

**The association between specific genetic,
demographic and lifestyle factors related to
homocysteine concentrations in black South Africans
undergoing an epidemiological transition**



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This thesis is dedicated with love to the memory of my mother Joey Nienaber who passed away during my Ph.D studies, who had supported and encouraged me, as well as to my father Pieter C. Nienaber for being the rock that carried me through this time.

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“As we express our gratitude, we must never forget that the highest appreciation is not to utter words, but to live by them.” - Hohn Fitzgerald Kennedy

OPSOMMING

Die assosiasie tussen spesifieke genetiese, demografiese en lewenstylfaktore verwant aan homosisteïen konsentrasies in swart Suid Afrikaners

Agtergrond: Homosisteïen (Hcy), wat in die ontwikkeling van kardiovaskulêre siektes (KVS) geïmpliseer word, word beïnvloed deur demografiese, omgewings- en genetiese faktore insluitende enkelnukleotied polimorfismes (SNPs) binne die metieltetrahidrofolaatreduktase (MTHFR), sistatioon- β -sintase (CBS) en metioniensintase (MTR) gene.

Doelwit: Die oorhoofse doelwit van hierdie ondersoek was om die moontlike rolle van verskeie dieet, omgewings en genetiese faktore op die regulasie van Hcy konsentrasies te verklaar. Spesifiek om interaksies tussen alkoholinnome en die MTHFR C677T genotipes en die voorkoms van die MTHFR C677T, MTR A2756G, CBS T833C/844ins68 en G9276A SNPs en hulle verhouding tot, en epistatiese interaksies met totale (t)Hcy konsentrasies te bepaal.

Studieontwerp en metodes: Hoofstukke 5 en 6 beskryf dwarsdeursnit data van 1,827 swart Suid-Afrikaners binne die internasionale Prospektiewe Stedelike en Landelike Epidemiologiese studie. Vastende plasma-tHcy-konsentrasies was bepaal met fluoressensie polarisasie immuno-analise tegnologie. Die SNPs was bepaal deur polimerase kettingreaksiegebaseerde restriksiefragmentlengte polimorfisme-analises. Dieetinnames was bepaal met 'n kwantitatiewe voedselfrekwensievraelys. Gammaglutamieltransferase (GGT) en persentasie koolhidraat wat 'n transferrien tekort het (%CDT) was bepaal om as alkoholbiomerkers te dien.

Resultate: Ouderdom en GGT het die beste met tHcy gekorreleer ($r = 0.26$ en $r = 0.27$; $p < 0.05$) terwyl %CDT en die B-vitamiene swak met tHcy gekorreleer het ($r < 0.1$ vir beide; $p < 0.05$). Ouderdom, GGT, geslag, MTHFR C677T en vitamien B₆ het 16.8% van die variasie in tHcy konsentrasies ($p < 0.01$) verklaar. Die frekwensies van die SNPs het voldoen aan die aannames van Hardy-Weinberg ewililibrium, maar het verskil van wat vir ander etniese groepe gerapporteer is. Daar was nie 'n interaksie tussen alkoholinnome en die MTHFR 677 CC/CT genotipes nie ($p > 0.05$). Die MTHFR 677 TT en MTR 2756 AA genotipes was geassosieer met betekenisvol hoër tHcy konsentrasies (16.6 en 10.1 $\mu\text{mol/L}$; $p < 0.05$) as in persone wat die MTHFR 677 CT/CC en die MTR 2756 AG genotipes (10.5, 9.7 en 9.5 $\mu\text{mol/L}$,

onderskeidelik) het. Tussen die CBS 844ins68, T833C of CBS G9276A en MTHFR C677T genotipes, was betekenisvolle twee-rigting interaksies ($p < 0.05$), hoewel daar nie 'n interaksie was tussen MTHFR C677T en MTR A2756G of tussen die CBS 844ins68/T833C of G9276A en MTR A2756G genotipes met verwysing tot tHcy konsentrasies nie.

Gevolgtrekking: Daar was nie 'n interaksie tussen alkoholiname en die MTHFR 677 CC/CT genotipes, hoewel MTHFR C677T genotipe, ouderdom, geslag en GGT belangriker determinante van tHcy was as B-vitamieniname. Geen-geen interaksies bestaan en beklemtoon die epistatiese karakter van KVS. Etnisiteit is 'n belangrike modulerende faktor wat genetiese vatbaarheid vir KVS bepaal.

ABSTRACT

The association between specific genetic, demographic and lifestyle factors related to homocysteine concentrations in black South Africans

Background: Homocysteine (Hcy) has been implicated in cardiovascular disease (CVD) and is influenced by demographic, environmental and genetic factors including single nucleotide polymorphisms (SNPs) within the genes encoding for methylenetetrahydrofolate reductase (MTHFR), cystathione- β -synthase (CBS) and methionine synthase (MTR).

Objective: The overall aim of this investigation was to elucidate the possible role of various dietary, environmental and genetic risk factors on the regulation of Hcy concentrations. Specifically, to determine interactions between alcohol consumption and the MTHFR C677T genotype and the prevalence of the MTHFR C677T, MTR A2756G and CBS T833C/844ins68 and G9276A SNPs and their relationship to and epistatic interactions with total (t)Hcy concentrations.

Study design and methods: Chapters 5 and 6 outline cross-sectional data of 1,827 black South Africans nested within the international Prospective Urban and Rural Epidemiology study. Fasting plasma tHcy concentrations were determined by fluorescence polarisation immunoassay technology. The SNPs were determined through polymerase chain reaction based restriction fragment length polymorphism analysis. Dietary intake was ascertained with a quantitative food frequency questionnaire. Gamma glutamyl transferase (GGT) and percentage carbohydrate deficient transferrin (%CDT) were measured as alcohol biomarkers.

Results: Age and GGT correlated best with tHcy ($r = 0.26$ and $r = 0.27$; $p < 0.05$) while %CDT and the B-vitamins were weakly associated with tHcy concentrations ($r < 0.1$ for both; $p < 0.05$). Age, GGT, gender, MTHFR genotype status and vitamin B₆ explained 16.8% of the variation in tHcy concentrations ($p < 0.01$). The frequencies of SNPs adhered to the assumptions of Hardy-Weinberg equilibrium, but differed when compared to those reported for other ethnic groups. There was no interaction between alcohol consumption and the MTHFR 677 CC/CT genotypes ($p > 0.05$). The MTHFR 677 TT and MTR 2756 AA genotypes were associated with significantly higher tHcy concentrations (16.6 and 10.1 $\mu\text{mol/L}$; $p < 0.05$) than

subjects harbouring the MTHFR 677 CT/CC and the MTR 2756 AG (10.5, 9.7 and 9.5 $\mu\text{mol/L}$, respectively). Between the CBS 844ins68, T833C or CBS G9276A and MTHFR C677T genotypes, there were significant two-way interactions ($p < 0.05$), however, there was not an interaction between MTHFR C677T and MTR A2756G or between the CBS 844ins68/T833C or G9276A and MTR A2756G genotypes with regard to tHcy concentrations.

Conclusions: There is no interaction between alcohol intake and the MTHFR 677 CC/CT genotypes, however, MTHFR C677T genotype status, age, gender and GGT are more important determinants of tHcy concentrations than B-vitamin intake. Gene-gene interactions exist thus highlighting the epistatic nature of CVD. Ethnicity is a major modulating factor in genetic susceptibility to CVD.

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LIST OF ABBREVIATIONS

844ins68	insertion of 68 base pairs at nucleotide position 844
A	adenine (nucleotide)
A	alanine (amino acid)
A222V	alanine replacement with a valine amino acid at amino acid number 222
A1298C	adenine to cytosine replacement at nucleotide position 1298
A2756G	adenine to guanine substitution at position 2756
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANCOVA	analysis of covariance
ANOVA	analysis of variances
ATP	adenosine triphosphate
AT III	glycosaminoglycan-antithrombin III
AUTHeR	Africa Unit for Transdisciplinary Health Research
BBM	brush border membrane
BHMT	betaine-homocysteine methyltransferase
BLM	basolateral membrane
BMI	body mass index
bp	base pairs
C	cytosine (nucleotide)
C677T	cytosine to thymine substitution at nucleotide position 677
CBS	cystathionine β -synthase
CBVD	cerebrovascular disease
CEN	Centre of Excellence for Nutrition
CHD	coronary heart disease
CI	confidence interval
CV	coefficient of variance
CVA	cerebrovascular accident

CVD	Cardiovascular disease
Cys	cysteine
%CDT	percentage carbohydrate deficient transferrin
D	aspartic acid (amino acid)
D919G	an aspartic acid to glycine substitution at amino acid residue 919
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide-5'-triphosphate
E	glutamate (amino acid)
E429A	a glutamate to alanine substitution at amino acid number 429
ecto-ADPase	ecto-adenosine diphosphatase
EDTA	ethylenediamine tetra-acetic acid
eNO	endothelial NO
F	phenylalanine (amino acid)
F1 + 2	prothrombin fragments 1 and 2
FAD	flavin adenine dinucleotide
FMN	flavin adenine mononucleotide
G	guanine (nucleotide)
G	glycine (amino acid)
GCP II	glutamate carboxypeptidase II
gDNA	genomic deoxyribonucleic acid
GGT	gamma glutamyl transferase
H	histidine (amino acid)
H475Y	a histidine to a tyrosine amino acid substitution
HAART	highly active antiretroviral therapy
Hcy	homocysteine
HHcy	hyperhomocysteinaemia
HIV	human immunodeficiency virus

hs-CRP	high sensitivity C-reactive protein
HWE	Hardy-Weinberg equilibrium
I	isoleucine (amino acid)
I22M	substitution of an isoleucine with methionine at codon 22
I278T	substitution of an isoleucine to threonine substitution at amino acid residue 278
ins	insertion
ISAK	International Society for the Advancement of Kinanthropometry
LDL	low density lipoprotein
Lp(a)	lipoprotein(a)
M	methionine (amino acid)
MLT	methionine loading tests
MRC	Medical Research Council
mRNA	messenger ribonucleic acid
MS	methionine synthase
MSE	mean square error
MT	mutant type
5-MTHF	5-methyltetrahydrofolate
5,10-MTHF	5,10-methylenetetrahydrofolate
MTHFR	methylenetetrahydrofolate reductase
MTR	gene coding for methionine synthase
MTRR	methionine synthase reductase
n	number of; sample size
NCD	non-communicable disease
NO	nitric oxide
NRF	National Research Foundation
NWU	North-West University

OR	odds ratio
P	praline (amino acid)
PCR	polymerase chain reaction
PhASRec	Physical Activity, Sport and Recreation
PHRI	Population Health Research Institute
PI	protease inhibitor
PLP	pyridoxal 5'-phosphate
PhASRec	Physical Activity, Sport and Recreation
PURE	Prospective Urban and Rural Epidemiology study
Q	glutamine (amino acid)
QFFQ	quantitative food frequency questionnaire
R	arginine (amino acid)
R27H	a substitution of an arginine to histidine at amino acid number 27
RFC I	reduced folate carrier 1
RR	relative risk
S	serine (amino acid)
SAH	s-adenosylhomocysteine
SAM	s-adenosylmethionine
SANPAD	South Africa - Netherlands Research Programme on Alternatives in Development
SD	standard deviation
SMAC	Sequential Multiple Analyser Computer
SNP	single nucleotide polymorphism
T	thymine (nucleotide)
T	threonine (amino acid)
T833C	thymine to cytosine substitution at base 833
TAT	thrombin-antithrombin complex

TC	transcobalamin
tHcy	total homocysteine
tPA	tissue type plasminogen activator
THF	tetrahydrofolate
UK	United Kingdom
V	valine (amino acid)
Vit	vitamin
VNTR	variable number of tandem repeats
WT	wild-type
Y	tyrosine (amino acid)

LIST OF SYMBOLS AND UNITS

β	beta
χ^2	Chi square
r	correlation
$\downarrow$	decrease
$^{\circ}\text{C}$	degrees, Celcius or centigrade
=	equal
$-\text{CH}_3$	methyl group
γ	gamma
g	gram
g	gravitational force
>	greater than
$\geq$	Greater than or equal to
$\uparrow$	increase
L	litre
-	negative; minus
p	p-value, indicates statistical significance
pH	indicator of acidity or alkalinity
kat	katal
kg	kilogram
kg/m^2	kilograms per meter squared; unit of body mass index
km	kilometer
%	percentage
$\pm$	plus minus
p	p-value
MgCl_2	magnesium choloride
♂	men
μ	micro: 10^{-6}
μmol	micromole
m	mili
mL	mililitre
-	minus

mol	mole
M	molecular weight
x	multiply
x g	multiplied by gravitational force
-	negative
n	number of subjects; sample size
n	nano: 10^{-9}
ng	nanogram
$\leftrightarrow$	normal or adequate
+	positive
<	smaller than
$\leq$	smaller than or equal to
-SH	thiol
U	unit
♀	women
yrs	years

CHAPTER 1

GENERAL INTRODUCTION

1.1 CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is a group of circulatory disorders that can roughly be grouped into coronary heart disease (CHD), also known as coronary artery disease, cerebrovascular disease (CBVD) and other uncommon vascular diseases *inter alia* occlusions of peripheral arteries or veins, congenital, infectious and rheumatoid heart diseases which are not within the scope of this thesis (De Bree *et al.*, 2002). The process leading up to the critical stage of CVD in adult life starts decades earlier during childhood or young adulthood, yet an individual is asymptomatic and generally remains undiagnosed until culmination in the CVD event (Cunnane, 1993; McGill *et al.*, 2002). Angina pectoris (commonly known as chest pain), myocardial infarction (also known as a heart attack), cerebrovascular accident (CVA) *i.e.* stroke in colloquial language, or ischaemic heart disease are all CVD events.

1.1.1 Cardiovascular disease as a multifactorial disease

CVD events were once deemed to be inevitably fatal, but are now considered to be both treatable and partially preventable (Jackson *et al.*, 2000; Stamler *et al.*, 1998). This can be attributed to the establishment of multiple variables causally involved in CVD development, such as cigarette smoking, physical inactivity, dyslipidaemia and hypertension (Jackson *et al.*, 2000; Hennekens, 2000). The significance of the mentioned CVD risk factors is well established, but cardiovascular events occur in many individuals without overt hyperlipidaemia or the other mentioned traditional risk factors (Greenland *et al.*, 2003; Ridker *et al.*, 2004). This has focused attention on CVD as a multifactorial disease *i.e.* a disease that results due to the interplay of multiple pathogenic mechanisms, including multiple genes, acting either alone or in concert with one another, which display effect modification in the presence of certain environmental and behavioural factors which are modestly associated with CVD or the main risk factors of CVD (Gelehrter *et al.*, 1998).

Causality in CVD development has also been suggested for several other variables, including the accumulation of the amino acid homocysteine (Hcy), in the body. Hcy seems to mediate atherosclerotic and thrombogenic events that cause the development of CVD (see Chapter 2 Section 2.3 for a complete review of the plausible mechanisms). However, Hcy is currently shrouded in a cloud of controversy (Chambers & Kooner, 2001; Faeh *et al.*, 2006; Ueland *et al.*, 2000) as researchers are debating whether it is a risk marker (merely an innocent bystander not causally involved in CVD development), a predisposing risk factor (a variable that affects other risk factors that act directly) or a risk factor (a variable causally involved in CVD aetiology). In Chapter 2 the literature regarding this matter is reviewed. Resolving the question of causality is important, since Hcy can be lowered by supplementation with folic acid, vitamin B₂, B₆ and B₁₂ (Powers, 2003; Schnyder *et al.*, 2002; Wald *et al.*, 2001; Zee *et al.*, 2007) and the proscription against *inter alia* binge alcohol drinking and cigarette smoking (De Bree *et al.*, 2002). This raises the prospect of a simple, inexpensive and safe means to prevent or reverse the rise of CVD in developed and developing countries.

Many variables associated with CVD, including Hcy, have their own set of environmental and genetic triggers that play a key role and thus complicate CVD aetiology (Gelehrter *et al.*, 1998). The focus of this thesis will be on the lifestyle factors (as reviewed in Chapter 3) especially alcohol consumption (as investigated in Chapter 5) and several of the genetic (as reviewed in Chapter 4) determinants of Hcy concentrations and their interactions with each other (as investigated in Chapter 6).

1.1.2 Cardiovascular disease, the epidemiological transition and urbanisation in South Africa

Despite the invaluable contributions of research in identifying risk factors and developing strategies to reduce risk, CVD remains one of the leading causes of death worldwide (Hennekens, 2000; Murray & Lopez, 1997; Yusuf *et al.*, 2001a). Researchers have reason to believe that the prevalence of CVD will rise due to the epidemiologic transition as well as urbanisation (exodus from rural to urban areas), which in turn results in the nutrition transition (Stewart *et al.*, 2006).

The epidemiologic transition is characterised by a decrease in childhood deaths due to decreases in infectious, parasitic and under nutrition related diseases thus extending life expectancy in those that escape infection from the human immunodeficiency virus. This allows a greater percentage of the population to reach the age at which CVD and other non-communicable diseases manifest (Chopra *et al.*, 2002; Steyn & Bradshaw, 2001; Yusuf *et al.*, 2001a), most likely due to the lengthened exposure to genetic, environmental and behavioural disease risk factors.

CVD seems to be increasing in developing countries, including South Africa, due to rapid industrialisation and urbanisation with subsequent adoption of higher risk lifestyles, including unhealthy dietary changes (Chopra *et al.*, 2002; Temple *et al.*, 2001; Tibazarwa *et al.*, 2009; Vorster, 2002). Since physical inactivity, obesity, hypertension, elevated total blood cholesterol and high fibrinogen concentrations, which are all associated with CVD, are prevalent among adult black South Africans undergoing transition (Kruger *et al.*, 2001; Kruger *et al.*, 2002; Tibazarwa *et al.*, 2009) and the CVD process tends to be slowly progressive over decades, an imminent CVD epidemic in this developing population could be expected.

1.1.3 Cardiovascular disease differences between ethnic groups

Even though CVD is a leading cause of death in the world, not everyone is targeted equally. CVD prevalence as well as the type of CVD present differs between the ethnic groups in the world (Yusuf *et al.*, 2001b) and between groups within South Africa (Bourne *et al.*, 2002; Mollentze *et al.*, 1995; Sliwa *et al.*, 2008; Steyn *et al.*, 1992). CHD, including ischaemic heart disease, seems to be considerably lower in most Africans compared to Caucasians (Yusuf *et al.*, 2001b). However, the high incidence of CBVD with resultant CVA (Kahn & Tollman, 1999; Steyn *et al.*, 1992) and heart failure attributable to dilated cardiomyopathy or hypertensive heart disease, or both (Sliwa *et al.*, 2008) among Africans are of concern.

The underlying basis for the observed ethnic disparities in CVD morbidity and mortality is likely multifactorial and, therefore, could be attributed to cultural or genetic differences or to interactions between genes and environmental factors as well

as differences in socio-economic circumstances. It is unknown whether differences in biomarkers of inflammation, haemostasis or Hcy concentrations contribute to the observed disparity. In most urban and virtually all rural regions of Sub-Saharan Africa, the prevalence of traditional CVD risk factors among black individuals is low, but with urbanisation an increase in conventional CVD risk factors and CHD rates is anticipated (Sliwa *et al.*, 2008; Tibazarwa *et al.*, 2009; Yusuf *et al.*, 2001b). Subsequently, the possible influence of Hcy in the variations of CVD by ethnicity and the influence of urbanisation will be discussed.

From a limited number of studies conducted in South Africa, Hcy concentrations seem to be significantly lower in traditional living African men than in men of European descent (Ubbink *et al.*, 1996; Vermaak *et al.*, 1991). Several genetic and lifestyle factors are known to influence Hcy concentrations, including variations in the efficiency of Hcy metabolism due to differences in genetic background and/or differences in B-vitamin consumption (De Bree *et al.*, 2002).

Genetic differences may, in part, explain the ethnic differences in Hcy metabolism. Reported genetic determinants of Hcy are *inter alia* polymorphisms within genes expressing the enzymes involved in Hcy metabolism. Methylenetetrahydrofolate reductase (MTHFR) and methionine synthase (MS) are two key enzymes involved in the folate and vitamin B₁₂-dependent transmethylation of Hcy into methionine, whereas cystathionine β -synthase (CBS) is a key enzyme in the catabolic transsulphuration pathway (Finkelstein, 2001). Differences in Hcy concentrations between ethnic groups could be related to the low frequency of the cytosine (C) to thymine (T) substitution at nucleotide position 677 (C677T) in the MTHFR gene of black South Africans as compared to Caucasians (Loktionov *et al.*, 1999). C677T within the MTHFR gene leads to thermolability of the MTHFR enzyme, which reduces its activity and, therefore, limits Hcy metabolism and increase circulating Hcy concentrations (Frosst *et al.*, 1995). It appears that there is no significant increase in Hcy in individuals harbouring the MTHFR adenine (A) to C replacement at nucleotide position 1298 (A1298C) (Friedman *et al.*, 1999; Van der Put *et al.*, 1998; Weisberg *et al.*, 1998). Therefore, the MTHFR A1298C polymorphism was not investigated within this investigation, but was merely discussed in the literature

review presented in Chapter 4. The frequencies of the other common genetic polymorphisms that influence Hcy, including variations within the CBS gene and the gene coding for the MS enzyme (MTR), have not been extensively researched or as widely documented as the genetic variation of the MTHFR gene. In addition, among black South Africans, to our knowledge, these genetic variations have not been investigated. Since, this population is part of the larger African L mitochondrial deoxyribonucleic acid macrohaplogroup, the most ancient human group from which all modern humans arose, they harbour a high level of genetic variation (Chen *et al.*, 1995). A recent study conducted by Schuster *et al.* (2010) corroborated the view that southern Africans are among the most divergent human populations. Therefore, it is important to investigate the distribution of the above mentioned genetic variations within this group. It is expected that the frequencies of these genetic variations will differ from other groups that have been investigated previously and that due to micro-evolutionary effects different phenotypic effects may also be expected for the same genetic variation between the different ethnic groups.

The insertion of 68 base pairs (bp) at position 844 (844ins68) in the CBS gene that usually co-exists with the CBS T to C substitution at base 833 (T833C) seems to be related to lower fasting and incremental post-methionine load Hcy concentrations in CVD patients (Tsai *et al.*, 2000). It still needs to be clarified whether the A to guanine (G) substitution at position 2756 (A2756G) within the MTR gene has functional consequences for enzymatic activity or the levels of MS. Some studies have reported no association between the MTR A2756G polymorphism and Hcy concentrations (Amouzou *et al.*, 2004; Hyndman *et al.*, 2000; Jacques *et al.*, 2003), contrastingly, several studies have reported that the genetic variant was associated with decreased Hcy concentrations (Harmon *et al.*, 1999; Silaste *et al.*, 2001).

Additionally, differences in Hcy concentrations between the ethnic groups could partly be due to differences in the stages of transition and differences in lifestyle including dietary habits, physical activity levels and alcohol consumption (Bourne *et al.*, 2002; Steyn *et al.*, 2005). Differences in dietary intakes of the B-vitamins between the ethnic groups would also contribute to differences in Hcy concentrations (De Bree *et al.*, 2002). In addition, cultural differences in food preparation methods and, therefore, folate cooking losses between Caucasian and African individuals could

also influence Hcy concentrations. However, during urbanisation, traditional African diets rich in fibre and grain, but low in fats, are replaced by imprudent Westernised diets that include increased levels of refined sugars, animal protein and fat associated with non-communicable diseases (Vorster, 2002). The effects of dietary changes during urbanisation are also exacerbated by a parallel decline in energy expenditure due to a reduction in daily physical activity which results in an increased prevalence of obesity (Kruger *et al.*, 2002; Steyn *et al.*, 2005).

Differences in alcohol consumption, which are associated with Hcy concentrations in a J-shaped fashion, would also explain differences in Hcy concentrations between the ethnic groups (De Bree *et al.*, 2002). Alcohol consumption plays a paramount role in both the epidemiological and nutrition transition associated with urbanisation of black South Africans. South Africans are among the leading consumers of alcohol in the world, consuming 20 litres of absolute alcohol per drinker per annum (Parry *et al.*, 2005). What is of greater concern is that a third of the reported drinkers in South Africa are binge drinkers *i.e.* drinking large amounts in a short period (Parry *et al.*, 2005). While moderate alcohol consumption is associated with decreased mortality from CVD, binge drinking and chronic alcohol abuse are associated with increased CVD morbidity particularly CBVD (Puddey *et al.*, 1999).

Furthermore, the presence of the MTHFR C677T alteration augments the effect that alcohol has on tHcy concentrations in Caucasian women (Chiuve *et al.*, 2005). To our knowledge, studies investigating the effect of alcohol consumption in the presence of variations in the MTHFR gene on Hcy concentrations in relation to CVD in men and women, are scarce and non-existent for African populations.

In summary, inadequate diets providing insufficient amounts of the B-vitamins, reduced physical activity levels and imprudent alcohol consumption, seem to be associated with higher Hcy concentrations in most populations (De Bree *et al.*, 2002). If these factors are associated with tHcy in Africans the urbanisation process and constantly shrinking gap between the rural and urban lifestyles could possibly result in elevated Hcy concentrations in black South Africans similar to those currently observed in Caucasians. Since elevated Hcy concentrations are associated with CHD, this could result in CHD rates that approach those observed in Caucasians.

1.2 MOTIVATION TO STUDY LIFESTYLE AND GENETIC VARIABLES POSSIBLY PREDISPOSING TO CARDIOVASCULAR DISEASE IN AN AFRICAN POPULATION UNDERGOING TRANSITION

South Africa is undergoing profound changes in disease burden. Critical assessment of the current health state, including surveillance of risk markers, predisposing risk factors as well as actual risk factors is essential, since it underpins health promotion and disease prevention (Beaglehole & Bonita, 2001). However, to date, no large epidemiological study has been conducted to investigate both lifestyle and genetic factors that influence Hcy or the interactions that could predispose to CVD in an African population undergoing transition. The cross-sectional epidemiological studies presented in this thesis are nested within the North West Province South African leg of the multi-centre twelve year Prospective Urban and Rural Epidemiology study (PURE). The PURE-study is a large international prospective study set up to collate and analyse several CVD risk markers as well as the health transition in urban and rural subjects of 16 developing countries using a multidisciplinary approach (Teo *et al.*, 2009). The data presented in this thesis were collected and determined during the baseline investigation of the PURE-study. Data were collected from approximately 1,002 subjects from an urban setting around Potchefstroom and 1,008 from three different rural villages 450 km from Vryburg *en route* to Botswana during 2005.

Data regarding Hcy concentrations in different ethnic groups is presently lacking and this study will provide valuable information pertaining to the risk of CVD through variable Hcy concentrations in a black South African group. This research will explore the association of specific Hcy-related genetic variants, B-vitamin status and other lifestyle factors with Hcy concentrations within a large black South African population.

Research has not fully characterised the polymorphic phenotype outcomes of the MTR A2756G alteration, since inconsistent results regarding the association between this genetic variant and Hcy concentrations have inhibited conclusions (see Chapter 4 Section 4.3.3 for a complete review of the literature regarding this genetic alteration) and this research will add to this area. Little has also been reported regarding the

effect of the CBS T833C/844ins68 and/or CBS G9276A on Hcy concentrations in any population group (Griffioen *et al.*, 2005). Therefore, this research will also shed light on the prevalence of these genetic variations and their influence on Hcy concentrations within the black South African population. There is a paucity of studies investigating the effect of alcohol consumption in the presence of variations in the MTHFR, CBS and MTR genes on Hcy concentrations and studies on Africans are non-existent. This thesis also contributes to this area of science by investigating the interaction between the MTHFR C677T genetic variation and alcohol consumption. In addition, the interactions between the determined genetic variations in relation to Hcy concentrations in black South Africans were also investigated.

1.3 AIMS AND OBJECTIVES OF THE STUDY

The main aim of this thesis was to evaluate the prevalence of hyperhomocysteinaemia *i.e.* elevated Hcy concentrations and to determine if the state is associated with the genetic variants, B-vitamin intake and lifestyle factors investigated in this study. This will improve insight into the determinants of Hcy concentrations in black South Africans.

The overarching postulated hypothesis is that specific genetic, environmental and lifestyle factors (physical activity and nutrition) and transitions secondary to urbanisation and industrialisation are associated with increased risk to non-communicable disease. In this thesis Hcy concentrations will be investigated as a variable for the possible development of CVD.

The objectives of this study were:

- To assess the major lifestyle determinants of Hcy concentrations in black South Africans;
- To determine whether B-vitamin intake (folate, vitamins B₂, B₆ and B₁₂) modulate the association between genetic factors and Hcy concentrations;
- To determine the superimposed effects of alcohol consumption on Hcy in the presence of the MTHFR C677T genetic variation which influence Hcy metabolism on Hcy concentrations in black South Africans;

- To determine the genotype distributions of the C677T alteration within the MTHFR gene, the A2756G within the MTR gene as well as the T833C/844ins68 and G9276A alterations within the CBS gene in a black South African cohort; and
- To determine the presence of any associations between the different genotypes to Hcy concentrations (cross-sectional) and whether there are interactions between these genotypes.

1.4 STRUCTURE OF THE THESIS

This thesis is presented in article format and is a compilation of chapters written to comply with the requirements of the North-West University (Chapters 1 – 4 and 7) and the journals to which the article manuscripts included were prepared for submission (Chapters 5 and 6). Directives in terms of language usage, formatting and quoting sources were strictly followed. All chapters and manuscripts have their own reference list provided at the end of each chapter. The content of each chapter is briefly described below.

Chapter 1 provides a general introduction which includes a discussion of CVD as a multifactorial disease, CVD risk differences between ethnic groups as well as exploring the influence of urbanisation on CVD risk and Hcy concentrations. It also delimits the research problem, gives a description of the PURE-study, indicates the aims and objectives, and presents an overview of the thesis format and content. In addition, Chapter 1 presents the contributions of the authors to the articles presented in the thesis.

Chapters 2, 3 and 4 are review articles with the purpose of conveying an integrated view of the literature available on Hcy in order to facilitate the understanding and interpretation of the ensuing research articles presented in this thesis. Chapter 2 focuses on the biochemistry of Hcy and investigates the role of Hcy in CVD aetiology and provides possible mechanisms of involvement of Hcy in CVD. Chapter 3 alternately highlights the lifestyle, environmental and biological determinants of Hcy, including alcohol consumption. Chapter 4 presents the common genetic determinants

of Hcy, describes their molecular aspects and functional consequences as well as their distribution within various regions and ethnic groups.

Chapter 5 is an article with the title: “No interaction effect established between alcohol intake and the genetic polymorphism methylenetetrahydrofolate reductase (MTHFR) C677T in relation to homocysteine concentrations in a black South African population” prepared for publication in Genetic epidemiology. However, only subjects harbouring the MTHFR 677 CC or CT genotypes could be included in the statistical analyses of this article, since only three subjects harbouring the TT genotype reported to consume alcohol.

Chapter 6 is an original research paper with the title: “Genotype distributions of C677T in the MTHFR gene, the A2756G in the MTR gene as well as the T833C/844ins68 and G9276A alterations in the CBS gene and their interactions in relation to Hcy concentrations in Africans” prepared for submission in the Journal of thrombosis and haemostasis. The research for this article included the determination of various polymorphisms and environmental factors and explored possible interactions in relation to tHcy concentrations in black South Africans.

Chapter 7 provides a recapitulation, discussion, conclusion and recommendation regarding the research conducted. This chapter will complete this thesis.

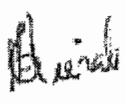
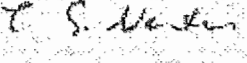
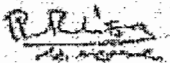
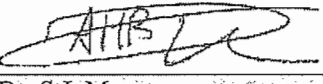
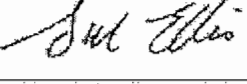
1.5 RESEARCH OUTPUTS

PISA, P.T., LOOTS, D.T. & NIENABER, C. Alcohol metabolism and health hazards associated with alcohol abuse in a South African context: a narrative review. *South African journal of clinical nutrition*, in press.

1.6 CONTRIBUTIONS OF THE AUTHORS TO THE ARTICLES PRESENTED IN THE THESIS

This collection of research articles was planned and executed by a team of researchers. The contributions of the researchers involved in the studies presented in this thesis are given in Table 1.1.

Table 1.1 List of members within the research team and their contributions to this study

Name and signature	Affiliation	Role in the study
Miss C. Nienaber (Ph.D candidate) 	CEN within the School for Physiology, Nutrition and Consumer Science of the NWU	Was responsible for the literature reviews, DNA extractions, all the genotypings including independent interpretation of the agarose gel photos, statistical analyses, data interpretation of the results and writing up of publications (first author of Chapter 1 - 7 and co-author of the paper of Pisa <i>et al.</i> , in press), critical revision, planning, writing and compilation of this thesis.
Dr. G.W. Towers (promoter) 	CEN within the School for Physiology, Nutrition and Consumer Science of the NWU	Supervisor of C. Nienaber and assisted with trouble shooting of laboratory analyses, independently interpretation of the agarose gel photos of the genotypes investigated, statistical analyses, interpretation of the results and co-authored Chapters 5 and 6.
Dr. T. Hoekstra (co-promoter) 	Julius Center for Health Sciences and Primary Care University Medical Center, Utrecht, the Netherlands	Co-promoter of C. Nienaber, involved in DNA extractions, initial laboratory supervision and guidance regarding the writing up of the articles and co-authored Chapters 5 and 6.
Prof. C.S. Venter (assistant promoter) 	CEN within the School for Physiology, Nutrition and Consumer Science of the NWU	Assistant promoter of C. Nienaber, involved in the interpretation of the results and guidance regarding the writing up of the data and co-authored Chapters 5 and 6.
Prof. A. Kruger 	AUTHeR within the Faculty of Health Science of the NWU	Planned (designed the study and approved the final protocol) and coordinated data collection of the PURE-study and co-authored Chapters 5 and 6.
Dr. P.T. Pisa 	CEN within the School for Physiology, Nutrition and Consumer Science of the NWU	Was responsible for the laboratory analysis of biological markers of alcohol consumption and co-authored Chapter 5.
Dr. A. Melse-Boonstra 	Division of Human Nutrition, Wageningen University, the Netherlands	Involved in the planning phase of the project, in securing funding, preparation for Hcy analysis and co-authored Chapters 5 and 6.
Dr. S.J. Moss 	PhASRec within the School of Biokinetics, Recreation and Sport Science of the NWU	Involved in the planning phase of the project, in securing funding, preparation for Hcy analysis and co-authored Chapters 5 and 6.
Dr. S.M. Ellis 	Statistical Consultation Service, North-West University, Potchefstroom Campus, South Africa	Was involved in all the statistical analyses and interpretation thereof and co-authored Chapters 5 and 6.
With my signature I declare that I have approved the above mentioned articles, that my role in the study, as indicated above, is representative of my actual contribution and that I hereby give consent that it may be published as part of the Ph.D thesis of Miss C. Nienaber.		

AUTHeR = Africa Unit for Transdisciplinary Health Research; CEN = Centre of Excellence for Nutrition; DNA = deoxyribonucleic acid; PhASRec = Physical Activity, Sport and Recreation, NWU = North-West University

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CHAPTER 2

LITERATURE REVIEW

HOMOCYSTEINE AND CARDIOVASCULAR DISEASE

2.1 INTRODUCTION

Cardiovascular disease (CVD) is one of the leading causes of death worldwide (Murray & Lopez, 1997; Yusuf *et al.*, 2001). Several established risk factors such as hypertension, high concentrations of low-density lipoprotein (LDL) cholesterol and smoking are causally related to CVD (Yusuf *et al.*, 2001). A causal role in the development of CVD is also suggested for other factors, including an elevated plasma homocysteine (Hcy) concentration (De Bree *et al.*, 2002). The association between Hcy concentration and the risk of CVD has been a topic of intense debate. Researchers are debating whether Hcy is a risk marker *i.e.* an innocent bystander not causally involved in CVD development, a predisposing risk factor *i.e.* presumed to work, at least in part, through an impact on other risk factors that act directly or a risk factor, *i.e.* directly responsible for CVD development and thus causative (Brattström & Wilcken, 2000; Faeh *et al.*, 2006; Ueland *et al.*, 2000). Hcy stands indicted of being involved in CVD. Settling this dispute of CVD causality is necessary, since Hcy can be lowered by the adequate intake of folate, vitamin B₂, B₆ and B₁₂ (Brattström, 1996; Powers, 2003; Schnyder *et al.*, 2002; Wald *et al.*, 2001; Zee *et al.*, 2007) as well as the proscription of *inter alia* heavy irregular (binge) alcohol drinking (Carmel & James, 2002) and cigarette smoking (Bamonti *et al.*, 2007) thus raising the prospect of a simple, inexpensive and safe means of CVD prevention.

This review provides an overview of the biochemistry and metabolism of Hcy. In addition this review critically analyses the various published studies with regard to the debate on whether Hcy is just an innocent bystander or a culprit in the development of CVD. Epidemiological, genetic and basic research as well as Hcy-lowering intervention trials are discussed within this review with regard to the possible pathological mechanisms through which Hcy could be involved in CVD thus elucidating whether the Hcy concentration is a disease marker, a predisposing risk factor or if Hcy is causally related to the development of CVD.

2.2 AN OVERVIEW OF THE BIOCHEMISTRY AND METABOLISM OF HOMOCYSTEINE

Hcy is a sulphur-containing amino acid, *i.e.* a thiol (-SH), with the chemical formula: $\text{HSCH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{CO}_2\text{H}$ (Jacobsen, 2001). Hcy is not present in food, but is formed by the body during the breakdown of methionine, an essential amino acid derived from dietary and recycled endogenous proteins (Scott, 2003). Hcy might, therefore, be increased in diets high in animal protein, specifically those containing the amino acid methionine. These diets are also usually high in LDL as well, therefore, the reason for an association between Hcy and CVD could be related to the high Hcy itself and/or to the associated high LDL intake (Scott, 2003).

The structures of methionine and Hcy are identical except for a conversion that results in the removal of a one-carbon methyl group (-CH₃) from the former (Scott & Weir, 1998). While methionine is chemically stable, the free thiol of Hcy renders it highly reactive in cells and within the circulation (Scott, 2003). In human plasma 70% to 80% of Hcy is bound to the thiol groups of plasma proteins such as albumin (Scott, 2003). Only 1% to 4% circulates as free Hcy in its reduced form, whilst the remainder auto-oxidises to form homocystine dimers (molecules formed by the combination of two smaller identical molecules) or it combines with other thiols such as cysteine (Cys) and glutathione to form mixed disulfides (Jacobsen, 2001; Mansoor *et al.*, 1992). It is not yet clear which complexes are formed by the reduced Hcy molecules within cells, but it seems probable with the reactivity of the thiol group of Hcy, that similar complexes will form at a neutral pH (Scott, 2003).

To enhance clarity the nomenclature regarding Hcy will be subsequently discussed. *Hcy* and *homocystine* refer to the reduced and oxidised forms of Hcy, respectively. The term *homocyst(e)ine* with parentheses around the 'e' has been used for unspecified forms *i.e.* the combined pool of free, bound, reduced and oxidised forms of Hcy in mammalian cells and in the plasma (Mudd & Levy, 1995), whereas the word *hyperhomocyst(e)inaemia* (HHcy) has been used for elevated Hcy concentrations to emphasise the pathogenic significance of Hcy as opposed to the disulfide homocystine. As the parentheses cannot be pronounced in speech it is

difficult to distinguish *homocyst(e)ine* from *homocysteine* (Mudd & Levy, 1995) and, therefore, the use of these words is becoming less popular (Jacobsen, 2001). For the purpose of this thesis the term *homocyst(e)ine* will be substituted with total Hcy or ‘*tHcy*’ (Mudd & Levy, 1995) and the abbreviation *Hcy* will be used for the term *homocysteine*.

The metabolism of B-vitamins, methyl groups and Hcy is inextricably linked as illustrated in Figure 2.1. According to Scott (2003), the clearance of Hcy from the circulation is dependent on folate (also referred to as pteroylglutamic acid whereas the synthetic form of folate is termed folic acid or folacin), vitamin B₂ (riboflavin), vitamin B₆ (pyridoxine, pyridoxal or pyridoxamine) and vitamin B₁₂ (cobalamin or cyanocobalamin).

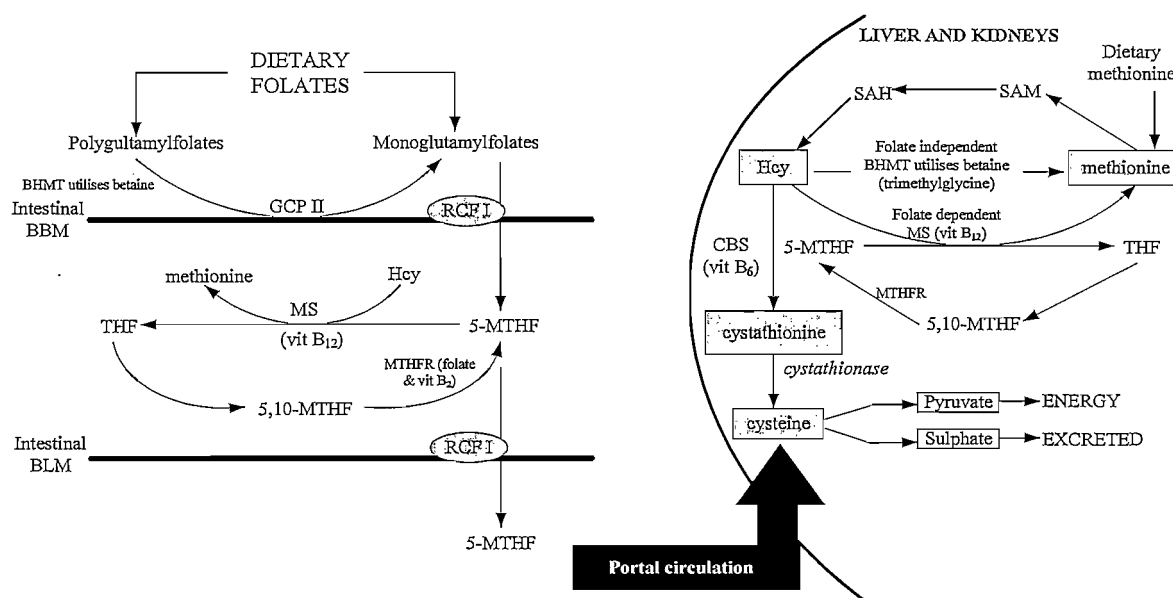


Figure 2.1 Homocysteine and folate metabolism [adapted from Devlin *et al.* (2006)]

BBM = brush border membrane; BHMT = betaine-homocysteine methyltransferase; BLM = basolateral membrane; CBS = cystathionine β -synthase; GCP II = glutamate carboxypeptidase II; Hcy = homocysteine; MS = methionine synthase; 5-MTHF = 5-methyltetrahydrofolate; 5,10-MTHF = 5,10-methylenetetrahydrofolate; MTHFR = methylenetetrahydrofolate reductase; RFC I = reduced folate carrier 1; SAH = s-adenosylhomocysteine; SAM = s-adenosylmethionine; THF = tetrahydrofolate; Vit = vitamin

In cells there are approximately 28 enzymes that can be identified as methyltransferases (Clarke & Banfield, 2001). Methyltransferases are involved in the donation (or transfer) of a methyl group from the activated form of methionine, namely s-adenosylmethionine, hereafter abbreviated as SAM, to the synthesis of thymine, choline, creatine, epinephrine, the protein 3-methylhistidine or for deoxyribonucleic acid (DNA) methylation (Scott, 2003). Methionine adenosyltransferase converts methionine to SAM by a reaction that includes the addition of a methyl group and the purine base adenine [from adenosine triphosphate (ATP) or diphosphate (ADP)]. According to Clarke and Banfield (2001), when SAM donates a methyl group, it is converted to s-adenosylhomocysteine (SAH). All SAM-dependent methyltransferase reactions result in the production of SAH, which can be catabolised immediately *in vivo* by SAH hydrolase to produce the nucleotide adenosine and Hcy (Scott, 2003; Williams & Schalinske, 2007). While the accumulation of SAH might in itself impair cellular function by inhibiting methyltransferase enzymes, thereby preventing the repair of aged or damaged cells (Coppola *et al.*, 2000), Hcy with its reactive free thiol group, could also be toxic to cells in its own right (Scott, 2003). Scott (2003) articulated that methyltransferase activity is responsible for the balance between the concentration of the methyl donor SAM and the concentration of the product SAH and that these activities are regulated by potent feedback inhibition mechanisms.

Hcy can follow three possible pathways within cellular metabolism. First, Hcy may be remethylated back to methionine by either folate-dependent or folate-independent mechanisms (Williams & Schalinske, 2007). During the folate-dependent remethylation, methionine synthase (MS) uses a methyl group from 5-methyltetrahydrofolate (5-MTHF) while methylcobalamin (the biologically active form of vitamin B₁₂) acts as the coenzyme. The methyl group for this reaction is produced by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR). MTHFR in turn uses flavin adenine dinucleotide (the biologically active form of vitamin B₂) as cofactor (Guenther *et al.*, 1999). In an alternative folate-independent remethylation route, the enzyme betaine-homocysteine methyltransferase (BHMT) catalyses the remethylation of Hcy using betaine (trimethylglycine which is a generator of methionine and SAM), a methyl group donor derived from choline oxidation, to convert Hcy to methionine and produce dimethylglycine as a byproduct.

BHMT catalyses the synthesis of methionine from betaine and Hcy, using a zinc ion to activate Hcy (Evans *et al.*, 2002). SAM-dependent transmethylation occurs in nearly all tissues, but the transsulphuration pathway and the remethylation of Hcy by BHMT are tissue specific, restricted to the liver and kidneys (Williams & Schalinske, 2007). SAM seems to function as a switch between the methionine cycle and the transsulphuration pathway. When SAM concentrations become limited, SAH as well as Hcy concentrations increase and there is an accompanying reduction in the methylation cycle (Finkelstein, 2001). High SAM concentrations, seem to limit Hcy remethylation by inhibiting MTHR and betaine methyltransferase. Transsulphuration seems to be enhanced by the stimulatory effect of SAM on CBS activity (Finkelstein, 2001).

Secondly, Hcy can be catabolised further to Cys by transsulphuration using pyridoxal 5'-phosphate (the biologically active form of vitamin B₆) as a cofactor, beginning with the irreversible conversion to cystathionine by cystathionine β -synthase (CBS). The enzyme γ -cystathionase then uses cystathionine to form Cys, which is required for the synthesis of many compounds, including glutathione, or can be further converted to pyruvate which can be used for energy and sulphate which is excreted through urine (Scott, 2003). While the transsulphuration pathway contributes to the maintenance of normal postprandial Hcy concentrations, the remethylation pathway maintains normal fasting Hcy concentrations (Rosenblatt, 1989).

Thirdly, excess Hcy can be exported from the cell into the circulation thereby regulating the intracellular Hcy concentrations (Finkelstein, 2001; Scott, 2003). Earlier studies seemed to indicate that the normal reference range for plasma tHcy was between 5 to 15 $\mu\text{mol/L}$ (to convert tHcy concentration to $\mu\text{g/mL}$ multiply by 0.1352), mild to moderate HHcy between 16 to 100 $\mu\text{mol/L}$ and severe HHcy $> 100 \mu\text{mol/L}$ (Eikelboom *et al.*, 1999; Malinow *et al.*, 1999). According to Ubbink and Delport (2000), a universally accepted reference range for tHcy does not exist for it cannot exist without proper standardisation of the Hcy analytical method. In 2007, Castañón *et al.* calculated that tHcy concentrations of $> 12 \mu\text{mol/L}$ significantly increased the risk of developing venous thrombosis. Since the definition of elevated circulating tHcy

concentrations should ideally be based on a health outcome, they proposed the use of 12 $\mu\text{mol/L}$ as a cutoff value of HHcy with clinical significance relating to CVD.

2.3 HOMOCYSTEINE AND CARDIOVASCULAR DISEASE

The ‘Hcy theory’ of CVD started with the observations of McCully (1969), but has been met with skepticism (Larkin, 1998). Mudd *et al.* (1964) observed that a deficiency of CBS resulted in Hcy accumulation in blood as well as in urine (homocystinuria). Shortly after this discovery, McCully (1969) reported that a MS deficiency also resulted in homocystinuria and that a patient with CBS deficiency had arterial damage comparable to a patient with MS deficiency. Since both abnormalities only shared the accumulation of Hcy, McCully (1969) postulated that Hcy itself or one of its derivatives, was culpable for the arterial damage and resultant premature CVD.

Wilcken and Wilcken (1976) were the first to test the above hypothesis and noted that patients with CVD suffered more often from abnormal Hcy metabolism than control individuals. Many studies have investigated the role of elevated Hcy concentrations in CVD development following these findings. Epidemiological, genetic, Hcy-lowering intervention trials and basic research have been conducted and have given insight into whether the Hcy concentration is a marker of disease, a predisposing risk factor or a direct cause of CVD. In the subsequent subsections these studies will be discussed as well as their contribution to the body of evidence implicating Hcy in CVD risk.

2.3.1 Evidence from observational epidemiological data

Data from cross-sectional studies strongly allude to an association between plasma Hcy concentrations and CVD (Clarke *et al.*, 1991; Graham *et al.*, 1997; Selhub *et al.*, 1995). Inconsistent results have been reported from prospective observational studies, with some reporting highly significant results (Stehouwer *et al.*, 1998) whereas others indicate no significant relationship between tHcy and CVD (Alfthan *et al.*, 1994).

The latter study which presented no significant association only included 265 CVD cases and, therefore, had limited statistical power (Alfthan *et al.*, 1994). Meta-analyses of prospective epidemiological studies, which provided more statistical power, indicated that elevated Hcy concentrations preceded the development of CVD, thus suggesting causality (Bautista *et al.*, 2002; Boushey *et al.*, 1995; Verhoef & Stampfer, 2001; Wald *et al.*, 2002). Recently, Humphrey *et al.* (2008) conducted a meta-analysis incorporating 26 articles using a random-effects model to determine the summary estimates of the risk of coronary heart disease (CHD) associated with each 5 $\mu\text{mol/L}$ increase in Hcy concentration. Their results yielded a combined risk ratio for coronary events of 1.18 [95% confidence interval (CI): 1.10 - 1.26] for each increase of 5 $\mu\text{mol/L}$ in Hcy concentration and each increase of 5 $\mu\text{mol/L}$ increased the risk of CHD events by approximately 20%, independent of traditional CHD risk factors.

Results from prospective studies seem to be generally weaker when compared to those of retrospective and cross-sectional studies (Boushey *et al.*, 1995; Christen *et al.*, 2000; Homocysteine Studies Collaboration, 2002). However, in a systematic review by Danesh and Lewington (1998), the relative risk (RR) for prospective and retrospective studies was calculated to be 1.6 (95% CI: 1.4 - 1.7) and 1.3 (95% CI: 1.1 - 1.5), respectively. Several theories exist to explain the differences in the associations between Hcy and CVD risk between the different epidemiological studies. Most explanations pertain to the chronological sequences of collecting data, including blood samples, on the exposures and the occurrence of the CVD event (*vide infra*).

Cross-sectional studies assess exposure and CVD at the same point in time (IFIC, 2001). Therefore, cross-sectional studies cannot distinguish between whether the exposure preceded the CVD event or whether the disease somehow affected the exposure.

In prospective studies, the exposure status of healthy individuals is assessed at the commencement of the study. The participants are followed over a period of time to observe the potential effect of the exposure *i.e.* the occurrence of CVD (IFIC, 2001). Healthy individuals are compared to those who developed CVD over the follow-up

period. In prospective studies, participants need not recall their habits of the past as exposure data are collected before the CVD event, therefore, limiting the influence of the disease on lifestyle, dietary habits and blood parameters (De Bree *et al.*, 2002; IFIC, 2001). Prospective studies have the best study design to detect associations between exposures and health outcome, as this design has various advantages that include limited bias over that of retrospective studies (De Bree *et al.*, 2002; IFIC, 2001).

In retrospective studies, the exposure data and blood samples are collected after a CVD incident. This is a major disadvantage as these studies cannot distinguish between whether elevated Hcy is a cause or a consequence of CVD events. tHcy elevation seems to occur following tissue injury in myocardial infarction and stroke (Egerton *et al.*, 1996; Landgren *et al.*, 1995; Lindgren *et al.*, 1995). The elevated Hcy concentrations observed in atherosclerotic patients are often associated with standard vascular risk, including early decline in renal function, which is common in atherosclerosis patients (Brattström & Wilcken, 2000). Decline in renal function on its own, causes elevated Hcy and Cys (Brattström & Wilcken, 2000). Therefore, the higher tHcy concentrations observed among cases in case-control studies could be the result rather than the cause of acute CVD events, therefore, reflecting reverse causality bias. This could possibly explain why the risk estimates of retrospective and cross-sectional studies are generally higher than those of prospective studies.

Another disadvantage of retrospective studies is that the recall of exposures in the past could be influenced by poor memory. The possibility that certain exposures such as lifestyle habits changed in individuals from the time when the disease was diagnosed until measurement of exposures cannot be ruled out. Diagnosis of the CVD event might, therefore, distort the recall of certain lifestyle and dietary habits (De Bree *et al.*, 2002). Medical treatments including lipid-lowering drugs, anti-hypertensive therapies and the proscription of smoking may also have altered the levels of CVD risk factors as well as tHcy concentrations after diagnosis. However, diagnosis of the disease and/or medical treatment that modify/ies the levels of CVD risk factors will also affect risk estimates of retrospective and cross-sectional studies (De Bree *et al.*, 2002).

According to De Bree *et al.* (2002), the duration of follow-up and the inherent risk of the chosen population (*inter alia* age > 60 years, pre-existing diseases including CVD, diabetes and renal insufficiency) must also be considered. In prospective studies, older subjects as well as subjects with preclinical disease will, in general, die earlier than those without preclinical disease.

The association between the tHcy concentration and CVD risk (Stehouwer *et al.*, 1998) or total CVD mortality (Kark *et al.*, 1999) was the strongest in the first few years of follow-up. Studies with a shorter follow-up period (< 5 years) in general revealed more statistically significant associations between the tHcy concentration and the risk of CVD, attesting to the probability that tHcy concentration might be a short-term risk factor (De Bree *et al.*, 2002). After 5 years of follow-up in the Physicians' Health Study, a significant increased risk of CHD was reported in men with elevated tHcy concentrations (Stampfer *et al.*, 1992), but when extending the follow-up to 7.5 years it yielded a non-significant RR (Chasan-Taber *et al.*, 1996) and further extension to 9 years resulted in no significant association between the tHcy concentration and the risk of angina pectoris in this well-nourished population (Verhoef *et al.*, 1997).

An additional feature of prospective studies with longer follow-ups is that a reduction in the risk estimate may occur as a result of changes in diet, lifestyle or medical treatment during the follow-up (De Bree *et al.*, 2002). These factors may alter the tHcy concentration in such a way that the baseline tHcy concentration is no longer representative of the concentration at the time of the CVD event (De Bree *et al.*, 2002).

Due to a combined effect of measurement errors and intra-individual variation, the 'typical' concentration of tHcy that is related to the risk of CVD might be difficult to determine with a single tHcy measurement. This bias can be estimated and corrected for by collecting more blood samples over the period of follow-up and determining several tHcy values (Clarke *et al.*, 2001). In addition, the intra-individual variability in tHcy measurements may dilute the association of 'usual' concentrations of tHcy with CVD risk, referred to as 'regression dilution' (Clarke *et al.*, 2001). Failure to correct for increasing regression dilution by using lower regression dilution ratios for longer

follow-up periods may underestimate the RR of CVD associated with tHcy by about one-fifth after 2 years and one-half after 10 years (Clarke *et al.*, 2001).

In elderly populations, the number of subjects with silent preclinical CHD and existing risk factors will be larger than in adult populations, therefore prospective studies performed in selected high-risk populations will consistently report that the tHcy concentration is a strong predictor of CVD mortality and morbidity (De Bree *et al.*, 2002). However, in the meta-analysis conducted by Bautista *et al.* (2002) on prospective studies, it was revealed that HHcy moderately increased the risk of a first CVD event, regardless of age and follow-up duration. The recent meta-analysis of Humphrey *et al.* (2008) also described that the association between Hcy and CHD was similar when analysed by gender, length of follow-up, outcome, study quality and study design.

Associations reported in observational studies between elevated tHcy concentrations and CVD could be an epiphenomenon *i.e.* a coincidental observation. This hypothesis has, however, been refuted given the large number of studies presenting with an association (Scott, 2003).

More epidemiological evidence, including longitudinal tracking of Hcy concentrations, needs to be collected to understand the influence of Hcy concentrations on CVD. Such trials must have sufficient sample sizes to allow conclusions to be drawn, bearing in mind the likely risk reduction in CVD that lower plasma Hcy concentrations would be expected to induce.

2.3.2 Evidence of homocysteine predicting future cardiovascular events in cardiovascular patients

Plasma Hcy concentration strongly predicts future mortality in patients with angiographically established CVD and, therefore, has prognostic value (Nygård *et al.*, 1997). Stehouwer *et al.* (1998) reported Hcy concentration to be more strongly associated with the recurrence of a CVD event than with the first episode of

myocardial infarction. Similar results have recently been reported by Zhang *et al.* (2010) for the risk of cerebrovascular accident recurrence and mortality in Chinese patients with pre-existing cerebrovascular disease. However, Vollset *et al.* (2001) observed that after stratification for high and low baseline risk (high risk was defined as a history of myocardial infarction, stroke, angina pectoris, diabetes or hypertension), the tHcy concentration was only a significant risk factor for cardiovascular mortality in high-risk persons.

2.3.3 Evidence from genetic studies

If raised tHcy truly increased the risk for CVD, inborn errors of Hcy metabolism should themselves be related to CVD risk proportional to the difference in tHcy concentration attributable to the genetic variant (Smith & Ebrahim, 2003). Moreover, genetic variations of the enzymes that metabolise Hcy are fixed characteristics that cannot be altered by disease development. Regression dilution bias can also be ruled out in studies investigating the association of genetic variations and CVD (Ebrahim & Smith, 2008). Consequently, genetic association with CVD (Mendelian randomisation) would permit to imply causality. However, considering the genetic basis of CVD, it is important to keep in mind that it is not the disease *per se* that is genetically determined, but rather the susceptibility to the disease (Gelehrter *et al.*, 1998). Therefore, genetically susceptible individuals may or may not develop CVD, depending on the interaction of various genetic and environmental factors, including dietary habits (Gelehrter *et al.*, 1998).

There is compelling evidence that severe elevations of plasma Hcy (200 - 300 $\mu\text{mol/L}$) reported in inborn errors of metabolism, do cause CVD. When patients with a genetically determined CBS deficiency (Mudd *et al.*, 1985; Wilcken & Wilcken, 1997), MTHFR deficiency (Rosenblatt, 1989) or defects in cobalamin metabolism (McCully, 1969) are left untreated they suffer from CVD at a very young age.

It is not yet clear whether moderate increases in tHcy cause CVD. Moderate increases can occur *inter alia* as a result of a point mutation in the gene coding for the enzyme

MTHFR in which cytosine (C) is replaced by thymidine (T) at base position 677 of the gene. The 677 TT genotype leads to an approximately 20% higher Hcy concentration compared with 677 CC subjects, due to reduced MTHFR activity (Brattström *et al.*, 1998). The C677T genetic variant was extensively studied in relation to CVD. However, this genotype has not consistently been associated with CVD (Brattström & Wilcken, 2000; Castañon *et al.*, 2007; Kluijtmans *et al.*, 1997). This produces evidence disproving the hypothesis, but this lack of association could also be attributed to the small sample sizes used in most studies which would have resulted in a lack of statistical power (Blom & Verhoef, 2000). The results could, therefore, be false negatives (Kothekar, 2007). An earlier meta-analysis on this subject also lacked sufficient power to reach statistically significant results and concluded that mild HHcy was not causally related to the pathogenesis of vascular disease (Brattström *et al.*, 1998). The meta-analyses of Wald *et al.* (2002), which included studies investigating the MTHFR gene as well as prospective studies of Hcy and disease events, and that of Casas *et al.* (2005) using the principle of mendelian randomisation to determine causality had adequate statistical power to detect moderate changes in Hcy concentration and its association with CVD risk. Results from both the genetic and prospective studies indicated a significant association between Hcy and CVD. Since genetic and prospective studies do not share the same potential sources of error, the findings provide strong evidence to support a causal relationship between Hcy and CVD. Ueland *et al.* (2000) opined that the TT genotype may even modulate CVD risk independently of Hcy, but this has not been confirmed.

2.3.4 Intervention trials lowering homocysteine concentrations

The 'Hcy theory' placed the genesis of CVD in the context of nutritional deficiency. The question of whether Hcy is a risk marker, a predisposing factor or a risk factor could also be resolved by properly conducted placebo-controlled intervention trials with Hcy-lowering B-vitamins and clinical endpoints. If increased CVD were due to a direct effect of elevated Hcy, it would be expected that protocols that lower tHcy, would ameliorate the risk. However, since some data suggest that the B-vitamins could be associated with CVD and vascular function independent of Hcy (Doshi *et al.*, 2002),

researchers must investigate to what extent reduced Hcy *per se* could account for the clinical and vascular effects.

There is ample evidence from studies that have investigated the intermediary stages of CVD or surrogate markers, that B-vitamins reduce risk of CVD. Various trials have indicated that folic acid supplementation, with or without vitamin B₆ and B₁₂, to be associated with improved blood vessel function (Brown & Hu, 2001; Moat *et al.*, 2004). In addition, reduced thickness of the carotid artery wall was observed in people at high risk of CVD after supplementation with B-vitamins (Till *et al.*, 2005).

Several studies have investigated CVD as an endpoint after fortification or supplementation with B-vitamins. The Food and Drug Administration mandated the fortification of food products with folic acid in America in 1998 (Anderson *et al.*, 2004). After implementation of the fortification rule, median tHcy declined and it was reported to be an independent, graded risk factor for mortality (adjusted RR = 1.03 per $\mu\text{mol/L}$; 95% CI: 1.01 - 1.05; $p = 0.006$). Treating patients with inherited CBS deficiency with Hcy-lowering nutrients seems to prevent vascular events (Kluijtmans *et al.*, 1999; Yap *et al.*, 2000). Albert *et al.* (2008) investigated the effect of folic acid, vitamin B₆ and B₁₂ on CVD risk and mortality among 5,442 high-risk women in a randomised, double-blind, placebo-controlled trial over 7.3 years and stated that the vitamins did not reduce total CVD events, despite significant Hcy-lowering. The Vitamins in Stroke Prevention trial reported that lowering tHcy by 2 $\mu\text{mol/L}$, with high-dose B-multivitamin therapy, failed to prevent recurrent stroke in 3,680 patients with recent ischaemic stroke (Toole *et al.*, 2004). However, the Vitamins in Stroke Prevention study was statistically under-powered and the results could have been a false negative. The possibility that the B-multivitamin therapy reduced the RR of stroke could, therefore, not be excluded (Hankey & Eikelboom, 2004). In a meta-analysis of randomised controlled trials Mei *et al.* (2010) evaluated the effects of Hcy-lowering on the risk of cardiocerebrovascular events and all-cause mortality among individuals with preexisting cardiocerebrovascular disease. They concluded that supplementation with folic acid as

a Hcy-lowering agent was not recommended for the secondary prevention of cardiocerebrovascular diseases.

The randomised trials of dietary supplementation with B-vitamins to lower tHcy have not yet provided clear evidence of benefit on vascular risk or secondary prevention. Reliable evidence will accrue from ongoing randomised trials as well as from meta-analyses of randomised trials that will maximise the power to assess the epidemiologically predicted differences in risk (Clarke *et al.*, 2007). However, homozygosity for MTHFR C677T is the most common genetic cause of HHcy and it is responsive to dietary folate supplementation, however, other causes of HHcy might not respond to folate or other B-vitamin supplementation, or might respond without altering the root cause of CVD risk (Matthews & Elmore, 2007).

2.3.5 Evidence that homocysteine augments the risk associated with established risk factors

Hcy powerfully increased the CVD risk associated with smoking and hypertension, thereby giving evidence that Hcy could augment the effect of other important CVD risk factors (Graham *et al.*, 1997). Hcy also increases the atherosclerotic properties of LDL and lipoprotein(a), hereafter abbreviated as Lp(a). Refer to Sections 2.3.6.14 and 2.3.6.15 for a complete review on the influence of Hcy on LDL and Lp(a), respectively. This evidence attests to Hcy being a predisposing risk factor as it is presumed to impact on other risk factors known to act directly on CVD development.

2.3.6 Evidence of possible mechanisms implicating homocysteine as a risk factor

Numerous mechanisms exist through which the accumulation of Hcy could result in a series of events that culminate in CVD. Many of these mechanisms involve the chemical structure of Hcy. Scott (2003) suggested that Hcy could not have a direct extracellular toxic effect by arguing that while very high Hcy concentrations could reach toxic levels, the reported Hcy concentrations associated with CVD are not that high (Castañon *et al.*, 2007) and, therefore, a direct toxic effect is not easy to envisage. It is more likely that plasma Hcy serves to increase the intracellular concentrations of Hcy and that this either has direct toxic effects inside the cells or the

toxicity results from a concomitant rise in SAH, which may be an actual risk factor (Scott, 2003).

Vascular endothelial cells play a crucial role in regulating and maintaining vascular health. The endothelium possesses numerous properties that are essential for the haemostatic processes of cell adhesion and migration, coagulation and fibrinolysis (Colman *et al.*, 2000). According to Colman *et al.* (2000), endothelial cells maintain a non-thrombogenic surface *via* the glycosaminoglycan-anti-thrombin III (AT III) and thrombomodulin-protein C anti-coagulant pathways and the inhibition of platelet activation by ecto-adenosine diphosphatase (ecto-ADPase), prostacyclin and nitric oxide (NO). The endothelium also synthesises clotting factors and, therefore, helps to balance pro-coagulant and anti-coagulant mechanisms in order to maintain haemostasis. The 'response to injury' hypothesis proposes that the primary event in atherogenesis is endothelial damage and dysfunction which disrupts these properties resulting in platelet and leukocyte adhesion, thrombosis, smooth muscle proliferation, vasospasm, lipid accumulation and ultimately atheroma (Thambyrajah & Townend, 2000). A wealth of data has been accumulated which suggests that Hcy may upset these pathways mentioned and predispose to thrombogenesis *via* a number of mechanisms (*vide infra*). Eventhough these mechanisms are interlinked, for the sake of this discussion it was divided in several subdivisions.

2.3.6.1 Homocysteine and inflammation

Hcy is regarded as a marker of inflammation (Refsum *et al.*, 1998). Eghlim *et al.* (2006) revealed HHcy to induce an inflammatory response, which could lead to tissue injury in the pathogenesis of the atherosclerotic process. *In vitro* studies have demonstrated that Hcy enhances the production of several pro-inflammatory cytokines (Dalal *et al.*, 2003; Gori *et al.*, 2005; Su *et al.*, 2005) as well as increasing the expression of the monocyte chemoattractant protein-1, involved in the pathogenesis of atherosclerosis by promoting recruitment of inflammatory cells to the vessel wall, in cultured human vascular endothelial cells, smooth muscle cells and monocytes (Poddar *et al.*, 2001; Wang, 2001).

In vitro, Hcy leads to an increase in cell proliferation and cell death through apoptosis and necrosis in smooth muscle cells (Buemi *et al.*, 2001). The addition of folic acid to the culture medium significantly reduced both Hcy concentrations *in media* and the effects of Hcy on the proliferation/apoptosis/necrosis balance of cells in culture (Buemi *et al.*, 2001).

2.3.6.2 Homocysteine oxidative stress and damage

A key regulatory system of endothelial cells involves NO synthase, which synthesises the inorganic compound NO and citrulline from L-arginine. Endothelium-derived NO (eNO) is anti-thrombotic because it regulates vessel tone, inhibits platelet activation, adhesion and aggregation, limits smooth muscle proliferation and modulates endothelial leukocyte interaction (Thambyrajah & Townend, 2000). Brachial artery flow-mediated dilation is endothelium dependent and largely mediated by NO. Hcy reacts with NO to form S-nitroso-homocysteine, which possesses some of the properties of NO (Ignarro & Gruetter, 1980). S-nitroso-homocysteine inhibits platelet aggregation, is a potent vasodilator, it does not support hydrogen peroxide generation and does not undergo conversion to Hcy thiolactone (Stamler *et al.*, 1993). Hydrogen peroxide and Hcy thiolactone are reaction products believed to contribute to endothelial toxicity (Stamler *et al.*, 1993). Therefore, S-nitroso-homocysteine represents a protective mechanism against the damaging effects of Hcy. However, prolonged exposure (Stamler *et al.*, 1993) and high Hcy concentrations (Upchurch *et al.*, 1996) impair NO production, thus, chronically elevated Hcy appears to result in a self-perpetuating cycle that progressively overwhelms the capacity of endothelial cells to reduce the toxicity of Hcy (Thambyrajah & Townend, 2000). Hcy also reduces NO activity by oxidative degradation (Jia & Furchgott, 1993; Starkebaum & Harlan, 1986) and by inhibiting glutathione peroxidase (Loscalzo, 1996; Upchurch *et al.*, 1997).

In vivo studies have demonstrated impairment of vascular NO activity by Hcy, probably due to oxidative stress. L-arginine seems to reduce mean blood pressure and lowers blood viscosity when infused in healthy subjects (Bode-Böger *et al.*, 1994). These responses were impaired in healthy volunteers by acute HHcy induced by methionine loading, an effect prevented by the co-administration of the anti-oxidant

vitamins (Nappo *et al.*, 1999). Inducing high Hcy concentrations by oral methionine loading caused transiently impaired flow-mediated vasodilatation in healthy subjects (Bellamy *et al.*, 1998; Chambers *et al.*, 1998). This *in vivo* evidence supports the view that Hcy results in endothelial dysfunction. This decreased flow-mediated vasodilatation was prevented by both pre-treatment with vitamin C (Chambers *et al.*, 1999) and folic acid (Usui *et al.*, 1999). It seems as though the anti-oxidant vitamins and folic acid can prevent Hcy-induced endothelial dysfunction by modulating cellular oxidative metabolism. Additionally, folic acid supplementation may be protective by stimulating NO production (Verhaar *et al.*, 1998). Hcy also leads to endothelial cell dysfunction by decreasing eNO bioavailability (Moat *et al.*, 2004). Extracellular matrix remodelling activates redox sensitive matrix metalloproteinases and decreases bioavailability of eNO which results in extracellular matrix fibrosis. Endothelial dysfunction may contribute to vasospasm, thrombosis and progression of atherosclerosis (Thambyrajah & Townend, 2000).

The oxidative stress induced by increased Hcy concentrations may be a key process in the pathogenesis of thrombosis in HHcy. Hcy might exert its harmful effects through mechanisms involving oxidative damage (Domagala *et al.*, 1997). The pro-oxidant nature of Hcy causes oxidative damage to the endothelium, thus altering the coagulant properties of the blood and impairing endothelium-dependent vasomotor regulation (Hankey & Eikelboom, 2001). In addition, Hcy seems to act as a pro-oxidant by inducing the formation of free radicals which facilitate oxidative arterial injury, thereby damaging the vascular matrix and augmenting the proliferation of vascular smooth muscle which forms the inner layer of blood vessels (Scott, 2003). Damage to these cells increases vulnerability to plaque build up, increases the tendency towards blood coagulation, reduces blood vessel function and increases blood vessel stiffness (Splaver *et al.*, 2004).

Endothelial glutathione peroxidase catalyses the reduction of both hydrogen and lipid peroxides to their corresponding alcohol (Freedman *et al.*, 1996; Loscalzo, 1996) and represents a mechanism for cellular defense against oxidative stress. It also prevents the oxidative inactivation of NO (Upchurch *et al.*, 1997). The activity of glutathione peroxidase in bovine aortic endothelial cells was reduced by up to 81% by Hcy. There was a parallel reduction in glutathione peroxidase messenger ribonucleic acid

levels, suggesting that the mechanism of action involves the suppression of the cellular expression of glutathione peroxidase (Upchurch *et al.*, 1997). Hcy is the only thiol with the ability to inhibit glutathione peroxidase activity *in vitro* and this may serve to explain its greater toxicity compared to Cys, which is also capable of generating free radicals and is present in serum at concentrations four times higher than Hcy (Upchurch *et al.*, 1997).

2.3.6.3 Homocysteine and intima-media thickness

Hcy appears to be associated with an increase in intima-media thickness (Adachi *et al.*, 2002; Durga *et al.*, 2004; Kelemen *et al.*, 2004; Malinow *et al.*, 1993). Contrastingly, in an Australian Aboriginal community, carotid intima-media thickness was not related to Hcy concentration (McDonald *et al.*, 2005).

Vascular intimal smooth muscle cell proliferation with subsequent formation of extracellular matrix collagen is an integral component of atherosclerosis (Ross, 1993). This process may be directly stimulated by Hcy or may be secondary to the mitogenic effect of the endothelial and/or platelet-derived growth factors released by Hcy-induced endothelial cell damage. Elevated Hcy significantly increases collagen production in a dose and time-dependent manner in smooth muscle cells (Majors *et al.*, 1997) as well as in vascular smooth muscle cells (Tyagi, 1998). Areas enriched in collagen correlate with increased platelet deposition (Van Zanten *et al.*, 1994). High Hcy concentrations increased DNA synthesis in both rat and human aortic smooth muscle cells and this was subsequently followed by proliferation (Tsai *et al.*, 1994; Tsai *et al.*, 1996). The same concentration of Hcy also resulted in a reduction of DNA synthesis in human umbilical vein endothelial cells (Tsai *et al.*, 1994). Smooth muscle cells are less susceptible to Hcy-induced damage compared to endothelial cells (Starkebaum & Harlan, 1986; Wall *et al.*, 1980) and these contrasting effects on the endothelial and smooth muscle cells may be an important mechanism in the development of atheroma.

2.3.6.4 Homocysteine and platelets (thromboxane)

The effects of Hcy on platelet function are not yet clear, but Hcy might increase thromboxane A₂ biosynthesis (Di Minno *et al.*, 1993; Durand *et al.*, 1997) and stimulate platelet aggregation (Durand *et al.*, 1997; Graeber *et al.*, 1982). During the auto-oxidation of Hcy in plasma, reactive oxygen species are generated which initiate lipid peroxidation in circulating lipoproteins, including LDL and arachidonic acid, which might trigger platelet activation as well as some of the haemostatic abnormalities reported in HHcy patients (Coppola *et al.*, 2000; Davì *et al.*, 2001).

An increased urinary excretion of a thromboxane metabolite was discovered in homocystinuric patients when compared to healthy controls (Di Minno *et al.*, 1993). Since thromboxane is a vasoconstrictor and thrombogenic agent, *in vivo* Hcy also seems to promote vasoconstriction and platelet activation.

Platelet activation results in thrombosis and smooth muscle proliferation and is, therefore, important in the pathogenesis of atherothrombosis (Ross, 1993). Ecto-ADPase catalyses the breakdown of the nucleotide ADP, which is the mediator responsible for the final common pathway of platelet activation. High Hcy concentrations have been reported to inhibit the ecto-ADPase activity of human umbilical vein endothelial cells, thereby decreasing the capacity of the endothelium to degrade ADP and thereby modulating platelet reactivity (Broekman *et al.*, 1994; Harpel *et al.*, 1996).

2.3.6.5 Homocysteine and adenosine

Another mechanism by which Hcy could induce CVD could be related to the ratio of the adenine nucleotide to adenosine, since extracellularly these nucleotides are associated with the modulation of processes such as platelet aggregation, vasodilation and coronary flow (Kahner *et al.*, 2006). Several authors have described the important role of these nucleotides in the process of haemostasis and thrombus formation (Born & Cross, 1963; Coade & Pearson, 1989; Kahner *et al.*, 2006). Hcy and adenosine are simultaneously produced *via* hydrolysis of SAH.

ATP and its breakdown products have been suggested to play a role in vascular tone, cardiac function and renal epithelial transport (Ralevic, 2000). Micromolar concentrations of ATP inhibit platelet aggregation, however, low concentrations are stimulatory (Soslau & Youngprapakorn, 1997). ADP is reported to induce changes in platelet shape and aggregation (Kahner *et al.*, 2006). Adenosine inhibits platelet aggregation and is capable of acting as a vasodilator, which is an important effect to consider when investigating the role of adenosine as a cardioprotector. Hcy seems to decrease the plasma and tissue adenosine concentrations associated with inhibition of SAH hydrolase (Chen *et al.*, 2002) and this could contribute to the cardiovascular complications of HHcy. Böhmer *et al.* (2006) evaluated whether Hcy can participate in the modulation of the extracellular adenine nucleotide hydrolysis in rat serum and reported that Hcy, at final concentrations of 5.0 mM, inhibits *in vitro* ATP, ADP and adenosine monophosphate (AMP) hydrolysis by 26%, 21% and 16%, respectively. Hcy at a concentration of 8.0 mM, inhibited the *in vitro* hydrolysis of ATP, ADP and AMP by 46%, 44% and 44%, respectively.

2.3.6.6 Homocysteine and tissue factor

Tissue factor plays a role in the initiation of the coagulation cascade (Colman *et al.*, 2000). Hcy seems to increase the activity of tissue factor due to an increase in tissue factor gene transcription (Fryer *et al.*, 1993).

2.3.6.7 Homocysteine and factor V

After treatment with Hcy, bovine aortic endothelial cells and human umbilical vein endothelial cells exhibited enhanced factor V activity, thereby activating the coagulation cascade (Carmel & Jacobsen, 2001). This mechanism of action involves the induction of a protease that is an endothelial cell activator of factor V and offers a mechanism for the promotion of coagulation by Hcy in the absence of thrombin (Rodgers & Kane, 1986).

2.3.6.8 Homocysteine and protein C

Protein C is activated by the interaction with thrombin that is bound to the glycoprotein thrombomodulin. In the absence of complex formation with thrombomodulin, thrombin is ineffective in activating protein C. After activation, protein C cleaves activated factors V and VIII to dampen their coagulant activity (Colman *et al.*, 2000). Exposure of human and bovine endothelial cells to Hcy resulted in a reduction of activated protein C (Rodgers & Conn, 1990). Hcy seems to inhibit the anti-coagulant pathway of protein C, presumably due to an interference in the thrombin pathway and the inhibition of cell surface expression of thrombomodulin (Lentz & Sadler, 1991; Rodgers & Kane, 1986).

2.3.6.9 Homocysteine, thrombin and thrombomodulin

Thrombomodulin binds thrombin and inhibits the ability of this enzyme to cleave fibrinogen and activate platelets and factors V and VIII, thereby regulating the coagulant activity of thrombin. After complex formation with thrombomodulin, thrombin fails to clot fibrinogen, aggregate platelets or be inhibited by AT III (Colman *et al.*, 2000). Hcy retards the thrombin cofactor activity of thrombomodulin, by affecting thrombomodulin glycosylation, thus preventing the expression of the thrombomodulin protein on the cell surface, as well as directly inactivating thrombomodulin (Lentz & Sadler, 1991).

2.3.6.10 Homocysteine and anti-thrombin III

AT III is a plasma protein that in the presence of heparin becomes a potent anti-coagulant by inhibiting the serine proteases involved in coagulation (Colman *et al.*, 2000). Endothelial cell surface heparin glycosaminoglycan, binds AT III and also enhances its anti-thrombin activity. However, Hcy reduces heparan sulphate proteoglycans that modulate AT III activity by decreasing the binding of AT III to the cells mediated by the generation of hydrogen peroxide through the alteration of the redox potential in the cells (Nishinaga *et al.*, 1993).

2.3.6.11 Homocysteine, pro-thrombin fragments 1 and 2, thrombin-anti-thrombin complex and fibrin degradation products

Klerk *et al.* (2002) examined the effect of lowering Hcy concentrations with B-vitamin supplementation (5 mg folic acid, 0.4 mg hydroxycobalamin and 50 mg pyridoxine daily for 8 weeks) on prothrombin fragments 1 and 2 (F1 + 2), thrombin-anti-thrombin complex (TAT) and fibrin degradation products (D-dimer). Although Hcy concentrations were significantly reduced in the B-vitamin group compared to the placebo group, no effect on F1 + 2 or TAT concentrations was observed. However, a 10.4% reduction was observed for D-dimer levels ($p = 0.08$). Therefore, it was concluded that in healthy subjects Hcy reduction by B-vitamin supplementation has a modest beneficial effect on haemostasis (Klerk *et al.*, 2002).

2.3.6.12 Homocysteine and fibrin

Metabolites such as Hcy can react with proteins (homocysteinylation) and thereby cause covalent modification (Hoffman, 2008). When fibrinogen is affected during such modifications it could be altered (Hoffman, 2008). Homocysteinylation results in clots that are composed of thinner more tightly-packed fibres and this increases clot resistance to fibrinolysis in rabbits (Sauls *et al.*, 2003; Sauls *et al.*, 2007). Elevated tHcy concentrations in human subjects have been reported to correlate with increased resistance of clots to fibrinolysis (Undas *et al.*, 2006). However, whether the above contribute to increased atherothrombotic risk must still be determined.

2.3.6.13 Homocysteine and prostacyclin

Prostacyclin production was inhibited by Hcy *ex vivo* in rat thoracic aorta (Panganamala *et al.*, 1986). The physiological relevance of these findings to humans is dubious as they are based on the effects of extremely high Hcy concentrations in animal tissue. Others, using human tissue have failed to detect any *in vitro* effect of Hcy or serum from homocystinuric patients, on prostacyclin production at lower Hcy concentrations (Graeber *et al.*, 1982; Wang *et al.*, 1993). It should be kept in mind that patients have a lifetime exposure to Hcy as opposed to the limited exposure period of the *in vitro* experiments. Consequently this may restrict the amount of

damage generated by Hcy underestimating the pathophysiology of HHcy (De Bree *et al.*, 2002).

2.3.6.14 Homocysteine and low-density lipoprotein

Acute methionine load-induced HHcy potentiated the platelet aggregation in response to thrombin and ADP as well as the thrombin-induced thromboxane synthesis (Durand *et al.*, 1997). In addition, Durand *et al.* (1997) revealed that it also stimulated the basal and lipopolysaccharide-induced tissue factor activity of peritoneal macrophages. All these prothrombotic effects were associated with an increased lipid peroxidation characterised by an elevation of plasma conjugated dienes, lipid hydroperoxides and thiobarbituric acid-reactive substances (Durand *et al.*, 1997).

Metal-catalysed (iron and copper) LDL oxidation is enhanced by the presence of Hcy (Hirano *et al.*, 1994; Pfanzagl *et al.*, 2003). Using the methionine loading test, Domagala *et al.* (1997) created an experimental model, which allowed them to assess oxidative stress *in vivo*. They reported a significant elevation in thiobarbituric acid reactive substances, four and six hours following the methionine loading test, which correlated with raised Hcy plasma concentrations. This study points to an association between HHcy and increased lipid peroxidation, which might be relevant to the development of atherosclerosis (Domagala *et al.*, 1997). During the auto-oxidation of Hcy in plasma, reactive oxygen species are generated which could initiate lipid peroxidation, both at the endothelial cell surface (potentially responsible for endothelial dysfunction) and within the lipoprotein particles in plasma (Schöneich *et al.*, 1992). Oxidised LDL may trigger platelet activation as well as some of the haemostatic abnormalities reported in such patients. According to Jackson *et al.* (2000), oxidised LDL has been determined to accelerate several steps in atherosclerosis, including endothelial damage, monocyte/macrophage recruitment, and increased uptake of LDL by foam cells, alteration in vascular tone, induction of growth factors and formation of autoantibodies to oxidised LDL. Thus the oxidative stress induced by Hcy may be a key process in the pathogenesis of thrombosis in HHcy (Coppola *et al.*, 2000).

2.3.6.15 Homocysteine and lipoprotein(a)

Lp(a) is an atherogenic lipoprotein which reduces fibrinolysis by competitively inhibiting the binding of plasminogen to endothelial cells, fibrin, fibrinogen and fibrin fragments, mononuclear cells and platelets, due to its structural homology with plasminogen (Harris, 1997). Hcy seems to increase the binding of Lp(a) to fibrin, thus reducing the activation of plasminogen. It also enhances the tissue factor stimulating activity of Lp(a), thereby increasing its thrombotic and atherogenic activities, especially when Lp(a) levels are elevated (Harpel *et al.*, 1992). Free apo(a) more readily inhibits the fibrinolytic system than complete Lp(a) and Hcy is reported to release apolipoprotein(a) from Lp(a) when tHcy exceeds 22 $\mu\text{mol/L}$ (Herrmann *et al.*, 2000). An *in vivo* study carried out by Delport *et al.* (2004) has verified that HHcy in individuals with high-molecular weight apo(a) isoforms was associated with increased CVD risk while the risk in subjects with low-molecular weight apo(a) isoforms was not significantly increased.

2.4 SUMMARY AND CONCLUSIONS

This review provides overwhelming evidence of Hcy being either a risk marker, a predisposing risk factor or a risk factor as previously outlined within this review. Hcy, therefore, still stands accused of being involved in CVD and in view of the studies discussed, Hcy cannot be exculpated.

Several researchers view Hcy as being a risk marker that merely indicates CVD. This theory is based on the observations that tHcy elevation has been determined to occur following tissue injury in myocardial infarction and stroke (Egerton *et al.*, 1996; Landgren *et al.*, 1995; Lindgren *et al.*, 1995) and in atherosclerotic patients with precocious decline in renal function (Brattström & Wilcken, 2000). Thus, mild HHcy could be an effect rather than a cause of CVD. Other arguments for this theory are that the observed association between Hcy and CVD could be due to the confounding effects of factors associated with HHcy (*e.g.* cigarette smoking or an atherogenic diet high in methionine and/or low in B-vitamins). Therefore, tHcy concentrations could merely reflect the status of B-vitamins, which may be the factors truly involved in the

risk for CVD (Scott, 2003). However, after reviewing the literature this theory is not supported by the evidence in the view of this author.

Other researchers view Hcy as being a predisposing risk factor, since Hcy seems to augment the effect of other risk factors including LDL, Lp(a), smoking and hypertension (Graham *et al.*, 1997). The possibility that Hcy is a predisposing risk factor that is causally related to an as yet unknown risk factor for CVD could thus not be ruled out (Jansson, 2003).

It may be that the relationship between CVD and elevated Hcy concentrations is both strong and independent of other risk factors. The role of Hcy in CVD development could thus be directly due to the chemical characteristics of Hcy and through the copious pathological mechanisms through which Hcy could negatively influence cardiovascular health. However, inconsistent findings prevent total verification that an elevated tHcy concentration results in increased CVD risk (Boushey *et al.* 1995; Eikelboom *et al.*, 1999). Hcy may have an effect on the risk of CVD by stimulating pro-coagulant factors and/or impairing anti-coagulant mechanisms or fibrinolysis. Excess Hcy seems to induce the formation of free radicals, which affect the endothelial and smooth muscle cells that form the inner layer of blood vessels (Scott, 2003). Damage to these cells increases vulnerability to plaque build up, increases the tendency towards blood coagulation, reduces blood vessel function and increases blood vessel stiffness (Splaver *et al.*, 2004). The numerous pathological mechanisms attest to Hcy possibly being a risk factor. A cause-and-effect relationship between Hcy and CVD, therefore, cannot be ruled out. It is the opinion of the author that Hcy is both a predisposing risk factor and a causal risk factor in CVD development.

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CHAPTER 3

LITERATURE REVIEW

LIFESTYLE AND DEMOGRAPHIC DETERMINANTS OF HOMOCYSTEINE CONCENTRATIONS

3.1 INTRODUCTION

Cardiovascular disease (CVD) is one of the leading causes of death in the world (Murray & Lopez, 1997; Yusuf *et al.*, 2001). Elevated plasma concentrations of the sulphur-containing amino acid, homocysteine (Hcy), a condition known as hyperhomocysteinaemia (HHcy), have been identified as a potential risk factor for CVD (Humphrey *et al.*, 2008). There are several possible pathological mechanisms through which Hcy could be involved in CVD (as discussed in Chapter 2). Elevated Hcy concentrations lead to dysfunction of vascular endothelial cells (Moat *et al.*, 2004), increased intima-media thickness (Durga *et al.*, 2004) and plaque formation (Schnyder *et al.*, 2002). Excess Hcy seems to induce the formation of free radicals, which affect endothelial and smooth muscle cells that form the inner layer of blood vessels. Damage to these cells increases vulnerability for plaque build up; increases tendency for blood coagulation; reduces blood vessel function and increases blood vessel stiffness (Splaver *et al.*, 2004).

The underlying causes for HHcy are due to the interplay between several genetic, biological and environmental factors. These factors can be roughly divided into two groups, the unchangeable factors such as genetic factors, gender and advancing age, as well as the modifiable factors *inter alia* lifestyle habits and behavioural factors such as smoking habits, dietary factors including alcohol consumption and physical activity levels (De Bree *et al.*, 2002). In addition there are acquired factors such as diseases [renal failure, rheumatoid arthritis, malignancies, psoriasis and infection with the human immunodeficiency virus (HIV)] and certain drugs (methotrexate, nitrous oxide, theophylline, thiazides) that can also lead to increased Hcy concentrations. All of these

determinants will be explored within this review except for the genetic factors that will be discussed in detail within the review outlined in Chapter 4 and among the acquired factors only renal failure and infection with HIV will be discussed in Section 3.3.2 and 3.3.3 of this Chapter, respectively.

3.2 LIFESTYLE, BEHAVIOURAL AND ENVIRONMENTAL DETERMINANTS OF HOMOCYSTEINE

Since a reduction in total Hcy (tHcy) concentrations may be clinically relevant to prevent CVD (Humphrey *et al.*, 2008), it is important to identify modifiable factors that influence Hcy concentrations and to guide individuals with elevated tHcy to reduce their tHcy concentrations as a strategy to prevent CVD. Several lifestyle habits and modifiable behavioural factors such as dietary intake, physical inactivity and smoking will be discussed within this section.

3.2.1 Dietary factors

It has been established that inadequate intake of the B-vitamins and excessive intake of methionine, coffee, tea and alcohol can lead to elevated Hcy concentrations. However, adequate intake and supplementation with B-vitamins, betaine or choline could lower Hcy concentrations. All these dietary determinants of Hcy concentrations will subsequently be discussed.

3.2.1.1 B-vitamin status

In addition to increasing Hcy concentrations, reduced B-vitamin status induces an imbalance of s-adenosylmethionine or SAM and s-adenosylhomocysteine or SAH (Fuso *et al.*, 2008), while supplementation with B-vitamins and their synthetic counterparts seems to reduce Hcy concentrations (Clarke & Armitage, 2000). Hcy has been proposed to be a highly sensitive indicator of folate and vitamin B₁₂ status (Bates *et al.*, 1997). Even within the normal ranges the concentrations of serum or red cell folate and serum

vitamin B₁₂ are strong determinants of plasma Hcy concentrations (Andersson *et al.*, 1992; Brattström *et al.*, 1994). The reason being is that the clearance of Hcy from the circulation is dependent on folate, a B-vitamin, with the chemical name pteroylglutamic acid (the synthetic form of folate is known as folic acid or folacin), riboflavin (vitamin B₂), vitamin B₆ [pyridoxine, pyridoxal, pyridoxamine or pyridoxal 5'-phosphate (PLP)] and vitamin B₁₂ (cobalamin or the synthetic form cyanocobalamin) (Scott, 2003).

3.2.1.1.1 Folate status

Folate is a micronutrient found in green leafy vegetables and in some animal products, such as egg yolk. Folate is an important cofactor and methyl donor in the conversion of Hcy to methionine (as discussed in Chapter 2 Section 2.2). Reduced folate status inhibits the methylation cycle by failing to keep it supplied with methyl groups, resulting in reduced Hcy remethylation (Beccia *et al.*, 2004). Folate deficiency, due to problems of malabsorption or inadequate dietary intake (malnutrition or alcoholism) is considered to be the most important nutritional cause of elevated tHcy concentrations (Verhoef & De Groot, 2005).

Although everyone should consume sufficient folate, the research of Chiuve *et al.* (2005) and Jacques *et al.* (1996) highlighted the necessity of adequate folate intake among drinkers as well as individuals harbouring the cytosine (C) to thymine (T) substitution at nucleotide position 677 (C677T) in the methylenetetrahydrofolate reductase (MTHFR) *i.e.* 677 TT genotype, respectively. Chiuve *et al.* (2005) reported that the adverse effects of moderate alcohol drinking on Hcy concentrations, but not excessive drinking may be overcome through adequate folate intake. MTHFR 677 TT homozygous individuals may require more folate for thermolabile MTHFR to function adequately and to ensure normal Hcy concentrations than individuals harbouring the wildtype alleles (Jacques *et al.*, 1996). In addition, alcohol drinkers who have the variant MTHFR 677 allele may require even more folate (Chiuve *et al.*, 2005). The negative effects of low intakes of the methyl-related nutrients including folate with high intakes of alcohol are additive in

relation to cancer risk and the MTHFR C677T genetic variant also seems to be related to this risk (Bailey, 2003).

A high intake of natural folate from food decreases plasma tHcy concentrations (Verhaar *et al.*, 2002), however, folic acid (a synthetic chemically stable form of folate used in supplements and fortified foods) has been reported to be more effective than dietary folate in this regard, probably due to the high bioavailability of folic acid (Winkels *et al.*, 2007). Mean fasting tHcy concentrations decreased modestly after mandatory folic acid fortification in America in 1998 (Anderson *et al.*, 2004). Supplementation with folic acid alone and in combination with other B-vitamins has been reported to reduce the plasma tHcy concentration (Homocysteine Lowering Trialists' Collaboration, 2005). In a meta-analysis of 25 randomised controlled trials (Homocysteine Lowering Trialists' Collaboration, 2005), it was concluded that ≥ 0.8 mg folic acid per day is required to achieve the maximal reduction in Hcy concentration and that 0.2 and 0.4 mg were associated with 60% and 90%, reduction of the maximal effect, respectively. Vitamin B₁₂ (0.4 mg/day) produced a further reduction of 7% [95% confidence interval (CI): 4% - 9%] in Hcy concentrations, but vitamin B₆ had no significant effect.

Evidence has accumulated that suggests that folic acid supplementation has a beneficial effect on the vascular endothelium by reducing Hcy concentrations and through other mechanisms *e.g.* by reducing oxidative stress (Wilmink *et al.*, 2000). In addition, acute administration of folic acid can restore impaired endothelial function induced by acute HHcy (Usui *et al.*, 1999). Folic acid was reported to have antioxidant properties and direct scavenging effects *in vitro* (Verhaar *et al.*, 1998) and may directly improve nitric-oxide production by enhancing enzymatic activity of nitric-oxide synthase (Stroes *et al.*, 2000). Hcy leads to an increase in cell proliferation and cell death through apoptosis and necrosis *in vitro* in smooth muscle cells (Buemi *et al.*, 2001), however adding folic acid to the culture medium lead to a significant reduction of Hcy concentrations *in media*. This is probably due to increased remethylation of Hcy to methionine and reduced effects of Hcy on proliferation/apoptosis/necrosis (Buemi *et al.*, 2001).

Mager *et al.* (2009) reported that long-term folate-based vitamin therapy was independently associated with lower all-cause mortality in HHcy patients with CVD and with reduced Hcy concentrations. Contrastingly, Albert *et al.* (2008) reported that long term supplementation with folic acid, vitamin B₆ and B₁₂ did not reduce total CVD events among high-risk women despite significant Hcy lowering.

3.2.1.1.2 Riboflavin status

The water-soluble vitamin, riboflavin or 7,8-dimethyl-10-ribityl-isalloxazine (vitamin B₂) is present in a wide variety of foods. Riboflavin is an essential precursor for the biosynthesis of the biologically active flavin adenine mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FMN and FAD participate in a range of redox reactions, some of which are absolutely key to the function of aerobic cells (Powers, 2003). FAD is an essential cofactor for the folate dependent enzyme MTHFR, which metabolises folate to the form used in Hcy methylation and for an enzyme that activates the vitamin B₆ precursor, pyridoxal, to the biologically active form, PLP (Scott, 2003). Thus in theory, inadequate intake of riboflavin might give rise to increased plasma Hcy (Scott, 2003). In addition, riboflavin deficiency may exert some of its effects on Hcy by reducing the metabolism of other B-vitamins, notably those of folate and vitamin B₆ (Powers, 2003). Researchers have largely ignored riboflavin as a possible Hcy-lowering nutrient in the past.

Riboflavin intake emerged as a factor influencing tHcy in men and women from the Framingham Offspring Cohort (Jacques *et al.*, 2001). In contrast Verhoef and De Groot (2005) reported that riboflavin has little influence on fasting Hcy concentrations. Riboflavin may be relevant in individuals with the MTHFR 677 TT genotype as riboflavin status was reported as being a modulator of Hcy in healthy adults, especially among those homozygous for the common MTHFR C677T mutation (Hustad *et al.*, 2000). Moat *et al.* (2003) recently confirmed a folate-riboflavin interaction in determining Hcy that is unrelated to the MTHFR genotype.

3.2.1.1.3 Vitamin B₆ status

Reduced vitamin B₆ is known to cause an accumulation of plasma Hcy because cystathionine β -synthase (CBS) and cystathionine- γ -lyase that catalyse the transsulphuration of Hcy are vitamin B₆ dependent (Finkelstein, 1990). The metabolism of vitamin B₆ is flavin-dependent and studies in humans and animals have shown impaired synthesis of PLP in the presence of a riboflavin deficiency (Lakshmi & Bamji, 1976). McKinley *et al.* (2001) reported that low-dose vitamin B₆ supplementation effectively lowers fasting plasma tHcy in healthy elderly persons who are both folate and riboflavin replete.

Vitamin B₆ supplementation has little influence on fasting tHcy concentrations, although it may improve tHcy catabolism in elderly individuals (Verhoef & De Groot, 2005). Vitamin B₆ supplementation significantly reduced the post methionine load increase in tHcy and also reduced plasma cystathionine concentrations probably due to enhanced cystathionine- γ -lyase activity (Bleie *et al.*, 2004).

3.2.1.1.4 Vitamin B₁₂ status

Folate and cobalamin (as methylcobalamin) are involved as substrate and coenzyme, respectively, in the remethylation pathway of Hcy to methionine. Plasma tHcy increases considerably when there is an intracellular deficiency of folate or cobalamin and is regarded as a sensitive marker of suboptimal vitamin concentrations (Bates *et al.*, 1997).

Vitamin B₁₂ deficiencies are most commonly due to problems of malabsorption [*inter alia* due to alcoholism (Lindenbaum, 1980)] or inadequate dietary intake especially in individuals following strict vegetarian diets, since vitamin B₁₂ is found only in animal-source foods (Herrmann & Geisel, 2002). Reduced vitamin B₁₂ status prevents the proper functioning of the methylation cycle, by directly reducing the activity of methionine synthase (MS), one of the enzymes needed for the methylation cycle to turn (Scott, 2003). The enzyme MS, is dependent on 5-methyltetrahydrofolate as a methyl

donor, but also on vitamin B₁₂ as methylcobalamin (Scott, 2003). Low vitamin B₁₂ status may alter plasma Hcy by reducing its remethylation cycle toward methionine resynthesis in the same manner that low folate status alter Hcy metabolism (Beccia *et al.*, 2004).

Certain rare and drastic genetic mutations such as the one described by Mudd *et al.* in 1969 can lead to impaired vitamin B₁₂ activation. This results in reduced MS activity, with a rise in urinary and plasma Hcy as well as reduction in the activity of methylmalonyl-coenzyme A mutase, the other vitamin B₁₂-dependent enzyme, causing accumulation of methylmalonic acid (Mudd *et al.*, 1969).

3.2.1.2 Dietary lipotropics

Methionine, betaine and choline belong to a group of compounds called lipotropics (Harper & Benton, 1956; Snyder *et al.*, 1957). Lipotropics are compounds that have an affinity for lipids and thus prevent or correct excess accumulation of fat in the liver by promoting the transport of fatty acids from the liver to the tissues or by accelerating the utilisation of fat in the liver itself.

3.2.1.2.1 Methionine

Methionine is an essential amino acid found naturally in dietary proteins (Krupková-Meixnerová *et al.*, 2002). The typical Western diet contains 1.6 - 2.8 g of methionine per day (Shoob *et al.*, 2001), which is more than required for protein biosynthesis thus the excess must be catabolised (Scott, 2003). The transamination of methionine only occurs at non-physiologically high concentrations and instead, methionine catabolism takes place mainly in the liver through its sequential conversion to SAM and SAH and then to Hcy, followed by the transsulphuration pathway (Scott, 2003).

Due to the above, methionine loading tests (MLT) are often used to induce 'stress' on Hcy metabolism and could reveal any defect in Hcy metabolism (methionine intolerance)

and cause a transient acute increase in Hcy concentrations. MLT permit the screening of 40 to 55% persons (Bostom *et al.*, 1995) who may have clinically relevant HHcy, which fasting tHcy determination alone may fail to identify. Ubbink *et al.* (1995) reported that the rise in Hcy after methionine loading is lower in black Africans than in caucasians, which would point towards a more effective Hcy metabolism. This seems to correlate with the lower prevalence of CVD seen in these subjects. Caucasian individuals with an elevated risk for CVD showed higher plasma tHcy concentrations after oral MLT (Simporè *et al.*, 2002; Van den Berg *et al.*, 1999). It is necessary to investigate whether this difference is attributed to genetic factors or whether other factors within the genotype influence Hcy concentrations in an African group. It has been suggested that methionine handling capacity is more dependent of (genetically determined) enzyme activities, but fasting (basal) Hcy concentrations are stronger influenced by environmental factors (Den Heijer *et al.*, 2005).

Oral MLT is associated with a small, but significant, enhancement of thrombin generation (Domagala *et al.*, 2002) and is associated with impaired flow-mediated endothelium-dependent vasodilatation (Bellamy *et al.*, 1998). Even low-dose methionine and animal protein intake was reported to increase plasma Hcy and lead to rapid onset of endothelial dysfunction (Chambers *et al.*, 1999) suggesting that even diet related increments in Hcy may contribute to the development and progression of atherosclerosis. It is unknown whether a diet rich in animal proteins, containing large amounts of methionine, might activate platelets, increase thrombin production or induce endothelial dysfunction.

3.2.1.2.2 Betaine and choline intake

Betaine and its precursor, choline are major sources of methyl groups (one-carbon nutrients) in the diet (Cho *et al.*, 2006) and thus can act as methyl donors in Hcy remethylation. Betaine generates methionine and SAM then converts Hcy to methionine using the enzyme betaine methyltransferase, independent of folate or vitamin B₁₂ (Scott, 2003). Betaine or trimethylglycine can be found in foods and it is estimated that a

normal diet contains 0.5 - 2 g/day or can be synthesised endogenously from choline, also found in food (Olthof & Verhoef, 2005). Choline is derived not only from the diet, but from *de novo* synthesis as well (Zeisel, 2007).

Consumption of betaine and choline can lower fasting tHcy concentrations to a similar extent as folic acid, particularly in the setting of a high intake of methionine (Verhoef & De Groot, 2005). High doses of betaine (6 g/day and higher) can be used as Hcy-lowering therapy for individuals with HHcy due to inborn errors in the Hcy metabolism. Betaine has been reported to lower plasma Hcy in vitamin B₆ resistant patients (Olthof & Verhoef, 2005). In addition, betaine supplementation seems to lower fasting Hcy dose-dependently up to 20% for a dose of 6 g/day in healthy volunteers with normal Hcy concentrations. Betaine also reduces the increase in Hcy after MLT by up to 50%, whereas folic acid has no effect (Olthof & Verhoef, 2005). Betaine could also lower tHcy independent of folate remethylation in the presence of ethanol (Barak *et al.*, 2003). In this regard Chiuve *et al.* (2007) reported that total choline and betaine intake was inversely associated with tHcy in 1,477 women and that the strongest dose response was observed in women with a low-methyl diet (high alcohol and inadequate folate intake). Therefore, Chiuve *et al.* (2007) concluded that the remethylation of tHcy may be more dependent on the betaine pathway when methyl sources in the diet are low. Betaine and choline depletion in animals play a role in the pathogenesis of homocystinuria due to deficiencies of the MTHFR enzyme (Schwahn *et al.*, 2004).

Betaine and choline can thus be important food components that attenuate Hcy rises after meals. If Hcy plays a causal role in the development of CVD, a diet rich in betaine or choline might benefit cardiovascular health through its Hcy-lowering effects. However, betaine and choline may adversely affect serum lipid concentrations, which can increase the risk of CVD. It is not yet clear whether the potential beneficial health effects of betaine and choline outweigh the possible adverse effects on serum lipids.

3.2.1.3 Coffee and tea consumption

Consumption of tea and coffee increases Hcy concentrations by up to 20% (Verhoef & De Groot, 2005). Habitual coffee drinking is positively associated with Hcy concentration in both men and women in most (De Bree *et al.*, 2001; Husemoen *et al.*, 2004; Jacques *et al.*, 2001; Nygård *et al.*, 1997; Stolzenberg-Solomon *et al.*, 1999), but not all (Nieto *et al.*, 1997; Rasmussen *et al.*, 2000; Saw *et al.*, 2001) observational studies. Intervention trials indicated that the increasing effect of coffee consumption on Hcy concentrations may be causal (Christensen *et al.*, 2001; Grubben *et al.*, 2000; Urgert *et al.*, 2000). In a randomised crossover trial the vitamin B₆ concentration was markedly lower when consuming coffee than during the period where no coffee was consumed, but since vitamin B₆ does not seem to influence fasting tHcy concentrations greatly, the significance of this finding is uncertain (Grubben *et al.*, 2000). The constituent caffeine, which is a methyl xanthine, might be the culprit (Grubben *et al.*, 2000; Jacques *et al.*, 2001), because methyl xanthines are known to act as vitamin B₆ antagonists which may inhibit the conversion of Hcy to cysteine (Cys) (Grubben *et al.*, 2000). The polyphenol, chlorogenic acid, which is present in coffee in the same amount as caffeine, may partly contribute to the increase in Hcy concentration. When polyphenols are metabolised, methyl groups from methionine are necessary, which results in a higher production of Hcy (Olthof *et al.*, 2001). Both caffeine and chlorogenic acid are also present in tea, although in smaller doses, which explains the absence of a clear association between Hcy and tea consumption (Olthof *et al.*, 2001). Consumption of 1 L strong coffee daily may affect diet composition and other lifestyle factors and, therefore, could influence Hcy concentrations indirectly (Vollset *et al.*, 2000), but this has not yet been explored in the literature. It is also possible that the Hcy response to coffee may be modulated by the genetic factors mentioned earlier (Vollset *et al.*, 2000). Since the habit of coffee drinking is widespread, the consequences at the population level may not be negligible. However, the effect of coffee consumption is modest and much less than the changes associated with variation in B-vitamin status.

3.2.1.4 Alcohol consumption

Light-moderate alcohol consumption is associated with decreased mortality from CVD (the French paradox) (Renaud & De Lorgeril, 1992). However, intermittent bouts of excessive consumption (binge drinking) and chronic high levels of alcohol consumption result in sequelae of health problems *inter alia* increased CVD (such as cardiomyopathy and arrhythmia) morbidity, neurological disorders, certain cancers, chronic pancreatitis and liver cirrhosis (Corrao *et al.*, 2004). Lifelong abstainers appear to be at a slightly higher risk than light or moderate consumers who are able to control their drinking (Yuan *et al.*, 1997). Therefore, total abstinence is unnecessary when the consumer can enjoy alcohol sensibly, but abstainers are not advised to start drinking in order to gain any claimed health benefits (Van Heerden & Parry, 2001).

In a meta-analysis, Bagnardi *et al.* (2008) reported that regular heavy drinkers and heavy irregular binge drinkers showed significantly different pooled relative risks (RR) of 0.75 (95% CI 0.64 – 0.89) and 1.10 (1.03 – 1.17), respectively as opposed to abstainers for developing CVD. Therefore, the pattern of drinking as well as the amount consumed plays a pivotal role in CVD development.

One of the myriad negative consequences of chronic alcoholism is elevated Hcy concentrations (Cravo *et al.*, 1996; Koehler *et al.*, 2001). Alcohol abuse is a common cause of HHcy (Carmel & James, 2002). The tHcy concentration seems to be twice as high and plasma B vitamins are lower among chronic alcoholics than among healthy controls. This is probably due to a combination of malnourishment, the direct effects of heavy alcohol intake on folate status and vitamin B₆ (acetaldehyde dislodges vitamin B₆ from its protective binding protein so that it is destroyed and alcohol interferes with thiamine, folate and vitamin B₁₂ absorption), decreased hepatic uptake and retention as well as increased urinary excretion of *inter alia* folate (Cravo *et al.*, 1996). A major difficulty in drawing conclusions about the effect of alcohol consumption on Hcy is in distinguishing the direct effects of alcohol from alcohol-induced malnutrition. Some studies have detected no association between alcohol and tHcy (Gudnason *et al.*, 1998;

Lussier-Cacan *et al.*, 1996), but several have shown that moderate consumption was inversely related to tHcy concentration compared with abstaining (Mayer *et al.*, 2001; Vollset *et al.*, 1997). However, in social drinkers moderate alcohol consumption was associated with increased Hcy (Bleich *et al.*, 2001) and wine consumption in particular was related to tHcy in a J-shaped manner *i.e.* tHcy is higher when alcohol consumption is high, lower when alcohol consumption is low or moderate, and tends to be slightly increased in individuals not consuming any alcohol (Husemoen *et al.*, 2004). Thus when reviewing the literature the cardioprotective effect of moderate alcohol consumption compared with nondrinking in relation to Hcy remains contentious.

It may be important to distinguish between effects that can be attributable directly to alcohol and those that may result from other constituents of alcoholic drinks. Therefore, different types of alcoholic beverages seem to influence Hcy in different ways. Results of studies to determine the effect of different alcoholic beverages were inconsistent (Bleich *et al.*, 2001; Van der Gaag *et al.*, 2000). Beer consumption might be responsible for the inverse or absence of an association with alcohol consumption and tHcy concentration (Husemoen *et al.*, 2004; Jacques *et al.*, 2001; Mayer *et al.*, 2001; Van der Gaag *et al.*, 2000), but some studies reported that it is positively associated with Hcy (Bleich *et al.*, 2001). Researchers have ascribed the beneficial effect of beer drinking on tHcy to its folate, riboflavin and vitamin B₆ content, all of which are important for the enzymatic Hcy conversion. Wine consumption seems to elevate tHcy concentrations (Bleich *et al.*, 2001; Van der Gaag *et al.*, 2000). In a large cross-sectional study Burger *et al.* (2004) revealed that Hcy among women showed a U-shaped curve with a minimum of 8.49 mmol/L at 10 - 20 g alcohol/day whereas an inverse association was observed for men. Spirits on the other hand seem to elevated Hcy concentrations (Bleich *et al.*, 2001; Van der Gaag *et al.*, 2000). In Africa, indigenous peoples brew traditional alcoholic beverages, such as sorghum, millet beers and *Mbamba*, the latter is a concoction of water, bread, oats, pineapple juice, sugar and yeast. Sorghum beer has been reported to make positive contributions to dietary intake, particularly when the beer is brewed with sorghum adjunct. Hence, the results for different types of alcoholic beverages are not clear.

The relationship between alcohol consumption and tHcy concentration is complex. Alcohol (ethanol) interferes with both the transmethylation and transulphuration pathways of Hcy metabolism, either directly (Barak *et al.*, 1993) or through its metabolite acetaldehyde (Hidioglou *et al.*, 1994; Shaw *et al.*, 1989) or through indirect effects mediated by interactions with vitamin metabolism. Alcohol studies conducted on humans are scarce, and results when using chronic alcoholics are often complicated by liver disease and/or dietary insufficiencies associated with alcohol consumption.

Acetaldehyde inhibits MS activity (Kenyon *et al.*, 1998), which could lead to HHcy independent of vitamin status. Animal studies indicated that alcohol consumption causes a compensatory increase in betaine, a Hcy methyltransferase to generate methionine from Hcy (Carmel & James, 2002). Although methionine levels seem to vary, methionine adenosyltransferase activity decreased in most, but not all studies (Carmel & James, 2002; Finkelstein *et al.*, 1974). In most animal studies the SAM:SAH ratio, an important determinant of methylation activity, has been decreased (Halsted *et al.*, 1996). The alcohol intermediate, acetaldehyde, accelerates the intracellular degradation of PLP reducing the PLP content of hepatic cells despite adequate dietary intake of vitamin B₆ (Lumeng *et al.*, 1978). This inhibits the PLP-dependent enzymes, CBS and cystathioninase compromising Hcy transsulphuration. Nitric oxide production, secondary to augmented Hcy inhibits MS activity possibly by inactivating cobalamin (Danishpajoo *et al.*, 2001), further disrupting MS activity despite cobalamin supplementation as well as by direct inhibition of MS due to acetaldehyde (Kenyon *et al.*, 1998). Studies conducted on ethanol-fed rats reported that the MS inhibition raises the “trapping” of folate as 5-methyltetrahydrofolate (Barak *et al.*, 1993; Barak *et al.*, 2001; Hidioglou *et al.*, 1994).

Folate intake as discussed in Section 3.2.1.1.1 seems to be inversely associated with fasting plasma tHcy concentration, but this relation seems to be modified by MTHFR C677T genotype and alcohol intake. Women with the thermolabile variant of MTHFR 677 or moderate alcohol intake had significantly higher tHcy concentration at low intakes of folate, but adequate folate intake minimised these differences (Chiuve *et al.*, 2005). In

addition, the elevation in tHcy among women who consumed low folate and drank moderate amounts of alcohol was greater in the presence of the variant MTHFR 677 T allele (Chiuve *et al.*, 2005).

3.2.2 Physical activity

It is well-known that a sedentary lifestyle directly and indirectly fosters the development of CVD. The protection afforded by exercise could in part be due to its effect on Hcy concentrations, since Nygård *et al.* (1995) reported that physical activity is weakly inversely associated with Hcy concentrations. One should keep in mind that an active lifestyle is generally associated with a healthy lifestyle, and the latter is associated with a lower tHcy concentration, therefore associating higher physical activity levels with lower Hcy concentrations is likely due to residual confounding. Various other studies do not support the findings of Nygård *et al.* (1995) and concluded that physical activity is probably not associated with Hcy concentrations (Gudnason *et al.*, 1998; Lussier-Cacan *et al.*, 1996; Saw *et al.*, 2001). An intervention study conducted by Wright *et al.* (1998) reported that acute exercise does not affect the tHcy concentration. More recently another intervention study by Boreham *et al.* (2005) reported that a 17.1% increase in maximal oxygen consumption relative to a control group after eight weeks of stair climbing in eight sedentary, healthy women with a mean age of 18.8 years did also not result in Hcy-lowering. However, the latter study only had sufficient power to detect a 1 $\mu\text{mol/l}$ difference between the control and intervention group.

3.2.3 Smoking and exposure to air pollution

Chronic tobacco smoking is one of the strongest risk factors for CVD (Jackson *et al.*, 2000). The precise mechanisms by which smoking increases risk of CVD are unclear. Studies indicate that smoking is positively associated with the tHcy concentration in patients with CVD and diabetes as well as with the general population (Adachi *et al.*, 2002; Bamonti *et al.*, 2007; Targher *et al.*, 2000). Several mechanisms have been suggested to account for the smoking-related increase in tHcy concentrations. The exact

mechanism behind the increase in the tHcy concentration is unidentified, but smoking may induce local effects in cells exposed to cigarette smoke (Piyathilake *et al.*, 1992), influence the tHcy concentration by changing the plasma thiol redox status, possibly because of the formation of more reactive oxygen species (Bergmark *et al.*, 1997; Mansoor *et al.*, 1995; Pryor & Stone, 1993), or inactivation of the enzymes of Hcy remethylation, such as MS (Blom, 1998); reduced intake of nutrients and vitamins; and lower levels of plasma folate, vitamin B₁₂ (Bamonti *et al.*, 2007) and plasma PLP, which is involved in Hcy metabolism. In a meta-analysis it was reported that smokers generally consume a less healthy diet (Dallongeville *et al.*, 1998), thus residual confounding of *inter alia* B-vitamin intake cannot be excluded and in 2001 Saw *et al.* reported that the effect of smoking on Hcy concentrations disappeared after correction for plasma folate in Asian men and women. Stein *et al.* (2002) reported that Hcy concentrations decreased by 11.6% in subjects who quit smoking, but significant changes in Hcy concentrations were not observed in subjects who reduced smoking or continued to smoke. In smokers harbouring the MTHFR 677 TT genotype HHcy is attributed to intracellular folate deficiency caused by a smoking-related reduction of NOS₃ activity which is further exacerbated when serum folate is low (Brown *et al.*, 2004). A case control study conducted by O'Callaghan *et al.* (2002) reported that current smokers had higher plasma Hcy than those who never smoked (11.7 $\mu\text{mol/L}$ vs. 10.07 $\mu\text{mol/L}$, $p < 0.05$ cases; 9.90 $\mu\text{mol/L}$ vs. 9.53 $\mu\text{mol/L}$ $p > 0.05$ for controls) and smokers with high plasma Hcy were at a 12-fold increased risk of CVD (odds ratio 12.4 95% CI 7.3 - 21.2) compared with non-smokers with a normal plasma Hcy. Therefore, O'Callaghan *et al.* (2002) advised smokers to cease smoking.

Research has demonstrated that ambient air pollution is also associated with increased risk of CVD (Brook *et al.*, 2004). It is possible that the inflammatory status induced by cigarette smoking produces an increased demand for methyl group donors that may be exacerbated in the presence of air pollution and will, therefore, result in an increase of tHcy (Baccarelli *et al.*, 2007).

3.2.4 Migration and urbanisation

Among immigrants who migrate from countries with low CVD mortality, the death rate is more similar to that of their adoptive country after acculturation than to that of their native country. Similarly a gradient of increased vascular risk with migration exists across the African diaspora. Kalra *et al.* (2008) noted that United Kingdom (UK) Afro-Caribbeans had higher tHcy and lower folate concentrations compared with West Indian African-Caribbeans that may contribute to the higher stroke risk seen in UK African-Caribbean people. This data suggests that environmental and lifestyle factors play a large role in the regulation of Hcy concentrations in Africans.

The recent changes in the political dispensation in South Africa have expedited the urbanisation of black South Africans, who predominate over other population sub-groups, from rural to urban areas. While the majority of black individuals reside in rural areas, the urban proportion is increasing steadily. This migration from traditional areas to areas where adaptation to a new Western-type lifestyle is necessary, exposes them to numerous noxious risks such as malnutrition (co-existing under- and overnutrition), abundant availability of tobacco and alcohol (Schutte *et al.*, 2003). There has been a shift over time from traditional diets rich in fibre and grain, but low in fat to more imprudent diets that include increased levels of sugars, oils and animal fat associated with non-communicable disease (NCD) (Bourne *et al.*, 2002). The effects of dietary changes during urbanisation are usually exacerbated by a parallel decline in energy expenditure associated with a reduction in daily physical activity increasing the prevalence of obesity (Chopra *et al.*, 2002; Kruger *et al.*, 2002; Steyn *et al.*, 2005). The rural exodus of Africans to urban areas is exposing them to Western lifestyles to the development of NCD formerly rare in this population group (Steyn *et al.*, 2005; Walker, 1963).

Hcy concentrations have been reported to differ between rural and urban living individuals. Beijing men living in a rural area had Hcy concentration 1.5 times significantly higher than urban living men, while Hcy concentration was 1.3 times higher in rural than urban women (Wang *et al.*, 2002). Zhan *et al.* (1997) also observed

significantly higher Hcy in rural than in urban pregnant women and attributed this difference to the level of vitamin B₁₂ that was lower in rural than in urban women. However, the level of folate was higher in rural than in urban women, but there was no significant difference in deficiency of folate between rural and urban residents. Similar results were reported by Kim *et al.* (2003) in subjects living in Puriscal, Costa Rica and by Tovar *et al.* (2003) in Mexican women. Tovar *et al.* (2003) determined that the nutrition status of the Mexican women was adequate for folate and vitamin B₁₂, however, they reported that plasma folate concentration was significantly lower in the rural than in the urban women.

3.3 DEMOGRAPHIC AND ACQUIRED DETERMINANTS OF HOMOCYSTEINE

3.3.1 Age and gender

Age and gender seem to be two of the stronger determinants of Hcy concentrations. Numerous studies have indicated that increasing age and male gender are biological factors associated with a higher tHcy concentration (Andersson *et al.*, 1992; Brattström *et al.*, 1994; Frick *et al.*, 2004). Jacques *et al.* (1999) observed significant age-gender interaction, indicating that the relation between age and Hcy differed between the genders. In females Hcy tended to diverge from those in males at younger ages and converge with those in males at older ages (Jacques *et al.*, 1999). This difference could partly be attributable to the strong correlation between Hcy and circulating creatinine and larger body size or muscle mass in men, since the formation of muscles is associated with simultaneous increased Hcy formation as a consequence of methyl group transfer during creatine-creatinine metabolism (Brattström *et al.*, 1994). Another culprit that could partly be responsible for the gender difference could be the influence of gender hormones on Hcy metabolism (Andersson *et al.*, 1992) which was confirmed in a study with transgendered males and females (Giltay *et al.*, 1998). Part of the relationship with age in women might be explained by menopausal status and oestrogen replacement therapy, since tHcy concentration was reported to be higher in post-menopausal women compared

with pre-menopausal women (Andersson *et al.*, 1992; Van der Mooren *et al.*, 1994; Wouters *et al.*, 1995). Hormone replacement therapies with combined oestrogen and progesterone (Van der Mooren *et al.*, 1994) seem to lower plasma tHcy in healthy pre- and postmenopausal women. The gender differences may also be explained in part by differences in folate, vitamin B₁₂ and B₆ status (Selhub *et al.*, 1993).

It has been suggested that raised Hcy concentrations seen in the elderly are due to an insufficient availability and/or deficiencies of folate, B₆ and/or B₁₂ [*inter alia* due to atrophic gastritis (Wolters *et al.*, 2003) and pernicious anaemia (Lindenbaum *et al.*, 1994)] (Selhub *et al.*, 1993; Brattström *et al.*, 1994), but not all studies are conclusive in this regard (Frick *et al.*, 2004). Progressive deterioration of kidney function with age (Ueland *et al.*, 1992), increased creatinine concentrations due to impaired renal function and impaired renal metabolism of Hcy (Guttormsen *et al.*, 1997) appear to play a role in the pathogenesis of increased tHcy in older persons. However, in West African subjects Amouzou *et al.* (2004) observed no significant difference in plasma tHcy in relation to age, but in other American ethnic groups Jacques *et al.* (1999) reported a significant association even within different ethnic groups. Kado *et al.* (2002) indicated that older persons with elevated Hcy are at increased risk of physical functional decline over three years and this association remained even after controlling for other determinants of Hcy including increased age. Since the normal aging process is accompanied by a decline in physical functioning (Bortz & Bortz, 1996) it is possible that the relationship between age and Hcy could be ascribed to the suggestion of Kado *et al.* (2002), however, Vilaseca *et al.* (1997) observed a linear relationship between age and Hcy in a paediatric population (2 months to 18 yrs) (Vilaseca *et al.*, 1997) thereby refuting this notion.

3.3.2 Renal function

A decline in renal function causes elevated plasma tHcy and Cys concentrations. Most researchers analyse creatinine concentrations as an indicator for renal function. Hcy concentrations seem to be inversely correlated with calculated glomerular filtration rate (McDonald *et al.*, 2005). According to Refsum *et al.* (1998), the HHcy in renal failure is

accounted for by a marked reduction in tHcy clearance, suggesting an important role of kidneys in elimination of Hcy from plasma. In contrast, Scott (2003) opined that this is not due to loss of the excretory function of the kidneys since little Hcy appears in the urine under normal circumstances. Earlier hypothesis was that the kidney was a major site of Hcy catabolism, but this was found to be true in rats and not in humans for the human kidney is responsible for the removal of very little, if not negligible, amounts of plasma Hcy (Van Guldener *et al.*, 1999). Thus, the explanation for an up to 100% increase in plasma Hcy in humans during renal failure remains unclear (Guttormsen *et al.*, 1997). It is only corrected to a minor degree by folic acid supplements and appears to be something intrinsic to an alteration in Hcy metabolism rather than due to impaired activity of the methylation cycle (Guttormsen *et al.*, 1997).

3.3.3 Human immunodeficiency virus infection

HIV infection has led to high morbidity and mortality prevalence globally. Few studies exist so far, investigating Hcy in HIV positive patients, and the results are controversial. Folate, vitamin B₁₂ [due to decreased absorption (Remacha & Cadafalch, 1999; Revell *et al.*, 1991)] and other micronutrient deficiencies are frequent in HIV-infected subjects (Bogden *et al.*, 1990) and might disrupt methyl-group metabolic pathways explicating in part the raise in plasma tHcy seen in HIV patients (Jacobson *et al.*, 2004; Vilaseca *et al.*, 2001). HIV infection combined with the MTHFR 677 CC genotype is associated with significantly lower vitamin B₁₂ than in uninfected individuals (Malavazi *et al.*, 2004) and could be a synergistic CVD risk pathway. Since HHcy is modifiable with B-vitamin supplementation, the determination of tHcy, as well as folate and vitamin B₁₂, in HIV-infected seems to be advisable. HHcy could aggravate CVD risk which is an important complication of the clinical course of HIV, especially after highly active antiretroviral therapy (HAART) has dramatically increased survival of HIV patients by reducing opportunistic infections, but causing several metabolic complications (Copur *et al.*, 2002; Périard *et al.*, 1999; Saber *et al.*, 2001). HIV infection is normally associated with hypocholesterolaemia, hypertriglyceridaemia, low plasma high density lipoprotein cholesterol as well as alterations in other CVD risk factors including Hcy,

inflammatory markers, clotting factors, apolipoproteins, lipoprotein(a), oxidative stress and non-esterified fatty acids (Crook, 2007; Kuritzkes & Currier, 2003). The use of HAART is associated with dyslipidaemia and lipodystrophy with underlying insulin resistance and associated glucose intolerance (Behrens *et al.*, 1999; Crook, 2007; Périard *et al.*, 1999). HAART seems to be associated with a decrease in vitamin B₁₂ status (Remacha *et al.*, 2003) and impaired folate status [especially protease inhibitor (PI) containing drugs (Vilaseca *et al.*, 2001)], but does not seem to be associated with HHcy (Uccelli *et al.*, 2006). Bernasconi *et al.* (2001) observed that Hcy is higher in adult HIV-positive patients on PI containing HAART with respect to healthy controls, however, vitamin B₁₂ or folate levels were not controlled for. The mechanism of action of PI on Hcy or folate metabolism is not yet known (Vilaseca *et al.*, 2001).

3.4 SUMMARY AND CONCLUSION

In conclusion, alteration in lifestyle habits such as dietary intake or use of B-vitamin, betaine and choline supplements can substantially lower tHcy concentrations. Individuals with HHcy can thus benefit from early tailored lifestyle modification and thereby decrease their risk of developing CVD. It is not known whether elevated Hcy concentrations in Africans can be attributed to the same factors as in Caucasians, such as low B-vitamin intake, high alcohol and coffee intake, low physical activity and smoking and whether the gene-environment interactions vary between different populations.

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CHAPTER 4

LITERATURE REVIEW

COMMON GENETIC DETERMINANTS OF HOMOCYSTEINE

4.1 INTRODUCTION

Elevated homocysteine (Hcy) concentrations have been associated with a myriad of health conditions, including cardiovascular disease, hereafter abbreviated as CVD (Chen *et al.*, 2001a; Humphrey *et al.*, 2008), which is one of the leading causes of death worldwide (Murray & Lopez, 1997; Yusuf *et al.*, 2001a). CVD prevalence as well as the type of CVD present differs between ethnic groups (Yusuf *et al.*, 2001b). It is unknown whether differences in Hcy concentrations contribute to this observed disparity in CVD prevalence.

Elevated total Hcy (tHcy) concentrations *i.e.* hyperhomocysteinaemia (HHcy) can arise from a combination of dietary and/or genetic-related disturbances in the transsulphuration or remethylation pathways of Hcy metabolism (Scott, 2003). Since environmental and genetic factors generally differ between ethnic groups, this could contribute to differences in Hcy concentrations and, possibly, to differences in CVD prevalence. Non-genetic factors affecting plasma Hcy concentrations (reviewed in Chapter 3) do not account for all cases of HHcy. Therefore, it is necessary to investigate the genetic factors that predispose an individual to the development of HHcy (Kang *et al.*, 1991).

Besides the rare well-documented mutations which underlie the severely diminished activity of the enzymes involved in Hcy metabolism, consequently leading to severe HHcy and increased risk towards CVD (McCully, 1969), there are also more common genetic variations or polymorphisms that have moderate effects on the activity of these enzymes and, therefore, on Hcy concentrations (Matthews & Elmore, 2007). Since these variations are more prevalent they are more likely to contribute to an increased population attributable risk for CVD than the rarer severe mutations (Castañon *et al.* 2007; Humphrey *et al.*, 2008), and thus these variations should also be investigated. Therefore, this review focuses on the common polymorphisms in the genes encoding the enzymes methylenetetrahydrofolate reductase (MTHFR),

cystathionine β -synthase (CBS) and methionine synthase (MTR), highlighting the ethnic differences in the distribution frequencies of these common genetic variations and briefly describing their associations with CVD. Several other mutations also influence Hcy concentrations and will briefly be discussed in Section 4.3.4.

4.2 CORE CONCEPTS USED WITHIN THIS REVIEW

Substantial genetic variation exists in the human genome *i.e.* all of the information contained in the deoxyribonucleic acid (DNA) of the chromosomes and mitochondria (Gelehrter *et al.*, 1998). Mutations, *inter alia*, contribute to this variation. A mutation can be defined as a change in the nucleotide sequence that may be neutral, deleterious or adaptive (Roche & Mensink, 2003). Point mutations include insertions, deletions, transitions {which are the substitutions of a pyrimidine [cytosine (C) to thymine (T)] or a purine [adenine (A) to guanine (G)] by the other pyrimidine or purine, respectively} and transversions (which result when a purine is replaced by a pyrimidine or *vice versa*).

A polymorphism can be defined as a variation in the DNA that is too common to be due to a new mutation. Therefore, when a mutation is present in a population at an allele frequency $\geq 1\%$, it could be defined as a polymorphism (Roche & Mensink, 2003). When the mutation is a change of a single nucleotide and the allele frequency of the altered allele is $\geq 1\%$ it is generally referred to as a single nucleotide polymorphism (SNP). It is often assumed that mutations are deleterious and polymorphisms benign, however, both terms refer to the prevalence and not the functional consequences of the genetic variation (Roche & Mensink, 2003). The allele or single nucleotide base which is the most 'commonly appearing' base in most populations is referred to as the wild-type, whereas the genetic sequence variant is referred to as the polymorphic or mutant allele (Roche & Mensink, 2003). In comparison to high penetrance mutations observed in single gene disorders, many SNPs are low penetrance, but due to their high prevalence these common SNPs may have a greater impact on the health of a population.

In certain circumstances the terms *mutation*, *point mutation*, *SNP* or *polymorphism* could be used interchangeably. For the purpose of this review the concepts provided here will be used as defined above.

4.3 COMMON GENETIC DETERMINANTS OF HOMOCYSTEINE CONCENTRATIONS

This section highlights the common genetic variations that have been reported to be associated with Hcy metabolism. In Sections 4.3.1, 4.3.2 and 4.3.3 certain polymorphisms within the genes encoding the enzymes MTHFR, CBS and MTR, respectively, are discussed. Section 4.3.4 briefly describes several other polymorphisms that also influence Hcy concentrations.

4.3.1 Polymorphisms within the methylenetetrahydrofolate reductase gene

The MTHFR flavoprotein fulfils a crucial role in Hcy metabolism by catalysing the irreversible conversion of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methyltetrahydrofolate (5-MTHF), which is the methyl donor in the folate-dependent remethylation of Hcy to methionine (Guenther *et al.*, 1999). The MTHFR gene which encodes the MTHFR enzyme has been mapped by Goyette *et al.* (1994) to chromosomal region 1p36.3 and has been extensively researched. To date, several different mutations have been identified within the MTHFR gene (Rozen, 1997). Certain polymorphisms in the MTHFR gene, critical to homocysteine regulation, will be discussed in the following section.

4.3.1.1 The C677T polymorphism in the methylenetetrahydrofolate reductase gene

This section describes the C677T polymorphism in the MTHFR gene, and is divided into two subsections. The first describes the molecular aspects and the functional consequences of this alteration, whereas the second explores its frequency in various population groups. Similar divisions are used when describing the other polymorphisms.

4.3.1.1.1 *Molecular aspects and functional consequences of the C677T alteration within the methylenetetrahydrofolate reductase gene*

The C677T alteration is designated as being at base pair (bp) position 677, however the actual location of this SNP is at nucleotide position 665 of the coding region if the nucleotide numbering starts from the first ATG site of the reported coding sequence (Van der Put & Blom, 2000). Since the C677T nomenclature is widely accepted in the literature its use will be retained. This C to T transition within exon 4 of the catalytic domain of the gene coding for MTHFR results in the replacement of an alanine (A) with a valine (V) amino acid at amino acid number 222 (A222V) in a conserved portion of the MTHFR enzyme (Goyette *et al.*, 1994).

The C677T missense mutation results in the enzyme being thermolabile at 47°C, thus resulting in a reduction in enzyme activity at 37°C (Frosst *et al.*, 1995; Kang *et al.*, 1991). The thermolabile form of the enzyme is readily altered or destroyed at high temperatures, thus MTHFR activity is reduced (Rozen, 1997). As a result, less 5,10-MTHF (precursor of folate) is converted to 5-MTHF (bioactive folate). Since bioactive folate is required in the metabolism of Hcy to methionine, this genotype results in the phenotype of elevated plasma tHcy concentrations.

In most populations investigated, the heterozygous CT genotype and the homozygous mutant TT genotype are both associated with increased tHcy concentrations when compared to individuals harbouring the CC wild-type genotype (Adjalla *et al.*, 2003; Anand *et al.*, 2000; Brattström *et al.*, 1998; Frosst *et al.*, 1995; Sun *et al.*, 2005; Tavares *et al.*, 2004). The TT genotype seems generally to be associated with much higher Hcy concentrations in Africans than those described in Caucasians, Hispanics and Mexicans (Adjalla *et al.*, 2003; Amouzou *et al.*, 2004; Guéant-Rodriguez *et al.*, 2006; Rady *et al.*, 2002). Furthermore, the association between the genotype and tHcy concentrations seems to be stronger in the presence of impaired folate or vitamin B₁₂ status (Amouzou *et al.*, 2004; Hanson *et al.*, 2001; Jacques *et al.*, 1996).

B-vitamins are theorised to play an integral role in the phenotypic expression of the genotype present at the MTHFR 677 locus as well as modulating the disease risk associated with the variant T allele. It has been reported that subjects with the T allele

who had adequate folate had a similar Hcy risk profile as individuals harbouring the homozygous wild-type CC genotype (Jacques *et al.*, 1996). A study by Guenther *et al.* (1999) in *Escherichia coli* bacteria reported that a mutation (A177V) orthologous to the human MTHFR C677T variation was associated with an enhanced dissociation of the riboflavin (vitamin B₂) cofactor form, *i.e.* flavin adenine dinucleotide (FAD), from its active site on the bacterial enzyme, leading to irreversible inactivation. However, optimal folate prevented the loss of FAD binding and in this way suppressed the inactivation of the enzyme (Guenther *et al.*, 1999). Riboflavin also seems to determine tHcy concentrations, but this relationship seems to be modified by the MTHFR genotype and is confined to subjects with the C677T transition (Hustad *et al.*, 2000). McNulty *et al.* (2002) surmised that for the C677T variant to induce a malfunction in enzymatic activity and result in increased Hcy concentrations, the homozygous or even heterozygous individuals must also have diminished riboflavin status. All the above data support the hypothesis that the mutant enzyme is unstable and that the instability may be overcome by adequate B-vitamin intake, thus the risk of the variant T allele seems to be neutralised by a diet rich in B-vitamins.

4.3.1.1.2 Population frequency of the C677T variation in the methylenetetrahydrofolate reductase gene

In Tables 4.1 to 4.4, information obtained from studies that reported the genotype frequencies of this polymorphism in both unaffected individuals as well as in individuals with CVD was tabulated. Several authors included in Tables 4.1 to 4.4, have not reported whether the genotype frequencies adhered to the assumptions of Hardy-Weinberg equilibrium (HWE), *i.e.* the population sampled is undergoing random mating, there is no migration in or out the population, no inbreeding, no selective survival among genotypes and the population size is sufficiently large (Hardy, 1908). Therefore, determining the amount of variation that is expected to be present in a population adhering to the assumptions of HWE is necessary when interpreting the distribution frequencies. HWE was calculated for the studies that did not report whether the frequencies adhered to the assumptions of HWE. The absolute allele frequencies were used as reported in these studies and the concordance of genotype frequencies to those expected if the population did indeed adhere to the

assumptions of HWE was tested by a Pearson χ^2 test (1 degree freedom). The outcome of these analyses is indicated within Tables 4.1 to 4.4. There exists considerable ethnic- and geographic-specific variation in the frequency of the C677T variant (Table 4.1). Several explanations will be outlined to explicate the observed differences in the frequency of the MTHFR C677T variant.

It has been surmised that the C677T alteration could be regarded as a polymorphism that has escaped natural selection, due to its high worldwide distribution throughout diverse ethnic groups (presented in Table 4.1) even though its phenotype follows an autosomal recessive pattern (Kang *et al.*, 1991); is associated with increased CVD risk (Wald *et al.*, 2002) as well as neural tube defects (Van der Put *et al.*, 1998) and Isotalo *et al.* (2000) further hypothesised that foetal viability was compromised in foetuses harbouring either 677 CT/1298 CC or 677 TT/1298 CC genotypes (see Sections 4.3.1.2 and 4.3.1.3 for discussion on the 1298 locus and compound heterozygosity for MTHFR C677T and A1298C, respectively). However, the last mentioned hypothesis is still controversial (Hanson *et al.*, 2001). One must bear in mind that the increased susceptibility to a CVD event, which usually occurs in the later years, would not affect fecundity of the individual harbouring the altered 677 genotype and, therefore, natural selection would not significantly affect its frequency. However, if susceptibility to neural tube defects is increased or foetal viability was compromised in individuals harbouring this genotype, natural selection would significantly affect its frequency.

It may be that the C677T polymorphism arose as a single ancestral mutation, because it is strongly associated with a haplotype common to populations from Israel, Japan and Ghana (Rosenberg *et al.*, 2002), however, this hypothesis has not yet been confirmed (Guéant-Rodriguez *et al.*, 2006). Rosenberg *et al.* (2002) suggested that the MTHFR 677 alteration occurred on a founder haplotype, with either the heterozygous or homozygous mutant genotypes, in certain circumstances, having a selective advantage over wild-type individuals.

Evidence has accumulated that suggests that there is an interaction between alcohol consumption, the MTHFR C677T variation and cancer risk. When folate intake is sufficient, 677 TT individuals appear to be protected against colon cancer (Eaton

et al., 2005; Hubner & Houlston, 2007; Ma *et al.*, 1997) and acute lymphatic leukemia (Skibola *et al.*, 1999) perhaps by increasing 5,10-MTHFR levels for DNA synthesis. However, the intake of folate and other methyl-related nutrients, *i.e.* methionine, vitamin B₆ and B₁₂, and alcohol may influence the effect of the MTHFR C677T polymorphism on cancer risk. Among folate deficient individuals harbouring the 677 TT genotype, the cancer protection associated with the mutation is negated (Ma *et al.*, 1997) and the risk for developing colorectal cancer is higher (Ulrich *et al.*, 2000). The negative effects of low intakes of the methyl-related nutrients, *i.e.* methionine, vitamin B₆ and B₁₂, with high intakes of alcohol are additive (Bailey, 2003). When folate status is low, individuals harbouring the MTHFR 677 TT will have increased Hcy concentrations and DNA hypomethylation (Friso & Choi, 2002). The higher tHcy concentrations observed in the CT and TT groups, might reflect aberrations in DNA methylation which are associated with increased risk for cancer (Wu & Wu, 2002). Individuals with the 677 TT genotype are especially sensitive to the carcinogenic effect of alcohol (Ma *et al.*, 1997; Chen *et al.*, 1999). Among postmenopausal women, there was an increase in breast cancer risk for women who were homozygote TT at the MTHFR 677 locus and had high lifetime alcohol intake [odds ratio (OR), 1.92; 95% confidence interval (CI), 1.13 - 3.28] and for individuals consuming a high number of drinks per drinking day (>1.91 drinks/day; OR, 1.80; 95% CI, 1.03 - 3.28) compared to non-drinkers who were homozygote CC (Platek *et al.*, 2009).

Engbersen *et al.* (1995) suggested that during times of famine decreased MTHFR activity could be beneficial, since the thermolabile form of the enzyme seems to decrease Hcy remethylation so that the one-carbon moieties of derivatives remain available for the vital synthesis of the purines and thymidine. Therefore, one could hypothesise that differences in the exposure to a famine between populations could contribute to differences in the distribution of the MTHFR 677 alteration, but this has not yet been determined.

From the above one can deduce that the increased incidence of disease associated with the C677T variation could be neutralised by its protective effects on cancer and beneficial effects during famine provided that the increase in morbidity and mortality is equal to the reduction in morbidity and mortality. This neutralising effect could

provide an explanation as to why certain researchers surmised that this polymorphism escaped natural selection (as discussed previously).

The T allele frequency at the 677 locus varies extensively in different populations and may partly explain the geographical or ethnic differences in the risk for CVD that this locus imparts. As reported in Table 4.1, the frequency of the mutant genotype is much rarer in African-Americans and Africans than in Caucasians, Asians, Ashkenazi Jews, Hispanics and Mexicans. Guéant-Rodriguez *et al.* (2006) noted that Mexicans harboured the highest prevalence of the 677 T allele, but the influence of the 677 TT genotype on Hcy concentrations was the lowest in this population, whereas the opposite was true in Africans. The low frequency of the T allele in Africans could explain the significantly lower mean tHcy concentrations generally observed in Africans when compared to Caucasians (Ubbink *et al.*, 1996; Vermaak *et al.*, 1991). However, HHcy was reported to be common in West African subjects from the coastal regions and from the savannah in Togo and Bénin, despite a very low MTHFR 677 T allele frequency (Amouzou *et al.*, 2004; Guéant-Rodriguez *et al.*, 2006). This implies that other factors such as folate, vitamin B₂, B₆ and B₁₂ status, physical activity, alcohol consumption, smoking or other genetic factors could further modulate the disease risk associated with the MTHFR 677 T allele (Husemoen *et al.*, 2004).

Franco *et al.* (1998a) hypothesised that the low prevalence of the mutation in Africans could be explained if the mutation occurred outside Africa, after the first human migration and thus attributed the presently small proportion of mutant genes observed in African populations to the evolution of this population. They also hypothesised that the difference in gene frequencies between African and non-African groups could be the consequence of a selective advantage of the mutation outside or a disadvantage inside Africa.

Table 4.1 Frequency of the MTHFR C677T alteration in different ethnic groups and regions around the world expressed as percentages

Citation	Ethnic group	Sample	677CC (%)	677CT (%)	677TT (%)	HWE
Adjalla <i>et al.</i> (2003)	Africans (West Africa)	240	83.3	15.9	0.8	= *
Amouzou <i>et al.</i> (2004)	Africans (West Africa savannah)	68	88.2	11.7	0.1	= *
	Africans (West Africa coast)	208	80.3	18.3	1.4	= *
Franco <i>et al.</i> (1998a)	Africans (Zaire and Cameroon)	67	89.6	10.4	0.0	= #
Guéant-Rodriguez <i>et al.</i> (2006)	Africans (West Africa Bénin and Togo)	465	90.2	9.0	0.8	= *
Loktionov <i>et al.</i> (1999)	Africans (South Africa)	100	80.0	20.0	0.0	= *
Scholtz <i>et al.</i> (2002)	Africans (South Africa)	60	92.0	8.0	0.0	= #
Yang <i>et al.</i> (2008)	Non-Hispanic blacks	2103	79.2	19.5	1.3	= *
Gebhardt <i>et al.</i> (2001)	Mixed ancestry (coloured South African pregnant women)	168	73.2	25.6	2.2	= #
Scholtz <i>et al.</i> (2002)	Mixed ancestry (South Africa)	73	65.0	34.0	2.0	= #
Djilley <i>et al.</i> (2001)	African-Americans					
	Myocardial infarction cases	110	83.0	15.0	2.0	= #
	Controls	185	83.0	15.0	2.0	≠ #
Conroy <i>et al.</i> (2000)	African-Americans (New York)	526	81.5	17.0	1.5	= #
McAndrew <i>et al.</i> (1996)	African-Americans	102	80.0	20.0	0.0	= #
Rady <i>et al.</i> (2002)	African-Americans	97	77.3	21.7	1.0	= #
Franco <i>et al.</i> (1998a)	Amerindians (Brazilian Amazonian tribes)	129	59.7	32.5	7.80	= #
Franco <i>et al.</i> (1998a)	Asians	40	40.0	40.0	20.0	= #
Kelemen <i>et al.</i> (2004)	South Asians (Canada)	283	70.3	25.2	4.20	= *
Franco <i>et al.</i> (1998a)	Brazilian blacks	50	78.0	20.0	2.0	= #
Tavares <i>et al.</i> (2004)	Brazilian Parkateje Indians	90	45.3	40.7	14.0	= *
Conroy <i>et al.</i> (2000)	Caucasians (New York)	503	44.5	47.5	8.0	≠ #
Franco <i>et al.</i> (1998a)	Caucasians	51	37.3	52.9	9.8	= #
Guéant-Rodriguez <i>et al.</i> (2006)	Caucasians (France)	366	49.7	36.1	14.2	= *
	Caucasians (Italy)	146	32.8	47.3	19.9	= *
Kelemen <i>et al.</i> (2004)	Caucasians (Canada)	260	42.6	46.0	11.4	= *
Loktionov <i>et al.</i> (1999)	Caucasians (Cambridge in UK)	107	32.0	56.0	12.0	= *
McAndrew <i>et al.</i> (1996)	Caucasians	101	49.0	43.0	9.0	= #
Rady <i>et al.</i> (2002)	Caucasians	159	45.9	42.8	11.3	= #
Scholtz <i>et al.</i> (2002)	Caucasians (South Africa)	76	39.0	50.0	11.0	= #
Yang <i>et al.</i> (2008)	Non-Hispanic whites	2622	45.8	42.6	11.7	= *
Kelemen <i>et al.</i> (2004)	Chinese from Canada	275	62.0	30.8	7.20	= *
Janosíková <i>et al.</i> (2003)	Czech patients with CHD	278	44.2	42.1	13.7	= *
	Czech controls	591	41.4	48.6	10.0	= *
Bathum <i>et al.</i> (2007)	Danish (Twins)	1136	49.0	42.0	9.0	= *
Husemoen <i>et al.</i> (2004)	Danish (Southern Copenhagen County)	2788	48.8	42.4	8.8	= #
Conroy <i>et al.</i> (2000)	Hispanics (New York)	520	41.1	45.2	13.7	= #
Rady <i>et al.</i> (2002)	Hispanics	96	30.2	43.8	26.0	= #
Friedman <i>et al.</i> (1999)	Jews (Israeli Jews) (Jerusalem)	401	40.6	44.1	15.2	= *
Rady <i>et al.</i> (2002)	Jews (Ashkenazi Jews)	155	31.0	42.6	26.4	= #
Guéant-Rodriguez <i>et al.</i> (2006)	Mexicans	300	6.3	58.0	35.7	= *
Mutchinick <i>et al.</i> (1999)	Mexicans (women)	250	17.6	47.6	34.8	= #
Yang <i>et al.</i> (2008)	Mexican-Americans	2068	29.9	49.8	20.3	= *
Hanson <i>et al.</i> (2001)	Mixed ancestry (Upper Midwestern region of the US)					
	CHD patients	772	42.0	47.2	10.9	= #
	Deep-vein thrombosis patients	137	42.3	46.0	11.7	= #
	Controls	329	39.5	48.0	12.5	= #

C = cytosine; CHD = coronary heart disease; HWE = Hardy-Weinberg equilibrium; T = thymine; UK = United Kingdom; US = United States; % = percentage distribution of the genotype; * Hardy-Weinberg equilibrium reported; # Hardy-Weinberg calculated; = adheres to assumptions of Hardy-Weinberg equilibrium; ≠ does not adhere to the assumptions of Hardy-Weinberg equilibrium

However, when investigating the geographic distribution of the ethnic groups the most likely explanation of the very low prevalence of the genetic variation among Africans and high prevalence among other ethnic groups could be due to a founder effect. The founder effect is a genetic bottleneck that occurs when a small group becomes reproductively separated from the main population which was the genetically diverse African population from which all other humans arose (Chen *et al.*, 1995). Small groups immigrated to other continents and lead to increased genetic homogeneity. The high prevalence of this genetic variation is ascribed to the high prevalence of the variation among the founder groups.

From the above it is clear that there is no simple explanation for the difference in gene frequencies observed between different ethnic groups. Even though these differences in distribution of the C677T exist between the ethnic groups, results from most of the studies included in Table 4.1 adhered to HWE.

In the study of Conroy *et al.* (2000) and Dilley *et al.* (2001), 503 caucasian newborns and the 185 African-American subjects (mean age 55; 65 and younger) that were used as controls for CVD patients, respectively, differed significantly from the frequencies assumed under the assumptions of HWE. The lack of HWE might have affected any associations detected between CVD patients and controls in the study of Dilley *et al.* (2001), in which it was concluded that neither the heterozygous nor the homozygous genotype was associated with myocardial infarction. Since the control group did not adhere to the assumptions of HWE, it was not only disease status that differed between these two groups, therefore, any association or lack thereof between the genes and disease could be due to the violation of the assumptions of HWE.

4.3.1.2 The A1298C polymorphism in the methylenetetrahydrofolate reductase gene

This section on common genetic variations within the MTHFR gene focuses on the A1298C alteration and includes an additional subdivision to those described in Section 4.3.1.1, namely Section 4.3.1.3. Within this section compound heterozygosity for MTHFR C677T and A1298C is discussed.

4.3.1.2.1 *Molecular aspects and functional consequences of the MTHFR A1298C alteration*

Another sequence change within the MTHFR locus was identified within exon 7. It consisted of an A to C transversion which results in a glutamate (E) to alanine (A) substitution at amino acid number 429 (E429A) in the C-terminal regulatory domain of the MTHFR enzyme (Viel *et al.*, 1997). The original published human MTHFR complementary DNA sequence (Goyette *et al.*, 1994) contains the C nucleotide at bp 1298, therefore, this mutation was initially reported as a C to A nucleotide change. However, after several studies investigating its frequency, it became clear that the more prevalent base at this position was the A nucleotide and the A1298C nomenclature has accordingly been adopted for this MTHFR variant (Rozen, 2001). Donnelly (2000) reviewed the MTHFR human messenger ribonucleic acid (mRNA) sequence and reported that the locus represented as being at nucleotide position 1298 was in fact at nucleotide position 1289. However, the 1289 nomenclature has not been used in the literature, since Van der Put *et al.* (1998) used the same method of numbering as that used for the C677T alteration, which was one of the first SNPs discovered in the MTHFR gene. As previously mentioned in Section 4.3.1.1.1, the C677T alteration is actually located at nucleotide position 665, but as the C677T nomenclature was widely accepted at that time, the designation A1298C was retained. In this review the A1298C naming will, therefore, be used when referring to this polymorphism.

In vitro data suggest that the A1298C SNP reduces MTHFR-specific activity, though to a lesser degree than the C677T SNP (Lieviers *et al.*, 2001a; Weisberg *et al.*, 1998; Weisberg *et al.*, 2001). Unlike the enzyme encoded by the MTHFR gene harbouring the C677T SNP, the enzyme harbouring the E429A alteration does not appear to be thermolabile (Van der Put *et al.*, 1998). The mechanism by which the A1298C SNP affects MTHFR activity remains speculative (Rozen, 2001).

It appears that there is no significant increase in Hcy in individuals harbouring the A1298C SNP (Friedman *et al.*, 1999; Van der Put *et al.*, 1998; Weisberg *et al.*, 1998). Hanson *et al.* (2001) confirmed that heterozygosity or mutant homozygosity for the A1298C polymorphism alone is not associated with increased fasting tHcy,

regardless of red blood cell folate concentrations, and furthermore that there is no significant association between the A1298C polymorphism and post methionine load increases in tHcy concentrations. Therefore, the MTHFR C677T polymorphism seems to be a stronger determinant of tHcy than the MTHFR A1298C polymorphism (Chiuve *et al.*, 2005; Friedman *et al.*, 1999; Van der Put *et al.*, 1998).

Several studies have reported that the MTHFR 1298 C allele has a synergistic effect with the MTHFR 677 T allele on Hcy concentrations, since harbouring both alterations has been associated with higher tHcy concentrations than those determined for the MTHFR 677 variant alone, however, not all results determined were statistically significant (Föding *et al.*, 2000; Hanson *et al.*, 2001; Weisberg *et al.*, 2001). In a study conducted by Weisberg *et al.* (1998), individuals who were compound heterozygotes for the MTHFR A1298C/C677T alterations, presented with Hcy concentrations similar to that observed among individuals harbouring the 677 TT genotype. This finding was, however, not present in another study of Jewish subjects who were compound heterozygotes (Friedman *et al.*, 1999). Friedman *et al.* (1999) ascribed this to the fact that the A1298C mutation is located within the region encoding the C-terminal regulatory domain of the MTHFR gene, whilst the C677T mutation is located within the region encoding the catalytic domain of MTHFR. This study also reported that the individuals with the 677 CC/1298 CC genotypes had significantly lower tHcy concentrations than those harbouring the 677 CC/1298 AA genotypes (Friedman *et al.*, 1999). Heterozygosity at these two loci was also not associated with significantly increased Hcy in an African population (Adjalla *et al.*, 2003). Adjalla *et al.* (2003) opined that unlike the previously reported results, the increased Hcy observed in the combined genotypes might only be due to the C677T mutation.

Currently, there is no evidence that the A1298C alteration, either on its own or in conjunction with C677T, increases the risk for CVD (Franco *et al.*, 1999; Hanson *et al.*, 2001; Janosíková *et al.*, 2003; Zetterberg *et al.*, 2002a). According to Scott (2003), however, it is possible that there could be an increased CVD risk associated with the A1298C alteration even in the absence of elevated Hcy concentrations *i.e.* if this SNP presents with tissue specific effects in the endothelial cells or cardiac cells, but not in liver cells. The former could result in CVD, whereas the absence of a

significant effect on the liver, which mainly controls Hcy concentrations, would result in no significant change in the tHcy concentrations. However, this theory has not yet been confirmed and since there is no evidence that the A1298C alteration is associated with CVD, it seems highly unlikely.

4.3.1.2.2 *Population frequencies of A1298C variant in the methylenetetrahydrofolate reductase gene*

The population frequency of the A1298C SNP varies among different populations as summarised in Table 4.2. It is clear that the MTHFR 1298 CC genotype is less common in Africans than in Caucasian individuals, individuals with mixed ancestry and individuals of Israeli descent.

It is noteworthy that the distribution of this genotype observed in West African individuals was 1.9% to 2% (Adjalla *et al.*, 2003; Amouzou *et al.*, 2004) *versus* 5% for black South Africans (Scholtz *et al.*, 2002). However, the distribution reported by Scholtz *et al.* (2002) for black Africans did not adhere to HWE and, therefore, one could infer that due to the small sample size the frequency of the mutant allele was not representative of the black African population in general.

4.3.1.3 *Compound heterozygosity for the C677T and A1298C alterations in the methylenetetrahydrofolate reductase gene*

The mutant allele of the A1298C polymorphism has rarely been seen in *cis* orientation with the mutant allele of the 677 polymorphism (Hanson *et al.*, 2001; Rozen, 2001). This observation could suggest that the two mutations arose separately on different haplotypes (Hanson *et al.*, 2001; Rozen, 2001).

Elevated Hcy concentrations due to various factors including genetic variations in the MTHFR gene and folate insufficiency have been associated with several obstetrical complications including placental pathology (Goddijn Wessel *et al.*, 1996), pre-eclampsia (Dekker *et al.*, 1995) and recurrent miscarriages (Coumans *et al.*, 1999). Periconceptional folic acid and riboflavin supplementation or fortification, which may lower the incidence of abruption placentae and spontaneous miscarriages

associated with compound heterozygosity for MTHFR C677T and A1298C (Gebhardt *et al.*, 2001; Isotalo & Donnelly, 2002), could be hypothesised to be responsible for the higher frequency of MTHFR mutated alleles observed in several countries (Zetterberg *et al.*, 2002b). This is especially true of developed countries such as North America, where riboflavin fortification has existed for > 50 years (McNulty *et al.*, 2006) and in Europe, since Europeans were reported to consume more B-vitamin supplements than South Asians or Chinese individuals (Kelemen *et al.*, 2004). For the same reason one could speculate that the frequencies of these mutations could also increase in future in the whole of the United States where folic acid fortification has been mandated since 1998. Future research should be focused on determining whether B-vitamins could have such a pronounced effect on allele frequencies in different populations.

Table 4.2 Frequency of the MTHFR A1298C alteration in different ethnic groups and regions around the world expressed as percentages

Citation	Ethnic group	Sample	% 1298AA	% 1298AC	% 1298CC	HWE
Adjalla <i>et al.</i> (2003)	Africans (West Africa)	240	70.0	28.0	2.0	= *
Amouzou <i>et al.</i> (2004)	Africans (West Africa savannah)	68	73.5	22.1	4.4	= *
	Africans (West Africa coast)	208	69.2	28.8	1.9	= *
Guéant-Rodriguez <i>et al.</i> (2006)	Africans (West Africa Bénin and Togo)	465	84.2	13.9	1.9	= *
Scholtz <i>et al.</i> (2002)	Africans (South Africa)	60	87.0	8.0	5.0	≠ #
Rady <i>et al.</i> (2002)	African-Americans	97	71.1	26.8	2.1	= #
Yang <i>et al.</i> (2008)	Non-Hispanic blacks	2100	67.6	29	3.4	= *
Rady <i>et al.</i> (2002)	Ashkenazi Jews	149	53.7	38.3	8.0	= #
Guéant-Rodriguez <i>et al.</i> (2006)	Caucasians (France)	366	52.8	35.7	11.5	= *
	Caucasians (Italy)	146	64.4	28.1	7.5	= *
Rady <i>et al.</i> (2002)	Caucasians	159	44.0	47.2	8.8	= #
Scholtz <i>et al.</i> (2002)	Caucasians (South Africa)	76	38.0	50.0	12.0	= #
Yang <i>et al.</i> (2008)	Non-Hispanic whites	2620	47.9	41.8	10.3	= *
Janosíková <i>et al.</i> (2003)	Czech Patients with CHD	278	51.4	38.9	9.7	= *
	Czech Controls	591	44.7	45.3	10.0	= *
Rady <i>et al.</i> (2002)	Hispanics	96	68.7	27.1	4.2	= #
Guéant-Rodriguez <i>et al.</i> (2006)	Mexicans	300	83.0	14.7	2.3	= *
Yang <i>et al.</i> (2008)	Mexican-Americans	2064	65.7	30.8	3.5	= *
Gebhardt <i>et al.</i> (2001)	Mixed ancestry (coloured South African pregnant women)	168	54.8	36.9	8.3	= #
Hanson <i>et al.</i> (2001)	Mixed ancestry (Upper Midwestern region of the United States)					
	CHD patients	772	46.6	41.7	11.7	= #
	Deep-vein thrombosis patients	137	43.8	45.3	10.9	= #
	Controls	329	49.9	42.2	7.9	= #
Scholtz <i>et al.</i> (2002)	Mixed ancestry (South Africa)	73	55.0	33.0	12.0	= #

A = adenine; C = cytosine; CHD = coronary heart disease; % = percentage distribution of the genotype; HWE = Hardy-Weinberg equilibrium * Hardy-Weinberg equilibrium reported; # Hardy-Weinberg calculated; = adheres to assumptions of Hardy-Weinberg equilibrium; ≠ does not adhere to the assumptions of Hardy-Weinberg equilibrium

In conclusion, genetic variations in the MTHFR gene can influence activity of the MTHFR enzyme and thereby influence Hcy concentrations. Differences in the distribution frequencies of these genetic variations among ethnic groups may in part explain differences in Hcy concentrations. However, there are several other genetic variations, that will be discussed subsequently, that can also influence Hcy concentrations.

4.3.2 Polymorphisms within the cystathionine β -synthase gene

The CBS enzyme plays a central regulating role in the vitamin B₆-dependent transsulphuration of Hcy, whereby CBS commits Hcy to its degradation pathway and its ultimate removal as sulphate (Scott, 2003). A reduction in enzyme activity may result in HHcy or homocystinuria (Kraus, 1998). The CBS gene was mapped to the subtelomeric region of chromosome 21q22.3 (Kraus *et al.*, 1998).

4.3.2.1 The cystathionine β -synthase gene T833C/844ins68 alterations

More than 60 mutations or polymorphisms have been reported in the CBS gene, some of which impair CBS activity (Kraus *et al.*, 1998). Among these, the two most prevalent mutations, *i.e.* a T833C point mutation in the 5' end of exon 8 and a 68-bp insertion (ins68) at position 844 in exon 8, may contribute to HHcy (Griffioen *et al.*, 2005; Sebastio *et al.*, 1995).

4.3.2.1.1 Molecular aspects and functional consequences of the CBS T833C/844ins68 alterations

The T833C transition SNP within exon 8 of the CBS gene causes an isoleucine (I) to threonine (T) substitution at amino acid residue 278 (I278T) and is highly prevalent among homocystinuric patients that have reduced CBS enzyme activity (Sebastio *et al.*, 1995; Sperandeo *et al.*, 1996). In tandem with the missense T833C SNP, a 68-bp insertion at nucleotide 844 (844ins68) has been identified (Sebastio *et al.*, 1995). It was initially hypothesised that this insertion encodes for eleven amino acids 'in frame' followed by a premature termination codon that resulted in the expression of a non-functional protein (Sperandeo *et al.*, 1996), however, it was later discovered

that the 844ins68 duplicates the intron–exon boundary between intron 7 and exon 8, and creates an alternative splicing site, which corrects for the effect of the T833C SNP (Griffioen *et al.*, 2005; Tsai *et al.*, 1996). According to Tsai *et al.* (1996), the net result is the generation of both quantitatively and qualitatively unaffected mRNA and CBS enzyme.

Hcy concentrations seem to be lower in individuals harbouring the 68-bp insertion as compared to those without it (De Stefano *et al.*, 1998; Tsai *et al.*, 1996; Tsai *et al.*, 1999a). It was reported that this decrease in Hcy was only observed in individuals who had low vitamin B₆ status, thus suggesting that the effect of the insertion was associated with low concentrations of pyridoxal 5'-phosphate. The reason for this observation remains to be established. Tsai *et al.* (2000) noted that the insertion was related to lower fasting and incremental post-methionine load Hcy concentrations in 1,031 Caucasian CVD patients and that the effect was modulated by vitamin B₆ status. Tsai *et al.* (1999a) speculated that the 68-bp insertion could be associated with somewhat higher levels of CBS enzyme activity, however, mRNA analysis provided evidence that the allele carrying the insertion is poorly transcribed (Sperandeo *et al.*, 1996). Another explanation could be that the presence of the 68-bp insertion may have abolished the Hcy-raising effect of the thermolabile MTHFR 677 TT genotype (De Stefano *et al.*, 1998).

Nair *et al.* (2002) have reported a nonsignificant higher frequency of the CBS 833 variant in subjects with CVD, but other studies have not reported an association between the T833C SNP and CVD (Dilley *et al.*, 2001; Janosíková *et al.*, 2003). The 844ins68 polymorphism has been reported to be more prevalent in atherosclerosis patients (Tsai *et al.*, 1996). In contrast, Giusti *et al.* (1999) reported no statistical difference in the allele frequencies and genotype distributions of the 844ins68 variant between the controls and coronary heart disease (CHD) patients. This insertion could be speculated to be pathogenic when associated with other CBS and/or MTHFR mutations, as in the case of the homozygous 677 TT mutation. On the other hand, Zhang and Dai (2001) have suggested that the 844ins68 alteration could provide protection to vascular thromboembolic disease and Janosíková *et al.* (2003) associated carriership of the 844ins68 with a significantly lowered risk of CHD. To explore the mechanisms by which the 844ins68 alteration protects against CVD, Janosíková *et al.*

(2003) examined the intermediates of Hcy metabolism. They reported that healthy carriers of the insertion exhibited significantly higher post methionine load ratios of blood s-adenosylmethionine to s-adenosylhomocysteine as well as the ratio of plasma total cysteine to Hcy when compared to the wild-type controls. Their results indicate that methylation status was improved in healthy individuals harbouring the 68-bp insertion, which alluded to enhanced activity of Hcy transsulphuration. Functional data regarding the variations within the CBS gene are still limited.

4.3.2.1.2 *Population frequencies of the cystathionine β -synthase gene T833C/844ins68 alterations*

Table 4.3 outlines that the variations in the CBS gene are fairly prevalent among the different populations, but that ethnic differences exist. The T833C/844ins68 mutation is very rare in Asian and Amerindian populations and rare in Caucasians. However, in an African-American population an 833 C allele frequency of 20% was reported (Dilley *et al.*, 2001). Documentation of the frequency of this CBS genetic variation in Africans is limited. Franco *et al.* (1998b) reported a high frequency of 37.7% and 4%, respectively for heterozygotes and mutant homozygotes of the T833C/844ins68 alterations, in a group of black Brazilians and Africans from Zaire, Cameroon and Angola. Since the T833C/844ins68 double mutation was not detected in earlier studies conducted in Asian populations, this allele has been hypothesised by Pepe *et al.* (1999) to be a reliable anthropogenetic marker for discriminating between two major human groups *i.e.* Africans and Asians. However, the proposal was rejected when the 844ins68 variant was observed in a Han Chinese (Zhang & Dai, 2001) and Indian population (Nair *et al.*, 2002). The 844ins68 frequency in healthy Thai children did not adhere to HWE (Sirachainan *et al.*, 2008). For the wildtype, heterozygote and mutant homozygote categories the observed amount of subjects were 94, 1 and 1 while the expected amount calculated using the Hardy-Weinberg formula was 93, 3 and 1, respectively ($\chi^2 \approx 42$ and $p < 0.01$). The healthy Thai children were most likely not representative of the Thai population due to a small sample size, however, the small sample ($n = 77$) of controls of mixed ancestry for CHD patients reported by Tsai *et al.* (1996) adhered to the HWE and, therefore, one could assume that the general population was represented even in their small sample.

Violation of any of the assumptions of HWE as described in Section 4.3.1.1.2 could also be the cause and cannot be ruled out.

Most screening studies report that the CBS T833C and the 844ins68 alterations cosegregate in a *cis* orientation (Giusti *et al.*, 1997; Giusti *et al.*, 1999), an association observed in Caucasian, white Brazilian, black Brazilian, black African and Amerindian populations (Franco *et al.*, 1998b). Franco *et al.* (1998b) speculated that this observation indicates that the double T833C/844ins68 CBS mutation firstly occurred prior to the divergence of non-Africans from Africans. Giusti *et al.* (1999) further reasoned that the insertion probably arose on the same haplotype as the mutated 833 allele (*i.e.* insertional mutation took place after the T833C mutation) and spread in the population (monophyletic origin). The frequency distribution of the isolated T833C mutation is unclear in healthy control populations, since little has been reported on the subject.

Individuals who are compound heterozygous at the T833C/844ins68 loci, a state which is reported to neutralise the effect of the T833C mutation, are extremely prevalent in black Brazilian and African populations. Therefore, Franco *et al.* (1998b) speculated that the expression of homocystinuria that was due to the CBS deficiency caused by homozygosity for the T833C mutation might have been suppressed or reduced during evolution by the CBS 844 insertion.

4.3.2.2 The G9276A single nucleotide polymorphism of the cystathionine β -synthase gene

Griffioen *et al.* (2005) discovered the novel G9276A transition in intron 8 of the CBS gene. If the G9276A mutation is present, a possible alternate splice acceptor site (CAG) is formed. In Caucasian volunteers, 0.2% was reported to be heterozygous at the G9276A alteration (Griffioen *et al.*, 2005). Since studies regarding this mutation are scarce, further studies should be undertaken to explore the functional effect of the CBS G9276A missense mutation as well as the distribution of the mutation in various ethnic groups.

Table 4.3 Frequency of the CBS T833C/844ins68 alteration in different ethnic groups and regions around the world expressed as percentages

Studies that investigated T833C	Ethnic group	Sample	% 833TT	% 833TC	% 833CC		HWE
Dilley <i>et al.</i> (2001)	African-Americans	107	60	34	6		= #
	Myocardial infarction cases	172	63	32	5		= #
Janosiková <i>et al.</i> (2003)	Nationality reported	278	99.64	0.36	0		= *
	Czech patients with coronary heart disease	591	100	0	0		= *
Tsai <i>et al.</i> (1999b)	Czech controls						
	Mixed ancestry (upper Midwestern region of the United States)	376	99.5	0.5	0		= #
	Coronary heart disease	82	100	0	0		Cannot calculate
	Controls						
Studies that investigated 844ins68	Ethnic group		% 844ins68 wild-type	% 844ins68 heterozygous	% 844ins68 homozygous insert		HWE
Yang <i>et al.</i> (2008)	Non-Hispanic blacks	2032	55	38.2	6.7		= *
Giusti <i>et al.</i> (1999)	Caucasians (Italy) with coronary heart disease	133	80.45	18.8	0.75		= *
	Caucasians (Italy) controls	595	85.55	13.61	0.84		= *
Yang <i>et al.</i> (2008)	Non-Hispanic whites	2566	84.2	15.3	0.5		= *
Janosiková <i>et al.</i> (2003)	Nationality reported	278	91	9	0		= *
	Czech patients with coronary heart disease	591	84.77	14.89	0.34		= *
Kluijtmans <i>et al.</i> (1997)	Czech controls						
	Caucasians	60	83.3	16.7	0		= *
Yang <i>et al.</i> (2008)	Dutch vascular disease patients	107	83.2	14.0	2.8		= *
	Dutch controls						
Tsai <i>et al.</i> (1996)	Mexican-Americans	2036	87.7	11.9	0.4		= *
	Mixed ancestry (healthy controls)	77	88.3	11.7	0		= #
	Mixed ancestry (coronary heart disease patients)	182	83.5	16	0.5		= #
Sirachainan <i>et al.</i> (2008)	Thai children with ischaemic stroke	29	97	3	0		= #
	Healthy Thai children	96	98	1	1		≠ #

Studies that investigated T833C and 844ins68 SNP	Ethnic group		% 833TT W/O 844ins68	% 833TC with 844ins68	% 833CC with 844ins68	833 TC W/O ins	HWE
Franco <i>et al.</i> (1998b)	Amerindians (Brazilian Amazon tribes)	110	99.1	0.9	0	0	= *
Franco <i>et al.</i> (1998b)	Blacks (50 Brazilian blacks, 101 Africans from Zaire, Cameroon and Angola)	151	58.3	37.7	4	0	= *
Franco <i>et al.</i> (1998b)	Whites (64 Brazilians, 41 Caucasians from Portugal)	104	86.5	13.5	0	0	= *
Giusti <i>et al.</i> (1997)	Caucasians (Northern region of Italy)	62	74.2	25.8	0	0	= #
	Caucasians (Southern region of Italy)	50	92	8	0	0	= #
Griffioen <i>et al.</i> (2005)	Caucasians (Dutch)	500	89.6	9.0	1.2	0.2	= *
Franco <i>et al.</i> (1998b)	Japanese	40	100	0	0	0	= *

C = cytosine; ins = insertion; % = percentage distribution of the genotype; T = thymine; HWE = Hardy-Weinberg equilibrium; W/O = without; * Hardy-Weinberg equilibrium reported;

Hardy-Weinberg calculated; = adheres to assumptions of Hardy-Weinberg equilibrium; ≠ does not adhere to the assumptions of Hardy-Weinberg equilibrium

4.3.3 The A2756G polymorphism of the methionine synthase gene

Methionine synthase (MS) is an enzyme involved in the methylation cycle of Hcy to methionine. MS functions to conserve Hcy as methionine and plays a key role in the folate-dependent one-carbon metabolism of Hcy by releasing 5-MTHF (Scott, 2003). Therefore, defects in the MS enzyme could result in altered Hcy metabolism. The human MTR gene coding for the MS enzyme has been mapped to chromosome 1q43 (Leclerc *et al.*, 1996).

4.3.3.1 Molecular aspects and functional consequences of the MTR A2756G alteration

Sequencing analysis of the MTR gene revealed an A to G transition at nucleotide position 2756 in the genetic region encoding the protein binding domain of the MS enzyme, which results in an aspartic (D) acid (which is believed to be part of a helix involved in cofactor binding) to glycine (G) substitution at amino acid residue 919 (D919G). The A2756G SNP has been identified to be in the vicinity of the binding domain of vitamin B₁₂ (cobalamin) on the apoenzyme MS. Since human MS has not been expressed *in vitro*, functional studies of this polymorphism have been performed in *Escherichia coli* and *Caenorhabditis elegans* (Li *et al.*, 1996). The mutated bacterial enzyme displayed a slight decrease in activity with impaired reductive activation (Rozen, 2001).

Since MS is a vitamin B₁₂-dependent enzyme which catalyses the remethylation of Hcy to methionine, reduction in its activity due to the MTR A2756G variant was hypothesised to increase Hcy concentrations and, hence, increase the risk for CVD (Klerk *et al.*, 2003; Leclerc *et al.*, 1996). *In vivo* studies on the biological function of this polymorphism, were mostly observational in nature with limited sample sizes, thus producing controversial results. Most studies have reported no association between the MTR A2756G polymorphism and Hcy concentrations (Adjalla *et al.*, 2003; Amouzou *et al.*, 2004; Hyndman *et al.*, 2000; Jacques *et al.*, 2003; Ma *et al.*, 1999; Tsai *et al.*, 1999b; Van der Put *et al.*, 1997; Wang *et al.*, 1998) even after methionine loading (Klerk *et al.*, 2003). Contrastingly, several other studies have reported that both fasting and post methionine load Hcy concentrations were decreased in an additive fashion dependant on the number of G alleles present in the genotype (Harmon *et al.*, 1999; Silaste *et al.*, 2001; Tsai *et al.*, 1999b; Wang *et al.*, 1999). The association between this genotype and Hcy concentrations, as described by Harmon *et al.* (1999), was independent of folate and vitamin B₁₂ status, however, the AA genotype had a larger Hcy-elevating effect in individuals with low vitamin B₆ status. Chen *et al.* (2001b) and Harmon *et al.* (1999) concluded that the lowering influence of the A2756G polymorphism on plasma tHcy concentration and its increasing influence on folate levels are significant albeit moderate. Klerk *et al.* (2003), who reported no association between the polymorphism and plasma Hcy, still

reported an OR of 4.0 [95% CI 1.4 – 11.6] for subjects harbouring the GG genotype towards CHD risk when compared to the AA genotype. They also reported that carriers of the GG genotype had 30% lower vitamin B₁₂ concentrations than the AA genotype. Klerk *et al.* (2003), therefore, opined that it is unlikely that this significant increase in CHD risk was related to altered Hcy metabolism, but could rather be ascribed to the association of an interaction between the MTR A2756G polymorphism, vitamin B₁₂, folate or other CVD risk factors. From the results of the study conducted by Chen *et al.* (2001b) it could be hypothesised that this variant could cause methyl-trapping and result in higher plasma folate concentrations, but this could not be confirmed in the study of Klerk *et al.* (2003). Since one would not expect that a polymorphism in a specific gene encoding an enzyme would affect the level of its co-factor, the lower vitamin B₁₂ concentrations among subjects harbouring the GG genotype was unexpected in the study of Klerk *et al.* (2003) and must have been due to a variable other than the GG genotype. Klerk *et al.* (2003) reasoned that even if the low vitamin B₁₂ concentrations of the subjects harbouring the GG genotype were a chance observation, then vitamin B₁₂ status could have confounded the association between the GG genotype and CHD risk, provided that low B₁₂ status is itself a CHD risk factor. However, an inverse association between vitamin B₁₂ and CHD is scarce in the literature (Siri *et al.*, 1998). Another possible confounding factor might have been linkage disequilibrium between this polymorphism and another genetic variation, which is the actual risk factor (Klerk *et al.*, 2003).

Several other studies suggest that the MTR GG genotype has a protective rather than an adverse effect on CHD (Morita *et al.*, 1999; Chen *et al.*, 2001b) and is rather associated with a reduced risk for a recurrent cardiovascular event (Hyndman *et al.*, 2000). However, the cardiovascular advantage observed in those harbouring the GG genotype could be neutralised by the increased susceptibility to malignant lymphoma that this genotype also seems to convey (Matsuo *et al.*, 2001). However, Ma *et al.* (1999) reported a possible association between the MTR polymorphism and lower risk toward colorectal cancer especially among subjects with low alcohol consumption, in the same direction as observed for the MTHFR polymorphism, however, these observations need to be confirmed in larger populations due to the limited statistical power of this study.

4.3.3.2 Population frequencies of the MTR A2756G alteration

The MTR A2756G transition is rare and the frequency of mutant homozygotes is less than 5% in most populations (as presented in Table 4.4). Data on black African groups is limited, but a study by Amouzou *et al.* (2004) reported that the G allele frequency of the MTR A2756G alteration is high in West Africans as compared to Caucasian populations. The A2756G genotype distribution of the CHD cases in the study of Klerk *et al.* (2003) did not adhere to the assumptions of HWE. This observation further supports the theory that this alteration has undergone negative selective forces towards CHD.

Table 4.4 Frequency of the MTR A2756G alteration in different ethnic groups and regions around the world expressed as percentages

Citation	Ethnic group	Sample	% 2756 AA	% 2756 AG	% 2756 GG	HWE
Adjalla <i>et al.</i> (2003)	Africans (West Africa)	240	51.7	42.5	5.80	= *
Amouzou <i>et al.</i> (2004)	Africans (West Africa savanna)	68	60.2	30.8	8.80	= *
	Africans (West Africa coast)	208	48.6	46.6	4.81	= *
Conroy <i>et al.</i> (2000)	African-Americans (New York)	528	58.7	35	6.3	= #
Chen <i>et al.</i> (2001b)	Caucasian-Americans (Men)					
	Myocardial infarction patients	387	67.7	30.2	2.1	= #
Conroy <i>et al.</i> (2000)	Controls	767	67.1	29.2	3.7	= #
	Caucasians (New York)	503	65	30.2	4.8	= #
Klerk <i>et al.</i> (2003)	Caucasians (Netherland)					
	CHD patients	123	65.9	26.8	7.3	≠ *
Ma <i>et al.</i> (1999)	Controls	540	69.6	28.2	2.2	= *
	Caucasian (American)	712	69.7	27.5	2.81	= *
Van der Put <i>et al.</i> (1997)	Caucasians (Netherland)	597	70.5	26.5	3.02	= *
Wang <i>et al.</i> (1998)	Caucasians (Australia)	745	61.9	33.8	4.30	= *
Bathum <i>et al.</i> (2007)	Danish (Adult twins)	1126	67	29	4	= *
Conroy <i>et al.</i> (2000)	Hispanics (New York)	521	65.5	31.5	3	= #
Hyndman <i>et al.</i> (2000)	Ethnic group not reported					
	Vascular disease patients	109	69.72	29.36	0.92	= #
Jacques <i>et al.</i> (2003)	Ethnic group not reported					
	(Framingham and Utah)	677	66.9	29.1	3.99	= #
Janosíková <i>et al.</i> (2003)	Czech CHD patients	278	65.83	29.5	4.67	= *
	Czech controls	591	65.3	30.8	3.9	= *
Morita <i>et al.</i> (1999)	Japanese (Tokyo)					
	CHD patients	215	67.4	31.2	1.4	= *
	Ischaemic stroke patients	251	64	33	3	= *
	Controls	257	67	32	2	= *

A = adenine; CHD = coronary heart disease; G = guanine; % = percentage distribution of the genotype; HWE = Hardy-Weinberg equilibrium; * Hardy-Weinberg equilibrium reported; # Hardy-Weinberg calculated; = adheres to assumptions of Hardy-Weinberg equilibrium; ≠ does not adhere to the assumptions of Hardy-Weinberg equilibrium

4.3.4 Several other common polymorphisms that influence homocysteine concentrations

Various other polymorphisms have been identified that are hypothesised to influence Hcy concentrations. Several of these alterations are within the genes already mentioned. However, an exhaustive analysis of all the mutations present is not within the scope of this review and, therefore, a brief mention of certain alterations will follow subsequently.

A variable number of tandem repeats (VNTR) of 31-bp which spans the exon 13-intron 13 boundary of the CBS gene, was first described by Kraus *et al.* (1998), and has been associated with post methionine load HHcy that may predispose to increased CVD risk (Liewers *et al.*, 2001b). Each repeat unit contains a consensus splice donor site. The CBS enzyme activity seems to decrease with increasing number of repeat units of the 31 bp VNTR, therefore, this VNTR can affect Hcy metabolism negatively.

The enzyme, methionine synthase reductase (MTRR), is indirectly involved in the methylation cycle. Normally, a small percentage of the vitamin B₁₂ cofactor that is attached to the active site of MS becomes oxidised (Ludwig & Matthews, 1997) and MTRR can reduce this back to the active form. Therefore, decreased MTRR activity would be expected to lead to reduced MS activity. A common variant *i.e.* the A66G transition within MTRR, has been reported and it results in the substitution of an isoleucine (I) with methionine (M) at codon 22 (I22M). Two studies have reported that there is no association between MTRR A66G genotype status and Hcy (Brown *et al.*, 2000; Wilson *et al.* 1999), however, higher Hcy concentrations were reported by Gaughan *et al.* (2001) for those who harboured the AA genotype at this locus. Recently, Yang *et al.* (2008) reported a significant interaction between the MTHFR C677T and the MTRR A66G alteration on the serum Hcy concentrations of a white non-Hispanic population. Individuals who were double mutant homozygotes *i.e.* individuals harbouring both the 677 TT and 66 GG genotypes, had a 25.6% lower serum tHcy concentrations than individuals who harboured the TT/AA genotypes.

Transcobalamin (TC) is the plasma transporter that delivers vitamin B₁₂ to cells, therefore, some of the genetic variation [*i.e.* I23V, G94 serine (S), proline (P) 259 arginine (R), S348 phenylalanine (F) and R399 glutamine (Q)] in the TC gene may affect intracellular vitamin B₁₂ availability and, consequently, tHcy concentrations (Lievers *et al.*, 2002b). However Lievers *et al.* (2002b) reported that the G94S, S348F and R399Q alterations had no association with tHcy, but that lower tHcy was observed in 259 PP and 23 VV carriers.

Folate-transporting proteins, such as reduced folate carrier-1 (RFC1), are also important in determining Hcy concentrations, since they are responsible for the amount of folate available in the cells. RFC1 protein is located in the intestinal mucosa membrane and plays a role in folate absorption, transporting 5-MTHF into the cells (Nguyen *et al.*, 1997). A common transition (G80A) in the RFC1, which results in a substitution of an arginine (R) to histidine (H) at amino acid number 27 (R27H) has also been identified. However, it seems to cause no change in tHcy or red blood cell folate concentrations (Chango *et al.*, 2000).

A polymorphism determined in the folate polyglutamate hydrolase gene, namely C1561T, results in a histidine (H) to a tyrosine (Y) amino acid substitution (H475Y). Study results regarding the effect of this variation on Hcy are inconsistent, since Devlin *et al.* (2000) associated the variation with higher Hcy, but Lievers *et al.* (2002a) observed lower tHcy concentrations in individuals with the 1561 TT genotype.

4.4 SUMMARY AND CONCLUSION

In Table 4.5 the molecular aspects and functional consequences of several of the polymorphisms which affect Hcy metabolism are summarised. Of the genetic variation described here, the MTHFR C677T seems to be the strongest determinant of Hcy concentrations (Bathum *et al.*, 2007). Therefore, it seems possible that the distribution of the different common point mutations affecting Hcy, especially for MTHFR C677T, among different ethnic groups may be one of several factors underlying the differences observed in the risk of CVD through increased Hcy concentrations. However, whether differences in the distribution of the genotypes

described within this review contribute to differences in CVD prevalence among ethnic groups remains to be established.

As Ubbink *et al.* (1995) speculated and as one can conclude from this review, Africans in general seem to have a favourable genetic background insofar as Hcy concentrations are concerned (*i.e.* low prevalence of 677 T risk allele and high prevalence of the CBS 844ins68 protective allele), which may protect them against CVD risk. However, among Africans the incidence of stroke (Kahn & Tollman, 1999) and heart failure attributable to dilated cardiomyopathy or hypertensive heart disease, or both (Sliwa *et al.*, 2008) remain high.

Table 4.5 Common polymorphisms in some of the enzymes involved in homocysteine metabolism [adapted from Scott (2003)]

Enzyme	Genetic alteration	Amino acid change	Phenotype of variant	Reference
MTHFR	C677T	A222V	Thermolabile enzyme Reduced enzyme activity Lower B-vitamin status Higher plasma Hcy	Frosst <i>et al.</i> (1995) and Rozen (2001)
MTHFR	A1298C	E429A	No changes	Van der Put <i>et al.</i> (1998) and Weisberg <i>et al.</i> (1998)
CBS	T833C	I278T	Reduced enzyme activity	Sebastio <i>et al.</i> (1995) and Sperandio <i>et al.</i> (1996)
CBS	844ins68	Frameshift	No change in basal Hcy Less accumulation of plasma Hcy after a methionine load	Tsai <i>et al.</i> (1999a) and De Stefano <i>et al.</i> (1998)
CBS	G9276A	Intronic region	No research has been conducted to determine the phenotype	Griffioen <i>et al.</i> (2005)
MS	A2756G	D919G	Higher plasma folate Higher red cell folate Lower plasma Hcy	Harmon <i>et al.</i> (1999) and Morita <i>et al.</i> (1999)

A = adenine (nucleotide); *Ala* = alanine (amino acid); *C* = cytosine (nucleotide); *CBS* = cystathionine β -synthase; *D* = aspartic (amino acid); *E* = glutamate (amino acid); *G* = guanine (nucleotide); *Gly* = glycine (amino acid); *Hcy* = homocysteine; *Ile* = isoleucine (amino acid); *ins* = insertion; *MS* = methionine synthase; *MTHFR* = methyltetrahydrofolate reductase; *T* = thymine (nucleotide); *Thr* = threonine (amino acid); *Val* = valine (amino acid)

Many cases of HHcy are not associated with micronutrient deficiencies, impaired renal function, and/or reported genetic mutations (Tsai *et al.*, 1999b). Further work is, therefore, needed to determine whether unknown mutations (particularly those residing in the intronic sequences of the genes involved in Hcy metabolism such as the CBS G9276A), other environmental factors, or interactions of genes, nutrients and

environmental factors may be the cause of the currently unexplained cases of mild HHcy. CVD has a multifactorial aetiology with multiple genes and environmental factors influencing its development, therefore, it is imperative to ascertain whether there are gene-gene interactions in the genes described previously, as such interactions could influence Hcy concentrations as well (Aléssio *et al.*, 2008). Researchers should further aim to document the genotype frequencies in large, population-based studies of the polymorphisms reported within this review as well as the additional, but less well studied, polymorphisms in these genes (*e.g.* SNPs in the CBS gene), including the prevalence of combinations of polymorphisms. The latter is particularly needed in less-investigated populations and ethnic groups, such as black South Africans, fill this gap in the literature and to determine their genetic susceptibility to CVD as related to Hcy concentration (Sharp & Little, 2004).

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CHAPTER 5

NO INTERACTION EFFECT ESTABLISHED BETWEEN ALCOHOL INTAKE AND THE GENETIC POLYMORPHISM METHYLENETETRAHYDROFOLATE REDUCTASE (MTHFR) C677T IN RELATION TO HOMOCYSTEINE CONCENTRATIONS IN A BLACK SOUTH AFRICAN POPULATION

Running title: Hcy, alcohol intake and the MTHFR C677T variation

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5.1 ABSTRACT

Background: It is unknown whether the effect of alcohol consumption on total homocysteine (tHcy) concentrations is modulated by the methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism.

Objective: To determine the interactions between alcohol consumption and the MTHFR 677 genotype on tHcy concentrations in a black South African population.

Design: This article outlines a cross-sectional epidemiological study of the baseline data of 1827 black individuals within the South African arm of the international Prospective Urban and Rural Epidemiology study.

Results: Subjects harboring the 677 TT genotype had the highest mean tHcy concentration. Among subjects harboring the 677 CC genotype, men had significantly ($p = 0.04$) higher tHcy concentrations than women. Age and γ -glutamyl transferase (GGT) correlated best with tHcy concentrations ($r = 0.26$ and $r = 0.27$; $p < 0.05$) while percentage carbohydrate deficient transferrin (%CDT) and the B-vitamins only correlated weakly ($r < 0.1$ for both; $p < 0.05$). Age, GGT, gender, MTHFR and vitamin B₆ explained 16.8% of the variation in tHcy with the following β -values 0.26, 0.23, 0.13, 0.10 and -0.09 ($p < 0.01$). tHcy concentrations were positively associated with reported alcohol intake ($p \leq 0.01$). There was no interaction between alcohol consumption and the MTHFR 677 CC or CT genotypes ($p > 0.05$).

Conclusions: There is no significant interaction between alcohol intake and the MTHFR 677 CC or CT genotypes, however, MTHFR C677T genotype status, age, gender and GGT are more important determinants of tHcy concentration than B-vitamin intake in the black South African population investigated.

KEY WORDS Africans, alcohol, gamma glutamyltransferase, homocysteine, methylenetetrahydrofolate reductase, MTHFR, polymorphism, percentage carbohydrate deficient transferrin

5.2 INTRODUCTION

Moderate alcohol consumption is regarded to be cardio-protective (Agarwal, 2002; Bagnardi et al., 2008). However, consuming large quantities in a short period *i.e.* binge drinking as well as chronic alcohol abuse are associated with increased cardiovascular disease (CVD) morbidity (Puddey et al., 1999) as well as increased risk for developing several cancers (Corrao et al., 2004).

One of the negative consequences of hazardous drinking is elevated concentrations of the sulfur-containing amino acid homocysteine (Hcy), *i.e.* hyperhomocysteinemia hereafter abbreviated as HHcy (Carmel & James, 2002) which is associated with CVD (Castañon et al., 2007; Humphrey et al., 2008) and several cancers (Wu & Wu, 2002). Besides alcohol consumption, total (t)Hcy concentrations are influenced by demographic determinants (*i.e.* age and gender), a combination of genetic determinants, especially the methylenetetrahydrofolate reductase (MTHFR) cytosine (C) to thymine (T) gene variant at base pair (bp) 677 (C677T) which seems to be among the strongest common genetic determinants of Hcy concentrations (Bathum et al., 2007), and other lifestyle factors. The latter include B-vitamin intake, tobacco use, and coffee and tea consumption (De Bree et al., 2002). Alcohol consumption amongst Caucasian women leads to increased tHcy concentrations and when the MTHFR C677T alteration is present and folate intake is inadequate, this effect of alcohol on tHcy concentrations seems to be exacerbated (Chuve et al., 2005).

It has been reported that South Africans annually consume approximately 20 litres of absolute alcohol per drinker and that a third of these drinkers binge drink (Parry et al., 2005). It would, therefore, be warranted to investigate the influence of alcohol intake and the presence of the 677 T allele on Hcy concentrations in South Africans. To our knowledge, no study has investigated the effect of alcohol consumption on tHcy concentrations in the presence of the MTHFR C677T alteration in a cohort including both men and women or in any African population. The present study was undertaken to determine the interaction and combined effects of alcohol intake [measured by the percentage carbohydrate deficient transferrin (%CDT), gamma (γ)-glutamyl transferase (GGT) concentrations and quantitative food frequency questionnaire (QFFQ) data] and the three different MTHFR C677T genotypes on

their concentrations in a black South African population including both men and women.

5.3 SUBJECTS AND METHODS

5.3.1 Study design and population selection

This cross-sectional epidemiological study was nested within the international multi-centre twelve-year Prospective Urban and Rural Epidemiology (PURE) study and was conducted on the baseline data collected in 2005 in South Africa (Koon et al., 2009). The sampling approach implemented ensured representative sampling of both urban and rural areas. The head of each randomly selected household within the selected communities provided voluntary, written informed consent (see Addenda A and B) before completing a detailed questionnaire (see Addendum G). Whenever the head of a household refused or was not present, the next randomly selected house was chosen. A total of 3750 questionnaires were completed, but only 2010 apparently healthy African volunteers, with no reported usage of medication for chronic diseases of lifestyle, tuberculosis or known infection with the human immunodeficiency virus (HIV) were eligible to be recruited.

5.3.2 Ethics

Permission to conduct this study was obtained from the North West Provincial Department of Health, tribal chiefs, community leaders, employers and mayors of the included towns. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Ethics Committee of the North-West University (Ethics number: 04M10). Confidentiality and anonymity of all results were assured. Recruited subjects were informed of the various aspects of the study and were required to provide written informed consent (see Addendum C) before they could participate.

5.3.3 Demographic characteristics

An interviewer-based questionnaire developed for the international PURE-study, adapted and standardized for use in South Africa was used (see Addendum G). Subjects provided information regarding ethnicity, a history of tobacco use and medical conditions, including a history of CVD, diabetes and cancer diagnosed by a doctor.

5.3.4 Biochemical analyses

Every subject identified with an anomaly in the tested markers was referred to the nearest health facility (see Addendum D).

5.3.4.1 Blood sampling and storage

Fasting venous blood samples were collected by registered nurses from the ante-cubital vein of the right arm of subjects. Collection tubes were mixed gently by inverting each tube five times. For the collection of serum, blood was allowed to clot in a tube without anti-coagulants (at room temperature for 30 minutes) and centrifuged at 2000 x g for 15 minutes at 10 °C. The resultant serum was aliquotted and stored at -70 °C until analysis. For plasma samples, blood was collected in ethylenediamine tetra-acetic acid (EDTA) tubes and centrifuged at 2000 x g for 15 minutes at 4 °C. The resultant supernatant was aliquotted and stored at -70 °C until analysis. For the collection of the buffy coat, citrate treated whole blood was centrifuged at 2000 x g for 15 minutes at room temperature and the leukocyte layer was transferred to a storage tube. All the above-mentioned samples were centrifuged within two hours after collection. However, plasma blood samples for Hcy were immediately placed on ice and separated within one hour of sampling to inhibit the *in vitro* cellular release of Hcy (Rasmussen & Møller, 2001).

5.3.4.2 Determination of human immunodeficiency virus status

HIV counseling and testing were done by research nurses trained in Voluntary Counseling and Testing, adhering to the UNAIDS/WHO Policy Statement on HIV-testing (UNAIDS/WHO, 2004) as well as to the protocol of the National

Department of Health of South Africa. Subjects signed informed consent (see Addendum C) to be tested for HIV after receiving pre-test counseling. Whole blood was used for the rapid first response HIV card test 1-2.0 (Transnational Technologies Inc.) and when the result was positive, it was confirmed with a Pareeshak test. Post-test counseling was provided for subjects who wanted to be informed of their HIV status.

5.3.4.3 Biochemical markers of alcohol intake

Serum GGT concentrations were measured by using a Sequential Multiple Analyzer Computer (SMAC), using the KonelabTM auto analyzer (Thermo Fisher Scientific Oy, Vantaa, Finland) which is a clinical chemistry analyzer for colorimetric, immunoturbidometric and ion-selective electrode measurements. The cut-off points used for men and women were 1.33 $\mu\text{kat/L}$ (80 U/L) and 0.83 $\mu\text{kat/L}$ (50 U/L) respectively. The SMAC methodology was also used for analyzing creatinine and high sensitivity C-reactive protein (hs-CRP) concentrations in the serum of the subjects.

Serum %CDT analyses were performed by using an *in vitro* heterogeneous immunoassay with column separation followed by turbidometric measurements (Axis-Shield % CDT kit, Oslo, Norway). The measuring range of this test is 1.5 to 24 mg/L of transferrin and the cut-off value for %CDT was set at 2.6% following the recommendations of the manufacturer. Percentage CDT indicates the relative amount of CDT isoforms in proportion to total transferrin and has been reported to be a better marker of chronic alcohol consumption than absolute CDT (Salaspuro, 1999; Sillanaukee, 1996; Viitala et al., 1998). The coefficient of variance (CV) for all the above-mentioned assays was < 10%.

5.3.4.4 Determination of total homocysteine concentrations

The sum of free and protein-bound Hcy, homocystine and homocysteine-cysteine mixed disulfide *i.e.* tHcy concentrations were determined from venous EDTA-treated plasma by a pathology firm using the Abbott automated immunoassay analyzer

(AxSYM) based on fluorescence polarization immunoassay technology (CV = 4.52%).

5.3.4.5 Deoxyribonucleic acid isolation and genotyping of the methylenetetrahydrofolate reductase C677T genetic alteration

Genomic deoxyribonucleic acid (gDNA) was isolated from citrate treated buffy coat using the QIAGEN FlexiGene DNA extraction kit according to the manufacturer instruction. Purity (A260 to A280 ratio) and yield were determined with the Nano-Drop spectrophotometer (ND-1000). Sample DNA was amplified by the polymerase chain reaction (PCR). The PCR mixture contained 0.125 nmol of each primer [MTHFR 677 forward 5'-TGA AGG AGA AGG TGT CTG CGG GA-3' and MTHFR 677 reverse 5'-AGG ACG GTG CGG TGA GAG TG-3' as described by Frosst et al. (1995)], 37.5 nmol MgCl₂, 0.5 nmol of each 2'-deoxynucleotide-5'-triphosphate (dNTP), 0.625 U polymerase and 50 ng DNA, with a final volume of 25 µL. The amplification was carried out in an iCycler thermal cycler (Bio-Rad, 582BR017217) according to the following program: one cycle for 5 minutes at 95 °C, 30 cycles at 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds, followed by a cycle of 5 minutes at 72 °C for final elongation. The MTHFR C677T, creates a *Hinf*I recognition sequence which is absent in individuals homozygous for the wild-type allele (677 CC). The presence of the alteration results in the generation of two restriction fragments *i.e.* 176 and 22 bp in length, from the 198-bp PCR amplified fragment. Quality control of the genotyping procedure was maintained by the use of both positive (to ensure digestion occurred) and negative controls (to detect contamination when present) in each experimental batch. The fragments were separated by 2% agarose gel electrophoresis and were visualized by ethidium bromide staining and ultraviolet transillumination. Two researchers, who were blinded to the status of the subjects, scored the genotypes of the 677 loci independently and the resulting electronic spreadsheets were merged. Any discrepancies in interpretation were resolved by re-analyzing the samples to confirm the true genotype.

5.3.5 Anthropometrical assessment

Level 2 International Society for the Advancement of Kinanthropometry (ISAK) accredited anthropometrists measured *inter alia* height and weight in duplicate. Subjects were examined in minimal clothing. Body mass was measured with a portable electronic scale (Precision Health Scale, A&D Company, Tokyo, Japan). Height was measured with a Stadiometer (IP 1465, Invicta, London, UK) without shoes, standing upright with the head in the Frankfort plane. Body mass index (BMI) was calculated as kilograms per meter squared (kg/m^2).

5.3.6 Dietary intake assessment and data analysis

B-vitamin, alcohol, tea and coffee intake were ascertained with a validated dietary QFFQ (see Addendum E) developed in the North West Province of South Africa (MacIntyre et al., 2001a; MacIntyre et al., 2001b) as well as from a 24-hour recall questionnaire (see Addendum F). The subjects were interviewed by trained fieldworkers using face-to-face interviews in their native language, to recall their usual food intake (foods and beverages) by reporting the frequency, amounts and preparation of the foods consumed during the previous month. The dietary data obtained from the QFFQ and the 24-hour recall were computerized using the *FoodFinder3*[®] program (Medical Research Council, Tygerberg, 2007) by two dietitians and sent to the Medical Research Council of South Africa for computerization, verification and nutrient analysis.

The QFFQ was used to distinguish the alcohol drinkers from the abstainers. Mean alcohol intake was determined from the QFFQ and expressed as reported intake of pure alcohol (ethanol) in grams (g) per day based on the most recent available South African Food Composition Tables for alcohol (Langenhoven et al., 1991). Beer, homemade brews (*Mbamba*: a concoction of water, bread, oats, pineapple juice, sugar and yeast), spirits and wine were considered to contain 3.6 g, 3 g, 36 g and 9.4 g of pure alcohol per 100 g of beverage, respectively. Consumption of coffee and tea was also recorded.

5.3.7 Statistical analysis

The computer software package *Statistica*® version 8 (Statsoft Inc., Tulsa, Oklahoma, USA) was used for the statistical analyses (Statistica, 2008). For all analyses a $p\text{-value} \leq 0.05$ was regarded as statistically significant. The sample size consisted of 2010 subjects, but subjects that had missing data for tHcy concentrations were excluded from the analyses and, therefore, only 1827 subjects were ultimately included in the analyses.

The expected genotype frequencies when a population adheres to the assumptions of Hardy-Weinberg equilibrium (HWE) were calculated and compared to the observed frequencies by using a Chi-square (χ^2) test to determine adherence to or significant differences in the genotypic distribution of the MTHFR C677T alteration (Hardy, 1908).

Initially, data were tested for normality using the Shapiro-Wilk W-test and the Kolmogorov-Smirnov test. Certain variables were not normally distributed (hs-CRP, creatinine, alcohol intake, GGT and tHcy concentrations) and were logarithmically transformed.

Descriptive statistics for all variables were calculated. Pearson and partial correlations were used to determine the association between measured continuous variables with tHcy concentrations. Pearson correlations were calculated on the parametric and logarithmically transformed non-parametric data.

Four subdivisions of alcohol consumption were made as defined by alcohol consumption reported in the QFFQ (abstainers; $> 0 - < 16$; $\geq 16 - < 30$; ≥ 30 g/day of pure alcohol), based on the recommendations that normal daily alcohol consumption is approximately 20 g and 15 g for men and women, respectively (Walmsley et al., 1998), and light to moderate intake < 30 g and heavy drinking > 30 g (Agarwal, 2002).

Differences between the MTHFR genotypes and gender groups were determined by means of cross-tabulation and analysis of variances (ANOVA). Where global

significant differences between the groups were indicated, the Tukey honest significant difference post hoc test for unequal n was used to determine between which groups the specific significant differences occurred.

Two- and three-way analyses of covariance (ANCOVA), adjusting for confounders, were performed to investigate the effect of alcohol intake, the MTHFR genotypes, gender on tHcy and their interactions with tHcy concentrations. This effect was further explored by regression analyses to determine the best predictors of tHcy concentrations. For these regression analyses the parametric and logarithmically transformed non-parametric data were used.

5.4 RESULTS

Characteristics of the subjects mostly reported to be of Tswana ancestry (as determined *via* self-report of the participant on the demographic questionnaire), as a whole and for the different genotype subdivisions, are presented in Table 5.1. The distribution of the mutant genotypes (677 TT) was 0.81% (15), of the heterozygous genotype (677 CT) was 15.24% (281) and the homozygous wild-type genotype (677 CC) was 83.95% (1548) which adhered to the assumptions of HWE ($\chi^2 = 0.32$; $p = 0.57$; $df = 1$).

Mean ages between the subjects harboring different genotypes did not differ except for the higher mean age of the men in the TT genotype subdivision. The mean BMI for all genotype groups was higher for women than for men indicating that the women within this population tended to be more overweight than the men. HIV status was determined in 1819 subjects, of whom 303 of these subjects were positive, 1516 were negative and 7 subjects' statuses were unknown. The HIV positive subjects were asymptomatic and did not use anti-retrovirals and, therefore, were not excluded from the statistical analysis.

The dietary data revealed that subjects only occasionally drank coffee and tea, therefore, neither was considered to be a possible confounder. Within this study sample it was previously reported that %CDT and GGT significantly correlated with

self-reported mean habitual alcohol intake (QFFQ) for both men and women [detailed results in Pisa et al. (in press)].

Pearson correlations to test linear relationship revealed that age and logarithmically transformed GGT correlated significantly ($p < 0.05$) with logarithmically transformed tHcy ($r = 0.26$ for age and $r = 0.27$ for logarithmically transformed GGT). Various other variables (BMI, alcohol intake, folate, vitamin B₁₂, vitamin B₆, pantothenic acid, biotin, methionine intake, logarithmically transformed alcohol intake and %CDT) correlated significantly with logarithmically transformed tHcy, but had correlations smaller than 0.1 indicating a weak association. These statistical significant results could be ascribed to the large sample size of this study (Hair et al., 1998) and are probably not of practical significance.

Results of the partial correlation analyses (data not shown), during which we corrected for age, indicated that several variables correlated significantly with logarithmically transformed tHcy values. Of these significant correlations only the correlation between logarithmically transformed GGT ($r = 0.26$) and logarithmically transformed tHcy for both genders was considered to be of potential clinical importance.

The ANOVA for %CDT in the four alcohol consumption subdivisions indicated that mean %CDT, increased for each alcohol intake category. The post hoc test revealed that for %CDT, the subdivision 0 (abstainers) differed significantly from all the other alcohol subdivisions and that subdivision 1 (subjects who consumed > 0 to < 16 g of alcohol per day) differed significantly from division 3 (those who consumed ≥ 30 g/day of pure alcohol) and that subdivision 3 differed from subdivision 1 and 2 (all $p < 0.01$). Similar results were obtained when GGT was analyzed according to the four alcohol intake subdivisions.

Only 14 subjects harbored the 677 TT genotype and had values for tHcy concentrations. Eleven of these subjects were abstainers falling within alcohol subdivision zero, two were included in subdivision one, while subdivision two had no data and only 1 subject with this genotype consumed enough alcohol to be included within subdivision three (see Figure 5.1).

Table 5.1 Selected characteristics of subjects across the different MTHFR C677T genotypes

	Whole group	677 CC		Whole group	677 CT		Whole group	677 TT	
		Men	Women		Men	Women		Men	Women
Number of subjects (n/%)	1533/84%	573/31%	960/53%	280/15.3%	109/6%	171/9.3%	14/0.8%	2/0.1%	12/0.7%
Age (y)	49.4 ± 10	49.9 ± 10.4	49.0 ± 10.4	49.2 ± 10.4	49.8 ± 10.3	48.7 ± 9.88	48.3 ± 11.5	60 ± 22.6	46.3 ± 8.93
BMI (kg/m ²)	24.6 ± 6.93	20.7 ± 3.97 [#]	26.9 ± 7.29 [#]	24.1 ± 6.7	20.8 ± 4.47 [#]	26.1 ± 7.03 [#]	27.1 ± 5.34	22.6 ± 2.91	27.8 ± 5.36
tHcy (μmol/L)	10.1 ± 4.41 [§]	11.2 ± 4.5 [#]	9.48 ± 4.22 [#]	11.1 ± 4.74 [§]	11.6 ± 4.44	10.7 ± 4.9	18.2 ± 9.95 [§]	19.2 ± 4.28	18 ± 10.7*
Dietary folate (μg)	382 ± 198	427 ± 219 [#]	355 ± 179 [#]	393 ± 187	431 ± 229 [#]	369 ± 151 [#]	306 ± 140	445 ± 183	283 ± 127
Dietary vitamin B ₁ (mg)	1.65 ± 0.84	1.86 ± 0.94 [#]	1.52 ± 0.74 [#]	1.71 ± 0.82	1.87 ± 0.99 [#]	1.61 ± 0.67 [#]	1.32 ± 0.58	2.23 ± 0.82 [#]	1.16 ± 0.4 [#]
Dietary vitamin B ₂ (mg)	1.22 ± 0.8	1.38 ± 0.83 [#]	1.12 ± 0.77 [#]	1.29 ± 0.81	1.41 ± 0.89 [#]	1.2 ± 0.74 [#]	0.89 ± 0.65	1.55 ± 0.06	0.78 ± 0.64
Dietary niacin (mg)	14.2 ± 7.64	16.1 ± 8.35 [#]	13.2 ± 6.96 [#]	15.1 ± 8.63	17 ± 10.3 [#]	13.8 ± 7.15 [#]	9.49 ± 4.68	18.4 ± 3.36 [#]	8 ± 2.83 [#]
Dietary pantothenic acid (mg)	3.78 ± 2.25	4.11 ± 2.45 [#]	3.59 ± 2.1 [#]	3.88 ± 2.25	4.23 ± 2.62 [#]	3.67 ± 1.96 [#]	2.62 ± 1.43	4.01 ± 1.7	2.38 ± 1.32
Dietary vitamin B ₆ (mg)	1.45 ± 0.82	1.6 ± 0.87 [#]	1.36 ± 0.77 [#]	1.5 ± 0.8	1.64 ± 0.97 [#]	1.41 ± 0.67 [#]	1.14 ± 0.61	1.64 ± 0.55	1.06 ± 0.6
Dietary biotin (μg)	35.2 ± 24.1	38.7 ± 26.5 [#]	33.1 ± 22.4 [#]	35.6 ± 22.6	39.1 ± 27.2 [#]	33.5 ± 18.9 [#]	26.5 ± 14.3	48.3 ± 8.04 [#]	22.9 ± 11.65 [#]
Dietary vitamin B ₁₂ (μg)	4.15 ± 5.08	4.42 ± 5.25	4 ± 4.97	4.57 ± 5.53	4.69 ± 5.06	4.49 ± 5.82	2.68 ± 1.96	4.95 ± 0.88	2.31 ± 1.83
Methionine (g/d)	0.85 ± 0.53	0.92 ± 0.57 [#]	0.81 ± 0.49 [#]	0.88 ± 0.56	0.94 ± 0.63	0.84 ± 0.51	0.56 ± 0.41	1.0 ± 0.42	0.48 ± 0.37
hsCRP (mg/L)	8.53 ± 12.0	8.23 ± 13.2	8.72 ± 12	8.13 ± 12.4	8.26 ± 13.8	8.05 ± 10.8	11.8 ± 17.2	4.36 ± 4.5	13.1 ± 18.3
Creatinine (μmol/L)	107 ± 105	111 ± 110	104 ± 102	103 ± 101	99.6 ± 98.1	105 ± 103	89.0 ± 74.9	67.6 ± 5.63	92.6 ± 80.8
Alcohol intake (g/day)	11.5 ± 22.7	18.4 ± 27.1 [#]	7.27 ± 18.4 [#]	14.9 ± 28	22.2 ± 31.1 [#]	10.3 ± 24.9 [#]	3.36 ± 12.2	22.9 ± 32.3 [#]	0.12 ± 0.25 [#]
abstainers:drinkers (n:n)	824 : 659	199 : 357	625 : 302	141 : 134	36 : 70	105 : 64	11 : 3	1 : 1	10 : 2
GGT (μkat/L)	1.57 ± 2.82	2.0 ± 2.73 [#]	1.31 ± 2.83 [#]	1.47 ± 2.22	1.62 ± 1.85	1.38 ± 2.43	0.91 ± 0.75	2.17 ± 0.06 [#]	0.70 ± 0.57 [#]
%CDT	2.95 ± 1.44	3.53 ± 1.7 [#]	2.61 ± 1.12 [#]	3.0 ± 1.24	3.33 ± 1.44 [#]	2.79 ± 1.04 [#]	2.86 ± 1.42	4.66 ± 3.14 [#]	2.56 ± 0.89 [#]
Smoking (n/%)									
Unknown	5/0.46%	3/0.72%	2/0.3%	1/0.49%	0/0%	1/0.82%	0/0%	0/0%	0/0%
Former smoker	45/4.14%	29/7%	16/2.38%	5/2.43%	3/3.57%	2/1.64%	0/0%	0/0%	0/0%
Current smoker	573/52.7%	246/59.3%	327/48.6%	129/62.6%	60/71.4%	69/56.6%	4/40%	1/100%	3/33.3%
Never smoked	465/42.7%	137/33%	328/48.7%	71/34.5%	21/25%	50/41%	6/60%	0/0%	6/66.7%
Caffeine intake (mg)	0.05 ± 0.66	0.02 ± 0.29	0.06 ± 0.81	0.01 ± 0.16	0.01 ± 0.8	0.02 ± 0.2	0 ± 0	0 ± 0	0 ± 0
HIV status (n)	1532	572	960	280	109	171	14	2	12
(-/+/do not know)	1269/256/7	485/85/2	784/171/5	234/46/0	88/21/0	146/25/0	13/1/0	2/0/0	11/1/0

Data is reported as means ± standard deviation (SD), or number and frequency. Analysis of variance revealed that the *p*-value for the differences between the MTHFR 677 CC, CT and TT for alcohol, niacin and tHcy was 0.03, 0.02 and <0.01, respectively. [§]Post hoc tests indicated the significant differences difference in tHcy concentrations between the MTHFR 677 CC and CT, CC and TT and between CT and TT groups i.e. *p* = 0.04, *p* < 0.01 and *p* < 0.01, respectively. [#]Significant difference between men and women harboring the MTHFR 677 CC genotype is *p* = 0.04. BMI = body mass index; HIV = human immunodeficiency virus; hs-CRP = high sensitivity C-reactive protein; GGT = gamma glutamyl transferase; n = sample size; %CDT = percentage carbohydrate deficient transferrin; tHcy = total homocysteine; vitamin B₁ = thiamin; vitamin B₂ = riboflavin; y = years

Due to the low frequency of subjects harboring this genotype, which is reflected in the wide confidence intervals (CI), some of the means could not be determined, therefore, the TT group will be excluded from subsequent analyses and was only included to portray the differences in tHcy concentrations for the abstainers. Since the subjects harboring the TT genotype differed significantly from the CC as well as the CT subjects, the TT genotype group was not analyzed together with the CT group.

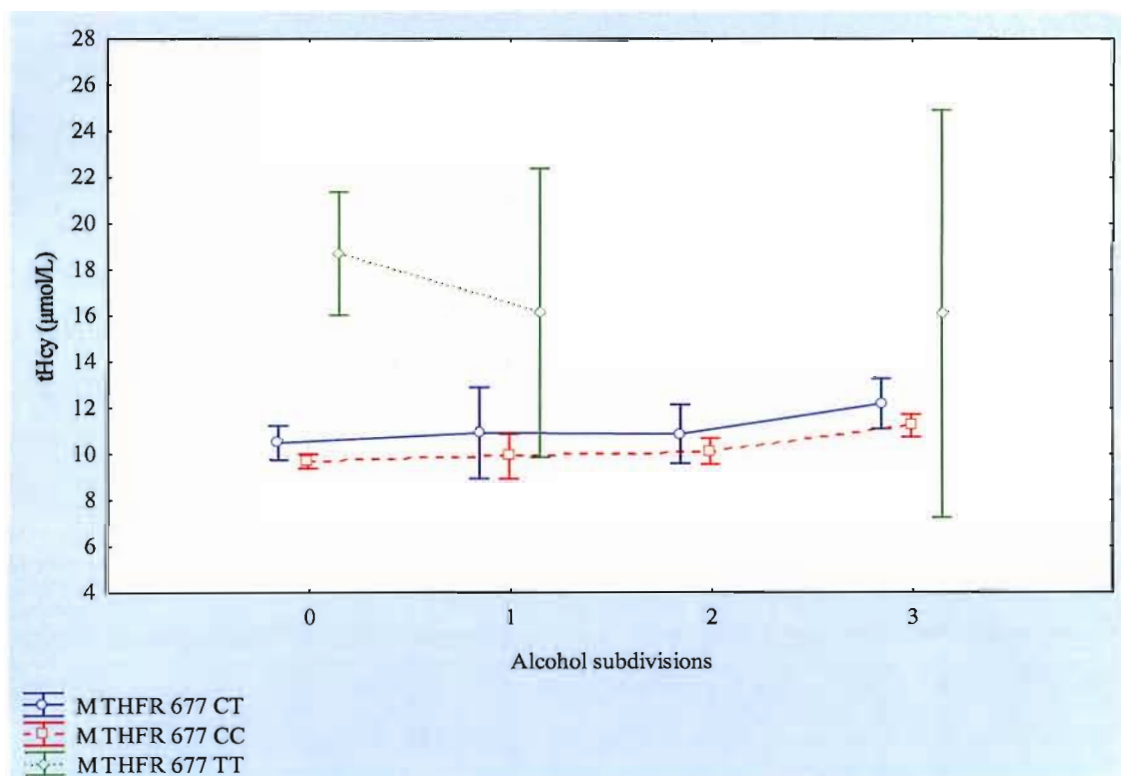


Figure 5.1 Total homocysteine concentrations for the three MTHFR C677T genotypes divided into four categories of alcohol intake while adjusting for age

Vertical bars indicate 95% confidence intervals (CI).

Alcohol subdivisions 0 → 0; 1 → > 0 – <16; 2 → ≥ 16 – < 30; 3 → ≥ 30 g/day of pure alcohol; MTHFR 677 = methylenetetrahydrofolate reductase C677T; tHcy = total homocysteine

To determine the manner in which the interaction of alcohol intake within the four different subdivisions and the two MTHFR genotypes (CC and CT) influences the tHcy concentrations, a two-way ANCOVA in which we adjusted for age was undertaken. No interaction existed between alcohol intake and the CC or the CT genotype subdivisions. For subjects in alcohol subdivision 3 (those who consumed ≥ 30 g/day of pure alcohol) tHcy concentrations were significantly ($p \leq 0.01$) higher

than that of subjects in subdivision 0 (abstainers) as well as between subdivision 3 and subdivision 2 (subjects who consumed $\geq 16 - < 30$ g/day of pure alcohol). Additionally, tHcy concentrations of the CT group were significantly ($p = 0.02$) higher than the CC group. Age that was adjusted for had a significant effect on tHcy concentrations. Similar results were obtained for the logarithmically transformed data.

In a one-way ANCOVA when adjusting for age and GGT there was not a significant difference ($p = 0.23$) in logarithmically transformed tHcy concentrations among those who abstained from alcohol drinking and those who drank alcohol. A three-way ANCOVA in which age was adjusted for, revealed that there were no significant interactions between gender and the alcohol intake subdivisions ($p = 0.2$), or between the alcohol subdivisions and MTHFR genotype ($p = 0.82$) or any three-way interactions between these variables ($p = 0.45$). However, there was an interaction between gender and the MTHFR CC genotype ($p = 0.04$) for tHcy concentrations. The tHcy concentrations and the logarithmically transformed tHcy data of men harboring the MTHFR 677 CC genotype were significantly higher than that of women with the same genotype.

Backward stepwise regression analyses were performed to determine the best predictors of tHcy concentration with age, GGT, riboflavin, folate, vitamin B₆ and B₁₂ intake as possible predictors as well as the MTHFR 677 alleles (where zero was assigned to the MTHFR 677 CC and one assigned to the CT and TT genotypes) and gender (where zero was assigned to women and one to men). Age, GGT, gender, MTHFR and vitamin B₆ explained 16.8% of the variation in logarithmically transformed tHcy with the following β -values 0.26, 0.23, 0.13, 0.10 and -0.09, that indicate the strength of their respective predictive values ($p < 0.01$).

5.5 DISCUSSION

Between all the MTHFR C677T genotypes tHcy concentrations differed significantly with subjects harboring the 677 TT having the highest mean tHcy concentrations. Among all genotypes, age and GGT seem to be among the more important determinants of tHcy concentrations. GGT, age, gender and MTHFR explained the

largest variation in tHcy concentrations. Even though no interaction existed between reported alcohol intake and the MTHFR 677 CC or the CT genotypes, tHcy concentrations increased significantly in the higher alcohol intake subdivisions. Subjects harboring the CT genotype seemed to have higher tHcy concentrations in the four categories for alcohol intake than those harboring the CC genotype, however, this trend was non-significant. To our knowledge, this is the first study that determined the superimposed effect of alcohol intake in the presence of the MTHFR C677T genetic variation on plasma tHcy concentrations in an African population of this magnitude.

Previous studies that reported the MTHFR C677T genotype distribution amongst black South Africans lacked statistical power to detect individuals harboring the 677 TT genotype (Loktionov et al., 1999; Scholtz et al., 2002). However, if the studies conducted on black South Africans investigated larger cohorts one would have expected to detect a similar distribution of the genetic variants to that observed in this study population. Gene distribution comparisons with studies conducted among other ethnic groups have reported that the frequency of the mutant genotype is much rarer in Africans and African-Americans than in Caucasians, Asians, Ashkenazi Jews, Hispanics and Mexicans (Conroy et al., 2000; Guéant-Rodriguez et al., 2006; Kelemen et al., 2004; Rady et al., 2002). Mexicans seem to have the highest prevalence of the 677 T allele, however, the influence of 677 TT genotype on tHcy concentrations seems to be the lowest in the Mexican population, whereas the opposite seems true for Africans (Guéant-Rodriguez et al., 2006). These differences in phenotypic expression of the genotype among the different ethnic groups might be explained by differences in B-vitamin intake and other lifestyle factors including alcohol intake. Due to differences in phenotypic expression between ethnic groups, extrapolating data from one ethnic group to another must be avoided and this also emphasizes the need for genetic research in the black South African population.

In this study an appreciable difference in plasma tHcy concentration was observed when the 677 CC wild-type subdivisions were compared to that of the heterozygous 677 CT and of the homozygous mutant 677 TT, respectively. These results are similar to previously reported results of other populations where the heterozygous CT genotype and the homozygous TT mutant genotype were both associated with

increased tHcy concentrations when compared to the CC genotype (Adjalla et al., 2003; Anand et al., 2000; Frosst et al., 1995; Tavares et al., 2004). In this study, the 14 individuals harboring the TT genotype had a mean tHcy concentration of 19.1 $\mu\text{mol/L}$. Earlier studies indicated that the normal reference range for plasma tHcy is between 5 to 15 $\mu\text{mol/L}$, mild to moderate HHcy between 16 to 100 $\mu\text{mol/L}$ and severe HHcy $> 100 \mu\text{mol/L}$ (Eikelboom et al., 1999; Malinow et al., 1999), however, in 2007, Castañón et al. proposed that tHcy concentrations above 12 $\mu\text{mol/L}$ must be regarded as clinically significant since it increased the risk of developing venous thrombosis. The elevated tHcy concentration in those harboring the 677 genetic variation is ascribed to the C677T missense mutation producing a thermolabile enzyme with reduced enzymatic activity (Frosst et al., 1995; Kang et al., 1991). As a result, less of the precursor of folate is converted to bioactive folate, which is required to convert Hcy to methionine, leading to elevated plasma tHcy concentrations. Probably due to chance, subjects harboring the MTHFR 677 TT genotype differed from those harboring the CC and CT genotypes in relation to higher mean BMI values, lower B-vitamin intake and higher hs-CRP values. However, for this population tHcy concentrations correlated weakly with BMI, B-vitamin intake and did not correlate with hs-CRP and, therefore, were not considered to contribute significantly to the tHcy difference observed between subjects harboring the different C677T genotypes.

Since none of the dietary B-vitamin intake data correlated strongly with tHcy concentrations, it seems as though genetic factors, age and GGT played a more important role in the African group than B-vitamin intake in determining tHcy concentrations. This may be due to the limitations of dietary data in that although we can measure the intake of nutrients we cannot necessarily determine their absorption or metabolism within the body. In future investigations of the PURE data, the biological concentrations of the B-vitamins should be measured to determine whether these concentrations correlate better with tHcy concentration than with the intake data. Currently, however, we only have access to the dietary information of these subjects.

Even though men harboring the MTHFR 677 CC genotype had higher intakes of folate, pantothenic acid, biotin, and vitamin B₁, B₂, B₃, and B₆, as opposed to women

harboring this genotype (see Table 5.1), they had significantly higher tHcy concentrations. Since there was no interaction between gender and alcohol intake, it seems as if gender is an important determinant of tHcy concentrations among individuals harboring the wild-type genotype in our study and that this difference was not due to differences in alcohol intake. Studies have reported that the male gender is associated with higher tHcy concentration (Andersson et al., 1992; Brattström et al., 1994). This difference could partly be attributable to the strong correlation reported between tHcy concentrations and circulating creatinine and larger body size or muscle mass in men, since the formation of muscles is associated with simultaneous increased Hcy formation as a consequence of methyl group transfer during creatine/creatinine metabolism (Brattström et al., 1994). However, in this black South African cohort tHcy concentrations were not associated with creatinine concentrations. The influence of gender hormones on Hcy metabolism could partly be responsible for this gender difference in tHcy concentrations (Andersson et al., 1992) and this hypothesis was confirmed in a study involving transgendered men and women (Giltay et al., 1998).

In our study, age and GGT presented with the strongest correlation and explained the largest percentage of the variation of the tHcy concentrations. Several studies have reported a positive linear association between tHcy concentrations and age (Bates et al., 1997; Brattström et al., 1994). It has been suggested that raised Hcy concentrations seen in the elderly are due to insufficient intakes of folate, B₆ and/or B₁₂ (Bates et al., 1997; Brattström et al., 1994; Selhub et al., 1993). Increased creatinine concentrations due to impaired renal function with age as well as impaired renal metabolism of Hcy (Guttormsen et al., 1997) also appear to play a role in the pathogenesis of increased tHcy in older persons.

Since subjects were apparently healthy, mild elevations of GGT values were probably not due to liver disease, but due to alcohol consumption which induces a rise in this membrane-bound glycoprotein enzyme (Johnston, 1999; Niemelä, 2007). Since alcohol intake has been reported to increase tHcy concentrations (Bleich et al., 2001; Cravo et al., 1996; Koehler et al., 2001; Van der Gaag et al., 2000), it could be expected that GGT would also correlate with tHcy concentrations. The increase in tHcy concentrations among drinkers is probably due to a combination of

malnourishment and the direct effects of heavy alcohol intake (Cravo et al., 1996). Alcohol (ethanol) interferes with both the transmethylation and transsulfuration pathways of Hcy metabolism, either directly (Barak et al., 1993) or through its primary metabolite acetaldehyde (Shaw et al., 1989; Hidioglou et al., 1994) and also through indirect effects mediated by interactions with vitamin metabolism.

The present study determined the interaction of alcohol intake (measured by the %CDT, GGT concentrations and QFFQ data), and MTHFR 677 genotype status on tHcy concentrations in a black South African population and established that tHcy concentrations increased significantly as alcohol intake increased. The low frequency of the MTHFR 677 CT (15.2%) and the very low frequency of the TT genotype (0.81%) in this black South African population and the percentage of these subjects harboring CT and CC who consumed alcohol (48.7% and 21.4%, respectively) lead to smaller sample sizes and thus may have limited our observations with regard to the modulating effect of the CC *versus* CT genotype upon the effect of alcohol on tHcy concentration and no conclusion could be drawn on the modulating effect of the MTHFR 677 TT genotype. Ideally, subjects homozygous for the C677T variation should also have been included in the analysis, however, because of the limited number of these subjects and since only three of them consumed alcohol we were unable to analyze these subjects. If we had combined the CT and TT groups, we could have assessed the combined effect, however, this would have lead to bias, since tHcy concentrations of subjects harboring the CT genotype was significantly lower than those of subjects harboring the TT genotype. Furthermore, it would not have been meaningful to combine three individuals harboring the 677 TT genotype with 134 subjects harboring the 677 CT genotype.

One of the limitations of the QFFQ data is that it cannot differentiate between regular heavy drinkers or heavy irregular drinkers (binge drinkers). A meta-analysis reported pooled relative risks for CVD of 0.75 [95% CI 0.64 – 0.89] for regular heavy drinkers and 1.10 (1.03 – 1.17) for irregular heavy drinkers upon comparison to abstainers (Bagnardi et al., 2008). It, therefore, seems as though the pattern of drinking as well as the amount consumed are also important to consider.

In conclusion, alcohol intake increased tHcy concentrations significantly, however, a modulating effect of the TT genotype could not be determined. Even if such an effect is determined it is not likely to be a relevant risk factor in the black South African population due to the low frequency of the TT genotype. In the future it would be warranted to investigate the influence of other genetic variations such as the A2756G [adenine (A) to guanine (G) at bp 2756] polymorphism in the methionine synthase (MTR) gene (Yamaji et al., 2009) in black South African populations, which are known to consume large amounts of alcohol (Parry et al., 2005).

Furthermore, from a public health viewpoint, it is important to identify ethnic-specific modifiable factors that influence plasma tHcy concentrations. Thus in the black South African population, age, gender, GGT and the MTHFR C677T genetic polymorphism are more important determinants of tHcy concentrations than B-vitamin intake. This has major implications for the development of intervention modalities to treat the increased CVD risk this population is currently experiencing. Since the PURE-study is a prospective study we will be able to determine the health outcomes with the emphasis on CVD within this group in the future. Elucidating ethnic specific strategies to nutrition-based disorders are, therefore, critical to the evolution of future dietetic and nutritional best practices.

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CHAPTER 6

GENOTYPE DISTRIBUTIONS OF SPECIFIC GENETIC VARIANTS IN THE MTHFR, MTR AND CBS GENES AND THEIR INTERACTIONS IN RELATION TO HOMOCYSTEINE CONCENTRATIONS IN A BLACK SOUTH AFRICAN POPULATION

Running title: Genetic variations in the MTHFR, MTR and CBS genes and Hcy

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6.1 ABSTRACT

Background: Several genes, including those encoding for methylenetetrahydrofolate reductase (MTHFR), cystathione- β -synthase (CBS) and methionine synthase (MTR), might interact with each other and influence homocysteine (Hcy) concentrations.

Objective: To determine the prevalence of specific single nucleotide polymorphisms (SNPs) within the aforementioned genes, their relationship to and epistatic interactions with total (t)Hcy concentrations in a black South African population.

Design: Cross-sectional study of black South Africans nested within the Prospective Urban and Rural Epidemiology study. Fasting plasma tHcy was determined by fluorescence polarisation immunoassay technology. The SNPs were determined through polymerase chain reaction based restriction fragment length polymorphism analysis.

Results: B-vitamin intake did not correlate strongly, while age, γ -glutamyl transferase and gender were associated with tHcy. The frequencies of investigated SNPs adhered to the assumptions of Hardy-Weinberg and differed when compared to those previously reported for other ethnic groups. The MTHFR 677 TT and the MTR 2756 AA genotypes were associated with significantly higher tHcy (16.6 and 10.1 $\mu\text{mol/L}$; $p < 0.05$) than subjects harbouring the MTHFR 677 CT/CC and the MTR 2756 AG (10.5, 9.7 and 9.5 $\mu\text{mol/L}$, respectively). Between the CBS 844ins68, T833C or CBS G9276A and MTHFR C677T genotypes, there were significant two-way interactions ($p < 0.05$), however, there was not an interaction between MTHFR C677T and MTR A2756G or between the CBS 844ins68/T833C or G9276A and MTR A2756G genotypes with regard to tHcy concentrations.

Conclusions: The gene-gene interactions determined in this study highlight the epistatic nature of cardiovascular disease risk conveyed through tHcy concentrations in black South Africans.

KEY WORDS Africans, cystathionine β -synthase, CBS, gene-gene interactions, homocysteine, methylenetetrahydrofolate reductase, MTHFR, methionine synthase, MTR, polymorphism

6.2 INTRODUCTION

Cardiovascular disease (CVD) seems to be increasing in developing countries, including South Africa, due to urbanisation with the subsequent adoption of higher risk lifestyles, including unhealthy dietary habits [1]. Elevated homocysteine (Hcy) concentrations *i.e.* hyperhomocysteinaemia have been implicated in the development of CVD [2,3]. Specific single nucleotide polymorphisms (SNPs) in the genes encoding for methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR) and cystathionine β -synthase (CBS), which are enzymes involved in the intracellular metabolism of Hcy, can alter Hcy metabolism and can lead to variations in Hcy concentrations.

A common SNP in the MTHFR gene (C677T) has been associated with depleted 5-MTHFR levels [4], elevated Hcy concentrations [5,6] and an increased risk toward CVD development [7]. Results are inconsistent regarding the association between an A to G transition at nucleotide 2756 within the MTR gene and Hcy concentrations. Some studies reported no association [5,8,9] while others reported lowered Hcy concentrations in those harbouring this genetic variation [10-12]. The reason for these variable results regarding the effects of this SNP is not clear due to the complex nature of the determinants of Hcy concentrations. The CBS T833C/844ins68 have also been included in this investigation and these variations seem to be associated with reduced tHcy concentrations [13,14]. All the aforementioned polymorphisms are considered important genetic determinants of Hcy concentrations and were investigated within this article. The recently described CBS G9276A within the intronic sequence of the CBS gene was also determined, since the method developed by Griffioen *et al.* [13] to determine the CBS T833C/844ins68 loci also enabled the genotyping of the G9276A SNP. In addition to investigating these genetic variations, it is necessary to determine whether there are gene-gene interactions of importance that influence Hcy concentrations significantly [15].

The MTHFR C677T, MTR A2756G and CBS T833C/844ins68 genetic alterations have been widely studied in Caucasian populations, but have rarely been reported separately or simultaneously in a large black African population. Since the genetic make-up of the latter population might be different from the usually studied

Caucasian population, it is important to investigate these genetic alterations in a black South African population.

The present study was undertaken to describe the frequencies of the MTHFR C677T, MTR A2756G, CBS T833C/844ins68 and G9276A SNPs in a black South African population and to investigate their respective influences on total plasma (t)Hcy concentrations as well as to determine whether there are epistatic interactions between these genotypes.

6.3 SUBJECTS AND METHODS

6.3.1 Study design and population selection

The 2005 baseline data of the South African leg of the multi-centre twelve year Prospective Urban and Rural Epidemiology study (PURE) were analysed for this cross-sectional study. As the study focuses on the epidemiological transition, apparently healthy black South African volunteers were recruited from an urban setting (n = 1,002) and from three rural villages (n = 1,008). To ensure long-term follow-up potential of subjects, the main selection criterion of these communities was migration stability. A census (demographic and health profiles) was undertaken within these communities starting from a randomly selected address. Individuals with reported chronic diseases, tuberculosis or known infection with the human immunodeficiency virus (HIV) and individuals who reported chronic medicine use were not eligible for inclusion.

6.3.2 Ethics

This study was performed in accordance with the protocol approved by the Ethics Committee of the North-West University (Potchefstroom Campus) according to the principles outlined in the Declaration of Helsinki (Ethics number: 04M10). Permission to conduct this study was obtained from the North West Provincial Department of Health, tribal chiefs, community leaders, employers and mayors of the included communities. Recruited eligible subjects were informed of all the aspects of the study and were required to provide written informed consent before inclusion (see Addenda A-C).

6.3.3 Biochemical analyses

When an anomaly in the tested biochemical markers was identified the subject was referred to the nearest medical facility (see Addendum D).

6.3.3.1 Blood sampling and storage

Registered nurses collected fasting venous blood samples. After collection, sample tubes were mixed gently by inversion and stored on ice until centrifugation. Serum for analyses was obtained by allowing blood to clot at room temperature for 30 minutes after which the tube was centrifuged for 15 minutes at 2000 x g at 10°C and stored at -70°C. Plasma for the analyses was collected in ethylenediamine tetra-acetic acid (EDTA) containing tubes and centrifuged for 15 minutes at 2000 x g at 4°C followed by storage at -70°C. Buffy coat for genomic deoxyribonucleic acid (gDNA) extraction was collected by transferring the leukocyte layer from citrate treated whole blood centrifuged for 15 minutes at 2000 x g at room temperature to a 1.5 ml collection tube. All samples were centrifuged within two hours after collection, however, for the collection of the plasma samples for the tHcy analyses, the blood was separated as soon as possible to prevent the artifactual increase in plasma tHcy concentrations due to the continuous production and release of Hcy *in vitro* [16].

6.3.3.2 Human immunodeficiency virus testing

For the determination of HIV status, additional informed consent (see Addendum C) was required. Researchers trained in Voluntary Counselling and Testing, adhering to the UNAIDS/WHO Policy Statement on HIV-testing [17] and to the protocol of the National Department of Health of South Africa were responsible for the HIV testing and counselling. Subjects were counselled before HIV-testing. Whole blood was used for the rapid First Response antibody HIV card test and when positive, the Pareeshak test was undertaken to confirm the outcome. Post-test counselling for subjects who wanted to be informed of the outcome of the HIV-test was provided in a confidential, one-on-one manner.

6.3.3.3 Biochemical markers of alcohol intake

For the analyses of gamma (γ)-glutamyl transferase (GGT) concentrations a Sequential Multiple Analyser Computer (SMAC) analysis was undertaken, using the KonelabTM auto analyser (Thermo Fisher Scientific Oy, Vantaa, Finland). The cut-off values for men and women were set at 80 U/L and 50 U/L, respectively. The SMAC methodology was also used for analysing serum high sensitivity C-reactive protein (hs-CRP) and serum creatinine.

Percentage carbohydrate deficient transferrin (%CDT) which expresses the relative amount of CDT isoforms in proportion to total transferrin [18,19] was measured with an *in vitro* heterogeneous immunoassay with column separation followed by a turbidometric measurement (Axis-Shield % CDT kit, Oslo, Norway). The measuring range for transferrin was between 1.5 and 24 mg/L. The cut-off value for %CDT was set at 2.6%, as suggested by the manufacturer. For all assays the coefficient of variance (CV) was < 10%.

6.3.3.4 Determination of homocysteine concentrations

Total Hcy concentrations were determined from EDTA-treated plasma on the Abbott automated immunoassay analyser (AxSYM). The determination of tHcy concentration was based on fluorescence polarisation immunoassay technology (CV = 4.52%).

6.3.3.5 Deoxyribonucleic acid isolation and genotyping

QIAGEN FlexiGene DNA extraction kits were used to extract gDNA from citrate treated buffy coat following the instructions of the manufacturer. The absorbance ratio (260 to 280 nm) and yield of the extracted sample DNA were determined with the Nano-Drop spectrophotometer (ND-1000). Polymerase chain reaction (PCR) based restriction fragment length polymorphism analysis was used to determine the genotypes. The PCR mixtures for the genotypes contained 0.125 nmol of each primer (the primers used are outlined in Table 6.1), 37.5 nmol MgCl₂, 0.5 nmol of each of the 2'-deoxynucleotide-5'-triphosphate (dNTPs), 0.625 U GoTaq® DNA polymerase and

50 ng DNA to a final volume of 25 μ l. The amplification was carried out in an iCycler thermal cycler (Bio-Rad, 582BR017217) according to the following programme: one cycle for 5 minutes at 95°C for initial denaturation, 30 cycles at 95°C for 30 seconds for denaturation, the specific optimised annealing temperature for the primer set for 30 seconds and 72°C for 30 seconds for elongation, followed by a cycle of 5 minutes at 72°C for final elongation.

Table 6.1 Primers, PCR profiles and restriction enzymes used to determine the genotypes

MTHFR-C677T	Forward primer	5'-TGA AGG AGA AGG TGT CTG CGG GA-3' [6]		
	Reverse primer	5'-AGG ACG GTG CGG TGA GAG TG-3' [6]		
	Annealing temperature	55.0°C		
	Restriction enzyme	<i>HinfI</i> produces 176 and 22 bp fragments from the 198 bp PCR fragment in the presence of the variation		
MTR-A2756G	Forward primer	5'-GAA CAT CCC AAG CCC ACT GAG-3' (own design)		
	Reverse primer	5'-GAC ACT GAA GAC CTC TGA TTT GAA C-3' [20]		
	Annealing temperature	58.0°C		
	Restriction enzyme	<i>BsuRI</i> (<i>HaeIII</i>) produces 260 and 180 bp fragments from the 440 bp PCR fragment in the presence of the variation		
CBS T833C, 844ins68 and G9276A	Forward primer	5'-ATA GAA TAT CGA GGC ATG TCC AGG CG-3' (13)		
	Reverse primer	5'-TGG GGC CCA GGG TCA GCC AGG CTC C-3' (13)		
	Annealing temperature	66.5°C		
	Restriction enzyme	<i>BseNI</i> (<i>BsrI</i>) Fragment lengths taken from Griffioen <i>et al.</i> [13]		
		Calculated fragments	Non-insert allele	Insert allele
		PCR product	282 bp	350 bp
		T833C wild-type	257,25 bp	325,25 bp *
		T833C mutant type	180,77,25 bp	248,77,25 bp
		G9276A mutant type	225,32,25 bp	293,32,25 bp *
		T833C and G9276A mutant type	148,77,32,25 bp	216,77,32,25 bp

A = adenine; *bp* = base pair; *C* = cytosine; *CBS* = cystathionine β -synthase; *G* = guanine; *ins* = insertion; *MTHFR* = methylenetetrahydrofolate reductase; *MTR* = methionine synthase gene; *PCR* = polymerase chain reaction; *T* = thymine

* no natural existence

For genotype determinations, each batch of sample DNA was analysed in parallel with the appropriate positive and negative controls to avoid misinterpretation from any lack of digestion or presence of contamination. For the determination of the MTHFR and MTR genotypes only the resultant fragments of the restriction enzyme

digested PCR products were analysed *via* electrophoresis and visualisation of an ethidium bromide-stained 2% agarose gel under ultraviolet light. However, for the three CBS alterations both the PCR products together with the digested PCR products were loaded on the gel.

Two researchers scored the genotypes at the MTHFR C677T, MTR A2756G, CBS T833C/844ins68 and G9276A loci independently and merged the electronic spreadsheets with the outcomes. Discrepancies in interpretation were resolved by re-determination of the sample DNA genotypes.

6.3.4 Anthropometrical assessment

Qualified level 2 International Society for the Advancement of Kinanthropometry (ISAK) accredited anthropometrists measured subjects wearing minimal clothing. Body mass was measured with a portable electronic scale (Precision Health Scale, A&D Company, Tokyo, Japan) while height was measured with a Stadiometer (IP 1465, Invicta, London, UK). Body mass index (BMI, kg/m^2) was calculated by dividing body mass in kilograms by the height squared in metre.

6.3.5 Questionnaires

Trained fieldworkers recruited from the selected communities interviewed each subject to complete a structured, validated demographic, socio-economic, lifestyle and a quantitative food frequency questionnaire (QFFQ).

6.3.5.1 Demographic characteristics

The questionnaire developed for the international PURE-study was adapted and standardised for use in South Africa (see Addendum G). Demographic, health and lifestyle information including a history of tobacco use and information regarding medical conditions, including a history of CVD, diabetes and cancer diagnosed by a doctor, were obtained from this interviewer based questionnaire.

6.3.5.2 Dietary intake assessment

Food and beverage (including alcohol, coffee and tea) consumption were assessed with a validated QFFQ (see Addendum E) developed in South Africa [21]. Subjects were interviewed in a relaxed atmosphere in their home language, to recall their regular food and beverage consumption of the previous month by reporting the frequency, portion sizes and preparation method. The dietary questionnaire data were computerised using the *FoodFinder3*[®] software [22] by dieticians and sent to the Medical Research Council of South Africa for verification and nutrient analyses. Average alcohol intake was expressed as the intake of pure alcohol (ethanol) in grams (g) per day based on the most recent available South African Food Composition Tables [23].

6.3.6 Statistical analysis

Data was analysed using the computer software package *Statistica*[®] version 9 (Statsoft Inc., Tulsa, Oklahoma, USA) [24]. A p-value < 0.05 was considered significant. Individuals (n = 138) who had missing data for tHcy concentrations were excluded from the analysis. The HIV positive subjects were asymptomatic and did not use anti-retrovirals, therefore they were not excluded from the analysis. The Shapiro-Wilk W-test and the Kolmogorov-Smirnov test were used to test for normality. Variables not normally distributed (hs-CRP, creatinine, alcohol intake, GGT and tHcy concentrations) were normalised through logarithmical transformation.

Descriptive statistics for the group as a whole and for men and women respectively were calculated. Independent t-tests and cross-tabulation were performed to determine whether there were significant differences in measured variables between the genders. Concordance of the observed genotype frequencies with those expected according to Hardy-Weinberg equilibrium (HWE) was tested for using Pearson Chi-square (χ^2) analysis to ensure adherence to the assumptions of HWE [25]. Pearson correlations were calculated to determine the linear relationships between logarithmically transformed tHcy concentrations and other variables. Possible confounders were identified from the results of the Pearson correlation analyses.

Potential joint influences between the MTHFR, MTR and CBS genotypes (*i.e.* gene-gene interactions) and gender in relation to tHcy concentrations were determined through one-, two- and three-way analyses of covariance (ANCOVA) while adjusting for age and logarithmically transformed GGT. To adjust for multiple comparisons where significant differences between the groups were indicated, the Tukey honest significant difference *post hoc* test for unequal *n* was used to establish between which groups the specific significant differences occurred.

6.4 RESULTS

Descriptive statistics for the study population, most self-reported to be of Tswana ancestry according to the demographic questionnaire, and for men and women separately are summarised in Table 6.2. The mean BMI was higher for women than for men indicating that women within this population tended to be more overweight than the men. Total Hcy concentrations, B-vitamin, methionine and alcohol intake as well as the biomarkers of alcohol intake GGT and %CDT were significantly higher in men than in women. The dietary data revealed that subjects only occasionally drank coffee and tea, therefore, neither was considered to be a possible confounder and thus were not included in Table 6.2. The subjects did not use dietary supplements. The diets of the subjects provided adequate amounts of most of the B-vitamins, however, the intake of folate among women and pantothenic acid for both men and women was inadequate.

Results of the cross-tabulation between CBS 844ins68 and CBS T833C indicated that all 746 subjects harbouring the CBS 833 TC were also heterozygous for the CBS 844 68-bp insertion, while 995 of the 997 subjects (99.8%) harbouring CBS 833 TT were also homozygous for the CBS 844ins68 wild-type genotype and 136 of 138 subjects (98.6%) harbouring the CBS 833 CC were homozygous for the CBS 844 insertion. In our cohort all the determined SNP distributions were in agreement with those expected under HWE [MTHFR C677T $p = 0.61$; MTR A2756G $p = 0.15$; CBS 844ins68 $p = 0.80$; CBS T833C $p = 0.92$ and CBS G9276A $p = 0.97$; degrees of freedom = 1].

Table 6.2 Selected characteristics and dietary intakes of subjects

	Total group	Men	Women
Number of subjects (n)	1825	684	1141
Age (y)	49.3 ± 10.5	49.9 ± 10.4 [#]	48.9 ± 10.3 [#]
Rural (n/%)	945	329/34.8%	616/65.2%
Urban (n/%)	880	355/40.3%	525/59.7%
BMI (kg/m ²)	24.5 ± 6.92	20.8 ± 4.04 [#]	26.8 ± 7.28 [#]
tHcy (μmol/L)	10.3 ± 4.6	11.3 ± 4.51 [#]	9.74 ± 4.55 [#]
Dietary folate (μg)	383 ± 196	428 ± 220 [#]	356 ± 174 [#]
Dietary vitamin B ₁ (mg)	1.66 ± 0.83	1.87 ± 0.95 [#]	1.53 ± 0.73 [#]
Dietary vitamin B ₂ (mg)	1.22 ± 0.8	1.39 ± 0.83 [#]	1.13 ± 0.76 [#]
Dietary niacin (mg)	14.3 ± 7.8	16.2 ± 8.68 [#]	13.2 ± 7 [#]
Dietary pantothenic acid (mg)	3.79 ± 2.25	4.13 ± 2.48 [#]	3.58 ± 2.08 [#]
Dietary vitamin B ₆ (mg)	1.46 ± 0.81	1.61 ± 0.88 [#]	1.37 ± 0.76 [#]
Dietary biotin (μg)	35.2 ± 23.8	36.7 ± 26.4 [#]	33 ± 21.8 [#]
Dietary vitamin B ₁₂ (μg)	4.2 ± 5.14	4.46 ± 5.21	4.05 ± 5.1
Dietary methionine (g)	0.85 ± 0.53	0.93 ± 0.6 [#]	0.81 ± 0.5 [#]
hs-CRP (mg/L)	8.5 ± 12.4	8.22 ± 13.3	8.67 ± 11.9
Creatinine (μmol/L)	106 ± 104	109 ± 108	104 ± 101
Alcohol intake (g/day)	12 ± 23.6	19 ± 27.8 [#]	7.68 ± 19.5 [#]
abstainers:drinkers (% : %)	55% : 45%	36% : 64%*	67% : 33%*
GGT	92.7 ± 163	116 ± 157 [#]	79 ± 165 [#]
%CDT	2.95 ± 1.41	3.5 ± 1.66 [#]	2.63 ± 1.11 [#]

Data is reported as means ± standard deviation (SD), or number and frequency. [#]Significant difference between men and women. *Significantly more men consumed alcohol than women. BMI = body mass index; GGT = gamma glutamyl transferase; hs-CRP = high sensitivity C-reactive protein; n = number of subjects; %CDT = percentage carbohydrate deficient transferrin; tHcy = total homocysteine; vitamin B₁ = thiamin; vitamin B₂ = riboflavin; y = years

Pearson correlations indicated that age and logarithmically transformed GGT correlated significantly with logarithmically transformed tHcy ($r = 0.27$ and $r = 0.28$, respectively; $p < 0.05$). Although significant, the correlations for alcohol intake, folate, vitamin B₁₂, vitamin B₆, pantothenic acid, biotin, methionine intake, BMI and %CDT with logarithmically transformed tHcy were weak ($r \leq 0.1$; $p < 0.05$). Therefore, only age and GGT were considered to be of potential clinical importance and were adjusted for during the ANCOVA analyses. Several two- and three-way interactions were considered including interactions between different genotypes and genders. There was no interaction between gender and any of the determined genotypes ($p > 0.05$), but gender had a main effect on logarithmically transformed tHcy concentrations whereby men had higher tHcy concentrations than women.

The results of the ANCOVA analyses for logarithmically transformed tHcy concentrations are displayed in Table 6.3. Subjects living in rural areas had significantly higher tHcy concentrations than the urban living subjects. HIV-uninfected subjects had significantly higher tHcy concentrations than HIV-infected subjects. When adjusting for age and GGT, there was not a significant difference in tHcy concentrations amongst those who abstained and those who drank alcohol.

A one-way ANCOVA indicated that logarithmically transformed tHcy concentrations differed significantly between the MTHFR C677T genotypes. Individually, the CBS 844ins68 and the T833C genotypes did not have a significant effect on tHcy concentrations, but together with the MTHFR C677T genotype there was a significant two-way interaction on tHcy concentrations ($p = 0.04$ and $p = 0.05$, respectively). The CBS 844ins68 genetic variation occurred together with the CBS T833C variation, therefore, the observed interactions were similar. The Tukey test revealed that the subjects harbouring the MTHFR 677 TT in combination with the CBS 833 TT (or the homozygous CBS 844 non-insert genotype) had significantly higher tHcy concentrations than subjects with any other MTHFR 677 CC or 677 CT and the CBS T833C/844ins68 genotype combinations.

The concentrations of tHcy did not differ significantly between the three CBS G9276A genotypes ($p = 0.10$). There was, however, a significant two-way interaction between the MTHFR C677T and CBS G9276A genotypes with regard to tHcy concentrations ($p < 0.01$). The Tukey test revealed that the significant interaction on logarithmically transformed tHcy concentrations was between subjects harbouring the MTHFR 677 TT in combination with the CBS 9276 GA genotype ($p = 0.01$) whereby subjects harbouring this combination had significantly higher tHcy concentrations when compared to those harbouring any other combination of these genotypes except compared to subjects harbouring MTHFR TT in combination with CBS 9276 GG ($p = 0.26$). Within this population there were no subjects that harboured the MTHFR 677 TT in combination with the CBS 9276 AA genotype.

Table 6.3

Total homocysteine concentrations of subjects in different subdivisions

		tHcy (μmol/L)		tHcy (μmol/L)		tHcy (μmol/L)	
		Total group	n/%	Men	n	Women	n
Rural		10.4* (10.2; 10.7)	945	10.9 [#] (10.5; 11.3)	319	9.61 [#] (9.34; 9.9)	596
Urban		9.09* (8.88; 9.31)	880	9.64 [#] (9.31; 9.98)	345	8.88 [#] (8.63; 9.14)	519
Abstainers		9.69 (9.44; 9.96)	974	10.3 [#] (9.83; 10.1)	233	9.15 [#] (8.9; 9.41)	718
Alcohol drinkers		9.76 (9.51; 10)	796	10.5 [#] (10.1; 10.8)	411	9.1 [#] (8.77; 9.46)	362
Tobacco use	Former smoker	8.79 (7.94; 9.73)	50	9.38 (8.42; 10.5)	332	8.60 (7.47; 9.90)	18
	Current smoker	9.84 (9.58; 10.1)	705	10.5 [#] (10.1; 10.9)	30	9.28 [#] (8.95; 9.62)	398
	Never smoked	10.0 (9.68; 10.3)	542	10.3 [#] (9.71; 10.8)	158	9.29 [#] (8.96; 9.62)	384
HIV status	HIV negative	9.64* (9.46; 9.82)	1514	10.5 [#] (10.2; 10.8)	575	9.44 [#] (9.23; 9.66)	938
	HIV positive	9.03* (8.66; 9.41)	303	9.11 [#] (8.53; 9.72)	106	8.42 [#] (8.02; 8.83)	197
MTHFR	CC	9.74* (9.56; 9.91)	1579/84%	10.2 [#] (9.89; 10.5)	573	9.06 [#] (8.86; 9.26)	958
C677T	CT	10.5* (10; 10.9)	286/15.2%	10.5 (9.79; 11.2)	109	10.1* (9.54; 10.7)	170
	TT	16.6* (12.9; 21.5)	15/0.8%	22.0 (8.28; 58.7)	2	13.4* (7.77; 23)	12
MTR	AA	10.1* (9.86; 10.3)	1194/63.7%	10.4 [#] (10.1; 10.8)	439	9.44 [#] (9.19; 9.69)	719
A2756G	AG	9.5* (9.22; 9.79)	590/31.5%	9.95 [#] (9.51; 10.4)	203	8.85 [#] (8.55; 9.16)	365
	GG	9.5 (8.79; 10.2)	89/4.8%	9.83 (8.74; 11)	35	9.68 (8.75; 10.7)	49
CBS	TT	9.91 (9.69; 10.1)	997/53%	10.5 [#] (10.1; 10.9)	356	9.27 [#] (9; 9.54)	603
T833C	TC	9.62 (9.37; 9.88)	746/39.7%	9.96 [#] (9.57; 10.4)	278	9.24 [#] (8.95; 9.54)	447
	CC	9.65 (9.07; 10.2)	138/7.3%	10.2 (9.32; 11.1)	47	9.3 (8.7; 9.94)	87
CBS	Homozygous non-insert (WT)	9.91 (9.69; 10.1)	998/53%	10.5 [#] (10.1; 10.9)	356	9.27 [#] (9; 9.55)	603
844ins68	Heterozygous	9.64 (9.39; 9.89)	748/39.8%	10 [#] (9.6; 10.4)	279	9.23 [#] (8.94; 9.53)	448
	Homozygous insert (MT)	9.66 (9.08; 10.3)	136/7.2%	10.2 (9.33; 11.2)	46	9.31 (8.71; 9.95)	87
CBS	GG	9.85 (9.63; 10.1)	977/51.9%	10.3 [#] (9.92; 10.6)	373 ^{\$}	9.38 [#] (9.12; 9.65)	577 ^{\$}
G9276A	GA	9.71 (9.45; 9.97)	757/40.3%	10.3 [#] (9.86; 10.8)	245 ^{\$}	9.14 [#] (8.85; 9.44)	480 ^{\$}
	AA	9.4 (8.88; 9.96)	146/7.8%	9.78 (8.94; 10.7)	62 ^{\$}	9.09 (8.39; 9.84)	80 ^{\$}

Data is reported as geometric means adjusted for age and GGT (95% confidence intervals), or number and frequency. [#]Significant difference between men and women. ^{\$}Significant difference between the amount of men and women harbouring the different CBS G9276A variations. *Significant differences between the subdivisions. A = adenine; C = cytosine; CBS = cystathionine β-synthase; G = guanine; GGT = gamma glutamyl transferase; HIV = human immunodeficiency virus; ins = insertion; MSE = mean square error between men and women; MT = mutant type; MTR = methionine synthase gene; MTHFR = methyltetrahydrofolate reductase; n = sample size; T = thymine; tHcy = total homocysteine; WT = wild-type

The ANCOVA indicated that there was not a significant ($p = 0.66$) two-way interaction between the MTHFR C677T and MTR A2756G loci on logarithmically transformed tHcy concentrations. There was a main effect on logarithmically transformed tHcy concentrations among the three different MTHFR C677T genotypes ($p < 0.01$), but not between the different MTR A2756G genotypes ($p = 0.40$). Even though tHcy concentrations differed significantly between the MTR 2756 AA and AG genotypes, the lack of a main effect on tHcy when including MTHFR C677T in the analyses could indicate that the MTHFR variation has a more pronounced effect on tHcy concentrations than the MTR SNP. In our population there were no subjects that harboured the MTHFR 677 TT and MTR 2756 AA genotype combination.

There was no significant two-way interaction between the CBS 844ins68 or CBS G9276A and MTR A2756G genotypes ($p = 0.75$ and $p = 0.10$, respectively). None of the above genotypes had a significant main effect on tHcy concentrations.

Three-way interactions between the determined SNPs could not be calculated due to the low frequency (0.8%) of the MTHFR 677 TT genotype in the black South African population. As mentioned above, there were no subjects who harboured the MTHFR 677 TT and MTR 2756 AA genotypes in combination within this cohort. In all the above-mentioned ANCOVAs conducted, both age and logarithmically transformed GGT, which were adjusted for, had significant effects on logarithmically transformed tHcy concentrations.

6.5 DISCUSSION

Due to the involvement of Hcy in several diseases including CVD [3] establishing gene-gene and gene-environment interactions with regard to Hcy may provide insight into disease development. The MTHFR 677 TT and the MTR 2756 AA genotypes were associated with significantly higher tHcy concentrations than subjects harbouring the MTHFR 677 CT/CC and the MTR 2756 AG genotypes, respectively. Several significant gene-gene interactions were revealed through the statistical analyses, however, no gene-gender interactions were discovered. To our knowledge, this is the first study to investigate the MTHFR C677T, CBS T833C/844ins68, or

G9276A and MTR A2756G together as well as their interactions in relation to tHcy concentrations in a black South African population.

Several studies indicated that Hcy can be lowered by the adequate intake of the B-vitamins [26]. However, among black South Africans B-vitamin intake did not correlate strongly with tHcy concentrations, while age, GGT and gender influenced tHcy concentrations more strongly. Therefore, it seems as though demographic factors are more important determinants of tHcy concentrations than B-vitamin intake within this population.

When investigating tHcy concentrations within the different subdivisions in our population, men had significantly higher tHcy concentrations than women, which is in keeping with observations of other studies [27,28]. This difference was not due to gene-gender interactions. It was hypothesised that the higher tHcy observed in men could partly be attributable to the strong correlation reported between Hcy and circulating creatinine and larger muscle mass of men [28]. The latter is associated with simultaneous increased Hcy formation as a consequence of methyl group transfer during creatine/creatinine metabolism [28]. However, in this cohort creatinine was not associated with tHcy concentrations. The men consumed more alcohol when compared to the women which could have contributed to this gender difference.

When comparing tHcy concentrations between the rural and urban residents irrespective of their genetic make-up, rural living subjects had significantly higher tHcy concentrations (10.4 $\mu\text{mol/L}$) than urban subjects (9.09 $\mu\text{mol/L}$). Factors contributing to this difference are probably multi-factorial and should be explored in future research. This discrepancy reinforces the importance of the influence of environmental factors on tHcy concentrations and is similar to results reported by Kim *et al.* [29] in men and women residing in Puriscal, Costa Rica and by Tovar *et al.* [30] in Mexican women.

Besides genetic and environmental factors, tHcy is also influenced by physiological conditions. After adjusting for age and GGT, subjects within the PURE-study who were HIV-uninfected had significantly higher tHcy concentrations (9.64 $\mu\text{mol/L}$) than the HIV-infected subjects (9.03 $\mu\text{mol/L}$). Raiszadeh *et al.* [31] reported no

statistically significant difference in Hcy concentrations between HIV-infected subjects and HIV-uninfected subjects. In a multivariate statistical analysis that did not include micronutrients, age, serum creatinine and lower CD4 counts were significantly associated with plasma Hcy concentrations in HIV-infected women [31]. Few studies have investigated Hcy in relation to HIV status and their results are controversial. There is a need for future studies to elucidate possible mechanisms and to determine whether this observation is of practical significance.

In our sample of black South African subjects the prevalence of subjects harbouring the MTHFR 677 CC genotype was 84%, for the 677 CT genotype the prevalence was 15.2% and for the 677 TT it was 0.8%. However, previous studies that determined the distribution of the MTHFR C677T genotype amongst this ethnic group lacked statistical power to detect individuals harbouring the homozygous 677 TT genotype [32,33]. Subjects in our population harbouring the C677T genetic variation displayed elevated tHcy concentrations, with those homozygous for the 677 T allele having the highest concentrations, heterozygous individuals having lower concentrations and subjects homozygous for the wild-type exhibiting the lowest mean tHcy concentrations. These differences in tHcy are similar to previously reported results of other populations [5,6,34]. The increased tHcy concentrations could be attributed to lowered stability and activity of the MTHFR enzyme associated with the MTHFR C677T variation that results in the diminished conversion of precursor folate to bioactive folate, which is necessary for the conversion of Hcy to methionine [6,35].

In this cohort, the MTR 2756 AA genotype frequency was 63.7%, the 2756 AG frequency was 31.5% and the 2756 GG was 4.8% which is similar to those reported by Adjalla *et al.* [5] and Amouzou *et al.* [8] for other African subjects. The G allele frequency of the MTR A2756G alteration seems to be higher in Africans when compared to those reported for Caucasian populations [20,36]. Since Klerk *et al.* [20] reported increased risk for subjects harbouring the GG genotype towards coronary heart disease when compared to the AA genotype, the higher prevalence of the variant allele could, therefore, result in an increase of CVD events. Subjects included in the present study harbouring the MTR 2756 AA had significantly higher tHcy concentrations than those harbouring the MTR AG genotype. However, several other

studies reported that Hcy concentrations were decreased in an additive manner dependant on the number of G alleles present in the genotype [10,12,37,38].

With regard to the CBS gene variants, previous studies have reported that the CBS T833C and the 844ins68 alterations cosegregate in a *cis* orientation in all individuals harbouring this insertion [39]. However, in our population two subjects who harboured the CBS 833 TT genotype were not homozygous for the CBS 844ins68 locus and another two that harboured the CBS 833 CC were not homozygous for the CBS 844 insertion. Therefore, it remains uncertain whether the insertion arose after the mutation or *vice versa*. In our population the frequencies of the different CBS 844ins68/T833C genotypes were similar to those reported by Franco *et al.* [40] who also reported a high frequency of 37.7% and 4%, respectively for heterozygotes and mutant homozygotes of the T833C/844ins68 alterations, in a group of black Brazilians and Africans from Zaire, Cameroon and Angola. The T833C/844ins68 mutation is very rare (heterozygous genotype prevalence < 1%) in Asian and Amerindian populations [40] and rare in Caucasian populations (heterozygous genotype prevalence < 10%) [13]. This inter-ethnic variability could be due to the founder effect *i.e.* if a small group with a low prevalence of the variation became reproductively separated (*i.e.* immigration to other continents) from the genetically diverse African population from which they arose [41].

In our study, the CBS 833C/T844ins68 and G9276A genotypes, respectively, did not have significant effects on tHcy concentrations. In other investigations, the CBS T833C genetic variation seems to reduce CBS enzyme activity and thus to elevate Hcy concentrations [42,43]. However, the elevated Hcy associated with the CBS T833C variation seems to be suppressed by the 844ins68 CBS insertion in individuals who are compound heterozygotes at the T833C/844ins68 loci, since the latter is associated with lower tHcy concentrations when compared to individuals without it [14,44-45]. As all the individuals in this black South African cohort harbouring the CBS 833 TC genotype were determined to harbour the 844ins68 heterozygote genotype, it is possible that this mechanism applies to this cohort.

Griffioen *et al.* [13] discovered the novel G9276A transition in intron 8 of the CBS gene. In Caucasian volunteers, 0.2% of the subjects were reported to be heterozygous

for the G9276A alteration [13]. This current study is the first study to determine the CBS G9276A missense mutation in a black South African population wherein it was observed that the frequency of the 9276 GG genotype was 51.9%, the GA genotype was 40.3% and that the AA genotype was 7.8%. The prevalence of this genetic variation is considerably higher among black South African than Caucasian subjects and this may also be due to the previously discussed founder effect.

Analysis of the current black South African cohort indicated that subjects harbouring the MTHFR 677 TT in combination with the CBS 833 TT (or the homozygous CBS 844 non-insert genotype) had significantly higher tHcy concentrations than all other genotype combinations of the MTHFR 677 CC/CT and the CBS T833C/844ins68 loci. These results are congruent with the observations made by Dekou *et al.* [46] and De Stefano *et al.* [44] who reported that the CBS 844 68 bp insertion, which when occurring together with the CBS 833 C allele, is associated with lower Hcy concentrations and that this lowering effect was more pronounced in subjects homozygous for the MTHFR 677 T allele.

Black South African subjects harbouring MTHFR 677 TT in combination with the CBS 9276 GA were determined to have significantly higher tHcy concentrations than subjects harbouring any other combination of the MTHFR 677 and CBS 9276 genotypes, except for between those subjects harbouring MTHFR TT in combination with CBS 9276 GG. However, the implications with regard to CVD of these two genotypes occurring together should be determined in observational studies, but would probably be associated with an increased risk. There was no significant interaction between the CBS 844ins68 or CBS G9276A and MTR A2756G genotypes. Our results indicated no significant interaction between MTHFR C677T and MTR A2756G on tHcy concentrations, however, Dekou *et al.* [46] reported that the Hcy-raising effect of the MTHFR 677T and MTR 2756A homozygosity was additive. All the observations reported within this article highlight the fact that future research endeavours which aim to determine causality between genetic factors and CVD risk, should take gene-gene interactions into consideration.

6.6 CONCLUSIONS

This investigation included the determination of various polymorphisms within the CBS gene and the MTR gene (which have not been extensively researched in the black South African population) and environmental factors and explored possible interactions in relation to tHcy concentrations in the largest group of black South Africans studied to date. The very high prevalence of the CBS G9276A variation in this black South African group when compared to Caucasians [13] highlights the importance of conducting genetic research in an ethnic-specific manner and not to extrapolate data between different ethnic groups. Of the determined genotypes, the MTHFR C677T and to a lesser extent the MTR A2756G polymorphisms have the most pronounced influence on tHcy concentrations. However, since significant gene-gene interactions between all the genetic variations determined within the CBS gene and the MTHFR C677T polymorphism were established, the importance of the influence of CBS gene on tHcy concentrations must not be ruled out.

Gene-gene interactions on tHcy concentrations between several of the genetic loci investigated in this analysis highlight the epistatic nature of cardiovascular disease (CVD) risk in the black South African population. Furthermore, these gene-gene interactions determined, highlight the importance of investigating multiple genetic variations simultaneously rather than investigating single polymorphisms individually. Gene-gene interactions are important sources of population-specific variation in tHcy concentrations and thus CVD risk conveyed through tHcy and as such, studies such as these are integral in unravelling the multifactorial aetiology of CVD.

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CHAPTER 7

CONCLUSION

7.1 INTRODUCTION

Since non-genetic factors affecting plasma homocysteine (Hcy) concentrations do not account for all cases of hyperhomocysteinaemia (HHcy), which is implicated in various diseases including cardiovascular diseases abbreviated as CVD (Castañón *et al.*, 2007; Humphrey *et al.*, 2008), it is important to investigate genetic determinants of Hcy and also to follow more holistic research strategies integrating both non-genetic and genetic determinants of Hcy. Therefore, the focus of this thesis was on Hcy and several determinants thereof, including alcohol consumption and several common single nucleotide polymorphisms (SNPs). Five SNPs were investigated in total within this thesis in relation to total (t)Hcy concentrations in the largest group of black South Africans studied to date. One of the SNPs investigated is located within the gene encoding for the methylenetetrahydrofolate reductase (MTHFR) enzyme in which cytosine (C) is replaced by thymidine (T) at base pair (bp) position 677 of the gene (Frosst *et al.*, 1995). Three SNPs within the cystathionine β -synthase (CBS) were determined including the T833C transition within exon 8 that generally occurs in tandem with a 68 bp insertion (ins) at nucleotide 844 (Sebastio *et al.*, 1995) as well as an adenine (A) to guanine (G) transition at position 9276 (G9276A) in intron 8 (Griffioen *et al.*, 2005). The fifth SNP is an A to G transition at nucleotide position 2756 in the methionine synthase (MTR) gene (Leclerc *et al.*, 1996). From the literature review all the SNPs mentioned were considered important genetic determinants of Hcy, however, to our knowledge the effect of the CBS G9276A on Hcy concentrations has not previously been determined and since the method developed by Griffioen *et al.* (2005) to determine CBS T833C/844ins68 also enabled the genotyping of the G9276A SNP, it was also investigated within this thesis.

Multi-factorial approaches on Hcy research such as the work presented within this thesis will lead to a better understanding of HHcy and the diseases related to HHcy including CVD and ultimately to better disease prevention and management

strategies. The ongoing progress in the identification of gene variants that influence Hcy concentrations will ultimately enhance our understanding of CVD aetiology. In addition, this thesis also explored possible gene-diet and gene-gene interactions.

7.2 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Figure 7.1 provides a summary of the literature and original research presented within this thesis in Chapters 5 and 6. This figure also portrays the areas where the work within this thesis contributed to the literature regarding Hcy.

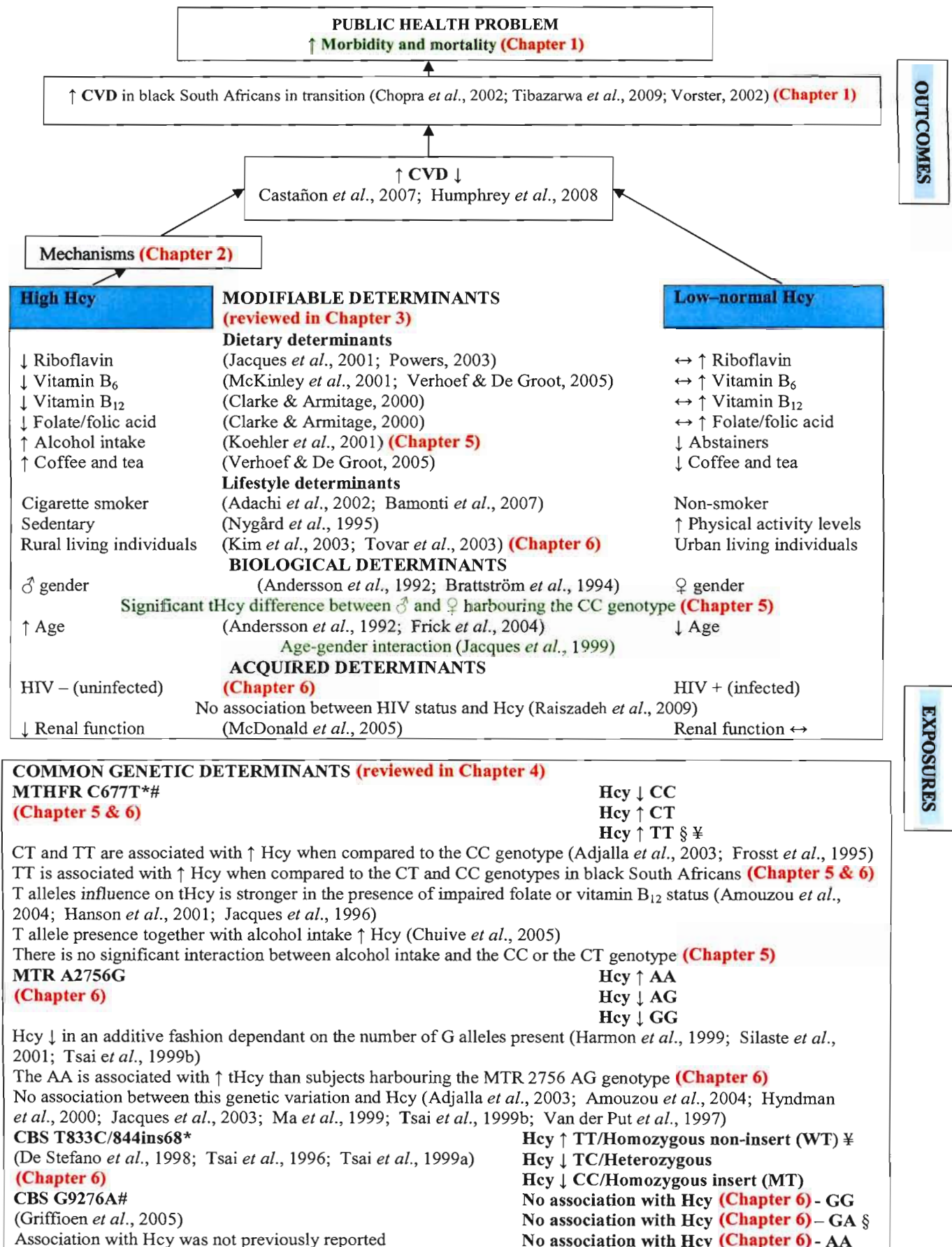
In Chapter 2 the author concluded that elevated Hcy concentrations are associated with the development of CVD, due to the growing evidence supporting this hypothesis (see Section 2.3). For several researchers it remains unresolved whether Hcy is causative in CVD development, however, in a recently published letter by Sacco and Carolei (2010), it was stated that the debate as to whether Hcy is a risk factor or not is over and that Hcy is now largely accepted as a risk factor towards CVD development including the development of cerebrovascular accidents. Research on Hcy is, therefore, warranted and necessary to contribute to public health.

In Chapters 3 and 4 in which the literature was reviewed it became clear that Hcy concentrations are influenced by several determinants including determinants that can be modified to lower Hcy concentrations and thereby possibly lower the risk for developing CVD. It seems as though alteration in lifestyle habits such as dietary intake or use of B-vitamin, betaine and choline supplements can lower tHcy concentrations. However, results of the original research presented in Chapters 5 and 6 indicated that B-vitamins were not strongly associated with tHcy concentrations in the black South African individuals studied. Therefore, one can speculate that the black South African population will not benefit from B-vitamin supplementation to lower their risk towards CVD development. However, to address B-vitamin deficiencies in South African communities known to be deficient of these micronutrients must remain an important priority. Other modalities that will induce a reduction in Hcy concentrations in the black South African population include the proscription against imprudent alcohol drinking.

Individuals with certain genetic characteristics respond better to B-vitamin intake and supplementation in terms of lowering Hcy than others especially among those homozygous for the common MTHFR C677T mutation (Chiuve *et al.*, 2005; Hustad *et al.*, 2000; Jacques *et al.*, 1996). It is the opinion of the author that future trials which use B-vitamins as Hcy-lowering agents should take the genetic makeup of the participants in consideration when interpreting their data. The latter could also be the reason for the lack of a strong association between B-vitamins and Hcy in the black South African population where the prevalence of the homozygous MTHFR 677 TT genotype was 0.8%.

The research presented in Chapter 5 falls within the scope of nutrigenetics as the objective was to determine whether alcohol consumption modulated the effect of the MTHFR C677T genetic polymorphism on tHcy concentrations. However, no significant gene-diet interaction was observed between the MTHFR C677T polymorphism and alcohol intake on tHcy concentrations. However, alcohol intake *per se* remains an important cause of HHcy (Carmel & James, 2002) and abuse thereof should be addressed because of its association with CVD (Puddey *et al.*, 1999) and several cancers (Corrao *et al.*, 2004).

In contrast to what was hypothesised in Chapter 1, urban living subjects as presented within the cross-sectional epidemiological studies presented in Chapter 6, were observed to have lower tHcy concentrations when compared to rural living subjects. Putative explanations for this significant difference are speculative and should be explored in more detail in the future. After completion of the twelve year Prospective Urban and Rural Epidemiology study it will be possible to determine whether this difference is of clinical importance with regard to CVD development or not.



* Between the CBS T833C/844ins68 and MTHFR C677T genotypes, there were significant two-way interactions (**Chapter 6**); # Between the CBS G9276A# and MTHFR C677T genotypes, there were significant two-way interactions (**Chapter 6**); § Subjects harbouring MTHFR 677 TT genotype with the CBS 9276 GA genotype had significantly ↑ tHcy concentrations when compared to those harbouring any other combination of these genotypes except compared to those subjects harbouring MTHFR TT with CBS 9276 GG genotype (**Chapter 6**); ¥ Subjects harbouring the MTHFR 677 TT genotype in combination with the CBS 833 TT/homozygous 844 non-insert genotype had significantly ↑ tHcy concentrations than subjects with any other MTHFR 677 CC or CT and the CBS T833C/844ins68 genotype combinations (**Chapter 6**)

Figure 7.1 *Model of modifiable and genetic determinants, gene-environment and gene-gene interactions in relation to Hcy concentrations and cardiovascular disease*

A = adenine; C = cytosine; CBS = cystathionine β -synthase; CVD = cardiovascular disease; ↓ = decrease; G = guanine; Hcy = homocysteine; HIV = human immunodeficiency virus; ↑ = increase; ins = insertion; ♂ = men; MT = mutant type; MTR = gene coding for methionine synthase; MTHFR = methyltetrahydrofolate reductase; - = negative; ↔ = normal or adequate; + = positive; T = thymine; tHcy = total homocysteine; WT = wild-type; ♀ = women

Chapter 6 endeavoured to elucidate the possible roles of these genetic alterations in the regulation of tHcy concentrations in a black South African cohort to understand better the CVD risk imparted by changes in tHcy concentrations in this population. Of the determined genotypes, the MTHFR C677T and to a lesser extent the MTR A2756G polymorphisms seem to have the most pronounced influence on tHcy concentrations. However, since significant gene-gene interactions between all the genetic variations determined within the CBS gene and the MTHFR C677T polymorphism were established, the importance of the influence of CBS gene on tHcy concentrations must not be ruled out.

Furthermore, the gene-gene interactions observed within Chapter 6 highlight the importance of investigating multiple genetic variations together rather than investigating one SNP when determining genetic influences. When several SNPs associated with increased tHcy concentrations are harboured together the negative effects seem to be additive. Subjects harbouring the MTHFR 677 TT genotype in combination with the CBS 833 TT/homozygous 844 non-insert genotype or the MTHFR 677 TT genotype in combination with the CBS 9276 GA/GG displayed significantly higher tHcy concentrations. The practical significance of these

observations should be established in future research, however, one could speculate that the low prevalence of the MTHFR 677 TT genotype among the black South African population should afford some protection to this population as a whole against the CVD risk conveyed through tHcy concentrations. However, in ethnic groups where the prevalence of this SNP is much higher such as those reported for Mexicans (Guéant-Rodriguez *et al.*, 2006), Mexican-Americans (Yang *et al.*, 2008), Hispanics (Rady *et al.*, 2002), Ashkenazi Jews (Rady *et al.*, 2002), Asians (Franco *et al.*, 1998a) and Caucasians of Italian nationality (Guéant-Rodriguez *et al.*, 2006), gene-gene interactions with the MTHFR C677T variation could be of clinical importance due to its effects on tHcy concentrations. Researchers that have access to conduct genetic research on the aforementioned ethnic groups should also investigate the prevalence of the CBS G9276A SNP to determine whether the observed gene-gene interaction between the MTHFR C677T and CBS G9276A variations is of practical importance in these groups.

7.3 NOVEL ASPECTS OF THIS THESIS

Strengths of this thesis include methodological rigor, the use of fasting blood samples, and assessment of nutrient intake on the basis of a validated quantitative food frequency questionnaire. The large sample size of unique subjects allowed accurate estimation of determinants of tHcy concentrations.

Several of the contributions of this research are novel. The research presented within this thesis is one of few in the South African setting to report plasma tHcy concentrations and simultaneously measure lifestyle, demographic, biological and genetic determinants of Hcy and accounted for their interactions. It is the first to report genetic determinants of Hcy concentrations other than MTHFR C677T variation and also the first of this magnitude to be conducted on black South African individuals ($n = 1,827$). Research on the polymorphisms within the CBS gene and gene encoding for the MS enzyme (MTR) is scarce within African individuals and to our knowledge, several of these genetic variations have not been investigated until now.

Africa is one of the most ethnically and genetically diverse regions of the world (Schuster *et al.*, 2010) and is hypothesised to harbour the ancestral population from which all modern humans arose (Chen *et al.*, 1995). The black South African population is part of the larger African L mitochondrial deoxyribonucleic acid macrohaplogroup (Chen *et al.*, 1995). Since the frequencies of these genetic variations investigated in these black South African individuals differed from other ethnic groups that have been investigated previously (Chapters 5 and 6) and due to micro-evolutionary effects different phenotypic effects may also be expected for the same genetic variation between the different ethnic groups, this work is of importance and contributes significantly to the existing literature.

Researchers have not paid enough attention to ethnicity and thus we could not extrapolate previous study observations to the black South African population. Researchers are at a pivotal time in the field of human genetics as various new tools to answer important questions are available, but to answer all these questions researchers need to study a wider variety of human populations, especially those from Africa (Tishkoff & Williams, 2002). The value of studying diverse ethnic groups is not only due to the diversity in genes, but also to the diversity in lifestyles.

Previous studies investigating the MTHFR C677T in the African population group were limited to a small number of cases and, therefore, had limited statistical power. When studying genotypes with low frequencies (prevalence < 5%) large numbers of individuals must be included to detect any gene-environment interactions or gene-gene interactions when they exist.

The very high prevalence of the CBS G9276A variation in this black South African group when compared to Caucasian individuals (Griffioen *et al.*, 2005) highlights the importance of conducting genetic research in Africans, who are reported to have the highest genetic diversity of any population (Schuster *et al.*, 2010), and not to extrapolate data between different ethnic groups. The observed differences between the black South Africans and Caucasians may help to pinpoint genetic adaptations to the unique lifestyle of each group and environmental exposures. The associations of certain SNP with certain phenotypes as reported in the literature for other ethnic

groups should be used with care as one should be sceptical about the validity of untested associations within a specific ethnicity (Schuster *et al.*, 2010).

Research has not fully characterised the polymorphic phenotypic outcomes of the MTR A2756G alteration, as inconsistent results regarding the association between this genetic variant and Hcy concentrations have inhibited conclusions from being definitively determined. This research has contributed to the elucidation of this polymorphisms association with tHcy concentrations (Figure 7.1). According to the results of Chapter 6, the AA is associated with significantly higher tHcy than subjects harbouring the MTR 2756 AG genotype.

Little has also been reported regarding the effect of the CBS T833C/ins68bp and/or CBS G9276A on Hcy concentrations in any population group (Griffioen *et al.*, 2005). This research has shed light on the prevalence of these genetic variations and their influence on Hcy concentrations within the black South African population.

Many basic questions on the role of the genetic determinants of Hcy concentrations in health and disease remain unanswered. To identify SNPs that associate with Hcy concentrations and are influenced by the environment and dietary intake that is relevant to health and disease can prove instrumental in clarifying CVD aetiology, as well as pointing to preventive dietary or lifestyle modifications tailored to the genetic make-up of an individual. The different susceptibility between the ethnic groups to vascular disease has yet to be settled, but could be due to genetic and epistatic differences as observed in Chapter 6. This thesis highlights the need for a strong systems biological approach in investigating nutrition-related disorders such as CVD as well as the confounding nature of the genetic background of a population on developing effective treatment strategies for these types of disorders.

7.4 REFERENCES

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ADDENDA

ADDENDUM A

PURE-SA Project
INFORMED CONSENT FORM (PHASE 1)

I, the undersigned(full names)
 understand that the only information that will be asked from me is the family census and household questionnaires. I understand that a field worker from the PURE-study will ask me the questions and that all the information gained from me will be kept confidential.

I indemnify the University, also any employee or student of the University, of any liability against myself, which may arise during the course of the project.

I will not submit any claims against the University regarding personal detrimental effects due to the project, due to negligence by the University, its employees or students, or any other subjects.

.....
 (Signature of the subject)

Signed at on

Witnesses

1.

2.

Signed at on

ADDENDUM B

PURE-SA project
INFORMATION TO COMMUNITIES

Dear Participant

Thank you for being willing to help us in this very important project. We are sure that the project will contribute to improve health of all the people of the North West Province.

The aim of the project is to get enough information regarding the development of chronic diseases like Diabetes, Stroke, Lung disease and heart disease with urbanisation to plan appropriate health and nutrition intervention strategies.

For this study we need 2 000 subjects whom we can follow for 12 years. The baseline survey will be done from April 2005 to November 2005. The subjects must be from rural as well as urban communities. Therefore, 500 subjects from 4 different levels of urbanisation will be needed. Ganyesa and Tlaskgameng were chosen for the rural and semi-rural areas because they are still under tribal law with a good infra structure and stability. We also spoke to Chief M. Letlhogile and the mayor Mr. E. Tladinyane and both gentlemen gave us permission to do the research in these two communities. Ikageng and the informal Ikageng were chosen as they are convenient and near the University. Cllr GG Megalanyane and Cllr Mahesh Roopa are informed about the study.

All the questionnaires will be filled out at your houses by trained research field workers who are from your communities. After a household survey and a family census on most of the households in your community to give us an overview of the total community, 250 men and 250 women from all four sites (Ganyesa, Tlaskgameng, Ikageng, and the Informal Ikageng) will be asked to proceed with the study. These subjects should be:

- Older than 35 years
- Healthy – which means that they must not be aware of any disease and do not take any chronic medication.

These 2 000 subjects will be asked to fill out the adult questionnaire, the food frequency questionnaire, the health questionnaire and the physical activity questionnaire. We will also make an appointment with each subject to take some measurements such as weight, height, skinfold thicknesses, ECG (test for heart abnormalities), lung functions, blood pressure, blood glucose, blood samples and a urine sample.

It is very important that we gather quality data and knowledge. Because HIV/AIDS is such a devastating illness and affects almost all aspects of health, it is necessary to know if HIV is absent before we analyse the data. Therefore, we will ask questions about your HIV status which you are allowed not to answer.

It is also very important to us that you feel free to participate in this study and that you understand what the study is all about. The fieldworker will ask you to sign this form after you have read and understood it.

Kind regards

Dr ANNAMARIE KRUGER

Contact details: 082 7715778 / 018 2994037(W) / 018 2907024(E)

ADDENDUM C

PURE-SA Project
INFORMED CONSENT FORM (Phase 2)

I, the undersigned(full names) read / listened to the information on the project in PART 1 and PART 2 of this document and I declare that I understand the information. I had the opportunity to discuss aspects of the project with the project leader and I declare that I participate in the project as a volunteer. I hereby give my consent to be a subject in this project.

I agree to be tested for HIV

Yes

No

I want to know my HIV-status

Yes

No

I agree to give a blood sample

Yes

No

I hereby also declare that I am aware that:

1. this blood sample will be used for the purpose of
 - a. Isolating DNA to look at genetic factors that are currently associated with Type 2 Diabetes (i.e. the Calpain10, Adiponectin, Leptin and Leptin Receptor genes), or genetic factors that may be associated with non-communicable diseases in the future. We give the assurance that all genetic tests and experiments will only focus on genotypes suspected to contribute to an increased risk of non-communicable diseases of lifestyle.
 - b. Testing for liver function by determining liver enzymes such as AST, GGT,
 - c. Analyses of other than genetic parameters for Diabetes Mellitus such as HbA_{1c}, Blood glucose and Insulin
 - d. Analyses of clotting factors and hypertension markers
 - e. Analyses of bone health, iron and nutrition status
 - f. And may be stored until such time as the above measurements/analyses will be done.
2. A two hour glucose tolerance test will be done
3. Body measurements such as height, weight, skinfold thicknesses, arm and leg circumferences will be taken
4. Electrocardiograph be taken
5. Blood pressure to be taken
6. Pulse wave velocity measurements will be made
7. A urine sample to be collected to analyse for the presence of heavy metals such as lead and mercury,
8. A Spirometer test to be performed to determine lung function
9. A handgrip test to be performed to test muscle strength
10. A hair sample to be taken to test for fumonisin mycotoxins.

.....
 (Signature of the subject)

Signed at ... Potchefstroom / Ganyesa ... (delete not applicable option) on/...../ 2005

Witnesses

1. 2.

Signed at ... Potchefstroom / Ganyesa ... (delete not applicable option) on/...../ 2005

PART 1

1. School/Institute:
Faculty of Health Sciences, North-West University

2. **Title of project/trial:**
PURE: Prospective Urban and Rural Epidemiological study

3. **Full names, surname and qualifications of project leader:**
Dr. Annamarie Kruger, Ph.D. (Nutrition)

4. **Rank/position of project leader:**
Research Manager

5.. Aim of this project

PURE's aim is that understanding the different lifestyle and health transitions of individuals in response to societal changes will elucidate societal and individual adaptive strategies that could diminish the adverse health effects of industrialisation and urbanisation on health, while retaining its benefits.

6. Explanation of the nature of all procedures, including identification of new procedures:

Each participant will have to fill in a number of questionnaires (Adult questionnaire, Physical activity questionnaire, Food frequency questionnaire, Health questionnaire) with the help of field workers. A blood and urine sample will be taken. Physical measures will be performed, including anthropometric measures (such as weight, height, and waist circumference), blood pressure, lung capacity and lung volume and an ECG will be performed.

7. Description of the nature of discomfort or hazards of probable permanent consequences for the subjects which may be associated with the project: (Including possible side-effects of and interactions between drugs or radio-active isotopes which may be used.)

It will take each participant quite a while (about two hours) to complete all the tests and discomfort may be experienced with the taking of blood samples. No measures will have permanent damage or consequences for the participants.

8. Precautions taken to protect the subjects:

The research nurse will be present at all times, and will be responsible for the blood sampling. She is very experienced and has performed these procedures numerous times in previous studies.

9. Description of the benefits which may be expected from this project:

When measures with immediate results are taken, such as blood glucose levels or blood pressure, the information will be communicated to the individual to seek professional help. Since this study is a longitudinal study, subjects that are high at risk will be identified from the dataset and personal feedback will be given.

10. Alternative procedures which may be beneficial to the subjects:

There will be tested for HIV/AIDS, therefore pre-test counselling will be given. If the subject wants to know his/her status and he/she tests positive, post counselling will also be given.

PART 2**To the subject signing the consent:**

You are invited to participate in a research project. It is important that you read/listen to and understand the following general principles, which apply to all participants in our research project:

1. Participation in this project is voluntary.
2. It is possible that you personally will not derive any benefit from participation in this project, although the knowledge obtained from the results may be beneficial to other people.
3. You will be free to withdraw from the project at any stage without having to explain the reasons for your withdrawal. However, we would like to request that you would rather not withdraw without a thorough consideration of your decision, since it may have an effect on the statistical reliability of the results of the project.
4. The nature of the project, possible risk factors, factors which may cause discomfort, the expected benefits to the subjects and the known and the most probable permanent consequences which may follow from your participation in this project, are discussed in Part 1 of this document.
5. We encourage you to ask questions at any stage about the project and procedures to the project leader or the personnel, who will readily give more information. They will discuss all procedures with you.
6. The University staff will use standardised procedures and take all possible precaution to protect the subject from risks.
7. All information will be kept **CONFIDENTIAL** and no personal information will be published without my consent.

Dr ANNAMARIE KRUGER

Contact details: **082 7715778 / 018 2994037(W) / 018 2907024(H)**

ADDENDUM D

**PURE-SA Project
REFERRAL LETTER**

To whom it may concern

Dear Doctor/Sister

Mr/Ms participated
in a project of our research group on

His/her fasted/random blood glucose was mmol/L

His/her resting blood pressure was mmHg

Will you please be so kind to attend to this patient?

Thank you and warm regards

.....

Dr ANNAMARIE KRUGER

Contact details: 082 7715778 / 018 2994037(W) / 018 2907024(H)

ADDENDUM E

The PURE-SA project
QUANTITATIVE FOOD FREQUENCY QUESTIONNAIRE

Subject ID

Subject Initials

Centre #

Community #

Household #

Subject #

F M L

Today's date:

year

month

day

1. Name: _____

2. Not applicable in South Africa

3. National identity # or equivalent _____ N/A ☐4. DOB:

OR

Age

years

5. Sex: ☐ Female ☐ Male

Please think carefully about the food and drink you have consumed during the *past month* (four weeks). I will go through a list of foods and drinks with you and I would like you to tell me:

- If you eat the food
- How the food is prepared
- How much of the food you eat at a time
- How many times a day you eat it and if you do not eat it everyday, how many times a week or a month you eat it.

To help you to describe the amount of a food you eat, I will show you pictures of different amounts of the food. Please say which picture is the closest to the amount you eat, or if it is smaller, between the sizes or bigger than the pictures.

There are no right or wrong answers.

Everything you tell me is confidential. Only your subject number appears on the form.

Is there anything you want to ask now?

Are you willing to go on with the questions?

FOOD FREQUENCY QUESTIONNAIRE

INSTRUCTIONS: Circle the subject's answer. Fill in the amount and times eaten in the appropriate columns.

I shall now ask you about the type and the amount of food you have been eating in the last few months. Please tell if you eat the food, how much you eat and how often you eat it. We shall start with maize meal porridge.

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
PORRIDGE AND BREAKFAST CEREALS AND OTHER STARCH								
Maize-meal porridge	Stiff (pap)						3400	
Maize-meal porridge	Soft (slappap)						3399	
Maize-meal porridge	Crumbly (phutu)						3401	
Ting								
Mabella	Stiff						3437	
Mabella	Soft							
Oats							3239	
Other cooked porridge	Type: _____							
Breakfast cereals	Brand name of cereals at home now: _____ _____ _____ _____							
Do you pour milk on your porridge or cereal? Yes No								
If yes, what type of milk (whole fresh, sour, 1%, fat free, milk blend, etc) _____								
If yes, how much milk								
Do you put sugar on your porridge or cereal? Yes No								

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
If yes, how much sugar							3989	
							3989	
							3989	
Samp	Bought Self ground						3250	
Samp and beans	Give ratio of samp:beans						3402 (1:1)	
Samp and peanuts	Give ratio of samp:peanuts						3250 (samp)	
Rice	White						3247	
	Brown						3315	
	Maize Rice						3250	
Pasta	Macaroni Spaghetti Other specify: _____ _____						3262	
Pizza	Home made: Specify topping _____ _____						3353 (base+ch)	
	Bought: Specify topping _____ _____						3353 (base+ch)	
You are being very helpful. Can I now ask you about meat? <u>CHICKEN, MEAT, FISH</u> How many times do you eat meat (beef, mutton, pork, chicken, fish) per week? _____								
Chicken (codes with	Boiled						2926	

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
skin)	Fried: in batter/crumbs Eg Kentucky						3018	
	Fried: Not coated							
	Bought: Chicken Licken						2925	
	Bought: Nando's							
	Roasted / Grilled						2925	
	Other: _____							
Do you eat chicken skin? Always Sometimes Never								
Chicken bones stew								
Chicken feet							2997	
Chicken offal								
Red meat	How do you like meat? With fat Fat trimmed							
Red meat	Fried							
	Stewed							
	Mince with tomato and onion						2987	
	Other:							
Beef Offal	Intestines: boiled nothing added						3003	
	Stewed with vegetables							
	Liver						2920	
	Kidney						2923	
	Other: Specify _____ _____							
Goat meat	Boiled						4281	
	Stewed with vegetables							
	Grilled / Roasted						4281	

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
<i>What type of vegetables is usually put into meat stews?</i>								
Wors / Sausage							2931	
Bacon							2906	
Cold meats	Polony						2919	
	Ham						2967	
	Vienna						2936	
	Other: Specify 							
Canned meat	Bully beef							
	Other: Specify 							
Meat pie	Beef						2939	
	Steak and kidney						2957	
	Cornish						2953	
	Chicken						2954	
	Other							
Hamburger	Bought							
Dried beans/peas/lentils	Soup						3145	
	Salad							
Soya products eg. Toppers	Brands at home now: 						3196 (Toppers)	
Pilchards in tomato/chilli/brine	Whole						3102	
	Mashed with fried onion							
Fried fish	With batter/crums							
	Without batter/crums							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
Other canned fish	Tuna						3056 (oil)	
	Pickled fish							
	Other: Specify _____							
Fish cakes	Bought: Fried						3080	
	Home made with potato						3098	
Fish fingers	Bought						3081	
Eggs	Boiled/poached						2867	
	Scrambled: milk + fat							
	Fried: Fat							

Now we come to vegetables and fruit

VEGETABLES AND FRUIT

Cabbage	How do you cook cabbage?							
	Boiled, nothing added						3756	
	Boiled with potato and onion and fat							
	Fried, nothing added							
	Fried in							
	Boiled, then fried with potato, onion							
	Other:							
	Don't know							
Spinach/morogo/ beetroot leaves other green leafy	How do you cook spinach?							
	Boiled, nothing added						3913	
	Boiled with fat added							
	Type of fat							
	With onion, tomato, potato							
	With peanuts							
	Other:							
	Don't know							
Tomato and onion gravy	Home made with fat							
	Type of fat							
	Without fat						3925	
	Canned						4192	
Pumpkin (yellow)	How do you cook pumpkin?							
	Boiled, nothing added						4164	

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
	Cooked in fat and sugar Fat							
	Boiled, little sugar and fat Fat							
	Other							
	Don't know							
Carrots	How do you cook carrots?							
	Boiled, nothing added Fat						3757	
	Boiled, sugar and fat Fat							
	With potato and onion: Fat							
	Raw, salad						3709	
	Chakalaka							
	Other							
	Don't know							
Mealies/ Sweet corn	How do you eat mealies?							
	On cob – fat added Fat							
	On cob – no fat added						3725	
	Creamed sweet corn / canned						3726	
	Whole kernel/canned						3942	
Beetroot	Salad						3699	
	Boiled, nothing added						3698	
Potatoes	How do you cook potatoes?							
	Boiled/baked with skin						4155	
	Boiled/baked without skin						3737	
	Mashed							
	Roasted Fat							
	French fries (chips)						3740	
Sweet potatoes	How do you cook sweet potatoes?							
	Boiled/baked with skin						3748	
	Boiled/baked without skin						3903	
	Mashed							
	Other:							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
	Don't know							
Salad vegetables	Mixed salad: tomato, lettuce and cucumber						3921	
	Raw tomato						3750	
	Other salad vegetables: _____ _____							
Other vegetables, specify + preparation	_____ _____ _____							
Do you like fruit?			☐ Yes	☐ No				
Apples							3592	
Pears							3582	
Oranges							3560	
Naartjie							3558	
Grapes							3550	
Peaches	Fresh						3565	
	Canned						3567	
Apricots	Fresh						3534	
	Canned						3535	
Mangoes							3556	
Guavas	Fresh						3551	
	Canned						3553	
Avocado							3656	
Wild fruit/berries	Specify type: _____							
Dried fruit	Types: _____							
Other fruit								

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
If subject eats canned fruit: Do you have custard with the canned fruit?			1	☐ Yes	2	☐ No		
Custard	Home made: Milk							
	Commercial eg Ultramel						2716	
<u>BREAD AND BREAD SPREADS</u>								
Bread / Bread rolls	White						3210	
	Brown						3211	
	Whole wheat						3212	
Do you spread anything on the bread?			☐ Always	☐ Sometimes	☐ Never			
Margarine	What brand do you have at home now?							
	Don't know _____							
Peanut butter							3485	
Jam/syrup/honey							3985	
Marmite / Fray bentos / Oxo							4058	
Fish/meat paste							3109	
Cheese	Type: _____ _____							
Achaar								
Other spreads	Specify: _____							
Dumpling								

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
Vetkoek	White flour						3257	
	Whole wheat flour						3324	
Provita, crackers, etc							3235	
Mayonnaise / salad dressing	Mayonnaise						3488	
	Other: Specify _____							
<u>DRINKS</u>								
Tea	English (normal)						4038	
	Rooibos						4054	
Coffee							4037	
Sugar/cup tea or coffee	Tea:						3989	
	Coffee:						3989	
Milk/cup tea or coffee	What type of milk do you use in tea and coffee?							
	Fresh/long life: whole/full						2718	
	Fresh/long life: 2%/low fat						2772	
	Fresh/long life: fat free						2775	
	Whole milk powder Brand: _____						2721 (powder)	
	Low fat milk powder Brand: _____						2825 (powder)	
	Skimmed milk powder Brand: _____						2825 (powder)	
	Milk blend Brand: _____						2770 (powder)	
	Whitener: type _____							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
	Condensed milk						2714	
	Evaporated milk						2715	
	None							
Milk as such	What type of milk do you drink milk as such?							
	Fresh/long life: whole/full						2718	
	Fresh/long life: 2%/low fat						2772	
	Fresh/long life: fat free						2775	
	Condensed milk						2714	
	Sour/maas						2787	
	Other: _____ _____							
Milk drinks	Nestle: _____							
	Milo: _____							
	Flavoured milk: _____							
	Other:							
Yoghurt	Drinking yoghurt						2756	
	Thick yoghurt						2734	
	Low fat sweetened with fruit						2732	
Squash	Sweet O						4027	
	Six O							
	Oros/Lecol – with sugar						3982	
	- artificially sweetener						3990	
	KoolAid						4027	
	Other: _____ _____							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
Fruit juice	Fresh/Liquifruit/Ceres						2866	
	Tropica (Dairy –fruit juice mix)						2791	
	Other:							

Fizzy drinks	Sweetened						3981	
Coke, fanta, etc	Diet							
Maueu/Motogo							4056	
Home brew								
Tlokwe							4039	
Beer							4031	
Spirits							4035	
Wine red							4033	
Wine White							4033	
Other specify								

SNACKS AND SWEETS								
Potato crisps							3417	
Peanuts	Raw						4285	
	Roasted						3458	
Cheese curls, Niknaks, etc							3267	
Raisins							3552	
Peanuts and raisins								
Chocolates	Name:							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
Candies	Sugus, gums, hard sweets, etc						4000	
Sweets	Toffees, fudge, caramels						3991	
Biscuits/cookies	Type: 							
Cakes and tarts	Type: 							
Scones								
Rusks	Type: 							
Savouries	Sausage rolls						2939	
	Samoosas: Meat filling						3355	
	Samoosas: Vegetable filling						3414	
	Biscuits eg bacon kips							
	Other specify: 							
Jelly							3983	
Baked pudding	Type: 							
Instant pudding	Milk type: 							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
Ice cream							3483	
Sorbet							3491	
Other specify	_____							

SAUCES, GRAVIES AND CONDIMENTS

Tomato sauce / Worcester sauce							3139	
Chutney							3168	
Pickles							3866	
Packet soups							3165	
Other:	_____							

WILD BIRDS, ANIMALS OR INCECTS (hunted in rural areas or on farms)

Wild fruit								

MISCELLANEOUS: Please mention any other foods used more than once/two times a week which we have talked about:

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
INDIGENOUS/TRADITIONAL FOODS/PLANTS/ANIMALS <i>Please tell me if you use any indigenous plants OR other indigenous foods like mopani worms, locusts ect to eat</i>								
Specify								

ADDENDUM F

The PURE-SA project
24-HOUR RECALL DIETARY INTAKE

Subject ID

Subject Initials

Centre #

Community #

Household #

Subject #

F M L

Today's date:

year

month

day

1. Name: _____

2. Not applicable in South Africa

3. National identity # or equivalent _____ N/A ☐

4. DOB:

OR

Age

years

5. Sex:

☐

Female

☐

Male

6. What day was yesterday? (tick correct one)

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
--------	---------	-----------	----------	--------	----------	--------

7. Would you describe the food that you ate yesterday as typical of your usual food intake?

Yes	1
-----	---

No	2
----	---

Greetings!

Thank you for giving up your time to participate in this study. I hope you are enjoying it so far. Here we want to find out what people living in this are eat and drink. This information is important to know as it will tell us if people are eating enough and if they are healthy.

There are no right or wrong answers.

Everything you tell me is confidential. Only your subject number appears on the form.

Is there anything you want to ask now?

Are you willing to go on with the questions?

I want to first ask you a few general questions about your food intake, the preparation of food and the type of food that you use in your home.

Instruction

Circle the subject's answer.

8. What type of pot do you usually use to prepare food in? (may answer more than one)

- Iron pot 1 ☐
- Stainless steel pot 2 ☐
- Aluminium pot 3 ☐
- Glass ware 4 ☐
- Other (specify) 5 ☐

9. Do you eat maize meal porridge?

1 ☐ Yes 2 ☐ No

If YES, what type do you have at home now?

Brand name: _____

Don't know: _____ 2

Grind self: _____ 3

If brand name is given, do you usually use this brand? 1 ☐ Yes 2 ☐ No 3 ☐ Don't know

Where do you get your maize meal from? (may answer more than one)

- Shop 1 ☐
- Employer 2 ☐
- Harvest and grind self 3 ☐
- Other (specify) 4 ☐
- Don't know 5 ☐

10. Do you eat fat/margarine or use it in the preparation of food?

1 ☐ Yes 2 ☐ No

If YES, what type do you have at home now?

Brand name: _____

Don't know: _____ 2

If brand name is given, do you usually use this brand? 1 ☐ Yes 2 ☐ No 3 ☐ Don't know

11. Do you use oil in the preparation of food?

1 ☐ Yes 2 ☐ No

If YES, what type do you have at home now?

Brand name: _____

Don't know: _____ 2

If brand name is given, do you usually use this brand?

1

Yes

2

No

3

Don't know

What type of oil do you buy for deep frying?

Brand name: _____

Do you use the same oil more than once?

1

Yes

2

No

If yes, how many times will you use the same oil? _____

12. What type of salt do you use?

Give brand names _____

Do you add salt to food while it is being cooked?

Always

1

Sometimes

2

Never

3

Don't know

4

Do you add salt to your food after it has been cooked?

Always

1

Sometimes

2

Never

3

Do you like salty foods eg salted peanuts, crisps, chips, fritos, biltong, dried sausage, etc

Very much

1

Like it

2

Not at all

3

13. Do you use any of the following:

	Name of product	Amount per day
Vitamins/vitamins and minerals		
Tonics		
Health foods		
Body building preparations		
Dietary fibre supplement		
Other: Specify		

ADDENDA

I want to find out about everything you ate or drank yesterday, including water or food you pick from the veld. Please tell me everything you ate from the time you woke up yesterday up to the time you went to sleep. I will also ask you where you ate the food and how much you ate.

To help you to describe the amount of a food you eat, I will show you pictures and examples of different amounts of the food. Please say which picture or example is the closest to the amount you eat, or if it is smaller, between the sizes or bigger than the pictures.

[illegible]

ADDENDUM G**The PURE-SA project
ADULT QUESTIONNAIRE**

We are very grateful to you for your participation in this study. All information given by you will be held in strict confidence, and will be used for the purpose of this study only after removing any personal identifying information.

Adult Questionnaire

INSTRUCTIONS

Please answer EACH question by marking
an X in ONE BOX on each line:
(unless otherwise instructed)



OR

By writing number(s) in the spaces provided:



OR

By specifying the answer on the line(s) provided

Adult Questionnaire

Subject Initials- F= first letter of first name
M= first letter of middle name
L= first letter of last name

3. National I.D#

If not applicable please mark the N/A box

Ethnicity Codes

- 01 - South Asian (India, Sri Lanka, Pakistan, Bangladesh)
- 02 - Chinese (China, Hong Kong, Taiwan)
- 03 - Japanese
- 04 - Malays
- 05 - Other Asian (Korea, Malaysia, Papua New Guinea, Thailand, Philippines, Indonesia, Nepal, Vietnam, Cambodia, Laos, Myanmar/Burma, Bhutan, Singapore)
- 06 - Persian
- 07 - Arab
- 08 - Black African
- 09 - Coloured African (Subsaharan African only)
- 10 - European
- 11 - Native North/South American or Australian Aborigine
- 12 - Latin American (Latino)
- 13 - Bantu/Semi Bantu
- 14 - Hemitic/Semi Hemitic
- 15 - Nilotic/Hausa
- 16 - Pygmie
- 17 - Swahili
- 18 - Other (any other ethnoracial group not listed above)

Subject ID

Centre #

Community#

Household #

Subject #

Subject
Initials

F M L

Today's date:

year

month

day

1. Name: _____
Given name Surname

2. Not applicable in South Africa

3. National identity # or equivalent: _____ N/A ☐

4. DOB: OR Age yrs
year month day

5. Sex: ☐ Female ☐ Male

6. Marital status: (check one only)

☐ Never married ☐ Currently married ☐ Common law/Living with partner
☐ Widowed ☐ Separated ☐ Divorced

7. Ethnicity: → (Please refer to facing page for codes)

8. Caste/Tribe: _____

9. What level of formal education have you completed? (check highest level only):

- ☐ None
☐ Primary
☐ Secondary/highschool/higher secondary
☐ Trade School
☐ College/University
☐ Unknown

Adult Questionnaire

11. Occupation

Group 1: Legislators, senior officials and managers

Legislators and senior officials
Corporate managers
General managers
Businessman

Group 2: Professionals

Physical, mathematical and engineering science professionals
Life science and health professionals
Teaching professionals
Other professionals

Group 3: Technicians and associate professionals

Physical, mathematical and engineering-
science associate professionals/technicians
Life science and health associate professionals/technicians
Teaching associate professionals/technicians
Other associate professionals/technicians

Group 4: Clerks

Clerks
Customer service clerks

Group 5: Service workers and shop and market sales workers

Personal and protective services workers
Models, salespersons and demonstrators

Group 6: Skilled agricultural and fishery workers

Market-oriented skilled agricultural and fishery workers
Subsistence agricultural and fishery workers

Group 7: Craft and related trade workers

Extraction and building trade workers
Metal, machinery and related trades workers
Precision, handicraft, printing and
related trades workers
Other craft and related trades workers

Group 8: Plant and machine operators and assemblers

Stationary plant and related operators
Machine operators and assemblers
Drivers and mobile plant operators

Group 9: Elementary occupations

Sales and services elementary occupations
Agricultural, fishery and related labourers
Labourers in mining, construction,
manufacturing and transport

Group 10: Armed forces

Armed forces

Group 11: Homemaker

Housewife/Househusband

Subject ID

Centre #

Community#

Household #

Subject #

Subject
Initials

F M L

10. Not applicable in South Africa

11a) Not applicable in South Africa

b) Please indicate which group best describes your main occupation.

(Please refer to facing page for definitions of groups and instruction manual for detailed definitions)

☐

Group 1

☐

Group 2

☐

Group 3

☐

Group 4

☐

Group 5

☐

Group 6

☐

Group 7

☐

Group 8

☐

Group 9

☐

Group 10

☐

Group 11

c) Not applicable in South Africa

d) What is your main source of income? _____

If occupation is group 11 (homemaker) go to question 13

12. Are you currently employed?

☐

No → (answer 12a - 12b)

☐

Yes → Go to #13

a) Are you retired/stopped work from your primary occupation due to old age?

☐

No

☐

Yes

b) Have you stopped working due to illness?

☐

No

☐

Yes

Subject ID

Centre #

Community#

Household #

Subject #

Subject
Initials

F M L

13. CURRENT DISABILITY:

- | | No | Yes |
|--|--------------------------|--------------------------|
| a) Do you have any problems using your fingers to grasp or handle? | ☐ | ☐ |
| b) Do you have any trouble walking about? | ☐ | ☐ |
| c) Do you have any trouble bending down and picking up an object from the floor? | ☐ | ☐ |
| d) Do you require a walking stick cane/walker to move about? | ☐ | ☐ |
| e) Do you have any trouble reading or seeing the individual grains of rice/corn on your plate? (with glasses worn) | ☐ | ☐ |
| f) Do you have trouble seeing a person from across the room? (12 feet/3.5 meters) (with glasses worn) | ☐ | ☐ |
| g) Do you have trouble speaking and being understood? | ☐ | ☐ |
| h) Do you have any trouble hearing what is said in a normal conversation? | ☐ | ☐ |

Subject Medical History

14. Have you experienced any of the following in the last six months?

- | | No | Yes | | No | Yes |
|--|--------------------------|--------------------------|-------------------------------------|--------------------------|--------------------------|
| a) Chest pain or tightness with usual activity | ☐ | ☐ | i) Vomiting | ☐ | ☐ |
| If Yes, —→ does the pain spread to the back, neck or inner border of arm | ☐ | ☐ | j) Loss of appetite | ☐ | ☐ |
| b) Breathlessness with usual activity | ☐ | ☐ | k) Painful or bleeding teeth/gums | ☐ | ☐ |
| c) Cough for at least 2 weeks | ☐ | ☐ | l) Jaundice | ☐ | ☐ |
| d) Any sputum while coughing | ☐ | ☐ | m) Burning while passing urine | ☐ | ☐ |
| e) Blood in sputum | ☐ | ☐ | n) Swelling of feet | ☐ | ☐ |
| f) Wheezing or whistling in the chest | ☐ | ☐ | o) Swelling of face | ☐ | ☐ |
| g) Early morning cough with chest tightness | ☐ | ☐ | p) Blood in urine | ☐ | ☐ |
| h) Loose stools/diarrhea for at least 3 days | ☐ | ☐ | q) Involuntary weight loss of > 3kg | ☐ | ☐ |

15. Not applicable in South Africa

16a) Do you use glasses/spectacles/contact lenses at present? No ☐ Yes ☐b) Do you use a hearing aid? No ☐ Yes ☐

Adult Questionnaire

Cancer Sites

- 1= Mouth
- 2= Esophagus
- 3= Stomach
- 4= Small intestine
- 5= Large intestine including rectum
- 6= Pancreas
- 7= Liver
- 8= Lung
- 9= Breast
- 10= Cervical/uterine/ovarian
- 11= Prostate
- 12= Head and neck
- 13= Other, specify

Subject ID

Centre #

Community#

Household #

Subject #

Subject
Initials

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17. Have you ever been diagnosed with any of the following?(check all that apply)

	No	Yes	#of yrs since diagnosis		No	Yes	#of yrs since diagnosis
a) Diabetes	☐	☐		i) COPD	☐	☐	
b) Hypertension/ high blood pressure	☐	☐		j) Asthma	☐	☐	
c) Stroke	☐	☐		k) Tuberculosis	☐	☐	
d) Angina/heart attack/ Coronary artery disease	☐	☐		l) Malaria	☐	☐	
e) Heart failure	☐	☐		m) Chagas	☐	☐	
f) Other heart disease	☐	☐		n) HIV/AIDS	☐	☐	
h) Hepatitis/Jaundice	☐	☐					
g) Cancer	☐	☐					

Not answered ☐

Please refer to facing page for cancer sites

↓

site other, specify _____

18. Have you been taking any medications regularly (ie. at least once per week) in the last month? ☐ No → go to 19 ☐ Yes

a) If yes, for what conditions:

	No	Yes
Blood pressure	☐	☐
Cholesterol lowering drugs	☐	☐
Stroke	☐	☐
Diabetes	☐	☐
Asthma	☐	☐
Chinese medicine	☐	☐
Others	☐	☐
Unknown	☐	☐

→ If Yes, specify _____

Adult Questionnaire

18b) If name of medication is unknown, please list as unknown.

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18b) List all the medications you are currently consuming at least once a week for the last month?

i) _____ ii) _____

iii) _____ iv) _____

v) _____ vi) _____

vii) _____ viii) _____

Men go to question #23

For Women Only (Questions 19 - 22)

19. Are you currently pregnant ? ☐ No ☐ Yes → Go to #21

20. Do you still have periods? ☐ No → (answer 20a) ☐ Yes → Go to #21

a) How many years since you stopped menstruating? years

21. Have you ever used an oral/ injectable contraceptive? ☐ No ☐ Yes

22a) How many live children have you given birth to? Boys Girls

b) Did you breast feed any of your children? ☐ No ☐ Yes

Adult Questionnaire

23. Accidents and Injuries

Location of Injury

- 1= Factory/industrial place
- 2= Office
- 3= Agriculture field/farm
- 4= Home
- 5= Road
- 6= Sport/game e.g. track, court, field, etc.
- 7= Public building
- 8= Mine/quarry
- 9= Construction site e.g. building, road-works, etc.
- 10 = Other

Type of Injury

- 1= Burns
- 2= Scalds
- 3= Fractures
- 4= Muscle and ligament sprains/tears
- 5= Cuts and lacerations
- 6= Bruises and abrasions
- 7= Suffocation
- 8= Head injury (where person did not lose consciousness)
- 9= Head injury (where person lost consciousness for some time)

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23. During the past 12 months, have you had any injuries that were serious enough to limit your normal activities? (check all that apply)

☐ No → Go to #24 ☐ Yes → (answer 23a - 23s)

If yes, please provide details:

Please refer to facing page for Location and Type Codes
Absence from work or usual activities (Days)

Cause of injury

Cause of injury	☐ No	☐ Yes	Location	Type	Absence from work or usual activities (Days)
a) Motor vehicle accident (as a passenger)	☐	☐			
b) Motor vehicle accident (as a pedestrian)	☐	☐			
c) Struck by an object	☐	☐			
d) Explosion	☐	☐			
e) Natural/environmental factors (gales/cyclones/lightning, etc.)	☐	☐			
f) Suffocation	☐	☐			
g) Poisoning	☐	☐			
h) Snake/scorpion bite	☐	☐			
i) Fall	☐	☐			
j) Fire/flames, resultant fumes	☐	☐			
k) Physical assault (gun, kidnapping, etc.)/violent crime	☐	☐			
l) Domestic violence (beaten by a family member)	☐	☐			
m) Drowning/submersion	☐	☐			
n) Hot or corrosive liquids/floods/substances	☐	☐			
o) Crush injuries (boulders, building materials, etc.)	☐	☐			
p) Accident caused by machinery	☐	☐			
q) Attempted suicide	☐	☐			
r) Armed conflict	☐	☐			
s) Other(specify) _____	☐	☐			

Adult Questionnaire

Location of Fractures

- 1= Hip/pelvis
- 2= Thigh
- 3= Leg
- 4= Forearm
- 5= Wrist
- 6= Hand/finger
- 7= Vertebrae (back)
- 8= Other

Fractures: In situations where subjects are in a cast and cannot differentiate between ligament tear or fracture, include as fracture only if doctor confirmed it as a broken bone

25c) Tobacco: Regular use is defined as consuming at least one tobacco product per day.

Duration of use:

For those that have consumed tobacco for <1 year, please enter "0"

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24. Have you ever fractured a bone? ☐ No (go to #25) ☐ Yes (if yes, answer a),b) and c)

a) Number of fractures

b) Years since last fracture

(yrs)

c) Bone (s) broken in the most recent fracture(if more than 3, list most severe sites)

(location)

If other, specify

→

Please refer to facing page for fracture locations

→

→

Tobacco

25. Which best describes your history of tobacco use?

- a) ☐ Formerly used tobacco products ☐ Currently use tobacco products ☐ Never used tobacco products → Go to #26

b) At what age did you start? yrs

c) Have you ever regularly used any of the following tobacco products? (check all that apply)

Past users only

	Average amount/day	Duration (years)	When Stopped (years ago)	If less than 1 yr (months ago)
(i) Cigarettes (all kinds)	number			
(ii) Beedies	number			
(iii) Cigars	number			
(iv) Pipes	number			
(v) Sheesha/water pipe Hookah	# of times			
(vi) Chewing tobacco	# of times			
(vii) Snuff	# of times			
(x) Other _____ Specify				

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Question 26 to be answered by non-smokers and former smokers only

26. During the past 12 months, have you been regularly (at least once per week) exposed to other people's tobacco smoke?

("Exposed" is defined as a minimum of 5 consecutive minutes, during which you inhale other people's smoke.)

☐

No

→ Go to #27

☐

Yes

→ Please answer questions 26a

a) Over the past 12 months, what has been your typical exposure to other people's smoke?

("Exposed" is defined as a minimum of 5 consecutive minutes, during which you inhale other people's smoke)

Select ONE only

☐

1-2 times/week

☐

3-6 times/week

☐

at least once a day

☐

2-3 times/day

☐

4 or more times/day

27. Not applicable in South Africa

Adult Questionnaire

28c) **Alcoholic Beverage:** Regular use is defined as at least once a month.

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28. Which best describes your history of alcohol use?

a) ☐ Formerly used alcohol products ☐ Currently use alcohol products ☐ Never used alcohol products → Go to #29

b) At what age did you start? yrs

c) What forms of alcohol have you regularly used? (check all that apply)

Form of Alcohol	Approx. size of one "drink"	Frequency			Average # of drinks	Duration (years)	Past users only When Stopped (years ago)
		Daily	Weekly	Monthly			
(i) Spirits(rum,whisky, gin,vodka etc)	30ml	☐	☐	☐			
(ii) Wine	125ml	☐	☐	☐			

(vi) Beer 375ml ☐ ☐ ☐

(vii) Country liquor/arrack/
sugar cane spirit 30ml ☐ ☐ ☐

d) At least once a month, do you consume >5 alcoholic drinks/day? ☐ No → Go to #29 ☐ Yes

i) How many times per month do you consume >5 alcoholic drinks in a day?

ii) What is the average number of drinks that you consume each time?

↓ If yes,(i,ii)

29 a) During your longest or nocturnal sleep period, what time do you normally go to bed? : (00:00-23:59)

b) During your longest or nocturnal sleep period, what time do you normally wake up? : (00:00-23:59)

c) Do you usually take naps/siestas? ☐ No ☐ Yes → Total nap duration → mins

Adult Questionnaire

33. Civic organization: are defined as non-profit, voluntary organization societies, self help groups and clubs.

Religious organization: are defined as different types of formal and informal groups set up on a religious basis.

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30. Are you a member of any of the following:

How often do you participate in
the activities of this group?

Per Month OR Per Year

(i) Self help group, Co-operative, Social club,
Sports club,☐ No☐ Yes(ii) Religious Group
(e.g: church group, etc.)☐ No☐ Yes(iii) Other _____
Specify☐ No☐ Yes

31. Please answer the following: (choose only one option for each)

Strongly Disagree Somewhat Disagree Somewhat Agree Strongly Agree

(i) People are generally honest and want to help others.

☐☐☐☐(ii) If I do nice things for someone, I can anticipate that they will
respect me and treat me just as well as I treat them.☐☐☐☐32a) The television, radio, newspaper or magazine advertisements
help me decide to buy the type of: (choose only one option for each)Not
Applicable

(i) Cooking oil

☐☐☐☐☐

(ii) Flour

☐☐☐☐☐

(iii) Rice/ Maize meal

☐☐☐☐☐b) The television, radio, newspaper or magazine advertisements
influence whether I buy: (choose only one option for each)

(i) Soft drinks

☐☐☐☐☐

(ii) Snacks

☐☐☐☐☐

(iii) Cigarettes

☐☐☐☐☐

(iv) Alcohol

☐☐☐☐☐

33. In a difficult situation, whose help can you count on from?(Please see facing page for definitions)

(i) Civic organizations: specify _____

☐ none☐ little☐ moderate/average☐ a great deal

(ii) Religious organizations: specify _____

☐ none☐ little☐ moderate/average☐ a great deal

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34. Have you experienced any of the following events during the last 12 months?

	No response	No	Yes	
(i) Loss of job	☐	☐	☐	
(ii) Retirement	☐	☐	☐	
(iii) Loss of crop/business failure	☐	☐	☐	
(iv) Household break in	☐	☐	☐	
(v) Marital separation/divorce	☐	☐	☐	
(vi) Other major intra-family conflict	☐	☐	☐	→ Please specify _____
(vii) Major personal injury or illness	☐	☐	☐	
(viii) Violence	☐	☐	☐	
(ix) Armed conflict/war	☐	☐	☐	
(x) Death of a spouse	☐	☐	☐	
(xi) Death/major illness of another close family member	☐	☐	☐	
(xii) Other major stress	☐	☐	☐	→ Please specify _____
(xiii) Wedding of family member	☐	☐	☐	
(xiv) New job	☐	☐	☐	
(xv) Birth in the family	☐	☐	☐	
(xvi) Separation from family	☐	☐	☐	
(xvii) Unavailability of food/ food insecurity	☐	☐	☐	

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35. Please answer the following: (Choose only one option for each)

For the following question, stress is defined as feeling irritable or filled with anxiety, or as having sleeping difficulties as a result of conditions at work or at home.

No response Never Experienced Stress Some Period of Stress Several Periods of Stress Permanent Stress

- a) How often have you felt stress at work in the last 12 months?
(Mark here if not applicable: i.e. no longer working ☐)

☐☐☐☐☐

- b) How often have you felt stress at home in the last 12 months?

☐☐☐☐☐

36. What level of financial stress have you felt in the last 12 months?

☐

No response

☐

Little/none

☐

Moderate

☐

High/severe

37. During the past twelve months, was there ever a time when you felt sad, blue, or depressed for two weeks or more in a row?

☐

No

☐

Yes

→ If yes, during those times, did you:

No response

No

Yes

- a) Lose interest in most things like hobbies, work or activities that usually give you pleasure?

☐☐☐

- b) Feel tired or low on energy?

☐☐☐

- c) Gain or lose weight?

☐☐☐

- d) Have more trouble falling asleep than you usually do?

☐☐☐

- e) Have more trouble concentrating than usual?

☐☐☐

- f) Think a lot about death (either your own, someone else's, or death in general)

☐☐☐

- g) Feel down on yourself, no good or worthless?

☐☐☐

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38. Please answer the following: (Choose only one option for each)

	Strongly Disagree	Somewhat Disagree	Somewhat Agree	Strongly Agree
a) I can do most of my regular shopping (food, household necessities, etc.) at stores within easy walking distance (less than 15 minutes) of my home.	☐	☐	☐	☐
b) Walking or bicycling in my neighbourhood is difficult because of the speed and/or amount of traffic.	☐	☐	☐	☐
c) My neighbourhood is generally free from pollution (litter, air pollution and noise pollution).	☐	☐	☐	☐
d) My neighbourhood streets are well lit at night.	☐	☐	☐	☐
e) I can see other people when I am walking in my neighbourhood.	☐	☐	☐	☐
f) I can speak to other people when I am walking in my neighbourhood.	☐	☐	☐	☐
g) There is a high crime rate in my neighbourhood.	☐	☐	☐	☐
h) There is a problem with unattended dogs in my neighbourhood.	☐	☐	☐	☐

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38a) Please answer the following: (Please check all that apply)

i) Has your household been a victim of the following crime(s) in the last 12 months?

	No	Yes
1. Armed robbery	☐	☐
2. Violent attacks	☐	☐
3. Murder	☐	☐
4. Vehicle hijacking	☐	☐
5. House breaking	☐	☐
6. Theft	☐	☐
7. Rape	☐	☐
8. Women abuse eg. (beat,swear-words,sexual) please specify _____	☐	☐
9. Child abuse eg. (burn,swear-words,rejection) please specify _____	☐	☐
10. Child sexual abuse	☐	☐
11. Other, please specify _____	☐	☐

ii) Do you think that crime in your area has increased in the past 5 years? ☐ No ☐ Yes

if yes, which of the following crime(s)?

- ☐ Armed robbery
- ☐ Violent attacks
- ☐ Murder
- ☐ Vehicle hijacking
- ☐ House breaking
- ☐ Theft
- ☐ Rape
- ☐ Women abuse
- ☐ Child abuse
- ☐ Child sexual abuse
- ☐ Other, please specify _____

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38b) Questions on HIV:

i) Do you know people who have HIV/AIDS? ☐ No ☐ Yes

if yes, which of these people: (please mark all that apply)

- ☐ Your children
- ☐ Your grandchildren
- ☐ Your spouse
- ☐ Your family members
- ☐ Your friends
- ☐ People in the community

ii) What would you consider the mean age of the people who are ill/have died of HIV/AIDS?

- ☐ Younger than 10 years ☐ Between 11-20 years ☐ Between 21-30 years
- ☐ Between 31-40 years ☐ Between 41-50 years ☐ Over 50 years

iii) If someone in your household is HIV positive, who is the primary caregiver?

- ☐ Spouse
- ☐ Parents
- ☐ Family member
- ☐ Child.children
- ☐ Friends
- ☐ Volunteer

38c) Do you care for any orphans in your family? ☐ No ☐ Yes

Adult Questionnaire

40b) Health History:

Cancer Sites

- 1= Mouth
- 2= Esophagus
- 3= Stomach
- 4= Small intestine
- 5= Large intestine including rectum
- 6= Pancreas
- 7= Liver
- 8= Lung
- 9= Breast
- 10= Cervical/uterine/ovarian
- 11= Prostate
- 12= Head and neck
- 13= Other, specify

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39. How long would it take you to get from your house to the nearest facility if you walked?

	Minutes	Don't know		Minutes	Don't know
i) grocery/convenience store		☐	iv) video store		☐
ii) bank		☐	v) non-fast food restaurant		☐
iii) post office		☐	vi) fast food restaurant		☐

40a) Total number of siblings

b) Health History: Complete for all parents and siblings, alive or dead

	Father			Mother			Siblings			
	Unknown	No	Yes	Unknown	No	Yes	Unknown	No	Yes	# of siblings with the condition*
Diabetes	☐	☐	☐	☐	☐	☐	☐	☐	☐	If yes →
Coronary Heart Disease	☐	☐	☐	☐	☐	☐	☐	☐	☐	
High Blood Pressure	☐	☐	☐	☐	☐	☐	☐	☐	☐	
Stroke	☐	☐	☐	☐	☐	☐	☐	☐	☐	
Cancer	☐	☐	☐	☐	☐	☐	☐	☐	☐	
	Please refer to facing page for cancer sites									
	if Yes, indicate site									
	↓			↓			↓			
	Other, Specify			Other, Specify			Other, Specify			

Adult Questionnaire

If subject refuses to provide any of the measures, enter a value of "0" into each of the boxes for that question

For more detailed instructions please refer to the instruction manual

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41. Physical Measurements

Sitting
 a) Right arm blood pressure
 #1 mmHg
 Systolic Diastolic
 #2 mmHg
 Systolic Diastolic

b) Heart Rate
 #1 beats/min
 #2 beats/min

c) Waist
 #1 . cm → ☐ minimal/no clothing
 #2 . cm ☐ full clothing

d) Weight . kg → ☐ minimal/no clothing
 ☐ full clothing

e) Hip
 #1 . cm → ☐ minimal/no clothing
 #2 . cm ☐ full clothing

f) Height . cm (without shoes)

42a) Circumference of mid upper right arm: . cm

b) Circumference of right calf: . cm

c) Head Circumference: . cm

d) Upper flexed arm circumference . cm

43a) Right arm triceps skinfold:
 #1 . mm
 #2 . mm
 #3 . mm

b) Right calf skinfold:
 #1 . mm
 #2 . mm
 #3 . mm

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c) Biceps
skinfold #1 . mm
#2 . mm
#3 mm

d) Subscapular
skinfold #1 . mm
#2 . mm
#3 mm

e) Supra spinal
skinfolds #1 . mm
#2 . mm
#3 mm

44 a) Humerous breadth . cm

b) Femur breadth . cm

45. Grip Strength (Maximal contraction):

a) Non-dominant hand: #1 kg.

#2 kg.

#3 kg.

b) Dominant hand: #1 kg.

#2 kg.

#3 kg.

Adult Questionnaire

If subject refuses to provide any of the measures, enter a value of "0" into each of the boxes for that question

For more detailed instructions please refer to the instruction manual

46. Spirometry:

American Thoracic Society criteria for acceptable spirometers:
Spirometers are acceptable if they are free from:

1. Cough during exhalation
2. Early termination or cut-off
3. Variable effort
4. Leaks
5. Obstructed mouth piece

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46. Spirometry:

a) FEV1 (Litre): #1 . #2 . #3 .

b) Does FEV1 obtained meet ATS criteria?

☐

No → (answer (i) to (iii))

☐

Yes → Go to c)

Reasons for not meeting the ATS criteria: (check all that apply)

i) Cough ☐ii) Values not within 0.2L of each other ☐iii) Less than 3 values ☐c) FVC (Litre): #1 . #2 . #3 .

d) Does FVC obtained meet ATS criteria?

☐

No → (answer (i) to (iii))

☐

Yes → Go to e)

Reasons for not meeting the ATS criteria: (check all that apply)

i) Cough ☐ii) Values not within 0.2L of each other ☐iii) Less than 3 values ☐e) PEFR (Litre/min): #1 #2 #3

f) Does PEFR obtained meet ATS criteria?

☐

No → (answer (i) to (ii))

☐

Yes → Go to Q#47

Reasons for not meeting the ATS criteria: (check all that apply)

i) Cough ☐ii) Less than 3 values ☐

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47. Not applicable in South Africa

48. ECG obtained? No ☐ → Go to #49 Yes ☐a)
year month dayPlace
ECG :File
Label Hereb) Please print ECG label #: 49 a) Blood sample obtained? No ☐ → Go to #50 Yes ☐b) ☐ Fasting sample ☐ Non-fasting samplec)
year month dayTime :
(00:00-23:59)Hours since any
food/beverage
consumed (excluding water) d) Please print Blood label #: Place
Blood label
here50 a) Urine sample obtained? No ☐ → Go to #51 Yes ☐b) ☐ Fasting sample ☐ Non-fasting samplec) Please print Urine label #: Place
Urine label
here51. Name of Interviewer:
(please print)

First Initial

Last Name

Interviewer Code: