Gene-diet interactions in relation to circulating homocysteine concentrations

JP Van Schalkwyk

orcid.org 0000-0001-7706-4967

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Supervisor:	Prof. C Nienaber-Rousseau
Co-Supervisor:	Dr L Zandberg

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The author

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"For I know the plans I have for you," declares the Lord, "plans to prosper you and not to harm you, plans to give you hope and a future" – Jeremiah 29:11

ABSTRACT

Gene–diet interactions in relation to circulating homocysteine concentrations

Background: Elevated homocysteine (Hcy) is associated with several disease pathologies and can be manipulated by modifiable factors such as diet, nutritional status, physical activity and smoking, but can also be altered by non-modifiable factors such as age, gender and the genetic susceptibility of an individual. Although both dietary factors and genetic make-up influence plasma Hcy concentrations, very few investigations have examined the interactive effects *i.e*. gene–diet interactions.

Objective: The overall aim of this study was to elucidate the interactive effects between six known single-nucleotide polymorphisms (SNPs) of the Hcy metabolism (i.e. *methylenetetrahydrofolate reductase* (*MTHFR*) C677T, *MTHFR* A1298C, *methionine synthase* (*MTR*) A2756G, *cystathionine β synthase* gene (*CBS*) T833C, *CBS* 844ins68 and *CBS* G9276A) and markers of nutritional status (anthropometry, biochemical variables i.e. blood lipids, and dietary components) in relation to Hcy concentrations.

Study design and methods: As explained in detail in Chapter 3, six SNPs of Hcy-metabolising enzymes were analysed in 2010 black South Africans nested within the North-West arm of the Prospective Urban and Rural Epidemiology (PURE) study. Fasting Hcy concentrations were determined by fluorescence polarisation immunoassay technology and five of the SNPs through polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) analysis. The *MTHFR* A1298C variant was genotyped using competitive allele-specific PCR (KASP) technology. Dietary intake was assessed by means of quantitative food frequency questionnaires and serum lipids were measured by using a sequential multiple analyser computer.

Results: Hcy presented positive correlations with age ($r = 0.28$; $p < 0.0001$) and gamma glutamyl transferase (GGT) ($r = 0.24$; $p < 0.0001$) and was adjusted accordingly. Hcy increased with each addition of the *MTHFR* C677T minor allele, but decreased in the *MTR* 2756AA genotype compared with the heterozygote genotype. Individuals harbouring the *CBS* C833T/844ins68 polymorphism had the lowest Hcy concentrations of all the SNPs. Significant interactions were observed for *MTHFR* C677T*high density lipoprotein cholesterol (HDL-c) ($p = 0.02$), *CBS* T833C/844ins68*HDL-c ($p = 0.001$), *CBS* T833C/844ins68*protein as % of total energy intake (%TE) ($p < 0.001$), *CBS* T833C/844ins68*animal protein intake ($p = 0.02$), *MTHFR* C677T*added sugar intake as % of total carbohydrate (%T CHO) ($p = 0.004$) and *CBS* T833C/844ins68*biotin intake ($p = 0.04$) and Hcy. Both *MTHFR* C677T and *CBS* T833C/844ins68 minor allele carriers were inversely associated with HDL-c. In terms of the

CBS T833C/844ins68 interaction with protein, the homozygote minor allele carriers displayed an increase in Hcy as protein intake increased, whereas Hcy decreased significantly in the major homozygote TT ($p < 0.01$) and heterozygote TC ($p = 0.01$) alleles when consumption of animal protein was high. Sugar and the *MTHFR* 677TT genotype presented an increase in Hcy as sugar intake increased. In *CBS* T833C major allele carriers, elevated biotin intake was associated with lowered Hcy whereas Hcy was elevated in those harbouring the homozygous minor allele.

Conclusion: The SNPs associated with Hcy concentrations are modulated by diet and this opens up the possibility of establishing dietary interventions to treat hyperhomocysteinaemia. Future intervention trials should explore the observed gene–diet and gene–blood lipid interactions further.

Keywords: hyperhomocysteinaemia; homocysteine; blood lipid–gene interactions; nutrient–gene interactions; precision nutrition

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LIST OF ABBREVIATIONS

844*ins*68	insertion of 68 base pairs at nucleotide position 844
A	adenine (nucleotide)
A1298C	adenine to cytosine replacement at nucleotide position 1298
A2756G	adenine to guanine substitution at position 2756
Ala	Alanine (amino acid)
ALDH1L1	*aldehyde dehydrogenase 1 family member L1* gene
AMP	adenosine monophosphate
ANCOVAs	analysis of covariance
ANOVA	analysis of variance
Asp	Aspartic acid (amino acid)
ATP	adenosine triphosphate
AUTHeR	Africa Unit for Transdisciplinary Health Research
bp	base pairs
BMI	body mass index
BHMT	*betaine homocysteine methyltransferase* gene
C	cytosine (nucleotide)
C677T	cytosine to thymine substitution at nucleotide position 677
CBS	*cystathionine β synthase* gene
CEN	Centre of Excellence for Nutrition
CI	confidence interval
CoA	coenzyme A
CSE	cystathionine γ-lyase
CV	coefficient variation
CVD(s)	cardiovascular disease(s)
Cys	cysteine
DNA	deoxyribonucleic acid

ES	effect sizes
FA(s)	fatty acid(s)
FAD	flavin adenine dinucleotide
FMN	flavin adenine mononucleotide
G9276A	adenine to guanine substitution at position 2756
G	guanine (nucleotide)
G6PDH	glucose-6-phosphate dehydrogenase
GGT	gamma glutamyl transferase
Glu	glutamate (amino acid)
GNMT	*glycine N-methyltransferase* gene
Hcy	homocysteine
HHcy	hyperhomocysteinaemia
HDL-c	high density lipoprotein cholesterol
$HbA1_C$	glycated haemoglobin
HIV	human immunodeficiency virus
HW	Hardy–Weinberg
HWE	Hardy–Weinberg equilibrium
Ins	insertion
Ile	Isoleucine (amino acid)
IR	insulin resistance
ISAK	International Society for the Advancement of Kinantropometry
KASP	competitive allele-specific PCR
kJ	kilojoule, energy
LCAT	lecithin: cholesterol acyltransferase
LD	linkage-disequilibrium
LDL-c	low density lipoprotein cholesterol
MAF	minor allele frequency

MRC	Medical Research Council
M.Sc	Magister Scientiae
MS	methionine synthase (enzyme)
MSE	mean square error
MT	methyltransferase
MTHFR	*methylenetetrahydrofolate reductase* gene
MTR	*methionine synthase* (gene)
MTRR	*methionine synthase reductase* (gene)
MUFA(s)	monounsaturated fatty acid(s)
n	number of; sample size
n-3	omega 3
n-6	omega 6
NAFLD	non- alcoholic fatty liver disease
NCD(s)	non-communicable disease(s)
NWU	North-West University
NRF	National Research Foundation
NWU-RERC	Research Ethics Regulatory Committee of North-West University
%CDT	percentage carbohydrate deficient transferrin
%TE	percentage total energy
%TCHO	percentage energy from carbohydrates
PA	physical activity
PCR	polymerase chain reaction
PEMT	phosphatidylethanolamine methyltransferase
PHRI	Population Health Research Institute
Pi	orthophosphate
PLP	pyridoxal 5-phosphate
PPi	pyrophosphate
PSPH	phosphoserine phosphatase

PUFA(s)	polyunsaturated fatty acid(s)
PURE	Prospective Urban and Rural Epidemiology study
PRIMER	Profiles in Resistance to Insulin in Multiple Ethnicities and Regions
QFFQ	quantitative food frequency questionnaire
R	acceptor
RCT	reverse cholesterol transport
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rs	reference number
-SH	thiol
SAH	S-adenosyl-l-homocysteine
SAM	S-adenosyl-l-methionine
SANPAD	South- Africa – Netherlands Research Programme on Alternatives in Development
SD	standard deviations
SE	standard error
SFA	saturated fatty acids
SNP(s)	single nucleotide polymorphism(s)
SR-B1	hepatic class B, type 1 scavenger receptor (1)
T	thymine
T_2DM	type 2 Diabetes Mellitus
T833C	thymine to cytosine transition at nucleotide position 833
TC	total cholesterol
TE	total energy
TG	triglycerides
THF	tetrahydrofolate
Thr	Threonine (amino acid)
Val	Valine (amino acid)
VLDL-c	very low density lipoprotein cholesterol

WT	wild type

LIST OF ABBREVIATIONS

β	beta
χ^2	Chi square
r	correlation
°C	degrees, Celsius or centigrade
=	equal
$-CH_3$	methyl group
γ	gamma
g	gram
g/day	gram per day
g	gravitational force
>	greater than
≥	Greater than or equal to
L	litre
-	negative; minus
p	p-value, indicates statistical significance
pH	indicator of acidity or alkalinity
kat	katal
kg	kilogram
kg/m^2	kilograms per meter squared; unit of body mass index
km	kilometre
%	percentage
±	plus minus
p	p-value

$MgCl_2$	magnesium choloride
µ	micro: 10^{-6}
µg	microgram
µmol/L	micromole per litre
m	mille
mg	milligram
mL	millilitre
-	minus
mol	mole
M	molecular weight
x	multiply
x g	multiplied by gravitational force
-	negative
n	number of subjects; sample size
n	nano: 10^{-9}
ng	nanogram
+	positive
®	registered trade mark
<	smaller than
≤	smaller than or equal to
-SH	thiol
U	unit
yrs	years

CHAPTER 1

GENERAL INTRODUCTION

1.1 Prevalence of non-communicable diseases in South Africa

South Africa is so burdened with non-communicable diseases (NCDs) that the quality of life many of its people have is reduced and they succumb early to disorders that could have been prevented (Mayosi *et al.*, 2012). The origins of most NCDs are multifaceted and complex, with many subtle role players, including diet and genetic variants – where each on its own might have a nearly imperceptible effect, but together have a cumulative measurable consequence. It is critical to unravel and understand the risk factors causing these conditions in order to address the NCDs that currently blight the country. To this end, this research will consider circulating homocysteine (Hcy), which is such a risk factor/marker (Hogeveen *et al.*, 2012; Huang *et al.*, 2013; Kohaar *et al.*, 2010; Numata *et al.*, 2015; Peng *et al.*, 2015; Wang *et al.*, 2014; Zhang *et al.*, 2014; Zintzaras, 2010).

1.2 Homocysteine and its determinants

Hcy is a sulphur-containing amino acid, *i.e*. a thiol, with the chemical formula $HSCH_2CH_2CH(NH_2)CO_2H$ (Carmel & Jacobsen, 2001). Hcy is synthesised in the liver as a response to the breakdown of the essential dietary amino acid methionine (Deminice *et al.*, 2016). The structures of methionine and Hcy are almost identical except for a one-carbon methyl group (-CH3) which is removed from the former (Scott & Weir, 1998).

Elevated Hcy, also known as hyperhomocysteinaemia (HHcy), plays a role in several NCDs, including Alzheimer's disease (Wang *et al.*, 2014), mental disorders such as schizophrenia (Numata *et al.*, 2015), impaired bone health (Zhang *et al.*, 2014), type 2 diabetes (Huang *et al.*, 2013) and inflammatory bowel disease (Zintzaras, 2010), as well as adverse obstetrical outcomes, (Hogeveen *et al.*, 2012) and cancer (Kohaar *et al.*, 2010). However, historically, HHcy was viewed as a risk factor/marker for cardiovascular disease (CVD) (McCully, 1969; Wilcken & Wilcken, 1976). HHcy is currently considered to be a strong predictor of cardiovascular and all-cause mortality (Peng *et al.*, 2015). Earlier studies established the range of normal plasma Hcy concentrations at between 5 and 15 µmol/L. HHcy appears with mild to moderate concentrations of Hcy, which range between 16 and 100 µmol/L, and severe HHcy when concentrations rise above 100 µmol/L (Eikelboom *et al.*, 1999; Malinow *et al.*, 1999). According to Deminice *et al.* (2016), a Hcy concentration of 14.3 µmol/L or greater was independently associated with relative risk of mortality, at rates of 54% for all-

cause mortality and 52% for cardiovascular mortality. Earlier investigation used 12 µmol/L as a cut-off value for HHcy because of its proposed clinical relevance relating to CVD (Eikelboom *et al.*, 1999; Malinow *et al.*, 1999). Hcy cut-off values for disease-specific cases other than CVD, which is associated with HHcy, have yet to be established (Deminice *et al.*, 2016).

A recent meta-analysis of prospective studies indicated that Hcy is one of the independent risk factors for atherosclerosis (Peng *et al.*, 2015). HHcy is associated with reduced nitric oxide bioavailability and endothelial function; it also promotes the formation of toxic Hcy adducts (*e.g.*, Hcy thiolactone) and favours oxidative stress, all of which can increase an individual's susceptibility to atherosclerosis (Peng *et al.*, 2015), thrombotic processes (Deminice *et al.*, 2016) and the formation of CVDs (Zhang *et al.*, 2014). Some of the effects of the CVD mechanisms include an increase in proliferation of vascular smooth muscle cells, an increase in synthesis of collagen and also deterioration of arterial wall elastic material (Zhang *et al.*, 2014). Several cross-sectional and case-control studies have indicated a clear correlation between total circulating Hcy and the incidence of coronary, carotid, and peripheral vascular disease (Peng *et al.*, 2015).

What complicates the disease aetiology of pathologies contingent on Hcy is the fact that this amino acid has its own set of environmental and genetic determinants that influence it. Hcy can be manipulated by modifiable factors such as lifestyle, which includes diet or nutritional status, physical activity and smoking (Deminice *et al.*, 2016; Nienaber-Rousseau, 2014). According to a review by Nienaber-Rousseau (2014), Hcy can be lowered by adequate intake of folate, vitamin B_2, B_6 and B_{12}, as well as the proscription of alcoholic drinks, especially in heavy irregular (binge) drinking. It can also be influenced by non-modifiable factors such as age, gender and the genetic make-up of an individual (Nienaber-Rousseau, 2014; Nienaber-Rousseau *et al.*, 2013b). Elevated Hcy can, therefore, arise from a combination of dietary and/or genetically related disturbances in the *trans*-sulphuration or remethylation pathways of Hcy metabolism.

Recently, nutrition research focused attention on the importance of several nutrients that seem to play a role in regulating the genome machinery. Some of these vitamins and micronutrients are substrates and cofactors in the metabolic pathways which are responsible for the control of deoxyribonucleic acid (DNA) synthesis and repair and also, importantly, the expression of multiple genes (Fenech & Ferguson, 2001). Furthermore, a response to a certain nutrient seems, in many cases, to be specific for each genotype, and losses of specific nutrients can result in different gene expressions, depending on the genotype. The deficiency of nutrients may lead to the disruption of genomic integrity and alteration of DNA

methylation, resulting in a link between nutrition and modulation of gene expression (Friso & Choi, 2002). The field of gene–nutrient interactions is, therefore, a fascinating model that helps elucidate the impact of dietary exposures on gene regulation at a molecular level.

There are some common single-nucleotide polymorphisms (SNPs) associated with Hcy and Hcy metabolism. Some of these more well-known polymorphism variants are *methylenetetrahydrofolate reductase (MTHFR)* c.C677Tand c.A1298C, *methionine synthase* (*MTR)* c.A2756G, *cystathionine β-synthase* (*CBS*) c.T833C, and *CBS* c.844ins68. Owing to monetary constraints, we were limited to 6 SNPs in the study reported in Chapter 3 and chose the better known variations, except for the *CBS* g.G9276A variant, which we determined by the same method as *CBS* c.T833C/844ins68.

Because neither genetic nor dietary factors are solely responsible for altering Hcy concentrations, it is also important to investigate the gene–diet interactions where the two factors are combined. Such studies taking this approach are limited and examples in the Hcy field will be briefly discussed. Hustad *et al.* (2000) reported that riboflavin modulated Hcy in healthy *MTHFR* c.677TT homozygote adults. Silaste *et al.* (2001) observed that high folate intake decreased Hcy concentrations for variants of the *MTHFR* C677T and *MTR* G2756A SNPs; however, *CBS* 844ins68 did not show any relationship with Hcy concentrations. Additionally, Kluijtmans *et al.* (2003) showed that folate modulated Hcy in healthy homozygous 677TT adults. Nilsson *et al.* (2014) on the other hand, did not find an interaction between *MTHFR* C677T and decreasing folate in influencing Hcy concentrations.

A study based on the population we described in this research has previously observed no interaction between alcohol consumption and the *MTHFR* 677 CC or CT genotypes in relation to Hcy concentrations; however, an interaction was determined for the marker of liver function gamma glutamyl transferase (GGT) and the *MTHFR* genotype, where Hcy increased more prominently in those carrying the variant allele as GGT increased (Nienaber-Rousseau *et al.*, 2013a). Nilsson *et al.* (2014) showed that those with the MTHFR677TT genotype raised their Hcy concentrations quantitatively more with concomitantly lower vitamin B_{12} (cobalamin) than those harbouring the 677CC or CT genotypes. A Taiwanese intervention study showed that even though both *CBS* mutant carriers (p.D47E, c.T141A) and non-carriers were folate-deficient compared with the control group, only the mutant carriers had elevated Hcy. However, the difference in Hcy concentrations disappeared after folate was supplemented *via* a daily regimen of 5 mg of folic acid for 6 months. This study found that *CBS* carriers tend to present with higher Hcy concentrations in the presence of folate deficiency than to non-carriers (Lu *et al.*, 2015).

We hypothesise that nutrition status and dietary intake of certain nutrients, especially those that act as cofactors (folate, vitamins B_2, B_6 and B_{12}) within the metabolism of Hcy and other dietary-related factors, might interact with certain genotypes in genes coding for Hcy-metabolising enzymes, and in doing so, modulate Hcy concentrations. Consequently, we investigated whether there are interactions between dietary and diet-related components which have been previously associated with Hcy in the literature, together with genetic variants formerly associated with Hcy. This approach may increase our understanding of nutritional modulation that impacts susceptibility to HHcy-contingent diseases. Moreover, observational studies, such as the one reported here (in the article presented in Chapter 3), exploring the existence of interactions between gene and diet or diet-related factors, might pave the way for experimental studies in which cause and effect can be established.

Updated future experimental studies, especially those in relation to Hcy concentrations, are needed since they are extremely scarce. Together, observational and experimental studies might lead to an improvement in our understanding of gene–diet interactions related to Hcy, which could lead to discovering context-dependent risk factors for HHcy, thus enabling us to give customised dietary advice to individuals based on their genetic make-up in the future.

1.3 Aims and objectives of this study

The aim of this project, affiliated to the South African North-West arm of the Prospective Urban and Rural Epidemiological (PURE) study, was to explore some nutrition-related and specific genetic determinants (*MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, *CBS* T833C, *CBS* 844ins68 and *CBS* G9276A) that have been previously investigated in relation to Hcy concentrations. In addition, we also established the *MTHFR* A1298C genotype frequencies in the cohort of black South African adults, self-reported to be mainly Tswana-speakers. The overall aim was to analyse the interactive effects between the previously mentioned SNPs (*i.e*. *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, *CBS* T833C, *CBS* 844ins68 and *CBS* G9276A) and markers of nutritional status (anthropometry, biochemical variables *i.e*. blood lipids, HbA1c and fasting glucose and dietary components) in relation to Hcy concentrations.

The specific objectives are:

- To genotype the *MTHFR* A1298C polymorphism and to determine the genotype distributions of this alteration within the *MTHFR* gene in a black South African cohort;
- To determine whether nutritional status (anthropometry, biochemical variables *i.e*. blood lipids, HbA1c and fasting glucose and dietary components, among others [energy (kJ),

alcohol intake (g/day), protein (% total energy, TE), protein (g), dietary methionine, dietary cysteine, dietary fat, dietary folate (µg), dietary vitamin B_1 (mg) (thiamin), dietary vitamin B_2 (mg) (riboflavin), dietary biotin (µg), dietary pantothenic acid (mg), dietary vitamin B_3 (mg) (niacin), dietary vitamin B_6 (mg), dietary vitamin B_{12} (µg) (cobalamin), fruit and vegetables (g), pulses, nuts and seeds (g)]), modulate the association between genetic factors (*MTHFR* C677T, *MTR* A2756G, *CBS* T833C*, CBS* 844ins68 and *CBS* G9276A) previously genotyped in this cohort *and MTHFR* A1298C, which was genotyped for the work presented in Chapter 3) and Hcy concentrations.

1.4 Structure of this mini-dissertation

This mini-dissertation is presented in article format and was technically edited in the style as well as the language that complies with the requirements of the North-West University (Chapter 1, 2 and 4). Chapter 3, however, was edited in the style and language of the journal, *Nutrients*, for which the article manuscript was prepared (Chapter 3). The manuscript was also revised by a competent language editor. Chapter 1 is a general introduction which delimits the research problem, indicates the aims and objectives, presents the structure of the mini-dissertation and outlines the contributions of the research team to the mini-dissertation.

Chapter 2 is a review of the literature entitled “Genetic factors, dietary intake and their associations with / effects on homocysteine metabolism / concentrations”, with the purpose of conveying the current research available on Hcy and gene–diet interactions. This chapter captures an overview of Hcy metabolism and biochemistry, modifiable and non-modifiable dietary determinants of Hcy, genetic determinants of Hcy, gene–diet or diet-related interactions, combined diet interactions and nutritional genomics.

Chapter 3 is a research article with the title: “Gene interactions observed with blood lipids, intakes of protein, sugar and biotin in relation to circulating homocysteine concentrations” prepared for submission to the journal *Nutrients*. Our main finding from this work was that relationships of polymorphisms with Hcy concentrations were modulated by the blood lipid, high-density lipoprotein cholesterol (HDL-c), as well as dietary intake of added sugar, non-animal and animal protein and biotin. This is the first study, to our knowledge, to explore blood lipids, as well as dietary factors other than coffee, alcohol, folate, vitamin B_{12} and riboflavin intake, with these specific gene variants. It is also the first time the *MTHFR* A1298C variant was genotyped for this particular population group.

Chapter 4 is a brief summary of the entire manuscript, which includes our conclusions as well as future recommendations regarding the research conducted and presented in this mini-dissertation.

1.5 The research team and their contributions to the mini-dissertation

Table 1-1: List of members within the research team and their contributions to the mini-dissertation

Team member	Affiliation	Role
Miss J.P. Van Schalkwyk (M.Sc. candidate)	Centre of Excellence for Nutrition, North-West University	Applied for ethical approval; genotyped the *MTHFR* A1298C within the South African arm of the PURE study's DNA samples collected in 2005 under supervision of Dr L Zandberg; performed the statistical analyses under supervision of Prof. C. Nienaber-Rousseau, interpreted the results and wrote up a manuscript that will be submitted for publication; first authored Chapter 1 to 4; planned, wrote and compiled the dissertation.
Prof. C. Nienaber-Rousseau (Supervisor)	Centre of Excellence for Nutrition, North-West University	Genotyped the *MTHFR* C677 *CBS*, *MTR* A2756G, *CBS* T833C, *CBS* 844ins68 and *CBS* G9276A SNPs; conceptualised the M.Sc. project; supervised the statistical analyses and interpretation of results with the student; co-author of the manuscript that will result from this work (Chapter 3); supervised and guided the writing up of the mini-dissertation and critically reviewed the content.
Dr L. Zandberg	Centre of Excellence for Nutrition, North-West University	Designed and optimised the method used to genotype the *MTHFR* A1298C SNP and supervised the student during the genotyping; co-authored the resulting manuscript (Chapter 3); critically reviewed Chapter 1 to 4.

A, adenine; C, cytosine; *CBS,* cystathionine β-synthase gene; DNA, deoxyribonucleic acid; G, guanine; ins, insertion; *MTHFR,* methylenetetrahydrofolate reductase gene; *MTR*, gene coding for methionine synthase; PURE, Prospective Urban and Rural Epidemiology study; SNP, single-nucleotide polymorphism; T, thymine.

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CHAPTER 2

LITERATURE REVIEW

GENETIC FACTORS, DIETARY INTAKE AND THEIR ASSOCIATIONS WITH / EFFECTS ON HOMOCYSTEINE METABOLISM / CONCENTRATIONS

2.1 Introduction

Homocysteine (Hcy) is classified as a non-proteinogenic, non-essential, sulphur-containing amino acid, *i.e*. a thiol (-SH), which is synthesised mostly in the liver as a response to the *trans*-methylation of the essential dietary amino acid, methionine. Circulating Hcy concentrations have gained attention in various research domains because of their association with several disease pathologies that can increase the risk of mortality (Huang *et al.*, 2013; Numata *et al.*, 2015; Peng *et al.*, 2015; Wang *et al.*, 2014; Zhang *et al.*, 2014; Zintzaras, 2010). Earlier studies indicate that normal plasma Hcy concentrations should not exceed 15 µmol/L (Eikelboom *et al.*, 1999; Malinow *et al.*, 1999) and that an elevation of plasma Hcy, classified as hyperhomocysteinaemia (HHcy), can range between moderate: 16 to 30 µmol/L, intermediate: 31 to 100 µmol/L, and severe: HHcy >100 µmol/L (Ji & Kaplowitz, 2003). According to Humphrey *et al.* (2008), each 5 µmol/L increase in Hcy concentrations will increase the risk of cardiovascular disease (CVD) by approximately 20%, independently of any additional CVD risk factors present. Hcy concentrations >14.3 µmol/L have already been independently associated with a 54% relative risk of all-cause mortality and 52% of cardiovascular mortality (Deminice *et al.*, 2016). There is controversy among studies, however, regarding the significance of Hcy concentrations and their association with specific diseases.

HHcy has a complex set of underlying causes which can also be subjected to interactions among each other, such as those between various genetic and/or dietary-related disturbances in the *trans*-sulphuration and remethylation pathways. Hcy determinants can be divided into two main groups, namely modifiable and non-modifiable factors. Age (Jung & Pfeifer, 2015; Nienaber-Rousseau *et al.*, 2013a), sex (Nilsson *et al.*, 2014) and genetic variations (Burdennyy *et al.*, 2017; Nienaber-Rousseau *et al.*, 2013a; Williams *et al.*, 2014) involved in the Hcy metabolism are classified as non-modifiable factors, whereas physical activity (Chrysohoou *et al.*, 2004; Deminice *et al.*, 2016; Oliveira *et al.*, 2017; Sinha &

Dwivedi, 2017), dietary intake (Nienaber-Rousseau, 2014; Oliveira *et al.*, 2017) and smoking (Chrysohoou *et al.*, 2004; Oliveira *et al.*, 2017) are some of the modifiable factors that could be manipulated. There are some studies to prove that dietary intake and nutritional status have direct effects on Hcy concentrations (Assies *et al.*, 2015; Berstad *et al.*, 2007; Brude *et al.*, 1999; Clarke *et al.*, 2014; Cravo & Camilo, 2000; Czajkowska *et al.*, 2009; Dawson *et al.*, 2016; Haulrik *et al.*, 2002; Huang *et al.*, 2015; Huang *et al.*, 2011). On the other hand, other evidence exists that certain genetic factors influence an individual's Hcy status (Williams *et al.*, 2014). Very few of these studies focused on the combined interactive effects of diet and genetic factors *i.e*. gene–diet interactions (Amouzou *et al.*, 2004; Burdennyy *et al.*, 2017; Hustad *et al.*, 2000; Kluijtmans *et al.*, 2003; Lu *et al.*, 2015).

Well-known dietary intake factors influencing Hcy concentrations, as well as lesser known dietary aspects, will be considered in this review. Regarding the genetic factors, the focus will be mainly on specific genotypes that are involved in Hcy's metabolism and their interactions with diet in relation to Hcy, as identified in previous literature. An elaborate discussion of all genetic factors involved in Hcy metabolism or associated with Hcy is not within the scope of this mini-dissertation. Additionally, 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, but these will not be discussed here.

2.2 Homocysteine metabolism and biochemistry: an overview

With regard to Hcy metabolism, it is known that Hcy can be cleared from or transformed in the body. Hcy is synthesised by the *trans*-methylation of the essential, diet-derived amino acid, methionine (Figure 2–1). It is the only way through which Hcy can be produced. This conversion of methionine involves three phases catalysed by different enzymes: S-adenosyl-l-methionine (SAM) synthetase/l-methionine adenosyltransferase, methyltransferase (MT) and S-adenosyl-l-homocysteine (SAH) hydrolase. Methionine is activated by SAM synthetase in reaction with adenosine triphosphate (ATP), leading to SAM synthesis. SAM is known and used as a universal methyl donor not only in a variety of cellular biosyntheses of different compounds (creatine, epinephrine, carnitine, phospholipids, proteins, nucleic acids and polyamines), but also in epigenetic modulations, such as regulation of DNA methylation (nuclear and mitochondrial), chromatin re-modelling, ribonucleic acid (RNA)

editing, noncoding RNA, micro RNA and post-translational modification of histones. The end product of all SAM-dependent *trans*-methylation reactions is SAH (Škovierová *et al.*, 2016).

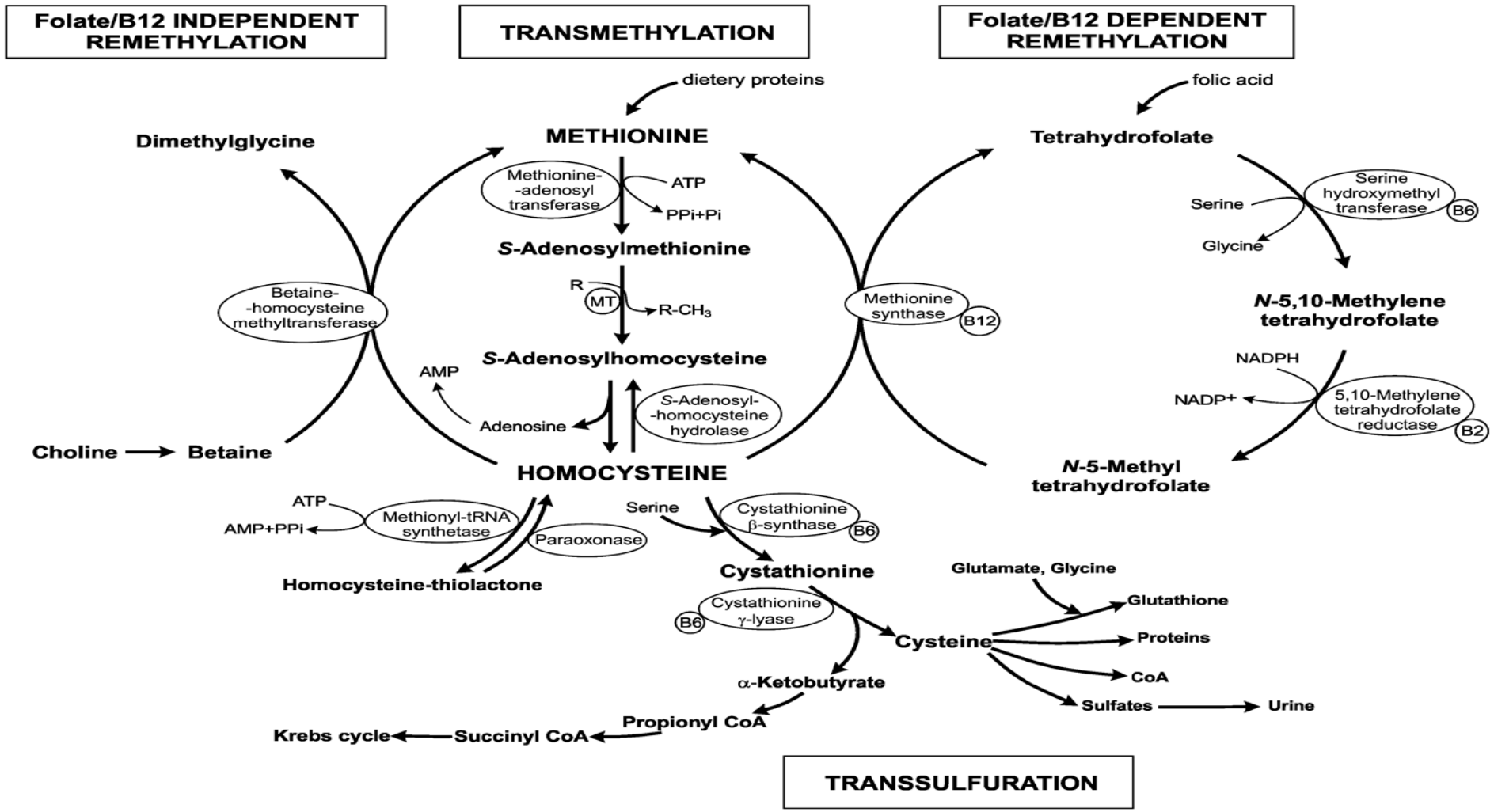
The fundamental pathways of Hcy were previously thought to be threefold: (i) remethylation to methionine by means of folate, vitamin B_{12}-dependent/independent pathways; (ii) *trans*-sulphuration to cystathionine; and (iii) regulation of intracellular Hcy concentrations by exporting excess Hcy from the cell into the circulation (Scott, 2003). However, recently, a fourth pathway has been identified as Hcy resynthesis to SAH through reversal activity of SAH hydrolase, which happens right after the second pathway (Figure 2–1) (Škovierová *et al.*, 2016).

The first pathway, identified as remethylation of Hcy back to methionine, happens by using either folate-dependent or folate-independent mechanisms (Škovierová *et al.*, 2016; Williams & Schalinske, 2007). During the folate-dependent remethylation, methionine synthase (MS) uses one methyl group from 5-methyltetrahydrofolate (5-MTHF) while the biologically active form of vitamin B_{12} (methylcobalamin) acts as a coenzyme. When the methyl group is produced by the enzyme 5,10-MTHFR, *methylenetetrahydrofolate reductase* (*MTHFR*), in turn, uses the biologically active form of vitamin B_2 (flavin adenine dinucleotide or FAD) as a cofactor. When using the alternative folate-independent remethylation route, Hcy is converted to methionine and dimethylglycine by using betaine, a methyl group donor derived from choline oxidation (Evans *et al.*, 2002). This remethylation is catalysed by the enzyme betaine-homocysteine methyltransferase (BHMT) by using a zinc ion to activate Hcy (Evans *et al.*, 2002).

During the *trans*-sulphuration process, which is the second pathway, the biologically active form of vitamin B_6 (pyridoxal 5-phosphate; PLP) is used as co-factor, causing the irreversible conversion to *cystathionine β-synthase* (CBS) (Scott, 2003). Hcy can also be further catabolised to cysteine (Cys) by using the enzyme cystathionine γ-lyase (CSE), which is required for the synthesis of various other compounds as well. Cys can also be converted to pyruvate, which is used for energy and sulphate and cleared through urine excretion (Scott, 2003).

With the third pathway, Hcy concentrations can be intracellularly regulated by being exported out of the cell and into the circulation (Scott, 2003).

The newly identified fourth pathway happens right after SAM-dependent *trans*-methylation. SAH is rapidly metabolised by SAH hydrolase to adenosine and Hcy, which potentially increases Hcy concentrations. When the methylation status is not regulated, it causes



ATP, adenosine triphosphate; AMP, adenosine monophosphate; PPi, pyrophosphate; Pi, orthophosphate; B2/B6/B12, vitamins B_2/B_6/B_{12}; CoA, coenzyme A; R, acceptor; R-CH3, methylated product; MT, methyltransferase.

Figure 2-1: A schematic overview of homocysteine metabolism [with permission from Škovierová *et al*. (2016)].

hypomethylation due to the reduced synthesis of SAM. This then results in the negative effect of HHcy (Jung & Pfeifer, 2015). It seems that Hcy in itself poses a potential risk, whether intracellular or extracellular. Scott (2003) also suggested that Hcy may be found in biological association with SAH, which is an actual risk factor for disease due to its inhibition of methyltransferases. The *trans*-sulphuration pathway of Hcy metabolism contributes to the maintenance of normal postprandial Hcy concentrations, while the remethylation pathway is responsible for maintenance of normal fasting Hcy concentrations.

From the role that dietary factors play in the Hcy metabolism, it is clear that they have the potential to directly influence Hcy concentrations. In the subsequent section, these factors will be considered.

2.3 Dietary determinants of homocysteine (modifiable)

2.3.1 Vitamin intake

It has been shown by various studies that Hcy and dietary methyl groups are interactively linked with each other (Deminice *et al.*, 2016; Evans *et al.*, 2002; Nienaber-Rousseau, 2014; Scott, 2003; Williams & Schalinske, 2007). As mentioned in section 2.2 of this chapter, the Hcy metabolism is dependent on four B vitamins that act as cofactors: folate and vitamin B_{12} for methylation of Hcy to methionine, vitamin B_6 for the irreversible *trans*-sulphuration to cysteine and vitamin B_2 and B_6 for the recycling of folate cofactors, which is necessary for activating vitamin B_6 to PLP (Apeland *et al.*, 2003). Vitamin B_2, B_6, B_{12} and folate thus play a key role in the clearance of Hcy from the circulation. Since vitamins are crucial to the Hcy metabolism, one can see the connection between dietary intake and Hcy concentrations. Insufficient vitamin B intake may increase Hcy concentrations.

Hcy has especially been identified as a sensitive indicator of vitamin B_{12} and folate status (Scott, 2003). Vitamin B_{12} deficiencies are commonly caused by inadequate dietary intake, especially in those who follow a vegetarian or vegan diet, because vitamin B_{12} is found in animal-source foods only (Obersby *et al.*, 2013). It can also be caused by the malabsorption commonly caused by alcoholism (Cravo & Camilo, 2000; Lakshmi & Bamji, 1976). An optimal vitamin B_{12} status promotes the proper functioning of the methylation cycle. This enzyme is not only dependent on 5-MTHF as a methyl donor, but is also dependent on vitamin B_{12} as coenzyme. Therefore, a low vitamin B_{12} status may increase Hcy concentration because of the reduction of the remethylation cycle, in the same way that low folate status influences Hcy metabolism.

As mentioned above, folate is an important co-factor and methyl donor when Hcy is converted to methionine (Bailey, 2003; Škovierová *et al.*, 2016). This micronutrient can be found in multiple green leafy vegetables and even in some animal products. An optimal folate status will

ensure a working methylation cycle by supplying adequate methyl groups, resulting in optimal Hcy remethylation. Folate deficiency, like vitamin B_{12} deficiency, is usually caused by inadequate dietary intake. Alternatively, serum folate is not affected by an inadequate diet alone, but also by intestinal malabsorption, altered hepatobiliary metabolism, and increased renal excretion (Wani *et al.*, 2013). Even though everyone should have a sufficient folate intake and status, research highlights the importance of adequate folate intake in individuals who harbour the *MTHFR* 677 TT genotype (Amouzou *et al.*, 2004; Bailey, 2003).

Riboflavin, 7,8-dimethyl-10-ribityl-isoalloxazine, commonly known as vitamin B_2, is a water-soluble B vitamin, which means that riboflavin is not stored in the body and can be sourced only through the diet by consuming food like animal protein, whole grains and certain vegetables, such as mushrooms and spinach. A low riboflavin status may cause increased Hcy concentrations because vitamin B_2 is a precursor of FAD, which acts as a cofactor of the MTHFR enzyme. The MTHFR enzyme interacts with folate, which suggests that a high folic acid intake may increase the riboflavin requirement (Apeland *et al.*, 2003).

Adequate intake of vitamin B_6 is also important for normal Hcy metabolism. Researchers of a Japanese study indicated that higher B_6 intake in young women was associated with lower Hcy concentrations (Murakami *et al.*, 2013). Investigators also observed that a higher intake of dairy products and lower intake of green and oolong tea was associated with decreased plasma Hcy concentrations (Murakami *et al.*, 2013). This goes to show that several nutrients and bioactive substances are integrated throughout the diet and that proper proportions are very important to maintain optimal Hcy status.

To our knowledge, research on interactions between Hcy and other vitamins is scarce or non-existent. The vitamin biotin, which is also a water-soluble vitamin found mostly in animal food sources like egg yolks, liver and salmon or non-animal sources like avocados and nuts, has not yet been studied in relation to gene–diet interactions modulating Hcy. Consequently, future research studies should focus on interactions between a variety of nutrients, micronutrients and different dietary combinations, with Hcy as the outcome variable.

2.3.2 Protein intake

As mentioned previously, dietary methionine is needed to synthesise Hcy. Some observational studies (Bailey, 2003; Stolzenberg-Solomon *et al.*, 1999) initially hypothesised that methionine-loading tests, which use animal protein to increase methionine content, may raise plasma Hcy concentrations because of the high methionine levels that are metabolised to Hcy. However, it now seems that the increased protein intake had an inverse effect on plasma Hcy concentrations (Bailey, 2003; Stolzenberg-Solomon *et al.*, 1999). In an older report of 1997, it

was shown that Hcy was not associated with methionine or protein intake (Shimakawa *et al.*, 1997). A long-term intervention study investigated the effects of a high-protein/high-methionine diet *versus* a low-protein/low-methionine diet on total plasma Hcy concentrations (Haulrik *et al.*, 2002). They observed a decrease in Hcy concentrations in the high-protein/high-methionine group from baseline, but no differences in Hcy after the intervention period between the high- and low-protein intake groups (Haulrik *et al.*, 2002). The results of these studies are, therefore, conflicting, and since the mechanism behind the inverse relationship between protein intake and methionine load with fasting Hcy concentrations is speculative, future studies are needed to resolve this issue. Methionine loading is characterised as a short-term, extreme situation, where methionine is trans-methylated through Hcy metabolism into Hcy (Stolzenberg-Solomon *et al.*, 1999). In contrast, protein intake usually represents long-term consumption, which can be seen as a more constant exposure to methionine. High-protein foods also contain other nutrients that influence Hcy concentrations, like vitamin B_{12} which, as mentioned, is derived only from animal-sourced food (Obersby *et al.*, 2013). Additionally, protein intake is often accompanied by increased intake of saturated fatty acids (SFAs), especially when the protein is of animal origin, and could lead to an increase in low-density lipoprotein cholesterol (LDL-c) concentrations (Scott, 2003). Intake of both SFAs and concentrations of LDL-c have previously been positively associated with elevated Hcy concentrations (see section 2.3.4).

Previous animal studies observed that the *trans*-sulphuration pathway and, to a lesser extent, the remethylation route, are triggered when animals are fed excessive amounts of methionine (Finkelstein, 1990; Han *et al.*, 2018). This suggests that high methionine intakes encourage activation of Hcy catabolising enzymes, which lead to more efficient Hcy catabolism and faster Hcy clearance from circulation (Han *et al.*, 2018). These findings are also supported by human studies indicating that an increase of plasma 5-MTHFR concentrations is a marker of an increased Hcy remethylation rate (Loehrer *et al.*, 1997). The mechanisms which indicate that protein intake may or may not produce a decrease in plasma Hcy concentration should be investigated in depth in future research to settle the dispute.

2.3.3 Hyperglycaemia, carbohydrate and sugar intake

Insulin is responsible for the strict regulation of hepatic glucose production (Finkelstein, 1990). Insulin resistance (IR), which is common in cases of metabolic syndrome and type 2 diabetes mellitus (T_2DM), invokes hyperglycaemia (Finkelstein, 1990). It is speculated by previous studies that IR causes a decrease in methionine transmethylation, hepatic Hcy *trans*-sulphuration and Hcy clearance, leading to increased concentrations of circulating Hcy (Chiang *et al.*, 2009; Han *et al.*, 2018; Tessari *et al.*, 2005). One of these studies also suggested that Hcy accumulation may be caused regardless of methionine status, but that insulin might also increase the Hcy remethylation flux when methionine is restricted (Chiang *et al.*, 2009). The

same study observed that some of the enzymes involved in Hcy remethylation, including MS, MTHFR, SAH and BHMT, were significantly induced by glucose and suggested that high cellular glucose may promote methionine synthesis (Chiang *et al.*, 2009).

Furthermore, impaired pancreatic β-cell function has also been linked to plasma Hcy, which may affect insulin signalling in peripheral tissues (Patterson *et al.*, 2007). A recent study proposed that, because Hcy has been linked with IR, elevated Hcy concentrations may be both a cause and consequence of metabolic syndrome since the activity of enzymes involved in Hcy metabolism might be affected by hyperglycaemia (Lind *et al.*, 2018). Alterations in Hcy metabolic enzymes have also been observed, where plasma insulin levels correlated positively with Hcy and MTHFR activity in rats that were fed a high-fat sucrose diet (Fonseca *et al.*, 2000).

The literature suggests that MTHFR activity decreased and cysteine production increased as glucose concentrations increased in hepatic cells and that the high glucose levels may have caused enhanced Hcy clearance owing to an elevation in Hcy *trans*-sulphuration (Dicker-Brown *et al.*, 2001) while another report observed that elevated glucose levels had no effect on Hcy *trans*-sulphuration, nor did they increase cysteine production in hepatic cells (Chiang *et al.*, 2009). Future studies are needed, therefore, to resolve issues of conflicting results and help understand the precise regulatory mechanisms by which insulin and glucose affect the Hcy metabolism, and thereby, Hcy concentrations.

When investigating a specific dietary component of nutritional intake, such as sugar intake, one should not ignore other nutritional components that accompany daily intake. Previous studies in black South Africans indicated that an elevated intake of sugar and saturated fat, which usually increase together, suggested a higher socio-economic status that, in turn, led to an improved micronutrient status and better overall diet quality (Teo *et al.*, 2009; Vorster *et al.*, 2007). The improved diet quality and micronutrient intake may together assist in lowering Hcy concentrations even though added sugar and saturated fat intake on their own are viewed as risk factors for various non-communicable diseases (Mendoza *et al.*, 2018).

We need to determine whether sugar intake and circulating glucose levels influence Hcy metabolism and/or concentrations and what the underlying mechanisms of such an influence might be. Additionally, no evidence on interactions between sugar intake and Hcy concentrations is available, according to our knowledge, especially for gene–sugar intake, which creates a possible research opportunity for future studies that may lead to better management of secondary complications accompanying IR, metabolic syndrome and diabetes.

2.3.4 Fat intake and blood lipids

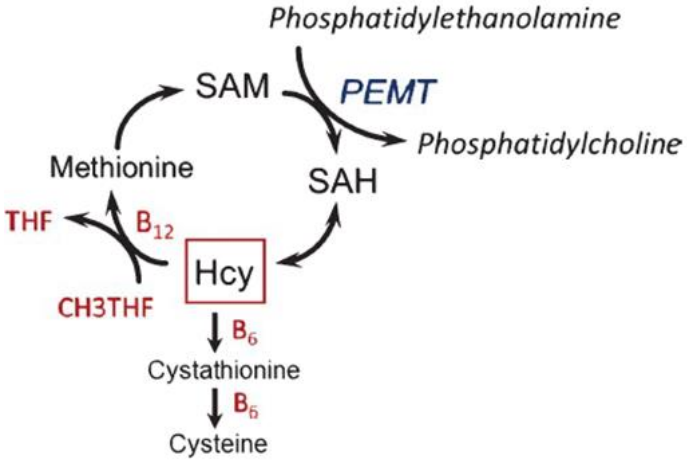
2.3.4.1 Dietary fat intake

Research regarding the association between plasma Hcy and dietary fat intake is scarce and findings have been inconsistent. Some investigations did not find any relationship between fat intake and Hcy concentrations (Brude *et al.*, 1999; Grundt *et al.*, 1999), whereas more recent studies observed interactions between omega-3 fatty acid (n-3 FA) intake and Hcy (Huang *et al.*, 2011; Li *et al.*, 2007; Pooya *et al.*, 2010). Other investigations also observed associations between n-3 FA intake and Hcy, but suggested that a combination of n-3 FAs and B-group vitamins is superior at lowering Hcy than n-3 FAs alone (Berstad *et al.*, 2007; Dawson *et al.*, 2016; De Bree *et al.*, 2004).

There are even fewer studies that investigated consumption of other types of dietary fat and Hcy. The Hordaland Hcy study observed significant positive associations between monounsaturated fatty acids (MUFAs) and plasma Hcy levels, as well as between polyunsaturated fatty acids (PUFAs) and omega 6 (n-6) PUFAs. The only group that was inversely associated with Hcy was the intake of marine n-3 FAs and the association was strong only in a younger age group (Berstad *et al.*, 2007). The Hordaland study, among other observations, confirmed that lower Hcy concentrations are associated with lower consumption of SFAs when compared with those who have a higher intake of SFA (Berstad *et al.*, 2007; Nygård *et al.*, 1995; Villegas *et al.*, 2004). The consumption of skimmed milk in comparison with full cream milk has also shown promising results in lowering plasma Hcy (Oshaug *et al.*, 1998), explained by the fact that skimmed milk has lower SFAs. When considering the role of the liver in lipid and Hcy metabolism, these observations can be expected. Although the mechanism has not yet been fully determined, the simultaneous occurrence of non-alcoholic fatty liver disease (NAFLD) and HHcy has been previously observed and Gulsen *et al.* (2005) indicated that HHcy was significantly higher in NAFLD subjects than others. An animal study observed that a high-fat diet elevated total cholesterol levels and doubled Hcy concentrations (Wang *et al.*, 2003).

There is a biochemical link between the lipid and Hcy metabolism (Figure 2–2), which could explain the relationship between plasma Hcy and fat intake (Noga *et al.*, 2003; Oulhaj *et al.*, 2016). Hcy is formed during SAH-dependent methylation of phosphatidylethanolamine to phosphatidylcholine, which is catalysed by the phosphatidylethanolamine methyltransferase (PEMT) enzyme and facilitated by B vitamins. During this metabolic action, phosphatidylethanolamine and phosphatidylcholine are enriched by n-3 FAs. This can also explain why some studies saw a more prominent decrease in plasma Hcy when n-3 and B vitamins were combined in the diet. PEMT may also explain why Hcy concentrations are

elevated in animals which are fed a phosphatidylethanolamine-rich diet (Noga *et al.*, 2003). However, a study has observed that phosphatidylethanolamine/choline supplementation lowered Hcy concentrations (Olthof *et al.*, 2005). The association between different types of fat intake and Hcy concentrations need to be investigated comprehensively in future experimental studies, seeing that different types of fat might have altered effects on phosphatidylcholine synthesis and Hcy concentrations.



Hcy, homocysteine; PEMT, phosphatidylethanolamine N-methyl transferase; SAH, S-adenosyl-l-homocysteine; SAM, S-adenosyl-l-methionine; THF, tetrahydrofolate.

Figure 2-2: Metabolic interactions between Hcy methylation-cycle and n-3 FAS [adapted from Oulhaj *et al.* (2016)].

2.3.4.2 Circulating blood lipids

Blood lipids are not dietary factors; however, they are directly associated with dietary fat intake (Mensink *et al.*, 2003), which is why blood lipids will be included in the discussion of Hcy and dietary factors. Research on HHcy and lipid metabolism is currently limited since most studies are conducted on mice and the tumour hepatic cell lines. Studies need to be performed on human patients with HHcy to confirm the associations between HHcy and high density lipoprotein cholesterol (HDL-c), as well as HHcy and lipid dysregulation, to identify the underlying mechanisms involved. There are several studies that observed an inverse association between plasma Hcy and HDL-c (Liao *et al.*, 2007; Mikael *et al.*, 2006; Momin *et al.*, 2017; Obeid & Herrmann, 2009; Samara *et al.*, 2010).

It is suggested that HHcy inhibits HDL-c biosynthesis and reverse cholesterol transport (RCT). There are three mechanisms identified which lead to the negative correlation between HDL-c and Hcy. The first mechanism is a reduction in HDL-c large particle formation as a result of hepatic apoA-I protein synthesis or secretion inhibition, which, in turn, suppresses

lecithin:cholesterol acyltransferase (LCAT) activity. The second mechanism enhances HDL-c clearance *via* hepatic class B, type 1 scavenger receptor (SR-B1) up-regulation; and the third limits HDL-c synthesis further *via* inhibition of HDL-c function and cholesterol efflux (Liao *et al.*, 2007). One investigation reported a positive correlation between total Hcy and LDL-c (Qujeq *et al.*, 2001) and another found a positive association between Hcy and triglycerides (TG) and very low-density lipoprotein cholesterol (VLDL-c) (Gulsen *et al.,* 2005). Both of these intervention studies also observed a negative association with Hcy and HDL-c, as previously reported. Other researchers observed similar results and reported that Hcy was associated only with TG and HDL-c, but not with total cholesterol (TC) or LDL-c (Mahalle *et al.*, 2013; Momin *et al.*, 2017). A few investigations reported that no significant correlations were found between HHcy and lipid profiles (De Luis *et al.*, 2005; Lupton *et al.*, 2016; Yadav *et al.*, 2006). The relationship of HHcy and dyslipidaemia, including hypercholesterolaemia and hypertriglyceridaemia, especially those including TC, LDL-c, VLDL-c and TG, has not been thoroughly researched and should be included in future studies.

2.3.5 Malnutrition

Malnutrition has also been related to HHcy and researchers observed that poor nutritional status resulted in HHcy (Choi *et al.*, 2015; Salles-Montaudon *et al.*, 2003). Most of the malnourished participants, whose weights varied between normal, overweight and obese, had an insufficient intake of protein and folate compared with participants with a healthy nutritional status, which could explain why Hcy concentrations were elevated (Choi *et al.*, 2015). Because of the malnourished state, it is probable that intake of all food groups, including meat and vegetables might be insufficient, leading to deficiencies of other essential vitamins and nutrients needed for Hcy metabolism. In society most of the malnourished cases are observed in the elderly who have lower calorie, protein and fat intake compared with younger well-nourished age groups. Advancing age has been associated with increased Hcy concentrations (Nienaber-Rousseau *et al.*, 2013a), which put this group at a particularly high risk of HHcy if malnutrition is also considered. Ingenbleek *et al.* (2002) proposed that the elevated Hcy concentrations in subjects with an inadequate nutritional status could be a result of the malnourished body's attempt to preserve methionine homeostasis. It still remains unclear why people with poor nutritional status develop HHcy and details regarding malnutrition and Hcy should be further investigated.

2.4 Non-modifiable determinants of homocystiene

2.4.1 Age and gender

Age and gender are considered to be among the stronger determinants of Hcy concentrations (Nienaber-Rousseau *et al.*, 2013a). Various studies have indicated that increasing age and the male sex are demographic factors associated with a higher Hcy concentration and treat these factors as possible confounders and/or stratify according to sex (Nienaber-Rousseau *et al.*, 2013a; Nilsson *et al.*, 2014).

Hcy increases with age, partly because increasing vitamin B_{12} deficiency is observed in the elderly as a result of poor absorption from food sources, and also because of declining renal function (Floyd & Hensley, 2002). Several body functions that are known to increase Hcy concentrations deteriorate with age, such as glomerular filtration rate and tubular function (Arnadottir *et al.*, 1996). Also, drug use increases with age (Ham *et al.*, 2017) and that could affect Hcy, as described in the introduction.

Brattström *et al.* (1994) suggested that the difference in Hcy concentrations between men and women could be explained by the larger body size and heavier muscle mass of men when compared with women. They observed a strong correlation between circulating Hcy and creatinine concentrations caused by a methyl-group transfer in the creatinine-creatinine metabolism during muscle formation. Therefore, muscle formation is associated with increased creatinine and Hcy formation.

Sex hormones might also play a role in Hcy concentrations (Andersson *et al.*, 1992). A study on transgender males and females proved that hormones do have an effect on the Hcy metabolism (Giltay *et al.*, 1998). Selhub *et al.* (1993) suggested that differences in Hcy concentrations between sexes could be explained by vitamin status *i.e*. the folate, vitamin B_6 and vitamin B_{12} status that differs between sexes. Other studies reported that age and hormone replacement therapy of menopausal women had an effect on Hcy concentrations (Andersson *et al.*, 1992; Lakryc *et al.*, 2015; Mooren *et al.*, 1994; Walsh *et al.*, 2000; Wouters *et al.*, 1995). These studies observed that circulating plasma Hcy was lower in pre-menopausal than in post-menopausal women, whether on oestrogen replacement therapy or not. Combined hormone replacement therapies that consisted of oestrogen and progesterone appeared to lower Hcy concentrations in both pre- and post-menopausal groups (Mooren *et al.*, 1994). Lower Hcy was also observed in pregnant, pre-menopausal and post-menopausal women undergoing hormone replacement therapy by Dimitrova *et al.* (2002). As mentioned in subsection 2.3.4, PEMT catalyses the synthesis of phosphatidylcholine, a betaine precursor, and betaine is source of methyl groups in the remethylation process of Hcy. Some argue that, since oestrogen induces

the expression of PEMT, women may supply choline from endogenous biosynthesis and, therefore, have reduced Hcy (Fischer *et al.*, 2010).

Some evidence is also emerging on gene–nutrient interactions that differ depending on the age and sex of the individual (Kluijtmans *et al.*, 2003; Nilsson *et al.*, 2014). Nilsson *et al*. (2014) demonstrated that, when stratifying according to age by using 52 years (which is considered to be the mean age of menopause) for both sexes, a more pronounced Hcy-lowering effect of the *MTHFR* 1793GA was observed in men below 52 years than in males with the1793GG genotype – a difference not observed in older men. Additionally, Nilsson *et al.* (2014) observed that the interaction between the *MTHFR* C677T genotype and cobalamin leading to increased Hcy was more pronounced with decreasing cobalamin in 677TT homozygote men than women. Kluijtmans *et al.* (2003) subdivided a population in quartiles according to folate status, stratified for genders, and found a divergent impact of the *MTHFR* 677TT genotype on Hcy concentrations, with men having higher Hcy than women in the lowest quartile of folate status.

2.5 Genetic determinants of homocysteine

Research on interactions between genes and other factors is still emerging and in-depth investigations are needed to determine the specific combinations of dietary and genetic factors that predispose individuals to HHcy. Studies should preferably include the commonly known polymorphisms of genes that have been identified within the Hcy metabolism cycle: *MTHFR, CBS* and *MTR,* as well as other lesser known genes such as the glycine N-methyltransferase (*GNMT)* (rs10948059), *phosphoserine phosphatase* (*PSPH*) (rs4948102), *aldehyde dehydrogenase 1 family member L1* (*ALDH1L1*) (rs10934753), *carbamoyl-phosphate synthase 1* (*CPS1*) (rs1047891), betaine-homocysteine S-methyltransferase (*BHMT)* (rs3733890) and *methionine synthase reductase* (*MTRR)* (rs1801394), which was identified in a genome-wide meta-analysis by (Williams *et al.*, 2014) and other researchers (Burdennyy *et al.*, 2017). A more detailed discussion follows on six of the better known single-nucleotide polymorphisms (SNPs) associated with Hcy.

Of the genetic determinants of Hcy, variations in the *MTFHR* gene, which codes for a key enzyme in the remethylation cycle, is the most researched and well described. The *MTHFR* C677T (rs1801133) polymorphism resulting in the phenotype of HHcy leads to a reduced MTHFR enzyme activity that is related to inadequate methionine metabolism. This is caused by the missense transversion mutation of the C677T that causes the enzyme to be thermolabile at 47°C and its activity to reduce at 37°C (Frosst *et al.*, 1995). Both the CT and the TT genotypes at the 677 locus have been associated with elevated Hcy concentrations when compared with those harbouring the CC wild-type genotype (Adjalla *et al.*, 2003; Frosst *et al.*, 1995). The TT genotype, however, has been shown to be prone to much higher Hcy concentrations in black

Africans compared with Caucasians, Mexicans and Hispanics (Adjalla *et al.*, 2003; Amouzou *et al.*, 2004). Higher Hcy concentrations and an impaired vitamin B_{12} and folate status have also been associated with the T allele at the *MTHFR* 677 locus (Amouzou *et al.*, 2004).

Another sequence change within the *MTHFR* locus, A1298C (rs1801131), consists of an A to C transversion, which results in a glutamate (Glu) to alanine (Ala) substitution at amino acid number 429 (Viel *et al.*, 1997). The C677T (rs1801133) and A1298C (rs1801131) have been investigated in various other population groups. However, other less well studied variations also exist, such as the G1793A, which seems to be in linkage with A1298C (1793A-allele in complete linkage disequilibrium with 1298C-allele) (Nilsson *et al.*, 2014). *In vitro* data suggests that the A1298C SNP also reduces MTHFR-specific activity and does not result in a thermolabile protein (Friedman *et al.*, 1999). The A1298C polymorphism is also more pronounced in the homozygous minor allele than in the heterozygous or homozygous major allele states (Friedman *et al.*, 1999; Lievers *et al.*, 2001; Weisberg *et al.*, 1998; Weisberg *et al.*, 2001). While studying the two *MTHFR* SNPs, researchers found that the combined *MTHFR* 677CC/1298CC genotype significantly lowered Hcy concentrations compared with those harbouring the 677CC/1298AA combined genotype (Friedman *et al.*, 1999). Further studies are required to determine the importance of the A1298C mutation and the role of the combined 677CC/1298CC genotype in Hcy metabolism.

The *CBS* gene plays a role in Hcy clearance by regulating the vitamin B_6-dependent *trans*-sulphuration, where the CBS enzyme contributes to the degradation pathway of Hcy and, ultimately, its removal as sulphate (Scott, 2003). Thus, a reduction in vitamin B_6 status will lead to a reduction in the enzyme activity with HHcy as a result (Scott, 2003). Of all the polymorphisms connected to the *CBS* gene, the two most prevalent mutations to cause HHcy are a T833C (rs5742905) point mutation in the 5' end of exon 8 and a 68-base pair insertion (ins68) at position 844, also in exon 8 (Griffioen *et al.*, 2005). Another variation of interest at the *CBS* locus is the G9276A SNP. Griffioen *et al.* (2005) stated that if the mutation is present, a possible alternate splice acceptor site is formed. However, limited studies regarding this specific mutation have been published and future studies should be conducted to further explore this variation.

The *MTR* A2756G (rs1805087) variant has been identified in the area where vitamin B_{12} (cobalamin) binds on the apoenzyme, methionine synthase (MS). Because MS catalyses the remethylation of Hcy to methionine and is a vitamin B_{12}-dependent enzyme, the reduction in its activity due to *MTR* A2756G may possibly be the cause of increased Hcy concentrations (Klerk *et al.*, 2003).

As mentioned in Chapter 1, with our constrained budget, in the study reported in Chapter 3, we were limited to six SNPs and chose the most well-known variations except for the *CBS* G9276A, which we determined by the same method as the well-known *CBS* T833C/844ins68.

2.6 Gene–diet and diet-related interactions

2.6.1 Individual nutrient interactions

2.6.1.1 Interactions with riboflavin

Riboflavin (vitamin B_2) is an essential precursor for the biosynthesis of the biologically active flavin adenine mononucleotide (FMN) and FAD. FAD is vital to the MTHFR enzyme and acts as co-factor to metabolise folate into a form used during Hcy methylation. FAD is also essential for an enzyme that activates the vitamin B_6 precursor, pyridoxal, to the biologically active form, PLP (Scott, 2003). When observing the Hcy metabolism, one could predict that in theory, inadequate vitamin B_2 intake might cause increased Hcy concentrations. Riboflavin status has been observed to be important in individuals with the *MTHFR* 677TT genotype, as vitamin B_2 status was previously reported to be a determinant of plasma Hcy in those harbouring the homozygous variant of *MTHFR* C677T (Hustad *et al.*, 2000). Studies on riboflavin are scarce and have not shown any strong interactions with genes other than *MTHFR*.

2.6.1.2 Interactions with folate

Silaste *et al.* (2001) determined that a high-folate diet increased serum folate concentrations by 85% for the CC, 77% for the CT and 55% for those with the homozygous TT variant of the *MTHFR* C677T polymorphism. Plasma Hcy concentrations also decreased during the high-folate intake period by 11%, 15% and 18% for the genotypes respectively. Participants harbouring the minor G allele of the *MTR* 2756 gene had a more prominent reduction in Hcy concentrations during the high-folate period when compared with those with the homozygous major allele (Silaste *et al.*, 2001). The *CBS* 844ins68 SNP did not show any significance when examined for plasma Hcy or diet responsiveness (Silaste *et al.*, 2001). Although Nilsson *et al.* (2014) did not find interactions between *MTHFR* C677T and decreasing folate levels in Hcy concentrations, Ni *et al.* (2017) observed a significant increase in Hcy concentrations in individuals that were folate-deficient, especially those harbouring the homozygote *MTHFR* 677TT genotype.

2.6.1.3 Interactions with vitamin B_{12} (cobalamin)

Nilsson *et al.* (2014) observed an interaction between the well-known *MTHFR* C677T genotype and cobalamin in relation to Hcy concentrations, where an increase in Hcy was more

pronounced with decreasing cobalamin than with decreasing folate in Spanish adults. To our knowledge, gene–diet interactions finding modulating effects of vitamin B_{12} are scarce and more research is needed.

2.6.1.4 Interactions with lipids and blood lipids

Even though there are a few studies investigating the associations between different lipid intakes and Hcy (section 2.3.4.1), none have combined lipid intake, Hcy and genetic polymorphism, to our knowledge. This creates a perfect research opportunity for future studies investigating gene–lipid intake interactions in relation to Hcy.

Hcy has been significantly and inversely correlated with HDL-c (Liao *et al.*, 2007; Mikael *et al.*, 2006; Momin *et al.*, 2017; Obeid & Herrmann, 2009; Samara *et al.*, 2010). However, not many considered genes when investigating the relationship. One study did find a significant correlation between Hcy concentrations and plasma HDL-c, where subjects with the TT genotype of the *MTHFR* C677T gene mutation had higher plasma Hcy values in association with lower HDL-c levels (Real *et al.*, 2010). No investigations exist, to our knowledge, that took any of the other polymorphisms into account when observing the correlation between Hcy and HDL-c. Some examinations did focus on CBS enzyme deficiency and determined that HHcy was more prominent when serum HDL-c concentrations were low (Liao *et al.*, 2007; Mikael *et al.*, 2006; Vanzin *et al.*, 2015).

2.6.1.5 Interactions with alcohol

Chiuve *et al.* (2005) observed that the elevation in Hcy among women who consumed low folate and drank moderate amounts of alcohol was greater in the presence of the variant *MTHFR* 677 T allele than the wild-type C allele. A study based on the population we described in this research previously reported that no interaction existed between alcohol consumption and the *MTHFR* 677 CC or CT genotypes in relation to Hcy concentrations; however, an interaction was determined for the marker of liver function, gamma glutamyl transferase (GGT), and the MTHFR genotype, where Hcy increased more prominently in those carrying the variant allele as GGT increased (Nienaber-Rousseau *et al.*, 2013b).

2.6.2 Combined diet interactions

Mediterranean diet

Where most studies focused on the interactions between certain genes and single nutrients in relation to Hcy, one study investigated the effect of interactions between the Mediterranean diet as a whole and the *MTHFR* C677T SNP on Hcy concentrations (Dedoussis *et al.*, 2004).

Dedoussis *et al.* (2004) observed that adherence to the Mediterranean diet was associated with reduced Hcy concentrations in those harbouring the homozygote TT and heterozygote CT alleles. Even though the Mediterranean diet consists mostly of foods of plant-based origin with abundant sources of folic acid, fibre and carotenoids, which are already associated with lowered risk for CVDs (Pitsavos *et al.*, 2003; Renaud *et al.*, 1995), the gene–diet interaction on Hcy concentrations was independent of fruit and vegetable intake. Therefore, a combined nutrient approach such as adherence to the traditional Mediterranean diet may have a positive effect on lowering Hcy concentrations. Research such as this one is valuable because nutrients are co-consumed and might influence each other and thereby change Hcy concentrations.

Data are currently lacking describing nutrient patterns and whether co-consumption interacts with gene variants in relation to Hcy concentrations. This will be an important area of investigation as we eat diets and the individual components often interact with each other. Because of these gene–diet interaction effects described here, Hcy is often considered the poster child for nutritional genomics and personalised nutrition. For this reason, it will be briefly discussed in the following section.

2.7 Nutritional genomics

Nutrients interact in many molecular mechanisms and contribute to the modulation of physiological functions in the body (Farhud *et al.*, 2010). Nutritional genetics and genomics are relatively new in the world of science: they study interactions between the human genome and food components to ultimately prevent or treat diseases through nutritional intervention (Javier Torrent & Armengol Rosell, 2013).

This science consists of two fields: nutrigenetics and nutrigenomics, which, in recent years, have become more common in preventative medicine. Nutrigenetics is known to focus more on how individual modifications in genes can cause heterogeneous responses to dietary components and specific nutrients (Farhud *et al.*, 2010). The nutrigenetic field is described as a genetic profile which influences how the body responds to food components by influencing the absorption, metabolism and site of action after consumption (Farhud *et al.*, 2010). Through nutrigenetics, personalised nutrition can be used to prevent certain diseases by analysing how genetic variants play a role in the susceptibility to an illness.

Nutrigenomics, in turn, study the effects of several nutrients, including macronutrients and micronutrients, on the human genome (Mutch *et al.*, 2005). Nutrigenomics help provide an understanding of how, by altering the expression of an individual's genetic make-up through dietary components, one can affect the balance between health and disease (Farhud *et al.*, 2010).

From the perspective of nutritional genomics, the investigation of Hcy and HHcy, together with diet and diet-related factors, becomes crucial because of the associations that Hcy has with various non-communicable health problems. Genetic modifications of certain genes *i.e*. *MTHFR*, *CBS* and *MTR*, together with deficiencies in or overexposure to a certain nutrient, can influence Hcy concentrations in a positive or negative way since some of these factors are directly or indirectly involved in the Hcy metabolism. This raises the possibility of using the human genome for precision nutrition based on individual genetic make-up and optimising for health and preventing disease. However, much more research is needed to make this a reality. With direct to consumer testing becoming more popular (Allyse *et al.*, 2018), researchers need to unravel the science to assist consumers interested in genetic testing and genetically based diets by giving advice founded on evidence and information that is ethically sound.

2.8 Conclusion

From this review it is clear that genetics and nutrition play an important role in the regulation of Hcy concentrations; there is also a scarcity of investigations that combine dietary intake and genetic factors. A great deal is known about folate and vitamin B_{12} intake and Hcy concentrations. Less is known about the interactions of folate and vitamin B_{12} with genes related to Hcy. Folate seems to be the most popular micronutrient for researchers to investigate in this field; however, different dietary factors, such as other micronutrients (including riboflavin and biotin) are also crucial in the clearance of Hcy, and, together with macronutrients (protein, carbohydrate, and lipids), are infrequently researched, with the result that studies of their interaction effects are negligible. These limited nutritional genomic studies investigating gene–diet interactions are of utmost importance in an era where direct to consumer genetic testing is becoming ubiquitous. The study that is reported in Chapter 3, investigating possible gene–diet interactions, is thus very timely and necessary to fill the current gaps in our knowledge.

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CHAPTER 3

GENE INTERACTIONS OBSERVED WITH BLOOD LIPIDS, INTAKES OF PROTEIN, SUGAR AND BIOTIN IN RELATION TO CIRCULATING HOMOCYSTEINE CONCENTRATIONS

3.1 Instructions to the author – *Nutrients*

For instructions to the authors of *Nutrients* journal to which this chapter will be submitted, please refer to **ADDENDUM A.**

3.2 Article

Running title: Gene Interactions Observed with Blood Lipids, Intakes of Protein, Sugar and Biotin in Relation to Circulating Homocysteine Concentrations

Jacomina P. Van Schalkwyk[1], Lizelle Zandberg[1] and Cornelie Nienaber-Rousseau[1]*

[1] Centre of Excellence for Nutrition, North-West University, Private bag x6001, Nutrition, Box 594, Potchefstroom, 2520, South Africa; jvjanele104@gmail.com; 12257656@nwu.ac.za; cornelie.nienaber@nwu.ac.za

***** Correspondence: cornelie.nienaber@nwu.ac.za; Tel.: +27-18-299-4169; Fax: +27-18-299-2464.

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3.2.1 Abstract

Elevated homocysteine (Hcy) is associated with several pathologies. Gene–diet interactions related to Hcy might be used to customize dietary advice to reduce disease incidence. To explore this possibility, we investigated interactions between diet and single-nucleotide polymorphisms (SNPs) in relation to Hcy concentrations. Six SNPs of Hcy-metabolizing enzymes were analyzed in 2010 black South Africans. Hcy increased as the *MTHFR* C677T minor allele increased, but was lower in *MTR* 2756AA homozygotes than heterozygotes. Individuals harboring *CBS* 833T/844ins68 had lower Hcy concentrations than others. *MTHFR* C677T and *CBS* T833C/844ins68 homozygote minor allele carriers presented with lower Hcy as HDL-c increased. Hcy concentrations rose as total dietary protein and animal protein intake increased in the CC genotype, but fell in the TT and TC genotypes of *CBS* T833C/844ins68. Hcy was elevated prominently in TT homozygotes of *MTHFR* C677T as added sugar intake increased. In *CBS* T833C/844ins68 major allele carriers, biotin intake was associated with lowered Hcy; however, those harboring the homozygous minor allele had elevated Hcy. The Hcy-SNP associations are modulated by diet and open up the possibility of invoking dietary interventions to treat hyperhomocysteinemia. Future intervention trials should further explore the observed gene–diet and gene–blood lipid interactions.

Keywords: hyperhomocysteinemia; homocysteine; blood lipid–gene interactions; nutrient–gene interactions; nutrigenetics; precision nutrition

3.2.2 Introduction

Circulating homocysteine (Hcy) is a sulfur-containing amino acid, i.e. a thiol (-SH), which is synthesized in the liver as a response to the breakdown of the essential dietary amino acid, methionine [1]. Elevated Hcy (hyperhomocysteinemia or HHcy) has been associated with several pathologies, including Alzheimer's disease [2], mental disorders such as schizophrenia [3], impaired bone health [4], type 2 diabetes [5], inflammatory bowel disease [6], adverse obstetrical outcomes [7], cancer [8] and cardiovascular diseases [9]. A total Hcy concentration of 14.3 µmol/L or greater is independently associated with a relative risk of mortality, with rates of 54% for all-cause mortality and 52% for cardiovascular mortality. HHcy is also associated with reduced nitric oxide bioavailability and endothelial function, promotes the formation of toxic Hcy adducts (e.g., Hcy thiolactone), and favors oxidative stress, all of which can increase an individual's susceptibility to atherosclerosis and thrombotic processes [1].

What complicates the disease etiology of pathologies contingent on Hcy is the fact that the protein has its own set of environmental and genetic determinants. Of these determining factors, the exposure to environmental agents is modifiable, thus raising the prospect of

intervention to reduce the likelihood of disease; however, non-modifiable factors such as age and genotype should also be taken into account. By exploiting the dietary aspect of Hcy metabolism, which can be easily manipulated, various studies have demonstrated that Hcy and dietary methyl groups are interactively linked with each other. Vitamin B_2, vitamin B_6, vitamin B_{12} and folate play key roles in the clearance of Hcy from the circulation [10]. Evidence also indicates the importance of dietary fats in influencing Hcy [11].

Among the non-modifiable factors that are inherent in the functioning of Hcy, the methylenetetrahydrofolate reductase (*MTHFR*) C677T (rs1801133) polymorphism is a well-known genetic variant that results in an amino acid substitution (Alanine222Valine). This cytosine (C) to thymine (T) transition at nucleotide position 677 (c.C677T) causes a reduction of enzyme activity, which leads to inadequate methionine metabolism that has the HHcy phenotype a result. Another sequence change within the *MTHFR* locus, an adenine (A) to C transversion, results in a glutamate to alanine substitution (rs1801131) [12]. *In vitro* data suggest that the A1298C variation reduces MTHFR-specific activity, though to a lesser degree than the C677T SNP [13-15]. Other SNPs – such as the insertion of 68 base pairs (bp) at position 844 (c.844ins68) in the cystathionine β synthase (*CBS*) gene that usually co-exists with the *CBS* T to C substitution at base 833 (c.T833C) (rs5742905), and the A to guanine (G) substitution at position 2756 (c.A2756G) (rs1805087) within the methionine synthase (*MTR*) gene – also influence Hcy concentrations. CBS plays a role in Hcy clearance by regulating vitamin B_6-dependent *trans*-sulfuration. The A2756G polymorphism of the *MTR* gene decreases vitamin B_{12}-dependent remethylation of Hcy to methionine, which can possibly cause an increase in Hcy concentrations. Li *et al*. [16] reported that *MTHFR* 677TT, *MTHFR* 1298AA, and *MTR* 2756AG + GG are independently correlated with high risk of folate deficiency and increased Hcy concentrations.

Elevated Hcy can arise from a combination of dietary and/or genetic disturbances in the *trans*-sulfuration or remethylation pathways of Hcy metabolism. Although both dietary factors and genetic susceptibility have major effects on Hcy status, very few investigations integrate genetic and dietary exposures. Such studies include that of Hustad *et al*.[17], who reported that vitamin B_2 (riboflavin) modulated Hcy in healthy homozygous *MTHFR* 677TT adults, and of Silaste*, et al.* [18] and Kluijtmans*, et al.* [19] that folate did so and of Nilsson*, et al.* [20] that vitamin B_{12} (cobalamin) did so. The minor G allele carriers of the MTR A2756G gene had a more prominent reduction in Hcy concentrations during high-folate intake when compared to those with the homozygous major allele [18]. The intervention study by Lu *et al*. [21] showed that folate-deficient *CBS* mutant carriers (p.D47E, c.T141A) had elevated Hcy compared with non-carriers, but that this difference disappeared after folate replacement. Studies that investigated possible

interactions focused on folate and vitamin B_2 only and neglected other dietary factors also involved in Hcy metabolism [10].

Our overall aim was to report on whether there are interactions between nutritional status and specific polymorphisms, coding for enzymes involved in Hcy metabolism, in relation to Hcy status, of which we had no prior knowledge. Specifically, this study explored interactions between the most important dietary factors relating to Hcy and genotypes *MTHFR C*677T and A1298C, *CBS* T833C/844ins68, *CBS* G9276A and *MTR* A2756G, which influence Hcy metabolism in relation to Hcy concentrations and status in a group of black South Africans. Proper understanding of gene–diet interactions may in future increase our ability to identify at-risk individuals who are particularly susceptible to HHcy as a result of environmental insult. Furthermore, it should provide evidence, when considering an individual's genetic make-up, for predicting the possible therapeutic success of lifestyle changes to prevent HHcy and treating, after appropriate intervention, the complex diseases contingent on Hcy.

Here we report that the relationships of polymorphisms with Hcy concentrations were modulated by dietary intake of sugar, protein and biotin (vitamin B_7), as well as one of the blood lipids, making this the first study, to our knowledge, to explore dietary factors other than coffee, alcohol, folate, vitamin B_{12} and vitamin B_2 intake, and paving the way for future experiments exploring the newly identified gene–diet interactions.

3.2.3 Materials and Methods

This study was conducted on the baseline data of the South African arm of the Prospective Urban and Rural Epidemiology (PURE) study, which examined the prevalence of non-communicable disease risk factors in several countries experiencing urbanization [22]. For our study, only the data from the South African arm of the PURE study were used; sampling procedures and study design are described in detail elsewhere [23]. In short, selection of ostensibly healthy black participants, stratified according to urbanization level from a census including 6000 households, resulted in the recruitment of 4000 eligible individuals. Of those meeting the inclusion criteria, 2792 (1348 = urban, 1444 = rural) gave consent to take part in the study and 2010 (1004 = urban, 1006 = rural) attended the measurement day. Ethical approval was granted by the Research Ethics Regulatory Committee of North-West University (NWU-RERC) (ethics number: 04M10; NWU-00332-16-S1). Written informed consent was obtained from all volunteers prior to enrollment.

Anthropometrical measurements were taken in accordance with the guidelines of the International Society for the Advancement of Kinantropometry (ISAK) by ISAK-trained

researchers. Measurements included height, weight and skinfolds as well as hip, waist and mid-upper arm circumferences.

All participants completed questionnaires verbally by means of interviews in the language of their choice. A standardized questionnaire was used to collect detailed demographic, health and lifestyle information. The dietary intake was assessed by means of a validated, interview-based quantitative food frequency questionnaire (QFFQ) which was developed in South Africa for the transition and health during urbanization in South Africa (THUSA) study [24]. The QFFQ used for this study was validated against 7-day weighed food records, 24-hour urinary nitrogen excretion, as well as the estimated basal metabolic rate [25]. Food portion photograph books were specifically designed and standardized for the South African population. All the participants were asked to recall their usual food intake, including drinks, by reporting the frequency, amounts (models and food labels were used to demonstrate portion sizes) and preparation methods for the foods consumed during the previous month. The data obtained from the QFFQ were computerized, using the FoodFinder3 ® program (Medical Research Council i.e. MRC, Tygerberg, 2007) and sent to the MRC of South Africa for nutrient analyses.

Blood samples were drawn after a 12-hour overnight fast. Plasma for the quantification of total Hcy was separated with minimal delay and stored at –80°C until analysis. Hcy concentrations were quantified by a pathology firm using the Abbott automated immunoassay analyzer (AxSYM), which is based on fluorescence polarization immunoassay technology (coefficient variation (CV) = 4.52%).

Serum lipids were measured by using a sequential multiple analyzer computer, using the KonelabTM auto analyzer (Thermo Fisher Scientific Oy, Vantaa, Finland), a clinical chemistry analyzer for colorimetric, immunoturbidometric and ion-selective electrode methods [26]. Low-density lipoprotein cholesterol (LDL-c) was calculated using the Friedewald–Levy–Fredrickson formula [27].

Fasting glycated hemoglobin ($HbA1_C$) was determined by using whole ethylenediamine tetra-acetic acid blood for measuring $HbA1_C$ values, with a D-10 hemoglobin testing system (Bio-Rad Laboratories, Hercules, CA, USA). The Bio-Rad D-10 uses a cation exchange chromatography principle to estimate glycated hemoglobin.

Fasting plasma glucose was quantified by using a hexokinase method of the SynchronR System (Beckman Coulter Co., Fullerton, CA, USA). During this method, hexokinase catalyzes the transfer of a phosphate group from adenosine triphosphate to glucose, forming adenosine diphosphate and glucose-6-phosphate. The glucose-6-phosphate is then oxidized to 6-phosphogluconate, with the concomitant reduction of β-nicotinamide adenine dinucleotide to

reduced β-nicotinamide adenine dinucleotide by catalytic activity of glucose-6-phosphate dehydrogenase. Spectroscopic change at 340 nm was observed and used to calculate plasma glucose levels.

Genomic DNA was isolated from buffy coat using an established method. All polymorphic variants, except for the *MTHFR* A1298C, were analyzed using polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP), details of which are described elsewhere [23]. The *MTHFR* A1298C (rs18001131) genotyping was performed using competitive allele-specific PCR (KASP) technology. The KASP system consisted of universal KASP 2x master mix V4.0 containing 50 mM $MgCl_2$ and SNP-specific KASP Assay mix. Assays were designed in-house and validated by LGC Ltd. The *MTHFR* A1298C-specific assay consisted of a common primer (5'-GGTAAAGAACGAAGACTTCAAAGACACTT-'3) and two labeled allelic discriminating primers with 5'-GGGGGAGGAGCTGACCAGT-'3_{FAM}, 5'-GGGGAGGAGCTGACCAGG-'3_{HEX}, respectively. Synthetic double-stranded DNA, gBlocks (IDT, Whitehead Scientific, South Africa), were included as positive controls. The gBlock synthetic DNA sequences were identical, discriminating at the point of variation only. Their sequences were as follows: *MTHFR* 1298CC (5'-aagcttGGTAAAGAACGAAGACTTCAAAGACACTTTCTTC[C]CTGGTCAGCTCCTCCCCCCggatccgc-'3), *MTHFR* 1298AA (5'-aagcttGGTAAAGAACGAAGACTTCAAAGACACTTTCTTC[A]CTGGTCAGCTCCTCCCCCCggatccgc-'3) and *MTHFR* 1298AC (5'- aagcttGGTAAAGAACGAAGACTTCAAAGACACTTTCTTC[A/C]CTGGTCAGCTCCTCCCCCCggatccgc-'3). Negative controls, i.e. no-template controls, were also included in each run. The genotyping was performed using the BioRad (CFX96) thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). The following thermocycler conditions were applied: step 1 comprised a cycle at 94°C for 15 minutes, followed by step 2, which entailed a cycle at 94°C for 20 seconds, at 61°C for 60 seconds (drop –0.6°C per cycle) for a total of 10 cycles, achieving a final annealing temperature of 55°C, followed by step 3 at 94°C for 20 seconds and 55°C for 60 seconds repeated for a total of 26 cycles. The final step, step 4, consisted of a cycle at 37°C for 60 seconds. The data were viewed and analyzed using the CFX manager software.

Haploview software version 4.2 (developed in Mark Daly's laboratory at the Broad Institute; http://www.broad.mit.edu/mpg/haploview) was used to calculate the level of pairwise linkage-disequilibrium (LD) between the *MTHFR* and *CBS* SNPs, using both D' and r^2 values [28]. Additionally, Haploview was used to compare the expected genotype frequencies according to the assumptions adhering to Hardy–Weinberg (HW) equilibrium (HWE) with those observed in our study. One limitation of this software is that it does not allow for the entering of indels; therefore, LD between the *CBS* T833C and *CBS* 844ins68 was determined through Statistica® (Statsoft Inc., Tulsa, OK, USA).

Statistical analyses were performed using the computer software programs Statistica® and SAS System for Windows (SAS Institute Inc., Cary, NC, USA). The data contained both categorical and quantitative variables. The quantitative variables were subjected to normality testing by using the Shapiro–Wilks Normality test to help estimate the distribution of the data, as well as visual inspection using Q-Q plots. Non-parametric variables were log transformed and then re-tested for normality. In the cases where log transformation increased normality, parametric statistics were performed.

Descriptive statistics were calculated and continuous variables were presented as means ± standard deviations (SD) or medians (interquartile ranges) for normal and median with the 25th and 75th percentiles skewed data, respectively. Spearman correlations were computed to determine the relationships between two variables, depending on the normality of the data.

Differences in Hcy concentrations and other biochemical and nutritional variables between the genotypic subgroups were analyzed using one-way analysis of variance (ANOVA) and Kruskal–Wallis ANOVA, respectively, followed by post-hoc tests. Differences in genotype frequencies among different Hcy strata and deviations from HWE were assessed by X^2 analysis. Analyses of covariance (ANCOVAs) were used to determine visually how the genotypes should be coded. For the *MTHFR* C677T, a stepwise (upward) association was observed and an additive genetic model of action was constructed by dummy coding (0/1/2) to indicate the number of copies of the variant allele. For the *MTR* A2756G genotype, a dominant genetic mode of action for those containing the variant allele (minor allele) was observed and, therefore, we combined the heterozygotes with those homozygous for the variant alleles (0/1/1). No distinct patterns were present for the remaining genotypes, and as a result, dummy coding was done by adding the heterozygotes to the homozygotes for the minor alleles similar to the dominant genetic mode of action. Subsequent correlations to determine the association of the genotypes with the Hcy phenotype were calculated using the above genetic coding.

To investigate whether factors (anthropometry, biochemical variables and dietary components) modulated Hcy polymorphisms and influenced Hcy concentrations, ANCOVAs (factorial), which allowed the assessment of any interaction effect over and above the main effects in the model being tested, were performed. Parametric statistical tests are robust and only slightly influenced by violations of the assumptions. Interactions that remained after excluding possible statistical outliers were reported as being significant.

Effect sizes (ES) were calculated as estimations of meaningfulness for t-tests as well as for the ANCOVAs, using Cohen's formulae $d = |x1 - x2| / Smax$ and $d = |x1 - x2| / \sqrt{MSE}$, respectively, where d = the ES; MSE = the mean square error; x1 = the mean of one of the groups; x2 = the mean of the other group; and Smax = the maximum standard deviation of the

two means [29]. D-values of 0.2 or less are regarded as small, 0.5 as moderate and 0.8 or more as large ES [29]. ES is the standardized difference between two groups and is used as an estimate of meaningfulness.

3.2.4 Results

For details of the descriptive characteristics of our population, see Table 1. Mean ± SD Hcy of the population and for men and women were 11.3 and 9.78 µmol/L respectively, which differed significantly ($p < 0.0001$). According to the definition of HHcy determined by Castañon *et al*. [30] (i.e. fasting plasma Hcy concentrations more than 12 µmol/L), 25.1% of our participants were hyperhomocysteinemic, which may bode negatively for them in the future as HHcy is associated with a range of pathologies.

Correlations between different variables and Hcy can also be seen in Table 3–1. Hcy correlated weakly with several dietary components and biomarkers; however, the positive correlations with age ($r = 0.28$; $p < 0.0001$) and gamma glutamyl transferase (GGT) ($r = 0.24$; $p < 0.0001$) are noteworthy. Therefore, in subsequent statistical analyses they were adjusted for.

Table 3-1: Characteristics of the study participants and correlations with Hcy.

Variables			Whole group (n = 2010) Median (25th – 75th) or Mean ± SD or n (%)	Correlations with Hcy (Spearman) r	p
Gender, n (%)		Men	749 (37.3)	–	
		Women	1261 (62.7)	–	
Age (yr)			48.0 (41.0 – 56.0)	0.28	<0.0001
Urbanization level, n (%)		Urban	1006 (49.9)	–	
		Rural	1004 (50.1)	–	
Tobacco use, n (%)		Current	1042 (52.1)	–	
		Former	77 (3.85)	–	
		Never	881 (44.1)	–	
Anthropometrical markers	BMI (kg/m^2)		22.98 (19.3 – 28.9)	–0.13	<0.0001
	Waist circumference (cm)		77.45 (70.2 – 87.7)	–0.03	0.24
	Hip circumference (cm)		93.13 (84.8 – 106)	–0.14	<0.0001
	Waist-to-hip ratio		0.83 (0.78 – 0.88)	0.17	<0.0001
Biochemical markers	HIV status, n (%)	Sero negative	1668 (83.1)	–	
		Sero positive	326 (16.2)	–	
		Status unknown	14 (0.70)	–	

	TC (mmol/L)		4.82 (4.01 – 5.87)	0.05	0.02
	LDL-c (mmol/L)		2.77 (2.07 – 3.63)	–0.05	0.03
	HDL-c (mmol/L)		1.42 (1.06 – 1.87)	0.19	<0.0001
	Triglycerides (mmol/L)		1.08 (0.82 – 1.55)	0.001	0.96
	Fasting glucose (mmol/L)		4.80 (4.30 – 5.30)	0.002	0.92
	HbA1c (%)		5.50 (5.30 – 5.80)	–0.053	0.02
	GGT (ukat/L)		46.0 (29.7 – 88.0)	0.24	<0.0001
	%CDT		2.67 (1.97 – 3.57)	0.089	0.0001
	Hcy (µmol/L)		9.18 (7.50 – 12.1)	-	-
Dietary intake	Energy (kJ)		7175 (5268 – 10001)	–0.05	0.04
	Alcohol intake (g/day)		11.8 ± 27.7	0.16	<0.0001
	Abstainers:drinkers (n:n)		1077:872	-	-
	Abstainers:drinkers (%n:%n)		55.3:44.7	-	-
	Protein (%TE)		11.64 (10.4 – 12.9)	–0.05	0.04
	Protein intake (g)		49.27 (34.8 – 71.5)	–0.05	0.02
	Methionine		0.76 (0.49 – 1.15)	–0.07	<0.01
	Cysteine		0.31 (0.18 – 0.50)	0.08	<0.001
	Carbohydrate (%TE)		60.3 (54.2 – 67.5)	–0.04	0.11
	Added sugar (%TCHO)		15.2 (9.51 – 21.8)	–0.10	<0.01
	Total fat (%TE)		22.5 (17.4 – 27.7)	–0.10	<0.0001
	SFA (%TE)		5.29 (3.63 – 7.06)	–0.10	<0.0001
	MUFA (%TE)		5.77 (3.75 – 7.74)	–0.08	<0.001
	MUFA (g):SFA (g) ratio		1.13 (0.97 – 1.27)	0.03	0.15
	PUFA	PUFA (%TE)	6.78 (5.06 – 8.60)	–0.06	<0.01
		Omega-6 FA (g)	12.0 (6.98 – 18.5)	–0.09	<0.001
		Omega-3 FA (g)	0.34 (0.20 – 0.52)	–0.09	<0.001
		Omega-6:omega-3 (g) FA ratio	36.1 (26.1 – 50.3)	–0.001	0.98
	Cholesterol (mg)		150 (80.0 – 259)	–0.07	<0.01
	Dietary folate (µg)		356 (248 - 481)	–0.04	0.07
	Dietary vitamin B_1 (mg) (Thiamin)		1.49 (1.09-2.05)	–0.02	0.48
	Dietary vitamin B_2 (mg) (Riboflavin)		1.01 (0.65 – 1.63)	0.003	0.90
	Dietary biotin (µg)		30.2 (19.1 – 46.3)	–0.08	<0.01
	Dietary pantothenic acid (mg)		3.67 (2.21 – 4.97)	–0.08	<0.001
	Dietary niacin (mg)(Vitamin B_3)		12.7 (8.88 – 18.6	–0.001	0.97
	Dietary vitamin B_6 (mg)		1.29 (0.92 – 1.84)	–0.06	<0.01
	Dietary vitamin B_{12} (µg)		2.69 (1.23 – 5.07)	–0.07	<0.01

	Magnesium (mg)	285 (199 – 406)	0.04	0.14
	Iron (mg)	12.2 (8.77 – 16.63)	–0.03	0.27
	Zinc (mg)	8.90 (6.49 – 12.6)	–0.03	0.25
	Selenium	21.9 (12.09 – 34.6)	–0.09	<0.001
	Carotenoids (µg)	1117 (303 – 2305)	–0.06	<0.01
	Fruit and vegetables (g)	86.6 (51.9 – 154)	–0.07	0.01
	Pulses, nuts and seeds (g)	4.29 (0 – 19.3)	–0.04	0.10

Numbers are slightly different for several variables because of missing data.

BMI, body mass index; FA, fatty acid; GGT, gamma glutamyl transferase; Hcy, homocysteine; HDL-c, high density lipoprotein cholesterol; HbA1c, glycated hemoglobin; HIV, human immunodeficiency virus; LDL-c, low density lipoprotein cholesterol; MUFA, monounsaturated fatty acids; %CDT, percentage carbohydrate-deficient transferrin; %TE, percentage total energy; %TCHO, percentage energy from carbohydrates; PA, physical activity; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acids; TC, total cholesterol.

3.2.4.1 Genotyping and the Individual Influence of the SNPs on Hcy Concentrations

The LD patterns as obtained from Haploview are presented in Figure 3–1. Additionally, the CBS T833C and CBS 844ins68 SNPs were found to be in linkage [23] and will be reported as CBS T833C/844ins68.

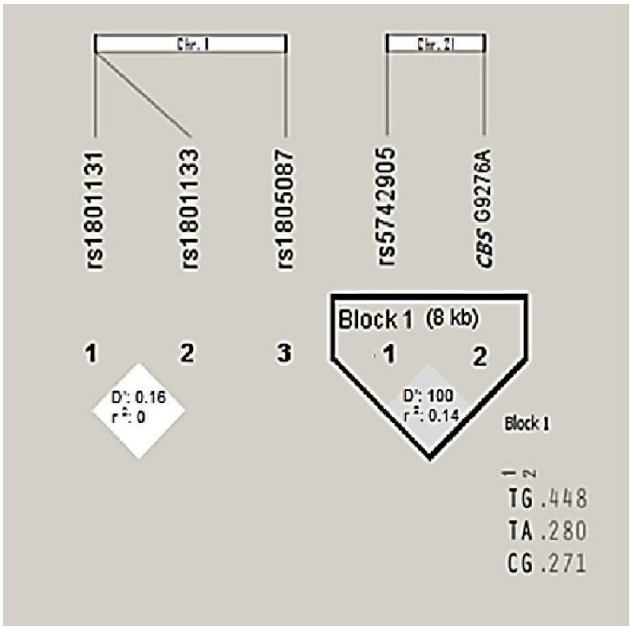

Figure 3-1: **Pair-wise LD represented as D' and r^2 values and the colour gradient (based on the r^2) from black, indicating complete, to white, indicating linkage equilibrium. The haplotype block was defined according to the CI method of Gabriel *et al*. [31] and the frequencies provided.**

CI, confidence interval; LD, linkage disequilibrium; D', a measure of LD indicating ranges from −1 to 1 for any set of allele frequencies for a pair of polymorphic biallelic markers; r^2, a measure of LD indicating the square of the correlation coefficient between two indicator variables

Genotype information of the participants is presented in Table 3–2. All genotype distributions were in accordance with HW predictions, except for the *MTHFR* A1298C SNP. The HW disequilibrium was not due to sampling problems since the genotype frequencies at this locus fell in the 95% confidence interval (CI) expected to be present in the target population. Most of the minor allele frequencies (MAFs) are comparable with those reported for African populations, whereas the MAF for the *CBS* T833C/844ins68 deviated from what has previously been reported [32]. For a full discussion of the genotypes' distributions, please refer to Nienaber-Rousseau *et al*. [23]. Additionally, Table 3–2 provides details on the frequencies of the investigated SNPs and their relationships with Hcy. When assuming the stepwise genetic model, *MTHFR* C677T correlated positively with Hcy concentrations. The only other significant, albeit weak, correlation with Hcy was a negative association with *MTR A*2756G when assuming a dominant genetic mode.

ANCOVAs indicated that Hcy concentrations increased with each addition of the T allele at the *MTHFR* 677 locus (see Table 3–2). The genotypes of the *MTHFR* A1298C SNP did not influence Hcy. The *MTR* 2756AA homozygotes presented with significantly higher Hcy than their 2756AG peers, but heterozygotes did not differ from those homozygous for the minor allele. When combining the heterozygotes with the homozygote variants for this polymorphism, a definite reduction in Hcy concentrations was observed. Although statistically insignificant, *CBS* 833T/844ins68 carriers had slightly lower Hcy concentrations than non-carriers. The difference in Hcy concentrations between the *CBS* 9276GG, 9276GA and 9276AA genotypes did not reach statistical significance although a trend toward higher Hcy in 9276G carriers was observed when compared with the other genotypes. Even though the *MTHFR* 1298, *CBS* T833C/844ins68 and *CBS* 9276 polymorphisms did not associate with Hcy, they were taken into consideration when determining possible interactions with diet.

3.2.4.2 Interaction Effects

3.2.4.2.1 Gene–gender Interactions in Relation to Hcy Concentrations

We assessed the potential interactions between gender and the Hcy-metabolizing SNPs; however, no gene–gender interaction was observed (data not shown).

3.2.4.2.2 Associations of Individual Dietary Components with Hcy as well as Gene–Diet and Gene–Lipid Interactions in Relation to Hcy

As mentioned before, several dietary components were associated with Hcy; however, the Spearman's rho values were weak and exceeded 0.1 only for alcohol intake, added sugar expressed as a % of carbohydrate intake, total fat and SFA intake ($p < 0.01$). To explore the interaction identified further, correlations were done stratified according to genotype. These associations per subgroup can by compared to those provided in Table 3–1 for the whole population, irrespective of genetic make-up. However, our focus is on the interactions and these will be described subsequently.

We observed HDL-c blood lipid interactions with the *MTHFR* C677T and *CBS* T833C/844ins68 SNPs. At both loci, minor allele carriers presented with lower Hcy than the heterozygote and homozygote major allele carriers (interaction $p = 0.02$; $p = 0.001$, respectively) as HDL-c increased. Significant interactions occurred between the *CBS* T833C/844ins68 polymorphisms and total dietary protein intake as well as dietary animal protein intake (interaction $p < 0.001$; $p = 0.02$, respectively). In terms of the interaction, the homozygote minor allele carriers displayed an increase in Hcy as total dietary protein intake and animal protein intake increased, whereas Hcy decreased in the major homozygote TT and heterozygote TC alleles when consumption was high.

Added sugar as % of energy from carbohydrates and the *MTHFR* C677T polymorphism interacted so that the TT genotype carriers, when compared with the other allele carriers (interaction $p = 0.004$), presented with a prominent increase in Hcy as sugar intake increased.

Significant interactions were observed for the *CBS* T833C/844ins68 polymorphisms and biotin (interaction $p = 0.04$) in modulating Hcy concentrations. In carriers of the *CBS* T833C major allele, elevated biotin intake was associated with lowered Hcy whereas Hcy was elevated in those harboring the homozygous minor allele.

Table 3-2: Frequencies of SNPs in the *MTHFR*, *MTR* and *CBS* genes and their relationship with Hcy

Gene	SNP (SNP ID; SNP location)	Genotype (genotype frequency)	Genotype frequency %	95% CI of genotype frequency (%)	MAF	Spearman Correlation with Hcy		Hcy (µmol/L)	ES* (d)
						r	P		
MTHFR	C677T; Ala222Val (rs1801133; 1:11796321)	CC (1579) CT (286) TT (15) X^2 HW test p = 0.70	84 15.2 0.8	82.2–85.6 13.7–17.0 0.37–1.17	0.08	0.10	<0.0001	10.1 (9.88; 10.3)$^{\partial\gamma}$ 11.1 (10.6; 11.6)$^{\partial\gamma}$ 18.5 (16.2; 20.7)$^{\gamma\gamma}$ ANCOVA p < 0.00001	0.22 1.70 1.93
						Genetic model: 0/1/2			
	A1298C; Glu429Ala (rs1801131; 1:11794419)	AA (1270) AC (339) CC (201) X^2 HW test p = 7.10^{-60}	70.2 18.7 11.1	68.1–72.3 16.9–20.5 9.66–12.5	0.20	0.01	0.82	10.3 (10.1; 10.6) 10.4 (9.97; 10.8) ANCOVA p = 0.75	0.02
						Genetic model: 0/1/1			
MTR	A2756G; Asp919Gly (rs1805087; 1:236885200)	AA (1194) AG (590) GG (89) X^2 HW test p = 0.16	63.7 31.5 4.8	61.1–65.4 29.2–33.4 3.76–5.68	0.21	–0.06	0.01	AA: 10.6 (10.3; 10.8)$^{\partial}$ AG/GG: 9.88 (9.54; 10.2)$^{\partial}$ ANCOVA p = 0.004	0.16
						Genetic model: 0/1/1			
CBS	T833C; Ile278Thr (rs5742905; 21:43063074)	TT (997) TC (746) CC (138) X^2 HW test p = 0.97	53 39.7 7.3	50.6–55.1 37.3–41.7 6.14–8.49	0.27	–0.02	0.41	10.5 (10.2; 10.7) 10.2 (9.87; 10.5) ANCOVA p = 0.16	0.07
						Genetic model: 0/1/1			
	844ins68 indel (no rs#)	Homozygous non-insert (WT) (998) Heterozygous (748) Homozygous insert (MT) (136) X^2 HW test p = 0.97	53 39.8 7.2	50.7–55.2 37.4–41.8 6.04–8.38	0.27	–0.02	0.41	10.5 (10.2; 10.7) 10.2 (9.87; 10.5) ANCOVA p = 0.16	0.07
						Genetic model: 0/1/1			
	G9276A (novel SNP no rs#) 21:43071860	GG (977) GA (757) AA (146) X^2 HW test p = 0.89	51.9 40.3 7.8	49.5–54.0 37.9–42.3 6.53–8.95	0.28	–0.02	0.44	10.5 (10.2; 10.7) 10.2 (9.88; 10.5) ANCOVA p = 0.17	0.07
						Genetic model: 0/1/1			

Hcy concentrations are means adjusted for age and GGT (95% CI).

Significant ($p < 0.01^{\partial}$; $p < 0.001^{\gamma}$) differences between the subdivisions as indicated by post hoc test i.e. t tests, corrected for multiple comparisons.

A, alanine; Ala, alanine; ANCOVA, analyses of covariance; Asp, aspartic acid; C, cytosine; *CBS*, *cystathionine β synthase*; CI; confidence intervals; ES, effect size; G, guanine; Glu, glutamic acid; Gly, glycine; HW, Hardy Weinberg; Ile, isoleucine; ins, insertion; MAF, minor allele frequency; MT, mutant type; *MTHFR*, *methylenetetrahydrofolate reductase*; *MTR*, *methionine synthase*, rs, reference number; SNP, single-nucleotide polymorphism; T, thymine; Thr, threonine; Val, valine; WT, wild type.

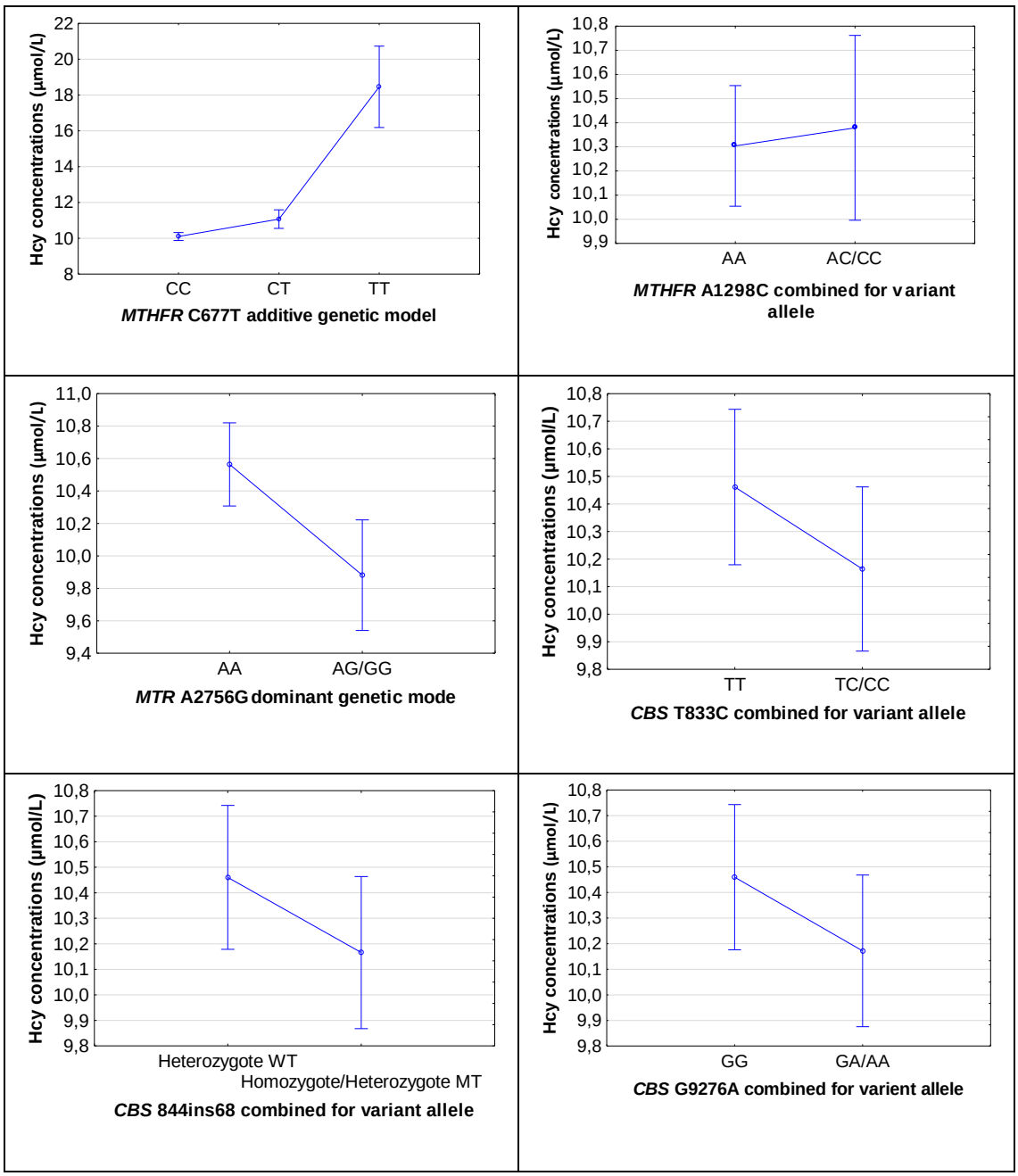

Figure 3-2: Visual representation of the relationship between specific SNPs involved in Hcy metabolism and total Hcy concentrations

A, adenine; C, cytosine; CBS, cystathionine β synthase; G, guanine; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; T, thymine.

3.2.5 Discussion

We hypothesized that certain individuals might be more susceptible to HHcy when their genetic make-up was combined with adverse dietary factors. Here we analyzed six genetic polymorphisms of Hcy-metabolizing enzymes [i.e. *MTHFR* C677T and A1298C, *MTR* A2756G, *CBS* T833C/844ins68 and G9276A] in approximately 2010 individuals, and quantified their interactions with dietary components in modulating Hcy concentrations. To our knowledge, this is the first time that the interacting relations of *MTHFR* A1298C and Hcy concentrations in a black South African adult cohort have been investigated. A brief description of our main findings follows. While examining the frequencies of certain SNPs in the *MTHFR*, *MTR* and *CBS* genes and their relationship with Hcy, we determined that individuals harboring the *MTHFR* 677TT and *MTR* 2756 AA genotypes presented with significantly higher Hcy concentrations when compared with other SNPs. We also observed that, when the *MTR* 2756 heterozygotes and homozygote minor alleles were combined, a definite reduction in Hcy concentrations occurred when compared with homozygote major allele carriers. Several gene–diet and gene–blood lipid interactions were observed during the statistical analysis [i.e *CBS* T833C/844ins68*HDL-c, *CBS* T833C/844ins68*protein intake (expressed as % of total energy), *CBS* T833C/844ins68*animal protein intake, *MTHFR* C677T*added sugar intake (expressed as % total carbohydrate intake) and *CBS* T833C/844ins68*biotin intake]. In our study, the Spearman's rho values showed a positive correlation with alcohol intake and Hcy concentrations. For a detailed discussion on this matter please refer to Nienaber-Rousseau *et al.* [33].

Even though the gender difference of Hcy was significant and men had higher Hcy concentrations than women did, no gene–gender interactions were observed in our population. Our finding is in contrast to the study by Kluijtmans *et al*. [19]. They reported that, when subdividing the population in quartiles based on folate status for the different genders, a divergent impact of the *MTHFR* 677TT genotype on Hcy concentrations was observed [19]. Men had higher Hcy concentrations than women in the lowest quartile of folate status [19]. Some studies [19,34] suggest that there may be fundamental differences in the interactions between nutritional and genetic variables between the sexes with respect to the elicited biochemical phenotypes.

The Hordaland Hcy study [35,36] reported that high intakes of SFA were associated with high plasma Hcy concentrations; we, however, found that added sugar expressed as a % of carbohydrate intake, total fat and SFA intake ($p < 0.01$) had a negative correlation with Hcy, where a higher dietary intake led to lower Hcy concentrations. To explain this unexpected phenomenon, we reviewed previous studies, which indicated that a higher sugar and saturated fat intake in this particular population indicated a higher socio-economic status which, in turn,

led to an improved micronutrient status and better overall diet quality [22,37]. Improved diet quality and micronutrient intake assists in lowering Hcy concentrations, which explains the negative correlation observed in our study.

The *CBS* T833C/844ins68 polymorphisms had the most interactions with blood lipids, protein and vitamin intake in relation to Hcy when compared with the other SNPs. The minor allele carriers of *CBS* T833C/844ins68 presented with lower Hcy concentrations as HDL-c blood lipids increased in the study participants. In the same minor allele carriers, an increase in Hcy concentrations was observed as total dietary protein and animal protein intake increased ($p<0.001$; $p = 0.02$), respectively; however, Hcy decreased in the major homozygote TT and heterozygote CT groups when consumption was high. Biotin was the only vitamin in our investigation that had a significant interaction in modulating Hcy concentrations ($p = 0.04$), where homozygotes of the major T allele indicated that elevated biotin intake was associated with lowered Hcy, whereas Hcy was elevated in those harboring the homozygous CC minor allele genotype.

Previous studies support our findings that plasma Hcy is significantly and inversely correlated with HDL-c [38-42]. However, to our knowledge, none exist which took into account the *CBS* polymorphism when investigating the correlation between Hcy and HDL-c. Some examinations focused on CBS enzyme deficiency and determined that HHcy was more pronounced when serum levels of HDL-c were low [39,40,43]. Three mechanisms have been identified which inhibit HDL-c biosynthesis in HHcy and reverse cholesterol transport, which leads to the negative correlation with Hcy. The first is a reduction in HDL-c large particle formation as a result of hepatic apoA-I protein synthesis or secretion inhibition, which, in turn, suppresses lecithin:cholesterol acyltransferase activity. The second mechanism enhances HDL-c clearance via hepatic class B, type 1 scavenger receptor (SR-B1) up-regulation; and the third limits HDL-c synthesis *via* inhibition of HDL-c function and cholesterol efflux. As with the *CBS* T833C/844ins68, the well-known *MTHFR* C677T polymorphism minor allele carriers presented with lower Hcy concentrations as HDL-c blood lipids increased. A previous study also found a significant correlation between plasma Hcy concentrations and plasma HDL-c, where subjects with the TT genotype had higher plasma Hcy values in association with lower HDL-c levels [44], which supports our observation. Therefore, high HDL-c concentrations could alleviate HHcy often observed in those harboring the 677TT genotype.

The literature indicated that serum Hcy concentrations are inversely correlated with daily total protein intake [45], which is in line with what we observed in the major homozygote TT and heterozygote CT allele carriers of *CBS* T833C/844ins68. We hypothesize that, for those harboring the T allele, prudent daily intake of protein has a protective effect on plasma Hcy concentrations as a result of protein-originated vitamin action. This postulation is supported by

our finding that elevated biotin intake, which is derived mainly from protein intake, decreases Hcy concentrations in the major TT allele of the *CBS* T833C/844ins68 polymorphism.

One of the other interactions observed for *MTHFR* C677T and Hcy was with added sugar as a % of energy intake from carbohydrates, where the same minor TT allele carriers presented with a noticeable increase in Hcy concentrations as sugar intake increased, which again underscores the potential benefits that may be gained by improving the dietary control of the glycemic milieu and a decreased intake of added sugar. Limited evidence on interactions between sugar intake and Hcy concentrations is available, especially for gene–sugar intake, which creates a possible research opportunity for future studies. Studies exploring nutrigenetic effects with regard to *MTHFR* C677T have to date focused on vitamin B_2, folate and vitamin B_{12} intake and their effects on Hcy concentrations. We, however, explored other dietary factors which are less known or have not been previously investigated.

Here we contribute to the body of evidence underpinning Hcy nutrigenetics in a black South African population by using a large data set. However, our Tswana population is not representative of all black South Africans and future studies should include other black South African ethnicities as well and preferably increase the sample size. While previous international research focused on vitamin B_2, folate and vitamin B_{12}, our paper investigated biotin and other neglected dietary factors as possible modulators of Hcy concentrations, thereby extending existing knowledge. To complement our work, researchers should use blood measures of vitamin intake in order to account for differences in bioavailability, absorption and metabolism, which is not possible when using only intake from questionnaire data.

It is important to identify factors that can affect the balance of Hcy concentrations to help us understand the pathophysiology of diseases dependent on Hcy. By understanding gene–diet interactions in terms of prediction of therapeutic efficacy, nutrigenetic studies can provide valuable information to tailor dietary advice for those who are more genetically susceptible to disease. Our study supports the use of genetic information to guide an individual's diet therapy; such information may have implications for health care workers, especially doctors and dieticians, when they treat HHcy.

3.2.6 Conclusion

The associations of the polymorphisms on Hcy are modulated by diet, which implies that genotype-guided dietary intake might be warranted and can be used to treat HHcy. For the general population, biotin consumption reduces Hcy concentrations, whereas for those homozygous for the rare minor allele of *MTHFR* and *CBS* polymorphisms, consumption of this vitamin does not seem to have this benefit. The minority of the population harboring the minor

allele of the *MTHFR* and *CBS* SNPs will, however, benefit in terms of Hcy from increasing levels of HDL-c, which is inversely associated with Hcy concentrations and interacts with these SNPs to lower Hcy. Moderate to high protein intake as well as a healthy nutritional status presented with a protective action against increasing Hcy concentrations. Whether dietary manipulations of Hcy in the presence of certain genetic characteristics will result in disease reduction still needs to be determined in future studies. As described here, new opportunities for improved risk-stratification tailored to treat HHcy in population subtypes according to their genotype are likely to emerge in the future.

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L.Z. performed the genotyping of the *MTHFR* A1298C SNP together with J.P.vS., helped with the interpretation of the results, read and corrected the manuscript;

C.N-R. isolated the DNA, performed the genotyping of the *MTHFR* C677T, *MTR A*2756G, *CBS* T833C/844ins68 and CBS G9276A, conceptualized the paper, performed the statistical analysis with J.P.vS, and was involved in the writing of the manuscript.

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CHAPTER 4

SUMMARY AND RECOMMENDATIONS

4.1 Introduction

From a gene–diet perspective, the investigation of diet and dietary-related factors in relation to homocysteine (Hcy) is of cardinal importance because Hcy has been associated with various non-communicable health problems (Huang *et al.*, 2013; Numata *et al.*, 2015; Peng *et al.*, 2015; Wang *et al.*, 2014; Zhang *et al.*, 2014; Zintzaras, 2010). Since Hcy is of clinical significance in preventing disease, gene–diet interactions in relation to Hcy open up a treatment modality for clinicians and dietitians. However, little is known about the gene–diet interaction in modulating Hcy and even less information is available on persons of African descent. To this end, we aimed to clarify the impact of nutrigenetics on Hcy concentrations, taking into consideration certain single-nucleotide polymorphisms (SNPs) in the *methylenetetrahydrofolate reductase* (*MTHFR*), *cystathionine β-synthase* (*CBS)* and *methionine synthase* (*MTR*) genotypes, together with dietary intake and related factors, in 2010 mainly Tswana-speaking black South African adults. We addressed the question of whether interactive effects between certain genetic variants (*i.e*. *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, *CBS* T833C, *CBS* 844ins68 and *CBS* G9276A) and markers of nutritional status (anthropometry, biochemical variables *i.e*. blood lipids, glycated haemoglobin (HbA1c) and fasting glucose and dietary components) existed in relation to Hcy concentrations. Five of the SNPs used in this mini-dissertation have previously been genotyped for this particular population group belonging to the North West arm of the Prospective Urban and Rural Epidemiological (PURE) study; however, the *MTHFR* A1298C polymorphism was not included. Consequently, we established the *MTHFR* A1298C SNP distribution and included it in our investigation with the other five genetic variations previously genotyped. Thus, our study is the first to investigate the interacting relations of the five SNPs and *MTHFR* A1298C in diet, excluding alcohol intake (previously reported alcohol-*MTHFR* C677T interaction in (Nienaber-Rousseau *et al.*, 2013)) in modulating Hcy concentrations in a group of Tswana adults.

Multi-faceted approaches on Hcy research such as the work presented within this mini-dissertation will assist in leading to a better understanding of gene–diet interactions related to hyperhomocysteinaemia (HHcy) and might in future assist in identifying those who are at risk of or particularly susceptible to HHcy as a result of genetic manifestations combined with environmental insult. Additionally, the research presented here raises the possibility of using the human genome for precision nutrition therapy based on individual needs and optimising for health by preventing or treating diseases influenced by HHcy.

4.2 Summary, conclusions and recommendations

In our initial hypothesis in Chapter 1, which was based on earlier research, we estimated that certain vitamins essential for Hcy metabolism, together with other nutrients, dietary factors and diet-related factors (HbA1c, blood lipids) of nutrition status, might interact with certain genotypes in genes coding for Hcy-metabolising enzymes, and in doing so, influence Hcy concentrations.

Chapter 2 offered a review of the relevant literature. In this chapter we named the diseases that are related to HHcy. The biochemical functions and metabolism of Hcy were also revised and a fourth fundamental pathway of Hcy was identified in the literature. We also indicated that a variety of nutrition and genetic factors are important determinants of Hcy concentrations. During the review we discovered that limited data were available on gene–diet interactions in relation to Hcy and that research such as we proposed and presented here is lacking.

Chapter 3 presented a nutrigenetic approach to bridge the gap in current research and to extend the body of literature to include results on individuals of African descent. Our research highlighted the fact that certain genes were associated with altered Hcy concentrations and observed that, with increasing numbers of the minor T allele of the *MTHFR* C677T SNP, Hcy increased significantly. Hcy was lower, however, in the *MTR* 2756AA homozygotes than in AC heterozygotes. Overall, those individuals harbouring the *CBS* 833T/844ins68 SNP had the lowest Hcy concentrations when compared with the other SNPs. Therefore, individuals who are carriers of the *MTHFR* 677TT and *MTR* 2756AC genotypes are more prone to HHcy when compared with those harbouring the *MTR* 2756AA genotypes and any variation of the *CBS* 833T/844ins68 polymorphism.

We also observed that Hcy concentrations were further modulated by diet. Even though the *MTHFR* 677 homozygous minor carriers are more inclined to HHcy, we established an inverse association between Hcy concentrations and HDL-c levels. The CC variant of *CBS* T833C/844ins68 had the same association with HDL-c, which indicates that those individuals harbouring the *MTHFR* 677TT and *CBS* 833CC genotypes, compared with other genetic modulations, will possibly benefit from elevated HDL-c concentrations in lowering plasma Hcy. The CC genotype of the *CBS* 833 gene was associated with elevated Hcy concentrations with increasing non-animal and animal protein intakes, but was lowered in those harbouring the major allele at this locus. Those homozygous for the minor allele of the *MTHFR* C677T SNP had elevated Hcy concentrations when sugar intake increased. Also, when biotin intake increased, *CBS* 833 TT carriers' plasma Hcy concentrations decreased in comparison with the CC genotype, where biotin intake had a positive relation with Hcy concentrations.

Contrary to our expectations (as hypothesised in Chapter 1 and observed in previous literature presented in Chapter 2), no associations were found for any of the well-known vitamins (folate, vitamins B_2, B_6 and B_{12}) needed for metabolism of Hcy; however, significant associations were observed for another vitamin, biotin, which has never been investigated in relation to Hcy and genetic factors involved in Hcy metabolism. Other dietary factors that are similarly less known to modulate Hcy (protein and sugar intake) and a diet-related factor (HDL-c) were also of crucial importance in our findings. This opens the possibility for future investigations to build on our findings and expand the nutrigenetic observation by conducting follow-up clinical trials.

These gene–diet and gene dietary-related results indicate that different genotypes will benefit from different dietary treatment. Increased intakes of polyunsaturated fatty acids (PUFAs), especially through fish oil, have a positive correlation with HDL-c levels (Bernstein *et al.*, 2011; Dewailly *et al.*, 2001; Eslick *et al.*, 2009; Nilsen *et al.*, 2001). Therefore, those harbouring the *CBS* 833CC genotype might benefit by consuming more omega 3 fatty acid sources in their diet to increase HDL-c and thereby exert positive effects on Hcy. However, an omega 3 rich diet will benefit most individuals. Similarly, lowering protein in their diet and thereby indirectly also lowering biotin intake (protein is a source of biotin) will also be beneficial in decreasing Hcy concentrations. However, protein intake should not be lower than RDA of 0.8g/kg and the appropriate level to which protein should be decreased to exert beneficial effects in terms of Hcy has to be determined in intervention trails. To possibly decrease plasma Hcy in those with the *MTHFR* TT genotype, a diet high in omega 3 PUFA combined with lowered sugar intake could be followed. The *CBS* 833TT genotype, in turn, may benefit from high intakes of lean protein and biotin in the diet to lower Hcy concentrations. These dietary suggestions are based on the research findings presented in Chapter 3 of our study although further investigations are needed to support our data.

In conclusion, elevated Hcy concentrations play a crucial role in the pathogenesis of various diseases, which makes this amino acid an ideal target for future investigations. In-depth studies are needed to: (i) understand the important role of precision nutrition which will help reduce Hcy concentrations by adding or removing certain nutrients from the diet; (ii) predict the significance of Hcy concentrations as biomarker or risk factor (along with other factors such as age, gender, diet and genetic variants) for development or progression of certain diseases; (iii) increase the body of evidence for gene–nutrient interactions with Hcy to assist in correctly utilising information given by direct to consumer tests.

Even though the field is still not fully explored, most direct to consumer genetic testing includes Hcy-related SNPs and the fact that information about individuals of African descent is especially lacking creates a problem for giving evidence-based advice to patients. In this regard, Gillies (2003) warned that we should temper the excitement and promise of molecular nutrition with the

need to validate the scientific data emerging from the nutrigenomic and nutrigenetic disciplines. He added that the need to educate practitioners and communicate the value to consumers – and to do it all within a socially responsible bioethical framework – will be a challenge. In order to heed the warning of Gillies (2003), more research, such as the work presented here, is needed to give evidence-based nutrigenetic advice.

4.3 References

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ADDENDUM A

Instructions to the author – *Nutrients*

MANUSCRIPT SUBMISSION OVERVIEW

Types of Publications

Nutrients has no restrictions on the length of manuscripts, provided that the text is concise and comprehensive. Full experimental details must be provided so that the results can be reproduced. *Nutrients* requires that authors publish all experimental controls and make full datasets available where possible (see the guidelines on Supplementary Materials and references to unpublished data).

Manuscripts submitted to *Nutrients* should neither been published before nor be under consideration for publication in another journal. The main article types are as follows:

- *Articles:* Original research manuscripts. The journal considers all original research manuscripts provided that the work reports scientifically sound experiments and provides a substantial amount of new information. Authors should not unnecessarily divide their work into several related manuscripts, although Short *Communications* of preliminary, but significant, results will be considered. Quality and impact of the study will be considered during peer review.
- *Reviews:* These provide concise and precise updates on the latest progress made in a given area of research. Systematic reviews should follow the PRISMA guidelines.
- *Case reports:* Case reports present detailed information on the symptoms, signs, diagnosis, treatment (including all types of interventions), and outcomes of an individual patient. Case reports usually describe new or uncommon conditions that serve to enhance medical care or highlight diagnostic approaches.

Submission Process

Manuscripts for *Nutrients* should be submitted online at susy.mdpi.com. The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting author must ensure that all eligible co-authors have been included in the author list (read the criteria to qualify for authorship) and that they have all read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to the submission website. All co-authors can see the manuscript

details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

Accepted File Formats

Authors must use the Microsoft Word template or LaTeX template to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. The total amount of data for all files must not exceed 120 MB. If this is a problem, please contact the editorial office nutrients@mdpi.com. Accepted file formats are:

- *Microsoft Word:* Manuscripts prepared in Microsoft Word must be converted into a single file before submission. When preparing manuscripts in Microsoft Word, the *Nutrients* Microsoft Word template file must be used. Please insert your graphics (schemes, figures, *etc.*) in the main text after the paragraph of its first citation.
- *LaTeX:* Manuscripts prepared in LaTeX must be collated into one ZIP folder (include all source files and images, so that the Editorial Office can recompile the submitted PDF). When preparing manuscripts in LaTeX, please use the *Nutrients* LaTeX template files. You can now also use the online application writeLaTeX to submit articles directly to *Nutrients*. The MDPI LaTeX template file should be selected from the writeLaTeX template gallery.
- *Supplementary files:* May be any format, but it is recommended that you use common, non-proprietary formats where possible (see below for further details).

Cover Letter

A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work and why it fits the scope of the journal. Confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal. Any prior submissions of the manuscript to MDPI journals must be acknowledged. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

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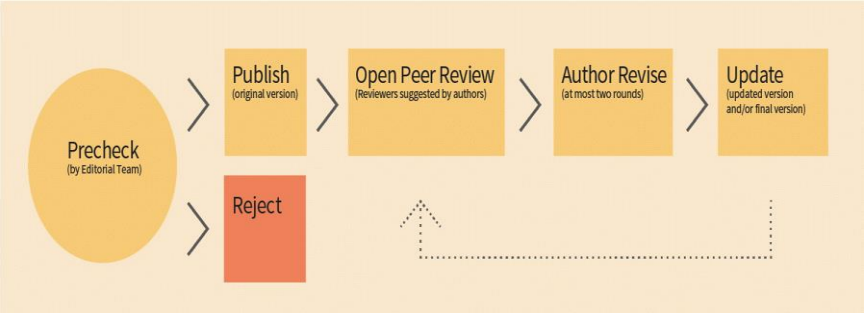
This journal automatically deposits papers to PubMed Central after publication of an issue. Authors do not need to separately submit their papers through the NIH Manuscript Submission System (NIHMS, http://nihms.nih.gov/).

Publishing Process



It is an open publishing model. Papers will be published online immediately once the Editorial Team—including our Advisory Board comprised of high level experts in particular fields—approve a pre-check. All original versions, final versions and/or updated versions, together with the review reports, will be published and can be tracked and indexed. For peer review, authors must provide at least three review candidates for their papers during online submission with our Editorial Team.

Below are the criteria for peer reviewers.

1. Hold at least a Ph.D. degree (there as some fields, e.g. medicine and the arts, where this is not strictly required).
2. Have published >5 papers in last 5 years in the field of the submitted paper
3. No conflict of interest with authors (see below for further details)
4. Have a verified email address, i.e., one that appears on their institution's website or a published paper
5. Reviewers cannot belong to the same university or institution

We consider the following situations a conflict of interest:

- The referee has published with the authors or one of the co-authors in the last three years
- The referee has worked on or financially supported research projects where the author or one of the co-authors was involved in the last three years
- The referee is from the same institution as the author or one of the co-authors
- The referee is related to the author or one of the authors (e.g. spouse, child, etc.)

MANUSCRIPT PREPARATION

General Considerations

- **Research manuscripts** should comprise:
    - Front matter: Title, Author list, Affiliations, Abstract, Keywords
    - Research manuscript sections: Introduction, Materials and Methods, Results, Discussion, Conclusions (optional).
    - Back matter: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, References.
- **Review manuscripts** should comprise the front matter, literature review sections and the back matter. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure. Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the PRISMA guidelines.
- **Case reports** should include a succinct introduction about the general medical condition or relevant symptoms that will be discussed in the case report; the case presentation including all of the relevant de-identified demographic and descriptive information about the patient(s), and a description of the symptoms, diagnosis, treatment, and outcome; a discussion providing context and any necessary explanation of specific treatment decisions; a conclusion briefly outlining the take-home message and the lessons learned.
- **Graphical abstract:** Authors are encouraged to provide a graphical abstract as a self-explanatory image to appear alongside with the text abstract in the Table of Contents. Figures should be a high quality image in any common image format. Note that images displayed online will be up to 11 by 9 cm on screen and the figure should be clear at this size.
- **Abbreviations** should be defined in parentheses the first time they appear in the abstract, main text, and in figure or table captions and used consistently thereafter.
- **SI Units** (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible
- **Accession numbers** of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on Deposition of Sequences and of Expression Data.

- **Equations:** If you are using Word, please use either the Microsoft Equation Editor or the MathType add-on. Equations should be editable by the editorial office and not appear in a picture format.
- **Research Data and supplementary materials:** Note that publication of your manuscript implies that you must make all materials, data, and protocols associated with the publication available to readers. Disclose at the submission stage any restrictions on the availability of materials or information. Read the information about Supplementary Materials and Data Deposit for additional guidelines.
- **Preregistration:** Where authors have preregistered studies or analysis plans, links to the preregistration must be provided in the manuscript.
- **Guidelines and standards:** MDPI follows standards and guidelines for certain types of research. See http://www.mdpi.com/editorial_process for further information.

Front Matter

These sections should appear in all manuscript types

- **Title:** The title of your manuscript should be concise, specific and relevant. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used.
- **Author List and Affiliations:** Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, country, and all email addresses. At least one author should be designated as corresponding author, and his or her email address and other details should be included at the end of the affiliation section. Please read the criteria to qualify for authorship.
- **Abstract:** The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used. 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the article: it must not contain results which are not presented and substantiated in the main text and should not exaggerate the main conclusions.

- **Keywords:** Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

Research Manuscript Sections

- **Introduction:** The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance, including specific hypotheses being tested. The current state of the research field should be reviewed carefully and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the main conclusions. Keep the introduction comprehensible to scientists working outside the topic of the paper.
- **Materials and Methods:** They should be described with sufficient detail to allow others to replicate and build on published results. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited. Give the name and version of any software used and make clear whether computer code used is available. Include any pre-registration codes.
- **Results:** Provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.
- **Discussion:** Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible and limitations of the work highlighted. Future research directions may also be mentioned. This section may be combined with Results.
- **Conclusions:** This section is not mandatory, but can be added to the manuscript if the discussion is unusually long or complex.
- **Patents:** This section is not mandatory, but may be added if there are patents resulting from the work reported in this manuscript.

Back Matter

- **Supplementary Materials:** Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.
- **Acknowledgments:** All sources of funding of the study should be disclosed. Clearly indicate grants that you have received in support of your research work and if you

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- **Author Contributions:** Each author is expected to have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; or have drafted the work or substantively revised it; AND has approved the submitted version (and version substantially edited by journal staff that involves the author's contribution to the study); AND agrees to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature.
  For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "X and Y conceived and designed the experiments; X performed the experiments; Y analyzed the data; Y wrote the paper." **Authorship must include and be limited to those who have contributed substantially to the work. Please read the section concerning the criteria to qualify for authorship carefully.**

- **Conflicts of Interest:** Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results must be declared in this section. If there is no role, please state "The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results".

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material. Include the digital object identifier (DOI) for all references where available. If available online, you may use reference style 9. below.

- Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

The reference list should include the full title, as recommended by the ACS style guide. Style files for Endnote and Zotero are available.

References should be described as follows, depending on the type of work:

Journal Articles:
1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* **Year**, *Volume*, page range, DOI. Available online: URL (accessed on Day Month Year).

Books and Book Chapters:
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3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A., Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196; ISBN.

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Conference Proceedings:
7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In *Title of the Collected Work* (if available), Proceedings of the Name of the Conference, Location of Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).

Thesis:
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- File for Figures and schemes must be provided during submission in a single zip archive and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.
- *Nutrients* can publish multimedia files in articles or as supplementary materials. Please contact the editorial office for further information.
- All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, *etc.*).
- All Figures, Schemes and Tables should have a short explanatory title and caption.
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Supplementary Materials, Data Deposit and Software Source Code

Data Availability

In order to maintain the integrity, transparency and reproducibility of research records, authors must make their experimental and research data openly available either by depositing into data repositories or by publishing the data and files as supplementary information in this journal.

Computer Code and Software

For work where novel computer code was developed, authors should release the code either by depositing in a recognized, public repository or uploading as supplementary information to the publication. The name and version of all software used should be clearly indicated.

Supplementary Material

Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. Any file format is acceptable, however we recommend that common, non-proprietary formats are used where possible.

Unpublished Data

Restrictions on data availability should be noted during submission and in the manuscript. "Data not shown" should be avoided: authors are encouraged to publish all observations related to the submitted manuscript as Supplementary Material. "Unpublished data" intended for publication in a manuscript that is either planned, "in preparation" or "submitted" but not yet accepted, should be cited in the text and a reference should be added in the References section. "Personal Communication" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

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Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of repositories specialized in scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal Data also accepts submissions of data set papers.

Deposition of Sequences and of Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- *New nucleic acid sequences* must be deposited in one of the following databases: GenBank, EMBL, or DDBJ. Sequences should be submitted to only one database.
- *New high throughput sequencing (HTS) datasets* (RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the GEO database or in the NCBI's Sequence Read Archive.
- *New microarray data* must be deposited either in the GEO or the ArrayExpress databases.The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.
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All sequence names and the accession numbers provided by the databases should be provided in the Materials and Methods section of the article.

References in Supplementary Files

Citations and References in Supplementary files are permitted provided that they also appear in the reference list of the main text.

RESEARCH AND PUBLICATION ETHICS

Research Ethics

Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/), revised in 2008. According to point 23 of this declaration, an approval from an ethics committee should have been obtained before undertaking the research. At a minimum, a statement including the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods Section of the article. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). Editors reserve the right to reject any submission that does not meet these requirements.

Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

A written informed consent for publication must be obtained from participating patients who can be identified (including by the patients themselves). Patients' initials or other personal identifiers must not appear in an image. For manuscripts that include any case details, personal information, and/or images of patients, authors must obtain signed informed consent from patients (or their relatives/guardians) before submitting to an MDPI journal. Patient details must be anonymized as far as possible, e.g., do not mention specific age, ethnicity, or occupation where they are not relevant to the conclusions.

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Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

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minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods section.

Nutrients endorses the ARRIVE guidelines (www.nc3rs.org.uk/ARRIVE) for reporting experiments using live animals. Authors and reviewers can use the ARRIVE guidelines as a checklist, which can be found at www.nc3rs.org.uk/ARRIVEchecklist.

1. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Used in Scientific Procedures. Available online: http://www.official-documents.gov.uk/document/hc8889/hc01/0107/0107.pdf.

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

Publication Ethics Statement

Nutrients is a member of the Committee on Publication Ethics (COPE). We fully adhere to its Code of Conduct and to its Best Practice Guidelines.

The editors of this journal enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *Nutrients* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *Nutrients* must abide to the following:

- Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.
- Authors should accurately present their research findings and include an objective discussion of the significance of their findings.
- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.
- Simultaneous submission of manuscripts to more than one journal is not tolerated.
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Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

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Each author is expected to have made substantial contributions to the conception or design of the work; acquisition, analysis, or interpretation of data; the creation of new software used in the work; and/or writing or substantively revising the manuscript. In addition, all authors must have approved the submitted version (and any substantially modified version that involves the author’s contribution to the study); AND agrees to be personally accountable for the author’s own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even those in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature. Note that acquisition of funding, collection of data, or general supervision of the research group do not, by themselves, justify authorship.Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgements.

More detailed guidance on authorship is given by the International Council of Medical Journal Editors (ICMJE). The journal also adheres to the standards of the Committee on Publication Ethics (COPE) that "all authors should agree to be listed and should approve the submitted and

accepted versions of the publication. Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication (e.g. answering reviewers' comments)." [1]. We reserve the right to request confirmation that all authors meet the authorship conditions.

1. Wager, E.; Kleinert, S. Responsible research publication: international standards for authors. A position statement developed at the 2nd World Conference on Research Integrity, Singapore, July 22-24, 2010. In Promoting Research Integrity in a Global Environment; Mayer, T., Steneck, N., eds.; Imperial College Press / World Scientific Publishing: Singapore; Chapter 50, pp. 309-16.

EDITORIAL PROCEDURES AND PEER-REVIEW

Initial Checks

All submitted manuscripts received by the Editorial Office will be checked by a professional in-house *Managing Editor* to determine whether they are properly prepared and whether they follow the ethical policies of the journal, including those for human and animal experimentation. Manuscripts that do not fit the journal's ethics policy or do not meet the standards of the journal will be rejected before peer-review. Manuscripts that are not properly prepared will be returned to the authors for revision and resubmission. After these checks, the *Managing Editor* will consult the journals' *Editor-in-Chief*, *Associate Editor*, or *Guest Editor* (or an *Editorial Board member* in case of a conflict of interest) to determine whether the manuscript fits the scope of the journal and whether it is scientifically sound. No judgment on the significance or potential impact of the work will be made at this stage. Reject decisions at this stage will be verified by the *Editor-in-Chief*.

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind review is applied, where authors' identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

In the case of regular submissions, in-house assistant editors will invite experts, including recommendations by an academic editor. These experts may also include *Editorial Board members* and Guest Editors of the journal. In the case of a special issue, the *Guest Editor* will advise on the selection of reviewers.

Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past five years and should not currently work or collaborate with any of the institutions of the co-authors of the submitted manuscript.

Editorial Decision and Revision

Based on the comments and advice of the peer-reviewers, an external editor–usually an Editorial Board Member or a *Guest Editor*–will make a recommendation to accept, reject, or to ask authors to revise the manuscript. The final decision is made by an Associate Editor or the Editor-in-Chief.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments. The *Managing Editor* of the journal will forward the manuscript and related information (including the identities of the referees) to the Editor-in-Chief, Associate Editor, or Editorial Board member. The academic Editor being consulted will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. A reject decision at this stage is final and cannot be reversed.

In the case of a special issue, the *Managing Editor* of the journal will forward the manuscript and related information (including the identities of the referees) to the *Editor-in-Chief* who will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. A reject decision at this stage will be final and cannot be reversed.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the www.mdpi.com website.

CLINICAL TRIALS REGISTRATION

Registration

Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register or and to cite a reference to the registration in the Methods section. Suitable databases include clinicaltrials.gov, the EU Clinical Trials Register and those listed by the World Health Organisation International Clinical Trials Registry Platform.

CONSORT Statement

Nutrients requires a completed CONSORT 2010 checklist and flow diagram as a condition of submission when reporting the results of a randomized trial. Templates for these can be found here or on the CONSORT website (http://www.consort-statement.org) which also describes several CONSORT checklist extensions for different designs and types of data beyond two group parallel trials. At minimum, your article should report the content addressed by each item of the checklist. Meeting these basic reporting requirements will greatly improve the value of your trial report and may enhance its chances for eventual publication.

ADDENDUM B

PURE-SA Project
INFORMED CONSENT FORM (PHASE 1)

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

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

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

……..
(Signature of the subject)

Signed at ..... on ……..

Witnesses

1. .....

2. ….....

Signed at on ……

ADDENDUM C

PURE-SA Project
INFORMATION TO THE COMMUNITIES

Dear Participant

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

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

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

ADDENDUM D

PURE-SA project

INFORMATION TO COMMUNITIES

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Kind regards

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

ADDENDUM E

PURE-SA Project
INFORMED CONSENT FORM (Phase 2)

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

I agree to be tested for HIV	Yes	No
I want to know my HIV-status	Yes	No
I agree to give a blood sample	Yes	No

I hereby also declare that I am aware that:

1. this blood sample will be used for the purpose of
    a. Isolating DNA to look at genetic factors that are currently associated with Type 2 Diabetes (i.e. the Calpain10, Adiponectin, Leptin and Leptin Receptor genes), or genetic factors that may be associated with non-communicable diseases in the future. We give the assurance that all genetic tests and experiments will only focus on genotypes suspected to contribute to an increased risk of non-communicable diseases of lifestyle.
    b. Testing for liver function by determining liver enzymes such as AST, GGT,
    c. Analyses of other than genetic parameters for Diabetes Mellitus such as HbA_1C, Blood glucose and Insulin
    d. Analyses of clotting factors and hypertension markers
    e. Analyses of bone health, iron and nutrition status
    f. And may be stored until such time as the above measurements/analyses will be done.
2. A two hour glucose tolerance test will be done
3. Body measurements such as height, weight, skinfold thicknesses, arm and leg circumferences will be taken
4. Electrocardiograph be taken
5. Blood pressure to be taken
6. Pulse wave velocity measurements will be made
7. A urine sample to be collected to analyse for the presence of heavy metals such as lead and mercury,
8. A Spirometer test to be performed to determine lung function
9. A handgrip test to be performed to test muscle strength
10. A hair sample to be taken to test for fumonisin mycotoxins.

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

Witnesses

1. ..... 2. .....

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

PART 1

1. School/Institute:
   5 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

6 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.

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

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

8 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.)
9
10 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.

11 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.

12 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.

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

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

PART 2

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

1. Participation in this project is voluntary.

2. It is possible that you personally will not derive any benefit from participation in this project, although the knowledge obtained from the results may be beneficial to other people.

3. You will be free to withdraw from the project at any stage without having to explain the reasons for your withdrawal. However, we would like to request that you would rather not withdraw without a thorough consideration of your decision, since it may have an effect on the statistical reliability of the results of the project.

4. The nature of the project, possible risk factors, factors which may cause discomfort, the expected benefits to the subjects and the known and the most probable permanent consequences which may follow from your participation in this project, are discussed in Part 1 of this document.

5. We encourage you to ask questions at any stage about the project and procedures to the project leader or the personnel, who will readily give more information. They will discuss all procedures with you.

6. The University staff will use standardised procedures and take all possible precaution to protect the subject from risks.

7. All information will be kept CONFIDENTIAL and no personal information will be published without my consent.

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

ADDENDUM F

The PURE-SA project

QUANTITATIVE FOOD FREQUENCY QUESTIONNAIRE

21 Subject ID Subject Initials

22 Centre #	**Community #**	**Household #**	**Subject #**	**F M L**
☐☐	☐☐☐☐	☐☐	☐☐	☐☐☐

Today's date: ☐☐☐☐ ☐☐ ☐☐
year *month* *day*

1. Name: _____ _____

2. Not applicable in South Africa

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

4. DOB: ☐☐☐☐ ☐☐ ☐☐ **OR Age** ☐☐ **years**

5. Sex: ☐ Female ☐ Male

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

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

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

There are no right or wrong answers.

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

Is there anything you want to ask now?

Are you willing to go on with the questions?

FOOD FREQUENCY QUESTIONNAIRE

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

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

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
24 PORRIDGE AND BREAKFAST CEREALS AND OTHER STARCH								
Maize-meal porridge	Stiff (pap)						3400	
Maize-meal porridge	Soft (slappap)						3399	
Maize-meal porridge	Crumbly (phutu)						3401	
Ting								
Mabella	Stiff						3437	
Mabella	Soft							
Oats							3239	
Other cooked porridge	Type: _____							
Breakfast cereals	Brand name of cereals at home now: _____ _____ _____ _____							
Do you pour milk on your porridge or cereal? Ye **1** No **2** If yes, what type of milk (whole fresh, sour, 1%, fat free, milk blend, etc) _____								
If yes, how much milk								

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
Do you put sugar on your porridge or cereal?		Ye 1		No 2				
If yes, how much sugar							3989	
							3989	
							3989	
Samp	Bought Self ground						3250	
Samp and beans	Give ratio of samp:beans						3402 (1:1)	
Samp and peanuts	Give ratio of samp:peanuts						3250 (samp)	
Rice	White						3247	
	Brown						3315	
	Maize Rice						3250	
Pasta	Macaroni Spaghetti Other specify: _____ _____						3262	
Pizza	Home made: Specify topping _____ _____						3353 (base+ch)	
	Bought: Specify topping _____ _____						3353 (base+ch)	

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

25 CHICKEN, MEAT, FISH

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

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
Chicken (codes with skin)	Boiled						2926	
	Fried: in batter/crumbs Eg Kentucky						3018	
	Fried: Not coated							
	Bought: Chicken Licken						2925	
	Bought: Nando's							
	Roasted / Grilled						2925	
	Other: _____							
Do you eat chicken skin?	Always **1**	Sometimes **2**	Never **3**					
Chicken bones stew								
Chicken feet							2997	
Chicken offal								
Red meat	How do you like meat? With fat Fat trimmed							
Red meat	Fried							
	Stewed							
	Mince with tomato and onion						2987	
	Other:							
Beef Offal	Intestines: boiled nothing added						3003	
	Stewed with vegetables							
	Liver						2920	
	Kidney						2923	
	Other: Specify _____ _____							
Goat meat	Boiled						4281	
	Stewed with vegetables							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
	Grilled / Roasted						4281	
27 What type of vegetables is usually put into meat stews? _____ _____								
Wors / Sausage							2931	
Bacon							2906	
Cold meats	Polony						2919	
	Ham						2967	
	Vienna						2936	
	Other: Specify _____ _____ _____							
Canned meat	Bully beef							
	Other: Specify _____ _____							
Meat pie	Beef						2939	
	Steak and kidney						2957	
	Cornish						2953	
	Chicken						2954	
	Other							
Hamburger	Bought							
Dried beans/peas/lentils	Soup						3145	
	Salad							
Soya products eg. Toppers	Brands at home now: _____ _____						3196 (Toppers)	
Pilchards in tomato/chilli/brine	Whole						3102	
	Mashed with fried onion							
Fried fish	With batter/crumbs							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
	Without batter/crumbs							
Other canned fish	Tuna						3056 (oil)	
	Pickled fish							
	Other: Specify _____							
Fish cakes	Bought: Fried						3080	
	Home made with potato						3098	
Fish fingers	Bought						3081	
Eggs	Boiled/poached						2867	
	Scrambled: milk + fat							
	Fried: Fat							
Now we come to vegetables and fruit **28 VEGETABLES AND FRUIT**								
Cabbage	How do you cook cabbage?							
	Boiled, nothing added						3756	
	Boiled with potato and onion and fat							
	Fried, nothing added Fried in ……							
	Boiled, then fried with potato, onion							
	Other:							
	Don't know							
Spinach/morogo/ beetroot leaves other green leafy	How do you cook spinach?							
	Boiled, nothing added						3913	
	Boiled with fat added Type of fat ……..							
	With onion, tomato, potato							
	With peanuts							
	Other:							
	Don't know							
Tomato and onion gravy	Home made with fat Type of fat ……..							
	Without fat						3925	

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
	Canned						4192	
Pumpkin (yellow)	How do you cook pumpkin?							
	Boiled, nothing added						4164	
	Cooked in fat and sugar Fat ……							
	Boiled, little sugar and fat Fat ……							
	Other							
	Don't know							
Carrots	How do you cook carrots?							
	Boiled, nothing added						3757	
	Boiled, sugar and fat Fat ……							
	With potato and onion: Fat							
	Raw, salad						3709	
	Chakalaka							
	Other							
	Don't know							
Mealies/ Sweet corn	How do you eat mealies?							
	On cob – fat added Fat ……							
	On cob – no fat added						3725	
	Creamed sweet corn / canned						3726	
	Whole kernel/canned						3942	
Beetroot	Salad						3699	
	Boiled, nothing added						3698	
Potatoes	How do you cook potatoes?							
	Boiled/baked with skin						4155	
	Boiled/baked without skin						3737	
	Mashed							
	Roasted Fat ……							
	French fries (chips)						3740	
Sweet potatoes	How do you cook sweet potatoes?							
	Boiled/baked with skin						3748	

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
	Boiled/baked without skin						3903	
	Mashed							
	Other: _____							
	Don't know							
Salad vegetables	Mixed salad: tomato, lettuce and cucumber						3921	
	Raw tomato						3750	
	Other salad vegetables: _____ _____							
Other vegetables, specify + preparation	_____ _____ _____							
Do you like fruit?		Ye 1	No 2					
Apples							3592	
Pears							3582	
Oranges							3560	
Naartjie							3558	
Grapes							3550	
Peaches	Fresh						3565	
	Canned						3567	
Apricots	Fresh						3534	
	Canned						3535	
Mangoes							3556	
Guavas	Fresh						3551	
	Canned						3553	
Avocado							3656	
Wild fruit/berries	Specify type: _____							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
Dried fruit	Types: _____							
Other fruit	_____ _____							
If subject eats canned fruit: Do you have custard with the canned fruit?			Ye		No			
Custard	Home made: Milk							
	Commercial eg Ultramel						2716	
29 BREAD AND BREAD SPREADS								
Bread / Bread rolls	White						3210	
	Brown						3211	
	Whole wheat						3212	
Do you spread anything on the bread?			Always **1**		Sometimes **2**		Never **3**	
Margarine	What brand do you have at home now?							
	Don't know _____							
Peanut butter							3485	
Jam/syrup/honey							3985	
Marmite / Fray bentos / Oxo							4058	
Fish/meat paste							3109	
Cheese	Type: _____ _____ _____							
Achaar								

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

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

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
	KoolAid						4027	
	Other: _____ _____							
Fruit juice	Fresh/Liquifruit/Ceres						2866	
	Tropica (Dairy –fruit juice mix)						2791	
	Other: _____ _____ _____							
Fizzy drinks	Sweetened						3981	
Coke, fanta, etc	Diet							
Maueu/Motogo							4056	
Home brew								
Tlokwe							4039	
Beer							4031	
Spirits							4035	
Wine red							4033	
Wine White							4033	
Other specify	_____ _____ _____							
31 SNACKS AND SWEETS								
Potato crisps							3417	
Peanuts	Raw						4285	
	Roasted						3458	
Cheese curls, Niknaks, etc							3267	

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
Raisins							3552	
Peanuts and raisins								
Chocolates	Name: _____ _____ _____							
Candies	Sugus, gums, hard sweets, etc						4000	
Sweets	Toffees, fudge, caramels						3991	
Biscuits/cookies	Type: _____ _____ _____							
Cakes and tarts	Type: _____ _____ _____							
Scones								
Rusks	Type: _____ _____							
Savouries	Sausage rolls						2939	
	Samoosas: Meat filling						3355	
	Samoosas: Vegetable filling						3414	
	Biscuits eg bacon kips							
	Other specify: _____							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
	_____							
Jelly							3983	
Baked pudding	Type: _____							
Instant pudding	Milk type: _____							
Ice cream							3483	
Sorbet							3491	
Other specify	_____ _____ _____							
32 SAUCES, GRAVIES AND CONDIMENTS								
Tomato sauce / Worcester sauce							3139	
Chutney							3168	
Pickles							3866	
Packet soups							3165	
Other:	_____ _____							
<u>WILD BIRDS, ANIMALS OR INCECTS</u> (hunted in rural areas or on farms)								
Wild fruit								
<u>MISCELLANEOUS:</u> Please mention any other foods used more than once/two times a week which we have talked about:								

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN				CODE	AMOUNT/ DAY
			Per day	Per week	Per month	Seldom / Never		
33 INDIGENOUS/TRADITIONAL FOODS/PLANTS/ANIMALS *34 Please tell me if you use any indigenous plants OR other indigenous foods like mopani worms, locusts ect to eat*								
Specify								