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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Assistant promoter: Prof. C.S. Venter

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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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The author
Cornelie Nienaber

“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 homosisteïn konsentrasies in swart Suid Afrikaners

Agtergrond: Homosisteïn (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 metieltetrahidrofolatreduktase (MTHFR), stasjon-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 alkoholinname 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. Vaste plasma-tHcy-konsentrasies was bepaal met fluoressensie polarisasie immuno-analise tegnologie. SNPs was bepaal deur polimerase kettingreaksiegebaseerde restriksiefragmentlengte polimorfisme-analises. Dieetinnames was bepaal met 'n kwantitatiewe voedselfabvraelys. Gammaglutamiettransferase (GGT) en persentasie koolhidraat wat 'n transferrithet 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 ekwilibrium, maar het verskil van wat vir ander etniese groepe gerapporteer is. Daar was nie 'n interaksie tussen alkoholinname en die MTHFR 677 CC/CT genotipes nie ($p > 0.05$). Die MTHFR 677 TT en MTR 2756 AA genotipes was geassocieer met betekenisvol hoër tHcy konsentrasies (16.6 en 10.1 µmol/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 µmol/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 alkoholintname en die MTHFR 677 CC/CT genotipes, hoewel MTHFR C677T genotipe, ouderdom, geslag en GGT belangriker determinante van tHcy was as B-vitamienintname. 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 μmol/L; p < 0.05) than
subjects harbouring the MTHFR 677 CT/CC and the MTR 2756 AG (10.5, 9.7 and 9.5 µmol/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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<tr>
<td>844ins68</td>
<td>insertion of 68 base pairs at nucleotide position 844</td>
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<tr>
<td>A</td>
<td>adenine (nucleotide)</td>
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<tr>
<td>A</td>
<td>alanine (amino acid)</td>
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<td>A222V</td>
<td>alanine replacement with a valine amino acid at amino acid number 222</td>
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<td>A1298C</td>
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<td>A2756G</td>
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<td>AUTHer</td>
<td>Africa Unit for Transdisciplinary Health Research</td>
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<td>BBM</td>
<td>brush-border membrane</td>
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<td>betaine-homocysteine methyltransferase</td>
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<td>C</td>
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<td>CI</td>
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<td>%CDT</td>
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<td>E429A</td>
<td>a glutamate to alanine substitution at amino acid number 429</td>
</tr>
<tr>
<td>ecto-ADPase</td>
<td>ecto-adenosine diphosphatase</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>eNO</td>
<td>endothelial NO</td>
</tr>
<tr>
<td>F</td>
<td>phenylalanine (amino acid)</td>
</tr>
<tr>
<td>F1 + 2</td>
<td>prothrombin fragments 1 and 2</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin adenine mononucleotide</td>
</tr>
<tr>
<td>G</td>
<td>guanine (nucleotide)</td>
</tr>
<tr>
<td>G</td>
<td>glycine (amino acid)</td>
</tr>
<tr>
<td>GCP II</td>
<td>glutamate carboxypeptidase II</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic deoxyribonucleic acid</td>
</tr>
<tr>
<td>GGT</td>
<td>gamma glutamyl transferase</td>
</tr>
<tr>
<td>H</td>
<td>histidine (amino acid)</td>
</tr>
<tr>
<td>H475Y</td>
<td>a histidine to a tyrosine amino acid substitution</td>
</tr>
<tr>
<td>HAART</td>
<td>highly active antiretroviral therapy</td>
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<tr>
<td>Hcy</td>
<td>homocysteine</td>
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<tr>
<td>HHcy</td>
<td>hyperhomocysteinaemia</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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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
<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>OR</td>
<td>odds ratio</td>
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<tr>
<td>P</td>
<td>praline (amino acid)</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PhASRec</td>
<td>Physical Activity, Sport and Recreation</td>
</tr>
<tr>
<td>PHRI</td>
<td>Population Health Research Institute</td>
</tr>
<tr>
<td>PI</td>
<td>protease inhibitor</td>
</tr>
<tr>
<td>PLP</td>
<td>pyridoxal 5’-phosphate</td>
</tr>
<tr>
<td>PhASRec</td>
<td>Physical Activity, Sport and Recreation</td>
</tr>
<tr>
<td>PURE</td>
<td>Prospective Urban and Rural Epidemiology study</td>
</tr>
<tr>
<td>Q</td>
<td>glutamine (amino acid)</td>
</tr>
<tr>
<td>QFFQ</td>
<td>quantitative food frequency questionnaire</td>
</tr>
<tr>
<td>R</td>
<td>arginine (amino acid)</td>
</tr>
<tr>
<td>R27H</td>
<td>a substitution of an arginine to histidine at amino acid number 27</td>
</tr>
<tr>
<td>RFC I</td>
<td>reduced folate carrier 1</td>
</tr>
<tr>
<td>RR</td>
<td>relative risk</td>
</tr>
<tr>
<td>S</td>
<td>serine (amino acid)</td>
</tr>
<tr>
<td>SAH</td>
<td>s-adenosylhomocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>s-adenosylmethionine</td>
</tr>
<tr>
<td>SANPAD</td>
<td>South Africa - Netherlands Research Programme on Alternatives in Development</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SMAC</td>
<td>Sequential Multiple Analyser Computer</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>T</td>
<td>thymine (nucleotide)</td>
</tr>
<tr>
<td>T</td>
<td>threonine (amino acid)</td>
</tr>
<tr>
<td>T833C</td>
<td>thymine to cytosine substitution at base 833</td>
</tr>
<tr>
<td>TAT</td>
<td>thrombin-antithrombin complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>TC</td>
<td>transcobalamin</td>
</tr>
<tr>
<td>tHcy</td>
<td>total homocysteine</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue type plasminogen activator</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofolate</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>V</td>
<td>valine (amino acid)</td>
</tr>
<tr>
<td>Vit</td>
<td>vitamin</td>
</tr>
<tr>
<td>VNTR</td>
<td>variable number of tandem repeats</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>Y</td>
<td>tyrosine (amino acid)</td>
</tr>
</tbody>
</table>
LIST OF SYMBOLS AND UNITS

\[ \beta \] beta
\[ \chi^2 \] Chi square
\[ r \] correlation
\[ \downarrow \] decrease
\[ ^\circ C \] degrees, Celcius or centigrade
\[ = \] equal
\[ -\text{CH}_3 \] methyl group
\[ \gamma \] 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
\[ \text{pH} \] indicator of acidity or alkalinity
\[ \text{kat} \] katal
\[ \text{kg} \] kilogram
\[ \text{kg/m}^2 \] kilograms per meter squared; unit of body mass index
\[ \text{km} \] kilometer
\[ \% \] percentage
\[ \pm \] plus minus
\[ p \] p-value
\[ \text{MgCl}_2 \] magnesium chloride
\[ \sigma \] men
\[ \mu \] micro: \(10^{-6}\)
\[ \mu\text{mol} \] micromole
\[ m \] mili
\[ mL \] mililitre
\[ - \] minus
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>mol</td>
<td>mole</td>
</tr>
<tr>
<td>M</td>
<td>molecular weight</td>
</tr>
<tr>
<td>x</td>
<td>multiply</td>
</tr>
<tr>
<td>x g</td>
<td>multiplied by gravitational force</td>
</tr>
<tr>
<td>-</td>
<td>negative</td>
</tr>
<tr>
<td>n</td>
<td>number of subjects; sample size</td>
</tr>
<tr>
<td>n</td>
<td>nano: $10^{-9}$</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>$\leftrightarrow$</td>
<td>normal or adequate</td>
</tr>
<tr>
<td>+</td>
<td>positive</td>
</tr>
<tr>
<td>$&lt;$</td>
<td>smaller than</td>
</tr>
<tr>
<td>$\leq$</td>
<td>smaller than or equal to</td>
</tr>
<tr>
<td>-SH</td>
<td>thiol</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>$\varphi$</td>
<td>women</td>
</tr>
<tr>
<td>yrs</td>
<td>years</td>
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</table>
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. Methylene tetrahydrofolate reductase (MTHFR) and methionine synthase (MS) are two key enzymes involved in the folate and vitamin B_{12}-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; Hyndmar 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


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

<table>
<thead>
<tr>
<th>Name and signature</th>
<th>Affiliation</th>
<th>Role in the study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miss C. Nienaber (Ph.D candidate)</td>
<td>CEN within the School for Physiology, Nutrition and Consumer Science of the NWU</td>
<td>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 et al., in press), critical revision, planning, writing and compilation of this thesis.</td>
</tr>
<tr>
<td>Dr. G.W. Towers (promoter)</td>
<td>CEN within the School for Physiology, Nutrition and Consumer Science of the NWU</td>
<td>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.</td>
</tr>
<tr>
<td>Dr. T. Hoekstra (co-promoter)</td>
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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
1.7 REFERENCES


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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: HSCH₂CH₂CH(NH₂)CO₂H (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).

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**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 I; **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 glutathionine, 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 μmol/L (to convert tHcy concentration to μg/mL multiply by 0.1352), mild to moderate HHcy between 16 to 100 μmol/L and severe HHcy > 100 μmol/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, Castañon et al. calculated that tHcy concentrations of > 12 μmol/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 μmol/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
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 μmol/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; Castañón 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 μmol/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 μmol/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 (Castañón 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). Even though 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; Davi 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 µmol/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 & Wicken, 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.

2.5 REFERENCES


CHAPTER 2


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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 $B_{12}$ status (Bates et al., 1997). Even within the normal ranges the concentrations of serum or red cell folate and serum
vitamin B$_{12}$ 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 B$_2$), vitamin B$_6$ [pyridoxine, pyridoxal, pyridoxamine or pyridoxal 5'-phosphate (PLP)] and vitamin B$_{12}$ (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-isoalloxazine (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 B\textsubscript{12} as methylcobalamin (Scott, 2003). Low vitamin B\textsubscript{12} 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 \textit{et al.}, 2004).

Certain rare and drastic genetic mutations such as the one described by Mudd \textit{et al.} in 1969 can lead to impaired vitamin B\textsubscript{12} 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 B\textsubscript{12}-dependent enzyme, causing accumulation of methylmalonic acid (Mudd \textit{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 \textit{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 \textit{et al.}, 2002). The typical Western diet contains 1.6 - 2.8 g of methionine per day (Shoob \textit{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 B$_6$ (acetaldehyde dislodges vitamin B$_6$ from its protective binding protein so that it is destroyed and alcohol interferes with thiamine, folate and vitamin B$_{12}$ 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 \( t^H \text{Hcy} \) (Bleich et al., 2001) and wine consumption in particular was related to \( t^H \text{Hcy} \) in a J-shaped manner i.e. \( t^H \text{Hcy} \) 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 \( \text{Hcy} \) 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 \( \text{Hcy} \) 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 \( t^H \text{Hcy} \) 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 \( \text{Hcy} \) (Bleich et al., 2001). Researchers have ascribed the beneficial effect of beer drinking on \( t^H \text{Hcy} \) to its folate, riboflavin and vitamin \( B_6 \) content, all of which are important for the enzymatic \( \text{Hcy} \) conversion. Wine consumption seems to elevate \( t^H \text{Hcy} \) concentrations (Bleich et al., 2001; Van der Gaag et al., 2000). In a large cross-sectional study Burger et al. (2004) revealed that \( \text{Hcy} \) 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 \( \text{Hcy} \) 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 (Hidiroglou 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 cystathioninélyase compromising Hcy transsulphuration. Nitric oxide production, secondary to augmented Hcy inhibits MS activity possibly by inactivating cobalamin (Danishpajooh 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; Hidiroglou 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).
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 Nygard 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 Nygard 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 µmol/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 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 μmol/L vs. 10.07 μmol/L, p < 0.05 cases; 9.90 μmol/L vs. 9.53 μmol/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 county 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 B_{12} 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 B_{12}, 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 (Gutormsen 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 lead 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; Pétiard et al., 1999). HAART seems to be associated with a decrease in vitamin $B_{12}$ 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 HHy (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 $B_{12}$ 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.

3.5 REFERENCES


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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 ≥ 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 ≥ 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 fulfills 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 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 *B*₂) 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 Hcy 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 $\chi^2$ 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

<table>
<thead>
<tr>
<th>Citation</th>
<th>Ethnic group</th>
<th>Sample</th>
<th>$677CC$ (%)</th>
<th>$677CT$ (%)</th>
<th>$677TT$ (%)</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjalla et al. (2003)</td>
<td>Africans (West Africa)</td>
<td>240</td>
<td>83.3</td>
<td>15.9</td>
<td>0.8</td>
<td>*</td>
</tr>
<tr>
<td>Amouzou et al. (2004)</td>
<td>Africans (West Africa savannah)</td>
<td>68</td>
<td>88.2</td>
<td>11.7</td>
<td>0.1</td>
<td>*</td>
</tr>
<tr>
<td>Alex et al. (2003)</td>
<td>Africans (West Africa coast)</td>
<td>208</td>
<td>80.3</td>
<td>18.3</td>
<td>1.4</td>
<td>*</td>
</tr>
<tr>
<td>Franco et al. (1998a)</td>
<td>Africans (Zaire and Cameroon)</td>
<td>67</td>
<td>89.6</td>
<td>10.4</td>
<td>0.0</td>
<td>*</td>
</tr>
<tr>
<td>Guéant-Rodriguez et al. (2006)</td>
<td>Africans (West Africa Bénin and Togo)</td>
<td>465</td>
<td>90.2</td>
<td>9.0</td>
<td>0.8</td>
<td>*</td>
</tr>
<tr>
<td>Loktionov et al. (1999)</td>
<td>Africans (South Africa)</td>
<td>100</td>
<td>80.0</td>
<td>20.0</td>
<td>0.0</td>
<td>*</td>
</tr>
<tr>
<td>Scholtz et al. (2002)</td>
<td>Africans (South Africa)</td>
<td>66</td>
<td>92.0</td>
<td>8.0</td>
<td>0.0</td>
<td>*</td>
</tr>
<tr>
<td>Yang et al. (2008)</td>
<td>Non-Hispanic blacks</td>
<td>2103</td>
<td>79.2</td>
<td>19.5</td>
<td>1.3</td>
<td>*</td>
</tr>
<tr>
<td>Gebhardt et al. (2001)</td>
<td>Mixed ancestry (coloured South African pregnant women)</td>
<td>168</td>
<td>73.2</td>
<td>25.6</td>
<td>2.2</td>
<td>*</td>
</tr>
<tr>
<td>Scholtz et al. (2002)</td>
<td>Mixed ancestry (South Africa)</td>
<td>73</td>
<td>65.0</td>
<td>34.0</td>
<td>2.0</td>
<td>*</td>
</tr>
<tr>
<td>Dilley et al. (2001)</td>
<td>African-Americans</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corroy et al. (2000)</td>
<td>African-Americans (New York)</td>
<td>526</td>
<td>81.5</td>
<td>17.0</td>
<td>1.5</td>
<td>*</td>
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<tr>
<td>McAndrew et al. (1996)</td>
<td>African-Americans</td>
<td>102</td>
<td>80.0</td>
<td>20.0</td>
<td>0.0</td>
<td>*</td>
</tr>
<tr>
<td>Rady et al. (2002)</td>
<td>African-Americans</td>
<td>97</td>
<td>77.3</td>
<td>21.7</td>
<td>1.0</td>
<td>*</td>
</tr>
<tr>
<td>Franco et al. (1998a)</td>
<td>AmerIndians (Brazilian Amazonian tribes)</td>
<td>129</td>
<td>59.7</td>
<td>32.5</td>
<td>7.8</td>
<td>*</td>
</tr>
<tr>
<td>Franco et al. (1998a)</td>
<td>Asians</td>
<td>40</td>
<td>40.0</td>
<td>40.0</td>
<td>20.0</td>
<td>*</td>
</tr>
<tr>
<td>Kelenen et al. (2004)</td>
<td>South Asians (Canada)</td>
<td>233</td>
<td>70.3</td>
<td>25.2</td>
<td>4.2</td>
<td>*</td>
</tr>
<tr>
<td>Franze et al. (1998a)</td>
<td>Brazilian blacks</td>
<td>50</td>
<td>78.0</td>
<td>20.0</td>
<td>2.0</td>
<td>*</td>
</tr>
<tr>
<td>Tavares et al. (2004)</td>
<td>Brazilian Parkateje Indians</td>
<td>90</td>
<td>45.3</td>
<td>40.7</td>
<td>14.0</td>
<td>*</td>
</tr>
<tr>
<td>Conroy et al. (2000)</td>
<td>Caucasians (New York)</td>
<td>503</td>
<td>44.5</td>
<td>47.5</td>
<td>8.0</td>
<td>*</td>
</tr>
<tr>
<td>Franco et al. (1998a)</td>
<td>Caucasians</td>
<td>51</td>
<td>37.3</td>
<td>52.9</td>
<td>9.8</td>
<td>*</td>
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<tr>
<td>Franco et al. (1998a)</td>
<td>Caucasians (France)</td>
<td>366</td>
<td>49.7</td>
<td>36.1</td>
<td>14.2</td>
<td>*</td>
</tr>
<tr>
<td>Kelenen et al. (2004)</td>
<td>Caucasians (Italy)</td>
<td>146</td>
<td>32.2</td>
<td>47.3</td>
<td>19.9</td>
<td>*</td>
</tr>
<tr>
<td>Lokochnov et al. (1999)</td>
<td>Caucasians (Canada)</td>
<td>260</td>
<td>42.6</td>
<td>46.0</td>
<td>11.4</td>
<td>*</td>
</tr>
<tr>
<td>McAndrew et al. (1996)</td>
<td>Caucasians (Cambridge in UK)</td>
<td>107</td>
<td>32.0</td>
<td>56.0</td>
<td>12.0</td>
<td>*</td>
</tr>
<tr>
<td>McAndrew et al. (1996)</td>
<td>Caucasians</td>
<td>101</td>
<td>49.0</td>
<td>43.0</td>
<td>9.0</td>
<td>*</td>
</tr>
<tr>
<td>Rady et al. (2002)</td>
<td>Caucasians</td>
<td>159</td>
<td>45.9</td>
<td>42.8</td>
<td>11.3</td>
<td>*</td>
</tr>
<tr>
<td>Scholtz et al. (2002)</td>
<td>Caucasians (South Africa)</td>
<td>76</td>
<td>39.0</td>
<td>50.0</td>
<td>11.0</td>
<td>*</td>
</tr>
<tr>
<td>Yang et al. (2008)</td>
<td>Non-Hispanic whites</td>
<td>2622</td>
<td>45.8</td>
<td>42.6</td>
<td>11.7</td>
<td>*</td>
</tr>
<tr>
<td>Kelenen et al. (2004)</td>
<td>Chinese from Canada</td>
<td>275</td>
<td>62.0</td>
<td>30.8</td>
<td>7.2</td>
<td>*</td>
</tr>
<tr>
<td>Janoskóvé et al. (2003)</td>
<td>Czech patients with CHD</td>
<td>278</td>
<td>44.2</td>
<td>42.1</td>
<td>13.7</td>
<td>*</td>
</tr>
<tr>
<td>Czech controls</td>
<td></td>
<td>591</td>
<td>41.4</td>
<td>48.6</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>Bathúm et al. (2007)</td>
<td>Danish (Twins)</td>
<td>1136</td>
<td>49.0</td>
<td>42.0</td>
<td>9.0</td>
<td>*</td>
</tr>
<tr>
<td>Husemøen et al. (2004)</td>
<td>Danish (Southern Copenhagen County)</td>
<td>2788</td>
<td>48.8</td>
<td>42.4</td>
<td>8.8</td>
<td>*</td>
</tr>
<tr>
<td>Conroy et al. (2000)</td>
<td>Hispanics (New York)</td>
<td>520</td>
<td>41.1</td>
<td>45.2</td>
<td>13.7</td>
<td>*</td>
</tr>
<tr>
<td>Rady et al. (2002)</td>
<td>Hispanics</td>
<td>96</td>
<td>30.2</td>
<td>43.8</td>
<td>26.0</td>
<td>*</td>
</tr>
<tr>
<td>Friedman et al. (1999)</td>
<td>Jews (Israeli Jews) (Jerusalem)</td>
<td>401</td>
<td>40.6</td>
<td>44.1</td>
<td>15.2</td>
<td>*</td>
</tr>
<tr>
<td>Rady et al. (2002)</td>
<td>Jews (Ashkenazi Jews)</td>
<td>155</td>
<td>31.0</td>
<td>42.6</td>
<td>26.4</td>
<td>*</td>
</tr>
<tr>
<td>Guéant-Rodriguez et al. (2006)</td>
<td>Mexicans</td>
<td>300</td>
<td>43.3</td>
<td>58.0</td>
<td>5.7</td>
<td>*</td>
</tr>
<tr>
<td>Mutchinick et al. (1999)</td>
<td>Mexicans (women)</td>
<td>250</td>
<td>17.6</td>
<td>47.6</td>
<td>34.8</td>
<td>*</td>
</tr>
<tr>
<td>Yang et al. (2008)</td>
<td>Mexican-Americans</td>
<td>2068</td>
<td>29.9</td>
<td>49.8</td>
<td>20.3</td>
<td>*</td>
</tr>
<tr>
<td>Hansen et al. (2001)</td>
<td>Mixed ancestry (Upper Midwestern region of the US)</td>
<td>772</td>
<td>42.0</td>
<td>47.2</td>
<td>10.9</td>
<td>*</td>
</tr>
<tr>
<td>CHD patients</td>
<td></td>
<td>137</td>
<td>42.3</td>
<td>46.0</td>
<td>11.7</td>
<td>*</td>
</tr>
<tr>
<td>Deep-vein thrombosis patients</td>
<td></td>
<td>329</td>
<td>39.5</td>
<td>48.0</td>
<td>12.5</td>
<td>*</td>
</tr>
</tbody>
</table>

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 (Lievers 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 (Födinger 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

<table>
<thead>
<tr>
<th>Citation</th>
<th>Ethnic group</th>
<th>Sample</th>
<th>% 1298AA</th>
<th>% 1298AC</th>
<th>% 1298CC</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjulla et al. (2003)</td>
<td>Africans (West Africa)</td>
<td>240</td>
<td>70.0</td>
<td>28.0</td>
<td>2.0</td>
<td>= *</td>
</tr>
<tr>
<td>Amouzou et al. (2004)</td>
<td>Africans (West Africa savannah)</td>
<td>68</td>
<td>73.5</td>
<td>22.1</td>
<td>4.4</td>
<td>= *</td>
</tr>
<tr>
<td></td>
<td>Africans (West Africa coast)</td>
<td>208</td>
<td>69.2</td>
<td>28.8</td>
<td>1.9</td>
<td>= *</td>
</tr>
<tr>
<td>Guéant-Rodriguez et al. (2006)</td>
<td>Africans (West Africa Benin and Togo)</td>
<td>465</td>
<td>84.2</td>
<td>13.9</td>
<td>1.9</td>
<td>= *</td>
</tr>
<tr>
<td>Scholtz et al. (2002)</td>
<td>Africans (South Africa)</td>
<td>60</td>
<td>87.0</td>
<td>8.0</td>
<td>5.0</td>
<td>≠  *</td>
</tr>
<tr>
<td>Rady et al. (2002)</td>
<td>African-Americans</td>
<td>97</td>
<td>71.1</td>
<td>26.8</td>
<td>2.1</td>
<td>≠  *</td>
</tr>
<tr>
<td>Yang et al. (2008)</td>
<td>Non-Hispanic blacks</td>
<td>2100</td>
<td>67.6</td>
<td>29.0</td>
<td>3.4</td>
<td>= *</td>
</tr>
<tr>
<td>Rady et al. (2002)</td>
<td>Ashkenazi Jews</td>
<td>149</td>
<td>53.7</td>
<td>38.3</td>
<td>8.0</td>
<td>≠  *</td>
</tr>
<tr>
<td>Guéant-Rodriguez et al. (2006)</td>
<td>Caucasians (France)</td>
<td>366</td>
<td>52.8</td>
<td>35.7</td>
<td>11.5</td>
<td>= *</td>
</tr>
<tr>
<td></td>
<td>Caucasians (Italy)</td>
<td>146</td>
<td>64.4</td>
<td>28.1</td>
<td>7.5</td>
<td>= *</td>
</tr>
<tr>
<td>Rady et al. (2002)</td>
<td>Caucasians</td>
<td>159</td>
<td>44.0</td>
<td>47.2</td>
<td>8.8</td>
<td>≠  *</td>
</tr>
<tr>
<td>Scholtz et al. (2002)</td>
<td>Caucasians (South Africa)</td>
<td>76</td>
<td>38.0</td>
<td>50.0</td>
<td>12.0</td>
<td>≠  *</td>
</tr>
<tr>
<td>Yang et al. (2008)</td>
<td>Non-Hispanic whites</td>
<td>2620</td>
<td>47.9</td>
<td>41.8</td>
<td>10.3</td>
<td>= *</td>
</tr>
<tr>
<td>Janoskova et al. (2003)</td>
<td>Czech Patients with CHD</td>
<td>278</td>
<td>51.4</td>
<td>38.9</td>
<td>9.7</td>
<td>≠  *</td>
</tr>
<tr>
<td></td>
<td>Czech Controls</td>
<td>391</td>
<td>44.7</td>
<td>45.3</td>
<td>10.0</td>
<td>= *</td>
</tr>
<tr>
<td>Rady et al. (2002)</td>
<td>Hispanics</td>
<td>96</td>
<td>68.7</td>
<td>27.1</td>
<td>4.2</td>
<td>≠  *</td>
</tr>
<tr>
<td>Guéant-Rodriguez et al. (2006)</td>
<td>Mexicans</td>
<td>300</td>
<td>83.0</td>
<td>14.7</td>
<td>2.3</td>
<td>= *</td>
</tr>
<tr>
<td>Yang et al. (2005)</td>
<td>Mexican-Americans</td>
<td>2064</td>
<td>65.7</td>
<td>30.8</td>
<td>3.5</td>
<td>= *</td>
</tr>
<tr>
<td>Gebhardt et al. (2001)</td>
<td>Mixed ancestry (coloured South African pregnant women)</td>
<td>168</td>
<td>54.8</td>
<td>36.9</td>
<td>8.3</td>
<td>= *</td>
</tr>
<tr>
<td>Hanson et al. (2001)</td>
<td>Mixed ancestry (Upper Midwestern region of the United States)</td>
<td>CHD patients</td>
<td>772</td>
<td>46.6</td>
<td>41.7</td>
<td>11.7</td>
</tr>
<tr>
<td></td>
<td>Deep-vein thrombosis patients</td>
<td>137</td>
<td>43.8</td>
<td>45.3</td>
<td>10.9</td>
<td>≠  *</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>329</td>
<td>49.9</td>
<td>42.2</td>
<td>7.9</td>
<td>= *</td>
</tr>
</tbody>
</table>

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 B_{6}-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 carrihership 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

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

<table>
<thead>
<tr>
<th>Studies that investigated T833C</th>
<th>Ethnic group</th>
<th>Sample</th>
<th>% 833TT</th>
<th>% 833TC</th>
<th>% 833CC</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilley et al. (2001)</td>
<td>African-Americans</td>
<td>107</td>
<td>60</td>
<td>34</td>
<td>5</td>
<td>= #</td>
</tr>
<tr>
<td></td>
<td>Myocardial infarction cases</td>
<td>172</td>
<td>63</td>
<td>32</td>
<td>5</td>
<td>= #</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Janosiková et al. (2003)</td>
<td>Nationality reported</td>
<td>278</td>
<td>99.64</td>
<td>0.36</td>
<td>0</td>
<td>= *</td>
</tr>
<tr>
<td></td>
<td>Czech patients with coronary heart disease</td>
<td>591</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>= *</td>
</tr>
<tr>
<td></td>
<td>Czech controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tsai et al. (1999b)</td>
<td>Mixed ancestry (upper Midwestern region of the United States)</td>
<td>376</td>
<td>99.5</td>
<td>0.5</td>
<td>0</td>
<td>= #</td>
</tr>
<tr>
<td></td>
<td>Coronary heart disease</td>
<td>82</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>Cannot calculate</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Studies that investigated 844ins68</th>
<th>Ethnic group</th>
<th>% 844ins68 wild-type</th>
<th>% 844ins68 heterozygous</th>
<th>% 844ins68 homozygous insert</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yang et al. (2008)</td>
<td>Non-Hispanic blacks</td>
<td>2032</td>
<td>55</td>
<td>38.2</td>
<td>6.7</td>
</tr>
<tr>
<td>Giusti et al. (1999)</td>
<td>Caucasians (Italy) with coronary heart disease</td>
<td>133</td>
<td>80.45</td>
<td>18.8</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>Caucasians (Italy) controls</td>
<td>595</td>
<td>85.55</td>
<td>13.61</td>
<td>0.84</td>
</tr>
<tr>
<td>Yang et al. (2008)</td>
<td>Non-Hispanic whites</td>
<td>2566</td>
<td>84.2</td>
<td>15.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Janosiková et al. (2003)</td>
<td>Nationality reported</td>
<td>278</td>
<td>91</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Czech patients with coronary heart disease</td>
<td>591</td>
<td>84.77</td>
<td>14.89</td>
<td>0.34</td>
</tr>
<tr>
<td>Kluinmans et al. (1997)</td>
<td>Caucasians</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Dutch vascular disease patients</td>
<td>60</td>
<td>83.3</td>
<td>16.7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Dutch controls</td>
<td>107</td>
<td>83.2</td>
<td>14.0</td>
<td>2.8</td>
</tr>
<tr>
<td>Yang et al. (2008)</td>
<td>Mexican-Americans</td>
<td>2036</td>
<td>87.7</td>
<td>11.9</td>
<td>0.4</td>
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<tr>
<td>Tsai et al. (1996)</td>
<td>Mixed ancestry (healthy controls)</td>
<td>77</td>
<td>88.3</td>
<td>11.7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mixed ancestry (coronary heart disease patients)</td>
<td>182</td>
<td>83.5</td>
<td>16</td>
<td>0.5</td>
</tr>
<tr>
<td>Sirachainan et al. (2008)</td>
<td>Thai children with ischaemic stroke</td>
<td>29</td>
<td>97</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Healthy Thai children</td>
<td>96</td>
<td>98</td>
<td>1</td>
<td>1</td>
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</tbody>
</table>
### 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).

<table>
<thead>
<tr>
<th>Studies that investigated T333C and 844ins68 SNP</th>
<th>Ethnic group</th>
<th>C/Tins68</th>
<th>% 832901 W/O 844ins68</th>
<th>% 83310C with 844ins68</th>
<th>% 8331CC with 844ins68</th>
<th>8331CC W/O ins</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Franco et al. (1998b)</td>
<td>Amerindians (Brazilian Amazon tribes)</td>
<td>110</td>
<td>99.1</td>
<td>0.9</td>
<td>0</td>
<td>0</td>
<td>= *</td>
</tr>
<tr>
<td>Franco et al. (1998b)</td>
<td>Blacks (50 Brazilian blacks, 101 Africans from Zaire, Cameroon and Angola)</td>
<td>151</td>
<td>58.3</td>
<td>37.7</td>
<td>4</td>
<td>0</td>
<td>= *</td>
</tr>
<tr>
<td>Franco et al. (1998b)</td>
<td>Whites (64 Brazilians, 41 Caucasians from Portugal)</td>
<td>104</td>
<td>86.5</td>
<td>13.5</td>
<td>0</td>
<td>0</td>
<td>= *</td>
</tr>
<tr>
<td>Giusti et al. (1997)</td>
<td>Caucasians (Northern region of Italy)</td>
<td>62</td>
<td>74.2</td>
<td>25.8</td>
<td>0</td>
<td>0</td>
<td>= #</td>
</tr>
<tr>
<td>Giusti et al. (1997)</td>
<td>Caucasians (Southern region of Italy)</td>
<td>50</td>
<td>92</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>= #</td>
</tr>
<tr>
<td>Griffioen et al. (2005)</td>
<td>Caucasians (Dutch)</td>
<td>500</td>
<td>89.6</td>
<td>9.0</td>
<td>1.2</td>
<td>0.2</td>
<td>= *</td>
</tr>
<tr>
<td>Franco et al. (1998b)</td>
<td>Japanese</td>
<td>40</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>= *</td>
</tr>
</tbody>
</table>

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
4.3.3.1 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 dependent 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 B_{12} 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 B_{12}, 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 B_{12} 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 B_{12} concentrations of the subjects harbouring the GG genotype were a chance observation, then vitamin B_{12} status could have confounded the association between the GG genotype and CHD risk, provided that low B_{12} status is itself a CHD risk factor. However, an inverse association between vitamin B_{12} 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.

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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

<table>
<thead>
<tr>
<th>Citation</th>
<th>Ethnic group</th>
<th>Sample</th>
<th>% 2756 AA</th>
<th>% 2756 AG</th>
<th>% 2756 GG</th>
<th>% 2756 HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjalla et al. (2003)</td>
<td>Africans (West Africa)</td>
<td>240</td>
<td>51.7</td>
<td>42.5</td>
<td>5.80</td>
<td>=*</td>
</tr>
<tr>
<td>Amouzou et al. (2004)</td>
<td>Africans (West Africa savanna)</td>
<td>68</td>
<td>60.2</td>
<td>30.8</td>
<td>8.80</td>
<td>*=</td>
</tr>
<tr>
<td></td>
<td>Africans (West Africa coast)</td>
<td>208</td>
<td>48.6</td>
<td>46.6</td>
<td>4.81</td>
<td>*=</td>
</tr>
<tr>
<td>Conroy et al. (2000)</td>
<td>African-Americans (New York)</td>
<td>528</td>
<td>58.7</td>
<td>35</td>
<td>6.3</td>
<td>=#</td>
</tr>
<tr>
<td>Chen et al. (2001b)</td>
<td>Caucasian-Americans (Men)</td>
<td>387</td>
<td>67.7</td>
<td>30.2</td>
<td>2.1</td>
<td>=#</td>
</tr>
<tr>
<td></td>
<td>Myocardial infarction patients</td>
<td>767</td>
<td>67.1</td>
<td>29.2</td>
<td>3.7</td>
<td>=#</td>
</tr>
<tr>
<td>Conroy et al. (2000)</td>
<td>Caucasian-Americans (Men)</td>
<td>503</td>
<td>65</td>
<td>30.2</td>
<td>4.8</td>
<td>=#</td>
</tr>
<tr>
<td>Klerk et al. (2003)</td>
<td>Caucasians (Netherland)</td>
<td>413</td>
<td>65.9</td>
<td>28.3</td>
<td>6.3</td>
<td>=*</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>540</td>
<td>69.6</td>
<td>28.2</td>
<td>2.2</td>
<td>=*</td>
</tr>
<tr>
<td>Ma et al. (1999)</td>
<td>Caucasian (American)</td>
<td>712</td>
<td>69.7</td>
<td>27.5</td>
<td>2.8</td>
<td>=*</td>
</tr>
<tr>
<td>Van der Put et al. (1997)</td>
<td>Caucasians (Netherlands)</td>
<td>597</td>
<td>70.5</td>
<td>26.5</td>
<td>3.0</td>
<td>=*</td>
</tr>
<tr>
<td>Wang et al. (1998)</td>
<td>Caucasians (Australia)</td>
<td>745</td>
<td>61.9</td>
<td>33.3</td>
<td>4.3</td>
<td>=*</td>
</tr>
<tr>
<td>Badium et al. (2007)</td>
<td>Danish (Adult twins)</td>
<td>1126</td>
<td>67</td>
<td>29</td>
<td>4</td>
<td>=*</td>
</tr>
<tr>
<td>Conroy et al. (2000)</td>
<td>Hispanics (New York)</td>
<td>521</td>
<td>65.5</td>
<td>31.5</td>
<td>3</td>
<td>=#</td>
</tr>
<tr>
<td>Hyndman et al. (2000)</td>
<td>Ethnic group not reported</td>
<td>109</td>
<td>69.72</td>
<td>29.36</td>
<td>0.92</td>
<td>=#</td>
</tr>
<tr>
<td>Jacques et al. (2003)</td>
<td>Ethnic group not reported (Pramingham and Utah)</td>
<td>677</td>
<td>65.9</td>
<td>29.1</td>
<td>3.99</td>
<td>=#</td>
</tr>
<tr>
<td>Janosiková et al. (2003)</td>
<td>Czech CHD patients</td>
<td>278</td>
<td>65.83</td>
<td>29.5</td>
<td>4.67</td>
<td>=*</td>
</tr>
<tr>
<td></td>
<td>Czech controls</td>
<td>591</td>
<td>63.3</td>
<td>30.8</td>
<td>3.9</td>
<td>=*</td>
</tr>
<tr>
<td>Morita et al. (1999)</td>
<td>Japanese (Tokyo)</td>
<td>215</td>
<td>67.4</td>
<td>31.2</td>
<td>1.4</td>
<td>=*</td>
</tr>
<tr>
<td></td>
<td>CHD patients</td>
<td>251</td>
<td>64</td>
<td>33</td>
<td>3</td>
<td>=*</td>
</tr>
<tr>
<td></td>
<td>Ischaemic stroke patients</td>
<td>257</td>
<td>67</td>
<td>32</td>
<td>2</td>
<td>=*</td>
</tr>
</tbody>
</table>

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 (Lievers 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. 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 B\textsubscript{12} to cells, therefore, some of the genetic variation \textit{[i.e. L23V, G94 serine (S), proline (P) 259 arginine (R), S348 phenylalanine (F) and R399 glutamine (Q)]} in the TC gene may affect intracellular vitamin B\textsubscript{12} availability and, consequently, tHcy concentrations (Lievers \textit{et al.}, 2002b). However Lievers \textit{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 \textit{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 \textit{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 \textit{et al.} (2000) associated the variation with higher Hcy, but Lievers \textit{et al.} (2002a) observed lower tHcy concentrations in individuals with the 1561 TT genotype.

\textbf{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 \textit{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)]

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Genetic alteration</th>
<th>Amino acid change</th>
<th>Phenotype of variant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR</td>
<td>C677T</td>
<td>A222V</td>
<td>Thermolabile enzyme</td>
<td>Frosst et al. (1995) and Rozen (2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reduced enzyme activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower B-vitamin status</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Higher plasma Hcy</td>
<td></td>
</tr>
<tr>
<td>MTHFR</td>
<td>A1298C</td>
<td>E429A</td>
<td>No changes</td>
<td>Van der Put et al. (1998) and Weisberg et al. (1998)</td>
</tr>
<tr>
<td>CBS</td>
<td>T833C</td>
<td>I278T</td>
<td>Reduced enzyme activity</td>
<td>Sebastio et al. (1995) and Sperandeo et al. (1996)</td>
</tr>
<tr>
<td>CBS</td>
<td>844ins68</td>
<td>Frameshift</td>
<td>No change in basal Hcy</td>
<td>Tsai et al. (1999a) and De Stefano et al. (1998)</td>
</tr>
<tr>
<td>CZ</td>
<td>G9276A</td>
<td>Intronic region</td>
<td>No change in basal Hcy</td>
<td>Griffioen et al. (2005)</td>
</tr>
<tr>
<td>MS</td>
<td>A2756G</td>
<td>D919G</td>
<td>Higher plasma folate</td>
<td>Harmon et al. (1999) and Morita et al. (1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Higher red cell folate</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower plasma Hcy</td>
<td></td>
</tr>
</tbody>
</table>

$A =$ adenine (nucleotide); $A =$ alanine (amino acid); $C =$ cytosine (nucleotide); $CBS =$ cystathionine $\beta$-synthase; $D =$ aspartic (amino acid); $E =$ glutamate (amino acid); $G =$ guanine (nucleotide); $G =$ glycine (amino acid); $Hcy =$ homocysteine; $I =$ isoleucine (amino acid); $ins =$ insertion; $MS =$ methionine synthase; $MTHFR =$ methyltetrahydrofolate reductase; $T =$ thymine (nucleotide); $T =$ threonine (amino acid); $V =$ 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).

4.5 REFERENCES


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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: Hey, 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 ≤ 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 (Castañón 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 (Batham 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 (Chuive 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


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 & Möller, 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 Konelab™ 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 μkat/L (80 U/L) and 0.83 μkat/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 Hinfl 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/m²).

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 *FoodFinders®* program (Medical Research Council, Tygerberg, 2007) by two dieticians 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-value ≤ 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 ($\chi^2$) 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; ≥ 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 $\geq 30$ 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

<table>
<thead>
<tr>
<th></th>
<th>Whole 677CC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men</td>
</tr>
<tr>
<td>Number of subjects</td>
<td>1533/84%</td>
</tr>
<tr>
<td>Age (y)</td>
<td>49.4±10</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.6±6.93</td>
</tr>
<tr>
<td>fHcy (μmol/L)</td>
<td>10.1±4.41</td>
</tr>
<tr>
<td>Dietary folate (μg)</td>
<td>382±198</td>
</tr>
<tr>
<td>Dietary vitamin B1 (mg)</td>
<td>1.65±0.84</td>
</tr>
<tr>
<td>Dietary vitamin B2 (mg)</td>
<td>1.22±0.8</td>
</tr>
<tr>
<td>Dietary niacin (mg)</td>
<td>14.2±7.64</td>
</tr>
<tr>
<td>Dietary pantothenic acid (mg)</td>
<td>3.78±2.25</td>
</tr>
<tr>
<td>Dietary vitamin B3 (ng)</td>
<td>1.45±0.82</td>
</tr>
<tr>
<td>Dietary vitamin B12 (μg)</td>
<td>35.2±24.1</td>
</tr>
<tr>
<td>Methionine (g/d)</td>
<td>0.85±0.53</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>8.3±12.0</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>107±105</td>
</tr>
<tr>
<td>Alcohol intake (g/day)</td>
<td>11.5±22.7</td>
</tr>
<tr>
<td>%CDT</td>
<td>1.57±2.82</td>
</tr>
<tr>
<td>Smoking (n%)</td>
<td>1.17±0.37</td>
</tr>
<tr>
<td>Unknown</td>
<td>5/0.46%</td>
</tr>
<tr>
<td>Former smoker</td>
<td>45/4.14%</td>
</tr>
<tr>
<td>Current smoker</td>
<td>573/52.7%</td>
</tr>
<tr>
<td>Never smoked</td>
<td>465/42.7%</td>
</tr>
<tr>
<td>Caffeine intake (mg)</td>
<td>0.05±0.66</td>
</tr>
<tr>
<td>HIV status (n)</td>
<td>1552</td>
</tr>
</tbody>
</table>

| Whole 677CT          | 677CT       | Whole 677TT |
|----------------------|-------------|
|                     | Men         | Women       |
| Number of subjects  | 280/15.3%   | 109/6%      |
| Age (y)             | 24.1±6.7    | 20.8±4.47   |
| BMI (kg/m²)         | 9.48±4.22   | 11.1±4.74   |
| fHcy (μmol/L)       | 382±198     | 573/31%     |
| Dietary folate (μg)| 26.9±7.29   | 24.1±6.7    |
| Dietary vitamin B1 (mg)| 1.22±0.8  | 1.12±0.77   |
| Dietary vitamin B2 (mg)| 14.2±7.64 | 13.2±6.96   |
| Dietary niacin (mg) | 3.78±2.25   | 4.11±2.45   |
| Dietary pantothenic acid (mg)| 1.45±0.82 | 1.6±0.87    |
| Dietary vitamin B3 (ng)| 35.2±24.1 | 38.7±26.5   |
| Methionine (g/d)    | 0.85±0.53   | 0.92±0.57   |
| hsCRP (mg/L)        | 8.3±12.0    | 8.23±13.2   |
| Creatinine (μmol/L)| 107±105     | 111±110     |
| Alcohol intake (g/day)| 11.5±22.7 | 18.4±27.1   |
| %CDT                | 1.57±2.82   | 2.0±2.73    |
| Smoking (n%)        | 1.17±0.37   | 0.91±0.75   |
| Unknown             | 5/0.46%     | 3/0.72%     |
| Former smoker       | 45/4.14%    | 29/77%      |
| Current smoker      | 573/52.7%   | 246/93.9%   |
| Never smoked        | 465/42.7%   | 137/33%     |
| Caffeine intake (mg)| 0.05±0.66   | 0.02±0.29   |
| HIV status (n)      | 1552        | 572         |

<table>
<thead>
<tr>
<th>Whole 677TT</th>
<th>677TT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men</td>
</tr>
<tr>
<td>Number of subjects</td>
<td>171/9.3%</td>
</tr>
<tr>
<td>Age (y)</td>
<td>24.1±6.7</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>9.48±4.22</td>
</tr>
<tr>
<td>fHcy (μmol/L)</td>
<td>382±198</td>
</tr>
<tr>
<td>Dietary folate (μg)</td>
<td>26.9±7.29</td>
</tr>
<tr>
<td>Dietary vitamin B1 (mg)</td>
<td>1.22±0.8</td>
</tr>
<tr>
<td>Dietary vitamin B2 (mg)</td>
<td>14.2±7.64</td>
</tr>
<tr>
<td>Dietary niacin (mg)</td>
<td>3.78±2.25</td>
</tr>
<tr>
<td>Dietary pantothenic acid (mg)</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>hsCRP (mg/L)</td>
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</tr>
<tr>
<td>Smoking (n%)</td>
<td>1.17±0.37</td>
</tr>
<tr>
<td>Unknown</td>
<td>5/0.46%</td>
</tr>
<tr>
<td>Former smoker</td>
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</tr>
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<td>Caffeine intake (mg)</td>
<td>0.05±0.66</td>
</tr>
<tr>
<td>HIV status (n)</td>
<td>1552</td>
</tr>
</tbody>
</table>

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 fHcy was 0.03, 0.02 and <0.01, respectively. §Post hoc tests indicated the significant differences in fHcy 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 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; fHcy = 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 ≤ 0.01) higher.
than that of subjects in subdivision 0 (abstainers) as well as between subdivision 3 and subdivision 2 (subjects who consumed ≥ 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; Gueant-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 (Gueant-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 μmol/L. Earlier studies indicated that the normal reference range for plasma tHcy is between 5 to 15 μmol/L, mild to moderate HHcy between 16 to 100 μmol/L and severe HHcy > 100 μmol/L (Eikelboom et al., 1999; Malinow et al., 1999), however, in 2007, Castañon et al. proposed that tHcy concentrations above 12 μmol/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
habilitating 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; Hidiroglou 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.

5.6 ACKNOWLEDGEMENTS

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   Corneliie Nienaber was responsible for the DNA extractions, all the genotypings including independent interpretation of the agarose gel photos, data interpretation
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Pedro T Pisa was responsible for the laboratory analysis of biological markers of alcohol consumption. He is a post-doctoral fellow at the NWU.

Christine S Venter was involved in the interpretation of the results and provided guidance regarding the writing up of the data. She is an employee of the NWU.

Suria M Ellis was responsible for all the statistical analyses and assisted in the interpretation thereof. She is an employee of the NWU.

Annamarie Kruger planned and coordinated the PURE-study (design, approval of final protocol and data collection). She is an employee of the NWU.

Tiny Hockstra was involved in DNA extractions and laboratory training. At the time the research was conducted she was a post-doctoral fellow at the NWU.

Sarah J Moss was involved in the planning phase of the project, in securing funding and preparation of the samples for Hcy analysis. She is an employee of the NWU.

Alida Melse-Boonstra was involved in the planning phase of the project, in securing funding, and the preparation of samples for Hcy analysis. At the time the research was conducted she was a post-doctoral fellow at the NWU.

G Wayne Towers assisted with troubleshooting of laboratory analyses, performed quality control of the laboratory analyses and gave guidance in the writing of the manuscript. He is an employee of the NWU.

5.7 REFERENCES


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), cystathionine-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 μmol/L; p < 0.05) than subjects harbouring the MTHFR 677 CT/CC and the MTR 2756 AG (10.5, 9.7 and 9.5 μmol/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
CHAPTER 6

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 Pareesha 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 (\(\gamma\))-glutamyl transferase (GGT) concentrations a Sequential Multiple Analyser Computer (SMAC) analysis was undertaken, using the Konelab™ 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 immunocassay 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 MgCl\(_2\), 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 μl. 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

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Annealing temperature</th>
<th>Restriction enzyme</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-TGA AGG AGA AGG TGT CTG CGG GA-3' [6]</td>
<td>5'-AGG ACG GTG CGG TGA GAG TG-3' [6]</td>
<td>55.0°C</td>
<td>HinfI produces 176 and 22 bp fragments from the 198 bp PCR fragment in the presence of the variation</td>
<td></td>
</tr>
<tr>
<td>5'-GAA CAT CCC AAG CCC ACT GAG-3' (own design)</td>
<td>5'-GAC ACT GAA GAC TCT TGT TTT GAA C-3' [20]</td>
<td>58.0°C</td>
<td>BsuRI (HaeIII) produces 260 and 180 bp fragments from the 440 bp PCR fragment in the presence of the variation</td>
<td></td>
</tr>
<tr>
<td>5'-ATA GAA TAT CGA GGC ATG TCC AGG CG-3' (13)</td>
<td>5'-TGG GGC CCA GGG TCA GCC AGG CTC C-3'(13)</td>
<td>66.5°C</td>
<td>BseNI (BsrI)</td>
<td></td>
</tr>
<tr>
<td>Fragment lengths taken from Griffioen et al. [13]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Calculated fragments**

<table>
<thead>
<tr>
<th>PCR product</th>
<th>Non-insert allele</th>
<th>Insert allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>T833C wild-type</td>
<td>282 bp</td>
<td>350 bp</td>
</tr>
<tr>
<td>T833C mutant type</td>
<td>257,25 bp</td>
<td>325,25 bp *</td>
</tr>
<tr>
<td>G9276A mutant type</td>
<td>180,77,25 bp</td>
<td>248,77,25 bp</td>
</tr>
<tr>
<td>T833C and G9276A mutant type</td>
<td>225,32,25 bp</td>
<td>293,32,25 bp *</td>
</tr>
</tbody>
</table>

* 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

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

**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

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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/m²) 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 FoodFinders® 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 ($\chi^2$) 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

<table>
<thead>
<tr>
<th></th>
<th>Total group</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects (n)</td>
<td>1825</td>
<td>684</td>
<td>1141</td>
</tr>
<tr>
<td>Age (y)</td>
<td>49.3 ± 10.5</td>
<td>49.9 ± 10.4</td>
<td>48.9 ± 10.3</td>
</tr>
<tr>
<td>Rural (n/%)</td>
<td>945</td>
<td>329/34.8%</td>
<td>616/56.2%</td>
</tr>
<tr>
<td>Urban (n/%)</td>
<td>880</td>
<td>355/40.3%</td>
<td>525/59.7%</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.5 ± 6.92</td>
<td>20.8 ± 4.04</td>
<td>26.8 ± 7.28</td>
</tr>
<tr>
<td>tHcy (μmol/L)</td>
<td>10.3 ± 4.6</td>
<td>11.3 ± 4.51</td>
<td>9.74 ± 4.58</td>
</tr>
<tr>
<td>Dietary folate (μg)</td>
<td>383 ± 196</td>
<td>428 ± 220</td>
<td>356 ± 174</td>
</tr>
<tr>
<td>Dietary vitamin B₆ (mg)</td>
<td>1.66 ± 0.83</td>
<td>1.87 ± 0.95</td>
<td>1.53 ± 0.73</td>
</tr>
<tr>
<td>Dietary vitamin B₂ (mg)</td>
<td>1.22 ± 0.8</td>
<td>1.39 ± 0.83</td>
<td>1.13 ± 0.76</td>
</tr>
<tr>
<td>Dietary niacin (mg)</td>
<td>14.3 ± 7.8</td>
<td>16.2 ± 8.68</td>
<td>13.2 ± 7.4</td>
</tr>
<tr>
<td>Dietary pantothenic acid (mg)</td>
<td>3.79 ± 2.25</td>
<td>4.13 ± 2.48</td>
<td>3.58 ± 2.08</td>
</tr>
<tr>
<td>Dietary vitamin B₆ (mg)</td>
<td>1.46 ± 0.81</td>
<td>1.61 ± 0.88</td>
<td>1.37 ± 0.76</td>
</tr>
<tr>
<td>Dietary vitamin B₁ (µg)</td>
<td>35.2 ± 23.8</td>
<td>36.7 ± 26.4</td>
<td>33 ± 21.8</td>
</tr>
<tr>
<td>Dietary vitamin B₁₂ (µg)</td>
<td>4.2 ± 5.14</td>
<td>4.46 ± 5.21</td>
<td>4.05 ± 5.1</td>
</tr>
<tr>
<td>Dietary methionine (g)</td>
<td>0.85 ± 0.53</td>
<td>0.93 ± 0.6</td>
<td>0.81 ± 0.5</td>
</tr>
<tr>
<td>hs-CRP (mg/L)</td>
<td>8.5 ± 12.4</td>
<td>8.22 ± 13.3</td>
<td>8.67 ± 11.9</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>106 ± 104</td>
<td>109 ± 108</td>
<td>104 ± 101</td>
</tr>
<tr>
<td>Alcohol intake (g/day)</td>
<td>12 ± 23.6</td>
<td>19 ± 27.8</td>
<td>7.68 ± 19.5</td>
</tr>
<tr>
<td>abstainers : drinkers (% : %)</td>
<td>55% : 45%</td>
<td>36% : 64%*</td>
<td>67% : 33%*</td>
</tr>
<tr>
<td>GGT</td>
<td>92.7 ± 163</td>
<td>116 ± 157</td>
<td>79 ± 165</td>
</tr>
<tr>
<td>%CDT</td>
<td>2.95 ± 1.41</td>
<td>3.5 ± 1.66</td>
<td>2.63 ± 1.11</td>
</tr>
</tbody>
</table>

Data is reported as means ± standard deviation (SD), or number and frequency. *Significant difference between men and women. ¹Significant 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 ≤ 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

<table>
<thead>
<tr>
<th></th>
<th>tHcy (μmol/L)</th>
<th>tHcy (μmol/L)</th>
<th>tHcy (μmol/L)</th>
<th>tHcy (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total group</td>
<td>n/%</td>
<td>Men</td>
<td>n</td>
</tr>
<tr>
<td>Rural</td>
<td>10.4* (10.2; 10.7)</td>
<td>945</td>
<td>10.5* (10.5; 11.3)</td>
<td>319</td>
</tr>
<tr>
<td>Urban</td>
<td>9.09* (8.88; 9.31)</td>
<td>880</td>
<td>9.64* (9.31; 9.98)</td>
<td>345</td>
</tr>
<tr>
<td>Abstainers</td>
<td>9.69 (9.44; 9.96)</td>
<td>974</td>
<td>10.3* (9.83; 10.1)</td>
<td>233</td>
</tr>
<tr>
<td>Alcohol drinkers</td>
<td>9.76 (9.51; 10)</td>
<td>796</td>
<td>10.5* (10.1; 10.8)</td>
<td>411</td>
</tr>
<tr>
<td>Tobacco</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Former smoker</td>
<td>8.79 (7.94; 9.73)</td>
<td>50</td>
<td>9.38 (8.42; 10.5)</td>
<td>332</td>
</tr>
<tr>
<td>Current smoker</td>
<td>9.84 (9.58; 10.1)</td>
<td>705</td>
<td>10.5* (10.1; 10.9)</td>
<td>30</td>
</tr>
<tr>
<td>Never smoked</td>
<td>10.0 (9.68; 10.3)</td>
<td>542</td>
<td>10.3* (9.71; 10.8)</td>
<td>158</td>
</tr>
<tr>
<td>HIV status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV negative</td>
<td>9.64* (9.46; 9.82)</td>
<td>1514</td>
<td>10.5* (10.2; 10.8)</td>
<td>575</td>
</tr>
<tr>
<td>HIV positive</td>
<td>9.03* (8.66; 9.41)</td>
<td>303</td>
<td>9.11* (8.53; 9.72)</td>
<td>106</td>
</tr>
<tr>
<td>MTHFR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>9.74* (9.56; 9.91)</td>
<td>1579/84%</td>
<td>10.2* (9.9; 10.5)</td>
<td>573</td>
</tr>
<tr>
<td>CT</td>
<td>10.5* (10; 10.9)</td>
<td>286/15.2%</td>
<td>10.5 (9.79; 11.2)</td>
<td>109</td>
</tr>
<tr>
<td>TT</td>
<td>16.6* (12.9; 21.5)</td>
<td>15/0.8%</td>
<td>22.0 (8.28; 58.7)</td>
<td>2</td>
</tr>
<tr>
<td>MTR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>10.1* (9.86; 10.3)</td>
<td>1194/63.7%</td>
<td>10.4* (10.1; 10.8)</td>
<td>439</td>
</tr>
<tr>
<td>A2756G</td>
<td>9.5* (9.22; 9.79)</td>
<td>590/31.5%</td>
<td>9.95* (9.51; 10.4)</td>
<td>203</td>
</tr>
<tr>
<td>GG</td>
<td>9.5 (8.79; 10.2)</td>
<td>894/4.8%</td>
<td>9.83 (8.74; 11)</td>
<td>35</td>
</tr>
<tr>
<td>CBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>9.91 (9.69; 10.1)</td>
<td>997/53%</td>
<td>10.5* (10.1; 10.9)</td>
<td>356</td>
</tr>
<tr>
<td>CC</td>
<td>9.65 (9.07; 10.2)</td>
<td>138/7.3%</td>
<td>10.2 (9.32; 11.1)</td>
<td>47</td>
</tr>
<tr>
<td>CBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygous non-insert (WT)</td>
<td>9.91 (9.69; 10.1)</td>
<td>998/53%</td>
<td>10.5* (10.1; 10.9)</td>
<td>356</td>
</tr>
<tr>
<td>844ins68</td>
<td>9.64 (9.39; 9.89)</td>
<td>748/29.8%</td>
<td>10* (9.6; 10.4)</td>
<td>273</td>
</tr>
<tr>
<td>Heterozygous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygous insert (MT)</td>
<td>9.66 (9.08; 10.3)</td>
<td>1367/2.2%</td>
<td>10.2 (9.33; 11.2)</td>
<td>46</td>
</tr>
<tr>
<td>CBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>9.85 (9.63; 10.1)</td>
<td>977/31.9%</td>
<td>10.3* (9.92; 10.6)</td>
<td>373</td>
</tr>
<tr>
<td>G9276A</td>
<td>9.71 (9.45; 9.97)</td>
<td>757/40.3%</td>
<td>10.3 (9.86; 10.8)</td>
<td>245</td>
</tr>
<tr>
<td>AA</td>
<td>9.4 (8.88; 9.96)</td>
<td>1467/8.8%</td>
<td>9.78 (8.94; 10.7)</td>
<td>62</td>
</tr>
</tbody>
</table>

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 μmol/L) than urban subjects (9.09 μmol/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 μmol/L) than the HIV-infected subjects (9.03 μmol/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 [5,35].

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 dependent 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.

6.7 ACKNOWLEDGEMENTS

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6.8 REFERENCES


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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 (Chiave 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.
CHAPTER 7

PUBLIC HEALTH PROBLEM

↑ Morbidity and mortality (Chapter 1)

↑ CVD in black South Africans in transition (Chopra et al., 2002; Tibazarwa et al., 2009; Vorster, 2002) (Chapter 1)

↑ CVD

Castañón et al., 2007; Humphrey et al., 2008

Mechanisms (Chapter 2)

High Hey

MODIFIABLE DETERMINANTS

(reviewed in Chapter 3)

Dietary determinants

↓ Riboflavin

(Jacques et al., 2001; Powers, 2003)

↓ Vitamin B₆

(McKinley et al., 2001; Verhoef & De Groot, 2005)

↓ Vitamin B₁₂

(Clarke & Armitage, 2000)

↓ Folate/folic acid

(Clarke & Armitage, 2000)

↑ Alcohol intake

(Koehler et al., 2001) (Chapter 5)

↑ Coffee and tea

(Verhoef & De Groot, 2005)

Lifestyle determinants

Cigarette smoker

(Adachi et al., 2002; Bamonti et al., 2007)

Sedentary

(Nygård et al., 1995)

Rural living individuals

(Kim et al., 2003; Tovar et al., 2003) (Chapter 6)

BIological determinants

♂ gender

(Andersson et al., 1992; Brattström et al., 1994)

♀ gender

Significant Hcy difference between ♂ and ♀ harbouring the CC genotype (Chapter 5)

↑ Age

(Andersson et al., 1992; Frick et al., 2004)

Age-gender interaction (Jacques et al., 1999)

ACQUIRED DETERMINANTS

HIV – (uninfected)

(Chapter 6)

No association between HIV status and Hcy (Raiszadeh et al., 2009)

↓ Renal function

(McDonald et al., 2005)

COMMON GENETIC DETERMINANTS (reviewed in Chapter 4)

MTHFR C677T*#

(Chapter 5 & 6)

CT and TT are associated with ↑ Hcy when compared to the CC genotype (Adjalla et al., 2003; Frosst et al., 1995)

TT is associated with ↑ Hcy when compared to the CT and CC genotypes in black South Africans (Chapter 5 & 6)

T alleles influence on tHcy is stronger in the presence of impaired folate or vitamin B₁₂ status (Amouzou et al., 2004; Hanson et al., 2001; Jacques et al., 1996)

T allele presence together with alcohol intake ↑ Hcy (Chuive et al., 2005)

There is no significant interaction between alcohol intake and the CC or the CT genotype (Chapter 5)

MTR A2756G

(Chapter 6)

Hcy ↓ in an additive fashion dependent on the number of G alleles present (Harmon et al., 1999; Silaste et al., 2001; Tsai et al., 1999b)

The AA is associated with ↑ tHcy than subjects harbouring the MTR 2756 AG genotype (Chapter 6)

No association between this genetic variation and Hcy (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)

CBS T833C/844ins68*

(De Stefano et al., 1998; Tsai et al., 1996; Tsai et al., 1999a)

(Chapter 6)

CBS G9276A#

(Griffioen et al., 2005)

Association with Hcy was not previously reported

No association with Hey (Chapter 6) - GG

No association with Hey (Chapter 6) - GA

No association with Hey (Chapter 6) - AA

Low-normal Hey

↑ Riboflavin

↓ Vitamin B₆

↓ Vitamin B₁₂

↓ Folate/folic acid

↓ Abstainers

↓ Coffee and tea

Non-smoker

↑ Physical activity levels

Urban living individuals

HIV + (infected)

Renal function

HIV – (uninfected)

No association between HIV status and Hcy (Raiszadeh et al., 2009)

↓ Renal function

No association between HIV status and Hcy (Chapter 6)

No association between this genetic variation and Hcy (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)

No association with Hey (Chapter 6) - GG

No association with Hey (Chapter 6) - GA

No association with Hey (Chapter 6) - AA
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 = methylenetetrahydrofolate 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


CHAPTER 7


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 ..............................................................
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 Tlakgameng were chosen for the rural and semi-rural areas because they are still under tribal law with a good infrastructure and stability. We also spoke to Chief M. Lethlhogile 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, Tlakgameng, 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(H)
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 HbA1C, 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. ..............................................................................

(Signature of the subject)
Signed at ... Potchefstroom / Ganyesa ... (delete not applicable option) on ........./........./ 2005
PART 1

1. School/Iнстitute:
   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 EEG 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)
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 ANAMARIE 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 #

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.

<table>
<thead>
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<th>DESCRIPTION</th>
<th>AMOUNT</th>
<th>TIMES EATEN</th>
<th>CODE</th>
<th>AMOUNT/DAY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Per day</td>
<td>Per week</td>
<td>Per month</td>
<td>Seldom/Never</td>
</tr>
<tr>
<td>PORRIDGE AND BREAKFAST CEREALS AND OTHER STARCH</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Maize-meal porridge</td>
<td>Stiff (pap)</td>
<td></td>
<td></td>
<td></td>
<td>3400</td>
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<tr>
<td>Maize-meal porridge</td>
<td>Soft (slappap)</td>
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<td></td>
<td></td>
<td>3399</td>
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<tr>
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<td>Crumbly (phutu)</td>
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<td>Mabella</td>
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<td>Breakfast cereals</td>
<td>Brand name of cereals at home now:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you pour milk on your porridge or cereal?</td>
<td>Yes</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>If yes, what type of milk (whole fresh, sour, 1%, fat free, milk blend, etc)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>If yes, how much milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you put sugar on your porridge or cereal?</td>
<td>Yes</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOOD</td>
<td>DESCRIPTION</td>
<td>AMOUNT</td>
<td>TIMES EATEN</td>
<td>CODE</td>
<td>AMOUNT/ DAY</td>
</tr>
<tr>
<td>---------------</td>
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<td>-------------</td>
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<td>-------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Per day</td>
<td>Per week</td>
<td>Per month</td>
<td>Seldom / Never</td>
</tr>
<tr>
<td>If yes, how much sugar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Samp</td>
<td>Bought</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Self ground</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Samp and beans</td>
<td>Give ratio of samp:beans</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Samp and peanuts</td>
<td>Give ratio of samp:peanuts</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Rice</td>
<td>White</td>
<td></td>
<td></td>
<td>3247</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brown</td>
<td></td>
<td></td>
<td>3315</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Maize Rice</td>
<td></td>
<td></td>
<td>3250</td>
<td></td>
</tr>
<tr>
<td>Pasta</td>
<td>Macaroni</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spaghetti</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other specify:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pizza</td>
<td>Home made: Specify topping</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bought: Specify topping</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

You are being very helpful. Can I now ask you about meat?

**CHICKEN, MEAT, FISH**

How many times do you eat meat (beef, mutton, pork, chicken, fish) per week? 

<table>
<thead>
<tr>
<th>Chicken (codes with)</th>
<th>Boiled</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2926</td>
<td></td>
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<tr>
<td>FOOD</td>
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<td>AMOUNT</td>
<td>TIMES EATEN</td>
<td>CODE</td>
<td>AMOUNT/ DAY</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------</td>
<td>--------</td>
<td>------------------------------</td>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Per day</td>
<td>Per week</td>
<td>Per month</td>
</tr>
<tr>
<td>skin)</td>
<td>Fried: in batter/crumbs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eg Kentucky</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fried: Not coated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bought: Chicken Licken</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bought: Nando's</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Roasted / Grilled</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other:</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

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  |        |          | 3003       |
|                    | added                       |        |          |            |
|                    | Stewed with vegetables      |        |          |            |
|                    | Liver                       |        |          | 2920       |
|                    | Kidney                      |        |          | 2923       |
|                    | Other: Specify              |        |          |            |

<p>| Goat meat          | Boiled                      |        |          | 4281       |
|                    | Stewed with vegetables      |        |          |            |
|                    | Grilled / Roasted           |        |          | 4281       |</p>
<table>
<thead>
<tr>
<th>FOOD</th>
<th>DESCRIPTION</th>
<th>AMOUNT</th>
<th>TIMES EATEN</th>
<th>CODE</th>
<th>AMOUNT/DAY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Per day</td>
<td>Per week</td>
<td>Per</td>
<td>Seldom/</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>month</td>
<td>Never</td>
</tr>
</tbody>
</table>

**What type of vegetables is usually put into meat stews?**

<table>
<thead>
<tr>
<th>Foods</th>
<th>Amount</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wors / Sausage</td>
<td></td>
<td>2931</td>
</tr>
<tr>
<td>Bacon</td>
<td></td>
<td>2906</td>
</tr>
<tr>
<td>Cold meats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polony</td>
<td></td>
<td>2919</td>
</tr>
<tr>
<td>Ham</td>
<td></td>
<td>2967</td>
</tr>
<tr>
<td>Vienna</td>
<td></td>
<td>2936</td>
</tr>
<tr>
<td>Other: Specify</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canned meat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bully beef</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other: Specify</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meat pie</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef</td>
<td></td>
<td>2939</td>
</tr>
<tr>
<td>Steak and kidney</td>
<td></td>
<td>2957</td>
</tr>
<tr>
<td>Cornish</td>
<td></td>
<td>2953</td>
</tr>
<tr>
<td>Chicken</td>
<td></td>
<td>2954</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hamburger</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bought</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dried beans/peas/lentils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soup</td>
<td></td>
<td>3145</td>
</tr>
<tr>
<td>Salad</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soya products eg. Toppers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brands at home now:</td>
<td></td>
<td>3196</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Toppers)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pilchards in tomato/chilli/brine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole</td>
<td></td>
<td>3102</td>
</tr>
<tr>
<td>Mashed with fried onion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fried fish</td>
<td></td>
<td></td>
</tr>
<tr>
<td>With batter/crums</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without batter/crums</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOOD</td>
<td>DESCRIPTION</td>
<td>AMOUNT</td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other canned fish</td>
<td>Tuna</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pickled fish</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other: Specify</td>
<td></td>
</tr>
<tr>
<td>Fish cakes</td>
<td>Bought: Fried</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Home made with potato</td>
<td></td>
</tr>
<tr>
<td>Fish fingers</td>
<td>Bought</td>
<td></td>
</tr>
<tr>
<td>Eggs</td>
<td>Boiled/poached</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Scrambled: milk + fat</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fried: Fat</td>
<td></td>
</tr>
</tbody>
</table>

Now we come to vegetables and fruit

**VEGETABLES AND FRUIT**

<table>
<thead>
<tr>
<th>Cabbage</th>
<th>How do you cook cabbage?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Boiled, nothing added</td>
</tr>
<tr>
<td></td>
<td>Boiled with potato and onion and fat</td>
</tr>
<tr>
<td></td>
<td>Fried, nothing added</td>
</tr>
<tr>
<td></td>
<td>Fried in ..................</td>
</tr>
<tr>
<td></td>
<td>Boiled, then fried with potato, onion</td>
</tr>
<tr>
<td></td>
<td>Other:</td>
</tr>
<tr>
<td></td>
<td>Don’t know</td>
</tr>
</tbody>
</table>

**Spinach/morogo/ beetroot leaves other green leafy**

<table>
<thead>
<tr>
<th>How do you cook spinach?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boiled, nothing added</td>
</tr>
<tr>
<td>Boiled with fat added</td>
</tr>
<tr>
<td>Type of fat ................</td>
</tr>
<tr>
<td>With onion, tomato, potato</td>
</tr>
<tr>
<td>With peanuts</td>
</tr>
<tr>
<td>Other:</td>
</tr>
<tr>
<td>Don’t know</td>
</tr>
</tbody>
</table>

**Tomato and onion gravy**

<table>
<thead>
<tr>
<th>How do you cook pumpkin?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Home made with fat</td>
</tr>
<tr>
<td>Type of fat ................</td>
</tr>
<tr>
<td>Without fat</td>
</tr>
<tr>
<td>Canned</td>
</tr>
</tbody>
</table>

**Pumpkin (yellow)**

<table>
<thead>
<tr>
<th>How do you cook pumpkin?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boiled, nothing added</td>
</tr>
<tr>
<td>FOOD</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Carrots</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
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<tr>
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<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Mealies/Sweet corn</td>
</tr>
<tr>
<td>Sweet corn</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Beetroots</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Potatoes</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
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<td></td>
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<tr>
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<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Sweet potatoes</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>FOOD</td>
</tr>
<tr>
<td>-----------------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Salad vegetables</td>
</tr>
<tr>
<td>Mixed salad: tomato, lettuce and cucumber</td>
</tr>
<tr>
<td>Raw tomato</td>
</tr>
<tr>
<td>Other salad vegetables:</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Do you like fruit?</td>
</tr>
<tr>
<td>Apples</td>
</tr>
<tr>
<td>Pears</td>
</tr>
<tr>
<td>Oranges</td>
</tr>
<tr>
<td>Naartjie</td>
</tr>
<tr>
<td>Grapes</td>
</tr>
<tr>
<td>Peaches</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Apricots</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Mangoes</td>
</tr>
<tr>
<td>Guavas</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Avocado</td>
</tr>
<tr>
<td>Wild fruit/berries</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Dried fruit</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Other fruit</td>
</tr>
<tr>
<td>FOOD</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Custard</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>BREAD AND BREAD SPREADS</td>
</tr>
<tr>
<td>Bread / Bread rolls</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Do you spread anything on the bread?</td>
</tr>
<tr>
<td>Margarine</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Peanut butter</td>
</tr>
<tr>
<td>Jam/syrup/honey</td>
</tr>
<tr>
<td>Marmite / Fray bentos / Oxo</td>
</tr>
<tr>
<td>Fish/meat paste</td>
</tr>
<tr>
<td>Cheese</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Achaar</td>
</tr>
<tr>
<td>Other spreads</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Dumpling</td>
</tr>
</tbody>
</table>

If subject eats canned fruit: Do you have custard with the canned fruit? 1 Yes 2 No
## ADDENDA

### FOOD DESCRIPTION

<table>
<thead>
<tr>
<th>FOOD</th>
<th>DESCRIPTION</th>
<th>AMOUNT</th>
<th>TIMES EATEN</th>
<th>CODE</th>
<th>AMOUNT/DAY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Per day</td>
<td>Per week</td>
<td>Per month</td>
<td>Seldom</td>
</tr>
<tr>
<td>Vetkoek</td>
<td>White flour</td>
<td></td>
<td></td>
<td></td>
<td>3257</td>
</tr>
<tr>
<td></td>
<td>Whole wheat flour</td>
<td></td>
<td></td>
<td></td>
<td>3224</td>
</tr>
<tr>
<td>Provita, crackers, etc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3235</td>
</tr>
<tr>
<td>Mayonnaise / salad dressing</td>
<td>Mayonnaise</td>
<td></td>
<td></td>
<td></td>
<td>3488</td>
</tr>
<tr>
<td></td>
<td>Other: Specify</td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>

### DRINKS

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<thead>
<tr>
<th>DRINKS</th>
<th></th>
<th>Code</th>
</tr>
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<tbody>
<tr>
<td>Tea</td>
<td>English (normal)</td>
<td>4038</td>
</tr>
<tr>
<td></td>
<td>Rooibos</td>
<td>4054</td>
</tr>
<tr>
<td>Coffee</td>
<td></td>
<td>4037</td>
</tr>
<tr>
<td>Sugar/cup tea or coffee</td>
<td>Tea:</td>
<td>3989</td>
</tr>
<tr>
<td></td>
<td>Coffee:</td>
<td>3989</td>
</tr>
<tr>
<td>Milk/cup tea or coffee</td>
<td>What type of milk do you use in tea and coffee?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fresh/long life: whole/full</td>
<td>2718</td>
</tr>
<tr>
<td></td>
<td>Fresh/long life: 2%/low fat</td>
<td>2772</td>
</tr>
<tr>
<td></td>
<td>Fresh/long life: fat free</td>
<td>2775</td>
</tr>
<tr>
<td></td>
<td>Whole milk powder</td>
<td>2721</td>
</tr>
<tr>
<td></td>
<td>Brand:</td>
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</tr>
<tr>
<td></td>
<td>Low fat milk powder</td>
<td>2825</td>
</tr>
<tr>
<td></td>
<td>Brand:</td>
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</tr>
<tr>
<td></td>
<td>Skimmed milk powder</td>
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<tr>
<td></td>
<td>Brand:</td>
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</tr>
<tr>
<td></td>
<td>Milk blend</td>
<td>2770</td>
</tr>
<tr>
<td></td>
<td>Brand:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Whitener: type</td>
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216
<table>
<thead>
<tr>
<th>FOOD</th>
<th>DESCRIPTION</th>
<th>AMOUNT</th>
<th>TIMES EATEN</th>
<th>CODE</th>
<th>AMOUNT/DAY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Per day</td>
<td>Per week</td>
<td>Per month</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Condensed milk</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Evaporated milk</td>
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<td></td>
</tr>
<tr>
<td>None</td>
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<td></td>
<td>Milk as such</td>
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<tr>
<td></td>
<td>What type of milk do you drink milk as such?</td>
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<td></td>
<td>Fresh/long life: whole/full</td>
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<tr>
<td></td>
<td>Fresh/long life: 2%/low fat</td>
<td></td>
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<tr>
<td></td>
<td>Fresh/long life: fat free</td>
<td></td>
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<td></td>
<td>Condensed milk</td>
<td></td>
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<td></td>
<td>Sour/maas</td>
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<td>Other:</td>
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<td>Milk drinks</td>
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<td></td>
<td>Nestle:</td>
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<td>Milo:</td>
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<td></td>
<td>Flavoured milk:</td>
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<td>Other:</td>
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<td>Yoghurt</td>
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<td>Drinking yoghurt</td>
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<td>Thick yoghurt</td>
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<td>Low fat sweetened with fruit</td>
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<td>Squash</td>
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<td>Sweet O</td>
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<td>Six O</td>
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<td>Oros/Lecol – with sugar</td>
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<td></td>
<td>- artificially sweetener</td>
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<td>KoolAid</td>
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<td>Other:</td>
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<td>FOOD</td>
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<td>Per day</td>
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<td>Per month</td>
<td>Seldom/Never</td>
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<tr>
<td>Fruit juice</td>
<td>Fresh/Liquifruit/Ceres</td>
<td></td>
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<td>2866</td>
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<tr>
<td></td>
<td>Tropica (Dairy –fruit juice mix)</td>
<td></td>
<td></td>
<td>2791</td>
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<td>Other:</td>
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<td>Fizzy drinks</td>
<td>Sweetened</td>
<td></td>
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<td>3981</td>
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<td>Coke, fanta, etc</td>
<td>Diet</td>
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<td>Maewe/Motogo</td>
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<td>4056</td>
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<td>Home brew</td>
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<td>Tlokwe</td>
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<td>Beer</td>
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<td>4031</td>
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<td>Spirits</td>
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<td>4035</td>
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<td>Wine red</td>
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<td>Wine White</td>
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<td>4033</td>
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<td>Other specify</td>
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<td>Other specify</td>
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<td>SNACKS AND SWEETS</td>
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<tr>
<td>Potato crisps</td>
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<td>3417</td>
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<tr>
<td>Peanuts</td>
<td>Raw</td>
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<td>4285</td>
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<td></td>
<td>Roasted</td>
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<td>3458</td>
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<td>Cheese curls,</td>
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<td>Niknaks, etc</td>
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<tr>
<td>Raisins</td>
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<td>3552</td>
<td></td>
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<tr>
<td>Peanuts and raisins</td>
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<td>Chocolates</td>
<td>Name:</td>
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<td>FOOD</td>
<td>DESCRIPTION</td>
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<td>Per day</td>
<td>Per week</td>
<td>Per month</td>
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<tr>
<td>Candies</td>
<td>Sugus, gums, hard sweets, etc</td>
<td>4000</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Sweets</td>
<td>Toffees, fudge, caramels</td>
<td>3991</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biscuits/cookies</td>
<td>Type:</td>
<td></td>
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<tr>
<td>Cakes and tarts</td>
<td>Type:</td>
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<td>Scones</td>
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<tr>
<td>Rusks</td>
<td>Type:</td>
<td></td>
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</tr>
<tr>
<td>Savouries</td>
<td>Sausage rolls</td>
<td>2939</td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>Samoosas: Meat filling</td>
<td>3355</td>
<td></td>
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<tr>
<td></td>
<td>Samoosas: Vegetable filling</td>
<td>3414</td>
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<tr>
<td></td>
<td>Biscuits eg bacon kips</td>
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<td></td>
<td>Other specify:</td>
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<tr>
<td>Jelly</td>
<td></td>
<td>3983</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baked pudding</td>
<td>Type:</td>
<td></td>
<td></td>
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<tr>
<td>Instant pudding</td>
<td>Milk type:</td>
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<tr>
<td>FOOD</td>
<td>DESCRIPTION</td>
<td>AMOUNT</td>
<td>TIMES EATEN</td>
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<tr>
<td>Ice cream</td>
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<tr>
<td>Sorbet</td>
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<tr>
<td>Other specify</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>SAUCES, GRAVIES AND CONDIMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato sauce / Worcester sauce</td>
</tr>
<tr>
<td>Chutney</td>
</tr>
<tr>
<td>Pickles</td>
</tr>
<tr>
<td>Packet soups</td>
</tr>
<tr>
<td>Other:</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>WILD BIRDS, ANIMALS OR INSECTS (hunted in rural areas or on farms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild fruit</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MISCELLANEOUS: Please mention any other foods used more than once/two times a week which we have talked about:</th>
</tr>
</thead>
<tbody>
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<td></td>
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</tbody>
</table>
### INDIGENOUS/TRADITIONAL FOODS/PLANTS/ANIMALS

Please tell me if you use any indigenous plants OR other indigenous foods like mopani worms, locusts ect to eat

<table>
<thead>
<tr>
<th>FOOD</th>
<th>DESCRIPTION</th>
<th>AMOUNT</th>
<th>TIMES EATEN</th>
<th>CODE</th>
<th>AMOUNT/DAY</th>
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<td></td>
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<td>Per day</td>
<td>Per week</td>
<td>Per month</td>
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</tbody>
</table>

Specify

Specify
The PURE-SA project
24-HOUR RECALL DIETARY INTAKE

Subject ID

Subject Initials

Centre # Community # Household # Subject #

F M L

Today’s date: [ ] [ ] [ ]

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)

<table>
<thead>
<tr>
<th>Monday</th>
<th>Tuesday</th>
<th>Wednesday</th>
<th>Thursday</th>
<th>Friday</th>
<th>Saturday</th>
<th>Sunday</th>
</tr>
</thead>
</table>

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

[ ] Yes [ ] No

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 area 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 2 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 2 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?

What type of oil do you buy for deep frying?

Brand name:

Do you use the same oil more than once?

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

12. **What type of salt do you use?**

Give brand names

<table>
<thead>
<tr>
<th></th>
<th>Always</th>
<th>Sometimes</th>
<th>Never</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

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

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

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

<table>
<thead>
<tr>
<th></th>
<th>Very much</th>
<th>Like it</th>
<th>Not at all</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

13. **Do you use any of the following:**

<table>
<thead>
<tr>
<th>Name of product</th>
<th>Amount per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamins/vitamins and minerals</td>
<td></td>
</tr>
<tr>
<td>Tonics</td>
<td></td>
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<tr>
<td>Health foods</td>
<td></td>
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<tr>
<td>Body building preparations</td>
<td></td>
</tr>
<tr>
<td>Dietary fibre supplement</td>
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<tr>
<td>Other: Specify</td>
<td></td>
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</tbody>
</table>
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 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.

<table>
<thead>
<tr>
<th>Time</th>
<th>Place</th>
<th>Description of food and preparation method</th>
<th>Amount</th>
<th>Amount in gram</th>
<th>Code (office use)</th>
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</thead>
<tbody>
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</table>

225
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)

X

OR

By writing number(s) in the spaces provided:

1 8

OR

By specifying the answer on the line(s) provided

April 28, 2005
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 (Latinu)
13 - Bantu/Semi Bantu
14 - Hemitic/Semi Hemitic
15 - NilotiC/Hausa
16 - Pygmie
17 - Swahili
18 - Other (any other ethnорacial group not listed above)
Subject ID

Centre #  Community#  Household #  Subject #

Today’s date: [ ] [ ] [ ]

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
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 service 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
13. CURRENT DISABILITY:

   a) Do you have any problems using your fingers to grasp or handle? [No] [Yes]

   b) Do you have any trouble walking about? [No] [Yes]

   c) Do you have any trouble bending down and picking up an object from the floor? [No] [Yes]

   d) Do you require a walking stick cane/walker to move about? [No] [Yes]

   e) Do you have any trouble reading or seeing the individual grains of rice/corn on your plate? (with glasses worn) [No] [Yes]

   f) Do you have trouble seeing a person from across the room? (12 feet/3.5 meters) (with glasses worn) [No] [Yes]

   g) Do you have trouble speaking and being understood? [No] [Yes]

   h) Do you have any trouble hearing what is said in a normal conversation? [No] [Yes]

Subject Medical History

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

   a) Chest pain or tightness with usual activity [No] [Yes]

      If Yes, does the pain spread to the back, neck or inner border of arm [No] [Yes]

   b) Breathlessness with usual activity [No] [Yes]

   c) Cough for at least 2 weeks [No] [Yes]

   d) Any sputum while coughing [No] [Yes]

   e) Blood in sputum [No] [Yes]

   f) Wheezing or whistling in the chest [No] [Yes]

   g) Early morning cough with chest tightness [No] [Yes]

   h) Loose stools/diarrhea for at least 3 days [No] [Yes]

15. Not applicable in South Africa

16a) Do you use glasses/spectacles/contact lenses at present? [No] [Yes]

16b) 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
17. Have you ever been diagnosed with any of the following? (check all that apply)

<table>
<thead>
<tr>
<th>Condition</th>
<th>No</th>
<th>Yes</th>
<th># of yrs since diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension/ high blood pressure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stroke</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angina/heart attack/ Coronary artery disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart failure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other heart disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis/Jaundice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COPD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tuberculosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malaria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chagas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV/AIDS</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Please refer to facing page for cancer 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:

<table>
<thead>
<tr>
<th>Condition</th>
<th>No</th>
<th>Yes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol lowering drugs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stroke</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chinese medicine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

If Yes, specify: ________________________________
18b) If name of medication is unknown, please list as unknown.
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)
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)

a) Motor vehicle accident (as a passenger)  ☐ No ☐ Yes ➔ 

b) Motor vehicle accident (as a pedestrian)  ☐ No ☐ Yes ➔ 

c) Struck by an object  ☐ No ☐ Yes ➔ 

d) Explosion  ☐ No ☐ Yes ➔ 

e) Natural/environmental factors (gales/cyclones/lightning, etc.)  ☐ No ☐ Yes ➔ 

f) Suffocation  ☐ No ☐ Yes ➔ 

g) Poisoning  ☐ No ☐ Yes ➔ 

h) Snake/scorpion bite  ☐ No ☐ Yes ➔ 

i) Fall  ☐ No ☐ Yes ➔ 

j) Fire/flames, resultant fumes  ☐ No ☐ Yes ➔ 

k) Physical assault (gun, kidnapping, etc.)/violent crime  ☐ No ☐ Yes ➔ 

l) Domestic violence (beaten by a family member)  ☐ No ☐ Yes ➔ 

m) Drowning/submersion  ☐ No ☐ Yes ➔ 

n) Hot or corrosive liquids/floods/substances  ☐ No ☐ Yes ➔ 

o) Crush injuries (boulders, building materials, etc.)  ☐ No ☐ Yes ➔ 

p) Accident caused by machinery  ☐ No ☐ Yes ➔ 

q) Attempted suicide  ☐ No ☐ Yes ➔ 

r) Armed conflict  ☐ No ☐ Yes ➔ 

s) Other (specify)  ☐ No ☐ Yes ➔ 

[Blank spaces for location and type codes]
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"
24. Have you ever fractured a bone?  
   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)  
      If other, specify  

Tobacco  
25. Which best describes your history of tobacco use?  
   a) Formerly used tobacco products  
   b) Currently use tobacco products  
   c) Never used tobacco products  

b) At what age did you start?  

Past users only  

<table>
<thead>
<tr>
<th>Tobacco Product</th>
<th>Average amount/day</th>
<th>Duration (years)</th>
<th>When Stopped (years ago)</th>
<th>If less than 1 yr (months ago)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Cigarettes (all kinds)</td>
<td>number</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ii) Beedies</td>
<td>number</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(iii) Cigars</td>
<td>number</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(iv) Pipes</td>
<td>number</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(v) Sheesha/water pipe Hookah</td>
<td># of times</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(vi) Chewing tobacco</td>
<td># of times</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(vii) Snuff</td>
<td># of times</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(x) Other Specify</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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.
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)

<table>
<thead>
<tr>
<th>Form of Alcohol</th>
<th>Approx. size of one “drink”</th>
<th>Frequency</th>
<th>Average # of drinks</th>
<th>Duration (years)</th>
<th>Past users only When Stopped (years ago)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Spirits (rum, whisky, gin, vodka etc)</td>
<td>30ml</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>(ii) Wine</td>
<td>125ml</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>(vi) Beer</td>
<td>375ml</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>(vii) Country liquor/arrack/ sugar cane spirit</td>
<td>30ml</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

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?

29 a) During your longest or nocturnal sleep period, what time do you normally go to bed?

b) During your longest or nocturnal sleep period, what time do you normally wake up?

c) Do you usually take naps/siestas? □ No □ Yes Total nap duration □ □ mins
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.
30. Are you a member of any of the following:

(i) Self help group, Co-operative, Social club, Sports club, 
   Yes [ ] No [ ]

(ii) Religious Group (e.g.: church group, etc.)
   Yes [ ] No [ ]

(iii) Other (Specify)
   Yes [ ] No [ ]

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

(i) People are generally honest and want to help others.
   Strongly Disagree [ ] Somewhat Disagree [ ] Somewhat Agree [ ] Strongly Agree [ ]

(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.
   Strongly Disagree [ ] Somewhat Disagree [ ] Somewhat Agree [ ] Strongly Agree [ ]

32a) The television, radio, newspaper or magazine advertisements help me decide to buy the type of: (choose only one option for each)

(i) Cooking oil
   [ ] Strongly Disagree [ ] Somewhat Disagree [ ] Somewhat Agree [ ] Strongly Agree [ ]

(ii) Flour
   [ ] Strongly Disagree [ ] Somewhat Disagree [ ] Somewhat Agree [ ] Strongly Agree [ ]

(iii) Rice/ Maize meal
   [ ] Strongly Disagree [ ] Somewhat Disagree [ ] Somewhat Agree [ ] Strongly Agree [ ]

32b) The television, radio, newspaper or magazine advertisements influence whether I buy: (choose only one option for each)

(i) Soft drinks
   [ ] Strongly Disagree [ ] Somewhat Disagree [ ] Somewhat Agree [ ] Strongly Agree [ ]

(ii) Snacks
   [ ] Strongly Disagree [ ] Somewhat Disagree [ ] Somewhat Agree [ ] Strongly Agree [ ]

(iii) Cigarettes
   [ ] Strongly Disagree [ ] Somewhat Disagree [ ] Somewhat Agree [ ] Strongly Agree [ ]

(iv) Alcohol
   [ ] Strongly Disagree [ ] Somewhat Disagree [ ] Somewhat Agree [ ] Strongly Agree [ ]

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

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

Please specify

Please specify
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.

<table>
<thead>
<tr>
<th>No response</th>
<th>Never Experienced Stress</th>
<th>Some Period of Stress</th>
<th>Several Periods of Stress</th>
<th>Permanent Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) How often have you felt stress at work in the last 12 months? (Mark here if not applicable: i.e. no longer working □)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b) How often have you felt stress at home in the last 12 months?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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:

<table>
<thead>
<tr>
<th>No response</th>
<th>No</th>
<th>Yes</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Lose interest in most things like hobbies, work or activities that usually give you pleasure?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b) Feel tired or low on energy?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c) Gain or lose weight?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d) Have more trouble falling asleep than you usually do?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e) Have more trouble concentrating than usual?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>f) Think a lot about death (either your own, someone else's, or death in general)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g) Feel down on yourself, no good or worthless?</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
38. Please answer the following: (Choose only one option for each)

<table>
<thead>
<tr>
<th></th>
<th>Strongly Disagree</th>
<th>Somewhat Disagree</th>
<th>Somewhat Agree</th>
<th>Strongly Agree</th>
</tr>
</thead>
<tbody>
<tr>
<td>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.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b) Walking or bicycling in my neighbourhood is difficult because of the speed and/or amount of traffic.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c) My neighbourhood is generally free from pollution (litter, air pollution and noise pollution).</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d) My neighbourhood streets are well lit at night.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e) I can see other people when I am walking in my neighbourhood.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>f) I can speak to other people when I am walking in my neighbourhood.</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>g) There is a high crime rate in my neighbourhood.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>h) There is a problem with unattended dogs in my neighbourhood.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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?

<table>
<thead>
<tr>
<th>Crime</th>
<th>No</th>
<th>Yes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Armed robbery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Violent attacks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Murder</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Vehicle hijacking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. House breaking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Theft</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Rape</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. Women abuse eg. (beat, swear-words, sexual)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>please specify</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. Child abuse eg. (burn, swear-words, rejection)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>please specify</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. Child sexual abuse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. Other, please specify</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ii) Do you think that crime in your area has increased in the past 5 years?  
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
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
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
39. How long would it take you to get from your house to the nearest facility if you walked?

<table>
<thead>
<tr>
<th>Facility</th>
<th>Minutes</th>
<th>Don't know</th>
<th>Minutes</th>
<th>Don't know</th>
</tr>
</thead>
<tbody>
<tr>
<td>i) grocery/convenience store</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ii) bank</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>iii) post office</td>
<td></td>
<td></td>
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<tr>
<td>iv) video store</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>v) non-fast food restaurant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>v) fast food restaurant</td>
<td></td>
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</tr>
</tbody>
</table>

40a) Total number of siblings

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

- **Diabetes**
- **Coronary Heart Disease**
- **High Blood Pressure**
- **Stroke**
- **Cancer**
  - Please refer to facing page for cancer sites
  - If yes, indicate site
  - Other, Specify

# of siblings with the condition: [ ]
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.
Subject ID

Centre #  Community#  Household #  Subject #

Subject Initials  F  M  L

41. Physical Measurements

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

- Heart Rate
  - #1 beats/min
  - #2 beats/min

- Waist
  - #1 cm
  - #2 cm

- Hip
  - #1 cm
  - #2 cm

- Weight
  - kg

- Height
  - cm (without shoes)

- Circumference of mid upper right arm:
  - cm

- Circumference of right calf:
  - cm

- Head Circumference:
  - cm

- Upper flexed arm circumference
  - cm

- Right arm triceps skinfold:
  - #1 mm
  - #2 mm
  - #3 mm

- Right calf skinfold:
  - #1 mm
  - #2 mm
  - #3 mm
### Subject ID

<table>
<thead>
<tr>
<th>Centre #</th>
<th>Community #</th>
<th>Household #</th>
<th>Subject #</th>
</tr>
</thead>
</table>

- **Subject Initials**: F M L

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td></td>
<td></td>
<td>mm</td>
</tr>
<tr>
<td>#2</td>
<td></td>
<td></td>
<td>mm</td>
</tr>
<tr>
<td>#3</td>
<td></td>
<td></td>
<td>mm</td>
</tr>
</tbody>
</table>

#### c) Biceps skinfold

<table>
<thead>
<tr>
<th>#1</th>
<th>#2</th>
<th>#3</th>
</tr>
</thead>
</table>

#### d) Subscapular skinfold

<table>
<thead>
<tr>
<th>#1</th>
<th>#2</th>
<th>#3</th>
</tr>
</thead>
</table>

#### e) Supra spinal skinfolds

<table>
<thead>
<tr>
<th>#1</th>
<th>#2</th>
<th>#3</th>
</tr>
</thead>
</table>

- **44 a) Humerous breadth**:   cm
- **b) Femur breadth**:   cm

- **45. Grip Strength (Maximal contraction):**
  - **a) Non-dominant hand**:   kg.   kg.   kg.
  - **b) Dominant hand**:   kg.   kg.   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 spirometry:
   Spiromgrams 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
Subject ID

Centre # | Community# | Household # | Subject #

Subject Initials: F M L

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 □
Subject ID

Centre # Community# Household # Subject #

47. Not applicable in South Africa

48. ECG obtained? No → Go to #49 Yes
   a) 2 0 2
      year month day
   b) Please print ECG label #:

49 a) Blood sample obtained? No → Go to #50 Yes
   b) Fasting sample Non-fasting sample
   c) 2 0 2
      year month day
      Time : 00:00-23:59
   d) Please print Blood label #:

50 a) Urine sample obtained? No → Go to #51 Yes
   b) Fasting sample Non-fasting sample
   c) Please print Urine label #:

51. Name of Interviewer: (please print) First Initial Last Name
    Interviewer Code: 51