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**A COMPARATIVE STUDY BETWEEN THE PREVALENCE OF MTHFR A1298C  
SNP AND HOMOCYSTEINE METABOLISM IN AN ELDERLY BLACK SOUTH  
AFRICAN POPULATION**

Submitted in fulfilment for the requirements of the degree Magister Technologiae:  
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## ABSTRACT

**Background:** Cardiovascular diseases are one of the most common causes of death worldwide. This is not only a problem in developed countries, it is of major concern for public health in developing countries as well. Increased homocysteine is an independent risk factor for cardiovascular diseases. Nutritional deficiencies of folate, vitamin B6 and vitamin B12 are associated with hyperhomocysteinemia. MTHFR A1298C, a single nucleotide polymorphism, is similarly linked with higher concentrations of homocysteine. The aim of this study was to determine the prevalence of MTHFR A1298C in a black elderly population, along with folate, vitamin B6 and vitamin B12 and to evaluate the effect on homocysteine levels.

**Methodology:** The research design was an observational cross-sectional study and was ethically approved. A total of 84 elderly who attend a day-care centre (also met inclusion criteria) were purposively selected. DNA was extracted and frozen on the day of blood collection. The MTHFR A1298C genotype was determined with real time PCR. Homocysteine, folate, vitamin B6 and vitamin B12 serum levels were detected with commercial assay kits.

**Results:** Homocysteine was found to be elevated with a median of 17.78  $\mu\text{mol/L}$  (interquartile range 13.98-21.03  $\mu\text{mol/L}$ ). Serum folate, vitamin B6 and vitamin B12 medians were in the normal range. Although, 5.95% and 22.62% of the population were deficient and possibly deficient for vitamin B12, respectively. MTHFR A1298C frequency was as follow: 89.29% (AA), 9.52% (AC) and 1.19% (CC), with no significant correlation ( $p>0.05$ ) with homocysteine. Vitamin B12 correlated significantly with homocysteine levels.

**Conclusion:** Vitamin B12 deficiency had an effect on homocysteine levels. Overall, nutritional deficiencies are not responsible for the hyperhomocysteinemia in this population. In conclusion from this study showed MTHFR A1298C frequency in black South Africans does not contribute to homocysteine as a risk factor for cardiovascular disease.

**Keywords:** Cardiovascular disease, elderly, folate, homocysteine, MTHFR A1298C, vitamin B6, vitamin B12

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## DECLARATION

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## ABBREVIATIONS

AHA	American Heart Association
AIDS	Acquired immune deficiency syndrome
ATP	Adenosine triphosphate
BHMT	Betaine homocysteine methyltransferase
bp	Base pair
CAD	Coronary artery disease
CBS	Cystathionine $\beta$ -synthase
CpG	Cytosine-phosphate guanine
CSIR	Council for Scientific and Industrial Research
CSL	Centre of Sustainable Livelihoods
CV	Coefficient of variance
CVD	Cardiovascular disease
CVDs	Cardiovascular diseases
CVR	Cardiovascular risk
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELSD	Evaporative light scattering detector
FAD	Flavin adenine dinucleotide
FBP	Folate binding protein
FOLH1	Folate hydrolase
FR	Folate receptor
FRET	Fluorescence resonance energy
H <sub>2</sub> S	Hydrogen sulphide
Hcy	Homocysteine
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
hs-CRP	High sensitivity C-reactive protein
ICAM	Intracellular adhesion molecule-1
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IQR	Interquartile range



LDL	Low density lipoprotein
MTHFD1	Methylenetetrahydrofolate dehydrogenase
MTHFR	5, 10-Methylenetetrahydrofolate reductase
MTR	Methionine synthase
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NCDs	Non-communicable diseases
NH <sub>3</sub>	Ammonia
NO	Nitric oxide
NTC	No template control
PAI-1	Plasminogen activator inhibitor-1
PL	Pyridoxal
PLP	Pyridoxal-5-phosphate
QC	Quality control
qPCR	Real time polymerase chain reaction
RFLP	Restriction fragment length polymorphism
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SANAS	South African National Accreditation System
SD	Standard deviation
SHMT	Serine hydroxymethyl transferase
SLC19A1	Solute carrier family 19 member 1
SLC46A1	Solute carrier family 46 member 1
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
SNPs	Single nucleotide polymorphisms
SPSS	Statistical Package for the Social Sciences
SSTs	Serum separator tubes
TAFI	Thrombin-activatable fibrinolysis inhibitor
TF	Tissue factor
TGF-β	Transforming growth factor beta
tHcy	Total homocysteine
THF	Tetrahydrofolate
TNF	Tumour necrosis factor
tPA	Tissue plasminogen activator

TYMS	Thymidylate synthase
USA	United States of America
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule-1
VLDL	Very low density lipoprotein
VUT	Vaal University of Technology
WHO	World Health Organisation
$\alpha$ 2-AP	$\alpha$ 2-antiplasmin

## GENERAL ABBREVIATIONS

<i>et al.</i>	Et alii
e.g.	Exempli gratia
etc.	Et cetera.
i.e.	Id est
Inc.	Incorporated
n.d.	Not dated
No.	Number

## MEASUREMENT UNITS

°C	Degree Celsius
$\mu$ g/day	Microgram per day
$\mu$ L	Microlitre
$\mu$ mol/L	Micromole per litre
A	Absorbance
g/day	Gram per day
kb	Kilobites
mg	Milligram
mg/dL	Milligram per decilitre
mL/min	Millilitre per minute
mM	Millimolar
mmHg	Millimetre of mercury
mmol/L	Milimole per litre
ng/ $\mu$ L	Nanogram per microlitre
ng/mL	Nanogram per millilitre

nm	Nanometre
nmol/L	Nanomole per litre
pg/mL	Picogram per millilitre
pmol/L	Picomole per litre
µg/L	Microgram per litre

## **SYMBOLS**

%	Percentage
/	Or
+	Plus or more than
<	Less than
>	More than
±	Plus, minus
≤	Less or equal than

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# CHAPTER 1

## PROBLEM AND SETTINGS

### 1.1. INTRODUCTION

In general, life expectancy is increasing in many parts of the world. There are numerous reasons for this including readily available health care and a better understanding of diseases (Collins and Varmus, 2015:793; Department of health and human services (USA) and WHO, 2011:8). This increase in life expectancy is accompanied by a concomitant growth in the elderly population group (WHO, 2014b:43). Leading causes of death departed from infection and parasitic diseases, towards cardiovascular diseases (CVDs) with more people dying annually from CVDs than any other cause (WHO, 2016a). CVDs can be caused by a prolonged unhealthy lifestyle and is therefore classified as a disease of lifestyle (Oldewage-Theron and Kruger, 2009:300; Labarthe, 2011:7-8; Lv *et al.*, 2017:1117). Increased serum homocysteine (Hcy) is an independent risk factor for CVD (Kuebler *et al.*, 2013:1382). There are two core genetic polymorphisms that act on Hcy regulatory enzymes, namely MTHFR A1298C and MTHFR C677T (Kadhim and Clement, 2011:41; Fekih-Mrissa *et al.*, 2013:465). Little knowledge exists for the prevalence of these genetic factors in any South African population groups (Nienaber-Rousseau *et al.*, 2013a:116; Atadzhanov *et al.*, 2014:194).

### 1.2. GERIATRIC PROFILE

According to the World Health Organisation (WHO), life expectancy increased by more than 3 years per decade since the 1950s (the 1990s being the exception due to the increase in the HIV epidemic and collapse of the soviet Union) (WHO, 2016b:7). The mean life expectancy in 2012 was 70.4 years (WHO, 2014b:42), while in 2015 it was 71.4 (WHO, 2016b:9). Overall, since the year 2000, life expectancy increased in an accelerated fashion in most areas; with a 5 year increase from 2000 to 2015 (WHO, 2016b:7). This trend is also seen in South Africa with life expectancy increasing from 55.2 to 62.4 years between 2004 and 2016 (Statistics South Africa, 2016a).

As mentioned before, cause of death has changed. The leading cause of death in the United States of America (USA) in 1904 was pneumonia and influenza, with tuberculosis being the

2nd leading cause (Centres for Disease Control and Prevention, 2009:66). A similar trend was seen in Australia where infectious diseases caused the most deaths in the early 1900s (Australian Bureau of statistics, 2006). In the past century, there was a decrease in famine, malnutrition, infectious outbreaks and war, all leading to increased life expectancy (Finch, 2010:1719; Kontis *et al.*, 2017:1323). One of the leading causes of death in Southern Africa from the 1980s was the human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS). This had a negative impact on life expectancy that decreased in comparison to the rest of the world (Bor *et al.*, 2013:961). From early 2004 antiretroviral therapies were made available in South Africa with financial assistance from the USA (Bor *et al.*, 2013:965; WHO, 2014b:42). This resulted in a decline in AIDS-mortality rates between 2006 (48%) and 2016 (28%) (Statistics South Africa, 2016a). Life expectancy in South Africa has been increasing since 2004, indicating that a well-designed antiretroviral treatment programme can have an impact on a population (Zaidi *et al.*, 2012; Bor *et al.*, 2013:965). The size of the elderly population in South Africa is increasing (Statistics South Africa, 2016a). In 2003 the elderly (older than 60) constituted about 6.4% of the population in South Africa and increased to 8% in 2016 (Statistics South Africa, 2013; Statistics South Africa, 2016a).

Although the increase in the elderly population is observed in both developed and developing countries the elderly contribute a higher percentage (Table 1) to the total population in developed countries (World Heart Federation, 2014:166). As indicated in Table 1, Japan had the biggest population of elderly (26%) and the highest life expectancy (84 years) in 2015. Angola and Uganda had the smallest elderly population (2%). The life expectancy gains of some developing countries has increased tremendously. For example, Zambia (49 to 61), Gabon (59 to 65) and Sierra Leone (44 to 51).

**Table 1. Comparison of elderly and life expectancy between developed and developing countries between 2005 and 2015 (The World Bank, 2017a; The World Bank, 2017b)**

Developed country					Developing country				
Country	Frequency > 65 (%)		Life Expectancy (years)		Country	Frequency > 65 (%)		Life Expectancy (years)	
	2005	2015	2005	2015		2005	2015	2005	2015
Austria	16	19	79	82	Angola	2	2	49	53
Canada	13	16	80	82	Bahamas	6	8	74	75
Czech Republic	14	18	76	79	Cameroon	3	3	52	56
Denmark	15	19	78	81	Egypt	5	5	69	71



Finland	16	20	79	81	Equatorial Guinea	3	3	54	58
France	17	19	80	83	Gabon	6	5	59	65
Germany	19	21	79	81	Guinea	3	3	53	59
Greece	18	21	79	82	Honduras	4	5	71	73
Ireland	11	13	79	82	Iraq	3	3	68	70
Italy	19	22	81	83	Liberia	3	3	55	61
Japan	20	26	82	84	Maldives	4	5	74	77
Malta	14	19	79	82	Pakistan	4	4	64	66
Netherlands	14	18	79	82	Samoa	5	5	71	74
Norway	15	16	80	82	Saudi Arabia	3	3	73	74
Singapore	8	12	80	82	Sierra Leone	2	3	44	51
Slovenia	15	18	78	81	South Africa	5	5	52	57
Spain	17	19	80	83	Uganda	3	2	52	59
Switzerland	16	18	81	83	Zambia	3	3	49	61
USA	12	15	78	79	Zimbabwe	3	3	42	59
<b>Mean</b>	15.2	18.4	89.3	81.8	<b>Mean</b>	3.7	3.8	59.2	64.2

This increase in the elderly population is due to various factors (health care availability, absence of famine/malnutrition, lack of war) affecting that country's life expectancy (Blagosklonny, 2010:179; Kontis *et al.*, 2017:1328). In some countries, the elderly group 85 years and older will constitute the largest part of the total population. Between 2010 and 2050 it is estimated that the population aged 85 and older will increase by 351% compared to the increase of 188% for age group 65-84 and 22% for age group under 6 (United Nations, 2011).

The elderly play a very important role in the South African society. A person above the age of 60, who meet the criteria, qualifies for the older persons grant (previously old age pension) (South African Government, 2015). In rural households, this money may be the sole income. This completely changes family living standards, causing an unemployed adult to move in with their elderly parents or never move out (Hamoudi and Thomas, 2014:30-31). Some households also provide multigenerational care of young (Finch, 2010:1718; Statistics South Africa, 2016a). The higher incidence of HIV/AIDS in South Africa behoves the elderly (more often women) to take on the role as primary care-giver, looking after orphaned children (Scahtz *et al.*, 2012:1865; Statistics South Africa, 2016a).

### 1.3. PREVALENCE OF CARDIOVASCULAR DISEASE

CVDs, the most common cause of death worldwide (Cramer *et al.*, 2014:170), is responsible for 17.3 million deaths annually (Oluyombo *et al.*, 2016:322). The annual mortality from CVDs

is projected to increase to 22.2 million in 2030 signifying its position as the main cause of death and disability globally (WHO, 2014a). CVDs have replaced infectious diseases as a leading cause of non-communicable disease (NCDs) due to the increase in unhealthy lifestyles. This is part of the theory of epidemiological transition (Labarthe, 2011:7-8; Murray *et al.*, 2015:2145). NCDs are chronic diseases that are not caused by infections and cannot be transmitted between people (Lim *et al.*, 2012:2226; WHO, 2017).

### **1.3.1. Prevalence of CVD globally**

Europe has a mortality rate of 46% for cardiovascular disease (CVD) (Nichols *et al.*, 2013:3029). Coronary heart disease constitutes 20% and cerebrovascular diseases 12%, while 14% is caused by other CVDs (Nichols *et al.*, 2013:3029). A study in Lebanon found that 45% of mortalities due to NCDs were CVDs (Deek *et al.*, 2015:333). In India, CVD is the leading reason for adult and elderly mortality rates (Joshi *et al.*, 2013:140). The prevalence of CVD in India is estimated to be 10 to 12% in urban and 4 to 5% in rural adults (Joshi *et al.*, 2013:140). In the USA one in four deaths are related to heart disease, with coronary heart disease being the most common (Centres for Disease Control and Prevention, 2017).

It is estimated that 80% of elderly at the age of 75 and older suffer from CVD in the USA (Rich, 2014). The American heart and stroke association (2013) estimated that 71% of elderly in the age group 60 to 79 suffer from CVD. In the 80+ age group estimates of CVD are 85%. The risk for CVD increases with age (Rich, 2014). In Europe 35% of the deaths due to CVD are in the age group above 65 (Nichols *et al.*, 2013:3029). A study in Beijing found that in a study group consisting of 2010 individuals, 49% passed away from some form of CVD (Zhou *et al.*, 2014:2203).

### **1.3.2. Prevalence of CVD in Africa**

In sub-Saharan Africa evidence is accumulating that a demographic shift is occurring from infectious diseases to NCDs, mainly CVD, as leading causes of death (Ngoungou *et al.*, 2012:78; Kavishe *et al.*, 2015:127). This in part is due to Sub-Saharan Africans tendency to adopt a western lifestyle and diet, reduce physical activity and potassium intake, with increased salt consumption (Muna, 2013:364). A study in Tanzania and Uganda found that the age-standardized prevalence of hypertension ranged between 19% and 44% (Kavishe *et al.*, 2015:136). This study also reported that in Sub-Saharan Africa the main cause of heart

failure is hypertension and the prevalence of heart failure will increase due to the high burden of uncontrolled arterial hypertension (Kavishe *et al.*, 2015:141).

Africa has limited quantity and quality empirical data available regarding NCDs. There are many needs for an African health ministry to address; a health data information system and country wide surveys are not necessarily priority (Cooper and Bovet, 2013:271). Although in the 2016 World Health Statistics, The World Health Organization did find that a few countries made progress with detailed death registrations (South Africa being one of them), even though 53% of deaths are still unregistered (WHO, 2016b:12).

### **1.3.3. Prevalence of CVD in South Africa**

Six of the top ten causes of death in 2013 were found to be NCDs in South Africa (Statistics South Africa, 2016b). In both 2011 and 2016 the main cause of death was due to infections, but CVD mortality rates are increasing (Statistics South Africa, 2014; Statistics South Africa, 2016b). According to The Heart and Stroke Foundation South Africa (2016:8) heart disease and stroke constitute most of the CVD burden and that the demands on the health care system and economy amounts to millions of Rands annually. The increased CVDs morbidity is seen especially in the elderly age group, although CVD mortality rates in the age group 45 to 64 is also on the rise (Statistics South Africa, 2014; Statistics South Africa, 2016b). A study done by Klug *et al.* (2012:106) supported the information gathered by Statistics South Africa showing that CVD is a major contributor to morbidity in South Africa. Approximately six million South Africans are hypertensive and prior to the study many were unaware of their blood pressure (74% men and 49% women) (Grace and Semple, 2012:375).

Urban black South Africans are more prone to unhealthy behaviours (lower physical activity higher smoking rates and alcohol abuse) when compared to Caucasians (Hamer *et al.*, 2011:240). Furthermore, the black population also have higher levels of sub-clinical vascular disease and other cardiovascular risk (CVR) factors (Hamer *et al.*, 2011:240). Black South Africans are at a higher risk of developing high blood pressure, diseases of the heart muscle or to suffer a stroke (Steyn, 2007:2). A study done by van Zyl *et al.* (2011:1) in the Free State province found that the rural population was at a 40.1% higher risk of getting CVDs than the urban population (34.4%).

CVDs are a problem in the elderly age group (60 and older). NCD caused 62.5% of deaths

in the elderly in 2013 (Statistics South Africa, 2016b), which is an increase from 61.9% in 2011 (Statistics South Africa, 2014). The prevalence of CVD will not be fully known in South Africa as the cause of death on medical certificates are ill-defined (Burger *et al.*, 2012:309). The WHO (2016b:7) did find an improvement in cause of death documentation in South Africa. The increase and growth in the elderly population will result in new challenges that must be addressed. An aging population experiences old age dependency, social and economic vulnerability and increased strain on health and social care (Statistics South Africa, 2016a).

#### **1.4. CONTEXT OF RESEARCH**

This study was conducted on a black elderly population who attend a day care centre in Sharpeville, Gauteng, South Africa. The study sample is part of a multi-nutritional programme at the Vaal University of Technology (VUT); an initiative of the Centre of Sustainable Livelihoods (CSL) led by Prof WH Oldewage-Theron. The Sharpeville day care centre was established in 2004 and in 2009 it received a funding from the National Lottery Foundation of South Africa. The facility provides the elderly with 2 nutritious meals daily as well as various activities including religious activities, skills training and a social programme (National Lotteries Board of South Africa, 2009). The CSL was involved with the centre from its commencement, with Prof Oldewage-Theron managing an integrated nutrition program.

Sharpeville falls within the Sedibeng District Municipality (Sedibeng District Municipality, 2011; Sedibeng District Municipality, 2015). The discovery of the large deposits of coal on the banks of the Vaal River in 1878 led to the industrialization of this area (Acton, 2008). There are iron, steel, coal and oil corporations. The multi-nutritional programme was approved by the ethical committee of the University of the Witwatersrand, Johannesburg (M070126) (Annexure B). For this specific study, additional ethical approval was received from the VUT ethical committee (20140827-1ms) (Annexure C).

#### **1.5. MOTIVATION FOR THE STUDY**

The Sharpeville elderly community has an increased risk for CVD (Oldewage-Theron *et al.*, 2008a:3). Grobler (2015) also found that the same population has a high serum Hcy level. Serum Hcy in the body has been indicated as a causal risk factor or biomarker for CVD (Kuebler *et al.*, 2013:1382). The 5, 10-methylenetetrahydrofolate reductase (MTHFR)

enzyme plays a key role in Hcy metabolism (Blom and Smulders, 2011:77; Kadhim and Clement, 2011:41; Collin, 2013:16; Kirsch *et al.*, 2013:497; Jennings and Willis, 2015:224). There are multiple single nucleotide polymorphisms (SNPs), but the two that reduce the function of the MTHFR enzyme the most (and therefore increase Hcy levels) are MTHFR A1298C and MTHFR C667T (Kim *et al.*, 2011:466; Izmirli, 2013:325).

There is limited information about the prevalence of MTHFR polymorphisms in Southern Africa (Nienaber-Rousseau *et al.*, 2013a:116; Atadzhanov *et al.*, 2014:194). This study is part of the route for eliminating some of the causal factors for hyperhomocysteinemia.

## **1.6. AIM OF THE STUDY**

The aim of the study was to determine the prevalence of the MTHFR A1298C SNP in an elderly black population and to evaluate the correlation of the SNP with serum Hcy metabolic markers (serum Hcy, serum vitamin B6, serum vitamin B12, and serum folate).

## **1.7. OBJECTIVES OF THE STUDY**

The main objectives of this study were:

1. To determine serum Hcy, folate, vitamin B6 and vitamin B12 of the sample population.
2. To determine the prevalence of MTHFR A1298C SNP in the sample population.
3. To establish the correlation of the serum folate, vitamin B6 and vitamin B12 levels with serum Hcy levels.
4. To establish the correlation of the presence of MTHFR A1298C with serum Hcy, folate, vitamin B6 and vitamin B12.

## 1.8. OUTLINE OF DISSERTATION

The dissertation covers the background and problem settings of this study in chapter 1. It also illustrates the gap in scientific knowledge that exists. Aims and objectives are clarified. A reporting of relevant literature is done in chapter 2. CVDs are explained on a pathological level. Risk factors for CVD are also further explored. Hcy, vitamin B6, vitamin B12, folate and the remethylation pathway are also described in more detail. Chapter 3 illustrates the various methodologies that were utilized during the study. In chapter 4 the results obtained from this study are stated. The statistical analysis is also reported here along with the discussion thereof. Main findings are summarised in chapter 5. Recommendations and limitations of this study is also in this chapter. The bibliography contains all the references that were utilized in the writing of this dissertation. The flow of the research project was as illustrated in Figure 1.

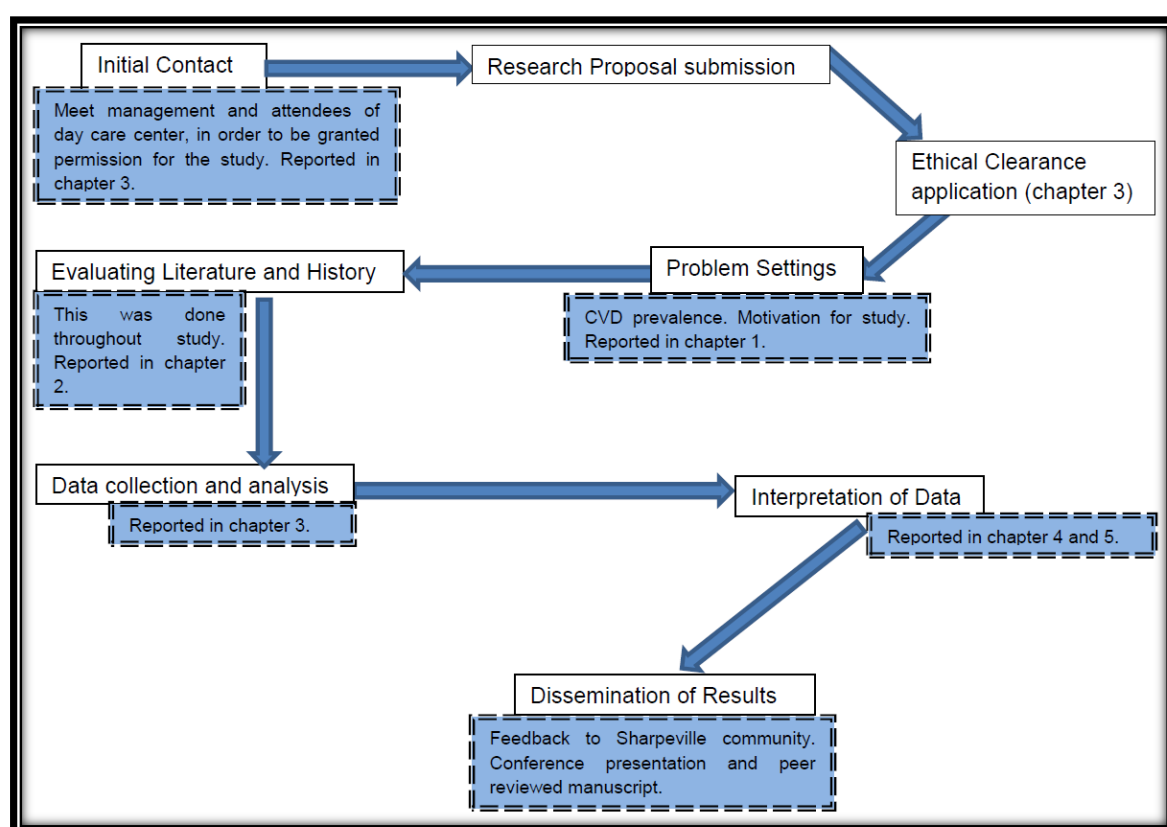


Figure 1. Flow of research project

## 1.9. SIGNIFICANCE OF THE STUDY

The elderly population in South Africa is increasing. Their role in the well-being of South Africa is vital. CVDs have become a fundamental problem worldwide; addressing it is imperative.

Developing countries are also showing this high incidence of CVDs. It is thus important to evaluate the contributing factors that have an impact on the health of the elderly. Elevated levels of serum Hcy has been indicated as a CVR. SNPs in the MTHFR gene play a role in high Hcy levels. This study assessed the prevalence of these SNPs' in a South Africa elderly black population.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1. INTRODUCTION

Life expectancy is increasing globally (see Table 1) due to numerous factors such as more readily available health care and improvements in the understanding of diseases (WHO, 2014b:42). The leading causes of death has shifted from infections towards CVDs (Labarthe, 2011:7-8; Cramer *et al.*, 2014:170; Department of health and human services (USA) and WHO, 2011:6). Understanding the development of CVD and the risk factors that contribute to this disease is key in prevention and treatment of CVDs (Sakakura *et al.*, 2013:399; Zhou *et al.*, 2014:2194). The main factor for majority of CVDs is atherosclerosis (Kampoli *et al.*, 2009:323; Otsuka *et al.*, 2015:773). In Africa, CVDs pose a great problem (due to limited research and implementation of few preventative programmes) (Mocumbi, 2012:74; Cooper and Bovet, 2013:271; WHO, 2016b:12). Hcy is an independent risk factor for the development of CVD (Gotlieb and Liu, 2012:454; Kuebler *et al.*, 2013:1382). Hyperhomocysteinemia is a condition characterised by increased serum Hcy levels (Kaur and Sekhon, 2013:1). Hcy levels are influenced by deficiencies of folate, vitamin B6 and vitamin B12 to which the elderly are susceptible (Ng *et al.*, 2012:1362). There are two core SNPs that affect Hcy regulatory enzymes, namely, methylenetetrahydrofolate reductase (MTHFR) A1298C and MTHFR C677T (Kadhim and Clement, 2011:41; Kim *et al.*, 2011:466; Izmirli, 2013:325). Information on the prevalence of these genetic factors in a South African population is very limited (Nienaber-Rousseau *et al.*, 2013a:116; Atadzhanov *et al.*, 2014:194).

#### 2.2. CARDIOVASCULAR DISEASE

CVD is the group name for diseases of the heart and/or blood vessels (WHO, 2011:3; WHO, 2018). Some examples of CVD are: stroke, congenital heart disease, rheumatic heart disease, cardiomyopathy, endocarditis, ischemic heart disease, hypertensive heart disease, atrial fibrillation, aortic aneurysms and peripheral artery disease (WHO, 2011; World Heart Federation, 2014; Mortality and Causes of Death, 2015).



### 2.2.1. Pathophysiology of cardiovascular disease

The pathophysiology of CVD depends on the specific disease: peripheral artery disease, ischemic heart disease and stroke, all involve atherosclerosis. Atherosclerosis is the major precursor for most CVDs (Kampoli *et al.*, 2009:323; Otsuka *et al.*, 2015:773). The word atherosclerosis broken down into its Greek constituents “athere” means gruel or porridge, while “scleros” means hard (Damjanov, 2012:137; Oxford dictionary, 2018). This describes the simultaneously hardening and weakening of the arteries (plaque formation).

Atherosclerosis is characterized by arterial wall thickening and a loss of elasticity (Kumar *et al.*, 2015:496; Yahagi *et al.*, 2017:191). An atherosclerotic plaque is a projection into the vessel lumen (Mudau *et al.*, 2012:222; Kumar *et al.*, 2015:496) and consists of a soft yellow lesion that is made mostly of lipids and covered with a white fibrous cap (Gallagher and van der Wal, 2013:249). The atherosclerotic plaque leads to clinically important complications, including mechanical obstruction of the blood vessel, thrombosis and weakening of the underlying media – leading to the formation of an aneurysm (Kumar *et al.*, 2015:496).

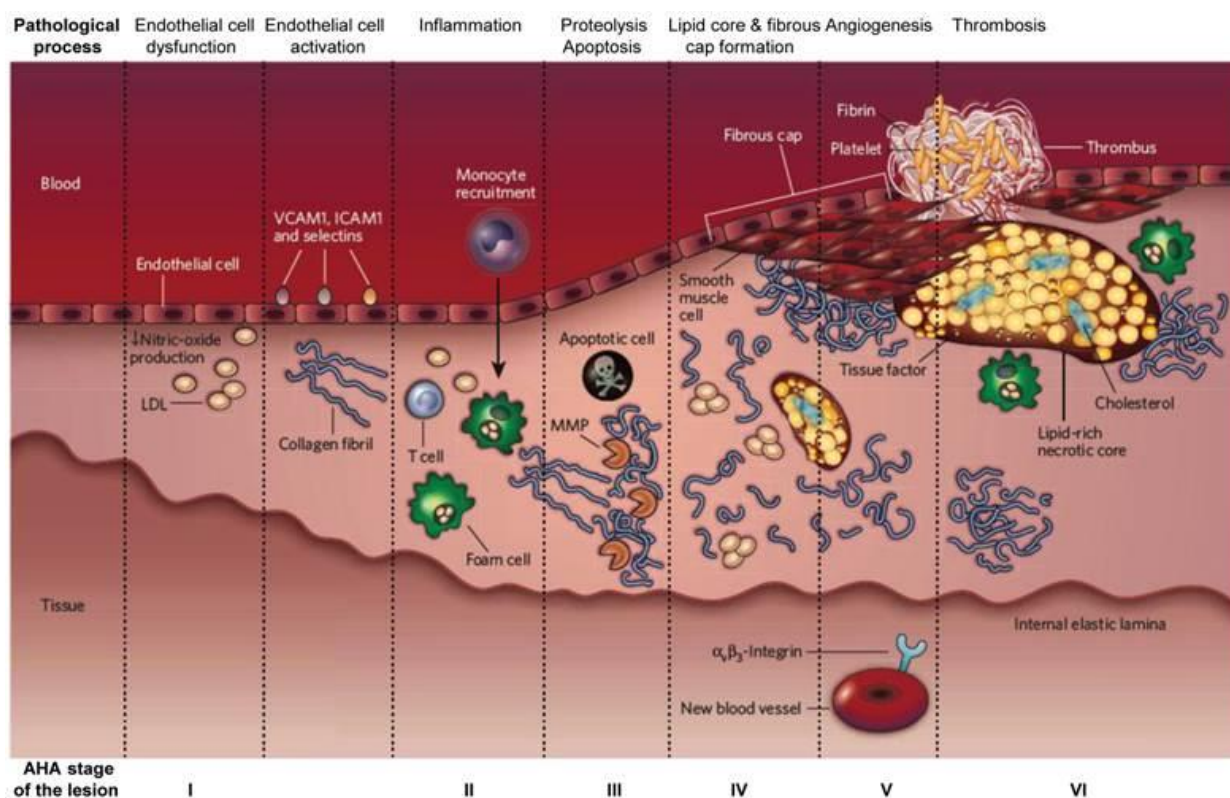
### 2.2.2. Atherosclerosis pathogenesis

In the past there were two hypotheses for the pathogenesis of atherosclerosis. The one focuses on intimal cellular proliferation while the other emphasizes repetitive formation and organisation of thrombi (Kumar *et al.*, 2015:499). These have been combined to form the response-to-injury hypotheses, which also considers the effect of CVD contributing risk factors (Winckers *et al.*, 2013:120; Kumar *et al.*, 2015:499). This model proposes atherosclerosis as a chronic inflammatory and healing response of the arterial wall to endothelial injury (Mudau *et al.*, 2012:222; Gallagher and van der Wal, 2013:252). There are interactions between modified lipoproteins, T-lymphocytes and monocyte-derived macrophages with the normal elements of the blood vessel wall (Kumar *et al.*, 2015:499).

Certain arteries (i.e. coronary, carotid, abdominal and descending aorta, and iliac artery) are more prone to plaque development (Sakakura *et al.*, 2013:400). One reason for this is the hemodynamic disturbances in these vessels (especially at vessel bifurcation) as flow of blood is more turbulent, resulting in endothelial dysfunction (Kumar *et al.*, 2015:500). Endothelial dysfunction is also caused by the chronic exposure to CVR factors. This along with the harmful circulating stimuli associated

with these conditions, overwhelm defence mechanisms of the vascular endothelium, compromising integrity (Mudau *et al.*, 2012:222).

Plaque formation is a two-step process: firstly there is an injury to the arterial wall and secondly the response of the blood vessel wall to the injury causing agent (Gallagher and van der Wal, 2013:252). Figure 2 describes the progression of atherosclerosis. In response to an injury the endothelial cells release adhesion molecules (e.g. vascular cell adhesion molecule-1 (VCAM), P-selectin and intracellular adhesion molecule-1 (ICAM)) (Hirase and Node, 2012:H499; Jager *et al.*, 2012:622; Winckers *et al.*, 2013:120). The endothelial wall becomes more permeable due to the release of inflammatory mediators, allowing inflammatory factors and lipids to enter and accumulate in the intima (Gallagher and van der Wal, 2013:252; Kumar *et al.*, 2015:500). The cell adhesion molecules attracts monocytes to the site of injury (Gallagher and van der Wal, 2013:252) which migrate into intima where they proliferate to macrophages (Kumar *et al.*, 2015:499).



**Figure 2. Progression of atherosclerosis. American Heart Association (AHA) stages of the disease is indicated at the bottom of each corresponding column (Orbay *et al.*, 2013:895).**

Toxic oxygen free radicals generated during the inflammatory process results in oxidation of low density lipoprotein (LDL) which has accumulated in the tunica intima of the vessel (Brasher, 2012:595). These are also sometimes referred to as pro-inflammatory lipids (Brasher, 2012:595).

Macrophages phagocytise the oxidised LDL (Damjanov, 2012:137). These macrophages will proliferate to foam cells (Brasher, 2012:595). When significant amounts of these foam cells are present, a lesion known as a fatty streak is formed (Brasher, 2012:595). These lesions are in the arteries of most people, even young children (Kampoli *et al.*, 2009:323; Torzewski and Bhakdi, 2013:22). More toxic oxygen free radicals are produced by the fatty streaks, resulting in recruitment of T-cell leading to autoimmunity. Additional inflammatory factors are produced (macrophages secrete cytokines [interleukin-1 (IL-1) and tumour necrosis factor (specifically TNF- $\alpha$ )] and chemokines [monocyte chemotactic protein-1] affecting the vessel wall; causing progressive damage (Brasher, 2012:595; Winckers *et al.*, 2013:120).

The released Platelet Derived Growth Factor and macrophage growth factor will result in Smooth Muscle Cell (SMC) migration from the tunica media to the tunica intima (middle and inner layers of a blood vessel), over the fatty streak (Brasher, 2012:595; Kumar *et al.*, 2015:500). The SMC in healthy arteries are found in the tunica media and are primarily quiescent and highly differentiated (Allahverdian *et al.*, 2018:540). These cells retain a high degree of dedifferentiation potential and plasticity and can shift from a contractile to a so-called synthetic phenotype. SMC will proliferate to foam cells resulting in the fibrous plaque formation (Allahverdian *et al.*, 2018:540). The SMC are stimulated by the growth factors to produce elastin, collagen and mucopolysaccharide (Gallagher and van der Wal, 2013:252).

The components of the fibrous cap are SMC, macrophages, foam cells, lymphocytes, collagen, elastin and proteoglycans (Kumar *et al.*, 2015:496). The necrotic centre/lesion consists of cell debris, foam cells, cholesterol crystals and calcium (Kumar *et al.*, 2015:496). The collagen results in the hardening of the arteries (Damjanov, 2012:137). The foam cells and lipid laden macrophages will undergo apoptosis, spilling their lipids into a growing lipid core, resulting in a continuous lipid accumulation occurring intracellularly as the macrophages will phagocytise the spilled lipids (Kumar *et al.*, 2015:499). The fibrous plaque can sometimes calcify and protrude into the blood vessel, possibly blocking blood flow to tissues and cause symptoms like angina (Brasher, 2012:595). The formation of the foam cells stimulate the release of TNF, cytokines, prothrombic factors, proteases and transforming growth factor beta (TGF- $\beta$ ) (Damjanov, 2012:137; Tavakoli and Asmis, 2012:1786). These factors result in the production of more inflammatory factors and SMC migrating to the site, causing the plaque to expand (Tavakoli and Asmis, 2012:1786).

Acute and chronic inflammation results in local acidification (Rajamaki *et al.*, 2013:13410; Oorni *et al.*, 2015:203). This is due to inflammatory cell infiltration and activation in the affected tissue, which

demands an increase in energy and oxygen (Rajamaki *et al.*, 2013:13410; Oorni *et al.*, 2015:203). Alternative energy is produced via glycolysis, resulting in acidification of extracellular fluid as both aerobic and anaerobic glycolysis cause lactic acid secretion (Rajamaki *et al.*, 2013:13410; Oorni *et al.*, 2015:203). Another factor that results in an acidic pH is the proliferation of SMC which stimulates glycolysis (Oorni *et al.*, 2015:203). The thicker intima (due to atherogenesis) may also have an effect on the pH of an atherosclerotic plaque, by often being hypoxic (due to a lack of capillaries and lymphatics), resulting in a restricted supply of oxygen and nutrition – cells are thus more reliant on glycolysis for energy production (Oorni *et al.*, 2015:203). In freshly removed human carotid atherosclerotic plaques the pH was found to be as low as 6.8 (Rajamaki *et al.*, 2013:13410).

Haemorrhaging of microvessels (within the plaque) enhances rapid growth of the plaque (Gallagher and van der Wal, 2013:252). A plaque that is prone to rupture is known as an unstable plaque whereas a ruptured one is referred to as a complicated plaque (Brasher, 2012:595). At a point where the fibrous plaque cap is thin (thinned by the inflammatory mechanisms) the necrotic core will rupture, initiating the coagulation cascade (Tavakoli and Asmis, 2012:1786). Tissue factor (TF) is a glycoprotein, on the cell's surface, that activates the extrinsic pathway of coagulation and is not found in healthy endothelial cells (found on the inner cells of blood vessel, only damaged vessels will have TF), binding to activated factor VII (VIIa) to form TF-VIIa, in turn activating factor X to factor Xa (Winckers *et al.*, 2013:120).

A small amount of thrombin is produced (from prothrombin) as a result of factor Xa, in turn activating factor XI which results in factor IX being active. Factor IXa (along with co-factor VIIIa) creates more factor Xa and consequently enough thrombin is produced to convert fibrinogen to fibrin (Winckers *et al.*, 2013:120). This results in a fibrous gel formation: a meshwork tangling red blood cells and ultimately a thrombus is formed (Tavakoli and Asmis, 2012:1786; Marieb, 2014:373). TF is overexpressed by the macrophages and SMC, enhancing pro-coagulation activity (Sueishi *et al.*, 1998:120; Winckers *et al.*, 2013). Other factors (discussed in 2.2.3.2.k.) can also cause hypercoagulability, increasing arteriosclerosis formation and thus increasing the risk for CVD.

During the healing of the injury site fibrinolysis (breaking and removal of the clot) will occur (Rote, 2012:135). The fibrinolytic pathway is activated by tissue plasminogen activator (tPA), which converts plasminogen into plasmin (Kleinegris *et al.*, 2012:558). Plasmin will in turn degrade fibrin and fibrinogen, thus removing the blood clot as the vessel heals (Kleinegris *et al.*, 2012:558). This pathway is regulated by plasminogen activator inhibitor-1 (PAI-1), thrombin-activatable fibrinolysis inhibitor (TAFI) and  $\alpha$ 2-antiplasmin ( $\alpha$ 2-AP) (Kleinegris *et al.*, 2012:558). tPA is inhibited by PAI-1, plasmin is inhibited by  $\alpha$ 2-AP and TAFI modifies fibrin, making it more resistant to plasmin activity

(Kleinegris *et al.*, 2012:558). If the thrombus dislodges it is known as an embolus, which if not dissolved can lead to blockage of a distal vessel (resulting in an embolism) (Kumar *et al.*, 2015:126). Decreased fibrinolytic factors (or when there is no balance between coagulation and fibrinolysis pathways) contributes to atherogenesis and is another risk factor for CVD.

### 2.2.3. Cardiovascular risk factors

A range of risk factors contribute to CVD and are grouped as non-modifiable and modifiable factors as indicated in Table 2 (Brasher, 2012: 599; Damjanov, 2012: 138; Gallagher and van der Wal, 2013:258; World Heart Federation, 2014; Kumar *et al.*, 2015: 496). Psychosocial and geographic factors are additional groupings (Kralova Lesna *et al.*, 2015:192; Lagraauw *et al.*, 2015:2; Shivappa *et al.*, 2015:665; Mishra, 2017:427; Psaltopoulou *et al.*, 2017:33).

**Table 2. Cardiovascular risk factors (World Heart Federation, 2014; Grobler, 2015:23; Kralova Lesna *et al.*, 2015:192; Lagraauw *et al.*, 2015:2; Shivappa *et al.*, 2015:665; Mishra, 2017:427; Psaltopoulou *et al.*, 2017:33).**

Non-modifiable risk factors	<ul style="list-style-type: none"> <li>• Male gender</li> <li>• Age</li> <li>• Genetically inherited factors</li> </ul>
Modifiable risk factors	<ul style="list-style-type: none"> <li>• Hypertension</li> <li>• Smoking</li> <li>• Obesity</li> <li>• Diabetes mellitus</li> <li>• Physical inactivity</li> <li>• Hyperlipidaemia</li> <li>• Hyperhomocysteinemia</li> <li>• Inflammatory status</li> <li>• Infection</li> <li>• Thrombophilia (or reduced fibrinolysis)</li> </ul>
Psychosocial factors	<ul style="list-style-type: none"> <li>• Low socioeconomic status</li> <li>• Diet</li> <li>• Stressful environment</li> </ul>
Geographic	<ul style="list-style-type: none"> <li>• Seasonal changes</li> <li>• Environmental pollution</li> </ul>

#### 2.2.3.1. Non-modifiable risk factors

These are risk factors that are congenital and cannot be changed and includes age, gender and genetics (Kumar *et al.*, 2015: 496).

### **a. Age**

Atherosclerosis is a progressive disorder and does not clinically manifest until middle age or later (Kumar *et al.*, 2015:496). An elderly person has poor endothelial healing with prolonged exposure to various risk factors (Brasher, 2012:598). Apart from the poor healing there is also alterations in blood vessels, which increases the probability of a cardiovascular event (North and Sinclair, 2012:1098).

Some of the consequences of vascular aging is a progressive loss of elasticity, tunica intima- and media thickening (Wang and Bennett, 2012:245). Vascular stiffness and hypertension is caused by a decrease in elastin and increased deposition of collagen in aged vessels (Wang and Bennett, 2012:245). With age there is also a progression in the dysfunction of SMC and vascular endothelium; SMC have an increased migration and proliferation pattern, while vascular endothelium is more prone to vasospasms, inflammatory reaction, thrombosis and penetration of macrophages, contributing to atherosclerosis progression (Herrera *et al.*, 2010:142; Monk and George, 2015:417).

### **b. Gender**

Atherosclerosis is slowed down by estrogen as it alters lipid profile by increasing high density lipoprotein (HDL), nitric oxide (NO) production is increased (a vasodilator), decreases oxidative stress and SMC proliferation is slowed down (Gill, 2015:541; Mathur *et al.*, 2015:321). Estrogen is responsible for this protective effect in females against CVD (Ski *et al.*, 2014:255) and therefore premenopausal women have a lower risk in comparison to men and postmenopausal women (Damjanov, 2012:139; Kumar *et al.*, 2015:496). On average a CVD event occurs 10 years later in premenopausal women than in men (Mathur *et al.*, 2015:321).

Hormone therapy trials have shown that the atheroprotective effect of estrogen is related to the age at which therapy was started, although some hormone therapy clinical trials in vascular disease prevention have failed (Kumar *et al.*, 2015:497). A testosterone deficiency leads to adipocyte deposition and even obesity which is a risk factor for CVD as it negatively affects the lipid profile, results in chronic inflammation and diabetes mellitus development (Heyman *et al.*, 2010:94; Fuentes *et al.*, 2013:2; Rosen *et al.*, 2015:49). Hypogonadism (underactive gonads; decreased levels of testosterone or estrogen and progesterone) has a correlation with insulin resistance and CVR (Heyman *et al.*, 2010:94; Tambo *et al.*, 2016:2).

Pregnancy complications increases a women's risk for a CVD event. For example, multiple



miscarriages are an independent risk factor for myocardial infarction, while 15-60% of women diagnosed with gestational diabetes develop type 2 diabetes within five years (Gill, 2015:542). Moreover, preeclampsia (hypertension with signs of damage to organs, often kidneys; resulting in protein in urine and fluid retention) increases a women's risk four times for hypertension and doubles the risk for stroke postpartum (Mayo Clinic, 2014; Gill, 2015:542). Furthermore, polycystic ovarian syndrome has been correlated with atherosclerosis, a higher incidence of obesity, dyslipidaemia and diabetes mellitus, are all factors related to increased risk for CVD (Rosen *et al.*, 2015:49).

### **c. Genetics**

Genetics is one of the most significant non-modifiable risk factor. Many Mendelian disorders including familial hypercholesterolemia, hyperhomocysteinemia, hypertension, obesity and diabetes mellitus are associated with atherosclerosis (Kumar *et al.*, 2015:497). An individual that has a father or brother who had myocardial infarction before the age of 55 or a mother or sister before the age of 65, has an increased risk for developing CVD (World Heart Federation, 2014). Familial predisposition to atherosclerosis is usually multi-factorial, with inheritance of various genetic polymorphisms (Kumar *et al.*, 2015:497) and inheritance is polygenic (Damjanov, 2012:139). Multiple polymorphisms have been identified as contributing factors to CVR and include MTHFR C667T/A1298C (hyperhomocysteinemia), AGT M235T (hypertension), TFN- $\alpha$  G308A (pro-inflammatory), PCSK9 A522T (increased LDL levels) and rs9939609 FTO (obesity predisposition)) (Davignon *et al.*, 2010:309; Evans and Goedecke, 2011:5; Badr *et al.*, 2012:1226; Zappacosta *et al.*, 2014:1; Smolková *et al.*, 2015:20).

### **2.2.3.2. Modifiable risk factors**

#### **a. Hypertension**

Hypertension is a major risk factor for CVD (Gallagher and van der Wal, 2013:255) and considerably contributes to atherosclerosis causing thickening of the tunica media (Gallagher and van der Wal, 2013:258; Kumar *et al.*, 2015:492). Smaller arteries and arterioles are more affected by hypertension (Gallagher and van der Wal, 2013:255). Hypertension also results in protein deposition in the arterial walls which includes fibrinogen (Gallagher and van der Wal, 2013:255). Hypertension increases the risk for coronary artery disease (CAD), ischaemic heart disease, heart failure, ventricular arrhythmias and atrial fibrillation (Gallagher and van der Wal, 2013:255). Control of blood pressure significantly reduces the risk (Gotlieb and Liu, 2012:453).

The sodium-potassium pump and sodium-calcium exchanger are two membrane proteins which removes sodium/imports potassium and remove calcium/import sodium, respectively, into and from the cell (Jaitovich and Bertorello, 2010:73; Hauck and Frishman, 2012:130). Increased sodium levels result in endogenous ouabain (a cardiac glycoside) to be secreted by the adrenal cortex inhibiting the sodium-potassium pump, resulting in increased sodium intracellularly. The sodium-calcium exchanger will be inhibited, calcium levels will increase in the cell, leading to increased vascular contraction and essential hypertension (Jaitovich and Bertorello, 2010:73; Hauck and Frishman, 2012:130).

When hypertension is the only risk factor it increases the risk for ischaemic heart disease by 60% (Kumar *et al.*, 2015:497). If hypertension develops at an early age and not controlled atherosclerosis formation is accelerated (Damjanov, 2012:139). Hypertension is classified as grade 1 (mild: 140/90-159/99mmHg), grade 2 (moderate: 160/100-179/109mmHg) and grade 3 (severe: more than 180/110mmHg) (Gallagher and van der Wal, 2013:255). Diastolic and systolic pressures both contribute to the increased risk for CVD (Kumar *et al.*, 2015:492). Blood pressure tends to increase with age while many black communities have a high incidence of blood pressure (Kumar *et al.*, 2015:492). There is a positive correlation between obesity and high blood pressure, while weight loss significantly correlates with a decrease in blood pressure (Gallagher and van der Wal, 2013:256).

## **b. Dyslipidaemia**

Lipids are found in the blood in various forms (cholesterol, triglycerides and phospholids) and as they are insoluble in water, they bind to proteins (known as lipoprotein) in order to move through the cell membrane (Mandal, 2012). Dyslipidaemia (modified circulatory lipids and lipoproteins) markedly increases the risk for CVD by stimulating plaque lesion formation. For example, when LDL accumulates within the tunica intima (due to increased plasma levels) and is oxidised (Brasher, 2012:595; Kumar *et al.*, 2015:497). High intake of cholesterol and saturated fats from sources such as egg yolks, butter, animal fats raises plasma cholesterol levels, while diets low in cholesterol, high in polyunsaturated fats and omega-3 fatty acids decreases plasma cholesterol levels (Kumar *et al.*, 2015:497).

Low density lipoprotein cholesterol carries the majority of cholesterol in the blood and delivers cholesterol to the peripheral tissues, while HDL causes cholesterol to move from tissues to the liver for excretion in bile (Kumar *et al.*, 2015:497). HDL is an important anti-atherogenic and is inversely



correlated with CVR (Soran *et al.*, 2015:248). Exercise and moderate alcohol use increases HDL levels, while obesity and smoking decreases it and vice versa (Kumar *et al.*, 2015:497).

### **c. Hyperhomocysteinemia**

McCully was the first to find a correlation between increased serum Hcy levels and CVD incidence in 1969 (McCully, 1969; Kaur and Sekhon, 2013), Hcy is considered an independent risk factor for CVD (Gotlieb and Liu, 2012:454) and implicated as a promoter of early atherosclerosis pathogenesis (Shenoy *et al.*, 2014:341; Ganguly and Alam, 2015:5). Hyperhomocysteinemia is a condition where serum Hcy levels are more than 15  $\mu\text{mol/L}$  (Kaur and Sekhon, 2013:1). Moderately increased Hcy can also be caused by diseases such as renal and thyroid dysfunction, cancer, psoriasis, and diabetes, drugs including coffee, tobacco, and alcohol, while older age and menopause also have an influence on its levels (Ganguly and Alam, 2015:2). The kidney is the organ that is mainly responsible for removal of Hcy from the blood, which would explain why patients with atherothrombotic vascular disease with chronic renal failure have higher Hcy concentrations than patients without chronic renal failure (Ganguly and Alam, 2015:2). Pathophysiology of Hcy will be discussed in more detail in 2.3.3.

### **d. Obesity**

Obesity leads to a chronic inflammatory status with the activation of several pro-inflammatory signalling pathways (Fuentes *et al.*, 2013:2). Obesity is a contributing factor to insulin resistance (due to insulin receptor damage by adiponectin, growth factors and cytokines), decreased HDL levels, hypertension and changes in adipokine levels, thus increasing risk for a CVD event occurring (Heyman *et al.*, 2010:93; Brasher, 2012:599; Fuentes *et al.*, 2013:2). Adipose tissue, once considered only as storage for excess calories, also has an endocrine function and secretes hormones (e.g. leptin and adiponectin), growth factors and cytokines, all of which actively contributes to development of CVD and type 2 diabetes mellitus (Heyman *et al.*, 2010:93; Brasher, 2012:599; Fuentes *et al.*, 2013:2).

Obesity therefore results in increased levels of leptin and decreased levels of adiponectin, contributing to hypertension and diabetes, reduced anti-inflammatory effect and endothelial protection (Brasher, 2012:599). Adipokine levels can be improved with a healthy diet, exercise and weight loss (Brasher, 2012:599). Adipocytes also release inflammatory factors (e.g. TNF- $\alpha$ , interleukin-6 (IL-6)) resulting in chronic inflammation (Heyman *et al.*, 2010:94; Parto and Lavie,

2017:378). Non-esterified fatty acid, another product of adipocytes, is transported to the liver, where it is converted to triglycerides and very low density lipoprotein (VLDL) resulting in hyperlipidaemia and inflammation (Heyman *et al.*, 2010:94; Nelson, 2013). Inflammatory mediators contribute to insulin resistance. For example, the pancreas increases insulin production and stimulates the sympathetic nervous system causing sodium and water retention, fuelling vasoconstriction, and contributing to hypertension (Heyman *et al.*, 2010:94; Parto and Lavie, 2017:378).

An overweight person has a higher risk of developing type 2 diabetes mellitus as adiponectin, growth factors and cytokines contribute to damage of insulin receptors on the cell surface thus leading to insulin resistance (Heyman *et al.*, 2010:93; Brasher, 2012:599; Fuentes *et al.*, 2013:2). A diabetic has two to four fold increased risk of a CVD event, with 80% of diabetic associated deaths being caused by a CVD (Funk *et al.*, 2012:2).

#### **e. Inflammatory status**

The inflammatory response recruits active immune cells and factors to the site of injury in order to remove pathogens/damaged cells and is involved in the pathogenesis of CVD, steatohepatitis (fatty liver), insulin resistance and diabetes mellitus type 2 (Fuentes *et al.*, 2013:2). Inflammation also plays a role in all the stages of atherosclerosis (Willerson and Ridker, 2004:II2; Koenig, 2013:5126); from the inflammatory mediators attaching to the dysfunctional endothelium to eventually assisting in the formation of the atherosclerotic lesion (Willerson and Ridker, 2004:II2; Libby, 2012:2046). Macrophages also contribute to thrombotic complications of atherosclerosis by releasing proteinases that catabolize collagen, a significant component of the plaque's fibrous cap, making it vulnerable to rupture (Willerson and Ridker, 2004:II2; Libby, 2012:2047).

C-reactive protein (CRP) is mostly produced in the liver (Brasher, 2012:599) and has a high affinity for phospholipids; increasing 20-30 fold during inflammation/infection and is thus a good marker for systematic inflammation (Koenig, 2013:5127). The function of CRP is to assist with opsonisation (singling out of an antigen for the immune system) of pathogens by binding to phosphorylcholine on its surface (Bray *et al.*, 2016:319). TNF- $\alpha$  and IL-6 (pro-inflammatory cytokines) stimulate the production of CRP (Bray *et al.*, 2016:319; Möller *et al.*, 2016:40). Increased levels of TNF- $\alpha$ , CRP and IL-6 are thus independent risk factors for CVD (Möller *et al.*, 2016:40). CRP stimulates TF from SMC, PAI-1 activity increases and tPA inhibition occurs resulting in a pro-thrombotic state and impaired fibrinolysis (Koenig, 2013:5127).

## **f. Smoking**

Prolonged smoking (one or more pack/s a day) doubles the risk for ischaemic heart disease (Kumar *et al.*, 2015:498). Indirect smoking (from environment) also increases the risk for CAD (Brasher, 2012:599). Smoking increases blood pressure by stimulating the release of epinephrine and norepinephrine, and in turn increasing heart rate and peripheral vessel constriction (Brasher, 2012:599). Smoking increases LDL, decreases HDL and generates oxygen free radicals (Brasher, 2012:599). Anti-smoking campaigns have markedly decreased atherosclerosis, but in far Eastern countries, where there are no campaigns and smoking is more prevalent, atherosclerosis incidence is still increasing (Damjanov, 2012:140).

## **g. Diabetes mellitus**

Diabetes mellitus is the group name for diseases that result in a high blood glucose level caused by the pancreatic cells not producing enough insulin (type 1) or cells not responding to insulin (type 2) (Kolluru *et al.*, 2012:3). Aretaeus of Cappadocia (ancient Greek physician) coined the term diabetes in connection with the copious urine production and Thomas Willis (English physician in 1645) coined diabetes mellitus, literally honey-sweet urine due to glucose in the urine (Funk *et al.*, 2012:2). Diabetes mellitus causes hypercholesterolemia and increases the risk for atherosclerosis (Kumar *et al.*, 2015:498). Insulin resistance and diabetes mellitus causes endothelial damage, inflammation, thrombosis and decreases endothelial-derived vasodilators production (Brasher, 2012:599). There is an increase in the production of reactive oxygen species (vascular production of superoxide) in diabetes mellitus which results in vascular dysfunction (Kolluru *et al.*, 2012:4). In children with type 1 diabetes, intimal medial thickness of carotid arteries is enhanced in contrast with non-diabetic children, thus contributing to arterogenesis from an early age (Funk *et al.*, 2012:3). When atherosclerotic plaques of a type 1 and type 2 are compared, it is found that type 2 diabetes enhances atherosclerosis formation with an increased plaque burden in distal vessels (Funk *et al.*, 2012:3).

## **h. Physical inactivity**

Physical inactivity increases the risk for obesity and is also an independent risk factor for CAD (Brasher, 2012:599). By being physically active a person not only reduces the risk for cancer, CVD and mental illness, but also improves quality of life (Rangul *et al.*, 2012:2). Low physical activity results in reduced CVD mortality when compared to complete inactivity (Murer *et al.*, 2012:3). Exercise increases HDL levels and also improves triglyceride levels (Rangul *et al.*, 2012:5). In a

meta-analysis, yoga was found to decrease both systolic and diastolic blood pressures (reduced by 5.85/4.12mmHg) and improved heart rate (Cramer *et al.*, 2014:178).

### **i. Infection**

Some infectious agents contribute to atherosclerosis (Gotlieb and Liu, 2012:454). *Chlamydia pneumonia*, *Helicobacter pylori*, herpes virus and cytomegalovirus' deoxyribonucleic acid (DNA) have been found in human atherosclerotic plaques (Gotlieb and Liu, 2012:454). An untreated *streptococcal* infection of the throat may result in rheumatic heart disease (WHO, 2011; Sika-Paotonu *et al.*, 2017). Periodontal disease also increases CVR (Brasher, 2012:599). Serum antibodies that are bound to microorganisms have also been linked to increased risk of CAD (Brasher, 2012:599). Infections stimulate the immune system and there is thus a pro-inflammatory state in the body (Lim *et al.*, 2015:551), which increases plaque formation and growth (Willerson and Ridker, 2004:II2; Koenig, 2013:5127).

### **j. Hypercoagulability**

Hypercoagulability is the proneness to develop a blood clot when there are abnormalities in the coagulation cascade (Synonyms.net, 2015:21; Marschner *et al.*, 2018) and it can be caused either by a congenital or acquired condition (Cohoon and Heit, 2014:254). Examples of acquired factors that adversely influence coagulation are active cancer, autoimmune disorders (e.g. celiac disease, inflammatory bowel disease, multiple sclerosis, pernicious anaemia and thyroiditis), nephrotic syndrome, chemotherapy, dehydration, dyslipidemia, obesity, pregnancy/post-partum, hyperhomocysteinemia, heparin-induced thrombocytopenia and infection (Cohoon and Heit, 2014:255). Examples of congenital factors include deficiencies of anti-thrombin, protein S, protein C, Factor V leiden (resistant to protein C); increased levels of fibrinogen, prothrombin, factor VII, IX and XI; sickle cell disease and dysfibrinogenemia (Cohoon and Heit, 2014:256). The fibrinolysis pathway can also be disrupted and can be due to reduced tPA, increased PAI-1, TF pathway inhibitor deficiency, increased TAFI and factor XIII polymorphisms (Cohoon and Heit, 2014:256).

Smoking, dyslipidaemia, hyperhomocysteinemia and hypertension alters the thrombus by making it more compact with thinner fibres and causes faster fibrin formation. Therefore the clot is less permeable to fibrinolytic factors and takes a longer time to lyse (Undas, 2011:313; Barua and Ambrose, 2013:1462). A correlation exists between thrombophilia and systemic inflammation. Furthermore IL-6 has a prothrombic effect and decreases anti-thrombin III, promoting platelet

aggregation and increasing tPA and PAI-1 production by endothelium (Iwaki *et al.*, 2012:292). Moreover, PAI-1 is an aggravating factor in systemic inflammation and is also known as an acute phase protein (Iwaki *et al.*, 2012:292).

### **2.2.3.3. Psychosocial risk factors**

#### **a. Socioeconomic status**

Socioeconomic status is an economic and sociological combined total measure of a person's work experience and of an individual's or family's economic and social position in relation to others, based on income, education, and occupation (National Center for Educational Statistics, 2008). Low socioeconomic status is a CVR factor as people of this income group are experiencing high rates of CVD burden and mortality (Ski *et al.*, 2014:256). Cardiovascular risk factors used to be more prevalent among people of higher socioeconomic status in developed countries, but in the past 50 years this was reversed (Mendis *et al.*, 2011; Psaltopoulou *et al.*, 2017:33). Disadvantaged groups are more exposed to CVD risks e.g. smoking, alcohol consumption, physical inactivity and poor diet (Ski *et al.*, 2014:256). The illiterate are also at a higher risk for CVDs (Zhou *et al.*, 2014:2199). CVD mortality is lower for elderly residing in a suburban area when compared to elderly in a rural residence (Zhou *et al.*, 2014:2199). Moderate and high levels of education, with higher than average income, leads to healthier life choices i.e. higher levels of physical activity (Murer *et al.*, 2012:4).

#### **b. Diet**

A diet rich in saturated fatty acids, trans fatty acids and cholesterol increases LDL and atherosclerosis, while a diet enriched with fruits, vegetables, omega-3 and -6 fatty acids and fibre lowers CVR (Oliveira *et al.*, 2011:246; Shivappa *et al.*, 2015:665). Additionally, a diet comprising of the aforementioned food items with a low intake of alcohol and red meat is correlated with a reduction in inflammatory markers (Oliveira *et al.*, 2011:246; Shivappa *et al.*, 2015:665). There is a well-established correlation between salt intake and hypertension, with a clear dose-response relationship (Morrison and Ness, 2011:71; Cobb *et al.*, 2014:1173). Sodium is an essential nutrient and is mostly obtained from salt (sodium chloride). Controversy exists here, because a correlation has been found between CVD mortality and the recommended daily allowance (RDA) of salt, as too little sodium results in a risk of developing hyponatremia, a condition with its own clinical manifestations and complications (O'Donnell *et al.*, 2014:529; Sahay and Sahay, 2014:760). Setting an ideal RDA for salt is difficult and needs more randomised trial studies. Antioxidant rich foods have been shown

according to studies to reduce risk for a CVD event. Good examples of antioxidants and antioxidant rich foods are red wine (reduces reactive oxygen species), vitamin C (decreases pro-inflammatory cytokines, endothelial dysfunction improvement and reduces oxidative stress), vitamin E (increases NO) and olive oil (prevents vascular remodelling and endothelial dysfunction) (Siti *et al.*, 2015:46).

### **c. Stress**

People experience and respond to stressful situations/increased demands differently (von Kanel, 2012:2). This experience is influenced by the genome, social support and stress perception/response, making some people more vulnerable to distress, especially if coping mechanisms fail (von Kanel, 2012:2). When a person is overwhelmed, they are more prone to make bad lifestyle decisions. The elderly who live alone are more prone to depression disorders, making them more likely to suffer a CVD event (van Marwijk *et al.*, 2015:3&5). Takotsubo cardiomyopathy (“broken heart syndrome”) patients have experienced a severe emotional upheaval (e.g. death of a spouse) and present with myocardial infarction, caused by excess stress hormone affecting the myocardium (von Kanel, 2012:1).

Chronic stress results in a maladaptive change of hypothalamus-pituitary axis and sympathetic nervous system resulting in increased levels of catecholamines, growth hormones and neuropeptides, thus contributing to atherogenesis by increasing heart rate, blood pressure and causing endothelial dysfunction (Lagraauw *et al.*, 2015:2). Stress also activates the inflammatory response with increased plasma concentrations of CRP, IL-6 and TNF- $\alpha$ , further assisting in atherogenesis (Lagraauw *et al.*, 2015:2).

### **2.2.3.4. Geographic risk factors**

#### **a. Seasonal changes**

In winter months, even in warmer climates, there is an increased incidents of CVD complications and stroke (Kralova Lesna *et al.*, 2015:192). The body is protected physiologically against internal temperature changes with the assistance of various homeostatic responses (Marieb, 2014:353). When the body responds to cold, the blood coagulates easier and blood is more viscous (Vasconcelos *et al.*, 2013:14) due to higher levels of erythro- and thrombocytes and a higher concentration of plasma fibrinogen, all atherogenesis contributing factors (Kralova Lesna *et al.*, 2015:192). Other responses to cold weather include increased blood pressure, heart rate, glucose

and plasma lipids (Vasconcelos *et al.*, 2013:14; Kralova Lesna *et al.*, 2015:192). Epidemiological studies have indicated the effect of winter on CVDs is more pronounced in the elderly (Vasconcelos *et al.*, 2013:16).

## **b. Environmental pollution**

Epidemiological studies have indicated air pollution as a contributing factor to CVD pathogenesis (Mostafavi *et al.*, 2015:1). Air pollution results in a pro-inflammatory state as it stimulates the alveolar macrophages and bronchial epithelial cells in the lungs to secrete inflammatory mediators (increased interleukin-6 and CRP) which can be detected in the systematic circulation (Mostafavi *et al.*, 2015:2). In third world countries many still use solid fuels (wood, dung and crops) for cooking and heat. The effects of these household air pollutants should not be underestimated as it results in more deaths than unsafe water and poor sanitation worldwide (McCracken *et al.*, 2012:223). Solid fuel air pollution has been correlated with hypertension, endothelial dysfunction and increased activated platelets, coagulation factor VIII and TF (McCracken *et al.*, 2012:225-228; Rajagopalan and Brook, 2012:210-211).

Lead is a heavy metal to which humans can be exposed to in nature or their work/daily environment i.e. mining/processing of lead/zinc/copper-zinc ore, car battery production, car emissions (before 2005 when lead petrol was still being used) and contaminated drinking water when pipes contain lead/lead junctions (Poreba *et al.*, 2011:268; Solenkova *et al.*, 2014:812). Increased physiological lead levels cause dyslipidaemia (increased total cholesterol, LDL and triglycerides; decreased HDL), hypertension (disturbs renin–angiotensin–aldosterone system), endothelial dysfunction (lead molecules interact with endothelium) and oxidative stress (Poreba *et al.*, 2011:269-272; Solenkova *et al.*, 2014:813).

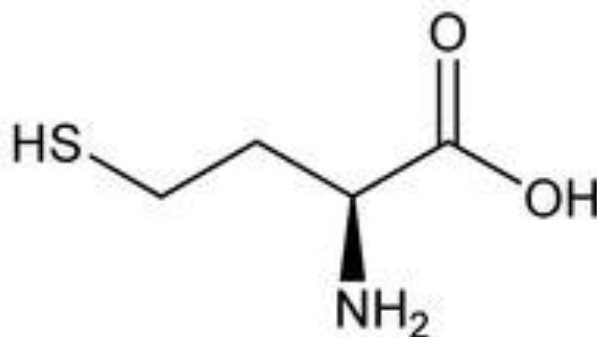
As mentioned before, atherogenesis is already present in childhood, and progresses further in adulthood while being completely asymptomatic (Sun and Jia, 2012:251). There is thus an important time period where prevention is key as a CVD event only occurs decades later (Sun and Jia, 2012:251). Therefore, identifying risk markers in patients is important as an early preventative measure. When two risk factors are present the probability for CVD increases fourfold (Kumar *et al.*, 2015: 496).



## 2.3. HOMOCYSTEINE

### 2.3.1. Hcy physical characteristics

Hcy was discovered in 1932 and Vincent du Vigneaud managed to synthesise it by heating methionine with sulphuric acid, for which he won a Nobel prize in 1955 (du Vigneaud and Butz, 1932; Ganguly and Alam, 2015:1). In 1964 Gibson, Carson and Neill found that individuals with vascular anomalies and arterial thrombosis had homocystinuria (increased Hcy levels in urine) (Gibson *et al.*, 1964; Ganguly and Alam, 2015); while in 1969 McCully found a correlation between hyperhomocysteinemia and arteriosclerosis (McCully, 1969; Ganguly and Alam, 2015:2). Homocysteine is a non-protein amino acid (Chmurzynska *et al.*, 2013:841) and is depicted in Figure 3. Cysteine and Hcy's chemical structure is very similar, with the difference being Hcy has an extra methylene group (Leesutthiphonchai *et al.*, 2011:871; Xiao *et al.*, 2013:145). There are three forms of Hcy in human plasma, in descending concentration: albumin-bound, free circulating disulfide, and sulfhydryl forms (Maron and Loscalzo, 2009:39; Ganguly and Alam, 2015:1; Bharathselvi *et al.*, 2016:756). Total Hcy (tHcy) is a sum of free thiol and disulfide bound Hcy, which are both used as biomarkers for diseases related to the Hcy metabolism (Jakubowski, 2013:9).



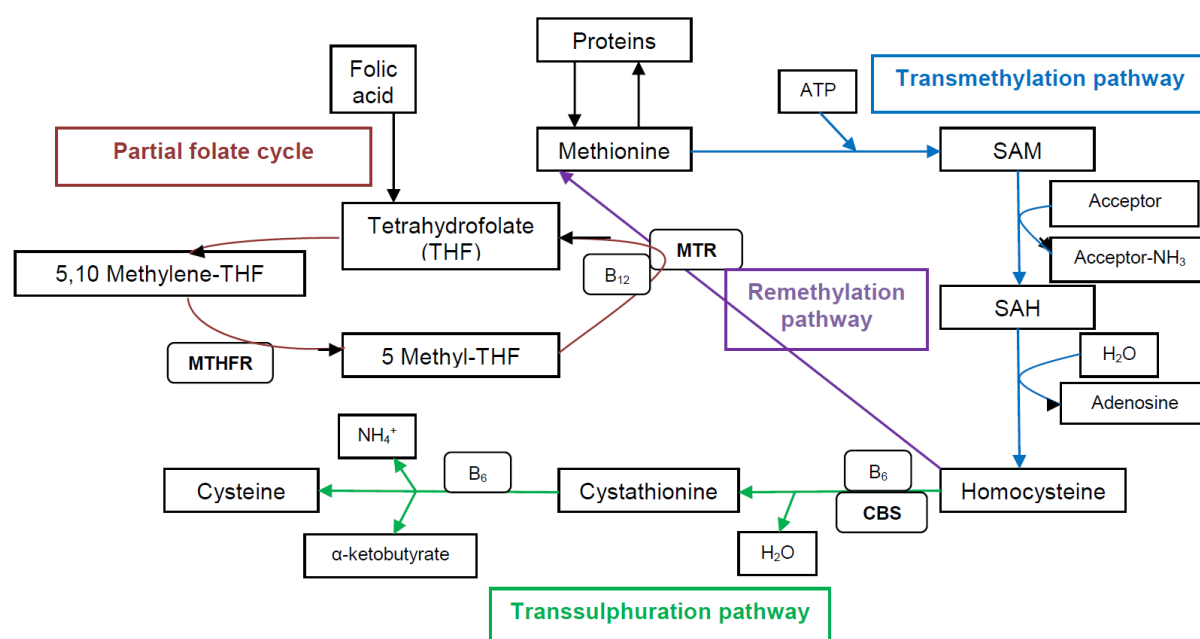
**Figure 3. Chemical structure of Hcy (C<sub>4</sub>H<sub>9</sub>NO<sub>2</sub>S) (The Merck Index. 2015).**

### 2.3.2. Hcy biochemical metabolism

Homocysteine is not found in human diets, but is rather a biochemical derivative in the body (Janani *et al.*, 2012:1). It is not utilized as an amino acid in protein synthesis; its role is as an intermediate product in methionine metabolism (Hirmerová, 2013:e249). In Hcy metabolism (Figure 4), there are three distinct pathways: transmethylation pathway (indicated in blue), the transsulphuration pathway



(indicated green) and the remethylation pathway (indicated in purple) (Blom and Smulders, 2011:75; Coppola and Mondola, 2017:1).



SAM - S-adenosyl-methionine  
 SAH - S-adenosyl-homocysteine  
 MTR – methionine synthase  
 MTHFR - 5, 10-Methylenetetrahydrofolate reductase  
 CBS - cystathionine β-synthase  
 B<sub>6</sub> - Vitamin B<sub>6</sub>  
 B<sub>12</sub> - Vitamin B<sub>12</sub>

**Figure 4. Homocysteine metabolism (Based on Blom and Smulders, 2011:76; Skovierova *et al.*, 2016:2)**

The transmethylation pathway results in the formation of S-adenosylhomocysteine (SAH) from methionine (Hcy is hydrolysed from SAH). In the transsulphuration pathway Hcy is converted to cystathionine. The remethylation pathway produces methionine from Hcy (Main *et al.*, 2010:1598-1599; Blom and Smulders, 2011:75-77; Kadhim and Clement, 2011:41; Hirmerová, 2013:e249).

### 2.3.2.1. Transmethylation pathway

Methionine is an essential amino acid attained from dietary products including milk, dairy products, meat, etc. (Bassett *et al.*, 2013:182). Once absorbed in the gastrointestinal tract, methionine is dispersed to organs by erythrocytes for utilization in protein synthesis and to serve as a biochemical precursor (Jakubowski, 2013:7). During transmethylation (indicated in blue in Figure 4) methionine (catalysed by methionine adenosyltransferase) condenses with adenosine triphosphate (ATP) to

form S-adenosylmethionine (SAM), an active form of methionine (Kadhim and Clement, 2011:41; Skovierova *et al.*, 2016:1). A methyl group is donated from SAM to an acceptor molecule (examples are DNA, RNA, proteins and neurotransmitters) and SAH is produced, catalysed by methionine adenosyltransferase (Main *et al.*, 2010:1598; Newsholme and Leech, 2010:336; Blom and Smulders, 2011:76; Skovierova *et al.*, 2016:2). An alternative pathway to the production of SAM to SAH is catalysed by glycine N-methyltransferase, a liver enzyme, and generates sarcosine from glycine (Wang *et al.*, 2011:486; Kumar *et al.*, 2016:12). SAH is then hydrolysed to Hcy when it loses an adenosine by adenosylhomocysteine hydrolase (Newsholme and Leech, 2010:336; Blom and Smulders, 2011; Bednarska-Makaruk *et al.*, 2016:84).

### **2.3.2.2. Transsulphuration pathway**

Cystathionine  $\beta$ -synthase (CBS) (a vitamin B6 dependant enzyme) catalyses the condensation of Hcy (Figure 4 – shown in green) and serine to form cystathionine (Kadhim and Clement, 2011:41; Hirmerová, 2013:e249). Cystathionine is converted to cysteine and  $\alpha$ -ketobutyrate by cystathionine  $\gamma$ -lyase, another vitamin B6 dependant enzyme (Blom and Smulders, 2011:77; Gregory *et al.*, 2016:21).

### **2.3.2.3. Remethylation pathway**

Homocysteine has another pathway apart from Transmethylation. During remethylation (Figure 4 – in purple) Hcy is converted back to methionine by methionine synthase (MTR) with vitamin B12 as a co-factor (Main *et al.*, 2010:1598; Blom and Smulders, 2011:76; Bednarska-Makaruk *et al.*, 2016:84). This pathway only occurs when methionine levels are low (Blom and Smulders, 2011:77; Ganguly and Alam, 2015:1). The remethylation pathway interacts with the folate metabolism (discussed in more detail in 2.4.2.) from which a methyl group is received (partial folate pathway shown in red in Figure 4) and firstly attached to the MTR-vitamin B12 complex then to Hcy (Blom and Smulders, 2011:77; Wang *et al.*, 2011:486; Bednarska-Makaruk *et al.*, 2016:84). The methyl is donated by 5-methyl-tetrahydrofolate to produce tetrahydrofolate (THF) (Newsholme and Leech, 2010:336; Bednarska-Makaruk *et al.*, 2016:84). Methylenetetrahydrofolate reductase catalyses the reaction from which 5-methyl THF is formed (Newsholme and Leech, 2010:336; Collin, 2013:23). When MTHFR is not functioning properly it will result in the methyl group not being available for attachment to Hcy, which results in increased Hcy levels (Main *et al.*, 2010:1598; Aneji *et al.*, 2012:2). A loss of function SNP can cause this and will be discussed in more detail in 2.7.4. (Aneji *et al.*, 2012:2).

Apart from the folate cycle another methyl donor can be utilized, namely, betaine (obtained from plant sources or oxidation of choline from an animal source) and is catalysed by betaine homocysteine methyltransferase (BHMT) (Dasarathy *et al.*, 2010:357; Blom and Smulders, 2011:77; Collin, 2013:486). The BHMT enzyme is found predominately in the liver and functions independently of folate (Collin, 2013:486). When methionine levels are increased, SAM levels are also higher and decreases MTHFR enzyme activity and increases CBS activity (Blom and Smulders, 2011:77; Wang *et al.*, 2011:487; Ganguly and Alam, 2015:2). Once Hcy is degraded to methionine it is irreversible and will not be converted again to Hcy (Wang *et al.*, 2011:487).

### 2.3.3. Hcy role in pathophysiology of CVD

Hyperhomocysteinemia as indicated in section 2.2.3.2.c. is an independent risk factor for CVD and can be the result of a mutation in the Hcy metabolism enzymes (namely MTHFR, MTR and CBS) or nutritional deficiencies of folate, vitamin B6 (Ganguly and Alam, 2015:2), Table 3 expands on the causes including diseases, medications and factors of lifestyle.

**Table 3. Causes of hyperhomocysteinemia (adopted from Chmurzynska *et al.*, 2013:842; Hirmerová, 2013:e250)**

<b>Genetic causes</b>	<ul style="list-style-type: none"> <li>• Defective folate metabolism</li> <li>• Congenital defects of cobalamin (Vitamin B12) absorption, transport and metabolism</li> <li>• Polymorphisms of folate metabolism enzymes: <ul style="list-style-type: none"> <li>• CBS</li> <li>• MTHFR</li> <li>• Methionine synthase</li> <li>• Methionine synthase-reductase</li> </ul> </li> </ul>
<b>Acquired factors</b>	<ul style="list-style-type: none"> <li>• Folate deficiency</li> <li>• Vitamin B6 deficiency</li> <li>• Vitamin B12 deficiency</li> <li>• Some diseases and disorders: <ul style="list-style-type: none"> <li>○ Kidney diseases</li> <li>○ Psoriasis</li> <li>○ Rheumatoid arthritis</li> <li>○ Systemic lupus erythematosus</li> <li>○ Hypothyroidism</li> </ul> </li> <li>• Certain medications: <ul style="list-style-type: none"> <li>• Steroid hormones</li> <li>• Insulin</li> <li>• Antiepileptic drugs</li> <li>• Fibrates</li> <li>• Metformin</li> <li>• D-penicillamine</li> </ul> </li> </ul>

	<ul style="list-style-type: none"> <li>• Proton pump inhibitors</li> <li>• Methotrexate</li> <li>• L-dop</li> <li>• 6-Mercaptopurin,</li> <li>• Sulfasalazin,</li> <li>• Cyclosporin,</li> <li>• High Vitamin C dose</li> <li>• Factors of lifestyle <ul style="list-style-type: none"> <li>• Smoking</li> <li>• Alcohol</li> <li>• High caffeine intake</li> <li>• Sedentary lifestyle</li> <li>• Methionine rich diet (animal protein)</li> </ul> </li> <li>• Other: <ul style="list-style-type: none"> <li>• Age</li> <li>• Male sex (larger muscle mass)</li> <li>• Gastroplasty</li> <li>• Down syndrome</li> <li>• Post-menopause</li> </ul> </li> </ul>
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Kuebler *et al.* (2013) suggested that stress can have an accumulative effect on Hcy levels. A diet rich in orange juice, eggs, milk, fish, fruits, non-processed meat, chicken, whole-grain bread lowers tHcy, whereas a high intake of cookies, sweets, pies and cakes increase tHcy. Thus the intake of folate, vitamin B6, vitamin B12, complex carbohydrates and riboflavin is inversely related to tHcy, while a diet high in fat is correlated with high Hcy levels (Chmurzynska *et al.*, 2013:842).

Hyperhomocysteinemia contributes to endothelial dysfunction by reducing NO to peroxynitrite essentially inhibiting the endothelium's vasodilation functionality and contributes to hypertension, a CVR factor to which hyperhomocysteinemia has been correlated (Kaur and Sekhon, 2013:2; Wang *et al.*, 2014:1; Ganguly and Alam, 2015:6). Hyperhomocysteinemia further enhances atherogenesis by the increase in SMC proliferation, extracellular matrix accumulation, LDL oxidation (Gotlieb and Liu, 2012:454), increased uptake of oxidised-LDL by macrophages and increase monocyte adhesion to endothelium, as hyperhomocysteinemia stimulates VCAM and E-selectin (Kaur and Sekhon, 2013:2; Wang *et al.*, 2014:1). Endothelial cells are vulnerable to hyperhomocysteinemia and as it does not produce CBS (important for transsulphuration pathway) or BHMT (alternative remethylation pathway in liver). The only enzymatic clearance of Hcy in endothelial cells are MTHFR and MTR, hence normal functioning of these enzymes inhibit pathological Hcy levels in vascular endothelial cells (Debrececi and Debrececi, 2014:132).

A pro-coagulation state is induced by hyperhomocysteinemia as it increase the following: platelet reactivity (inhibition thrombomodulin and ecto-adenosine diphosphataseinhibition on endothelium's cell surface), thromboxane synthesis, TF production, activity of factor VII, activation of factor V,

thrombin generated and modifies fibrinogen (Gotlieb and Liu, 2012:454; Hirmerová, 2013:e251). It also decreases the following: platelet aggregation, inactivation of factor Va, activity of antithrombin, thrombomodulin activity and inhibits protein C activation (Hirmerová, 2013:e251; Kaur and Sekhon, 2013:2). Increased levels of Hcy impairs fibrinolysis by reducing the binding of tPA and reduced plasmin concentration and increases TAFI levels (Hirmerová, 2013:e251).

Inflammation is amplified by hyperhomocysteinemia as it stimulates the endothelium's release of cytokines, interleukin-8 (IL-8), monocyte chemotactic protein-1 and cyclins (Kaur and Sekhon, 2013:2), and additionally stimulates monocyte chemotactic protein-1 and CRP release from SMC hyperhomocysteinemia. Furthermore this results in changing the connective tissue in plaques eventually causing fibrosis, proteoglycan deposition and calcification (Kaur and Sekhon, 2013:2 & 4). Moreover, dyslipidaemia is also increased when HMG Co A reductase activity is increased, thus increasing cholesterol synthesis. The kidneys are responsible for Hcy clearance and it has higher concentrations in patients with chronic renal disease when compare to patients with atherothrombotic vascular disease. This is a possible explanation for vascular complications observed with chronic renal failure (Ganguly and Alam, 2015:7).

Apart from risk for CVD, there are other clinical manifestations correlated with hyperhomocysteinemia: carcinogen, neural tube and other defects (congenital heart defects, Down's syndrome, cleft palate), diabetes, leukemia, migraine, depression, schizophrenia, Alzheimer's disease, recurrent pregnancy loss, neuropsychiatric disease, chemotherapy toxicity and preeclampsia (Rao *et al.*, 2010:5; Safarinejad *et al.*, 2012:270; Hickey *et al.*, 2013:153; Izmirli, 2013:326).

#### **2.3.4. Normal Hcy serum levels**

Serum Hcy levels are classified as follow: normal ( $\leq 15 \mu\text{mol/L}$ ) and mild hyperhomocysteinemia ( $16-3 \mu\text{mol/L}$ ), whereas moderate and severe hyperhomocysteinemia are specifically classified when serum levels are  $31-100 \mu\text{mol/L}$  and  $>100 \mu\text{mol/L}$ , respectively (Abraham and Cho, 2010:912; Hirmerová, 2013:e250; Ganguly and Alam, 2015:2).

### **2.4. FOLATE**

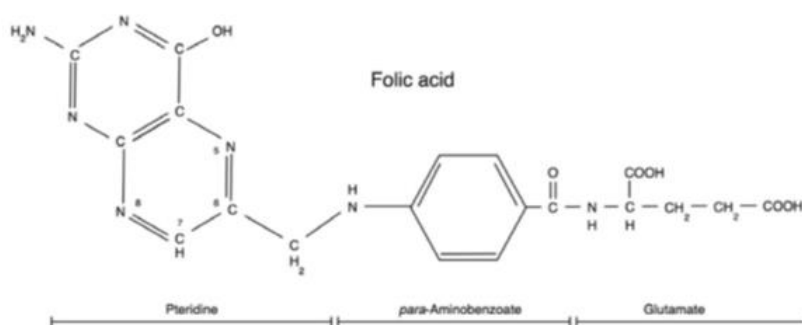
#### **2.4.1. Folate physical characteristics**

Folate was discovered in 1937 when Lucy Wills examined yeast and liver extracts as effective treatments for pernicious anaemia and tropical macrocytic anaemia. Furthermore folate was

extracted from spinach in 1941 and folic acid was synthesized for the first time in 1943 (Wills *et al.*, 1937; Mitchell *et al.*, 1941; Pfiffner *et al.*, 1943; Collin, 2013:16; Jennings and Willis, 2015). Mammals, including humans, do not have the ability to synthesise folate, but plants have the ability to synthesise folate anew (Collin, 2013:15; Jennings and Willis, 2015:224). Thus humans obtain folate from green vegetables (e.g. spinach), legumes, oranges and the liver of animals who ate these plants (Blom and Smulders, 2011:77; Collin, 2013:15; Nazki *et al.*, 2014:13).

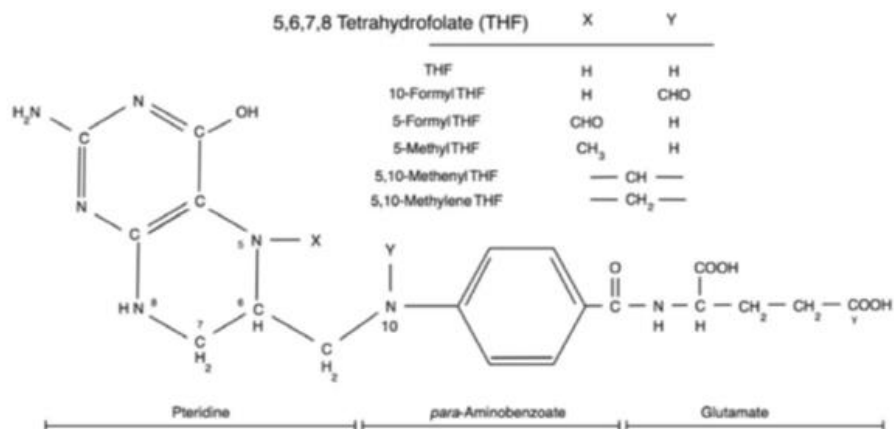
Vitamin B9 or folate is the family name for a group of molecules sharing a structure comprising of three parts, namely, a pteridine ring, a para-aminobenzoic acid and one or more glutamates and an amino acid (Figure 5) (Stover and Field, 2011:325; Collin, 2013:13; Nazki *et al.*, 2014:12). The pteridine ring is composed of a pyrimidine ring and a pyrazine ring with the para-aminobenzoic acid attached at C6 of the pteridine ring (Collin, 2013:14; Gueant *et al.*, 2013:281). Monoglutamate forms of folate is just one of L-glutamic acid attached to the para-aminobenzoic acid, while eight g-linked glutamates can attach to the first glutamate to create monoglutamate forms of folate (Collin, 2013:15).

Apart from the number of glutamates, folates also have variants in the following: level of reduction of the pyrazine ring, N5 position of pteridine ring and N10 para-aminobenzoic acid has variants of the methyl and formyl groups (number, position, and reduction status) (Stover and Field, 2011:326; Collin, 2013:15). Figures 5 and 6 depict the chemical structure of folic acid and variants of STHF, respectively. The various forms of folate each play a specific role during folate metabolism (Collin, 2013:15), although 5-methyl THF is the most predominant comprising 82-93% of all folate in human serum (Blom and Smulders, 2011:77; Gueant *et al.*, 2013:281; Kirsch *et al.*, 2013:497).



**Figure 5. Chemical structure of folic acid (Collin, 2013:14)**

One synthetic form of folate is known as folic acid where the pteridine ring is completely oxidized and is stable for years, as it does not lose activity and is not chemically labile like natural folates harvested from plant sources (Blom and Smulders, 2011:77; Stover and Field, 2011:325; Collin, 2013:16). In the gastrointestinal mucosal cells folic acid is first converted to THF and then 5-methyl THF, although the high consumption of folic acid overwhelms this mechanism resulting in circulation of un-metabolized folic acid (Stover and Field, 2011:325; Collin, 2013:16). The un-metabolized folic acid can still be transported into cells, but it competes with DHF for reduction by dihydrofolate reductase (DHFR) (Collin, 2013:16). Homocysteine and folate have an inverse relationship; a person with low folate levels tend to have high Hcy levels and thus increased CVR (Carroll *et al.*, 2012:1531; Kirsch *et al.*, 2013:498). Folates are hydrophilic anionic molecules which cannot cross biological membranes without the assistance of membrane transport systems (Jager *et al.*, 2012:624; Hou and Matherly, 2014:177).



**Figure 6. Chemical structure of THF (Collin, 2013:14)**



## 2.4.2. Folate biochemical metabolism

Folate has an essential role as a one carbon donor and co-enzyme in methylation and various reactions (Carroll *et al.*, 2012:1531; Kirsch *et al.*, 2013:497; Nazki *et al.*, 2014:12). The folate metabolism alternatively referred to as the one-carbon metabolism is regulated by at least 30 enzymes (Nazki *et al.*, 2014:13). Figure 7 is a simplified diagram of folate metabolism starting at the intestines, a summary of this metabolism follows as per the diagram. Foliates are digested and absorbed in the duodenum and upper part of the jejunum. Folate hydrolase (FOLH1) removes the polyglutamyl chain of 5-methyl THF polyglutamate as only monoglutamatic forms can be transported across cell membranes (Blom and Smulders, 2011:77; Collin, 2013:15; Nazki *et al.*, 2014:13). A proton-coupled folate transporter is responsible for the transport of folates into the duodenum/jejunum cells (encoded by solute carrier family 46 member 1 (SLC46A1)). SLC46A1 functions optimally in the acidic pH of the duodenum (Collin, 2013:15; Gueant *et al.*, 2013:281). Reduced folate carrier (encoded by solute carrier family 19 member 1 (SLC19A1)) transports folates out of the intestinal cells and across mammalian cell membranes (Collin, 2013:15; Gueant *et al.*, 2013:281).

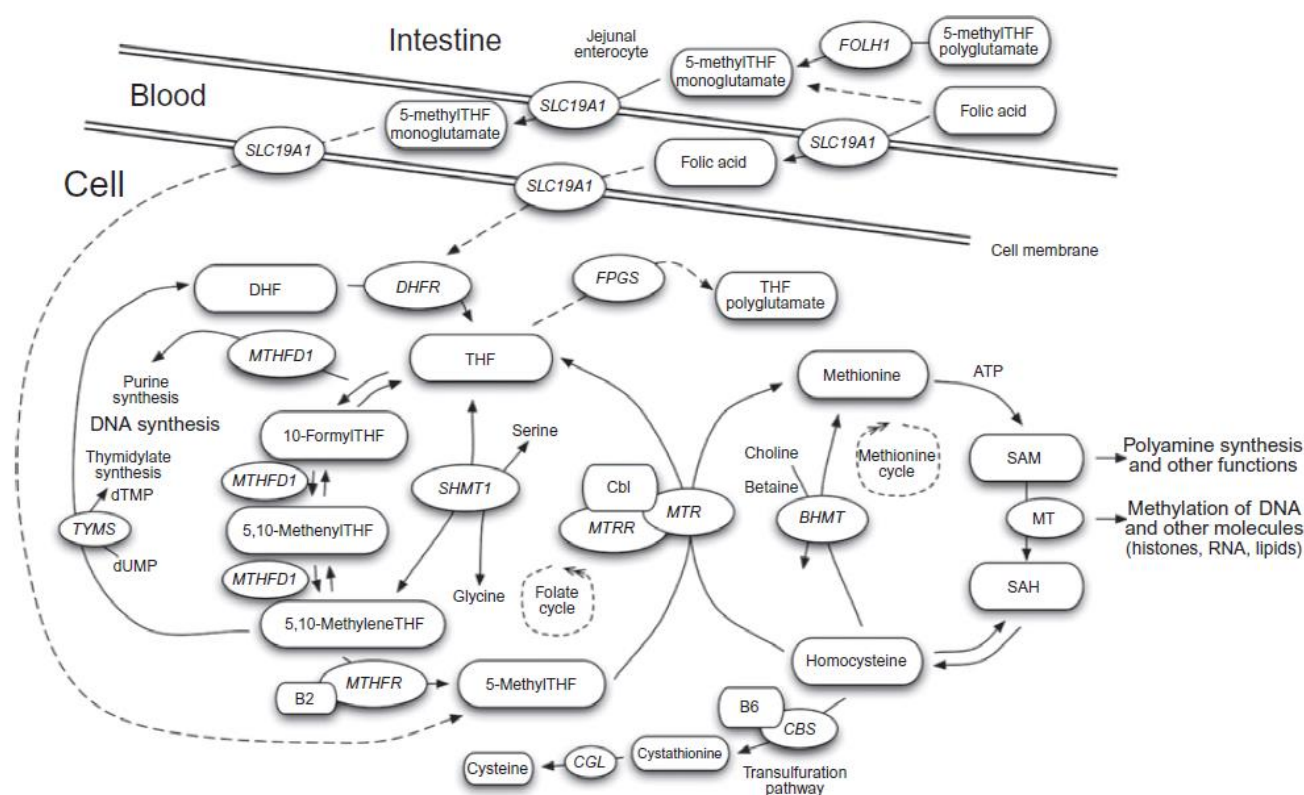


Figure 7. Simplified overview of folate one carbon metabolism (Collin, 2013:4)



Endocytosis of folate into a cell by a folate receptor (FR) is another means of folate transport, but this is mainly expressed in the placenta, the choroid plexus and the kidney (Collin, 2013:15; Gueant *et al.*, 2013:281).

Essentially there are 3 pathways in folate metabolism, namely, Hcy remethylation, catalysation of purine (guanosine and adenosine) and thymidylate (thymidine monophosphate (dTMP)) synthesis (Stover and Field, 2011:326; Field *et al.*, 2016:27). Inside the cell folic acid and dihydrofolate (DHF) is reduced to THF by DHFR, although DHFR has a higher affinity for folic acid (Nazki *et al.*, 2014:13; Wahl *et al.*, 2015:108). THF is regenerated by the folate cycle (to a certain extent) or by the diet (Figure 7), and is at the core of the folate cycle functionality (Field *et al.*, 2016:27). THF is oxidised to 5,10-methylene THF by two reactions. Firstly by serine hydroxymethyl transferase (SHMT), a vitamin B6 dependant enzyme, converts glycine to serine; donating a methyl to THF (as formaldehyde) (Blom and Smulders, 2011:77; Collin, 2013:23; Field *et al.*, 2016:28). Methylenetetrahydrofolate dehydrogenase (MTHFD1) is a trifunctional enzyme with activities of formyltetrahydrofolate synthetase, methenyltetrahydrofolate cyclohydrolase and MTHFD1 (Blom and Smulders, 2011:77; Stover and Field, 2011:327; Collin, 2013:23). MTHFD1 catalyses the conversion of THF to 5,10-methylene THF in a 10-step process, donating two carbon-groups (through a possible enzyme complex) for purine synthesis (Blom and Smulders, 2011:77; Stover and Field, 2011:327; Collin, 2013:23).

The 5,10-methylenetetrahydrofolate is converted to DHF (once again available for DHFR) by thymidylate synthase (TYMS) enzyme and results in the conversion of deoxyuridine monophosphate (dUMP) into dTMP (Blom and Smulders, 2011:77; Nazki *et al.*, 2014:14; Field *et al.*, 2016:29). dTMP is of physiological importance as it is the precursor for thymine, a nucleotide, a significant component of DNA (Collin, 2013: 23; Field *et al.*, 2016:28). The other fate of 5,10-methylene THF, apart from dTMP synthesis, is to be reduced to THF in a few reactions (Blom and Smulders, 2011:77; Collin, 2013:16; Kirsch *et al.*, 2013:497; Jennings and Willis, 2015:224), which is important for Hcy remethylation as discussed in section 2.3.2. Of note though, MTR is the key enzyme that links Hcy metabolism and folate one-carbon metabolism (Collin, 2013:22).

### **2.4.3. Folate deficiency**

A folate deficiency is caused by decreased intake of folate rich products, impaired absorption (due to celiac- or inflammatory bowel disease), increased metabolism of folate (e.g. pregnancy, haemolysis, dermatitis), smoking, alcoholism, lactation, genetic defects, bariatric surgery, chemotherapy and antiepileptic medications (Okumura and Tsukamoto, 2011:523; Okada *et al.*,

2014:273; Gudgeon and Cavalcanti, 2015:56). A deficiency results in neurological and haematological complications (Okada *et al.*, 2014:273). The haematological complications are characterised by megaloblastic anaemia, while the neurological complications has a variety of conditions including neuropathy, depression, dementia, psychosis and myelopathy. In newborns it may result in neural tube defects (Okada *et al.*, 2014:273). In the absence of folate dUMP levels will increase and cause increased integration of uracil instead of thymine in DNA. Results in chromosome breakage, point mutations, micronucleus formation, generation of single and double stranded DNA breaks (Fenech, 2012:22; Collin, 2013:25).

#### **2.4.4. Folate role in cardiovascular risk**

A deficiency of folate causes an increase in plasma Hcy levels as the remethylation of Hcy decrease (Okumura and Tsukamoto, 2011:523; Ganguly and Alam, 2015:2). As previously discussed, hyperhomocysteinemia plays a role in atherosclerosis pathogenesis as it is associated with LDL modification, lipid peroxidation, thrombosis etc. (Okumura and Tsukamoto, 2011:523; Kaur and Sekhon, 2013:2; Wang *et al.*, 2014:1). Therefore, folate fortification can reduce Hcy levels by 25%, although it does not significantly reduce risk for a CVD event (Okumura and Tsukamoto, 2011:523; Santilli *et al.*, 2015:1). A correlation of folic acid with increased HDL, apolipoprotein A and endothelial function also exists (Okumura and Tsukamoto, 2011:523). Furthermore, a correlation exists between folate and inflammatory state with a decrease in inflammatory markers (high sensitivity C-reactive protein [hs-CRP], IL-6 and TNF- $\alpha$ ) after folate supplementation (Kolb and Petrie, 2013:164).

#### **2.4.5. Normal folate levels**

Erythrocytes' folate levels represent tissue stores and can remain stable for 3-4 months in the absence of dietary folate (Gudgeon and Cavalcanti, 2015:56). The normal range for erythrocytes' folate is 317-1422 nmol/L (Ghadban and Almourani, 2013). The normal range for serum folate is between 6.0-28.0 nmol/L (3–17 ng/ml) (Burtis and Bruns, 2015:848). The recommended daily intake of folate to achieve optimal serum values is 400  $\mu$ g/day (Fenech, 2012:21).

## 2.5. VITAMIN B6

### 2.5.1. Vitamin B6 physical characteristics

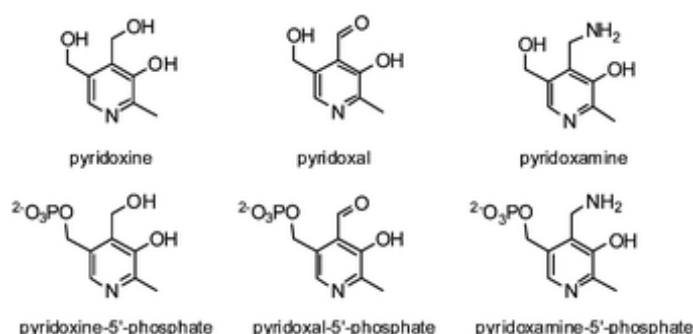
Vitamin B6's formula was first described by Ohdake in 1932 when he discovered it as a by-product during the isolation of vitamin B1 from rice-polishings, although he was unaware that it was a vitamin or of its physiological importance (Ohdake, 1932; Mooney *et al.*, 2009:330; Živković, 2016). In 1934 Paul György described vitamin B6 as the attributed factor in a yeast elute responsible for curing rat acrodynia (György, 1934; Mooney *et al.*, 2009:330; Rosenberg, 2012:236; Gazeley and Newell, 2015). The crystalline vitamin B6 was isolated in 1938 by Samuel Lepkovsky (Lepkovsky, 1938; Rosenberg, 2012:236). Leslie Harris and Karl Folkers, as well as Richard Kuhn and his associates independently demonstrated in 1939 that vitamin B6 was a pyridine derivative, namely 3-hydroxy-4,5-dihydroxy-methyl-2-methyl-pyridine (Harris and Folkers, 1939; Kuhn *et al.*, 1939; Rosenberg, 2012:236). Vitamin B6 was named pyridoxine by György due to its homology to pyridine (György, 1938; Mooney *et al.*, 2009:330; Rosenberg, 2012:236).

Vitamin B6 is a water-soluble (Corken and Porter, 2011:623; van der Ham *et al.*, 2012:108; Kjeldby *et al.*, 2013:13) essential nutrient in humans that cannot be synthesized (Mooney *et al.*, 2009:331; van der Ham *et al.*, 2012:108; Cellini *et al.*, 2014:159; Fratoni and Brandi, 2015:2180). Dietary sources for vitamin B6 is very diverse and includes fish, poultry, meat, liver, eggs, potatoes, whole grain, legumes, lentils, avocado, leafy vegetables, bananas, non-citrus fruit, nuts, cheese, milk, soybean and soy products (Corken and Porter, 2011:623; van der Ham *et al.*, 2012:108; Fratoni and Brandi, 2015:2180).

Vitamin B6 exists in various chemical forms called vitamers and is comprised of three different pyridine derivatives: pyridoxine, pyridoxal (PL) and pyridoxamine (Rosenberg, 2012:236; van der Ham *et al.*, 2012:108; Wu and Lu, 2012:5333; Jankowska *et al.*, 2013:58; Kjeldby *et al.*, 2013:13). The difference between these forms is a variable group present at the 4<sup>th</sup> position (Mooney *et al.*, 2009:330; Wu and Lu, 2012:5333; Ueland *et al.*, 2017:11). Pyridoxine carries a hydroxyl group, while PL has an aldehyde and pyridoxamine has an amine as illustrated in Figure 8 (van der Ham *et al.*, 2012:108; Wu and Lu, 2012:5333; Ueland *et al.*, 2017:11).

A requirement for all three to function as co-factors in enzymatic reactions is phosphorylation (Figure 8) by kinases (Rosenberg, 2012:236; Wu and Lu, 2012:5333). Pyridoxal-5-phosphate (PLP) is

biologically the most active and most abundant form in plasma (Rosenberg, 2012:236; Wu and Lu, 2012:5333; Jankowska *et al.*, 2013:58; Kjeldby *et al.*, 2013:13).

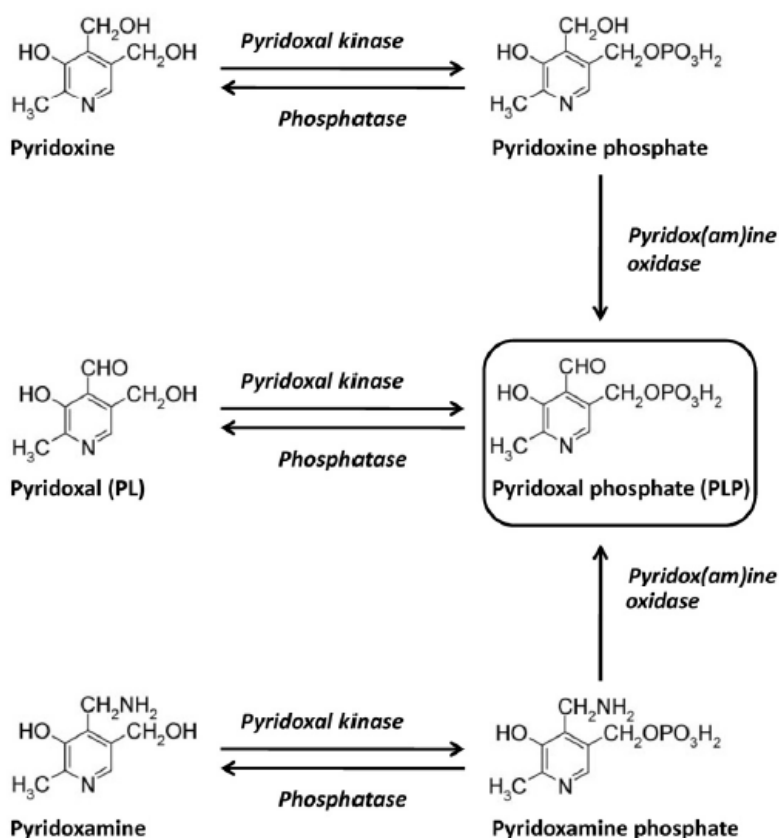


**Figure 8. Chemical structures of vitamin B6 (Samuel *et al.*, 2012:7279)**

Pyridoxamine-5-phosphate reflects tissue stores, although PLP is used most often to measure vitamin B6 status in humans (Jankowska *et al.*, 2013:58). Plant sources have a unique form of vitamin B6 known as pyridoxine glucoside with only half the bioavailability of vitamin B6 from other sources or supplements (Fratoni and Brandi, 2015:2180). Another chemical form of vitamin B6 is 4-pyridoxic acid, which is the end product of vitamin B6 metabolism and is produced in the liver while being excreted through urine (Jankowska *et al.*, 2013:58). Pyridoxic acid increases under two conditions, namely, vitamin B6 supplementation and impaired renal function (Jankowska *et al.*, 2013:58).

### 2.5.2. Vitamin B6 biochemical metabolism

The small intestine plays a major role in human metabolism of vitamin B6 (Albersen *et al.*, 2013:e54119). Phosphorylated vitamin B6 vitamers are hydrolysed by alkaline phosphatase, as shown in Figure 9, in the jejunum and ileum lumen (Said, 2011:361; Combs Jr, 2012:316; van der Ham *et al.*, 2012:108; Albersen *et al.*, 2013:e54114). Previously it was thought that these products and non-phosphorylated vitamin B6 vitamers diffuse into the jejunum and ileum cells, but in 2003 the first carrier mediated mechanism was reported (van der Ham *et al.*, 2012:108; Albersen *et al.*, 2013:e54114). The study found that pyridoxine uptake was inhibited by pyridoxamine but not by PL or PLP making the carrier protein selective for two of the three non-phosphorylated B6 vitamers (Mooney *et al.*, 2009:334; Said, 2011:361; Albersen *et al.*, 2013:e54114). Another study in 2008 found a different pyridoxine uptake mechanism which had different characteristics and was inhibited by both pyridoxamine and PL (Albersen *et al.*, 2013:e54114). Although vitamin B6 intestinal transportation has been characterized to an extent, their transporter proteins and encoding genes have not been identified (Said, 2011:361; Albersen *et al.*, 2013:e54114).



**Figure 9. Vitamin B6 metabolism or conversions (adapted from van der Ham *et al.*, 2012:109)**

There are two hypotheses for the location of vitamin B6 metabolism. All B6 vitamers must be converted to PLP for utilization as it is the biologically the most active (Rosenberg, 2012:236; Wu and Lu, 2012:5333; Jankowska *et al.*, 2013:58; Kjeldby *et al.*, 2013:13). There is no clear consensus in the literature if vitamin B6 is metabolized in intestinal cells or only absorbed and transported to the liver for conversion to PLP (Albersen *et al.*, 2013:e54114). For instance, Combs Jr (2012) and di Salvo *et al.* (2011) report the liver as the major organ for vitamin B6 metabolism, while Cellini *et al.* (2014) report intestinal cells. van der Ham *et al.* (2012) avoids stating a specific location and reports conversions as intracellular. Regardless of the location of metabolism, as illustrated by Figure 9, pyridoxal kinase phosphorylates pyridoxine, pyridoxamine and PL, resulting in pyridoxine-5-phosphate, pyridoxamine-5-phosphate and PLP, respectively (Combs Jr, 2012:316; Albersen *et al.*, 2013:e54114; Cellini *et al.*, 2014:159). Pyridoxine-5-phosphate and pyridoxamine-5-phosphate are converted to PLP by the flavin mononucleotide dependent pyridox(am)ine oxidase (Albersen *et al.*, 2013:e54114; Cellini *et al.*, 2014:159). The PLP is dephosphorylated by an ecto-enzyme phosphatase (Albersen *et al.*, 2013:e54114; Cellini *et al.*, 2014:159).

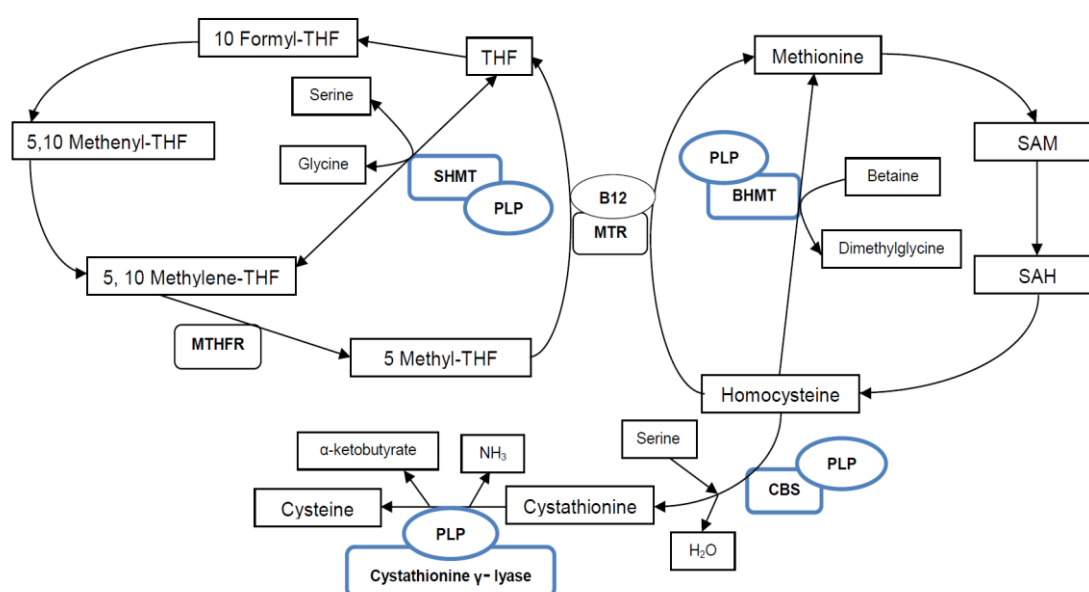
The degradation of vitamin B6 occurs when PL is oxidised by pyridoxal oxidase of which the major product is pyridoxic acid (Albersen *et al.*, 2013:e54114). Approximately 40-60% of all ingested

vitamin B6 is converted to pyridoxic acid and excreted in urine (Lotto *et al.*, 2011:183; Combs Jr, 2012:316). Vitamin B6 deficient patients have undetectable urine pyridoxic acid concentration (Combs Jr, 2012:316). When high doses of vitamin B6 doses have been given, other products of vitamin B6 metabolism can be detected in the urine, including pyridoxine, pyridoxamine, PL and their respective phosphorylated forms (Combs Jr, 2012:316; Ueland *et al.*, 2017:13). The liver releases PLP in the circulatory system bound to albumin by a lysine residue and it remains tightly bound while in the plasma (di Salvo *et al.*, 2011:1598; Ueland *et al.*, 2017:11). Although PLP is biologically the most active form of vitamin B6, it cannot enter cells and is dephosphorylated by tissue-nonspecific alkaline phosphatase found within the cellular membrane (Cellini *et al.*, 2014:159). Once inside the cell, PL is phosphorylated and available to function as a co-enzyme (di Salvo *et al.*, 2011:1598; Ueland *et al.*, 2017:11).

There are 160 distinct enzyme activities that utilizes PLP as a co-enzyme or co-factor (Cellini *et al.*, 2014:159). This is approximately 4% of all catalytic activities and is distributed across five of the six enzyme classes (Mooney *et al.*, 2009:330; di Salvo *et al.*, 2011:1598; Gregory, 2014). Metabolisms PLP are involved in are amino acid metabolism, neurotransmitter synthesis, gluconeogenesis, histamine synthesis, haemoglobin synthesis and function, lipid metabolism and gene expression (Combs Jr, 2012:319; van der Ham *et al.*, 2012:108; Gregory, 2014). In both amino acid and neurotransmitter synthesis, PLP is needed for decarboxylation, transamination and desulphuration (Mooney *et al.*, 2009:330; Wu and Lu, 2012:5333; Gregory, 2014). The role PLP has in Hcy metabolism is also classified as part of amino acid synthesis, which will be discussed later. More than half of vitamin B6 in the human body is used as a co-factor for glycogen phosphorylase to release glucose from glycogen stores in muscle (Dhalla *et al.*, 2012:535; Chikwana *et al.*, 2013:20976; Gregory *et al.*, 2013:63545). Aminolevulinic acid synthase functions with PLP to produce aminolevulinic acid, a haem precursor (Wu and Lu, 2012:5333; Gregory, 2014). Furthermore, vitamin B6 also binds to two sites of haemoglobin, increasing oxygen binding capabilities (Combs Jr, 2012:321; Gregory, 2014).

### 2.5.2.1. Role of PLP in HCY metabolism

In Hcy metabolism there are four enzymes that are PLP dependant, namely, CBS, SHMT, BHMT and cystathionine  $\gamma$ -lyase. As seen in Figure 10, Hcy condenses with serine to form cystathionine catalysed by the CBS enzyme (Kadhim and Clement, 2011:41; Hirmerová, 2013:e249). More specifically, CBS is responsible for the  $\beta$ -replacement of the hydroxyl group on serine with the thiolate group of Hcy (Wu and Lu, 2012:5334). The CBS enzyme exists as a tetramer with four potential binding sites for CBS (Gregory *et al.*, 2016:22). Cystathionine  $\gamma$ -lyase enzyme converts cystathionine to cysteine,  $\alpha$ -ketobutyrate and ammonia ( $\text{NH}_3$ ) (Blom and Smulders, 2011:77; Gregory *et al.*, 2016:21). Apart from their role in the transsulphuration pathway, CBS and Cystathionine  $\gamma$ -lyase also generate hydrogen sulphide ( $\text{H}_2\text{S}$ ), a physiologically active signalling molecule (Gregory *et al.*, 2016:21).



**Figure 10. Hcy metabolism highlighting the four PLP dependant enzymes**

The BHMT enzyme is an alternative to MTR for the remethylation of Hcy to methionine, as shown in Figure 10 (Wu and Lu, 2012:5335; Collin, 2013:21). Betaine is the substrate for BHMT, donating a methyl group to Hcy resulting in the formation of dimethylglycine and methionine (Blom and Smulders, 2011; Wu and Lu, 2012:5335; Collin, 2013:21). The enzyme is responsible for up to half of Hcy remethylation (Wu and Lu, 2012:5335). Remethylation by BHMT is independent of folate (Collin, 2013:21) and is confined to the human liver, kidney and lens of the eye (Wu and Lu, 2012:5335). The SHMT enzyme plays a role in the folate cycle (Wu and Lu, 2012:5334; Field *et al.*, 2016:28). SHMT is responsible for the conversion of serine to glycine (Figure 10) producing a methyl group (in the form of formaldehyde) which is donated to THF to produce 5,10-methyleneTHF (Blom and Smulders, 2011:77; Wu and Lu, 2012:5334; Collin, 2013:23). The resulting 5,10-methylene-THF



is used for methionine and thymidylate biosynthesis (Wu and Lu, 2012:5334; Field *et al.*, 2016:28). This simultaneous reaction is reversible (Wu and Lu, 2012:5334; Collin, 2013:23).

### **2.5.3. Vitamin B6 deficiency**

A vitamin B6 deficiency can have its origins from malnutrition, malabsorption in the small intestine, altered phosphorylation of PL on PLP, dialysis membranes causing increased vitamin B6 loss, alcoholism, liver cirrhosis, increased degradation of vitamin B6, drug-nutrient interactions or drug influence on enzymes involved in PLP metabolism (Corken and Porter, 2011:619; van der Ham *et al.*, 2012:109; Vrolijk *et al.*, 2017:206). The elderly are at a higher risk for vitamin B6 shortage, apart from the aforementioned causes, they also eat smaller portions, as well as food containing less vitamin B6 (Jankowska *et al.*, 2013:58). Elderly also have a higher requirement for vitamin B6 owing to increased catabolism, impaired phosphorylation and decreased absorption (Kjeldby *et al.*, 2013:13). Low levels of vitamin B6 has been associated with increased risk for cancer and CVD, migraine, depression, seizures, angular stomatitis, immune deficiency, scaly dermatitis, chronic pain, cognitive failure, confusion and glossitis (Corken and Porter, 2011:623; Wu and Lu, 2012:5335; Kjeldby *et al.*, 2013:13).

### **2.5.4. Vitamin B6 role in cardiovascular risk**

PLP functions as a co-enzyme in numerous metabolic pathways including Hcy metabolism as discussed in section 2.5.2. A vitamin B6 deficiency causes ineffective Hcy metabolism resulting in an increase in Hcy concentration (Corken and Porter, 2011:623; Wu and Lu, 2012:5335; Kjeldby *et al.*, 2013:13). Low PLP concentrations have been inversely correlated with CRP (Lotto *et al.*, 2011:183; Ueland *et al.*, 2017:12), a marker of inflammation (section 2.2.3.2.e.) which in turn is a CVD risk factor. Decreased PLP in combination with increased hs-CRP and elevated LDL:HDL ratio increases the risk for a cardiovascular event in a graded manner (Lotto *et al.*, 2011:184). Decreased PLP concentrations hastens the atherosclerotic process, as mentioned PLP is crucial in pathways related to thrombosis, inflammation and immune function (Lotto *et al.*, 2011:189; Ueland *et al.*, 2017:12).



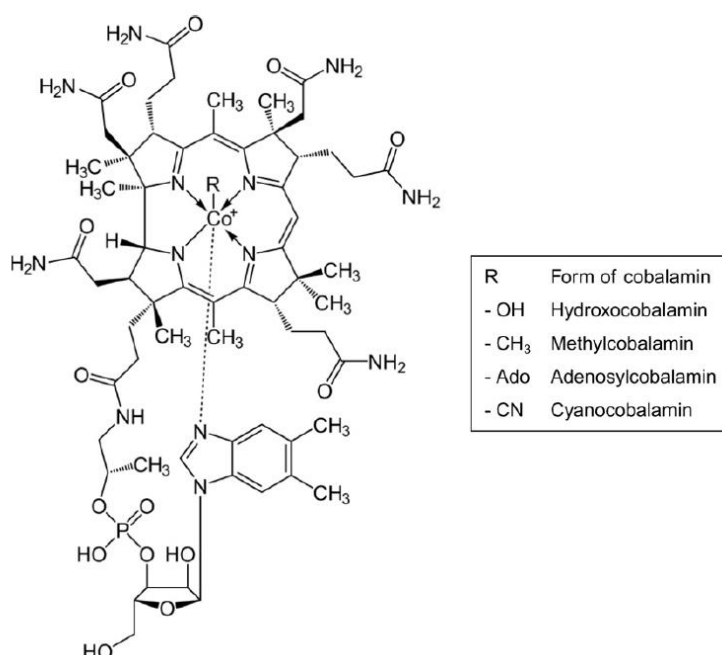
### 2.5.5. Normal vitamin B6 serum levels

The normal range for PLP is between 5 µg/L and 30 µg/L, while less than 5 µg/L is considered low and more than 30 µg/L as high (Lotto *et al.*, 2011:183; Gregory *et al.*, 2013:e63545; Burtis and Bruns, 2015:473).

## 2.6. VITAMIN B12

### 2.6.1. Vitamin B12 physical characteristics

Vitamin B12 is produced only by microorganisms, e.g. bacteria and algae, including bacteria in the gastrointestinal tract of animal (Collin, 2013:18). For humans the primary source of vitamin B12 is animal protein, eggs and dairy products (Chatthanawaree, 2011:227; Collin, 2013:18). Cobalamin is the family name for a group of molecules, including vitamin B12, that have a corrin ring with a central cobalt ion, with variations on this ion (Chatthanawaree, 2011:227; Collin, 2013:17; Hughes *et al.*, 2013:316). Figure 11 depicts the chemical structure of cobalamin and indicates the central cobalt ion where the variants are attached. Also indicated in Figure 11 are some examples of the variations on the central cobalt ion (indicated by an R) are as follow: CN<sup>-</sup> – cyanocobalamin, CH<sub>3</sub> – methylcobalamine, Ado – adenosylcobalamin (synonym: deoxyanosincobalamin) and OH<sup>-</sup> – hydroxocobalamin (Collin, 2013:17). The synthetic form of vitamin B12 is known as cyanocobalamin and is very stable and therefore used for food fortification (Hughes *et al.*, 2013:316).



**Figure 11. Chemical structure of vitamin B12 (Collin, 2013:17)**

### 2.6.2. Vitamin B12 biochemical metabolism

Vitamin B12 is bound to protein and is released by pepsin and hydrochloric acid in the stomach where it is bound to haptocorrin (also known as HC, R-protein and transcobalamin I) which is produced by salivary glands and parietal cells (Collin, 2013:18; Hughes *et al.*, 2013:317). Vitamin B12 is protected against denaturation in the stomach when part of this holohaptocorrin complex (Collin, 2013:18). Once, as illustrated by Figure 12, in the duodenum vitamin B12 is released from haptocorrin (which is digested by pancreatic proteases) and binds to intrinsic factor, travelling to the terminal portion of the ileum (Collin, 2013:18; Hughes *et al.*, 2013:317). Here, vitamin B12-intrinsic factor complex enters ileum cells via cubilin or megalin receptors through calcium dependant endocytosis (Collin, 2013:18; Hughes *et al.*, 2013:317). Another absorption pathway that occurs in the absence of intrinsic factor is diffusion (Hughes *et al.*, 2013:317). In the ileal cell B12 binds to transcobalamin (now known as holotranscobalamin) and is transported across the basal membrane into portal blood (Collin, 2013:18; Shipton and Thachil, 2015:145).

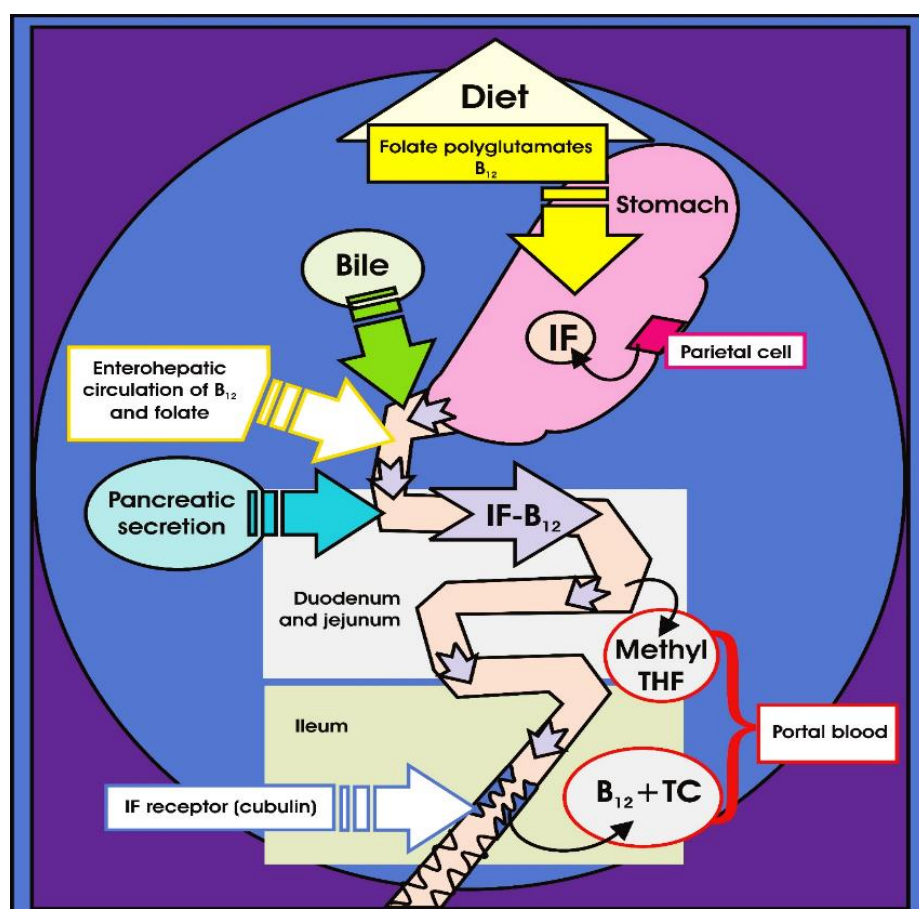
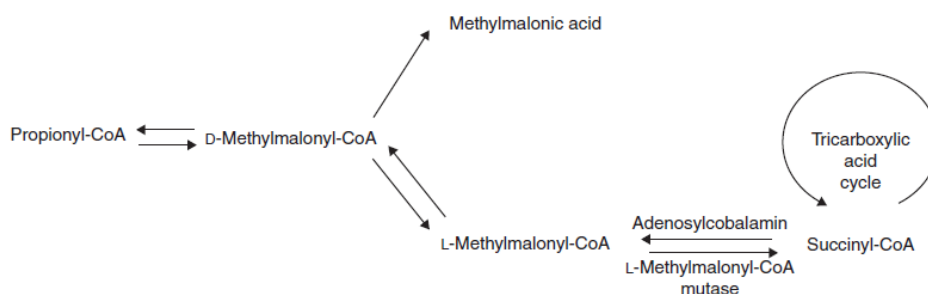


Figure 12. Digestion and absorption of vitamin B12 (Grobler, 2015:82)

The liver stores majority of vitamin B12 and excretes 1-10 mg a day into bile, although 90% of this is reabsorbed (Chatthanawaree, 2011:227; Hughes *et al.*, 2013:317). Only 15% of vitamin B12 is bound to holotranscobalamin the rest is bound to holohaptocorrin, although holotranscobalamin is the only bioavailable portion and holohaptocorrin transports vitamin B12 to the liver, where it enters hepatocytes via asialoglycoprotein receptor (Collin, 2013:18&20). When intracellular levels of methylcobalamin are low in proliferating cell, a receptor called transcobalamin receptor (part of the low-density lipoprotein receptor family) is upregulated and holotranscobalamin is absorbed into cells via endocytosis (Collin, 2013:20).

Inside the cell, transcobalamin is degraded and cobalamin is converted to methylcobalamin and adenosylcobalamin (Chatthanawaree, 2011:228; Collin, 2013:20). During the folate cycle methylcobalamin acts as a co-factor for MTR for the conversion of Hcy to methionine (as seen in Figure 4), while adenosylcobalamin enters the mitochondrion to act as a co-factor, as illustrated in Figure 13, for methylmalonyl CoA mutase for the conversion of methylmalonyl CoA to succinyl CoA in the tricarboxylic acid cycle (or Krebs cycle) (Chatthanawaree, 2011:228; Collin, 2013:20; Hughes *et al.*, 2013:316).



**Figure 13. Vitamin B12 (i.e. adenosylcobalamin) dependant conversion of methylmalonyl-CoA to succinyl-CoA (Hughes *et al.*, 2013:316)**

### 2.6.3. Vitamin B12 deficiency

Vitamin B12 deficiency can be due to any of the following: vegetarianism, veganism, low dietary intake and alcohol abuse, while the following leads to malabsorption: pernicious anaemia, Zollinger–Ellison syndrome, drug–nutrient interactions (e.g. Proton pump inhibitors, H2 antagonists, metformin, nitrous oxide interactions) (Hughes *et al.*, 2013:320). In the elderly the main reason for malabsorption of vitamin B12 can be due to any of the following: atrophic gastritis, bacterial overgrowth in the small intestine, degradation of R-protein (due to pancreatic insufficiency), total gastrectomy (or ileal resection/bypass), Chron's disease, tropical and ileac spree (Mirkazemi *et al.*, 2012:227). Aging

causes a decrease in protein-bound vitamin B12 absorption, but crystalline vitamin B12 absorption does not have this problem, making it ideal for fortification and supplementation (Hughes *et al.*, 2013:322). It takes years for a vitamin B12 deficiency to develop, as the daily requirement is so little when compared to body stores, even if a complete stop in vitamin B12 absorption occurs (Hughes *et al.*, 2013:317).

Moreover, a deficiency leads to 5-methyl THF accumulation and decrease of THF which in turn results in a cellular folate deficiency, thus DNA biosynthesis will not occur causing an anaemia similar to that of a true folate deficiency (Chatthanawaree, 2011:228; Bito and Watanabe, 2016:1665). The effects of vitamin B12 deficiency can again be classified, like folate, as haematological and neurological. The former is a result of the aforementioned impaired DNA synthesis and includes megaloblastic anaemia, which is characterised by hypersegmented neutrophils, leukopenia, thrombocytopenia and large immature blood cells with low haemoglobin levels (Hughes *et al.*, 2013:322). The neurological features vary, but can be irreversible if the deficiency is not treated, with the most severe manifestation being degeneration of the spinal cord (Hughes *et al.*, 2013:322). Apart from risk for CVD, attention is also given to cognitive function, osteoporosis and fracture risk in correlation with vitamin B12 deficiency (Hughes *et al.*, 2013:322).

#### **2.6.4. Vitamin B12 role in cardiovascular risk**

Depleted vitamin B12 leads to inhibition of MTR enzyme, thus reducing methylation reactions and increasing Hcy levels (Hughes *et al.*, 2013:316). Methylmalonic acid levels increase in the absence of vitamin B12, destabilizing mitochondrial metabolism and leading to the formation of reactive oxygen species further contributing to CVR (Fenech, 2012:22; Bito and Watanabe, 2016:1665). Moreover, vitamin B12 supplementation enhances endothelial function and improves heart variability (suggesting sympathetic involvement) in elderly (Kwok *et al.*, 2012:571; Sucharita *et al.*, 2012:70).

#### **2.6.5. Normal vitamin B12 serum levels**

There are several definitions for vitamin B12 deficiency, but these depend on population studies and the assay utilized (Scarpa *et al.*, 2013:628). Apart from the different direct detection methods (section 2.6.6.), other biomarkers can be used as an indication of possible vitamin B12 deficiency indirectly (section 2.6.7.), namely, plasma holotranscobalamin, serum methylmalonic acid, Hcy levels and red blood cell mean corpuscular volume (Chatthanawaree, 2011:228). The normal range cut-points for vitamin B12 vary between methods, laboratories and in literature. This disparity alters data

interpretation and sensitivity-specificity balance and results in conflicting conclusions (Carmel, 2013:1050).

As there is no gold standard for the ranges of a vitamin B12 deficiency, an upward shift in cut-off points was seen in the 1980s (150 pmol/L – 250-3600 pmol/L) to increase sensitivity, leading to a misdiagnosis of deficiency for individuals with normal levels (Carmel, 2013:1051). Furthermore, the lack of a global standard also makes it problematic to compare different studies. Burtis and Bruns (2015:982) suggests that a value of less than 110 pmol/L (149.05 pg/ml) be considered deficient and more than 147 pmol/L (199.19 pg/mL) as acceptable. MacFarlane *et al.* (2011:1081) classified a deficiency as less than 148 pmol/L, marginal between 148 and 220 pmol/L and adequate as more than 220 pmol/L. Chatthanawaree (2011:229) also found range classification for serum vitamin B12 is method dependant, but can be classified according to deficiency: more than 300 pg/mL is deficiency unlikely, 200-300 pg/mL indicates possible deficiency and less than 200 pg/mL indicates deficiency.

## **2.7. MTHFR ENZYME**

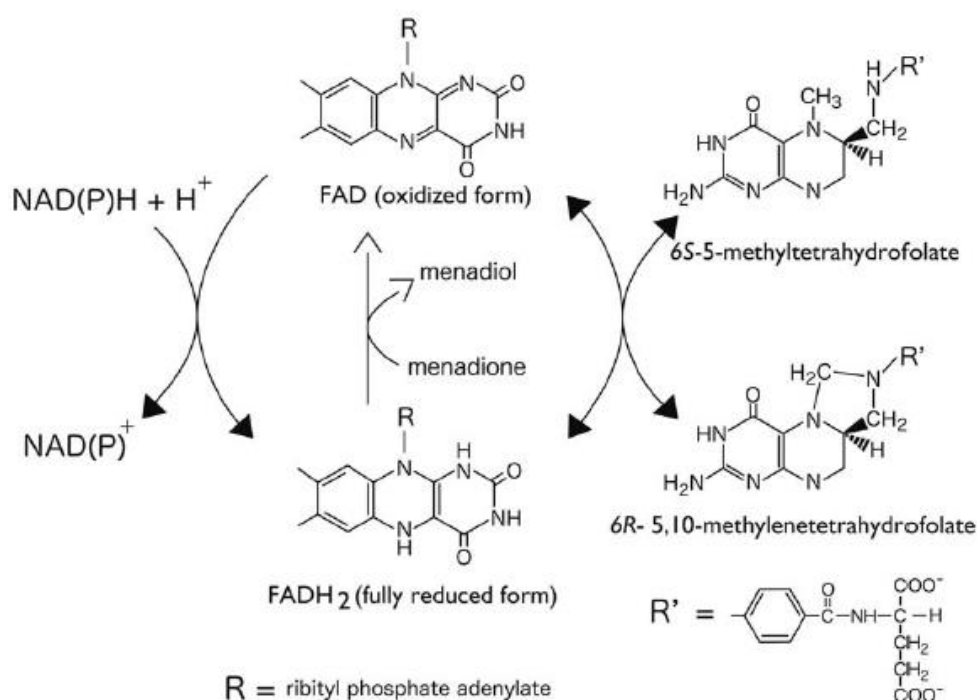
### **2.7.1. MTHFR enzyme physical characteristics**

The enzyme Methylenetetrahydrofolate reductase consists of 656 amino acids which is encoded by the MTHFR gene (section 2.7.3.) (Igari *et al.*, 2011:2; Akram *et al.*, 2012:1599; Shahzad *et al.*, 2013:929). In eukaryotic cells MTHFR exists as a homodimer with each subunit consisting of both a regulatory and a catalytic domain (Igari *et al.*, 2011:2; Shahzad *et al.*, 2013:929). The catalytic domain is at the N-terminal while the regulatory domain is at the C-terminal (Forges *et al.*, 2010:143). The N-terminal interacts with 5,10-methylene THF, Nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) (the electron donor) and flavin adenine dinucleotide (FAD), the coenzyme (Forges *et al.*, 2010:143). At the C-terminal SAM can bind essentially inhibiting the enzyme (Forges *et al.*, 2010:143; Shahzad *et al.*, 2013:930). It can also be phosphorylated which reduces the enzyme's activity by 20% and is also more easily inhibited by SAM (Shahzad *et al.*, 2013:930). The enzymes' optimal temperature is 24-37°C with a pH of 4.3 (Igari *et al.*, 2011:2).

### **2.7.2. MTHFR enzyme function**

As previously mentioned in both folate and Hcy (section 2.3. and 2.4.) the MTHFR enzyme is important for the methylation of Hcy (Blom and Smulders, 2011:77; Igari *et al.*, 2011:1; Collin,

2013:16; Kirsch *et al.*, 2013:497; Jennings and Willis, 2015:224). More specifically MTHFR is responsible for the conversion of 5,10-methylene THF to 5-methyl THF (Blom and Smulders, 2011:77; Collin, 2013:16; Kirsch *et al.*, 2013:497; Jennings and Willis, 2015:224). The MTHFR enzyme has a non-covalent bound co-factor, namely FAD, as illustrated in Figure 14, which is reduced with NAD(P)H and  $H^+$  (forming NAD(P) $^+$ ) (Igari *et al.*, 2011:2). This results in the formation of fully reduced FAD (now FADH<sub>2</sub>) which donates two  $H^+$  molecules. Menadione accepts one  $H^+$  as well as a methyl group (the other  $H^+$  acceptor) from 5,10-methylene THF resulting in the production of 5-methyl THF, menadiol and FAD (Igari *et al.*, 2011:2). The enzyme is inhibited by a negative feedback loop when there is a high concentration of SAM (produced using methionine as a substrate) which binds to the C-terminal of enzyme as previously mentioned (Forges *et al.*, 2010:143; Shahzad *et al.*, 2013:930).



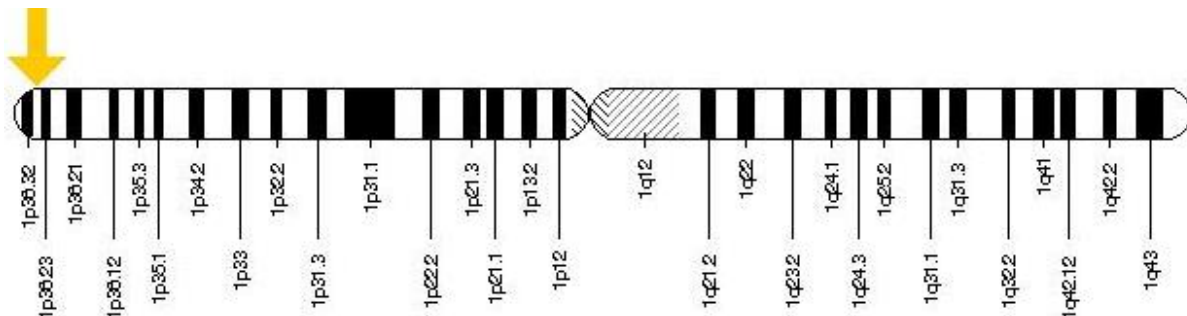
**Figure 14. Function of MTHFR for the conversion of 5,10-methylene THF to 5-methyl THF (Igari *et al.*, 2011:2)**

### 2.7.3. MTHFR gene

The MTHFR gene is located on the short arm of chromosome one (Figure 15), specifically at position 36.1, which is abbreviated as 1p36.3 (Rao *et al.*, 2010:6; Aneji *et al.*, 2012:85; Safarinejad *et al.*, 2012:269; Izmirli, 2013:625). The precise location on chromosome one is from base pair (bp) 11,769,246 to bp 11,788,568 (Rao *et al.*, 2010:6) making it 2.2 kb in length (Aneji *et al.*, 2012:85; Safarinejad *et al.*, 2012:269; Izmirli, 2013:325). The initial number of exons was thought to be 11 as



first reported by Goyette *et al.* (1998), but subsequent studies have proved that there are 14 exons as published on the The National Center for Biotechnology Information (2015b) database (gene ID: 4524, reference sequence: NM\_005957.4). The MTHFR gene does not have a TATA box region; rather it has cytosine-phosphate guanine (CpG) islands which acts as a binding site for multiple transcription factors. Moreover, there are two of these promoter regions with 2 isoforms of the enzyme (Akram *et al.*, 2012:1599; Baroudi *et al.*, 2013:1).



**Figure 15. Location of the MTHFR gene on chromosome 1's short arm indicated by yellow arrow (Genetics Home Reference, 2015)**

#### 2.7.4. MTHFR single nucleotide polymorphisms

The two polymorphisms with the highest impact are MTHFR A1298C and C677T, although other polymorphisms include T1317C and G1793A (Izmirli, 2013:325). A deficiency in MTHFR enzyme (Online Mendelian Inheritance in Man, 2014. ID: MIM 236250) is an autosomal recessive condition which is classified as part of Hcy remethylation disorders (includes MTR deficiency) with the following features: homocystinuria, low plasma methionine and hyperhomocysteinemia (Forges *et al.*, 2010:143; Tsuji *et al.*, 2011:521; Richard *et al.*, 2017:2). There are approximately 52 SNPs in the MTHFR gene, although this number can increase with continuing research (Miyaki, 2010:267). The C677T SNP (The National Center for Biotechnology Information 2015a. ID: rs1801133) is located on exon 4 and causes a replacement of alanine to valine at codon 222 on the protein (Forges *et al.*, 2010:144; Safarinejad *et al.*, 2012:270; Izmirli, 2013:325). This substitution is located at the N-terminal of the enzyme which binds FAD, causing a 35% and 70% reduction in function in heterozygotes and homozygotes, respectively, in comparison to the wild type (Forges *et al.*, 2010:144; Izmirli, 2013:325).

#### **2.7.4.1. MTHFR A1298C**

The MTHFR A1298C SNP (The National Center for Biotechnology Information 2015a. ID: rs1801131) is located on the 7<sup>th</sup> exon of the gene and causes a substitution of an adenine nucleotide to cytosine (Safarinejad *et al.*, 2012:270; Izmirli, 2013:325). In the protein this SNP will cause a glutamate to be substituted with an alanine at position 429 (Akram *et al.*, 2012:1600; Izmirli, 2013:325). Position 429 of the protein is found within the C-domain (regulatory site) where SAM will inhibit the enzyme and it causes conformational change that adversely affects enzymatic activity (Izmirli, 2013:325). The clinical consequences of A1298C SNP are less documented than C677T SNP (Forges *et al.*, 2010:144; Hickey *et al.*, 2013:154). Homozygous for 1298CC individuals' enzyme activity is 40% less than individuals who are homozygous for 1298AA (Safarinejad *et al.*, 2012:270).

#### **2.7.4.2. MTHFR A1298C role in cardiovascular risk**

In summary, MTHFR reduces 5,10-methylene THF to 5-methyl THF, which is further reduced to THF by MTR (Blom and Smulders, 2011:77; Collin, 2013:16; Kirsch *et al.*, 2013:497; Jennings and Willis, 2015:224). This donated methyl group attaches to MTR along with co-factor vitamin B12 (Blom and Smulders, 2011:77; Wang *et al.*, 2011:486; Bednarska-Makaruk *et al.*, 2016:84) in remethylation of Hcy to produce methionine (Main *et al.*, 2010:1598; Blom and Smulders, 2011:76; Collin, 2013:23). The MTHFR A1298C SNP reduces MTHFR enzyme activity (Safarinejad *et al.*, 2012:270), causing the methyl group not to be readily available for attachment to Hcy to produce methionine (Main *et al.*, 2010:1598; Aneji *et al.*, 2012:2). Therefore, increasing Hcy levels and risk for CVD.

### **2.8. CONCLUSION**

Atherosclerosis is the predecessor for majority of CVDs and a variety of risk factors contribute to pathogenesis thereof. Hcy is a well-established independent risk factor and is influenced by folate, vitamin B6 and vitamin B12 levels. Furthermore, SNP of Hcy regulatory enzymes can result in hyperhomocysteinemia. The two SNPs that has the most influence on Hcy levels is MTHFR A1298C and MTHFR C677T. Thus it is important to consider these when evaluating Hcy levels.



## **CHAPTER 3**

### **METHODOLOGY**

#### **3.1. INTRODUCTION**

This study aimed to gain knowledge of the prevalence of specific genetic factors, their effect on Hcy, and contribution to CVD risk in the study population group. In chapter 3 the methods utilized during this study as well as ethical considerations were discussed. Furthermore, study design and the principles of each method has been described. Special attention was given to reliability, validity and reproducibility of results.

#### **3.2. ETHICAL CONSIDERATIONS**

This research project was done within the borders of the Constitutional Law of the Republic of South Africa by adhering to Section 12(2)(c) of the Constitution of South Africa Act, no. 108 of 1996: “Everyone has the right to bodily and psychological integrity, which includes the right not to be subjected to medical or scientific experiments without their informed consent” (South African Department of Health, 2015:6). Furthermore, principles and guidelines dictated by the Belmont report (The United States' Department of Health & Human services, 1979), Declaration of Helsinki (World Medical Association, 2013) and the South African Department of Health (2015) was adhered to for the duration of this project. The Medical Research Council of South Africa's ethical principles were also followed; they are: autonomy (respect should be upheld for the participants and their human dignity), beneficence (study participants should benefit from the study), non-maleficence (the participants should not be harmed in the study) and justice (risk and benefits should be equally distributed among communities) which are adapted from the Helsinki Declaration (Medical Research Council of South Africa, 2004:9).

As mentioned in chapter 1 the multi-nutritional programme that is led by Prof WH Oldewage-Theron at the centre was approved by the ethical committee of the University of the Witwatersrand, Johannesburg (M070126) (Annexure B). For this specific study additional ethical approval was applied for at the VUT ethical committee (20140827-1ms): please see Annexure C.

The elderly who attend the Sharpeville Day Care centre live in vulnerable circumstances as they earn a low income. They thus need to be respected at all times with the necessity of not having them feel like they are being taken for granted. All analysis were done on blood and such the blood withdrawal process can be very stressful to certain individuals. A qualified phlebotomist was employed to ensure as little harm as possible was inflicted. The blood collection was conducted in the correct prescribed manner.

Communication with the elderly was done in a language that they understand best. All the following was communicated to them before written consent (Annexure D) was obtained.

- The nature and purpose of the study.
- The identity, institution and contact details of the study leader and student supervisor.
- Participation being voluntary and that all responses will be treated confidentially.
- Furthermore, confidentiality will be insured by using designated laboratory numbers which was blinded to the researcher and laboratory personnel.
- And lastly, that they are free to withdraw from the study at any point without any negative consequences to themselves.

An initial visit was made to the day care centre in Sharpeville. The participants and management of the centre were informed about the study, and permission was given for the study to be conducted with elderly volunteers who attend the centre. A follow-up visit was made to explain the aims/objectives of the study and the elderly gave their consent. The elderly that were illiterate signed their consent forms with an x. All the data will be stored for 5 years by the student and by the supervisor.

### **3.3. SAMPLE SELECTION**

#### **3.3.1. Sample size**

In order to establish the prevalence of MTHFR 1298CC (homozygous for SNP) in the Sharpeville population the following power calculation was done for sample size (Charan and Biswas, 2013:122):

$$n = \frac{Z_{1-\alpha/2}^2 p (1 - p)}{d^2}$$

Where:

$Z_{1-\alpha/2} = 1.96$  at 5% type 1 error ( $P < 0.05$ )

$p = 5\%$  expected proportion in the population = 0.05 (Scholtz *et al.*, 2002:466)

$d = 0.05$  precision/absolute error

Then:

$$n = \frac{1.96^2 \times 0.05 (1 - 0.05)}{0.05^2}$$

$$n = 72.99$$

A total of 73 individuals were needed for statistical representation for this study.

### **3.3.2. Inclusion criteria**

The participants fell within the same age grouping (older than 60), were of the same race (black), demographic (Sharpeville) and socio-economic status (unemployed or pensioner).

Other inclusion criteria that were considered:

- Participants were attendees at the day care centre.
- All the participants gave informed consent.
- If the participants suffered from diagnosed diabetes and megaloblastic anaemia, they were not excluded from this study. These conditions are considered as CVR markers and therefore, relevant to this study.

### **3.3.3. Exclusion criteria**

- Participants who could not provide substantial information to complete the consent process/form were excluded from this study.

### 3.4. STUDY DESIGN

The research was an analytical observational study (describes associations and analyses them for possible cause and effect) and cross-sectional (measurements were made on a single occasion) (Fathalla and Fathalla, 2004:44). A purposive sampling strategy was used (Martinez-Mesa *et al.*, 2016:328). As a result, 84 individuals met the inclusion criteria and was selected from all the elders attending the Sharpeville day care centre. Blood collection from some of the elderly proved difficult and sufficient blood could not be collected. The result was that some analytes had less than 84 samples.

### 3.5. DATA COLLECTION

The study aimed to meet reliability (consistency of measurements) and validity (accuracy of measurements) criteria to be of a high quality. The following procedures were put in place to combat error thus ensuring that results were reliable, valid and reproducible (Pietersen and Maree, 2010:217; Surbhi, 2017):

- Face validity – logic is used to decide if an instrument appears to be measuring what it should: This is not quantifiable, but through a proper literature study, all laboratory instruments and procedures were ascertained to have a high level of face validity.
- Content validity – the degree by which an instrument represents every single element of conceptual construct: literature was used to confirm the laboratory instruments and procedures covered all possibilities to achieve accurate measurements.
- Criterion validity – the degree to which an instrument measures what it is supposed to: This was ensured by measuring a control serum throughout the analysis.
- Construct validity – a correlation between different instruments' measurements of an analyte: Variables were identified from literature that could possibly have an influence on results and these were measured.

The data collection was done in May 2015. Each of the participants were allocated a sample number and a fieldworker had a file number corresponding to each participant. All the blood collection tubes were labelled with this sample number. The phlebotomist measured blood pressure of the participants in duplicate and recorded it in the file, followed by blood collection using a vacutainer system from the vena cephalica. During the blood collection, the phlebotomist checked that the sample number on file corresponded with the tube labels for

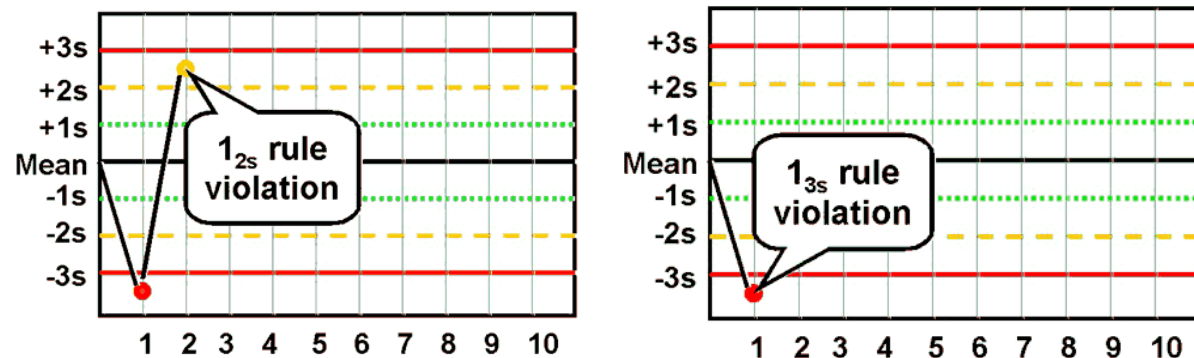
each person. Dural variation was avoided by collecting blood between 07:00 and 10:00. Two 7 ml serum separator tubes (SSTs) and one 5 ml ethylenediaminetetraacetic acid (EDTA) tube was drawn from each of the participants. All the blood tubes were placed in a cooler box with ice packs to be cooled to 8°C and protected from direct sunlight. The samples were transported to the laboratory within two hours for centrifugation and DNA extraction. The SST tubes were centrifuged for 10 minutes at 3000 rpm at room temperature. The serum was aliquoted into test tubes labelled with the same sample numbers. The EDTA tubes were used for molecular analysis; thus upon arrival at the lab, DNA was extracted and stored in micro-centrifuge tubes at -80°C.

Errors such as mismatch were eradicated as the aliquot tubes and DNA micro-centrifuge tubes were labelled with the sample number and stored numerically. Before any transfer of serum or DNA occurred, face validity verification was utilized and number on test tube was double checked for correspondence with the aliquot tubes and DNA micro-centrifuge tubes. All laboratory analysis was done alongside a commercially available validated control serum. An instrument is considered valid when a specified parameter's minimum and maximum is measured accurately and precisely (Pietersen and Maree, 2010:215; Surbhi, 2017). The reliability of an instruments was determined by mean, standard deviation (SD) and coefficient variance (CV), while validity was determined by a standard curve. The Konelab™ used in this study is programmed with the Westgard rules for the interpretation of the Levey-Jennings chart (mean and SD) for quality control (QC) and the assurance of reliability. The rules were manually applied to the Tosoh AIA®-600 II and Agilent 1260 Infinity high-performance liquid chromatography (HPLC) system. The  $1_{2s}$  rule is when the control limits with a Levey-Jennings chart is set at the mean  $\pm 2SD$ , meaning a run is rejected when a single control was outside of this 2SD range of the indicated control value as seen in Figure 16. The  $1_{3s}$  rule is when a single control falls 3SD outside of control limit – the run is then rejected, also illustrated in Figure 16. The  $1_{2s}$  rule is more often used even though it may result in a high level of false rejections, as it has a high error detection. The  $1_{3s}$  rule has a lower rate of false rejections, but it also has a lower error detection rate (Barry, 2009).

The previous two rules are applied in a single-rule QC procedure, where a single set of control limits are used. A multi-rule QC uses a combination of control rules to decide if an analytical run is inside or outside of the control. The well-known Westgard multi-rule QC procedure applies five different control rules to analyse an analytical run as acceptable namely:  $1_{3s}$ ,  $2_{2s}$ ,  $R_{4s}$ ,  $4_{1s}$  and  $10_x$  (described in Table 4) where the  $1_{2s}$  is used as a warning rule to trigger careful inspection of control data by the other rules. These rules are generally applied when two or

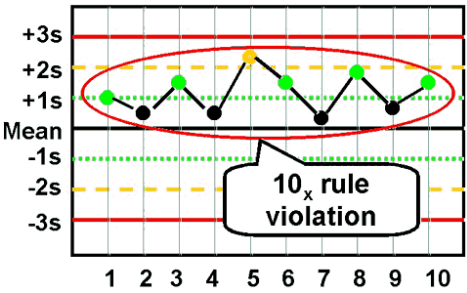
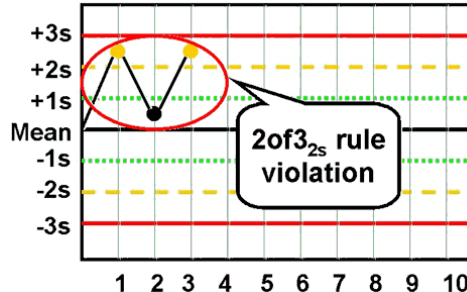
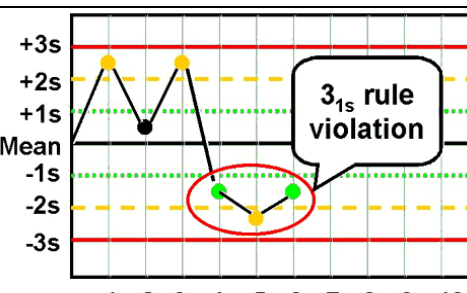
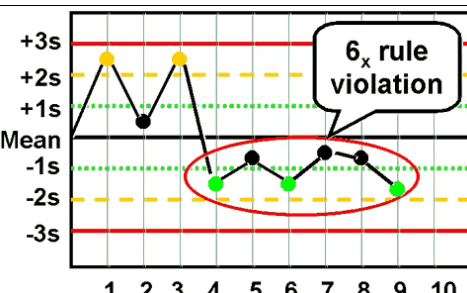
four control measurements are used per run (meaning two different control materials are measured once or twice each). Other control rules are applied when three control materials are utilized namely:  $2\text{of}3_{2s}$ ,  $3_{1s}$  and  $6_x$  (also described in Table 4). (Westgard, 2009a)

**Table 4. Other Westgard rules (Westgard, 2009a)**



**Figure 16. Levey-Jennings chart indicating  $1_{2s}$  and  $1_{3s}$  rules respectively (Westgard, 2009a)**

Name of rule	Rule	Levey-Jennings chart
$2_{2s}$	Reject run if two consecutive control measurements are 2SDs from the mean either plus or minus.	
$R_{4s}$	Reject if 2 consecutive control measurements are +2SD and -2SD respectively, within the same run.	
$4_{1s}$	Run is rejected if four consecutive measurements exceed the same mean with +1SD or -1SD control limit.	

10 <sub>x</sub>	The run is rejected when 10 consecutive control values fall on one side of the mean.	 <p>A control chart with a mean line at 0 and standard deviation limits at ±1s, ±2s, and ±3s. Ten consecutive data points (green dots) are plotted above the mean line, enclosed in a red oval. A callout box points to the oval with the text '10<sub>x</sub> rule violation'.</p>
2of3 <sub>2</sub>	Run is rejected when 2 out of 3 control values exceed the same mean plus 2SD or mean minus 2SD control limit.	 <p>A control chart with a mean line at 0 and standard deviation limits at ±1s, ±2s, and ±3s. Three data points are plotted: two are above the +2s limit (yellow dots) and one is below the mean (black dot). A red oval encloses the two points above +2s, with a callout box pointing to it saying '2of3<sub>2s</sub> rule violation'.</p>
3 <sub>1s</sub>	Run is rejected if three consecutive control measurements exceed mean +1SD or -1SD control limit.	 <p>A control chart with a mean line at 0 and standard deviation limits at ±1s, ±2s, and ±3s. Three consecutive data points (green dots) are plotted below the -1s limit, enclosed in a red oval. A callout box points to the oval with the text '3<sub>1s</sub> rule violation'.</p>
6 <sub>x</sub>	The run is rejected when six consecutive control values fall on one side of the mean.	 <p>A control chart with a mean line at 0 and standard deviation limits at ±1s, ±2s, and ±3s. Six consecutive data points (green dots) are plotted below the mean line, enclosed in a red oval. A callout box points to the oval with the text '6<sub>x</sub> rule violation'.</p>

For this study 1<sub>2s</sub> (used as a warning rule to trigger careful inspection of control data by the other rules), 1<sub>3s</sub>, 2of3<sub>2s</sub>, 3<sub>1s</sub> and 6<sub>x</sub> rules were used to judge control limit values.

### 3.6. BLOOD ANALYSIS

The laboratory analyses were performed at the Vaal University of Technology's laboratories, strictly following standard operating procedures, to comply with the South African National Accreditation System (SANAS). Furthermore, this was done under the supervision of Dr C.J.

Grobler (a HPCSA registered medical technologist) and the assistance of available enrolled BTech Biomedical Technology students. Although DNA extraction was also done at VUT, the molecular analysis was done under the supervision of Dr P.J. Lebea at the molecular laboratory at the Council for Scientific and Industrial Research (CSIR) in Pretoria, Gauteng.

### **3.6.1. Measuring instruments**

The results reported in chapter 4 were measured with automated laboratory equipment, namely, Thermo Konelab™ 20i (Replamed, Pretoria, South Africa), Tosoh AIA®-600 II (Replamed, Pretoria, South Africa), Agilent 1260 Infinity HPLC system (Chemetrix (Pty) Ltd., Johannesburg, South Africa) and LightCycler® 96 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany). There were no other measuring instruments e.g. questionnaires, anthropometric instruments etc.

#### **3.6.1.1. Automated Konelab™**

The Konelab™ 20i is an automated clinical chemistry system manufactured by Thermo Scientific. The instrument was used to measure Hcy by photometric principles. The automated system uses colorimetric and turbidimetric principles by using a single channel interference filter photometer. A halogen lamp with linear absorbance (A) range of 0-25 and resolution of 0.0001 A is utilized by the instrument. The spectral range is 340-800 nm and measure the reactions in sequence in the cells (Thermo Scientific, 2009:12-13).

#### **3.6.1.2. Tosoh AIA®-600 II**

The Tosoh AIA®-600 II system is capable of performing three different assays, namely, a competitive binding immunoassay, an immunoenzymetric immunoassay and a two-step immunoenzymetric immunoassay (Tosoh Biosciences, 2008:10). All these assays occur in a test cup (including measurements) which contains the necessary reagents and analytes for analysis of one specimen. The concentration of the analyte is measured and calculated with a fluorescent rate method and a predetermined calibration curve. (Tosoh Biosciences, 2008:11) The system can use up to 6 calibrator sets for each analyte and can store two different sets (Tosoh Biosciences, 2008:14). For this study the Tosoh AIA®-600 II system was used for the detection of vitamin B12 and folate concentrations in serum.



### **3.6.1.3. Agilent 1260 Infinity HPLC system**

The Agilent Infinity HPLC system consists of an ultra-low carryover autosampler, a quaternary pump and thermostatic column compartment. The system has a flow rate of 5 mL/min with a pressure range of up to 600 bar. Furthermore, the system has an ultraviolet (UV) light detector, evaporative light scattering detector (ELSD) or fluorescence detector (LabX, 2017).

### **3.6.1.4. Roche Diagnostics LightCycler® 96 Real-Time PCR System**

The LightCycler® 96 system is capable of standard analysis i.e. absolute-, relative quantification and endpoint genotyping, although it also has dedicated modules for qualitative detection and an advanced high-resolution melting analysis (Roche Diagnostics, 2013:2). The main components of the block cycler unit consist of the silver thermal block cycler, the heated block cycler cover, the block cycler fan, and the electroformed silver mount (Roche Diagnostics, 2013:4). The system has a optic system comprised of two sets of fibre optics – one for excitation light and one for collecting the emitted light to and from the well (Roche Diagnostics, 2013:4). The temperature range is between 37 and 98°C with  $\pm 0.2^\circ\text{C}$  accuracy of target temperature (Roche Diagnostics, 2013:10). The LightCycler® 96 system was used for the detection of MTHFR 1298AA, MTHFR 1298CC and MTHFR 1298AC.

## **3.6.2. Blood parameters**

As mentioned before the following analytes were measured in this study: Hcy, folate, vitamin B6 and vitamin B12, with the genotype of MTHFR A1298C also determined. Commercially available kits are available for Hcy, folate, vitamin B6 and vitamin B12, and the manufacturer's instructions were followed for the determination of concentrations. Sample genotype for MTHFR A1298C was determined with a commercial MTHFR real time polymerase chain reaction (qPCR) kit using a commercial qPCR mastermix.

### **3.6.2.1. Homocysteine**

DiaSys Diagnostic System's Hcy fluid stable reagents (Replamed, Pretoria, South Africa) was used to determine serum Hcy concentrations. The assay is an enzymatic cycling method that determines serum Hcy concentrations by measuring a co-substrate converted from Hcy. The reagents are comprised of SAH, nicotinamide adenine dinucleotide (NADH), tris (2-

carboxyethyl) phosphine (TCEP), 2-oxolutarate, glutamate dehydrogenase, SAH hydrolase, adenosine deaminase and homocysteine methyltransferase. (DiaSys Diagnostic Systems, 2012)

#### **a. Principle**

This assay detects a co-substrate that is converted from Hcy. An EDTA collection tube of blood is centrifuged and the plasma is used for the assay (Axis-Shield, 2011; Demeditec Diagnostics, 2013; Diazyme, 2014). Bound Hcy is reduced to free Hcy, which is then reacts with SAM to form methionine and SAH (catalysed by S-methyltransferase) (Axis-Shield, 2011; Demeditec Diagnostics, 2013; Diazyme, 2014). The resulting SAH is catalysed by SAH-hydrolyse to produce adenosine and Hcy (Axis-Shield, 2011; Demeditec Diagnostics, 2013; Diazyme, 2014). This Hcy is cycled back into the reaction with SAM, causing amplification. The formed adenosine is catalysed by adenosine deaminase to inosine and  $\text{NH}_3$  (Axis-Shield, 2011; Demeditec Diagnostics, 2013; Diazyme, 2014). Glutamate dehydrogenase causes  $\text{NH}_3$ , NADH and 2-oxoglutaraate to form glutamate,  $\text{NAD}^+$  (oxidised NADH) and water. The decreased NADH concentration is measured at 340 nm and is directly proportional to the amount of Hcy in the sample (Axis-Shield, 2011; Demeditec Diagnostics, 2013; Diazyme, 2014).

#### **b. Validation**

Hcy calibrators (TruCal) from DiaSys Diagnostic System (Replamed, Pretoria, South Africa) was used to determine the Konelab™'s range for quantifying Hcy. Level 0 concentration was 0  $\mu\text{mol/L}$ , level 1 was 6.00  $\mu\text{mol/L}$  and level 2 was 29.5  $\mu\text{mol/L}$ . A linear calibration curve was produced from these readings. The reliability of instrument was evaluated with Hcy controls (TruLab): level 1 (range: 10.6-15.8  $\mu\text{mol/L}$  and mean: 13.2  $\mu\text{mol/L}$ ) and 2 (range: 26.4-35.8  $\mu\text{mol/L}$  and mean: 31.1  $\mu\text{mol/L}$ ) also from DiaSys Diagnostic System (Replamed, Pretoria, South Africa).

If the controls were not in line with the Westgard rules (as discussed in section 3.5.), the control values were rejected. All the reagents in the instrument were replaced with fresh ones and instrument was re-calibrated with fresh calibrators after which controls were re-run. This resolved the issue of controls and no further action was needed.

### **c. Reference value**

Hcy concentration is classified as normal ( $\leq 15 \mu\text{mol/L}$ ), mild ( $16\text{--}30 \mu\text{mol/L}$ ), moderate ( $31\text{--}100 \mu\text{mol/L}$ ) and severe ( $>100 \mu\text{mol/L}$ ) (Abraham and Cho, 2010:912; Hirmerová, 2013:e250; Ganguly and Alam, 2015:2).

### **3.6.2.2. Folate**

Folate concentration was determined with an AIA-PACK FOLATE manufactured by Tosoh Biosciences (Replamed, Pretoria, South Africa). The method is a competitive enzyme immunoassay and uses serum. The reaction occurs in a test cup with lyophilized magnetic beads. (Tosoh Biosciences, 2009b:1).

#### **a. Principle**

The sample is pre-treated with dithiothreitol and sodium hydroxide to release folate from its protein bound form. The folate in the sample competes with an enzyme labelled folate for fluorescein labelled bovine folate binding protein (FBP) for limited binding sites. The FBP is allowed to bind to a mouse monoclonal antibody labelled with fluorescein isothiocyanate that is immobilized on the magnetic beads. Another reagent immobilized on the beads is an alkaline phosphatase bound folate that has been preserved with 0.1% sodium azide. The beads are washed to remove all unbound enzyme labelled folate. The beads are then incubated with 4-methylumbelliferyl phosphate (a fluorogenic substance). The amount of enzyme labelled folate bound to the beads are inversely proportional to the serum folate concentration. (Tosoh Biosciences, 2009b:1)

#### **b. Validation**

Commercial folate calibrators produced by Tosoh Biosciences (Replamed, Pretoria, South Africa) were used to determine the Tosoh AIA®-600 II instrument's minimum and maximum for folate by calibrating and producing a standard curve. A 6-point calibration (0 ng/mL, 1.46 ng/mL, 2.74 ng/mL, 5.38 ng/mL, 11.6 ng/mL and 23.0 ng/mL) was done in duplicate to produce the calibration curve. The calibration curve illustrates inverse relationship between the rate and concentration where the rate decreases with an increase in concentration.

The reliability of the instrument was evaluated with commercial controls (a multi analyte control [Replamed, Pretoria, South Africa]) and appraised with a control data sheets' expected ranges at 3 levels (level 1: 1.25-2.61 ng/mL [mean: 1.93 ng/mL], level 2: 3.42-6.36 ng/ml [mean: 4.89 ng/mL] and level 3: 8.10-15.05 ng/mL [mean: 11.57 ng/mL]), and was ran in triplicate. The mean for each level are known as x, y and z, respectively. If the controls were not in line with the Westgard rules (as discussed in section 3.5.), the control values were rejected. Process discussed in section 3.5.1.1.b. was repeated.

### **c. Reference value**

The normal range for serum folate is between 3 ng/mL and 17 ng/mL (6.0-28.0 nmol/L) (Burtis and Bruns, 2015:848). In this study a serum concentration of less than 5.21 ng/mL was considered as low and above 20 ng/mL as high (Shenzhen New Industries Biomedical Engineering, 2013:4).

### **3.6.2.3. Vitamin B6**

A commercial HPLC kit for PLP was acquired from Recipe ClinRep® (Microsep (Pty) Ltd., Sandton, South Africa). The kit contained Reagent N, Precipitant, mobile phase, standard solution and serum calibrator. There are five components in a HPLC system, namely, a pump (responsible for forcing liquid through the system at a certain flow rate), injector (introduces the sample into the liquid flowing in the system), the column (consists of the stationary phase which separates the sample into different components), detector (detects the eluted components) and the computer (analyses information from the detector to produce a chromatogram) (Agilent Technologies, 2015). A solvent is called the mobile phase, held in a reservoir and flows through the system by a high pressured pump usually in millilitres per minute (Waters, 2015). An autosampler injects the sample into the continuously flowing mobile phase carrying it into the HPLC column with the packing material (known as the stationary phase) which will result in separation (Wang and Abate, 2013). The continuously flowing mobile phase will elucidate separated compound bands from the HPLC column and move past a detector, after which it will be sent to waste, or alternatively collected (Waters, 2015).

### **a. Principle**

Plasma is combined and incubated with precipitation reagent to remove high molecular substances. HPLC was loaded with 50 µL of the resulting analyte. The column temperature was 25°C, with an isocratic pump flow rate of 1.0 mL/min (mobile phase) and a peristaltic pump flow rate of 0.2 mL/min (reagent N). The injection interval was 10 minutes. Analyte to standard was analysed at 370 nm excitation and 470 nm emission by a fluorescent detector; based upon a six point calibration (Recipe ClinRep®, 2012).

### **b. Validation**

The commercial vitamin B6 calibrators included in the Recipe ClinRep® HPLC kit was used for a six-point calibration. Commercial controls (levels I, II and III) from Recipe ClinRep® (Microsep (Pty) Ltd., Sandton, South Africa) were used for reliability. The control ranges were 9.04 – 13.6 µg/L, 16.9 - 25.3 µg/L and 24.7–37.1 µg/L for levels I, II and III, respectively. Samples were assayed in batches of 10 along with the controls. If controls were not in range, the assay was repeated with only the controls after it was flushed. If issue was not resolved, the instrument was re-calibrated with fresh calibrators and controls. If the controls were not in line with the Westgard rules (as discussed in section 3.5.), the control values were rejected.

### **c. Reference value**

The normal range for PLP is 5-30 µg/L, while less than 5 µg/L is considered low and more than 30 µg/L as high (Lotto *et al.*, 2011:183; Burtis and Bruns, 2015:473).

#### **3.6.2.4. Vitamin B12**

Only direct methods of detection was employed for vitamin B12 concentration. Serum vitamin B12 concentration was determined with AIA-PACK B12 available from Tosoh Biosciences (Replamed, Pretoria, South Africa). As with the folate assay principle, vitamin B12 concentration was measured by a competitive enzymatic immunoassay. The reagents consisted of test cups with lyophilized magnetic beads coated with mouse monoclonal fluorescein labelled procine intrinsic factor and enzyme linked vitamin B12 preserved with sodium azide. (Tosoh Biosciences, 2009a:1)

## **a. Principle**

The sample is pre-treated to release vitamin B12 from serum binding proteins with potassium cyanide, sodium hydroxide and dithiothreitol. Furthermore, the pre-treatment converts cyanocobalamin to a stable and detectable form of vitamin B12. The sample vitamin B12 is allowed to compete with an enzyme labelled vitamin B12 for a limited number of binding sites on a fluorescein labelled porcine intrinsic factor. The porcine intrinsic factor is allowed to bind to a fluorescein isothiocyanate labelled antibody. Sample is incubated with 4-methylumbelliferyl phosphate (a fluorogenic substance). The amount of enzyme labelled folate bound to the beads are inversely proportional to the serum folate concentration. (Tosoh Biosciences, 2009a:1)

## **b. Validation**

Tosoh AIA®-600 II instrument's minimum and maximum detection of vitamin B12 was evaluated with vitamin B12 calibrators from Tosoh Biosciences (Replamed, Pretoria, South Africa) from which a 6-point calibration curve was constructed. The six calibrators were assayed in duplicate and the concentrations were as follow 0 pg/mL, 97.9 pg/mL, 243 pg/mL, 499 pg/mL, 991 pg/mL and 2190 pg/mL. The calibration curve was accepted if an inverse relationship between the rate and concentration existed, with the rate decreasing with increasing concentration.

A multi analyte control from Tosoh Biosciences (Replamed, Pretoria, South Africa) was used to evaluate the reliability of the instrument's measurement of vitamin B12. The controls have three levels and were run in triplicate and compared with a control data sheets' expected ranges, namely: level 1 (308-463 pg/mL [mean: 385 pg/mL]), level 2 (705-1058 pg/mL [mean: 882pg/mL]) and level 3 (944-1416 pg/mL [mean: 1180 pg/mL]).

If the controls were not in line with the Westgard rules (as discussed in section 3.5.), the control values were rejected. Process discussed in section 3.5.1.1.b. was repeated.

## **c. Reference value**

Vitamin B12 was classified as adequate (>300 pg/mL), possibly deficient (200-300 pg/mL) and deficient (<200 pg/mL) (Chatthanawaree, 2011:229; MacFarlane *et al.*, 2011:1081).

### 3.6.2.5. MTHFR A1298C

DNA was extracted within 2 hours from EDTA blood with the use of Quick-DNA™ Universal kit manufactured by Zymo Research (Inqaba Biotechnical (Pty) Ltd., Pretoria, South Africa). The reagents in the kit were used for lysis of EDTA blood (BioFluid and Cell buffer), digestion of proteins (proteinase K), DNA extraction (Genomic Binding Buffer), DNA washing buffer and DNA elution buffer (Zymo Research, 2014). All the DNA was stored at -80°C. A NanoDrop™ 2000c spectrophotometer (NanoDrop Technologies®, Wilmington, USA) was used to evaluate DNA concentration at 260 nm. All the DNA samples had concentrations within the acceptable range (5–100 ng/μL) and there was no need to dilute. Genotyping was done with the use of a SNP kit (MTHFR A1298C Catalogue no.: 40-0269-64, Tib Molbiol, Berlin, Germany) and the LightCycler® Faststart DNA Master Hybridization Probes kit (Roche Diagnostics, Mannheim, Germany) containing LightCycler® Faststart enzyme, Faststart Taq DNA polymerase, reaction buffer, dNTPs (dUTP instead of dTTP) and MgCl<sub>2</sub>. A LightCycler® 96 instrument was utilized.

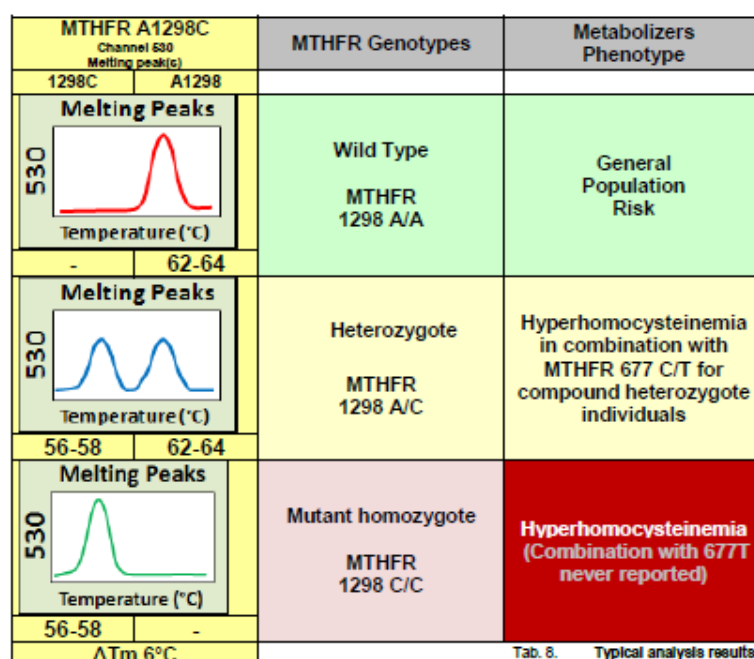
#### a. Principle

The SNP was detected with qPCR instead of restriction fragment length polymorphism (RFLP) PCR for its convenience and lower risk of cross contamination. A standardised kit was more efficient than designing a new assay with the available time and resources. A hybridization probe system in a 10 μL reaction volume with specific primers resulting in a 269 bp amplicon was employed. Two oligonucleotides labelled with fluorescent dyes made up the probe system. During annealing the probes hybridized to the amplicon in a head-to-tail arrangement. They are designed to hybridize on 2 adjacent sites on the amplicon. One of the oligonucleotides is the donor labelled with a fluorescein at its 3' end, while the other is an acceptor labelled with a red fluorophore at its 5' end. The acceptor oligonucleotide is also phosphorylated at its 3' end, so it cannot be extended. The two fluorescent dyes are bought close together, with fluorescein excited by the light source, causing it to emit green fluorescent light. FRET (fluorescence resonance energy) occurs, resulting in the excitement of the red fluorophore by the green fluorescent light. The two dyes can only interact when both are bound to the amplicon. (Roche Diagnostics, 2011:18; Premier Biosoft, 2016; TIB MOLBIOL, 2017:18)

The probes sets are displaced during elongation due to the temperature increase. After elongation the amplicon is double stranded and the displaced probes sets are back in solution

and too far apart for FRET to occur. The samples are genotyped by reading the melting curves as compared to the supplied internal standards, known as melt curve genotyping. The probes dissociate at a specific temperature (melting temperature) resulting in a reduction of fluorescence. (Roche Diagnostics, 2011:19; TIB MOLBIOL, 2017:18) Genotype is assessed by melt curve analysis. The emitted fluorescence is measured after every annealing step, when fluorescent intensity is at its greatest. Any mismatch covered by the probe destabilizes the hybrid and lowers the melting temperature. Detection format is 470/514 FAM. Results are interpreted by comparing to the supplied standards of the kit.

The melt curve was interpreted according to Figure 17. Homozygous wild type (MTHFR 1298AA) has a peak fluorescence between 62 and 64°C, while homozygous (MTHFR 1298CC) peaks between 56 and 58°C. Heterozygous MTHFR 1298AC has two peaks at both the temperature ranges. (Roche Diagnostics, 2011:19; TIB MOLBIOL, 2017:23).



**Figure 17. Interpretation of melting curve (TIB MOLBIOL, 2017:23)**

## b. Validation

The commercially available kit included genotyping standards of both the wildtype and mutant which was used to calibrate the genotyping module. Reliability of test was checked using a no template control (NTC) and the positive heterozygous control. The NTC serves to demonstrate no contamination occurred with genomic DNA or MTHFR PCR product. The positive heterozygous control in turn serves to identify specific melting temperatures.



Every run was done with these controls. Furthermore, good molecular techniques were used to eradicate contamination. These include working in a laminar flow cabinet that has been thoroughly sterilized by a bleach solution, an alcohol solution as well as a UV light. No template DNA was open in the cabinet in conjunction with items that could cause contamination by aerosol (i.e. standards and control). To ensure consistent volumes and concentrations of reagents, a reaction mastermix for 64 reactions was prepared with all the reagents except the template DNA/controls. A volume of 8  $\mu\text{L}$  mastermix was used for every reaction and 2  $\mu\text{L}$  DNA template. Furthermore, gentle up and down pipetting of every reaction was done to ensure a homogenous reaction mixture. Every reaction sample was also checked for bubbles before being loaded into the instrument. SNP genotyping was done according to the qPCR protocol seen in Table 5.

Melt curve genotyping by instrument was used allelic discrimination of each sample and classified as 1298 AA (or homozygous AA), 1298 AC (or heterozygous) and 1298 CC (or homozygous CC), although every sample was viewed for deviating curves and intermediate melting point temperatures. If the automated genotyping failed to report consistent genotype results, the genotype was deducted from the melt temperatures.

**Table 5. qPCR protocol for MTHFR A1298C SNP genotyping**

Sample Reaction			
Component	Volume (μL)	Concentration	
Parameter specific reagents	1	Premixed primers and probes	
Mg <sup>2+</sup>	0.8	2.5mM	
LightCycler® Faststart DNA Master Mix	1	/	
DNA sample/template	2	1 to 20ng/μL	
PCR-grade water	5.2	/	
Total	10	/	
Thermal Cycler Settings			
	Temperature (°C)	Time (sec)	Cycles
Step 1 Denaturing	95	600	1
Step 2 Cycling	95	5	45
	60	10	
	72	15	
Step 3 Melting	95	30	1
	43	120	
	75	1	
Step 4 Cooling	40	30	1

### **3.7. DATA ANALYSIS**

Hcy, folate, vitamin B6 and vitamin B12 results were captured on Microsoft Excel and exported to SPSS 25 software (formerly known as Statistical Package for the Social Sciences) for descriptive statistics. MTHFR A1298C data was coded into three groups and assigned a number, namely: 1298AA (1), 1298AC (2), 1298CC (3). All the missing data in each variable was statistically imputed by the software before analyses. Vitamin B6 had two missing variables; vitamin B12 and folate each had five. The MTHFR A1298C SNP had 15 missing variables. The distribution of the data was checked with a skewness calculation and Shapiro-Wilk normality test ( $p < 0.05$  was significant), although the frequency graph was also analysed. This information was used to establish if mean or median was to be used to describe data (Ghasemi and Zahediasl, 2012:487).

Frequency distribution was determined with SPSS 25. Inferential statistics in SPSS 25 was also used to seek a relationship as follow:

1. Hcy with folate, vitamin B6 (PLP) and vitamin B12 levels
2. MTHFR A1298C with serum Hcy.

### **3.8. CONCLUSION**

The research study was ethically approved and throughout the study the elderly attending the Sharpeville day care centre were accommodated as well as treated fairly. The sample size was representative of the population group as determined by the power calculation. Several measuring instruments were used for the determination of the blood parameters (Hcy, folate, vitamin B6, vitamin B12 and MTHFR A1298C genotype). The methods used in this study were all done under strict QC criteria for valid and reliable results.

## CHAPTER 4

### RESULTS AND DISCUSSION

#### 4.1. INTRODUCTION

The elderly of Sharpeville are part of a vulnerable socioeconomic group, a risk factor for CVD. This group has a high risk for CVDs with known hyperhomocysteinemia, as previously discussed in chapter 1. The mean age was  $77 \pm 8.65$  years. The prevalence of the MTHFR A1298C SNP in this group and the correlation of this SNP with serum Hcy metabolic markers (serum Hcy, serum vitamin B6, serum vitamin B12, and serum folate) are discussed in this chapter.

#### 4.2. QUALITY ASSURANCE

The results reported in this study are reliable and accurate as the controls were analysed with each batch of samples on all measuring instruments. Apart from adhering to the Westgard rules, the instrument's accuracy was further analysed by determining the coefficient of variance (CV) and the SD of the controls. The SD was also used to gage the precision of the instrument. In Table 6 the CV and SD values of the Konelab™, Tosoh AIA®-600 II and Agilent 1260 Infinity HPLC system for each analyte is represented.

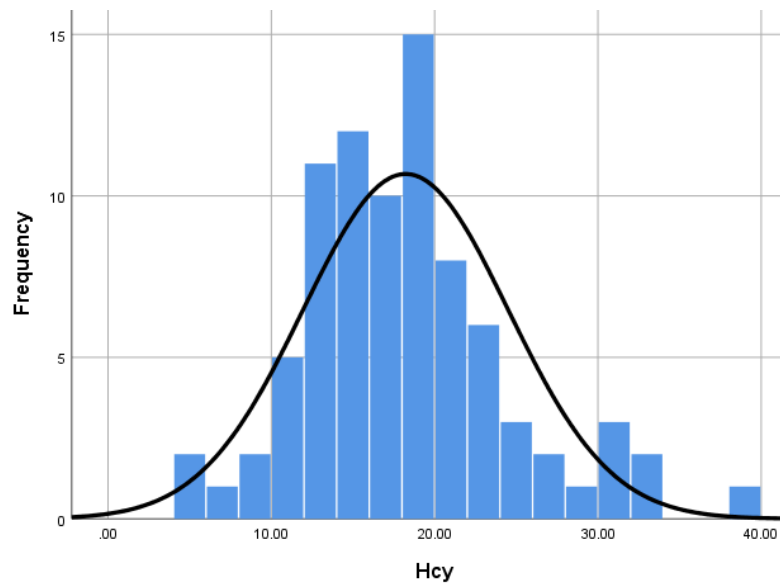
**Table 6. CV and SD values of controls of each analyte for automated analytical instruments**

Instrument	Analyte	CV	SD
Konelab™	Homocysteine	6.69%	2.59
Tosoh AIA®-600 II	Vitamin B12	3.93%	32.71
	Folate	11.88%	0.74
Agilent 1260 Infinity HPLC system	Vitamin B6 (PLP)	9.5%	1.72

The results were accepted as accurate, as all the CV-values are less than 15% and the SD value was within a 2SD range (Westgard, 2009b).

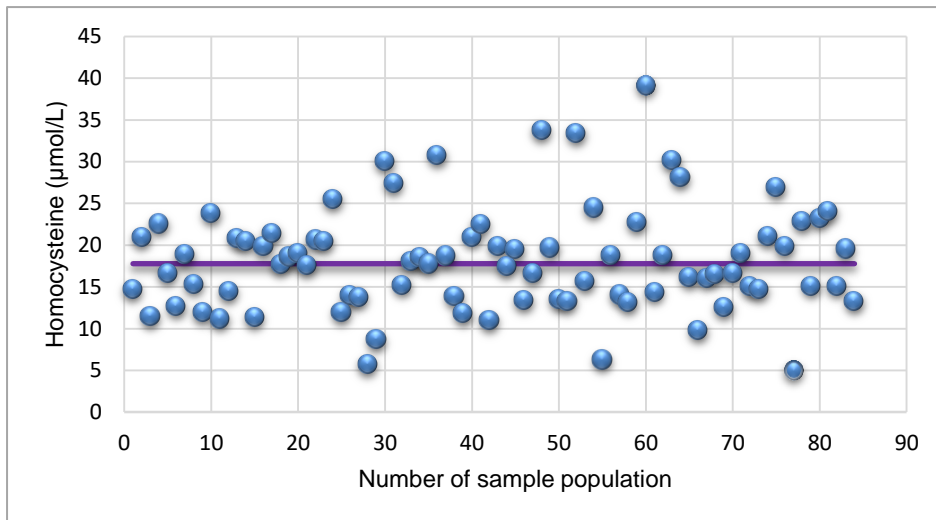
### 4.3. HOMOCYSTEINE STATUS

The median and interquartile range (IQR) are reported for Hcy as the results are skewed (also confirmed by the Shapiro-Wilk test for normality,  $p=0.015^*$ ) as seen by the bell-shaped curve in Figure 18.

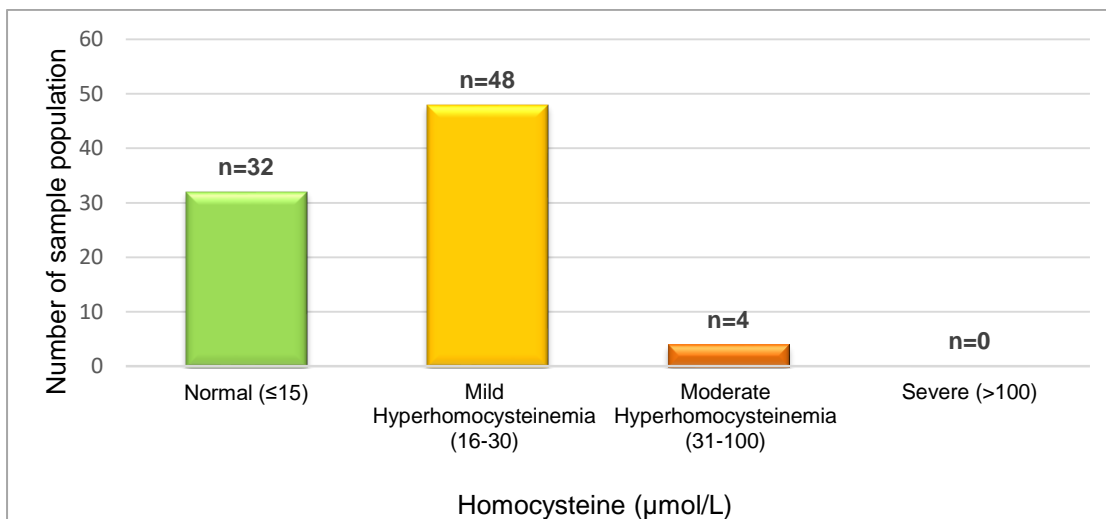


**Figure 18. Frequency distribution of Hcy concentrations**

The median for Hcy was 17.78  $\mu\text{mol/L}$  with an IQR of 13.98-21.03  $\mu\text{mol/L}$ . As seen in Figure 19, the homocysteine values are scattered around the 17.78  $\mu\text{mol/L}$  median (purple line). Figure 19 also shows that only five samples have a concentration of less than 10  $\mu\text{mol/L}$ . According to the Hcy concentrations classification in section 3.6.2.1. the median fell within mildly elevated Hcy. As illustrated in Figure 20, 38.1% ( $n=32$ ) of the sample had normal Hcy, 57.14% ( $n=48$ ) had mild hyperhomocysteinemia, 4.76% ( $n=4$ ) had moderate hyperhomocysteinemia and 0% had severe hyperhomocysteinemia.



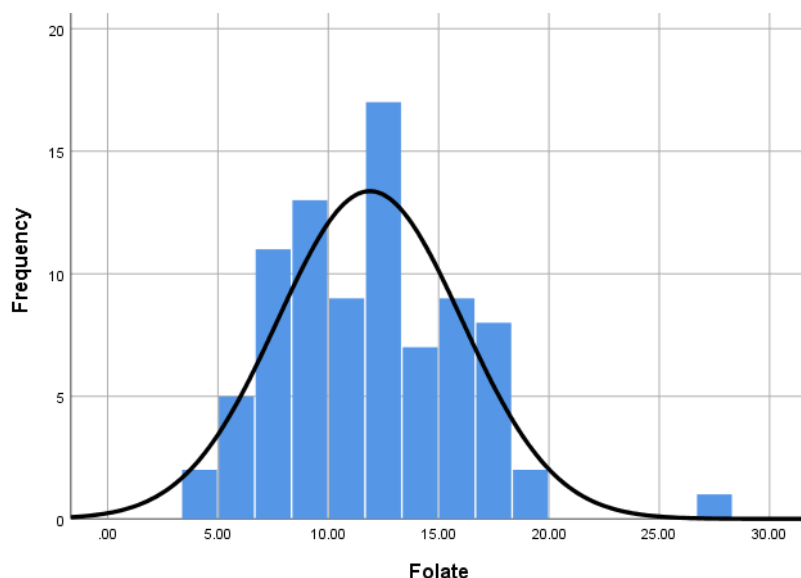
**Figure 19. Scatterplot of the Hcy concentrations around the 17.78 µmol/L median**



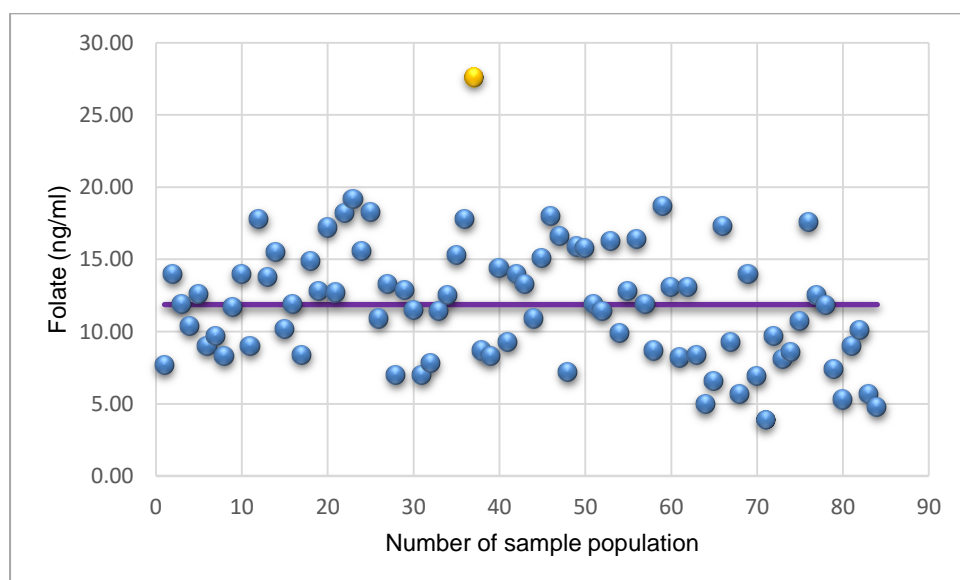
**Figure 20. Samples according to Hcy concentration classification**

#### 4.4. FOLATE STATUS

Folate did not have normally distributed data as seen in the frequency graph in Figure 21; confirmed by the Shapiro-Wilk test for normality ( $p=0.026^*$ ) as well.



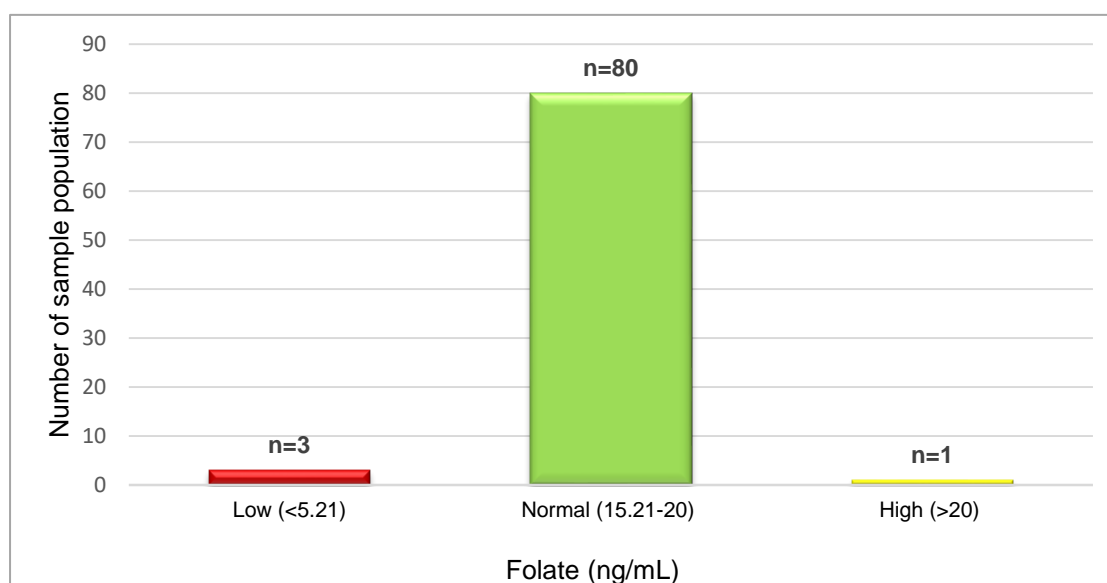
**Figure 21. Frequency distribution of folate concentrations**



**Figure 22. Scatterplot of the folate concentrations around the 11.60 ng/mL mean**

Folate had a median of 11.60 ng/mL with an IQR of 8.63-14.78 ng/mL. Figure 22 is a scatterplot of the folate concentrations of sample population with the median indicated in purple. This Figure also indicated that the highest concentration is an outlier (yellow dot), which was diluted and repeated. According to the classification of folate levels in section 3.6.2.2.c. the median for folate fell well within the normal range. As seen in Figure 23, 95.24% (n=80) of the samples were classified as normal, 3.57% (n=3) were classified as low and

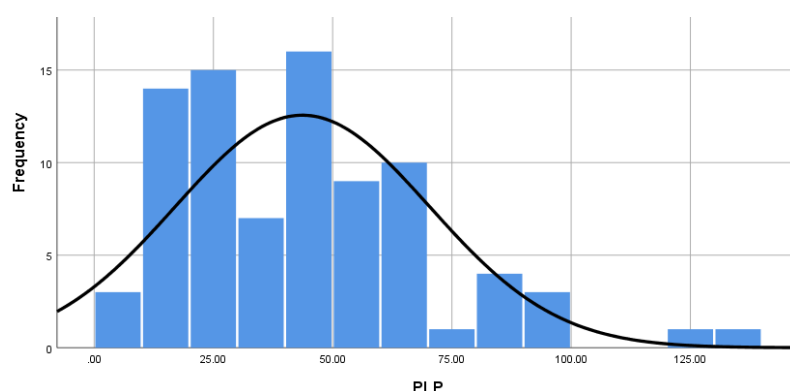
1.19% (n=1) were classified as high. In Figure 22 it can also be seen that majority of samples fell within the normal range of 5.21 ng/mL and 20 ng/mL.



**Figure 23. Samples according to Folate concentration classification**

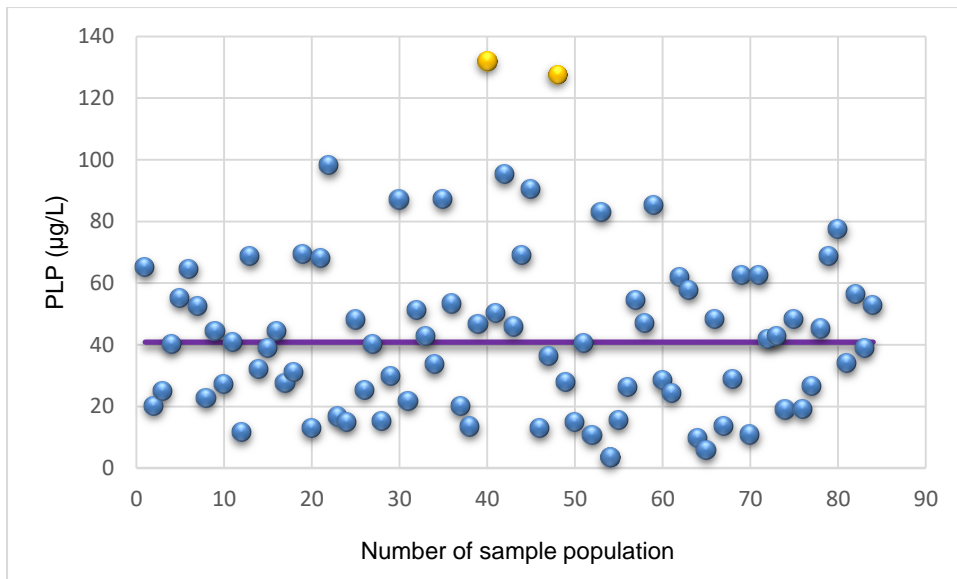
#### 4.5. VITAMIN B6 (PLP) STATUS

The vitamin B6 samples were skewed as illustrated in Figure 24's bell graph. This was also confirmed by the Shapiro-Wilk test ( $p=0.000^*$ ).

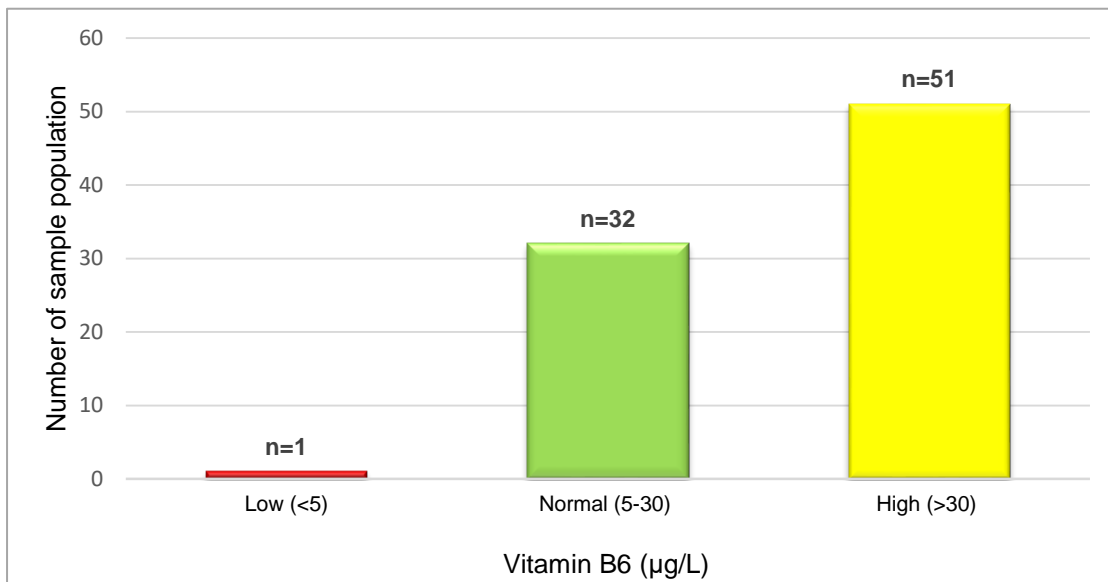


**Figure 24. Frequency distribution of sample Vitamin B6 (PLP) concentrations**

In Figure 25 the vitamin B6 (PLP) concentrations scattered around the 40.25  $\mu\text{g/L}$  median (purple line) which had IQR of 21.91-57.4  $\mu\text{g/L}$ . There are also 2 outliers (yellow dots) that can be seen on Figure 25. Of the sample population (as illustrated in Figure 26) 1.19% (n=1) was classified as PLP deficient, with 38.1% (n=32) being normal and the majority as high with 60.71% (n=51).



**Figure 25. Scatterplot of the PLP concentrations around the 40.25 µg/L median**

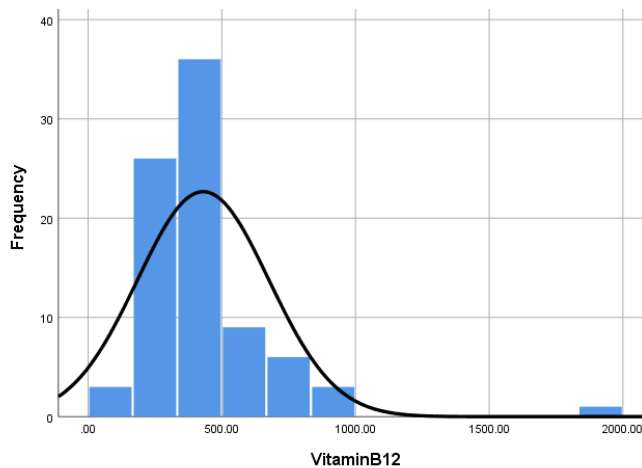


**Figure 26. Samples according to Vitamin B6 (PLP) concentration classification**

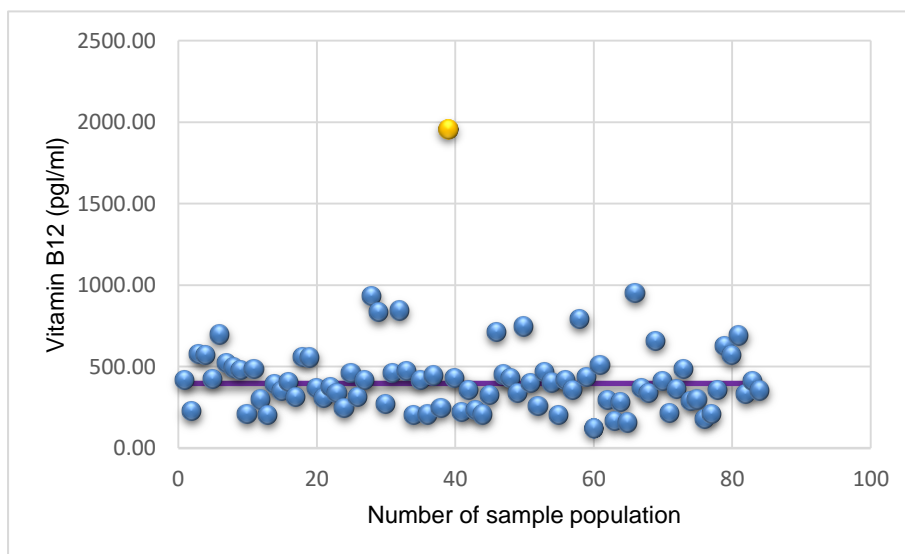


## 4.6. VITAMIN B12 STATUS

The Shapiro-Wilk test for normality indicated non-normal ( $p=0.000^*$ ) distribution for vitamin B12 (as supported by the skewed bell graph in Figure 27).



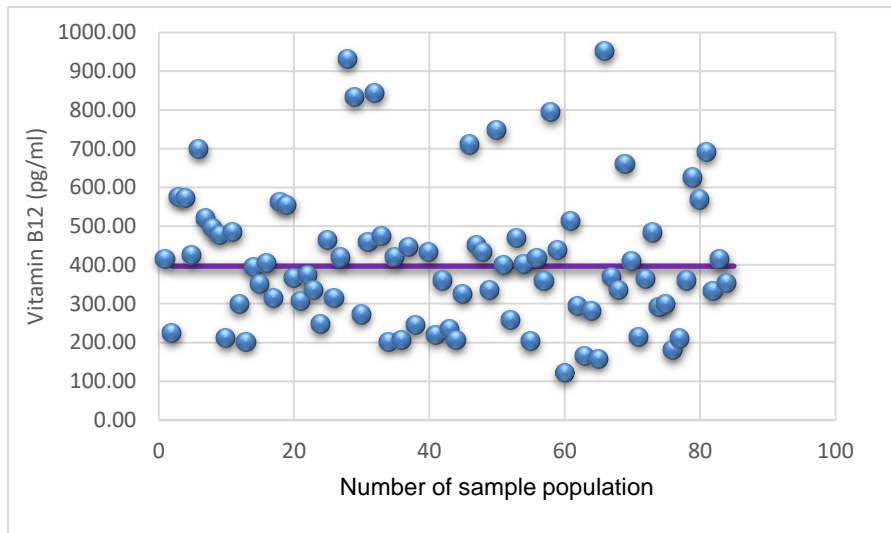
**Figure 27. Frequency distribution of vitamin B12 concentrations**



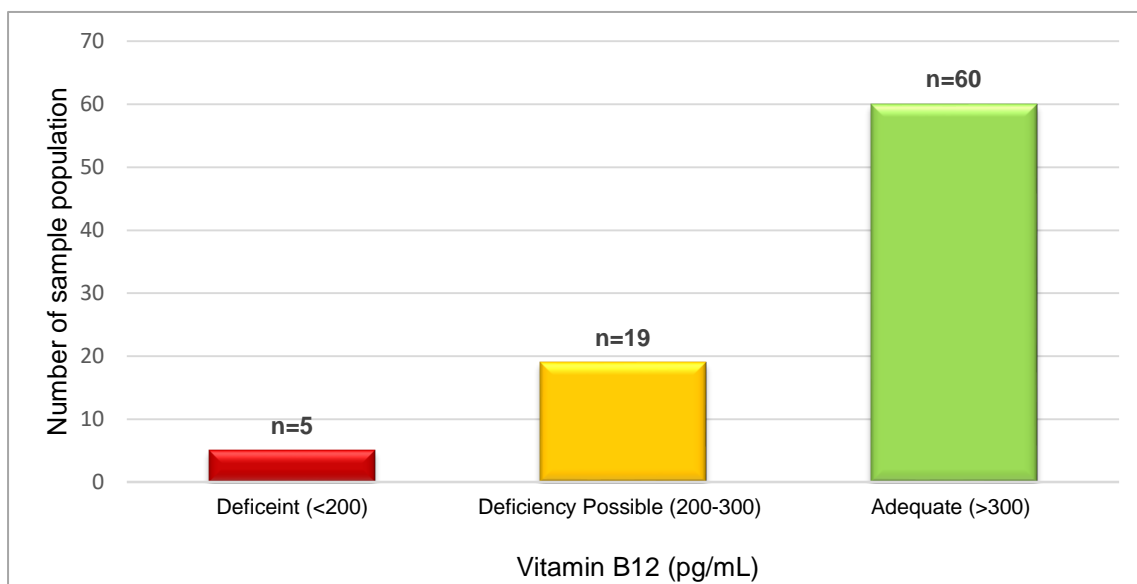
**Figure 28. Scatterplot of the vitamin B12 concentrations around the 384 pg/mL median**

The median for vitamin B12 was 384.00 pg/mL with an IQR of 276.75-483.00 pg/mL. The scatterplot seen in Figure 28 depicts the sample population's vitamin B12 serum concentrations around the median (seen in purple). The Figure also illustrates the highest vitamin B12 concentration, 1957 pg/mL (yellow dot); an outlier. In order to see the distribution of the other concentrations, it has been omitted from Figure 29 (note: not discarded, median is unchanged). The median is within the normal classification (according to the classification

discussed in section 3.6.2.4.c.). Majority of samples were above 300 pg/mL (vitamin B12 adequate concentration), as seen in Figure 30 as well as Figure 26. The population was classified as follows (as illustrated in Figure 30); 5.95% (n=5) were deficient, 22.62% (n=19) had a possible deficiency and 71.43% (n=60) were adequate for vitamin B12. Figure 29 also clearly shows the 5 samples that fell below 200 pg/ml.



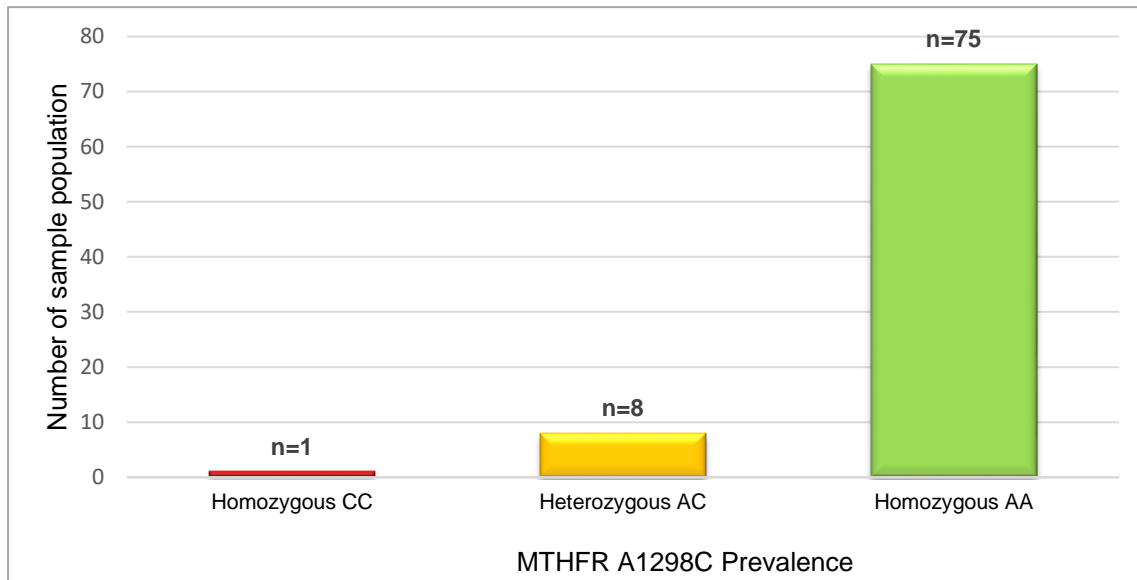
**Figure 29. Scatterplot of the vitamin B12 concentrations around the 384 pg/mL median, without outlier**



**Figure 30. Samples according to Vitamin B12 concentration classification**

#### 4.7. PREVALENCE OF MTHFR A1298C

The allele frequency of MTHFR 1298AC was 9.52% (n=8), with majority of population being homozygous for the AA wildtype 89.29% (n=75). Only 1.19% (n=1) was homozygous for the CC mutation as seen in Figure 31.



**Figure 31. Frequency distribution of MTHFR A1298C**

#### 4.8. CORRELATIONS

Data on levels of serum folates and Hcy related to selected genotype were compared using the ANOVA (die is die regte manier om Mthfr met sy numerical maters te correllate, nie spearmans nie).

All the continuous data had a non-normal distribution; thus, Spearman's rank correlation was used. A p-value of less than 0.05 was considered significant. To test the effect of the MTHFR A1298C on Hcy; Spearman's rank correlation was used. This failed to show a relationship upon which Odds Ratio was used.

##### 4.8.1. Homocysteine, folate, vitamin B6 and vitamin B12

As seen in Table 7 the only significant correlation was between Hcy and vitamin B12. The p-value was 0.0001 and it was an inversely proportional relationship.

**Table 7. Correlation coefficient between Hcy biochemical markers ( \*\* p<0.01)**

	Hcy	Folate	Vitamin B12	Vitamin B6
Hcy				
Folate	0.011 (p=0.918)			
Vitamin B12	-0.0407 (p=0.0001) **	-0.027 (p=0.806)		
Vitamin B6	0.011 (p=0.922)	0.030 0.785	0.163 (p=0.139)	

#### 4.8.2. MTHFR A1298C, homocysteine, folate, vitamin B6 and vitamin B12

MTHFR A1298C and vitamin B12 correlated according to Spearman's rank correlation (Table 8). None of the other Hcy biochemical markers, including Hcy, has a relationship with MTHFR A1298C.

**Table 8. Correlation coefficient between MTHFR A1298C and Hcy biochemical markers (\* p<0.05)**

	MTHFR A1298C
Hcy	-0.163 (p=0.138)
Folate	-0.009 (p=0.935)
Vitamin B6	0.054 (p=0.628)
Vitamin B12	0.223 (p=0.042) *

Odds Ratio indicated an inverse relationship between MTHFR A1298C and Hcy (-2.186), but it was not significant with p=0.254.

Table 9 shows the MTHFR A1298C genotypes with their respective Hcy medians, further illustrating that this SNP has an insignificant effect on Hcy as the heterozygous genotype has a lower Hcy value than the wildtype.

**Table 9. MTHFR A1298C genotypes with Hcy levels**

MTHFR A1298C Genotype	1298AA	1298AC	1298CC
Hcy	18.58 µmol/L	15.26 µmol/L	18.94 µmol/L

## 4.9. DISCUSSION

The aim of the study was to determine the frequency of MTHFR A1298C and determining its effect along with Hcy metabolites on Hcy concentration in an elderly black South African population. This specific population group has been found to have a high risk for a CVD event by Oldewage-Theron *et al.* (2008a:3) as well as Grobler (2015). As mentioned before, an increased serum Hcy concentration is an independent risk factor for CVD (Gotlieb and Liu, 2012:454). Hcy has a wide inter-individual variation and is influenced by multiple factors (Table 3), including SNPs of Hcy regulating enzymes (Huang *et al.*, 2011:1805; Chmurzynska *et al.*, 2013:842; Hirmerová, 2013:e250) and nutritional deficiencies of folate, vitamin B6 and vitamin B12 (Ganguly and Alam, 2015:2). Single nucleotide polymorphisms account for approximately 6% of hyperhomocysteinemia incidences (Chmurzynska *et al.*, 2013:841). When hyperhomocysteinemia is combined with another risk factor, the probability of a CVD event occurring increases fourfold (Kumar *et al.*, 2015: 496).

An intervention study done by Grobler (2015:151) on the same sample population found that 66.36% of the population had hyperhomocysteinemia ( $>15 \mu\text{mol/l}$ ), which decreased to 32% after an intervention. In this study 57.14% of samples had hyperhomocysteinemia, thus Hcy levels have worsened since the intervention was discontinued in 2011 (as reported in figure 20). The mean and median for folate and vitamin B12 were found to be normal if compared to reference range, although 3.57% of elderly had low folate levels and 5.95% were possibly deficient for vitamin B12. In comparison to the 2011 study, 9.62% of the study population were classified as having low folate levels, thus the prevalence of folate deficiency has improved, while vitamin B12 has increased from 4.81% (Grobler, 2015:151). The vitamin B6 mean was  $0.74 \pm 0.47 \mu\text{g/L}$  in 2011 and 98% of samples were deficient (Grobler, 2015:151), while in this study only 1.19% of the sample population were deficient. It must be noted that in the 2011 study  $8.6 \mu\text{g/L}$  served as the cut off for normal, while in this study  $5 \mu\text{g/L}$  served as the lower limit. An intervention study with vitamin B6 supplements were done prior to the current study. The results have not been published and consequently can't be compared to the present research.

Ankrah *et al.* (2012:314) reported in a South African population with a mean age of 56 years, 33% had elevated Hcy. Although, hyperhomocysteinemia was classified as more than  $12 \mu\text{mol/L}$ . A study done in Togo, Western Africa, (Yayehd *et al.*, 2012:4) found 52.2% of the samples have elevated levels of hyperhomocysteinemia, but the mean age was  $57.8 \pm 9.9$  with the youngest being 24 years old. The similarity between the Sharpeville and Togo

observations can be attributed to the fact that 33.3% of the Togo sample had coronary heart diseases. Tiahou *et al.* (2009:321) found 58.6% of participants in Ivory Coast to have elevated levels of Hcy, with a median age of 45. Creatinine levels related to protein intake was one of the possible contributing factors to hyperhomocysteinemia.

In Beijing, China one study found that 58.4% of 370 elderly (all older than 65) were hyperhomocysteinemic ( $>15 \mu\text{mol/l}$ ) (Zhang *et al.*, 2014:492). The Chinese sample population were all diagnosed as CVD patients. In contrast to the Chinese and Sharpeville results, in Korea, only 24.25% of an elderly study population ( $71.1 \pm 5.2$  years) were hyperhomocysteinemic (Song *et al.*, 2010:20). In a study involving elderly Polish females (older than 60 years) it was found that mean Hcy levels were  $7.94 \pm 0.3 \mu\text{mol/L}$  with 20% of the group being hyperhomocysteinemic, although normal was considered as less than  $10.0 \mu\text{mol/L}$  (Chmurzynska *et al.*, 2013:842-843).

A study by Kirsch *et al.* (2013:499), elderly Germans (mean age of 74) had a median Hcy concentration of  $14.1 \mu\text{mol/L}$  with 63% of the study population's Hcy levels being more than  $12 \mu\text{mol/L}$ . There was a correlation between age, vitamin B12 and folate with Hcy, all contributing factors to hyperhomocysteinemia, which may explain the higher incidence when compared to the Sharpeville elderly. In Brazil Vital *et al.* (2016:30) found that 70% of an elderly study population (mean age:  $78.8 \pm 7$  years) had elevated Hcy ( $>15 \mu\text{mol/L}$ ), although there was only 30 samples and a power calculation for sample size was not reported (Vital *et al.*, 2016:30).

As previously mentioned, the incidence of folate deficient elders has improved in the study population compared to that of 2011, decreasing from 9.62% to 3.57%. In a study on the same sample population, Oldewage-Theron *et al.* (2008b:26) reported 20.8% of the men and 32.5% of the women (average 30.4%) to be folate deficient (low classified as less than  $5.03 \text{ ng/ml}$ , reported as  $16 \text{ nmol/L}$ ), indicating a drastic improvement compared to current, declining to a 3.57% deficiency. The 2008 study found that the elder's diet was insufficient for animal products, dairy, fruits and vegetables, which could possibly result in the deficiency. Dietary intake measurements were not an objective in the current study, but the decreased incidence of folate deficiency is possibly the result of the elders having access to more diverse food sources.

In South Africa published data is focused on folate intake rather than serum folate. A study by Kruger *et al.* (2011) is an example of this as biochemical analysis was done, but only folate

intake for the elderly was reported. The present study had similar results as Song *et al.* (2010:20), who found 3% elderly in Korea to be folate deficit. When comparing the Sharpeville results to elderlies in Austria and Southern Iran with a 10% and 39.4% folate deficiency, respectively, the incidence of folate deficient elders in Sharpeville is lower (Fabian *et al.*, 2011:498; Lankarani *et al.*, 2015:1910). A possible reason for the lower incidence, compared to Southern Iran, is the 2003 South African legislation mandating maize meal and wheat flour folate fortification (Metz, 2013:979). In contrast, in Singapore 0% of an elderly sample population had a deficiency, although 0.3% were classified as borderline deficit (Ng *et al.*, 2012:1365).

The vitamin B6 mean in the Sharpeville elderly in 2011 was  $0.74 \pm 0.47$  µg/L, which can be considered a great deal lower than that of the current study (40.25 µg/L IQR: 21.91-57.4 µg/L). As noted before the number of the population that was deficit for vitamin B6 decreased drastically from 98% in 2011 to 1.19% in the current study. This drastic improvement can be attributed to various causes, including: improved access to vitamin B6-rich foods, vitamin B6 supplementation, lack of kidney disease, healthy livers, low alcohol consumption (Corken and Porter, 2011:619; van der Ham *et al.*, 2012:109). A study on Norway elderly in a nursing home had a high frequency of vitamin B6 deficiency (49.2%), further supporting an event occurring with the Sharpeville elderly's improved vitamin B6 status (Kjeldby *et al.*, 2013:3). It is worth noting that the authors found that elderly who used vitamin B6 supplementation all had a normal vitamin B6 status (Kjeldby *et al.*, 2013:4). In Poland Elderly were found to not be deficient for vitamin B6, although 29% had a concentration higher than the upper limit for vitamin B6 (Jankowska *et al.*, 2013:60). The former study was on kidney transplant patients, who were taking vitamin supplementation, which would explain why there were no vitamin B6 deficiencies.

In this study sample 22.62% were possibly deficient for vitamin B12, while in the 2011 study by Grobler (2015) reported 4.81% as being possibly deficient. Oldewage-Theron *et al.* (2008b:26) reported a vitamin B12 deficiency as less than 156 pmol/L and a possible deficiency as 156-250 pmol/L. They found that 29.2% of the men and 10.5% of the women (13%) had a vitamin B12 deficiency, while 25% men and 15.8% women (21%) were possibly deficient. Thus, the vitamin B12 status of this population improved from 2008 to 2011 but worsened again when comparing 2011 with 2015.

In both Singapore and Austria 5% of elderly were found to be vitamin B12 deficient, although in Austria 28% were possibly deficient, which is higher than the Sharpeville incidence (Fabian

*et al.*, 2011:498; Ng *et al.*, 2012:1365). MacFarlane *et al.* (2011) also found a similar trend in Canada as in Singapore and Austria, with 4.5% of elderly being vitamin B12 deficient and 17.1% possibly deficient. In Southern Iran 19.12% of the elderly were possibly deficient for vitamin B12, while 36.4% had a deficiency (Lankarani *et al.*, 2015:1909), a much higher incidence than in the Sharpeville community. According to Fabian *et al.* (2011:499) 30-40% of elderly on average are affected by an inadequate vitamin B12 status. He concluded that this may be due to an age-related decrease in gastric acid secretion, an intrinsic factor deficiency or a slight increase in gastric pH, which is not the case in the Sharpeville population. In contrast Lachner *et al.* (2012:7) stated that 3-40% of elderly may be affected by a vitamin B12 deficiency, with lower rates observed in communities and higher rates in institutional settings. The last statement is true for the Sharpeville elderly, with elderly being 5.95% vitamin B12 deficient and 7.07% possibly deficient. The variation between studies can also be due to the fact that there are several range definitions for vitamin B12 deficiency (Scarpa *et al.*, 2013:628) and a lack of consensus as discussed in section 2.6.5.

Only 1.19% of the elderly in Sharpeville was found to have the CC homozygous mutation. MTHFR 1298AC was detected in 9.52%; while majority of the group (89.29%) do not have the MTHFR A1298C mutation. In South-Africa there are few studies that looked at the MTHFR A1298C mutation, regardless on which ethnicity study was done. The most prominent studies on MTHFR and Hcy are by Dr C. Nienaber-Rousseau and her colleagues (Nienaber-Rousseau *et al.*, 2013a; Nienaber-Rousseau *et al.*, 2013b; Nienaber-Rousseau *et al.*, 2015), who assessed only the MTHFR C677T, and not MTHFR A1298C. A comprehensive study done on a South African population by Scholtz *et al.* (2002) compared prevalence of MTHFR A1298C of different ethnic groups, in order to understand contributing factors to explain CVD event differences between these groups. The frequency distribution is similar to the current study, although with a higher incidence of MTHFR 1298CC (AA=87%, AC=8%, CC=5%) (Scholtz *et al.*, 2002:466). Interestingly, the white and coloured (mixed race/ancestry) people of South Africa have a higher incidence of MTHFR 1298CC (12%). MTHFR 1298AA for both white and coloured people were lower (38%, 55%) when compared to the current study. The difference between the frequency of CC homozygous mutation of current study and that found by Scholz *et al.* (2002:466) could be due to sample size and needs further investigation. Frequency distribution studies for MTHFR A1298C in Sub-Saharan Africa are lacking.

A regional study in middle-Southern Italy also had a higher frequency of MTHFR 1298CC (7.6%) than the Sharpeville elderly (Zappacosta *et al.*, 2014:2), while AA was lower with group frequency of 48.7%. A pulmonary embolism study in Turkey compared genotypes of patients



and a control group (Basol *et al.*, 2016:36). The elderly black people of the current study have a completely different frequency distribution when compared with the previous two studies. A frequency of 7.6% was seen for MTHFR 1298CC in patients with pulmonary embolism, while the control group had a frequency of 3.2% (Patients: AA 48.3%, AC 44.1%, Control AA 58.7%, AC 38.1%) (Basol *et al.*, 2016:38). A similar study was done with ischaemic stroke patients in The Republic of Tunisia by Fekih-Mrissa *et al.* (2013). The study's control group is comparable to the present study as MTHFR A1298C wildtype is the most frequent (93%) (Fekih-Mrissa *et al.*, 2013:467), with 0% incidence of CC and 7% AC. While the CVD event group had the following frequency distribution: AA 69%, AC 17.9%, CC 13.1% (Fekih-Mrissa *et al.*, 2013:467).

Lower levels of vitamin B12 was found to be partially responsible for the increased serum Hcy levels, even though the median of vitamin B12 fell within the deficiency unlikely grouping with a median of 384.00 pg/mL (IQR 276.75-483.00 pg/mL). Vitamin B6 and folate were found to not have any effect on Hcy levels. Nazef *et al.* (2014) found comparable results to the current study with an inverse relationship between Hcy and vitamin B12, with folate having no effect (vitamin B6 was not analysed). Four other studies found that both folate and vitamin B12 had an inverse relationship with Hcy, as well as a relationship between folate and vitamin B12 (King *et al.*, 2012; Zappacosta *et al.*, 2013; Barnabe *et al.*, 2015; Cai *et al.*, 2016). Very few studies incorporate the effect of vitamin B6 on Hcy, which is surprising considering the role it has in the Hcy metabolism. As elucidated in chapter 2, four enzymes in the Hcy metabolism are dependent on vitamin B6 as a co-factor. Furthermore, vitamin B12 is the co-factor for only one enzyme in the Hcy metabolism. In the current study vitamin B6 had no relationship with Hcy, but two others found vitamin B6, vitamin B12 and folate had an inverse correlation with Hcy (Lindqvist *et al.*, 2012:320; Nienaber-Rousseau *et al.*, 2013b). Nienaber-Rousseau *et al.* (2013b) found a weak correlation, which they noted may be due to the big sample population consisting of more than 1800 participants.

The MTHFR 1298AC and CC genotypes were found not to have a significant effect on Hcy; moreover, the wild type genotype's median was higher than the heterozygous median (Table 7). Vitamin B12 and MTHFR A1298C were also indicated to have a relationship; however, at the time of examination of this dissertation, comparable results in literature were not found. Cai *et al.* (2016), de Lau *et al.* (2010) and Zappacosta *et al.* (2014) found similar results to the current study with Hcy levels higher in MTHFR 1298AA genotype than in AC or CC, with no significant correlation. Fekih-Mrissa *et al.* (2013), Mahfouz *et al.* (2012) and Tanaka *et al.* (2009) have also found no significant difference between the MTHFR A1298C genotypes'

Hcy levels. Mahfouz *et al.* (2012) also stated MTHFR A1298C may not be associated with hyperhomocysteinemia, but only with decreased MTHFR enzyme activity.

#### **4.10. CONCLUSION**

The serum Hcy median (17.78  $\mu\text{mol/L}$ ) fell in the mild hyperhomocysteinemia classification, while folate, vitamin B6 and vitamin B12 medians indicated no deficiencies. Although mild, a decrease of 3–5  $\mu\text{mol/L}$  in serum Hcy may reduce the incidence of CVD and venous thrombosis by up to 25% (Fabian *et al.*, 2011:499). Only 1.19% of the study population was homozygous for the MTHFR A1298C mutation with 9.52% (1298AC) being heterozygous. Vitamin B12 was the only variable which significantly correlated with elevated Hcy levels, even though the median indicated that a deficiency was unlikely. MTHFR A1298C and Hcy levels also had a relationship, however not statistically significant. Bailey *et al.* (2002) found that supplementation with vitamin B12 and/or folate can weaken the correlation between the MTHFR SNP and Hcy levels, effectively masking it. They also stated that an increase in folate and vitamin B12 levels counter the effect of the MTHFR SNP on Hcy levels. The relationship between MTHFR genotype and Hcy changes with age and is most significant in younger people (Chmurzynska *et al.*, 2013:842). Normal folate and vitamin B6 as well as the elderly as a study population can as a result both be a plausible reason that MTHFR A1298C failed to show a significant relationship with hyperhomocysteinemia.

## **CHAPTER 5**

### **CONCLUSION AND RECOMMENDATIONS**

#### **5.1. INTRODUCTION**

The aim of the project was to determine the prevalence of MTHFR A1298C polymorphism in an elderly black population and to evaluate the correlation of this MTHFR SNP with serum Hcy metabolic markers (serum Hcy, serum vitamin B6, serum vitamin B12, and serum folate). The research was motivated by an increased incidence of hyperhomocysteinemia in the sample population as reported in a previous study. Furthermore, there is limited information available about the frequency of the MTHFR A1298C SNP in black South Africans.

#### **5.2. RESEARCHER'S CONTRIBUTION**

This study was part of a multi-nutritional programme at VUT – an initiative of CSL led by Prof WH Oldewage-Theron. The aim of the programme is to address malnutrition among elderly attending a day care centre in Sharpeville. A multidisciplinary team is involved in the project: an administrator, nutritionists, a dietician, nursing sisters (phlebotomists), medical technologists and a statistician. Ethical clearance (University of the Witwatersrand and VUT) as well as funding (National research foundation and VUT) was acquired before data collection. The researcher is a registered student medical scientist with the HPCSA and was responsible for the project design and writing of the research proposal. The researcher was also actively involved in fieldwork preparations, as well as data collection during fieldwork. Blood analysis was carried out by the researcher along with other team members. Researcher designed and optimised molecular assays, extracted DNA and analysed molecular samples, with the technical assistance of Dr PJ Lebea and the CSIR. The researcher did statistical analyses in consultation with a statistician. Finally, the researcher wrote the dissertation and articles with the guidance of the supervisor. Part of the study was also presented by the researcher at a national conference before the time of examination (Medical Laboratory Professionals Congress 2015).

### 5.3. LIMITATIONS OF THE STUDY

There was a challenge with blood extraction from some of the elderly and insufficient blood was drawn to analyse all the parameters, specifically folate (five missing), vitamin B6 (two missing), vitamin B12 (five missing) and MTHFR A1298C (fifteen missing). Statistical imputation during data analysis overcame this limitation. No gender-based analysis was done for any of the variables. The study also did not take into consideration any other factors that negatively affects Hcy levels i.e. age, diseases, pharmaceutical medications, smoking, alcohol intake, sedentary lifestyle or animal protein intake (Chmurzynska *et al.*, 2013:842; Hirmerová, 2013:e250). This would be vital for a regression analysis to have the true correlation between vitamin B12 and Hcy. This will give the true extent of the influence vitamin B12 has on Hcy. As previously mentioned, a vitamin B6 supplement intervention study was done with the elderly prior to the current study. These results have not yet been published, and could therefore not have been compared to this study.

### 5.4. MAIN FINDINGS

#### 5.4.1. Problem and settings

- CVDs are the most common cause of death worldwide (Cramer *et al.*, 2014:170).
- The Sharpeville elderly community have an increased risk for CVD (Oldewage-Theron *et al.*, 2008a:3). Grobler (2015) also found that the same populations have a high serum Hcy level.
- Serum Hcy in the body has been indicated as a causal risk factor or biomarker for CVD (Kuebler *et al.*, 2013:1382).
- The MTHFR enzyme plays a key role in Hcy metabolism (Kadhim and Clement, 2011:41).
- There is limited information available about the prevalence of MTHFR polymorphisms in Southern Africa (Nienaber-Rousseau *et al.*, 2013a:116; Atadzhanov *et al.*, 2014:194).

#### 5.4.2. Objectives' findings

The serum Hcy median was found to be 17.78  $\mu\text{mol/L}$  (IQR 13.98-21.03  $\mu\text{mol/L}$ ) which was above the 15  $\mu\text{mol/L}$  cut-off for normal Hcy levels. However, folate fell within the normal range

(5.21-20.00 ng/mL) with a median of 11.60 ng/mL (IQR 8.63-14.78 ng/mL). Vitamin B6 was found to be elevated with a median of 40.25 µg/L (IQR 21.91-57.4 µg/L), falling well above the 30 µg/L normal cut off. The vitamin B12 median fell within the adequate grouping with a median of 384.00 pg/mL (IQR 276.75-483.00 pg/mL). The frequency of MTHFR A1298C was as follow: 89.29% (AA), 9.52% (AC) and 1.19% (CC). The only significant correlation between the Hcy metabolites was found to be between Hcy and vitamin B12 ( $p=0.0001$ ), indicating an inverse relationship. This indicates that even though the vitamin B12 median fell within the adequate category, it does influence Hcy serum levels of the study population. Odds ratio illustrated a relationship between MTHFR A1298C and serum Hcy levels, although it was not significant.

## 5.5. RECOMMENDATIONS

The Sharpeville community can benefit from further research, as proven for instance when comparing serum levels of vitamin B12 in 2008 and 2011 to the current study. The CSL is constantly giving feedback to the community as well as various stakeholders about the findings of studies, which results in specific nutritional implementations to address needs. The results from the current study along with those of other team members will assist the nutritionists and dietician in identifying specific shortcomings that exist now, which can be addressed through the meals the centre provide.

The recommendations for future Hcy studies in Sharpeville would be to include a questionnaire to identify medications elderly are taking that may increase Hcy levels. Serum markers for hypothyroidism, diseases of the kidney and rheumatoid arthritis should also be determined and correlated with Hcy, as these are known to increase Hcy levels. Apart from these, other factors e.g. smoking, alcohol, alcohol intake, sedentary lifestyle, animal protein intake that negatively affect Hcy levels, should be incorporated. A control group can also be incorporated into the study, to compare the various Hcy markers of the Sharpeville elderly with a younger healthy population. Diseases are multidimensional, and various literature indicate that risk factors for CVD interact with one another. Future studies can seek to elucidate the relationship between these risk factors. Especially as Sharpeville is in the Vaal Triangle, an area with heavy air pollution. South Africa has a history of racial discrimination, which has been shown to act as a psychosocial stressor, a risk factor for CVD. The effects of apartheid on the elderly as a CVD risk factor could be determined. Furthermore, a relationship can be sought between this risk factor and Hcy. MTHFR is not the only important Hcy metabolising enzymes; CBS and MTR also influence Hcy. The prevalence of SNPs for these

two enzymes can also be determined in this population group. In South Africa studies on the frequency of SNPs of Hcy metabolising enzymes are scarce. A larger study can compare frequencies between urban, rural and peri-urban communities, as well as different races.

## **5.6. CONCLUSION**

The study achieved its objectives and eliminated several factors (MTHFR A1298C SNP, vitamin B6, folate) that may be the cause of the Sharpeville elderly's hyperhomocysteinemia. In doing so, it contributed to scientific knowledge of Hcy as a risk factor for CVD in an elderly black South African population. MTHFR A1298C frequency in a black South African population in Gauteng were determined. The results in this study are reliable and reproducible due to the QC standard of laboratory analysis. The findings of these results (as well as other findings by my peers) will still be disseminated and communicated with the Sharpeville elderly and management at the day care centre.



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## ANNEXURES

### Annexure A: Turnitin report of dissertation

## Draft Submission

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### ORIGINALITY REPORT

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## Annexure B: Ethical approval University of Witwatersrand

**UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG**

Division of the Deputy Registrar (Research)

**HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)**  
R14/49 Oldewage-Theron

**CLEARANCE CERTIFICATE** **PROTOCOL NUMBER M070126**

**PROJECT** Multi-Micronutrient Supplementation to Address Malnutrition amongst the Elderly Attending the Sharpeville Care of the Aged


**INVESTIGATORS** Prof W Oldewage-Theron

**DEPARTMENT** Inst. of Sustainable Livelihoods

**DATE CONSIDERED** 07.01.26

**DECISION OF THE COMMITTEE\*** Approved Unconditionally (The Committee suggest-  
delay the quality of life information to the end of the study so that it does not confound findings)

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

**DATE** 07.01.30 **CHAIRPERSON**   
(Professors PE Cleaton-Jones, A Dhali, M Vorster, C Feldman, A Woodiwiss)

\*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof W O-Theron

**DECLARATION OF INVESTIGATOR(S)**

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10005, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress report.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

## Annexure C: Ethical approval Vaal University of Technology



### VAAL UNIVERSITY OF TECHNOLOGY RESEARCH & INNOVATION ETHICAL CLEARANCE CERTIFICATE

Applicant:	Mrs. Christa Grobler
Project:	To establish the effect of vitamin B6 supplementation on the cardiovascular risk (CVR) and to determine the prevalence of genetic polymorphisms in correlation with CVR markers, in a black elderly community in Sharpeville.
Institution:	Vaal University of Technology
Date Approved:	21 <sup>st</sup> August 2014
Ethical Clearance Number:	20140827-1ms
Approved: Yes/No	Approved

Approved by: B.J. Johnson

Date: 21/8/14

Dr. B.J. Johnson  
Chairperson: Research & Innovation Ethics Committee

## Annexure D: Written informed consent form

### Informed Consent Form

#### Person to contact in the event of any problems or queries:

Dr C.J. Grobler

Prof W.H. Oldewage-Theron

016 950 9210

016 930 5085

#### Statement of agreement to participate in the research study:

I \_\_\_\_\_ (full name), ID number \_\_\_\_\_, have read this document in its entirety and understand its contents. Where I have had any questions or queries, these have been explained to me by \_\_\_\_\_ (fieldworker name) to my satisfaction. Furthermore, I fully understand that I may withdraw from this study at any stage without any adverse consequences and my future health care will not be compromised. I, therefore, voluntarily agree to participate in this study.

Name: \_\_\_\_\_ Signature: \_\_\_\_\_

Date: \_\_\_\_\_

Researcher's Name: \_\_\_\_\_ Signature: \_\_\_\_\_

Date: \_\_\_\_\_

Witness Name: \_\_\_\_\_ Signature: \_\_\_\_\_

Date: \_\_\_\_\_