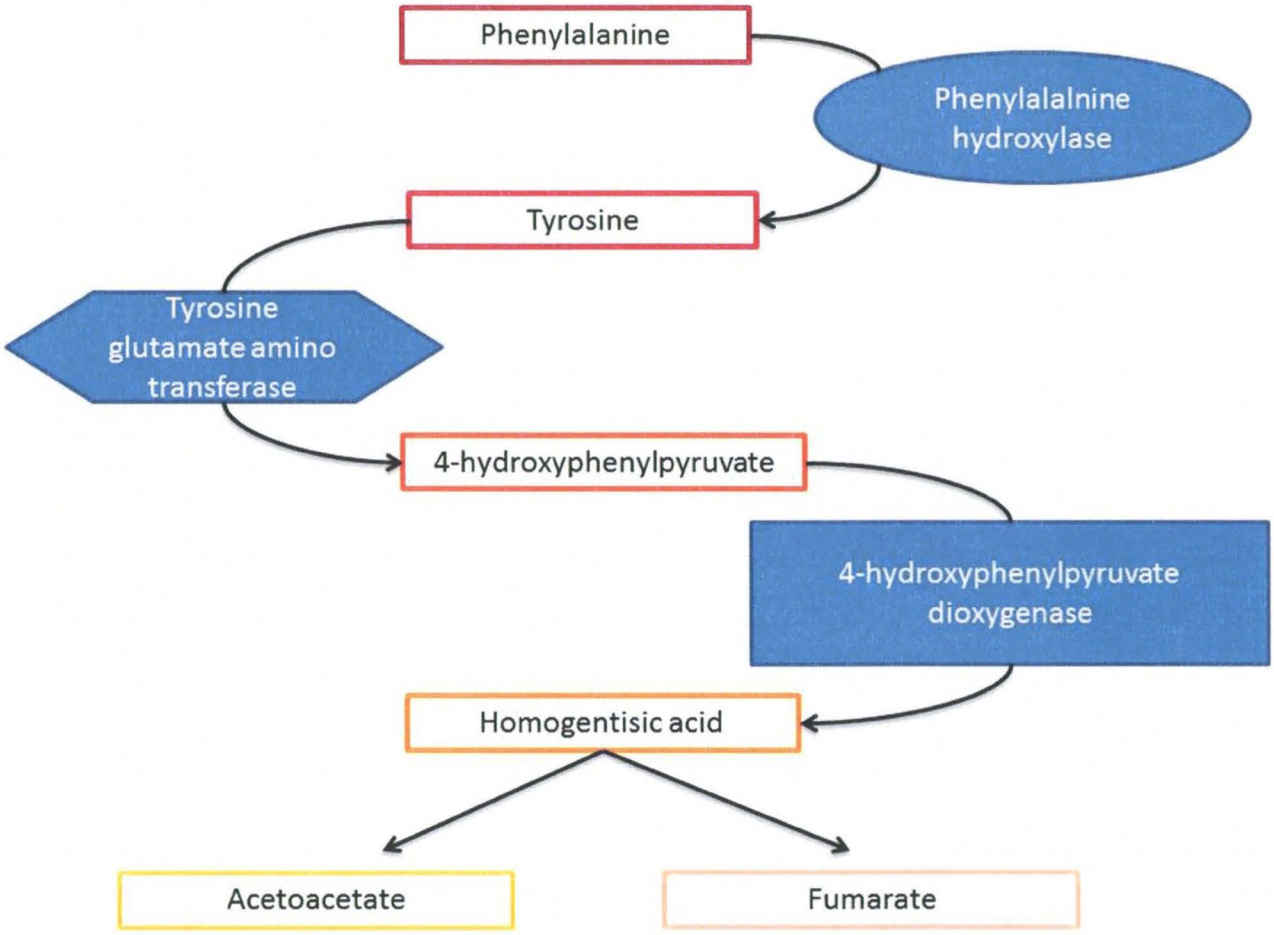

Figure 2.5 Summarized metabolic pathway of phenylalanine and tyrosine. Firstly, phenylalanine is hydroxylated to form tyrosine catalyzed by phenylalanine hydroxylase. This is followed by the breakdown of tyrosine which forms 4-hydroxypyruvate, catalyzed by tyrosine glutamate amino transferase. 4-hydroxyphenylpyruvate catalyzes the next step which breaks down the tyrosine intermediate into homogentisic acid. The homogentisic acid goes through a series of other reactions until the formation of acetoacetate and fumarate.

2.2.3 Impaired BCAAs Metabolism

Impairment of BCAA metabolism which is evident in metabolically unhealthy individuals is proposed to induce insulin resistance and β-cell destruction (Newguard *et al.*, 2009 and Newgard *et al.*, 2012). Accumulation of BCAA intermediates such as acylcarnitines causes

mitochondrial oxidative stress in β-cells which may lead to β-cell toxicity and mitochondrial pore opening and ultimately mitochondrial mediated cell apoptosis (Olson *et al.*, 2014). This could explain why acylcarnitines in particular were associated with pancreatic failure in the Huffman *et al.* (2009) study. In addition, oxidative stress that is caused by the BCKA accumulation can cause lipid peroxidation, and lipid toxicity in adipose tissue.

Although acylcarnitine mediated mitochondrial dysfunction was observed in adipose tissue (Lackey *et al.*, 2013; Olson *et al.*, 2014) and in skeletal muscles (Roe *et al.*, 1998 and Lefort *et al.*, 2010), it is uncertain whether this process is tissue specific or affects the whole body. Furthermore, animal models may differ in organ size which may affect the interpretation of enzymatic activity of the BCAA metabolism enzymes. For example, liver organs of obese Zucker rats are twofold bigger than those of lean Zucker rats (She *et al.*, 2013), indicating that the results may show false positives if liver size was not adjusted.

2.3 BCAA STIMULATION OF THE MAMMALIAN TARGET OF RAPAMYCIN SIGNALING PATHWAY

The significant association of BCAAs and AAAs with obesity, metabolic syndrome and insulin resistance has led to speculations that BCAAs and AAAs play a role in inducing metabolic dysfunction. One proposed model is the BCAA/mTOR signaling that affect insulin signaling pathway.

The mammalian target of rapamycin (mTOR) is a serine-threonine kinase that behaves as a nutrient sensor. The function of the mTOR signaling pathway is to relay signals related to nutrition, cell growth and cell survival. The mTOR can be present as two complexes, the mTORC1 and mTORC2. The mTORC1 is mostly associated with cell growth and

proliferation, protein synthesis, inhibition of autophagy and hypoxia and transcription. The mTORC2 pathway, on the other hand is mainly involved in cytoskeleton organization (Leibowitz *et al.*, 2008a; Adeva *et al.*, 2012). The mTOR signaling pathway is regulated by various number of stimuli related to nutrition, cell growth and survival. For example amino acids, insulin and the insulin like growth factor (IGF) are upstream activators of the mTOR signaling pathway. The mTORC1signaling pathway can be regulated by either, the class III phosphoinositide 3-kinase (PI3K) or the small GTPase called Ras homolog enriched in brain (Rheb). In turn, the Rheb GTPase is regulated by the tuberous sclerosis complex protein (TSC2) and its binding protein TSC1. The TSC1-TSC2 complex converts the active Rheb-GTP to the inactive Rheb-GDP (Adeva *et al.*, 2011).

2.3.1 Insulin or IGF-1 activation of the mTOR

The binding of insulin or insulin growth factor (IGF)-1 to the insulin receptor, changes the conformation of the receptor, consequently initiating the mTOR signal transduction. Protein kinase B (PKB)/Akt phosphorylation activated by the insulin receptor substrate (IRS)-1will then disrupt the TSC1 and TSC2 dimerization or induce the degradation of the proteins to prevent the TSC1-TSC2complex from deactivating Rheb GTPase/mTOR signaling (Figure 2.6).

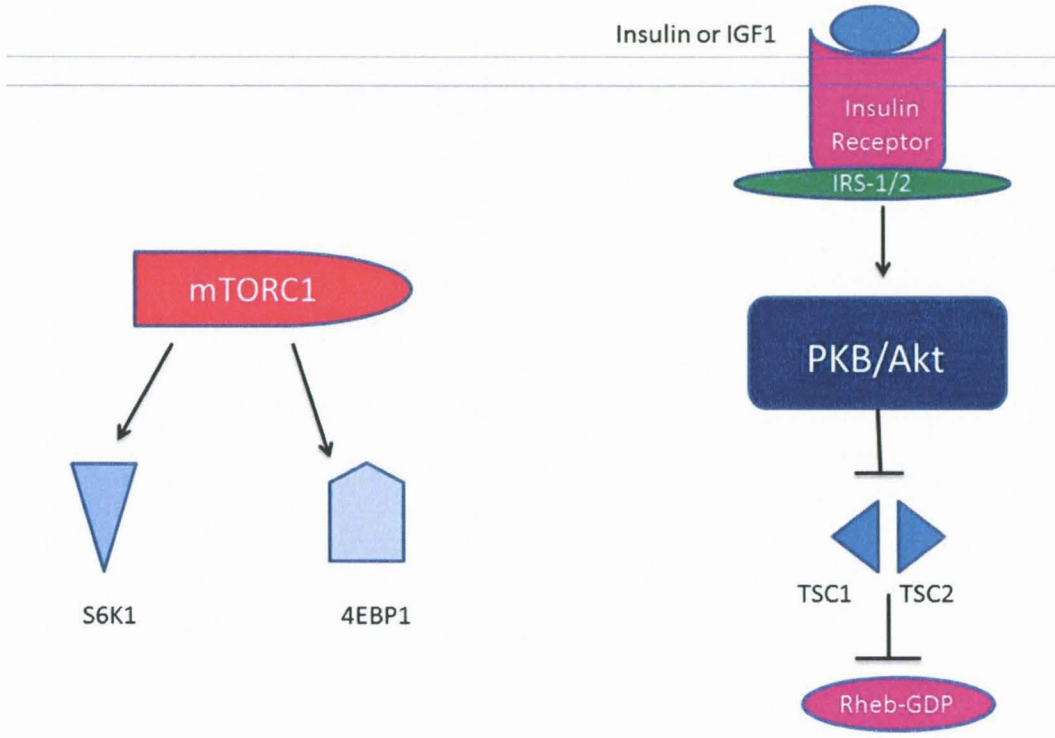

Figure 2.6 Insulin or insulin like growth factor-1 (IGF-1) stimulation of the mammalian target of rapamycin (mTOR). The binding of insulin or IGF-1 onto the insulin receptor changed the confirmation of the receptor followed by the activation of insulin receptor substrate-1(IRS-1). The signal transduction is carried on to Protein kinase B (PKB)/Akt which will inhibit the tuberous sclerosis complex protein 2 (TSC2) and its binding protein (TSC1) from reacting. The inhibition of TSC1/2 complex inhibits the Ras homolog enriched in brain (Rheb) from deactivating the mTOR signaling pathway. The mTOR is then capable of relaying signals through the ribosomal substrate 6 kinase 1 (S6K1) and the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1).

2.3.2 Amino acid activation of the mTOR

The amino acids ability to regulate metabolic function through the mTORC1 pathway is well documented (Tremblay and Marette, 2001; Drummond et al., 2008; Leibowitz et al., 2008b; Lynch and Adams, 2014). Amino acids activate the mTORC1 pathway through the activation of the class III phosphoinositide 3-kinase (PI3K) and consequently mTORC1 phosphorylation (Figure 2.6). The activation of mTORC1 leads to the phosphorylation of two main downstream effectors, namely, the ribosomal substrate 6 kinase 1 (S6K1) and the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (Hay and Sonenberg, 2004).

Studies have proposed that amino acids/mTORC1 signaling causes insulin resistance by disrupting the insulin signaling pathway (Tremblay and Marette, 2001; Khamzina *et al.*, 2005). The mechanism behind the proposed amino acid/ mTOR mediated insulin resistance is as follows; ingestion of high fat and high protein diet causes a sustained hyperglycemia and hyper-aminoacidemia over time. The accumulation of BCAAs over-stimulated the mTORC1 pathway in skeletal muscles, liver, pancreas and adipose tissue(Briaud *et al.*, 2005; Khamzina *et al.*, 2005; Krebs *et al.*, 2007; Ruffolo *et al.*, 2007). Studies suggest that over-stimulation of the mTORC1 pathway by amino acids creates a negative feedback loop which inhibits insulin signaling through IRS-1 serine phosphorylation as demonstrated in Figure 2.7 (Tremblay and Marette, 2001; Um *et al.*, 2004; Shah *et al.*, 2012).

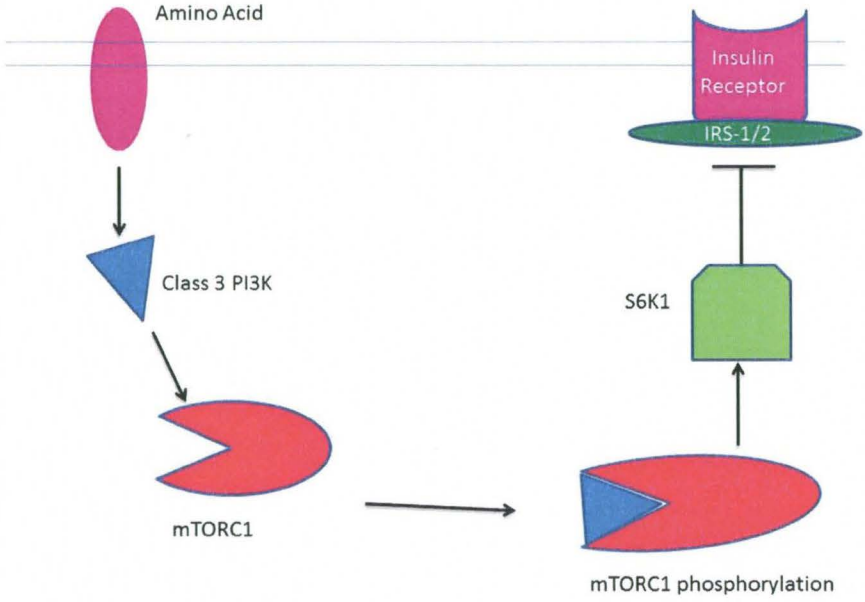

Figure 2.7 The amino acid/ mammalian target of rapamycin (mTOR) mediated insulin resistance model. Accumulation of amino acids leads to the activation of the mTOR signaling pathway through the class III phosphoinositide 3-kinase (PI3K). Followed by the phosphorylation of the ribosomal substrate 6 kinase 1 (S6K1). Insulin receptor substrate-1 signaling is inhibited by serine phosphorylation stimulated by the S6K1.

IRS-1 serine phosphorylation impairs the ability of IRS-1 to relay insulin/IGF-1 mediated signaling. Consequently, deactivation of the TSC1-TSC2 heterodimer by the PKB/Akt complex is inhibited. The active TSC1-TSC2 heterodimer can then convert Rheb-GTP (active) to Rheb-GDP (inactive) which ultimately prevent the activation of the mTORC1 signaling pathway.

Numerous studies on animal and humans were performed to test this proposed amino acid/mTOR mediated insulin resistance model. Um and colleagues (2004) showed that the

knockout of S6K1 gene in high fat fed mice enhances insulin signaling. Furthermore, the S6K1 knockout resulted in reduced serine phosphorylation of IRS-1. This indicates that S6K1 is strongly involved in IRS-1 serine phosphorylation. Human studies showed that a combination of hyperinsulinemia and hyper aminoacidemia increases S6K1 phosphorylation and serine phosphorylation of IRS-1 serine residues in skeletal muscles (Tremblay *et al.,* 2005 and Krebs *et al.,* 2007). Rapamycin, an mTOR specific inhibitor, partially reversed the amino acid mediated insulin resistance in humans (Krebs *et al.,* 2007). However, the opposite was observed in diet induced T2DM mice given a calorie-rich laboratory chow (Fraenkel *et al.*, 2008). This suggests that rapamycin action on the mTOR is dependent on the presence of amino acids. This was further supported by the Tremblay *et al.* (2001) study where rapamycin did not impair IRS-1 signaling in absence of amino acids in mice skeletal muscle cells. However, when amino acids were introduced into the rapamycin treated cells, IRS-1 serine phosphorylation was increased. The amino acid dependent mTORC1 activation was also observed in human skeletal cells (Drummond *et al.,* 2008).

There are many uncertainties with the amino acid mediated insulin resistance model. For instance, it is not known if the concentration of BCAAs observed in obese and T2DM patients is enough to activate the negative feedback loop and consequently cause insulin resistance. In contrast to the amino acid/ mTOR mediated metabolic dysfunction, mTORC1 activation increased islet and β-cell mass in patients undergoing rapamycin therapy(Dandel *et al.*, 2010; Gyurus *et al.*, 2011; Lopes *et al.*, 2013). In addition, mTORC1 phosphorylation remains unchanged in subjects who had improved metabolic function along with a decrease in plasma BCAAs after gastric bypass surgery (Magkos *et al.*, 2013). Considering the entire evidence reviewed above showing that the elevation of amino acids induces insulin resistance, it seems

that this is the body's way to conserve β-cells by reducing insulin secretion signaling. More studies are needed to confirm the observations above.

2.4 METABOLIC SYNDROME

There is an alarming increase in death caused by cardiovascular diseases (CVD) in Africa. Forty one percent of deaths from CVD in South African occur in individuals at the age between 35 and 64 (Leeder *et al.,* 2004). The metabolic syndrome which is a clustering of cardio-metabolic risk factors is used to predict the risk of developing CVD. The criteria used to diagnose the metabolic syndrome states that an individual should present with three or more of the following risk factors; elevated fasting plasma glucose, elevated triglycerides, low HDL-cholesterol, hypertension and abdominal obesity to be diagnosed with the metabolic syndrome, using ethnic specific cut-off levels (Alberti *et al.*, 2009). The diagnosis can be complicated because the ability of the metabolic syndrome to predict T2DM and CVD risk is unequal among the different ethnic groups. For example, African Americans have a higher mortality rate of T2DM and coronary heart disease than white Americans yet they have a lower prevalence of metabolic syndrome (Ford et al., 2002a; Meis et al., 2006; Ervin, 2009).

2.4.1 The Ethnic Differences in the Metabolic Syndrome Components

2.4.1.1 Obesity and hyperglycemia

Development of the metabolic syndrome is often dependent on an increase in BMI however some lean individuals have the metabolic syndrome and there are obese individuals without the metabolic syndrome (Alberti *et al.*, 2006). This was demonstrated in a study by Wildman *et al.*(2008) in which 24% of lean subjects with body mass index (BMI) of < 25 kg/m^2 had the metabolic syndrome (defined by having 2 or more of the metabolic syndrome components)

and 31.7% of obese individuals (BMI > 30 kg/m^2) did not have the metabolic syndrome. The body mass index is a value derived from the mass (weight) and height of an individual. Similarly, a study by Batch *et al.* (2014) observed that 67% of lean individuals were metabolically unwell (defined by having 2 or more of the metabolic syndrome components) and 13% of obese individuals were metabolically well (one or none of the metabolic syndrome components). More evidence of the obesity paradox is shown in a BMI-mortality study where obese individuals had better chances of survival when compared to lean individuals and BMI–mortality association was significantly higher in lean African Americans (HR 1.95 [95% CI 1.44-2.63]) than in Caucasians (HR 1.53 [95% CI 1.0-2.1]) (Kokkinos *et al.*, 2012). These finding were consistent with those from the Translating Research into Action for Diabetes (TRIAD) study which showed that overweight men had lower mortality risk (HR, 0.43 [95% CI, 0.32-0.59]) compared to underweight men. Forty four percent of these were African-American (McAuley *et al.*, 2010). Similarly, lean individuals had a higher risk of mortality when compared to obese individuals in Asian and Bangladesh populations (Zheng *et al.*, 2011). Although the studies on BMI-mortality association do not specify the cause of mortality, the results however, suggest that obesity cannot be used independently to predict cardio-metabolic risk.

Obesity is mainly a contributor to CVD and T2DM risk because of adipose dysfunction associated with weight gain. Abdominal obesity, determined by the visceral and subcutaneous fat measurement, is a more accurate measure of obesity associated with the risk of developing CVD and T2DM than BMI (Phillips and Prins, 2008). Adipose tissues function as an endocrine organs and releases pro-inflammatory or anti-inflammatory substances known as

adipokines which are associated with insulin resistance (Arita *et al.*, 1999) and coronary artery disease (Ouchi *et al.*, 1999). However there are limitations to the use of visceral fat measurements. For example, Asian Indians and African-American women have relatively high visceral fat when compared to Caucasians women (Lovejoy *et al.*, 1996; Banerji *et al.*, 1999; Ferris *et al.*, 2005; Lear *et al.*, 2007). A South African study compared the adipose tissue of black obese women with white obese women using a five-level computed tomography (CT) scan. Both groups had similar mean subcutaneous fat mass (black obese women 555 ±9.0 vs. white obese women 532 ±6.0 cm; not significant), however, black obese women had less visceral fat (90 ± 3.0 vs. 121 ± 3.1 cm^2; $p < 0.05$) (van der Merwe *et al.*, 2000). The differences in visceral fat in ethnic groups make the ability of predicting CDV risk using abdominal obesity less effective.

2.4.1.2 High triglycerides and low HDL-cholesterol

High triglycerides are usually associated with the increased risk of developing CVD and T2DM. However, the elevation of triglycerides in an individual with metabolic dysfunction varies with ethnicity. Africans with the metabolic syndrome whether from America, Africa or Caribbean, all seem to present with favorable lipid profile. Unlike white and South Asians who are likely to present with high triglycerides, majority of Africans present with normal triglycerides (Zoratti et al., 2000; Ford et al., 2002b; Park et al., 2003; Sumner et al., 2010). Zoratti *et al.* (2000) investigated the contribution of high lipid levels to insulin resistance and coronary heart disease in healthy African- Caribbeans, South Asians and Europeans. African-Caribbean men were more insulin-resistant than Europeans ($p < 0.01$) despite their beneficial lipid profile of lower levels of very-low-density lipoprotein (VLDL) cholesterol (0.21 v 0.40 mmol/L, $p < 0.01$) and triglycerides (0.34 v 0.74 mmol/L, $p < 0.01$), lower serum total

triglycerides, higher high-density lipoprotein 2 (HDL2) cholesterol, and larger low-density lipoprotein (LDL) particle size.

HDL-cholesterol is known as the good cholesterol due to its anti-thrombotic, anti-inflammatory and anti-oxidant properties (Remaley *et al.*, 2008). Therefore, individuals with low HDL-cholesterol have less protection from cardiovascular impairment compared to individuals with high HDL-cholesterol. Several studies have shown that South Asians have lower HDL-cholesterol than Caucasians (Bhalodkar *et al.*, 2005; Ajjan *et al.*, 2007; Chandalia *et al.*, 2008). HDL-cholesterol in black Africans is generally lower than in West Africans and African Americans (Sumner *et al.*, 2010). This may be explained by the socioeconomic transition in African countries which is associated with high fat diets and sedentary life styles (Onyegbutulem *et al.*, 2009).

2.4.1.3 Hypertension

Hypertension is a major risk factor for CVD with a prevalence of 40% in individuals over the age of 25 in Africa ("WHO | Raised blood pressure,"). Africans are more susceptible to having hypertension than other ethnic groups. For example, African-Caribbeans had higher mean blood pressure when compared to Europeans (Chaturvedi *et al.*, 1993; Lane and Lip, 2001). A study focusing on ethnic and racial differences in diabetic care showed that although all subjects had medical insurance and had a similar socioeconomic status, 60% of African Americans receiving treatment for hypertension had blood pressure >140/90 mm/Hg, as compared with 38% of non-Hispanic whites ($p \leq 0.001$) (Bonds *et al.*, 2003). This suggests that the hypertension disparities were not influenced by poor management or social income. Hypertension in black people may be less driven by the differences in genetic makeup and

mainly related to environmental and lifestyle factors such as high salt and high fat diet consumption and high alcohol consumption (Saunders, 1991; Ibrahim and Damasceno, 2012).

Table 2.1 below shows details the findings of some studies that have compared some of the metabolic syndrome components across various ethnic groups.

Table 2.1.The ethnic comparisons in relation to metabolic syndrome components

AUTHOR	ETHNIC COMPARISONS
Bonds *et al.*, 2003 (n= 1624)	African Americans had a higher hypertension prevalence (%) than non-Hispanic whites whites (60% vs. 38%, $p \leq 0.001$)
Zorrati *et al.*, 2000 (n= 92)	South Asians had higher triglycerides compared to African Caribbean (1.59 mmol/L vs. 0,34 mmol/L, $P < 0.01$) and European (1.59 mmol/L vs. 0,74 mmol/L, $p < 0.01$)
van der Merwe *et al.*, 2000 (n=20)	South African White women had more visceral adipose than South African black women (123±3.1 cm vs. 90±3.0 cm, $p < 0.05$)
Banerji *et al.*, 2009 (n= 35)	Asian Indians had a lower waist circumference compared to African Americans (86.2±8.4 cm vs. 90.9±7.1 cm, $p < 0.0001$)
Lear *et al.*, 2007 (n=800)	South Asians had a lower waist circumference than Europeans (88.6±12.3 cm vs. 89.3±12.5 cm, $p < 0.0001$)
Bhalodkar *et al.*, 2005 (n=119)	Asian Indians had a lower HDL-C particle size than African Americans (8.9 nm vs. 9.4 nm, $p < 0.0001$)
Ajjan *et al.*, 2007 (n=490)	Caucasians had high HDL-C (1.43 mmol/L vs. 1.10 mmol/L, $p <0.001$) and low triglycerides (1.2mmol/L vs. 1.5mmol/L, $p <0.001$) compared to South Asians.

2.5 BCAAs AND AAAs ARE POTENTIAL BIOMARKERS FOR CARDIO-METABOLIC RISK

The limitation with using the components of the metabolic syndrome is that they predict cardio-metabolic risk when there is already metabolic dysfunction. Ideally biomarkers should

detect risk long before it becomes clinically evident. The predictability of BCAAs and AAAs will be discussed below.

2.5.1 BCAAs and AAAs association with Insulin Resistance and T2DM

BCAAs and AAAs have been shown to predict insulin resistance and T2DM years before the disease is clinically apparent (Wang *et al.*, 2011; Wurtz *et al.*, 2013; Tillin *et al.,* 2015). In the Wang *et al.* (2011) study, metabolic profiling was performed at baseline with two large longitudinal studies, namely; the Framingham Offspring study and the Malmö Diet and Cancer study. The fasting concentrations of the BCAAs, tyrosine and phenylalanine were significantly higher in Caucasians who developed new-onset diabetes compared to those who did not during the 12 year follow up ($p< 0.05$ for all the amino acids). The study further stratified the subjects into upper and lower amino acids scores (summation of the BCAAs, tyrosine and phenylalanine) and the individuals with a high amino acid score had approximately five times higher risk of developing T2DM when compared to individuals with low amino acid score ($p<0.007$). Furthermore, the BCAAs elevation was sustained throughout the 12 year follow up. Tillin *et al* (2015) performed a prospective study on British population-based cohort of 1,279 European and 1,007 South Asian non-diabetic men. Follow-up of 19 years was done in 801 European and 643 South Asian participants. The study agreed with the notion that BCAAs, tyrosine and phenylalanine were predictors of T2DM as was shown in Framingham Offspring study and the Malmö Diet and Cancer study (Wang *et al.,* 2011). However, the study showed that the associations of BCAAs, tyrosine and phenylalanine with T2DM were stronger in South Asians than in Europeans, tyrosine having the strongest association even after adjusting metabolic risk factors including obesity and insulin resistance ($p<0.05$). In addition, BCAAs, tyrosine and phenylalanine predicted homeostatic model

assessment index –insulin resistance (HOMA-IR) in a 6 year follow up study (Wurtz *et al.*, 2013). BCAAs were associated with insulin sensitivity (S_i), acute insulin response (AIR) and metabolic clearance of insulin (MCRI) in 290 Caucasians, 165 African Americans and 230 Hispanics ($p<0.0001$) (Lee *et al.*, 2016). The ability of BCAAs to predict T2DM was only evident in the Caucasian and Hispanic group (OR 1.67 [95%CI 1.21, 2.29], $p=0.002$) and not in African Americans.

2.5.2 BCAAs and AAAs association with Obesity and hyperglycemia

Fasting plasma BCAAs, tyrosine and phenylalanine have been shown to be higher in obese individuals compared with non-obese individuals (She *et al.*, 2007; Huffman *et al.*, 2009; Newgard *et al.*, 2009). Among 75 metabolites, BCAAs, tyrosine, phenylalanine and C3 and C5 acylcarnitines showed the strongest difference in obese and lean individuals. Similarly, BCAAs, tyrosine and phenylalanine were significantly and inversely associated with insulin sensitivity ($p< 0.0001$) in sedentary, overweight to obese subjects with dyslipidemia (Huffman *et al.*, 2009). Moreover, acylcarnitines which are intermediate of BCAAs were associated with pancreatic failure.

2.5.3 BCAAs and AAAs association with high triglycerides and low HDL-cholesterol

Dyslipidemia (hyper triglycerides and low HDL-cholesterol) is been shown as a major predictor of CVD and T2DM. However, research on BCAAs and AAAs association with dyslipidemia is limited. Therefore, the role of BCAAs and AAAs is not fully understood. Yang and colleagues (2016) investigated the association of dyslipidemia with BCAAs in 1310 Chinese subjects, stratified into pre-diabetic, impaired glucose regulation and normal glucose

tolerance groups. The pre-diabetic group had significantly higher BCAA levels compared to the normal glucose tolerance group ($p<0.001$). Furthermore, elevated BCAAs were associated with triglycerides and inversely associated with HDL-cholesterol after adjustment for BMI, systolic BP, diastolic BP, fasting blood glucose and smoking.

Although tyrosine has been shown to be associated with CVD and T2DM (Wang *et al.*, 2011; Wurtz *et al.*, 2013; Tillin *et al.,* 2015), it is unclear whether it is associated with high triglycerides. A study in 775 healthy individuals from Germany showed that leucine, isoleucine valine, arginine, proline, phenylalanine, and lysine levels were significantly associated with hypertriglyceridemia ($p<0.05$) within a seven year follow up (Mook-Kanamori *et al.*, 2014). Leucine had the strongest significance ($p = 0.009$) among the amino acids. Interestingly, tyrosine was not significantly associated with high triglycerides .In contrast, results from a Japanese study showed that tyrosine was associated with dyslipidemia after a 4 year follow up (Yamakado *et* al., 2015). More studies are needed to better understand the BCAAs and AAAs association, particularly tyrosine with dyslipidemia.

2.5.4 BCAAs and AAAs association with Hypertension

Hypertension has been shown to be major predictor of CVD and T2DM. However, research on BCAAs and AAAs association with hypertension is limited. Therefore, the role of BCAAs and AAAs in hypertension is not fully understood.

Yamakado *et al.* (2015) showed that plasma free amino acids were associated with hypertension in Japanese subjects ($p<0.001$) in a four year follow up study. However, no significant associations were found between plasma free amino acids and future hypertension development after adjusting for age, gender, BMI, FPG, and HOMA-IR.

Similar to BCAAs and AAAs association with dyslipidemia, more studies are warranted to fully understand the role of BCAAs and AAAs in developing hypertension.

CHAPTER 3: METHOD DEVELOPMENT

Amino acid analysis has been performed for many years and analytical methods keep on improving to date. There are various ways that amino acids can be analyzed such as nuclear magnetic resonance spectroscopy (NMR), capillary electrophoresis (CE), liquid chromatography (LC) and gas chromatography (GC). The challenge with amino acid analysis is finding a reproducible analytical method that will analyze the polar compounds with high sensitivity and specificity while also allowing for high throughput. GC is a very robust method which produce highly reproducible retention times (Kaspar *et al.*, 2008a), however the samples have to be derivatized before analysis. Derivatization is a process where the functional groups of compounds are modified so that the compound can be analyzed using the specific method. In GC derivatization, the motive is to make the compound less polar and volatile, sometimes fluorescent. This allows for better resolution of the analyte. Disadvantages of derivatization are long sample preparation time, possibility of crosstalk by analytes other than amino acids that may also interfere with the derivatization reagent and instability of the analytes after derivatization (Gehrke *et al.*, 1969). Another method that also requires derivatization is LC coupled with optical detection. CE and NMR can analyze amino acids without derivatization, however at a cost of losing sensitivity. Moreover, NMR requires a large sample volume and usually has long analysis time (Kaspar et al., 2008b).

LC coupled with mass spectrometry (LCMS) detectors have high sensitivity and specificity; therefore have a lower limit of detection (LOD) than CE and NMR methods. LCMS methods may or may not require analyte derivatization. Recent advances in amino acid analytical method have made available kits that can allow for amino acid analysis by various detectors such as AccQ-Tag Ultra Derivatization kit from Waters (Milford, MA, U.S.A) and EZ:

FAAST Amino Acid Analysis kit from Phenomenex (Torrance, U.S.A) that can be used for UV, fluorimetry, amperometry, and mass spectrometry detection.

This chapter will present the method developed for BCAAs and AAAs analysis, the method employed for the present study is one that is simple, quick and reproducible for BCAAs and AAAs. Moreover, the method was modified and optimized to obtain optimum sensitivity and reproducible results.

3.1 SAMPLE AND SOLVENT PREPARATION

3.1.1 Sample Collection and Storage

The serum samples used in this study were from a previous study carried out by Prof. Jaya George (George *et al.,* 2013). Serum samples were collected from a mixed population of 730 Black African and Asian Indian adults. The samples were collected through the caregivers of the Birth to Twenty Cohort. The Cohort is a longitudinal analysis of more than 3200 children and their caregivers which began in 1990 and is representative of longstanding urban inhabitants (Richter *et al.*, 2004). The exclusion criteria consisted of:

- Pregnancy
- Breast feeding
- History of renal failure
- Age below 18 or above 70
- People of mixed ancestry or race other than Asian Indians and Black African.

Data on fasting blood glucose, lipid profile, fasting insulin, hemoglobin A1c (HbA1c) and body mass index (BMI) as well as measurements of visceral, subcutaneous abdominal fat and

total body fat were obtained from Prof. Jaya George's study (George *et al.,* 2013). Social data such as age, gender, smoking and alcohol intake was obtained from subjects using a questionnaire. Ethics clearance for this study was obtained from the University of Witwatersrand Human Research Ethics Committee (Ethic clearance no: M150669 Appendix A). Patient informed consent was obtained from all participants (Appendix B).

After an overnight fast of at least 8 hours, peripheral venous blood was collected into EDTA tubes for glycated hemoglobin (HbA1c), fluoride tubes for fasting glucose analysis as well as tubes with no anticoagulant for fasting BCAAs and AAAs analysis. The fasting blood samples were kept on ice for 30 minutes and centrifuged at 3,500 g for 10 minutes. Glucose, HbA1c and lipid profile measurements from fasting blood samples were performed on the automated Siemens ADVIA 1800 analyzer (Tarrytown, USA) on the day of collection within the NHLS Chemical Pathology routine laboratory unit of the Charlotte Maxeke Johannesburg Academic Hospital. The sera from the fasting blood samples were isolated into clean plastic tubes and stored at -80°Celcius until the time of BCAAs and AAAs analysis (Brinc *et al.*, 2012; Paltiel *et al.*, 2008)

3.1.2 Preparation of Amino acid calibrators.

A 2, 5mmol/L stock solution of lyophilized amino acid standard was prepared in 0, 1% formic acid in deionized water. The stock solution was stored at -20°C. The amino acids were stable at room temperature (± 25 °C) during analysis.

On the day of analysis a seven point calibration curve was prepared by diluting the stock solution \ 0, 1% formic acid at a concentration range of 16- 400µmol/L. The concentration range chosen for the calibration was guided by previous studies that have performed BCAAs

and AAAs analysis to investigate the association of BCAAs and AAAs with one or more of the cardio-metabolic risk factors (Newgard *et al.,* 2009; Würtz *et al.*, 2013; Yang *et al.*, 2014). Matrix matched amino acid calibrators are available commercially; however, they are available in a lower concentration range than that which is required of this method. Therefore, pure standards that fit the concentration were applied in this study. We spiked our standards with plasma controls to test linearity when pure standards are matrix matched. The calibrators showed a linear relationship.

3.1.3 Preparation of Internal standards.

Internal standards are part of the extraction procedure and are subjected to everything that the analytes are exposed to in the sample preparation and analysis process. Therefore, internal standards are expected to behave similar to the target analytes given that internal standard are structurally and chemically similar to the analyte. The use of internal standards corrects for analyte loss and minimizes matrix effects (Stokvis *et al.*, 2005). In this study, the internal standard used for Isoleucine (ILE) and Leucine (LEU) was Leucine-d3 (135µmol/L), for Phenylalanine (PHE) and Tyrosine (TYR) was Homo-phenylalanine (50µmol/L), and for Valine (VAL), Methionine-d3 (50µmol/L) was used.

On the day of analysis, methionine-D3 and homo-phenylalanine internal standards were prepared by diluting the 200µmol/L stock solution to 50µmol/L with 0, 1% formic acid in deionized water and pipetted into 5mL polypropylene tubes. Lyophilized leucine-D3 internal standard stock solution was prepared by diluting\ 0, 1% formic acid to a concentration of 134µmol/L. All internal standards stock solutions were stored at 4°C until time of analysis.

3.1.4 Preparation of Plasma controls

Amino acid plasma controls of two concentration levels were used to ensure that the method is stable and analysis is correct. Table 3.1 indicates the expected concentration range and mean value of valine, leucine, isoleucine, phenylalanine and tyrosine in the plasma control analysis. The lyophilized plasma controls were prepared by reconstituting with 3mL of deionized water and lightly mixing for 15 minutes. Aliquots of 120 µL from the stock solution were pipetted into 200 µL eppendorf tubes and stored at -20°C until time of analysis. The plasma controls are stable for at least 10 days when stored below -18 °C.

Table 3.1 The expected mean concentration and concentration range of amino acid plasma controls.

Analyte	Mean Concentration Level I (µmol/L)	Control Concentration range Level I (µmol/L)	Mean Concentration level II (µmol/L)	Control Concentration range Level II (µmol/L)
Valine	212	170- 254	402	322- 482
Leucine	187	150- 224	304	243- 365
Isoleucine	68.3	54.6- 82	116	92.8- 139
Phenylalanine	78.8	67- 90.6	413	351- 475
Tyrosine	57.4	45.9- 68.9	234	187- 281

3.1.5 Sample Extraction

Sample extraction is an essential step in analytical methods which analyze complex matrices such as plasma and serum like the present study. Unlike urine, plasma and serum have more proteins which indicate that there is more interference compared to urine samples. Liquid chromatography integrated with mass spectrometry is a sufficient tool for amino acid analysis however it is prone to matrix effects. To minimize matrix effects one should perform a sample extraction technique that will isolate the target analytes with minimum co-eluting compounds.

The commonly used sample extraction technique for amino acids is protein precipitation (PPT) because it is simple, fast, cost effective and does not require a large sample volume (Kaspar *et al.*, 2008b). The conventional protein precipitates used almost four decades ago was sulfosalicylic acid (SSA), perchloric acid, trifluoroacetic acid (TFA), acetonitrile, ethanol and acetone (Qureshi and Qureshi, 1989). In the present times, researchers have moved away from using strong acids as precipitators to organic solvents mainly because of their high compatibility with the liquid chromatography instruments (ref).

The calibrators, controls and serum samples were all extracted on the same day of analysis. The organic solvent protein precipitator used was methanol (CH_3OH) as it is known to be more effective and reproducible than acetonitrile (Want *et al.*, 2006; Michopoulos *et al.*, 2009) in protein precipitation. Frozen samples were thawed at room temperature. 100µL of calibrators, controls and serum samples were pipetted into their respective extraction tubes. 200µL of the internal standard mixture was then added to the mixture. The samples were lightly mixed and thereafter 600µL of methanol was added. The emulsion was then vortexed for 10 seconds. Thereafter, samples were centrifuged at 3, 148 g for 10 minutes. An aliquot of 100µL of the supernatant was transferred into 5mL glass tubes and evaporated to dryness at 80°C in the multi block heater. The resultant dried eluent was then reconstituted with the mobile phase and was transferred into 2ml light protected glass vials with inserts. Figure 3.1 shows a summary of the sample preparation method.

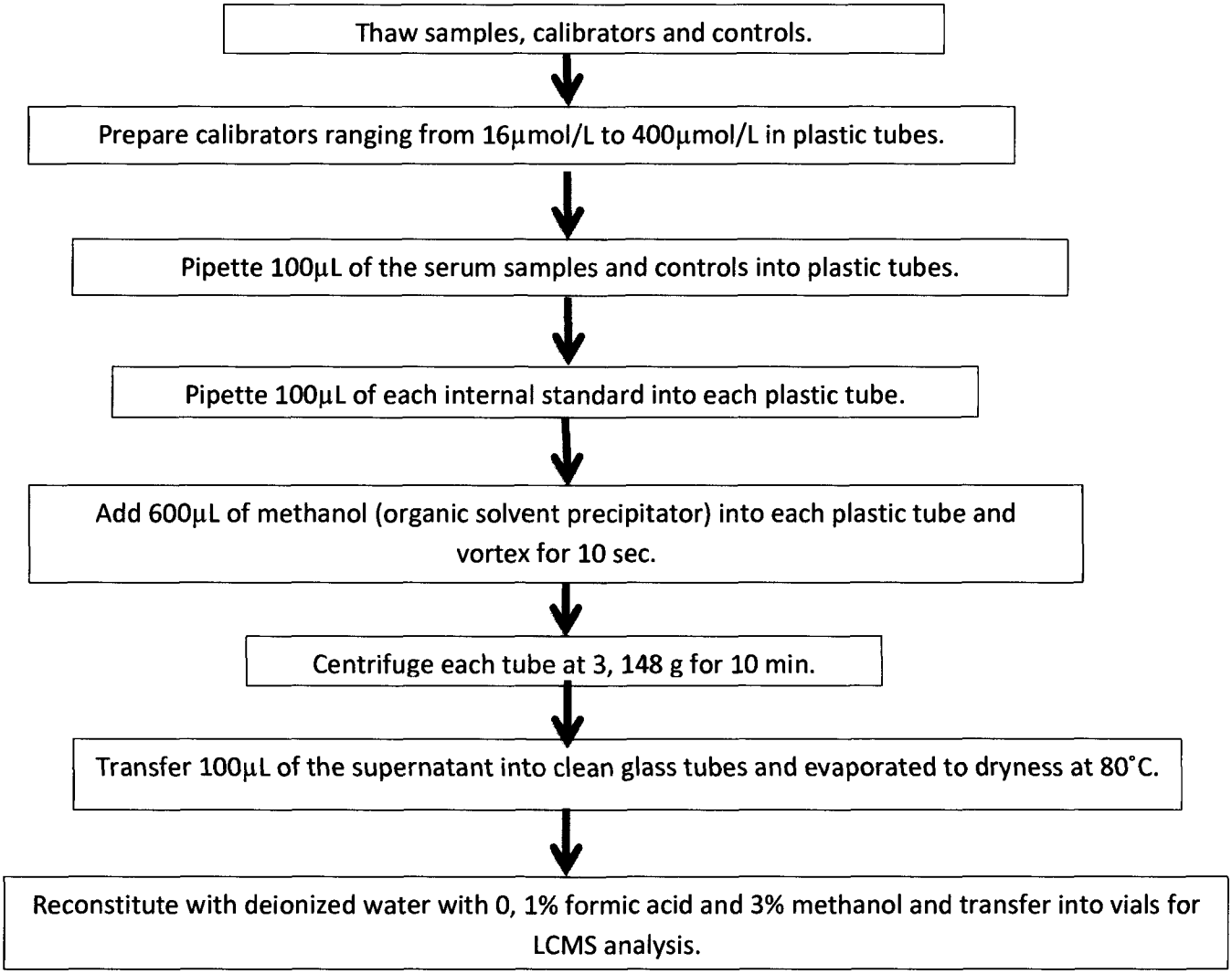

Figure 3.1 Flow diagram of the sample preparation method. A cocktail of internal standards and samples calibrators or plasma controls were pipetted into plastic tubes and precipitated with methanol. The supernatant after centrifugation was evaporated then reconstituted with deionized water with 0, 1% formic acid and 3% methanol.

3.2 LIQUID CHROMATOGRAPHY

Upon completion of sample extraction, the samples were further separated by liquid chromatography. Chromatography is defined as the process where a mixture is separated by passing it in solution or vapor through a medium that will elute compounds in different rates.

In chromatography, two phases are manipulated to separate compounds, namely, the stationary phase and the mobile phase. The mobile phase can either be in liquid or gas form depending on the chromatographic technique employed. The mobile phase carries the sample and together maneuver through the particles of the stationary phase. The stationary phase commonly known as the column in gas and liquid chromatography is the solid particle that is modified to enable specific separation of target analytes. Compounds with less affinity for the stationary phase will elute first while compounds with high affinity for the stationary phase will remain in the stationary phase much longer. The compounds eluted from the column are then detected and quantitation of the compounds is applied if necessary.

Separation of the compounds of interest can be achieved by using two phases, the normal phase (NP) and the reversed-phase (RP) separation. Normal phase separation is based on the polarity of the compounds and is commonly employed for the separation of polar compounds like amino acids. However, normal phase columns are commonly made from silica which is likely to absorb water. This can lead to inconsistent retention rates and incorrect quantitation. On the other hand, RP- separation is based on hydrophobic interactions of the compound with the stationary phase and favored over NP-separation because aqueous mobile phases can be used and gradient elusions are far less complicated compared to NP-separation. It is not likely

that non-derivatized amino acids are analyzed with reversed-phase separation due to their high polarity (Armstrong *et al.*, 2007). Amino acid liquid chromatography analytical methods are constantly improving, resulting in analytical methods such as hydrophilic-interaction chromatography (HILIC) and ion-pair liquid chromatography which both use the polar stationary phases in combination with reverse-phase separation. The limitation of HILIC and ion-pair chromatography is the inconsistent retention rates due to prolonged equilibration of reagents on the stationary phase (Kato *et al.*, 2009; Harder *et al.*, 2011). The choice of ion-pair reagent may affect analyte retention therefore careful planning should be done before analysis. Additionally, the use of strong acids and bases as ion-pair reagents may not be compatible with certain liquid chromatography instruments.

The present study modified and optimized the analytical method developed by Yang *et al.*(2014) which could rapidly and precisely measure serum BCAAs and AAAs. The method employed RP-separation and amino acids were not derivatized which shortened the sample preparation time. More on the analytical method optimization is given in section 4.4 below.

3.2.1 Ultra Performance Liquid Chromatography analysis

Studies have shown that ultra-performance liquid chromatography (UPLC) enhances performance and has increased sensitivity, selectivity and speed of analysis than HPLC (Zhou *et al.*, 2005). The reason for the improved sensitivity, selectivity and rapid rate of analysis is in the smaller columns designed to have smaller particles that can withstand higher pressures (Swartz, 2005).Polymeric columns are designed in such a way as to allow for high speed analysis without sacrificing analyte sensitivity and resolution. An example of such a column is the polymeric bridged ethane hybrid (BEH) column. The column can withstand pressures of

up to 15000 psi and wider pH range. Furthermore, UPLC was proven to give higher accuracy and reproducibility than HPLC (Bruce *et al.*, 2008; Chambers *et al.*, 2007; Swartz, 2005).

3.2.1.1 Ultra-performance Liquid Chromatography (UPLC) conditions

The BCAAs and AAAs were chromatically separated by the Acquity (Waters, Massachusetts, USA) Ultra-Performance Liquid Chromatography (UPLC) system with integrated auto-sampler, binary solvent manager, a column heater, and interfaced to a Waters Xevo triple quadruple mass spectrometer (TQ-MS) (Fig. 3.2). The Acquity (Waters, Massachusetts, USA) Reversed Phase UPLC BEH C18 column (1.7 µm, 2.1 x 100 mm) was used in this study.

Figure 3.2 Acquity UPLC system integrated with Xevo TQ-S-MS

The C8 or C18 columns are the most commonly used stationary phases for non-polar amino acid separation (Fig. 3.3) due to their carbon chains that can trap the non-polar part of the

amino acids. The C18 column has a longer carbon chain than the C8 column therefore it can trap more compounds than the C8 column, enhancing peak resolution.

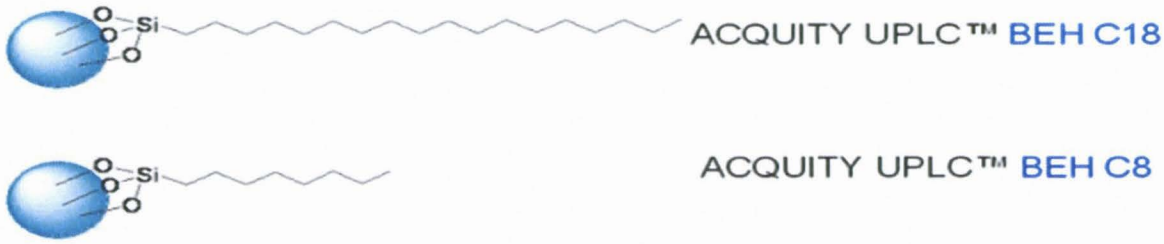

Figure.3.3 The difference between BEH C18 and BEH C8 column stationary phase. The BEHC18 has a longer carbon chain compared to the BEHC8.

Separation of the amino acid was achieved by gradient elution (Table 3.2). The run time was 5, 30 minutes per sample with a sample injection volume of 2, 5 µL.

Table 3.2 The mobile phase gradient elution for BCAAs and AAAs

Time (min)	Flow Rate (µL/min)	%A	%B
Initial	0.3	98	2
2.00	0.3	95	5
3.00	0.3	90	10
4.00	0.3	95	5
5.50	0.3	98	2

3.2.1.2 Preparation of Mobile phases and Wash solvents.

All mobile phases were changed every two days to avoid contamination and changes that might influence the chromatography. Separation of the amino acids was achieved by gradient elution on a binary pump. The Mobile phase A was made up of 99.99% deionized water with 0, 01% formic acid and the mobile phase B was 99.99% methanol with 0, 01% formic acid. The strong wash solvent was made up of 70% acetonitrile in deionized water with 0, 01%

formic acid and the weak wash was 10% acetonitrile in deionized water with 0, 01% formic acid.

3.3 AMINO ACID DETECTION AND QUANTITATION

The final process of amino acid analysis by using liquid chromatography involves the detection and quantitation of the target analytes. There are various HPLC detectors available that can be applied to achieve this. These include detectors that measure the sample's ability to absorb light (ultraviolet and photodiode array), the wavelength at which a compound emits light (fluorescent and light scattering detectors), the current resulting from any oxidation–reduction reaction coming from the compound (electrochemical potential detector), and mass spectrometry (MS) detectors that measure the mass of the compound.

The use of UV detection, fluorimetry and amperometry for amino acid analysis is only possible after derivatization. The efficiency of the detector is dependent on the reagent employed for derivatization. Derivatization with *o*-phthalaldehyde (OPA) can be performed post column and pre-column. OPA reacts with amino compounds in the presence of a thiol such as mercaptoethanol to form a fluorescent derivative (Joseph and Davies, 1983). OPA derivatives can be detected with UV, fluorimetry and amperometry. Derivatization with ninhydrin can also form fluorescent amino acid derivatives. Disadvantages with using UV and fluorescent detector are lack of specificity, derivatization process is time consuming and stability of the derivatives is not assured.

On the other hand, mass spectrometry has been showed as a highly sensitive and specific technique and recommended for amino acid analysis in a metabolomics review by Bain *et al.* (2009). Mass spectrometry can be integrated with various analyzers that assist with the

specificity of the instrument. The most commonly applied analyzers for amino acid analysis are tandem mass (MS/MS) and time-of-flight (TOFMS) spectrometers. The TOFMS analyzer as the name suggest, separates ions by their time of flight over a known distance. Ions with a smaller mass will travel faster and therefore will have a short time of flight. TOFMS is not capable of separating isobaric ions such as leucine and isoleucine however using a column with smaller solid phase particle, 1,8 μ and not 3,5 μ, can allow for the complete resolution of leucine and isoleucine (Armstrong *et al.*, 2007). The MS/MS uses quadruple mass filters that apply a charge on the ions and filter out the ions that are not of the specified mass. There are three quadruple cells within the MS/MS analyzer. The first chamber (MS1) is the first mass analyzer where ions can be scanned and filtered according to their mass to charge ratio (*m/z*). The isolated ion will enter into the collision chamber where the ions are fragmented by gas collision into daughter ions. The daughter ions will then go through the second mass analyzer (MS2) where further filtered according to their specified *m/z*.

3.3.1 Mass Spectrometry Conditions

The present study use the tandem mass spectrometer as the analyzer operated on electronic spray ionization positive mode (ESI+) using multiple reaction monitoring (MRM) mode. Table 3.3 shows the instrument parameters applied for BCAAs and AAAs analysis. The MRM ion transitions as well as the cone voltage and collision energy for each BCAA and AAA are indicated on Table 3.4.

Table 3.3 Instrumental parameters for the BCAAs, AAAs and their internal standards.

Voltages	Settings
Capillary (kV)	2.50
Cone (V)	70.00
Source offset (V)	50.00
Gas Flow	
Desolvation (L/hr)	800.00
Cone (L/hr)	150.00
Nebulizer (Bar)	7.00
Analyzer	
LM Resolution 1	3.08
LM Resolution 2	3.00
HM Resolution 1	15.19
HM Resolution 2	14.78
Ion Energy 1	0.40
Ion Energy 2	1.10
Collision gas flow (ml/min)	0.15
Collision	2.00
Temperature (°C)	
Desolvation	400.00
Column	30.00
Sample	10.00

Table 3.4 The Ion transitions and instrumental parameters for the triple quadruple detection in MRM mode.

Amino acid / internal standard	Ion Transition (m/z)	Dwell (s)	Cone voltage(v)	Collision energy(Ev)
Valine	118> 72.1	0.003	20	30
Methionine-D3	153> 107.1	0.003	2	10
Leucine	132.1> 43.1	0.003	22	36
Isoleucine	132.1> 69.2	0.003	15	32
Leucine-D3	135> 46.1	0.003	20	36
Tyrosine	182> 136	0.003	20	30
Phenylalanine	166> 120.1	0.003	20	40
Homo-phenylalanine	180> 91.1	0.003	5	22

3.3.2 Amino acid quantitation

The amino acids concentrations were quantified using calibration curve and internal standardization which was automatically calculated by the in-house MassLynx TargetLynx software program. The calibration curve consisted of a blank sample (sample extracted without internal standards) and a zero sample (sample extracted with internal standards). The blank sample was used to observe the intensity of the background noise; the background noise should not have a higher intensity than the actual samples to be quantified. Zero samples were used as the zero mark in the calibration curve. A calibration curve was plotted automatically using a least square regression analysis.

3.4 MATERIALS

All chemicals and reagents used in this study were analytical HPLC grade quality while BCAAs and AAAs standards were obtained from Industrial Analytical (Kyalami, R.S.A). The isotopic-labeled DL-Leucine-D3 internal standard with isotopic purity of 99.0% and DL-Leucine and DL-Isoleucine standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany), while methionine-D3 and homophenylalanine internal standards were purchased from Phenomenex (Torrance, U.S.A). Lyophilized plasma controls were purchased from Recipe (Munich, Germany). Sterilized 5mL glass tubes were purchased from Glassblowing Industries (Gauteng, R.S.A) while polypropylene 5mL tubes were purchased from Tools and Carbide Plastics (Gauteng, R.S.A). Light protected glass vials were purchased from Phenomenex (Torrance, U.S.A).

3.5 ANALYTICAL METHOD OPTIMIZATION

The present study modified and optimized the analytical method developed by Yang *et al.*(2014). The upper calibration point was increased to 400µmol/L from 300µmol/L because of the high concentration of valine quantitated in various studies (Newgard *et al*; 2009, Wurtz *et al*; 2013 and Yang *et al*; 2013) as mentioned above.

3.5.1 Sample preparation optimization

Protein precipitation is prone to matrix effects, mainly because other compounds either than protein are not precipitated, for instances phospholipids from serum samples may interfere with the amino acid analysis. The protein precipitator was changed from acetonitrile to methanol due to the enhanced sensitivity and peak resolution when using methanol. The sample to precipitator ration was reduced from 1:4 to 1:2. The reason for the modification is that the 1:4 sample: precipitator ratio had high interferences which resulted to low peak resolution. To improve the sensitivity and peak resolution of target analytes, the sample: precipitator ratio to 1:2 and samples were reconstituted with 100µL and not 200µL 0, 1% formic acid in deionized water recommended by Yang *et al.*(2014). As a result of the changes made, the peak resolution and analyte sensitivity was increased.

3.5.2 Leucine and Isoleucine differentiation.

Considering that leucine and isoleucine have the same molecular weight and a similar chemical structure, both amino acids are eluted at the same time. This indicates that the peak observed during analysis before optimization, is a representation of both LEU and ILE.

The present amino acid method was optimized for LEU and ILE analysis. Firstly, IntelliStart (in-house method development software program) was used to identify the optimal daughter fragments of LEU and ILE. The amino acids were analyzed separately using pure LEU and ILE standards (Dr. Ehrenstorfer). As expected, the daughter fragments of LEU and ILE were identical which indicates that the daughter fragments are not specific for LEU and ILE.

The peak intensity LEU and ILE MRM transitions 132 *m/z* > 43.1 and 132 *m/z* > 69.2 were compared against each other to identify the optimal MRM transition for each amino acid. Table 4.5 shows the MRM transition used for LEU and ILE analysis. Repeat analysis of LEU and ILE were performed for three consecutive days at concentrations of 46, 92, 138, 184, and 230µmol/L. To correct the peak height of each amino acid transition, the mean peak height ratio of 132 *m/z* > 43.1 to 132 *m/z* > 69.2 for LEU and 132 *m/z* > 69.2 to 132 *m/z* > 43.1 for ILE were calculated. For simplicity, peak height ratio for LEU and ILE will be abbreviated as LEU_{HR} and ILE_{HR}, respectively. The peak height ratios were used to correct the peak height of each amino acid by using the formula:

LEU corrected peak height = 132 *m/z* > 43.1 peak height – (LEU_{HR} x 132 *m/z* > 69.2 peak height)

ILE corrected peak height =132 *m/z* > 69.2 peak height – (ILE_{HR} x 132 *m/z* > 43.1 peak height)

3.5.2.1 Leucine and Isoleucine Peak Height Correction Results

The ion transition used for LEU and ILE were 132 > 43.1 and 132 > 69.2, respectively. This was chosen because the ion transitions had higher peak height response than the other (Figure 3.3). This was also observed in the study done by Yang *et al.*(2014). The peak height ratio

which was used for LEU (Table 3.5) and ILE (Table 3.6) peak height correction was 0.06 and 0.03, respectively.

Table 3.5 Leucine Peak Height Correction

Leucine Peak Height correction										
Concentration (µmoles/L)	**230**		**184**		**138**		**92**		**46**	
Daughter Fragment	69.2	43.1	69.2	43.1	69.2	43.1	69.2	43.1	69.2	43.2
Peak Height	1978	37586	1878	28763	1282	24706	1310	19332	710	10418
	2831	37444	1797	33330	2005	28136	1073	18235	621	11121
	2336	34750	1885	30311	1731	24610	1611	19654	613	9692
Peak Height Mean	2381.	36593	1853	30801	1672	25817	1331	19073	648	10410
Peak Height Ratio	**0.0650**		**0.060**		**0.064**		**0.069**		**0.062**	
Peak Height Ratio Mean	**0.06**									

Table 3.6 Isoleucine Peak Height Correction

Isoleucine Peak Height correction										
Concentration (µmoles/L)	**230**		**184**		**138**		**92**		**46**	
Daughter Fragment	69.2	43.1	69.2	43.1	69.2	43.1	69.2	43.1	69.2	43.1
Peak Height	17935	646	49040	1967	35092	1610	23132	703	0	0
	65628	1448	46625	2032	0	0	0	0	0	0
	58126	1414	46072	2737	35625	902	24684	1042	11508	515
Peak Height Mean	47229	1169	47245	2245	35358	1256	23908	872	11508	515
Peak Height Ratio	**0.024**		**0.0476**		**0.036**		**0.036**		**0.045**	
Peak Height Ratio Mean	**0.03**									

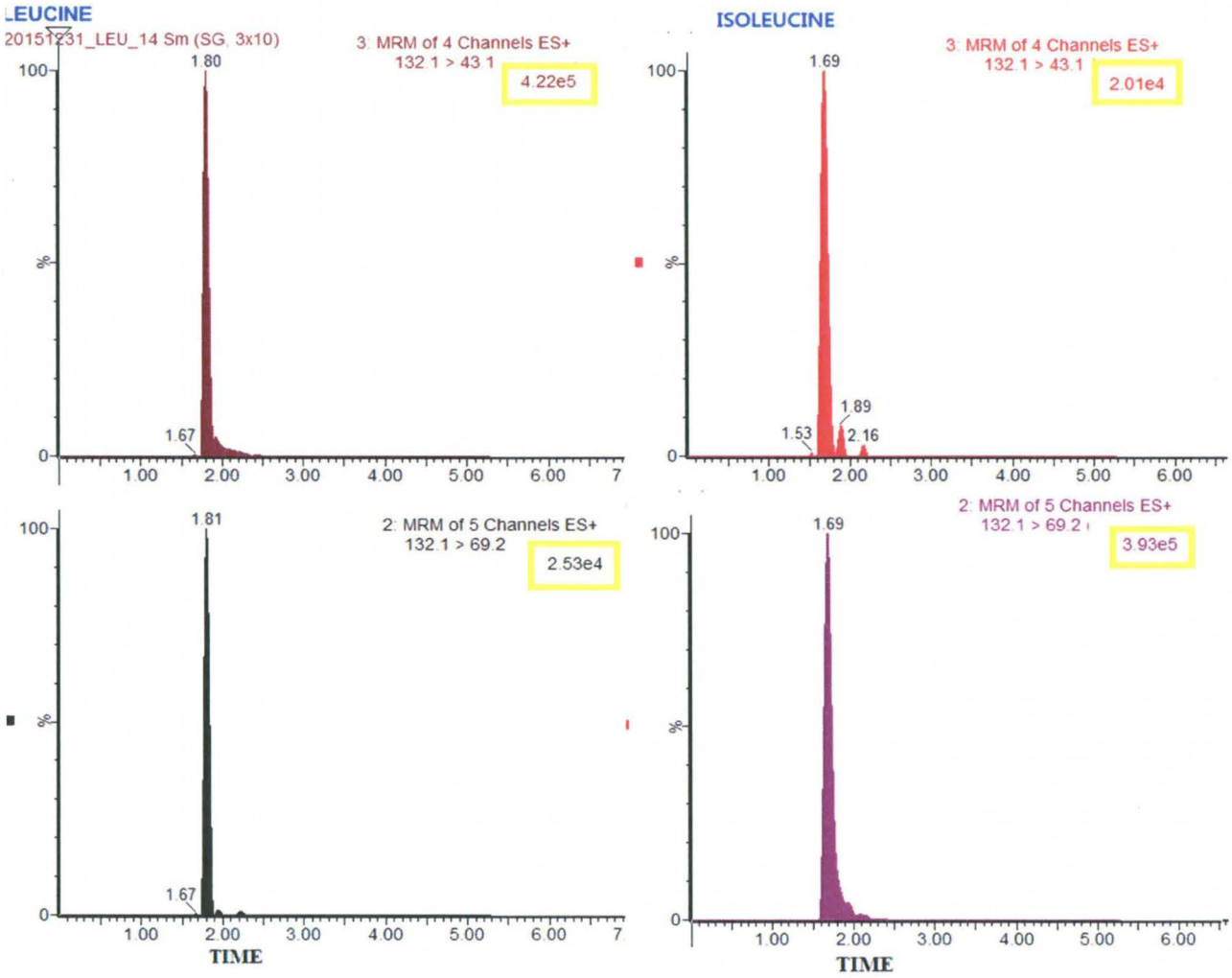

Figure 3.4 The ion transitions of leucine and isoleucine. Leucine has a higher intensity for the ion transition 132 > 43.1 than 132 > 69.2. Isoleucine on the other hand has a higher intensity for the ion transition 132 > 69.2 than 132 > 43.1.

3.5.3 Strategic sample listing.

The amino acid sample list was arranged in a way that after each run a blank solution was run. The blank solution was run with an inlet constructed to flush the column and thereafter re-stabilizing the pressure. This procedure was performed to prevent the column from blocking and to minimize foreign matter from building up inside the column. The same mobile phases were used to flush the column. Table 3.7 indicates the inlet used to flush the column after each

sample run. The plasma controls were run after every 10 samples to see if the method is continuously working efficiently.

Table 3.7 The mobile phase gradient elution for washing the column.

Time (s)	Flow (mL/min)	Mobile Phase A%	Mobile Phase B%
Initial	0.2	80	20
0.5	0.2	30	70
2.5	0.2	20	80
3.5	0.2	50	50
4.5	0.2	98	2

3.6 STATISTICAL ANALYSIS

The data was presented in median and interquartile range for skewed continuous variables, mean and ±standard deviation (SD) for normally distributed continuous variables, and percentage for categorical variables. We performed t-tests for normally distributed data and the Wilcoxon rank-sum test for skewed data when comparing data.

We also performed an ANOVA test with Bonferroni correction to compare the differences in amino acid levels in the three BMI categories (normal, overweight and obese) within each ethnic group.

Correlations between the amino acid levels and the cardiometabolic variables (fasting glucose, insulin, HOMA-IR, HDL-cholesterol, LDL-cholesterol, systolic and diastolic blood pressure, BMI, waist circumference, visceral and subcutaneous fat was determined using Pairwise correlation analysis.

Logistic regression analysis was performed to analyze the association of the total amino acids and then for each individual amino acid with the metabolic syndrome adjusted for age, gender and BMI.

We then carried out further logistic regression to investigate the associations of the total amino acids and individual amino acids with each metabolic syndrome component excluding waist circumference. In the model, age, gender and BMI were adjusted for. Waist circumference was excluded because of possibility of co-linearity with BMI. Metabolic syndrome variables were used as categorical variables using cut offs as defined by the harmonized definition of the metabolic syndrome (Alberti *et al.* 2009).

Statistical analysis was conducted using STATA 12 software (college Station, TX, USA).Statistical significance was defined as $p \leq 0.05$.

3.7 METABOLIC SYNDROME DEFINITION

Cardio metabolic abnormalities were defined according to the harmonized definition (Alberti *et al.* 2009) of the metabolic syndrome, with metabolic syndrome diagnosed in subjects with 3 or more of the following 5 cardio metabolic risk factors:

1. Fasting glucose ≥ 5.6mmol/L,
2. HDL-C < 1.0 mmol/L in men or < 1.3 mmol/L in women,
3. TG ≥ 1.7 mmol/L
4. Waist circumference ≥ 94cm for African males and ≥ 80cm for African women and ≥ 80cm for Asian Indian females and ≥ 90cm for Asian males.

5. Systolic blood pressure ≥ 130mmHg and/or diastolic blood pressure ≥ 80mmHg.

Incident diabetes was diagnosed based on the fasting serum glucose of ≥7.0 mmol/L while prevalent diabetes was based on past physician diagnosis with the subject currently receiving anti-diabetic therapy. Insulin resistance was determined using homeostatic model assessment of insulin resistance index (HOMA-IR) where HOMA-IR = (fasting insulin in μUI/mL X fasting glucose in mmol/L) / 22.5) (Matthews *et al.*, 1985).

CHAPTER 4: AMINO ACID ANALYSIS METHOD VALIDATION

Method validation is performed after method development to ensure that the method does what it was intended to do. The analytical method should be capable of producing accurate, reproducible, highly specific and sensitive results. Analytical method validation guidelines have been published invented to assist in making methods that are in compliance with the Food and Drug Administration regulations (CDER, 2002)

The present amino acid analytical method was validated as described in the Center for Drug Evaluation and Research (CDER) guidelines (CDER, 2002). Linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision, selectivity, specificity and robustness was evaluated.

All analysis was performed on amino acid standards together with internal standards at seven concentration levels (12, 30, 60, 120, 180, 240 and 300μmols/L). The amino acid standards were prepared and analyzed using the method above. All analysis was performed in triplicate for five consecutive days with newly prepared amino acid standards each time. In addition to the amino acid standard analysis, serum and plasma samples of five subjects were ran to compare the differences in amino acid concentration levels between plasma samples and serum sample.

4.1 LINEARITY

Linearity is the ability of the method to produce peak response (peak height or area) ratios that is directly proportional to analyte concentration within a given range (CDER, 2002). The range is the interval between the upper and lower concentration levels (inclusive) that have proven to be accurate and precise when using the analytical method to be tested. The

analytical method linearity is accepted when the regression co-efficient (R^2) is greater than 0.98.

Calibration curves were constructed using a blank sample, a zero sample and amino acid standards with a concentration range of 12-300nmols/L. A peak height response (*y-axis*) versus concentration (*x-axis*) plot was automatically constructed by TargetLynx in-house software program using a least square regression analysis. The linear regression between the peak height ratios and amino acid concentrations was used to draw a calibration curve.

4.1.1 Linearity of Valine

Valine peak height response ratios showed a linear relationship with a concentration range of 9-300µmol/L (Figure 4.1). The mean standard deviation of the slope and intercept for the calibration curves constructed on five consecutive days were 0.0004 and 0.0036, respectively. The mean regression co-efficient (R^2) was greater than 0.98 (Table 4.1).

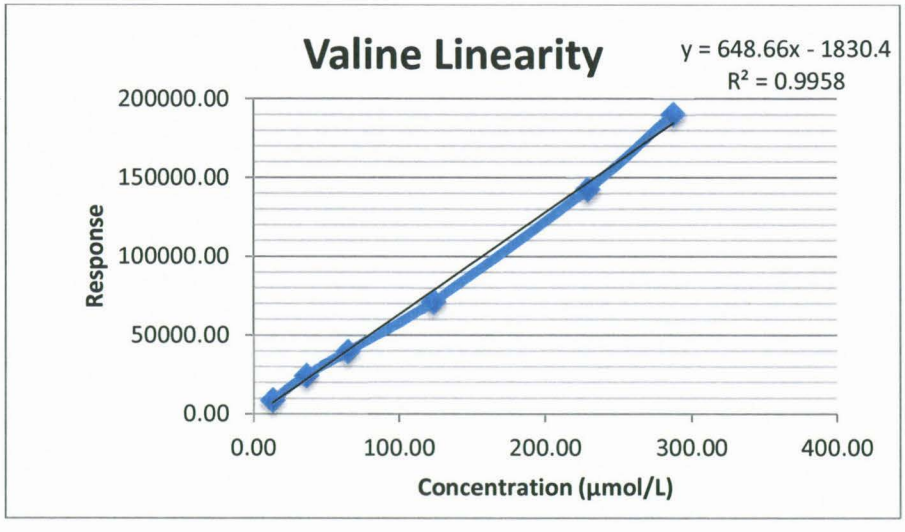

Figure 4.1 Calibration curve of Valine with regression co-efficient of > 0.99.

4.1.2 Linearity of Leucine

Leucine peak height response ratios showed a linear relationship with a concentration range of 9-300µmol/L (Figure 4.2). The mean standard deviation of the slope and intercept for the calibration curves constructed on five consecutive days were 119.88 and 8274, respectively. The mean regression co-efficient (R^2) was greater than 0.98 (Table 4.1).

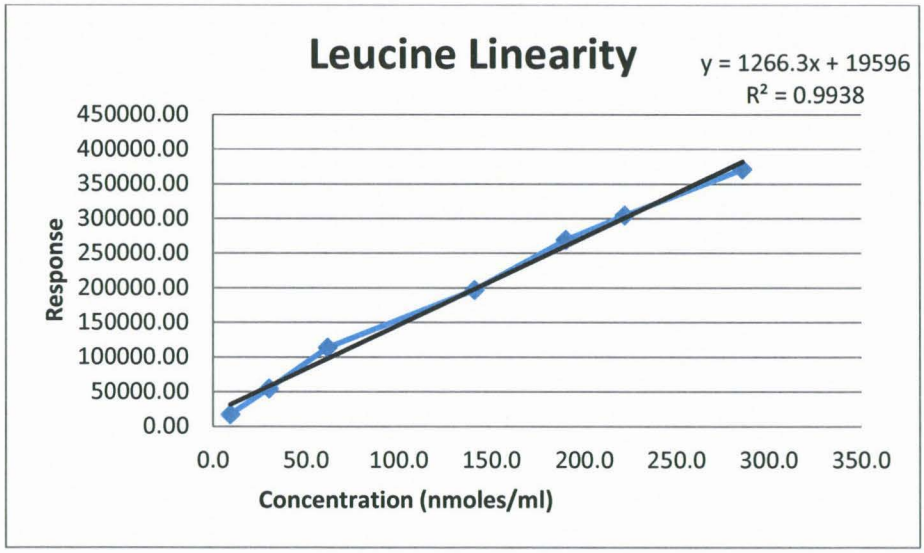

Figure 4.2 Calibration curve of Leucine with regression co-efficient of > 0.99

4.1.3 Linearity of Isoleucine

Isoleucine peak height response ratios showed a linear relationship with a concentration range of 9-300µmol/L (Figure 4.3). The mean standard deviation of the slope and intercept for the calibration curves constructed on five consecutive days were 141.82 and 6183, respectively. The mean regression co-efficient (R^2) was greater than 0.98 (Table 4.1).

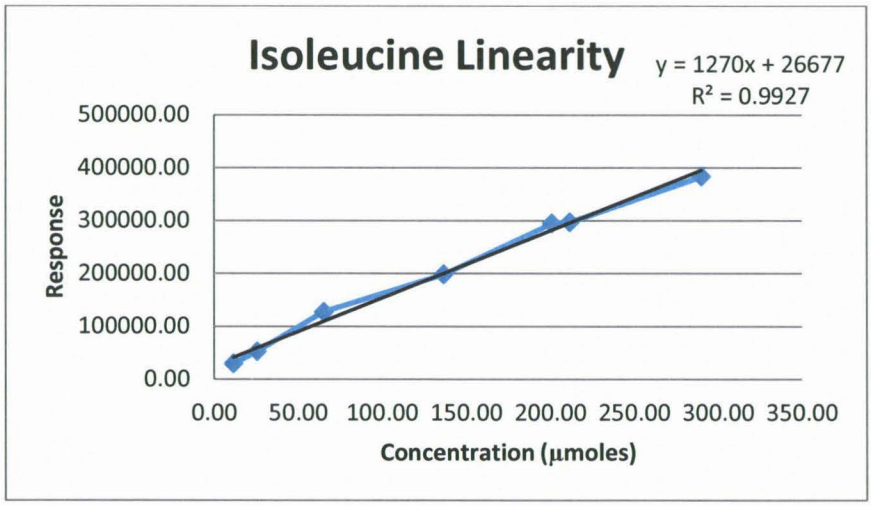

Figure 4.3 Calibration curve of Isoleucine with regression co-efficient of > 0.99.

4.1.4 Linearity of Tyrosine

Tyrosine peak height response ratios showed a linear relationship with a concentration range of 9-300µmol/L (Figure 4.4). The mean standard deviation of the slope and intercept for the calibration curves constructed on five consecutive days were 0.0005 and 0.0095, respectively. The mean regression co-efficient (R^2) was greater than 0.98 (Table 4.1).

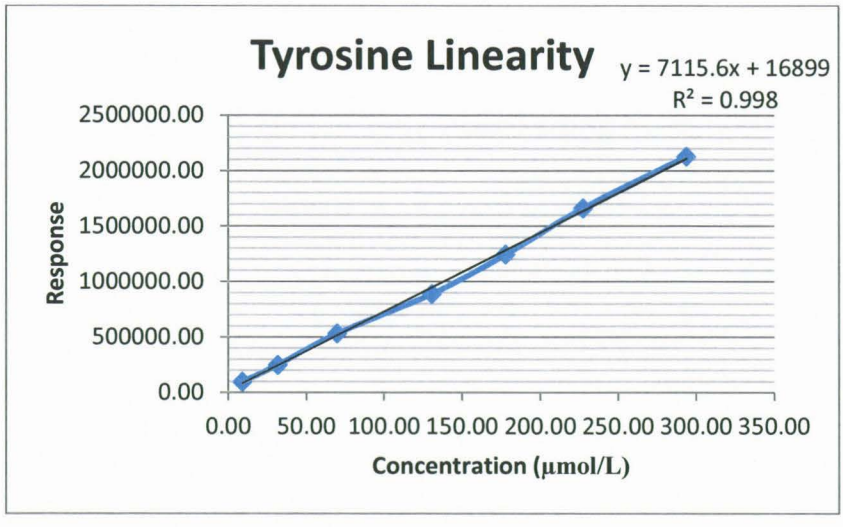

Figure 4.4 Calibration curve of Tyrosine with regression co-efficient of > 0.99.

4.1.5 Linearity of Phenylalanine

Phenylalanine peak height response ratios showed a linear relationship with a concentration range of 9-300µmol/L (Figure 4.5). The mean standard deviation of the slope and intercept for the calibration curves constructed on five consecutive days were 0.0001 and 0.0076, respectively. The mean regression co-efficient (R^2) was greater than 0.98 (Table 4.1).

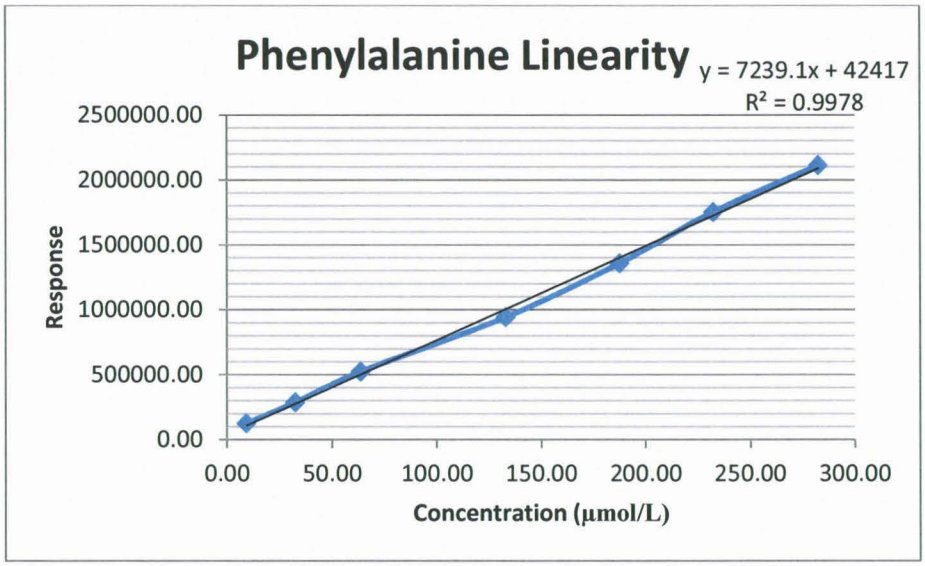

Figure 4.5 Calibration curve of Phenylalanine with regression co-efficient of > 0.99.

Table 4.1 The regression analysis of BCAAs and AAAs

Calibration Parameters	Valine		Tyrosine		Phenylalanine		Leucine*		Isoleucine*	
	Mean	±S.D. (*n*=5)	Mean	±S.D. (*n*=5)	Mean	±S.D. (*n*=5)	Mean	±S.D. (*n*=5)	Mean	±S.D. (*n*=5)
Slope	0.0028	0.0004	0.0035	00005	0.0030	0.0001	1293	119.8	1350.	141.82
Intercept	0.0085	0.0036	0.0227	0.0095	0.0428	0.0076	24882	8274	28895	6182
Regression co-efficient r^2	0.9801	0.0023	0.9867	0.0071	0.9826	0.0077	0.9873	0.01	0.987	0.01

*The amino acids calibration curves were constructed on Excel after peak height was corrected (see section 3.5.2 for more details on the peak height correction method)

4.2 DETECTION AND QUANTITATION LIMITS

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected whereas the limit of quantitation (LOD) is the lowest concentration of an analyte in sample that can be quantitated with acceptable precision and accuracy.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined from the lowest concentration peak to the background baseline noise (S/N) ratio defined by S/N > 3, S/N>10 respectively, which means the peak height of the LOQ and LOD is supposed to be at least 10x and 3x higher than the baseline noise, respectively.

The estimated LOQ for VAL, TYR, PHE, LEU and ILE are 11.07, 3.16, 3.71, 1.24 and 0.7μmoles/L, respectively based on the above criteria. The estimated LOD for VAL, TYR, PHE, LEU and ILE are 3.32, 0.95, 1.11, 0.37 and 0.32μmoles/L, respectively based on the criteria above (Table 4.2). The signal to noise was visually obtained from the chromatograms

Table 4.2 The estimated LODs and LOQs of BCAA and AAA determined from the signal to noise ratio.

AMINO ACID		Blank Signal	Lowest Std. Signal	S/N	True concentration (µmoles/L)	LOQ (µmoles/L)	LOD (µmoles/L)
VALINE	DAY 1	1389.67	18825.00	13.55	12	8.86	2.66
	DAY 2	1.00	25047.50	25047.50	12	0.00	0.00
	DAY 3	557.00	6631.33	11.91	12	10.08	3.02
	DAY 4	1023.33	20786.33	20.31	12	5.91	1.77
	DAY 5	3965.00	15599.67	3.93	12	30.50	9.15
					Mean LOQ (µmoles/L)		**11.07**
					Mean LOD (µmoles/L)		**3.32**
TYROSINE	DAY 1	3890.00	152537.33	39.21	12	3.06	0.92
	DAY 2	7126.00	151903.00	21.32	12	5.63	1.69
	DAY 3	15095.33	361113.33	23.92	12	5.02	1.50
	DAY 4	2297.33	132938.33	57.87	12	2.07	0.62
	DAY 5	1.00	99518.00	99518.00	12	0.00	0.00
					Mean LOQ (µmoles/L)		**3.16**
					Mean LOD (µmoles/L)		**0.95**
PHENYLALANI-NE	DAY 1	3726.33	131729.00	35.35	12	3.39	1.02
	DAY 2	1.00	58937.50	58937.50	12	0.00	0.00
	DAY 3	1609.00	21523.33	13.38	12	8.97	2.69
	DAY 4	960.00	18609.33	19.38	12	6.19	1.86
	DAY 5	1	14802.33	14802.33	12	0.01	0.00
					Mean LOQ (µmoles/L)		**3.71**
					Mean LOD (µmoles/L)		**1.11**
LEUCINE	DAY 1	1.00	31385.00	31385.00	12	0.00	0.00
	DAY 2	1.00	58937.50	58937.50	12	0.00	0.00
	DAY 3	1.00	21523.33	21523.33	12	0.00	0.00
	DAY 4	960.00	18609.33	19.38	12	6.19	1.86
	DAY 5	1.00	14802.33	14802.33	12	0.00	0.00
					Mean LOQ (µmoles/L)		**1.24**
					Mean LOD (µmoles/L)		**0.37**
ISOLEUCINE	DAY 1	712.67	15960.67	22.40	12	5.36	1.61
	DAY 2	1.00	57474.50	57474.50	12	0.00	0.00
	DAY 3	1.00	16794.67	16794.67	12	0.01	0.00
	DAY 4	1.00	22752.50	22752.50	12	0.01	0.00
	DAY 5	1.00	24364.00	24364.00	12	0.00	0.00
					Mean LOQ (µmoles/L)		**0.7**
					Mean LOD (µmoles/L)		**0.32**

4.3 RECOVERY

Recovery of an analyte describes the capability of the method to recover the analyte after extraction. Therefore, recovery is related to the methods extraction efficiency.

Recoveries of the analytes were calculated by comparing the true concentration of the analyte to the measured concentration of the analyte. The plasma controls, amino acid standards were run in triplicate. The recovery was calculated in percentage using the formula:

$$\%\ Recovery = \frac{Measured\ concentration}{True\ concentration} \times 100$$

All the amino acids and internal standards had recovery between 75 and 125% (Table 4.3). The internal standards recovery was evaluated in plasma controls because the samples to be analysed using the validated method have a similar matrix.

Table 4.3 Recovery results of BCAAs and AAAs along with their internal standards.

Amino Acid	Sample ID	True Conc. (µmol/L)	Measured Conc.(µmol/L)				Recovery (%)
Valine			RUN1	RUN2	RUN3	MEAN	
	Standard	12	15.9	8.3	15	13.07	108.89
	Standard	30	33.6	27.9	35.9	32.47	108.22
	Standard	60	68.5	77	61.1	68.87	114.78
	Standard	120	136.1	132.5	137	135.20	112.67
	Standard	180	169.5	165.8	171.5	168.93	93.85
	Standard	240	234.3	265.6	213.4	237.77	99.07
	Standard	300	311.9	288	269.5	289.80	96.60
	Plasma Ctrl I	212	193	213.3	188.3	198.20	93.49
	Plasma Ctrl II	402	311.6	385	349	348.53	86.70
Tyrosine	Standard	12	9	9.1	10.4	9.50	79.17
	Standard	30	33.1	31.5	34.6	33.07	110.22
	Standard	60	70.1	61.6	67.9	66.53	110.89
	Standard	120	129.3	133.3	117.9	126.83	105.69
	Standard	180	153.6	184.8	173	170.47	94.70
	Standard	240	261.1	240.1	241	247.40	103.08
	Standard	300	313.8	259.9	290.7	288.13	96.04
	Plasma Ctrl I	57.4	53.5	64.77	46.95	55.07	95.95
	Plasma Ctrl II	234	239.87	222.7	215.37	225.98	96.57
Phenylalanine	Standard	12	9.2	11.6	7.2	9.33	77.78
	Standard	30	34.7	35.2	28.6	32.83	109.44
	Standard	60	72.2	58.5	61.2	63.97	106.61
	Standard	120	126.7	129.9	142.9	133.17	110.97
	Standard	180	189.3	195.1	178.7	187.70	104.28
	Standard	240	219.1	238	240.2	232.43	96.85
	Standard	300	278.9	266.5	302.4	282.60	94.20
	Plasma Ctrl I	78.8	78.57	89.63	67.03	78.41	99.51
	Plasma Ctrl II	413	333.73	335.33	323.5	330.85	80.11
Leucine	Standard	12	12	7.2	12.8	10.67	88.89
	Standard	30	32.3	39.1	42.4	37.93	124.44
	Standard	60	68.2	66.5	60.8	65.17	108.61
	Standard	120	99.3	128.6	135.1	121.00	100.83
	Standard	180	161.3	157.8	181.3	166.80	92.67
	Standard	240	257.3	244.6	252.7	251.53	104.81
	Standard	300	308	291.7	268.1	289.27	96.42
	Plasma Ctrl I	187	176.23	169.83	172.3	172.79	92.40
	Plasma Ctrl II	304	260.7	279.63	265.23	268.52	88.33
Isoleucine	Standard	12	10.4	15.1	12	12.50	104.17
	Standard	30	23.1	31.2	31.1	28.47	94.89
	Standard	60	59.7	64.2	72.7	65.53	109.22
	Standard	120	118.3	111.5	108.5	112.77	93.97
	Standard	180	177.6	206	170.6	184.73	102.63
	Standard	240	237.7	255.7	223.4	238.93	99.56
	Standard	300	307.8	291.5	294.7	298.00	99.33
	Plasma Ctrl I	68.3	72.07	66.97	66.53	68.52	100.33
	Plasma Ctrl II	116	123.53	114.8	103.2	113.84	98.14
Homo-phenylalanine	Plasma Ctrl I	50	52.0	47.5	50.1	49.85	99.69
	Plasma Ctrl II	50	47.0	46.3	42.7	45.33	90.67
Methionine-D3	Plasma Ctrl I	50	36.5	37.5	38.2	37.41	74.82
	Plasma Ctrl II	50	35.2	38.5	39.1	37.60	75.20
Leucine-D3	Plasma Ctrl I	100	154.9	102.9	96.8	118.19	118.19
	Plasma Ctrl II	100	93.8	86.2	85.0	88.34	88.34

4.4 ACCURACY AND PRECISION

4.4.1 Accuracy

Accuracy of an analytical method is described as the closeness of the mean concentration obtained by the method to the true concentration of the analyte.

The accuracy of the analytical method was evaluated by triplicate analysis of amino acid standards and plasma control samples containing know amounts of valine, tyrosine, phenylalanine, leucine and isoleucine. Accuracy was calculated as the percent deviation between the true concentration and the mean calculated concentration using the formula:

$$\%\ Recovery = \frac{Measured\ concentration}{True\ concentration} \times 100$$

$$\%\ Accuracy = 100\% - Recovery$$

The mean value should be within 15% of the actual value except at LOQ, where it should not deviate by more than 20% for the method to be declared accurate.

4.4.2 Precision

Precision of analytical method is described as the closeness of individual concentrations of analytes in a sample that is repeatedly analyzed under the same conditions. This test is performed to see if the analytical method is reproducible.

Two types of precision tests were performed, namely, intra-day and inter-day precision. Intra-day precision tests for the repeatability of the analytical method to be used within the lab at short period of time. Inter-day precisions tests for intermediate repeatability of the analytical method therefore is assessed in a course of minimum of three days.

Intra-day precision data was from a single batch run in triplicate on a single day and inter-day precision was evaluated from a single batch run in triplicate on five consecutive days. Five subject serum samples were analyzed in triplicate to test for intra-day precision of the amino acid method when serum samples are analyzed. The relative standard deviation (RSD) and coefficient of variation (CV) was used to determine the method precision. The precision determined at each concentration level should not exceed 15% of the CV except for the LOQ, where it should not exceed 20% of the CV for the method to be declared precise. The RSD should be ≤ 1 % where RSD and CV were calculated using the formula:

$$RSD = \frac{Standard\ Deviation\ (SD)}{Mean}$$

$$\%\ CV = \frac{Standard\ Deviation\ (SD)}{Mean} \times 100$$

4.4.3 Accuracy and Precision results

Accuracy and precision calculations of the inter-day run are given in Table 4.4 and intra-day run in Table 4.5.The inter-day accuracy for valine, tyrosine, leucine and isoleucine ranged from -13.64 to 18, 67%. However, tyrosine's lowest concentration level had the accuracy of 21.84 and phenylalanine's lowest and third concentration levels were of 29.17 and 23.46, respectively. The intra-day accuracy for all five amino acids ranged from -14.78 to 20, 3%. However, phenylalanine's lowest concentration level had the accuracy of 22.22% and leucine second concentration level was -26.44%. The inter-day precision (CV) of all five amino acids were < 16%, respectively and intra-day precision were <20%, respectively. However, phenylalanine, valine and leucine lowest concentration level intra-day precision were 24, 28 and 32%, respectively. These results are not clinically relevant. The slightly poor accuracy

estimated in the lowest concentrations of leucine, phenylalanine and valine do not badly affect the methods ability to quantify the fore mentioned amino acids.

Table 4.4 The analytical method inter-day accuracy and precision calculations of BCAAs and AAAs

Amino Acid	True Conc. (µmol/L)	Mean measured Conc.(µmol/L)	Accuracy (%)	SD	RSD	%CV
Valine						
	12	9.76	18.67	1.53	0.16	15.70
	30	32.62	-8.72	3.25	0.10	9.97
	60	66.29	-10.48	2.46	0.04	3.71
	120	126.67	-5.56	10.38	0.08	8.19
	180	183.49	-1.94	9.07	0.05	4.95
	240	245.92	-2.47	21.09	0.09	8.58
	300	269.67	10.11	11.66	0.04	4.32
Tyrosine						
	12	9.42	21.48	0.52	0.05	5.48
	30	33.21	-10.69	1.46	0.04	4.41
	60	68.18	-13.64	2.22	0.03	3.25
	120	127.43	-6.19	8.58	0.07	6.73
	180	186.79	-3.77	4.61	0.02	2.47
	240	231.32	3.62	1.97	0.01	0.85
	300	285.53	4.82	14.00	0.05	4.90
Phenylalanine						
	12	8.50	29.17	0.47	0.06	5.55
	30	32.46	-8.20	0.93	0.03	2.85
	60	74.08	-23.46	2.82	0.04	3.80
	120	131.19	-9.32	2.87	0.02	2.18
	180	186.39	-3.55	11.30	0.06	6.06
	240	230.36	4.02	4.27	0.02	1.85
	300	277.13	7.62	9.60	0.03	3.47
Leucine						
	12	11.34	5.46	1.73	0.15	15.24
	30	31.69	-5.64	2.54	0.08	8.01
	60	64.04	-6.73	6.18	0.10	9.66
	120	132.19	-10.16	11.83	0.09	8.95
	180	192.68	-7.04	12.52	0.06	6.50
	240	239.20	0.33	10.24	0.04	4.28
	300	278.82	7.06	13.21	0.05	4.74
Isoleucine						
	12	11.03	8.11	1.22	0.11	11.02
	30	28.97	3.44	2.47	0.09	8.53
	60	65.86	-9.77	3.24	0.05	4.92
	120	128.59	-7.16	8.06	0.06	6.27

	180	185.11	-2.84	12.13	0.07	6.55
	240	237.55	1.02	16.56	0.07	6.97
	300	285.59	4.80	12.45	0.04	4.36

Table 4.5 The analytical method intra-day accuracy and precision calculations of BCAAs and AAAs

Amino Acid	True Conc. (µmol/L)	Measured Conc. (µmol/L)				Accuracy (%)	SD	RSD	CV (%)
		RUN 1	RUN2	RUN3	Mean				
Valine	12	15.9	8.3	15	13.07	-8.89	4.15	0.32	31.78
	30	33.6	27.9	35.9	32.47	-8.22	4.12	0.13	12.69
	60	68.5	77	61.1	68.87	-14.78	7.96	0.12	11.55
	120	136.1	132.5	137	135.20	-12.67	2.38	0.02	1.76
	180	169.5	165.8	171.5	168.93	6.15	2.89	0.02	1.71
	240	234.3	265.6	213.4	237.77	0.93	26.27	0.11	11.05
	300	311.9	288	269.5	289.80	3.40	21.26	0.07	7.34
Plasma Control I	212.0	193.3	213.3	188.83	198.5	6.38	13.03	0.07	6.57
Plasma Control II	402.0	311.6	385	349.03	348.5	13.30	36.70	0.11	10.53
Tyrosine									
	12	9	9.1	10.4	9.5	20.30	0.78	0.08	8.22
	30	33.1	31.5	34.6	33.1	-10.22	1.55	0.05	4.69
	60	70.1	61.6	67.9	66.5	-10.89	4.41	0.07	6.63
	120	129.3	133.3	117.9	126.8	-5.69	7.99	0.06	6.30
	180	153.6	184.8	173	170.5	5.30	15.75	0.09	9.24
	240	261.1	240.1	241	247.4	-3.08	11.87	0.05	4.80
	300	313.8	259.9	290.7	288.1	3.96	27.04	0.09	9.39
Plasma Control I	57.4	53.5	64.77	46.95	55.1	4.05	9.01	0.16	16.37
Plasma Control II	234	239.87	222.7	215.37	226.0	3.43	12.58	0.06	5.56
Phenylalanine									
	12	9.2	11.6	7.2	9.3	22.22	2.20	0.24	23.60
	30	34.7	35.2	28.6	32.8	-9.44	3.67	0.11	11.19
	60	72.2	58.5	61.2	64.0	-6.61	7.26	0.11	11.34
	120	126.7	129.9	142.9	133.2	-10.97	8.58	0.06	6.44
	180	189.3	195.1	178.7	187.7	-4.28	8.32	0.04	4.43
	240	219.1	238	240.2	232.4	3.15	11.60	0.05	4.99
	300	278.9	266.5	302.4	282.6	5.80	18.23	0.06	6.45
Plasma Control I	78.8	78.57	89.63	67.03	78.4	0.49	11.30	0.14	14.41
Plasma Control II	413.0	337.73	335.33	323.5	332.2	19.57	7.62	0.02	2.29
Leucine									
	12	12	7.2	12.8	10.7	11.11	3.03	0.28	28.39
	30	32.3	39.1	42.4	37.9	-26.44	5.15	0.14	13.58
	60	68.2	66.5	60.8	65.2	-8.61	3.88	0.06	5.95
	120	99.3	128.6	135.1	121.0	-0.83	19.07	0.16	15.76
	180	161.3	157.8	181.3	166.8	7.33	22.68	0.08	7.60
	240	257.3	244.6	252.7	251.5	-4.81	6.43	0.03	2.56
	300	308	291.7	268.1	289.3	3.58	20.06	0.07	6.94
Plasma Control I	187.0	176.23	169.83	172.3	172.8	7.60	3.23	0.02	1.87
Plasma Control II	304.0	260.7	279.63	265.23	268.5	11.67	9.88	0.04	3.68
Isoleucine									
	12	10.4	15.1	12	12.5	-4.17	2.39	0.19	19.12
	30	23.1	31.2	31.1	28.5	5.11	4.65	0.16	16.33

	60	59.7	64.2	72.7	65.5	-9.22	6.60	0.10	10.07
	120	118.3	111.5	108.5	112.8	6.03	5.02	0.04	4.45
	180	177.6	206	170.6	184.7	-2.63	18.75	0.10	10.15
	240	237.7	255.7	223.4	238.9	0.44	16.19	0.07	6.77
	300	307.8	291.5	294.7	298.0	0.67	8.64	0.03	2.90
Plasma Control I	68.3	72.07	66.97	66.53	68.5	-0.33	3.08	0.04	4.49
Plasma Control II	116.0	123.53	114.8	103.2	113.8	1.86	10.20	0.09	8.96

4.5 SELECTIVITY AND SPECIFICITY

An analytical method is declared a selective and specific method if the method is capable of correct quantification of analyte in the presence of other components in the sample.

The selectivity and specificity of the amino acid method was evaluated by comparing the peak resolution of the analyzed plasma control samples to the resolution of all other sample components.

Figure 4.6 and 4.7 shows the LC-MS/MS chromatogram of the BCAAs and AAAs at the lowest and highest calibration concentration level, along with their internal standards. The peak resolution of the analytes is very high and the background noise resolution is very low at both concentrations which indicate that the analytical method is very sensitive to the BCAAs, AAAs and their internal standards. Additionally, the analytical method is very specific to the BCAAs, AAAs and their internal standards because the multi-reaction mode (MRM) was applied for the analyte analysis.

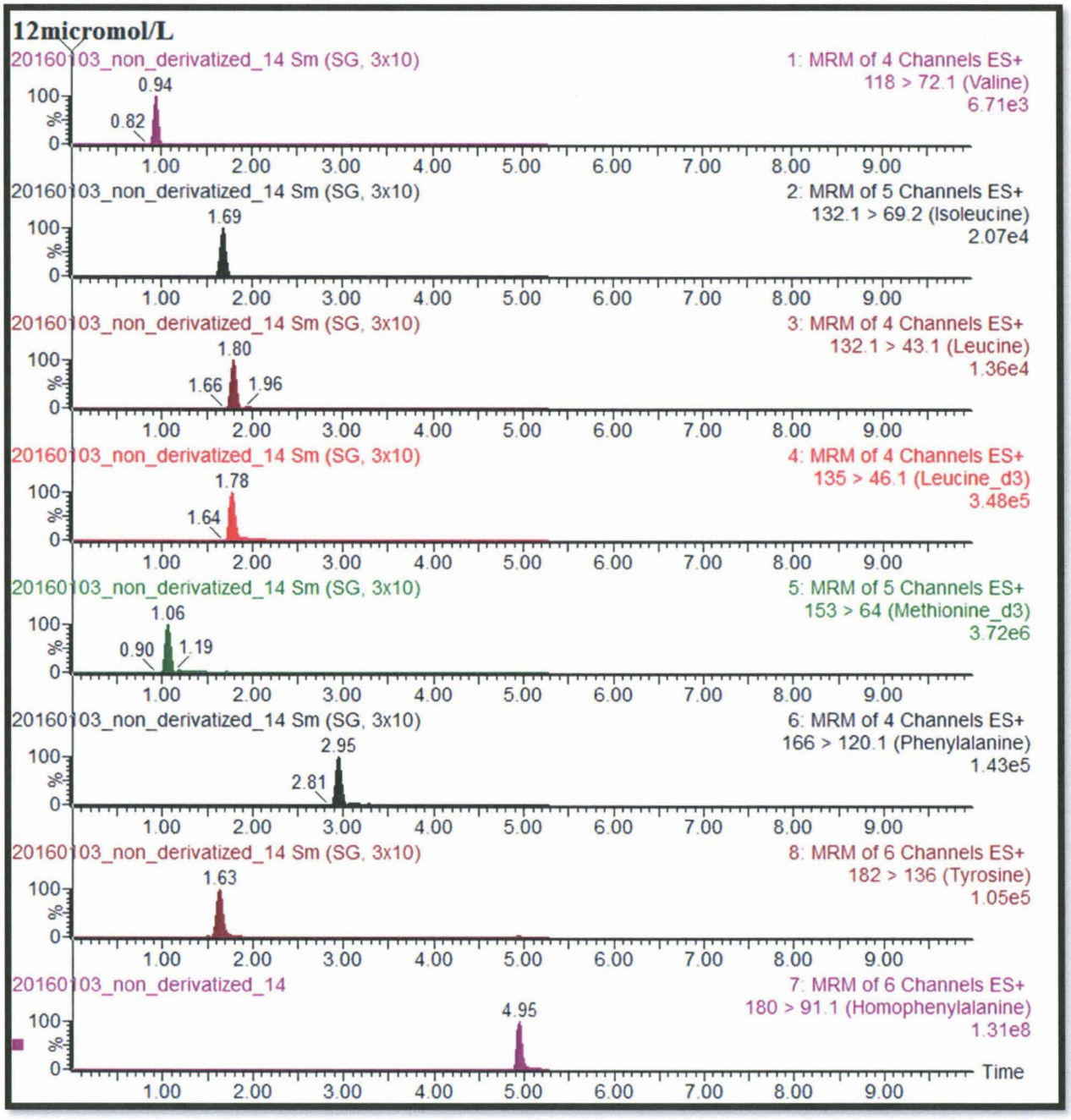

Figure 4.6 LC-MS/MS chromatograms of the BCAAs and AAAs along with their internal standards at 12µmol/L

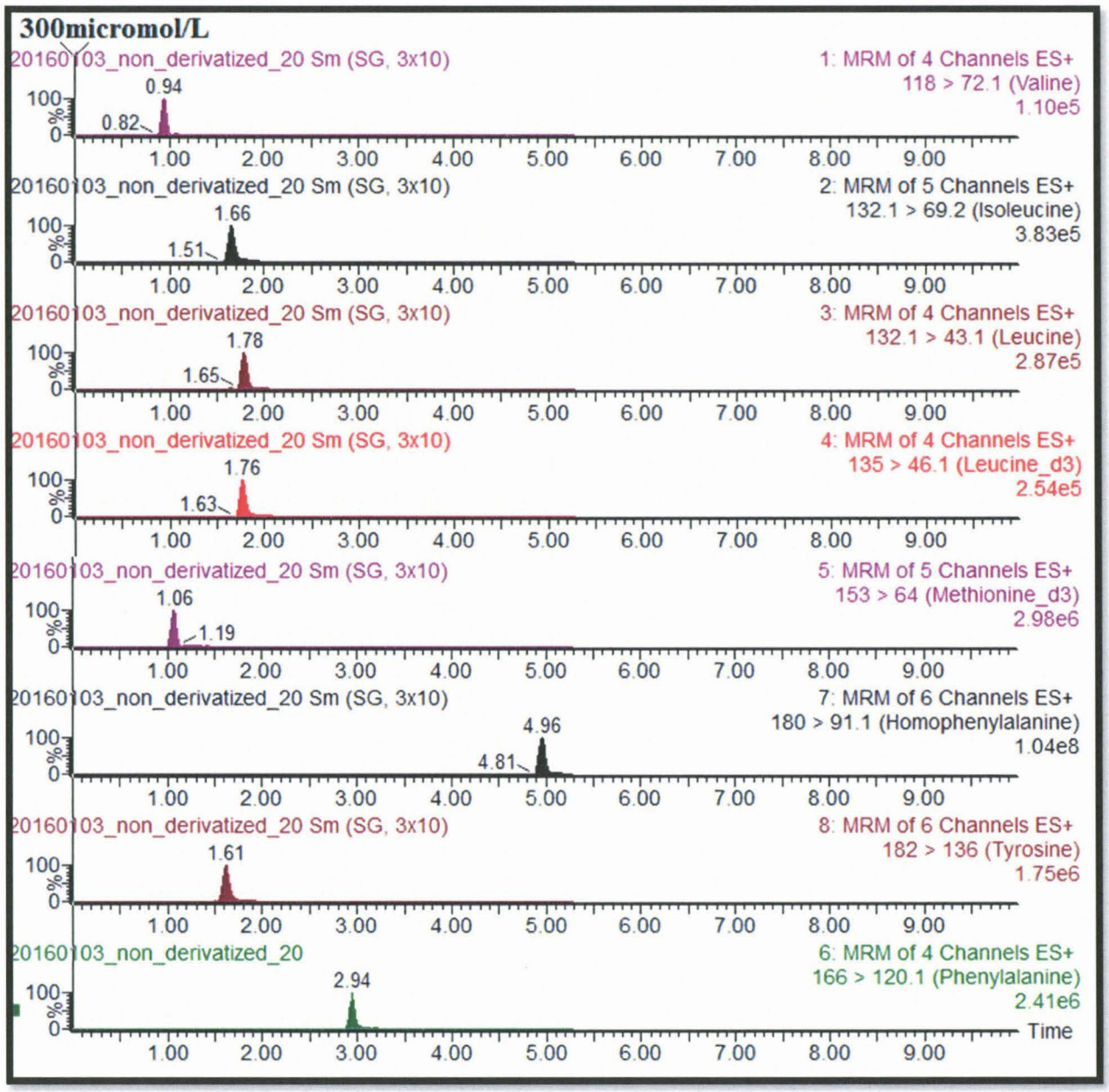

Figure 4.7 LC-MS/MS chromatograms of the BCAAs and AAAs along with their internal standards at 300µmol/L

4.6 PLASMA AND SERUM COMPARISON

The major differences between plasma and serum blood samples are the clotting factors. Plasma samples consist of clotting factors such as fibrinogen whereas serum is the plasma sample without fibrinogen. Plasma and serum samples were analyzed to identify amino acid level differences between plasma and serum samples that could have arisen from extraction of the two types of samples from whole blood.

Serum and plasma samples from five healthy subjects were collected into vacutainer blood collection tubes, the tubes for which plasma was to be extracted were the EDTA-treated tubes. The samples from these particular subjects were not part of the present study's aim and objectives investigation. The serum and plasma samples were immediately analyzed in duplicate using the present analytical method. The mean of the amino acid concentration was calculated for both serum and plasma samples. The standard deviation of plasma and serum samples was calculated.

All of the amino acids analyzed had standard deviation ranging from 0.07 to 14.4 (Table 4.6). This is an indication that differences in amino acid concentrations in plasma and serum are negligible.

Table 4.6 Differences in amino acid concentration of plasma and serum

AMINO ACID	SUBJECT	SAMPLE CONC. (μmol/L)		SD (plasma and serum)
		PLASMA	SERUM	
VALINE	1	29.15	28.25	0.64
	2	25.05	22.35	1.91
	3	25.40	21.70	2.62
	4	18.65	22.95	3.04
	5	21.90	20.15	1.24
TYROSINE	1	134.00	126.50	5.30
	2	100.60	120.45	14.04
	3	164.90	167.20	1.63
	4	213.60	221.55	5.62
	5	160.05	169.70	6.82
PHENYLALANINE	1	42.55	42.80	0.18
	2	30.90	33.95	2.16
	3	45.30	40.20	3.61
	4	30.25	42.05	8.34
	5	31.95	31.20	0.53
LEUCINE	1	51.03	46.63	3.11
	2	22.53	21.00	1.08
	3	32.73	26.30	4.55
	4	24.93	29.93	3.54
	5	10.97	10.43	0.38
ISOLEUCINE	1	100.45	100.35	0.07
	2	61.00	79.20	12.87
	3	43.95	51.55	5.37
	4	52.15	61.60	6.68
	5	43.55	37.25	4.45

CHAPTER 5: RESULTS

5.1 BASELINE CHARACTERISTICS OF PARTICIPANTS

Data is presented for 718 participants for whom we have complete data. Of the 718 participants 51% were black Africans (n=369) and 49% were Asian Indians (n=349). The mean age of the study population was 43 (±13) years. The median BMI in black Africans was 26.2 (21.7, 31.7) kg/m^2 and 26.7 (23.3, 31.0) kg/m^2 for Asian Indians.

Asian Indians had significantly higher fasting glucose, fasting insulin, HOMA-IR measurement, triglycerides, LDL-cholesterol, waist circumference and subcutaneous fat mean (p= <0.0001, for all) than the black Africans. However, black Africans had significantly higher systolic (p<0.0001) and diastolic blood pressure (p<0.0001) than the Asian Indians. There was no significant difference in smokers when stratified by ethnicity. Furthermore, there was no significant difference in BMI, HDL-cholesterol and mean visceral fat when the two ethnic groups were compared (Table 5.1)

Table 5.1 Baseline characteristics of Black Africans and Asian Indians

Baseline Characteristics	Ethnicity		*p*-values for ethnic differences
	Black African *n*=369	Asian Indians *n*=349	
Age (years) Mean ±SD	42±13.1	43±12.95	0.029
Fasting glucose (mmol/L)	4.9 (4.6, 5.4)	5.2 (4.8, 5.7)	<0.0001
Fasting insulin (mU/L)	7.77 (4.88, 12.2)	13.7 (9.24, 20.4)	<0.0001
HOMA-IR	1.83 (1.10, 2.90)	3.23 (2.13, 5.01)	<0.0001
Triglycerides (mmol/L)	0.85 (0.62, 1.27)	1.26 (0.83, 1.85)	<0.0001
HDL-cholesterol (mmol/L)	1.28 (1.08, 1.53)	1.26(1.07, 1.53)	0.8096
LDL-cholesterol(mmol/L)	2.30 (1.73, 2.92)	2.91 (2.40, 3.50)	<0.0001
Systolic BP (mmHg)	131 (120, 146)	123 (113, 135)	<0.0001
Diastolic BP (mmHg)	83.0 (76, 93)	80.0 (73, 87)	<0.0001
BMI(kg/m^2)	26.2 (21.7, 31.7)	26.7 (23.3, 31)	0.387
Waist circumference(cm)	89 (78, 102)	95 (85, 106)	0.0001
Smoker *n* (%)	102 (27.72)	80 (23.32)	0.1801
Visceral fat (cm) Mean ±SD	5.04 (±1.83)	4.95 (±2.01)	0.7383
Subcutaneous fat (cm) Mean ±SD	2.61 (±1.31)	3.19 (±1.29)	<0.0001

Data are presented in median (interquartile range), *n* (%) unless otherwise stated

The total amino acid levels were significantly higher in Asian Indians (23.0±1.40) when compared with black Africans (22.6±1.35), p=0.0001. The amino acids responsible for the significant difference were leucine (p=0.0232), isoleucine (p<0.0001) and tyrosine (p<0.0001) (Table 5.2). Valine and phenylalanine were not significantly different amongst the two ethnic groups.

Table 5.2 Fasting amino acid levels according ethnicity

Amino Acids (µmol/L)	Ethnicity		*p*-values for ethnic differences
	Black African *n*=369	Asian Indians *n*=349	
Total Amino Acids	506 (441, 615)	538 (459, 675)	0.0038
Valine	154 (116, 206)	160 (117, 224)	0.1352
Leucine	133 (109, 168)	148 (111, 186)	0.0245
Isoleucine	52.1 (40.7, 70.9)	60.0 (44.8, 80.4)	<0.0001
Phenylalanine	83.3 (68.0, 103)	81.6 (67.1, 98.7)	0.2097
Tyrosine	74.4 (58.9, 94.8)	84.9 (67.1, 105.7)	<0.0001

Data are presented in median (interquartile range), *n* (%) unless otherwise stated

5.2 AMINO ACID LEVELS BY METABOLIC SYNDROME

We compared total amino acid levels and individual amino acid levels in participants with the metabolic syndrome and those without the metabolic syndrome for both ethnic groups. There was no significant difference in the total amino acid levels between participants with the metabolic syndrome (22.8±0.13) and those without the metabolic syndrome (22.6±0.08) in black Africans (p=0.25) (Figure 5.1). The total amino acid levels among Asian Indians with the metabolic syndrome (23.4±0.10) were significantly higher than those without the metabolic syndrome (22.7±0.10) (p<0.0001) (Table 5.3). All the individual amino acids levels in Asian Indians with the metabolic syndrome were significantly higher than those without the metabolic syndrome (Table 5.3 and Fig. 5.1).

Table 5.3 Amino acid levels according to ethnic group and metabolic syndrome status

Amino Acid (µmol/L)	Black Africans (n=364)			Asian Indians (n=349)		
	Without Metabolic syndrome n=257	With Metabolic syndrome n=107	p-values	Without Metabolic syndrome n=189	With Metabolic syndrome n=160	p-values
Total Amino Acids	505 (434, 606)	524 (455, 647)	0.1294	516 (434, 615)	592 (497, 724)	<0.0001
Valine	154 (114, 202)	147 (110, 198)	0.4738	147 (110, 279)	174 (131, 244)	0.0008
Leucine	129 (107, 167)	137 (102, 181)	0.1895	137 (102, 181)	162 (119, 192)	0.0014
Isoleucine	52.3 (39.7, 64.4)	56.4 (42.5, 71.3)	0.9843	56.4 (42.5, 71.3)	69.9 (51.3, 86.4)	0.0001
Tyrosine	72.8 (59.0, 94.9)	76.6 (58.3, 94.7)	0.7093	82.2 (63.6, 100)	89.3 (70.2, 113)	0.0011
Phenylalan -ine	81.4 (67.2, 102)	90.5 (69.2, 113)	0.0775	79.7 (66.4, 94.8)	83.6 (69.4, 101)	0.0418

Data are presented in median and (interquartile range)

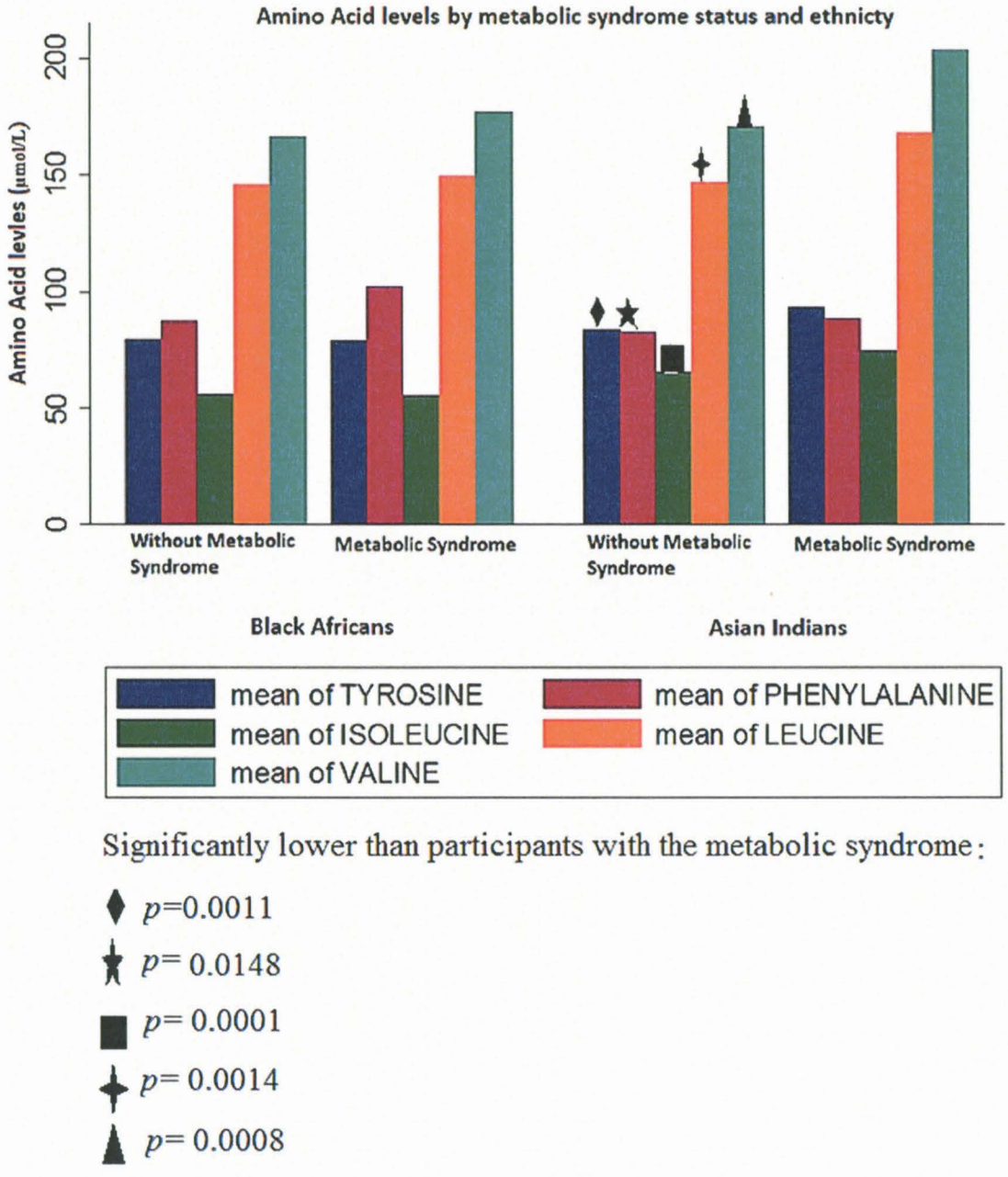

Figure 5.1 Comparisons of amino acid levels with metabolic syndrome by ethnicity. All individual amino acids were significantly lower in Asian Indians without the metabolic syndrome than those with the metabolic syndrome.

We compared total amino acid levels by the number of metabolic syndrome components. There was no significant difference between those with one or two component of the

metabolic syndrome compared to those with no components of the metabolic syndrome in black Africans (Figure 5.2). In Asian Indians, the total amino acid levels of individuals with no components were significantly lower than those with two or three components (p=0.042).

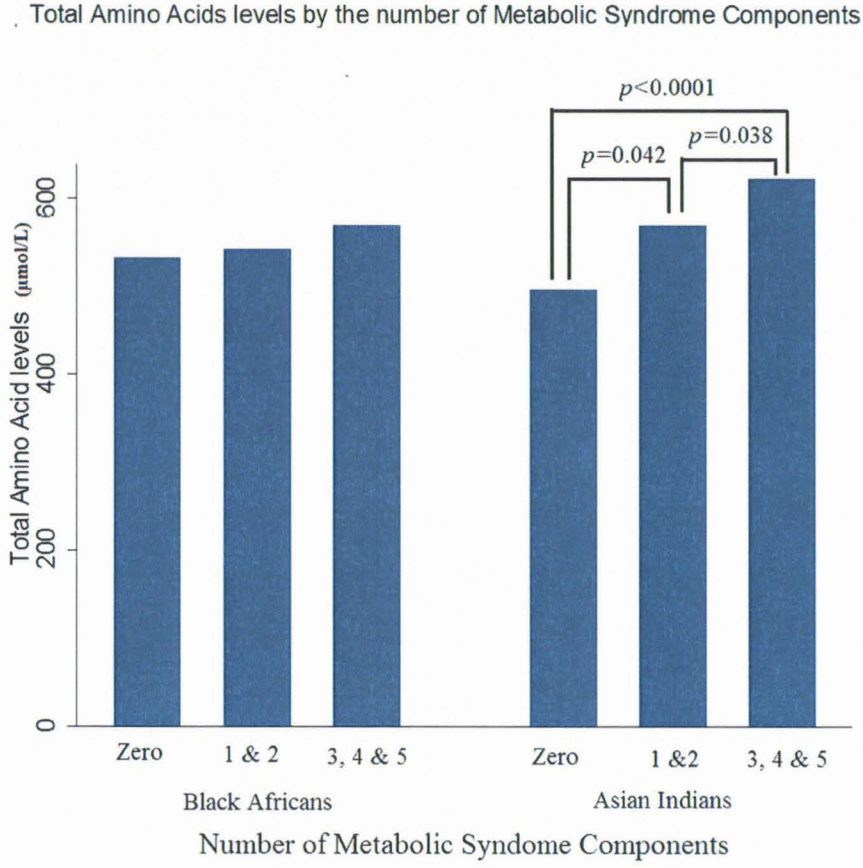

Figure 5.2 The total amino acid levels compared by the number of metabolic syndrome components in black Africans and Asian Indians.

5.3 AMINO ACID LEVLES BY BMI CATEGORIES

We categorized BMI as normal (<24,9kg/m^2), overweight (25-29,9kg/m^2) and obese (≥30kg/m^2) and then compared total amino acid levels in the three BMI categories by

ethnicity. The total amino acid levels in overweight and obese participants were significantly higher in Asian Indians than in black Africans (p-value=0.0295; 0.00497), respectively (Table 5.4), even though BMI was not different by category (Table 5.6).

Table 5.4 Total amino acid levels according to ethnicity and BMI categories

BMI Category	**Ethnicity**		***p*-value**
	Black Africans n**=369**	**Asian Indians** n**=349**	
Normal	502 (426, 596)	509 (424, 613)	0.5229
Overweight	503 (446, 603)	557 (446, 690)	0.0295
Obese	543 (461, 632)	585 (513, 725)	0.0038

Data are presented as median and IQR

Normal = <24kg/m^2, Overweight =25-29kg/m^2, Obese = ≥30kg/m^2

Within stratification by ethnicity, there was no significant difference in total amino acid levels by BMI categories in black Africans (Table 5.5 and Fig. 5.3). However, the total amino acid levels in normal participants were significantly lower when compared with overweight (p<0.002) and obese (p <0.0001) participants in Asian Indians (Table 5.5 and Fig.5.3).

Table .5.5 ANOVA test comparing the total amino acid levels of normal, overweight and obese individuals in black Africans and Asian Indians

BMI Comparisons	**Ethnicity**	
	Black Africans (n= 369)	**Asian Indians (n= 349)**
	***p*-values**	***p*-values**
Normal to Overweight	NS	0.002
Normal to Obese	NS	<0.0001
Overweight to Obese	NS	NS

Normal = <24kg/m^2, Overweight =25-29kg/m^2, Obese = ≥30kg/m^2

NS= Not significant.

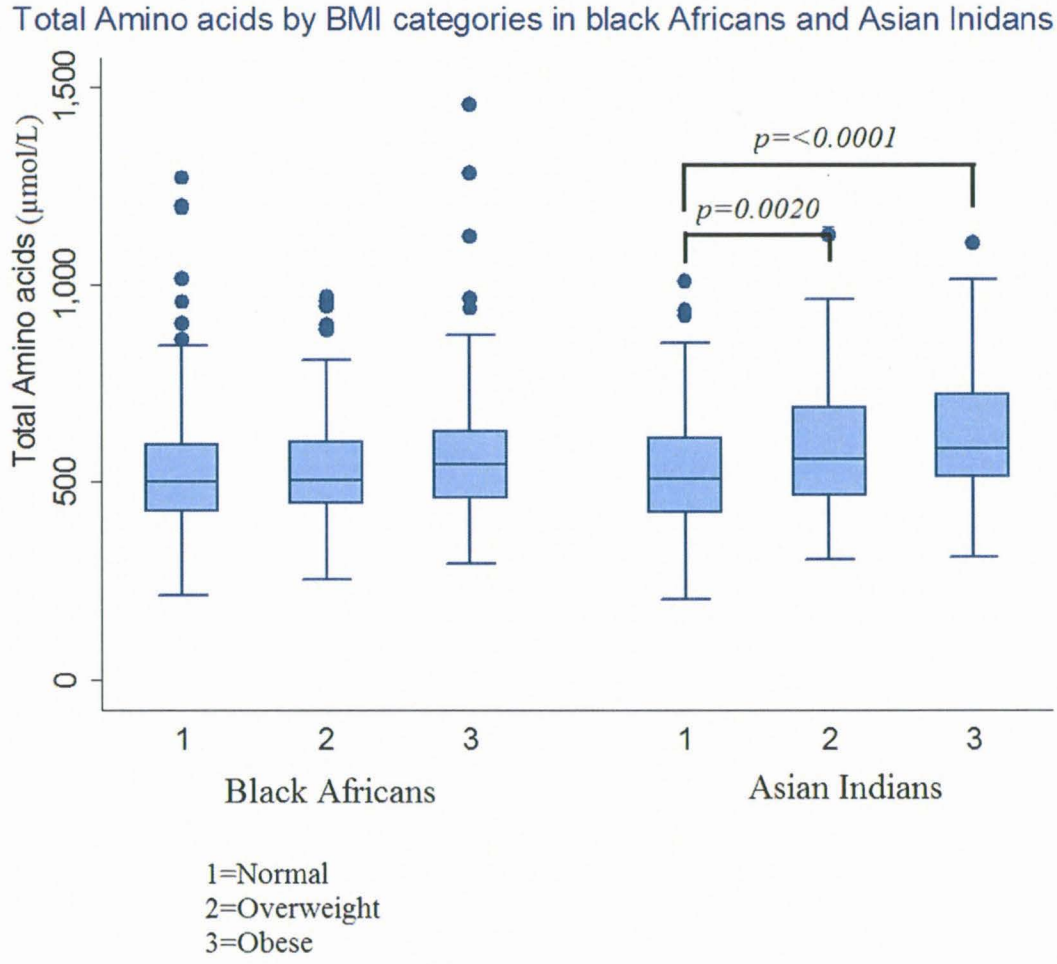

Figure 5.3 The total amino acid levels compared by BMI categories black Africans and Asian Indians. There was no significant difference in total amino acid levels in black Africans. Total amino acid levels in Asian Indians with normal weight were significantly lower than overweight (*p=0.0020)* and obese (*p<0.0001)* Asian Indians.

We compared individual amino acid concentrations across BMI category in each ethnic group and noted that in black Africans phenylalanine levels were higher in obese individuals compared normal weight individuals ($p< 0.05$) while in Asian Indians all amino acids were higher in obese compared to normal individuals ($p<0.05$) and all except phenylalanine were

higher in overweight compared to normal individuals ($p<0.05$) (Table 5.6). We would have liked to look at a group of lean individuals i.e. BMI < 18 but there were too few individuals in each group to make any meaningful comparison. (13 in Black Africans and 16 in Asian Indians)

Table 5.6 Amino acid levels according to ethnicity and BMI categories

Amino Acid (μmol/L)	Black Africans (n=364)			Asian Indians (n=349)		
	Normal Weight	**Overweight**	**Obese**	**Normal Weight**	**Overweight**	**Obese**
Valine	153 (110, 119)	143 (110, 199)	160 (120, 238)	144 (108, 196)	167 (117, 238)*	175 (135, 241)**
Leucine	131 (110, 169)	144 (113, 180)	131 (105, 164)	137 (99.8, 180)	144 (109, 189)*	158 (123, 206)**
Isoleucine	53.1 (40.8, 65.5)	53.6 (40.6, 64.0)	48.2 (39.6, 63.0)	54.7 (41.6, 71.5)	65.3 (50.0, 86.2)*	65.2 (50.6, 86.3)**
Tyrosine	80.0 (56.3, 91.1)	71.0 (59.0, 91.6)	78.8 (59.9, 105)	76.7 (60.8, 96.9)	85.8 (70.7, 106)*	92.4 (70.3, 116)**
Phenylala-nine	79.9 (67.7, 99.9)	79.9 (64.4, 101)	92.1 (69.7, 111)**	76.8 (66.2, 92.3)	81.5 (70.0, 94.8)*	86.9 (70.5, 106)**

Data are presented as median and IQR

Normal = <24kg/m^2, Overweight =25-29kg/m^2, Obese = ≥30kg/m^2

*$p<0.05$ normal weight vs. overweight

**$p<0.05$ normal vs. obese

5.4 PAIRWISE CORRELATION OF AMINO ACID LEVELS WITH CARDIO-METABOLIC RISK FACTORS

In black Africans, total amino acid levels significantly correlated with triglycerides (p= 0.001) and visceral fat (p= 0.001). When we looked at individual amino acids, isoleucine and tyrosine did not significantly correlate with any of the cardio-metabolic risk factors in black Africans. Phenylalanine levels significantly correlated with the following cardio-metabolic risk factors; fasting insulin, HOMA-IR, triglycerides, BMI index, waist circumference, visceral and subcutaneous fat ($p<0.05$ for all factors) (Table 5.7). Valine levels significantly

correlated with triglycerides (p= 0.002), BMI (p= 0.044), visceral (p= 0.004) and subcutaneous fat (p< 0.0001) and leucine correlated significantly with triglycerides (p<0.0001) visceral fat (p=0.02) and subcutaneous fat (p=0.02)

In Asian Indians, total amino acids were significantly correlated with all the cardio-metabolic risk factors with the exception for HDL-cholesterol and LDL-cholesterol, (p<0.05) for all factors (Table 5.7). Phenylalanine was significantly associated with diastolic BP, BMI, visceral and subcutaneous fat. Leucine levels were significantly associated will all of the cardio-metabolic risk factors with the exception of subcutaneous fat and LDL-cholesterol. Furthermore, isoleucine levels were significantly correlated with all cardio-metabolic risk factors excluding subcutaneous and visceral fat, HDL-cholesterol and LDL-cholesterol, p <0.05 for all factors (Table 5.7). Tyrosine levels also significantly correlated with insulin, HOMA-IR, systolic BP, diastolic BP, BMI, waist circumference, visceral and subcutaneous fat (p<0.05 for all) but not with glucose, triglycerides, HDL-cholesterol and LDL-cholesterol, (p<0.05 for all) (Table 5.7).

It was interesting to note that HDL-cholesterol and LDL-cholesterol were not correlated with any of the amino acids in both ethnic groups with the exception of leucine in Asian Indians.

Table 5.7 Pairwise correlations between amino acids and cardio-metabolic risk factors after log transformation

Cardio-metabolic risk factor		Total Amino Acids (μmol/L)		Valine (μmol/L)		Leucine (μmol/L)		Isoleucine (μmol/L)		Phenylalanine (μmol/L)		Tyrosine (μmol/L)	
Ethnicity, African (*n*=369), Asian (*n*=349)		**Black African**	**Asian Indians**	**Black African**	**Asian Indians**	**Black African**	**Asian Indians**	**Black African**	**Asian Indians**	**Black African**	**Asian Indians**	**Black African**	**Asian Indians**
Fasting glucose (μmol/L)	*r*	0.003	0.214	-0.033	0.149	0.032	0.201	0.011	0.146	0.046	0.097	-0.035	0.144
	p-value	0.954	**0.001***	0.524	**0.005***	0.543	**<0.001***	0.833	**0.007***	0.385	0.069	0.499	**0.007***
Fasting insulin (mU/L)	*r*	0.072	0.179	0.062	0.089	0.027	0.154	-0.017	0.117	0.148	0.086	0.052	0.165
	p-value	0.171	**0.001***	0.236	0.096	0.613	**0.004***	0.752	**0.03***	**0.004***	0.108	0.318	**0.002***
HOMA-IR	*r*	0.065	0.216	0.044	0.122	0.034	0.193	-0.011	0.144	0.146	0.104	0.034	0.184
	p-value	0.220	**0.001***	0.401	**0.023***	0.518	**<0.001***	0.832	**0.007***	**0.005***	0.053	0.515	**<0.001***
Triglycerides (μmol/L)	*r*	0.177	0.176	0.158	0.156	0.176	0.151	0.097	0.143	0.105	0.078	0.038	0.063
	p-value	**0.001***	**0.001***	**0.002***	**0.004***	**<0.001***	**0.005***	0.065	**0.009***	**0.045***	0.144	0.463	0.244
HDL-cholesterol (μmol/L)	*r*	-0.048	-0.049	-0.049	-0.011	-0.060	-0.128	-0.025	0.058	-0.003	0.060	-0.042	-0.020
	p-value	0.358	0.369	0.349	0.834	0.252	**0.017***	0.630	0.282	0.952	0.267	0.425	0.741
LDL-cholesterol (μmol/L)	*r*	-0.032	0.030	-0.025	<-0.001	-0.001	0.030	-0.030	0.021	0.002	0.050	-0.028	0.015
	p-value	0.544	0.579	0.639	0.997	0.991	0.574	0.571	0.705	0.977	0.357	0.591	0.830
Systolic BP (mmHg)	*r*	0.009	0.245	-0.034	0.175	0.005	0.183	-0.021	0.182	0.103	0.100	0.052	0.165
	p-value	0.861	**<0.001***	**0.518***	**0.001***	0.927	**<0.001***	0.680	**<0.001***	0.051	0.062	0.320	**0.002***
Diastolic BP (mmHg)	*r*	-0.045	0.205	-0.096	0.124	-0.027	0.124	-0.013	0.121	0.041	0.148	0.014	0.174
	p-value	0.397	**0.001***	0.069	**0.021***	0.614	**0.021***	0.808	**0.025***	0.439	**0.006***	0.796	**0.001***
BMI(kg/m2)	*r*	0.064	0.270	0.105	0.175	-0.028	0.194	-0.017	0.145	0.111	0.147	0.085	0.263
	p-value	0.226	**<0.001***	**0.044***	**0.001***	0.594	**<0.001***	0.748	**0.007***	**0.034***	**0.006***	0.105	**<0.001***
Waist circumference (cm)	*r*	0.087	0.234	0.092	0.164	0.036	0.208	0.012	0.145	0.106	0.086	0.071	0.187
	p-value	0.099	**<0.001***	0.079	**0.002***	0.491	**<0.001***	0.818	**0.007***	**0.043***	0.110	0.174	<0.001*
Mean Visceral fat (cm)	*r*	0.185	0.364	0.149	0.253	0.122	0.329	0.069	0.292	0.172	0.146	0.085	0.205
	p-value	**0.001***	**<0.001***	**0.004***	**<0.001***	**0.020***	**<0.001***	0.195	<0.001	**0.001***	**0.006***	0.105	**<0.001***
Mean Subcutaneous fat (cm)	*r*	0.118	0.199	0.192	0.211	-0.021	0.096	-0.024	0.105	0.161	0.072	0.071	0.166
	p-value	0.025	**0.001***	**<0.001***	**<0.001***	**0.020***	0.076	0.648	0.051	**0.002***	0.182	0.177	**<0.001***

All data was log transformed to normality before correlation analysis

*significant *p*-values

5.5 ASSOCIATION OF AMINO ACID LEVELS WITH THE METABOLIC SYNDROME AND ITS COMPONENTS

We created several models for regression analysis. First we carried out a logistic regression analysis to test association of metabolic syndrome with the total amino acids and then for individual amino acids. In black Africans neither total amino acids nor individual amino acids were associated with the metabolic syndrome. In the Asian Indian group, the total amino acids were close to being significant with the metabolic syndrome (p= 0.054) (Table 5.8).

Thereafter, we tested the association of the individual metabolic syndrome components with each of the amino acids. Phenylalanine was significantly associated with systolic BP in black Africans (p=0.018) but none of the other cardio-metabolic risk factors that were looked at were associated with any of the amino acids (Table 5.8). In Asian Indians, glucose was significantly associated with tyrosine (p= 0.027) and triglycerides with valine (p=0.009) (Table 5.9).

Neither total BCAA nor any individual amino acids were significantly associated with HOMA-IR after adjustment for age, BMI and ethnicity (data not presented).

Table 5.8 Estimated logistic regression odds ratios (95% CI) on the association of metabolic syndrome and total amino acids and individual amino acids.

Dependent variable	Independent Variable	Black Africans (*n*=369)	*p*-values	Asian Indians (*n*=349)	*p*-values
Metabolic Syndrome	Model 1	1.36 (0.55, 3.39)	0.510	2.41 (0.98, 5.92)	**0.054**
	Model 2	1.04 (0.62, 1.74)	0.886	1.55 (0.90, 2.68)	0.116
	Model 3	1.78 (0.87, 3.64)	0.115	1.53 (0.78, 3.01)	0.219
	Model 4	1.00 (0.59, 1.71)	0.986	1.66 (0.94, 2.92)	0.08
	Model 5	0.68 (0.34, 1.35)	0.268	1.69 (0.79, 3.62)	0.268
	Model6	1.36 (0.63, 2.94)	0.433	1.00 (0.46, 2.17)	0.992

All data was adjusted for age, gender and BMI
Data presented in odds ratios (95% Confidence Intervals)
Model 1= Total amino acids
Model 2= Valine,
Model 3= Leucine,
Model 4= Isoleucine,
Model 5= Tyrosine
Model 6= Phenylalanine

Table 5.9 Estimated logistic regression odds ratios (95% CI) on the association cardio-metabolic risk factors with each amino acid.

Dependent Variable	Independent Variable	Black Africans (*n*=369)	*p*-values	Asian Indians (*n*=349)	*p*-values
Glucose	Model 1	1.24 (0.71, 2.18)	0.447	1.21 (0.69, 2.11)	0.505
	Model 2	1.00 (0.46, 2.16)	0.997	1.98 (0.97, 4.06)	0.063
	Model 3	1.22 (0.68, 2.19)	0.505	1.67 (0.93, 3.00)	0.085
	Model 4	0.62 (0.29, 1.32	0.212	**2.51 (1.10, 5.67)**	**0.027**
	Model 5	1.38 (0.61, 3.11)	0.436	1.48 (0.62, 3.56)	0.379
Triglycerides	Model 1	1.50 (0.82, 2.75)	0.192	**2.00 (1.19, 3.37)**	**0.009**
	Model 2	1.28 (0.57, 2.87)	0.552	1.89 (0.97, 3.67)	0.062
	Model 3	1.47 (0.73, 2.83)	0.249	1.51 (0.89, 2.56)	0.127
	Model 4	0.80 (0.34, 1.81)	0.577	1.03 (0.50, 2.12)	0.945
	Model 5	1.22 (0.51, 2.94)	0.659	0.83 (0.39, 1.77)	0.626
HDL-cholesterol	Model 1	0.90 (0.57, 1.42)	0.649	0.978 (0.61, 1.54)	0.89
	Model 2	1.11 (0.60, 2.06)	0.744	0.77 (0.42, 1.42)	0.405
	Model 3	0.88 (0.55, 1.42)	0.606	0.88 (0.54, 1.44)	0.617
	Model 4	1.05 (0.59, 1.88)	0.871	1.08 (0.55, 2.14)	0.819
	Model 5	1.73 (0.88, 3.39)	0.112	0.80 (0.39, 1.64)	0.538
Systolic BP	Model 1	1.03 (0.65, 1.64)	0.903	1.25 (0.73, 2.16)	0.417
	Model 2	1.08 (0.58, 2.01)	0.799	1.04 (0.52, 2.09)	0.905
	Model 3	0.92 (0.56, 1.51)	0.738	1.48 (0.84, 2.62)	0.179
	Model 4	1.20 (0.66, 2.18)	0.545	1.50 (0.69, 3.27)	0.303
	Model 5	**2.44 (1.17, 5.11)**	**0.018**	1.35 (0.60, 3.05)	0.474
Diastolic BP	Model 1	0.82 (0.52,	0.402	1.16 (0.69, 1.93)	0.576

	1.30)			
Model 2	1.13 (0.62, 2.07)	0.686	1.13 (0.58, 2.18)	0.713
Model 3	1.27 (0.79, 2.04)	0.315	1,20 (0.70, 2.05)	0.513
Model 4	0.78 (0.43, 1.41)	0.407	1.40 (0.67, 2.93)	0.369
Model 5	0.93 (0.47, 1.81)	0.825	1.39 (0.63, 3.08)	0.415

All data was adjusted for age, gender and BMI index
Data presented in odds ratios (95% Confidence Intervals)
Model 1= Valine,
Model 2= Leucine,
Model 3= Isoleucine,
Model 4= Tyrosine
Model 5= Phenylalanine

CHAPTER 6: DISCUSSION

We set out to develop a method for the analysis of amino acids in our laboratory and this was done successfully. Using an ultra-performance liquid chromatography (UPLC) coupled with tandem mass spectrometry has allowed for a highly sensitive and highly specific analysis of the amino acids in our study. Sample preparation did not involve derivatization of the amino acids which reduced the sample preparation time and allowed for quick analysis. We also used isotopically labeled internal standards which reduced matrix effects. We were also able to analyze the isobaric amino acids (leucine and isoleucine) with peak specificity by firstly detecting the transition with the highest intensity in each isobaric amino acid and then corrected the peak height to allow for specific detection of each amino acid. This method was taken from (Yang *et al.*, 2013) and optimized. The method was validated before it was applied to study samples.

Furthermore we have reported ethnicity specific data in relation to BCAAs and AAAs and the following cardio-metabolic risk factors; hyperglycemia, high triglycerides, low HDL-cholesterol, hypertension and abdominal obesity. The hypothesis that plasma BCAAs, tyrosine and phenylalanine are elevated in individuals with the metabolic syndrome was noted in Asian Indians but not in black Africans. We have also shown that phenylalanine levels were significantly associated with systolic blood pressure in black Africans and that valine and tyrosine levels were significantly associated with high triglycerides and hyperglycemia, in the local Asian Indians population. Furthermore, we have shown that total amino acids and individual amino acids are higher in overweight and obese Asian Indians compared with normal weight Asian Indians, but this was not the case for black Africans. . In black Africans, phenylalanine was the only amino acid that was different between normal weight and obese individuals. We noted that Asian Indians had significantly higher total amino acid (BCAAs, tyrosine and phenylalanine) levels than black Africans even for subjects with similar BMI.

6.1 COMPARISONS WITH OTHER STUDIES

6.1.1 BCAAs and AAAs Association with the Metabolic Syndrome

In Asian Indians, total amino acids (BCAAs, tyrosine and phenylalanine) and individual amino acids were increased among subjects with the metabolic syndrome compared to those without. This was similar to results obtained from a mixed race population where BCAAs were elevated in individuals with the metabolic syndrome (2 or more of the metabolic syndrome components) compared to those without (Batch *et al.*, 2013).

In contrast, black Africans did not show significant difference in amino acids levels in subjects with the metabolic syndromes and those without. The possible reason for these findings is that black Africans seem to be less affected by two out of five of the metabolic syndrome components (obesity and high triglycerides). For example, Africans were shown to have lower triglycerides than Asians and Caucasians (Zoratti *et al.*, 2000; Ford *et al.*, 2002). Secondly, although there was no difference in visceral fat between both ethnic groups, Asian Indians were more insulin resistant than black Africans and had higher total amino acid levels than black Africans. This suggests that the metabolic pathways of the two ethnic groups differ and it seems that black Africans are less prone to metabolic dysfunction than Asian Indians.

In addition to the elevated total amino acid levels observed in Asian Indians with the metabolic syndrome, the present study has shown that total amino acid levels are significantly higher in Asian Indians with one or two of the metabolic syndrome components compared to those with zero metabolic syndrome components. Therefore, longitudinal studies should be carried out to determine if total amino acids predict cardio-metabolic risk before the individual has the metabolic syndrome (individual with three or more of the metabolic syndrome components) in Asian Indians. Whether elevated BCAA can be used to predict future cardiovascular risk remains to be determined in longitudinal studies. The early prediction of the increased risk of developing CVD and T2DM will allow for an early intervention to prevent the development of CVD and T2DM in patients (Association, 2004; Van Buren and Tibbs, 2014).

6.1.2 BCAAs and AAAs Association with Obesity

In Asian Indians, total amino acid and individual amino acid levels were elevated in overweight and in obese subjects compared to normal weight subjects. These findings are consistent with one of the earliest studies in metabolomics (Felig *et al.*, 1969).In this study, out of the 20 plasma amino acids that were analyzed, BCAAs, phenylalanine and tyrosine levels increased in healthy obese subjects compared to aged-and-sex matched controls. Similarly, Newgard *et al.* (2009) showed that healthy obese individuals had significantly higher BCAA, phenylalanine tyrosine, methionine, glutamate/glutamine C3 and C5 acylcarnitines levels than lean individuals ($p<0.0001$).

In black Africans, total amino acids were not different between BMI categories (normal weight, overweight and obese). However phenylalanine levels were significantly higher in obese individuals compared to individuals with normal weight.

In a single study from South Africa; Dugas *et al.* (2016) showed that in obese black African women, valine and tyrosine correlated with BMI whilst in our study we found that valine correlated with BMI and leucine with visceral and subcutaneous fat. The amino acids profiles were different in Asian Indians; all the amino acids correlated significantly with measures of body fat namely BMI and visceral fat.

Our findings suggest that there are different profiles of amino acids in both of these populations. Whilst the reasons for these were not investigated a possibility is that dietry differences may result in the difference in amino acids profiles (Newgard *et al.*, 2009; Pal *et al.*, 2010; Chiu *et al.*, 2014).

6.1.3 BCAAs and AAAs Association with Hyperglycemia

In our study, fasting plasma glucose was associated with tyrosine in Asian Indians. Tyrosine has been associated with increased risk of T2DM in several studies. For example tyrosine and 18 other metabolites including BCAAs and phenylalanine were predictors of increase in fasting plasma glucose and the increase in 2-h glucose in a normoglycaemic Finnish population within the 6.5 year follow up (Würtz *et al.*, 2012). Similarly, in the Framingham Offspring study tyrosine along with BCAAs and phenylalanine were significant predictors of T2DM within the 12 year follow up (Wang *et al.*, 2011). Moreover, the amino acids were elevated from baseline up until the onset of T2DM in those individuals who had incident diabetes. These longitudinal studies indicate that tyrosine along with the other interlinked amino acids are elevated long before the onset of T2DM. Therefore, the amino acids are potential early predictors of metabolic dysfunction.

Lee *et al.* (2016) have shown ethnic specific differences in the association of BCAAs with incident diabetes, with higher BCAAs in Caucasians and Hispanics but not in African-Americans. Phenylalanine as well as tyrosine was associated with T2DM in the Europeans. Leucine, on the other hand was significantly higher in diabetics when compared to non-diabetics in African Americans (Fiehn *et al.*, 2010). The observations in our study suggest that the association of amino acids with hyperglycemia is ethnicity dependent.

Tai *et al.* (2010) suggests that elevated BCAAs, phenylalanine and tyrosine levels are related to insulin resistance and not to obesity. This was shown in a BMI- matched South Asian population from Singapore in which insulin resistance correlated with BCAAs, phenylalanine, tyrosine and other amino acids. In this study, subjects were categorized into upper and lower tertiles of insulin resistance, as measured by HOMA and the group in the top tertile had high BCAA, tyrosine and phenylalanine levels compared to the low tertile. BCAAs, tyrosine and

phenylalanine have been associated with insulin resistance and inversely associated with insulin sensitivity in both cross sectional studies (Huffman *et al.*, 2009; Lee *et al.*, 2016) and longitudinal study (Würtz *et al.*, 2013; Chen *et al.*, 2016). In our study insulin resistance (HOMA-IR) correlated with total amino acids and individual amino acids excluding phenylalanine in Asian Indians ($p< 0.05$ for all). This was consistent with the study by Tai *et al.* (2010) However after adjustment for age, BMI and ethnicity this was no longer significant. Similarly, phenylalanine correlated with insulin resistance in univariate analysis in black Africans, however; this association was no longer significant after multivariate analysis.

6.1.4 BCAAs and AAAs Association with Hypertension

We have shown that phenylalanine levels are significantly associated with systolic blood pressure in black Africans. There is a few data on amino acids association with blood pressure globally; Mels *et al.* (2013) showed that systolic blood pressure is significantly associated with leucine and isoleucine in black Africans and Caucasians with hyperglycemia (HbA1c glycated hemoglobin ≥ 5.6 %) after adjustments for age, gender, ethnicity and BMI.

Elevated phenylalanine levels were associated with oxidative stress in patients with phenylketonuria (Sanayama *et al.*, 2011) and oxidative stress is the formation of uncontrollable levels of reactive oxygen species (ROS) during energy production. The association of oxidative stress with phenylalanine could explain in part the link between phenylalanine and systolic blood pressure seen in black Africans in our study because elevated ROS formation can lead to nitrogen oxide disruption. Nitrogen oxide stimulates guanylyl cyclase to form 3′,5′-cyclic guanosine monophosphate, which results in vasodilatation of

vascular smooth muscle cells and the prevention of arterial stiffness (Moncada *et al.*, 1991; Förstermann and Münzel, 2006; Hermann *et al.*, 2006).

BCAAs and AAAs are not the only metabolites that are associated with hypertension. A longitudinal study performed in the general population from Potsdam, Germany showed that out of 127 metabolites, serine and glycine along fatty acid derivatives were predictors for the development of hypertension within the follow up of ten years (Dietrich *et al.*, 2016). Another longitudinal study showed that out of 204 metabolites that were analyzed, 4-hydroxyhippurate and a sex steroid pattern were significantly associated with incident hypertension within a follow up of more or less ten years (Zheng *et al.*, 2013).

Longitudinal studies should be carried out to determine if phenylalanine can predict cardiovascular risk before the onset of hypertension.

6.1.5 BCAAs and AAAs Association with High Triglycerides

In Asian Indians, triglycerides were significantly associated with valine in our study. There is little knowledge available about the relationship of valine with high triglycerides. BCAAs were associated with triglycerides and inversely associated with HDL-C in pre-diabetic Chinese subjects (Yang *et al.*, 2016). In a 12 year longitudinal study, BCAAs, tyrosine, phenylalanine and alanine were associated with dyslipidemia and hypertension in two large human cohort studies (Framingham Heart Study and the Malmö Diet and Cancer Study). Similarly, BCAAs, arginine, proline, phenylalanine, and lysine levels were significantly associated with hypertriglyceridemia ($p<0.05$) within a seven year follow up Mook-Kanamori *et al.*, 2014.

6.2 STRENGTH AND LIMITATION

Our study contributes to the existing knowledge of elevated BCAAs, tyrosine and phenylalanine association with cardio-metabolic risk factors by showing that elevated BCAAs are associated with cardio-metabolic risk such as hyperglycemia, hypertension and high triglycerides in both ethnic groups but the associations were stronger in Asian Indians than in black Africans.

The strengths of this study are the large sample size and the use of specific and sensitive amino acid analysis. The limiting factor of the study was the cross-sectional nature of the study which did not investigate the possible mechanism that can lead to elevated BCAAs and AAAs in metabolically unwell individuals.

6.3 FUTURE WORK AND CONCLUSION

Future studies should be directed to longitudinal studies which will investigate whether BCAAs and AAAs are predictive of cardiovascular risk factors in Asian Indians and whether phenylalanine levels are predictive of hypertension in black Africans.

Furthermore, analyzing genes encoding for BCAA and AAA catabolism enzymes such as *BCAT2*, *BCKDHB, PAH* will show whether the BCAA and AAA metabolic pathway is impaired in those individuals who are obese and/or have the metabolic syndrome in the two ethnic groups as seen in co-twins with obesity (Pietiläinen *et al.*, 2008). In addition, gene analysis will investigate the differences, if any, in BCAA and AAA metabolism between the two ethnic groups.

It is possible that the elevated BCAAs and AAAs observed in overweight, obese and metabolically unwell Asian Indians subjects in the present study is an indication of BCAA metabolism overload. In this case, the amino acid overload would attenuate the activity of the

enzymes such as the BCAT and BCKD enzymes resulting in oxidative stress and increased acylcarnitine formation, similar to the lipid and glucose metabolism overload demonstrated in obese individuals (Furukawa *et al.*, 2004; Lefort *et al.*, 2010; Serra *et al.*, 2013). Although this study did not investigate acylcarnitines levels, previous studies have shown that elevated BCAAs are accompanied by elevated acylcarnitines which lead to oxidative stress and β-cell mitochondrial dysfunction in humans (Adams *et al.*, 2009; Huffman et al., 2009; Newgard *et al.*, 2009; Newgard *et al.*, 2012; Shah *et al.*, 2012) and in mice (Walajtys-Rode *et al.,* 1980; Amaral *et al.,* 2010). More studies are needed to investigate these mechanisms in black Africans and Asian Indians.

In conclusion we have shown that BCAA are higher in obese Asian Indians and are elevated in those Asian Indians who have one or two more components of the metabolic syndrome compared to those who do not have any components of the metabolic syndrome and to those who had the metabolic syndrome. BCAA were not different in obese black Africans compared to overweight or normal weight black Africans and did not differ by the number of components of the metabolic syndrome the subjects had. Longitudinal studies are required to confirm these findings and to investigate other mechanisms for metabolic dysfunction in black Africans.

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APPENDIX A

Ethics Clearance Certificate

R14/49 Miss Lungile Khambule

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150669

NAME: (Principal Investigator)	Miss Lungile Khambule
DEPARTMENT:	Chemical Pathology Chemical Pathology Development Laboratory
PROJECT TITLE:	Association of Branched Chain Amino Acids and Aromatic Amino Acids with Cardio-Metabolic Risk Factors
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Jaya Anna George
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	17/06/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.**

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX B

Subject Consent Form

PATIENT INFORMATION.

1. INTRODUCTION

My name is Jaya George and I am a doctor working in the Department of Chemical pathology You are invited to consider participating in a research study. Your participation in this study is entirely voluntary.

Before agreeing to participate, it is important that you read and understand the following explanation of the purpose of the study, the study procedures, benefits, risks, and precautions as well your right to withdraw from the study at any time.

This information leaflet is to help you to decide if you would like to participate. You should fully understand what is involved before you agree to take part in this study.

If you have any questions, do not hesitate to ask me..

1. PURPOSE OF THE STUDY

The project is aimed at studying the vitamin D levels in various ethnic groups in this province and to compare these levels will internationally recommended levels. Vitamin D is important for normal bone strength as well as for normal immune function.

We will also measure the levels of calcium, magnesium, parathyroid hormone, creatinine and alkaline phosphate. Changes in vitamin D levels will affect the levels of these in blood. Some blood will also be stored to check for DNA for changes in the vitamin D receptor.

2. LENGTH OF STUDY AND NUMBER OF PARTICIPANTS

Approximately 600 participants will be recruited to participate in the study.
The total amount of time required for your participation will be a maximum of 4 hours and you will not be expected return for follow up.

PROCEDURES

If you agree to take part in this study, you will first be asked questions and examined to see if you qualify for this study.

A few tubes of blood will be collected from a vein in your arm. This is equal to about two tablespoons (30 mls) of blood. The samples will be given a unique number to protect your privacy.

Thereafter, you will have your weight, height, waist circumference and blood pressure measured and recorded. You will also have a scan to assess the density of your bones and an ultrasound scan for fat.This information will be used to assess if body mass index has any impact on vitamin D levels.

3. WILL ANY OF THESE STUDY PROCEDURES RASULT IN DISCOMFORT OR INCONVENIENCE?

Drawing of blood may cause some discomfort associated with pain, bruising or bleeding at the puncture site. The procedure will be performed under sterile conditions to ensure your protection.

4. BENEFITS

Your participation in this study will assist in assessing the status of vitamin D in the different population groups in this province.

5. RIGHTS AS A PARTICIPANT IN THIS STUDY.

Voluntarity

Your participation in the study is entirely voluntary and you may decline to participate.

Withdrawal

You may withdraw at any time and your withdrawal will not affect access to other medical care.

6. FINANCIAL ARRANGMENTS

You will not be required to pay for any of the study related procedures.

7. REIMBURSMENT FOR STUDY PARTICIPATION

You will not be paid to participate in the study.

8. ETHICAL APPROVAL.

This study has been approved by the Human Ethics Committee of the University of the Witwatersrand, the clearance number

9. SOURCES OF ADDITIONAL INFORMATION.

If at any time after you feel that you have questions regarding the study, please do not hesitate to the principal investigator , Dr Jaya George, at this number: 0828820132.

10. CONFIDENTIALITY

All information obtained during the course of the study including hospital records, personal data and research data will be kept strictly confidential. Data that may be reported in scientific or medical journals will not include any information that identifies you as a participant in this study.

Any information uncovered regarding your test results or state of health as a result of your participation in the study will only be made known to your treating doctors. You will be informed of any findings of importance to your health during participation in this study.

I hereby confirm that the researcher, has explained the nature, conduct, benefits and risks of this research project.

- I have also received, read and understood the above written information regarding the study.
- I am aware that the results of the study, including personal details regarding my age, sex and diagnosis will anonymously be processed into a study report.
- In view of the requirements of research, I agree that the data collected during this study can be processed in a computerized system by the researcher appearing on the first page of this document.
- I may at any stage, without prejudice, withdraw my consent and participation in the study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study

PARTICIPANT.

Printed Name	**Signature / Name or Thumbprint**	**Date**

WITNESS (if applicable)

Printed Name	**Signature / Name or Thumbprint**	**Date**

Researcher Name **Signature**
Date