TO ESTABLISH THE PREVALENCE OF MTHFR C677T POLYMORPHISM IN CORRELATION WITH HOMOCYSTEINE METABOLIC MARKERS IN A BLACK ELDERLY COMMUNITY, IN SHARPEVILLE, GAUTENG PROVINCE IN SOUTH AFRICA

Thesis submitted in accordance with requirements for the degree Magister Technologiae: Biotechnology in Faculty of Applied & Computer, Sciences at the Vaal University of Technology

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DEDICATION

This dissertation is dedicated to my girlfriend, Mafahla Hlongwane. I thank her for the immense contribution and everlasting support in achieving my life goal.

ACKNOWLEDGEMENTS

I thank all those who directly and indirectly contributed to the compilation of this dissertation. My deepest gratitude is to:

- My family, which is my mother, who always believed that I can achieve more. My uncle, who forever shows me a different perspective in approaching life and my young sister for always looking up to me and thus ensuring that I set a good standard.
- This dissertation would not be completed if it was not for Dr Christa Grobler. Not only is the compilation of this work indebted to her, I am who I have become because she discovered me. Dr Grobler did not only just develop me from a novice researcher, she has been my safety belt through the rollercoaster ride in the world of research. I would like to thank her for being a mother first before being a supervisor, the motherly approach that she used contributed enormously to completion of this dissertation. I will forever be indebted to the knowledge she has developed in me during the compilation of this project.
- I am sincerely grateful to Prof. Wilna Oldewage-Theron for making it possible for us to execute this research. It was a privilege to be part of research team that is led by the calibre of her stature. I am thankful for the confidence she demonstrated in me to complete this project and, the unlimited resources she contributed towards this study. Her work ethic has been an inspiration and a driving force towards achieving this objective.
- I am thankful to Prof. Abdul Egal for his significant contribution in various aspects. He provided advice and statistical knowledge, as well as technical advice. His words of wisdom encouraged me and brought me strength to counter the challenges encountered in the research environment.
- Without the technical expertise of Dr Justice Lebea completion of this research would have been almost impossible. I thank him for the knowledge he shared, the constructive criticism which developed me technically upon conclusion of this research.
- Research is a team effort, one cannot achieve their objectives working in a silo, and therefore I thank the CARE team founded by Dr Grobler. I thank them all for their brotherly and sisterly support.

- My gratitude is extended to the Replamed team particularly, Mr Cornel Pretorius and Mr Dietrich. They not only went beyond their line of duty to be service providers but also provided academic support whenever needed.
- I am sincerely grateful to Lulama Dube for not only her logistical contribution but also for the support she provided throughout this project in the best way she saw it appropriate.
- I also like to thank all the staff of Health Science for their encouragement and making me believe that the quest I was on is attainable irrespective of the challenges encountered.
- I thank friends, Samuel, Kaone, Steven and Dr Cornelius Ssemakalu for their contribution and support.
- This research would not have been possible without the support of the Vaal University of Technology, particularly the Institution's research department which works tirelessly to facilitate and create a conducive environment for research.

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ABSTRACT

Background: Increased serum homocysteine is well known as an independent cardiovascular risk factor. Hyperhomocysteinemia may be due to several factors such as nutritional deficiencies and genetics. The *MTHFR C677T* polymorphism is associated with increased serum homocysteine. Folate and vitamin B_{12} play essential roles in lowering homocysteine levels. Limitations have been identified using serum vitamin B_{12} as a marker for vitamin B_{12} status due to lack an efficient of test. Holotranscobalamin has been reported as a more accurate marker for vitamin B_{12} status. Cardiovascular risk due to hyperhomocysteinemia has been confirmed among the elderly in Sharpeville. Knowledge of the prevalence of *MTHFR C677T* polymorphism among Black elderlies in South Africa is limited.

Objectives: The main aim of the study was to evaluate the prevalence of *MTHFR C677T* polymorphism as a cardiovascular risk in an elderly black population in Sharpeville. Correlations between the presence of *MTHFR C677T* polymorphism and homocysteine metabolic markers were evaluated. Holotranscobalamin as a diagnostic test for vitamin B_{12} status was also assessed in this study.

Materials and methods: This study was ethically approved by the Vaal University of Technology ethics committee (20140827-1ms). It was an observational, experimental study conducted in 102 elderly (≥60 years) attending the day-care centre in Sharpeville. Real-Time PCR was used to determine *MTHFR* genotypes. Folate and vitamin B_{12} were measured with AIA-PACK. Homocysteine levels were determined with an automated Konelab™ 20i and holotranscobalamin by ELISA. STATA 12 software was used for analysis of descriptive and inferential statistics.

Results: The prevalence of *MTHFR C677T* polymorphism in this sample population was 19%. Heterozygous *CT* single nucleotide polymorphism was 17% and mutant homozygous *TT* was 2%. The majority (81%) of the subjects had wild type homozygous *CC* genotypes. No associations were found between *MTHFR C677T* genotypes and homocysteine and folate levels. Hyperhomocysteinemia was high (54%) and low (5%) folate deficiency found. No vitamin B_{12} deficiency was found however 7% were on the category of likely to be deficient. Sensitivity and specificity of holotranscobalamin were 100% and 95% respectively.

Conclusion: The conclusions drawn from the study is that the prevalence of *MTHFR C677T* polymorphism is low within elderly in Sharpeville. There is a high risk of cardiovascular disease as a result of high prevalence of hyperhomocysteinemia. An intervention to lower homocysteine concentration of elderlies residing in Sharpeville is needed. Other genetic predisposing factors of increased homocysteine levels should be investigated.

LIST OF ABBREVIATIONS

4-MUP	4-methylbelliferyl phosphate
AdoHcy	S-Adenosylhomocysteine
AIDS	Acquired immune deficiency syndrome
ALPCO	American Laboratory Products Company
Anti-FITC	Antibody fluorescein isothiocyanate
ATP	Adenosine triphosphate
BHMT	Betaine-homocysteine methlytransferase
BMI	Body mass index
bp	Base pair
C	Cytosine
CAD	Congenital heart disease
CBS	Cystathionine-β-synthase
CETP	Cholesterol ester transfer protein
CHD	Coronary heart disease
CLIA	Chemiluminescent immunoassay
CpG	Cytosine-phosphate-Guanine
CRP	C-reactive protein
CSL	Centre of Sustainable Livelihoods
CV	Coefficient variance
CVD	Cardiovascular disease
CVR	Cardiovascular risk
Cys	Cysteine
DHF	Dihydrofolate
DNA	Deoxyribonucleic acid
dTMP	Deoxythymidine monophosphate
DTT	Dithiothreitol
dUMP	Deoxyuridine monophosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAD	Flavin adenine dinucleotide

FBP	Folate binding proteins
FN	False negative
FP	False positive
FRET	fluorescence resonance energy transfer
FVII	Factor VII
FVII	Factor VII activated
FX	Factor X
FXa	Factor X activated
GC-MS	Gas chromatography–mass spectrometry
GCPII	Glutamtecarboxypeptidase II
GLDH	2-oxolutarate, glutamate dehydrogenase
HAS	Homocysteine sulfinic acid
HCA	Homocysteic acid
HCl	Hydrochloric acid
Hcy-S-S-Cys	Disulphide of homocysteine and cysteine
Hcy-S-S-Hcy	Dislufide homocysteine
Hcy-thiolactone	Homocysteine-Thiolactone
HDL-C	High density lipoprotein cholesterol
*Hin*fl	HinfI restriction enzyme
HIV	Human immunodeficiency virus
HPCSA	Health Professions Council of South Africa
HPLC	High performance liquid chromatography
Hs-CRP	High sensitivity C-reactive protein
HT	Positive heterogeneous control
IBL	Immuno-Biological Laboratories Co,Ltd
ICAM-1	Intracellular adhesion molecule-1
IF	Intrinsic factor
IL	Interleukin
kDA	Kilodalton
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDL-C	Low-density lipoprotein cholesterol
Lp- PLA_2	Lipoprotein-associated phospholipase A_2
MAC	Multi-analyte control

MMA	Methylmalonic acid
MMP	Matrix metalloproteinase
MONICA	Multinational Monitoring of trends and determinants in Cardiovascular diseases
MORGRAM	Multinational Monitoring of trends and determinants in Cardiovascular diseases, Risk Genetics Archiving and Monograph
MRC	Medical Research Council
MT	Mutant
MTHFR	Methylenetetrahydrofolate reductase
MTR	Methionine synthase
NADH	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NCD	Non-communicable disease
Nε-Hcy-Lys	Nε-Homocysteinyl-Lysine
NHANES	National Health and Nutrition Examination Survey
N-Hcy-protein	N-Homocysteinyl-Protein
NTC	No template control
PABA	Pteridine, *p*-aminobenzoic acid
PCR	Polymerase chain reaction
PECAM-1	Platelet endothelial cell adhesion molecule-1
Pte	Pteroic acid
r	Pearson's correlation
RBM	Radio-binding method
RIA	Radioimmunoassay
RLFP-PCR	Restriction length fragment polymerase - polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Real time polymerase chain reaction
SAH	*S*-adenosyl-homocysteine
SAM	*S*-adenosyl-methionine
SANAS	South African National Accreditation System
SBD-F	1,3-diazole-4-sulphonate
SD	Standard deviation
S-Hcy-protein	Homocysteine with plasma protein

SMC	Smooth muscle cells
SNP	Single nucleotide polymorphism
SST	Serum separating tube
T	Thymine
TC II	Transcobalamin II
TCEP	tris (2-carboxyethyl) phosphine
TF	Tissue factor
TGF-β	Transforming growth factor beta
tHcy	Total homocysteine
THF	Tetrahydrofolate
Tm	Temperature
TN	True negative
TNF	Tumour necrosis factor
TNF-α	Tumour necrosis factor-alpha
TP	True positive
tPA	Tissue plasminogen activator
US	United States of America
VCAM-1	Vascular cell adhesion molecule-1
VUT	Vaal University of Technology
WHO	World Health Organization
WT	Wild type

LIST OF SYMBOLS

±	Plus, minus
-	Till
%	Percentage
/	or
+	Plus or more than
<	Smaller than
>	Greater than
≤	Smaller than or equal
≥	Greater than or equal
α	Alpha
~	Approximately
X	Multiply
^{o}C	degrees Celsius
®	Registered trademark
=	Equals
ρ	Rho

MEASUREMENT UNITS

°C	Degree Celsius
µL	Microlitre
µmol/L	Micromole per litre
A	Absorbance
kb	Kilobites
ng/µl	Nanogram per microlitre
ng/mL	Nanogram per millilitre
nm	Nanometre
nmol/L	Nanomol per litre
pg/mL	Picogram per millilitre
pmol/L	Picomol per litre

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CHAPTER 1

PROBLEMS AND SETTINGS

1.1 INTRODUCTION

The representation of elderly people (≥60 years) in the South African population continues to grow, from 7.3% in 2009 to 8.1% in 2015 (Oldewage-Theron & Kruger, 2009; Statistics South Africa Social profile of older persons 2011-2015 report 2017). Previous studies reported that the elderly in Sharpeville (Vaal region, Gauteng, South Africa) live in poverty (Oldewage-Theron & Kruger, 2009; Marumo-Ngwenya, 2014). Nutritional deficiencies and chronic diseases are hallmarks in elders of low-socio-economic communities (German *et al*., 2011; Selvamani & Singh, 2018). Elevated plasma homocysteine level is a known risk factor for cardiovascular disease (Ford *et al*., 2011; Ganguly & Alam, 2015). Increased levels of homocysteine are age-related and common in the elderly (Herrmann & Obeid, 2011; Bickel *et al*., 2017).

1.2 PREVALENCE OF CARDIOVASCULAR DISEASE

1.2.1 Globally

Cardiovascular disease (CVD) is regarded as a problem of high-income countries whereas the consequences of the disease are felt globally, irrespective of economic status (Fuster & Kelly, 2010; Ioannidis, 2014). In the past 30 years, the burden of CVD has been decreasing in developed countries while the developing countries are experiencing an increase (Mendis *et al*., 2011; Ioannidis, 2014).

Deaths due to cardiovascular disease are greater than the total deaths caused by other diseases such as diabetes mellitus, cancer, respiratory disease and nutritional disorders (Sun & Jia, 2012; Nichols *et al.*, 2014). Global mortality is estimated at 57 million per annum in which 36 million of the deaths are caused by non-communicable diseases (Mendis *et al.*, 2011; Ding *et al.*, 2016). Cardiovascular disease alone account for over 17.3 million of the deaths caused by non-

communicable diseases, making it the largest leading cause of global deaths and is expected to increase to 26.3 million in 2030 (Smith *et al.*, 2012; Townsend *et al.*, 2016).

1.2.2 Africa and South Africa

There is lack of comprehensive and representative data on non-communicable disease risk factors in sub-Saharan Africa due to the scarcity of important statistics and adequate systems to monitor causes of deaths in the whole population among these countries; where such data is available there are technical challenges such as lack of data collection systems (McCarthy *et al.*, 2010; Negin *et al.*, 2011; Bovet & Paccaud, 2012; Alkema *et al.*, 2016). However, studies conducted project that deaths caused by non-communicable diseases in Africa will surpass those caused by communicable diseases in the next 20 years. Notable among these communicable diseases include malaria, HIV/AIDS and tuberculosis (Gersh *et al.*, 2010; Mendis *et al.*, 2011; Alkema *et al.*, 2016). Despite these projections, African states together with donors and research-funders have dedicated their resources towards communicable diseases and nutritional challenges (McCarthy *et al.*, 2010; Bovet & Paccaud, 2012; Kruk *et al.*, 2016)

In the 2011 South African health report, it was reported that national strategies implemented to combat challenges of non-communicable diseases from primary through tertiary health care levels are ineffective and therefore need for robust strategies (Gray *et al.*, 2011; Nyaaba *et al.*, 2017). Similar to any other industrialized country, the emergence of non-communicable diseases in South Africa is mainly influenced by vulnerability of the ageing population developing chronic diseases and coexistence epidemics such as HIV/AIDS resulting in marginalization of prevention and treatment of non-communicable diseases (Mayosi *et al*., 2009; Angotti *et al.*, 2018).

Risk factors for cardiovascular disease in black Africans are aggravated by myths, such as the belief that effects of risk factors are exerted more in Western populations who are relatively more atherogenic thus proposing that black Africans have genetic resistances (Onen, 2013; Tshuma, 2017). The increasing burden of CVD in African states is profound and is contributed by rapid growth of diabetes and obesity (McCarthy *et al.*, 2010; Sliwa *et al.*, 2016). Black communities are gradually becoming wealthier, causing a nutritional transition which enhances the occurrence of non-communicable diseases (Mayosi *et al.*, 2009; Battersby, 2017). Black

South Africans mimic Western culture thus the increase in prevalence of hypertension, almost similar to that of the US population (Tibazarwa, *et al.*, 2009; Ferris & Crowther, 2016).

1.2.3 The Elderly

Life expectancy in high-income countries has increased by approximately 30 years resulting in a rapid increase in ageing populations (Christensen *et al.,* 2009; Fabian *et al.,* 2012; WHO, 2014; Mathers *et al.,* 2015). It is projected that in 40 years there will be a historic turning point in which the majority of the population will be those of the age ≥65 and the minority will be of the age range ≤5 years (Suzman, 2010; Buckinx *et al.,* 2015). South Africa is amongst the developing countries which are experiencing population growth of elderly people regardless of HIV/AIDS reducing life expectancy (Mayosi *et al.,* 2009; Statistics South Africa, 2017; Angotti *et al.,* 2018).

The health impact of the ageing is better anticipated in developed countries, putting such nations as the Europeans at the forefront of ageing awareness and policy adaptation whereas developing nations lag behind (Suzman, 2010; Biggs, 2018). There is a disproportionate global age-standardized CVD mortality rate distribution. In Australia, France and United States age-adjusted CVD mortality rates are between 100 and 200 per 100 000; about 300 for Brazil and China; for India, Saudi Arabia and South Africa they are between 400 and 500 whereas Russia and Egypt exceeds 500 per 100 000 (Fuster & Kelly, 2010; Roth, 2013).

There are inequalities in developing countries when it comes to addressing health challenges that emerge among young and ageing population, thus leaving healthcare gaps on the latter population despite is the fast growth (Suzman 2010; Biggs 2018). Nutritional deficiencies, HIV/AIDS and child deaths in sub-Saharan Africa are some of the reasons for prioritizing the young and overlooking the aged (Negin *et al.,* 2011; Audain *et al.,* 2015). Approximately 30% to 40% of the elderly population in rural areas of Western Africa are suffering from hypertension and about 50% of semi-urban dwellers have hypertension (Onen, 2013).

1.3 BACKGROUND OF THE STUDY

This study forms part of a multi-micronutrient program conducted by the Centre of Sustainable Livelihoods (CSL) at the Vaal University of Technology (VUT), under the leadership of Prof.

W.H Oldewage-Theron. It was conducted with the approval of Vaal University of Technology ethical committee (20140827-1ms).

The study was conducted in a free living community attending a day care centre in Sharpeville, Gauteng, South Africa. The centre was established in 2004 to provide community elders with skills training and religious activities. Breakfast and lunch are also provided for attendees of the centre. A high risk for cardiovascular disease was reported in the same community (Oldewage-Theron, *et al.*, 2008; Grobler, 2015).

1.4 SIGNIFICANCE OF THE STUDY

Correlations have been found in literature between homocysteine levels, vitamin B_{12} status, presence of *methylenetetrahyrofolate reductase* (*MTHFR) C677T* polymorphism and cardiovascular risk (Waśkiewicz *et al.*, 2011; Liew & Gupta 2015). Limitations have been identified using serum vitamin B_{12} (cobalamin) as a marker for vitamin B_{12} status due to lack of a gold standard test. Serum vitamin B_{12} can be falsely elevated when affected by pathological conditions such as liver disease (Fragasso *et al.*, 2011; Moll & Davis 2017). Holotranscobalamin (active vitamin B_{12}) has been reported as a more accurate marker for vitamin B_{12} status (Doets *et al.*, 2014).

This study scientifically contributes to the better comprehension of the risk and management of cardiovascular disease in Africa. It also scientifically contributes to a better understanding of the interaction between vitamin B_{12}, homocysteine and cardiovascular risk. Furthermore it contributes to new knowledge, as the first conducted study amongst the elderly in South Africa.

1.5 AIMS AND OBJECTIVES

1.5.1 Aims

The aim of the study was to evaluate the prevalence of the *MTHFR C677T* polymorphism. Furthermore the study evaluates the correlation of folate status and its effect on homocysteine metabolism, correlation of vitamin B_{12} status and its effect on homocysteine metabolism as

cardiovascular risks in the elderly black population in Sharpeville, Gauteng, South Africa as related to the presence of the *MTHFR C677T* polymorphism.

1.5.2 Objectives

The specific objectives of the study are:

1) To determine the prevalence of the *MTHFR C677T* polymorphism in an elderly black South African population.
2) To determine homocysteine status of the elderly black South African population.
3) To determine the folate concentrations in the elderly black South African population.
4) To evaluate the vitamin B_{12} status by using serum vitamin B_{12}, and holotranscobalamin parameters.
5) To evaluate the correlation between the *MTHFR C677T* polymorphism and homocysteine status.
6) To evaluate the correlation between the *MTHFR C677T* polymorphism and folate status.
7) To evaluate the correlation between the *MTHFR C677T* polymorphism and vitamin B_{12} status.
8) To correlate holotranscobalamin levels with serum vitamin B_{12} concentrations.

1.6 STRUCTURE OF THE DISSERTATION

- **Chapter 1- Background and problem setting**

Chapter 1 will provide information background of the study, outline and limitations.

- **Chapter 2- Literature**

A comprehensive literature review on the general background of the studywill be reported in Chapter 2.

❖ **Chapter 3- Methodology**

In Chapter 3 methods followed during the study will be outlined.

❖ **Chapter 4- Results**

Reporting and discussing of results obtained will be described in Chapter 4.

❖ **Chapter 5- Conclusion and recommendation**

Conclusions will be made to accomplish the objectives of the study and recommendations will be made for application.

❖ **Chapter 6-References**

As prescribed by VUT, the "Harvard" method of referencing will be used to compile a reference list.

1.7 CONCLUSION

This study pays attention to the link between cardiovascular risk and homocysteine status, cardiovascular risk (CVR) and genetic polymorphism as well as the interaction between homocysteine status genetic polymorphism.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Like other Sub-Saharan countries, there is limited data on the prevalence of polymorphisms associated with diseases such as cardiovascular diseases in South Africa (Atadzhanov *et al.*, 2014). Decrypting disease associated polymorphisms in African regions is a challenge because of an inadequate knowledge of the prevalence of such polymorphisms in specific populations (Atadzhanov *et al.*, 2014). Elderly people are prone to pathophysiological conditions of homocysteine biomarkers such as deficiencies in vitamin B_{12} and folate (Ng *et al.*, 2012; Wong, 2015).

The mutation *methylenetetrahydrofolate reductase* (*MTHFR*) *C667T* affects the physiological functional activity of methylenetetrahydrofolate reductase (MTHFR), an enzyme which catalyzes the reduction of 5,10-methylenetetrahydrofolate (5,10-MTHF) to a coenzyme 5-methyltetrahydrofolate which is required for remethylation of homocysteine to methionine (McEwen 2016). Elevated plasma homocysteine concentrations is a multifactorial risk factor of non-communicable diseases (NCDs) such as cardiovascular diseases, hypertension, stroke, cognitive and dementia and diabetes (Chen *et al.*, 2010; Burdennyy *et al.*, 2017).

This research evaluates the prevalence of *MTHFR* C677T polymorphism with special reference to homocysteine metabolic markers in a black elderly population in the community.

2.2 CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is a group of pathological conditions of the circulatory system such as the heart, blood vessels and the cerebrovascular system (Sun & Jia, 2012; Bonnefont-Rousselot, 2016). Acute coronary syndromes, angina, congenital heart disease (CAD), coronary heart disease (CHD), ischaemic heart disease, rheumatic heart disease and heart failure are some of heart-associated cardiovascular diseases (World Heart Federation, 2012; Bonnefont-Rousselot, 2016). Cerebrovascular disease (stroke), haemorrhagic and ischemic strokes are all categorized by the World Heart Federation (2012) as brain-related

cardiovascular diseases (Chandra *et al.,* 2017). The third category of cardiovascular disease is the circulatory system which is made up of hypertensive heart disease, deep vein thrombosis, peripheral artery disease and pulmonary embolism (World Heart Federation 2012).

2.2.1 Atherosclerosis

Atherosclerosis is described by Apple *et al.,* (2015) as an inflammatory disease due to the triggered inflammatory response to endothelium injury. It is a pathological condition of the arteries characterized by progressive development of a focal lesion termed atherosclerotic plaque (Bentzon *et al.,* 2014). Atherosclerosis mechanism is central to the development of CVDs (Kampoli *et al.,* 2009; Jaipersad *et al.,* 2014:1; Barquera 2015). Stimuli of endothelium inflammatory response are known as cardiovascular risk factors, as they initiate endothelial dysfunction, an early predictor of atherosclerosis (Mudau *et al.,* 2012).

Atherosclerosis manifest in a three-phase procedure, viz, endothelium injury phase, tissue response and tissue repair phases (Gimbrone & García-Cardeña 2016). Endothelium injury is caused by chronic exposure to cardiovascular risk factors (2.2.1.1) triggering pathophysiological changes of endothelial cells (Mudau *et al.,* 2012). As a response to the atherogenic stimuli such homocysteine (2.3.3.1), dyslipidaemia, oxidative stress and diabetes mellitus (tissue response phase), endothelial cells express vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1(ICAM-1) adhesion (Hirase & Node 2012; Kolodziejczyk-Czepas *et al.,* 2012).

Adhesion molecules recruit monocytes which mature into macrophages responsible for retaining low-density lipoprotein cholesterol (LDL-C) (Libby *et al.,* 2011; Jaipersad *et al.,* 2014). Following invasion of the arterial wall by circulating LDL-C particles, oxidation of LDL-C takes place in the intima (Rocha & Libby, 2009; Rafieian-Kopaei *et al.,* 2014). Macrophages adhere to Low-density lipoprotein (LDL) via LDL receptors present on the surface of macrophages, this results in lipid accumulation (Newsholme & Leech, 2009; Santovito *et al.,* 2016).

These lipid-loaded macrophages known as form cells target lesions formed by injured smooth muscle cells (SMCs) and stimulate the secretion of cytokines, tumour necrosis factor (TNF) and transforming growth factor beta (TGF-β), prothrombic factors, proteases and reactive

oxidative stress (Libby *et al.*, 2011; Tavakoli & Asmis 2012; Gimbrone & García-Cardeña, 2016; Tabas & Bornfeldt, 2016). These substances attract migration of additional inflammatory cells and SMCs resulting in expansion of the plaque size (Tavakoli & Asmis, 2012; Rafieian-Kopaei *et al.*, 2014).

The rate at which SMCs proliferate during atherogenesis is increased as a response to a platelet-derived growth factor and collagen synthesis resulting in formation of a fibrous cap (Wang & Bennett, 2012). Senescence of SMCs in atherosclerosis is an effect of apoptosis caused by death ligands (present on surface of macrophages) and accelerated enzyme activity in matrix (fibrous cap) degradation (Wang & Bennett, 2012). Efferocytosis forms a necrotic core which augment inflammatory response within a mature atherosclerotic plaque (Tavakoli & Asmis, 2012).

Necrotic core rapture at a thin fibrous cap location which releases thrombogenic substances to the coagulation cascade resulting in development of arterial thrombosis (Tavakoli & Asmis, 2012). As opposed to normal endothelial cells which lack tissue factor (TF) (a glycoprotein that initiates extrinsic pathway of coagulation), atherosclerotic endothelial cells produce this glycoprotein that binds to factor VII (FVII) (Borissoff *et al.*, 2011; Winckers *et al.*, 2013). The complex (TF-FVII) converts FVII to activated factor VIIa (FVIIa) forming a TF-FVIIa complex (Kleinegris *et al.*, 2012).

Factor X (FX) is activated to factor Xa (FXa) and subsequently generates thrombin as a result of TF-FVIIa complex formation (Winkers *et al.*, 2013). Thrombin signalling activity converts fibrinogen to fibrin, this activates platelets which ultimately forming thrombus (Breitenstein *et al.*, 2010; Borissoff *et al.*, 2011; Weitz, 2014).

Nitric oxide protects vascular endothelium against atherosclerosis by inhibiting processes of atherothrombosis namely; monocyte chemotaxis and adhesion, SMCs proliferation and platelet aggregation (Ķēniņa *et al.*, 2009; Tabas & Bornfeldt, 2016).

In the tissue repair phase, platelet endothelial cell adhesion molecule-1 (PECAM-1) induces transmigration of platelets (Privratsky & Newman, 2014). The platelets secrete growth factors which stimulate proliferation of SMCs at the area of injury (Golebiewska & Poole, 2015).

Not only is endothelial dysfunction a predictor but also a mechanistic connection between cardiovascular risk factors and the pathological condition (Sitia *et al.*, 2010; Gimbrone &

García-Cardeña, 2016). Although the endothelial dysfunction process is multifactorial, it is hallmarked by decreased nitric oxide and oxidative stress (Mudau *et al.*, 2012). Nitric oxide which is derived from amino acid L-arginine and released by endothelium functions as a vasodilator factor (Herrera *et al.*, 2010). Reactive oxygen species, particularly superoxide increase oxidative stress causing reduction in bioavailability of nitric oxide (Herrera *et al.*, 2010:145; Rafieian-Kopaei *et al.*, 2014).

An elevated state of homocysteine plays a role in advancement of atherosclerosis via various mechanisms, namely; impairing of endothelial function, inducing oxidative stress and enhancing inflammation and thrombosis (Seo *et al.*, 2010; Karababa *et al.*, 2017). When there is oxidative stress induction, hyperhomocysteinemia alters the molecular structure and activation mechanism of factors involved in oxidative process of atherosclerosis (Seo *et al.*, 2010; Lai & Kan, 2015). The end products of the atherosclerosis process accumulate and generate what is known as atherosclerotic plaque (Gimbrone & García-Cardeña, 2016).

The atherosclerotic plaque can cause several complications which vary in respect to the area of the plaque formation (Hansson *et al.*, 2015) A plaque formation in cerebral area causes a complication known as cerebral infarction, whereas carotid atheroma and myocardial infarction are caused by plague formation on the carotid and the heart respectively (Caplann, 2015; Hansson *et al.*, 2015).

2.2.1.1 Predisposing factors

A spectrum of predisposing factors have been related to a cardiovascular event. These factors are usually grouped as modifiable and non-modifiable (World Heart Federation, 2012; Frohlich & Al-Sarraf, 2013). Modifiable risk factors range from diabetes mellitus, obesity, smoking, poor diet, declined physical activity, to dyslipidaemia (Maredza *et al.*, 2011; Buchan *et al.*, 2012; Gupta *et al.*, 2013). Uncontrollable predisposing factors namely age, gender and family history cause cardiovascular disease (World Heart Federation 2012; Gupta *et al.*, 2013).

Psychosocial and geographic factors are two other categories of cardiovascular predisposing factor (Grobler, 2015). Psychosocial predisposing risk factors are the product of low socioeconomic class, stressful environment and types of personalities whereas geographic factors consist of soft drinking water, environmental pollution and climatic change (Grobler, 2015).

The following are mechanistic links between predisposing factors and manifestation of cardiovascular disease.

a) Diabetes mellitus

Chronic hyperglycaemia and insulin deficiency in diabetes mellitus are mechanisms thought to responsible for cardiovascular event (Wannamethee *et al.,* 2011; Wang *et al.,* 2016). Hyperglycaemia causes endothelial dysfunction by scavenging nitric oxide (reduced nitric oxide bioactivity) and inducing advanced glycation end-products (nitric oxide activity inhibitors) (Sánchez-Quesada & Pérez, 2013).

b) Obesity

This pathological condition primarily increases risk of cardiovascular disease via activation of inflammatory response (Gustafsson *et al.,* 2013). In obese conditions, secretion of a bioactive molecule (adiponectin) with inflammatory, oxidative and atherogenic protective properties is reduced (Gustafsson *et al.,* 2013; Neupane *et al.,* 2014).

Secondary predisposing property of obesity is by association with traditional cardiovascular risk factors such as diabetes mellitus, altered lipid metabolism and high blood pressure (Tzotzas *et al.,* 2010; Yatsuya *et al.,* 2014).

c) Smoking

Smoking promotes the occurrence of cardiovascular event as a result of oxidative stress from inhaled reactive oxides (Perk *et al.,* 2012). Cigarettes constitutes of compounds that have adverse effects on the endothelium, such as nicotine and carbon monoxide (Di Stefano *et al* 2010; George, 2014). These compounds cause both physiological alterations (impaired nitric oxide release and impaired flow mediated dilation and vasoconstriction) and anatomical alterations (platelet aggregation, fibrinolysis cascade activation and lipid metabolism) on the vascular endothelium (Di Stefano *et al.,* 2010; Li *et al.,* 2014).

d) *Nutritional Status*

According to a World Health Organisation (WHO) report (Mendis, 2015), in 2010 approximately 1.7 million of cardiovascular worldwide deaths resulted from excessive intake of salt (sodium). Excessive dietary salt consumption reduces insulin sensitivity and activates both sympathetic nervous system and renin-angoitensin-aldesterone system, ultimately leading to vascular dysfunction (Lastra *et al.,* 2010; Feng *et al.,* 2017).

Intake of saturated fat increase LDL-C levels therefore increasing the risk of cardiovascular events (Siri-Tarino *et al.,* 2010; Boekholdt *et al.,* 2014). Carbohydrate-rich diet is associated with metabolic state (overweight and obesity) that aggravates dyslipidaemia (Siri-Tarino *et al.,* 2010; Dehghan *et al.,* 2017). Deficiency of vitamins especially vitamin $B_{6,}$ vitamin B_{12} and folate increases risk of cardiovascular disease because of their roles as cofactors in metabolism of homocysteine (an independent cardiovascular risk factor) (Cui *et al.,* 2010; Duthie *et al.,* 2015).

Contamination of drinking water by chemical elements such as arsenic, increases cardiovascular mortality (Medrano *et al.,* 2010; D'Ippoliti *et al.,* 2015). During arsenic-induced atherosclerosis there is vascular matrix reduction, endothelium damage and increased platelet aggregation (Lisabeth *et al.,* 2010; Newman *et al.,* 2017). Air pollutants (gaseous and particulate matter) are atherogenic as they may cause systematic inflammation, hypercoagulability, and oxidative stress (Franchini & Mannucci, 2012; Franchini *et al.,* 2016).

e) *Physical inactivity*

A sedentary lifestyle has a high cardiovascular risk due to lack of beneficial effects of exercising such as anti-inflammatory effect, decreased fat accumulation and improved lipid profile (increased high density lipoprotein cholesterol (HDL-C) particles, lowered triglycerides and low density lipoprotein cholesterol (LDL-C) particles) (Gleeson *et al.,* 2011; Lessiani *et al.,* 2016).

f) Aging

With age advancement the likelihood of a cardiovascular event increases due to alterations on the vasculature (North & Sinclair, 2012). Progressive loss of elasticity in the arteries and vascular remodelling (intimal and medial thickening) alterations are consequences of vascular aging (Wang & Bennett, 2012).

Decreased elastin content and increased collagen deposit in aged vessels enhance matrix metalloproteinase activity and stimulation of angiotensin II signal which causes vascular stiffness and ultimately hypertension (Wang & Bennett, 2012).

g) Hypertension

Intima-medial thickness, modified endothelial-dependent vasodilation are consequences of prolonged hypertensive condition (Wang *et al.,* 2010; Weaver & Mitsnefes, 2016). Early detection of atherosclerosis is reliably indicated by Intima-medial thickness (Ntaios *et al.,*

2010; Weaver & Mitsnefes, 2016). Among the aging, these structural changes are accelerated by hypertension. Thick intima-medial thickness of the internal carotid artery predicts a cardiovascular risk in the presence of a plaque (Polak *et al.,* 2011; Naqvi & Lee, 2014).

h) Dyslipidaemia

Elevated triglycerides, decreased high density lipoprotein (HDL) particles and increased LDL particles are attributes of dyslipidaemia with atherogenic properties which are cardiovascular risk (Musunuru, 2010; Chang & Robidoux, 2017).

i) Psychosocial factors

Cardiovascular incidence is more likely to occur among people living in a low socioeconomic state as a result of limited interpersonal and intrapersonal resources (education, occupation and income) required to cope with stress encountered in life (Matthews & Gallo, 2011; Lazzarino *et al.,* 2013). The mechanistic link between psychological distress and CVD is through the effect that psychological distress has on the function of vascular endothelium, C-reactive protein (CRP), proinflammatory cytokines and haemostatic factors (Lazzarino *et al.,* 2013).

j) Seasonal changes

Altered coronary diameter and systematic vasoconstriction are direct response to cold season (Cuspidi *et al.,* 2012). During this response to cold weather there is elevation of blood viscosity and concentration, hypercoagulability and elevation plasma lipids (Cuspidi *et al.,* 2012; Vasconcelos *et al.,* 2013).

k) Gender

In general cardiovascular disease in an elderly population is more prevalent in females than males due to longer life expectancy of the women (Mosca *et al.,* 2011; Manson & Bassuk, 2015). Independent cardiovascular risk factors such as hyperhomocysteinemia is more prevalent in males as compared to their counterparts (2.3.3). Premenopausal women are relatively less likely to experience an onset of atherosclerosis compared to men of the same age due to reproductive hormones (Vaccarino *et al.,* 2010; El Khoudary *et al.,* 2017). Oestrogens stimulates a protective action against atherosclerosis by rapidly increasing nitric oxide production, promoting re-endothelialization and inhibiting proliferation of smooth cells as well as deposition of matrix (Vaccarino *et al.,* 2010; Tabas & Bornfeldt, 2016).

2.2.1.2 Cardiovascular risk markers

A wide range of biomarkers identified in the Multinational Monitoring of trends and determinants in Cardiovascular diseases (MONICA), Risk Genetics Archiving and Monograph (MORGRAM) biomarker project were clustered in different cardiovascular risk markers as indicated in Table 1 (Blankenberg 2010; Buchan *et al.*, 2012:59; Herder *et al.*, 2017).

TABLE 1 CARDIOVASCULAR RISK MARKERS

CARDIOVASCULAR RISK MARKER	BIOMARKERS
1. Markers of oxidative stress and antioxidants	• Homocysteine • Vitamin B_{12} • Holotranscobalamin • Myeloperoxidase
2. Lipid profile	• Apolipoprotein A1 • Apolipoprotein B100 • Lipoprotein • Paraoxonase-1
3. Metabolic markers	• Glucose • Insulin • Ferritin • Adiponectin • Leptin
4. Inflammatory markers	• C-reactive protein • Interleukin-18 • Interleukin-11 receptor antagonist • Neoptenin
5. Coagulation markers	• D-Dimer
6. Renal function markers	• Creatinine • Cystain-C
	• Troponin 1

7. Necrosis markers	• Creatine kinase-MB
8. Angiogenesis markers	• Cardiac placental growth factor
9. Markers of vascular function and neurohumoral activity	• B-type natriuretic pepetide • C-terminal pro adrenomedulin • C-terminal pro-endothelin-1 • Mild regional pro-adrenomedulin • Mild regional pro-atrial natriuretic peptid • Tissue inhibitor of metalloproyeinase-1

Source: Blankenberg *et al.,* 2010; Graversen *et al.,* 2017.

Furthermore other biomarkers have been identified and reported, they are; glutathione peroxidase, superoxide dismutase isoprostanes, malondialdehyde, nitrotyrosine, S-gluthionylation oxidized low density lipoprotein and oxidized phospholipids (markers of oxidative stress and antioxidants) (Ho *et al.*, 2013). Additional to the lipid profile is triglycerides, HDL-C, lipoprotein (a), cholesterol ester transfer protein (CETP), lipoprotein-associated phospholipase A_2 (Lp- PLA_2) (Apple *et al.*, 2015).as cardiovascular risk predictors.

Other inflammatory markers identified as cardiovascular risk markers include high sensitivity C-reactive protein (Hs-CRP) that measure lower concentrations of serum CRP (down to 0.3 mg/l), interleukin (IL)-6 and tumour necrosis factor-alpha (TNF-α) (Nishida *et al.*, 2011; Silva & Lacerda 2012). The procoagulant state of cardiovascular risk can also be predicted by plasminogen activator inhibitor-1, fibrinogen and P-selectin (Goldberg, 2009; Tofler *et al.*, 2016).

2.3 HOMOCYSTEINE

2.3.1 Physical characteristics

Homocysteine is non-essential, non-proteinogenic, thio-containing metabolite formed during synthesis of essential methionine (Fabian *et al.*, 2011; Škovierová *et al.*, 2016). There are three forms which plasma homocysteine can be structured, it can either exist as free sulfhydryl group,

a disulfide or as mixed disulfide attached to cysteine residues (Bock, 2011; Škovierová *et al.*, 2016). The protein-bound homocysteine is about 70%, approximately 30% of the total homocysteine is bound to thiol via disulfide bonds and only 1% is the reduced form (Cheng *et al.*, 2009; Sawuła *et al.*, 2009; Zinellu *et al.*, 2017). Free thiol and disulfide-bound homocysteine are collectively termed total homocysteine (tHcy) and are plasma biomarkers of diseases related to homocysteine metabolism (Jakubowski, 2013). Homocysteine and cysteine have similar chemical structures with a difference of one methylene (Figure 1) (Xiao *et al.*, 2011; Xiao *et al.*, 2013).

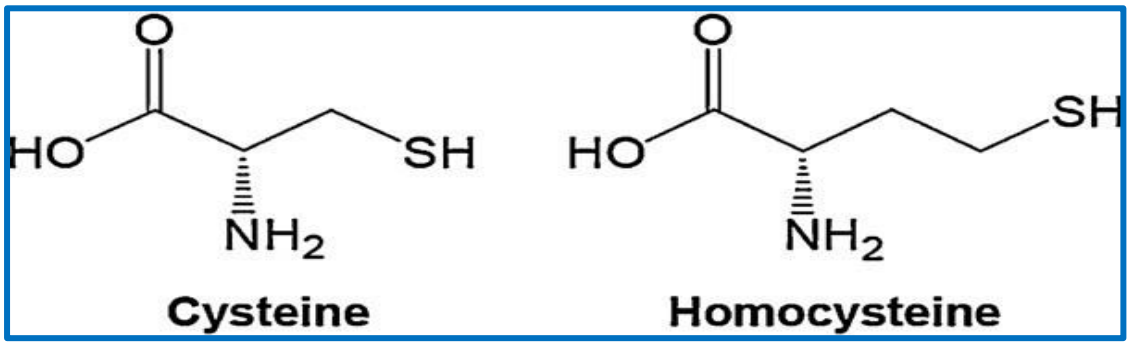

FIGURE 1: CYSTEINE AND HOMOCYSTEINE STRUCTURES

Source: Adapted from Xiao *et al.*, 2013.

2.3.2 Biochemical metabolism

Homocysteine in humans is solely sourced from demethylation methionine (Cheng *et al.*, 2009; Mao *et al.*, 2014). During metabolic process of methionine, homocysteine primarily function as a significant intermediate metabolite (Chen *et al.*, 2010; Luft, 2015). Unlike bacteria and plants which synthesize their own methionine, mammals obtain this essential amino acid only from food in the form of proteins (Jakubowski, 2013). Homocysteine is situated at a biochemical junction at which can be either irreversibly degraded to cysteine or remethylated to methionine via respective metabolic pathways (Blom & Smulders, 2011; Luft, 2015).

Homocysteine metabolism involves multiple, interlinked processes catalyzed by enzymes such as MTHFR, methionine synthase and cystathionine-β-synthase (Schalinske & Smazal, 2012; Codoñer-Franch & Alonso-Iglesias, 2015). Accumulation of homocysteine in the serum is prevented by the MTHFR activity which generates a pool of systemic folate (Aneji *et al.*, 2012;.

According to King *et al.,* (2012), physiological metabolism of homocysteine occurs in two pathways, the remethylation and transsulfuration pathways (Figure 2).

The transsulfuration pathway which occurs mainly in the kidney and liver cells is catalyzed by vitamin B_6-depedent enzymes (Sabetisoofyani *et al.,* 2010; DeRatt *et al.,* 2014). Every organ is a site for the remethylation pathway whereas the transsulfuration exclusively takes place in the liver and kidneys (Jakubowski, 2013).

2.3.2.1 Remethylation

In the remethylation pathway, the folate cycle interacts with homocysteine metabolism by enzymatic action of methionine synthase (Creus *et al.,* 2013). It is in this pathway that the two direct methyl donor compounds are found, these are methlyltetrahydrofolate (MTHF) and *S*-adenosyl-methionine (SAM) (Newsholme & Leech, 2009; McEwen, 2016). Amino acid methionine is known as SAM when it is in an active form and it is the removal of the methyl group from the active form that *S*-adenosyl-homocysteine (SAH) is produced (Figure 2) (Manolescu *et al.,* 2010; Ganguly & Alam, 2015). S-adenosyl-homocysteine metabolite further losses adenosine to adenosine triphosphate (ATP) conversion subsequently producing homocysteine (Newsholme & Leech, 2009; Ganguly & Alam, 2015).

Methlyltetrahydrofolate compound which is primarily from folate, donates methyl group to homocysteine to form an inactive form of methyl donor, methionine (Newsholme & Leech, 2009; Santilli *et al.,* 2016). Homocysteine methylation betaine-homocysteine methlytransferase (BHMT) is confined whereas the vitamin B_{12} mediated homocysteine methylation occurs in all tissues (Brustolin *et al.,* 2010; Blom & Smulders, 2011; Santilli *et al.,* 2016). Once the methyl group is released to homocysteine, methionine synthase (MTR) is revitalized to accept another methyl group (Blom & Smulders, 2011; Ganguly & Alam, 2015).

2.3.2.2 Transsulfuration pathway

In the transsulfuration biochemical pathway, homocysteine is one of the components of one-carbon metabolism system and the other two components interacting with the one-carbon metabolism network are *S*-adenosyl-methionine (SAM) and *S*-adenosyl-homocysteine (SAH) metabolic pathways (King *et al.,* 2012). Deprivation of homocysteine metabolite SAM, inhibits methyl group transfer and the latter process is essential for synthesis of deoxyribonucleic acid

(DNA) (Newsholme & Leech, 2009: Mentch *et al.*, 2015). Glutathionine, an antioxidant needed in detoxification of xenobiotics is derived from cysteine (Blom & Smulders, 2011; Robaczewska *et al.*, 2016).

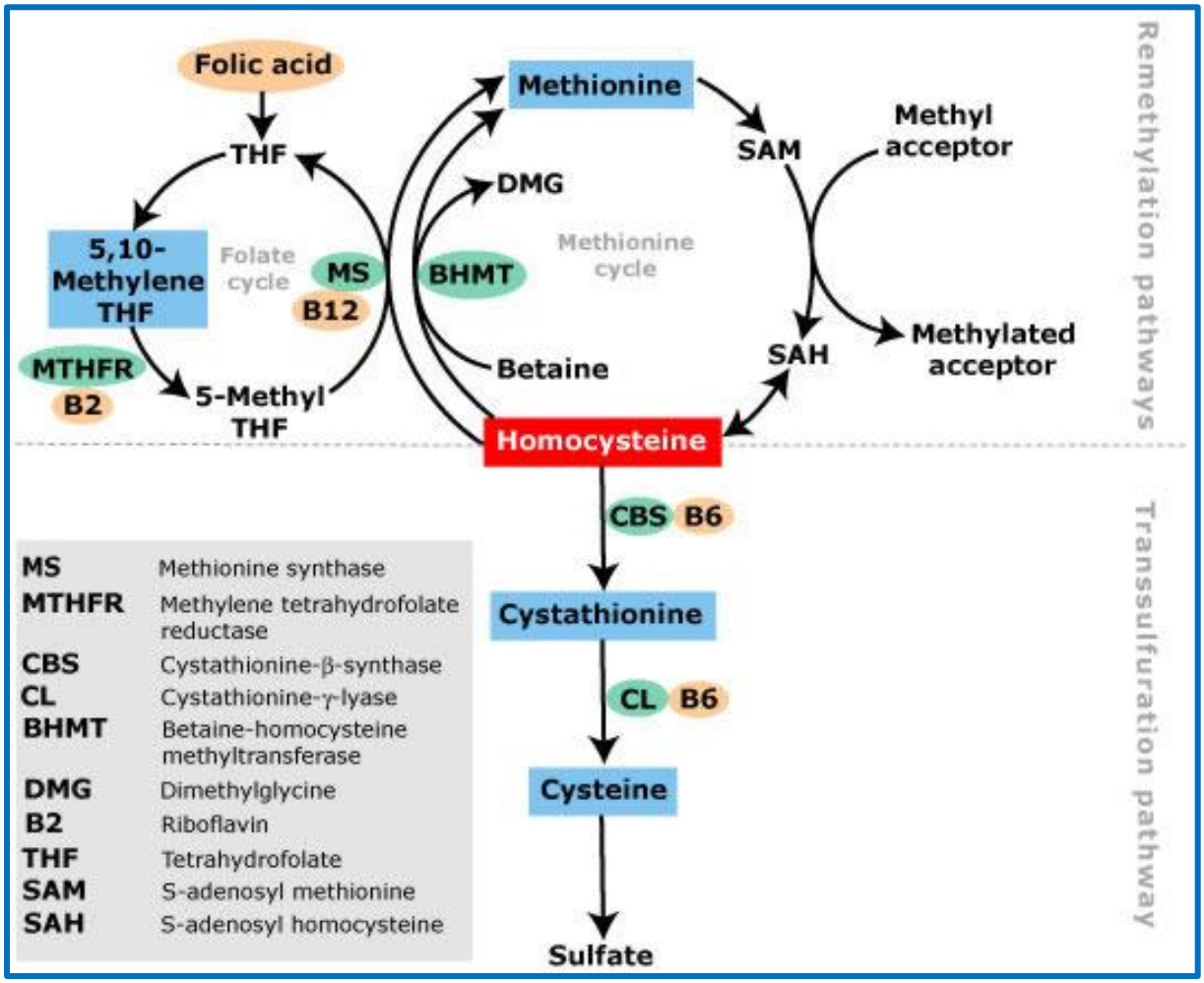

FIGURE 2: HOMOCYSTEINE METABOLISM

Source: Adapted from http://www.homocysteine2011.com/homocysteine-metabolism

Homocysteine easily enters the plasma membrane in contrast with its metabolite SAH elucidating greater homocysteine concentration in plasma as compared to SAH (King *et al.*, 2012). The conversion reaction of homocysteine to methionine is dependent on water-soluble B vitamins that act as essential coenzymes during the degradation (MacFarlane *et al.*, 2011;

Yeh *et al.,* 2018). Once absorbed in the alimentary tract, methionine is distributed by blood cell to various organs where it utilized in protein synthesis and as a precursor (Jakubowski, 2013).

Methylation potential is reduced by elevation of homocysteine and this is corrected by increased folate and vitamin B_{12} (Sabetisoofyani *et al.,* 2010; Yeh *et al.,* 2018). Both tHcy and methylmalonic acid (MMA) are increased in vitamin B_{12} deficiency whereas in folate deficiency only tHcy is increased, this phenomenon makes tHcy and MMA to be sensitive biomarkers of vitamin B_{12} and folate status (Fragasson *et al.,* 2012).

The pathway which homocysteine is metabolized is determined by methionine levels. In principle, an increase in methionine is an increase in *S*-adenosyl-methionine (Blom and Smulders, 2011; Aggrey *et al.,* 2018). The latter inhibits MTHFR (a remethylation pathway enzyme) activity when elevated and when decreased, *S*-adenosyl-methionine activates cystathionine-β-synthase (CBS) (transsulphuration pathway enzyme) to irreversibly degrade the methionine precursor, homocysteine (Wang *et al.,* 2011; Aggrey *et al.,* 2018).

Homocysteine methylation is regulated by methionine and CBS in the liver (Blom & Smulders, 2011; McEwen, 2016). The more homocysteine is present the more the methionine is synthesized, increased methionine subsequently increases SAM which inhibits MTHFR and activates CBS (Blom & Smulders 2011; Yeh *et al.,* 2018). Decreased methionine results in low *S*-adenosyl-methionine thus neither activating its inhibitory nor activation effects on MTHFR and CBS respectively (Blom & Smulders, 2011; Mentch *et al.,* 2015).

Several homocysteine metabolites are produced during metabolism of homocysteine, these include; tHcy, Homocysteine-Thiolactone (Hcy-thiolactone), N-Homocysteinyl-Protein (N-Hcy-protein), Nε-Homocysteinyl-Lysine (Nε-Hcy-Lys), S-Adenosylhomocysteine (AdoHcy), homocysteic acid (HCA), homocysteine sulfinic acid (HAS) and cystathionine (Jakubowski, 2013). Hcy-thiolactone is produced when a small amount of homocysteine is converted to thioster by methionyl-tRNA synthase. This reaction accelerated when there is; increased methionine consumption, decreased methyltetrahydrofolate supply or presence of pathological conditions of homocysteine metabolism (Jakubowski, 2013).

Hcy-thiolactone immediately reacts with lysine residue via amide (isopeptide) bonds to form N-Hcy-protein product, this product is degraded by enzymatic hydrolysis to produce Nε-Hcy-Lys (Sikora *et al.*, 2010; Jakubowski, 2013). This modifies lysine structure and function thus triggering an autoimmune response targeting the amide bond (Sikora *et al.,* 2010; Codoñer-

Franch & Alonso-Iglesias, 2015). Excessive homocysteine favours cell membrane damaging thiolactone in a spontaneous metabolism (Newsholme & Leech, 2009; McCully, 2015).

In methionine metabolism, the immediate precursor of Hcy is AdoHcy species and cystathionine species is a metabolite of conversion of Hcy to cysteine (Jakubowski, 2013). Other species that exist in human plasma are disulfide homocysteine (Hcy-S-S-Hcy), mixed disulfide of homocysteine and cysteine (Hcy-S-S-Cys) and mixed disulfide of homocysteine with plasma protein (S-Hcy-protein).

Optimal homocysteine methylation is only possible with adequate presence of folate and cofactors, vitamin B_6 and vitamin B_{12} (Herrmann & Obeid, 2011; Santilli *et al.,* 2016). Homocysteine methylation is initiated by 5-methyl tetrahydrofolate, a MTHFR catalyzed folate metabolite (Das & Kaul, 2008; McCully, 2015).

2.3.3 Hyperhomocysteinemia

Increase in plasma homocysteine is known as hyperhomocysteinemia (Cheng *et al.,* 2009; McCully 2015). Various factors including age, gender, nutritional deficiencies, genetic and environmental factors, lifestyle and chronic diseases may lead to hyperhomocysteinemia (Nilsson *et al.,* 2010; Codoñer-Franch & Alonso-Iglesias, 2015). Age related hyperhomocysteinemia may be due to decreased folate concentration and deterioration of renal physiology are common in age advancement (Kirsch *et al.,* 2013).

Males have higher homocysteine as a result of sex hormones and relatively large muscle mass (Chmurzynska *et al.,* 2013). Muscles produce creatinine, a product that requires methylation by SAM to form creatine (Malinow *et al.,* 2012). Larger muscle mass produce high creatinine levels, therefore requiring increased methyl donation from SAM.

Excessive intake of coffee, alcohol and smoking are some lifestyle factors influencing homocysteine elevation (Chmurzynska *et al.,* 2014). Coffee contains caffeine which has structural similarities with theophylline, a vitamin B_6 antagonist (van Dam, 2008; Mitra *et al.,* 2018). Alcohol inhibits methionine synthase expression and activity (Halsted, 2013).

S-adenosyl-homocysteine, a DNA methyltransferase inhibitor is excreted by intracellular and extracellular mechanisms (Zawada *et al.,* 2014). The intracellular mechanism converts SAH to

homocysteine whereas the extracellular mechanism regulates plasma SAH (Zawada *et al.*, 2014:21). Approximately 70 % of homocysteine is excreted by kidneys therefore compromised renal function resulting in low glomerular filtration rate thus resulting in an elevation of homocysteine (Abraham & Cho, 2010; McCully, 2015).

Intriguing environmental factor influencing plasma homocysteine is that of nutrition, particularly the B-vitamins (B_6, B_{12} and folate) which are directly linked to homocysteine metabolism (Truswell, 2010; Barroso *et al.*, 2017). Homocysteine can elevate at different magnitudes, resulting in mild (15-30 µmol/L), moderate (30-100 µmol/L) or severe (≥100 µmol/L) elevation (Abraham & Cho, 2010; Liu *et al.*, 2017). Severe folate deficiency and moderate vitamin B_{12} deficiency are some of the causes of moderately elevated homocysteine (Abraham & Cho, 2010; Barroso *et al.*, 2017). Mild elevation of homocysteine may be caused by renal failure, drugs (such as antiepileptic and immunosuppressive drugs), hypothyroidism, vitamin B_{12} or folate deficiencies, *MTHFR C677T*, ageing and high protein intake (Abraham & Cho, 2010; Codoñer-Franch & Alonso-Iglesias, 2015). Serum homocysteine level of ≥100 µmol/L may be due to severe vitamin B_{12} deficiency or cystathionine beta-synthase deficiency (Abraham & Cho, 2010; Liu *et al.*, 2017).

Homocysteine metabolism is mediated key enzymes, namely, MTHFR and methionine synthase of the remethylation pathway and CBS of the transsulfuration pathway. Mutation of genes coding for these enzymes are causes of elevation of homocysteine (Veeranki & Tyagi, 2013). Two common *MTHFR gene* mutations are *C677T* and *A1298C*, which code for a heat sensitive enzyme thus reducing enzyme activity (Nagele *et al.*, 2011; Liew & Gupta, 2015). Polymorphism *MTR A2756G* coding alters the cofactor binding domain (Weiner *et al.*, 2014). Single nucleotide *CBS gene* mutations, C260A and G700A alter the catalytic site of the enzyme CBS thus impairing the binding site of the substrate or cofactor (Casique *et al.*, 2013).

The adverse effects of elevated homocysteine are yet to be distinguished according to which species is more detrimental and which has no significant effect (Jakubowski, 2013). Folate, vitamin B_6 and vitamin B_{12} are B vitamins that have a noteworthy effect on decreasing concentrations of plasma homocysteine, which is a cardiovascular risk factor (Fabian *et al.*, 2012).

2.3.3.1 Hyperhomocysteinemia as a cardiovascular risk factor

Studies associating homocysteine with CVD orbit McCully's1969 'Homocysteine Theory', in which he stated that homocysteine and its derivatives are toxic for the vascular wall (McCully, 1969; Blom & Smulders, 2011; Hainsworth *et al.,* 2016). The primary mechanism by which homocysteine causes CVD is that of endothelial injury (Cheng *et al.,* 2009; Hainsworth *et al.,* 2016). Homocysteine induced endothelium injury is caused by thiolactone, a metabolite produced during homocysteine metabolism (Newsholme & Leech, 2009; Obradovic *et al.,* 2018). An early indicator progressing CVD is endothelial dysfunction, which is an impairment of endothelium-dependent relaxation of blood vessels (Cheng *et al.,* 2009; Obradovic *et al.,* 2018). Subsequent to endothelial dysfunction is development of atherosclerotic plaque (2.2.1) (Cheng *et al.,* 2009; Hainsworth *et al.,* 2016).

Hyperhomocysteinemia results in endothelial cell injury via oxidative stress which activates coagulation, inactivates glutathione peroxidase, reduces bioavailability of nitric oxide and increases NADPH expression (Michelini *et al.,* 2012). Glutathione peroxidase is an antioxidant enzyme, inhibition of this enzyme by hyperhomocysteinemia induces endothelial damage (Antoniades *et al.,* 2009; Michelini *et al.,* 2012).

Hyperhomocysteinemia decreases nitric oxide supply by uncoupling nitric oxidase synthase thus resulting in the formation of superoxides (Antoniades *et al.,* 2009; Toda & Okamura, 2016). Reaction of the by-products with nitric oxide form peroxynitrite radicals thus reducing nitric oxide bioavailability causing endothelial injury (Antoniades *et al.,* 2009:6; Michelini *et al.,* 2012). Decreased bioavailability of nitric oxide activates matrix metalloproteinase (MMP)-2 and MMP-9 with subsequently accelerating aggregation of platelets and increase interaction between platelets and endothelium. (Michelini *et al.,* 2012).

Homocysteine-thiolactone, a reactive form of homocysteine is primarily responsible for the atherogenic property of homocysteine (Banecka-Majkutewicz *et al.,* 2012). This disruptive form promote thrombosis by increasing expression VCAM-1 and ICAM-1 (adhesion molecules), cytokines, factor V, tissue factor, inhibiting fibrinolysis, disrupting metabolism of nitric oxide and elevating reactivity of platelets (Fay, 2012).

In a physiological state, plasminogen bond to fibrin clot is activated by tissue plasminogen activator to plasmin but in hyperhomocysteinemia, fibrinolytic enzymes bind to lysine residues

(binding sites) in fibrinogen (Sauls, *et al.,* 2011: Lai & Kan, 2015). Homocysteine-thiolactone affinity to lysine causes homocysteine induced structural change of fibrinogen thus resulting in resistance to tissue plasminogen activator (tPA) initiated fibrinolysis (Sauls *et al.,* 2011; Yao *et al.,* 2016).

2.3.4 Methods of detecting serum homocysteine

Quantification of homocysteine can be analysed by various analytical methods but they all implement a reduction step that converts homocysteine metabolites to a reduced form (Manolescu *et al.,* 2010; Głowacki *et al.,* 2016). These analytical techniques can be categorized as enzymatic method, enzymatic immunoassay and chromatographic methods (Manolescu *et al.,* 2010; Ghassabian *et al.,* 2014). Amongst the available analytical techniques, high performance liquid chromatography (HPLC) method is regarded as the best when determining serum homocysteine (Barbosa *et al.,* 2014; Costa, 2014).

Enzyme-linked immunosorbent assay (ELISA)

a) Principle

The initial step of quantification of this enzyme immunoassay involves reduction of homocysteine with dithiothreitol (DTT) and enzymatic action of SAH hydrolase to convert protein-bound homocysteine to SAH in the presence of excessive adenosine (American Laboratory Products Company (ALPCO), 2011; Lee *et al.,* 2018). Subsequent to homocysteine reduction is competition for binding sites on anti-SAH antibodies between converted SAH in the sample and synthetic SAH coated on the walls of the microplate (Demeditec Diagnostic GmbH, 2012). The wash step removes unbound anti-SAH antibodies, followed by addition of a peroxidase (horse radish peroxidase) and a substrate resulting in chemiluminescence (ALPCO, 2011; Li *et al.,* 2016). Absorbance and homocysteine concentration in the sample has an inverse relationship (Immuno - Biological Laboratories Co. Ltd. (IBL), 2010; Głowacki *et al.,* 2016).

b) Advantages

- Relatively low-cost high method.
- Rapid procedure with quality because of automation (Barbosa *et al.,* 2014).
- Correlates with HPLC technique (Martens *et al.,* 2008; Espina *et al.,* 2015).

*c) **Disadvantages***

- Enzymatic technique has a relatively lower sensitivity as compared to HPLC (Martens *et al.,* 2008; Espina *et al.,* 2015).

2.3.4.2 Chromatographic assay

There are several chromatographic applications available for homocysteine assessment, these are HPLC, gas chromatography–mass spectrometry (GC-MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Stockdale & Bowron, 2013).

In high performance liquid chromatography (HPLC) molecules are separated according to their elusion capacity in the mobile phase (Mallik *et al.,* 2012). In the chromatographic technique, samples are deriviatized with derivatization agents either in the pre-column or post column passage depending on the detection system (Manolescu *et al.,* 2010; Li *et al.,* 2016).

a) ***Principle***

With HPLC assay, homocysteine is first unbound from albumin and reduced to two forms (ALPCO, 2010). Determination of serum homocysteine by the HPLC method is achieved using a reverse-phased column to separate derivatized homocysteine molecules in an isocratic flow at 30^{o}C depending on retention times (ALPCO, 2010; Diazyme, 2014). The molecules are detected on a fluorescence detector set at specific wavelength (Hortin & Burtis, 2015).

b) ***Advantages***

- Has high sensitivity specificity (Barbosa *et al.,* 2014).
- Biological thiol (homocysteine) analyte derived with ammonium 7 fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) is stable, easily separated and strongly fluoresce.

c) ***Disadvantages***

- Equipment and maintenance is very expensive.
- Time consuming.
- Labour intensive process of derivatization is required.
- Requires technical expertise (Barbosa *et al.,* 2014).

2.3.5 Normal value

According to DiaSys Diagnostic Systems GmbH (2012), the normal range of homocysteine varies with age and gender. Homocysteine normal interval in both men and women below 30 years old range from 6-14 µmol/L. Men aged 31-59 have homocysteine concentration levels of 6-16 µmol/L whereas in women of the same age the homocysteine levels are relatively lower with intervals of 5-13 µmol/L. In elderly (>60 years) men, homocysteine levels range from 6-17 µmol/L and elderly women normal concentrations are 7-14 µmol/L (DiaSys Diagnostic Systems GmbH, 2012; Yao, *et al*., 2017; Moretti & Caruso, 2019).

2.4 FOLATE

2.4.1 Characteristics

Folate is also known as pteroyglutamicacidis, a water-soluble B crystalline that is yellow in colour and has a molecular weight of 44 (Hoffbrand *et al.*, 2011; Navarro-Pérez *et al.*, 2016). Folate is a pteroic acid (Pte) derivative and it consists of three structural components (figure 3) pteridine, *p*-aminobenzoic acid (PABA) and L-glutamic acid (Rosenthal & Glew, 2009; Leamon *et al.*, 2017). Folic acid is a completely oxidized L-glutamic acid form of folate and usually used for fortification (Zempleni *et al.*, 2013). Folate and its derivatives function as coenzymes in one-carbon metabolism (Kirsch *et al.*, 2013).

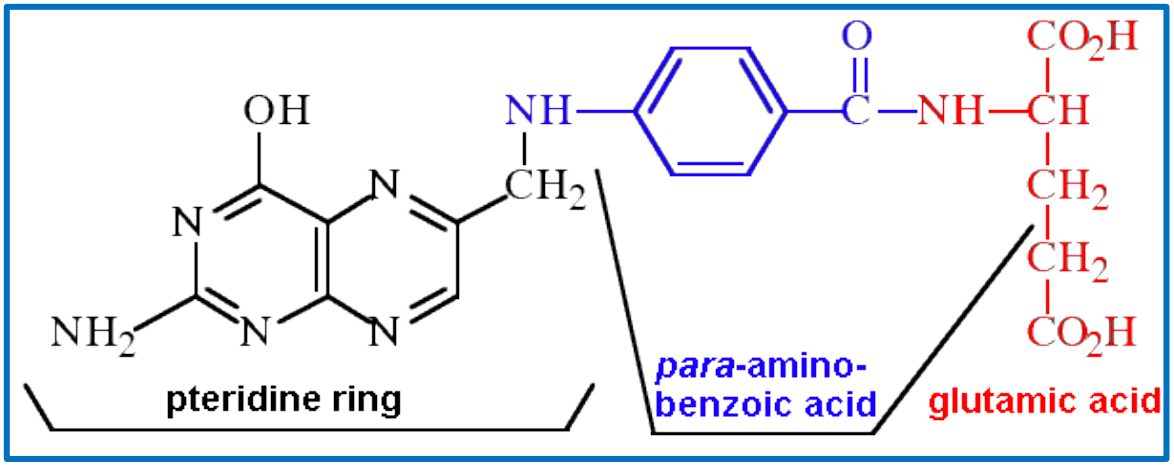

FIGURE 3: FOLIC ACID STRUCTURE

Source: Adapted from: http://www.chm.bris.ac.uk/motm/folic-acid/folich.htm

2.4.2 Biochemical metabolism

The source of dietary folate is either natural or synthetic, the former is relatively unstable and shows partial bioavailability thus foods and supplements are fortified with the synthetic form, a monoglutamate that is easily metabolized following ingestion (McNulty *et al.,* 2012). In the process of fortification, folate is used as an artificial oxidized provitamin (Smulders & Blom, 2011; Davison, 2017).

Folate is rich in food sources such as the liver, kidneys, green leafy vegetables like spinach, lima beans and orange juice (Shenkin, & Roberts, 2015). Cereals, oil seed and beans or peas (pulses) are average source of folate (Shetty, 2008; Hefni *et al.,* 2015). Dietary folate requirements are influenced by the body mass index (BMI), the bigger the BMI the higher the demand (Messika *et al.,* 2010; Pinto 2015).

Folate is metabolised at the proximal third of the small intestine during which polyglutamate is uncoupled from the pteridine and *para*-aminobenzoic acid structural complex to form a monoglutamate structure of folate (Lieberman & Marks, 2013). The initial process of metabolism of dietary folate occurs in a microvilli of the small intestine known as the brush border and it is at this site that the enzyme glutamtecarboxypeptidase II (GCPII) converts dietary pteroylpolyglutamate to pteroylmonoglutamate (Halsted, 2013). Enzyme dihydrofolatereductase converts folic acid to tetrahydrofolate and a methyl group is added to it, forming 5-MTHF (Shetty, 2008; Servy & Menezo, 2017). Folate enters the circulation as a reduced form 5-MTHF, the only biologically active form of folate (Shenkin & Roberts, 2015). The membrane of enterocytes has reduced folate carrier (RFC) that transport 5-MTHF across the brush border (Halsted, 2013).

Availability of 5-Methyl-THF for homocysteine methylation depends on MTHFR activity (Aneji *et al.,* 2012). The Polymorphism *MTHFR C677T* reduces enzyme activity involved in the conversion of folate into bioactive form (Siqueira *et al.,* 2011; Liew & Gupta, 2015). Some authors suggest that, the polymorphism *MTHFR C677*T can only affect enzyme activity of MTHFR in the presence of low folate (Sharp *et al.,* 2008; Ince *et al.,* 2016).

Serum circulatory folate pre-exist as a monoglutamate derivative, following receptor-mediated endocytosis, a polyglutamate is formed by gamma-peptide linkages with one-carbon functional group at the N5 and/or N10 position thus retaining folate within the cell (Stover & Field, 2011; Shenkin, & Roberts, 2015). The liver is the predominate site which folate is vastly utilized in

its methyltetrahydofolate form as received from the intestinal cells. (Lieberman & Marks, 2013). Non-bound folate is excreted in the kidney, filtration and reabsorption occurs in the glomerulus and proximal tubules respectively (Shenkin & Roberts, 2015).

2.4.3 Physiology

The existence of multiple forms of folate within a cell enables it to function as a coenzyme in several folate-dependent metabolisms (Table 2) (Hoffbrand, *et al.*, 2011; Liu *et al.*, 2011; Servy & Menezo, 2017). The majority circulating folate form is methyltetrahydrofolate, occupying 82-93% ratio of total plasma folate (Kirsch *et al.*, 2013). In conjunction with vitamin B_{12}, B_6, and B_2 derived coenzymes, folate functions as a coenzyme in one-carbon metabolism (Shenkin & Roberts, 2015).

Tetrahydrofolate-folate derivative, is a carbon carrier from one unit to another in at least five carbon-transfer-dependent biochemical reactions such as serine metabolism, glycine synthesis and cleavage, purine synthesis and homocysteine metabolism (Shetty, 2008; Ducker & Rabinowitz, 2017) Purines and thymidylate are synthesized on the availability of folate, stability and integrity of processes such as synthesis of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), repairing and methylation of DNA are also entirely folate-dependent (Qi *et al.*, 2010; Kirsch *et al.*, 2013).

The methyltetrahydrofolate precursor, methylenetetrahydrofolate participates in the transition of uracil to thymine during DNA Synthesis (Balderrábano-Saucedo *et al.*, 2012). Enzyme thymidylate synthase binds to cosubstrate 5,10-MTHF as it regulates deoxyuridine monophosphate (dUMP) and deoxythymidine monophosphate (dTMP) levels therefore maintaining DNA nucleotide equilibrium (Halsted, 2013). Absence of 5,10-MTHF causes an imbalance between dUMP and dTMP, these conditions favour dUMP subsequently uracil is incorporated into DNA instead of thymine (Fenech, 2012). Folate also functions as a coenzyme in homocysteine metabolism and it is the foremost micronutrient influencing metabolism of this amino acid (Shenkin & Roberts, 2015).

TABLE 2 BIOCHEMICAL REACTIONS OF FOLATE COENZYMES.

Reaction	Coenzyme form of folate involved	Importance
Formate activation	THF	Generation of 10-formyl-THF
Purine synthesis	5,10-MTHF	Formation of purines needed for DNA, RNA synthesis
Pyrimidine synthesis	5,10-MTHF	Rate limiting DNA synthesis Oxidizes THF to DHF Some breakdown of folate at the C-9-N10 bond
Amino acid interconversion	THF 5-Methyl-THF	Entry of single carbon units into active pool Demethylation of 5,10-MTHF to THF

DHF, dihydrofolate; THF, tetrahydrofolate.

Source: Hoffbrand *et al.,* 2011; Ebara, 2017

2.4.4 Deficiency

Several factors contribute to folate deficiency. It may arise from inadequate dietary intake, drug-induced, increased demand or pathological disorders. Some drugs such as anticonvulsant, folate antagonist, anti-cancer drugs and antibiotics decrease folate by either inhibiting intestinal bacterial to synthesize folate or dihyrofolatereductase activity (Shetty 2008; Hesdorffer & Longo, 2017). Other factors include increased demand without compensation in situation such as pregnancy and haemolytic anaemia; malabsorption caused by gastrointestinal tract pathological conditions such as celiac disease and sprue (Shetty 2008; Green, 201l; Golani *et al.*, 2016).

2.4.4.1 Folate deficiency as a cardiovascular risk factor

The essential role of folate in the remethylation pathway of homocysteine metabolism signifies the importance of low folate as risk factor for atherosclerosis (Imamura *et al.*, 2010; Ducker & Rabinowitz, 2017).

2.4.5 Methods of detection

According to Shenkin & Roberts (2015), folate concentration can be determined in serum, red blood cells and whole blood. The latter is indicative of folate levels stored in tissue whereas serum folate is indicative of recent folate intake (Shenkin & Roberts, 2015). Historically, serum folate was determined by microbiological assay (Shane, 2011; Zhang *et al.*, 2017). This method has been replaced by chemiluminescent immunoassay (CLIA) assay (Nakazato *et al.*, 2012). Other modern procedures such as the radio-binding method (RBM) and HPLC were also introduced but HPLC is currently restricted for research use only (Nakazato, *et al.*, 2012).

2.4.5.1 Microbiological assay

In this method a bacterium known as *Lactobacillus rhamnosus* is used to quantify folate (Nakazato, *et al.*, 2012). The microbiological method was discovered around 1930s by Möller, three decades later the method was improved by removing the need for the sterilization step (Snell & Peterson, 1939; Pfeiffer *et al.*, 2011; Shane, 2011; Zhang *et al.*, 2017). This improvement was achieved by using a chloramphenicol-resistant strain of *Lactobacillus rhamnosus* (Pfeiffer *et al.*, 2011; Zhang *et al.*, 2017). Improvements were not limited to a better strain but also standard growth curves could be used because of the ability to preserve inoculum below sub-zero temperatures, microtiter plate technology was introduced (Pfeiffer *et al.*, 2011; Zhang *et al.*, 2017).

a) *Principle*

Two tubes are used in folate microbiological assay, serum test tube which contains all growth requirements of with an exception of folate and the second tube has all growth ingredients and a known concentration of folate (Sood, 2009; Zhang *et al.*, 2017). The growth of *L. rhamnosus* is directly proportional to the amount of total folate present in serum or whole blood samples, the total folate level can be assessed by measuring the turbidity of the inoculated medium (National Health and Nutrition Examination Survey (NHANES), 2012).

b) *Advantages*

- Microbiological assay has the advantage of a good response to a vast range of folate monoglutamane forms.

c) ***Disadvantages***

- ❖ Poor precision and low throughput
- ❖ Labour intensive
- ❖ Time consuming

(Shane, 2011; Nakazato, *et al.*, 2012; Pfeiffer, *et al.*, 2012).

2.4.5.2 ELISA

There are various designs of folate ELISA. The difference lies on the coating of microtiter plates, some are coated with *L. rhamnosus* and other with folate specific monoclonal antibodies. (Immundiagnostik 2012; Diagnostic Automation/Cortez Diagnostics, Inc., 2014).

a) ***Principle***

In *Lactobacillus rhamnosus* designed ELISA, serum added to a microtiter plate wells coated with *L. rhamnosus* trigger folic acid-dependent growth of the bacteria. Following 48 hours of incubation at 37^{o}C, growth turbidity is measured at 540-550 nm. Turbidity has a direct relationship with the folic acid concentration of the sample. (Immundiagnostik, 2012).

Folate specific monoclonal antibody ELISA uses the competitive inhibition enzyme immunoassay method. Biotin labelled folic acid competes with sample folic acid (unlabelled folic acid) for binding sites of the monoclonal antibody immobilized on the wells. After incubation free conjugate is washed off and horseradish peroxidase is added then incubated. Subsequent to incubation is the addition of a substrate that produces colour. The developed colour is indirectly proportional to concentration of folic acid present in the sample. (Cloud-Clone Corp., 2013).

b) ***Advantages***

- ❖ The assay responds to various forms of folate in the sample (WHO, 2015).

c) ***Disadvantages***

- ❖ Radioactivity and false elevated results are drawbacks of this method that justified the phasing out radio-binding assay utilization (Nakazato *et al.*, 2012).

2.4.5.3 Chemiluminescent immunoassay (CLIA)

Automatic folate determination emerged in the 1990s, a test method that applied the chemiluminescent immunoassay principle (Nakazato *et al.,* 2012). This assay involves an immunological reaction with a light emitting molecule used as an indicator to determine folate concentration (Kricka *et al.,* 2015).

a) ***Principle***

Commercially available folate chemiluminescent immunoassay involves a two-step procedure to determine folate concentration (Abbott laboratories 2010; Liu *et al.,* 2018). The first step is sample pre-treatment to free folate from serum binding protein (Abbott laboratories, 2010; Dudley, 2016). The subsequent step involves free folate from the sample to bind with micro-particles coated with folate binding proteins (FBP) and washing (Liu *et al.,* 2018). A chemiluminescent conjugate is added to bind with unbound micro-particles coated with folate binding proteins, followed by a solution triggering light emission from the reaction (Abbott laboratories 2010; Liu *et al.,* 2018). Folate is quantified by an inverse relationship of between the light emitted by chemiluminescent conjugate-FBP complex and sample folate (Abbott laboratories, 2010; Dudley, 2016).

b) ***Advantages***

Unlike the microbiological method, specificity exist in chemiluminescent immunoassay and as a result Nakazato *et al.,* (2012) suggested that this method may fail to completely detect particular folates.

c) ***Disadvantages***

- Lack specificity (Nakazato *et al.,* 2012).

2.4.5.4 HPLC

New methods are emerging to simultaneously quantify folate derivatives (Shane, 2011; Espina *et al.,* 2015). Developing methods such as affinity HPLC measure folate distribution to the extent of deterring unmetabolized folic acid (Kalmbach *et al.,* 2011; Espina *et al.,* 2015). Due to the ability to specifically and accurately isolate various forms of folate, liquid

chromatography tandem mass spectrometry (LC-MS/MS) is a popular platform for rapidly determining folate concentrations (Liu *et al.,* 2011; Espina *et al.,* 2015).

a) ***Principle***

Purified (using high-affinity folate binding protein) folate held in a reverse-phase column is eluded, separated and measured by an ultraviolet detector (Kalmbach *et al.,* 2011; Espina *et al.,* 2015).

b) ***Advantages***

- Folate metabolites are easily distinguishable with this method.

c) ***Disadvantages***

- Ultraviolet detector is relatively less selective (Espina *et al.,* 2015).

2.4.6 Normal values

Normal serum folate concentrations are between 6.0-28 nmol/L, deficient are below 3.2nmol/L (Burtis, 2008; Pfeiffer *et al*., 2015).

2.5 VITAMIN B_{12}

2.5.1 Characteristics

The term "Vitamin B_{12}" describes compounds based on corrin frame and has biological activity of cynacobalamin (Watanabe *et al.,* 2014). By definition vitamin B_{12} which is also known as cobalamin is a complex organic compound containing cobalt, metallic atoms surrounded by a corrin ring (Chatthanawaree 2011; Giedykb *et al.,* 2015). As compared to other vitamins it is the largest vitamin with a more complex structure ($C_{63}H_{88}CoN_{14}O_{14}P$) (Figure 4).

Molecular structure of vitamin B_{12} comprises of two halves; a planar group which is a corrin and 90^{o} to it is a nucleotide consisting of a base, 5,6-dimethylbenzimidazole and a phosphorylated sugar (Hoffbrand *et al.,* 2011; Kandemir *et al.,* 2016). The normal molecular

weight of vitamin B_{12} is 1355 Da and it gradually decreases when subjected to light (Shenkin & Roberts, 2015) The corrin system consist of a cobalt atom surrounded by four pyrrole rings (Rosenthal & Glew 2009; Shenkin & Roberts 2015).

Several chemical structures of cobalamin exist in nature of side groups bound to cobalt, naturally two forms exist in humans, the 5'-deoxyadenosyl (ado) found in the mitochondria and methylcobalaminform found in plasma and cell cytoplasm sugar (Hoffbrand, 2011; Clarke, 2014). Either methyl group or dexoyadenosine binds to position 6 of the cobalamin coordination hence the existence of methylcobalamin and deoxyadenosincobalamin (Reed, 2009; Kamath & Pemminati, 2017).

Methylcobalamin is a coenzyme utilized in the remethylation pathway of homocysteine degradation whereas 5'-deoxyadenosyl acts in an isomerazation reaction where L-methylmalonyl-coenzyme A is converted to succinyl-coenzyme A (Singh and Sachan, 2011; Cohen, 2014). Other forms are: hydroxocobalamin, aquocobalamin and cyanocobalamin. According to Shenkin & Roberts, (2015) cyanocobalamin is the most stable amongst these compounds. For fortification, the cyanocobalamin form is used and it is rapidly converted to hydroxocobalamin then finally to active 5'-deoxyadenosyl form (Rosenthal & Glew, 2009; Kadjo *et al.*, 2015).

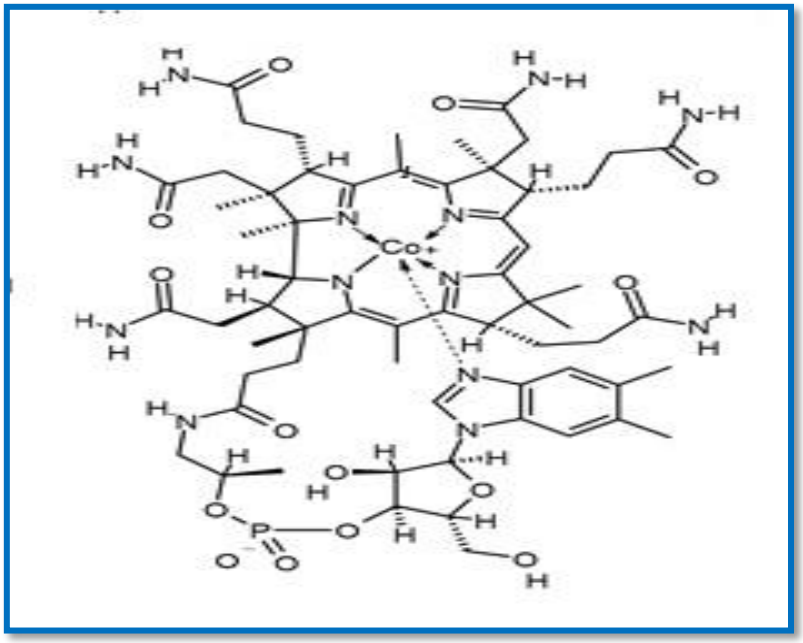

FIGURE 4: COBALAMIN STRUCTURE

Source: Adapted From: Watanabe *et al.*, 2014

2.5.2. Biochemical metabolism

Vitamin B_{12} is exclusively synthesized by microorganisms and the main dietary source of vitamin B_{12} for humans is of animal origin (Shenkin & Roberts, 2015). Cobalamin can be also obtained from dairy products, chicken, fish and shellfish but cannot be obtained from any other foods of non-animal origin (Singh & Sachan, 2011; Watanabe & Bito, 2018). There are two mechanisms in which vitamin B_{12} is absorbed; 1) passive mechanism which is rapid but inefficient and 2) active mechanism which is regarded as a normal, efficient mechanism in humans (Hoffbrand, 2011; Watanabe & Bito, 2018).

Metabolism of cobalamin is mediated by hydrochloric acid (HCl), haptocorrins (R proteins) and intrinsic factor (IF) found in the digestive system (Rosenthal & Glew, 2009; Lukaszuk *et al.,* 2015). Digestion of vitamin B_{12} as shown in figure 5, begins in the stomach through hydrolytic actions of pepsin and HCl thus liberating it from food proteins (Kozyraki & Cases, 2013). Prior to leaving the stomach, free cobalamin binds to R proteins that are secreted by salivary glands. The R protein bound cobalamin enters the duodenum together with IF, a crucial glycoprotein of 45 kDA (Chatthanawaree, 2011; Lukaszuk *et al.*, 2015). The intrinsic factor has a strong affinity for cobalamin and it is released from parietal cells of the stomach due stimulation by histamine, gastrin and pentagastrin as well as food presence (Chatthanawaree, 2011; Manolis *et al.,* 2013).

Pancreatic enzymes digest R proteins in an alkaline milieu of the duodenum, this enzymatic action generates vitamin B_{12}-IF complexes (Burtis *et al.*, 2008; Kozyraki & Cases, 2013). The vitamin is shielded from stomach acid attack by these complexes (Newsholme & Leech 2009; Watanabe & Bito, 2018). Vitamin B_{12} degradation by intestinal bacteria and proteolytic enzyme is prevented by IF-vitamin B_{12} complexes therefore enhancing absorption (Chatthanawaree, 2011; Lukaszuk *et al.,* 2015). Absorption occurs at the distal ileum, at this site the vitamin B_{12}-IF complex binds to specific receptors called cubulins and are found on the mucosal epithelial cells (Figure 5). (Rosenthal & Glew, 2009; Grobler, 2015).

Vitamin B_{12}-IF complex enters the ileal cell by endocytosis, it is here that cobalamin is disassociates from the IF and binds with transcobalamin II (TcII) before being exported into the portal circulation (Bain *et al.*, 2012). Not more than 30% of cobalamin released from enterocytes binds to transcobalamin II (TC II), forming complexes known as

holotranscobalamin (2.6) and excessive cobalamin is transported to the liver by transcobalamin I (Chatthanawaree, 2011; Watkins *et al.*, 2016).

Since the portal vein passes through the liver, vitamin B_{12} stored in parenchymal liver cells is released into the plasma according to physiological demand (Shenkin & Roberts, 2015). Circulatory vitamin B_{12} exceeding the binding capacity of transcobalamins I and II is excreted via urination (O'Leary & Samman, 2010; Watkins *et al.*, 2016). Bacteria synthesized vitamin B_{12} and unabsorbed vitamin B_{12} from biliary, gastrointestinal cells as well as secretions are excreted via faeces (O'Leary & Samman, 2010; Watanabe & Bito, 2018).

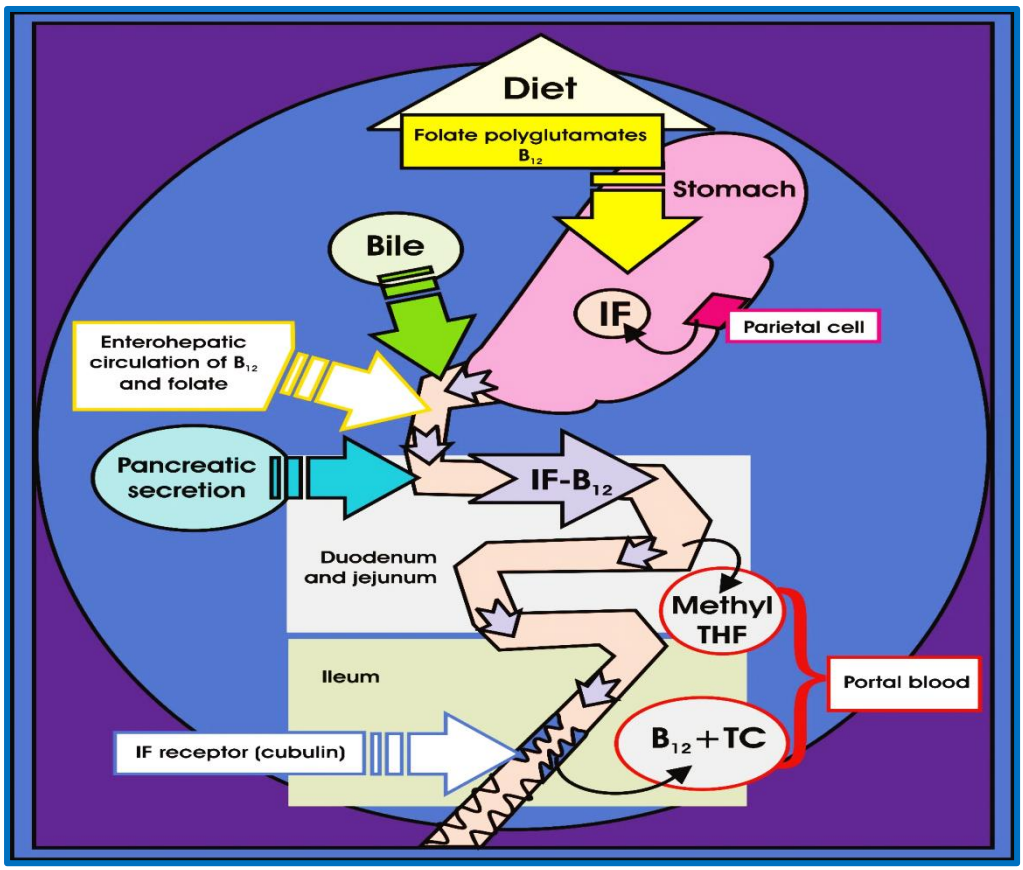

FIGURE 5: COBALAMIN METABOLISM

Source: Adapted from: Grobler, 2015

2.5.3 Function

The physiological role of vitamin B_{12} is as a coenzyme in two enzymatic reactions that involve methionine synthase and methymalonyl CoA mutase enzymes (Hughes *et al.*, 2013). As a co-

factor for methionine synthase, vitamin B_{12} is essential for the transfer of methyl group in metabolism of homocysteine to methionine (O'Leary & Samman, 2010; Shenkin & Roberts, 2015). Vitamin B_{12} facilitated methionine production is pivotal in the physiological synthesis of purines and pyrimidines required for DNA synthesis (Figure 6) (Singh & Sachan, 2011; Kibirige & Mwebaze, 2013; Grobler, 2015).

In an enzymatic pathway that involves methymalonyl CoA mutase, vitamin B_{12} assist the enzyme to convert L-methymalonyl coenzyme to succinyl coenzyme (Riedel *et al.*, 2011:1; Lieberman & Marks, 2013). Furthermore vitamin B_{12} has a protective role against oxidative stress induced by hydrogen peroxide and also behaves as an antioxidant in homocysteine-independent pathways (Moreira *et al.*, 2011; Ekim *et al.*, 2014).

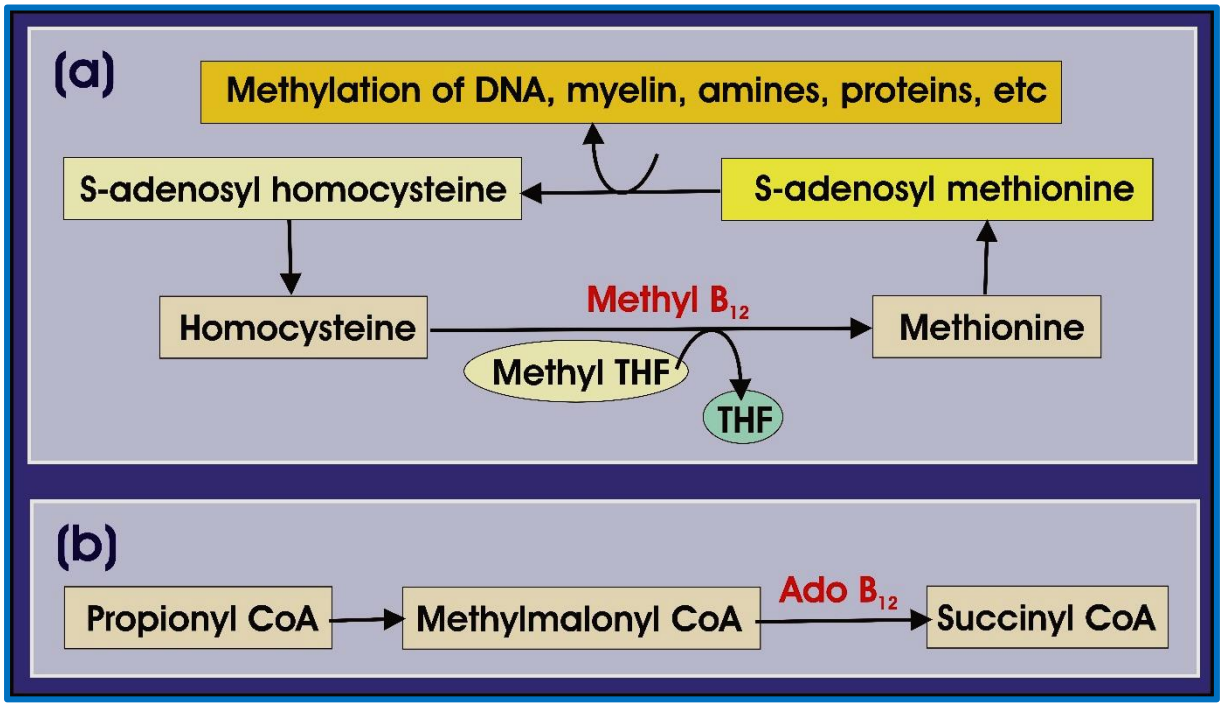

FIGURE 6: COBALAMIN METABOLISM

Source: Adapted from: Grobler, 2015

2.5.4 Deficiency

Vitamin B_{12} deficiency in elderly people is usually caused by malabsorption and inadequate dietary intake (Leishear *et al.,* 2013). The signs and symptoms of vitamin B_{12} deficiency can be subtle particularly in deficiency associated with dietary insufficiency (Newsholme & Leech, 2009: Mirkazemi *et al.,* 2012). Dietary deficiency can manifest for at least 10 years before signs surface because it is compensated by vitamin $B_{12,}$ produced by microorganisms in the colon Newsholme & Leech 2009; Csapó *et al.,* 2017).

Malabsorption associated vitamin B_{12} deficiency among elderly population may be caused by conditions such as pernicious anaemia, atrophic gastritis and drug-nutrient interactions (Hughes *et al.,* 2013). Pernicious anaemia is an autoimmune disease that arises when there is deficiency of an intrinsic factor which leads to malabsorption of vitamin B_{12} (Singh & Sachan, 2011; Wong *et al.,* 2015).

Another cause of vitamin B_{12} deficiency is vegetarianism and it is most common in developed countries (Mirkazemi *et al.,* 2012). Development of deficiency among vegetarians is relatively slower than that caused by malabsorption (Gibson, 2005: Rizzo *et al.,* 2016). Although this deficiency progresses over a long period of time drastic conditions such as pernicious anaemia can cause death (Stein *et al.,* 2016). Accumulation of MMA due to cobalamin deficiency destabilizes metabolism of the mitochondria thus leading to formation of reactive oxygen species (Fenech, 2012).

2.5.4.1 Vitamin B_{12} deficiency as a cardiovascular risk factor

The interrelationship between vitamin B_{12} and cardiovascular risk is via homocysteine levels. In vitamin B_{12} deficiency there is an elevation homocysteine and therefore increasing the risk of a cardiovascular event (Elmadfa & Singer 2009; Pawlak 2015).

2.5.5 Methods of detection

2.5.5.1 Microbiological assay

a) Principle

This method measures concentration of serum vitamin B_{12} using the organism *Lactobacillus leishmani* or *Euglena gracilis* (Karmi *et al.,* 2011). *Lactobacillus leishmani* grows well in the presence of vitamins (HiMedia Laboratories, 2015). Following 48 hours incubation, vitamin

B12 assay medium appear cream to yellow with soft lump in the presences of vitamin B_{12}. Absorbance of the prepared suspension is measured at 620 nm and the concentration is determined against the standard curve (HiMedia Laboratories, 2015).

2.5.5.2 Radioimmunoassay (RIA)

a) Principle

Quantitation of vitamin B_{12} is based on radioisotopic competitive binding of intrinsic factor (IF), a cobalamin-binding protein (Carmel, 2011; Tsiminis *et al.,* 2017). Radio-labeled IF factor competes with endogenous IF (patient's serum) for cobalamin binding site (Karmi *et al.,* 2011; Kozyraki & Cases, 2013). IF-cobalamin complex is separated from free IF and the free IF remains in the supernatant. Radioactivity of the free IF is measured following optimal incubation (Karmi *et al.,* 2011; Kozyraki & Cases, 2013).

2.5.5.3 Chemiluminescent immunoassay (CLIA)

a) Principle

Similar to folate analysis, vitamin B_{12} analysis by chemiluminescent methods are widely used on automated platforms (Kotte-Marchant & Davis, 2012). Analysis of vitamin B_{12} by this method employs competitive steps between serum vitamin B_{12} and labelled cobalamin vitamin B_{12} (Bain *et al.,* 2012). Serum cobalamin and labelled cobalamin compete for limited binding sites on chemiluminescent tagged intrinsic factor (Tosoh Cooperation, 2010; Kamruzzaman *et al.,* 2013). Antibody fluorescein isothiocynate restrained on magnetic beads forms a complex with fluorescent-labelled intrinsic factor and incubated with fluorogenic substrate. Enzymatic action triggers generation of chemiluminescent signal (Bain, *et al.,* 2012). Enzyme labelled vitamin B_{12} concentration forming a complex with beads is inversely proportional to the concentration of vitamin B_{12} contained a test sample (Tosoh Cooperation, 2010; Kamruzzaman *et al.,* 2013).

2.5.6 Normal values

Normal serum concentration of vitamin B_{12} ranges depend on the method applied, hitherto gold standard is yet to be established vitamin B_{12} concentration (Carmel, 2011; Akini, 2014).

Although there is lack of a gold standard, vitamin B_{12} levels can be categorized according to deficiency depending on the concentration. Levels >300 pmol/L are interpreted as cobalamin deficiency unlikely, ranges between 200-300 pmol/L indicate possibility of deficiency and levels < 200 pmol/L are indicative of vitamin B_{12} deficiency (Chatthanawaree, 2011; Palacios *et al.,* 2013; National Institute for Health and Care Excellence, 2015).

2.6 HOLOTRANSCOBALAMIN

2.6.1 Characteristics

Holotranscobalamin is a complex of transcobalamin and cobalamin (Riedel *et al.,* 2011; Sobczyńska-Malefora *et al.,* 2016). Transcobalamin is a single stranded peptide of 43 kilodalton (kDA) which is highly synthesized of enterocytes (McCaddon, 2013; Kozyraki & Cases, 2013). Unlike other cobalamin transporting proteins (intrinsic factor and haptocorrin) which are glycosylated, transcobalamin is a non-glycosylated protein (Obeid *et al.,* 2011; Brokner *et al.,* 2017).

This transporting protein has a high affinity for cobalamin with a binding ratio of 1:1 (McCaddon, 2013). Although transcobalamin has a high affinity for cobalamin, it has a relatively low affinity when compared to haptocorrin (Green, 2010; Bloch *et al.,* 2017). This phenomenon is due to the relatively greater cobalamin specificity of transcobalamin in contrast with haptocorrin specificity (Quadros & Sequeira, 2013). Two transcobalamin forms are of existence, 1) cobalamin-free transcobalamin, an apo form and 2) cobalamin-bound form known as holotranscobalamin (McCaddon 2013). The latter form has a half-life 1-2 hours (Quadros, 2010; Golding, 2016).

2.6.2 Biochemical metabolism

Cellular metabolism of holotranscobalamin is facilitated by receptors on cell surfaces which is known as transcobalamin receptors (Watkins & Roesnblatt, 2013). These receptors are specific and select binding of transporting proteins based on glycation status of transporting proteins, therefore favouring transcobalamin because of its non-glycosylation status (Obeid *et al.,* 2011; Velkova *et al.,* 2017)

Transcobalamin receptors function as an entry portal for holotranscobalamin to be assimilated into the cell (Green, 2010; Bloch *et al.*, 2017). Present in the cell are lysosomes which dissociates holotranscobalamin subsequent to internalization into the cell (Figure 7) (Green, 2010; Watkins & Roesnblatt, 2013). The release of cobalamin from the holotranscobalamin complex is mediated by lysosomal hydrolases (Gherasim *et al.*, 2013).

Transcobalamin is degraded by proteolysis and free cobalamin is transferred into the cytoplasm whereas transcobalamin receptors are go back to the cell surface to be re-used (Watkins & Roesnblatt, 2013; Zhao *et al.*, 2014). Cobalamin is then converted into methyl cobalamin and adenosyl cobalamin used respectively in homocysteine degradation and the synthesis of succinyl-coenzyme A (Zhao *et al.*, 2014).

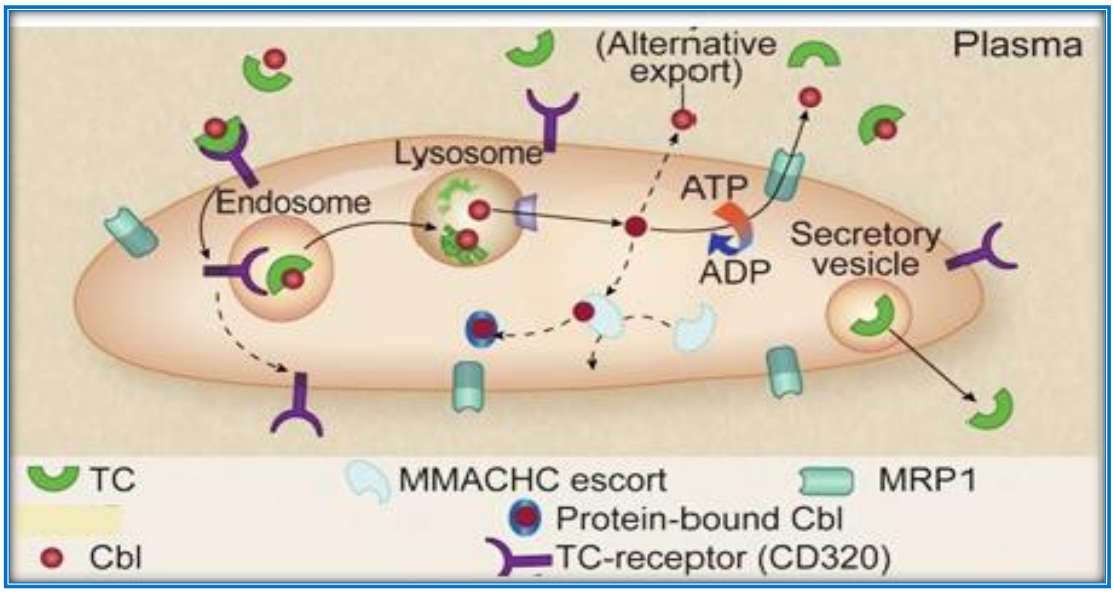

FIGURE 7: COBALAMIN ENDOCYTOSIS

Source: Adapted from: Green, 2010

2.6.3 Deficiency

Transcobalamin deficiency is rare and a hereditary condition caused by genetic mutations (Trakadis *et al.*, 2013). The common transcobalamin deficiency causing mutation is single nucleotide polymorphism of *TCN2* gene (Castro *et al.*, 2010; Kurnat-Thoma *et al.*, 2015).

2.6.3.1 Transcobalamin deficiency as a cardiovascular risk factor

Transcobalamin delivers vitamin B_{12} into the cells which is required for homocysteine metabolism (2.3.2). Deficiency of this protein leads to insufficient bioactive cobalamin thus leading to increase of homocysteine and therefore a higher risk of cardiovascular disease.

2.6.4 Methods of detection

Holotranscobalamin assay is proposed to be an early detector of vitamin B_{12} decline (Valente *et al.,* 2011; Nexo & Hoffmann-Lucke, 2011; Hughes & McNulty, 2018). Holotranscobalamin concentration was previously determined by radioimmunoassay until 2008 when ELISA and enzyme immunoassay became alternatives (Carmel, 2011; Afyoncu *et al.,* 2016).

2.6.4.1 Radioimmunoassay

Radioimmunoassay involves competition of a radio-labelled antigen with an unlabelled antigen for binding site on a specific antibody (Burtis & Bruns, 2015). In this assay, holotranscobalamin monoclonal antibodies are coated on magnetic microspheres (Bain, *et al.,* 2012). A denaturing agent uncouples holotrancobalamin bonds allowing competition between free vitamin B_{12} and radioactive isotopes for binding site of intrinsic factor microspheres (Bain, *et al.,* 2012). Holotrasncobalamin is determined by measuring trapped active vitamin B_{12} in an isotope dilution assay (Nexo & Hoffmann-Luckes, 2011; Höller *et al.,* 2018).

2.6.4.2 ELISA

Similar to radioimmunoassay, specific monoclonal antibodies are used for determining holotranscobalamin by ELISA. Commercial ELISA kits and platforms such as Abbott ASYM are available for holotranscobalamin assay (Valente *et al.,* 2011; Nexo & Hoffmann-Lucke, 2011; Höller *et al.,* 2018).

2.6.5 Normal Values

There is inconsistency in the normal reference range of serum holotranscobalamin. Bain *et al.,* (2012) consider a range of 19-134 pmol/L as normal whereas Al Aisari, *et al.,* (2010:9) regards the lower limit as 9 pmol/L and the upper limit to be 123 pmol/L (Golding 2016). For commercially available ELISA from IBL the reference range is 21-123 pmol/L (2012).

2.7 MTHFR ENZYME

2.7.1 Characteristics

Methylenetetrahydrofolate reductase (MTHFR) is a non-covalently bound protein with a prosthetic group Flavin (Igari *et al.,* 2011; Alam, 2016). It is a flavoprotein enzyme which therefore requires coenzyme flavin adenine dinucleotide (FAD) (Araki *et al.,* 2013). Mammalian MTHFR-FAD is a homodimer formed by two subunits with each comprising of catalytic and regulatory sites (Igari *et al.*, 2011; Alam, 2016).

Each protein structural unit consist of 77 kDa, of which 40 kDa is N-terminal (catalytic region) and 37 kDa is C-terminal (regulatory region) (Forges *et al.,* 2010; Burda *et al.,* 2015). The regulatory region has an S-Adenosylmethionine-binding site which functions as a regulator of methionine in cells (Shahzad *et al.,* 2013). Optimum enzymatic function of MTHFR is achieved when favourable physiological conditions such as temperature and pH are 24~37 ^{o}C and 4.3 respectively (Igari *et al.*, 2011; Al-Batayneh *et al.,* 2018). Enzyme MTHFR is encoded by *MTHFR* gene (2.7.3) (Gao *et al.*, 2014).

2.7.2 Function

MTHFR is an enzyme responsible for the bioavailability of active form of folate (Arzaghi *et al.,* 2011; Dattilo *et al.,* 2016). It is one of the key enzymes required in homocysteine metabolism. The other two enzymes are methionine synthase and cystathionine beta-synthase (CBS) (Miyaki, 2010; Dattilo *et al.*, 2016).

As previously mentioned (2.4.2), MTHFR converts 5,10-methylelenetetrahydrofolate to 5-methyltetrahydrofolate, a product required for methionine synthesis which is further utilized for DNA methylation (Rao *et al.,* 2010; Al-Batayneh *et al.,* 2018). The metabolite 5-methyltetrahydrofolate catalysed by MTHFR also plays an important role in homocysteine metabolism as a co-substrate in remethylation of homocysteine (Kalkan *et al.,* 2013).

2.7.3 *MTHFR* gene

2.7.3.1 Characteristics

Cytogenetic location of human *MTHFR* gene place it on the short arm of chromosome 1 at positioned 36.3 (1p36.3) (Figure 8), with the exact location being from base pair 11,769,246 to 11,788,568 of the chromosome (Rao *et al.*, 2010; Aneji *et al.*, 2012). Goyette *et al.*, (1994) pioneered the isolation, mapping and polymorphism identification of human *MTHFR*, they described the gene to consist of 11 exons.

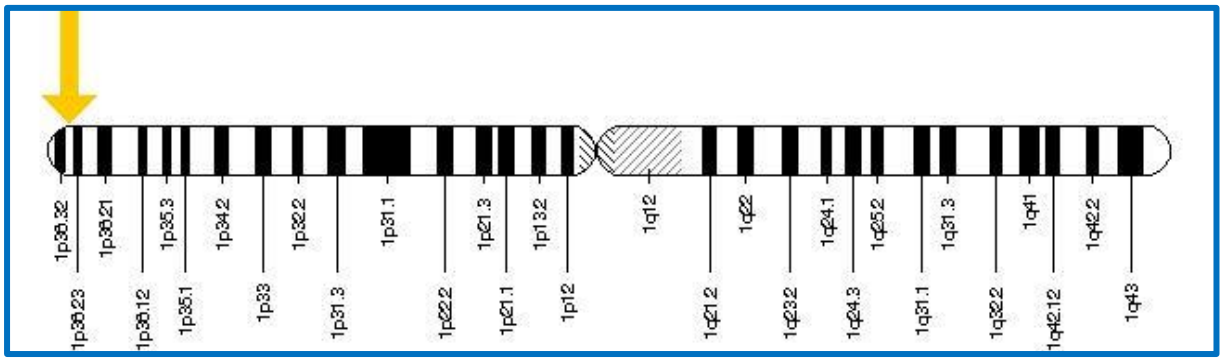

FIGURE 8: MTHFR GENE LOCATION

Source: Adapted from: http://ghr.nlm.nih.gov/gene/MTHFR

Gao *et al.*, (2014) characterized the human *MTHFR* gene to consist of 11 exons and 10 introns with a length of 19.3 kb. However according to the National Center for Biotechnology Information (NCBI) (2016) the exon count of human *MTHFR* gene (gene ID: 4524) is 13.

2.7.3.2 Transcription

The *MTHFR* coding sequence of human species has similarities to that of a mouse, the amino acid of both species have a 90 % match when aligned (Lévesque *et al.*, 2014). The *MTHFR* gene consist of two promoters known as upstream and downstream (Pickell *et al.*, 2011; Nunes *et al.*, 2017).

According to Akram *et al.*, (2012), the *MTHFR* gene has two promoters consisting of 77 kDa subunits and isoform that encompasses 70 kDa. There is no TATA box within the promoter regions of *MTHFR* but there are numerous -Cytosine-phosphate-Guanine- (CpG) islands present (Baroudi *et al.*, 2014).

2.7.3.3 *MTHFR* Function

During protein synthesis, specific genetic information for enzyme MTHFR is transmitted by *MTHFR* gene (Akram *et al.,* 2012). Other 655 amino acids are also coded by the *MTHFR* gene (Shahzad *et al.,* 2013).

2.7.3.4 *MTHFR* polymorphism

The common type genetic polymorphism of *MTHFR* is that of single nucleotide polymorphism (SNP). Single nucleotide polymorphisms are defined as the difference in single nucleotides between two DNA molecules that also functions as genetic markers (Klug *et al.,* 2012). Among a wide range of 52 *MTHFR* SNPs identified, *MTHFR* C677T and *MTHFR* A198C are the two well-studied functional polymorphisms with relevant information (Miyaki, 2010; Jin *et al.,* 2017). Noteworthy of the two variants is *MTHFR C677T* variant (rs1801133) because it is regarded as the most important genetic determinant of homocysteine (Nagele *et al.,* 2011; Jin *et al.,* 2017).

In *MTHFR* C677T polymorphism, cytosine (C) is replaced by thymine (T) at position 677 resulting in the gene to code for valine as opposed to alanine (Miyaki, 2010; Safarinejad, *et al.,* 2011; Balderra´bano-Saucedo *et al.,* 2012). Cytosine to thymine mutation at nucleotide 677 occurs at exon 4 (Izmirli, 2012). Substitution between alanine and valine occur on codon 222 hence *MTHFR* C677T polymorphism is sometimes referred to as Ala222Val polymorphism (Safarinejad *et al.,* 2011; Ince *et al.,* 2016).

There are two mutant alleles of *MTHFR C677T* namely, heterozygous *C677T*, homozygous *T667T*, whereas homozygous *C677C* is a wild type allele (Ibrahim & El Dessokiy, 2009; Al-Batayneh *et al.,* 2018). The magnitude which polymorphism *MTHFR C677T* reduces enzyme activity varies according to genotype (Sawuła *et al.,* 2009; Ince, 2016). Enzyme activity in *MTHFR C677T* and *MTHFR T677T* polymorphisms is reduced by approximately 30% and 60 % respectively (Sawuła *et al.,* 2009; Servy & Menezo, 2017).

2.7.3.4.1 *MTHFR C677T* as a cardiovascular risk factor

Alanine replacement by valine takes place at the N-terminal catalytic domain of MTHFR enzyme (Akram *et al.,* 2012). This substitution result in synthesis of a thermolabile (sensitivity to thermal denaturation) *MTHFR* variant which has a decreased enzyme activity (Dulin & Guisasola, 2012). The other consequences of Ala222Val polymorphism is loss of flavin cofactor and subunit dissociation of the enzyme (Igari *et al.,* 2011; McEwen, 2016). These structural alterations of MTHFR enzyme lead to diseases such cardiovascular disease and neural tube defects (Shahzad *et al.,* 2013).

Decreased enzymatic activity reduces the availability of the bioactive form of folate (5-methyltetrahyfrofolate) ultimately leading to increased levels of homocysteine (Blom & Smulders, 2011; Dulin & Guisasola, 2012; Burdennyy *et al.,* 2017). A direct relationship between *MTHFR C677T* polymorphism and hyperhomocysteinemia is commonly reported (Yafei *et al.,* 2012; Burdennyy *et al.,* 2017).

2.7.3.4.2 Prevalence of *MTHFR C677T* polymorphism

Global prevalence of polymorphism *MTHFR* differs according to geographic location and ethnicity (Somarajan *et al.,* 2011; Liew & Gupta, 2015). The *T677T* genotype is well reported because of a clear association with reduced enzyme activity of MTHFR (Seremak-*et al.,* 2013). In the European region the prevalence of homozygous *T677T* variant ranges between 24.1% and 64.3% whereas the range in North American region is 2% to 48% and among the South American region is 7% (Zidan *et al.,* 2013). Asian and African region the prevalence of *MTHFR T677T* ranges from 2% to 63.1% and 0% to 35.5% respectively (Zidan *et al.,* 2013).

Prevalence by ethnicity of *MTHFR T677T* genotype was found to range from 1.2% among non-Hispanic blacks and 11.6% in non-Hispanic whites, whereas in Mexican Americans was 19.4% (Yang *et al.,* 2012). Among Black Africans the prevalence of this genotype was 0.8 % (Nienaber-Rousseau *et al.,* 2013).

2.7.3.4.3 Analysis of *MTHFR C677T* polymorphism

The Polymerase chain reaction (PCR) method is used to identify *MTHFR C677T* polymorphism. In this method, two techniques can be used, namely; 1) restriction length

fragment polymerase - polymerase chain reaction (RLFP-PCR) and 2) real time polymerase chain reaction (RT-PCR).

2.7.3.4.3.1 RLFP-PCR

a) *Principle*

Restriction fragment length polymerase PCR analysis identifies SNPs using restriction enzymes that recognize the site of mutation (Rasmussen, 2012). Subsequent to PCR amplification (Figure 9 A), the presence of *MTHFR C677T* mutation is determined by The *Hin*fl restriction enzyme (Singh *et al.,* 2015; Tiwari *et al.,* 2015).

An electrophoresis gel is used to visualize the digested DNA. Wild type C genotype on codon 677 which is undigested by the enzyme is characterised by a single band of 198 base pair (bp) whereas heterozygous (*CT*) and homozygous (*TT)* mutant variants are characterized by three bands at 198,175 and 23 bp or only 175 and 23 bp (homozygous *TT*) (Figure 9 B), (Deeparani *et al.,* 2009; Tiwari *et al.,* 2015).

b) *Advantages*

This technique is easy to design and relatively cheap as it does not require advanced instruments (Rasmussen, 2012).

c) *Disadvantages*

Restriction fragment length polymerase PCR analysis is time consuming due to additional steps involved such as electrophoresis required for separation (Rasmussen, 2012).

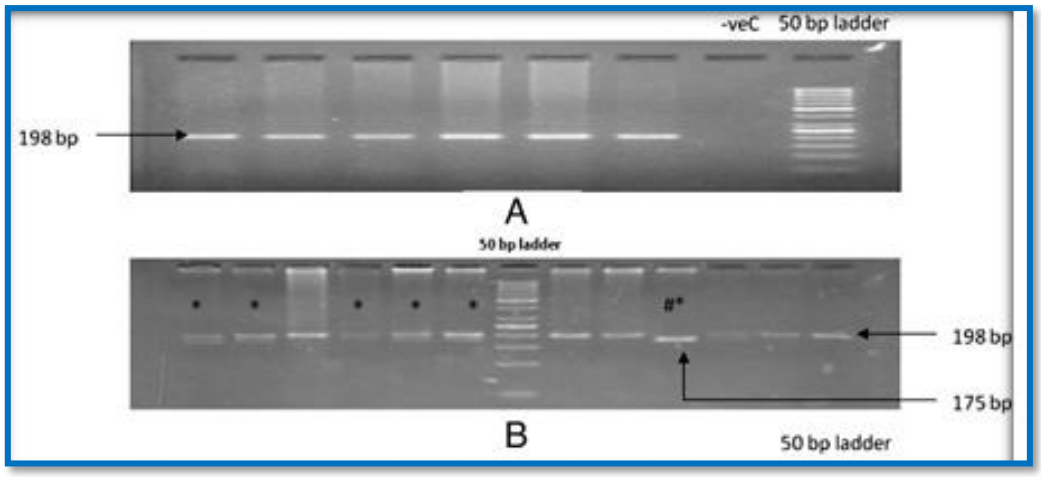

FIGURE 9: *MTHFR* GENE LOCATION AGAROSE GEL ELECTROPHORESIS FOR PCR AMPLIFICATION OF *MTHFR* GENE FRAGMENT. B: representation of restriction digestion products of MTHFR gene showing the presence of either wild type or heterozygous (characterized by presence of 198 + 175 + 23 bp and marked by *) or homozygous condition (characterized by the presence of 175 + 23 bp marked by #*)

Source: Adapted from: Tiwari *et al.*, 2015.

2.7.3.4.3.2 Real time PCR

a) Principle

Analysis of *MTHFR C677T* polymorphism using this techniques involves amplification using fluorescence detection (Hernádez-Rodríguez & Ramirez, 2012). Real time PCR employs two detection methods dye-based and probe-based (Sigma, 2008; Liu *et al.*, 2014). With the dye-based method, viz, the dye intercalates with double-stranded DNA and produces fluorescence (Eurogenetec, 2009; Liu *et al.*, 2014).

The probe-based method involves probes designed with a fluorescent reporter dye at the 5’ end and a quencher at the 3’ end kept in proximity via fluorescence resonance energy transfer (FRET) (Popa, Bojincá *et al.*, 2009; Eurogenetec, 2009; Baris *et al.*, 2013). In the process of PCR amplification, a fluorogenic 5’ nuclease DNA polymerase cleaves the probe at the 5’end releasing the flourophore thus increasing fluorescence (Eurogenetec, 2009; Baris *et al.*, 2013).

In probe-based, two specific dye-labelled probes for genotypes *TT* and *CC* are used (Liferiver 2012).

i. *Advantages of the dye-based method*

Results are rapidly obtained using this technique and there is minimum risk of contamination due to relatively less steps (Hernádez-Rodríguez & Ramirez, 2012). It is relatively cheaper as compared to the probe-based method and can be used for melt analysis (Sigma, 2008; Baris *et al.,* 2013).

ii. *Disadvantages of the dye-based method*

The dye based method lacks specificity as it can detect both specific and non-specific DNA products (Popa *et al.,* 2009). This method cannot be adapted to multiplex and is not suitable for qualitative real time PCR (Eurogenetec, 2009; López-Rojas *et al.*, 2017).

iii. *Advantages of the probe-based method*

Results are rapidly obtained using this technique and there is minimum risk of contamination due to relatively less steps (Hernádez-Rodríguez & Ramirez 2012). It is relatively cheaper as compared to probe-based method and can be used for melt analysis (Sigma, 2008; Baris *et al.,* 2013).

iv. *Disadvantages of the probe-based method*

The dye dye based method lacks specificity as it can detect both specific and non-specific DNA products (Popa *et al.,* 2009). This method cannot be adapted to multiplex and is not suitable for qualitative real time PCR (Eurogenetec, 2009; López-Rojas *et al.*, 2017).

2.8 CONCLUSION

Increased serum homocysteine is well established as an independent cardiovascular risk factor. The literature indicates that hyperhomocysteinemia due to *MTHFR T677T* is concomitant with low dietary folate intake (Frost *et al.,* 2012). This genotype is regarded as a cardiovascular risk factor (Ibrahim & El Dessokiy, 2009; Liew & Gupta, 2015). Prevalence of homozygous *T/T* genotype varies with ethnicity. Asians have a relatively low prevalence whereas recent data on Africans remain inadequate to estimate an accurate prevalence. Mutation of the *MTHFR* gene has been shown to be consistent determinant of homocysteine levels therefore there is a need to consider *MTHFR C677T* polymorphism when evaluating homocysteine levels.

Fundamental causes of both cobalamin and folate deficiencies are inadequate consumption, malabsorption and increased demand. The suggestion is that analysis of un-metabolized folate will be valuable to polymorphism studies affecting folate status (Shane, 2011; Pfeiffer *et al.,* 2014).

Nutritional status in an elderly population is compromised by chronic diseases and malabsorption (Fabian *et al.,* 2012). Age-related CVD is due to vascular changes, the elasticity and strength of vascular wall declines with increasing age (Cross, 2012). Vascular endothelium and smooth muscle cells functions decline as age increases (Herrera *et al.,* 2010; Gimbrone & García-Cardeña, 2016).

The dynamics of cardiovascular disease are influenced by lifestyle particularly smoking, physical inactivity and unhealthy dietary intake but age is the most influential risk factor (WHO, 2011; Perk *et al.,* 2012). Elderly people are at high risk of CVD due to declined physical activity and habitual intake of unbalanced diet such as, consuming a lot of solid fats, added sugars and sodium (Cottell *et al.,* 2011; Feng *et al.,* 2017).

Some of the identified challenges in the combat of cardiovascular risk are; lack of quality information and accurate predictors (Gersh *et al.,* 2010; Graversen *et al.,* 2016). This study addresses these epidemiological challenges by progressively adding advanced scientific knowledge on the risk and management of cardiovascular disease in Africa. New knowledge about the interaction between vitamin B_{12}, homocysteine and cardiovascular risk amongst South African elderly emerge in this study as it is the first to be conducted in this group.

The study also provides a comprehensive understanding of homocysteine status in the elderly as it extensively explores homocysteine biomarkers. That is evaluating *MTHFR C677T* polymorphism in the coding gene an enzyme responsible for folate metabolism, the study intends to also scrutinize vitamin B_{12} status by evaluating holotranscobalamin, an essential form of vitamin B_{12}.

CHAPTER 3

DESIGN AND METHODOLOGY

3.1 INTRODUCTION

Previous studies conducted on the selected sample population of the elderly people of Sharpeville indicated that they are at high risk for CVD (Oldewage-Theron *et al.*, 2008; Grobler 2015). The studies reported the elderly in the Sharpeville community to be challenged by malnutrition, undesirable health conditions as well as food insecurity. Majority of the study participants had low formal education (Marumo-Ngwenya, 2014).

The same population group was also reported to have elevated homocysteine, a cardiovascular risk factor (Grobler 2015). It is within the same study group that the knowledge on how genetics contribute to epidemiological transition of cardiovascular disease will be explored.

The methods followed to measure the objectives outlined in chapter 1 will be reported in this chapter. Procedural aspects such as principles, reliability, validity and reproducibility are reported in depth. Quality control and assurance are also explained in this chapter. Furthermore, this chapter reports on research ethics adhered.

3.2 ETHICAL CONSIDERATION

This study was conducted within the boundaries of the Constitutional Law of The Republic of South Africa by upholding Section 12(2)(c) of the Constitution of South Act, No 108 of 1996, which states: ‘ 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'.

As previously mentioned, the study was conducted with the approval of the Vaal University of Technology ethical committee (20140827-1ms) (Annexure 1). Guidelines and principles prescribed by the Belmont Report (1979) and Helsinki Declaration (2008) were followed during the execution of this study. To ensure that the study was carried out in alignment to the prescribed principles, the Medical Research Council (MRC) of South Africa’s adopted ethics (from Helsinki Declaration) guidelines were also observed. The adapted principles promoted by MRC of South Africa are autonomy, beneficence, non-maleficence and justice.

To effectively incorporate the above mentioned principles into the study, a code of conduct guided by the Health Professions' Council of South Africa (HPCSA) guidelines for good practice (2008) was also observed (HPCSA, 2008).

In compliance this law and principles of the Belmont report and the previously mentioned Act of the South African constitution, the following principles of biomedical ethics defined by the MRC were practiced;

- *Autonomy*- respect for the participants and their human dignity. All study participants were given an informed consent to sign at free will (Annexure 2).
- *Beneficence*- the benefit to the study participants. Scientific knowledge gained in this study provides an enhanced insight of the role that genetics has on the previously reported cardiovascular risk within this study group (Oldewage-Theron *et al.,* 2008; Grobler, 2015)
- *Non-maleficence*- the study should be harm-free for participants. A qualified phlebotomist collected blood samples to ensure that the procedure is carried out without adverse aftermath on the participants. Safety protocols were followed such as the use of medical waste sharps containers were used to create an injury-free environment.
- *Justice*- risk and benefits of the study should be equally distributed amongst communities.

3.3 SAMPLING STRATEGIES

To assure that there is autonomy, privacy, confidentiality and anonymity, introductory visits were made to the day-care centre, explaining the objectives of the project to the management of the day-care centre and the attendees in order to obtain approval from both parties. The following actions were taken in order to assure that this study was conducted with high ethical practice:

- Participation in this study was voluntary and autonomous. Before informed consent was given, care was taken to ensure that the subject understands the aim, objectives and methodology of the study. To achieve this, communication was done through translation into Sotho in order to ensure participants understanding. Care was also taken to avoid interviewer's bias.

- ❖ Participants were treated sensitively, helpfully and with respect to ensuring their dignity.
- ❖ All actions at all times were non-maleficent.
- ❖ Confidentiality was assured at all times by using subject numbers, the subjects were double blinded to the laboratory personnel as well as for the data analyst.

3.4 STUDY DESIGN

This was an observational, experimental study design (Gallin & Ognibene 2012) in 102 purposively (power calculation n=84) selected samples, meeting the inclusion criteria from all the elders attending the day-care centre in Sharpeville. All subjects were equivalent in age (≥60 years), race (black), resident in Sharpeville, Vaal region, unemployed / pensioner (socio-demographic).

Verbal presentation was carried out in Sotho to inform participants and management of the day care centre about the study, subsequently requested to complete and sign a consent form voluntarily. In case of illiteracy, Sotho speaking fieldworkers explained and translated the informed consent requirement in the presence and observation of the day care management.

After ensuring that participants understood clearly in the presence of the management of the centre, a consent form was signed. Those who were illiterate signed the consent by marking an X.

3.4.1 Sample size

An adequate sample size required for this study on the prevalence of *MTHFR C677T* polymorphism was determined by using the calculations as indicated below. The confidence level was 95%, confidence interval set at 8 and the population at the centre was 192 as recommended for a cross sectional study (Charan & Biswas, 2013).

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

Where:

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

p = 5% expected proportion in the population = 0.05

d = 0.05 precision/absolute error

Therefore

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

$$n = 84$$

The population available as respondents was 192. Based on the confidence interval of 8 at 95% confidence level, it was calculated that the sample size required was 84 respondents. However on the day of sampling, there was more respondents who voluntarily participated in the study. This increased the number of respondents from 84 to 99 and consequently reducing the confidence interval from 8 to 6.87, which had a positive effect on the study as the margin of error reduced.

3.4.2 Inclusion criteria:

Participants aged 60 years or older, attending the day-care centre and most importantly gave a consent qualified for study.

3.4.3 Exclusion criteria:

Subjects who failed to provide substantial information to complete the consent process due to conditions like dementia did not form part of this study.

3.5 DATA COLLECTION

3.5.1 Fieldwork

The study was nested within a larger study evaluating vitamin B_6 status in the sample population. The larger study was conducted by Dr C.J Grobler in collaboration with the Centre of Sustainable Livelihoods (CSL), the centre conducts a multi-micronutrient program at the Sharpeville day-care centre of the aged.

Data was collected by a research team which consisted of the principal investigator, an HPCSA registered medical technologist, qualified dietician, qualified phlebotomist, HPCSA registered student medical technologists and recruited, trained fieldworkers. The roles of principal investigator and dietician in the field was to manage a systematic data collection process. Blood collection was conducted by a qualified phlebotomist with the assistance of student medical technologists under the supervision of the principal investigator who ensured that ethical protocols were safeguarded.

Fasted blood was collected in six different vacutainer collection tubes, two 7 ml serum separating tube (SST), 3ml glucose tube, two 5ml ethylenediaminetetraacetic acid (EDTA) and 5 ml sodium citrate. However for this study, only SST and EDTA tubes were used for biochemical and DNA analysis respectively.

During the data collection, subjects were requested to rotate between the following stations in order to collect data:

- **Station 1**-On arrival a subject number was allocated to a participant. A file containing field worker's control list was labelled with the participant's subject number. All questionnaires and blood collection tubes (in a Ziploc bag) were labelled with the same number and placed in the file. The participants were then requested to move to the next station.
- **Station 2** -Anthropometric measurements (weight, height and waist circumference), completion of socio-demographic, health, food frequency and 24-hour recall required for the larger study were done by trained fieldworkers.

- **Station 3 –** Before collecting blood, the phlebotomist took blood pressure with TENSOVAL® MOBIL wrist monitor and also observed clinical signs. Blood pressure readings were noted on a fieldworkers control list. Blood was collected from the fasted subject by using a vacutainer system from the vena cephalica in two 7 ml SST*, 3ml glucose tube, two 5ml EDTA*[1] and 5 ml sodium citrate. Tubes blood were collected from each subject. Blood was placed in a cooler box (8ºC), and protected against direct sunlight.

- Subjects were requested to return to the **reception desk (station 1)** where files were handed in and control list verified to assure that all the data collection processes were completed, after which breakfast was served.

3.5.2 Standardization and validation

Reliability is defined as getting the same outcome by an instrument in repeated measurements (Bernard, 2013). Validity is the accurate and precise assessment of the minimum and maximum measurement of a specified parameter of an instrument (Pietersen & Maree, 2010; Konieczka, & Namiesnik, 2016).

Reliability of methods used was determined by mean, standard deviation and coefficient variance (CV) and validity by a standard deviation (SD). Instrument's accuracy and precision of intended measurements (validity) was assessed in various contexts indicated in Table 3, depending on the application of the instrument, data acquired and the accuracy of response variable. In order to ensure that this study produced reliable, reproducible and valid results the following procedures were put into place.

TABLE 3 TYPES OF MEASUREMENT VALIDITY

Type	Description
Face validity	A validity in which logic is applied to decide whether an instrument appears to be measuring what is intended to.

[1] * For the purpose of this study, serum and EDTA blood was used.

Content validity	The degree to which a measurement instrument has an appropriate content of a conceptual definition.
Criterion-related validity	The extent which an indicator correlates with another measure produced by other instrument.
1. Concurrent validity	*The criterion variable exist in the present.*
2. Predictive validity	*The degree which an instrument accurately forecast the outcome.*
Constructive validity	The extent to which an instrument measurement conforms with theoretical predictions.

Source: (Portney, 2009; Kumar, 2011; Bernard, 2013; Leedy & Ormrod, 2014; Punch, 2014).

3.5.2.1 Standardization and validation of blood collection

Blood collection

Labelled collection tubes were firstly checked for number correspondence with the subject number on the participants' file. A qualified phlebotomist drew fasted blood from the study participants. The phlebotomist ensured that participants were comfortably seated before drawing blood from the vena cephalica using a vacutainer system and tourniquets. To ensure reliable results, blood collected in tubes containing anticoagulants (EDTA, Glucose and Sodium citrate) were gently mixed in figure 8 motion to evenly distribute the anticoagulant in the blood to prevent haemolysis.

Transportation

To ensure reliable results, care was taken during transportation of samples. Samples were placed in a cooler box with ice-packs to prevent temperature increase and also to protect them from direct sunlight. They were transported to the laboratory within two hours of collection.

Separation

To ensure stability of analytes SST blood was separated within four hours after collection by centrifuging at 3000 rpm for 10 minutes at room temperature upon arrival at the laboratory. Separated serum and plasma were transferred into 2 ml aliquot test tubes that were labelled with numbers.

To eliminate errors such as mismatch, separated samples on labelled aliquot tubes were numerically arranged. Before transferring of serum into aliquot tubes, face validity verification was used to double check if the number on the sample corresponded with the number on the aliquot tubes.

DNA was extracted from EDTA tubes on arrival at the laboratory. For DNA extraction, EDTA blood was numerically arranged before being extraction. The extracted DNA was transferred into 2 ml Eppendorf tubes. A similar process as discussed above was used for double-checking the numbers.

Storage

Aliquot tubes with serum were stored at -80^{o}C and DNA extract at -20^{o}C until they were analysed. Temperature was monitored daily and recorded on temperature charts. Information collected about the participants was locked in a cabinet situated in a restricted room.

Blood analysis

Standard laboratory procedures were followed during the analysis to comply with South African National Accreditation System (SANAS) requirements. The research was conducted under the supervision of Dr C.J Grobler (Health Profession Council of South Africa registered Biomedical Technologist). Reliability and validity procedures of analysing various parameters were conducted and will be reported as part of description of the test.

3.6 MEASURING INSTRUMENTS

This study used multiple measuring instruments in analyses of parameters. Homocysteine, folate, vitamin B_{12} and *MTHFR C677T* mutation were determined with standardized automated

instruments. Homocysteine concentration was determined using Konelab™ 20i instrument. Folate and vitamin B_{12} were both measured using Tosoh AIA®-600 II automated immunoassay analyser.

The variable holotranscobalamin was determined using a semi-automated system with internationally standardized ELISA kits. Single nucleotide polymorphism of *MTHFR C677T* was analysed with a Roche PCR LightCycler 96® Software version 1.6.

3.6.1 Automated Konelab™ *instrument*

Automated Konelab™ 20i instrument is a random access automated clinical chemistry system that was used to quantify homocysteine by photometric principle. This system uses two measuring principles, namely colorimetric and turbidimetric. Automated Konelab™ 20i measures the analytes at a temperature of 37°C and a spectral range of 340-800 nm. Modes of measurement include end-point and kinetic measurement. The latter occurs between 30 seconds to 1 hour with a maximum of 12 points. The light source is a halogen lamp with linear absorbance (A) range of 0-25 and resolution of 0.0001 A. Reproducibility of the light source has a standard deviation of ≤0.005 A at 2 A.

3.6.2 Tosoh AIA®-600 II

This is an automated enzyme immunoassay system that was used to measure folate and vitamin B_{12} using an AIA-PACK reagent. Tosoh AIA®-600 II assay principles are; immunoenzymatic (sandwich), competitive binding and a two immunoassays (discussed in 2.5.6.3).

Immunoreaction and fluorescent measurements occur in the AIA-PACK reagent cup containing magnetic beads, it is in this cup that the specimen and diluent are added and incubated at 37°C for a specific duration depending on the analyte. For fluorescent detection, substrate 4-methylbelliferyl phosphate (4-MUP) is added to change intensity of fluorescence and concentration measured by calculations derived from a predetermined calibration curve.

3.6.3 Enzyme Linked Immunosorbent Assays (ELISA)

Holotranscobalamin was determined by internationally standardized kits procured from Immuno - Biological Laboratories Co. Ltd. (IBL). The kits consisted of micro-titre wells coated with specific monoclonal antibodies for holotranscobalamin. Upon addition of samples into the micro-titre wells, holotranscobalamin was present in the sample is captured by surface antibodies of the wells.

An alkaline phosphatase labelled murine monoclonal antibody to human transcobalamin (conjugate) binds to the captured serum holotranscobalamin and unbounded material is washed off with phosphate buffer. A para-NitroPhenyl Phosphate, a substrate reacts with bound holotranscobalamin to generate a detectable coloured end-product. The colour generated is measured at absorbance of 405 nm and it is directly proportional to the concentration of holotranscobalamin present serum.

3.6.4 Roche PCR LightCycler 96®

Roche LightCycler® 96 is a real-time PCR system for rapid cycling. The Peltier-based thermal cycler has a fast ramp rate, that is 4.4 ^{o}C for heating and cooling is 2.2 ^{o}C. The Roche LightCycler® 96 system uses optical detection that is flexible and has multiplex as well as multicolour capabilities. The detection format is 470/514 FAM. The melting curve analysis and endpoint genotyping up to 96 samples applications of the systems allowed for single nucleotide polymorphism of *MTHFR C677T* analysis (Roche Diagnostics GmbH, 2016).

3.7 BLOOD PARAMETERS

3.7.1 Homocysteine

In this study homocysteine levels were determined with Homocysteine fluid stable (FS) reagents from DiaSys Diagnostic System. The reagents consisted of S-adenosylhomocysteine (SAH), nicotinamide adenine dinucleotide (NADH), tris (2-carboxyethyl) phosphine (TCEP), 2-oxolutarate, glutamate dehydrogenase (GLDH), SAH hydrolase, adenosine deaminase and homocysteine methyltransferase. An enzymatic cycling method was used to determine serum homocysteine concentrations.

3.7.1.1 Principle

Enzymatic cycling method involves reduction of oxidized total homocysteine resulting in protein-free homocysteine. Unbound homocysteine reacts with co-substrate SAM, a reaction catalyzed by homocysteine-methyltransferase to generate metabolites methionine and S-adenosylhomocysteine (SAH). The latter is hydrolyzed by enzyme SAH-hydrolase to form two products, homocysteine and adenosine.

The product homocysteine re-enters the conversion process (reaction catalyzed by homocysteine-methyltransferase) and the cycling reaction produces a considerable amount of detection signals. Conversely, adenosine is further hydrolysed by adenosine deaminase to form inosine and ammonia. The by-product ammonia reacts with NADH and 2-oxolutarate, glutamate dehydrogenase to generate glutamate, NAD^+ and water. This reaction is catalyzed by GLDH. Decrease in NADH due to reaction with ammonia is measured at 340 nm. The decrease of NADH is directly proportional to homocysteine concentration in the serum (DiaSys Diagnostic System).

3.7.1.2 Validation

The extent to which homocysteine can be quantified was determined by using TruCal Homocysteine level 0, 1 and 2 calibrators from the DiaSys Diagnostic System. The concentrations of level 0, 1 and 2 calibrators were 0, 6.00 and 29.5 µmol/L, respectively. The linear calibration curve of these calibrators was validated with TruLab Homocysteine level 1 and 2 controls. Control level 1 concentration ranged between 10.6 – 15.8 µmol/L with a target value of 13.2 µmol/L and level 2 concentration ranges were 26.4- 35.8 µmol/L and the assay value was 31.1 µmol/L.

3.7.1.3 Data analysis

Homocysteine results from the Konelab™ 20i system were captured on excel before being exported to STATA 12 software. The software was used for statistical analysis of descriptive and inferential statistics. Homocysteine concentrations were categorized into; normal level (≤15 µmol/L), mild elevation (16-30 µmol/L), moderate elevation (31-100 µmol/L) and severe elevation (>100 µmol/L).

Furthermore inferential statistics was also used to determine the relationship between; 1) homocysteine and folate levels; 2) homocysteine and vitamin B_{12} concentrations and homocysteine levels and *MTHFR C677T* genotypes.

3.7.1.4 Normal ranges

In elderly (>60 years) men, homocysteine levels range from 6-17 µmol/L and elderly women normal concentrations are 7-14 µmol/L.

3.7.2 Folate

This parameter was analysed with AIA-PACK FOLATE from Tosoh Biosciences. The folate reagents consisted of test cups containing lyophilized magnetic beads. Present on the surface of the magnetic beads was antibody fluorescein isothiocyanate (anti-FITC) mouse monoclonal and fluorescein labelled bovine folate binding protein. Also present on the surface of the beads is alkaline phosphatase bound folate that was preserved with 0.1 % sodium azide.

3.7.2.1 Principle

Folate was determined using the competitive enzyme immunoassay principle. In this assay, samples are pre-treated with reagents containing sodium hydroxide and dithiothreitol. Pre-treatment of samples dissociates folate from serum binding proteins. Free serum folate competes with alkaline phosphatase bound folate for limited binding sites on a fluorescein labelled bovine folate binding protein.

Antibody fluorescein isothiocyanate binds to the formed complex (folate and fluorescein labelled bovine folate binding protein). Subsequent to binding of anti-FITC is the wash phase that removes unbound labelled alkaline phosphatase bound folate. Incubation with 4-MUP, a fluorogenic substrate changes intensity of fluorescence. The amount of alkaline phosphatase bound folate that binds to anti-FITC is inversely proportional to serum folate concentration of a sample. The amount of serum folate was automatically determined using calculations derived from a constructed calibration curve (Tosoh Cooperation, 2010).

3.7.2.2 Validation

Automated AIA®-600 II instrument's minimum and maximum were determined by calibrating for the folate analyte in which a standard curve was generated with commercial folate calibrators (Tosoh Cooperation, 2010). Each of the six calibrators were run in duplicates in the following concentrations, viz, 0 ng/mL, 1.46 ng/mL, 2.74 ng/mL, 5.38, ng/mL, 11.6 ng/mL and 23.0 ng/mL. A calibration curve displayed an inverse relationship between the rate and concentration, the rate decreases with increasing concentration.

The reliability of serum folate was evaluated using known commercial control from Tosoh Bioscience and compared expected ranges from the control data sheet supplied. The expected concentrations were; 1.25 - 2.61 ng/ml with a target value of 1.93 ng/ml for level 1; level 2 range was 3.42-6.36 ng/ml with a target value of 4.89 ng/ml and level 3 control range was 8.10 – 15.05 ng/ml with a target value of 11.57 ng/ml.

3.7.2.3 Data analysis

Microsoft Office Excel was used to capture the data about the concentrations of serum folate and the file was imported to a STATA 12 statistical software. The STATA 12 software was used for descriptive statistical analysis and correlations. Both measures of tendencies and dispersion statistics was analysed. Associations between folate and *MTHFR* C677T polymorphism, and folate and homocysteine levels were evaluated using the statistical software. The mean concentration was determined and compared to normal concentration ranges.

3.7.2.4 Normal ranges

Physiological serum folate concentrations range between 6.0-28 nmol/L (Burtis, 2008; Pfeiffer *et al*., 2015).

3.7.3 Vitamin B_{12}

The AIA-PACK B12 used for serum vitamin B_{12} determination consisted of test cups containing twelve lyophilized magnetic beads. The beads were coated with mouse monoclonal

and fluorescein labelled porcine intrinsic factor. The beads were also coated with 50 µl of enzyme linked vitamin B_{12} preserved with sodium azide.

3.7.3.1 Principle

Similar to the folate test principle, vitamin B_{12} is determined by competitive enzyme immunoassay. Samples are pre-treated with reagents containing potassium cyanide, sodium hydroxide and dithiothreitol to detach vitamin B_{12} bound to serum proteins. During pretreatment vitamin B_{12} is also converted into a stable, measurable form, viz, cyanocobalamin.

Cyanocobalamin competes with alkaline phosphatase bound vitamin B_{12} for limited binding sites on fluorescein labelled porcine intrinsic factor. The generated complex binds to anti-FITC and subsequently washed to remove excess free enzyme linked vitamin B_{12}. After washing follows incubation with 4-MUP, substrate that has the ability to fluoresce. Enzyme-labeled vitamin B_{12} binding to magnetic beads is inversely proportional to concentration of converted serum vitamin B_{12}. The concentration of serum vitamin B_{12} was automatically determined using calculations derived from a constructed calibration curve (Tosoh Cooperation, 2010).

3.7.3.2 Validation

The minimum and maximum detection of the Tosoh instrument was determined with preparation of a calibration curve using commercial Vitamin B_{12} calibrators (Tosoh). The six calibrators were run in duplicates in the following concentrations, viz, 0 pg/mL, 97.9 pg/mL, 243 pg/mL, 499 pg/mL, 991 pg/mL and 2190 pg/mL. Calibration curve was accepted on the criteria that; there is an inverse relationship between the rate and concentration, the rate decreases with increasing concentration. Secondly the replicate values were ≤10% range.

The reliability of serum vitamin B_{12} was evaluated using commercial multi-analyte control (MAC) samples obtained from Tosoh Bioscience. Three known control levels for vitamin B_{12} were run in triplicate following calibration and the results were compared to those of the expected results from the control data sheet supplied. The predefined level 1 concentrations range was 166.3 -249.4 pmol/l with a mean value of 207.9 pmol/l; level 2 value range was 536.5 – 804.5 pmol/l with a mean value of 670.5 pmol/l and level 3 range was 770.3 -1155.5 pmol/l with a target concentration of 962.9 pmol/l.

3.7.3.3 Data analysis

Serum vitamin B_{12} data from the Tosoh AIA®-600 II was captured on Microsoft Office Excel and exported to a STATA 12 statistical software. With the software, measures of central tendencies and measures of dispersion (descriptive statistics) were analysed. Additionally inferential statistics were also analysed using the STATA 12 software. In this type of analysis, an associations between Serum vitamin B_{12} and holotranscobalamin concentrations were evaluated.

3.7.3.4 Normal ranges

The reference level of vitamin B_{12} is >200 pg/ml.

3.7.4 Holotranscobalamin

Serum holotranscobalamin was quantified using ELISA kits from IBL International. In the kit were microtitre strips coated with anti-hotranscobalamin murine monoclonal antibodies. Another component was alkaline phosphatase-labelled murine monoclonal antibody to human transcobalamin (conjugate), stabilized in Tris buffer with protein stabilizer and preserved in <0.1 % sodium azide. As part of the kit were also a Substrate (para-Nitro Phenyl phosphate in buffer solution) and a stop solution containing 1M sodium hydroxide (IBL International, 2013).

3.7.4.1 Principle

Determination of serum holotranscobalamin concentrations with IBL International ELISA kit is a two-phase incubation binding process. In the first phase of incubation, serum added to antibody-coated microtitre wells binds with monoclonal antibodies having high affinity to holotranscobalamin. The second phase of incubation involves binding of the conjugate with antibody-serum holotranscobalamin complex.

Following the second phase of incubation is removal of unbound components by washing. A substrate targeting bound holotranscobalamin is added followed by a stop solution which halts the reaction. The stop solution results in a coloured end-product which is measured for absorbance at a wavelength of 405 nm. The colour generated is directly proportional to

holtranscobalamin concentration in the serum sample. Serum concentrations were interpolated from a calibration curve generated using calibrations standards (IBL International, 2013).

3.7.4.2 Validation

This method was validated using standard calibrators provided within the kit. Six calibrators with varying concentrations of 0 pmol/L, 8 pmol/L, 16 pmol/L, 32 pmol/L, 74 pmol/L and 148 pmol/L were used. These calibrators were used in duplicates to generate a calibration curve used for the determination serum holotranscobalamin. Reliability of a calibration curve displaying a direct relationship between absorbance (405 nm) of the coloured end-product and concentration was determined with low and high controls. According to the kit insert an acceptable range of low control was 18 pmol/L to 32 pmol/L with a target value of 25 pmol/L. The high control had a lower limit of 42 pmol/L, an upper limit of 78 pmol/L and target value of 60 pmol/L. Control results obtained were compared with the previously mentioned reference values.

3.7.4.3 Data analysis

Raw data of holotranscobalamin concentration was captured in Microsoft Office Excel. The file was exported to a STATA 12 statistical program used for descriptive statistical analysis and correlations. The software was used to determine the strength of association between holotranscobalamin and vitamin B_{12} using p-values and confidence intervals.

3.7.4.4 Normal value

Normal serum holotranscobalamin has a lower limit of 21pmol/L and an upper limit of 23 pmol/L (Chatthanawaree, 2011; Nexo & Hoffmann-Lucke, 2011; IBL International, 2013).

3.7.5 *MTHFR C677T*

Analysis of MTHFR C677T involved two stages, viz, DNA extraction and amplification. Extraction of DNA was done using a Quick-DNA™ universal kit from Zymo Research Corporation. The kit consisted of a BioFluid and Cell buffer used to lyse EDTA blood. Another

component of the kit was proteinase K that digested unwanted proteins. The DNA was extracted with genomic binding buffer that was included in the kit and was stored at -20^{o}C (Annexure 4) (Zymo Research Corporation). The concentration of the extracted DNA was measured with a nanodrop.

A *LightMix® Kit MTHFR C677T* was used for amplification. The kit consisted of premixed and lypholized primers and probes. It also had <0.01pg unlabelled oligonucleotides and a <0.01pg SimpleProbe® 519 labelled probe.

3.7.5.1 *MTHFR C677T* detection principle

The *LightMix® Kit MTHFR C677T* detects the single nucleotide polymorphism of the gene *MTHFR C677T* (rs1801133) in the human genome. A 2µl of extracted and purified DNA containing 5 – 100 ng/µl of genomic DNA is required for the master mix. Primers bind to 288 base pairs fragment of the *MTHFR* gene that is amplified. The SimpleProbe® 519 labelled probe bridges at the region that consist of the mutation site.

Amplification protocol was set according to the programme settings in Annexure 5. The post amplification process consists of the melting curve analysis. In this analysis there is a gradual increase in temperature (Tm) up to a point at which the probe is cleaved and causes a decrease in fluorescence. The probe complements the wild type sequence whereas there is a mismatch in the presence of a mutation thus resulting in a relatively lower melting temperature.

3.7.5.2 Validation

The Roche LightCycler® 96 instrument was calibrated using the controls supplied with the *LightMix® Kit MTHFR C677T*. The *LightMix® Kit MTHFR C677T* consisted of three positive control DNA; 1) positive heterogeneous control (HT); 2) genotyping standard wild type (WT) and 3) genotyping standard mutant (MT). PCR-grade water was used as a no template control (NTC) which was a negative control.

An acceptance criteria used for quality control was that NTC produced negative results which were, no assay-specific peaks. If the HT positive control showed two melting peaks at 58-61 and 65-68 ^{o}C, WT genotyping standard depicted a single melting peak at 65-68 ^{o}C and MT had a single peak at a lower temperature of 58-61 ^{o}C.

3.7.5.3 Data analysis

The Roche LightCycler® 96 instrument “High Resolution Melting" software was used for genotyping. The data was categorized as heterozygous *CT*, homozygous *CC* and homozygous *TT*. Data was captured on Microsoft Excel before being transferred to STATA 12 software. The latter software was used for frequency and correlation statistical analysis. Data was analysed similarly to folate and homocysteine data analysis.

3.8 CONCLUSION

This was an ethically approved study and the selected sample was statistically representative of the elderly. Numerous measuring instruments were used to determine the blood parameters, they are; Automated Konelab™ 20i, Tosoh AIA®-600 II, ELISA and Bio-Rad CFX96™. The blood parameters tested were; serum homocysteine, folate, vitamin B_{12}, holotranscobalamin as well as DNA analysis from EDTA whole blood. Procedures followed in analysis of the blood parameters were standardized and validated to ensure reliable results were reported.

CHAPTER 4

RESULTS

4.1 INTRODUCTION

In this section the results obtained from the study by means of descriptive and inferential statistics are presented. Categorical variables are described by frequencies, reported in proportions and numbers presented either graphically or tabulated. Measures of central tendencies (means, median and mode) and measures of dispersion (standard deviation, range and inter-quartile range) describes numerical variables of this research.

Associations were investigated between homocysteine and its biomarkers (folate and vitamin B_{12}) as well as *MTHFR C677T* polymorphism, between folate and *MTHFR C677T* polymorphism, between vitamin B_{12} and holotranscobalamin.

4.2 QUALITY ASSURANCE

To ensure accurate and reliable results, controls were analysed together with the samples on each measuring instrument. To determine the accuracy of the instrument, the CV of the controls as well as SD were calculated. The latter was used to evaluate the precision of the instrument (Konieczka & Namiesnik, 2016).

The control values of homocysteine measured on the Konelab™ 20i instrument had a mean of 14.5 µmol/L, CV of 9.7 % and an SD of 1.41. Folate controls reported a mean of 6.29, CV of 11.9% and an SD of 0.74 whereas the vitamin B_{12} mean was 689 pmol/L, the CV was calculated to be 3.9% with an SD of 32.71. Holotranscobalamin control values had a mean of 23.8 pmol/L, CV of 7.9% and an SD value 1.48. The results were accepted as accurate because of the CV values that were below 15% and SD values did not vary more than 2SDs from the mean as per Levey-Jennings guidelines (Leedy & Ormrod, 2014).

4.3 SAMPLING

According to the power calculations, the required number of participants was 90 (refer to 3.4). There were 102 subjects that gave consent to participate in the study. There were some challenges during blood collection with some of the elderly men and women. Sufficient serum could not be collected from all samples. Statistical imputation was done in cases of insufficient samples. As blood was collected in 2014 and 2015, results reported on homocysteine status, folate levels and vitamin B12 status as well as their respective relationships are based on the 2014 collection. Prevalence of *MTHFR C677T* polymorphism and its association to homocysteine and folate were analysed using a 2015 serum.

4.4 HOMOCYSTEINE STATUS

Normally distributed data should have a skewness value of zero (Ghasemi & Zahediasl, 2012; Park, 2015). A normality test indicated that homocysteine distribution within the study participants was positively skewed by 3.84 (skewness). Therefore, the median and interquartile range were calculated, the median was found to be 16 µmol/L with an inter-quartile range of 13.50-19.10 µmol/L. The median homocysteine concentration was higher than normal serum concentration of ≤15 µmol/L (Chatthanawaree, 2011; Doğan *et al.*, 2016).

The measured homocysteine concentrations varied from a minimum value of 6 µmol/L to a maximum of 69 µmol/L. Homocysteine levels can be categorized into four groups, normal (≤ 15 µmol/L), mild (16-30 µmol/L), moderate (31-100 µmol/L) and severe (> 100 µmol/L) (Abraham & Cho, 2010; Bhargava, 2018)

As depicted on figure 10, there were 46.08 % (n = 47) of the participants with normal homocysteine concentrations. The majority of the participants' homocysteine concentration was mildly elevated, 50.98% (n = 52). The lowest frequency of 2.94% (n =3) was that of participants with moderately elevated homocysteine (Figure 10). In this study there were no participants with severely elevated homocysteine.

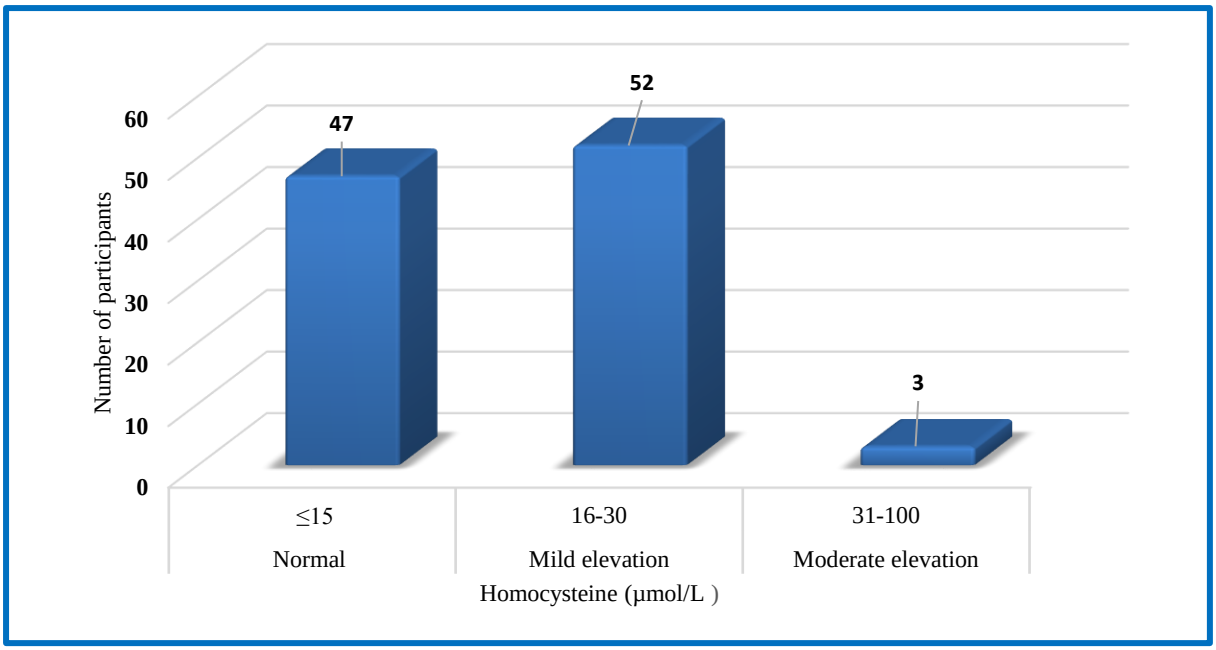

FIGURE 10: HOMOCYSTEINE CONCENTRATION FREQUENCY

4.5 FOLATE LEVELS

Folate concentration was determined in 99 participants from the 102 of the participants (as discussed in 4.3). The folate distribution was tested for normality and found to be normal. The normal density plot had a 0.16 value which is close to zero, therefore mean and standard deviation were reported. The mean folate concentration was 11.08 ng/ml with a standard deviation of 3.52 ng/ml.

Folate status of the participants was grouped as normal and low levels depending, on the lower limit of serum folate normal reference range (5.21-20 ng/ml). Folate concentrations that were below 5.21 ng/ml were grouped as low, those within the reference range as normal, there were no participants that had concentration readings above the upper limit of the reference range. Although folate mean of 11.08 ng/ml was within the normal range of 5.21-20 ng/ml, 5% (n =5) of the participants had low folate levels (Figure 11).

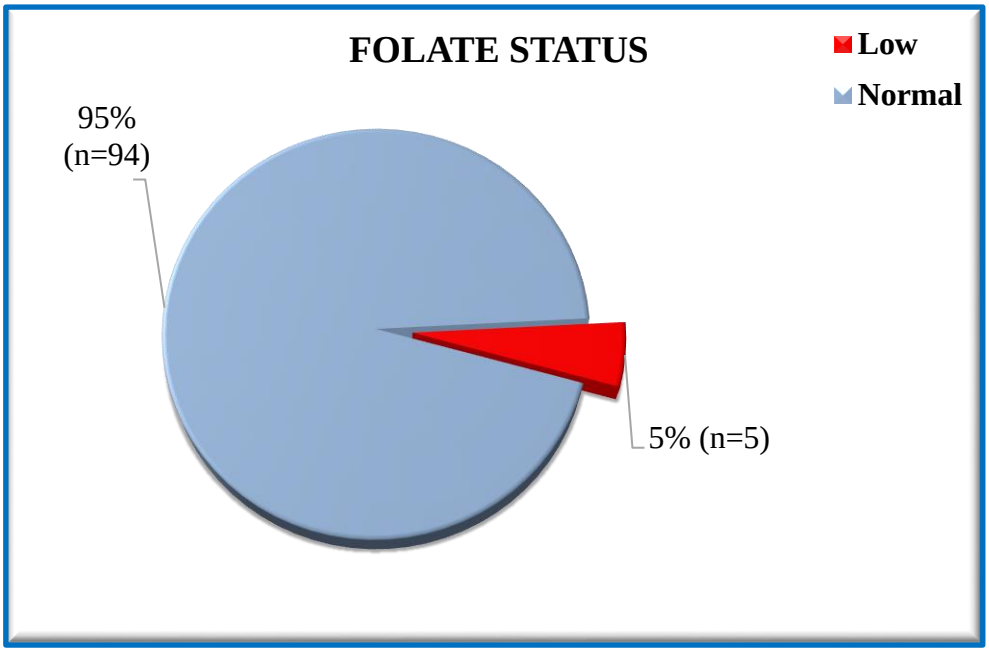

FIGURE 11: THE SERUM FOLATE STATUS RATIO IN THE ELDERLY

4.6 VITAMIN B_{12} STATUS

4.6.1 Serum vitamin B_{12}

Similar to folate, 99 participants were assessed for serum vitamin B_{12}. A normality test for serum vitamin B_{12} status indicated that distribution was skewed, the skewness value was 0.64. Therefore the median and interquartile ranges were determined. The median concentration of serum vitamin B_{12} of the participants was 595 pmol/L with a lower quartile of 449 pmol/L and upper quartile of 802 pmol/L.

Vitamin B_{12} concentrations of this sample group varied from 200 to 1334 pmol/L. Although none of the 99 participants had serum vitamin B_{12} levels below the reference range of ≥200 pmol/L, they were categorized according to the possibility of vitamin B_{12} deficiency. This possibility was selected on the criteria that serum vitamin B_{12} concentrations >300 pmol/L are categorized cobalamin deficiency unlikely and ranges between 200-300 pmol/L as likely (Chatthanawaree, 2011; Zagar & Longyhore, 2014). Table 4 indicates the frequency of vitamin B_{12} deficiency possibility of the study sample. The majority of the participants (93% (n =92)) were unlikely to be vitamin B_{12} deficient and 7 % were likely.

TABLE 4: SERUM VITAMIN B_{12} DEFICIENCY CATEGORIES

Vitamin B_{12} deficiency	Frequency (n)	Percentage (%)
Unlikely (>300 pmol/L)	92	92.93
Likely (200-300 pmol/L)	7	7.07
Total	99	100

4.6.2 Holotranscobalamin (Active serum vitamin B_{12})

The concentration of holotranscobalamin was determined on all participants (n=102). The data distribution normality test for holotranscobalamin had a skewness value of 0.56. Another characteristic of normally distributed data is that of the mean is almost equal to the median, therefore normality was verified using this characteristic. The mean and median concentrations were 69 and 69 pmol/L respectively thus qualifying holotranscobalmin to be normally distributed within the sample population. As abovementioned, the mean concentration was 69 pmol/L. with a standard deviation of ± 30.5. The mean concentration was within the holotranscobalamin reference range of 21-123 pmol/L (IBL, 2012; Aine *et al.*, 2016).

The minimum measured holotranscobalamin concentration in the sample was 10 pmol/L and the maximum was 163 pmol/L. There were 93% (n=95) of the participants who had normal holotranscobalamin levels, 5 % (n=5) had concentrations above the upper limit of the reference range (123 pmol/L) and 2 % (n =2) of the participants had reported concentrations below 21 pmol/L (Figure 12).

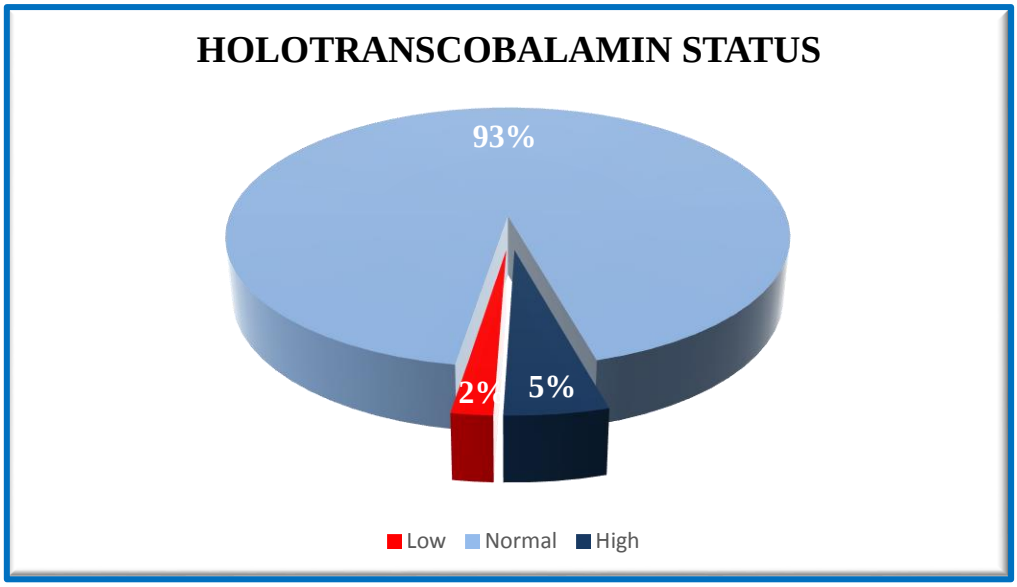

FIGURE 12: DISTRIBUTION OF HOLOTRANSCOBALAMIN STATUS OF THE PARTICIPANTS

4.7 PREVALENCE OF *MTHFR C677T*

Genotyping was done on samples collected in 2015 participants, as indicated earlier in chapter 3. The single nucleotide polymorphism *MTHFR C667T* distribution and allele frequency are presented in Figure 13 and 14, respectively. The gene counting method was used to determine the frequencies of *C* and *T* alleles within this sample population.

Within this sample population majority, 81 % (n = 62) had the normal homozygous *CC* genotype. As depicted in Figure 12, there was 17% (n= 13) participants with heterozygous *CT*, which is a single nucleotide polymorphism and only 2 % (n = 2) was homozygous *TT* genotype.

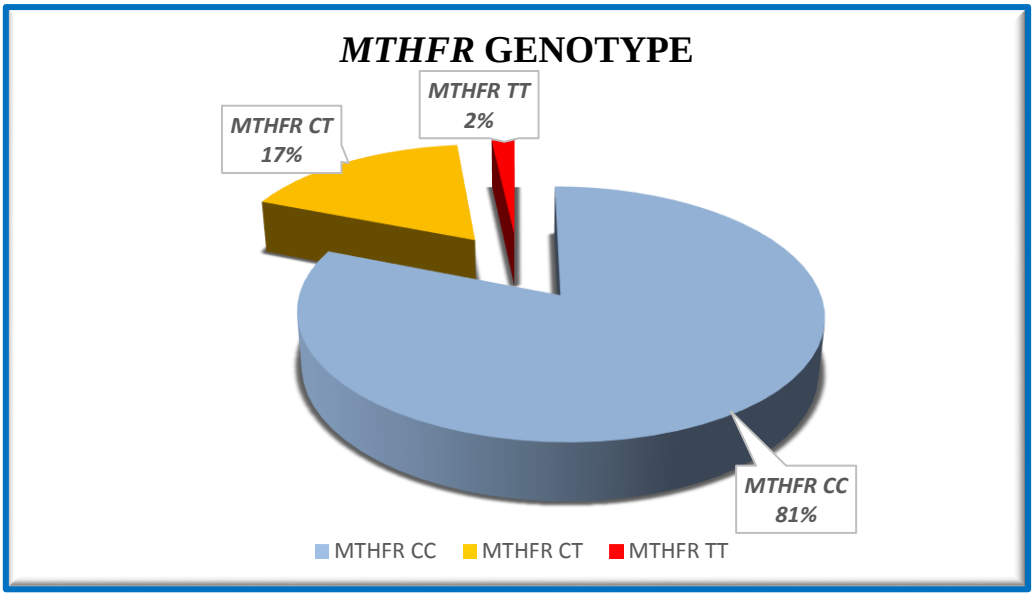

FIGURE 13: PREVALENCE OF *MTHFR C677T* IN A BLACK ELDERLY COMMUNITY, IN SHARPEVILLE

The C allele had the highest frequency of 89% as compared to the T allele frequency which was 11% (Figure 14).

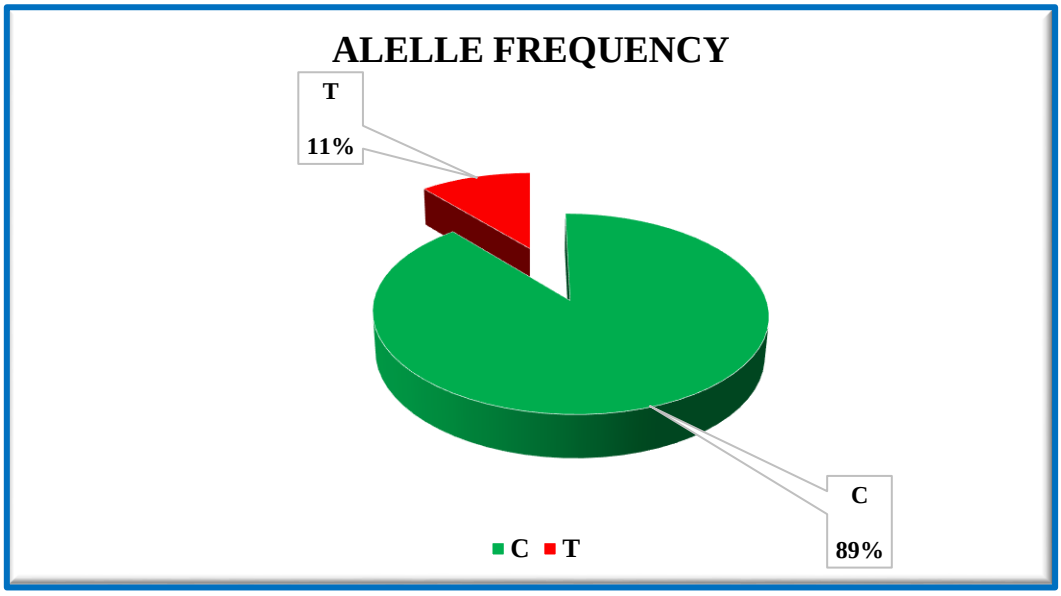

FIGURE 14: ALLELE FREQUENCY DISTRIBUTION *MTHFR C677T* POLYMORPHISM

4.8 CORRELATIONS

As previously indicated, variables folate and vitamin B_{12} had three participants less than other variables. Data was imputed before bivariate analysis was done. Imputations were done using a mean imputation technique with the assistance of a biostatistician.

4.8.1 Homocysteine

4.8.1.1 Homocysteine and folate

It was reported in 4.4 and 4.5, that homocysteine and folate distributions were skewed and normal respectively. Therefore, the statistical test used for bivariate analysis was Spearman's rank correlation. The test indicated that there is no relationship between homocysteine (dependent variable) and folate (independent variable) (Figure 15), Spearman's rho (ρ) was -0.0315 with a p-value of 0.75.

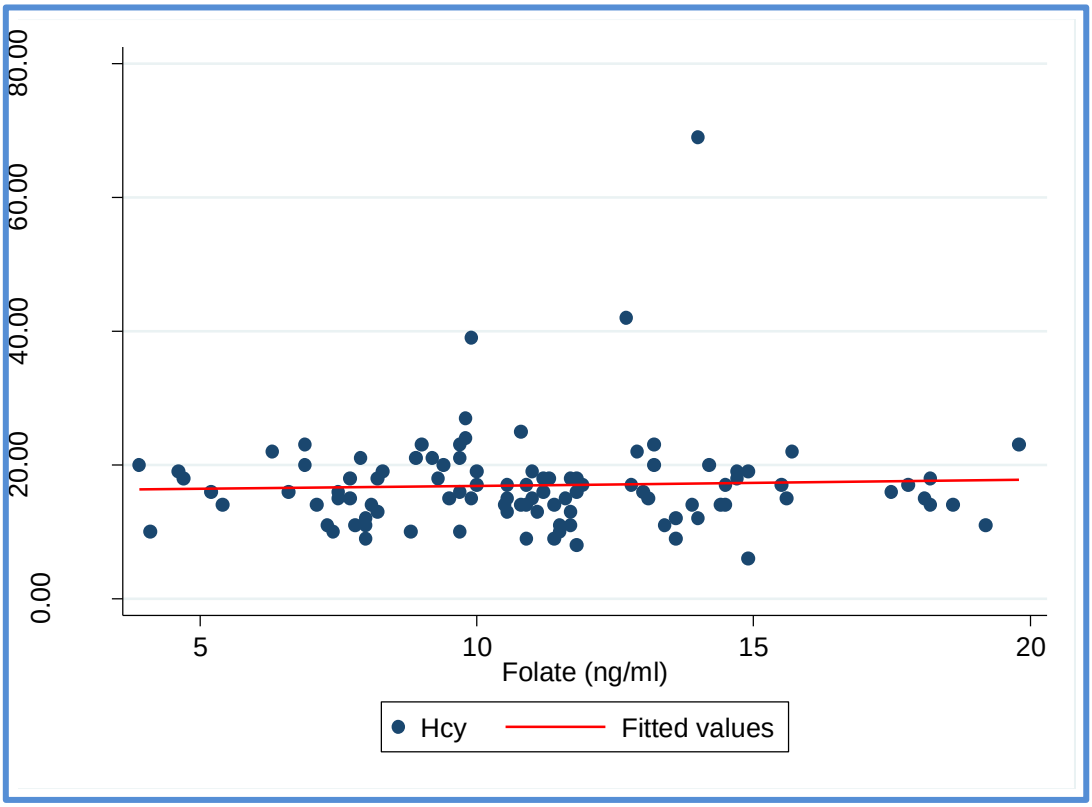

FIGURE 15: HOMOCYSTEINE AND FOLATE CORRELATION

As shown on Figure 15, there were three homocysteine outlier, two of the three were in the 40 µmol/L region and one read at 60 µmol/L region. The 3% of the deviating homocysteine concentrations increases the mean and standard, respectively by 1 µmol/ and ±3 µmol/L. Homocysteine mean and standard deviation with outliers are 16.98 and ±7.47 respectively, without outliers the mean is 15.98 µmol/L whereas standard deviation is ±4.22 µmol/L.

4.8.1.2 Homocysteine and vitamin B_{12}

As elucidated in 2.5.4, vitamin B_{12} functions as a cofactor in homocysteine metabolism therefore a correlation between the two variables was evaluated. Spearman's rank correlation was used to determine the relationship between homocysteine and vitamin B_{12}. There was a strong correlation between homocysteine and vitamin B_{12} with a ρ of -0.3453 and a p-value 0.0002.

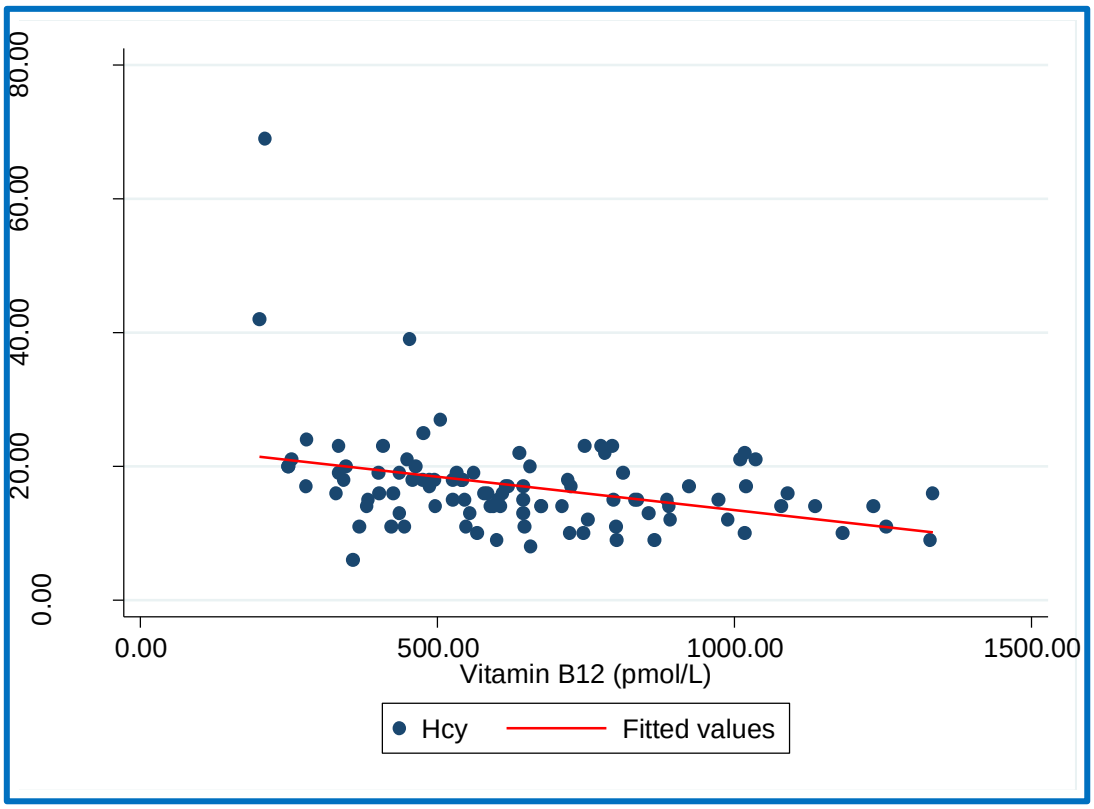

FIGURE 16: HOMOCYSTEINE AND VITAMIN B_{12} CORRELATION

As vitamin B_{12} concentrations increase homocysteine concentration demonstrates a declining slope (Figure 16). The correlation between vitamin B_{12} and homocysteine correlation also demonstrated three homocysteine outliers (Figure 16). Imputed and categorized vitamin B_{12} was tabulated against homocysteine categories.

TABLE 5: FREQUENCY TABLE OF VITAMIN B_{12} AND HOMOCYSTEINE CATEGORIES

Vitamin B_{12} category	Homocysteine category			
	Normal (≤ 15 µmol/L)	**Mild elevation (16-30 µmol/L)**	**Moderate elevation (31-100 µmol/L)**	**Total n %**
Likely (200-300 pmol/L)	0 *0.0 %*	5 *71.43 %*	2 *28.57 %*	7 *100 %*
Unlikely >300 pmol/L	47 *49.47 %*	47 *49.47 %*	1 *1.05 %*	95 *100 %*
Total	47 ***46.08%***	52 ***50.98%***	3 ***2.94%***	102 ***100 %***

In this study 71.43% (n =5) of the participants whom were likely to have vitamin B_{12} deficiency had mildly elevated homocysteine (Table 5). Among the 7 who were likely to be vitamin B_{12} deficient, 2 (28.57%) of them had moderately elevated homocysteine. None of the participants who were likely to be vitamin B_{12} deficient had normal homocysteine concentrations. Within participants who were unlikely to be vitamin B_{12} deficient, 49.47 % (n=47) had normal homocysteine, 49.47% (n = 47) and 1.05 % (n =1) had mild and moderate homocysteine increase respectively (Table 5).

4.8.1.3 Homocysteine and holotranscobalamin

Holotranscobalamin, which is the physiological active form of vitamin B_{12} was evaluated for correlation with homocysteine. As both homocysteine and holotranscobalamin were normally distributed, therefore the Pearson's correlation coefficient (*r*) was used for analysis, as

indicated on Figure 17, there was a strong correlation between the two variables with an *r* of -0.3622 and p-value of 0.0002.

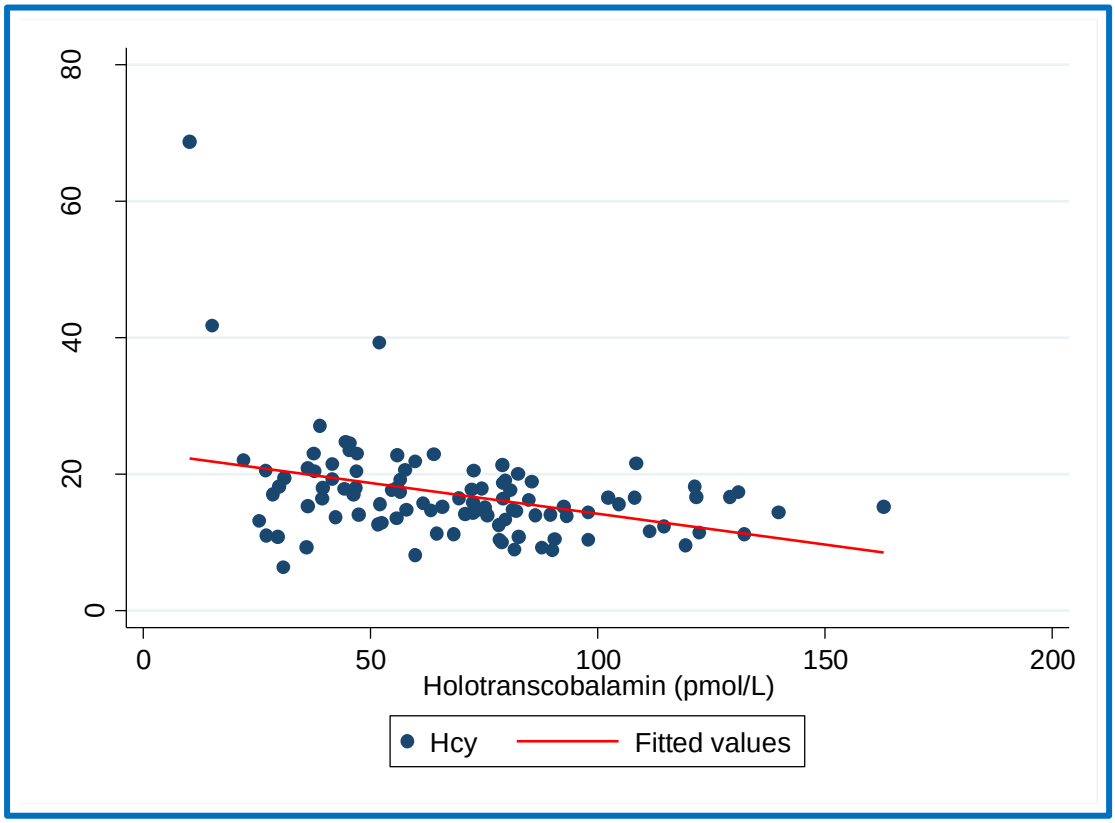

FIGURE 17: HOMOCYSTEINE AND HOLOTRANSCOBALAMIN CORRELATION

4.8.2 Folate

4.8.2.1 Folate and vitamin B_{12}

The correlation between serum folate and vitamin B_{12} which were both statistically imputed (4.8) was also evaluated with Spearman's rank. There was no relationship was found between folate and vitamin B_{12}. Spearman's rho was 0.1927 and with p-value of 0.0523.

There is no uniform spread of data between folate and vitamin B_{12} concentrations, this indicated that there is no relationship between the two variables. The vitamin B_{12} does not increase nor decrease with either folate elevation or decline (Figure 18).

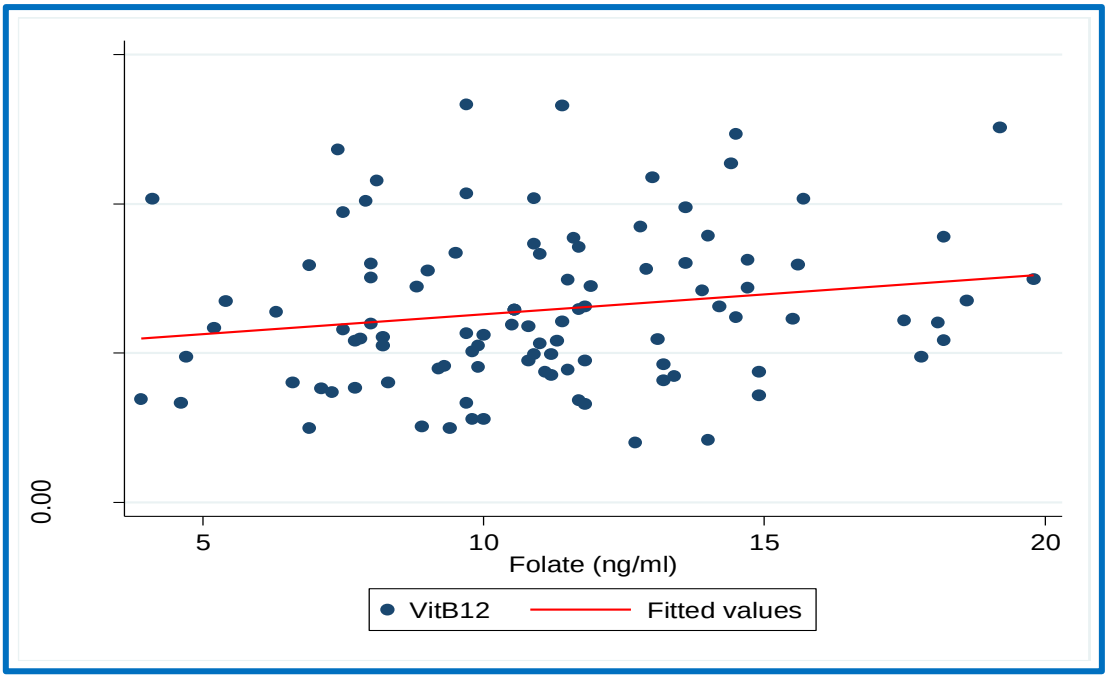

FIGURE 18: VITAMIN B_{12} AND FOLATE CORRELATION

4.8.2.2 Folate and Holotranscobalamin

Both folate and holotranscobalamin were normal distributed, therefore the statistical test used was Person's correlation. Bivariate analysis of folate and holotranscobalamin had a Person's correlation of 0.1454 and a p-value of 0.1448 (Figure 19). In this sample group, no relationship exists between folate and holotranscobalamin.

4.8.3 Vitamin B_{12}

4.8.3.1 Vitamin B_{12} and holotranscobalamin

Spearman's rank correlation test indicated that vitamin B_{12} and holotranscobalamin have a strong, positive relationship (Figure 20). The Spearman's rho between these variables was 0.6928 and there was statistical significance indicated by a p- value of 0.0000. Holotranscobalamin is directly proportional to vitamin B_{12} therefore a decrease in holotranscobalamin concentrations decreases levels of vitamin B_{12}.

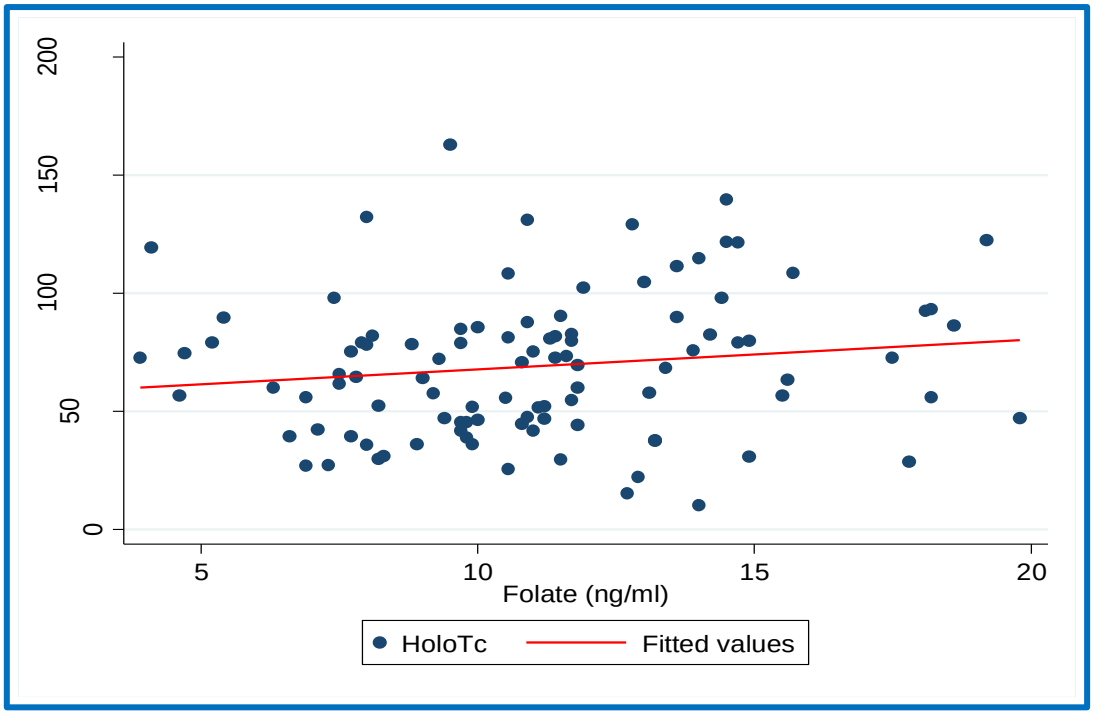

FIGURE 19: FOLATE AND HOLOTRANSCOBALAMIN CORRELATION

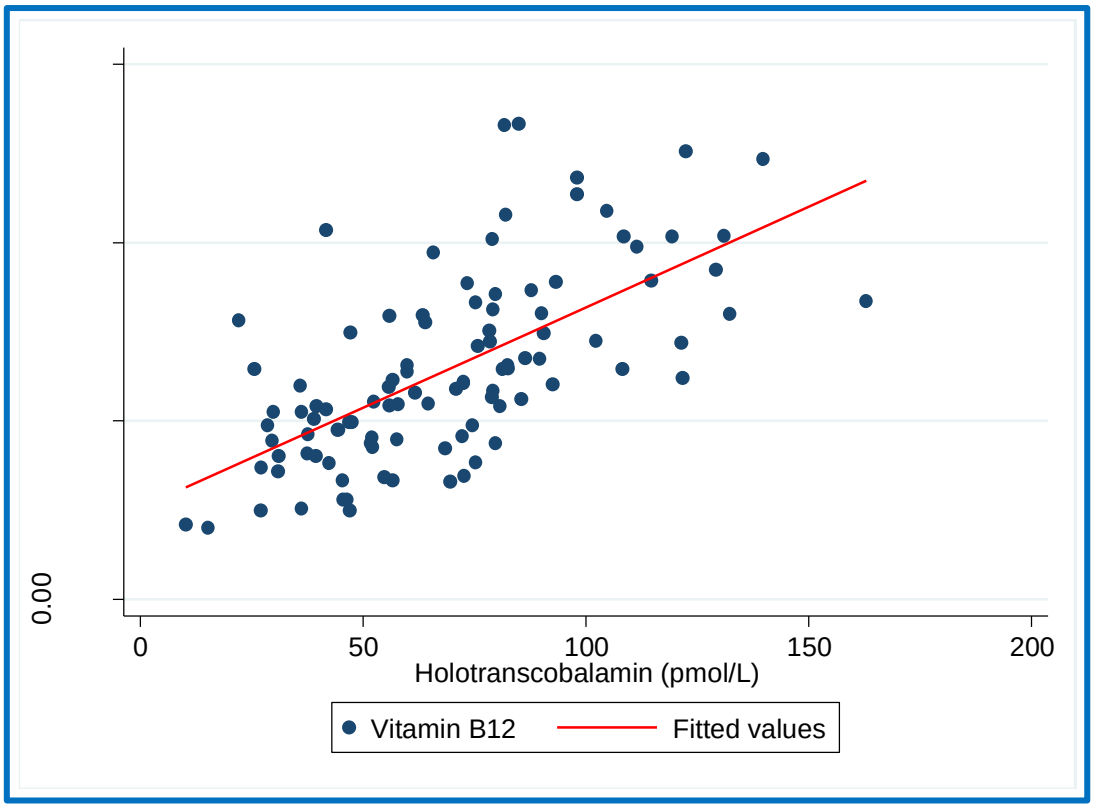

FIGURE 20: VITAMIN B12 AND HOLOTRANSCOBALAMIN CORRELATION

4.8.3.2 Holotranscobalamin sensitivity and specificity

To evaluate the accuracy of holotranscobalamin in detecting cobalamin deficiency, sensitivity and specificity were determined using two-by-two (2 x 2) table. Parikh *et al.*, (2008) and Hajian-Tilaki (2013) define sensitivity as the ability of a diagnostic test to correctly detect the presence of a disease. Sensitivity is defined as the ability of a diagnostic test to correctly exclude the presence of a disease when it is truly not present (Zhou *et al.*, 2011; Hajian-Tilaki, 2013.).

In determination of accuracy for a new diagnostic test, results obtained are compared against a routine test and are denoted as; true positive (TP); false positive (FP); true negative (TN) and false (FN) in a two-by-two table (Table 6) (Parikh *et al.*, 2008; Kampen *et al.*, 2013). According to Parikh *et al.*, (2008) and Kampen *et al.*, (2013), the criteria used in denoting test results is as follows;

- If both new and routine diagnostic tests are positive the test is designated as TP.
- If a positive detected by new diagnostic test and a negative detected by routine test, the test is regarded as FP.
- When both evaluated and routine test excludes the presence of a condition, the test is denoted TN.
- A negative detection by the evaluated test and a positive detection by a routine test is known as FN.

TABLE 6: ILLUSTRATES TWO-BY-TWO TABLE

	Disease present	Disease absent
Test positive	True positive (TP) a	False positive (FP) b
Test negative	False negative (FN) c	True negative (TN) d

a,b,c,d = number of participants

Holotranscobalamin sensitivity and specificity were calculated using the following formulae;

a) Sensitivity = $\frac{a}{(a + b)}$ X 100

b) Specificity = $\frac{d}{(c + d)}$ X 100

In this study, holotranscobalamin was compared with vitamin B_{12} in evaluation the of cobalamin status of the elderly. Holotranscobalamin concentrations below the lower limit of the reference range (21-123 pmol/L) were assigned as positive (deficiency present) whereas those within and above were negative (no deficiency). Vitamin B_{12} concentration ranging from 200 to 300 pmol/L, were labelled positive (deficiency present and >300 pmol/L readings were negative (no deficiency).

The test results obtained indicated that 2 % (n = 2) were TP, 93 % (n = 95) TN and 5 % (n = 5) were FN (See Table 7). In this study there were no false negative results. Sensitivity and specificity of holotranscobalamin calculated as the above formulae were, respectively 100% and 95%.

TABLE 7: FREQUENCY FOR TEST RESULTS OBTAINED

	Disease present	Disease absent
Test positive	True positive (TP) 2 (2 %)	False positive (FP) 0 (0 %)
Test negative	False negative (FN) 5 (5 %)	True negative (TN) 95 (93 %)

Sensitivity results indicate that there was a 100 % probability that holotranscobalamin will detect insufficient cobalamin status as positive and a 95 % probability that it will detect sufficient serum cobalamin as negative (specificity). Positive and negative likelihood ratios were calculated using the respective formulae;

Positive likelihood ratio: $\frac{\text{Sensitivity}}{100\text{- Specificity}}$

Negative likelihood ratio: $\frac{100\text{-Sensitivity}}{\text{Specificity}}$

The positive likelihood ratio of holotranscobalamin was 1.4 thus indicating that the holotranscobalamin is associated with cobalamin status whereas the negative likelihood was 0.

4.8.4 *MTHFR C677T* polymorphism

4.8.4.1 *MTHFR C677T* polymorphism and homocysteine

MTHFR plays an important role in homocysteine metabolism as described in 2.6.2. Single nucleotide polymorphism of the gene coding *MTHFR* such as *MTHFR C677T* has an adverse effect on homocysteine metabolism. A Chi-square test was used to determine the association between genotypes and the homocysteine levels using 2015 results, as Table 8.

As reported in 4.7 that 62 participants genotyped *CC*, the majority of them had mildly elevated homocysteine levels with a 53 % proportionality and 5% of them reported a moderately elevated homocysteine levels. The remaining 42% of the *CC* genotyped had normal concentrations of homocysteine. Participants coded with the *CT* genotype were highly prevalent within the mildly elevated homocysteine category with a 77% of the 13 *CT* genotype. There were only 2 participants that genotyped *TT*. One reported normal levels of homocysteine and the other had a mild elevation. The p- value was 0.584, therefore the differences are not statistically significant. No association was found between homocysteine levels and genotypes.

4.8.2.3 *MTHFR C677T* genotype and folate

Folate status was categorized as low (< 5.21 ng/ml) and normal (5.21-20 ng/ml). Table 9 indicates that 58 genotyped participants demonstrated that 66.67 % (n = 2) had homozygous CC genotype with low folate levels and 33.33 % (n =1) had heterozygous *CT* genotype within the same group. Participants with normal folate status, 81.82 % (n=45) were coded with homozygous CC genotype and 16.36 % (n=9) demonstrated single nucleotide polymorphism *MTHFR C677T* whereas 1.82 % (n =1) had a homozygous *TT* genotype.

TABLE 8: FREQUENCY TABLE OF HOMOCYSTEINE CATEGORIES AND *MTHFR* GENOTYPES

Homocysteine category				
	≤ 15 Normal	**16-30 Mild elevation**	**31-100 Moderate elevation**	**Total**
Genotype				
CC *n* (%)	*26* (**41.94**)	*33* (**53.2**)	*3* (**4.84**)	62 (**100**)
CT *n* (%)	*3* (**23.08**)	*10* (**76.92**)	*0* (**0**)	13 (**100**)
TT *n* (%)	*1* (**50**)	*1* (**50**)	*0* (**0**)	2 (**100**)
Total	30	44	3	77
	38.96%	57.14%	3.9%	100
	Pearson chi2(4) = 2.8455 Pr = 0.584			

TABLE 9 FREQUENCY TABLE OF FOLATE STATUS AND *MTHFR* GENOTYPES

Folate status				
	Low	**Normal**	**High**	**Total**
GENOTYPE				
CC n	1	60	1	62
%	1.61	96.77	1.61	100
CT n	1	12	0	13
%	7.69	92.31	0	100
TT n	0	2	0	2
%	0	100	0	100
Total n	2	74	1	77
%	2.6	96.1	1.3	100
	Pearson chi2(4) = 1.8500 Pr = 0.763			

CHAPTER 5

DISCUSSION

5.1 INTRODUCTION

Since 1969 when McCully postulated that even a small increase in homocysteine levels has the potential of initiating atherosclerosis, studies on homocysteine as a cardiovascular risk factor continue to increase (McCully, 1969; Bawaskar *et al.*, 2015; Grobler, 2015).

5.2 TO DETERMINE HOMOCYSTEINE STATUS OF THE ELDERLY BLACK SOUTH AFRICAN POPULATION.

The subjects in the present study demonstrated a mildly elevated hyperhomocysteinaemic status. A study conducted by Grobler (2015) on the same sample population reported that in 2011 the prevalence of hyperhomocysteinaemia was 32% (n =104). In the present study the prevalence of hyperhomocysteinaemia increased in the four years to 54 % (n = 102). This increase could be due to deficiency of either MTHFR, methionine synthase or cystathionine-ß-synthase, which are enzymes involved in homocysteine metabolism (Blom & Smulders, 2011).

The increase in hyperhomocysteinaemia observed in the present research is possibly caused by supplementation that was discontinued following an interventional study conducted by Dr C.J Grobler on the same sample population (Grobler, 2015). The sample population of the present study was previously reported to be suffering from metabolic syndrome (Grobler, 2015). This could have been an additional contributory factor to the hyperhomocysteine levels (Esteghamati *et al.*, 2014).

In a South African trial study conducted by Ankrah *et al.*, (2012), the prevalence of hyperhomocysteinemia was reported to be 33 % in a sample population with a median age of 56 years. However hyperhomocysteinemia in this trial study was defined as homocysteine levels > 12 µmol/L.

As a result of inconsistent definition of the age of elderlies, the prevalence of elevated homocysteine varies in African studies. In Tunisia a 52.4% prevalence of hyperhomocysteinaemia was reported among subjects with the mean age of 56 years ± 12.5 (SD) (Fekih-Mrissa *et al.,* 2013). The prevalence reported for Nigerians with a mean age of 58.8 ± 8.9 (SD) was 10.1 % (Okubadejo *et al., 2008*).

In comparison with hyperhomocysteinemia observed within the Sharpeville elderly sample population, studies conducted on elderlies in Shangai (China) and North-eastern Iran reported a relatively lower prevalence of hyperhomocysteinemia of 32.4% and 49.4%, respectively (Manavir *et al.,* 2012; Chen *et al.,* 2013). Hyperhomocysteinaemia in Shangai was linked to renal failure (Chen *et al.,* 2013) and a 30% prevalence was found on elderlies with renal dysfunction by Spence & Stampfer (2011). The study conducted in North-eastern of Iran on subjects aged ≥ 65 years found that low vitamin B_{12} and folate were contributing factors of hyperhomocysteinaemia (Manavir *et al.,* 2012). In a study conducted in Dublin, more than 50 % of the sample population reported a hyperhomocysteinaemia status (52.2 %) (Valente *et al.,* 2011). A similar observation was made from results obtained in the present study (Figure 10).

5.3 TO DETERMINE THE FOLATE CONCENTRATIONS IN THE ELDERLY BLACK SOUTH AFRICAN POPULATION

Folate deficiency is considered a cardiovascular risk factor because of its inverse relationship with homocysteine (Barnabé *et al.,* 2015). The results from the present study showed a 5% prevalence folate deficiency (Figure 11). This prevalence decreased as compared to the 9.6 % found by Grobler (2015) in 2011 on the same population. The reduced prevalence of folate deficiency could be a result of adequate supply of folate-rich food sources (2.4.2). Recent studies on folate status of South African elderlies focus on folate intake rather than levels of serum folate. Previous studies that evaluated folate concentration (red blood cell) on South African elderlies, reported a deficiency of 1.6 % (Charlton *et al.,* 1997), which is even lower than the results found in the current study. Mandatory folate fortification of maize and wheat was legislated by the South African government in 2003 (Metz, 2013). The low prevalence of folate deficiency observed in the present study reflects adequate folate intake (Odewole *et al.,* 2013).

During a period of pre-folic acid fortification, the average prevalence of folate deficiency in elderly people of Latin America and the Caribbean was 23% (Brito *et al.,* 2015). This prevalence significantly declined to an average of 2.5 % subsequent to folate fortification (Brito *et al.,* 2015). Spanish elderlies have much higher prevalence of 62.4 % than that of the Sharpeville elderlies of this study (Palacios *et al.,* 2013).

There is a well-established association between folate and homocysteine (Cacciapuoti, 2011; McCully, 2011; Mehlig *et al.,* 2013); however the findings within this sample population failed to demonstrate such a relationship ($P = 0.75$) as reported in 4.8.1.1. Anderson *et al.,* (2010) observed the same tendency in an elderly population. Some of the studies such as that of conducted among the Japaneseby Imamura *et al.,* (2010), reported an inverse relationship between folate and homocysteine but there was no statistical significance.

Available data on folate and homocysteine relationship is mostly in Caucasians and similar studies on black populations is limited (Osunkalu, *et al.,* 2010; Kolb & Petrie, 2013; Delport *et al.,* 2015). It is for this reason that conflicting results are reported in respect to the relationship between folate and homocysteine in black populations (Osunkalu *et al.,* 2010).

The results obtained from this study indicated that there was no association between folate and vitamin B_{12}. Similar finding was reported by Doets *et al.,* (2014) who also did not find an association between folate and vitamin B_{12}. Inconsistent with the present study, an association between folate and vitamin B_{12} ($P < 0.001$) has been previously reported (Semmler *et al.,* 2010). It can be concluded that the correlation between folate and vitamin B_{12} is inconsistent.

5.4 TO EVALUATE THE VITAMIN B_{12} STATUS BY USING SERUM VITAMIN B_{12}, AND HOLOTRANSCOBALAMIN PARAMETERS

Brito *et al.,* (2015) reported that vitamin B_{12} deficiency is common among elderly due to malabsorption. Studies have reported the prevalence of vitamin B_{12} deficiency in elderly varies from 3 % and 40 % (Lachner *et al.,* 2012; Palacios *et al.,* 2013; Wong, 2015). This variation is a result of population samples and lack of consensus on the definition of vitamin B_{12} deficiency (Chatthanawaree, 2010). The definition of vitamin B_{12} deficiency varies according to cut-off levels in different studies, consequently a variation on vitamin B_{12} deficiency exists (Bailey *et al.,* 2011; Zagar & Longyhore, 2014).

The serum vitamin B_{12} results reported demonstrated that there was no vitamin B_{12} deficiency within the Sharpeville sample population. Nonetheless the prevalence of a likely deficiency that is serum levels ranging between 200-300 pmol/L was 7%. Preliminary to vitamin B_{12} deficiency, inefficient vitamin B_{12} absorption, which is common in elderlies could be reason of the likelihood of deficiency. Grobler (2015) reported a 4.81 % prevalence of vitamin B_{12} deficiency in 2011 for the same sample population. Measurement of dietary intake was not a focus of this study; however, a possible cause of decreased prevalence of vitamin B_{12} deficiency could be a result of a better accessibility sources of vitamin B_{12} (2.5.2).

A relatively higher prevalence of likely deficiency (16 %) was reported among elderly Californian Latinos (Allen, 2008). In Brazilian elderlies, the prevalence of likely deficiency was double (14%) as compared to that reported in this study (Xavier *et al.*, 2010). The results obtained from this study, therefore, show that the likely deficiency of vitamin B_{12} is less frequent in black elderlies from Sharpeville, South Africa compared to other international populations.

Vitamin B_{12} as an independent variable in the present study was reported to have an inverse relationship with homocysteine (P = 0.0002) (4.8.1.2). A study by Raina *et al.*, (2015) also revealed a similar relationship (P =0.005). The association between vitamin B_{12} and homocysteine found in the present study confirms findings that, an increase in serum vitamin B_{12} decreases hyperhomocysteinemia. Vitamin B_{12} as a homocysteine biomarker was confirmed to be the contributing factor of the hyperhomocysteinaemia reported in the present study. Subjects who reported to be likely vitamin B_{12} deficient reported hyperhomocysteinaemia.

Additionally, observations in this research emphasized the relationship with an inverse correlation between homocysteine and the biologically active form of vitamin B_{12}, holotranscobalamin (P = -0.3622). Woo *et al.*, (2010) reported an inverse relationship between holotranscobalamin and homocysteine. A strong positive association between holotanscobalamin and vitamin B_{12} (r = 0.69; P = 0.000) reported in this study was also reported in a study by Palacios *et al.*, (2013) (r = 0.66; P < 0.001). A significant correlation (r = 0.66; P < 0.0001) between these two variables was reported by Doets *et al.*, (2014).

The prevalence of holotranscobalamin deficiency within the sample population was 2 %. Low serum holotranscobalamin is possibly caused by polymorphisms in *transcobalamin II* gene.

Genetic variations in *transcobalamin II* have an adverse effect on the binding of cobalamin as well as intracellular uptake of cobalamin (Garg *et al.,* 2011:2; Riedel *et al.,* 2011). A previous study by Valente *et al.,* (2011) observed a relatively higher prevalence of holotranscobalamin deficiency of 8 % among elderlies. In a Turkish sample population, prevalence of low holotranscobalamin was reported to be 78% (Serefhanoglu *et al.,* 2008). Several studies have been conducted on the status of vitamin B12 and recommend the assessment of holotranscobalamin. However, there is lack of such studies in an African or South African context, particularly on the elderly.

According to Wong *et al.,* (2015), there are different stages of vitamin B_{12} deficiency. Using the classification described by Wong *et al.,* (2015), holotranscobalamin results of the present study indicate that 2 % of the subjects have stage I vitamin B_{12} deficiency (serum depletion indicated by low serum holotranscobalamin (< 21 pmol/L)). Incorporation of holotranscobalamin in evaluation of vitamin B_{12} status detected deficiency within the sample population comparable to the evaluation with serum vitamin B_{12} concentration only.

Holotranscobalamin showed an inverse relationship with homocysteine (Spearman's rho = -0.3561 and p-value = 0.0002). Hooshmand *et al.,* (2011:206) reported a similar interaction in an elderly population (r = 0.46 and P < 0.001).

A threshold of 21 pmol/L was used to demonstrate that holotranscobalamin was highly sensitive (100 %) and specific (95 %) in detecting the possible vitamin B_{12} deficiency. Valente *et al.,* (2011:860) demonstrated that the sensitivity and specificity of holotranscobalamin varies depending on decision threshold, a 19.6 pmol/L threshold had 50.7 % and 96.4 % sensitivity and specificity, respectively. For a 29.9 pmol/L threshold, sensitivity and specificity were respectively reported to be 80.6 % and 84.5 %. Sensitivity and specificity of holotranscobalamin was also considered a better diagnostic assay than serum vitamin B_{12} as it reported deficiencies that were reported as sufficient by serum vitamin B_{12} (Oberley & Yang, 2013; Doets *et al.,* 2014). However Palacios *et al.,* (2014) reported a poor sensitivity of holotranscobalamin of 44 %.

In spite of favourable findings on sensitivity and specificity of holotranscobalamin, Brito *et al.,* (2015) suggested that to increase diagnostic accuracy both conventional serum vitamin B_{12} and holotranscobalamin assays should be used in assessing vitamin B_{12} status of the elderlies. A similar observation was made in the present study. However Carmel (2011) prefers serum

cobalamin as the first choice because most studies omit information on the influence that cofounders have on holotranscobalamin. Data obtained from this research indicated holotranscobalamin as a better marker for the interaction between vitamin B_{12} and homocysteine.

The relationship between folate and holotranscobalamin could not be observed in this research. The results are inconsistent with previous studies which reported a decreased holotranscobalamin concentrations in subjects with folate deficiency (Remacha *et al.*, 2014). Furthermore Lewerin *et al.*, (2014) demonstrated a correlation between folate and holotranscobalamin ($r = 0.19$, $p < 0.001$).

5.5 TO DETERMINE THE PREVALENCE OF THE *MTHFR C677T* POLYMORPHISM IN AN ELDERLY BLACK SOUTH AFRICAN POPULATION

Metabolic enzyme MTHFR can be coded by various functional variants such as *MTHFR C677C, MTHFR C677T* and MTHFR *T667T*. Genotype *MTHFR C677T* is a considered a risk factor of increased homocysteine concentrations (Roussotte, 2016). Enzyme activity of the homozygotes is reported to be at 30% efficiency whereas heterozygotes activity is 65% (McEwen, 2017).

Ethnicity and geographical position influences allelic frequency (Liew & Gupta, 2015). A study conducted within a Chinese elderly group reported the prevalence of the variants *CC*, *CT* and *TT* to be 25.2 %, 44.2% and 30.6%, respectively (Li *et al.*, 2017). The allele frequencies of the same population were 0.473 for C and 0.527 for T (Li *et al.*, 2017). The prevalence hyperhomocysteinemia risk factor variants (*CT* and *TT*) of this Chinese group were relatively higher than that of this study as reported in Table 5, as well as the *T* allele.

An Italian elderly sample population reported the prevalence of respective polymorphisms *CT* and *TT* as 49.25% and 17.17% with the wild type (*CC*) just constituting 33.58 % (Polito *et al.*, 2016). The prevalence of *MTHFR C677T* of the Italians is high compared to that of the sample population of Sharpeville. Contrary to their European counterparts, a Turkish study on the elderly indicated the prevalence of *MTHFR C677T* to be relatively lower mutant, the variants are reported at 32.2 % for *CT* and *TT* at 9.3% whereas the prevalence of the wild type was 58.5% (Basol *et al.*, 2016). Although the prevalence of the *MTHFR C677T* polymorphism in

the Turkish study is higher than the current study as reported in Table 5, both studies indicate that the majority of the genotype is the wild type. In this study 80.5% is the wild type and the Turkish sample population is 58.5%.

In a hypertensive Cameroonian elderly sample population, *MTHFR* genotypes *CC*, *CT* and *TT* were reported as 7.3%, 58.5% and 34.1% respectively (Ghogomu *et al.,* 2016). The allele frequencies of this Cameroonian population were 0.634 for *T* and 0.366 for *C* (Ghogomu *et al.,* 2016). These allelic frequencies are high, compared to those reported in this study (Figure 14). Nienaber-Rousseau *et al.,* (2013) conducted a study on 2000 black South African participants and reported the genotypes of the sample population as *CC* (84%), *CT* (15.2%) and *TT* (0.8%). The results as reported in table 5 that is *CC* (80.5%), *CT* (16.9%) and *TT* (2.6%) are consistent with those reported by Nienaber-Rousseau *et al.,* (2013).

5.6 TO EVALUATE THE CORRELATION BETWEEN THE *MTHFR C677T* POLYMORPHISM AND HOMOCYSTEINE STATUS

There was no statistically significant (p= 0.584) relationship found between *MTHFR C677T* polymorphism and homocysteine levels in this study. However, there was a trend identified as the majority, 77% (n=10) of the heterozygous *MTHFR C677T* variant was found in the mildly elevated group. Zappacosta *et al.,* (2014) report concur findings in the Sharpeville group, their report indicated increasing homocysteine values from CC to TT subjects. The findings of this study are similar to those by Nienaber-Rousseau *et al.,* (2013), in which participants carrying the *MTHFR C677T* variant had relatively high homocysteine concentration as compared to those with the wild type variant.

5.7 TO EVALUATE THE CORRELATION BETWEEN THE *MTHFR C677T* POLYMORPHISM AND FOLATE STATUS

The polymorphism *MTHFR C677T* codes for a thermolabile enzyme, therefore it decreases the enzyme activity of converting folate into its bioactive form (Zappacosta *et al.,* 2013). In the Sharpeville sample population there was no statistically significant (p = 0.763) association between *MTHFR C677T* and folate status. The majority, 92 % (n =12) of those with

heterozygous *MTHFR C677T* polymorphism had normal folate status and there was only one heterozygous carrier with low folate levels.

A study among Indian elderly subject reported that the presence of a *T* allele contributed to decreased folate levels (Chhillar *et al.*, 2014). The Indian study supports the literature in respect to the relationship between *MTHFR C677T* polymorphism and folate. In the Sharpeville population, the influence of the *T* allele was contradictory to the Indian study as 12 (92%) of the 13 heterozygous carriers had normal folate levels. Those with the homozygous *T* allele (n =2) also had normal folate concentrations (Table 9). Folate deficiency was low (n =2) within the Sharpeville study group of 77 participants.

5.8 CONCLUSION

Evidence gathered from this study indicated that hyperhomocysteinemia continues to be a challenge within the elderlies in Sharpeville. There was no relationship between folate and other biochemical parameters. A statistical relationship was found between vitamin B_{12} and holotranscobalamin. Vitamin B_{12} and holotranscobalamin demonstrated a statistically significant inverse relationship with homocysteine respectively. Prevalence of *MTHFR C677T* polymorphism is low among Black South African elderly. Reduced enzyme activity is not a genetic risk factor for hyperhomocysteinemia in group with sufficient folate.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 INTRODUCTION

This study intended to determine the prevalence of *MTHFR C677T* polymorphism in black elderly population in correlation with homocysteine metabolic markers. The results reported in previous studies in the same population confirmed the prevalence of increased homocysteine. Limited data is available on the prevalence of *MTHFR C677T* polymorphism in black elderlies in South Africa.

6.2 RESEARCHER'S CONTRIBUTION

The research was conducted under the leadership of Dr C.J Grobler in collaboration with the Centre of Sustainable Livelihoods (CSL) at the Vaal University of Technology. The research was a collective approach by a multidisciplinary team, these included; an administrator, phlebotomist, molecular biologist, medical technologist and a biostatistician. The researcher was responsible for;

- Procurement of blood collection materials and reagents.
- Labelling blood collection tubes with the assistance of the research team.
- Laboratory analysis.
- Data analysis
- Writing of this report

6.3 LIMITATIONS OF THE STUDY

Sufficient blood could not be drawn from all participants, due to phlebotomy challenges resulting in some parameters (vitamin B_{12} (n =3) and folate (n = 3)) that could not to be analysed on the same subjects. This was overcome by statistical imputations during data analysis. In spite of this, the sample size was adequate as it was above the power calculation (n =89). Prevalence of vitamin B_{12} deficiency in the Sharpeville population is unknown therefore

positive and negative predictive values of holotranscobalamin diagnostic test could not be evaluated.

The third limitation of this study is that it did not evaluate the effects of drugs on homocysteine, folate and vitamin B_{12} concentrations. The prevalence of *MTHFR C677T* polymorphism could not be done on all the subjects included in the biochemical analysis. Gender based analysis could not be conducted because a few males were attending the day care centre.

6.4 MAIN FINDINGS

The main findings of the present research are described as follows;

6.4.1 Prevalence of *MTHFR C677T*

- The prevalence of *MTHFR C677T* polymorphism found in this study is 17%.
- Homozygous wild type variant (*CC*) is predominant (81%) within the elderly sample group.
- Prevalence of homozygous mutant genotype was 2%.
- There is a high frequency of the C allele with an 89% presence in this study population and the T allele was 11%.

6.4.2 Homocysteine status

- Findings of this study confirm that hyperhomocysteinemia previously reported in this population increased since 2011.
- Mild hyperhomocysteinemia (16-30 µmol/L) is predominant (50.98% (n = 52) in the elderly residing from Sharpeville and attending a day-centre.
- Possible contributing factors of mild elevation of homocysteine in this group are; ageing, inflammatory status and metabolic syndrome (Grobler, 2015).
- There was no relationship between homocysteine and folate however there was an inverse relationship with vitamin B_{12} (a ρ of -0.3453 and a p-value 0.0002).
- No statistically significant association was found between *MTHFR C677T* polymorphism and homocysteine

- However, it was observed that the majority of those with *MTHFR C677T* polymorphism presented were dominantly (77%) in the mildly elevated homocysteine group and 23% of the heterozygous carriers were in the normal homocysteine group.
- Wild type homozygous carriers represented 53% in the mildly elevated homocysteine group, 5% in the moderately elevated group and 42 % in the normal homocysteine group.

6.4.3 Folate status

- The folate status of the majority (95% (n = 94) of participants is at ideal levels.
- An intervention such as supplementation of increased folate doses is required for those with folate deficiency.
- There was no correlation between folate and other measured biochemical variables.
- The association between *MTHFR C677T* genotypes and folate status was statistically insignificant.
- Only one heterozygous carrier was found to have low folate levels.
- Two of the subject with homozygous mutant genotype reported a normal folate concentration.

6.4.4 Vitamin B_{12} status

- No vitamin B_{12} deficiency was found in this sample population, however there were 7 % of the subjects who were likely to have the deficiency (200-300 pmol/L).
- A statistically significant (p- value of 0.0000) positive correlation between serum vitamin B_{12} and holotranscobalamin was demonstrated.
- Holotranscobalamin was highly sensitive (100%) and specific (95%).

6.4.5 Correlations between *MTHFR C677T* polymorphism and biochemical analysis

- No association was found between homocysteine levels and *MTHFR C677T* genotypes.
- Normal concentrations of homocysteine were found in 42% (n =26) of the 62 participants with homozygous wild type (*CC*).

- A 53 % (n=33) of the 62 participants genotyped *CC* had mildly elevated homocysteine levels.
- Moderately elevated homocysteine levels were found among 5% (n = 3) of the 62 participants genotyped *CC*.
- No statistically significant association between *MTHFR C677T* and folate status.
- Among subjects with normal folate status, 81.82% (n=45) had homozygous CC genotype and 16.36% (n=9) heterozygous *CT* genotype whereas 1.82 % (n =1) had homozygous TT genotype.
- Within low folate levels group, 66.67% (n = 2) had homozygous CC genotype and 33.33 % (n =1) had heterozygous CT genotype.

6.5 SIGNIFICANCE OF THE STUDY

This study addressed the limited knowledge on the prevalence of *MTHFR C677T* polymorphism among Black elderly South Africans. Furthermore, it also narrowed the knowledge gap of folate, vitamin B_{12} and homocysteine status of this population. The extent at which the elderlies are at risk of cardiovascular disease was demonstrated in this study by evaluating homocysteine, which is a well-established cardiovascular risk factor. Additionally, the study showed that emerging diagnostic tool, holotranscobalamin is a sensitive and specific diagnostic tool that can be used for detecting early vitamin B_{12} deficiency.

6.6 CONCLUSION

The focus of this research was an evaluation of the prevalence of *MTHFR C677T* polymorphism in correlation with homocysteine metabolic markers. The conclusions drawn from the study were: 1) there is a low prevalence of *MTHFR C677T* polymorphism within the Black South African elderly in Sharpeville, 2) there is a high risk of cardiovascular disease as a result of high prevalence of hyperhomocysteinemia, a predisposing factor, 3) the folate status in the sample population is favourable, however, attention is required for those with folate deficiency, 4) high levels of homocysteine found in this sample population are not a results of folate, vitamin B_{12} or *MTHFR C677T* polymorphism factors, 5) generally the vitamin B_{12} levels are normal but those who are likely to have a deficiency need to be monitored; 5) holotranscobalamin is a relatively more sensitive test for evaluating vitamin B_{12} status.

6.7 RECOMMENDATIONS

6.7.1 Community

An intervention to lower homocysteine concentration of elderlies residing in Sharpeville is recommended. A vitamin-B (folate, B_{12} and B_6) supplementation would be an ideal intervention to decrease the prevalence of hyperhomocysteinemia as it was demonstrated by Grobler (2015). Such an intervention will also reduce the prevalence of folate deficiency and prevent those with likelihood of vitamin B12 deficiency to become truly deficient.

6.7.2 Scientific community

More studies are required focusing on the status of homocysteine, folate and vitamin B_{12} in South African elderlies, as well as African elderly populations. Data from this study deduced that holotranscobalamin assay is a better diagnostic tool for evaluating vitamin B_{12} status.

6.7.3 Health policy makers

This study recommends that an acute intervention plan to lower homocysteine has to be included in the South African strategic plan for the prevention and control of non-communicable diseases. Monitoring of homocysteine concentration, particularly on elderlies should be incorporated as a routine test in in the health care system.

6.8 FURTHER RESEARCH NEEDED

Future research is required that should include clinical trials evaluating vitamin B_{12} status using holotranscobalamin as a diagnostic test. Furthermore, this study recommends that prospective studies should be conduct in known population to be able to evaluate positive and negative predictive values of holotranscobalamin. The *MTHFR C677T* polymorphism is excluded as a predisposing factor for hyperhomocysteinemia in this population, therefore other genetic predisposing factors of increased homocysteine levels should be investigated. Insight future studies can incorporate qualitative data on cardiovascular risk factors to be able to drawn

relatively more substantial conclusion in respect to *MTHFR C677T* polymorphism, hyperhomocysteinemia and cardiovascular risk.

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