EVALUATION OF HIGH-THROUGHPUT METHODOLOGY FOR MULTI-GENE SCREENING IN PATIENTS WITH NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

BY

LESLIE REGINALD FISHER

Thesis presented in partial fulfilment of the requirements for the degree of Master of Medical Sciences in the Faculty of Health Sciences at Stellenbosch University.

Supervisor: Prof Maritha J Kotze

Co-supervisor: Dr FC Kruger

Faculty of Health Sciences

Department of Pathology

December 2011

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 11 October 2011

Summary

Non-Alcoholic Fatty Liver Disease (NAFLD) is the most prevalent chronic liver disease in Western countries and is considered the hepatic manifestation of the Metabolic Syndrome (MetS). Its heterogeneous nature ranges from hepatic steatosis through steatohepatitis to advanced fibrosis and cirrhosis where the ingestion of significant amounts of alcohol has been excluded. The disease profile of NAFLD and its necro-inflammatory subset Nonalcoholic Steatohepatitis (NASH) were described in the parent study, which provided a clinically well-characterised patient cohort for the present investigation. South African patients with NASH had significantly higher mean serum cholesterol and triglyceride levels than those with fatty liver only.

The objective of this study was to implement a high-throughput real-time polymerase chain reaction (PCR) method in our laboratory to enable the assessment of cardiovascular genetic risk factors in NAFLD patients. The specific aims were to determine the clinical utility and perform analytical validation of each mutation included in the multi-gene cardiovascular disease (CVD) screening assay. The Pathology Supported Genetic Testing (PSGT) concept developed at our department provides a practical approach to personalized medicine. The CVD multi-gene screen analyses key metabolic pathways relating to atherogenic dyslipidaemia, chronic inflammation, hypercoagulation and iron dysregulation implicated in insulin resistance, which is known to be a universal factor in the pathogenesis of NAFLD. Deleterious low-penetrance mutations in the APOE (APOE2 and E4 alleles), *MTHFR* (677C>T and 1298A>C), *F2* (20210G>A), *FV* (1691G>A, Leiden) and *HFE* (C282Y and H63D) genes were included for analysis due to their important role as genetic contributors to these biological processes. A total of 178 patients diagnosed with NAFLD and 75 controls were studied using direct DNA sequencing and a RT-PCR system for mutation detection. In addition, two patients with high ferritin levels were included as case studies.

A significant association was found between *HFE* mutations and elevated Alanine Transaminase (ALT) levels in the NAFLD population ($p = 0.04$). This discovery is interpreted as the identification of a subset of patients at greater risk of developing progressive liver damage who would benefit most from genetic testing to direct more aggressive therapy at an earlier stage. The necessity of an integrative, systems-based network approach was demonstrated to more accurately distinguish between Hereditary Haemochromatosis (HH) and Insulin Resistance-associated Hepatic Iron Overload (IR-HIO) syndrome in obese patients. The PSGT approach to personalized medicine facilitates diagnosis of CVD subtypes, prevention of cumulative risk and the formulation of gene-based intervention programs tailored to the needs of the patient.

These findings support the clinical utility of the CVD multi-gene test to guide chronic disease risk management in patients with NAFLD. The *HFE* mutation detection component of this test is of particular relevance in directing an effective treatment strategy in patients with a medical history of CVD and/or high iron stores.

Opsomming

Nie-Alkoholiese Vettige Lewer Siekte (NAFLD) is die mees algemene kroniese lewer siekte in Westerse lande en word bestempel as die hepatiese manifestasie van die Metaboliese Sindroom (MetS). Die heterogene natuur van NAFLD strek van hepatiese steatose deur steatohepatietis tot gevorderde fibrose en sirrose waar grootskaalse alkohol inname uitgesluit is. Die siekte-profiel van NAFLD en sy nekro-inflammatoriese subtipe Nie-Alkoholiese Steatohepatietis (NASH) is reeds beskryf in die ouer studie, wat ‘n klinies goed-gekarakteriseerde pasiënt groep vir die huidige ondersoek daar gestel het. Suid-Afrikaanse pasiënte met NASH het beduidend hoër gemiddelde serum cholesterol en trigliseried vlakke in vergelyking met slegs vettige lewer.

Die doel van hierdie studie was om ‘n hoë deurvoer rieëltyd polimerase kettingreaksie (RT-PCR) metode in ons laboratorium te implimenteer om kardiovaskulêre genetiese risiko faktore in NAFLD pasiënte te ondersoek. Die spesifieke mikpunte was om die kliniese nut en analitiese geldigheid van elke mutasie wat ingesluit is in die multi-geen kardiovaskulêre siekte (KVS) siftings toets vas te stel. Die Patologie Ondersteunde Genetiese Toetsing (PSGT) konsep wat by ons departement ontwikkel is, verskaf ‘n praktiese benadering tot persoonlike medisyne. Die KVS multi-geen toets analiseer belangrike metaboliese weë verwant aan atherogene dyslipidemie, kroniese inflammasie, oormatige bloedstolling en yster disregulering wat betrokke is by insulien weerstand wat bekend is as ‘n universele factor in the patogenese van NAFLD. Nadelige lae-penetrasie mutasies in die APOE (APOE2 en E4 allele), *MTHFR* (677C>T en 1298A>C) *F2* (20210G>A), *FV* (1691G>A, Leiden) en *HFE* (C282Y en H63D) gene was ingesluit vir analise as gevolg van hul belangrike rol as genetiese bydraers tot die bogenoemde biologiese prosesse. ‘n Totaal van 178 pasiënte gediagnoseer met NAFLD en 75 kontroles is bestudeer deur gebruik te maak van direkte DNA volgordebepaling en ‘n RT-PCR metode vir mutasie opsporing. Twee pasiënte met verhoogde ferritien vlakke is ook as gevalle studies ingesluit.

‘n Beduidende assosiasie is gevind tussen *HFE* mutasies en verhoogde Alanien Transaminase (ALT) vlakke in die NAFLD studiepopulasie ($p = 0.04$) wat aanduidend is van ‘n subgroup van pasiënte wat die meeste baat sal vind uit genetiese toetsing om meer aggressiewe behandeling te rig op' n vroeër stadium. Die noodsaaklikheid van 'n geïntegreerde, stelsels-gebaseerde netwerk benadering is gewys om meer akkuraat te onderskei tussen Oorerflike Hemochromatose (HH) en Insulien Weerstand-geassosieerde Hepatiese Yster Oorlading (IR-HIO) sindroom in vetsugtige pasiënte. Die PSGT benadering tot persoonlike medisyne formuleer geen-gebaseerde intervensie

programme aangepas tot die behoeftes van die pasiënt ek maak diagnose van KVS-subtipes en voorkoming van kumulatiewe risiko moontlik.

Hierdie bevindinge ondersteun die kliniese nut van die KVS multi-geen toets om riglyne vir die risikobestuur van kroniese siektes soos NAFLD daar te stel. Die *HFE* mutasie opsporings komponent van hierdie toets is van besondere belang om 'n effektiewe strategie vir die behandeling van pasiënte met 'n mediese geskiedenis van KVS en/of hoë yster vlakke daar te stel.

Table of Contents

List of Abbreviations and Symbols I
List of Figures VIII
List of Tables XIII
Acknowledgements XIV
Dedications XV

Chapter 1: Literature Review 1
1.1. Non-Alcoholic Fatty Liver Disease 2

1.2. NAFLD and the Metabolic Syndrome 3

1.3. NAFLD and Cardiovascular Disease 4

1.4. NAFLD and Hereditary Haemochromatosis 9
1.4.1. Hereditary Haemochromatosis 10
1.4.1.1. Genetic Classification 11

1.5. Genetic Testing for NAFLD 19
1.5.1. Apolipoprotein E (MIM ID +107741) 20
1.5.2. 5, 10-Methylenetetrahydrofolate Reductase (MIM ID *607093) 27
1.5.3. Coagulation Factor II (MIM ID *176930) 31
1.5.4. Coagulation Factor V (MIM ID *612309) 34

1.6. NAFLD and Environmental Factors 38
1.6.1. Iron and NAFLD 38
1.6.2. Alcohol and NAFLD 41

1.7. Diagnosis 41

1.8. Treatment 42

1.9. Aims and Objectives of this Study 43

Chapter 2: Detailed Experimental Procedures 44
2.1. Study Population 45

2.2. DNA Extraction 46
2.2.1. DNA extraction from Whole Blood using the QIAGEN QIAamp® DNA Blood Mini Kit (Spin protocol) 46
2.2.2. DNA extraction from Buccal Swabs using QIAGEN QIAamp® DNA Blood Mini Kit (Spin protocol) 47
2.2.3. DNA extraction from Whole Blood using the QIAGEN QIAamp® DNA Blood Midi Kit (Spin Protocol) 48
2.2.4. DNA extraction from saliva using the Oragene-DNA / Saliva Kit 49

2.3. DNA Quantification 50

2.4. Polymerase Chain Reaction Amplification 50
2.4.1. Oligonucleotide Primers 50
2.4.2. PCR Reaction Mixture and Thermal Cycling Parameters 52

2.5. Gel Electrophoresis 53

2.6. DNA Sequencing and Analysis 53

2.7. Real-Time Polymerase Chain Reaction Amplification 54
2.7.1. Applied Biosystems® *Taq*Man® SNP Genotyping Assays 54
2.7.2. Applied Biosystems® 7900HT 54

2.8. Statistical Analysis 54

Chapter 3: Results 55
3.1. Conventional Sequencing – Gels and Electropherograms 56

3.2. RT-PCR Genotyping with the ABI™ 7900HT 62

3.3. Comparative Analysis in NAFLD Patients 79
3.3.1. Allelic and Genotype Distributions 80

3.3.2. Genotype-Phenotype Correlation 83

3.4. Clinical Application 84

Chapter 4: Discussion 86

4.1. Analytical Validation 88

4.1.1. Polymerase Chain Reaction Amplification 89

4.1.2. DNA Sequencing 92

4.1.3. Real-Time Polymerase Chain Reaction Amplification 94

4.1.4. Allele Frequencies and Genotype Distributions 96

4.2. Clinical Utility 97

4.2.1. APOE 98

4.2.2. MTHFR 99

4.2.3. HFE 100

4.2.4. Coagulation Factors 101

4.2.5. Genotype-Phenotype Correlation Analysis 102

4.3. Network Medicine 103

4.4. Translation Research 105

4.5. Personalized Medicine through Pathology Supported Genetic Testing 107

Chapter 5: Conclusions 111

Chapter 6: References 116

Appendix A: Confirmation Form for Approval of Project N04/02/033.

Appendix B: Confirmation Form for Approval of Project N09/08/244.

List of Abbreviations and Symbols

3’	3-prime
5’	5-prime
^{32}P	a radioactive isotope of Phosphorus
α	alpha
β	beta
κ	kappa
©	copyright
°C	degrees Celsius
=	equal to
>	greater than
≥	greater than or equal to
µg/L	microgram per litre
µl	micro litre
-	minus
%	percentage
+	plus
±	plus-minus
®	registered trademark
<	less than
≤	less than or equal to

A	Adenine
A (Ala)	Alanine
ABI	Applied Biosystems
ALD	Alcoholic Liver Disease
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
ATP	Adenosine 5’-Triphosphate
apoE	mouse apolipoprotein E
ApoE	human apolipoprotein E
APOE	human Apolipoprotein E gene
APOE2	Apolipoprotein E allele 2

APOE3	Apolipoprotein E allele 3
APOE4	Apolipoprotein E allele 4
apo-Tf	iron-depleted Transferrin
APRI	Aspartate Aminotransferase to Platelet Ratio Index
AST	Aspartate Transaminase
bp	base pair
BLAST	Basic Local Alignment Search Tool
C (Cys)	Cysteine
C	Cytosine
CCD	Charge Coupled Device
CRP	C-Reactive Protein
CT	Computerised Tomography
CVD	Cardiovascular Disease
D	Dalton
D (Asp)	aspartic acid
dATP	2'deoxy-adenosine-5'triphosphate
dbSNP rs# ID	Single Nucleotide Polymorphism database identification number
dCTP	2'deoxy-cytosine-5'triphosphate
ddATP	2',3'-dideoxy-adenosine-5'triphosphate
ddCTP	2',3'-dideoxy-cytosine-5'triphosphate
ddGTP	2',3'-dideoxy-guanosine-5'triphosphate
ddH_2O	double distilled water
ddTTP	2',3'-dideoxy-thymidine-5'triphosphate
dGTP	2'-deoxy-guanosine-5'-triphosphate
dH_2O	distilled water
dl	decilitre
DNA	Deoxyribonucleic Acid
DMT1	Divalent Metal Transporter 1
dsDNA	double stranded DNA
dTTP	2'-deoxy-thymidine-5'-triphosphate

EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
FH	Familial Hypercholesterolaemia
FLD	Fatty Liver Disease
FRET	Fluorescence Resonance Energy Transfer
g	gram
G (Gly)	Glycine
G	Guanine
GGT	Gamma-Glutamyl Transferase
H (His)	Histidine
H_2O	water
HAMP	Hepcidin Antimicrobial Peptide gene
H_3BO_3	boric acid
HDL	High-Density Lipoprotein
HDL-C	High-Density Lipoprotein Cholesterol
HFE	High iron protein
HFE	High iron gene
HFE1	Haemochromatosis Type 1
HFE2A	Haemochromatosis Type 2A
HFE2B	Haemochromatosis Type 2B
HFE3	Haemochromatosis Type 3
HFE4	Haemochromatosis Type 4
Hg	Mercury
HH	Hereditary Haemochromatosis
HJV	Hemojuvelin gene
HLA-A*3	major histocompatibility complex class I A3
HLP III	type III Hyperlipoproteinaemia
holo-Tf	iron-loaded Transferrin
HOMA-IR	Homeostasis Model Assessment for Insulin Resistance
HR	Hazard Ratio
HRM	High Resolution Melt

I (Ile)	Isoleucine
ID	Identification Document
IFNβ	Interferon beta
IR	Insulin Resistance
IRE(s)	Iron-Responsive Element(s)
IRP(s)	Iron-Regulatory Protein(s)
IRP1	Iron-Regulatory Protein 1
IRP2	Iron-Regulatory Protein 2
IVS	Intervening Sequence
JH	Juvenile Haemochromatosis
JNK	c-Jun N-terminal Kinase
k	kilo
kD	kilo-Dalton
l	Litre
L (Leu)	Leucine
LDL	Low-Density Lipoprotein
LDL-C	Low-Density Lipoprotein Cholesterol
M (Met)	Methionine
M	Molar
MetS	Metabolic Syndrome
mg	milligram
MGB	Minor Groove Binder
$MgCl_2$	magnesium chloride
MHC	Major Histocompatibility Complex
MIM	Mendelian Inheritance in Man
ml	millilitre
mm	millimetre
mM	milli-Molar
MR	Magnetic Resonance
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribonucleic Acid

MS	Multiple Sclerosis
N (Asn)	asparagine
NaCl	sodium chloride
NADH	Nicotinamide Adenine Dinucleotide
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Nonalcoholic Steatohepatitis
NCBI	National Centre for Biotechnology Information
NCEP	National Cholesterol Education Program
ng	nanogram
ng/µl	nanogram per micro litre
NHANES	National Health and Nutrition Examination Survey
NSAID	Non-Steroidal Anti-Inflammatory Drug
NTC(s)	Non-Template Control(s)
NF-κB	Nuclear Factor κB
OMIM	Online Mendelian Inheritance In Man
p	short arm of chromosome
P	Phosphorus
P (Pro)	Proline
PAGE	Polyacrylamide Gel Electrophoresis
PAI-1	Plasminogen Activator Inhibitor 1
PCR	Polymerase Chain Reaction
pmol	picomole
q	long arm of chromosome
Q (Glu)	glutamine
QUICKI	Quantitative Insulin sensitivity Check Index
R (Arg)	arginine
RefSeq	Reference Sequence
RFLP	Restriction Fragment Length Polymorphism
RGM	Repulsive Guidance Molecule
RNA	Ribonucleic Acid

ROS	Reactive Oxygen Species
rpm	revolution per minute
RT-PCR	Real-Time Polymerase Chain Reaction
rxn	reaction
S (Ser)	Serine
SLC40A1	Solute Carrier family 40 (iron regulated transporter) member 1 gene
SNP(s)	Single Nucleotide Polymorphism(s)
SOP	Standard Operating Procedure
ssDNA	single stranded DNA
SVR	Sustained Virological Response
T (Thr)	Threonine
T	Thymine
T_A	annealing Temperature
Taq	*Thermus aquaticus* polymerase enzyme
TBE	Tris-Borate-EDTA buffer
TE	Tris-EDTA buffer
Tf	Transferrin
TfR	Transferrin Receptor
TfR1	Transferrin Receptor 1 protein
TFR2	Transferrin Receptor 2 gene
TfR2	Transferrin Receptor 2 protein
TFRC	Transferrin Receptor 1 gene
TGFB1	Transforming Growth Factor Beta-1
T_M	melting Temperature
TM	Trademark
TNF α	Tumour Necrosis Factor alpha
U	Units
UTR	Untranslated Region
UV	Ultraviolet
V (Val)	Valine
V	Volts

VLDL	Very Low-Density Lipoprotein
v	volume
v/v	volume per volume
VWFBP	von Willebrand factor-binding protein
w	weight
WC	waist circumference
w/v	weight per volume
x	times
x g	times gravity
Y (Tyr)	Tyrosine
ZIC1	Zinc finger protein of the Cerebellum- 1
ZIC2	Zinc finger protein of the Cerebellum- 2

List of Figures

CHAPTER 1

Figure 1. CVD in NAFLD Patients – Possible Biological Mechanisms 6

CHAPTER 3

Figure 3.1.1. A 2% (w/v) agarose gel depicting the PCR amplicons synthesized with the *APOE* primer set, which screens for both APOE2 and APOE4, visualized with 0.0001% (v/v) EtBr 57

Figure 3.1.2. Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *APOE* primer set 57

Figure 3.1.3. Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *APOE* primer set 57

Figure 3.1.4. A 2% (w/v) agarose gel depicting the PCR amplicons synthesized with the *MTHFR* 677 primer set visualized with 0.0001% (v/v) EtBr 58

Figure 3.1.5. Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *MTHFR* 677 primer set 58

Figure 3.1.6. A 2% (w/v) agarose gel depicting the PCR amplicons synthesized with the *MTHFR* 1298 primer set visualized with 0.0001% (v/v) EtBr 58

Figure 3.1.7. Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *MTHFR* 1298 primer set 59

Figure 3.1.8. A 2% (w/v) agarose gel depicting the PCR amplicons synthesized with the *F2* 20210 primer set visualized with 0.0001% (v/v) EtBr 59

Figure 3.1.9. Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *F2* 20210 primer set 59

Figure 3.1.10. A 2% (w/v) agarose gel depicting the PCR amplicons synthesized with the *FV* Leiden primer set visualized with 0.0001% (v/v) EtBr..... 60

Figure 3.1.11. Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *FV* Leiden primer set 60

Figure 3.1.12. A 2% (w/v) agarose gel depicting the PCR amplicons synthesized with the *HFE* C282Y primer set visualized with 0.0001% (v/v) EtBr 60

Figure 3.1.13. Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *HFE* C282Y primer set..... 61

Figure 3.1.14. A 2% (w/v) agarose gel depicting the PCR amplicons synthesized with the *HFE* H63D primer set visualized with 0.0001% (v/v) EtBr..... 61

Figure 3.1.15. Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *HFE* H63D primer set 61

Figure 3.2.1. Typical amplification achieved using the ABI™ *Taq*Man® assay for APOE2 (ΔRn vs number of cycles)..... 63

Figure 3.2.2. Typical allelic discrimination analysis using the ABI™ *Taq*Man® assay for APOE2 [Allele Y (C_904973_10-T) vs Allele X (C_904973_10-C)] 63

Figure 3.2.3. Genotype distribution of 253 samples obtained using the ABI™ *Taq*Man® APOE2 assay 64

Figure 3.2.4. Typical amplification achieved using the ABI™ *Taq*Man® assay for APOE4 (ΔRn vs number of cycles)..... 65

Figure 3.2.5. Typical allelic discrimination analysis using the ABI™ *Taq*Man® assay for APOE4 [Allele Y (C_3084793_20-T) vs Allele X (C_3084793_20-C)] 65

Figure 3.2.6. Genotype distribution of 253 samples obtained using the ABI™ *Taq*Man® APOE4 assay 66

Figure 3.2.7. Typical amplification achieved using the ABI™ *Taq*Man® assay for *MTHFR* 677 (ΔRn vs number of cycles) 67

Figure 3.2.8. Typical allelic discrimination analysis using the ABI™ *Taq*Man® assay for *MTHFR* 677 [Allele Y (C_1202883_20-A) vs Allele X (C_1202883_20-G)]..... 67

Figure 3.2.9. Genotype distribution of 253 samples obtained using the ABI™ *Taq*Man® *MTHFR* 677 assay 68

Figure 3.2.10. Typical amplification achieved using the ABI™ *Taq*Man® assay for *MTHFR* 1298 (ΔRn vs number of cycles) 69

Figure 3.2.11. Typical allelic discrimination analysis using the ABI™ *Taq*Man® assay for *MTHFR* 1298 [Allele Y (C_850486_20-T) vs Allele X (C_850486_20-G)] 69

Figure 3.2.12. Genotype distribution of 253 samples obtained using the ABI™ *Taq*Man® *MTHFR* 1298 assay 70

Figure 3.2.13. Typical amplification achieved using the ABI™ *Taq*Man® assay for *F2* 20210 (ΔRn vs number of cycles) 71

Figure 3.2.14. Typical allelic discrimination analysis using the ABI™ *Taq*Man® assay for *F2* 20210 [Allele Y (C_8726802_20-G) vs Allele X (C_8726802_20-A)]..... 71

Figure 3.2.15. Genotype distribution of 253 samples obtained using the ABI™ *Taq*Man® *F2* 20210 assay 72

Figure 3.2.16. Typical amplification achieved using the ABI™ *Taq*Man® assay for *FV* Leiden (ΔRn vs number of cycles) 73

Figure 3.2.17. Typical allelic discrimination analysis using the ABI™ *Taq*Man® assay for *FV* Leiden [Allele Y (C_11975250_10-T) vs Allele X (C_11975250_10-C)]..... 73

Figure 3.2.18. Genotype distribution of 253 samples obtained using the ABI™ *Taq*Man® *FV* Leiden assay 74

Figure 3.2.19. Typical amplification achieved using the ABI™ *Taq*Man® assay for *HFE* C282Y (ΔRn vs number of cycles) 75

Figure 3.2.20. Typical allelic discrimination analysis using the ABI™ *Taq*Man® assay for *HFE* C282Y [Allele Y (C_1085595_10-A) vs Allele X (C_1085595_10-G)]..... 75

Figure 3.2.21. Genotype distribution of 253 samples obtained using the ABI™ *Taq*Man® *HFE* C282Y assay 76

Figure 3.2.22. Typical amplification achieved using the ABI™ *Taq*Man® assay for *HFE* H63D (ΔRn vs number of cycles) 77

Figure 3.2.23. Typical allelic discrimination analysis using the ABI™ *Taq*Man® assay for *HFE* H63D [Allele Y (C_1085600_10-G) vs Allele X (C_1085600_10-G)] 77

Figure 3.2.24. Genotype distribution of 253 samples obtained using the ABI™ *Taq*Man® *HFE* H63D assay..... 78

Figure 3.3.1. Comparison of log-corrected ALT levels [log10 (ALT)] between NAFLD patients with (n=10) and without (n=34) mutations in the *HFE* gene 83

Figure 3.4.1. Pedigree of the index case, GMX1, diagnosed with Hereditary Haemochromatosis . 84

List of Tables

CHAPTER 1

Table 1.1. A summary of the disorders that have been associated with defective APOE 22

Table 1.2. A summary of the disorders that have been associated with defective *MTHFR*..... 30

CHAPTER 2

Table 2.1. Details of the primers used in the conventional PCR and DNA sequencing 51

CHAPTER 3

Table 3.1. A synopsis of the mutations evaluated in this study and their metabolic associations.... 56

Table 3.2. Clinical Characteristics of Coloured and White patients included in this study 79

Table 3.3. P-values of Hardy Weinberg Equilibrium for the eight mutations studied..... 80

Table 3.4. Comparison of APOE genotype distribution and allele frequencies between the Control, White and Coloured study groups 81

Table 3.5. Comparison of *MTHFR* genotype distribution and allele frequencies between the Control, White and Coloured study groups..... 81

Table 3.6. Comparison of *HFE* genotype distribution and allele frequencies between the Control, White and Coloured study groups 82

Table 3.7. Clinical and lifestyle information documented at referral of GMX1 85

Acknowledgements

I would like to thank the following individuals and institutions for making this study possible:

The NAFLD patients for their participation in this research project.

The University of Stellenbosch, the Department of Pathology and the Division of Anatomical Pathology for supplying the infrastructure required to complete this study. The Harry Crossley Foundation, Medical Research Council of South Africa, National Research Foundation and the Technology Innovation Agency (formerly the Cape Biotech Trust) for providing the funding that made this project a reality.

Prof Maritha J Kotze for her enthusiastic support, wisdom, experience and guidance.

Dr FC Kruger for laying the foundation for this project with his PhD study in 2008.

Mrs Caroline Daniels for her vital role in every aspect pertaining to the NAFLD patients.

Prof Martin Kidd for his perseverance with the statistical analysis conducted in this project.

Ms Mahjoubeh Jalali Sefid Dashti for her encouragement and invaluable assistance with the techniques employed in this study.

Ms Johanna Grobbelaar for her guidance on laboratory protocol and all things molecular.

Mrs René Veikondis for her crucial role in the DNA sequencing and Real-Time PCR.

Mrs Lize van der Merwe for her contribution to the statistical analysis.

Mr Dieter H Geiger for granting me the opportunity to become part of a research community.

My family: Ohna, Kuiken, Neal, Kristi, the Breytenbachs and the rest of the Fishers for their encouragement, support and unwavering belief in me.

Dedications

To my father,

Leslie "Les" Peter Fisher

(29-10-1955 to 10-07-2000)

“I maintain there is much more wonder in science than in pseudoscience. And in addition, to whatever measure this term has any meaning, science has the additional virtue, and it is not an inconsiderable one, of being true.”

Carl Sagan

US astronomer & popularizer of astronomy (1934 - 1996)

Chapter 1

Literature Review

1.1. Non-Alcoholic Fatty Liver Disease

Non-Alcoholic Fatty Liver Disease (NAFLD) is the most prevalent chronic liver disease in Western countries, with an incidence of 25 to 37% among the general population. NAFLD is considered the hepatic manifestation of the metabolic syndrome and its heterogeneous nature ranges from hepatic steatosis through steatohepatitis to advanced fibrosis and cirrhosis where the ingestion of significant amounts of alcohol has been excluded (Farrell et al. 2005; Mishra et al. 2008). The histology of NAFLD is indistinguishable from alcoholic hepatitis (Matteoni et al. 1999), as it is characterized by macrovesicular hepatic steatosis (Sanyal, 2002). NAFLD is frequently associated with Insulin Resistance (IR) (Angelico et al. 2005; Svegliati-Baroni et al. 2007; Kruger, 2008; Tilg and Moschen, 2008) and hyperferriteinaemia (Lee D et al. 2007; Valenti et al. 2007), while a slight yet significant increase in overall mortality [hazard ratio (HR) = 1.038; $P < 0.0001$] and a considerable increase in liver-related mortality (HR = 9.32; $P < 0.0001$) have also been discovered (Ong et al. 2008).

Due to the heterogeneous nature of NAFLD, the true incidence and prevalence of the disorder remain highly debatable. An autopsy-based study found steatohepatitis in 2.7% of lean and 18.5% of obese, apparently non-alcoholic, patients (Wanless and Lents, 1990). A study of predominantly healthy young adults who were evaluated for adult living-related orthotropic liver transplantation found fatty liver disease (FLD) in 20% of the 126 subjects (Markos et al. 2000). The prevalence of NAFLD in the general population of the United States of America has been estimated at 20% to 30% based on data from the National Health and Nutrition Examination Survey (NHANES; Younossi, 2008). The universality of IR among NAFLD cases was corroborated in South Africa with an ethnically diverse, overweight/obese population from the Western Cape. Disease severity was not associated with the degree of obesity (Kruger et al. 2010). Studies performed in South Africa (Kruger et al. 2010) and elsewhere (Petersen et al. 2010; Tian et al. 2010) suggest a strong genetic component in the development and progression of NAFLD.

Nonalcoholic Steatohepatitis (NASH) is a subset of NAFLD, characterized by necro-inflammatory injury and steatosis (Brunt, 2001). NASH differs from simple, non-progressive steatosis by virtue of its frequent development to cirrhosis. A study conducted on a large, urbanized American population revealed that Hispanics have the highest frequency of hepatic steatosis (45%), followed by European-Americans (33%) and African-Americans (24%; Browning et al. 2004b). The prevalence of cirrhosis and steatohepatitis is also greatest amongst Hispanics, while the probability of liver

failure in African-Americans is reduced (Browning et al. 2004a). Steatohepatitis includes ballooning degeneration, lobular neutrophilic inflammation and sporadic Rappaport zone III perisinusoidal fibrosis, while Mallory bodies are less common in NASH than in alcoholic steatohepatitis (Sanyal, 2002). Atypical features such as lymphocytic inflammation or portal fibrosis are also seen in some individuals (Sanyal, 2002). The progression of steatosis to NASH may be induced by inflammatory cytokines produced by elevated rates of lipid peroxidation in the presence of IR (Angelico et al. 2005; Day and James, 1998).

1.2. NAFLD and the Metabolic Syndrome

The Metabolic Syndrome (MetS) is a collection of interconnected metabolic risk factors that directly contribute to the development of atherosclerotic cardiovascular disease (CVD), significantly increase the risk for developing type 2 diabetes mellitus and promote inflammation and thrombosis (Grundy et al. 2005).

According to the National Cholesterol Education Program (NCEP) of the United States, MetS is defined as the presence of at least three of the following five fundamental symptoms:

1.) Increased waist circumference (≥ 102 cm in men or ≥ 88 cm in women);
2.) Increased triglyceride concentrations (≥ 1.7 mmol/L) or active therapeutic drug intervention;
3.) Decreased high-density lipoprotein-cholesterol (HDL-C) concentrations (< 1.0 mmol/L in men or < 1.3 mmol/L in women) or active therapeutic drug intervention;
4.) Increased blood pressure (≥ 130mmHg systolic or ≥ 85mmHg diastolic) or on active therapeutic drug intervention;
5.) Increased fasting glucose (≥ 5.6 mmol/L) or on active therapeutic drug intervention (Grundy et al. 2005).

MetS may result from abnormal deposition of fat in the liver, muscles and pancreatic b-cells instead of compartmentalization to adipose tissue which in turn causes dyslipidaemia, steatosis, insulin secretory failure and both hepatic and peripheral IR (Lewis et al. 2002). The alterations in IR that are associated with MetS may provide the biochemical foundation for the link with NAFLD, for example IR may cause steatosis by promoting fatty acid flux from adipose tissue to the liver. The severity of steatosis has been found to increase in parallel with IR in a statistically significant manner while the five biochemical and clinical features of MetS are strongly associated ($P < 0.001$)

with severe steatosis in the absence of diabetes (Angelico et al. 2005). Presence of MetS considerably increases the probability of NAFLD development among men and women ($P < 0.001$ for both), while regression of the disorder is 50% less likely to occur (Hamaguchi et al. 2005). NAFLD, NASH, advanced fibrosis and diabetes are all associated with morbid obesity (Ong et al. 2005). Increased fasting plasma glucose levels are associated with greater prevalence of NAFLD and the levels of albumin, glycosylated haemoglobin (HbA_{1C}), liver enzymes, mean fasting plasma glucose, total protein and triglyceride are considerably elevated while the high-density lipoprotein-cholesterol (HDL-C) concentrations are reduced (Jimba et al. 2005). NAFLD may become an increasingly important clinical problem, especially in terms of increased CVD risk, due to the increasing prevalence of MetS and its close link with NAFLD. The metabolic abnormalities inherent in these disorders may also influence disease progression and response to treatment, for example in hepatitis C virus infection, where IR is associated with a reduced Sustained Virological Response (SVR) and SVR is associated with decreased IR (Romero-Gomez et al. 2005; Conjeevaram et al. 2007; Kawaguchi et al. 2007). SVR is also affected by the body mass index (BMI), fibrosis, steatosis and waist circumference (WC) which further illustrates the pervasive effects of metabolic abnormalities traditionally associated with MetS and NAFLD (Bressler et al. 2003; Tarantino et al. 2006; Poynard et al. 2003; Svegliati-Baroni et al. 2007).

1.3. NAFLD and Cardiovascular Disease

The close association between the symptoms of NAFLD and the diagnostic criteria of MetS confer numerous risk factors for cardiovascular disease (CVD) development and progression (Angulo, 2002; Marchesini et al. 2008; Kotronen and Yki-Järvinen, 2008; de Alwis and Day, 2008; Targher et al. 2008b). NAFLD induces the development of two reliable markers of subclinical atherosclerosis independently of obesity or other established risk factors, namely impaired flow-mediated vasodilatation (Villanova et al. 2005) and increased carotid-artery intimal medial thickness (Targher et al. 2004; Brea et al. 2005; Völzke et al. 2005a; Targher et al. 2006; Fracanzani et al. 2008; Kim et al. 2009). Carotid-artery intimal medial thickness differs according to the severity of steatosis and is lowest in the complete absence of the disorder, greater in the presence of simple steatosis and the greatest in NASH. The degree of carotid-artery intimal medial thickness is associated with the histologic severity of NASH independently of classic CVD risk factors, IR and MetS (Targher et al. 2006). Evidence against the association of NAFLD with either increased carotid-artery intimal medial thickness or increased prevalence of carotid-artery calcium

(McKimmie et al. 2008; Petit et al. 2009) has been strongly rebuked by the findings of a meta-analysis of seven cross-sectional studies involving 3497 subjects (Sookoian and Pirola, 2008). Cardiac phosphorus-31 magnetic resonance spectroscopy has revealed echocardiographic features of early left ventricular dysfunction (Goland et al. 2006) and impaired left ventricular energy metabolism (Perseghin et al. 2008) in young NAFLD patients independent of diabetes, hypertension and/or obesity.

Ischaemic heart disease is more prevalent in ultrasonographically diagnosed cases of NAFLD, independent of traditional risk factors (Lin et al. 2005). NAFLD is associated with a 2-fold increased prevalence of coronary heart disease among children (Schwimmer et al. 2005) and decreased myocardial perfusion independent of insulin sensitivity, traditional risk factors and visceral fat mass among patients with type-2 diabetes mellitus who were also known to have coronary artery disease (Lautamäki et al. 2006). The severity of coronary artery disease is greater among NAFLD patients repeatedly referred for elective coronary angiography, independently of established risk factors (Mirbagheri et al. 2007). NAFLD – concurrent with either type-1 or type-2 diabetes mellitus – is associated with increased prevalence of cerebrovascular, coronary and peripheral vascular disease independent of traditional risk factors, MetS, extent of glycaemic control, duration of diabetes and lipid-lowering, hypoglycaemic, antiplatelet or antihypertensive medications (Targher et al. 2007b; Targher et al. 2010).

The exact nature of the complex associations between abdominal obesity, IR and NAFLD which result in elevated CVD risk may be linked to the accelerated atherogenesis seen among NAFLD cases. In this regard, the liver may be both the target of the systemic abnormalities induced by expanded visceral adipose tissue and provide the pro-atherogenic molecules that amplify the arterial damage. The biological mechanisms potentially involved in these processes are summarized in figure 1.

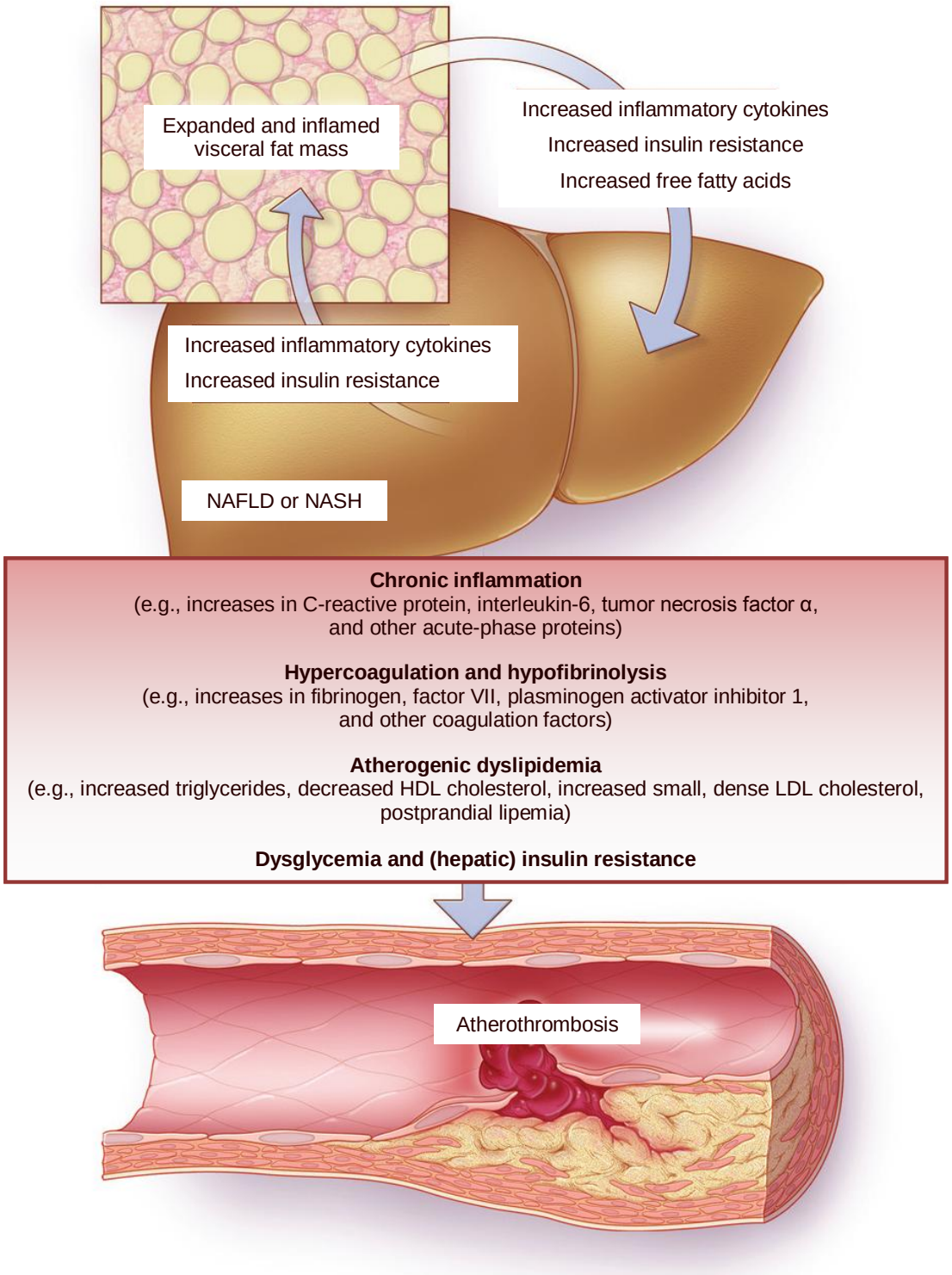

Figure 1: CVD in NAFLD Patients – Possible Biological Mechanisms.
The underlying mechanisms linking NAFLD and CVD might derive from the expanded and inflamed visceral adipose tissue, with the liver acting as both the source of several proatherogenic factors and the target of the resulting systemic abnormalities. NAFLD and especially NASH might affect the pathogenesis of CVD through the systemic release of several haemostatic, inflammatory and oxidative-stress mediators or through the contribution of NAFLD to atherogenic dyslipidaemia and IR.
(HDL = high-density lipoprotein, IR = insulin resistance, LDL = low-density lipoprotein, NAFLD = non-alcoholic fatty liver disease, NASH = nonalcoholic steatohepatitis)
[Used with the permission of Dr Giovanni Targher from Targher et al. (2010)]

Inflammation, Insulin Resistance and Obesity

The development of atherosclerosis and IR may be caused by any of a variety of molecules released by expanded and inflamed visceral adipose tissue, such as free fatty acids, interleukin- 6, monocyte chemotactic protein 1, Tumour Necrosis Factor α (TNF-α) and numerous other proinflammatory cytokines (Day, 2006; Badman and Flier, 2007; Shoelson et al. 2007; Stefan et al. 2008; Tilg and Moschen, 2008). Adipocytes, macrophages, or a combination of both may serve as the source of these cytokines (Day, 2006; Badman and Flier, 2007; Shoelson et al. 2007; Stefan et al. 2008; Tilg and Moschen, 2008). The adipose-tissue inflammation caused by these cytokines is among the most fundamental events resulting in IR, particularly in obese and overweight individuals (Shoelson et al. 2007; Tilg and Moschen, 2008). The gatekeepers of the innate immune system are cytokine receptors and pattern-recognition receptors, such as toll-like receptors and receptors for advanced glycation end products. Receptors of this nature mediate the activation of proinflammatory pathways (Kiechl et al. 2002; Shoelson et al. 2007; Tilg and Moschen, 2008), which converge on two primary intracellular transcription factor signalling pathways: the Nuclear Factor κB (NF-κB) pathway and the c-Jun N-terminal Kinase (JNK) pathway (Day, 2006; Shoelson et al. 2007; Stefan et al. 2008; Tilg and Moschen, 2008). The association of IR in the liver and activation of the JNK pathway in adipose tissue has been proven experimentally in mice (Sabio et al. 2008). Cellular lipid accumulation in skeletal muscle and the inhibition of the insulin-signalling cascade may be responsible for the apparent dissociation of IR from adipose-tissue inflammation in the earliest stages of the disorder among lean persons (Savage et al. 2007). IR in skeletal muscle is associated with hyperinsulinaemia in peripheral and portal veins, which causes hepatic IR and steatosis through several mechanisms, including inhibition of fatty acid oxidation and induction of hepatic lipogenesis mediated by sterol regulatory element-binding protein 1c (Petersen et al. 2007; Savage et al. 2007).

Inflammation, Insulin Resistance and Hepatic Steatosis

IR plays an instrumental role in the development and progression of CVD, MetS (Bonora, 2006) and NAFLD (Angulo, 2002; Day, 2006; Shoelson et al. 2007; de Alwis and Day, 2008; Kotronen and Yki-Järvinen, 2008; Marchesini et al. 2008; Stefan et al. 2008; Tilg and Moschen, 2008). Hepatic steatosis is caused by elevated uptake of free fatty acids by the liver, primarily from the hydrolysis of abundant adipose-tissue triglycerides due to IR, but also from dietary chylomicrons and hepatic lipogenesis (Angulo, 2002; Day, 2006; Shoelson et al. 2007; de Alwis and Day, 2008; Kotronen and Yki-Järvinen, 2008; Marchesini et al. 2008; Stefan et al. 2008; Tilg and Moschen, 2008). The over-production of proinflammatory cytokines, such as interleukin-6, by hepatocytes

and non-parenchymal cells is associated with hepatic steatosis (Day, 2006; Shoelson et al. 2007; Stefan et al. 2008; Tilg and Moschen, 2008). Carotid-artery intimal medial thickness is significantly increased in the presence of NASH and/or chronic viral hepatitis, which is consistent with the role of liver inflammation in the pathogenesis of CVD (Targher et al. 2007a). The liver is both a contributor to and the target of systemic inflammatory changes in the presence of increased free fatty acid flux and chronic, low-grade inflammation which is aggravated by the activation of the NF-κB pathway in the liver of patients with NASH which leads to amplified transcription of several proinflammatory genes (Stefan et al. 2008; Tilg and Moschen, 2008). Fat-derived factors and hepatocellular damage mediate the activation of the NF-κB pathway within the liver, which causes elevated intrahepatic cytokine expression that may be instrumental in the progression of both CVD (de Alwis and Day, 2008; Targher et al. 2008b) and NAFLD (Day, 2006; Shoelson et al. 2007; Stefan et al. 2008; Tilg and Moschen, 2008). NAFLD is associated with the overexpression of many genes involved in coagulation, fatty acid metabolism, inflammation, lipolysis, and both macrophage and monocyte recruitment (Targher et al. 2009). The serum concentrations of several inflammatory (including C-reactive protein [CRP], interleukin-6, TNF-α), procoagulant (such as Plasminogen Activator Inhibitor 1 [PAI-1], fibrinogen, factor VII) and oxidative stress markers (e.g. nitrotyrosine, oxidized Low-Density Lipoprotein Cholesterol [LDL-C], thiobarbituric acid-reacting substances,) are proportional to the severity of steatosis independently of traditional risk factors, with the absence of steatosis corresponding to the lowest marker levels, followed by elevated values in cases of simple steatosis and the greatest excess in the presence of NASH (Targher et al. 2009).

Histologic Severity

The histologic severity of NAFLD has been strongly associated with the intrahepatic messenger Ribonucleic Acid (mRNA) expression of CRP, interleukin-6 and PAI-1 (Yoneda et al. 2007; Wieckowska et al. 2008; Thuy et al. 2008). NASH is associated with elevated atherogenic risk beyond the contribution of visceral adiposity as increased plasma CRP, fibrinogen and PAI-1 activity levels with lower adiponectin levels have been reported, while visceral adiposity remained unaffected. Independent association of the histologic severity of NASH with the plasma concentrations of inflammatory and procoagulant markers support this additional pathogenic mechanism (Targher et al. 2008a). NASH is associated with a greater risk of CVD relative to simple steatosis as well as increased serum liver enzyme concentrations due to the necro-inflammatory nature of the disorder (Matteoni et al. 1999; Adams et al. 2005; Ekstedt et al. 2006; Rafiq et al. 2009; Söderberg et al. 2010). NAFLD and especially NASH can promote the development of atherogenic dyslipidaemia and the progression of both hepatic and systemic IR, which greatly

increase the risk of CVD (Targher et al. 2006; Shoelson et al. 2007; de Alwis and Day, 2008; Kotronen and Yki-Järvinen, 2008; Stefan et al. 2008; Targher et al. 2008b). Defective lipoprotein metabolism, particularly during the postprandial phase, is yet another mechanism by which NAFLD may elevate CVD risk (Musso et al. 2003; Matikainen et al. 2007). Iron and lipids are both vital to the processes that affect and are influenced by NAFLD, while the same is true for CVD. The combination of increased body iron stores and hypercholesterolaemia, as measured by transferrin saturation and LDL (respectively), was demonstrated to exacerbate CVD mortality risk by two independent studies (Salonen et al. 1992; Wells et al. 2004).

1.4. NAFLD and Hereditary Haemochromatosis

Excessive iron accumulation due to defective export is frequently related to inflammatory responses or the iron overload disorder known as Hereditary Haemochromatosis (HH). A fundamental element of NAFLD pathogenesis, IR, is also a common feature of HH (Valenti et al. 2003).

Detection of a genetic predisposition in the presence of high serum ferritin and transferrin saturation levels is usually sufficient to diagnose HH. However, care must be taken to prevent misdiagnosis of HH in patients with hyperferriteinaemia, which might be caused by the Insulin Resistance Hepatic Iron Overload (IR-HIO) syndrome, also known as dysmetabolic iron overload. Ferritin concentrations, which reflect iron stores, are independent predictors of vascular damage in NAFLD (Kruger, 2008). The mechanism may involve up-regulation of hepcidin by increased iron stores in patients not carrying *HFE* mutations and iron compartmentalization into macrophages (Valenti et al. 2010).

Deleterious *HFE* genotypes, which are responsible for approximately 80% of all HH cases, induce NAFLD progression irrespective of the severity of metabolic abnormalities (Valenti et al. 2003; Nelson et al. 2007). Genetic susceptibility to the development and progression of NAFLD is supported by the discovery of inter-ethnic variation of HH (de Villiers et al. 1999b), while the combined effects of various environmental and genetic risk factors could explain the divergent disease phenotype (Kruger, 2008).

The role of HFE in oxidative stress and inflammation is the primary reason for its inclusion as one of the candidate genes studied in the context of NAFLD.

1.4.1. Hereditary Haemochromatosis

HH was regarded as a clinically and genetically unique entity for much of the 20th century. A German pathologist named Von Recklinghausen first described the classic findings on presentation – including diabetes, bronze pigmentation of the skin and cirrhosis – in 1889 and coined the term "haemochromatosis" (Von Recklinghausen, 1889). By 1935 it was clear that the disease was hereditary in nature and was caused by excess deposits of iron in various tissues (Sheldon, 1935). In the 1970s and 1980s it was recognized as an autosomal recessive disorder linked to the region of the short arm of chromosome 6 encoding the major histocompatibility complex class 1 A. The "haemochromatosis gene" designated *HFE*, was finally identified in 1996 by Feder et al. *HFE* has since become known as the high-iron gene owing to its role in iron metabolism.

In the years since the discovery of *HFE*, our collective understanding of HH – and that of human iron metabolism in general – has improved dramatically. We know that mutations in other genes that control iron metabolism can cause similar forms of iron overload (defined in terms of excess body iron levels) that lead to deposits of iron in especially parenchymal tissues with distinct patterns and organ-damaging potential, as is often observed among NAFLD patients. The genetic age has revolutionized the diagnosis of HH and revealed that the phenotypic expression of any given mutation in an iron-metabolism gene may vary significantly. Such advances have stretched the limits of the historical definition of HH to the point where a new classification of this disorder has arisen. HH is defined today as a hereditary iron loading disorder of multigenic nature, caused by a genetically determined inability to prevent the excessive influx of iron into the circulatory pool. It is characterized by progressive parenchymal iron overload with the potential for significant multi-organ damage and disease (Pietrangelo, 2006).

HH is one of the most common forms of hereditary defects in iron metabolism among Caucasian populations of northern European descent on a global scale (Sheldon, 1935; Edwards et al. 1988). Approximately one in 100 individuals of European ancestry are affected in the South African population (Meyers et al. 1987; de Villiers et al. 1999a). Efficient DNA-diagnostics are made possible for patients of European descent due to the identification of two mutations (C282Y and H63D) in the *HFE* gene, cloned in 1996, which are the cause of HH in more than 80% of Caucasian HH patients (Feder et al. 1996; Potekhina 2005). The C282Y mutation is exceedingly rare in Asian, Australian, Amerindian and African populations, with documented cases of its complete absence (Beckman et al. 1997; Chang et al. 1997; Merryweather-Clarke et al. 1997; Agostinho et al. 1999; Rochette et al. 1999; Sodha et al. 1999; Barut et al. 2003; Zorai et al. 2003; Karimi et al. 2004;

Kotze et al. 2004a; Sassi et al. 2004; Leone et al. 2005). Novel mutations are constantly being identified in a number of genes that have been implicated in iron homeostasis and different forms of HH (Beutler, 2005). The development and implementation of rapid mutation detection tests is vital for the efficient identification of the principal causes of HH in patients without the typical C282Y homozygous status (Kotze et al. 2004a). This will also reduce the risk of HH misdiagnosis in NAFLD cases, thereby drastically improving clinical management and outcome for affected individuals.

1.4.1.1. Genetic Classification

There are four types of HH that are currently recognized by the international scientific and medical communities, as indicated by the Online Mendelian Inheritance in Man (OMIM) database. Haemochromatosis Type 1 (HFE1) is the most common form and is caused by mutations in the *HFE* gene on chromosome 6 (Sheldon, 1935; Simon et al. 1975; Simon et al. 1987; Feder et al. 1996). Type 2 haemochromatosis is divided into subtypes 2A (HFE2A) and 2B (HFE2B). HFE2A is the more common of the two and is caused by mutations in the Hemojuvelin (HJV) gene on chromosome 1 (Roetto et al. 1999; Papanikolaou et al. 2004). HFE2B is caused by mutations in the Hepcidin Anti-Microbial Peptide (*HAMP*) gene on chromosome 19 (Roetto et al. 2003; Merryweather-Clarke et al. 2003). Haemochromatosis Type 3 (HFE3) is caused by mutations in the Transferrin Receptor 2 (*TFR2*) gene on chromosome 7 (Camaschella et al. 2000b; Mattman et al. 2002; Girelli et al. 2002; Hattori et al. 2003). Type 4 haemochromatosis (HFE4) is also known as ferroportin disease and is caused by mutations in the Solute Carrier 40 (iron-regulated transporter) member 1 gene (*SLC40A1*) on chromosome 2 (Pietrangelo et al. 1999; Montosi et al. 2001; Njajou et al. 2001).

HFE1, 3 and 4 are further categorized as Adult-onset haemochromatosis. They are characterized by gradual iron loading, a relatively late onset of parenchymal iron deposition and predominantly hepatic organ damage (Pietrangelo, 1998). Juvenile Haemochromatosis (JH) is characterized by onset of more severe iron overload, occurring typically in the first to third decades of life (Cazzola et al. 1983). Affected individuals have been reported worldwide and both males and females are equally affected. HFE2A and 2B are the only current examples of this disease sub-class.

The adult and juvenile forms are merely two points on a phenotypic range with the same underlying syndrome as well as identical targets of iron toxicity: liver, heart and endocrine glands. The earlier onset of hypogonadism or cardiopathy in JH as compared to the “adult” forms is simply related to

the rapidity and extent of massive circulatory and tissue iron overload due to marked hepcidin loss. This also suggests that endocrine organs and the heart are particularly susceptible to iron toxicity and the rapid iron accumulation noted in these organs may be less tolerated than in other tissues. For example, the liver has superior protection against the toxic effects of iron due to its unique physiology.

Adult-Onset Haemochromatosis

HFE1 is the most common form of HH in most populations of European descent and is caused by deleterious mutations in several of the six exons of the *HFE* gene at locus 6p21.3 (Feder et al. 1996). The HFE protein encoded by this gene is a 343-residue type 1 transmembrane glycoprotein. It is similar, in both sequence and three-dimensional structure, to Major Histo-compatibility Complex (MHC) class I-type proteins (Lebron et al. 1998).

The HFE and MHC class 1 proteins contain three extracellular domains (α1, α2 and α3), consisting of a transmembrane domain and a short cytoplasmic tail. The α1 and α2 globular domains form an eight-stranded anti-parallel β-sheet platform topped by two α helices which is maintained on the surface of an immunoglobulin constant-like α3 domain. Cell surface expression of this molecule is made possible by the binding of the α3 domain to β2-microglobulin to form a heterodimer. While the α1 and α2 helices create a groove for peptide binding in the case of MHC proteins, HFE does not bind peptides. Crystallographic studies have proven that the HFE α1 helix is located close to the α2 helix, forming a shallower and narrower groove than the MHC peptide-binding groove. Differences in physical structure between these proteins indicate that each one has a different role in cellular transferrin-mediated iron uptake (Feder et al. 1998). A cluster of four histidine residues which resembles the structure of iron-binding sites in numerous proteins has been identified on the surface of the α1 domain (Lebron et al. 1998). The exact molecular mechanism by which HFE regulates iron uptake has not been determined, although it is thought to form a complex with the Transferrin Receptor 1 (TfR1) and influence intracellular iron delivery (Parkkila et al. 1997, Feder et al. 1998). The association of HFE with TfR1 significantly reduces the binding affinity of TfR1 for transferrin (Feder et al. 1998, Gross et al. 1998, Ikutu et al. 2000).

The “classic” form of HH, known as HFE1, is an autosomal recessive iron-overload disorder. In most documented cases the causative mutation is a guanine to adenine Single Nucleotide Polymorphism (SNP) in exon 4 of *HFE* (845G>A), resulting in the substitution of tyrosine for cysteine in the α3 domain at amino acid position 282. This mutation is designated Cys-282-Tyr, or

C282Y with a Single Nucleotide Polymorphism Database identification number (dbSNP rs# ID) of rs1800562 (Feder et al. 1996). C282Y seems to have originated by chance in a single Celtic or Viking ancestor in north-western Europe approximately 2000 years ago. This genetic defect, which evidently caused no serious obstacle to reproduction and may even have conferred some advantages (e.g. resistance to dietary iron deficiency and certain infectious diseases) was passed on and spread by population migration. Homozygosity for the C282Y mutation is now found in approximately 5 of every 1000 persons of northern European descent - a prevalence 10 times that of cystic fibrosis genotypes (Merryweather-Clarke et al. 1997, Rochette et al. 1999). Relative to wild type individuals, C282Y homozygotes have twice the risk of breast and colorectal cancer. The risk of developing hepatocellular carcinoma is increased 200-fold, with serum ferritin concentration >1000 µg/L identified as the strongest predictor of cirrhosis (Osborne et al. 2010).

Heterozygosity for the C282Y mutation is associated with increased risk of acute myocardial infarction in men (Tuomainen et al. 1999) and with cardiovascular death in postmenopausal women (Roest et al. 1999). This correlation was strengthened in the latter population in the presence of hypertension or smoking, while the combination of both factors resulted in a nearly 20-fold increased risk. This discovery emphasizes the importance of analysing multiple risk factors when low-penetrance mutations are investigated.

The H63D mutation (rs1799945) results from a cytosine to guanine base change in exon 2 at nucleotide position 187 (187C>G) which causes a substitution of aspartic acid for histidine at amino acid position 63 (His-63-Asp) of the protein (Feder et al. 1996). In the homozygous state, H63D rarely results in disease expression unless complications such as excessive alcohol intake, haemolytic anaemia or ineffective erythropoiesis are present (Best et al. 2001). When both H63D and C282Y are present in an individual, a state known as compound heterozygosity, a mild phenotype is observed (Bacon et al. 1999). The effect of the H63D mutation on HFE is localized in the α1 domain where the amino acid substitution (aspartic acid for histidine) interferes with the formation of a His-Asp salt bridge, disrupting the local protein structure (Waheed et al. 1997). H63D is expressed at the cell surface, but lacks the Tansferrin Receptor (TfR) interaction of the wild type protein (Feder et al. 1998). Under normal circumstances, cells depend on HFE to modulate iron intake, but the mutation results in deposition of excess iron in the cells. This supports the deduction that H63D disrupts the function of the wild type protein.

Many studies have been conducted to establish the effects of the C282Y and H63D mutations on protein structure and function (Feder et al. 1996, Waheed et al. 1997, Feder et al. 1998, Lebron et al. 1998). C282Y prevents the formation of a disulphide bond and modifies HFE protein folding, thereby rendering the mutant molecule incapable of binding β2-microglobulin (Waheed et al. 1997). For normal protein processing, transport and cell surface expression HFE must bind to β2-microglobulin. Consequently, mutant HFE remains confined to the endoplasmic reticulum and mid-Golgi compartments where it cannot undergo late Golgi processing and is degraded rapidly resulting in the loss of protein function.

A second mutation in exon 2 of the *HFE* gene, S65C, involves an adenine to thymine base change at nucleotide position 193 (rs1800730, 193A>T), resulting in a substitution of cysteine for serine at amino acid position 65 (Ser-65-Cys) (Mura et al. 1999). The allele frequency of S65C in Caucasians is highly variable, ranging from 1.6% to 5.5% (Rochette et al. 1999). S65C appears to be a benign polymorphism, as affected individuals homozygous for this mutation have not been reported. In the presence of C282Y, however, it may confer a slight disease risk resulting in a mild HH phenotype (Mura et al. 1999). Other *HFE* mutations which have been proven to cause HFE1 are I105T (Barton et al. 1999), G93R (Barton et al. 1999) and Q283P (Le Gac et al. 2003). Two polymorphisms have not yet been associated with development of a disease state, V53M and V59M (de Villiers et al. 1999a). Discovery of the *HFE* intronic polymorphism 5569G>A jeopardized the results of previous mutation detection studies of *HFE* (Jeffrey et al. 1999), as it is located within the binding area of one of the primers used by Feder et al. (1996) in the detection of the C282Y mutation in a sample population. The European Haemochromatosis Consortium (1999), representing 11 laboratories, retyped hundreds of samples with a new primer external to the 5569G>A polymorphism or by DNA sequencing. Non-amplification of the polymorphic allele was not found, thereby verifying their previous publications.

The vast majority of individuals that are clinically affected by HH are either homozygous for the C282Y mutation or are compound heterozygotes for the C282Y/H63D mutations (Feder et al. 1996). These individuals are genetically predisposed to a sequence of events that may end in severe damage to multiple organs, but it is currently impossible to predict whether or to what extent the mutation will be phenotypically expressed. The natural history of classic HH involves a gradual and highly variable stepwise progression that is dependent upon many variables (Pietrangelo, 2004). An example of the extreme variability in phenotypic expression of this disorder can be found in the laboratory evidence that a small percentage of C282Y homozygotes never develop altered iron

metabolism. This is true for an even greater percentage of C282Y/H63D compound heterozygotes. When symptomatic organ involvement does occur it generally begins in midlife, often with non-specific symptoms such as unexplained fatigue or joint pain (Tavill, 2001). Liver disease usually predominates in the later stages, ranging from slightly elevated amino-transferase levels – with or without hepatomegaly – to cirrhosis and even hepatocellular carcinoma. Endocrine disorders (diabetes, hypogonadotropic hypogonadism, hypothyroidism and impotence) and cardiac problems (arrhythmias and heart failure) as well as joint disease (destructive arthritis) are also found. Even though iron metabolism is abnormal, haematologic anomalies are not usually seen as erythropoiesis is not jeopardized by HH (Pietrangelo, 2004). Therapeutic phlebotomy is usually effective in reducing both plasma iron and tissue iron stores and even aggressive phlebotomy generally poses no risk of anaemia to the patient (Tavill, 2001).

HFE3 is a relatively rare iron-overload phenotype with symptoms that are very similar to those of HFE1 (Camaschella et al. 2000b; Mattman et al. 2002; Girelli et al. 2002; Hattori et al. 2003). It is caused by mutations in *TFR2* which maps to chromosome position 7q22 and contains 18 exons (Kawabata et al. 1999). The mutations associated with the development of HFE3 are Y250X (Camaschella et al. 2000b), M172K (Roetto et al. 2001), Q690P (Mattman et al. 2002) and a 1-bp insertion of a cytosine residue in exon 2 in a polyC tract (g.84_88insC). g.84_88insC causes a frameshift followed by a premature stop codon, a Glutamic acid-60-to-Termination or E60X mutation (Roetto et al. 2001). The R455Q mutation has been found to modify the progression and expression of the HFE1 disease state (Hofmann et al. 2002). *TFR2* is a member of the transferrin receptor-like family and encodes a single-pass type II membrane protein with a protease associated domain, an M28 peptidase domain and a transferrin receptor-like dimerization domain (Kawabata et al. 1999). This protein, TfR2, regulates cellular uptake of transferrin-bound iron (Fleming et al. 2000). Alternatively spliced variants of *TFR2* have been identified, with each one encoding a different protein isoform. The exact nature of these variants has yet to be fully elucidated.

Ferroportin-associated iron overload, also known as ferroportin-disease, is currently classified in the OMIM database as HFE4 and was first clinically recognized in 1999 (Pietrangelo et al. 1999). The disorder was linked to *SLC40A1*, which encodes ferroportin (Montosi et al. 2001). Ferroportin is a cell membrane protein involved in cellular iron export from duodenal epithelial cells and acts as the primary iron export protein in mammals (Njajou et al. 2001). *SLC40A1* encompasses 8 exons and maps to chromosome position 2q32 (Montosi et al. 2001). Mutations in *SLC40A1* associated with the development of HFE4 are N144H (Njajou et al. 2001), A77D (Montosi et al. 2001), D157G,

Q182H, G323V (Hetet et al. 2003), D181V, G80V, G267D (Cemonesi et al. 2005) and a 3-bp (TTG) deletion in exon 5 which results in the loss of a valine residue at amino acid position 162 (Wallace et al. 2002). These mutations cause a hereditary iron storage disease distinct from the other types of HH in that it is an autosomal dominant inherited disorder of iron metabolism which induces progressive iron retention primarily in reticulo-endothelial cells of the spleen and liver (Montosi et al. 2001). HFE4 is characterized by gradually increasing concentrations of serum ferritin, disproportionately high when compared to the degree of serum transferrin saturation, as well as marginal anaemia and minor organ damage (Pietrangelo, 2004).

This disorder has been reported in many countries worldwide and in many different ethnic groups which differs considerably from the apparently restricted distribution of *HFE* mutations among Caucasians of northern European ancestry. The current model for the pathogenicity of HFE4 involves mutations in *SLC40A1* which cause a slight yet substantial impairment to the iron recycling capacity of reticulo-endothelial macrophages (Montosi et al. 2001). These cells normally process and release large quantities of iron which they derive from the lysis of senescent erythrocytes. As the enterocyte manages relatively small quantities of iron daily, mutations in *SLC40A1* may not be limiting for iron transport and residual protein activity could be sufficient to maintain normal function. This possibility is reinforced by the discovery that absorbing enterocytes do not show excess iron deposits in HFE4 (Corradini et al. 2005). The retention of iron by macrophages results in tissue iron accumulation, as indicated by high serum ferritin concentrations. This reduces the availability of iron for bone marrow and circulating transferrin, which lowers transferrin saturation. As this condition progresses, the cellular iron retention combined with the activation of feedback mechanisms to increase intestinal absorption might contribute to more prominent symptoms of iron overload. This pathophysiologic model is supported by the discovery that patients with mutations in *SLC40A1* have significantly elevated reticulo-endothelial iron stores from those with other forms of HH (Pietrangelo, 2006).

Even though patients affected by HFE4 are not anaemic, signifying adequate iron availability for normal erythropoiesis, they have a reduced tolerance to phlebotomy and may become anaemic on therapy despite persistently elevated serum ferritin concentrations (Montosi et al. 2001; Pietrangelo, 1999). Different mutations in *SLC40A1* may affect the function of ferroportin in diverse ways and thereby indirectly cause variability in clinical expressivity. This view is partially supported by anecdotal evidence which implies that mutations in *SLC40A1* may also be linked to parenchymal iron overload similar to that observed in HFE1 (Sham et al. 2005). Recent *in vitro* studies propose

that a subgroup of *SLC40A1* mutations might induce hepcidin "resistance" and increased rather than diminished iron export (De Domenico et al. 2005; Drakesmith et al. 2005; Schimanski et al. 2005). This situation may result in a subgroup of HFE4 patients with "gain-of-function" mutations that enhance iron release from enterocytes and macrophages, resulting in a phenotype similar to HFE1. This hypothesis awaits confirmation from additional experimental data and more extensive clinical studies.

Juvenile Haemochromatosis

HFE2A is caused by mutations in the Hemojuvelin gene (*HJV*) at locus 1q21 (Roetto et al. 1999; Papanikolaou et al. 2004). It encompasses 4 exons and codes for the hemojuvelin protein, which contains 426 amino acids and is predicted to be approximately 41 kD in size. A 35-amino acid hydrophobic signal peptide was identified at the N-terminal through bioinformatic analyses, with a transmembrane domain and a glycophosphatidyl inositol addition signal sequence at the C-terminal end (Zhang et al. 2005). Significant discoveries in the structure of hemojuvelin include a tri-amino acid arginine-glycine-aspartic acid domain at position 98 which is believed to be vital to cell adhesion and a partial von Willebrand Factor-like domain that spans the central portion of the protein (from amino acid 167 to 253). Furin and Repulsive Guidance Molecule (RGM) autocatalytic sites are among numerous cleavage sites predicted in hemojuvelin. There are two isoforms of hemojuvelin: the full-length, membrane-bound protein and a soluble variant. This soluble form of hemojuvelin has disulphide bonded N- and C-terminal chains cleaved at the aspartic acid-proline RGM autocatalytic cleavage site and competes with the membrane-bound form (Lin et al. 2005). Known mutations in *HJV* associated with the development of HFE2A are G320V, R326X, I222N, I281T (Papanikolaou et al. 2004), C80R (Barton et al. 2002, Lee et al. 2004b), L101P (Lee et al. 2004b), C321X (Lee et al. 2004a), R54X (Murugan et al. 2008) and a 4-bp deletion at nucleotide position 980. This deletion is predicted to result in a premature termination codon at amino acid residue 337 (Gehrke et al. 2005).

HFE2B is caused by mutations in *HAMP* which encodes the protein hepcidin, also known as the iron-hormone. *HAMP* contains 3 exons and maps to chromosome position 19q13.1 (Roetto et al. 2003; Merryweather-Clarke et al. 2003). Expression of hepcidin mRNA occurs nearly exclusively in the liver, but has been detected in much lower concentrations in other tissues including the heart, brain and lung (Pigeon et al. 2001). This protein is produced by hepatocytes in response to inflammatory stimuli and iron (Lamon et al. 1979, Cazzola et al. 1983, Roetto et al. 2003). Hepdicin is vital to the maintenance of iron homeostasis, particularly in the regulation of iron storage in

macrophages and intestinal iron absorption. It has been classified as a member of the cysteine-rich, cationic, anti-microbial peptides which includes the thionins and defensins due to the significant anti-bacterial and anti-fungal activities of the C-terminal peptide. The 84 amino acid pre-proprotein is post-translationally cleaved into mature peptides of 20, 22 and 25 amino acids (Krause et al. 2000, Park et al. 2001). An N-terminal signal sequence and a penta-arginyl proteolysis site are used to produce the active C-terminal 25 amino acid peptide encoded by exon 3 (Park et al. 2001, Nemeth et al. 2006). It contains eight cysteines involved in disulfide bridge formation, with strong intra-molecular bonds that stabilize their β-sheet structures (Pigeon et al. 2001). Hepcidin is filtered by the kidneys and can be detected in urine due to its small size (Krause et al. 2000).

HAMP mutations associated with the development of HFE2B include R56X (Roetto et al. 2003), G71D (Merryweather-Clarke et al. 2003), two deletions and a 5-prime untranslated region (UTR) defect. One of these deletions was that of a guanine residue in exon 2 at amino acid position 93 (*HAMP*, 1-BP DEL, 93G) which caused a frameshift and generated an abnormally elongated (179 residues) pro-hepcidin peptide (Roetto et al. 2003). The second deletion results in a frameshift due to the loss of a 4-bp sequence (*HAMP*, 4-BP DEL, ATGG) containing the last codon of exon 2 (methionine-50) and the first base of the splice donor site of intron 2 [IVS+1(-G)]. Retention of the splice consensus site was predicted to be the effect of this mutation, but it was instead found to extend the reading frame beyond the end of the normal transcript (Merryweather-Clarke et al. 2003). Another cause of HFE2B involves a homozygous guanine-to-adenine transition at position +14 of the 5-prime UTR relative to the cap site of the mRNA for *HAMP* (*HAMP*, +14G-A, 5-PRIME UTR). A new initiation codon was thus created at position +14 of the 5-prime UTR, resulting in a shift of the reading frame and the subsequent translation of an abnormal protein (Matthes et al. 2004).

No particular ethnic background appears to have a higher frequency, however a clustering of *HJV* mutations occurs in Italy and Greece. A much smaller number of individuals of Arab, Greek, Italian and Portuguese descent with *HAMP*-related JH have been reported. Few documented cases exist though, as it is a very rare disease. Prominent clinical features include arthropathy, cardiomyopathy, hypogonadotropic hypogonadism and liver fibrosis or cirrhosis. Hepatocellular carcinoma has not been reported. The main cause of death is cardiac disease. Curiously, the parenchymal iron distribution remains similar to adult-onset haemochromatosis. If JH is detected early enough and blood is removed regularly through the process of phlebotomy to achieve iron depletion, morbidity and mortality are greatly reduced. Mutations in *HJV* represent the majority of worldwide cases of

JH (Roetto et al. 1999), while *HAMP* mutations account for the most severe form of any HH. Plasma iron loading (reflected by increased transferrin saturation values) and tissue iron excesses (indicated by increased serum ferritin levels) are evident early in life in both sexes.

Functional iron-metabolism data from patients with JH are limited, but estimated rates of iron accumulation markedly exceed those observed in the adult-onset forms (Cazzola et al. 1998). Liver biopsies and autopsies reveal a parenchymal iron distribution resembling that seen in both *HFE*- and *TFR2*-related disease (De Gobbi et al. 2002) even though symptomatic organ involvement occurs as early as the second decade of life. Although liver involvement is a constant feature, arrhythmias, cardiomyopathy, diabetes, heart failure and hypogonadotropic hypogonadism are far more evident than in the adult-onset form (Lamon et al. 1979, Cazzola et al. 1983). This difference may reflect different susceptibilities to massive iron overload among the developing organs. Death is usually caused by intractable heart failure and it is not uncommon for patients to pass on at the young age of 30 years. Rare cases of JH have been linked to homozygous for R56X (Roetto et al. 2003) mutation in *HAMP* (approximately 10%), but the vast majority of juvenile-onset cases are caused by deleterious mutations in *HJV* (approximately 90%).

1.5. Genetic Testing for NAFLD

While the clinical and biochemical markers for NAFLD are universally accepted and fairly well documented, the genetics underlying this disorder are still quite speculative. Due to the close association between CVD and NAFLD, the biochemical and genetic determinants for pathogenesis can be ascertained through similar testing protocols. Deleterious mutations in many genes have been implicated in CVD and by extension in NAFLD. Significant associations have been reported for the genes that encode Apolipoprotein E; 5, 10-Methylenetetrahydrofolate Reductase and both Coagulation Factors II and V. The clinical expression of these genes depends to a large extent on gene-gene and gene-environment interaction. The role of iron as an environmental trigger for expression of mutations in the *HFE* gene is well established and provides a model for a new approach in healthcare termed Pathology Supported Genetic Testing (PSGT; Kotze et. al. 2009). Variation in the *HFE* gene is associated with disease risks in the vascular and other biological systems, thus supporting its inclusion in the genetic analysis of NAFLD (see section 1.4).

1.5.1. Apolipoprotein E (MIM ID +107741)

Apolipoprotein E (Apo-E) is a major apoprotein of the chylomicron and binds to a specific receptor on liver and peripheral cells, facilitating the rapid removal of triglyceride-rich chylomicron- and Very Low-Density Lipoprotein (VLDL) remnants from the circulation by receptor-mediated endocytosis in the liver (Schaefer et al. 1986). It has been found to influence patient response to cholesterol-lowering drugs (e.g. reduced efficacy of statins) and antiretroviral therapy (e.g. development of dyslipidaemia). This protein is hyper-sensitive to lifestyle intervention, with differential effects on cholesterol and triglyceride levels stimulated by environmental triggers as diverse as alcohol, antiretroviral therapy, diabetes, high-calorie diet, hypothyroidism, obesity and physical inactivity (NCBI, Gene ID: 348). The mature protein is a 299-amino acid polypeptide with a molecular mass of approximately 34 kilo-Dalton (kD) (Rall et al. 1982a). Apo-E production and accumulation is greatly increased in response to peripheral nerve injury as well as during the regenerative process, indicating the significant role that it plays in the redistribution of cholesterol to the neurites for membrane biosynthesis during axon elongation and to the Schwann cells for myelin formation (Mahley, 1988).

Mapping

The APOE gene maps to 19q13.2 and was first theoretically localised to chromosome 19 by Olaisen et al. (1982), then definitely mapped by Southern blot analysis through the efforts of Das et al. (1985). Lusis et al. (1986) used a reciprocal whole arm translocation between the long arm of chromosome 19 and the short arm of chromosome 1 to map APOE to a cluster on the q-arm.

Molecular Genetics

The APOE gene is divided into several different isoforms. The 3 most significant variants (APOE2, -E3 and -E4) were initially identified by isoelectric focusing and are encoded by 3 alleles (Epsilon 2, 3 and 4). These isoforms differ in amino acid sequence at 2 sites, known as residue 112 (site A) and residue 158 (site B). At sites A/B, the APOE2, -E3 and -E4 alleles contain cysteine/cysteine, cysteine/arginine and arginine/arginine, respectively (Weisgraber et al. 1981, Rall et al. 1982b). The 3 alleles have varying electric charges (0, 1+ and 2+), which accounts for the observed electrophoretic differences (Margolis, 1982). APOE3 is the most common, or "wild type," isoform.

APOE4 differs from APOE3 by a cysteine-to-arginine change at amino acid position 112, earning it the designation of cys112-to-arg (C112R) and dbSNP rs# ID of rs429358 (Smit et al. 1990). Presence of the APOE4 allele results in decreased plasma concentrations of Apo-E with increased

plasma cholesterol, low density lipoprotein-cholesterol, apolipoprotein B, lipoprotein (a), a significantly increased risk of Alzheimer's disease development and more than 40% greater risk of coronary heart disease (Weisgraber et al. 1981; Das et al. 1985; Paik et al. 1985).

The APOE4 allele has been consistently associated with the development of Alzheimer's disease (Saunders et al. 1993a; Corder et al. 1993; Myers et al. 1996; Tang et al. 1996; Mori et al. 2002), general cognitive decline (Caselli et al. 2004; Farlow et al. 2004; Blair et al. 2005), worse response to cerebral trauma (Teasdale et al. 1997; Friedman et al. 1999; Crawford et al. 2002; Liberman et al. 2002; Koponen et al. 2004) and greater deterioration of multiple sclerosis patients (Enzinger et al. 2004; De Stefano et al. 2004).

A recent study by Genin et al. (2011) demonstrated that the APOE4 allele confers a significantly increased risk of developing Alzheimer's disease which resembles the dangers associated with Breast Cancer 1 Gene (BRCA1) mutations and cancer. The APOE4 allele displayed high penetrance in the homozygous state in a similar manner to traditionally major genes associated with Mendelian disorders even though individual deleterious variations in APOE are generally of the low-penetrance variety. This discovery emphasizes the complexities of biological systems and their interactions which determine the functionality of genes and their proteins. While individual mutations may not cause substantial dysfunction, the cumulative risk of multiple detrimental alterations to a particular biological system may result in both localized and diffuse disease.

A band at the APOE2 position, obtained through isoelectric focussing, has been ascribed to 4 different mutations: E2 (arg158-to-cys, R158C), E2 (lys146-to-gln, L146Q), E2 (arg145-to-cys, R145C) and E2-Christchurch (arg136-to-ser, R136S). The E2 arg158-to-cys (R158C) mutation, rs7412, is the most common, while all APOE2 variants are associated with dyslipidaemia in the presence of diabetes, hypothyroidism or obesity. Development of the genetic disorder familial dysbetalipoproteinaemia, or type III hyperlipoproteinaemia (HLP III) would depend on the level of unhealthy fats and refined carbohydrates in the diet (Rall et al. 1983a; Gill et al. 1985; Smit et al. 1990).

At least thirty APOE variants have been characterized, 14 of which are associated with familial dysbetalipoproteinaemia characterized by elevated plasma cholesterol and triglyceride levels with an increased risk for atherosclerosis development (de Knijff et al. 1994). Polymorphisms in the APOE gene have been associated with increased survival and longevity (Gerdes et al. 2000). Potent

transcriptional activation of APOE by the Zinc finger protein of the Cerebellum- 1 (ZIC1) and 2 (ZIC2) transcription factors have been linked to stimulation of binding sites in the APOE promotor (Salero et al. 2001). The differential abilities of the Apo-E isoforms to form a stable folding intermediate (known as a molten globule structure) may contribute to the isoform-specific effects of this in disease (Morrow et al. 2002), e.g. the 22 kD N-terminus of APOE4 forms a molten globule more readily than does APOE3 or APOE2. Transmission of the APOE2 allele, which is associated with a lower risk of CVD, is significantly reduced in babies born with growth restriction and provides a possible explanation for the higher risk generally associated with development for such disease later in life (Infante-Rivard et al. 2003). APOE and Transforming Growth Factor Beta-1 (TGFB1) are associated with obesity phenotypes (Long et al. 2003). Both the APOE2 and APOE4 alleles have been found to reduce the likelihood of chronic hepatitis C virus infection, possibly through facilitating increased clearance of the virus (Price et al. 2006). The APOE4 allele has been identified as a determinant of AIDS pathogenesis, with homozygosity for APOE4 facilitating an accelerated disease course and progression to death when compared with APOE3 homozygosity (Burt et al. 2008).

Disease Association

Genetic and/or structural variations within Apo-E have been identified as a major contributing factor in the development, progression and/or severity of many disorders, as illustrated by table 1.1.

Table 1.1. A summary of the disorders that have been associated with defective APOE.

Associated Disorder	References
Abnormalities of blood lipids	(see below)
Cardiovascular disease	(see below)
Abnormal immunologic response	Van den Elzen et al. 2005
Acute ischaemic stroke	Broderick et al. 2001
Alzheimer's disease	Saunders et al. 1993a; Agosta et al. 2009
Cerebral amyloid angiopathy	Greenberg et al. 1995; O'Donnell et al. 2000
Coagulation inhibition	Riddell et al. 1997
Cognitive impairment	Reiman et al. 1996; van Vliet et al. 2009
Creutzfeldt-Jakob disease	Saunders et al. 1993b; Amouyel et al. 1994
Dystonia	Matsumoto et al. 2003
Foetal iodine deficiency disorder	Wang et al. 2000
Frontotemporal dementia	Verpillat et al. 2002; Acciarri et al. 2006
Huntington disease	Kehoe et al. 1999
Inclusion body myositis	Garlepp et al. 1995
Ischaemic cerebrovascular disease	McCarron et al. 1999; Frikke-Schmidt et al. 2001
Medically intractable temporal lobe epilepsy	Busch et al. 2007
Multiple sclerosis	Chapman et al. 2001; Ghaffar et al. 2010
Non-Alzheimer dementia	Dufouil et al. 2005

Obstructive sleep apnea	Gottlieb et al. 2004; Gozal et al. 2007
Parkinson disease	Marder et al. 1994; Huang et al. 2004
Pick disease	Farrer et al. 1995
Progressive supranuclear palsy	Tabaton et al. 1995
Schizophrenia	Harrington et al. 1995
Sleep-disordered breathing	Kadotani et al. 2001
Subarachnoid haemorrhage	Lanterna et al. 2007
Traumatic brain injury	Teasdale et al. 1997; Koponen et al. 2004
Vascular dementia	Frisoni et al. 1994; Mahieux et al. 1994; Frikke-Schmidt et al. 2001
Primary open-angle glaucoma	Copin et al. 2002; Zetterberg et al. 2007
Age-related macular degeneration	Anderson et al. 2001; Bojanowski et al. 2006

The diverse spectrum of associated conditions and the link with longevity emphasize the multifunctional role of APOE in health and disease. This observation highlights the necessity of a systems approach when including this gene in disease risk assessments.

Role of APOE in Abnormalities of Blood Lipids and in CVD

In HLP III, impaired clearance of chylomicron- and VLDL remnants by defective Apo-E or its receptor results in increased plasma cholesterol and triglycerides (NCBI, Gene ID: 348). Accumulation of these remnants can also cause xanthomatosis and premature coronary and/or peripheral vascular disease. HLP III may develop from primary heritable defects in apolipoprotein metabolism or due to complications induced by secondary conditions such as diabetic acidosis, hypothyroidism or systemic lupus erythematosus. The majority of patients suffering from HLP III are homozygous for the APOE2 isoform (E2/E2) while the heterozygous phenotypes (E3/E2 and E4/E2) rarely result in the disorder (Breslow et al. 1982). Homozygosity for APOE2 results in poor binding efficiency of chylomicron- and VLDL remnants to hepatic lipoprotein receptors (Schneider et al. 1981; Rall et al. 1982b) and delayed clearance from plasma (Gregg et al. 1981), whereas the E3 and E4 isoforms bind well due to a positively charged amino acid residue at variable site B (Weisgraber et al. 1982). While nearly all HLP III patients are homozygous for the APOE2 allele, 95% to 99% of E2 homozygotes have neither the disorder nor increased plasma cholesterol levels. Furthermore, the APOE2 protein variant found in hypo-, normo- and hypercholesterolaemic subjects contains the same severe functional abnormalities (Rall et al. 1983a). This indicates the presence of additional environmental and/or genetic factors in the development of the disease and the potential for disease prevention. Examples of such factors include: menopause in women which renders the patients especially sensitive to oestrogen therapy; hypothyroidism and thyroid hormone are known to enhance receptor-mediated lipoprotein metabolism and exacerbate type III HLP; age, diabetes and obesity are associated with increased hepatic synthesis of VLDL and/or cholesterol

(HLP III in APOE2 homozygotes may be explained by these factors). Hepatic overproduction of cholesterol and VLDL may be the defect in familial combined hyperlipidaemia, which is combined with APOE2 homozygosity in the production of type III HLP (Utermann et al. 1979; Hazzard et al. 1981). While Familial Hypercholesterolaemia (FH), known to be highly prevalent in South Africa due to a founder effect (Kotze et al. 1991; Kotze et al. 2003), is a genetic defect of the Low-Density Lipoprotein (LDL) receptor, familial dysbetalipoproteinaemia is a genetic defect in a ligand (Brown and Goldstein, 1983). A possible explanation for the observation that all APOE2 homozygotes do not have extremely high plasma levels of Apo-E containing lipoproteins (intermediate density lipoprotein and chylomicron remnants) is that lipoprotein levels are highly sensitive to factors that reduce hepatic LDL receptors (such as age, the genetic defect of FH and reduced levels of thyroid hormone and oestrogen) and elevated concentrations of hepatic LDL receptors may compensate for the genetic binding defect inherent in E2 homozygotes.

The most characteristic biochemical feature of HLP III is the abnormal separation profile of Apo-E achieved by isoelectric focusing, especiall the absence of the E3 isoform. The fractional catabolic rate *in vivo* of Apo-E isolated from subjects with HLP III is decreased in both type III HLP patients and normal individuals (Gregg et al. 1981). Other biochemical features of HLP III include noticeably elevated VLDL and decreased LDL. Patients with this disease may show increased plasma cholesterol concentrations and the presence of an abnormal lipoprotein called beta-VLDL. As this disorder is induced by a defect in the exogenous cholesterol transport system, the level of hypercholesterolemia is affected by the dietary intake of cholesterol (Brown et al. 1981). Carbohydrate consumption induces or exacerbates the hyperlipidaemia which results in noticeable variability in plasma levels. This quality also provides the foundation for a dietary treatment option to manage the disorder. Planar, tendon and especially tuberous and tuberoeruptive xanthomas are particularly characteristic of HLP III, often in conjunction with abnormal glucose tolerance and precocious atherosclerosis (Levy and Morganroth, 1977). The genetically heterogeneous nature of this phenotype was proven by description of specific biochemical alterations in apolipoprotein structure and metabolism. Immunoassays employed in the analysis of apoprotein revealed that the arginine-rich variant of Apo-E is high in the VLDLs of HLP III (Kushwaha et al. 1977). While exogenous oestrogen typically stimulates triglyceride production in normal women and in those with endogenous hypertriglyceridaemia, a contradictory hypotriglyceridaemic effect occurs in patients suffering from HLP III (Kushwaha et al. 1977). Among VLDLs, the ratio of iso-apolipoprotein E3 to E2 is determined by two APOE3 alleles designated “d” and “n.” These alleles produce 3 phenotypes – namely apoE3-d, apoE3-nd and apoE3-n – which correspond to the low,

intermediate and high ratios of the isoforms, respectively (Hazzard et al. 1981). Deficiency of wild type Apo-E, or presence of high quantities of abnormal variants of the protein, has been identified as contributory factors in the development of HLP III (Ghiselli et al. 1981). Dominant expression of type III HLP has been associated with compound heterozygosity for the wild type APOE3 and a variant APOE3 with 2 substitutions: cystein-to-arginine at residue 112 and arginine-to-cysteine at residue 142. The latter APOE3 variant is defective in its ability to bind to lipoprotein receptors, a functional defect probably contributing to expression of type III HLP (Havel et al. 1983; Rall et al. 1989; Horie et al. 1992).

Apo-E deficiency may result from defective transcription or processing of the primary transcript, as well as instability on the part of the mRNA (Anchors et al. 1986). An example of such a defect is a mutation identified in the acceptor splice site in intron 3 of APOE (Cladaras et al. 1987). Premature CVD, tuberoeruptive xanthomas and HLP III are associated with familial Apo-E deficiency (Schaefer et al. 1986). Both the APOE2 and -E4 alleles are associated with an elevated risk of ischemic heart disease as compared with the E3 allele (Eto et al. 1989). The APOE4 allele promotes premature atherosclerosis (Schachter et al. 1994) and homozygosity is associated with coronary angioplasty (van Bockxmeer and Mamotte, 1992). Xanthomas of the elbows, interphalangeal joints and interdigital webs of the hands may be indicative of double heterozygosity for HLP III and FH (Feussner et al. 1996). APOE genotype and birth weight may be significant determinants for the development of atherosclerosis (Garces et al. 2002). Increasing age and the presence of the APOE4 allele may be significant predictors of aortic stenosis (Novaro et al. 2003). Apo-E may play a role in the modulation of embryonic development and malformations as maternal APOE genotype is associated with the efficiency of cholesterol transport from the mother to the embryo (Witsch-Baumgartner et al. 2004). Significantly increased risk of ischemic heart disease is associated with combinations of SNPs in APOE and the lipoprotein lipase genes beyond that bestowed by environmental factors such as diabetes, hypertension and smoking (Frikke-Schmidt et al. 2007). APOE genotype may be an important marker for clinical responses to statin drugs (Donnelly et al. 2008).

A study by Kathiresan et al. (2008) on several SNPs in 9 genes among 5 414 subjects from the cardiovascular cohort of the Malmo Diet and Cancer Study led to the development of a genotype score based on the number of unfavourable alleles. These SNPs were all associated with increased LDL or decreased High-Density Lipoprotein (HDL) concentrations, a combination of factors which greatly exacerbate CVD development and progression. Increasing genotype scores corresponded to

elevated LDL and reduced HDL-C. At 10-year follow-up, the genotype score proved to be an independent risk factor for incident CVD, including ischemic stroke, myocardial infarction or death from coronary heart disease. Risk discrimination was not improved by application of the scoring system, but the clinical risk reclassification for individual subjects was moderately improved beyond standard clinical factors.

Population Genetics

The APOE3 allele is the most common among all human populations, especially amid groups with a long-established agricultural economy such as those of the Mediterranean basin. A variable allele frequency is present in this region, ranging from 0.849 to 0.898. The ancestral allele, APOE4, has a higher frequency among populations where a foraging economy still exists, or in areas with relatively scarce or sporadically available food sources. Examples of such groups are the aborigines of Australia (0.26) and Malaysia (0.24), Khoi San (0.37), Lapps (0.31), some Native Americans (0.28), Papuans (0.368) and Pygmies (0.407). The scarcest of the alleles, APOE2, has a frequency which fluctuates in an undetermined fashion (0.145 - 0.02). It is also absent in Native Americans. It has been suggested that APOE4, due to several functional properties, may be a 'thrifty' allele. Exposure of APOE4 to certain environmental factors, such as a longer life expectancy than in many other world regions and a Western diet, may have rendered it a susceptibility allele for Alzheimer's disease and coronary artery disease. This hypothesis is strongly supported by the absence of the same association of APOE4 with either disorder among sub-Saharan Africans and the presence of the association in African Americans (Corbo and Scacchi, 1999). The allele frequencies among white South Africans have been reported as 0.08 for APOE2, 0.75 for APOE3 and 0.17 for APOE4 (Kotze et al. 1993). Although the APOE4 allele was shown to be associated with significantly higher cholesterol levels in the South African population, an additive effect could not be demonstrated due to the severely elevated levels already present in FH patients.

Animal Model Discoveries

Apo-E is a key regulator of cholesterol-rich lipoprotein metabolism and is primarily synthesized by the liver, but also by several extrahepatic tissues, including macrophages. As macrophages derive from hematopoietic cells, bone marrow transplantation may be a viable therapeutic approach in the treatment of defective or deficient Apo-E production (Boisvert et al. 1995; Linton et al. 1995). Isoforms of human Apo-E protein may reduce the aggregation or increase the clearance of amyloid-beta relative to a setting in which mouse apoE or no Apo-E is present (Holtzman et al. 1999). Beta-VLDL has been found to stimulate cholesteryl ester accumulation by macrophages which may

accelerate vascular disease as they are converted into the foam cells of atherosclerotic lesions (Bersot et al. 1983). The mechanism which stabilises the synapto-dendritic apparatus may be maintained by Apo-E (Masliah et al. 1995). Hypercholesterolemia and hypertriglyceridaemia have been associated with defective clearance of beta-migrating VLDL particles and spontaneously developed atherosclerotic plaques in APOE2 homozygotes on an average diet, while an atherogenic diet (high in cholesterol and fat) exacerbates development of atherosclerosis and xanthomas (Sullivan et al. 1998).

Secretion of the apoE protein in mice has been found to significantly decrease total plasma cholesterol concentrations and markedly reduce the development of atherosclerosis (Mitchell et al. 2000). DNA sequencing analysis and the pattern of haplotype relationships among the chimpanzee and human APOE genes have identified the human APOE4 variant as the closest homologous match to the chimpanzee counterpart. The evolutionary history of allelic divergence within humans, as inferred from sequence analysis, suggests that the APOE3 and APOE2 alleles were derived from the ancestral APOE4 and that the APOE3 group of haplotypes have increased in frequency relative to APOE4 in the past 200,000 years. Reduced levels of isoprostanes in artery walls, LDLs and urine have been attributed to the antioxidant properties of Apo-E (Tangirala et al. 2001). The lipid- and receptor-binding regions of the apoE protein, amino acids 241-272 and 135-150 respectively, have been associated with the mitochondrial dysfunction and neurotoxicity of the apoE4 isoform (Chang et al. 2005). The delta-9-tetrahydrocannabinol (THC) or cannabinoids with activity at the CB2 receptor have been identified as possible targets for treatment of atherosclerosis (Steffens et al. 2005).

1.5.2. 5, 10-Methylenetetrahydrofolate Reductase (MIM ID *607093)

Methylenetetrahydrofolate reductase (MTHFR) is a 150 kD homodimer spanning 656 amino acids that catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate which acts as a cosubstrate for homocysteine remethylation to methionine (Goyette et al. 1998). Defects in this protein are associated with MTHFR deficiency and increased risk of acute leukaemia, colon cancer, neural tube defects and occlusive vascular disease (NCBI, Gene ID: 4524). Transcripts of varying sizes are expressed in different tissues and at diverse concentrations due to alternative transcription start sites and polyadenylation signals (Gaughan et al. 2000).

Mapping

The *MTHFR* gene, which encodes the MTHFR protein, was first localized to chromosome position 1p36.3 by Goyette et al. (1994) using fluorescence *in situ* hybridization. The 11 exons of *MTHFR* were first identified by Goyette et al. (1998), while the close linkage with chloride channel 6 (CLCN6) was discovered by Gaughan et al. (2000). *MTHFR* does not contain a TATA box in its promoter region, but rather CpG islands and binding sites for several transcription factors including multiple potential SP1 sites (Gaughan et al. 2000).

Molecular Genetics

A reduction in the enzymatic activity of MTHFR is associated with several genetic variations in the encoding gene. The two most common, extensively studied and functionally relevant mutations are known as *MTHFR* 677 and *MTHFR* 1298.

MTHFR 677 is a cytosine to thymine transition at nucleotide position 677 in exon 4 of *MTHFR* (677C>T; rs1801133) that results in an alanine to valine substitution at amino acid position 222 (A222V). Presence of the mutant allele induces thermolability in the enzyme, decreases its activity and creates HinfI restriction site. This restriction site enables genotyping to be performed by Restriction Fragment Length Polymorphism (RFLP). The thermolabile feature of the mutant enzyme is neutralized by the stabilizing effect of folate. Homozygosity for *MTHFR* 677 results in significantly elevated concentrations of homocysteine in the plasma, representing a major genetic risk factor for CVD (Frosst et al. 1995).

MTHFR 677, in the presence of low folate status, may be the most prevalent cause of hyperhomocysteinemia and daily treatment with low-dosage folic acid as part of a B-vitamin complex may restore normal homocysteine levels (Guttormsen et al. 1996). The deleterious effects of *MTHFR* 677 may be neutralized by serum folate concentrations is excess of 15.4 nM (Jacques et al. 1996). Homozygosity for *MTHFR* 677 may protect against anaemia as well as explain the dissociation between haematologic and neurologic disease observed among some patients with vitamin B12 deficiency (Kvittingen et al. 1997). The apparent change in distribution of folate in red blood cells may be caused by the reduced *in vivo* activity of the thermolabile MTHFR generated in the presence of *MTHFR* 677 (Bagley and Selhub, 1998). The scarcity of the homozygous state among the elderly may be indicative of the predominantly detrimental effects associated with the mutant allele (Heijmans et al. 1999). *MTHFR* 677 alters DNA methylation via manipulation of folate status (Friso et al. 2002). Heterozygosity for this mutation reduces enzymatic activity to 65%

of normal, while homozygotes have only 30% activity (Tajouri et al. 2006). Furthermore, the homozygous state for *MTHFR* 677 may confer heightened sensitivity to disturbances in B vitamin status, necessitating a treatment centred on personalized nutritional intervention (Hustad et al. 2007).

MTHFR 1298 is an adenine to cytosine transition at nucleotide position 1298 in exon 7 of *MTHFR* (1298A>C; rs1801131) that results in a glutamic acid to alanine substitution at amino acid position 429 (E429A). Unlike *MTHFR* 677 which occurs within the catalytic domain of MTHFR, this mutation resides in the regulatory domain. Presence of *MTHFR* 1298 obliterates an MboII restriction site, which enables RFLP-based genotyping. Homozygosity for this mutation results in an appreciable reduction in enzymatic activity to 60% of normal (Weisberg et al. 1998), while the effect of the heterozygous state is less pronounced. Neither state is associated with the characteristic features of *MTHFR* 677, namely elevated plasma homocysteine or reduced plasma folate levels. However, compound heterozygosity for both mutations results in decreased enzymatic activity, plasma folate concentrations and increased plasma homocysteine levels (van der Put et al. 1998).

The *MTHFR* 677 mutation locus may in actuality be nucleotide 665 of the coding region, with the *MTHFR* 1298 locus at nucleotide position 1289 due to past inconsistencies in SNP naming conventions (Donelly, 2000; van der Put and Blom, 2000). Presence of three (677CT/1298CC) or four (677TT/1298CC) mutant alleles of *MTHFR* 677 and 1298 may reduce foetal viability (Isotalo et al. 2000). The magnitude of the selective disadvantage conferred by three mutant alleles (677CT/1298CC) may be significantly less than that induced by four (677TT/1298CC), as the latter has not yet been documented (Volcik et al. 2001). Enzymatic activity is decreased by 10% to 36% of normal in the presence of three (*MTHFR* 677CT/1298CC) mutant alleles (Sibani et al. 2003). While *MTHFR* 1298 does not appear to affect the biochemical properties of the encoded protein, *MTHFR* 677 does increase the dissociation of the protein into monomers and promote loss of its flavin adenine dinucleotide cofactor. Compound heterozygosity has no additive effects on the protein (Yamada et al. 2001), but enzymatic activity is decreased by 50% of normal (Tajouri et al. 2006). Embryogenesis may be negatively affected by mutant *MTHFR* alleles when the requirements for folate are high (Zetterberg et al. 2002). Treatment with betaine, hydroxocobalamin, methyltetrahydrofolic acid and pyridoxine may improve the activity of mutant MTHFR (Tonetti et al. 2001). Non-synonymous coding SNPs may influence the function of *MTHFR* by altering the concentration of protein available for metabolic activity (Martin et al. 2006).

Disease Association

Defects in MTHFR are associated with a wide variety of disorders, as illustrated by table 1.2.

Table 1.2. A summary of the disorders that have been associated with defective *MTHFR*.

Associated Disorder	References
Budd-Chiari syndrome	Li et al. 2002
Cancer	Paz et al. 2002; Castro et al. 2004; Hubner et al. 2007
Cleft lip/palate	Shaw et al. 1998; Gaspar et al. 2004; Zhu et al. 2006; Mostowska et al. 2006
Depression	Bjelland et al. 2003; Lewis et al. 2006
Down syndrome	Hobbs et al. 2000; O'Leary et al. 2002; Hobbs et al. 2002; Boduroglu et al. 2004
Glaucoma	Junemann et al. 2005
Hypertension	Nishio et al. 1996; Qian et al. 2007
Ischemic stroke	Casas et al. 2004
Migraine with aura	Kowa et al. 2000; Scher et al. 2006; Todt et al. 2006
Neural tube defects	Ou et al. 1996; Mornet et al. 1997; Speer et al. 1997; Christensen et al. 1999
Preeclampsia susceptibility	Sohda et al. 1997; Kobashi et al. 2000
Retinal artery occlusion	Talmon et al. 1997; Weger et al. 2002
Schizophrenia	Lewis et al. 2005; Muntjewerff et al. 2005; Muntjewerff et al. 2006; Allen et al. 2008; Roffman et al. 2008
Thrombosis	Tonetti et al. 2002; Zalavras et al. 2002

Homozygosity for *MTHFR* 677 results in a 7.2-fold increased risk for developing neural tube defects (Ou et al. 1996). Presence of the mutant alleles of both *MTHFR* 677 and FV Leiden increase the risk of recurrent venous thrombosis to a greater extent than either mutation alone (Keijzer et al. 2002). Severe MTHFR deficiency is associated with a wide variety of mutations throughout *MTHFR* (Rozen, 1996; Kluijtmans et al. 1998; Sibani et al. 2000; Sibani et al. 2003; Tonetti et al. 2003). Nitrous oxide anaesthesia in the presence of MTHFR deficiency, *MTHFR* 677, 1298 and 1755G>A (M581I) may be associated with neurologic deterioration and death (Selzer et al. 2003).

***MTHFR* 677 and 1298 in CVD**

Homozygosity for *MTHFR* 677 is associated with a 3-fold increased risk of premature CVD (Kluijtmans et al. 1996). The association of *MTHFR* 677 with coronary artery disease among the Japanese (Morita et al. 1997) appears to be absent among whites (van Bockxmeer et al. 1997; Schwartz et al. 1997). *MTHFR* 677 and hyperhomocysteinemia are strongly associated with an elevated risk of ischemic stroke (Kelly et al. 2002; Casas et al. 2004; Schürks et al. 2008). Homozygosity for *MTHFR* 677, especially in the presence of diminished folate status, is associated with a considerably elevated risk of coronary heart disease (Lee et al. 2011; Wald et al. 2011). This

discovery further strengthens the hypothesis that defective folate metabolism and high homocysteine concentrations are causally linked to greater risk of coronary heart disease (Klerk et al. 2002). *MTHFR* 1298 may be associated with protection against congenital heart defects (Hobbs et al. 2006).

Population Genetics

The frequency of 677T, which is the mutant allele of *MTHFR* 677, is approximately 0.30 to 0.35 among Caucasians and 0.10 among African Americans (McAndrew et al. 1996; Stevenson et al. 1997; van der Put et al. 1998). While *MTHFR* 677 has been observed in every population tested and the allele frequencies are relatively high throughout, there is significant variation between ethnic groups and homozygosity for this mutation is (McAndrew et al. 1996; Stevenson et al. 1997; Schneider et al. 1998). Homozygosity for this mutation is greatest in northern China (20%), southern Italy (26%) and Mexico (32%) while it is exceedingly rare among Africans (Wilcken et al. 2003). The distribution of *MTHFR* 677 is suggestive of a founder haplotype with a selective, evolutionary advantage (Rosenberg et al. 2002; Wilcken et al. 2003). The exceedingly rare *MTHFR* haplotype 677T/1298C may be increased in some regions of Canada and the United Kingdom due to a founder effect (Ogino and Wilson, 2003). Homozygosity for *MTHFR* 1298 appears to be significantly increased among Indians at 19.46%, which is considerably greater than has been reported for Caucasian (9.4%), Chinese (3.3%) or Japanese (1.6%) populations (Kumar et al. 2005).

Animal Model Discoveries

The amino acid sequence of MTHFR is approximately 90% identical between humans and mice, while significant homology exists with the bacterial "metF" genes and pigs. Human *MTHFR* is largely analogous to the murine version in terms of exon and intron sizes as well as intronic boundary locations (Goyette et al. 1998).

1.5.3. Coagulation Factor II (MIM ID *176930)

Coagulation factor II (also known as prothrombin) is a vitamin K-dependent glycoprotein produced in the liver as an inactive zymogen, prothrombin. Prothrombin is a 622 amino acid pre-propeptide with a molecular mass of approximately 70 kD while the mature circulating protein, thrombin, consists of 579 amino acid residues (Meeks and Abshire, 2008). Prothrombin contains 5 domains: the propeptide (residues -43 to -1), the Gla domain (residues 1 to 40), a kringle domain (residues 41 to 155), a kringle-2 domain (residues 156 to 271) and a serine protease domain (residues 272 to 579). Prothrombin is activated to the serine protease thrombin by factor Xa, which is the active

form of coagulation factor X, in the presence of phospholipids, calcium and factor Va, which is the active form of coagulation factor V (see section 1.5.4). The active enzyme, alpha-thrombin, consists of a light (alpha) and heavy (beta) chain covalently linked by a disulfide bond (Degen and Davie, 1987). The activated thrombin enzyme is essential to haemostasis and thrombosis: it proteolytically cleaves fibrinogen to fibrin for blood clot formation, promotes platelet aggregation, activates factor XIII to cross-link the fibrin clot, enhances clot stability by activating thrombin-activated fibrinolysis inhibitor, up-regulates its own synthesis by activating coagulation factors V, VIII and IX. Thrombin also inhibits coagulation by activating protein C (Goodnight and Hathaway, 2001; Davie and Kulman, 2006; Sambrano et al. 2001; Lancellotti and De Cristofaro, 2009).

The complete absence of prothrombin, a condition known as aprothrombinaemia, is considered to be incompatible with life (review by Meeks and Abshire, 2008). The alpha subunit of glycoprotein 1B (GP1BA), on the surface of platelets, has been found to form a complex with thrombin via analysis of the crystal structure at 2.3 angstrom resolution. Two sites that bind to exosite II and exosite I of 2 distinct alpha-thrombin molecules have also been defined. The interactions between these exosites and thrombin molecules may regulate alpha-thrombin function and limit fibrinogen clotting (Celikel et al. 2003). Crystal structure analysis at a resolution of 2.6 angstrom revealed a periodic arrangement of GP1BA-thrombin complexes in the crystal lattice which mirrors a scaffold that may serve as a driving force for tight platelet adhesion (Dumas et al. 2003). The von Willebrand factor-binding protein (VWFBP) produced by *Staphylococcus aureus* (*S. aureus*) has been identified as a strong non-enzymatic conformational activator of prothrombin. VWFBP is homologous to another prothrombin activator protein secreted by *S. aureus*, namely staphylocoagulase. The difference in activation mechanism between VWFBP and staphylocoagulase suggest a unique method for deposition of fibrin during *S. aureus* endocarditis (Kroh et al. 2009).

Mapping

The *F2* gene, which encodes prothrombin, was first localized to chromosome 11p11-q12 by Royle et al. (1987) through analysis of a panel of somatic cell hybrid DNAs and *in situ* hybridization with both cDNA and genomic probes. The approximately 27.3 kb nucleotide sequence (NCBI: NG_008953.1), consisting of 14 exons, 30 Alu repeats and 2 Kpn repeats which constitute about 40% of the gene, was ascertained by Degen and Davie (1987).

Molecular Genetics

A guanine to adenine substitution in the 3-prime untranslated (propeptide) region of the *F2* gene (20210G>A, rs1799963) results in increased plasma prothrombin concentrations and an elevated risk of venous thrombosis (Degen and Davie, 1987; Poort et al. 1996; Franco et al. 1999). This mutation (*F2* 20210) is associated with a 50% elevated risk of myocardial infarction in men and a 4-fold increase in women (Rosendaal et al. 1997; Doggen et al. 1998). Idiopathic cerebral vein thrombosis is associated with *F2* 20210 and the disease risk becomes elevated in the presence of oral contraceptive usage (Martinelli et al. 1998). This discovery emphasizes the importance of endogenous (genetic) and exogenous factors in disease pathogenesis (Bertina and Rosendaal, 1998). Compound heterozygosity for *F2* 20210 and the FV Leiden mutation significantly increases the risk of recurrent thrombosis relative to carriers of either mutation alone (De Stefano et al. 1999; Martinelli et al. 2000). The *F2* 20210 mutation is a gain-of-function mutation which counteracts the inefficient physiologic *F2* 3-prime end cleavage signal by increasing cleavage site recognition, 3-prime end processing, mRNA accumulation and protein synthesis (Gehring et al. 2001). The *F2* 20210 mutation induces a moderately thrombophilic state and is associated with defects in both the arterial and venous systems, unlike FV Leiden (Pihusch et al. 2001). The predictive value of genetic testing for *F2* 20210 in recurrent venous thromboembolism appears limited when used in isolation (Segal et al. 2009).

Disease Association

Presence of the mutant allele of *F2* 20210, 20210A, may increase the risk of developing Budd-Chiari syndrome (Bucciarelli et al. 1998) and perception deafness (Mercier et al. 1999). Heterozygosity for *F2* 20210 is associated with a greater risk of spontaneous abortions, possibly due to the elevated prothrombin concentrations which may affect placental function by altering essential mechanisms such as cell adhesion, smooth muscle proliferation and vasculogenesis (Pihusch et al. 2001). The *F2* 20210 mutation is associated with ischemic stroke (Casas et al. 2004). A greater risk of prothrombin deficiency exists among ethnic groups or in regions with elevated incidence of consanguinity, due to the autosomal recessive nature of the disorder (Acharya et al. 2004).

The rare autosomal recessive disorder hypoprothrombinaemia, or congenital prothrombin deficiency, is characterized by severe bleeding manifestations, decreased prothrombin antigen levels and reduced enzyme activity (below 10% of normal). Similarly, dysprothrombinaemia is characterized by a dysfunctional prothrombin molecule, but normal antigen levels. The latter

condition has greater variability in bleeding tendency and often displays a good correlation between the levels of prothrombin activity and clinical severity. Such abnormalities are typically caused by a defect in the activation of the protease or within the protease molecule itself (reviews by Girolami et al. 1998 and Lancellotti and De Cristofaro, 2009).

Population Genetics

The observed allele frequencies of *F2* 20210 indicate a single, relatively recent origin event after divergence of Africans from non-Africans and Caucasoids from Mongoloid subpopulations (Zivelin et al. 1998). *F2* 20210 has a prevalence of 3.0% in southern- and 1.7% in northern Europe (Rosendaal et al. 1998), while the variant is exceedingly rare among other ethnic groups, such as Africans and Asians (Zivelin et al. 1998; Rosendaal et al. 1998; Rees et al. 1999).

The age of *F2* 20210 has been estimated at 23 720 years through linkage disequilibrium analysis between the subject and several flanking SNPs and microsatellites, placing its origin toward the end of the last ice age. The age and prevalence of this prothrombin variant among whites may be explained by selective evolutionary advantages, such as diminished blood loss or protection against infections. The selected disadvantage from thrombosis would have been irrelevant to ancient mankind as a species due to the average human life expectancy which, until recent centuries, has not been long enough for the incidence of thrombosis to manifest in a significant manner (Zivelin et al. 2006).

Animal Model Discoveries

Inactivation of the *F2* gene and the subsequent complete deficiency of prothrombin has been shown to be incompatible with life in knockout mice, where embryonic death was most common and no homozygous mutants survived beyond a few days from birth. *F2* plays an essential role in the maintenance of vascular integrity, both during development and in postnatal life (Sun et al. 1998).

1.5.4. Coagulation Factor V (MIM ID *612309)

Coagulation factor V (also known as proaccelerin, labile factor, protein C cofactor, activated protein C cofactor), is a large 330 kD glycoprotein that circulates in the plasma with little or no activity. Conversion to the activated form of this protein, Va, is dependent on the release of thrombin (see section 1.5.3) during coagulation. Va is an essential cofactor of the blood coagulation cascade and consists of a heavy- and a light chain connected by calcium ions. Prothrombin is activated to thrombin by the combined action of Va and activated coagulation factor X (Xa). Va is inactivated

by activated protein C (Kane and Davie, 1986; Cripe et al. 1992). The amino acid sequence of Va consists of 2224 residues, which includes a 28 residue leader peptide, and contains a triplicate A domain and duplicated C domain which are nearly 40% identical to the corresponding regions in coagulation factor VIII. There are also 19 cysteine residues and 37 potential N-linked glycosylation sites, of which 25 are in the B domain (Jenny et al. 1987). The C2 domain of Va exists in two isoforms of a conserved beta-barrel framework which acts as a scaffold for three protruding loops. The binding mechanism of Va and activated factor VIII (VIIIa) to phospholipid membranes may be calcium-independent and stereospecific due to the favourable electrostatic contacts of basic side chains with negatively charged membrane phosphate groups, the immersion of hydrophobic residues at the peaks of the three protruding loops in the non-polar membrane core and the specific interactions with phosphatidylserine head groups in the groove enclosed by these loops (Macedo-Ribeiro et al. 1999).

Mapping

The *FV* gene, which encodes coagulation factor V, was first localized to chromosome 1q21-q25 by Riddell et al. (1987) and Wang et al. (1988) through Southern hybridization to somatic cell hybrid DNAs and *in situ* hybridization. The approximately 81.5 kb (NCBI: NG_011806.1) gene was finally assigned to position 1q23 through linkage data analysis of the *FV* locus by McAlpine et al. (1989). The 25 exons that constitute the *FV* gene were ascertained by Cripe et al. (1992).

Molecular Genetics

A guanine to adenine transition at nucleotide position 1691 in exon 10 of the *FV* gene (1691G>A, rs6025) results in an arginine to glutamine substitution at amino acid position 506 (R506Q). This mutation is also known as *FV* Leiden, named after the town in the Netherlands where it was first identified. *FV* Leiden causes a hypercoagulability disorder known as Activated Protein C (APC) resistance, in which APC becomes incapable of deactivating Va, which upsets the coagulation cascade and increases the incidence of thrombosis (Bertina et al. 1994). *FV* Leiden has been associated with recurrent thromboembolism (Voorberg et al. 1994). Heterozygosity for *FV* Leiden and a mutation in the protein C gene confers an elevated risk of thrombosis relative to either variant alone (Koeleman et al. 1994). *FV* Leiden is associated with a 4- to 5-fold greater risk of recurrent thrombosis (Ridker et al. 1995). Oral contraceptive use in the presence of prothrombotic conditions, such as *FV* Leiden, significantly increases the risk of cerebral venous sinus thrombosis in women (De Bruijn et al. 1998).

A pseudohomozygous state for *FV* Leiden, in which heterozygosity for the mutation is combined with the presence of a Va deficiency allele, is associated with thromboembolism (Castaman et al. 1997; Castoldi et al. 1998) and thrombophilia (Zehnder et al. 1999). Compound heterozygosity for *FV* Leiden and *F2* 20210 (see section 1.5.3) confer a significantly increased risk of thrombosis, greater than either mutation alone (Gerhardt et al. 2000). *FV* Leiden and *F2* 20210 are both associated with an approximate 3-fold increased risk of late foetal loss (Martinelli et al. 2000). Alteration to the molecular structure of *FV* which result in the absence or dysfunction of Va are associated with hemorrhagic disease, while mutations that increase the longevity of the active species are associated with thrombosis (Mann and Kalafatis, 2003). Thrombophilia due to APC resistance has been associated with three other mutations in the *FV* gene. These mutations are: an arginine to threonine substitution at amino acid residue 306 (R306T; Williamson et al. 1998), an isoleucine to threonine substitution at residue 359 (I359T; Mumford et al. 2003) and a nonsense mutation at amino acid position 119 (E119X; Mumford et al. 2003). Heterozygosity for *FV* Leiden confers an approximate 2.7-fold greater risk of thromboembolism, while homozygosity increases the risk to 18-fold, as compared with non-carriers (Juul et al. 2004). *FV* Leiden is associated with incidence and prevalence of ischemic stroke (Casas et al. 2004).

Disease Association

The Haemolysis, Elevated Liver enzymes and Low Platelet count (HELLP) syndrome may be associated with pseudohomozygosity for *FV* Leiden (Brenner et al. 1996). *FV* Leiden may play a significant role in the pathogenesis of Budd-Chiari syndrome (Mahmoud et al. 1997; Leebeek et al. 1998; Gurakan et al. 1999). Retinal arterial occlusion is associated with heterozygosity for *FV* Leiden combined with homozygosity for thermolabile methylenetetrahydrofolate reductase (*MTHFR* 677C>T; Talmon et al. 1997). Idiopathic venous thrombosis may be associated with double homozygosity for *FV* Leiden and *F2* 20210 (Meinardi et al. 1999).

Va deficiency has been ascribed to two specific genotypes for different mutations in the *FV* gene, namely: homozygosity for a 4 bp deletion in exon 13 which results in a frameshift and premature protein truncation (Guasch et al. 1998) and compound heterozygosity for two mutations known as FV Seoul-1 and -2. The former is an 8 bp deletion in exon 7 (nucleotides 1131 – 1139) which results in a frameshift and subsequent generation of a premature stop codon, while the latter is an adenine to guanine transition at nucleotide position 5279 in exon 15 resulting in a tyrosine to cysteine (Y1702C) substitution (van Wijk et al. 2001). Genetic variations in the *FV* gene other than *FV* Leiden may contribute to disease susceptibility, as may be the case with preeclampsia and the

FV SNP, R485K (Faisel et al. 2004). Deleterious variants of the *FV* gene are associated with preterm delivery (Hao et al. 2004).

Population Genetics

Positive selection pressure may explain the relatively high prevalence of *FV* Leiden among the Dutch (2% to 4%) and Swedish (7%) populations, possibly due to a slight advantage in foetal implantation conferred by elevated thrombotic tendency (Majerus et al. 1994). APC resistance, such as that conferred by *FV* Leiden, is associated with reduced risk of intrapartum bleeding complications. This may explain the abnormally high prevalence of hazardous mutations in the *FV* gene, as evolutionary selection mechanisms would inevitably retain genetic variants which confer such survival advantages (Lindqvist et al. 1998).

FV Leiden has been identified among African American (0.87%; Gregg et al. 1997), Hispanic American (1.65%; Gregg et al. 1997) as well as Ashkenazi Jewish and European populations (small, family studies only; Greenberg et al. 1994). Heterozygosity for the mutation within the general British population has been reported as 3.5% (Beauchamp et al. 1994), while 7.8% of a study population in southern Germany were mutation carriers (Braun et al. 1996). *FV* Leiden has not been reported among Asian- or Native Americans (Gregg et al. 1997), nor Hong Kong Chinese (Chan et al. 1998), indicating that it segregates in primarily Caucasian populations and is rare in genetically distant non-European groups.

The age of *FV* Leiden has been estimated at 21 340 years through linkage disequilibrium analysis between the subject and several flanking SNPs and microsatellites, placing its origin toward the end of the last ice age (Zivelin et al. 2006). The age and prevalence of *FV* Leiden among whites may be explained by selective evolutionary advantages, such as reduced mortality from postpartum haemorrhage, haemorrhagia associated with severe iron deficiency anaemia and port-traumatic bleeding (Lindqvist et al. 1998; Lindqvist et al. 2001). The selected disadvantage from thrombosis would have been irrelevant to ancient mankind as a species due to the average human life expectancy which, until recent centuries, has not been long enough for the incidence of thrombosis to manifest in a significant manner (Zivelin et al. 2006).

Animal Model Discoveries

Complete absence of Va in mice has been found to be incompatible with life. Embryonic death is commonplace, possibly due to abnormalities in the yolk-sac vasculature. Massive haemorrhage

results in death within hours after birth for any embryos that develop to term. The vital role of the coagulation pathway and the essential requirement for functional Va in prothrombinase activity is thus demonstrated, while alluding to additional functions of the coagulation system in early mammalian development (Cui et al. 1996). Homozygosity for the R504Q mutation in mice, equivalent to *FV* Leiden (R506Q), results in viable embryos and normal survival with a significant increase in spontaneous fibrin deposition in various tissues. Disseminated intravascular thrombosis, which develops during the perinatal period, causes substantial mortality shortly after birth. This may explain the extensive conservation of the R504/R506 APC cleavage site within the Va among mammals (Cui et al. 2000).

1.6. NAFLD and Environmental Factors

In addition to the elevated risk of CVD and the frequent coexistence of MetS, several environmental factors may affect the clinical severity of NAFLD. Impaired iron homeostasis and alcohol consumption are instrumental in liver disease progression. Liver damage results from a synergistic interaction between these two factors, which provides a possible explanation for the relative scarcity of Alcoholic Liver Disease (ALD) among individuals who consume large quantities of alcohol over extended periods of time (Harrison-Findik, 2009).

1.6.1. Iron and NAFLD

Iron is an essential nutrient involved in a multitude of physiological processes, with immune function and respiration chief among them. Deleterious changes in the homeostasis of this element – such as excessive accumulation and/or incorrect compartmentalisation – may result in production of Reactive Oxygen Species (ROS) which are hazardous to cells and tissues (Bothwell et al. 1979). ROS may cause damage by way of direct or indirect mutagenesis: the former presents as strand breakage or disruption of DNA structure while the latter disrupts immunological processes such as tumour surveillance and macrophage-mediated disposal of transformed cells (Brock et al. 1994). Cirrhosis and hepatocellular carcinoma are associated with iron deposition in the liver. Tissue damage and disease severity may be amplified when increased production of ROS is present in a liver already compromised by a disorder such as ALD or NAFLD. The oxidative stress induced by elevated levels of ROS may also inhibit hepatic hepcidin transcription, which increases liver iron storage and intestinal iron transport (Harrison-Findik, 2009). The exact mechanisms involved in the hepatic iron accumulation observed in NAFLD/NASH cases have not yet been fully elucidated and

may include defective iron-regulatory mechanisms/molecules, erythrophagocytosis by Kupffer cells, genetic factors and/or IR (Sumida et al. 2009). IR and serum transaminase activity in NAFLD/NASH patients may be reduced by iron reduction therapies, including dietary iron restriction and phlebotomy. The latter is used extensively to treat metabolic disturbances, resulting in improved liver function tests and reduced serum iron load (Aigner and Datz, 2008). Many environmental, genetic and nutritional factors are involved in a stringent regulatory system to protect against the potentially toxic effects of iron and to limit its biological availability (Papanikolaou et al. 2004).

Iron Homeostasis

Iron exists in a wide range of oxidation states, form -2 to +6. The most common and stable form is the trivalent Fe^{3+} also known as ferric iron. Fe^{3+} is obtained from the diet by way of digestion and is reduced to the divalent Fe^{2+} or ferrous form by vitamin C or duodenal cytochrome b which is located on the cell membranes of enterocytes. Fe^{2+} is absorbed via the Divalent Metal Transporter (DMT1) from food by the enterocytes lining the duodenum (Kemna et al. 2008).

Iron status is determined by a complex system of integrated proteins and relayed to affector proteins, which allows the absorption of iron within enterocytes to be stored in ferritin molecules if not required by other tissues of the body or prepared for transport if needed. When sufficient iron is available in the body, ferritin-bound iron may be excreted when the enterocytes slough off. If iron is needed, it can be loaded onto the iron-transporter molecule transferrin (Tf) through the combined action of ferroportin and haephestin. Ferroportin is the only known cellular iron exporter and haephestin is a copper-containing protein that oxidises Fe^{2+} to Fe^{3+} for incorporation into Tf molecules.

The regulation of iron absorption and storage is mediated by a small (25 amino acid) protein that is produced in the liver, circulates in the plasma and is excreted in the urine. This small regulatory protein is known as hepcidin and it is instrumental in the maintenance of optimal iron levels (Park et al. 2001). When iron overload takes place, hepcidin is secreted and modulates the plasma iron concentration by preventing iron uptake in the intestines and iron release from macrophages. In the case of clinical iron deficiency, hepcidin is suppressed to promote intestinal iron absorption. Hepcidin down-regulates iron efflux from the intestines and macrophages via its interaction with ferroportin. This interaction involves the internalization and consequent degradation of the ferroportin protein (Nemeth et al. 2004). This mechanism allows excess iron absorption by the

intestines to be prevented while promoting iron delivery to all body cells according to the functional requirements of the respective tissues.

Iron absorption from dietary sources is needed to establish and maintain a sufficient metabolic quantity of the element (approximately 4g to 5g in total), but very little is lost (approximately 1mg daily by men and 1.5mg - 2.0mg by women) through sweating, shedding of skin cells and mucosal cells lining the gastrointestinal tract. Most of the iron is recycled by the reticuloendothelial system which breaks down old red blood cells. The heme released in this manner is absorbed by enterocytes via heme-carrier protein-1 and the iron is liberated by heme oxygenase-1 (Shayeghi et al. 2005).

Irrespective of the initial source, the mechanism that binds iron to Tf is identical, while each molecule of Tf can bind two atoms of iron. Iron-depleted Tf is called apo-Tf and loaded Tf is known as holo-Tf. Holo-Tf molecules transport their cargo to cells in need of the iron for their metabolic activities. Entry into a cell requires Tf to be bound to a TfR. TfR is presented on the membrane surface of a target cell that is iron-deficient and the resultant Tf-TfR complex is endocytosed. The pH in the vesicles produced by the endocytosis process is lowered, which releases the iron and allows apo-Tf and TfR to be re-circulated to the blood and cell membrane, respectively. The exact sensory mechanisms that identify iron status and trigger hepcidin activity when required have not yet been ascertained. The transcription of hepcidin is upregulated by bone morphogenetic proteins, inflammatory cytokines and iron while hypoxia, ineffective erythropoiesis and iron deficiency induce downregulation (Lee and Beutler, 2009). The HFE protein forms a complex with TfR2 and is associated with transcriptional regulation of hepcidin by holo-TfR (Gao et al. 2009).

Cellular iron availability is optimised by the Iron-Regulatory Proteins 1 and 2 (IRP1 and IRP2), which regulate the expression of multiple iron metabolism genes via their interactions with Iron-Responsive Elements (IREs). Increased iron absorption and transport, as well as decreased storage are induced by the binding of IRPs to IREs on the mRNAs of ferritin, Tf and other iron metabolism transcripts in cells that are iron-deficient. This results in downregulation of ferritin synthesis and upregulation of TfR1, while high iron status triggers the reverse of this mechanism (Rouault, 2006).

1.6.2. Alcohol and NAFLD

Alcohol consumption increases iron uptake considerably, which may both amplify and mask the effects of disorders such as HH and NAFLD. In particular, the synthesis of hepcidin is inhibited by alcohol in hepatic parenchymal cells, which may induce liver disease progression (Harrison-Findik, 2009). Low to moderate alcohol consumption may be associated with reduced IR among obese patients with NAFLD, but no discernable effect on the stage or severity of liver disease has been reported (Cotrim et al. 2009).

1.7. Diagnosis

The initial diagnosis of NAFLD is based on the presence of MetS features, exclusion of significant alcohol consumption and the results of various laboratory tests and imaging studies. Confirmation of the diagnosis is achieved through liver biopsy. The use of nuclear medicine as a diagnostic tool has fallen into disfavour in recent times, due to the advancements in modern imaging systems such as ultrasound examination, Computerised Tomography (CT), Magnetic Resonance (MR) imaging and MR spectroscopy. The sensitivity, specificity and operational cost of these systems differ considerably in an increasing fashion from ultrasound to CT to MR. They often provide the first evidence that a patient has otherwise unsuspected NAFLD, but their use is generally restricted to identification of steatosis and cirrhosis while fibrosis and necro-inflammatory injury cannot be accurately determined by current instruments and protocols. Recent attempts at improving MR-imaging and spectroscopy in this regard have produced encouraging results (Aubé et al. 2007; Kato et al. 2007; Friedrich-Rust et al. 2010).

The consumption of as much as 20 grams (g) of alcohol per day is not considered sufficient for the development of liver disease, while an intake of 60 g/day may contribute to disease development. The effects of the intermediate range, >20 g/day but <60 g/day, are not yet well established.

Laboratory tests include measurement of aminotransferases (Alanine Transaminase [ALT]; Aspartate Transaminase [AST]; ALT/AST ratio), cholestatic enzymes (Alkaline Phosphatase [ALP]; Gamma Glutamyl Transferase [GGT]), lipids (total cholesterol; triglycerides) and IR using either the Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) or the Quantitative Insulin sensitivity Check Index (QUICKI). Supplementary assays are also employed to exclude other causes of liver disease such as auto-immune hepatitis, hepatitis C and Wilson's disease.

Aspartate Aminotransferase to Platelet Ratio Index (APRI) was shown to be a simple bedside marker for advanced fibrosis that can avoid liver biopsy in patients with NAFLD/NASH (Kruger et al. 2011).

1.8. Treatment

The pathogenesis and underlying mechanism of NAFLD remains elusive. Current therapeutic intervention strategies are focused on managing the underlying metabolic risk factors as there is no ideal pharmacotherapy available for effective treatment of this disorder. Lifestyle intervention to achieve weight loss and increase exercise is consistently associated with improved liver histology (Cheung and Sanyal, 2009). Loss of more than 5% of total body mass over a nine month period has been associated with improved IR and steatosis, while loss of 9% or more results in improved IR, steatosis and NASH-based inflammation (Harrison et al. 2009). Anti-obesity medications such as the enteric lipase inhibitor orlistat (Harrison et al. 2004) and the selective serotonin re-uptake inhibitor sibutramine (Sabuncu et al. 2003) show potential for future development of similar drugs as a viable treatment option. Bariatric surgery to achieve weight loss is employed in cases of morbid obesity and has been shown to reduce hepatic steatosis and even fibrosis (Silverman et al. 1995; Clark et al. 2005).

Experimental pharmacological treatments to improve IR, which is prevalent in NAFLD and has been associated with the grade of steatosis and even fibrosis (Matteoni et al. 1999), with members of the angiotensin II type 1 receptor blockers (Kudo et al. 2007), thiazolidinediones (Caldwell et al. 2001; Neuschwander-Tetri et al. 2003b; Promrat et al. 2004; Reynaert et al. 2005; Belfort et al. 2006) and metformin (Uygun et al. 2004) have yielded mixed results. Additional drug therapies with potential application in NAFLD include compounds that prevent fat accumulation in the liver (Song et al. 2007), antioxidants (Hasegawa et al. 2001; Kugelmas et al. 2003; Harrison et al. 2003; Ersöz et al. 2005) and cytokine response inhibitors (Buranawuti et al. 2007).

1.9. Aims and Objectives of this Study

The objective of this study was to implement a high-throughput real-time polymerase chain reaction (RT-PCR) method in our laboratory to enable the assessment of cardiovascular genetic risk factors in NAFLD patients.

The specific aims related to each mutation included in the multi-gene cardiovascular disease (CVD) screening assay were as follows:

1.) Evaluation of clinical utility based on the biological importance of the individual genes, functionality of the genetic variations, their allele frequencies in the population, replication of important disease associations, combined effects (gene-gene and gene-environment interaction) and potential risk reduction strategies that may be applicable in clinical practice.

2.) Genotyping to standardize mutation detection in the Pathology Research Facility laboratory, using direct sequencing of PCR amplified fragments for analytical validation of high-throughput RT-PCR SNP assays.

The Pathology Supported Genetic Testing (PSGT) concept developed at our department provides a practical approach to personalized medicine. The PSGT approach combines the vast knowledge and experience of specialists from all healthcare disciplines to enable effective, personalized disease management that would not be possible for any single discipline to achieve alone due to the complexities inherent to biological systems.

Chapter 2

Detailed Experimental Procedures

2.1. Study population

Study participants were recruited at the Gastroenterology Unit of the Department of Internal Medicine, Tygerberg Academic Hospital and the University of Stellenbosch as well as from clinicians working at other academic and private hospitals. Subjects were grouped according to those who fulfilled the criteria for NASH and fatty liver disease. Fatty liver disease is defined as steatosis only or steatosis with inflammation but not fulfilling the criteria for NASH.

The study population consisted of 178 patient samples diagnosed with NAFLD by liver biopsy, representing an extension (67) of the study population (111) used by Kruger et al. (2010) to describe the disease profile of NAFLD in the South African population. In addition, two patients (GMX1 and GMX2) with high ferritin levels were included as case studies for real world evaluation of the clinical utility of the CVD multi-gene test.

The sample size was increased relative to that used by Kruger et al. (2010) to augment the statistical power following DNA analysis (not performed in all participants during the initial study) and sub-classification of NAFLD patients into clinical subgroups for comparative analyses (Kleiner et al. 2005). Gene profiles in patients with type 1 and type 2 histological changes were compared with patients with more advanced liver disease. Clinical outcome was also correlated with relevant risk factors (genetic, biochemical parameters and lifestyle factors including alcohol intake).

A total of 75 DNA samples were included for analytical validation of eight mutations included in a multi-gene assay previously developed for application in patients at risk of CVD (Kotze et al. 2003, Kotze and Thiart, 2003; Kotze and Badenhorst, 2005). These controls were selected based on the presence or absence of metabolic syndrome features.

Clinical and biochemical assessments

High risk individuals were screened by standard liver function tests and liver ultrasonography. Blood, saliva and/or swab samples were collected from all the screened patients after obtaining informed consent, in cases where DNA was not already available from the parent study. Only patients with histology confirming NAFLD were enrolled in this study.

Inclusion criteria: Patients with written informed consent.
Histology with features of NAFLD.

Exclusion criteria: Women with alcohol intake above 20 g/day and males with alcohol intake above 30 g/day.
Histology and/or blood investigations suggestive of another liver disease.
Patients with a secondary cause for fatty liver disease.

A questionnaire was used to denote alcohol intake and other personal details (including lifestyle, drugs and dietary factors, family history, and clinical characteristics for patients not already included in the study). Disease severity was compared between NAFLD patients with no alcohol intake versus low-to-moderate alcohol intake of less than 20 g/day in women and less than 30 g/day in men in relation to genotype.

The following assays were performed using standard methods: Lipogram including total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, LDL particle size, serum iron status (ferritin, serum iron, Tf), glucose and insulin. Fasting glucose and insulin levels were determined (for patients not already done) and the results evaluated to exclude other liver diseases. Insulin resistance was determined for each patient by using HOMA-IR (fasting insulin [µU/ml] x [fasting glucose {mmol/l}/22.5]).

Ethical approval for research on the NAFLD samples used in this study was granted by the Ethics Review Committee of the University of Stellenbosch under project number N04/02/033 (appendix A) and the control samples used for analytical validation were approved by the same regulatory body under project number N09/08/244 (appendix B).

2.2. DNA Extraction

2.2.1. DNA extraction from Whole Blood using the QIAGEN QIAamp® DNA Blood Mini Kit (Spin protocol)

The DNA extraction procedure started with the addition of the QIAGEN Proteinase K stock solution (20 µl) into the bottom of a 1.5 ml microcentrifuge tube. The protease is an enzyme, which is responsible for lysing the cells in the sample to release their DNA into the solution. A blood sample was then added (200 µl) to the tube, followed by Buffer AL (200 µl) and mixed thoroughly (through pulse-vortexing) yield a homogeneous solution that ensures adequate lysis of the sample.

The homogenized solution was then incubated at 56°C on a dry block for 10 minutes. After removal from the dry block, the tube was briefly centrifuged to remove drops from the inside of the lid. Ethanol (96-100%) was added to the sample (200 µl) and mixed by pulse-vortexing for 15 seconds, then briefly centrifuged to remove drops from the inside of the lid. A homogeneous solution, obtained by thoroughly mixing the sample after addition of ethanol, is required to ensure efficient binding of the lysate to the membrane (QIAamp® Mini spin column). The resultant solution was then carefully transferred onto the QIAamp® Mini spin column (in a 2 ml collection tube) and centrifuged at 8000 rpm (6000 x g) for 1 minutes. The QIAamp® Mini spin column was transferred to a clean 2 ml collection tube and the tube containing the filtrate was discarded. This is essential to prevent the nozzle of the QIAamp® Mini spin column from being submerged in the filtrate, which would reduce the washing efficacy.

Wash buffer AW1 was added (500 µl) to the QIAamp® Mini spin column and centrifuged at 8000 rpm (6000 x g) for 1 minute. The QIAamp® Mini spin column was transferred to a clean 2 ml collection tube and the tube containing the filtrate was discarded. Thereafter, buffer AW2 was added (500 µl) to the QIAamp® Mini spin column and centrifuged at 14 000 rpm (20 000 x g) for 3 minutes. The filtrate was then discarded and the tube centrifuged again at 14 000 rpm (20 000 x g) for 1 minute. The QIAamp® Mini spin column was then placed in a clean 1.5 ml microcentrifuge tube and the tube containing the filtrate was discarded. Nuclease-free water (150 µl) was added directly onto the membrane of the QIAamp® Mini spin column and incubated at room temperature (15-25°C) for 5 minutes, followed by centrifugation at 8000 rpm (6000 x g) for 2 minutes. The final solution, containing purified DNA, was then incubated on a shaker at room temperature (15-25°C) overnight to ensure homogenization of the newly extracted DNA and then stored at 4ºC.

2.2.2. DNA extraction from Buccal Swabs using QIAGEN QIAamp® DNA Blood Mini Kit (Spin protocol)

Cotton and DACRON swabs were used in this study. For each sample, buccal swabs were placed into a 2 ml microcentrifuge tube and PBS solution (400 µl) added. QIAGEN Protease (20 µl) and then buffer AL (400 µl) were added and mixed by vortexing for 15 seconds. The tubes were then incubated for 10 minutes at 56ºC on a dry block, followed by brief centrifugation to remove drops from the lid. A measure of the swab mixture (700 µl) was added to a QIAamp® Mini spin column (in a 2 ml collection tube) and centrifuged at 8000 rpm (6000 x g) for 1 minute. The QIAamp® Mini

spin column, containing the unpurified DNA, was then transferred to a clean 2 ml collection tube and the previous step was repeated.

Wash buffer AW1 (500 µl) was applied to the QIAamp® Mini spin column and centrifuged at 8000 rpm (6000 x g) for 1 minute. The QIAamp® Mini spin column was placed in a clean 2 ml collection tube and wash buffer AW2 (500 µl) was added to it, followed by centrifugation at 14 000 rpm (20 000 x g) for 3 minutes. The QIAamp® Mini spin column was transferred to a 1.5 ml microcentrifuge tube and nuclease-free water (100 µl) was added. This was followed by incubation at room temperature (15-25°C) for 5 minutes and centrifugation for 2 minutes at 8000 rpm (6000 x g). The final solution, containing purified DNA, was then incubated on a shaker at room temperature (15-25°C) overnight to ensure homogenization of the newly extracted DNA and then stored at 4ºC.

2.2.3. DNA extraction from Whole Blood using the QIAGEN QIAamp® DNA Blood Midi Kit (Spin Protocol)

This protocol was used for purification of genomic DNA from larger volumes (2 ml) of whole blood. The DNA extraction procedure started with the addition of the QIAGEN Protease stock solution (200 µl) into the bottom of a 15 ml centrifuge tube. The protease is an enzyme, which is responsible for lysing the cells in the sample to release their DNA into the solution. A blood sample was then added (2 ml) and briefly mixed through vigorous shaking (i.e. vortexing) to distribute the protease throughout the solution, which increases the efficacy of the lysing reaction. Buffer AL was added to the tube (2.4 ml) and mixed thoroughly. Mixing was achieved by inverting the tube 15 times, followed by vortexing for approximately 1 minute. This degree of mixing was necessary to yield a homogeneous solution that ensures adequate lysis of the sample. The homogenized solution was then incubated at 70°C on a dry block for 10 minutes. After removal from the dry block, ethanol (96-100%) was added to the sample (2 ml) and mixed by inverting the tube 10 times followed by additional vigorous shaking. A homogeneous solution, obtained by thoroughly mixing the sample after addition of ethanol, is required to ensure efficient binding of the lysate to the membrane (QIAamp® Midi spin column).

Half of the solution was then carefully transferred onto the QIAamp® Midi spin column (in a 15 ml collection tube) and centrifuged at 3000 rpm (1850 x g) for 3 minutes. The filtrates were then discarded and the remainder of the solution was loaded on the QIAamp® Midi spin column and centrifuged for 3 minutes at 3000 rpm (1850 x g). The QIAamp® Midi spin column was transferred

to a clean 15 ml collection tube and the tube containing the filtrate was discarded. Removal of the filtrate is essential to prevent the nozzle of the QIAamp® Midi spin column from being submerged in the filtrate, which would reduce the washing efficacy.

Wash buffer AW1 was added (2 ml) to the QIAamp® Midi spin column and centrifuged at 5000 rpm (4500 x g) for 1 minute. Thereafter, buffer AW2 was added (2 ml) to the QIAamp® Midi spin column and centrifuged at 5000 rpm (4500 x g) for 15 minutes. The QIAamp® Midi spin column was then placed in a clean 15 ml collection tube and the tube containing the filtrate, was discarded. Nuclease-free water (300 µl) was equilibrated to room temperature (15-25°C) and added directly onto the membrane of the QIAamp® Midi spin column. Incubation was then carried out at room temperature (15-25°C) for 5 minutes, followed by centrifugation at 5000 rpm (4500 x g) for 2 minutes. To maximize the DNA concentration achieved through extraction, the eluate was reloaded onto the QIAamp® Midi spin column and incubated at room temperature (15-25°C) for 5 minutes. This was followed by centrifugation at 5000 rpm (4500 x g) for 2 minutes. The final solution, containing purified DNA, was then incubated on a shaker at room temperature (15-25°C) overnight to ensure homogenization of the newly extracted DNA and then stored at 4ºC.

2.2.4. DNA extraction from saliva using the Oragene-DNA / Saliva Kit

The Oragene-DNA/saliva sample in the Oragene-DNA vial was mixed thoroughly by inversion for several seconds and incubated at 50ºC for 2 hours on a dry block. A volume of the Oragene-DNA/saliva sample (500 µl) was then transferred to a 1.5 ml microcentrifuge tube. The Oragene-DNA purifier (OG-L2P) was added to the tube (20 µl) and was mixed by vortexing for a few seconds, followed by incubation on ice for 10 minutes. Centrifugation was then carried out at room temperature (15-25°C) for 5 minutes at 13 000 rpm (15000 x g), resulting in separation of the DNA-containing supernatant from the pelletized impurities. The clear supernatant was carefully transferred into a new 1.5 ml microcentrifuge tube and an equal volume of ethanol (95-100%) was added (500 µl), followed by gentle mixing by inverting the tube 10 times.

The sample was then incubated at room temperature (15-25°C) for 10 minutes to allow for DNA precipitation. The tube was loaded in a centrifuge in a known orientation (in order to position the resulting DNA pellet at the tip of the tube below the hinge as it is nearly invisible to the naked eye) and spun for 2 minutes at 13 000 rpm (15 000 x g). The supernatant was then carefully removed and discarded. Thereafter, ethanol (70%) was added (250 µl) and the resulting mixture incubated at room temperature (15-25°C) for 1 minute. The ethanol was then carefully removed without

disturbing the pellet. After addition of nuclease-free water (100 µl), the tube was vortexed for 5 seconds to dissolve the DNA pellet. Additional vigorous pipetting and vortexing was followed by overnight incubation on a shaker at room temperature (15-25°C) to ensure homogenization of the newly extracted DNA and then stored at 4ºC.

2.3. DNA Quantification

The Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies, USA) with the v3.5.2 software package was used to measure the quality and quantity of DNA in the extracts. All genomic DNA samples were diluted to a concentration of 10 ng/µl using nuclease-free water. The ratio absorbance reading at 260/280 for all the samples ranged from 1.6 to 1.9. Values within this range indicate absence of contaminants such as salts or phenols in a sample (http://www.nanodrop.com/Library/nd-1000-v3.8-users-manual-8%205x11.pdf).

2.4. Polymerase Chain Reaction Amplification

2.4.1. Oligonucleotide Primers

Oligonucleotide primers were designed to detect specific mutations in the *APOE* (2 & 4), *F2* (20210G>A), *FV* (1691G>A, Leiden), *HFE* (C282Y and H63D) and *MTHFR* (677 and 1298) genes using the LightCycler® Probe Design Software 2.0 (Version 1.0. R.36). The genomic reference sequences for *APOE* (NG_007084), *F2* (NG_008953), *FV* (NG_011806), *HFE* (NG_008720) and *MTHFR* (NG_013351) were obtained from the National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). The primers used in the conventional PCR experiments and direct DNA sequencing reactions are specified in table 2.1.

Table 2.1. Details of the primers used in the conventional PCR and DNA sequencing.

Gene	SNP	Primer	Nucleotide Sequence (5' to 3')	G/C Content (%)	T_M (°C)	T_A (°C)	SNP Position/s	Amplicon Size (bp)
APOE	rs7412 & rs429358	Forward	GAGACCATGAAGGAGTTG	50.00	49.80	50-62	4075	473
		Reverse	TCGCGGATGGCGCTGAG	70.50	61.20		3937	
F2	rs1799963	Forward	GGGATGGGAAATATGGCTTC	50.00	53.30	61	20210	432
		Reverse	GCCCTGCTCTGAAGATAGAT	50.00	53.90			
FV	rs6025	Forward	GCAGTTCAACCAGGGGAAA	52.60	55.50	61	1691	409
		Reverse	CACTCTAGACTTGCCTTCG	52.60	52.50			
HFE	rs1800562	Forward	TGGCAAGGGTAAACAGATCC	50.00	54.80	56	845	396
		Reverse	TACCTCCTCAGGCACTCCTC	60.00	57.90			
	rs1799945	Forward	ACATGGTTAAGGCCTGTTGC	50.00	55.90		187	208
		Reverse	GCCACATCTGGCTTGAAATT	45.00	53.90			
MTHFR	rs1801133	Forward	ATCCCTCGCCTTGAACA	52.90	53.60	56	677	256
		Reverse	TCACCTGGATGGGAAAGAT	47.30	53.10			
	rs1801131	Forward	CTCTGTCAGGAGTGTGC	58.80	52.40	61	1298	383
		Reverse	GGTGGAGGTCTCCCAACTTA	55.00	56.10			

2.4.2. PCR Reaction Mixture and Thermal Cycling Conditions

With the exception of the APOE2&4 primer set, amplification of the various amplicons was performed with the Promega GoTaq® Flexi DNA Polymerase PCR kit and the Applied Biosystems® 2700, 2720 and 9700 thermal cyclers in 25 µl reactions. For APOE2&4, the Roche FastStart PCR kit was used. The reaction mixture, universal for all the primer sets employed, consisted of 100 ng template DNA, 1x Colourless GoTaq® Flexi buffer (Promega), 0.2 mM of each dNTP (dATP, dTTP, dGTP, dCTP) (Fermentas), 1.5 mM $MgCl_2$ (Promega), 60 pmol of each primer and 1.25 U GoTaq® DNA Polymerase (Promega). For APOE2&4, the FastStart DNA Polymerase (Roche) was used instead of the GoTaq® DNA Polymerase.

APOE2 & E4 Thermal Cycling Conditions

The PCR conditions were as follows: an initial denaturation step at 95°C for 2 minutes; 40 cycles of denaturation at 95°C for 30 seconds, annealing at a gradient of 50 to 62°C for 30 seconds and extension at 72°C for 30 seconds; with a final extension step at 72°C for 4 minutes.

MTHFR 677 Thermal Cycling Conditions

The PCR conditions were as follows: an initial denaturation step at 95°C for 2 minutes; 40 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds; with a final extension step at 72°C for 5 minutes.

MTHFR 1298 Thermal Cycling Conditions

The PCR conditions were as follows: an initial denaturation step at 95°C for 2 minutes; 40 cycles of denaturation at 95°C for 30 seconds, annealing at 61°C for 30 seconds, and extension at 72°C for 30 seconds; with a final extension step at 72°C for 5 minutes.

F2 20210 Thermal Cycling Conditions

The PCR conditions were as follows: an initial denaturation step at 95°C for 2 minutes; 40 cycles of denaturation at 95°C for 30 seconds, annealing at 61°C for 30 seconds, and extension at 72°C for 30 seconds; with a final extension step at 72°C for 5 minutes.

FV Leiden Thermal Cycling Conditions

The PCR conditions were as follows: an initial denaturation step at 95°C for 2 minutes; 40 cycles of denaturation at 95°C for 30 seconds, annealing at 61°C for 30 seconds, and extension at 72°C for 30 seconds; with a final extension step at 72°C for 5 minutes.

HFE C282Y and H63D Thermal Cycling Conditions

The PCR conditions were as follows: an initial denaturation step at 95°C for 2 minutes; 40 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds; with a final extension step at 72°C for 5 minutes.

2.5. Gel Electrophoresis

PCR products were resolved on a 2% (w/v) agarose gel to test for successful amplification. The gel mixture consisted of 2g agarose in 100 ml 1xTBE buffer (90 mM Tris-HCl, 90 mM boric acid [H_3BO_3] and 2.2 mM Ethylenediaminetetraacetic Acid [EDTA] at a pH of 8.0). To enable visualization of the PCR products, 0.0001% (v/v) Ethidium Bromide (EtBr) was added to the 1xTBE electrophoresis buffer (70 µl of EtBr in 700 ml 1xTBE). A total volume of 8 µl was loaded onto a gel consisting of Ficoll Orange G loading buffer (0.1% [w/v] Orange G, 20% [w/v] Ficoll, 10 mM EDTA at pH 7.0) and PCR product (5 µl and 3 µl, respectively). To ascertain the amplification of the correct PCR product, a molecular size marker (100 bp DNA ladder, Promega, Wisconsin, USA) was loaded along with the PCR products on the agarose gel. Electrophoresis of the PCR products was performed for approximately 1 hour at 80 V in 1xTBE buffer. Visualization was achieved by ultraviolet light trans-illumination using a GibcoBRL Life Technologies TFX-35M UV Transilluminator (California, USA).

2.6. DNA Sequencing and Analysis

The PCR products were sent to the Central DNA Sequencing Facility of Stellenbosch University for post-PCR clean-up and sequencing. The electropherograms were analysed using FinchTV Version 1.4.0 (developed by the Geospiza Research Team).

2.7. Real-Time Polymerase Chain Reaction Amplification

2.7.1. Applied Biosystems® *Taq*Man® SNP Genotyping Assays

The ABI™ *Taq*Man® SNP Genotyping assays are standardised mixtures of PCR reagents, with unlabelled primers and *Taq*Man® Minor Groove Binder (MGB) probes (FAM™ and VIC® dye-labelled). These pre-designed assays are used for end-point genotyping by allelic discrimination analysis for SNPs and were employed in conjunction with two different RT-PCR instruments, namely the ABI™ 7900HT Fast Real-Time PCR System and the Corbett Rotor-Gene™ 6000 / QIAGEN® Rotor-Gene™ Q. The assays employed in this study were APOE2 (C_904973_10, rs7412), APOE4 (C_3084793_20, rs429358), *F2* 20210 (C_8726802_20, rs1799963), *FV* Leiden (C_11975250_10, rs6025), *HFE* C282Y (C_1085595_10, rs1800562), *HFE* H63D (C_1085600_10, rs1799945), *MTHFR* 677 (C_1202883_20, rs1801133) and *MTHFR* 1298 (C_850486_20, rs1801131). Prior to any reaction setup, the 40x *Taq*Man® SNP Genotyping assay mixture was diluted to 20x in sterile SABAX water (double distilled water).

2.7.2. Applied Biosystems® 7900HT

The ABI™ 7900HT Fast Real-Time PCR System with ABI™ *Taq*Man® SNP Genotyping assays was employed for genotyping of large sample batches obtained during this study. Total reaction volumes of 10 µl were used, consisting of: 10 ng/µl template DNA (2.0 µl), 5 µl of *Taq*Man® Genotyping Master Mix (P/N 4371355), 20x *Taq*Man® SNP Genotyping Assay (0.5 µl) and 2.5 µl nuclease-free water. The thermal cycling program used in the amplification run was as follows: an initial hold step at 95°C for 10 minutes followed by 45 cycles of denaturation at 92°C for 15 seconds and annealing/extension at 60°C for 1 minute. Thereafter, an end-point genotyping allelic discrimination scan was carried out.

2.8. Statistical Analysis

Population frequencies of the mutations studied were estimated from allele counts and deviation from Hardy-Weinberg equilibrium was assessed using the Chi-square test. All data were analysed using the StatSoft Inc. STATISTICA Data Analysis Software System (www.statsoft.com). For comparison of ordinal/continuous measurements between different genetic groupings, one-way ANOVA was used. Where necessary, log transformations were used when deviations from normality were deemed to be a problem. Comparison of categorical responses was done using cross tabulation and the Chi-square test.

Chapter 3

Results

Eight mutations in five genes (table 3.1) were evaluated in patients with NAFLD, which represents a significant CVD risk factor. The literature review supported the clinical utility of these mutations for subtyping of complex multi-factorial diseases such as CVD and NAFLD for more directed treatment and lifestyle intervention.

Table 3.1. A synopsis of the mutations evaluated in this study and their metabolic associations.

Biological Pathway	Gene	Genetic Variation
Lipid and Lipoprotein Metabolism	APOE	3937 T>C, allele E4 (rs429358)
		4075 C>T, allele E2 (rs7412)
Homocysteine and Folate Metabolism	*MTHFR*	677 C >T, A222V (rs1801133)
		1298 A>C, E429A (rs1801131)
Haemostasis	*F2*	20210 G>A (1799963)
	FV	1691 G>A, Leiden (rs6025)
Iron Overload	*HFE*	845 G>A, C282Y (rs1800562)
		187 C>G, H63D (rs1799945)

Following the analytical validation of each genotyping assay employed in the SNP analysis of the test using 75 DNA control samples, a total of 178 samples of patients diagnosed with NAFLD were genotyped using RT-PCR.

3.1. Conventional Sequencing – Gels and Electropherograms

The amplicons obtained through conventional PCR amplification of the control samples for the *APOE* (2 and 4), *MTHFR* 677, *MTHFR* 1298, *F2* 20210, *FV* Leiden, *HFE* C282Y and *HFE* H63D mutations were visualized with EtBr in an agarose gel after electrophoresis and are presented in figures 3.1.1, 3.1.4, 3.1.6, 3.1.8, 3.1.10, 3.1.12 and 3.1.14, respectively. An example of the sequencing results is presented as an electropherogram for one of the control samples in figures 3.1.2 (APOE2), 3.1.3 (APOE4), 3.1.5 (*MTHFR* 677), 3.1.7 (*MTHFR* 1298), 3.1.9 (*F2* 20210) 3.1.11 (*FV* Leiden), 3.1.13 (*HFE* C282Y) and 3.1.15 (*HFE* H63D). Forward (sense) and reverse (anti-sense) sequencing reactions were carried out after PCR clean-up, with only the former shown as the latter always corroborated the result obtained.

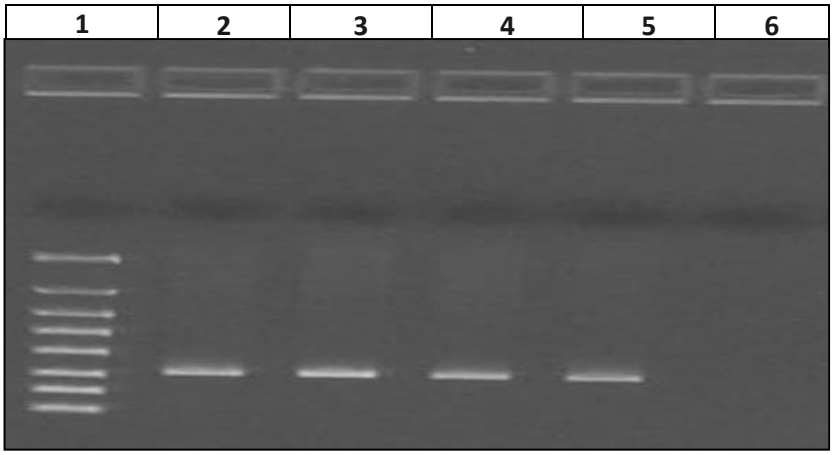

Figure 3.1.1. A 2% (w/v) agarose gel depicting the PCR amplicons synthesized with the *APOE* primer set, which screens for both APOE2 and APOE4, visualized with 0.0001% (v/v) EtBr. Lane 1 contains a 100-bp DNA ladder, lanes 2 to 5 contain amplicons of 473 bp and lane 6 contains the PCR blank. (Abbreviations: bp = base pairs)

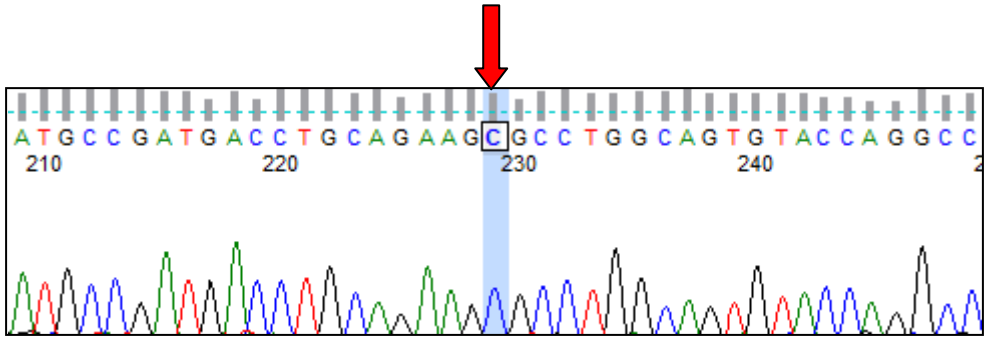

Figure 3.1.2. Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *APOE* primer set. The red arrow indicates the APOE2 mutation position (nucleotide 229). The "C" at the highlighted position corresponds to a Wild Type CC genotype.

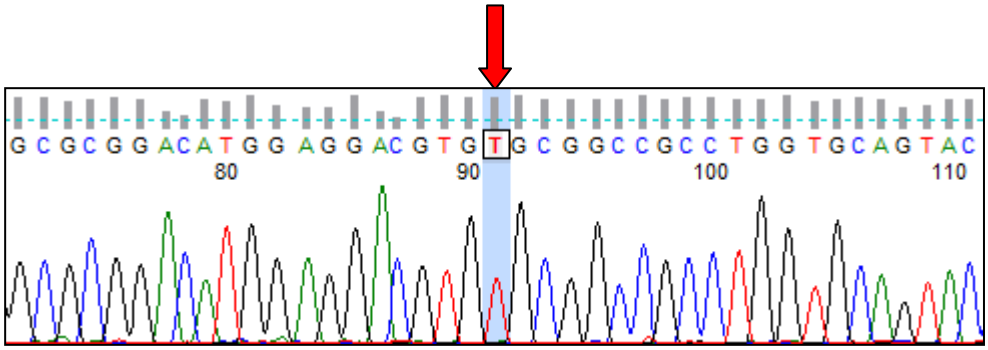

Figure 3.1.3. Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *APOE* primer set. The red arrow indicates the APOE4 locus (nucleotide 91). The "T" at the highlighted position corresponds to a Wild Type TT genotype.

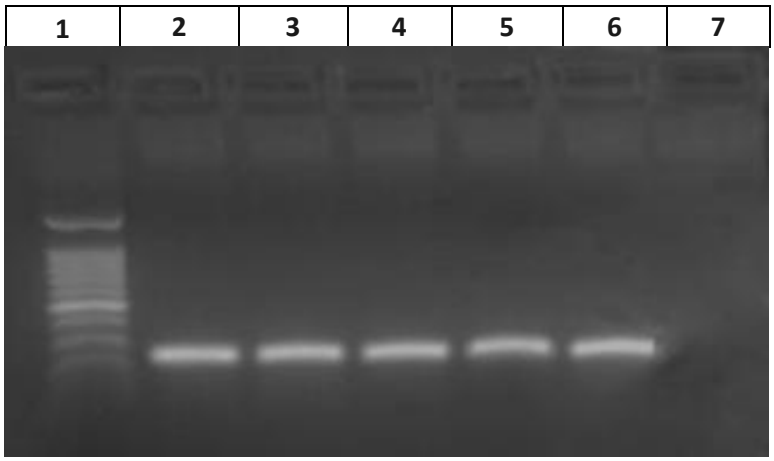

Figure 3.1.4. A 2% (w/v) agarose gel depicting the PCR amplicons synthesized with the *MTHFR* 677 primer set visualized with 0.0001% (v/v) EtBr. Lane 1 contains a 100-bp DNA ladder, lanes 2 to 6 contain amplicons of 256 bp and lane 7 contains the PCR blank. (Abbreviations: bp = base pairs)

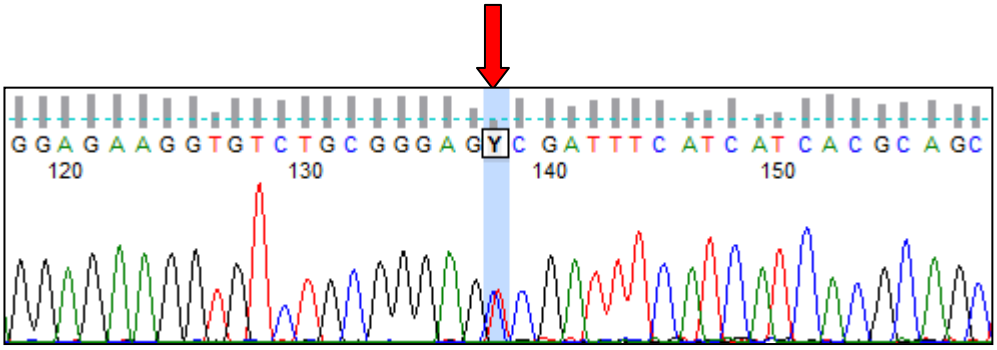

Figure 3.1.5. Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *MTHFR* 677 primer set. The red arrow indicates the mutation position (nucleotide 138). The "Y" at the highlighted position corresponds to a Heterozygous CT genotype.

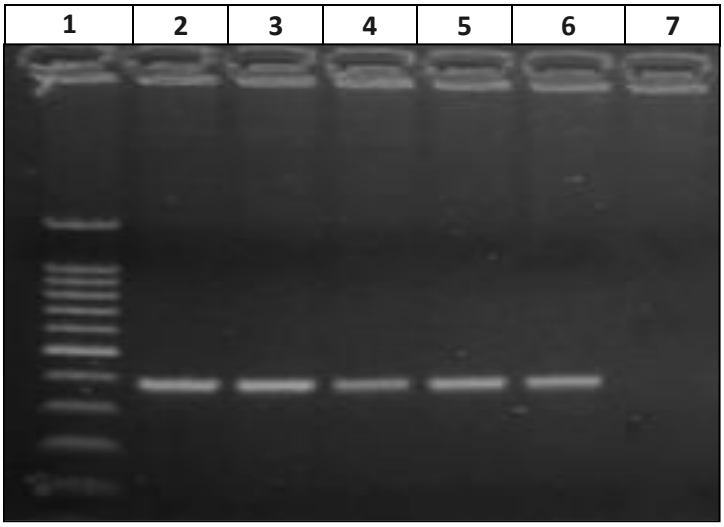

Figure 3.1.6. A 2% (w/v) agarose gel depicting the PCR amplicons synthesized with the *MTHFR* 1298 primer set visualized with 0.0001% (v/v) EtBr. Lane 1 contains a 100-bp DNA ladder, lanes 2 to 6 contain amplicons of 383 bp and lane 7 contains the PCR blank. (Abbreviations: bp = base pairs)

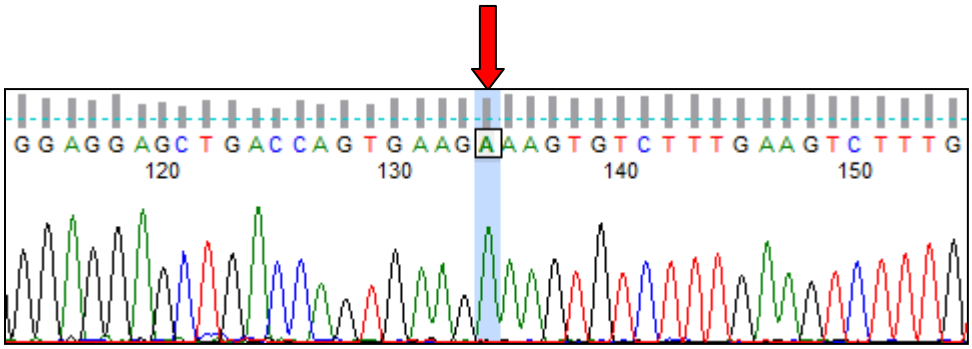

Figure 3.1.7. Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *MTHFR* 1298 primer set. The red arrow indicates the mutation position (nucleotide 134). The "A" at the highlighted position corresponds to a Wild Type AA genotype.

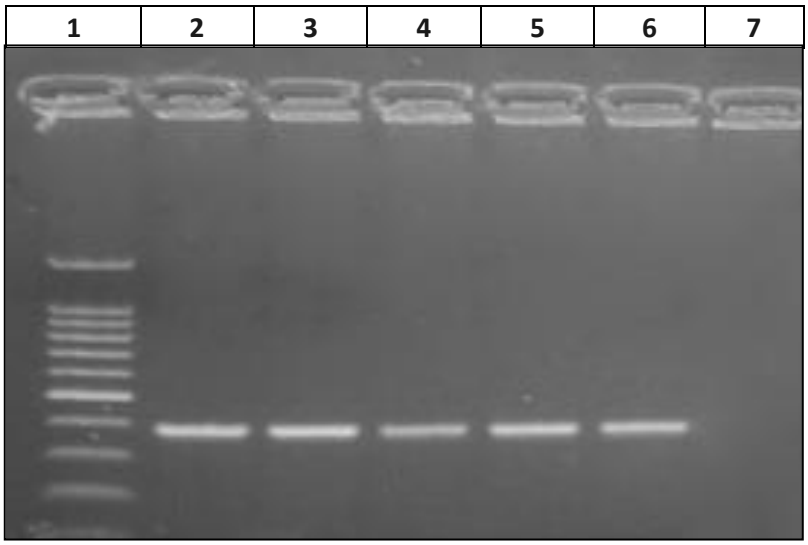

Figure 3.1.8. A 2% (w/v) agarose gel depicting the PCR amplicons synthesized with the *F2* 20210 primer set visualized with 0.0001% (v/v) EtBr. Lane 1 contains a 100-bp DNA ladder, lanes 2 to 6 contain amplicons of 432 bp and lane 7 contains the PCR blank. (Abbreviations: bp = base pairs)

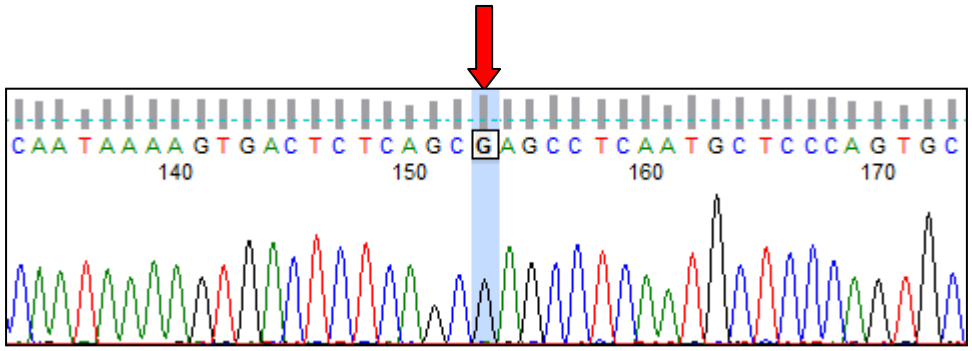

Figure 3.1.9. Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *F2* 20210 primer set. The red arrow indicates the mutation position (nucleotide 153). The "G" at the highlighted position corresponds to a Wild Type GG genotype.

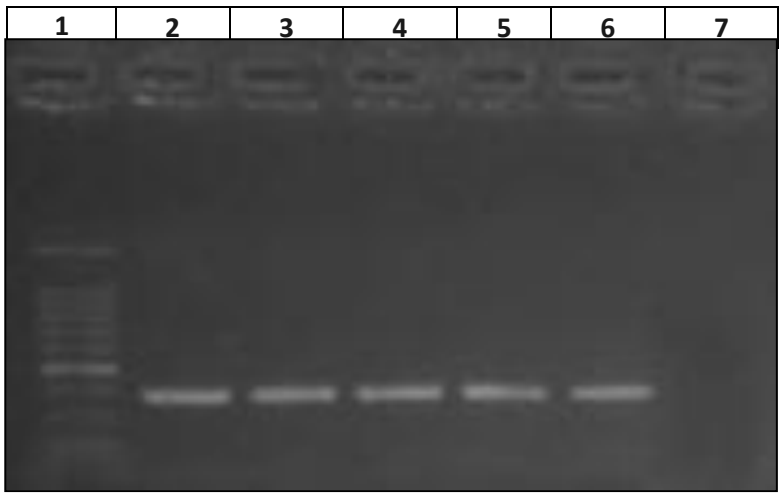

Figure 3.1.10. A 2% (w/v) agarose gel depicting the PCR amplicons synthesized with the *FV* Leiden primer set visualized with 0.0001% (v/v) EtBr. Lane 1 contains a 100-bp DNA ladder, lanes 2 to 6 contain amplicons of 409 bp and lane 7 contains the PCR blank. (Abbreviations: bp = base pairs)

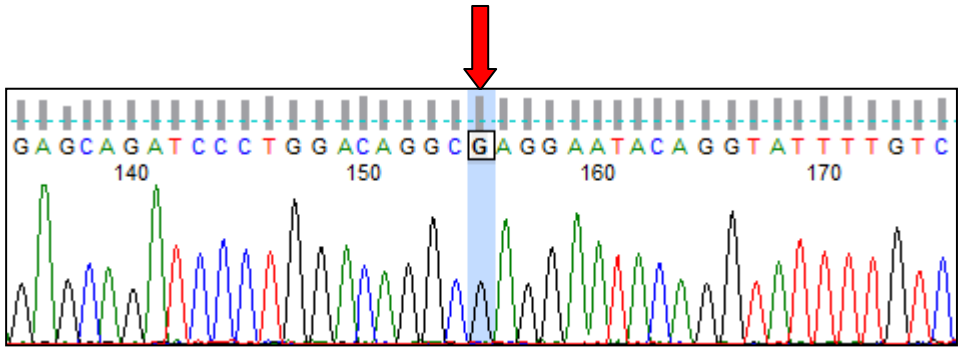

Figure 3.1.11. Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *FV* Leiden primer set. The red arrow indicates the mutation position (nucleotide 155). The "G" at the highlighted position corresponds to a Wild Type GG genotype.

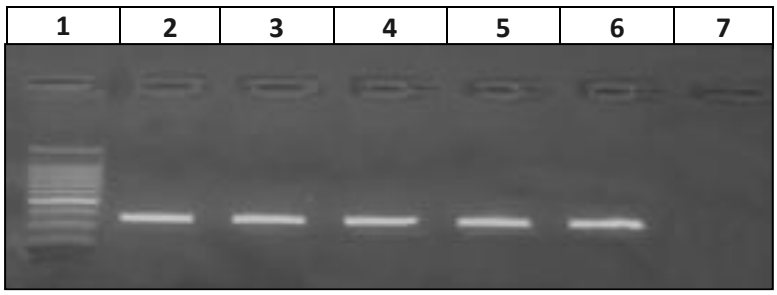

Figure 3.1.12. A 2% (w/v) agarose gel depicting the PCR amplicons synthesized with the *HFE* C282Y primer set visualized with 0.0001% (v/v) EtBr. Lane 1 contains a 100-bp DNA ladder, lanes 2 to 6 contain amplicons of 396 bp and lane 7 contains the PCR blank. (Abbreviations: bp = base pairs)

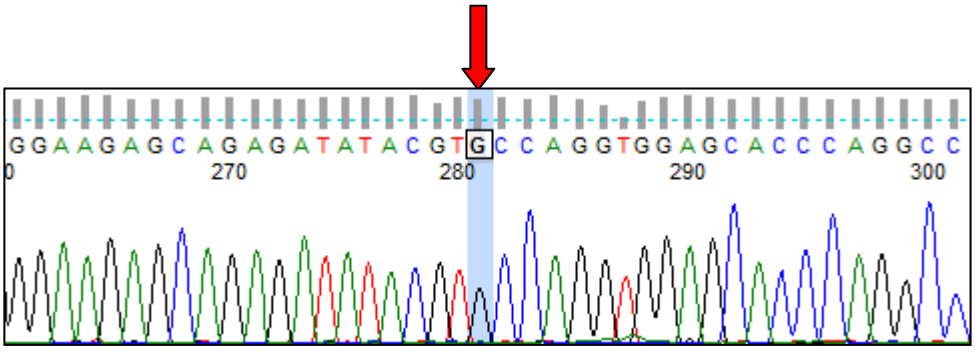

Figure 3.1.13. Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *HFE* C282Y primer set. The red arrow indicates the mutation position (nucleotide 281). The "G" at the highlighted position corresponds to a Wild Type GG genotype.

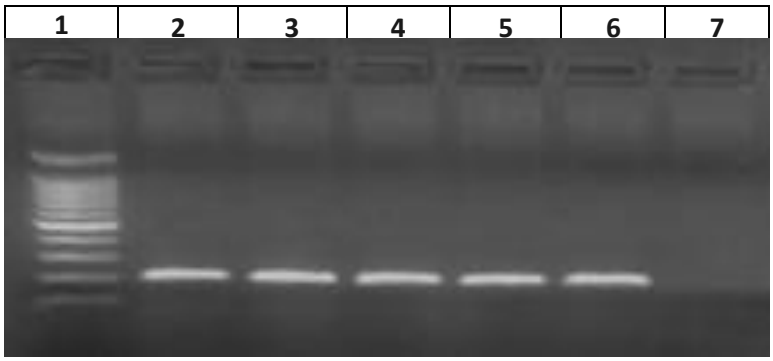

Figure 3.1.14. A 2% (w/v) agarose gel depicting the PCR amplicons synthesized with the *HFE* H63D primer set visualized with 0.0001% (v/v) EtBr. Lane 1 contains a 100-bp DNA ladder, lanes 2 to 6 contain amplicons of 208 bp and lane 7 contains the PCR blank. (Abbreviations: bp = base pairs)

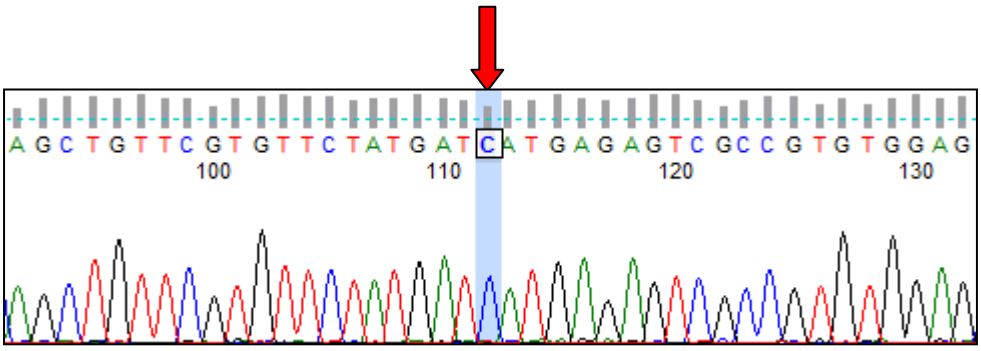

Figure 3.1.15. Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *HFE* H63D primer set. The red arrow indicates the mutation position (nucleotide 112). The "C" at the highlighted position corresponds to a Wild Type CC genotype.

3.2. RT-PCR Genotyping with the ABI™ 7900HT

The ABI™ 7900HT Fast Real-Time PCR System was used with ABI™ *Taq*Man® SNP Genotyping assays to determine the genotypes of the various control- and patient samples screened in this study. The instrument is controlled by the ABI™ SDS Software version 2.3 (Foster City, California, USA), which acts as the user interface and performs analysis of the fluorescence data obtained after amplification. Assay validation was achieved using the control samples with known genotypes and 12 Non-Template Controls (NTCs; with nuclease-free H_2O instead of DNA) per 96-well reaction plate. Thereafter, NAFLD patient samples were divided into three sample batches and screened for genetic variants by the same protocol. The RT-PCR runs also included 12 NTCs and five control samples that had previously been genotyped via conventional PCR and DNA sequencing for quality assurance purposes. Genotyping was achieved in three phases. The first involved an amplification run using the Standard Curve (AQ) setting, during which PCR products are exponentially synthesized and the associated fluorescence plotted as a graph by the ABI™ SDS software displaying "ΔRn (unit of fluorescence) versus cycles." The second phase was a post-amplification scan with the allelic discrimination setting. The SDS software presents the results on an allelic discrimination scatterplot by contrasting the fluorescence values obtained from the FAM™ and VIC® dyes. After signal normalization and multi-component analysis, the software plots the data obtained from every well on the 96-well plate, each as a single datapoint on the scatterplot. The allelic discrimination analysis displays the results as an "Allele Y ("Assay ID and Specific Allele") versus Allele X ("Assay ID and Specific Alternate Allele")" graph. Analysis and verification of the results constituted the final phase of the genotyping process.

All eight assays employed in the mutation screening yielded clear amplification of the polymorphic target sequence for all samples investigated. The specificity of the assays were verified by the internal control samples included in each of the RT-PCR runs, yielding identical genotype calls to those obtained through DNA sequencing. The tight and distinct clustering of the NTCs revealed that no contamination was present in the reaction setups, ensuring the credibility of the results. Due to the sheer volume of genotyping data generated by this study, only one sample batch for each assay is provided in this section to illustrate the results obtained through successful application of this RT-PCR genotyping system.

The amplification plot for APOE2 is presented in figure 3.2.1 and features the raw fluorescence data acquired during amplification. The APOE2 allelic discrimination plot is a graphical representation of the genotypes of each sample tested and can be found in figure 3.2.2, with black dots representing NTCs, red dots equivalent to wild types, green dots indicative of heterozygotes and the less prevalent blue dots signifying homozygotes.

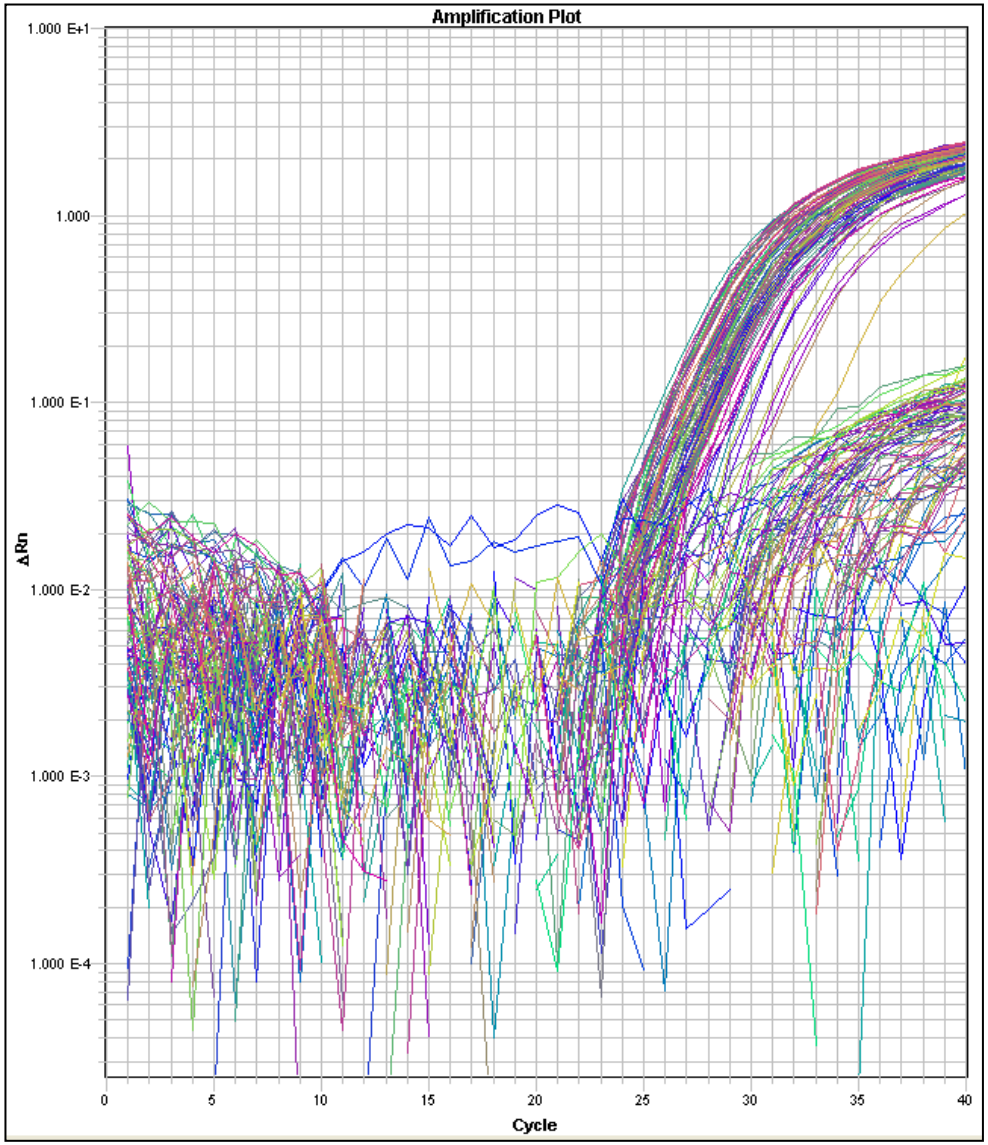

Figure 3.2.1. Typical amplification achieved using the ABI™ *Taq*Man® assay for APOE2 (ΔRn vs number of cycles). (Abbreviations: ΔRn = unit of fluorescence)

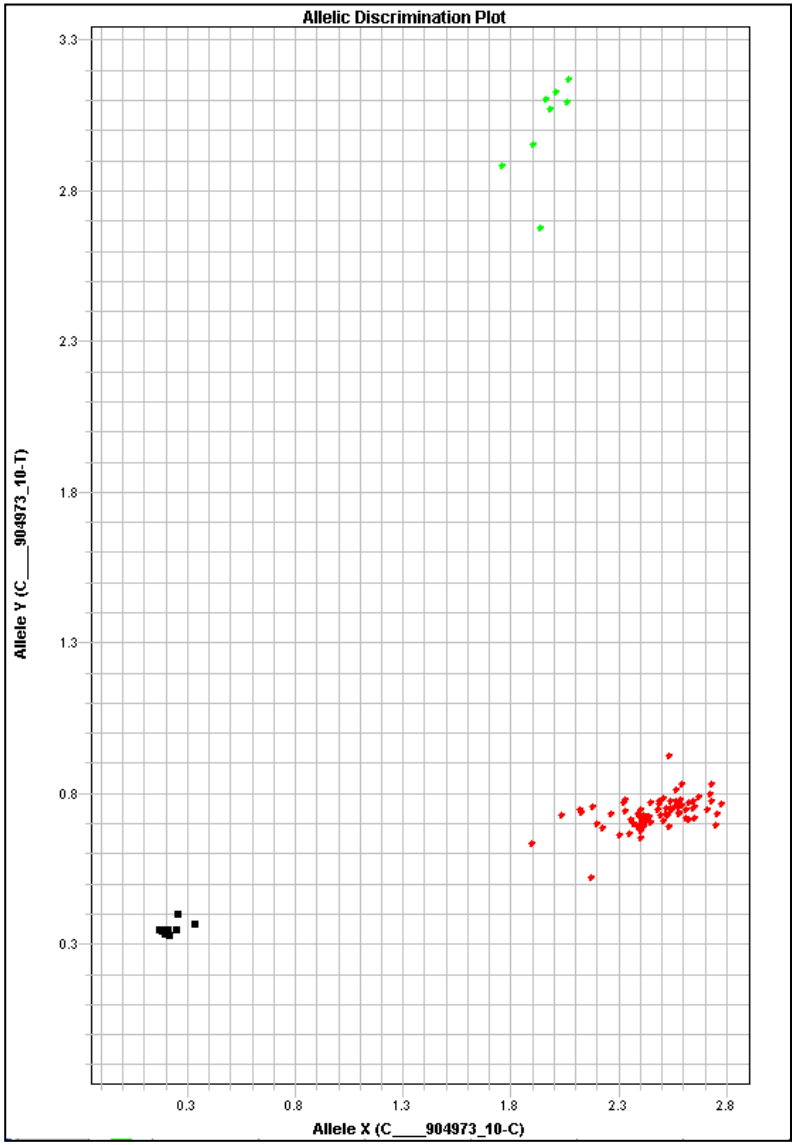

Figure 3.2.2. Typical allelic discrimination analysis using the ABI™ *Taq*Man® assay for APOE2 [Allele Y (C_904973_10-T) vs Allele X (C_904973_10-C)]. Black = NTC, Red = Wild Type CC and Green = Heterozygous CT.

The genotype distribution observed for APOE2 among all NAFLD (178) and control (75) samples is summarized and contrasted in figure 3.2.3. The total study population, which includes NAFLD patients and controls for a total of 253 samples, was found to be 86.96% Wild Type CC, 13.04% Heterozygous CT and 0.0% Homozygous TT.

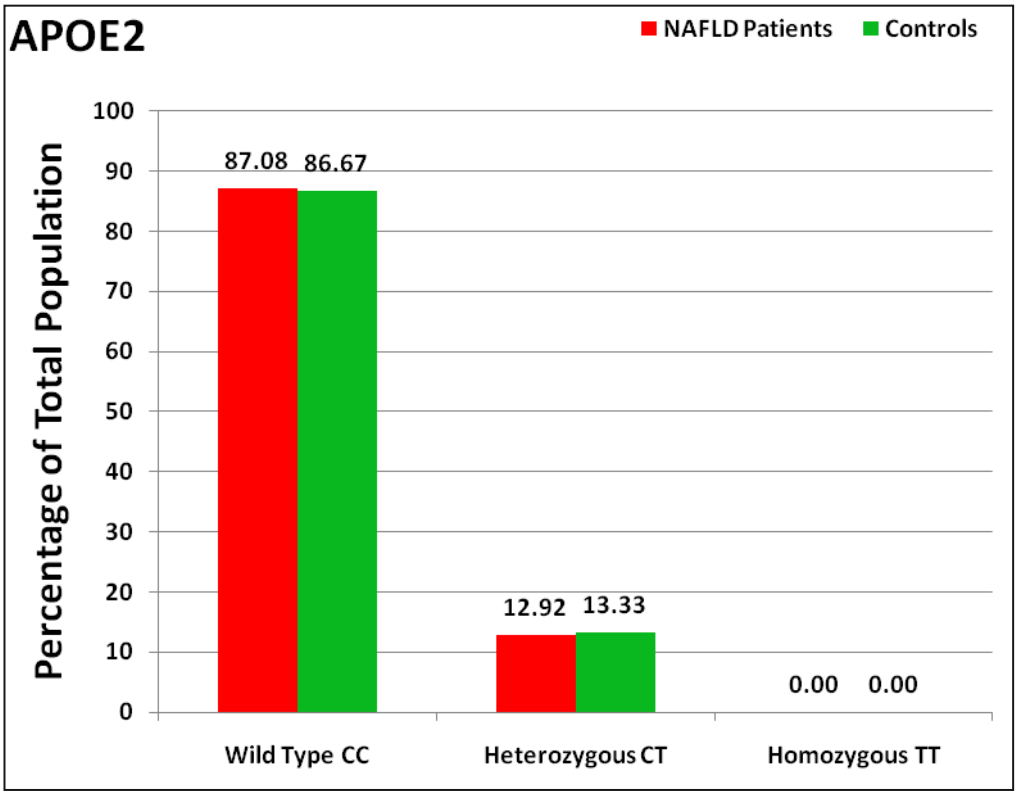

Figure 3.2.3. Genotype distribution of 253 samples obtained using the ABI™ *Taq*Man® APOE2 assay. 178 NAFLD samples presented with 155 Wild Type CC (87.08%), 23 Heterozygous CT (12.92%) and 0 (0.00%) Homozygous TT. Among the 75 control samples, 65 (86.67%) were Wild Type CC, 10 (13.33%) Heterozygous CT and 0 (0.00%) Homozygous TT.

The amplification plot for APOE4 is presented in figure 3.2.4 and features the raw fluorescence data acquired during amplification. The APOE4 allelic discrimination plot is a graphical representation of the genotypes of each sample tested and can be found in figure 3.2.5, with black dots representing NTCs, red dots equivalent to wild types, green dots indicative of heterozygotes and the less prevalent blue dots signifying homozygotes.

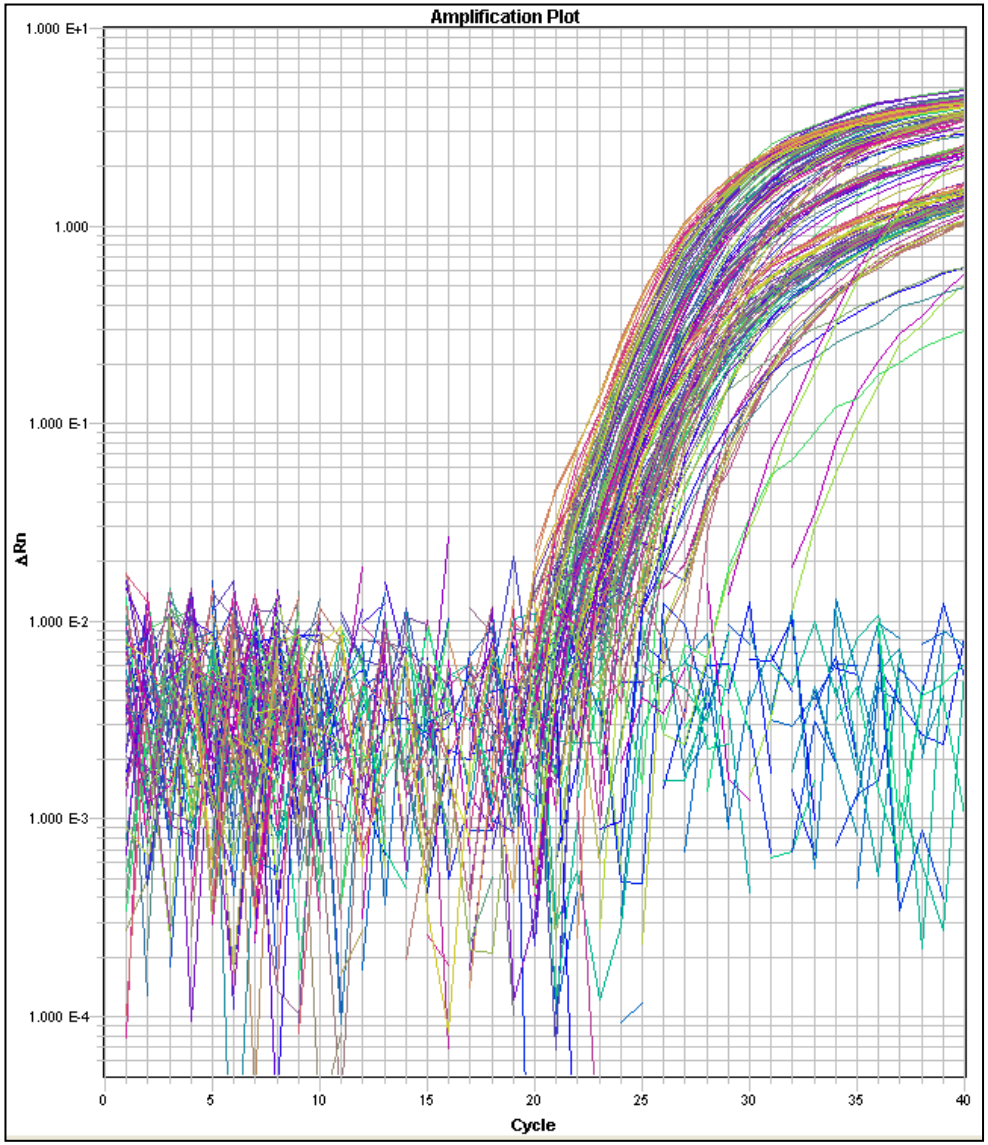

Figure 3.2.4. Typical amplification achieved using the ABI™ *Taq*Man® assay for APOE4 (ΔRn vs number of cycles). (Abbreviations: ΔRn = unit of fluorescence)

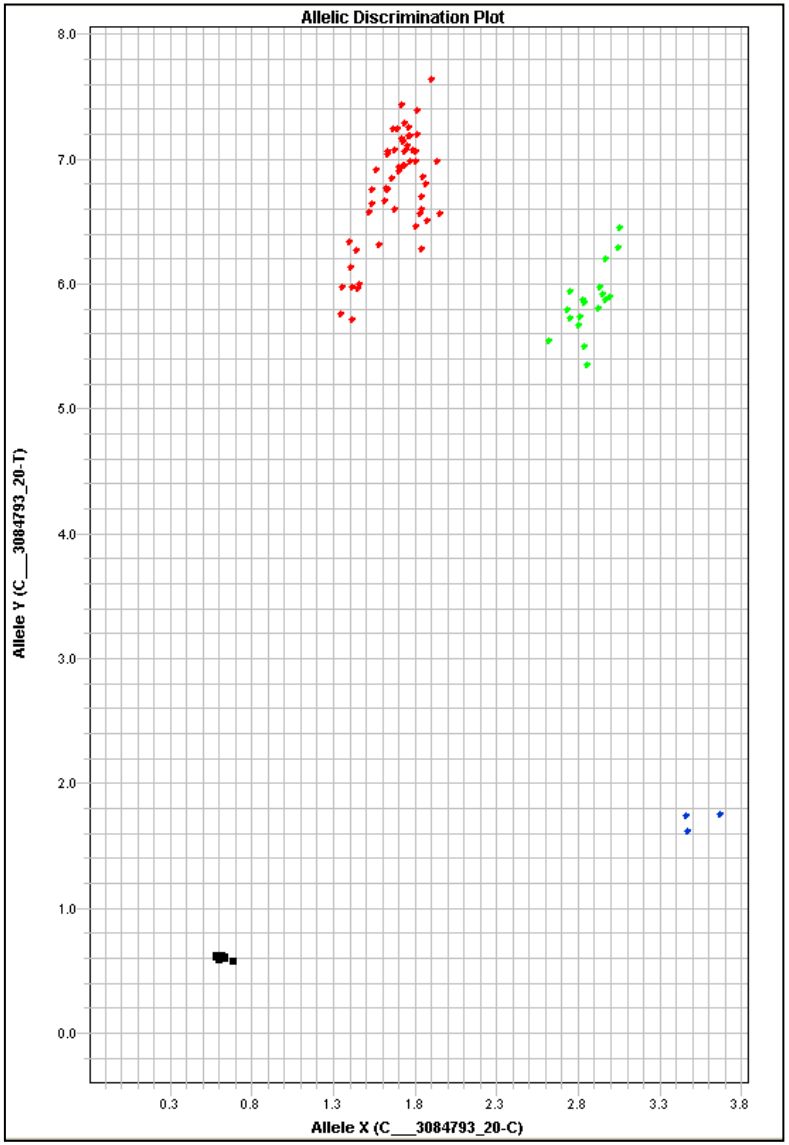

Figure 3.2.5. Typical allelic discrimination analysis using the ABI™ *Taq*Man® assay for APOE4 [Allele Y (C_3084793_20-T) vs Allele X (C_3084793_20-C)]. Black = NTC, Red = Wild Type TT, Green = Heterozygous TC and Blue = Homozygous CC.

The genotype distribution observed for APOE4 among all NAFLD (178) and control (75) samples is summarized in figure 3.2.6. The total study population, which includes NAFLD patients and controls for a total of 253 samples, was found to be 66.14% Wild Type TT, 30.28% Heterozygous TC and 3.59% Homozygous CC.

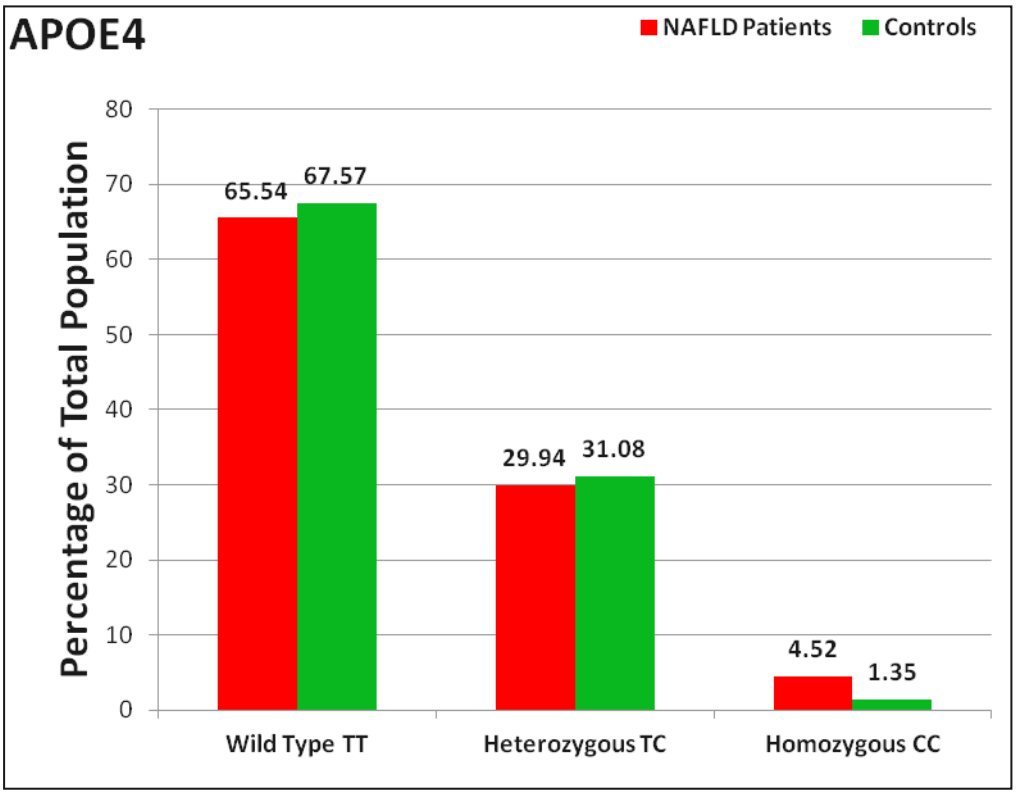

Figure 3.2.6. Genotype distribution of 253 samples obtained using the ABI™ *Taq*Man® APOE4 assay. 178 NAFLD samples presented with 116 Wild Type TT (65.54%), 53 Heterozygous CT (29.94%), 8 (4.52%) Homozygous TT and 1 could not be determined. Among the 75 control samples, 50 (67.57%) were Wild Type TT, 23 (31.08%) Heterozygous T, 1 (1.35%) Homozygous CC and 1 could not be determined.

The genotypes of one of the NAFLD patient samples and one of the control samples analysed could not be determined through the RT-PCR protocol employed in this study even though they were re-tested several times. These samples were subsequently excluded from further analysis after the DNA quality within the amplification loci were called into question. The amplification plot for *MTHFR* 677 is presented in figure 3.2.7 and features the raw fluorescence data acquired during amplification. The *MTHFR* 677 allelic discrimination plot in figure 3.2.8 presents the sample genotypes as groups, where black dots = NTCs, red = wild types, green = heterozygotes, blue = homozygotes. The *MTHFR* 677 assay probes bind to the reverse (anti-sense) strand. Therefore, the "C_1202883_20-G" and "C_1202883_20-A" alleles presented on the allelic discrimination plot in figure 3.2.8 correspond to the forward (sense) nucleotides "C" and "T," respectively.

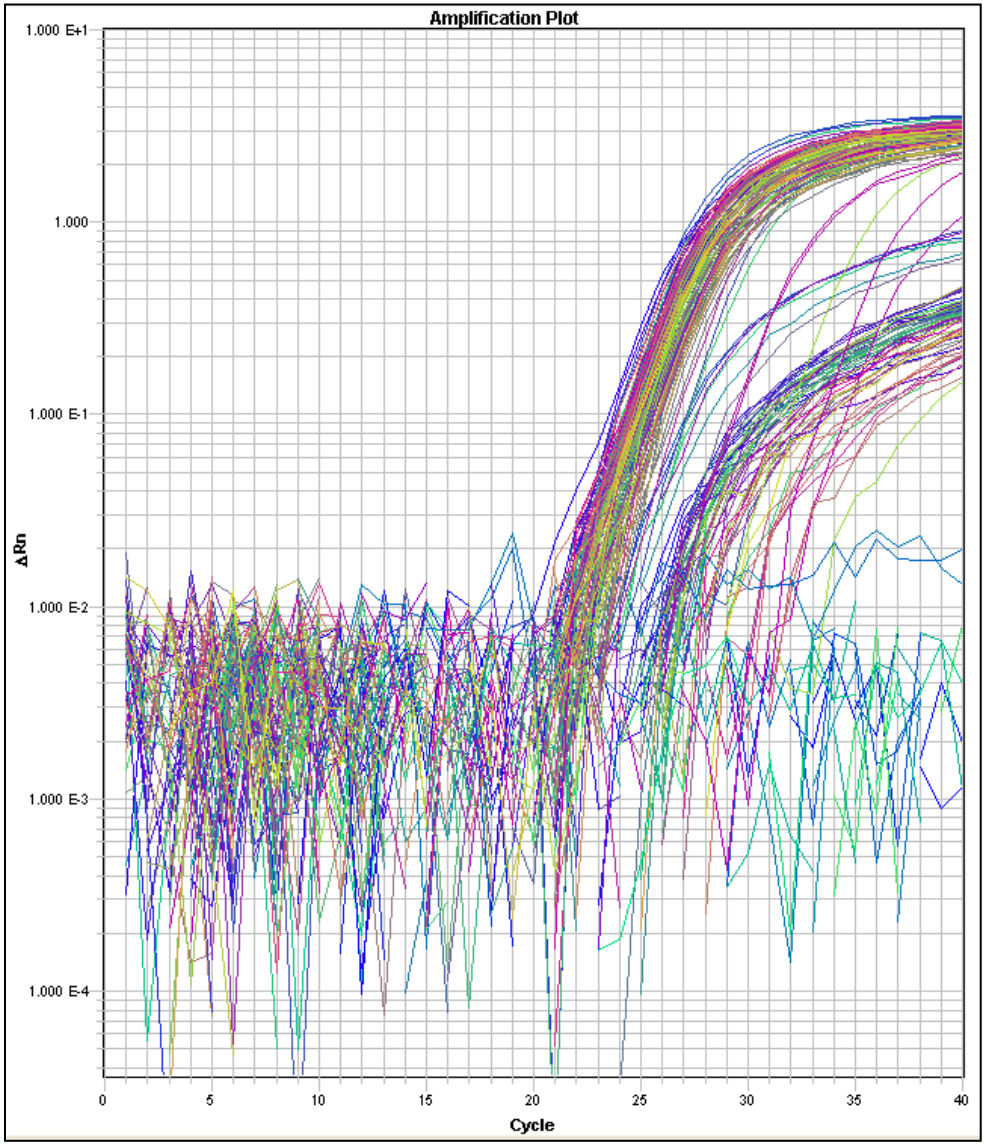

Figure 3.2.7. Typical amplification achieved using the ABI™ *Taq*Man® assay for *MTHFR* 677 (ΔRn vs number of cycles). (Abbreviations: ΔRn = unit of fluorescence)

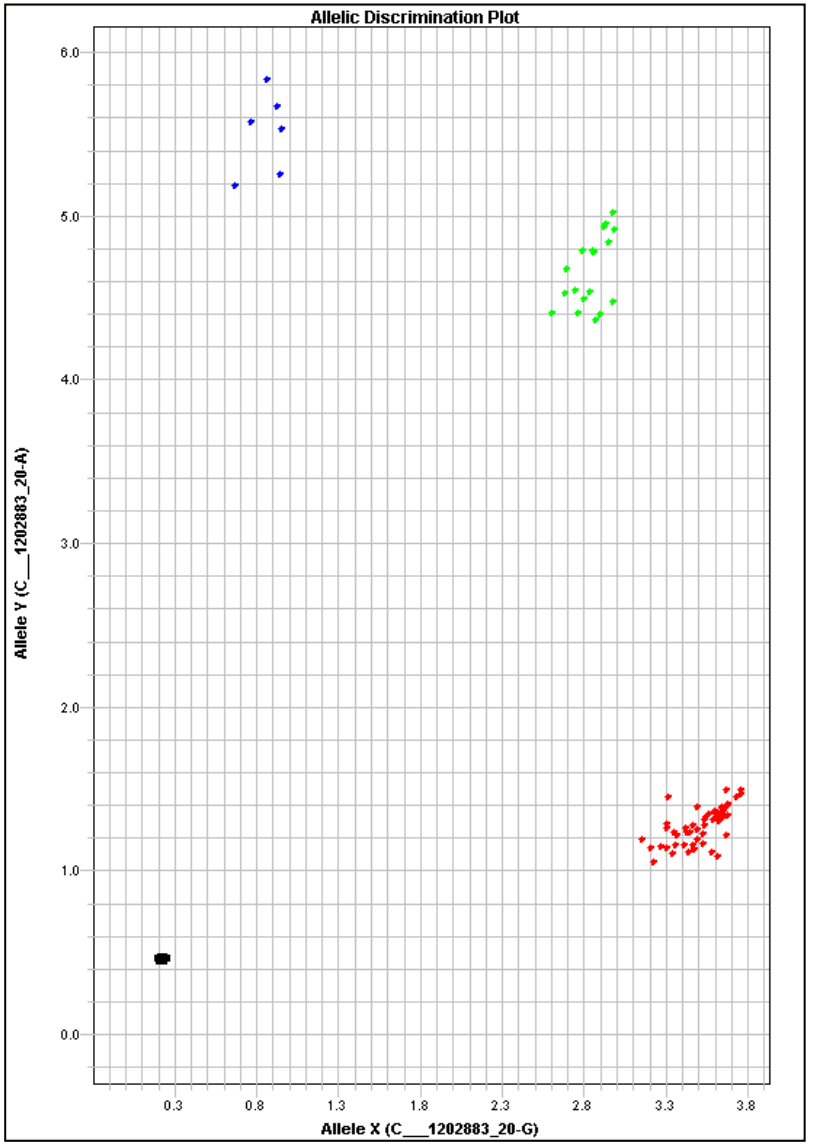

Figure 3.2.8. Typical allelic discrimination analysis using the ABI™ *Taq*Man® assay for *MTHFR* 677 [Allele Y (C_1202883_20-A) vs Allele X (C_1202883_20-G)]. Black = NTC, Red = Wild Type CC, Green = Heterozygous CT and Blue = Homozygous TT.

The genotype distribution observed for *MTHFR* 677 among all NAFLD (178) and control (75) samples is summarized in figure 3.2.9. The total study population, which includes NAFLD patients and controls for a total of 253 samples, was found to be 64.82% Wild Type CC, 28.46% Heterozygous CT and 6.72% Homozygous TT.

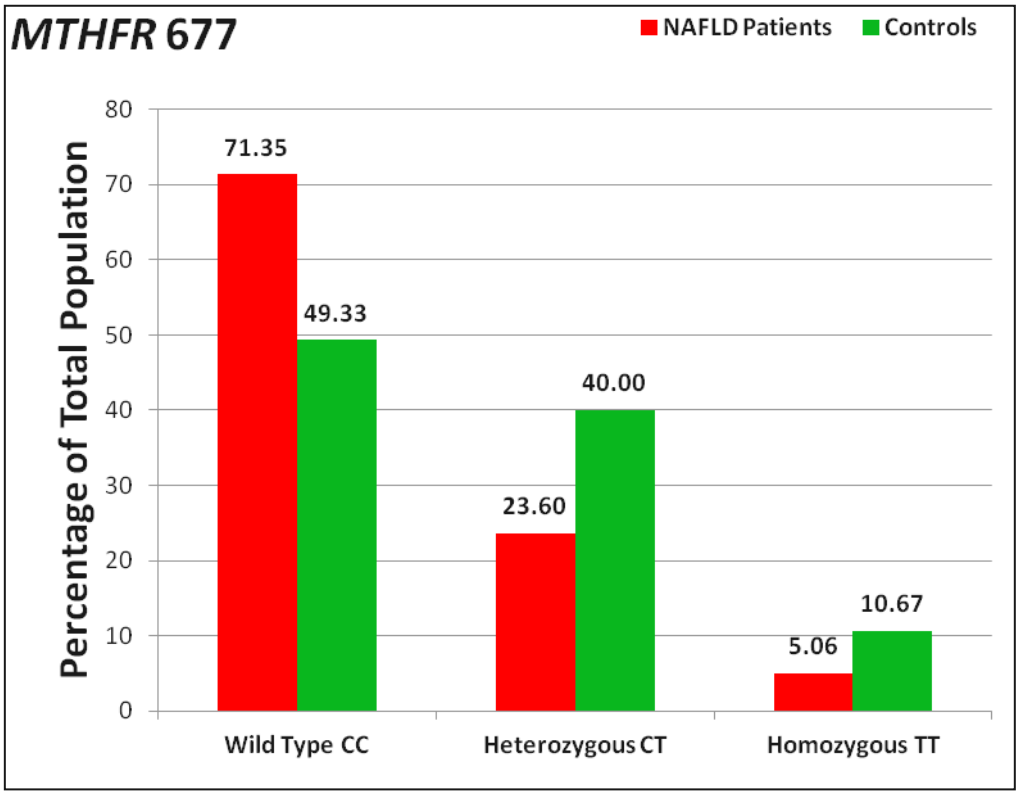

Figure 3.2.9. Genotype distribution of 253 samples obtained using the ABI™ *Taq*Man® *MTHFR* 677 assay. 178 NAFLD samples presented with 127 Wild Type CC (71.35%), 42 Heterozygous CT (23.60%) and 9 (5.06%) Homozygous TT. Among the 75 control samples, 37 (49.33%) were Wild Type CC, 30 (40.00%) Heterozygous CT and 8 (10.67%) Homozygous TT.

The amplification plot for *MTHFR* 1298 is presented in figure 3.2.10 and features the raw fluorescence data acquired during amplification. The *MTHFR* 1298 allelic discrimination plot is a graphical representation of the genotypes of each sample tested and can be found in figure 3.2.11, with black dots representing NTCs, red dots equivalent to wild types, green dots indicative of heterozygotes and the less prevalent blue dots signifying homozygotes.

The probes employed by the *MTHFR* 1298 assay are designed to bind to the reverse (anti-sense) strand. Therefore, the "C_850486_20-T" and "C_850486_20-G" alleles presented on the allelic discrimination plot in figure 3.2.11 correspond to the forward (sense) nucleotides "A" and "C," respectively.

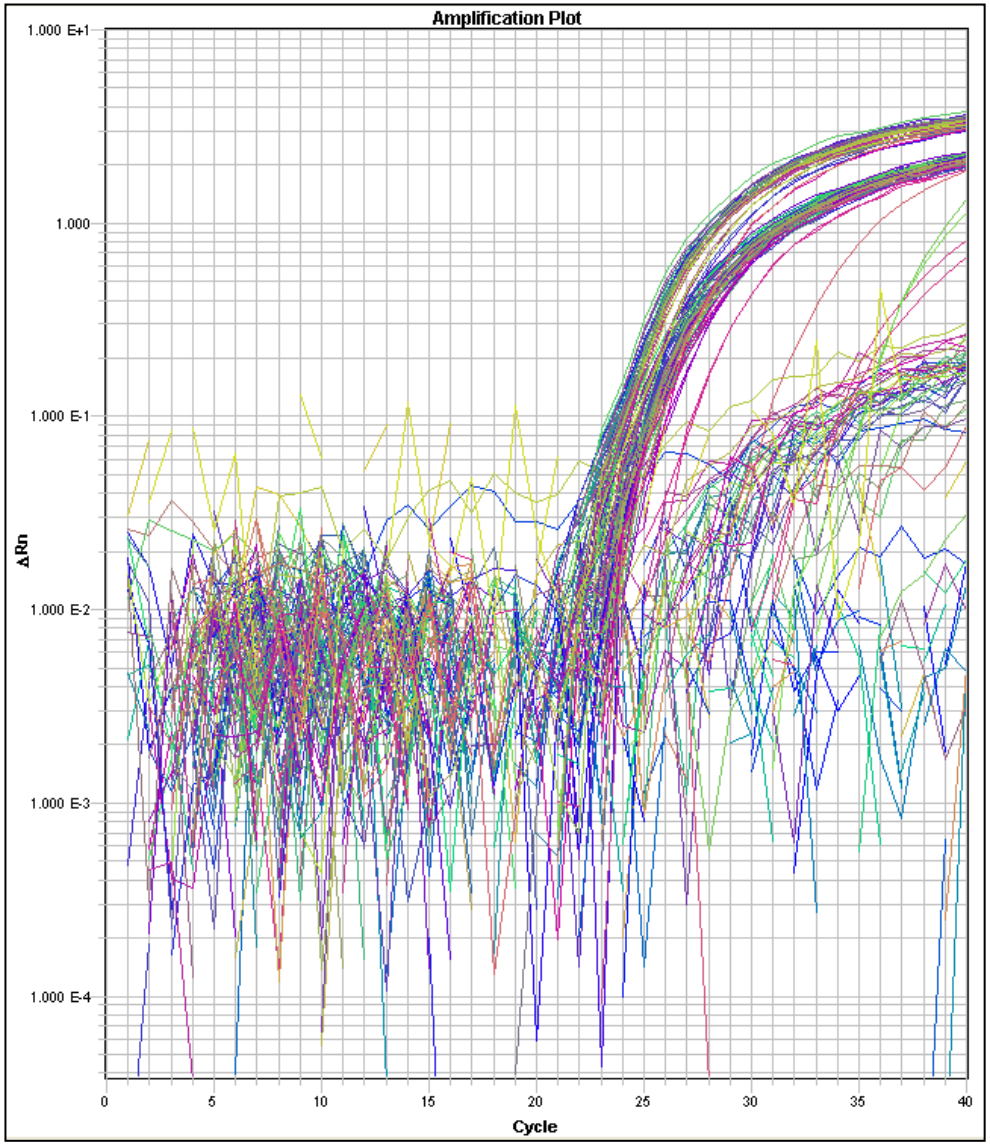

Figure 3.2.10. Typical amplification achieved using the ABI™ *Taq*Man® assay for *MTHFR* 1298 (ΔRn vs number of cycles). (Abbreviations: ΔRn = unit of fluorescence)

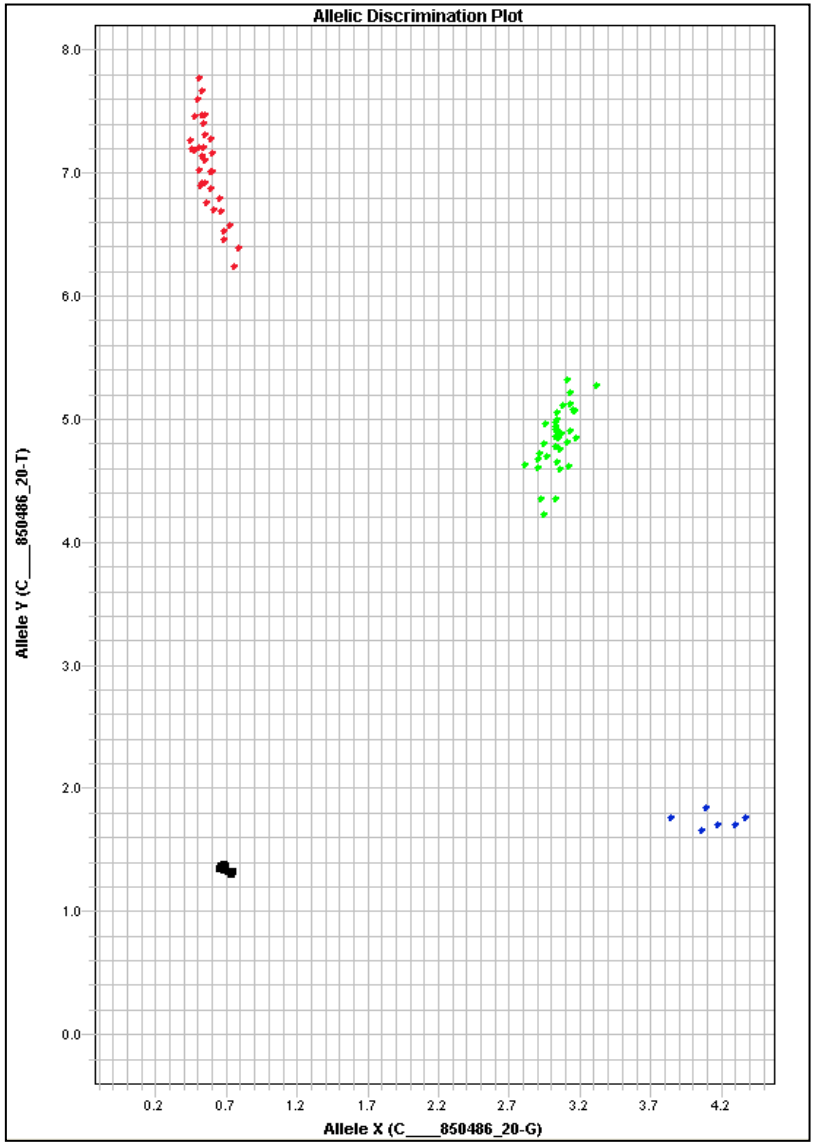

Figure 3.2.11. Typical allelic discrimination analysis using the ABI™ *Taq*Man® assay for *MTHFR* 1298 [Allele Y (C_850486_20-T) vs Allele X (C_850486_20-G)]. Black = NTC, Red = Wild Type AA, Green = Heterozygous AC and Blue = Homozygous CC.

The genotype distribution observed for *MTHFR* 1298 among all NAFLD (178) and control (75) samples is summarized in figure 3.2.12. The total study population, which includes NAFLD patients and controls for a total of 253 samples, was found to be 46.64% Wild Type AA, 43.87% Heterozygous AC and 9.49% Homozygous CC.

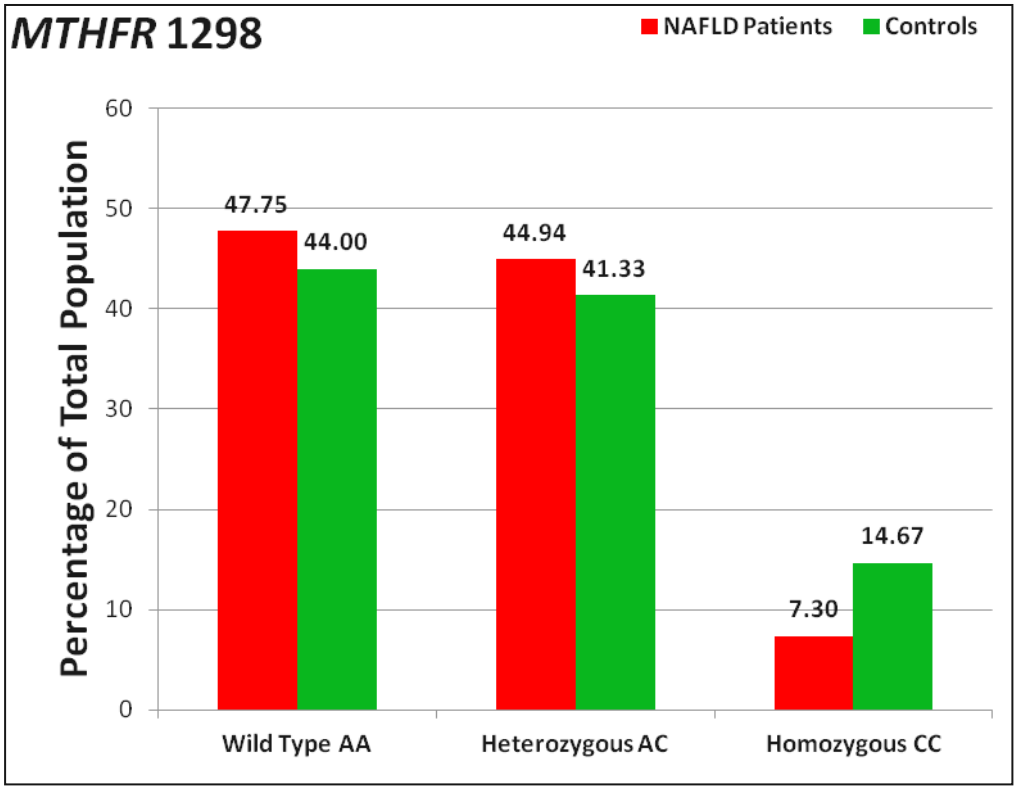

Figure 3.2.12. Genotype distribution of 253 samples obtained using the ABI™ *Taq*Man® *MTHFR* 1298 assay. 178 NAFLD samples presented with 85 Wild Type AA (47.75%), 80 Heterozygous AC (44.94%) and 13 (7.30%) Homozygous CC. Among the 75 control samples, 33 (44.00%) were Wild Type AA, 31 (41.33%) Heterozygous AC and 11 (14.67%) Homozygous CC.

The amplification plot for *F2* 20210 is presented in figure 3.2.13 and features the raw fluorescence data acquired during amplification. The *F2* 20210 allelic discrimination plot is a graphical representation of the genotypes of each sample tested and can be found in figure 3.2.14, with black dots representing NTCs, red dots equivalent to wild types, green dots indicative of heterozygotes and the less prevalent blue dots signifying homozygotes.

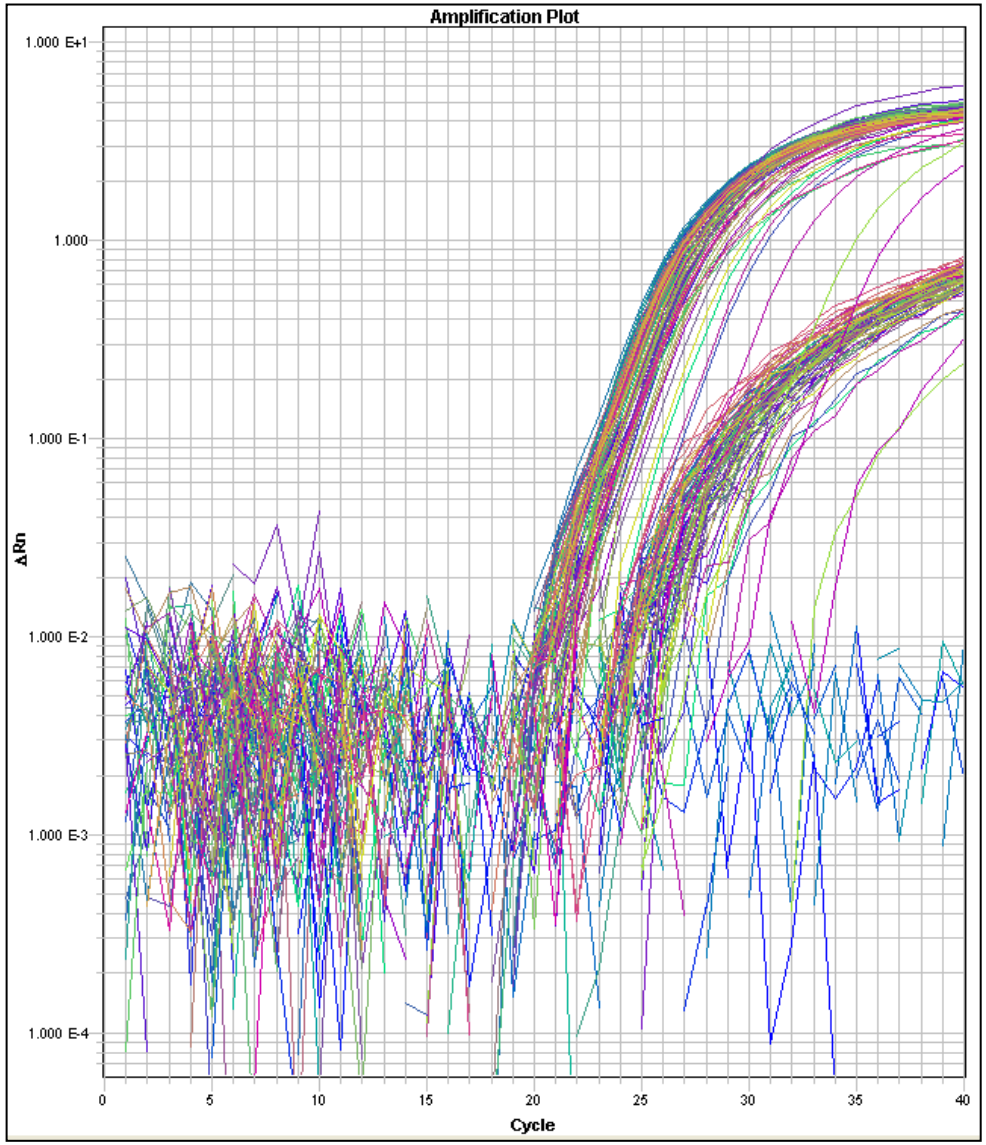

Figure 3.2.13. Typical amplification achieved using the ABI™ *Taq*Man® assay for *F2* 20210 (ΔRn vs number of cycles). (Abbreviations: ΔRn = unit of fluorescence)

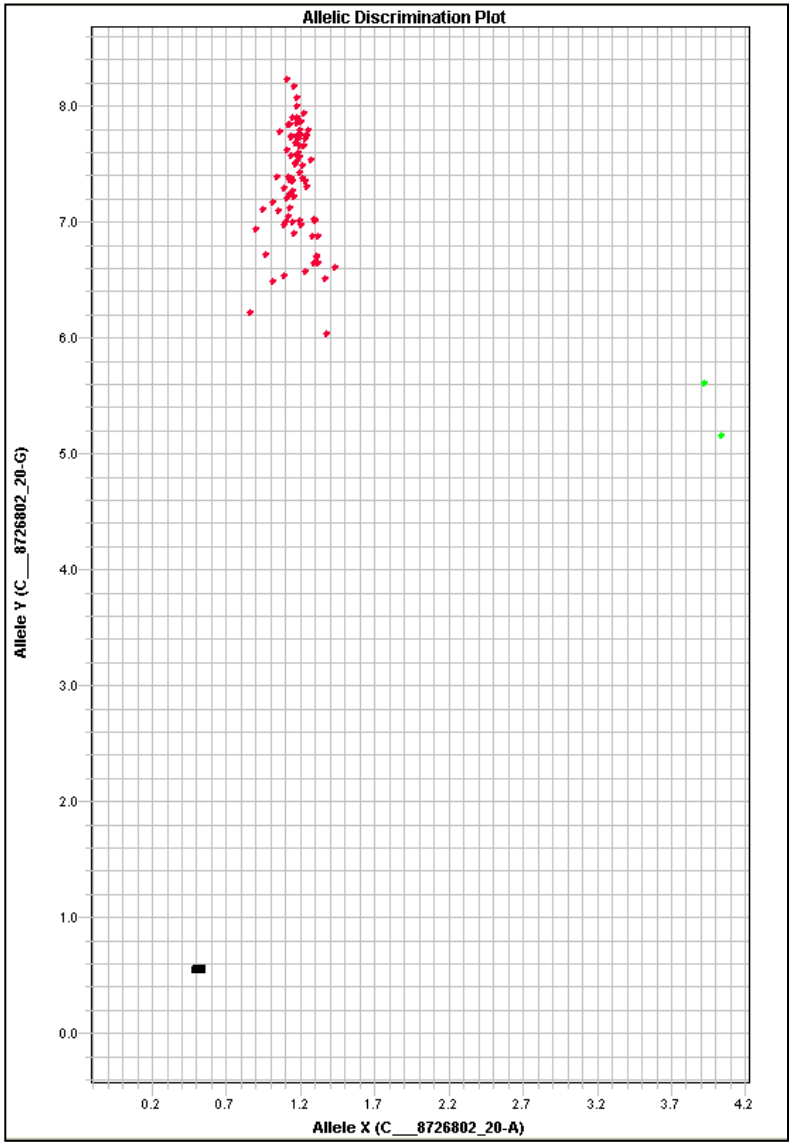

Figure 3.2.14. Typical allelic discrimination analysis using the ABI™ *Taq*Man® assay for *F2* 20210 [Allele Y (C_8726802_20-G) vs Allele X (C_8726802_20-A)]. Black = NTC, Red = Wild Type GG and Green = Heterozygous GA.

The genotype distribution observed for *F2* 20210 among all NAFLD (178) and control (75) samples is summarized in figure 3.2.15. The total study population, which includes NAFLD patients and controls for a total of 253 samples, was found to be 98.80% Wild Type GG, 1.20% Heterozygous GA and 0.00% Homozygous AA.

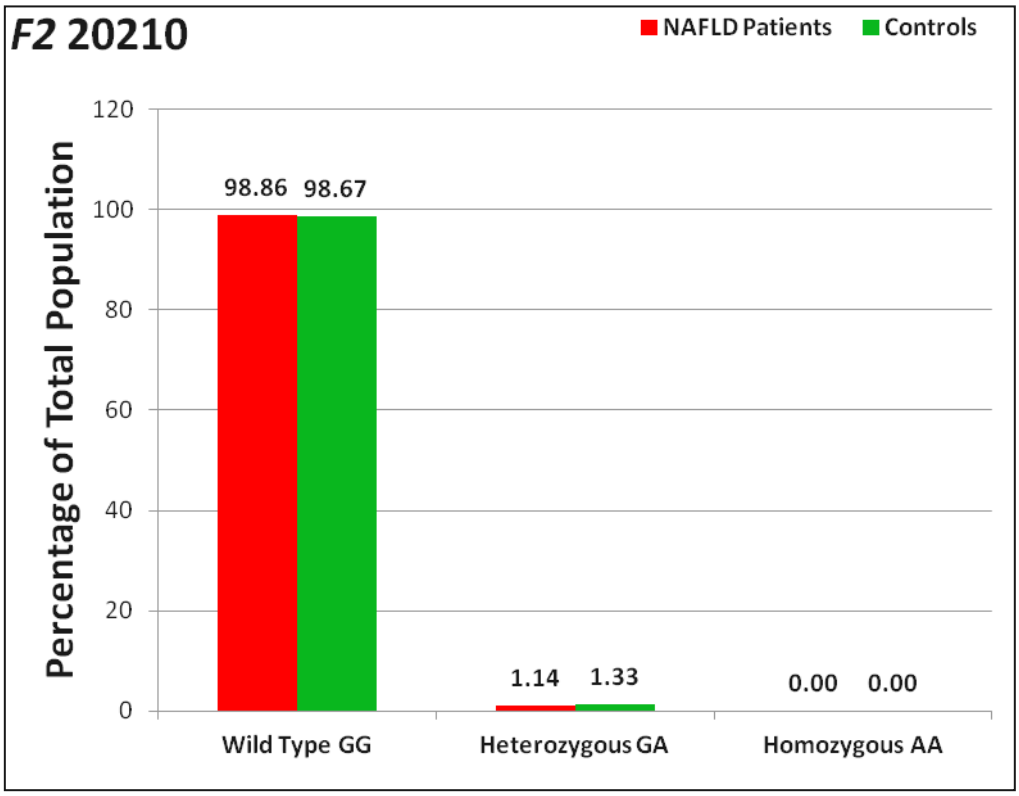

Figure 3.2.15. Genotype distribution of 253 samples obtained using the ABI™ *Taq*Man® *F2* 20210 assay. 178 NAFLD samples presented with 173 Wild Type GG (98.86%), 2 Heterozygous GA (1.14%), 0 (0.00%) Homozygous AA and 3 could not be determined. Among the 75 control samples, 74 (98.67%) were Wild Type GG, 1 (1.33%) Heterozygous GA and 0 (0.00%) Homozygous AA.

The genotypes of three of the NAFLD patient samples analysed could not be determined through the RT-PCR protocol employed in this study even though they were re-tested several times. These samples were subsequently excluded from further analysis after the DNA quality within the amplification loci were called into question. The amplification plot for *FV* Leiden is presented in figure 3.2.16 and features the raw fluorescence data acquired during amplification. The *FV* Leiden allelic discrimination plot is a graphical representation of the genotypes of each sample tested and can be found in figure 3.2.17, with black dots representing NTCs, red dots equivalent to wild types, green dots indicative of heterozygotes and the less prevalent blue dots signifying homozygotes. The *FV* Leiden assay probes bind to the reverse (anti-sense) strand. Therefore, the "C_11975250_10-C" and "C_11975250_10-T" alleles presented on the allelic discrimination plot in figure 3.2.17 correspond to the forward (sense) nucleotides "G" and "A," respectively.

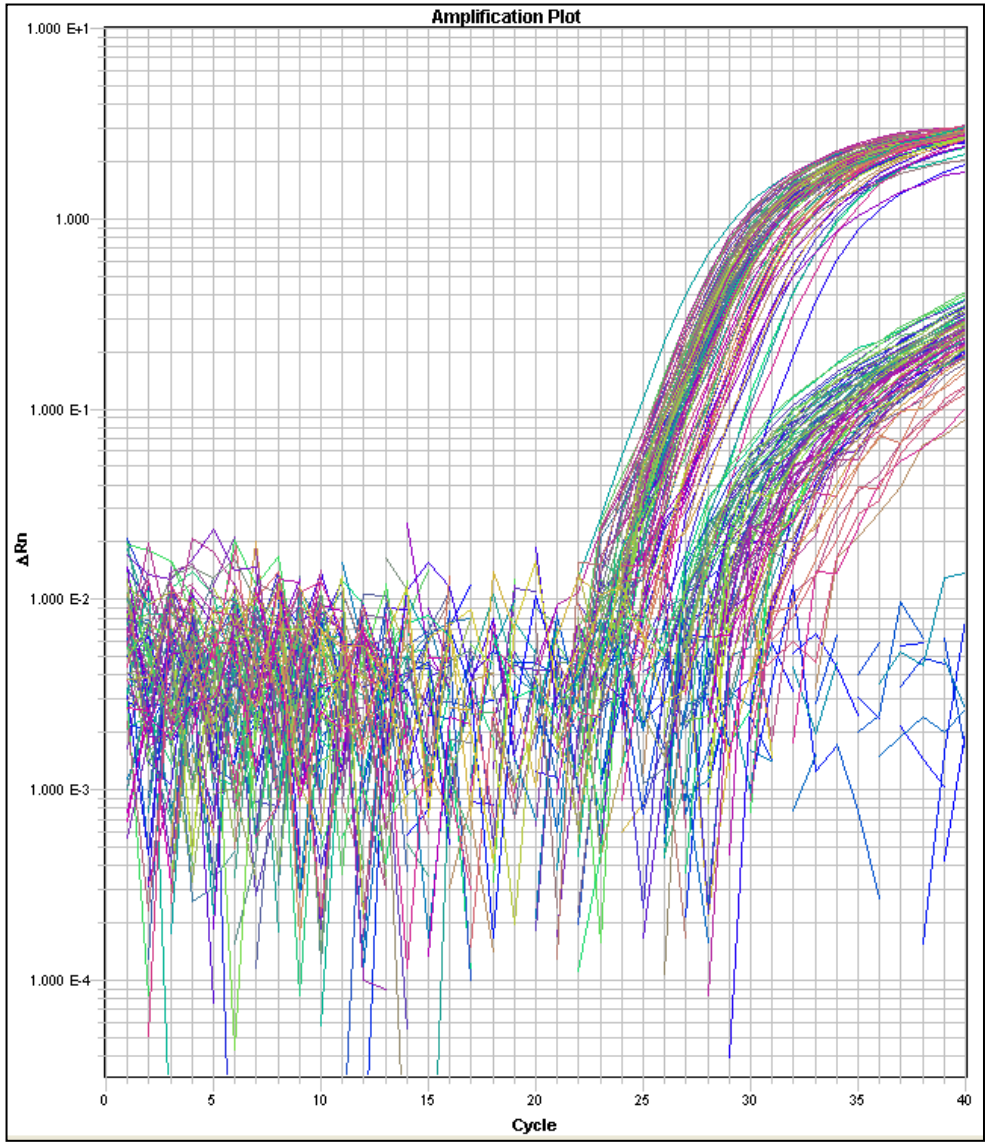

Figure 3.2.16. Typical amplification achieved using the ABI™ *Taq*Man® assay for *FV* Leiden (ΔRn vs number of cycles). (Abbreviations: ΔRn = unit of fluorescence)

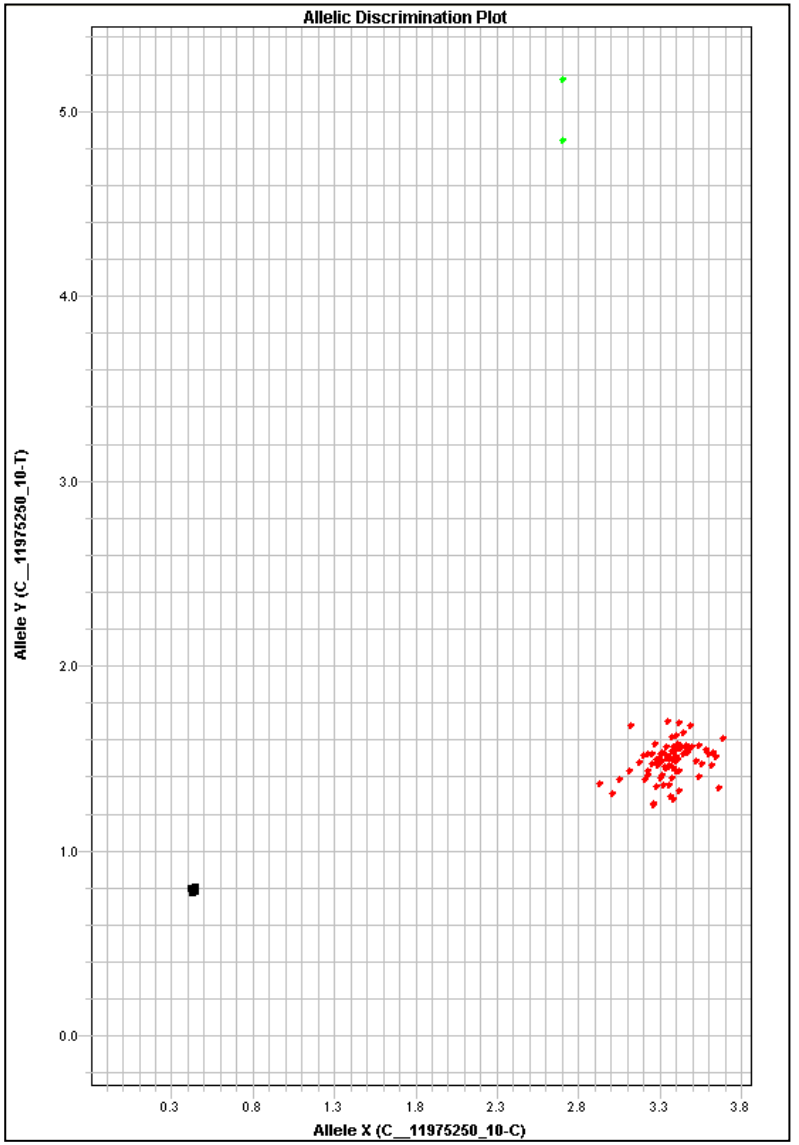

Figure 3.2.17. Typical allelic discrimination analysis using the ABI™ *Taq*Man® assay for *FV* Leiden [Allele Y (C_11975250_10-T) vs Allele X (C_11975250_10-C)]. Black = NTC, Red = Wild Type GG and Green = Heterozygous GA.

The genotype distribution observed for *FV* Leiden among all NAFLD (178) and control (75) samples is summarized in figure 3.2.18. The total study population, which includes NAFLD patients and controls for a total of 253 samples, was found to be 97.63% Wild Type GG, 2.37% Heterozygous GA and 0.00% Homozygous AA.

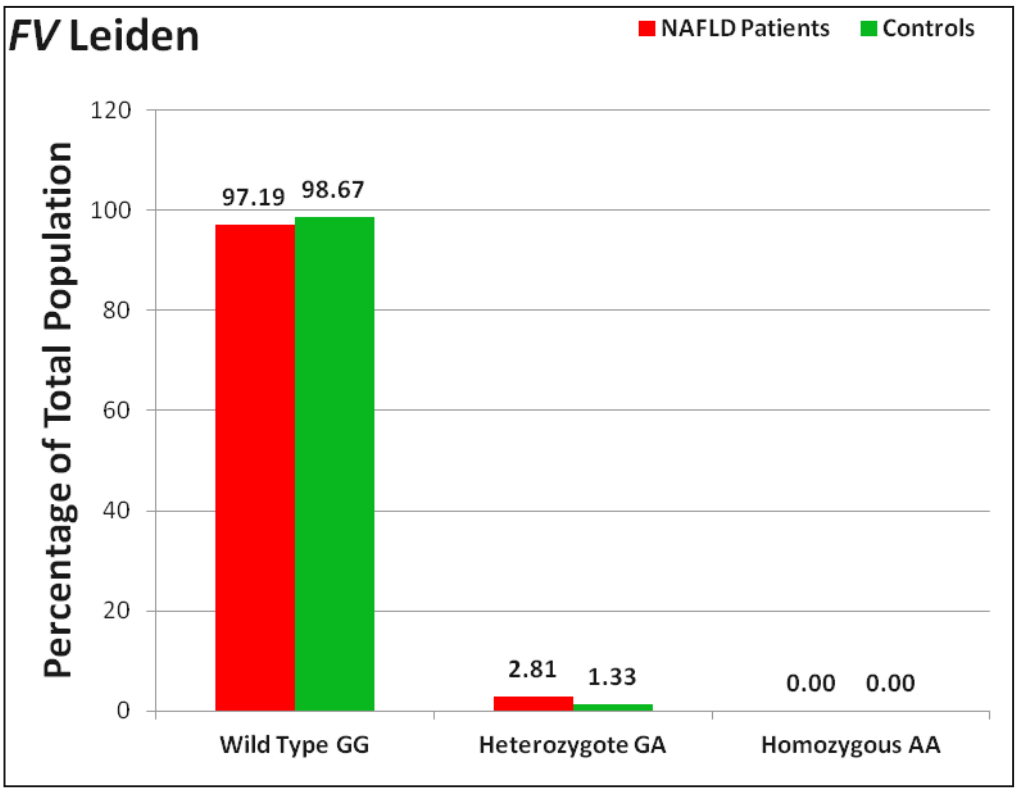

Figure 3.2.18. Genotype distribution of 253 samples obtained using the ABI™ *Taq*Man® *FV* Leiden assay. 178 NAFLD samples presented with 173 Wild Type GG (97.19%), 5 Heterozygous GA (2.81%) and 0 (0.00%) Homozygous AA. Among the 75 control samples, 74 (98.67%) were Wild Type GG, 1 (1.33%) Heterozygous GA and 0 (0.00%) Homozygous AA.

The amplification plot for *HFE* C282Y is presented in figure 3.2.19 and features the raw fluorescence data acquired during amplification. The *HFE* C282Y allelic discrimination plot is a graphical representation of the genotypes of each sample tested and can be found in figure 3.2.20, with black dots representing NTCs, red dots equivalent to wild types, green dots indicative of heterozygotes and the less prevalent blue dots signifying homozygotes.

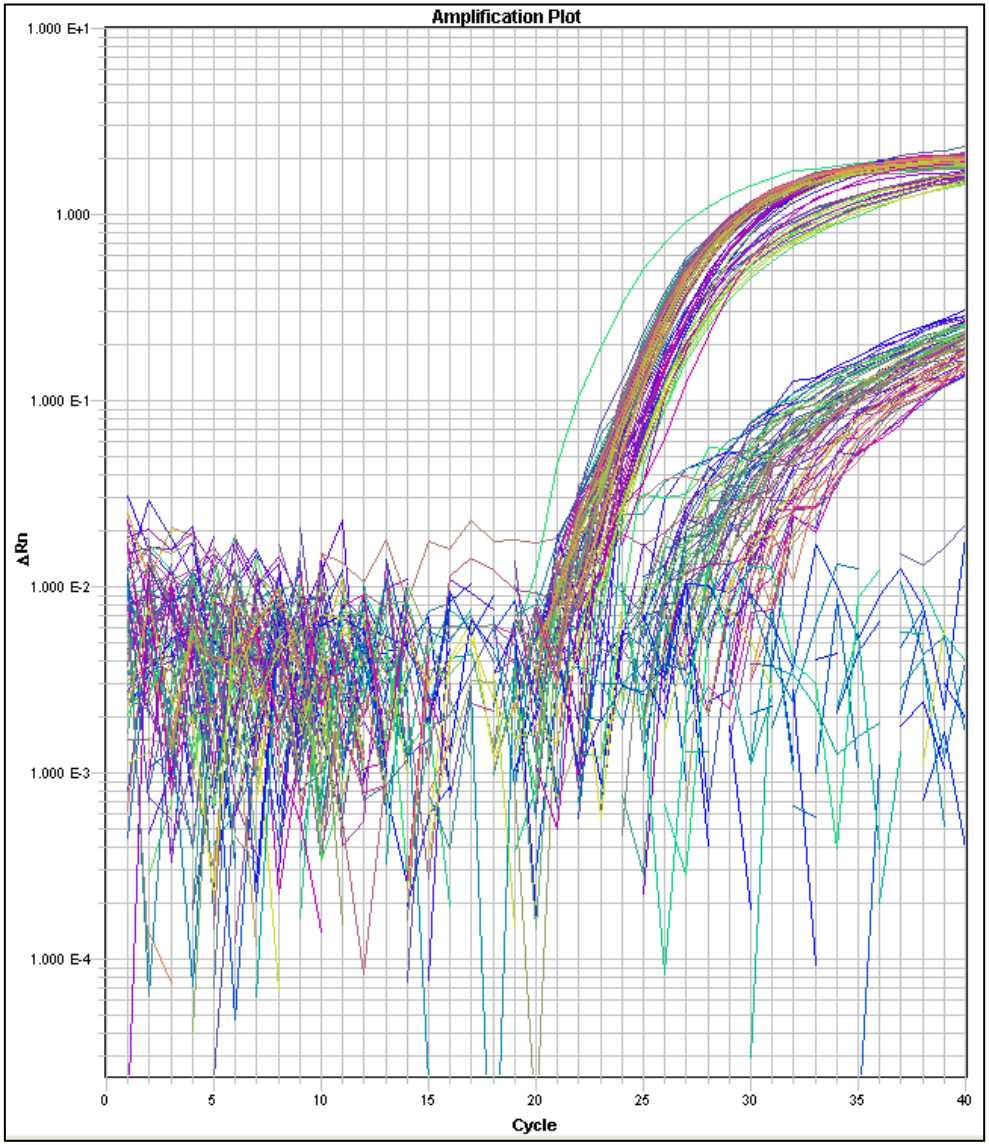

Figure 3.2.19. Typical amplification achieved using the ABI™ *Taq*Man® assay for *HFE* C282Y (ΔRn vs number of cycles). (Abbreviations: ΔRn = unit of fluorescence)

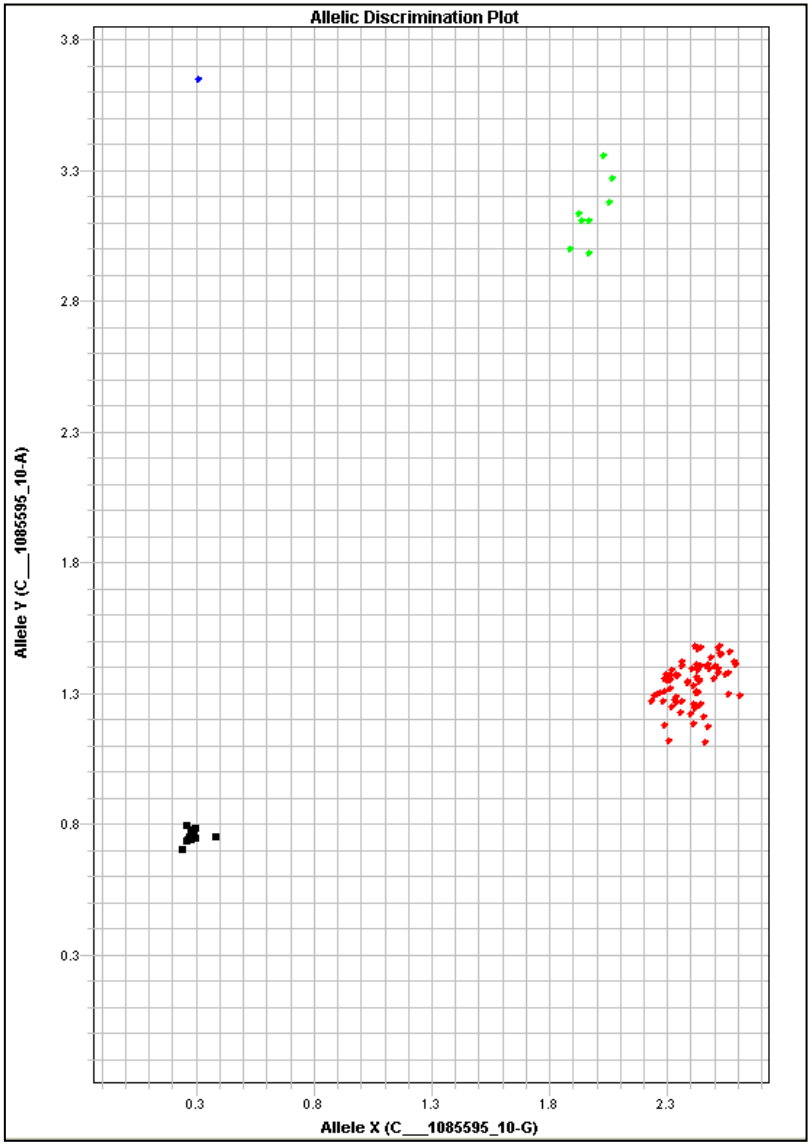

Figure 3.2.20. Typical allelic discrimination analysis using the ABI™ *Taq*Man® assay for *HFE* C282Y [Allele Y (C_1085595_10-A) vs Allele X (C_1085595_10-G)]. Black = NTC, Red = Wild Type GG, Green = Heterozygous GA and Blue = Homozygous AA.

The genotype distribution observed for *HFE* C282Y among all NAFLD (178) and control (75) samples is summarized in figure 3.2.21. The total study population, which includes NAFLD patients and controls for a total of 253 samples, was found to be 92.49% Wild Type GG, 6.72% Heterozygous GA and 0.79% Homozygous AA.

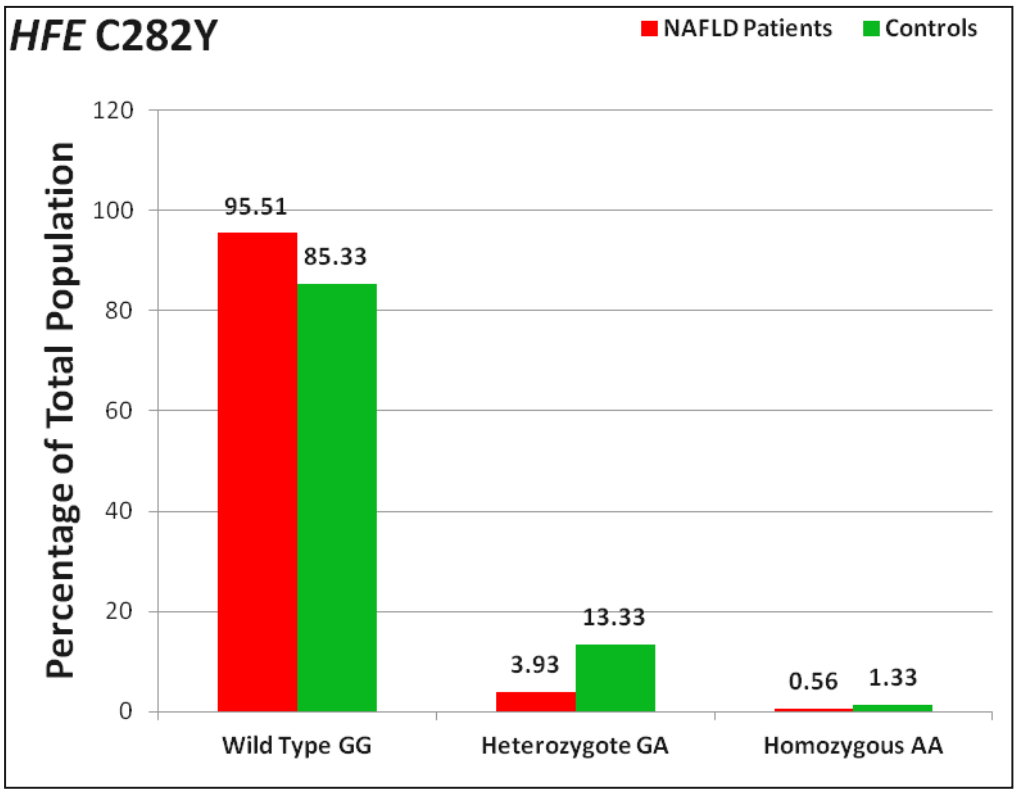

Figure 3.2.21. Genotype distribution of 253 samples obtained using the ABI™ *Taq*Man® *HFE* C282Y assay. 178 NAFLD samples presented with 170 Wild Type GG (95.51%), 7 Heterozygous GA (3.93%) and 1 (0.56%) Homozygous AA. Among the 75 control samples, 64 (85.33%) were Wild Type GG, 10 (13.33%) Heterozygous GA and 1 (1.33%) Homozygous AA.

The amplification plot for *HFE* H63D is presented in figure 3.2.22 and features the raw fluorescence data acquired during amplification. The *HFE* H63D allelic discrimination plot is a graphical representation of the genotypes of each sample tested and can be found in figure 3.2.23, with black dots representing NTCs, red dots equivalent to wild types, green dots indicative of heterozygotes and the less prevalent blue dots signifying homozygotes.

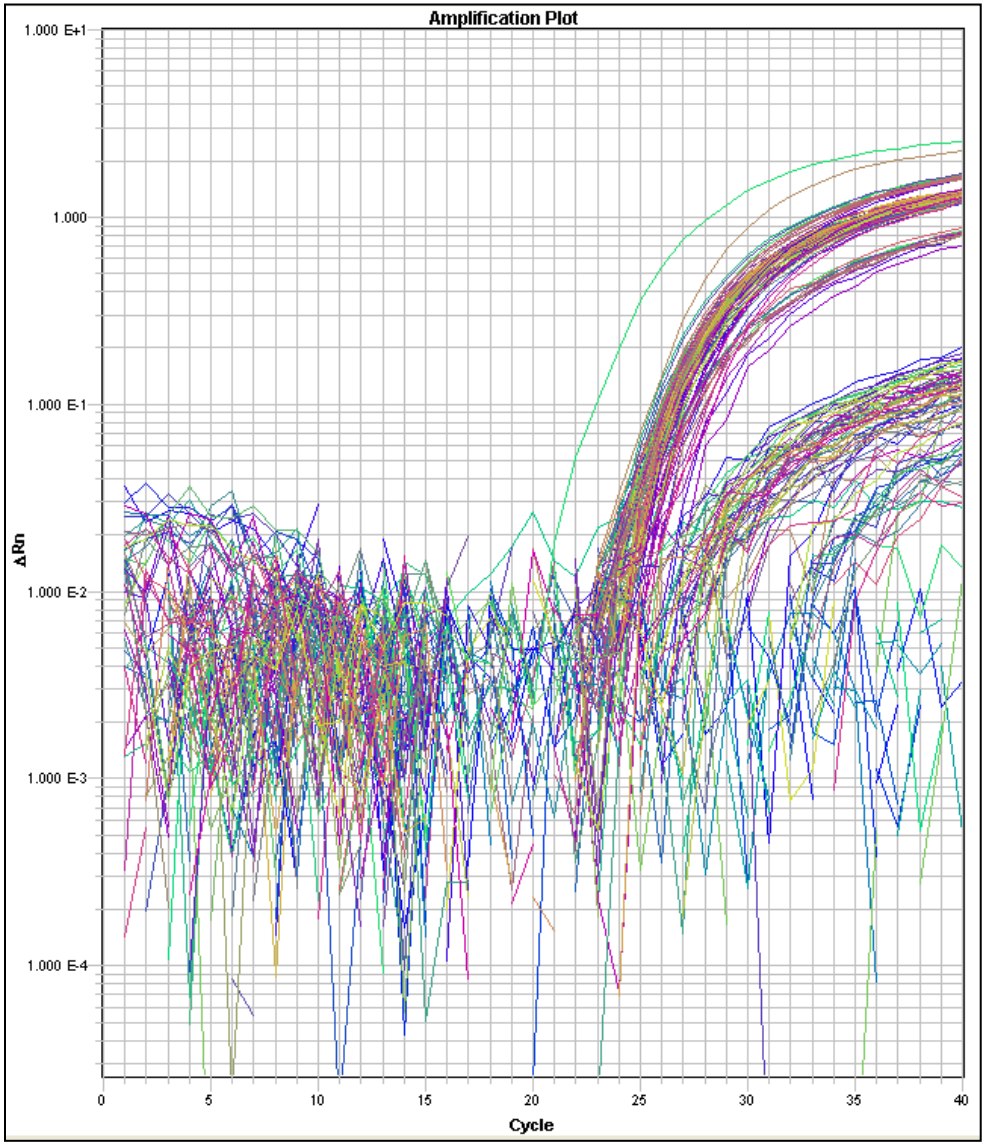

Figure 3.2.22. Typical amplification achieved using the ABI™ *Taq*Man® assay for *HFE* H63D (ΔRn vs number of cycles). (Abbreviations: ΔRn = unit of fluorescence)

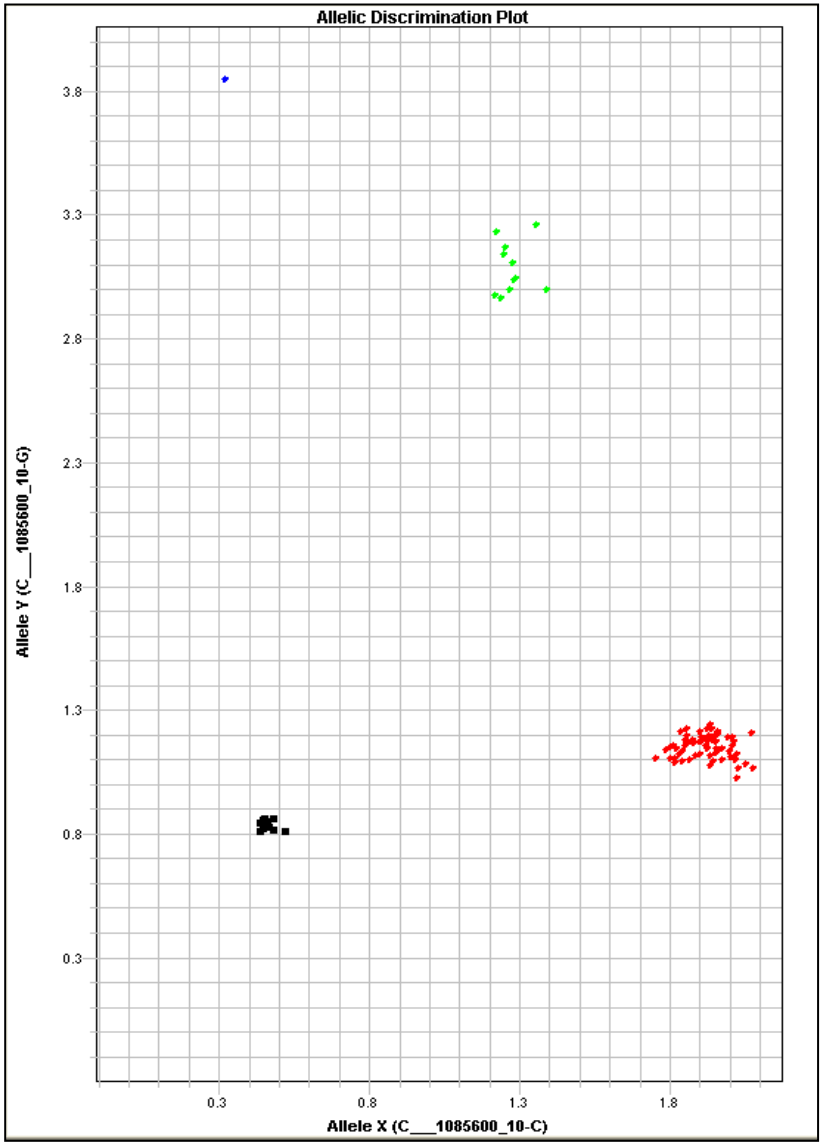

Figure 3.2.23. Typical allelic discrimination analysis using the ABI™ *Taq*Man® assay for *HFE* H63D [Allele Y (C_1085600_10-G) vs Allele X (C_1085600_10-G)]. Black = NTC, Red = Wild Type CC, Green = Heterozygous CG and Blue = Homozygous GG.

The genotype distribution observed for *HFE* H63D among all NAFLD (178) and control (75) samples is summarized in figure 3.2.24. The total study population, which includes NAFLD patients and controls for a total of 253 samples, was found to be 82.94% Wild Type CC, 15.87% Heterozygous CG and 1.19% Homozygous GG.

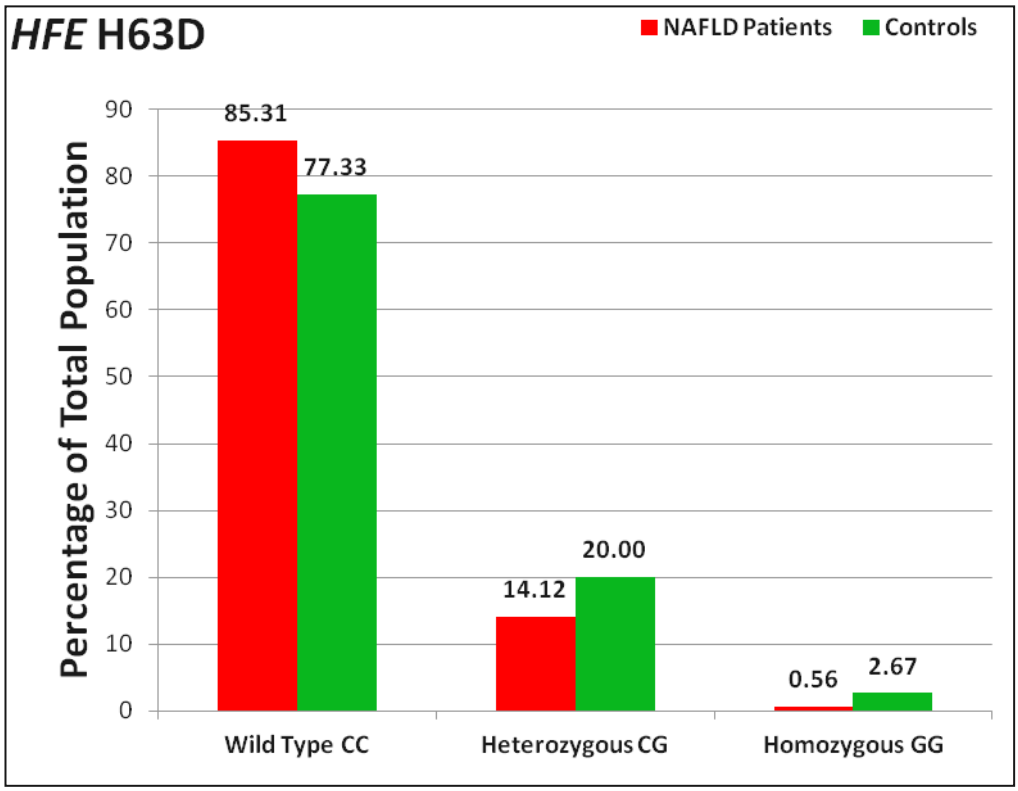

Figure 3.2.24. Genotype distribution of 253 samples obtained using the ABI™ *Taq*Man® *HFE* H63D assay. 178 NAFLD samples presented with 151 Wild Type CC (85.31%), 25 Heterozygous CG (14.12%), 1 (0.56%) Homozygous GG and 1 could not be determined. Among the 75 control samples, 58 (77.33%) were Wild Type CC, 15 (20.00%) Heterozygous CG and 2 (2.67%) Homozygous GG.

The genotype of one of the NAFLD patient samples analysed could not be determined through the RT-PCR protocol employed in this study even though it was re-tested several times. This sample was subsequently excluded from further analysis after the DNA quality in the amplification loci was called into question.

3.3. Comparative Analysis in NAFLD Patients

The clinical characteristics of the NAFLD patients included in the study are shown in Table 3.2, subdivided into the Coloured and White population groups.

Table 3.2. Clinical Characteristics of 107 Coloured and 49 White patients included in this study.

Characteristics*	Coloured Patients (Mean ± STD)	White Patients (Mean ± STD)	Total Patients (Mean ± STD)
Males	21 (19.63%)	27 (55.10%)	48 (30.77%)
Females	86 (80.37%)	22 (44.90%)	108 (69.23%)
Mean Age (years)	50.79 ± 11.19	52.25 ± 10.99	51.24 ± 11.11
Alcohol user (yes)	9 (8.82%)	17 (36.17%)	26 (17.45%)
Smoker (yes)	27 (26.21%)	10 (20.41%)	37 (24.34%)
Physically active (yes)	10 (9.80%)	11 (23.40%)	21 (14.09%)
Mean BMI (kg/m^2)	37.06 ± 7.75	32.66 ± 5.69	35.63 ± 7.42
Waist circumference (cm)	107.94 ± 14.77	109.73 ± 12.44	108.49 ± 14.08
LDL particle size (B/small)	24 (26.37%)	10 (27.78%)	34 (26.71%)
Total Cholesterol (mmol/l)	5.80 ± 1.30	5.61 ± 1.17	5.75 ± 1.26
LDL Cholesterol (mmol/l)	3.64 ± 0.96	3.46 ± 1.02	3.58 ± 0.98
HDL-Cholesterol (mmol/l)	1.34 ± 0.37	1.17 ± 0.29	1.29 ± 0.35
Triglycerides (mmol/l)	2.02 ± 2.33	2.21 ± 1.03	2.07 ± 2.04
C-Reactive Protein (mg/l)	11.99 ± 21.85	13.32 ± 23.64	12.52 ± 22.31
Fasting Glucose (mmol/l)	7.82 ± 3.67	6.84 ± 3.06	7.53 ± 3.52
Insulin (pmol/L)	26.98 ± 45.27	25.61 ± 32.84	26.55 ± 41.58
HbA_{1c} (%)	7.95 ± 2.38	6.66 ± 1.79	7.61 ± 2.30
AST/ALT ratio	0.82 ± 0.22	0.78 ± 0.33	0.81 ± 0.26

Tf Saturation (%)	18.70 ± 10.59	21.52 ± 10.84	19.44 ± 10.68
**Family history (yes)	90 (90.00%)	31 (81.58%)	121 (87.68%)
NAFLD Severity			
Fatty Liver	25 (40.98%)	17 (45.95%)	42 (42.86%)
NASH	23 (37.71%)	18 (48.65%)	41 (41.84%)
Cirrhosis	2 (1.85%)	8 (16.67%)	10 (6.41%)
***Heart Disease (yes)	3 (2.80%)	7 (14.29%)	10 (6.41%)

*NAFLD patient tallies differ between individual characteristics due to incomplete biochemical, clinical and environmental records. Calculations were performed on all available data.
**Family history of cardiovascular disease, hypertension, diabetes, liver disease.
***Heart Disease = angina and ischaemic heart disease.

3.3.1. Allelic and Genotype Distributions

Allele frequencies and genotype distributions were determined for all mutations investigated and were found to be in Hardy Weinberg Equilibrium in the patient and control groups studied (table 3.3).

Table 3.3. P-values of Hardy Weinberg Equilibrium for the eight mutations studied.

Mutations	**White Patients**	**Coloured Patients**	**White Controls**
APOE2, E4	0.2836	0.7212	0.4603
MTHFR 677, 1298	0.7664	0.7478	0.3544
FV Leiden	1.0000	1.0000	1.0000
F2 20210	1.0000	1.0000	1.0000
HFE C282Y, H63D	0.7806	0.4739	0.8788

In tables 3.4 (APOE gene), 3.5 (*HFE* gene) and 3.6 (*MTHFR* gene) the allele frequencies are compared between Coloured and White patients with NAFLD, and between the White NAFLD patients and White controls. A control group for the Coloured population was not available for this study.

Table 3.4. Comparison of APOE genotype distribution and allele frequencies between the Control, White and Coloured study groups.

APOE Genotype	**Control group (n=74)**	**White patient group (n=49)**	**Coloured patient group (n=106)**	**White patients to controls:**	**White to Coloured patients:**
E2/E2	0	0	0	P = 0.19985	P = 0.10894
E2/E3	10 (0.14)	5 (0.10)	10 (0.09)		
E3/E3	40 (0.54)	31 (0.63)	58 (0.55)		
E3/E4	23 (0.31)	9 (0.18)	30 (0.28)		
E4/E4	1 (0.01)	4 (0.08)	3 (0.03)		
E2/E4	0	0	5 (0.05)		
Allele					
E2	10 (0.07)	5 (0.05)	15 (0.07)	P > 0.05	
E3	113 (0.76)	76 (0.78)	156 (0.74)		
E4	25 (0.17)	17 (0.17)	41 (0.19)		

Table 3.5. Comparison of *MTHFR* genotype distribution and allele frequencies between the Control, White and Coloured study groups.

***MTHFR* Genotype**	**Control group (n=74)**	**White patient group (n=49)**	**Coloured patient group (n=107)**	**White patients to controls:**	**White to Coloured patients:**
W/W	13 (0.18)	9 (0.18)	34 (0.32)	P = 0.51299	**P = 0.07450**
W/C	13 (0.18)	14 (0.29)	40 (0.37)		
W/T	12 (0.16)	7 (0.14)	15 (0.14)		
C/C	10 (0.14)	5 (0.10)	6 (0.06)		
C/T	18 (0.24)	9 (0.18)	9 (0.08)		
T/T	8 (0.11)	5 (0.10)	3 (0.03)		
Allele					
W	51 (0.34)	39 (0.40)	123 (0.57)	P > 0.05	
C	51 (0.34)	33 (0.34)	61 (0.29)		
T	46 (0.31)	26 (0.27)	30 (0.14)		

Table 3.6. Comparison of *HFE* genotype distribution and allele frequencies between the Control, White and Coloured study groups.

HFE **Genotype**	**Control group (n=75)**	**White patient group (n=49)**	**Coloured patient group (n=107)**	**White patients to controls:**	**White to Coloured patients:**
W/W	49 (0.66)	34 (0.69)	92 (0.86)	P = 0.78570	P = 0.12349
W/G	13 (0.17)	11 (0.22)	12 (0.11)		
W/A	8 (0.11)	3 (0.06)	2 (0.02)		
G/G	2 (0.03)	0	1 (0.01)		
G/A	2 (0.03)	1 (0.02)	0		
A/A	1 (0.01)	0	0		
Allele					
W	121 (0.80)	82 (0.84)	198 (0.93)	P > 0.05	
A	12 (0.08)	4 (0.04)	2 (0.01)		
G	19 (0.12)	12 (0.12)	14 (0.07)		

3.3.2. Genotype-Phenotype Correlation

The effect of the individual mutations analysed was correlated with biochemical parameters previously found to be altered in the presence of relevant environmental risk factors.

A significant increase (p = 0.04) in Alanine Transaminase (ALT) levels was identified between *HFE* mutation carriers (hetero- and homozygous individuals for the C282Y and H63D mutations of the *HFE* gene) and wild types among stage 3 and 4 NAFLD patients (figure 3.3.1).

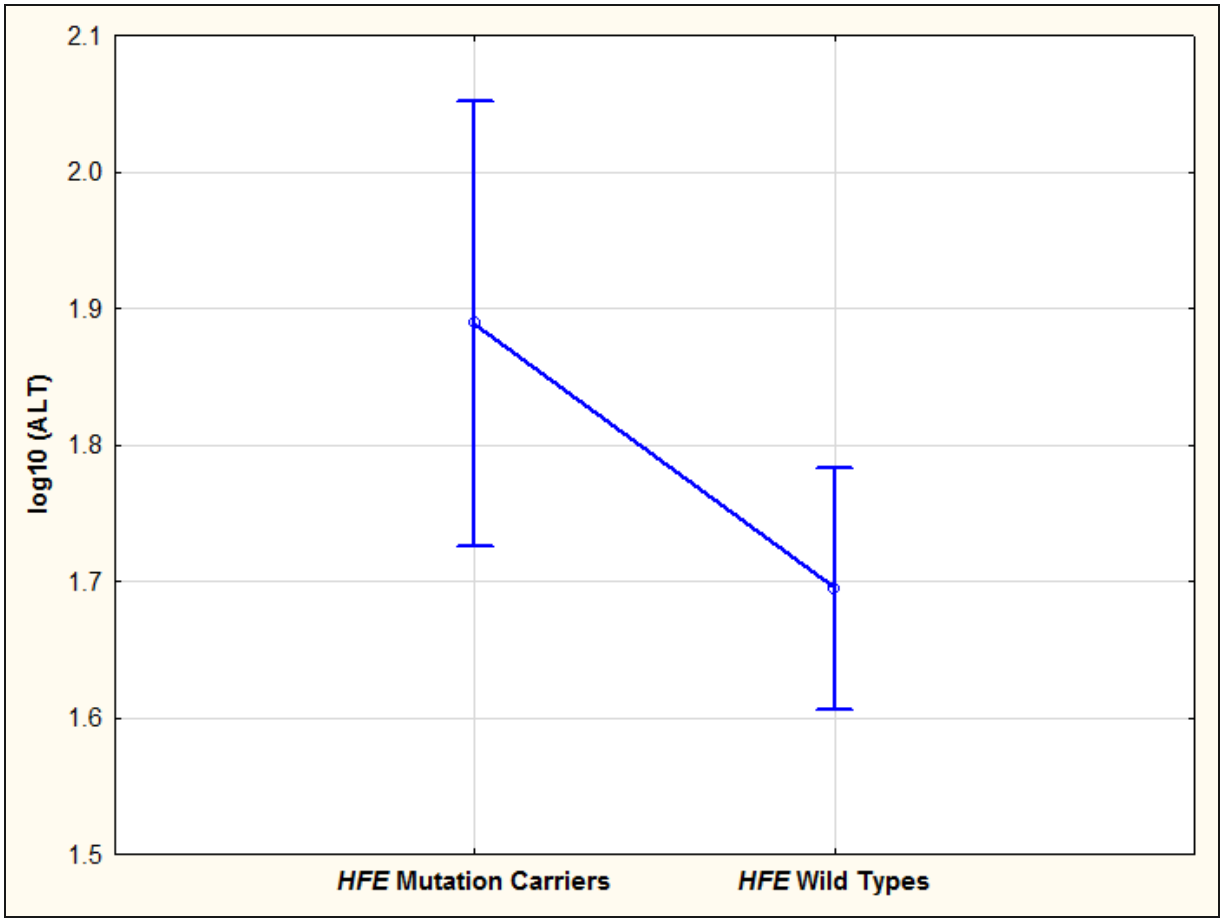

Figure 3.3.1. Comparison of log-corrected ALT levels [log10 (ALT)] between NAFLD patients with (n=10) and without (n=34) mutations in the *HFE* gene. Mean ± STD: HFE Mutation Carriers = 1.89 ± 0.25; Wild Types = 1.67 ± 0.26. F (1, 42) = 4.4499; **p = 0.04**; Mann-Whitney U p = 0.05.

Trends were observed in the prevalence of APOE2 and APOE4 mutations relative to the levels of triglyceride and cholesterol (respectively), while *MTHFR* mutations also tended to differ slightly in frequency between control individuals and patients. However, none of these observations reached statistical significance.

3.4. Clinical Application

The clinical utility of pathology supported genetic testing was demonstrated in an obese patient with a medical history of myocardial infarction. The pedigree of this patient (GMX1) with several family members that have been diagnosed with diseases ranging from Alzheimer's disease to CVD and kidney cancer to thalassemia, is presented in figure 3.4.1. He was referred by a gastroenterologist for the CVD multi-gene test that includes both a diagnostic and risk management component based on gene-environment and gene-gene interactions, using a combined service and research approach.

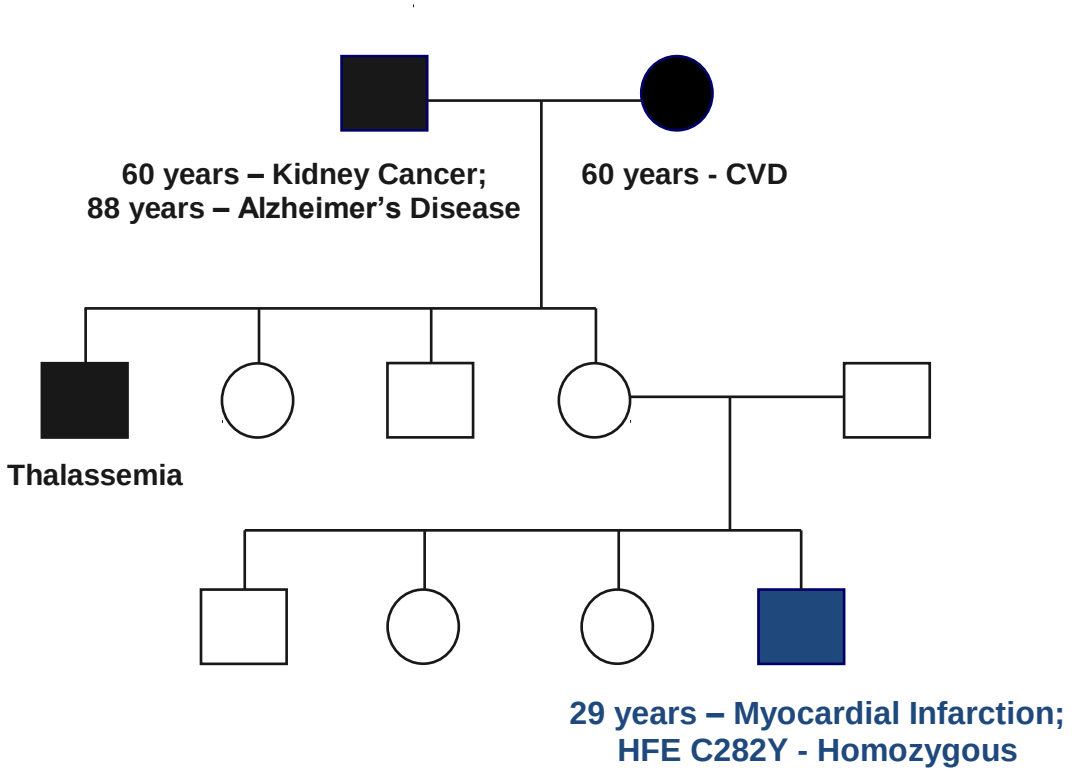

Figure 3.4.1. Pedigree of the 40 year old index case, GMX1, diagnosed with Hereditary Haemochromatosis.

The genetic test was performed in conjunction with a medical and lifestyle assessment to identify a combination of risk factors that, if left untreated, could cause or contribute to disease development or recurrence. Table 3.7 provides a summary of the clinical characteristics and lifestyle risk factors entered into the Gknowmix Database at referral. The patient tested positive for two copies of mutation C282Y in the *HFE* gene through application of the RT-PCR end-point genotyping method evaluated in this study. Due to the relative scarcity of homozygotes and the disease risks associated with this genotype, DNA sequencing was conducted to verify the RT-PCR result (data not shown).

Table 3.7. Clinical and lifestyle information documented at referral of GMX1.

GENETIC TEST RESULTS

RISK AREA	GENE	GENETIC VARIATION	RESULTS
Lipid and lipoprotein metabolism	APOE	3937 T > C, allele E4	Not Detected
		4075 C > T, allele E2	Not Detected
Homocysteine and folate metabolism	*MTHFR*	**677 C > T, A222V**	**Heterozygous**
		1298 A > C, E429A	Not Detected
Haemostasis and thrombophilia	*FV*	1691 G > A, Leiden	Not Detected
	F2	20210 G > A	Not Detected
Iron overload	*HFE*	**845 G > A, C282Y**	**Homozygous**
		187 C > G, H63D	Not Detected

FAMILY HISTORY

Family Medical Conditions	Diagnosis	Relationship
Alzheimer's Disease	88 years	Grandfather
Cardiovascular Disease	60 years	Grandmother
Kidney cancer	60 years	Grandfather
Thalassemia		Uncle

HEALTH STATUS

Personal Medical Conditions	Diagnosis	Therapy
Haemochromatosis / High Iron	40 years	Phlebotomy treatment
Myocardial Infarction	29 years	Bayer Cardio
Clinical Assessment	**Value**	**Evaluation Values**
Cholesterol, total	4.1 mmol/l	3.21-5.20 mmol/l
Glucose, fasting	4.9 umol/dl	3.3-5.5 umol/dl
Serum ferritin	**252 ng/ml (Pre-treatment: 671 ng/ml)**	15-300 ng/ml
Transferrin saturation	**75 % - High (Pre-treatment: 83 %)**	20-55 %
Blood pressure - Systolic	129 mmHg	< 140 mmHg
Blood pressure - Diastolic	79 mmHg	< 90 mmHg
Weight	92 kg	
Height	1.75 m	
Body mass index (BMI) Adult	**30.04 kg/m^2 - High**	18.5-24.9 kg/m^2
Lifestyle Assessment	**Score**	
Physical activity	4 or more times / week , Daily: Sedentary - High	
Smoker	Current: No , Previous: Not Applicable	
Alcohol consumption	1-2 Units Occasionally - Low	
Nutrition Assessment	**Score**	
Fat intake, saturated & *trans* fats	9 - Very Low (excellent)	
Fruit, vegetables, fibre intake	14 - Moderate	
Folate intake	**5 - Very Low**	

The index patient (GMX1) was one of the first two individuals with high ferritin levels referred for genetic testing during the course of this translational research project. In the second referral (GMX2) normal transferrin saturation (36%) in the presence of high ferritin (498 ng/ml) and fasting glucose levels (6.6 mmol/l) was consistent with wild type *HFE* mutation status (data not shown).

Chapter 4

Discussion

This study was based on the hypothesis that the simultaneous analysis of multiple Single Nucleotide Polymorphisms (SNPs) associated with an increased risk of Cardiovascular Disease (CVD) would increase the clinical utility of their individual applications and constitute an improved risk-profiling system for individuals with Non-Alcoholic Fatty Liver Disease (NAFLD). This possibility is supported by extensive literature, as reviewed in Chapter 1 of this thesis and further discussed hereafter in a more integrative manner.

The translation of scientific findings into therapeutic recommendations requires a multi-disciplinary approach, where pathological determinations are essential to measure gene expression and response to the intervention strategy advised. This study represents an extension of the work by Kruger et al. (2010), who were the first to describe the clinical characteristics of Non-Alcoholic Fatty Liver Disease (NAFLD) among individuals of the Western Cape province of South Africa. Insulin resistance was identified as the universal factor, while the degree of obesity was not associated with disease severity. Increasing age was associated with more advanced fibrosis, while no correlation was observed with Nonalcoholic Steatohepatitis (NASH). South African Patients with NASH showed significantly higher mean serum cholesterol ($p < 0.01$) and triglyceride ($p = 0.03$) levels than those with fatty liver only. Low-Density Lipoprotein (LDL) particle size was also found to decrease significantly from fatty liver disease to NASH ($p = 0.03$). The undefined nature of genetic risk factors on disease development and severity underscored the necessity of further studies on NAFLD. This mission was undertaken in the present study by building on a clinically well-characterized study population.

Genotyping using RT-PCR was performed for the APOE2, APOE4, *MTHFR* 677, *MTHFR* 1298, *F2* 20210, *FV* Leiden, *HFE* C282Y and *HFE* H63D mutations previously included in a CVD multi-gene strip assay test (Kotze et al. 2003; Kotze and Thiart, 2003) following optimization and standardization of high-throughput mutation detection to assess the genetic contributions to the associations identified by Kruger et al. (2010).

Analytical validation of the Real-Time Polymerase Chain Reaction (RT-PCR) mutation detection system was achieved by comparison with DNA sequencing and inclusion of the sequenced samples as internal controls during patient screening (see section 4.1). The clinical utility of the CVD multi-gene test in guiding chronic disease risk management in patients with NAFLD was evaluated by genotype-phenotype association testing with a special focus on the role of the *HFE* gene (see section 4.2). The necessity of an integrative, systems-based network approach was assessed in

context of the rapidly expanding fields of network medicine, translation research and personalized medicine (see sections 4.3, 4.4 and 4.5). A Pathology Supported Genetic Testing (PSGT) approach to the latter was shown to assist in clear differentiation between Hereditary Haemochromatosis (HH) and Insulin Resistance-associated Hepatic Iron Overload (IR-HIO) syndrome in obese patients (see section 4.5).

A significant association was found between *HFE* mutations and elevated Alanine Transaminase (ALT) levels in the NAFLD population, which might define a subset of patients who would benefit most from genetic testing to direct more aggressive therapy at an earlier stage. These findings emphasize the importance of the *HFE* mutation detection component of the CVD multi-gene test as it may facilitate an effective treatment strategy in patients with a medical history of CVD and/or high iron stores (see section 4.2.5).

4.1. Analytical Validation

Methods currently available for routine genotyping of patients include Polymerase Chain Reaction (PCR) Restriction Fragment Length Polymorphism (RFLP) analysis, PCR mediated site-directed mutagenesis, oligonucleotide ligation, reverse hybridization line-probe assay, DNA sequencing, allele-specific PCR and Real-Time PCR (RT-PCR) using Fluorescence Resonance Energy Transfer (FRET) probes or High-Resolution Melt (HRM) analysis.

The most popular modern technique consists of PCR amplification of the suspected site of a mutation and subsequent DNA sequencing. This combination has proven to be the most reliable, efficient and cost-effective for individual samples or small batches (dozens). RT-PCR has gained considerable support in recent years as a high-throughput alternative to the aforementioned standard, especially for analysis of larger sample batches (hundreds to thousands).

In this study, PCR amplification was used in conjunction with DNA sequencing to determine the genotypes of the control samples for each of the eight mutations investigated. Verified samples (wild type, heterozygous and homozygous) were then used as internal controls in the standardization of a high-throughput RT-PCR multi-gene screen and the subsequent implementation of this system in the genotyping of the NAFLD patient samples.

4.1.1. Polymerase Chain Reaction Amplification

A Polymerase Chain Reaction (PCR) is a laboratory procedure in which millions of copies of a specific section of DNA are synthesized (Brown, 2007: 2.3). It is an amplification method in which minute amounts of DNA obtained from blood, hair, saliva or tissue can be copied to provide sufficient quantities for analysis. The reaction is carried out in an automated device able to rapidly and precisely increase and decrease the temperature to exact values, known as a thermal cycler. This method is named after the key component which carries out the replication of the DNA, an enzyme known as a DNA polymerase. The most commonly used of which is *Taq* polymerase, obtained from the bacterium *Thermus aquaticus*. This enzyme functions optimally at a temperature of approximately 70°C. It can create a new DNA strand by using the original DNA as a template and employing DNA oligonucleotide primers. The primers used in PCR are short (20 to 30 nucleotides), artificial sequences of DNA that are designed to match the ends of the DNA region to be copied exactly. The reaction is initiated by heating until the two strands of DNA separate (denaturation phase), the primers then bind to their intended locations (annealing phase) and the DNA polymerase commences elongation of the primers (extension or elongation phase).

PCR has replaced previous methods of DNA replication that used bacteria and could take several weeks to produce adequate amounts of product for practical application. PCR is a very rapid assay and can accomplish the goals of the former methods in a matter of hours (typically two to three hours). Speed is often required in a diagnostic setting when urgent results are necessary. The PCR technique was developed around 1983 by Kary Mullis (Mullis et al. 1994), who won a Nobel Prize in Chemistry for the invention in 1993. Since then, PCR has been widely used as a diagnostic and research tool. The variety of applications for this technique are constantly expanding throughout many scientific disciplines, including clinical diagnostics, environmental science, forensic science, microbiology, molecular biology and paternity testing.

PCR is extensively used in analysing clinical specimens for the presence of infectious agents such as Human Immunodeficiency Virus (HIV), hepatitis, human papillomavirus (which causes genital warts and cervical cancer), Epstein-Barr virus (which causes glandular fever), malaria (Yang and Rothman, 2004) and anthrax (Hoffmaster et al. 2002). Cancer diagnostics have also been aided by the implementation of PCR in the identification of causative mutations, providing information on the prognosis of a patient as well as predictions concerning response or resistance to therapy (Bernard and Wittwer, 2002).

The sensitivity of PCR enables it to be used with great success in analysing mutations that occur in many genetic diseases (for example cystic fibrosis, sickle cell anaemia, phenylketonuria, muscular dystrophy). A DNA template for the amplification can be obtained from a single cell taken from an embryo before birth. Paternity tests are essentially performed by PCR. A cheek swab can for example be taken from inside the mouth of both parents and the child, the DNA extracted from the cells obtained and then analysed by PCR. The basic DNA sequence in every cell of the body is identical, varying in amount between somatic (non-reproductive) and gametic (reproductive) cells. Sequence variance between cells is caused by genetic alterations (polymorphisms, cytogenetic changes, etc.) that take place during the life of an organism, whether natural (error during replication, mitosis, meiosis) or induced by mutagens (UV light, chemicals such as ethidium bromide, etc.). The DNA of a child should be a combination of the genomes of its parents, with minimal variation. Several locations referred to as 'loci' on the child's DNA are examined and the sequences of these loci are compared to those of the mother and father. The conclusions regarding the paternity of the child are based on the degree of identity found.

For this study, it was essential to have an in-depth understanding of the PCR procedure to facilitate the optimization and standardization of the high-throughput mutation detection system evaluated for implementation of the CVD multi-gene test.

A PCR is conducted using a solution known as a reaction master mixture, which contains very specific amounts of all the essential reagents for the reaction to take place. All of the components are mixed together in minute volumes in a single tube. These reagents are nuclease-free water, a buffer solution, magnesium chloride ($MgCl_2$) salt solution, deoxyribonucleotide triphosphates (dNTPs), oligonucleotide primers, template DNA and a DNA polymerase. The nuclease-free water is very highly purified H_2O intended for laboratory research or diagnostic use. The buffer solution creates an optimized and chemically stabile environment for the reaction. $MgCl_2$ is used to provide Mg^{2+}-ions for use in the PCR. These divalent cations promote DNA/DNA interactions and form complexes with dNTPs which are the actual substrates for *Taq* Polymerase. When the concentration of Mg^{2+} in the PCR is too low, primers are unable to anneal to the target DNA. When Mg^{2+} is over-abundant the base-pairing becomes too strong and the amplicon fails to denature completely when heated to 95°C (Williams, 1989; Ellsworth et al. 1993). The dNTPs are the core components used by the polymerase to synthesize new DNA strands. The primers are used in sets, consisting of a forward ("sense") and reverse ("anti-sense") oligonucleotide. The template DNA contains the sequence to be amplified and is usually extracted and purified from blood, saliva or tissue. The

DNA polymerase is the enzyme that synthesizes the PCR products and is most often a member of the *Taq* polymerase group.

There are three basic steps involved in performing a PCR. These steps are repeated 30-40 times in cycles of heating and cooling, each step at a different temperature. Prior to the first step an initial denaturation is performed at a high temperature (about 95°C) for several minutes (usually two to five), then the three basic steps are repeated in cycles followed by a final round of extension at 72°C for several minutes (usually five to ten). Collectively, this protocol is known as the PCR- or thermal cycling programme.

The first step is known as the denaturation step and is carried out at approximately 95°C. The DNA molecule exists in nature in a double stranded confirmation, with the strands linked together by weak hydrogen bonds. To be able to copy any section of DNA, the helix must be separated into single strands. This process of separation is called denaturation and can be initiated by heating the molecule to a temperature above 90°C.

The second step is the annealing step and is typically carried out at 50°C - 60°C. During this step the oligonucleotide primers attach ("anneal") to their matching sequence on the original DNA strand. Excess amounts of primer preclude restoration of the double helix structure even though the temperature of the denatured DNA is lowered.

The third step is called the extension or elongation step and carried out at about 72°C. *Taq* DNA polymerase binds to the annealed primer and travels along the DNA strands, extending the primer sequence by adding complementary dNTPs and other components in the reaction mix. This step completes the replication process.

Once synthesis has been completed, the entire mixture is heated again to approximately 95°C to melt (denature) the newly formed DNA complexes, resulting in twice the amount of template available for the next round of amplification. Repeated heating and cooling quickly amplifies the DNA segment of interest with about one million copies synthesized after 20 cycles. PCR products can be used in a variety of applications, most notably separation by gel electrophoresis and/or DNA sequencing (see section 1.9.2). Gel electrophoresis is a widely used technique for the analysis of nucleic acids and proteins. It refers to the separation of charged particles (for example DNA, amino acids, peptides) located in an electrophoretic medium (the gel) when an electric current is applied.

A gel is a colloid, a suspension of minute particles in a medium, occurring in a solid form similar to gelatine. Agarose gel electrophoresis is routinely used for the analysis of DNA fragments where the success of a PCR experiment may be evaluated by the intensity, quantity and width of the observed bands.

4.1.2. DNA Sequencing

DNA sequencing was developed in 1975 and has since become a powerful technique in molecular biology (Brown, 2007: 4). It has been applied to many areas of research, as it enables the user to analyse genes at the nucleotide level. DNA sequencing is the determination of the precise sequence of nucleotides in a sample of DNA and is considered the "gold standard" for mutation detection. Unlike PCR, the starting material used for sequencing is not genomic DNA but mostly PCR fragments or cloned genes. There are two competing methods for determination of DNA sequence, namely chemical degradation and chain termination. The chain termination method has become the dominant DNA sequencing technique in use worldwide due to several major disadvantages of the chemical degradation method. These include the increased technical complexity which prohibits its use in standardized molecular biology kits, the extensive use of hazardous chemicals and difficulties in the expansion of the technique.

The chemical degradation (or Maxam - Gilbert) method was developed in 1976-1977 by Allan Maxam and Walter Gilbert (Maxam and Gilbert, 1977). It entails the end-labelling of a DNA sequence with ^{32}P-phosphate followed by selective, sequential removal of specific nucleotide bases from one end of the single-stranded DNA (ssDNA) molecules via specialized chemical treatments. This process generates a series of fragments, of different lengths, which are separated by electrophoresis on a gel. The relative lengths of these fragments are then determined by reading the DNA bands by autoradiography after visualization. Interpretation of these results then allows for the determination of the DNA sequence.

The chain termination method (also known as the Sanger method or cycle sequencing) was developed by Frederick Sanger in 1975 and refined in 1977 (Sanger and Coulson, 1975; Sanger et al. 1977). It involves the sequential addition of bases to an oligonucleotide primer annealed to a complementary strand of DNA. The template used in this technique is ssDNA, obtained by denaturing double-stranded DNA (dsDNA) at high temperature (above 90°C) during the initial denaturation step of the sequencing reaction. Precisely defined primers with radioactive ^{32}P-phosphate labels at their 5' ends were initially used in this method, but were replaced by non-

radioactive primers and chain-terminating dideoxynucleotide triphosphates (ddNTPs) labelled with fluorescent dyes in recent years. This technique is known as Dye Terminator Labelling. An alternative labelling method, Dye Primer Labelling, involves end-labelling of the oligonucleotide primers with four different fluorescent dyes and four separate synthetic reactions (adenine, A; cytosine, C; guanine, G; thymine, T) are carried out in the presence of appropriate dNTPs and ddNTPs. This technique is rarely employed because four different reactions, in four different tubes must be conducted and the results combined for interpretation to obtain the sequence for a single DNA template.

The sequencing reaction is identical to PCR, except that only one primer is used per reaction (not a set of two) and the extension step is carried out at a temperature of 60°C instead of 72°C. The temperature is lowered during extension to facilitate the incorporation of ddNTPs, which are chemically modified with a fluorescent label and require more time for the polymerase to successfully integrate them. The reaction mixture includes the template DNA, free nucleotides (dNTPs and ddNTPs), an enzyme (usually a variant of *Taq* polymerase) and a single primer. Each primer is then extended by the DNA polymerase using a mixture of dNTP and ddNTP molecules.

Four different colours of fluorescence are used to distinguish the four ddNTP molecules (ddATP, ddCTP, ddGTP and ddTTP). The bases (dNTPs or ddNTPs) are coupled to the 3'-end of the primer and thus added in the 5'-to-3' direction, which is complementary to the 3'-to-5' direction on the template DNA. When a ddNTP is incorporated, the extension reaction stops because a ddNTP contains a hydrogen atom (H) instead of a hydroxyl group (OH^-) on the third carbon of the molecule. This OH^--group is required for attachment of the next nucleotide. The fragments produced by this reaction are then separated by Polyacrylamide Gel Electrophoresis (PAGE). Polyacrylamide provides higher resolution separation than agarose and can resolve single nucleotide differences in DNA molecules. Since the ddNTPs are fluorescently labelled, it is possible to detect the colour of the last base of each fragment on an automated sequencer. The fluorescently labelled fragments that migrate through the gel pass a laser beam at the bottom of the electrophoretic medium. Excitation by the laser causes the fluorescent molecule to emit light of a distinct wavelength (colour). That light is collected and focussed by lenses into a spectrograph. Based on the wavelength, the spectrograph separates the light across a Charge Coupled Device (CCD) camera. The colour peaks are interpreted by the sequencer, which determines the order of the bases in the sequenced molecule and outputs the data in the form of an electropherogram.

Automated DNA sequencing utilizes the chain termination method as described above and has largely replaced the antiquated manual technique.

The greatest obstacle to researchers when converting from manual to automatic DNA sequencing is in learning how to effectively use the required computer software to interpret the results obtained. The vital importance of computers and information technology contributed to the development of bioinformatics to resolve the complications imposed by the advent of the digital age. Bioinformatics is the division of the biological sciences that facilitates efficient analysis of acquired data through application of computer science and information technology to living systems. The complex, systems-based research made possible by the effective implementation of bioinformatic models and theories has transformed the biological and medical sciences.

A comprehensive understanding of DNA sequencing facilitated the analytical validation of the high-throughput mutation detection system evaluated in this study, as the control samples employed were first genotyped by DNA sequencing.

4.1.3. Real-Time Polymerase Chain Reaction Amplification

The reproducible quantitation of amplification products has long been the goal for many scientists and researchers. The traditional process requires the end-point analysis of amplification products via gel electrophoresis. This method allows for the identification of target and competitor product sizes, estimation of purity and subjective measuring of band intensities. However, the reproducibility of amplification end products is highly variable due to limiting reagents, which compound the difficulties with this process. It is the exponential phase of amplification that provides the most useful and reproducible data. There is a quantitative relationship between the amount of starting target DNA and the amount of amplification product during the exponential phase of a cycling program. This is the very basis for Real-Time PCR (RT-PCR) amplification. Aided by intercalating DNA dyes and probe specific chemistries, the study of the amplification process has improved exponentially as a result of real-time detection.

The practical method of RT-PCR is nearly identical to that of conventional PCR, except that FRET- or hybridization probes are included in the reaction mixtures and more specialized instruments are employed in the thermal cycling (Lee et al. 1993; Livak et al. 1995). Two labelled oligonucleotide probes, traditionally referred to as FRET and anchor probes, bind to the PCR product in a head-to-tail fashion. One of these probes is labelled with a donor dye at the 3' end and the other is labelled

with an acceptor dye at the 5' end. As the probes hybridize to adjacent regions in the same strand, their fluorophores come into close proximity, which allows energy transfer from a donor to an acceptor fluorophore to take place. The acceptor fluorophore then emits light in a longer wavelength, which is used for signal detection (Hiyoshi, 1994; Chen et al. 1997). The requirement of a spectral overlap of donor emission and acceptor excitation results in an overlap of the emission bands, since it is a general feature of fluorescent dyes that they exhibit broad emission spectra. This cross-talk must be compensated in dual-colour experiments by a colour-compensation calibration.

Modern RT-PCR instruments consist of a fluorometer and a thermal cycler for the detection of fluorescence during the cycling process. A computer that communicates with the real-time machine collects fluorescence data. These data are displayed in a graphical format through software developed for real-time analysis. Popular RT-PCR instruments among researchers worldwide include the Applied Biosystems® (ABI™) 7900HT Fast Real-Time PCR System (Foster City, California, USA), the Corbett Rotor-Gene™ 6000 Series 5-Plex HRM Multiplexing System (originally by Corbett Research, Australia now the Rotor-Gene™ Q by QIAGEN®, Germany) and the Roche LightCycler® 480 II System (Roche Applied Science, Germany).

Fluorescence data are collected at least once during each cycle of amplification allowing for real-time monitoring of amplification. A user is able to determine which samples are amplifying on a cycle-by-cycle basis. This instant data allows them to see how individual samples amplify in relation to known standards, positive controls and negative controls. Not only is the user able to monitor the whole reaction during the amplification process, but they can truly optimize their protocols based on the information they receive. This leads to increased sensitivity, specificity and efficiency. After raw data are collected, the analysis can begin. The software for the real-time instrument normalizes the data to account for differences in background fluorescence. Once normalization is complete, a threshold level can be set. This is the level at which fluorescence data are analysed. The number of cycles it takes for a sample to reach the threshold level is the Ct-value (threshold cycle). The threshold is set at a level where the rate of amplification is the greatest during the exponential phase. This allows for the most accurate and reproducible results. If standards with corresponding concentrations are run, a linear regression analysis produces a standard curve from which the concentration of unknown samples can be determined.

The advantage of RT-PCR over other mutation detection methods lies in its ability to rapidly analyse many samples simultaneously at a relatively low cost per individual reaction. Depending on

the instrument employed, as many as 384 reactions (with e.g. the ABI™ 7900HT) can be performed simultaneously in a single device and no post-processing is required. This reduces the overall costs of performing mutation analyses. Conclusive results are obtained faster than with any other technique (typically one to two hours) and the progression of the reactions can be monitored in real-time which aids in the optimization of the protocol and in troubleshooting (if necessary). Quantitative and qualitative data can be obtained due to the versatility of both the software and hardware. The data collection and analysis phases of RT-PCR can be automated, while controls and standards can be built into the system to ensure objectivity. The cleaved FRET probes provide a permanent record of the amplification of any particular amplicon and can be stored for future reference or downstream applications. The dynamic range of detection attributed to RT-PCR is greater than in most other detection methods and the small amplicon size results in increased amplification efficiency. Samples with as little as 1 ng/µl of DNA can be used as a template for successful amplification. Once fully standardized, RT-PCR has proven to be a precise, highly sensitive and reproducible method with broad applicability.

All eight assays employed in the mutation screening yielded clear amplification of the polymorphic target sequence for all samples investigated. The specificity of the assays was verified by the internal control samples included in each of the RT-PCR runs, yielding identical genotype calls to those obtained through DNA sequencing. The tight and distinct clustering of the NTCs revealed that no contamination was present in the reaction setups, ensuring the credibility of the results.

4.1.4. Allele Frequencies and Genotype Distributions

Hardy Weinberg equilibrium was demonstrated for all mutations investigated through statistical analysis of the Coloured and White patient populations with NAFLD as well as the White control group (table 3.3). Allele frequencies and genotype distributions for the APOE mutations (table 3.4) among the White patient and control populations closely resembled those found by Kotze et al. (1993). In the case of the *HFE* mutations (table 3.5), the Coloured patients and both White patients and controls were found to be similar to those previously recorded for the South African population by de Villiers et al. (1999b). The coagulation factor mutations (those of the *F2* and *FV* genes) among the White controls and NAFLD patients were comparable to those observed by Schneider et al. (2000). The mutation frequencies of the *MTHFR* gene (table 3.6) matched up with those quantified by Scholtz et al. (2002) for three South African population subgroups investigated.

The heterogeneous distribution observed in allele frequencies and genotypes between the different ethnic groups as confirmed in this study may be caused by dietary, environmental and/or genetic factors influenced by cultural variation. Such variation must be accounted for through an integrative, systems-based approach incorporating all relevant factors if personalized medicine is to become a reality.

4.2. Clinical Utility

The limited sensitivity of preclinical disease identification and often inadequate specificity of unequivocal disease definition obtained through conventional, non-molecular techniques may be significantly enhanced through the use of genetic testing protocols, especially in developing countries (Pahwa et al. 2005). This will greatly improve disease prevention and therapeutic intervention to remove, reduce or defer the risk of disease development. The application of genetic testing is limited by its ability to improve the diagnostic and therapeutic capabilities of healthcare practitioners. This restriction may be overcome by the implementation of Pathology Supported Genetic Testing (PSGT) to integrate the diverse characteristics of an individual and direct effective management through prevention of cumulative risk (Kotze et al. 2009).

The genes and specific mutations evaluated in this study for their clinical utility are each associated with key elements in the pathogenesis of NAFLD and are proven risk factors for CVD and the Metabolic Syndrome (MetS). These key pathogenic elements include atherogenic dyslipidaemia, chronic inflammation, hypercoagulation and iron dysregulation implicated in insulin resistance.

Atherogenic dyslipidaemia is characterized by increased triglycerides, decreased HDL cholesterol, postprandial lipaemia and elevated levels of small, dense LDL cholesterol. APOE2 and E4 are associated with this disorder (see section 1.5.1), while the *MTHFR* variants (677 and 1298) may alter the functional qualities and bio-availability of the proteins involved through epigenetic manipulation of translational processes (see section 1.5.2).

Chronic inflammation involves elevated levels of C-reactive protein, interleukin-6, tumour necrosis factor α and other acute-phase proteins. APOE4 (see section 1.5.1) and both *MTHFR* 677 and 1298 (see section 1.5.2) are known to affect the inflammatory process which may initiate or contribute to the metabolic insult causing the chronic manifestation of the disorder.

Hypercoagulation increases levels of fibrinogen, factor VII, plasminogen activator inhibitor 1 and other coagulation factors. These conditions have been linked to genetic variations in the coagulation factor genes, especially *F2* 20210 (see section 1.5.3) and *FV* Leiden (see section 1.5.4), as well as *MTHFR* 677 (see section 1.5.2).

Dysglycaemia and hepatic insulin resistance have been correlated with functional deviations of iron-regulatory proteins such as HFE, in which the C282Y and H63D mutations induce considerable adverse effects (see section 1.4).

In this study, the clinical value of the CVD multi-gene test (Kotze et al. 2003; Kotze and Thiart, 2003) was evaluated in a South African NAFLD patient group with a special focus on the contribution of the *HFE* gene component of the test. To this end, the role of possible genetic determinants was assessed relative to biochemical deviations and their effect on the risk profile. A similar pattern was followed with all other markers and genes, first evaluating individual gene effects, then the impact of certain combinations on the risk profile and in relation to family history, personal medical history/status, environmental factors, genotypes and biochemistry. The relative lack of associations detected in this study with most of the metabolic pathways related to the genes discussed below, underscores the necessity for a PSGT approach to direct investigations in the South African population.

4.2.1. APOE

Defective ApoE is most strongly associated with abnormal lipid metabolism due to its crucial role in receptor-mediated endocytosis of chylomicron and Very Low-Density Lipoprotein (VLDL) remnants in the liver. Deleterious mutations in the APOE gene are often correlated with structural variations that disrupt the function of the protein. The two most prominent genetic variations in this regard, are APOE2 and APOE4. The APOE2 allele is known to be associated with elevated triglycerides, while the APOE4 allele is correlated with increased total- and LDL-cholesterol. Other significant associations include the effect of APOE polymorphism on LDL particle size and the impact of APOE4 on inflammation. The latter is especially apparent in the presence of environmental triggers such as alcohol use, diabetes, elevated glucose, insulin, obesity and smoking. These effects all impact directly on CVD and NAFLD risk.

Recent investigation into the effect of APOE polymorphism among NASH patients elsewhere (Sazci et al. 2008) has identified the wild type APOE3 allele as representative of the increased risk

group. This peculiarity emphasizes the limitations of the current predominant protocol for genetic research in the field of human disease and the necessity of expanding investigations to encompass wider biological systems. The impact of relevant disease-modifying genes and environmental factors must be evaluated in combination with the apparent principal molecular abnormalities, which together constitute a greater biological network that must be understood (Loscalzo et al. 2007).

In a recent animal study (Karavia et al. 2011) it was found that apoE-deficient mice displayed resistance to diet-induced obesity, which is a major risk factor for both NAFLD and CVD. This discovery highlights possible selective evolutionary mechanisms, whereby the immediate advantage (heightened metabolism) of the abnormal protein proved more advantageous to the species than the reduced life expectancy due to eventual CVD. This observation has yet to be confirmed among humans.

4.2.2. *MTHFR*

MTHFR is directly involved in homocysteine metabolism and deleterious structural variations in this enzyme significantly increase the risks for development of many different cancers as well as cardiovascular and neurological diseases. The two most prominent mutations associated with reduced enzymatic activity, elevated homocysteine levels and inflammation are *MTHFR* 677 and 1298. From the established literature it is clear that the *MTHFR* 677 genetic variant induces the greatest loss of function in the protein, especially in the homozygous state. Compound heterozygosity for both mutations constitutes the second most severe phenotypic expression, while the influence of homozygosity for *MTHFR* 1298 constitutes a 40% reduction in enzymatic activity.

In a study by Sazci et al. (2008), numerous associations were found between NASH and *MTHFR* 1298. The mutant allele of *MTHFR* 1298 was identified as significantly elevated among NASH patients, while homozygosity was especially prominent in the female patients and heterozygosity in the males. It was concluded that the deleterious allele of *MTHFR* 1298 increases the risk for development of NASH. While the association with homozygosity may be expected from the diminished functional capacity of the mutant protein, the significance of heterozygosity for *MTHFR* 1298 represents a deviation from the established literature. This may well be due to the limited scope of the study methodology, where relevant disease-modifying genes (e.g. all other homocysteine-related genes) and environmental factors (e.g. alcohol use, obesity and smoking) have not been included to account for the natural variations (both genetic and environmental)

observed between populations. To solve this dilemma an integrative, systems-based approach is needed to further genetic research, particularly in the field of human disease.

The importance of evaluating the clinical, environmental and genetic factors affecting certain metabolic pathways in a patient is illustrated by the interactions of MTHFR, folate and diet. High homocysteine levels are a marker of folate- and vitamin B12 deficiency, providing valuable information when determined in combination with *MTHFR* genotyping and nutritional assessment of folate status. However, the predictive value of mutations in susceptibility genes is limited when used in isolation, as dietary advice based on detection of variation in the *MTHFR* gene can be harmful when the clinical picture of the patient is not taken into account. For example, high-dose folate supplementation is inadvisable for cancer patients irrespective of the genetic status of the individual.

The effect of *MTHFR* on coagulation is discussed further in section 4.2.4.

4.2.3. *HFE*

The HFE protein plays an essential role in iron metabolism which impacts numerous metabolic processes with deleterious structural variations in this molecule influencing diverse diseases of highly variable phenotypic expression (from haemochromatosis to diabetes mellitus to hepatocellular carcinoma). The two most prominent genetic variations in the *HFE* gene, which directly affect the structure and function of the protein, are C282Y and H63D. These mutations are associated with increased ferritin and transferrin saturation levels, although this is primarily the case in C282Y homozygotes and to a lesser extent evident among C282Y/H63D compound heterozygotes. The phenotypic penetrance is so varied that heterozygous carriers for either variant are rarely influenced. The same is often true of H63D homozygotes. In stark contrast to the observed phenotype among many Whites, Lee et al. (2010) reported the presence of H63D mutations as an independent factor associated with NAFLD and elevated transferrin saturation in a Korean cohort. They concluded that H63D may increase the risk of NAFLD development, possibly through peripheral iron overload (especially among men). This observation again emphasizes the necessity of expanded investigations encompassing wider biological systems with careful attention to relevant disease-modifying genes (e.g. genes of the Iron Regulator Proteins and Iron Responsive Elements) and environmental factors (e.g. alcohol use, obesity and smoking). Such biological networks are at the core of all health and disease mechanisms which require a new research approach for their potential to be fully realized (Barabási, 2007).

Insulin resistance is also associated with abnormal HFE and measurements of fasting glucose, insulin and the Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) may be prudent when conducting detailed studies (Martínez-García et al. 2009). Liver enzymes (ALT and AST) may also be assessed in relation to *HFE* genotype due to the hepatic derangements caused by HFE-related damage (see section 1.4).

A recent mouse study by Tan et al. (2011) demonstrated that Hfe-deficiency promotes development of severe NAFLD resulting from defective hepatic-intestinal iron and lipid signalling. These iron and lipid abnormalities predispose subjects toward diet-induced hepatic lipotoxicity and an accelerated progression of injury to fibrosis through activation of Tnf-α, mitochondrial respiratory complex, hypoxia-related steatohepatitis and antioxidant dysfunction with early fibrogenesis. This discovery may have a significant impact on our understanding of the underlying mechanisms of not only NAFLD, but all similarly complex diseases once confirmed among humans.

4.2.4. Coagulation Factors

Prothrombin and coagulation factor V, the proteins encoded by the *F2* and *FV* genes respectively, are crucial cofactors in the coagulation cascade. The most prominent variations in these genes are *F2* 20210 and *FV* Leiden. These mutations are relatively rare, even in the heterozygous state, which may be explained by the severity of dysfunction in their proteins. The complete absence of prothrombin is considered to be incompatible with life while a similar observation has been reported for coagulation factor V among mice. Less extreme deleterious variations may contribute to severe cardiovascular abnormalities by disrupting thrombosis, which in turn could facilitate systems-wide damage. Important environmental risk factors are alcohol use, obesity and smoking. The literature study has shown that the risk of venous thrombosis imposed by the *FV* Leiden mutation is significantly increased in patients with high cholesterol levels, with dire implications for disease severity and age of onset for NAFLD patients (Völzke et al. 2005b).

Deleterious variations in *MTHFR* are also associated with abnormal coagulation in the context of elevated homocysteine levels while *F2* 20210 and *FV* Leiden may affect the strength of the *MTHFR*-induced disturbance. The hazardous influence posed by multiple mild genetic defects was illustrated by Talmon et al. (1997), where retinal arterial occlusion was discovered in a child heterozygous for the coagulation factor V R506Q mutation and homozygous for thermolabile MTHFR. The coexistence of these relatively mild hereditary thrombophilic contributors resulted in

severe thrombotic manifestations. Elevated triglycerides (possibly due to APOE2) and APOE4 are also associated with reduced coagulation tendency (Riddell et al. 1997).

In a study of the thrombotic risk factors in patients with NAFLD subdivided into three groups (those with fatty liver, NASH or chronic viral hepatitis only), Assy et al. (2005) discovered associations with protein S (lower in NASH patients than in fatty liver alone) and protein C levels (higher in patients with NAFLD and mild or severe fibrosis than in cases with only chronic viral hepatitis). No correlations between NAFLD and *F2* 20210, *FV* Leiden, *MTHFR* 677 or 1298 were found. These results again emphasize the need for expanded investigations guided by an integrative, systems-based approach to further genetic research by critically analysing relevant disease-modifying genes and environmental factors in concert with clinical characteristics and the principal molecular abnormalities.

4.2.5. Genotype-Phenotype Correlation Analysis

A significant increase ($p = 0.04$) in Alanine Transaminase (ALT) levels was identified between *HFE* mutation carriers (hetero- and homozygous individuals for the C282Y and H63D mutations of the *HFE* gene) and wild type individuals among stage 3 and 4 NAFLD patients (figure 3.3.1). ALT is a marker for liver damage as it is present only in hepatocytes and is thus expected to increase in the latter stages of NAFLD development with mounting cellular damage releasing more of the enzyme into the bloodstream. This correlation with adverse *HFE* genotypes indicates a greater degree or onset of liver damage in the presence of the mutations and demonstrates that a subset of NAFLD patients at increased risk of NASH may be identified through genetic testing. Such a pathology-supported genetic evaluation may improve the clinical outcome of NAFLD patients belonging to this elevated risk group as more aggressive therapy will be required at an earlier stage to prevent irreversible hepatic damage.

Alcohol consumption was evaluated as a possible environmental risk factor affecting disease severity in patients already diagnosed with NAFLD. However, as only eight of the 44 members of the stage 3 and 4 NAFLD patient population were alcohol users (18%), the association found was primarily relevant to abstinent individuals. Since the sample number of NASH patients was relatively small, the analysis was not done separately for the Coloured and White patients. This decision is justified as there were no significant differences in the allele frequencies between the ethnic groups (tables 3.4, 4.5 and 3.6).

A trend was observed in the prevalence of APOE2 mutation carriers among stage 3 and 4 NAFLD patients where triglyceride levels were elevated relative to the lower stage patients and control individuals (data not shown). Similar to the APOE2-triglyceride trend, cholesterol levels showed a marginal increase relative to the prevalence of APOE4 mutation carriers among the same severely affected patients when compared to the lower stage 1 and 2 patients, as well as the control individuals (data not shown). The more severely deleterious *MTHFR* genotypes, namely homozygosity for *MTHFR* 677 and compound heterozygosity for *MTHFR* 677/1298, tended to present more often among NAFLD patients (stages 1 – 4) than control subjects (data not shown). None of these inclinations were statistically significant, but they were shown to be independent of racial variation and are in compliance with expected observations from the established literature. The small percentage of the study population found to be carriers of the functionally detrimental genotypes reduced the statistical power available for examination of association.

4.3. Network Medicine

Recent advances in the mapping of networks has transformed our understanding of highly interconnected systems such as biology, society and technology resulting in the discovery that common designs governed by relatively simplistic and quantifiable organizing principles are universal (Barabási, 2003). When this development is considered in the current global context of an increased desire for interconnectedness, the pervasiveness of networks throughout all aspects of human health becomes apparent. The need for an integrative, systems-based approach is supported by Barabási (2007) in his conclusion that disease mechanisms can only be fully understood when the "detailed wiring diagram" of all cellular and molecular elements involved has been determined. The concept of network medicine has gained considerable support in recent years, partly due to its acknowledgement of the true complexities and diversity of associations inherent in biological systems that were historically considered and treated as isolated entities. As an emerging field, a framework for future research is currently being developed to encompass the totality of environmental, genomic, pathologic and proteomic factors affecting disease development (Pawson and Lindin, 2008; Zanzoni et al. 2009; Barabási et al. 2011). The structural variations affecting protein function were assessed through biochemical measurements in this study due to the limited availability of proteomic evaluations in South Africa.

The contemporary classification of human disease and medical diagnosis, which is based on inductive generalization predicated on Occam's razor, was challenged on numerous accounts by Loscalzo et al. (2007) for its universal acceptance in spite of its many inadequacies in the so-called "postgenomic" era.

While observational correlations between clinical syndromes and pathological analyses by Cartesian reductionism have proven to be the most effective past methodology, the dominant focus on observational skills and simple laboratory tools to define the disease phenotype are fundamentally lacking in risk management and disease prevention capabilities. Significant limitations in the sensitivity of preclinical disease identification and the specificity of explicit disease definition emphasize the need for a new classification of human disease that relies on a combination of conventional reductionism and the non-reductionist approach of systems biomedicine.

Medical science has relied on the structure provided by reductionism in much the same manner, largely due to its analytical and experimental viability resulting in many successful applications of basic molecular medicine over the past 50 years. However, genomics has transformed the biological sciences into a vast data-generating industry where the enormity of potentially valuable functional information cannot readily be analyzed optimally with the conventional approach.

This development has placed tremendous strain and high expectations on the crucial role of bioinformatics to store, analyse, interpret and predict every facet of living systems. As bioinformatics is itself a systems-based and highly networked field, it has transcended its origins as a mere informational database and now serves as the hub of all biological research and development while providing a model for all associated disciplines.

The advantages of an integrative, systems-based approach to human disease classification and treatment can be summarized in five key points (Loscalzo et al. 2007):

1. Such a protocol can determine those elements or combinations of elements with a significant effect on network behaviour and disease expression,
2. Reductionist principles may not be capable of reaching and confirming the associations of such elements which might result in a deeper understanding of disease mechanisms and the discovery of new targets for therapeutic intervention,

3. This approach allows all aspects of the network genome, environmental exposures and environmental effects on the posttranslational proteome to be methodically investigated for their role in determining the pathophenotype,
4. The exact disease phenotype can be defined more precisely by the application of this process through the analysis of the distinctive genetic and environmental factors that determine intermediate phenotypes which contribute to disease expression,
5. This method provides a novel perspective for discovering individual- or combinations of therapeutic targets that may influence the disease phenotype.

4.4. Translation Research

An initiative launched at the Department of Pathology at the University of Stellenbosch in April 2009 incorporates the ideals of an integrative, systems-based network approach to genetic testing. This enterprise seeks to integrate the service- and research sectors via the common thread of pathology with the development of Pathology Supported Genetic Testing (PSGT; www.gknowmix.com). The aim is to combine the clinical aspects, environmental risk factors, genetic characteristics and pathology of the patient to improve disease diagnosis and therapeutic design. PSGT holds great promise for the management of complex diseases, as proven in the case of haemochromatosis (Kotze et al. 2009). The genetic mechanisms and modifying factors influencing the development of Hereditary Haemochromatosis (HH) provide an excellent example of the complexities inherent to pathogenic processes. The current approach to the diagnosis and treatment of HH involves the initial determination of the exact biochemical, clinical and pathologic profile of a patient. An optimal treatment is then devised, implemented and the response is monitored according to the specific genetic and pathologic parameters provided by the testing phase. This information is made available to the patient and any relevant physicians or specialists as needed.

This study is modelled on the PSGT approach which is founded on the concept of translation research which was well-characterized and divided into four progressive phases by Khoury et al. (2007). While the types of research conducted within each of the individual phases often overlap in practice, the fundamental organizational structure employed to achieve the desired end result in each level remains accurate.

These four phases are:

- Phase 1 research, also designated Tier 1 or Type 1 (T1), focuses on the application of a genomic medicine discovery to the healthcare setting. The patented CVD multi-gene test is an example of such a genomic application.
- T2 research concerns the development of evidence-based practice guidelines to maximize the value of a specific healthcare application. The publications by Kotze et al. (2005) on genetic testing of HH and the CVD multi-gene test as described by Kotze and Badenhorst (2005) for chronic disease risk management, provide examples of guideline development based on established literature and extensive research in the local population.
- T3 research is centred on the evaluation of the evidence-based practice guidelines within health practice. Of the 75 control individuals (without NAFLD), at least 15 were referred from medical doctors who registered for the distance-learning Integrative Medicine course offered by the University of Stellenbosch during 2011. Their evaluation of the clinically-integrated patient reports resulted in requests from most of these doctors (and their spouses) to be tested using the CVD multi-gene test as part of a wellness screen (unpublished data).
- T4 research assesses the impact of the practice on the health of populations. This process is underway using a combined research and service approach (appendix B) as demonstrated in the two case studies representing a "real-world" situation.

The PSGT system encompasses several tests developed via the pathology supported protocol for genetic testing, with each of these tests focussed on a specific major health concern or CVD subtype. This system exists to improve patient care by furthering medical research and incorporating it in a practical manner within the contemporary healthcare system. The majority of biological research conducted throughout modern history is of the T1 or T2 variety, while T3 and T4 are exceedingly rare due in part to the complexities associated with the interconnectivity of information necessary for success at such a level (Khoury et al. 2007). New tests that form part of the PSGT system are constantly being developed for inclusion in T4 research, while existing protocols are amended and improved upon through T1, T2 and T3 research.

According to the above criteria, the PSGT system has reached T4-status in light of the degree of monitoring achieved through annual workshops that include questionnaire-based surveys, case studies and feedback sessions. The results of a questionnaire-based survey among physicians were presented at the Joint African and Southern African Congress for Human Genetics held in March of 2011 in Cape Town, South Africa (Vogel S et al. 2011).

A recent study by Kotze et al. (2011) employed the PSGT approach to investigate the genotype associations in South African patients with the MetS where a statistically significant association was observed between presence of the APOE2 allele and an increasing number of MetS features ($p < 0.03$). A similar correlation was noted with the presence of deleterious *MTHFR* genotypes ($p < 0.02$). Based on these results, it was concluded that biochemical and clinical anomalies could be explained by the combined effects of unfavourable genotypes and environmental factors. It was suggested that gene expression could be gauged by monitoring specific biochemical parameters and that evaluation of the response to treatment should be based on the overall risk profile of each individual. This study illustrates the complex interconnectivity of genetic and environmental factors, as well as the overlapping nature of the various phases of translation research and the progressive inclination of the healthcare industry to the establishment of personalized medical doctrine.

4.5. Personalized Medicine through Pathology Supported Genetic Testing

Personalized medicine is a medical model in which individual patients receive customized preventative and therapeutic care based on their own biochemical, clinical, environmental and genetic information. The standards and practices of the contemporary healthcare system are principally determined by epidemiological studies of large cohorts, resulting in a medical industry that is limited in both sensitivity and specificity in diagnosing, preventing or treating patients whose conditions fall outside the established disease definition. These deviations are caused by any number of discrete or interconnected factors that differ among individuals and are rarely fully accounted for in modern practice. Personalized medicine aims to resolve this dilemma by modifying healthcare through an integrative, systems-based approach to suit the needs of every patient. A diverse array of diagnostic systems will converge in this medical model to provide the data necessary to develop specific prophylactic strategies or therapeutic interventions to manage an individual's condition. This may be achieved by monitoring the disease status, determining the appropriate medication and tailoring dosages to a patient's specific requirements. Such diagnostic systems will include molecular assays to identify specific mutations, measure gene expression levels and determine the functional efficacy of key proteins related to the disease in question. A thorough risk assessment is also made possible by the application of these methods and individual preventative treatments for recognized disorders may thus be formulated and applied prior to symptomatic pathogenesis.

The case study (GMX1) presented in section 3.4 illustrates the clinical utility of the PSGT approach to personalized medicine in a 40 year old patient who had a myocardial infarction at the age of 29 years. Detection of two copies of mutation C282Y in the *HFE* gene in the presence of high serum ferritin and transferrin saturation levels confirms a diagnosis of HH. Therapeutic phlebotomy was recommended to reduce the abnormally elevated serum iron stores of the index patient, while regular monitoring of transferrin saturation, serum ferritin and haemoglobin levels is advisable due to the reported family history of thalassemia. As indicated in table 3.8, transferrin saturation decreased modestly (from 83% to 75%) following treatment while the serum ferritin was reduced (from 671 ng/ml to 252 ng/ml). This result was achieved within three months of therapeutic intervention, emphasizing the efficiency of the intervention. The swift reduction in serum ferritin is of greater immediate benefit to the prevention of organ damage than transferrin saturation, while the slower rate of normalization of the latter may be due to the functional role of the protein as a transport molecule. The risk of hepatocellular carcinoma is increased 200-fold when serum ferritin concentration exceeds 1000 ug/L; therefore it is important to keep the ferritin levels below this threshold representing a strong predictor of cirrhosis in HH patients (Osborne et al. 2010).

The index patient was particularly interested in a diet plan to minimize long-term iron absorption while avoiding any possible side-effects from pharmacological agents and improving his overall state of health. This goal was facilitated by the dieticians affiliated with the PSGT system, who then provided a personalized dietary strategy supported by his physician and the chief medical scientist overseeing the HH-test. General recommendations provided included the following:

- Increase calcium intake as it lowers body iron stores by inhibiting the absorption of haem and non-haem iron (Aranda et al. 2010). Foods rich in calcium include almonds, broccoli, canned salmon, cheese, figs, milk, sardines, tofu and yogurt. E.g. a single cup of skimmed milk contains 300 mg of calcium.
- Oxalates also impair the absorption of iron and can be found in foods such as beets, chocolates, kale, nuts, rhubarb, spinach, strawberries, tea, wheat bran and herbs such as basil, oregano and parsley (Benito and Miller, 1998).
- Phytate, a compound found in soy protein and fibre, affects the bioavailability of iron by reducing its absorption by 50 to 65% and is especially prevalent in almonds, cereals, dried beans, lentils, peas, sesame, walnuts and whole grains (Conrad and Umbreit, 1993).

- Polyphenols and tannins are also major inhibitors of iron absorption and may be found in cocoa, coffee, fruit (e.g. apples, black-, rasp- and blueberries), herbal teas (e.g. black tea), peppermint and walnuts (Hurrel et al. 1999).
- Ceramic and glass cookware should be used to avoid iron filings from contaminating the food, as cast iron skillets and some grills may provide contaminant iron in sufficiently bioavailable form to influence iron status (Adish et al. 1999).

Diagnosis of iron-related disorders is frequently complicated by MetS features and steatosis in the presence of increased iron levels and *HFE* mutations. The combination of these factors is indicative of the Insulin Resistance-associated Hepatic Iron Overload (IR-HIO) syndrome, also known as dysmetabolic iron overload (Riva et al. 2008). This disorder is characterized by the co-existence of hepatic steatosis, normal transferrin saturation levels and two or more components of the MetS. Inclusion of hyperferritinaemia in the diagnostic criteria for IR-HIO syndrome has fallen into disfavour in recent years due to the non-specific nature of elevated ferritin levels, especially in the presence of metabolic abnormalities (George et al. 1998; Fargion et al. 2001; Bugianesi et al. 2004).

Biochemical and clinical manifestation of HH, IR-HIO syndrome and NAFLD may sometimes be so similar that the risk of misdiagnosis is significantly increased (Kruger, 2008; Kotze et al. 2009). The index patient (GMX1) presented as a case study to demonstrate this point, was one of the first two individuals with high ferritin levels referred for participation in this translational research project. In the second patient referral (GMX2), normal transferrin saturation levels in the presence of high ferritin and fasting glucose levels was consistent with the wild type *HFE* mutation status found. These findings facilitated discriminative diagnosis of HH (GMX1) and NAFLD in the presence of the IR-HIO syndrome (GMX2), respectively.

HH patients are frequently identified as a result of being investigated for early, non-specific symptoms such as chronic fatigue, joint pain and liver disease, or as a result of having an affected family member. This was indeed the case for patient GMX1 diagnosed with HH, while patient GMX2 was referred for *HFE* mutation analysis due to a previous finding of C282Y heterozygosity in his father. The necessity of an integrative, systems-based network approach combining the clinical aspects, environmental risk factors, genetic characteristics and pathology of the patient to improve disease diagnosis and therapeutic intervention was thus exemplified by these cases.

The nature of the PSGT approach may nullify the requirement of clinical trials, similar to *HFE* mutation screening performed within a clinical context without any randomised clinical trials performed to first demonstrate the effectiveness of avoiding iron overload through treatment (phlebotomy) and/or diet intervention. The targets for PSGT as applied with use of the CVD multi-gene test are well established in the scientific continuum while the interventions are non-invasive and focussed on improving health by directed promotion of a healthier lifestyle. This may involve modification of diet, physical exercise regiments, nutritional supplements or contra-indication of pharmacological interventions. Several studies have for example indicated that metformin may increase homocysteine levels, which may be particularly relevant in patients with deleterious mutations in the *MTHFR* gene (de Jager et al. 2010; Palomba et al. 2010). Another key element of the PSGT approach to consider is the vast knowledge and experience of professionals from traditionally distinct fields of healthcare that are all combined by this system to provide a truly individualized service to a patient. From physicians to medical scientists and bioinformaticians to genetic counsellors, all could be actively involved in the diagnosis, treatment and monitoring stages, thereby providing dynamic medical support to each and every patient.

Chapter 5

Conclusions

As a result of this study, a high-throughput diagnostic test for detection of multiple mutations contributing to the development or progression of Non-Alcoholic Fatty Liver Disease (NAFLD) and Cardiovascular Disease (CVD) through gene-gene and gene-environment interactions is now available in South Africa. This detection system facilitates early diagnosis at the DNA level and allows for individual preventative treatments to be formulated and applied for recognized disorders prior to the development of severe symptoms.

The significant association between *HFE* mutations and elevated Alanine Transaminase (ALT) levels found in the NAFLD patient population studied with the severe form of the disease (NASH) resulted in the definition of a subset of patients who would benefit most from genetic testing to direct more aggressive therapy at an earlier stage. Previous studies have shown that NASH is an independent risk factor for CVD, and the risk is also increased in NAFLD patients with elevated ALT levels (Kruger et al. 2011). The association between *HFE* mutations and increasing ALT levels is therefore in agreement with evidence from the literature that mutations in the *HFE* gene may play a role in CVD risk (Tuomainen et al. 1999; Roest et al. 1999).

The necessity of an integrative, systems-based network approach was demonstrated where misdiagnosis of Insulin Resistance-associated Hepatic Iron Overload (IR-HIO) syndrome and NAFLD was prevented in an obese, Hereditary Haemochromatosis (HH) patient homozygous for the C282Y mutation in the *HFE* gene.

This study also illustrates the clinical utility of the Pathology Supported Generic Testing (PSGT) approach to personalized medicine. This approach may nullify the traditional requirement for clinical trials, as the targets for testing are well established in the scientific continuum while the interventions are non-invasive and focussed on improving health by directed promotion of a healthier lifestyle. This may involve modification of diet, physical exercise regiments, nutritional supplements or contra-indication of certain medication (e.g. prescription of Metformin in patients with deleterious *MTHFR* genotypes and high homocysteine levels). Another key element of the PSGT approach to consider is the vast knowledge and experience of professionals from traditionally distinct fields of healthcare that are all combined by this system to provide a truly individualized service to a patient. From physicians to medical scientists and bioinformaticians to genetic counsellors, all could be actively involved in the diagnosis, treatment and monitoring stages, thereby providing dynamic medical support to each and every patient.

Ethical Considerations

While the assessment of genetic variation may improve the diagnosis, treatment or prevention of a disease such as NAFLD, ethical issues may arise when a genetic predisposition is identified in a healthy individual without a family history of the risk factors for NAFLD or NASH. Such complications include the effect of a multi-gene screen on health insurance, as discussed in the South African context in a paper by Kotze et al. (2004b) where medical advisors of the insurance industry were approached on the subject. Effective Standard Operating Procedures (SOPs) and a secure digital database were developed in parallel to this study to ensure the confidentiality of patient information. This matter is vital to any medically related field and especially where genetic testing is concerned, as it may influence entire families.

The following issues should be explained to all prospective study participants before any specimens are collected for laboratory testing to alleviate the development of any ethical complications that may otherwise arise during the investigation:

- The genetic test will only screen for specific genetic alterations expected to provide useful information in relation to diagnosis and/or treatment strategies,
- Detection of genetic alterations (positive test) implies that other family members may also have the genetic change(s),
- Failure to detect a specific genetic alteration (negative test result) does not exclude undefined gene mutations or other risk factors not tested for,
- Genetic testing may result in better motivation for lifestyle changes or targeted treatment, or possibly anxiety when genetic risk factors are identified in an individual without clinical symptoms of a disease,
- Identification of genetic alterations in individuals with a family history or clinical features of the associated disease will not impact further on insurance, while exclusion of a genetic defect in a family member could be beneficial for insurance purposes in some instances,
- A positive genetic test does not mean that the person has a genetic disease or will develop the condition, but it can increase the risk of disease in the absence of appropriate risk reduction intervention or inappropriate medication,
- In the event that genetic testing is performed in families, non-paternity may be revealed and it is therefore important that adoption be reported at the time that specimens are obtained for genetic testing.

Study Limitations

Detailed information on clinical, biochemical and environmental factors relevant to the development of NAFLD were not available for all the patients investigated for genetic variations in this study. Similarly, patient DNA samples were not accessible in several cases where other valuable information was provided. These issues resulted in a severely reduced total study population for statistical analysis and may explain the scarcity of significant genotype-phenotype associations detected.

The ABI™ *Taq*Man® SNP Genotyping Assays employed in this study are only capable of detecting a single genetic variation per genotyping reaction, thereby necessitating separate reactions for each polymorphism screened. Each of the 253 samples was tested for eight different mutations, which resulted in a total of 2024 separate genotyping reactions, excluding positive and negative control reactions. This has a significant effect on the cost and turnover time of sample testing. Also, individual samples cannot be screened for all genetic variations simultaneously, necessitating the practice of sample batching which is not ideal for diagnostic purposes when patients require their genotyping results quickly as batching may delay the process substantially.

Recommendations and Future Prospects

All study participants should be evaluated for all relevant biochemical, clinical, environmental and genetic variables that may be associated with the central focus of any subsequent studies and the data should be stored in a secure, well-maintained digital database. Due to the high costs and large scale associated with clinical trials, they are incapable of demonstrating the effectiveness of such dynamic studies where careful individual monitoring of patients and a versatile database must be employed.

Multiplexing of the ABI™ *Taq*Man® SNP Genotyping Assays employed in this study was not possible, as all of the assays use the same fluorophores in their probes and thus have identical emission spectra. High-Resolution Melt (HRM)-based allelic discrimination could be investigated in future for its ability to screen for all eight mutations simultaneously. However, Jalali Sefid Dashti (2010) reported difficulties in the optimization and standardization of HRM assays, which suggests that multiplexing with similar probes may be impractical or even impossible for eight mutations. Alternatives include PCR arrays and SNP genotyping chips, which may each screen a sample for dozens (PCR arrays) to thousands (genotyping chips) of individual polymorphisms at the same time. The issue of rapid, individual sample turnover may also be addressed by application of such

methods, as previously employed for the CVD multi-gene assay using reverse-hybridisation strip-assay technology (Kotze et al. 2003, Kotze and Thiart, 2003). Although the CVD strip-assay enables simultaneous analysis of the eight mutations screened for in the NAFLD patients and controls included in this study, transferral to a new test platform using real-time TaqMan® technology for analytical validation of high-throughput genotyping proved to be more cost-effective.

Further investigations are required to corroborate the association found between *HFE* mutations and elevated Alanine Transaminase (ALT) levels within NAFLD patients and to expand the possible clinical utility of such a correlation.

Recent studies on the genetic basis of NAFLD have revealed that structural variations in Apolipoprotein C-III (Petersen et al. 2010) and Patatin-like Phospholipase domain-containing protein 3 (Romeo et al. 2008) may be instrumental in its pathogenesis. These proteins are encoded by the APOC3 and PNPLA3 genes, respectively. Further investigation of these determinants may prove vital to understanding, treating and even preventing this highly prevalent disorder.

Development of a risk score that includes the genetic contribution to multi-factorial diseases such as NAFLD may aid healthcare professionals in monitoring and managing the disorder in question. This may be realized once the limitations encountered in this study relating to the statistical analysis of complex systems can be overcome. Such a score should consist of all relevant biochemical, clinical, environmental and genetic factors with each contribution weighted for its individual and joint effects on the risk of pathogenesis. It is envisaged that the findings presented in this study would form an important component of such a genotype risk scoring system, to be developed for early diagnosis of treatable CVD subtypes and prevention of cumulative risk in NAFLD patients.

The clinical utility of the CVD multi-gene test to guide chronic disease risk management in patients with NAFLD, with particular relevance to the *HFE* mutation detection component of this test, has been demonstrated in this study. This extends the application of the CVD multi-gene test developed initially to better distinguish between hypercholesterolaemics with familial hypercholesterolaemia versus less severe forms of dyslipidaemia that can be treated effectively by diet and lifestyle modification alone.

Chapter 6

References

Acciarri A, Masullo C, Bizzarro A, Valenza A, Quaranta D, Marra C, Tiziano FD, Brahe C, Seripa D, Matera MG, Fazio VM, Gainotti G, Daniele A. APOE epsilon-2-epsilon-4 genotype is a possible risk factor for primary progressive aphasia. Ann Neurol. 2006; 59: 436-437.

Acharya SS, Coughlin A, Dimichele DM. Rare Bleeding Disorder Registry: deficiencies of factors II, V, VII, X, XIII, fibrinogen and dysfibrinogenemias. J Thromb Haemost. 2004; 2: 248-56.

Adams LA, Lymp JF, St Sauver J, Sanderson SO, Lindor KD, Feldstein A, Angulo P. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. Gastroenterology. 2005; 129: 113-121.

Adish AA, Esrey SA, Gyorkos TW, Jean-Baptiste J, Rojhani A. Effect of consumption of food cooked in iron pots on iron status and growth of young children: a randomised trial. Lancet. 1999; 353: 712-716.

Agosta F, Vossel KA, Miller BL, Migliaccio R, Bonasera SJ, Filippi M, Boxer AL, Karydas A, Possin KL, Gorno-Tempini ML. Apolipoprotein E epsilon-4 is associated with disease-specific effects on brain atrophy in Alzheimer's disease and frontotemporal dementia. Proc Nat Acad Sci. 2009; 106: 2018-2022.

Agostinho MF, Arruda VR, Basseres DS, Bordin S, Soares MC, Menezes RC, Costa FF, Saad ST. Mutations analysis of the HFE gene in the Brazilian population. Blood Cells Mol Dis. 1999; 25: 324-327.

Aigner E and Datz C. Iron Perturbations in Human Non-Alcoholic Fatty Liver Disease (NAFLD): Clinical Relevance and Molecular Mechanisms. Hepatitis Monthly. 2008; 8 (3): 213-220.

Allen NC, Bagade S, McQueen MB, Ioannidis JPA, Kavvoura FK, Khoury MJ, Tanzi RE, Bertram L. Systematic meta-analyses and field synopsis of genetic association studies in schizophrenia: the SzGene database. Nat Genet. 2008; 40: 827-834.

Amouyel P, Vidal O, Launay JM, Laplanche JL. The apolipoprotein E alleles as major susceptibility factors for Creutzfeldt-Jakob disease. Lancet. 1994; 344: 1315-1318.

Anchors JM, Gregg RE, Law SW, Brewer HB (Jr). ApoE deficiency: markedly decreased levels of cellular apoE mRNA. Biochem Biophys Res Commun. 1986; 134: 937-943.

Anderson DH, Ozaki S, Nealon M, Neitz J, Mullins RF, Hageman GS, Johnson LV. Local cellular sources of apolipoprotein E in the human retina and retinal pigmented epithelium: implications for the process of drusen formation. Am J Ophthal. 2001; 131: 767-781.

Angelico F, Del Ben M, Conti R, Francioso S, Feole K, Fiorello S, Cavallo MG, Zalunardo B, Lirussi F, Alessandri C, Violi F. Insulin resistance, the metabolic syndrome, and nonalcoholic fatty liver disease. J Clin Endocrinol Metab. 2005; 90: 1578-1582.

Angulo P. Nonalcoholic fatty liver disease. N Engl J Med. 2002; 346: 1221-1231.

Aranda N, Viteri FE, Montserrat C, Arija V. Effects of C282Y, H63D, and S65C HFE gene mutations, diet, and life-style factors on iron status in a general Mediterranean population from Tarragona, Spain. Ann Hematol. 2010; 89 (8): 767-773.

Aubé C, Moal F, Oberti F, Roux J, Croquet V, Gallois Y, Argaud C, Caron C, Calès P. Diagnosis and measurement of liver fibrosis by MRI in bile duct ligated rats. Dig Dis Sci. 2007; 52 (10): 2601-9.

Bacon BR, Olynyk JK, Brunt EM, Britton RS, Wolff RK. HFE genotype in patients with hemochromatosis and other liver diseases. Ann Intern Med. 1999; 15: 953-962.

Badman MK and Flier JS. The adipocyte as an active participant in the energy balance and metabolism. Gastroenterology. 2007; 132: 2103-2115.

Bagley PJ and Selhub J. A common mutation in the methylenetetrahydrofolate reductase gene is associated with an accumulation of formylated tetrahydrofolates in red blood cells. Proc Nat Acad Sci. 1998; 95: 13217-13220.

Barabási A-L. Linked. New York: Plume, 2003.

Barabási A-L. Network Medicine — From Obesity to the "Diseasome." N Engl J Med. 2007; 357 (4): 404-407.

Barabási A-L, Gulbahce N, Loscalzo J. Network medicine: a network-based approach to human disease. Nat Rev Genet. 2011; 12 (1): 56-68.

Barton JC, Rao SV, Pereira NM, Gelbart T, Beutler E, Rivers CA, Acton RT. Juvenile hemochromatosis in the Southeastern United States: a report of seven cases in two kinships. Blood Cells Molec Dis. 2002; 29: 104-115.

Barton JC, Sawada-Hirai R, Rothenberg BE, Acton RT. Two novel missense mutations of the HFE gene (I105T and G93R) and identification of the S65C mutation in Alabama hemochromatosis probands. Blood Cells Molec Dis. 1999; 25: 146-154.

Barut G, Balci H, Bozdayi M, Hatemi I, Ozcelik D, Senturk H. Screening for iron overload in the Turkish population. Dig Dis. 2003; 21: 279-285.

Beauchamp NJ, Daly ME, Hampton KK, Cooper PC, Preston E, Peake IR. High prevalence of a mutation in the factor V gene within the U.K. population: relationship to activated protein C resistance and familial thrombosis. Brit J Haemat. 1994; 88: 219-222.

Beckman LE, Saha N, Spitsyn V, Van Landeghem G, Beckman L. Ethnic differences in the HFE codon 282 (Cys/Tyr) polymorphism. Hum Hered. 1997; 47: 263-267.

Benito P and Miller D. Iron absorption and bioavailability: An updated review. Nutrition Research. 1998; 18 (3): 581-603.

Bernard PS and Wittwer CT. Real-time PCR technology for cancer diagnostics. Clin Chem. 2002; 48: 1178-1185.

Bersot TP, Innerarity TL, Mahley RW, Havel RJ. Cholesteryl ester accumulation in mouse peritoneal macrophages induced by beta-migrating very low density lipoproteins from patients with atypical dysbetalipoproteinemia. J Clin Invest. 1983; 72: 1024-1033.

Bertina RM, Koeleman BPC, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, Reitsma PH. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature. 1994; 369: 64-67.

Bertina RM and Rosendaal FR. Venous thrombosis - the interaction of genes and environment. N Engl J Med. 1998; 338: 1840-1841.

Best LG, Harris PE, Spriggs EL. Hemochromatosis mutations C282Y and H63D in 'cis' phase. Clin Genet. 2001; 60 (1): 68-72.

Beutler E. Haemochromatosis: Genetics and Pathophysiology. Ann Rev Med. 2005; 57: 5.1-5.17.

Bjelland I, Tell GS, Vollset SE, Refsum H, Ueland PM. Folate, vitamin B12, homocysteine, and the MTHFR 677C-T polymorphism in anxiety and depression: the Hordaland Homocysteine Study. Arch Gen Psychiat. 2003; 60: 618-626.

Blair CK, Folsom AR, Knopman DS, Bray MS, Mosley TH, Boerwinkle E. APOE genotype and cognitive decline in a middle-aged cohort. Neurology. 2005; 64: 268-276.

Boduroglu K, Alanay Y, Koldan B, Tuncbilek E. Methylenetetrahydrofolate reductase enzyme polymorphisms as maternal risk for Down syndrome among Turkish women. Am J Med Genet. 2004; 127A: 5-10.

Boisvert WA, Sprangenberg J, Curtiss LK. Treatment of severe hypercholesterolemia in apolipoprotein E-deficient mice by bone marrow transplantation. J Clin Invest. 1995; 96: 1118-1124.

Bojanowski CM, Shen D, Chew EY, Ning B, Csaky KG, Green WR, Chan C-C, Tuo J. An apolipoprotein E variant may protect against age-related macular degeneration through cytokine regulation. Environ Molec Mutagen. 2006; 47: 594-602.

Bonora E. The metabolic syndrome and cardiovascular disease. Ann Med. 2006; 38: 64-80.

Bothwell TA, Charlton JD, Cook JD, Finch CA. Iron metabolism in man. Blackwell Scientific, Oxford 1979.

Braun A, Muller B, Rosche AA. Population study of the G1691A mutation (R506Q, FV Leiden) in the human factor V gene that is associated with resistance to activated protein C. Hum Genet. 1996; 97: 263-264.

Brea A, Mosquera D, Martin E, Arizti A, Cordero JL, Ros E. Nonalcoholic fatty liver disease is associated with carotid atherosclerosis: a case-control study. Arterioscler Thromb Vasc Biol. 2005; 25: 1045-1050.

Brenner B, Lanir N, Thaler I. HELLP syndrome associated with factor V R506Q mutation. Brit J Haemat. 1996; 92: 999-1001.

Breslow JL, Zannis VI, SanGiacomo TR, Third JLHC, Tracy T, Glueck CJ. Studies of familial type III hyperlipoproteinemia using as a genetic marker the apoE phenotype E2/2. J Lipid Res. 1982; 23: 1224-1235.

Bressler BL, Guindi M, Tomlinson G, Heathcote J. High body mass index is an independent risk factor for nonresponse to antiviral treatment in chronic hepatitis C. Hepatology. 2003; 38: 639-644.
Brock JH. Iron in infection, immunity, inflammation and neoplasia. In: Brock JH, Halladay JW, Pippart MJ, Powell LW, eds. Iron metabolism in health and disease. Philadelphia: Saunders, 1994; 353-389.

Broderick J, Lu M, Jackson C, Pancioli A, Tilley BC, Fagan SC, Kothari R, Levine SR, Marler JR, Lyden PD, Haley EC (Jr), Brott T, Grotta JC, the NINDS t-PA Stroke Study Group. Apolipoprotein E phenotype and the efficacy of intravenous tissue plasminogen activator in acute ischemic stroke. Ann Neurol. 2001; 49: 736-744.

Brown MS and Goldstein JL. Lipoprotein receptors in the liver: control signals for plasma cholesterol traffic. J Clin Invest. 1983; 72: 743-747.

Brown MS, Kovanen PT, Goldstein JL. Regulation of plasma cholesterol by lipoprotein receptors. Science. 1981; 212: 628-635.

Brown TA. 2007. Genomes 3. New York: Garland Science Publishing.

Browning JD, Kumar KS, Saboorian MH, Thiele DL. Ethnic differences in the prevalence of cryptogenic cirrhosis. Am J Gastroent. 2004a; 99: 292-298.

Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. Hepatology. 2004b; 40: 1387-1395.

Bucciarelli P, Franchi F, Alatri A, Bettini P, Moia M. Budd-Chiari syndrome in a patient heterozygous for the G20210A mutation o the prothrombin gene. Thromb Haemost. 1998; 79: 445-446.

Bugianesi E, Manzini P, D'Antico S, Vanni E, Longo F, Leone N, Massarenti P, Piga A, Marchesini G, Rizzetto M. Relative contribution of iron burden, HFE mutations, and insulin resistance to fibrosis in nonalcoholic fatty liver. Hepatology. 2004; 39 (1): 179-187.

Buranawuti K, Boyle MP, Cheng S, Steiner LL, McDougal K, Fallin MD, Merlo C, Zeitlin PL, Rosenstein BJ, Mogayzel PJ Jr, Wang X, Cutting GR. Variants in mannose binding lectin and tumour necrosis factor- alpha affect survival in cystic fibrosis. J Med Genet. 2007; 44 (3): 209-214.

Burt TD, Agan BK, Marconi VC, He W, Kulkarni H, Mold JE, Cavrois M, Huang Y, Mahley RW, Dolan MJ, McCune JM, Ahuja SK. Apolipoprotein (apo) E4 enhances HIV-1 cell entry in vitro, and the APOE epsilon-4/epsilon-4 genotype accelerates HIV disease progression. Proc Nat Acad Sci. 2008; 105: 8718-8723.

Busch RM, Lineweaver TT, Naugle RI, Kim KH, Gong Y, Tilelli CQ, Prayson RA, Bingaman W, Najm IM, Diaz-Arrastia R. ApoE-epsilon-4 is associated with reduced memory in long-standing intractable temporal lobe epilepsy. Neurology. 2007; 68: 409-414.

Camaschella C, Roetto A, Cali A, De Gobbi M, Garozza G, Carella M, Majorano N, Totaro A, Gasparini P. The gene TFR2 is mutated in a new type of haemochromatosis mapping to 7q22. Nat Genet. 2000b; 25: 14-15.

Casas JP, Hingorani AD, Bautista LE, Sharma P. Meta-analysis of genetic studies in ischemic stroke: thirty-two genes involving approximately 18000 cases and 58000 controls. Arch Neurol. 2004; 61: 1652-1662.

Caselli RJ, Reiman EM, Osborne D, Hentz JG, Baxter LC, Hernandez JL, Alexander GG. Longitudinal changes in cognition and behavior in asymptomatic carriers of the APOE e4 allele. Neurology. 2004; 62: 1990-1995.

Castaman G, Lunghi B, Missiaglia E, Bernardi F, Rodeghiero F. Phenotypic homozygous activated protein C resistance associated with compound heterozygosity for arg506-to-gln (factor V Leiden) and his1299-to-arg substitutions in factor V. Brit J Haemat. 1997; 99: 257-261.

Castoldi E, Kalafatis M, Lunghi B, Simioni P, Ioannou PA, Petio M, Girolami A, Mann KG, Bernardi F. Molecular bases of pseudo-homozygous APC resistance: the compound heterozygosity for FV R506Q and a FV null mutation results in the exclusive presence of FV Leiden molecules in plasma. Thromb Haemost. 1998; 80: 403-406.

Castro R, Rivera I, Ravasco P, Camilo ME, Jakobs C, Blom HJ, de Almeida IT. 5,10-Methylenetetrahydrofolate reductase (MTHFR) 677C-T and 1298A-C mutations are associated with DNA hypomethylation. J Med Genet. 2004; 41: 454-458.

Cazzola M, Ascari E, Barosi G, Claudiani G, Daccó M, Kaltwasser JP, Panaiotopoulos N, Schalk KP, Werner EE. Juvenile idiopathic haemochromatosis: a life-threatening disorder presenting as hypogonadotropic hypogonadism. Hum Genet. 1983; 65: 149-154.

Cazzola M, Cerani P, Rovati A, Iannone A, Claudiani G, Bergamaschi G. Juvenile genetic hemochromatosis is clinically and genetically distinct from the classical HLA-related disorder. Blood. 1998; 92: 2979-2981.

Celikel R, McClintock RA, Roberts JR, Mendolicchio GL, Ware J, Varughese KI, Ruggeri ZM. Modulation of alpha-thrombin function by distinct interactions with platelet glycoprotein Ib-alpha. Science. 2003; 301: 218-221.

Cemonesi L, Forni GL, Soriani N, Lamagna M, Fermo I, Daraio F, Galli A, Pietra D, Malcovati L, Ferrari M, Camaschella C, Cazzola M. Genetic and clinical heterogeneity of ferroportin disease. Brit J Haemat. 2005; 131: 663-670.

Chan WP, Lee CK, Kwong YL, Lam CK, Liang R. A novel mutation of arg306 of factor V gene in Hong Kong Chinese. Blood. 1998; 91: 1135-1139.

Chang JG, Liu TC, Lin SF. Rapid diagnosis of the HLA-H gene Cys 282 Tyr mutation in hemochromatosis by polymerase chain reaction-A very rare mutation in the Chinese population. Blood. 1997; 89: 3492-3493.

Chang S, Ma T, Miranda RD, Balestra ME, Mahley RW, Huang Y. Lipid- and receptor-binding regions of apolipoprotein E4 fragments act in concert to cause mitochondrial dysfunction and neurotoxicity. Proc Nat Acad Sci. 2005; 102: 18694-18699.

Chapman J, Vinokurov S, Achiron A, Karussis DM, Mitosek-Szewczyk K, Birnbaum M, Michaelson DM, Korczyn AD. APOE genotype is a major predictor of long-term progression of disability in MS. Neurology. 2001; 56: 312-316.

Chen X, Zehnbauer B, Gnirke A, Kwok PY. Fluorescence energy transfer detection as a homogeneous DNA diagnostic method. Proc Natl Acad Sci USA. 1997; 94: 10756-10761.

Cheung O and Sanyal AJ. Recent Advances in Nonalcoholic Fatty Liver Disease. Curr Opin Gastroenterol. 2010; 26 (3): 202-208.

Christensen B, Arbour L, Tran P, Leclerc D, Sabbaghian N, Platt R, Gilfix BM, Rosenblatt DS, Gravel RA, Forbes P, Rozen R. Genetic polymorphisms in methylenetetrahydrofolate reductase and methionine synthase, folate levels in red blood cells, and risk of neural tube defects. Am J Med Genet. 1999; 84: 151-157.

Cladaras C, Hadzopoulou-Cladaras M, Felber BK, Pavlakis G, Zannis VI. The molecular basis of a familial apoE deficiency: an acceptor splice site mutation in the third intron of the deficient apoE gene. J Biol Chem. 1987; 262: 2310-2315.

Clark JM, Alkhuraishi AR, Solga SF, Alli P, Diehl AM, Magnuson TH. Roux-en-Y gastric bypass improves liver histology in patients with nonalcoholic fatty liver disease. Obes Res. 2005; 13: 1180-1186.

Conjeevaram HS, Kleiner DE, Everhart JE, Hoofnagle JH, Zacks S, Afdhal NH, Wahed AS. Race, insulin resistance and hepatic steatosis in chronic hepatitis C. Hepatology. 2007; 45: 80-87.

Conrad ME and Umbreit JN. A concise review: Iron absorption - the mucin-mobilferrin-integrin pathway. A competitive pathway for metal absorption. American Journal of Hematology. 1993; 42, 67.

Copin B, Brezin AP, Valtot F, Dascotte J-C, Bechetoille A, Garchon H-J. Apolipoprotein E-promoter single-nucleotide polymorphisms affect the phenotype of primary open-angle glaucoma and demonstrate interaction with the myocilin gene. Am J Hum Genet. 2002; 70: 1575-1581.

Corbo RM and Scacchi R. Apolipoprotein E (APOE) allele distribution in the world: is APOE*4 a 'thrifty' allele? Ann Hum Genet. 1999; 63: 301-310.

Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science. 1993; 261: 921-923.

Corradini E, Montosi G, Ferrara F, Caleffi A, Pignatti E, Barelli S, Garuti C, Pietrangelo A. Lack of enterocyte iron accumulation in the ferroportin disease. Blood Cells Mol Dis. 2005; 35: 315-318.

Cotrim HP, Freitas LA, Alves E, Almeida A, May DS, Caldwell S. Effects of light-to-moderate alcohol consumption on steatosis and steatohepatitis in severely obese patients. Eur J Gastroenterol Hepatol. 2009; 21 (9): 969-972.

Crawford FC, Vanderploeg RD, Freeman MJ, Singh S, Waisman M, Michaels L, Abdullah L, Warden D, Lipsky R, Salazar A, Mullan MJ. APOE genotype influences acquisition and recall following traumatic brain injury. Neurology. 2002; 58: 1115-1118.

Cripe LD, Moore KD, Kane WH. Structure of the gene for human coagulation factor V. Biochemistry. 1992; 31: 3777-3785.

Cui J, Eitzman DT, Westrick RJ, Christie PD, Xu ZJ, Yang AY, Purkayastha AA, Yang TL, Metz AL, Gallagher KP, Tyson JA, Rosenberg RD, Ginsburg D. Spontaneous thrombosis in mice carrying the factor V Leiden mutation. Blood. 2000; 96: 4222-4226.

Cui J, O'Shea KS, Purkayastha A, Saunders TL, Ginsburg D. Fatal haemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V. Nature. 1996; 384: 66-68.

Das HK, McPherson J, Bruns GAP, Karathanasis SK, Breslow JL. Isolation, characterization, and mapping to chromosome 19 of the human apolipoprotein E gene. J Biol Chem. 1985; 260: 6240-6247.

Davie EW and Kulman JD. An overview of the structure and function of thrombin. Semin Thromb Hemost, 2006; 32 (1): 3-15.

Day CP. From fat to inflammation. Gastroenterology. 2006; 130: 207-210.

Day CP, James OF. Steatohepatitis: a tale of two ‘‘hits’’? Gastroenterology. 1998; 114: 842-845.

de Alwis NMW and Day CP. Non-alcoholic fatty liver disease: the mist gradually clears. J Hepatol. 2008; 48: Suppl 1:S104-S112.

de Bruijn SF, Stam J, Koopman MMW, Vandenbroucke JP. Case-control study of risk of cerebral sinus thrombosis in oral contraceptive users who are carriers of hereditary prothrombotic conditions. Brit Med J. 1998; 316: 589-592.

De Domenico I, Ward DM, Nemeth E, Vaughn MB, Musci G, Ganz T, Kaplan J. The molecular basis of ferroportin-linked hemochromatosis. Proc Natl Acad Sci USA. 2005; 102: 8955-8960.

De Gobbi M, Roetto A, Piperno A, Mariani R, Alberti F, Papanikolaou G, Politou M, Lockitch G, Girelli D, Fargion S, Cox TM, Gasparini P, Cazzola M, Camaschella C. Natural history of juvenile haemochromatosis. Br J Haematol. 2002; 117: 973-979.

de Jager J, Kooy A, Lehert P, Wulffelé MG, van der Kolk J, Bets D, Verburg J, Donker AJ, Stehouwer CD. Long term treatment with metformin in patients with type 2 diabetes and risk of vitamin B-12 deficiency: randomised placebo controlled trial. BMJ. 2010; 340: c2181.

de Knijff P, van den Maagdenberg AMJM, Frants RR, Havekes LM. Genetic heterogeneity of apolipoprotein E and its influence on plasma lipid and lipoprotein levels. Hum Mutat. 1994; 4: 178-194.

De Stefano N, Bartolozzi ML, Nacmias B, Zipoli V, Mortilla M, Guidi L, Siracusa G, Sorbi S, Federico A, Amato MP. Influence of apolipoprotein E epsilon-4 genotype on brain tissue integrity in relapsing-remitting multiple sclerosis. Arch Neurol. 2004; 61: 536-540.

De Stefano V, Martinelli I, Mannucci PM, Paciaroni K, Chiusolo P, Casorelli I, Rossi E, Leone G. The risk of recurrent deep venous thrombosis among heterozygous carriers of both factor V Leiden and the G20210A prothrombin mutation. N Engl J Med. 1999; 341: 801-806.

de Villiers JNP, Hillermann R, de Jong G, Langenhoven E, Rossouw H, Marx MP, Kotze MJ. High prevalence of the Cys282Tyr HFE mutation facilitates an improved diagnostic service for hereditary haemochromatosis in South Africa. S Afri Med J. 1999b; 89: 279-282.

de Villiers JNP, Hillermann R, Loubser L, Kotze MJ. Spectrum of mutations in the HFE gene implicated in haemochromatosis and porphyria. Hum Molec Genet. 1999a; 8: 1517-1522.

Degen SJF and Davie EW. Nucleotide sequence of the gene for human prothrombin. Biochemistry. 1987; 26: 6165-6177.

Doggen CJM, Cats VM, Bertina RM, Rosendaal FR. Interaction of coagulation defects and cardiovascular risk factors: increased risk of myocardial infarction associated with factor V Leiden or prothrombin 20210A. Circulation. 1998; 97: 1037-1041.

Donnelly LA, Palmer CNA, Whitley AL, Lang CC, Doney ASF, Morris AD, Donnan PT. Apolipoprotein E genotypes are associated with lipid-lowering responses to statin treatment in diabetes: a Go-DARTS study. Pharmacogenetics Genomics. 2008; 18: 279-287.

Drakesmith H, Schimanski LM, Ormerod E, Merryweather-Clarke AT, Viprakasit V, Edwards JP, Sweetland E, Bastin JM, Cowley D, Chinthammitr Y, Robson KJ, Townsend AR. Resistance to hepcidin is conferred by hemochromatosis-associated mutations of ferroportin. Blood. 2005; 106: 1092-1097.

Dufouil C, Richard F, Fievet N, Dartigues JF, Ritchie K, Tzourio C, Amouyel P, Alperovitch A. APOE genotype, cholesterol level, lipid-lowering treatment, and dementia: the three-city study. Neurology. 2005; 64: 1531-1538.

Dumas JJ, Kumar R, Seehra J, Somers WS, Mosyak L. Crystal structure of the Gp1b-alpha-thrombin complex essential for platelet aggregation. Science. 2003; 301: 222-226.

Edwards CQ, Griffin LM, Goldgar D, Drummond C, Skolnick MK, Kushner JP. Prevalence of hemochromatosis among 11 065 presumably healthy blood donors. N Engl J Med. 1988; 318: 1355-1362.

Ekstedt M, Franzén LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, Kechagias S. Long-term follow-up of patients with NAFLD and elevated liver enzymes. Hepatology. 2006; 44: 865-873.

Ellsworth DL, Rittenhouse KD, Honeycutt RL. Artificial variation in randomly amplified polymorphic DNA binding patterns. Biotechniques. 1993; 14: 214–217.

Enzinger C, Ropele S, Smith S, Strasser-Fuchs S, Poltrum B, Schmidt H, Matthews PM, Fazekas F. Accelerated evolution of brain atrophy and 'black holes' in MS patients with APOE-epsilon-4. Ann Neurol. 2004; 55: 563-569.

Ersöz G, Günşar F, Karasu Z, Akay S, Batur Y, Akarca US. Management of fatty liver disease with vitamin E and C compared to ursodeoxycholic acid treatment. Turk J Gastroenterol. 2005; 16: 124-128.

Eto M, Watanabe K, Makino I. Increased frequencies of apolipoprotein E2 and E4 alleles in patients with ischemic heart disease. Clin Genet. 1989; 36: 183-188.

European Haemochromatosis Consortium. Polymorphism in intron 4 of HFE does not compromise haemochromatosis mutation results. (Letter) Nat Genet. 1999; 23: 271.

Faisel F, Romppanen E-L, Hiltunen M, Helisalmi S, Laasanen J, Punnonen K, Salonen JT, Heinonen S. Susceptibility to pre-eclampsia in Finnish women is associated with R485K polymorphism in the factor V gene, not with Leiden mutation. Europ J Hum Genet. 2004; 12: 187-191.

Fargion S, Mattioli M, Fracanzani AL, Sampietro M, Tavazzi D, Fociani P, Taioli E, Valenti L, Fiorelli G. Hyperferritinemia, iron overload, and multiple metabolic alterations identify patients at risk for nonalcoholic steatohepatitis. Am J Gastroenterol. 2001; 96 (8): 2448-2455.

Farlow MR, He Y, Tekin S, Xu J, Lane R, Charles HC. Impact of APOE in mild cognitive impairment. Neurology. 2004; 63: 1898-1901.

Farrer LA, Abraham CR, Volicer L, Foley EJ, Kowall NW, McKee AC, Wells JM. Allele epsilon-4 of apolipoprotein E shows a dose effect on age at onset of Pick disease. Exp Neurol. 1995; 136: 162-170.

Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, Dormishian F, Domingo Jr. R, Ellis MC, Fullan A, Hinton LM, Jones NL, Kimmel BE, Kronmal GS, Lauer P, Lee VK, Loeb DB, Mapa FA, McClelland E, Meyer NC, Mintier GA, Moeller N, Moore T, Morikang E, Prass CE, Quintana L, Starnes RC, Schatzman RC, Brunke KJ, Drayna DT, Risch NJ, Bacon BR, Wolff RK. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nat Genet. 1996; 13: 399-408.

Feder JN, Penny DM, Irrinki A, Lee VK, Lebron JA, Watson N, Tsuchihashi Z, Sigal E, Bjorkman PJ, Schatzman RC. The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding. Proc Nat Acad Sci USA. 1998; 95: 1472-1477.

Feussner G, Dobmeyer J, Nissen H, Hansen TS. Unusual Xanthomas in a young patient with heterozygous familial hypercholesterolemia and type III hyperlipoproteinemia. Am J Med Genet. 1996; 65: 149-154.

Fleming RE, Migas MC, Holden CC, Waheed A, Britton RS, Tomatsu S, Bacon BR, Sly WS. Transferrin receptor 2: continued expression in mouse liver in the face of iron overload and in hereditary hemochromatosis. Proc Nat Acad Sci USA. 2000; 97: 2214-2219.

Fracanzani AL, Burdick L, Raselli S, Pedotti P, Grigore L, Santorelli G, Valenti L, Maraschi A, Catapano A, Fargion S. Carotid artery intima-media thickness in nonalcoholic fatty liver disease. Am J Med. 2008; 121: 72-78.

Franco RF, Trip MD, ten Cate H, van den Ende A, Prins MH, Kastelein JJP, Reitsma PH. The 20210G-A mutation in the 3-prime-untranslated region of the prothrombin gene and the risk for arterial thrombotic disease. Brit J Haemat. 1999; 104: 50-54.

Friedman G, Froom P, Sazbon L, Grinblatt I, Shochina M, Tsenter J, Babaey S, Yehuda AB, Groswasser Z. Apolipoprotein E-epsilon-4 genotype predicts a poor outcome in survivors of traumatic brain injury. Neurology. 1999; 52: 244-248.

Friedrich-Rust M, Müller C, Winckler A, Kriener S, Herrmann E, Holtmeier J, Poynard T, Vogl TJ, Zeuzem S, Hammerstingl R, Sarrazin C. Assessment of liver fibrosis and steatosis in PBC with FibroScan, MRI, MR-spectroscopy, and serum markers. J Clin Gastroenterol. 2010; 44 (1): 58-65.

Frikke-Schmidt R, Nordestgaard BG, Thudium D, Moes Gronholdt M-L, Tybjaerg-Hansen A. APOE genotype predicts AD and other dementia but not ischemic cerebrovascular disease. Neurology. 2001; 56: 194-200.

Frikke-Schmidt R, Sing CF, Nordestgaard BG, Steffensen R, Tybjaerg-Hansen A. Subsets of SNPs define rare genotype classes that predict ischemic heart disease. Hum Genet. 2007; 120: 865-877.

Friso S, Choi S-W, Girelli D, Mason JB, Dolnikowski GG, Bagley PJ, Olivieri O, Jacques PF, Rosenberg IH, Corrocher R, Selhub J. A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. Proc Nat Acad Sci. 2002; 99: 5606-5611.

Frisoni GB, Geroldi C, Bianchetti A, Trabucchi M, Govoni S, Franceschini G, Calabresi L. Apolipoprotein E epsilon-4 allele frequency in vascular dementia and Alzheimer's disease. Stroke. 1994; 25: 1703.

Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJH, den Heijer M, Kluijtmans LAJ, van den Heuvel LP, Rozen R. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet. 1995; 10: 111-113.

Gao J, Chen J, Kramer M, Tsukamoto H, Zhang AS, Enns CA. Interaction of the hereditary hemochromatosis protein HFE with transferrin receptor 2 is required for transferrin-induced hepcidin expression. Cell Metab. 2009; 9 (3): 217-227.

Garces C, Benavente M, Ortega H, Rubio R, Lasuncion MA, Artalejo FR, Pardo JF, De Oya M. Influence of birth weight on the apo E genetic determinants of plasma lipid levels in children. Pediat Res. 2002; 52: 873-878.

Garlepp MJ, Tabarias H, van Bockxmeer FM, Zilko PJ, Laing B, Mastaglia FL. Apolipoprotein E epsilon-4 in inclusion body myositis. Ann Neurol. 1995; 38: 957-959.

Gaspar DA, Matioli SR, de Cassia Pavanello R, Araujo BC, Alonso N, Wyszynski D, Passos-Bueno MR. Maternal MTHFR interacts with the offspring's BCL3 genotypes, but not with TGFA, in increasing risk to nonsyndromic cleft lip with or without cleft palate. Europ J Hum Genet. 2004; 12: 521-526.

Gaughan DJ, Barbaux S, Kluijtmans LA, Whitehead AS. The human and mouse methylenetetrahydrofolate reductase (MTHFR) genes: genomic organization, mRNA structure and linkage to the CLCN6 gene. Gene. 2000; 257: 279-289.

Gehring NH, Frede U, Neu-Yilik G, Hundsdoerfer P, Vetter B, Hentze MW, Kulozik AE. Increased efficiency of mRNA 3-prime end formation: a new genetic mechanism contributing to hereditary thrombophilia. Nat Genet. 2001; 28: 389-392.

Gehrke SG, Peitrangelo A, Kascak M, Braner A, Eisold M, Kulaksiz H, Herrmann T, Hebling U, Bents K, Gugler R, Stremmel W. HJV gene mutations in European patients with juvenile hemochromatosis. Clin Genet. 2005; 67: 425-428.

Genin E, Hannequin D, Wallon D, Sleegers K, Hiltunen M, Combarros O, Bullido MJ, Engelborghs S, De Deyn P, Berr C, Pasquier F, Dubois B, Tognoni G, Fiévet N, Brouwers N, Bettens K, Arosio B, Coto E, Del Zompo M, Mateo I, Epelbaum J, Frank-Garcia A, Helisalmi S, Porcellini E, Pilotto A, Forti P, Ferri R, Scarpini E, Siciliano G, Solfrizzi V, Sorbi S, Spalletta G, Valdivieso F, Vepsäläinen S, Alvarez V, Bosco P, Mancuso M, Panza F, Nacmias B, Bossù P, Hanon O, Piccardi P, Annoni G, Seripa D, Galimberti D, Licastro F, Soininen H, Dartigues JF, Kamboh MI, Van Broeckhoven C, Lambert JC, Amouyel P, Campion D. APOE and Alzheimer disease: a major gene with semi-dominant inheritance. Mol Psychiatry. 2011; 16 (9): 903-907.

George DK, Goldwurm S, MacDonald GA, Cowley LL, Walker NI, Ward PJ, Jazwinska EC, Powell LW. Increased hepatic iron concentration in nonalcoholic steatohepatitis is associated with increased fibrosis. Gastroenterology. 1998; 114 (2): 311-318.

Gerdes LU, Jeune B, Ranberg KA, Nybo H, Vaupel JW. Estimation of apolipoprotein E genotype-specific relative mortality risks from the distribution of genotypes in centenarians and middle-aged men: apolipoprotein E gene is a 'frailty gene,' not a 'longevity gene'. Genet Epidemiol. 2000; 19: 202-210.

Gerhardt A, Scharf RE, Beckmann MW, Struve S, Bender HG, Pillny M, Sandmann W, Zotz RB. Prothrombin and factor V mutations in women with a history of thrombosis during pregnancy and the puerperium. N Engl J Med. 2000; 342: 374-380.

Ghaffar O, Reis M, Pennell N, O'Connor P, Feinstein A. APOE epsilon-4 and the cognitive genetics of multiple sclerosis. Neurology. 2010; 74: 1611-1618.

Ghiselli G, Schaefer EJ, Gascon P, Brewer HB (Jr). Type III hyperlipoproteinemia associated with apolipoprotein E deficiency. Science. 1981; 214: 1239-1241.

Gill LL, Peoples OP, Pearston DH, Robertson FW, Humphries SE, Cumming AM, Hardman N. Isolation and characterization of a variant allele of the gene for human apolipoprotein E. Biochem Biophys Res Commun. 1985; 130: 1261-1266.

Girelli D, Bozzini C, Roetto A, Alberti F, Daraio F, Colombari R, Olivieri O, Corrocher R, Camaschella C. Clinical and pathologic findings in hemochromatosis type 3 due to a novel mutation in transferrin receptor 2 gene. Gastroenterology. 2002; 122: 1295-1302.

Girolami A, Scarano L, Saggiorato G, Girolami B, Bertomoro A, Marchiori A. Congenital deficiencies and abnormalities of prothrombin. Blood Coagul Fibrinolysis. 1998; 9: 557-569.

Goland S, Shimoni S, Zornitzki T, Knobler H, Azoulai O, Lutaty G, Melzer E, Orr A, Caspi A, Malnick S. Cardiac abnormalities as a new manifestation of nonalcoholic fatty liver disease: echocardiographic and tissue Doppler imaging assessment. J Clin Gastroenterol. 2006; 40: 949-955.

Goodnight S, Hathaway W. Disorders of Hemostasis and Thrombosis – A Clinical Guide, 2nd edn. New York: The McGraw-Hill Companies, 2001.

Gottlieb DJ, DeStefano AL, Foley DJ, Mignot E, Redline S, Givelber RJ, Young T. APOE epsilon-4 is associated with obstructive sleep apnea/hypopnea: the sleep heart health study. Neurology. 2004; 63: 664-668.

Goyette P, Pai A, Milos R, Frosst P, Tran P, Chen Z, Chan M, Rozen R. Gene structure of human and mouse methylenetetrahydrofolate reductase (MTHFR). Mammalian Genome. 1998; 9: 652-656.

Goyette P, Sumner JS, Milos R, Duncan AMV, Rosenblatt DS, Matthews RG, Rozen R. Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification. Nat Genet. 1994; 7: 195-200.

Gozal D, Capdevila OS, Kheirandish-Gozal L, Crabtree VM. APOE epsilon-4 allele, cognitive dysfunction, and obstructive sleep apnea in children. Neurology. 2007; 69: 243-249.

Greenberg SM, Rebeck GW, Vonsattel JPG, Gomez-Isla T, Hyman BT. Apolipoprotein E epsilon-4 and cerebral hemorrhage associated with amyloid angiopathy. Ann Neurol. 1995; 38: 254-259.

Gregg JP, Yamane AJ, Grody WW. Prevalence of the factor V-Leiden mutation in four distinct American ethnic populations. Am J Med Genet. 1997; 73: 334-336.

Gregg RE, Zech LA, Schaefer EJ, Brewer HB (Jr). Type III hyperlipoproteinemia: defective metabolism of an abnormal apolipoprotein E. Science. 1981; 211: 584-586.

Gross CN, Irrinki A, Feder JN, Enns CA. Co-trafficking of HFE, a nonclassical major histocompatibility complex class I protein, with the transferrin recerptor implies a role in intracellular iron regulation. J Biol Chem. 1998; 273: 22068-22074.

Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SC, Spertus JA, Costa F. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Circulation. 2005; 112: 2735-2752.

Guasch JF, Cannegieter S, Reitsma PH, van't Veer-Korthof ET, Bertina RM. Severe coagulation factor V deficiency caused by a 4 bp deletion in the factor V gene. Brit J Haemat. 1998; 101: 32-39.

Gurakan F, Gurgey A, Bakkaloglu A, Kocak N. Homozygous factor V Leiden mutation in a child with Budd-Chiari syndrome. J Pediat Gastroent Nutr. 1999; 28: 516-517.

Guttormsen AB, Ueland PM, Nesthus I, Nygard O, Schneede J, Vollset SE, Refsum H. Determinants and vitamin responsiveness of intermediate hyperhomocysteinemia (equal to or greater than 40 micromole/liter): the Hordaland homocysteine study. J Clin Invest. 1996; 98: 2174-2183.

Hamaguchi M, Kojima T, Takeda N, Nakagawa T, Taniguchi H, Fujii K, Omatsu T, Nakajima T, Sarui H, Shimazaki M, Kato T, Okuda J, Ida K. The metabolic syndrome as a predictor of nonalcoholic fatty liver disease. Ann Intern Med. 2005; 143: 722-728.

Hao K, Wang X, Niu T, Xu X, Li A, Chang W, Wang L, Li G, Laird N, Xu X. A candidate gene association study on preterm delivery: application of high-throughput genotyping technology and advanced statistical methods. Hum Molec Genet. 2004; 13: 683-691.

Harrington CR, Roth M, Xuereb JH, McKenna PJ, Wischik CM. Apolipoprotein E type epsilon-4 allele frequency is increased in patients with schizophrenia. Neurosci Lett. 1995; 202: 101-104.

Harrison SA, Fecht W, Brunt EM, Neuschwander-Tetri BA. Orlistat for overweight subjects with nonalcoholic steatohepatitis: A randomized, prospective trial. Hepatology. 2009; 49 (1): 80-86.

Harrison SA, Fincke C, Helinski D, Torgerson S, Hayashi P. A pilot study of orlistat treatment in obese, nonalcoholic steatohepatitis patients. Ailment Pharmacol Ther. 2004; 20: 623-628.

Harrison SA, Torgerson S, Hayashi P, Ward J, Schenker S. Vitamin E and vitamin C treatment improves fibrosis in patients with nonalcoholic steatohepatitis. Am J Gastroenterol. 2003; 98: 2485-2490.

Harrison-Findik DD. Is the iron regulatory hormone hepcidin a risk factor for alcoholic liver disease? World J Gastroenterol. 2009; 14: 1186-1193.

Hasegawa T, Yoneda M, Nakamura K, Makino I, Terano A. Plasma transforming growth factor-beta1 level and efficacy of alpha-tocopherol in patients with nonalcoholic steatohepatitis: a pilot study. Ailment Pharmacol Ther. 2001; 15: 1667-1672.

Hattori A, Wakusawa S, Hayashi H, Harashima A, Sanae F, Kawanaka M, Tamada G, Yano M, Yoshioka K. AVAQ 594-597 deletion of the TfR2 gene in ajapanese family with hemochromatosis. Hepatol Res. 2003; 26: 154-156.

Havel RJ, Kotite L, Kane JP, Tun P, Bersot T. Atypical familial dysbetalipoproteinemia associated with apolipoprotein phenotype E3/3. J Clin Invest. 1983; 72: 379-387.

Hazzard WR, Warnick GR, Utermann G, Albers JJ. Genetic transmission of isoapolipoprotein E phenotypes in a large kindred: relationship to dysbetalipoproteinemia and hyperlipidemia. Metabolism. 1981; 30: 79-88.

Heijmans BT, Gussekloo J, Kluft C, Droog S, Lagaay AM, Knook DL, Westendorp RGJ, Slagboom EP. Mortality risk in men is associated with a common mutation in the methylene-tetrahydrofolate reductase gene (MTHFR). Europ J Hum Genet. 1999; 7: 197-204.

Hetet G, Devaux I, Soufir N, Grandchamp B, Beaumont C. Molecular analyses of patients with hyperferritinemia and normal serum iron values reveal both L ferritin IRE and 3 new ferroportin (slc11A3) mutations. Blood. 2003; 102: 1904-1910.

Hiyoshi M. Assay of DNA denaturation by polymerase chain reaction-driven fluorescent label incorporation and fluorescence resonance energy transfer. Anal Biochem. 1994; 221: 306-311.

Hobbs CA, Cleves MA, Lauer RM, Burns TL, James SJ. Preferential transmission of the MTHFR 677T allele to infants with Down syndrome: implications for a survival advantage. Am J Med Genet. 2002; 113: 9-14.

Hobbs CA, James SJ, Parsian A, Krakowiak PA, Jernigan S, Greenhaw JJ, Lu Y, Cleves MA. Congenital heart defects and genetic variants in the methylenetetrahydrofolate reductase gene. J Med Genet. 2006; 43: 162-166.

Hobbs CA, Sherman SL, Yi P, Hopkins SE, Torfs CP, Hine RJ, Pogribna M, Rozen R, James SJ. Polymorphisms in genes involved in folate metabolism as maternal risk factors for Down syndrome. Am J Hum Genet. 2000; 67: 623-630.

Hoffmaster AR, Meyer RF, Bowen MP, Marston CK, Weyant RS, Thurman K, Messenger SL, Minor EE, Winchell JM, Rasmussen MV, Newton BR, Parker JT, Morrill WE, McKinney N, Barnett GA, Sejvar JJ, Jernigan JA, Perkins BA, Popovic T. Evaluation and validation of a real-time polymerase chain reaction assay for rapid identification of Bacillus anthracis. Emerg Infect Dis. 2002; 8: 1179-1182.

Hofmann W-K, Tong X-J, Ajioka RS, Kushner JP, Koeffler HP. Mutation analysis of transferrin-receptor 2 in patients with atypical hemochromatosis. Blood. 2002; 100: 1099-1100.

Holtzman DM, Bales KR, Wu S, Bhat P, Parsadanian M, Fagan AM, Chang LK, Sun Y, Paul SM. Expression of human apolipoprotein E reduces amyloid-beta deposition in a mouse model of Alzheimer's disease. J Clin Invest. 1999; 103: R15-R21.

Horie Y, Fazio S, Westerlund JR, Weisgraber KH, Rall SC (Jr). The functional characteristics of a human apolipoprotein E variant (cysteine at residue 142) may explain its association with dominant expression of type III hyperlipoproteinemia. J Biol Chem. 1992; 267: 1962-1968.

Hubner RA, Lubbe S, Chandler I, Houlston RS. MTHFR C677T has differential influence on risk of MSI and MSS colorectal cancer. Hum Molec Genet. 2007; 16: 1072-1077.

Hurrell RF, Reddy M, Cook JD. Inhibition of non-haem iron absorption in man by polyphenolic-containing beverages. British Journal of Nutrition. 1999; 81: 289-295.

Hustad S, Midttun O, Schneede J, Vollset SE, Grotmol T, Ueland PM. The methylenetetra-hydrofolate reductase 677C-T polymorphism as a modulator of a B vitamin network with major effects on homocysteine metabolism. Am J Hum Genet. 2007; 80: 846-855.

Ikutu K, Fujimoto Y, Suzuki Y, Tanaka K, Saito H, Ohhiro M, Sasaki K, Kohgo Y. Overexpression of hemochromatosis protein, HFE, alters transferrin recycling process in human hepatoma cells. Biochem Biophys Acta. 2000; 1496: 221-231.

Infante-Rivard C, Levy E, Rivard G-E, Guiguet M, Feoli-Fonseca J-C. Small babies receive the cardiovascular protective apolipoprotein epsilon-2 allele less frequently than expected. J Med Genet. 2003; 40: 626-629.

Isotalo PA, Wells GA, Donnelly JG. Neonatal and fetal methylenetetrahydrofolate reductase genetic polymorphisms: an examination of C677T and A1298C mutations. Am J Hum Genet. 2000; 67: 986-990.

Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, Selhub J, Rozen R. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. Circulation. 1996; 93: 7-9.

Jalali Sefid Dashti M. 2010. Development of a Pathology-Supported Genetic Test for Improved Clinical Management of Patients Diagnosed with Multiple Sclerosis [dissertation]. [Stellenbosch (Western Cape)]: Stellenbosch University.

Jeffrey GP, Chakrabarti S, Hegele RA, Adams PC. Polymorphism in intron 4 of HFE may cause overestimation of C282Y homozygote prevalence in haemochromatosis. (Letter) Nat Genet. 1999; 22: 325-326.

Jenny RJ, Pittman DD, Toole JJ, Kriz RW, Aldape RA, Hewick RM, Kaufman RJ, Mann KG. Complete cDNA and derived amino acid sequence of human factor V. Proc Nat Acad Sci. 1987; 84: 4846-4850.

Jimba S, Nakagami T, Takahashi M, Wakamatsu T, Hirota Y, Iwamoto Y, Wasada T. Prevalence of nonalcoholic fatty liver disease and its association with impaired glucose metabolism in Japanese adults. Diabet Med. 2005; 22: 1141-1145.

Junemann AGM, von Ahsen N, Reulbach U, Roedl J, Bonsch D, Kornhuber J, Kruse FE, Bleich S. C677T variant in the methylentetrahydrofolate (sic) reductase gene is a genetic risk factor for primary open-angle glaucoma. Am J Ophthal. 2005; 139: 721-723.

Juul K, Tybjaerg-Hansen A, Schnohr P, Nordestgaard BG. Factor V Leiden and the risk for venous thromboembolism in the adult Danish population. Ann Intern Med. 2004; 140: 330-337.

Kadotani H, Kadotani T, Young T, Peppard PE, Finn L, Colrain IM, Murphy GM (Jr), Mignot E. Association between apolipoprotein E epsilon-4 and sleep-disordered breathing in adults. JAMA. 2001; 285: 2888-2890.

Kane WH and Davie EW. Cloning of a cDNA coding for human factor V, a blood coagulation factor homologous to factor VIII and ceruloplasmin. Proc Nat Acad Sci. 1986; 83: 6800-6804.

Karimi M, Yavarian M, Delbini P, Harteveld CL, Farjadian S, Fiorelli G, Giordano PC. Spectrum and haplotypes of the HFE hemochromatosis gene in Iran: H63D in beta-thalassemia major and the first E277K homozygous. Hematol J. 2004; 5: 524-527.

Kathiresan S, Melander O, Anevski D, Guiducci C, Burtt NP, Roos C, Hirschhorn JN, Berglund G, Hedblad B, Groop L, Altshuler DM, Newton-Cheh C, Orho-Melander M. Polymorphisms associated with cholesterol and risk of cardiovascular events. N Engl J Med. 2008; 358: 1240-1249.

Kato H, Kanematsu M, Zhang X, Saio M, Kondo H, Goshima S, Fujita H. Computer-aided diagnosis of hepatic fibrosis: preliminary evaluation of MRI texture analysis using the finite difference method and an artificial neural network. AJR Am J Roentgenol. 2007; 189 (1): 117-122.

Kawabata H, Yang R, Hirama T, Vuong PT, Kawano S, Gombart AF, Koeffler HP. Molecular cloning of transferrin receptor 2: a new member of the transferrin receptor-like family. J Biol Chem. 1999; 274: 20826-20832.

Kawaguchi T, Ide T, Taniguchi E, Hirano E, Itou M, Sumie S, Nagao Y, Yanagimoto C, Hanada S, Koga H, Sata M. Clearance of HCV improves insulin resistance, beta-cell function, and hepatic expression of insulin receptor substrate 1 and 2. Am J Gastroenterol. 2007; 102: 570-576.

Kehoe P, Krawczak M, Harper PS, Owen MJ, Jones AL. Age of onset in Huntington disease: sex specific influence of apolipoprotein E genotype and normal CAG repeat length. J Med Genet. 1999; 36: 108-111.

Keijzer MB, den Heijer M, Blom HJ, Bos GM, Willems HP, Gerrits WB, Rosendaal FR. Interaction between hyperhomocysteinemia, mutated methylenetetrahydrofolatereductase (MTHFR) and inherited thrombophilic factors in recurrent venous thrombosis. Thromb Hemost. 2002; 88: 723-728.

Kelly PJ, Rosand J, Kistler JP, Shih VE, Silveira S, Plomaritoglou A, Furie KL. Homocysteine, MTHFR 677C-T polymorphism, and risk of ischemic stroke: results of a meta-analysis. Neurology. 2002; 59: 529-536.

Kemna EH, Tjalsma H, Willems HL, Swinkels DW. Hepcidin: from discovery to differential diagnosis. Haematologica. 2008; 93 (1): 90-97.

Kiechl S, Lorenz E, Reindl M, Wiedermann CJ, Oberhollenzer F, Bonora E, Willeit J, Schwartz DA. Toll-like receptor 4 polymorphisms and atherogenesis. N Engl J Med. 2002; 347: 185- 192.
Kim HC, Kim D, Huh KB. Association between nonalcoholic fatty liver disease and carotid intima-media thickness according to the presence of metabolic syndrome. Atherosclerosis. 2009; 204: 521-525.

Klerk M, Verhoef P, Clarke R, Blom HJ, Kok FJ, Schouten EG, MTHFR Studies Collaboration Group. MTHFR 677C-T polymorphism and risk of coronary heart disease: a meta-analysis. JAMA. 2002; 288: 2023-2031.

Kluijtmans LA, van den Heuvel LP, Boers GH, Frosst P, Stevens EM, van Oost BA, den Heijer M, Trijbels FJ, Rozen R, Blom HJ. Molecular genetic analysis in mild hyperhomocysteinemia: a common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. Am J Hum Genet. 1996; 58: 35-41.

Kluijtmans LA, Wendel U, Stevens EM, van den Heuvel LP, Trijbels FJ, Blom HJ. Identification of four novel mutations in severe methylenetetrahydrofolate reductase deficiency. Europ J Hum Genet. 1998; 6: 257-265.

Kobashi G, Yamada H, Asano T, Nagano S, Hata A, Kishi R, Fujimoto S, Kondo K. Absence of association between a common mutation in the methylenetetrahydrofolate reductase gene and preeclampsia in Japanese women. Am J Med Genet. 2000; 93: 122-125.

Koeleman BPC, Reitsma PH, Allaart CF, Bertina RM. Activated protein C resistance as an additional risk factor for thrombosis in protein C-deficient families. Blood. 1994; 84: 1031-1035.

Koponen S, Taiminen T, Kairisto V, Portin R, Isoniemi H, Hinkka S, Tenovuo O. APOE-epsilon-4 predicts dementia but not other psychiatric disorders after traumatic brain injury. Neurology. 2004; 63: 749-750.

Kotronen A and Yki-Järvinen H. Fatty liver: a novel component of the metabolic syndrome. Arterioscler Thromb Vasc Biol. 2008; 28: 27-38.

Kotze MJ and Badenhorst CH. Chronic disease risk management: Combining genetic testing with medical and nutrition therapy. SA Fam Pract. 2005; 47 (4): 40-42.

Kotze MJ, de Villiers JNP, Bouwens CSH, Warnich L, Zaahl MG, van der Merwe S, Oberkanins C. Molecular diagnosis of hereditary hemochromatosis: application of a newly developed reverse-hybridization assay in the South African population. Clin Genet. 2004a; 565: 317-321.

Kotze MJ, de Villiers JNP, van der Merwe SJ. Preventing organ damage by genetic testing for hereditary haemochromatosis. SA Fam Pract. 2005; 47 (2): 44-45.

Kotze MJ, de Villiers WJ, Steyn K, Kriek JA, Marais AD, Langenhoven E, JS Herbert, Graadt van Roggen JF, van der Westhuyzen DR, Coetzee GA. Phenotypic Variation Among Familial Hypercholesterolemics Heterozygous for Either One of Two Afrikaner Founder LDL Receptor Mutations. Arterioscler Thromb. 1993; 13: 1460-1468.

Kotze MJ, Langenhoven E, Warnich L, du Plessis L, Retief AE. The molecular basis and diagnosis of familial hypercholesterolaemia in South African Afrikaners. Ann Hum Genet. 1991; 55 (2): 115-121.

Kotze MJ, Schorn D, Coetzer P. The impact of genetic testing on life insurance. J Genomics Afr Soc. 2004b; 1. [online article: www.africagenome.co.za/publications/genomics].

Kotze MJ and Thiart R. Genetics of dyslipidaemia. CME Journal. 2003; 21: 399-402.

Kotze MJ, Thiart R, de Villiers JNP, Scholtz CL, Joffe Y. Cardiovascular genetics: practical applications and gene-based intervention. Cardiovasc J S Afr. 2003; 14: 269-270.

Kotze M, Van Velden D, Kidd M, Marnewick J. 2011. Genotype associations in South African patients with the metabolic syndrome [abstract – P136]. In: Joint International Conference of the African and Southern African Societies of Human Genetics Conference Book; 2011 March 6-9; Cape Town. Joint International Conference of the African and Southern African Societies of Human Genetics.

Kotze MJ, van Velden DP, van Rensburg SJ, Erasmus R. Pathogenic mechanisms underlying iron deficiency and iron overload: New insights for clinical application. eJIFCC. 2009; 20 (2). [online article: http://www.ifcc.org/PDF/publications/eJIFCC/vol20/02/eJIFCC-02-01.pdf]

Kowa H, Yasui K, Takeshima T, Urakami K, Sakai F, Nakashima K. The homozygous C677T mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for migraine. Am J Med Genet. 2000; 96: 762-764.

Krause A, Neitz S, Mägert HJ, Schulz A, Forssmann WG, Schulz-Knappe P, Adermann K. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett. 2000; 480: 147–150.

Kroh HK, Panizzi P, Bock PE. Von Willebrand factor-binding protein is a hysteretic conformational activator of prothrombin. Proc Nat Acad Sci. 2009; 106: 7786-7791.

Kruger FC. 2008. Analysis of candidate genes implicated in insulin resistance and fatty acid oxidation in South African patients with non-alcoholic fatty liver disease (NAFLD) [dissertation]. [Stellenbosch (Western Cape)]: Stellenbosch University.

Kruger FC, Daniels C, Kidd M, Swart G, Brundyn K, van Rensburg C, Kotze MJ. A descriptive analysis of non-alcoholic fatty liver disease (NAFLD) in the Western Cape. S Afr Med J. 2010; 100: 168-171.

Kruger FC, Daniels CR, Kidd M, Swart G, Brundyn K, van Rensburg C, Kotze M. APRI: a simple bedside marker for advanced fibrosis that can avoid liver biopsy in patients with NAFLD/NASH. S Afr Med J. 2011; 101 (7): 477-480.

Kugelmas M, Hill DB, Vivian B, Marsano L, McClain CJ. Cytokines and NASH: A pilot study of the effects of lifestyle modification and vitamin E. Hepatology. 2003; 38: 413-419.

Kumar J, Das SK, Sharma P, Karthikeyan G, Ramakrishnan L, Sengupta S. Homocysteine levels are associated with MTHFR A1298C polymorphism in Indian population. J Hum Genet. 2005; 50: 655-663.

Kushwaha RS, Hazzard WR, Gagne C, Chait A, Albers JJ. Type III hyperlipoproteinemia: paradoxical hypolipidemic response to estrogen. Ann Intern Med. 1977; 87: 517-525.

Kvittingen EA, Spangen S, Lindemans J, Fowler B. Methionine synthase deficiency without megaloblastic anaemia. Europ J Pediat. 1997; 156: 925-930.

Lamon JM, Marynick SP, Roseblatt R, Donnelly S. Idiopathic hemochromatosis in a young female: a case study and review of the syndrome in young people. Gastroenterology. 1979; 76: 178-183.

Lancellotti S and De Cristofaro R. Congenital prothrombin deficiency. Semin Thromb Hemost. 2009; 35: 367-381.

Lanterna LA, Ruigrok Y, Alexander S, Tang J, Biroli F, Dunn LT, Poon WS. Meta-analysis of APOE genotype and subarachnoid hemorrhage: clinical outcome and delayed ischemia. Neurology. 2007; 69: 766-775.

Lautamäki R, Borra R, Iozzo P, Komu M, Lehtimäki T, Salmi M, Jalkanen S, Airaksinen KE, Knuuti J, Parkkola R, Nuutila P. Liver steatosis coexists with myocardial insulin resistance and coronary dysfunction in patients with type 2 diabetes. Am J Physiol Endocrinol Metab. 2006; 291: 282-290.

Lebron JA, Bennett MJ, Vaughn DE, Chirino AJ, Snow PM, Mintier GA, Feder JN, Bjorkman PJ. Crystal structure of the hemochromatosis protein HFE and characterization of its interaction with transferrin receptor. Cell. 1998; 93: 111-123.

Le Gac G, Dupradeau F-Y, Mura C, Jacolot S, Scotet V, Esnault G, Mercier A-Y, Rochette J, Ferec C. Phenotypic expression of the C282Y/Q283P compound heterozygosity in HFE and molecular modeling of the Q283P mutation effect. Blood Cells Molec Dis. 2003; 30: 231-237.

Lee D, Jeong SH, Lee M, Cho YA, Kim JW, Park Y, Hwang JH, Kim NY, Lee DH. The Prevalence of Peripheral Iron Overload and the Presence of HFE gene (H63D) Mutation among the Korean Patients with Nonalcoholic Fatty Liver Disease. Korean J Hepatol. 2007; 13 (2): 174-184.

Lee LG, Connell CR, Bloch W. Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res. 1993; 11: 3761-3766.

Lee PL, Barton JC, Brandhagen D, Beutler E. Hemojuvelin (HJV) mutations in persons of European, African-American and Asian ancestry with adult onset haemochromatosis. Brit J Haemat. 2004a; 127: 224-229.

Lee PL and Beutler E. Regulation of hepcidin and iron-overload disease. Annu Rev Pathol. 2009; 4: 489-515.

Lee PL, Beutler E, Rao SV, Barton JC. Genetic abnormalities and juvenile hemochromatosis: mutations of the HJV gene encoding hemojuvelin. Blood. 2004b; 103: 4669-4671.

Lee S-Y, Kim H-Y, Park KM, Lee SYG, Hong SG, Kim H-J, Yang DH. MTHFR C677T Polymorphism as a Risk Factor for Vascular Calcification in Chronic Hemodialysis Patients. J Korean Med Sci. 2011; 26 (3): 461–465.

Leebeek FWG, Lameris JS, van Buuren HR, Gomez E, Madretsma S, Sonneveld P. Budd-Chiari syndrome, portal vein and mesenteric vein thrombosis in a patient homozygous for factor V Leiden mutation treated by TIPS and thrombolysis. Brit J Haemat. 1998; 102: 929-931.

Leone PE, Gimenez P, Collantes JC, Paz-Y-Mino C. Analysis of HFE gene mutations (C282Y, H63D, and S65C) in the Ecuadorian population. Ann Hematol. 2005; 84: 103-105.

Levy RI, Morganroth J. Familial type III hyperlipoproteinemia. Ann Intern Med. 1977; 87: 625-628.

Lewis GF, Carpentier A, Adeli K, Giacca A. Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. Endocr Rev. 2002; 23: 201-229.

Lewis SJ, Lawlor DA, Smith GD, Araya R, Timpson N, Day INM, Ebrahim S. The thermolabile variant of MTHFR is associated with depression in the British Women's Heart and Health Study and a meta-analysis. Molec Psychiat. 2006; 11: 352-360.

Lewis SJ, Zammit S, Gunnell D, Smith GD. A meta-analysis of the MTHFR C677T polymorphism and schizophrenia risk. Am J Med Genet. 2005; 135B: 2-4.

Li X-M, Wei Y-F, Hao H-L, Hao Y-B, He L-S, Li J-D, Mei B, Wang S-Y, Wang C, Wang J-X, Zhu J-Z, Liang J-Q. Hyperhomocysteinemia and the MTHFR C677T mutation in Budd-Chiari syndrome. Am J Hemat. 2002; 71: 11-14.

Liberman JN, Stewart WF, Wesnes K, Troncoso J. Apolipoprotein E epsilon-4 and short-term recovery from predominantly mild brain injury. Neurology. 2002; 58: 1038-1044.

Lin L, Goldberg YP, Ganz T. Competitive regulation of hepcidin mRNA by soluble and cell-associated hemojuvelin. Blood. 2005; 106: 2884–2889.

Lin YC, Lo HM, Chen JD. Sonographic fatty liver, overweight and ischemic heart disease. World J Gastroenterol. 2005; 11: 4838-4842.

Lindqvist PG, Svensson PJ, Dahlback B, Marsal K. Factor V Q-506 mutation (activated protein C resistance) associated with reduced intrapartum blood loss: a possible evolutionary selection mechanism. Thromb Haemost. 1998; 79: 69-73.

Lindqvist PG, Zoller B, Dahlback B. Improved hemoglobin status and reduced menstrual blood loss among female carriers of factor V Leiden: an evolutionary advantage? Thromb Haemost. 2001; 86: 1122-1123.

Linton MF, Atkinson JB, Fazio S. Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation. Science. 1995; 267: 1034-1037.

Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. 1995; 4: 357-362.

Long J-R, Liu P-Y, Liu Y-J, Lu Y, Xiong D-H, Elze L, Recker RR, Deng H-W. APOE and TGF-beta-1 genes are associated with obesity phenotypes. J Med Genet. 2003; 40: 918-924.

Loscalzo J, Kohane I, Barabási A-L. Human disease classification in the postgenomic era: A complex systems approach to human pathobiology. Mol Syst Biol. 2007; 3 (124). Epub 2007 Jul 10.

Lusis AJ, Heinzmann C, Sparkes RS, Scott J, Knott TJ, Geller R, Sparkes MC, Mohandas T. Regional mapping of human chromosome 19: organization of genes for plasma lipid transport (APOC1, -C2, and -E and LDLR) and the genes C3, PEPD, and GPI. Proc Nat Acad Sci. 1986; 83: 3929-3933.

Macedo-Ribeiro S, Bode W, Huber R, Quinn-Allen MA, Kim SW, Ortel TL, Bourenkov GP, Bartunik HD, Stubbs MT, Kane WH, Fuentes-Prior P. Crystal structures of the membrane-binding C2 domain of human coagulation factor V. Nature. 1999; 402: 434-439.

Mahieux F, Couderc R, Bailleul S, Moulignier A. Apolipoprotein E epsilon-4 allele frequency in vascular dementia and Alzheimer's disease. Stroke. 1994; 25: 1703-1704.

Mahley RW. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science. 1988; 240: 622-630.

Mahmoud AEA, Elias E, Beauchamp N, Wilde JT. Prevalence of the factor V Leiden mutation in hepatic and portal vein thrombosis. Gut. 1997; 40: 798-800.

Majerus PW. Bad blood by mutation. Nature. 1994; 369: 14-15.

Mann KG and Kalafatis M. Factor V: a combination of Dr Jekyll and Mr Hyde. Blood. 2003; 101: 20-30.

Marchesini G, Moscatiello S, Di Domizio S, Forlani G. Obesity-associated liver disease. J Clin Endocrinol Metab. 2008; 93: Suppl 1:74-80.

Marcos A, Fisher RA, Ham JM, Olzinski AT, Shiffman ML, Sanyal AJ, Luketic VA, Sterling RK, Olbrisch ME, Posner MP. Selection and outcome of living donors for adult to adult right lobe transplantation. Transplantation. 2000; 69: 2410-1415.

Marder K, Maestre G, Cote L, Mejia H, Alfaro B, Halim A, Tang M, Tycko B, Mayeux R. The apolipoprotein E4 allele in Parkinson's disease with and without dementia. Neurology. 1994; 44: 1330-1331.

Margolis S. Personal Communication. 1982. Baltimore, Md.

Martin YN, Salavaggione OE, Eckloff BW, Wieben ED, Schaid DJ, Weinshilboum RM. Human methylenetetrahydrofolate reductase pharmacogenomics: gene resequencing and functional genomics. Pharmacogenet Genomics. 2006; 16: 265-277.

Martinelli I, Bucciarelli P, Margaglione M, De Stefano V, Castaman G, Mannucci PM. The risk of venous thromboembolism in family members with mutations in the genes of factor V or prothrombin or both. Brit J Haemat. 2000; 111: 1223-1229.

Martinelli I, Sacchi E, Landi G, Taioli E, Duca F, Mannucci PM. High risk of cerebral-vein thrombosis in carriers of a prothrombin-gene mutation and in users of oral contraceptives. N Engl J Med. 1998; 338: 1793-1797.

Martinelli I, Taioli E, Cetin I, Marinoni A, Gerosa S, Villa MV, Bozzo M, Mannucci PM. Mutations in coagulation factors in women with unexplained late fetal loss. N Engl J Med. 2000; 343: 1015-1018.

Martínez-García MA, Luque-Ramírez M, San-Millán JL, Escobar-Morreale HF. Body iron stores and glucose intolerance in premenopausal women: role of hyperandrogenism, insulin resistance, and genomic variants related to inflammation, oxidative stress, and iron metabolism. Diabetes Care. 2009; 32 (8): 1525-1530.

Masliah E, Mallory M, Ge N, Alford M, Veinbergs I, Roses AD. Neurodegeneration in the central nervous system of apoE-deficient mice. Exp Neurol. 1995; 136: 107-122.

Matikainen N, Mänttäri S, Westerbacka J, Vehkavaara S, Lundbom N, Yki-Järvinen H, Taskinen MR. Postprandial lipemia associates with liver fat content. J Clin Endocrinol Metab. 2007; 92: 3052-3059.

Matsumoto S, Nishimura M, Sakamoto T, Asanuma K, Izumi Y, Shibasaki H, Kamatani N, Nakamura T, Kaji R. Modulation of the onset age in primary dystonia by APOE genotype. Neurology. 2003; 60: 2003-2005.

Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu LC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. Gastroenterology. 1999; 116: 1413-1419.

Matthes T, Aguilar-Martinez P, Pizzi-Bosman L, Darbellay R, Rubbia-Brandt L, Giostra E, Michel M, Ganz T, Beris P. Severe hemochromatosis in a Portuguese family associated with a new mutation in the 5-prime-UTR of the HAMP gene. Blood. 2004; 104: 2181-2183.

Mattman A, Huntsman D, Lockitch G, Langlois S, Buskard N, Ralston D, Butterfield Y, Rodrigues P, Jones S, Porto G, Marra M, De Sousa M, Vatcher G. Transferrin receptor 2 (TfR2) and HFE mutational analysis in non-C282Y iron overload: identification of a novel TfR2 mutation. Blood. 2002; 100: 1075-1077.

Maxam AM and Gilbert W. A new method for sequencing DNA. Proc Natl Acad Sci USA. 1977; 74: 560–564.

McAlpine PJ, Coopland G, Guy C, James S, Komarnicki L, MacDonald M, Stranc L, Lewis M, Philipps S, Coghlan G, Kaita H, Cox DW, Guinto ER, MacGillivray R. Mapping the genes for erythrocytic alpha-spectrin 1 (SPTA1) and coagulation factor V (F5). Cytogenet Cell Genet. 1989; 51: 1042.

McAndrew PE, Brandt JT, Pearl DK, Prior TW. The incidence of the gene for thermolabile methylene tetrahydrofolate reductase in African Americans. Thromb Res. 1996; 83: 195-198.

McCarron MO, Delong D, Alberts MJ. APOE genotype as a risk factor for ischemic cerebrovascular disease: a meta-analysis. Neurology. 1999; 53: 1308-1311.

McKimmie RL, Daniel KR, Carr JJ, Bowden DW, Freedman BI, Register TC, Hsu FC, Lohman KK, Weinberg RB, Wagenknecht LE. Hepatic steatosis and subclinical cardiovascular disease in a cohort enriched for type 2 diabetes: the Diabetes Heart Study. Am J Gastroenterol. 2008; 103: 3029-3035.

Meeks SL and Abshire TC. Abnormalities of prothrombin: a review of the pathophysiology, diagnosis, and treatment. Haemophilia. 2008; (14): 1159-1163.

Meinardi JR, Pelsma PM, Koning H, van der Meer J, Middeldorp S, Buller HR, Hamulyak K. Double-homozygosity for factor V Leiden and the prothrombin gene G20210A variant in a young patient with idiopathic venous thrombosis. Blood. 1999; 94: 1828-1829.

Mercier E, Quere I, Chabert R, Lallemant J-G, Daures J-P, Berlan J, Gris J-C. The 20210A allele of the prothrombin gene is an independent risk factor for perception deafness in patients with venous thromboembolic antecedents. Blood. 1999; 93: 3150-3152.

Merryweather-Clarke AT, Cadet E, Bomford A, Capron D, Viprakasit V, Miller A, McHugh PJ, Chapman RW, Pointon JJ, Wimhurst VL, Livesey KJ, Tanphaichitr V, Rochette J, Robson KJ. Digenic inheritance of mutations in HAMP and HFE results in different types of haemochromatosis. Hum Mol Genet. 2003; 12 (17): 2241-2247.

Merryweather-Clarke AT, Pointon JJ, Shearman JD, Robson KJH. Global prevalence of putative haemochromatosis mutations. J Med Genet. 1997; 34: 275-278.

Meyers TE, Ballot D, Bothwell TH, Green A, Derman DP, Baynes RD, Jenkins T, Jooste PL, Du Toit ED, Jacobs PJ. The HLA linked iron laoding gene in an Afrikaner population. Br J Med Genet. 1987; 24: 348-356.

Mirbagheri SA, Rashidi A, Abdi S, Saedi D, Abouzari M. Liver: an alarm for the heart? Liver Int. 2007; 27: 891-894.

Mishra N, Rafiq N, Younossi ZM. Nonalcoholic fatty liver disease. In: Younessi ZM, ed. Practical Management of Liver Diseases. Cambridge, UK: Cambridge University Press, 2008; 77-97.

Montosi G, Donovan A, Totaro A, Garuti C, Pignatti E, Cassanelli S, Trenor CC, Gasparini P, Andrews NC, Piertangelo A. Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SLC11A3) gene. J Clin Invest. 2001; 108: 619-623.

Mori E, Lee K, Yasuda M, Hashimoto M, Kazui H, Hirono N, Matsui M. Accelerated hippocampal atrophy in Alzheimer's disease with apolipoprotein E E4 allele. Ann Neurol. 2002; 51: 209-214.

Morita H, Taguchi J, Kurihara H, Kitaoka M, Kaneda H, Kurihara Y, Maemura K, Shindo T, Minamino T, Ohno M, Yamaoki K, Ogasawara K, Aizawa T, Suzuki S, Yazaki Y. Genetic polymorphism of 5,10-methylenetetrahydrofolate reductase (MTHFR) as a risk factor for coronary artery disease. Circulation. 1997; 95: 2032-2036.

Mornet E, Muller F, Lenvoise-Furet A, Delezoide A-L, Col J-Y, Simon-Bouy B, Serre J-L. Screening of the C677T mutation on the methylenetetrahydrofolate reductase gene in French patients with neural tube defects. Hum Genet. 1997; 100: 512-514.

Morrow JA, Hatters DM, Lu B, Hochtl P, Oberg KA, Rupp B, Weisgraber KH. Apolipoprotein E4 forms a molten globule: a potential basis for its association with disease. J Biol Chem. 2002; 277: 50380-50385.

Mostowska A, Hozyasz KK, Jagodzinski PP. Maternal MTR genotype contributes to the risk of non-syndromic cleft lip and palate in the Polish population. Clin Genet. 2006; 69: 512-517.

Mullis KB, Ferre F, Gibbs, RA. 1994. The Polymerase chain reaction. Switzerland: Birkhauser Press.

Mumford AD, McVey JH, Morse CV, Gomez K, Steen M, Norstrom EA, Tuddenham EGD, Dahlback B, Bolton-Maggs PHB. Factor V I359T: a novel mutation associated with thrombosis and resistance to activated protein C. Brit J Haemat. 2003; 123: 496-501.

Muntjewerff JW, Hoogendoorn ML, Kahn RS, Sinke RJ, den Heijer M, Kluijtmans LA, Blom HJ. Hyperhomocysteinemia, methylenetetrahydrofolate reductase 677TT genotype, and the risk for schizophrenia: a Dutch population based case-control study. Am J Med Genet. 2005; 135B: 69-72.

Muntjewerff JW, Kahn RS, Blom HJ, den Heijer M. Homocysteine, methylenetetrahydrofolate reductase and risk of schizophrenia: a meta-analysis. Molec Psychiat. 2006; 11: 143-149.

Mura C, Raguenes O, Férec C. HFE mutations analysis in 711 hemochromatosis probands: evidence for S65C implication in mild form of hemochromatosis. Blood. 1999; 93(8): 2502-2505.

Murugan RC, Lee PL, Kalavar MR, Barton JC. Early age-of-onset iron overload and homozygosity for the novel hemojuvelin mutation HJV R54X (exon 3; c.160A-T) in an African American male of West Indies descent. Clin Genet. 2008; 74: 88-92.

Musso G, Gambino R, De Michieli F, Cassader M, Rizzetto M, Durazzo M, Fagà E, Silli B, Pagano G. Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. Hepatology. 2003; 37: 909-916.

Myers RH, Schaefer EJ, Wilson PWF, D'Agostino R, Ordovas JM, Espino A, Au R, White RF, Knoefel JE, Cobb JL, McNulty KA, Beiser A, Wolf PA. Apolipoprotein E epsilon-4 association with dementia in a population-based study: the Framingham study. Neurology. 1996; 46: 673-677.

Nelson JE, Bhattacharya R, Lindor KD, Chalasani N, Raaka S, Heathcote EJ, Miskovsky E, Shaffer E, Rulyak SJ, Kowdley KV. HFE C282Y mutations are associated with advanced hepatic fibrosis in Caucasions with non-alcoholic fatty liver disease. Hepatology. 2007; 46: 723-729.

Nemeth E, Preza GC, Jung CL, Kaplan J, Waring AJ, Ganz T. The N-terminus of hepcidin is essential for its interaction with ferroportin: structure–function study. Blood. 2006; 107: 328-33.

Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science. 2004; 306: 2090-2093.

Nishio H, Lee MJ, Fujii M, Kario K, Kayaba K, Shimada K, Matsuo M, Sumino K. A common mutation in methylenetetrahydrofolate reductase gene among the Japanese population. Jpn J Hum Genet. 1996; 41: 247-251.

Njajou OT, Vaessen N, Joosse M, Berghuis B, van Dongen JW, Breuning MH, Snijders PJ, Rutten WP, Sandkuijl LA, Oostra BA, can Duijn CM, Heutink P. A mutation in SLC11A3 is associated with autosomal dominant hemochromatosis. Nat Genet. 2001; 28: 213-214.

Novaro GM, Sachar R, Pearce GL, Sprecher DL, Griffin BP. Association between apolipoprotein E alleles and calcific valvular heart disease. Circulation. 2003; 108: 1804-1808.

O'Donnell HC, Rosand J, Knudsen KA, Furie KL, Segal AZ, Chiu RI, Ikeda D, Greenberg SM. Apolipoprotein E genotype and the risk of recurrent lobar intracerebral hemorrhage. N Engl J Med. 2000; 342: 240-245.

Ogino S and Wilson RB. Genotype and haplotype distributions of MTHFR 677C-T and 1298A-C single nucleotide polymorphisms: a meta-analysis. J Hum Genet. 2003; 48: 1-7.

Olaisen B, Teisberg P, Gedde-Dahl T (Jr). The locus for apolipoprotein E (apoE) is linked to the complement component C3 (C3) locus on chromosome 19 in man. Hum Genet. 1982; 62: 233-236.

O'Leary VB, Parle-McDermott A, Molloy AM, Kirke PN, Johnson Z, Conley M, Scott JM, Mills JL. MTRR and MTHFR polymorphism: link to Down syndrome? Am J Med Genet. 2002; 107: 151-155.

Ong JP, Elariny H, Collantes R, Younoszai A, Chandhoke V, Reines HD, Goodman Z, Younossi ZM. Predictors of nonalcoholic steatohepatitis and advanced fibrosis in morbidly obese patients. Obes Surg. 2005; 15: 310-315.

Ong JP, Pitts A, Younossi ZM. Increased mortality and liverrelated mortality in patients with non-alcoholic fatty liver disease. J Hepatol. 2008; 49: 608-612.

Osborne NJ, Gurrin LC, Allen KJ, Constantine CC, Delatycki MB, McLaren CE, Gertig DM, Anderson GJ, Southey MC, Olynyk JK, Powell LW, Hopper JL, Giles GG, English DR. HFE C282Y homozygotes are at increased risk of breast and colorectal cancer. Hepatology. 2010; 51 (4): 1311-1318.

Ou CY, Stevenson RE, Brown VK, Schwartz CE, Allen WP, Khoury MJ, Rozen R, Oakley GP (Jr), Adams MJ (Jr). 5,10 Methylenetetrahydrofolate reductase genetic polymorphism as a risk factor for neural tube defects. Am J Med Genet. 1996; 63: 610-614.

Pahwa R, Hedau S, Jain S, Jain N, Arora VM, Kumar N, Das BC. Assessment of possible tuberculous lymphadenopathy by PCR compared to non-molecular methods. J Med Microbiol. 2005; 54 (9): 873-878.

Paik Y-K, Chang DJ, Reardon CA, Davies GE, Mahley RW, Taylor JM. Nucleotide sequence and structure of the human apolipoprotein E gene. Proc Nat Acad Sci. 1985; 82: 3445-3449.

Palomba S, Falbo A, Giallauria F, Russo T, Tolino A, Zullo F, Colao A, Orio F. Effects of metformin with or without supplementation with folate on homocysteine levels and vascular endothelium of women with polycystic ovary syndrome. Diabetes Care. 2010; 33 (2): 246-251.

Papanikolaou G, Samuels ME, Ludwig EH, MacDonald ML, Franchini PL, Dubé MP, Andres L, MacFarlane J, Sakellaropoulos N, Politou M, Nemeth E, Thompson J, Risler JK, Zaborowska C, Babakaiff R, Radomski CC, Pape TD, Davidas O, Christakis J, Brissot P, Lockitch G, Ganz T, Hayden MR, Goldberg YP. Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. Nat Genet. 2004; 36: 77-82.

Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem. 2001; 276 (11): 7806-7810.

Parkkila S, Waheed A, Britton RS, Bacon BR, Zhou XY, Tomatsu S, Fleming RE, Sly WS. Association of the transferrin receptor in human placenta with HFE, the protein defective in hereditary hemochromatosis. Proc Natl Acad Sci USA. 1997; 94: 13198-13202.

Pawson T and Linding R. Network medicine. FEBS Lett. 2008; 582 (8): 1266-1270.

Paz MF, Avila S, Fraga MF, Pollan M, Capella G, Peinado MA, Sanchez-Cespedes M, Herman JG, Esteller M. Germ-line variants in methyl-group metabolism genes and susceptibility to DNA methylation in normal tissues and human primary tumors. Cancer Res. 2002; 62: 4519-4524.

Perseghin G, Lattuada G, De Cobelli F, Esposito A, Belloni E, Ntali G, Ragogna F, Canu T, Scifo P, Del Maschio A, Luzi L. Increased mediastinal fat and impaired left ventricular energy metabolism in young men with newly found fatty liver. Hepatology. 2008; 47: 51-58.

Petersen KF, Dufour S, Hariri A, Nelson-Williams C, Foo JN, Zhang X-M, Dziura J, Lifton RP, Shulman GI. Apolipoprotein C3 gene variants in nonalcoholic fatty liver disease. N Engl J Med. 2010; 362: 1082-1087.

Petersen KF, Dufour S, Hariri A, Nelson-Williams C, Foo JN, Zhang XM, Dziura J, Lifton RP, Shulman GI. Apolipoprotein C3 gene variants in nonalcoholic fatty liver disease. N Engl J Med. 2010; 362 (12): 1082-1089.

Petersen KF, Dufour S, Savage DB, Bilz S, Solomon G, Yonemitsu S, Cline GW, Befroy D, Zemany L, Kahn BB, Papademetris X, Rothman DL, Shulman GI. The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. Proc Natl Acad Sci U S A. 2007; 104: 12587-12594.

Petit JM, Guiu B, Terriat B, Loffroy R, Robin I, Petit V, Bouillet B, Brindisi MC, Duvillard L, Hillon P, Cercueil JP, Verges B. Nonalcoholic fatty liver is not associated with carotid intima-media thickness in type 2 diabetic patients. J Clin Endocrinol Metab. 2009; 94: 4103-4106.

Pietrangelo A. Hemochromatosis: is one gene enough? J Hepatol. 1998; 29: 502-509.

Pietrangelo A. Hereditary hemochromatosis--a new look at an old disease. N Engl J Med. 2004; 350 (23): 2383-2397.

Pietrangelo A. Hereditary haemochromatosis. Biochimica et Biophysica Acta. 2006; 1763: 700-710.

Pietrangelo A, Montosi G, Totaro A, Garuti C, Conte D, Cassanelli S, Fraquelli M, Sardini C, Vasta F, Gasparini P. Hereditary hemochromatosis in adults without pathogenic mutations in the hemochromatosis gene. N Engl J Med. 1999; 341: 725-732.

Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P, Loreal O. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. Journal of Biological Chemistry. 2001; 276: 7811-7819.

Pihusch R, Buchholz T, Lohse P, Rubsamen H, Rogenhofer N, Hasbargen U, Hiller E, Thaler CJ. Thrombophilic gene mutations and recurrent spontaneous abortion: prothrombin mutation increases the risk in the first trimester. Am J Reprod Immunol. 2001; 46: 124-131.

Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3-prime-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood. 1996; 88: 3698-3703.

Potekhina ES, Lavrove AV, Samokhodskaya LM, Efimenko AY, Balatskiy AV, Baev AA, Litvinova MM, Nikitina LA, Shipulin GA, Bochkov NP, Tkachuk TA, Bochkov VN. Unique genetic profile of hereditary hemochromatosis in Russians: High frequency of C282Y mutation in population, but not in patients. Blood Cells Mol Dis. 2005; 35: 182-188.

Poynard T, Ratziu V, Mchutchison J, Manns M, Goodman Z, Zeuzem S, Younossi Z, Albrecht J. Effect of treatment with peginterferon or interferon alfa-2b and ribavirin on steatosis in patients infected with hepatitis C. Hepatology. 2003; 38: 75-85.

Price DA, Bassendine MF, Norris SM, Golding C, Toms GL, Schmid ML, Morris CM, Burt AD, Donaldson PT. Apolipoprotein epsilon-3 allele is associated with persistent hepatitis C virus infection. Gut. 2006; 55: 715-718.

Qian X, Lu Z, Tan M, Liu H, Lu D. A meta-analysis of association between C677T polymorphism in the methylenetetrahydrofolate reductase gene and hypertension. Europ J Hum Genet. 2007; 15: 1239-1245.

Rafiq N, Bai C, Fang Y, Srishord M, McCullough A, Gramlich T, Younossi ZM. Longterm follow-up of patients with non-alcoholic fatty liver. Clin Gastroenterol Hepatol. 2009; 7: 234-238.

Rall SC (Jr), Newhouse YM, Clarke HRG, Weisgraber KH, McCarthy BJ, Mahley RW, Bersot TP. Type III hyperlipoproteinemia associated with apolipoprotein E phenotype E3/3: structure and genetics of an apolipoprotein E3 variant. J Clin Invest. 1989; 83: 1095-1101.

Rall SC (Jr), Weisgraber KH, Innerarity TL, Bersot TP, Mahley RW, Blum CB. Identification of a new structural variant of human apolipoprotein E, E2(lys146-to-gln), in a type III hyperlipoproteinemic subject with the E3/2 phenotype. J Clin Invest. 1983a; 72: 1288-1297.

Rall SC (Jr), Weisgraber KH, Innerarity TL, Mahley RW. Structural basis for receptor binding heterogeneity of apolipoprotein E from type III hyperlipoproteinemic subjects. Proc Nat Acad Sci. 1982b; 79: 4696-4700.

Rall SC (Jr), Weisgraber KH, Innerarity TL, Mahley RW, Assmann G. Identical structural and receptor binding defects in apolipoprotein E2 in hypo-, normo-, and hypercholesterolemic dysbetalipoproteinemia. J Clin Invest. 1983b; 71: 1023-1031.

Rall SC (Jr), Weisgraber KH, Mahley RW. Human apolipoprotein E: the complete amino acid sequence. J Biol Chem. 1982a; 257: 4171-4178.

Rees DC, Chapman NH, Webster MT, Guerreiro JF, Rochette J, Clegg JB. Born to clot: the European burden. Brit J Haemat. 1999; 105: 564-566.

Reiman EM, Caselli RJ, Yun LS, Chen K, Bandy D, Minoshima S, Thibodeau SN, Osborne D. Preclinical evidence of Alzheimer's disease in persons homozygous for the epsilon-4 allele for apolipoprotein E. N Engl J Med. 1996; 334: 752-758.

Riddell DC, Wang H, Royle NJ, Nigli M, Guinto E, Kochinsky ML, Irwin DM, Cool D, MacGillivray RTA, Hamerton JL. Regional assignment for the human genes encoding FII, FV, FXIII, ceruloplasmin and pseudoceruloplasmin. Cytogenet Cell Genet. 1987; 46: 682.

Riddell DR, Graham A, Owen JS. Apolipoprotein E inhibits platelet aggregation through the L-arginine:nitric oxide pathway. Implications for vascular disease. J Biol Chem. 1997; 272 (1): 89-95.

Ridker PM, Miletich JP, Stampfer MJ, Goldhaber SZ, Lindpaintner K, Hennekens CH. Factor V Leiden and risks of recurrent idiopathic venous thromboembolism. Circulation. 1995; 92: 2800-2802.

Riva A, Trombini P, Mariainni R, Salvioni A, Coletti S, Bonfadini S, Paolini V, Pozzi Matteo, Facchetti R, Bovo G, Piperno A. Revaluation of clinical and histological criteria for diagnosis of dysmetabolic iron overload syndrome. World J Gastroenterol. 2008; 14 (30): 4745-4752.

Rochette J, Pointon JJ, Fisher CA, Perera G, Arambepola M, Arichchi DS, De Silva S, Vandwalle JL, Monti JP, Old JM, Merryweather-Clarke AT, Weatherall DJ, Robson KJ. Multicentric origin of hemochromatosis gene (HFE) mutations. Am J Hum Genet. 1999; 64: 1056-1062.

Roest M, van der Schouw YT, de Valk B, Marx JJ, Tempelman MJ, de Groot PG, Sixma JJ, Banga JD. Heterozygosity for a hereditary hemochromatosis gene is associated with cardiovascular death in women. Circulation. 1999; 100 (12): 1268-1273.

Roetto A, Papanikolaou G, Politou M, Alberti F, Girelli D, Christakis J, Loukopoulos D, Camaschella C. Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. Nat Genet. 2003; 33: 21–22.

Roetto A, Totaro A, Cazzola M, Cicilano M, Bosio S, D'Ascola G, Carella M, Zelante L, Kelly AL, Cox TM, Gasparini P, Camaschella C. Juvenile hemochromatosis locus maps to chromosome 1q. Am J Hum Genet. 1999; 64: 1388-1393.

Roetto A, Totaro A, Piperno A, Piga A, Longo F, Garozzo G, Cali A, De Gobbi M, Gasparini P, Camaschella C. New mutations inactivating transferrin receptor 2 in hemochromatosis type 3. Blood. 2001; 97: 2555-2560.

Roffman JL, Weiss AP, Purcell S, Caffalette CA, Freudenreich O, Henderson DC, Bottiglieri T, Wong DH, Halsted CH, Goff DC. Contribution of methylenetetrahydrofolate reductase (MTHFR) polymorphisms to negative symptoms in schizophrenia. Biol Psychiat. 2008; 63: 42-48.

Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, Boerwinkle E, Cohen JC, Hobbs HH. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. (Letter) Nat Genet. 2008; 40: 1461-1465.

Romero-Gomez M, Del Mar Viloria M, Andrade RJ, Salmerón J, Diago M, Fernández-Rodríguez CM, Corpas R, Cruz M, Grande L, Vázquez L, Muñoz-de-Rueda P, López-Serrano P, Gila A, Gutiérrez ML, Pérez C, Ruiz-Extremera A, Suárez E, Castillo J. Insulin resistance impairs sustained response rate to peginterferon plus ribavirin in chronic hepatitis C patients. Gastroenterology. 2005; 128: 636-641.

Rosenberg N, Murata M, Ikeda Y, Opare-Sem O, Zivelin A, Geffen E, Seligsohn U. The frequent 5,10-methylenetetrahydrofolate reductase C677T polymorphism is associated with a common haplotype in whites, Japanese, and Africans. Am J Hum Genet. 2002; 70: 758-762.

Rosendaal FR, Doggen CJM, Zivelin A, Arruda VR, Aiach M, Siscovick DS, Hillarp A, Watzke HH, Bernardi F, Cumming AM, Preston FE, Reitsma PH. Geographic distribution of the 20210 G to A prothrombin variant. Thromb Haemost. 1998; 79: 706-708.

Rosendaal FR, Siscovick DS, Schwartz SM, Psaty BM, Raghunathan TE, Vos HL. A common prothrombin variant (20210 G to A) increases the risk of myocardial infarction in young women. Blood. 1997; 90: 1747-1750.

Rouault TA. The role of iron regulatory proteins in mammalian iron homeostasis and disease. Nat Chem Biol. 2006; 2: 406-414.

Royle NJ, Irwin DM, Koschinsky ML, MacGillivray RTA, Hamerton JL. Human genes encoding prothrombin and ceruloplasmin map to 11p11-q12 and 3q21-24, respectively. Somat Cell Molec Genet. 1987; 13: 285-292.

Rozen R. Molecular genetics of methylenetetrahydrofolate reductase deficiency. J Inherit Metab Dis. 1996; 19: 589-594.

Sabio G, Das M, Mora A, Zhang Z, Jun JY, Ko HJ, Barrett T, Kim JK, Davis RJ. A stress signaling pathway in adipose tissue regulates hepatic insulin resistance. Science. 2008; 322: 1539-1543.

Sabuncu T, Nazligul Y, Karaoglanoglu M, Ucar E, Kilic FB. The effects of sibutramine and orlistat on the ultrasonographic findings insulin resistance and liver enzyme levels in obese patients with nonalcoholic steatohepatitis. Rom J Gastroenterol. 2003; 12: 189-192.

Salero E, Perez-Sen R, Aruga J, Gimenez C, Zafra F. Transcription factors Zic1 and Zic2 bind and transactivate the apolipoprotein E gene promoter. J Biol Chem. 2001; 276: 1881-1888.

Salonen JT, Nyyssönen K, Korpela H, Tuomilehto J, Seppänen R, Salonen R. High stored iron levels are associated with excess risk of myocardial infarction in eastern Finnish men. Circulation. 1992; 86 (3): 803-811.

Sambrano GR, Weiss EJ, Zheng YW, Huang W, Coughlin SR. Role of thrombin signalling in platelets in haemostasis and thrombosis. Nature. 2001; 413: 74-78.

Sandholzer C, Delport R, Vermaak H, Uterman G. High frequency of the apo epsilon 4 allele in Khoi San from South Africa. Hum Genet. 1995; 95 (1): 46-48.

Sanger F and Coulson AR. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J Mol Biol. 1975; 94: 441–448.

Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inihibitors. Proc Natl Acad Sci USA. 1977; 74: 5463–5467.

Sanyal AJ. AGA technical review on nonalcoholic fatty liver disease. Gastroenterology. 2002; 123: 1705-1725.

Sassi R, Hmida S, Kaabi H, Hajjej A, Abid A, Abdelkefi S, Yacoub S, Mamaar M, Mojaat N, Ben Hamed L, Bellali H, Dridi A, Jridi A, Midouni B, Boukef MK. Prevalence of C282Y and H63D mutations in the haemochromatosis (HFE) gene in Tunisian population. Ann Genet. 2004; 47: 325-330.

Saunders AM, Schmader K, Breitner JCS, Benson MD, Brown WT, Goldfarb L, Goldgaber D, Manwaring MG, Szymanski MH, McCown N, Dole KC, Schmechel DE, Strittmatter WJ, Pericak-Vance MA, Roses AD. Apolipoprotein E epsilon-4 allele distributions in late-onset Alzheimer's disease and in other amyloid-forming diseases. Lancet. 1993b; 342: 710-711.

Saunders AM, Strittmatter WJ, Schmechel D, St. George-Hyslop PH, Pericak-Vance MA, Joo SH, Rosi BL, Gusella JF, Crapper-MacLachlan DR, Alberts MJ, Hulette C, Crain B, Goldgaber D, Roses AD. Association of apolipoprotein E allele E4 with late-onset familial and sporadic Alzheimer's disease. Neurology. 1993a; 43: 1467-1472.

Savage DB, Petersen KF, Shulman GI. Disordered lipid metabolism and the pathogenesis of insulin resistance. Physiol Rev. 2007; 87: 507-520.

Schachter F, Faure-Delanef L, Guenot F, Rouger H, Froguel P, Lesueur-Ginot L, Cohen D. Genetic associations with human longevity at the APOE and ACE loci. Nat Genet. 1994; 6: 29-32.

Schaefer EJ, Gregg RE, Ghiselli G, Forte TM, Ordovas JM, Zech LA, Brewer HB (Jr). Familial apolipoprotein E deficiency. J Clin Invest. 1986; 78: 1206-1219.

Scher AI, Terwindt GM, Verschuren WM, Kruit MC, Blom HJ, Kowa H, Frants RR, van den Maagdenberg AM, van Buchem M, Ferrari MD, Launer LJ. Migraine and MTHFR C677T genotype in a population-based sample. Ann Neurol. 2006; 59: 372-375.

Schimanski LM, Drakesmith H, Merryweather-Clarke AT, Viprakasit V, Edwards JP, Sweetland E, Bastin JM, Cowley D, Chinthammitr Y, Robson KJ, Townsend AR. In vitro functional analysis of human ferroportin (FPN) and hemochromatosis-associated FPN mutations. Blood. 2005; 105: 4096–4102.

Scholtz CL, Odendaal HJ, Thiart R, Loubser L, Hillermann R, Delport R, Hayward Vermaak WJ, Kotze MJ. Analysis of two mutations in the MTHFR gene associated with mild hyperhomocysteinaemia – heterozygous distribution in the South African population. S Afr Ned J. 2002; 92: 464-467.

Schneider DJ, Steyn PS, Mansvelt EP. Factor V Leiden mutation and the risk of thrombo-embolic disease in pregnancy: a case report. Eur J Obstet Gynecol Reprod Biol. 2000; 91 (2): 197-198.

Schneider JA, Rees DC, Liu Y-T, Clegg JB. Worldwide distribution of a common methylenetetrahydrofolate reductase mutation. Am J Hum Genet. 1998; 62: 1258-1260.

Schneider WJ, Kovanen PT, Brown MS, Goldstein JL, Utermann G, Weber W, Havel RJ, Kotite L, Kane JP, Innerarity TL, Mahley RW. Familial dysbetalipoproteinemia: abnormal binding of mutant apoprotein E to low density lipoprotein receptors of human fibroblasts and membranes from liver and adrenal of rats, rabbits, and cows. J Clin Invest. 1981; 68: 1075-1085.

Schürks M, Zee RY, Buring JE, Kurth T. Interrelationships among the MTHFR 677C>T polymorphism, migraine, and cardiovascular disease. Neurology. 2008; 71 (7): 505-513.

Schwartz SM, Siscovick DS, Malinow MR, Rosendaal FR, Beverly RK, Hess DL, Psaty BM, Longstreth WT (Jr), Koepsell TD, Raghunathan TE, Reitsma PH. Myocardial infarction in young

women in relation to plasma total homocysteine, folate, and a common variant in the methylenetetrahydrofolate reductase gene. Circulation. 1997; 96: 412-417.

Schwimmer JB, Deutsch R, Behling C, Lavine JE. Fatty liver as a determinant of atherosclerosis. Hepatology. 2005; 42: Suppl: 610A. abstract.

Segal JB, Brotman DJ, Necochea AJ, Emadi A, Samal L, Wilson LM, Crim MT, Bass EB. Predictive value of factor V Leiden and prothrombin G20210A in adults with venous thromboembolism and in family members of those with a mutation: a systematic review. JAMA. 2009; 301: 2472-2485.

Selzer RR, Rosenblatt DS, Laxova R, Hogan K. Adverse effect of nitrous oxide in a child with 5,10-methylenetetrahydrofolate reductase deficiency. N Engl J Med. 2003; 349: 45-50.

Sham RL, Phatak PD, West C, Lee P, Andrews C, Beutler E. Autosomal dominant hereditary hemochromatosis associated with a novel ferroportin mutation and unique clinical features. Blood Cells Mol Dis. 2005; 34: 157-161.

Shaw GM, Rozen R, Finnell RH, Todoroff K, Lammer EJ. Infant C677T mutation in MTHFR, maternal periconceptional vitamin use, and cleft lip. Am J Med Genet. 1998; 80: 196-198.

Shayeghi M, Latunde-Dada GO, Oakhill JS, Laftah AH, Takeuchi K, Halliday N, Khan Y, Warley A, McCann FE, Hider RC, Frazer DM, Anderson GJ, Vulpe CD, Simpson RJ, McKie AT. Identification of an intestinal heme transporter. Cell. 2005; 122: 789-801.

Sheldon JH. Haemochromatosis. 1935. London: Oxford University Press.

Shoelson SE, Herrero L, Naaz A. Obesity, inflammation, and insulin resistance. Gastroenterology. 2007; 132: 2169-2180.

Sibani S, Christensen B, O'Ferrall E, Saadi I, Hiou-Tim F, Rosenblatt DS, Rozen R. Characterization of six novel mutations in the methylenetetrahydrofolate reductase (MTHFR) gene in patients with homocystinuria. Hum Mutat. 2000; 15: 280-287.

Sibani S, Leclerc D, Weisberg IS, O'Ferrall E, Watkins D, Artigas C, Rosenblatt DS, Rozen R. Characterization of mutations in severe methylenetetrahydrofolate reductase deficiency reveals an FAD-responsive mutation. Hum Mutat. 2003; 21: 509-520.

Silverman EM, Sapala J, Appelman HD. Regression of hepaticsteatosis in morbidly obese persons after gastric bypass. Am J Clin Pathol. 1995; 104: 23-31.

Simon M, Le Mignon L, Fauchet R, Yaouang J, David V, Edan G, Bourel M. A study of 609 HLA haplotypes marking for the hemochromatosis gene: (1) mapping of the gene near the HLA-A locus and characters required to define a heterozygous population and (2) hypothesis concerning the underlying cause of hemochromatosis-HLA association. Am J Hum Genet. 1987; 41: 89-105.

Simon M, Pawlotsky Y, Bourel M, Fauchet R, Genetet B. Hemochromatose idiopathique maladie associee a l'antigene tissulaire HL-A 3. Nouv Presse Med. 1975; 4: 1432.

Smit M, de Knijff P, van der Kooij-Meijs E, Groenendijk C, van den Maagdenberg AMJM, Gevers Leuven JA, Stalenhoef AFH, Stuyt PMJ, Frants RR, Havekes LM. Genetic heterogeneity in familial dysbetalipoproteinemia: the E2(lys146-to-gln) variant results in a dominant mode of inheritance. J Lipid Res. 1990; 31: 45-53.

Söderberg C, Stål P, Askling J, Glaumann H, Lindberg G, Marmur J, Hultcrantz R. Decreased survival of subjects with elevated liver function tests during a 28-year follow-up. Hepatology. 2010; 51: 595-602.

Sodha T, Yanai J, Soejima H, Tamura K. Frequencies in the Japanese population of HFE gene mutations. Biochem Genet. 1999; 37: 63-68.

Sohda S, Arinami T, Hamada H, Yamada N, Hamaguchi H, Kubo T. Methylenetetrahydrofolate reductase polymorphism and pre-eclampsia. J Med Genet. 1997; 34: 525-526.

Song Z, Deaciuc I, Zhou Z, Song M, Chen T, Hill D, McClain CJ. Involvement of AMP-activated protein kinase in beneficial effects of betaine on high-sucrose diet-induced hepatic steatosis. Am J Physiol Gastrointest Liver Physiol. 2007; 293 (4): G894-902.

Sookoian S and Pirola CJ. Non-alcoholic fatty liver disease is strongly associated with carotid atherosclerosis: a systematic review. J Hepatol. 2008; 49: 600-607.

Speer MC, Worley G, Mackey JF, Melvin E, Oakes WJ, George TM, NTD Collaborative Group. The thermolabile variant of methylenetetrahydrofolate reductase (MTHFR) is not a major risk factor for neural tube defect in American Caucasians. Neurogenetics. 1997; 1: 149-150.

Stefan N, Kantartzis K, Häring HU. Causes and metabolic consequences of fatty liver. Endocr Rev. 2008; 29: 939-960.

Steffens S, Veillard NR, Arnaud C, Pelli G, Burger F, Staub C, Zimmer A, Frossard J-L, Mach F. Low dose oral cannabinoid therapy reduces progression of atherosclerosis in mice. Nature. 2005; 434: 782-786.

Stevenson RE, Schwartz CE, Du Y-Z, Adams MJ (Jr). Differences in methylenetetrahydrofolate reductase genotype frequencies, between whites and blacks. Am J Hum Genet. 1997; 60: 229-230.

Sullivan PM, Mezdour H, Quarfordt SH, Maeda N. Type III hyperlipoproteinemia and spontaneous atherosclerosis in mice resulting from gene replacement of mouse Apoe with human APOE*2. J Clin Invest. 1998; 102: 130-135.

Sumida Y, Yoshikawa T, Okanoue T. Role of hepatic iron in non-alcoholic steatohepatitis. Hepatology Research. 2009; 39: 213-222.

Sun WY, Witte DP, Degen JL, Colbert MC, Burkart MC, Holmback K, Xiao Q, Bugge TH, Degen SJF. Prothrombin deficiency results in embryonic and neonatal lethality in mice. Proc Nat Acad Sci. 1998; 95: 7597-7602.

Svegliati-Baroni G, Bugianesi E, Bouserhal T, Marini F, Ridolfi F, Tarsetti F, Ancarani F, Petrelli E, Peruzzi E, Lo Cascio M, Rizzetto M, Marchesini G, Benedetti A. Post-load insulin resistance is an independent predictor of hepatic fibrosis in virus C chronic hepatitis and in non-alcoholic fatty liver disease. Gut. 2007; 56: 1296-1301.

Tabaton M, Rolleri M, Masturzo P, Cammarata S, Angelini G, Hansen LA, Saitoh T, Petersen RB, Perry G, Richey P, Gambetti P, Bertolini S. Apolipoprotein E epsilon-4 allele frequency is not increased in progressive supranuclear palsy. Neurology. 1995; 45: 1764-1765.

Tajouri L, Martin V, Gasparini C, Ovcaric M, Curtain R,Lea RA, Haupt LM, Csurhes P, Pender MP, Giffiths LR. Genetic investigation of methylenetetrahydrofolate reductase (MTHFR) and catechol-O-methyl transferase (COMPT) in multiple sclerosis. Brain Research Bulletin. 2006; 69: 327-331.

Talmon T, Scharf J, Mayer E, Lanir N, Miller B, Brenner B. Retinal arterial occlusion in a child with factor V Leiden and thermolabile methylene tetrahydrofolate reductase mutations. Am J Ophthal. 1997; 124: 689-691.

Tang M-X, Maestre G, Tsai W-Y, Liu X-H, Feng L, Chung W-Y, Chun M, Schofield P, Stern Y, Tycko B, Mayeux R. Relative risk of Alzheimer disease and age-at-onset distributions, based on APOE genotypes among elderly African Americans, Caucasians, and Hispanics in New York City. Am J Hum Genet. 1996; 58: 574-584.

Tangirala RK, Pratico D, FitzGerald GA, Chun S, Tsukamoto K, Maugeais C, Usher DC, Pure E, Rader DJ. Reduction of isoprostanes and regression of advanced atherosclerosis by apolipoprotein E. J Biol Chem. 2001; 276: 261-266.

Tarantino G, Conca P, Sorrentino P, Ariello M. Metabolic factors involved in the therapeutic response of patients with hepatitis C virus-related chronic hepatitis. J Gastroenterol Hepatol. 2006; 21: 1266-268.

Targher G, Bertolini L, Padovani R, Rodella S, Arcaro G, Day C. Differences and similarities in early atherosclerosis between patients with nonalcoholic steatohepatitis and chronic hepatitis B and C. J Hepatol. 2007a; 46: 1126-1132.

Targher G, Bertolini L, Padovani R, Rodella S, Tessari R, Zenari L, Day C, Arcaro G. Prevalence of nonalcoholic fatty liver disease and its association with cardiovascular disease among type 2 diabetic patients. Diabetes Care. 2007b; 30: 1212-1218.

Targher G, Bertolini L, Padovani R, Rodella S, Zoppini G, Pichiri I, Sorgato C, Zenari L, Bonora E. Prevalence of non-alcoholic fatty liver disease and its association with cardiovascular disease in patients with type 1 diabetes. J Hepatol. 2010; 53: 713-718.

Targher G, Bertolini L, Padovani R, Rodella S, Zoppini G, Zenari L, Cigolini M, Falezza G, Arcaro G. Relations between carotid artery wall thickness and liver histology in subjects with nonalcoholic fatty liver disease. Diabetes Care. 2006; 29: 1325-1330.

Targher G, Bertolini L, Padovani R, Zenari L, Zoppini G, Falezza G. Relation of nonalcoholic hepatic steatosis to early carotid atherosclerosis in healthy men: role of visceral fat accumulation. Diabetes Care. 2004; 27: 2498-2500.

Targher G, Bertolini L, Rodella S, Lippi G, Franchini M, Zoppini G, Muggeo M, Day CP. NASH predicts plasma inflammatory biomarkers independently of visceral fat in men. Obesity (Silver Spring). 2008a; 16: 1394-1399.

Targher G, Chonchol M, Miele L, Zoppini G, Pichiri I, Muggeo M. Nonalcoholic fatty liver disease as a contributor to hypercoagulation and thrombophilia in the metabolic syndrome. Semin Thromb Hemost. 2009; 35: 277-287.

Targher G, Marra F, Marchesini G. Increased risk of cardiovascular disease in nonalcoholic fatty liver disease: causal effect or epiphenomenon? Diabetologia. 2008b; 51: 1947-1953.

Tavill AS. Diagnosis and management of hemochromatosis. Hepatology. 2001; 33: 1321-1328.

Teasdale GM, Nicoll JAR, Murray G, Fiddes M. Association of apolipoprotein E polymorphism with outcome after head injury. Lancet. 1997; 350: 1069-1071.

Thuy S, Ladurner R, Volynets V, Wagner S, Strahl S, Königsrainer A, Maier K-P, Bischoff SC, Bergheim I. Nonalcoholic fatty liver disease in humans is associated with increased plasma endotoxin and plasminogen activator inhibitor 1 concentrations and with fructose intake. J Nutr. 2008; 138: 1452-1455.

Tian C, Stokowski RP, Kershenobich D, Ballinger DG, Hinds DA. Variant in PNPLA3 is associated with alcoholic liver disease. Nat Genet. 2010; 42: 21-23.

Tilg H and Moschen AR. Insulin resistance, inflammation, and non-alcoholic fatty liver disease. Trends Endocrinol Metab. 2008; 19: 371-379.

Todt U, Freudenberg J, Goebel I, Netzer C, Heinze A, Heinze-Kuhn K, Gobel H, Kubisch C. MTHFR C677T polymorphism and migraine with aura. Ann Neurol. 2006; 60: 621-622.

Tonetti C, Amiel J, Munnich A, Zittoun J. Impact of new mutations in the methylenetetrahydrofolate reductase gene assessed on biochemical phenotypes: a familial study. J Inherit Metab Dis. 2001; 24: 833-842.

Tonetti C, Ruivard M, Rieu V, Zittoun J, Giraudier S. Severe methylenetetrahydrofolate reductase deficiency revealed by a pulmonary embolism in a young adult. Brit J Haemat. 2002; 119: 397-399.

Tonetti C, Saudubray J-M, Echenne B, Landrieu P, Giraudier S, Zittoun J. Relations between molecular and biological abnormalities in 11 families from siblings affected with methylenetetrahydrofolate reductase deficiency. Europ J Pediat. 2003; 162: 466-475.

Tuomainen TP, Kontula K, Nyyssönen K, Lakka TA, Heliö T, Salonen JT. Increased risk of acute myocardial infarction in carriers of the hemochromatosis gene Cys282Tyr mutation: a prospective cohort study in men in eastern Finland. Circulation. 1999; 100 (12): 1274-1279.

Utermann G, Pruin N, Steinmetz A. Polymorphism of apolipoprotein E. III. Effect of a single polymorphic gene locus on plasma lipid levels in man. Clin Genet. 1979; 15: 63-72.

Valenti L, Dongiovanni P, Fracanzani AL, Santorelli G, Fatta E, Bertelli C, Taioli E, Fiorelli G, Fargion S. Increased susceptibility to non-alcoholic fatty liver disease in heterozygotes for the mutation responsible for hereditary hemochromatosis. Dig Liver Dis. 2003; 35: 172-178.

Valenti L, Fracanzani AL, Dongiovanni P, Bugianesi E, Marchesini G, Manzini P, Vanni E, Fargion S. Iron depletion by phlebotomy improves insulin resistance in patients with nonalcoholic fatty liver disease and hyperferritinemia: evidence from a case-control study. Am J Gastroenterol. 2007; 102: 1251-1258.

Valenti L, Swinkels DW, Burdick L, Dongiovanni P, Tjalsma H, Motta BM, Bertelli C, Fatta E, Bignamini D, Rametta R, Fargion S, Fracanzani AL. Serum ferritin levels are associated with vascular damage in patients with nonalcoholic fatty liver disease. Nutr Metab Cardiovasc Dis. 2011; 21 (8): 568-575.

van Bockxmeer FM and Mamotte CDS. Apolipoprotein epsilon-4 homozygosity in young men with coronary heart disease. Lancet. 1992; 340: 879-880.

van Bockxmeer FM, Mamotte CDS, Vasikaran SD, Taylor RR. Methylenetetrahydrofolate reductase gene and coronary artery disease. Circulation. 1997; 95: 21-23.

van den Elzen P, Garg S, Leon L, Brigl M, Leadbetter EA, Gumperz JE, Dascher CC, Cheng T-Y, Sacks FM, Illarionov PA, Besra GS, Kent SC, Moody DB, Brenner MB. Apolipoprotein-mediated pathways of lipid antigen presentation. Nature. 2005; 437: 906-910.

van der Put NMJ and Blom HJ. Reply to Donnelly. Am J Hum Genet. 2000; 66: 744-745.

van der Put NMJ, Gabreels F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, van den Heuvel LP, Blom HJ. A second mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet. 1998; 62: 1044-1051.

van Vliet P, Oleksik AM, Mooijaart SP, de Craen AJM, Westendorp RGJ. APOE genotype modulates the effect of serum calcium levels on cognitive function in old age. Neurology. 2009; 72: 821-828.

van Wijk R, Nieuwenhuis K, van den Berg M, Huizinga EG, van der Meijden BB, Kraaijenhagen RJ, van Solinge WW. Five novel mutations in the gene for human blood coagulation factor V associated with type I factor V deficiency. Blood. 2001; 98: 358-367.

Verpillat P, Camuzat A, Hannequin D, Thomas-Anterion C, Puel M, Belliard S, Dubois B, Didic M, Lacomblez L, Moreaud O, Golfier V, Campion D, Brice A, Clerget-Darpoux F. Apolipoprotein E gene in frontotemporal dementia: an association study and meta-analysis. Europ J Hum Genet. 2002; 10: 399-405.

Villanova N, Moscatiello S, Ramilli S, Bugianesi E, Magalotti D, Vanni E, Zoli M, Marchesini G. Endothelial dysfunction and cardiovascular risk profile in nonalcoholic fatty liver disease. Hepatology. 2005; 42: 473-480.

Vogel S, Harvey J, Avenant H, Geiger D, Kotze MJ. 2011. A questionnaire-based survey to rate doctor's opinion on genetic testing in the era of personalised medicine [abstract – P236]. In: Joint International Conference of the African and Southern African Societies of Human Genetics Conference Book; 2011 March 6-9; Cape Town. Joint International Conference of the African and Southern African Societies of Human Genetics.

Volcik KA, Blanton SH, Northrup H. Examinations of methylenetetrahydrofolate reductase C677T and A1298C mutations--and in utero viability. Am J Hum Genet. 2001; 69: 1150-1152.

Völzke H, Robinson DM, Kleine V, Deutscher R, Hoffmann W, Ludemann J, Schminke U, Kessler C, John U. Hepatic steatosis is associated with an increased risk of carotid atherosclerosis. World J Gastroenterol. 2005a; 11: 1848-1853.

Völzke H, Wolff B, Grimm R, Robinson DM, Schuster G, Herrmann FH, Motz W, Rettig R. Interaction between factor V Leiden and serum LDL cholesterol increases the risk of atherosclerosis. Atherosclerosis. 2005b; 180 (2): 341-347.

Von Recklinghausen FD. Uber Haemochromatose. Tageblatt Versammlung Dtsche Naturforscher Arzte Heidelberg. 1889; 62: 324-325.

Voorberg J, Roelse J, Koopman R, Buller H, Berends F, ten Cate JW, Mertens K, van Mourik JA. Association of idiopathic venous thromboembolism with single point-mutation at arg506 of factor V. Lancet. 1994; 343: 1535-1536.

Waheed A, Parkkila S, Zhou XY, Tomatsu S, Tsuchihashi Z, Feder JN, Schatzman RC, Britton RS, Bacon BR, Sly WS. Hereditary hemochromatosis: effects of C282Y and H63D mutations on association with beta2-microglobulin, intracellular processing, and cell surface expression of the HFE protein in COS-7 cells. Proc Natl Acad Sci USA. 1997; 94: 12384-12389.

Wald DS, Morris JK, Wald NJ. Reconciling the Evidence on Serum Homocysteine and Ischaemic Heart Disease: A Meta-Analysis. PLoS One. 2011; 6 (2): e16473.

Wallace DF, Pedersen P, Dixon JL, Stephenson P, Searle JW, Powell LW, Subramaniam VN. Novel mutation in ferroportin1 is associated with autosomal dominant hemochromatosis. Blood. 2002; 100 (2): 692-694.

Wang H, Riddell DC, Guinto ER, MacGillivray RTA, Hamerton JL. Localization of the gene encoding human factor V to chromosome 1q21-25. Genomics. 1988; 2: 324-328.

Wang HY, Zhang FC, Gao JJ, Fan JB, Liu P, Zheng ZJ, Xi H, Sun Y, Gao XC, Huang TZ, Ke ZJ, Guo GR, Feng GY, Breen G, St. Clair D, He L. Apolipoprotein E is a genetic risk factor for fetal iodine deficiency disorder in China. Molec Psychiat. 2000; 5: 363-368.

Wanless IR and Lentz JS. Fatty liver hepatitis (steatohepatitis) and obesity: an autopsy study with analysis of risk factors. Hepatology. 1990; 11: 74-80.

Weger M, Stanger O, Deutschmann H, Leitner FJ, Renner W, Schmut O, Semmelrock J, Haas A. The role of hyperhomocysteinemia and methylenetetrahydrofolate reductase (MTHFR) C677T mutation in patients with retinal artery occlusion. Am J Ophthal. 2002; 134: 57-61.

Weisberg I, Tran P, Christensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab. 1998; 64 (3): 169-172.

Weisgraber KH, Innerarity TL, Mahley RW. Abnormal lipoprotein receptor-binding activity of the human E apoprotein due to cysteine-arginine interchange at a single site. J Biol Chem. 1982; 257: 2518-2521.

Weisgraber KH, Rall SC (Jr), Mahley RW. Human E apoprotein heterogeneity: cysteine-arginine interchanges in the amino acid sequence of the apo-E isoforms. J Biol Chem. 1981; 256: 9077-9083.

Wells BJ, Mainous AG 3rd, King DE, Gill JM, Carek PJ, Geesey ME. The combined effect of transferrin saturation and low density lipoprotein on mortality. Fam Med. 2004; 36 (5): 324-329.

Wieckowska A, Papouchado BG, Li Z, Lopez R, Zein NN, Feldstein AE. Increased hepatic and circulating interleukin-6 levels in human nonalcoholic steatohepatitis. Am J Gastroenterol. 2008; 103: 1372-1379.

Wilcken B, Bamforth F, Li Z, Zhu H, Ritvanen A, Redlund M, Stoll C, Alembik Y, Dott B, Czeizel AE, Gelman-Kohan Z, Scarano G, Bianca S, Ettore G, Tenconi R, Bellato S, Scala I, Mutchinick OM, López MA, de Walle H, Hofstra R, Joutchenko L, Kavteladze L, Bermejo E, Martínez-Frías ML, Gallagher M, Erickson JD, Vollset SE, Mastroiacovo P, Andria G, Botto LD. Geographical and ethnic variation of the 677C-T allele of 5,10 methylenetetrahydrofolate reductase (MTHFR): findings from over 7000 newborns from 16 areas world wide. J Med Genet. 2003; 40: 619-625.

Williams JF. Optimization strategies of the polymerase chain reaction. Biotechniques. 1989; 7: 762–768.

Williamson D, Brown K, Luddington R, Baglin C, Baglin T. Factor V Cambridge: a new mutation (arg306-to-thr) associated with resistance to activated protein C. Blood. 1998; 91: 1140-1144.

Witsch-Baumgartner M, Gruber M, Kraft HG, Rossi M, Clayton P, Giros M, Haas D, Kelley RI, Krajewska-Walasek M, Utermann G. Maternal apo E genotype is a modifier of the Smith-Lemli-Opitz syndrome. J Med Genet. 2004; 41: 577-584.

Yamada K, Chen Z, Rozen R, Matthews RG. Effects of common polymorphisms on the properties of recombinant human methylenetetrahydrofolate reductase. Proc Nat Acad Sci. 2001; 98: 14853-14858.

Yang S and Rothman RE. PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. Lancet Infect Dis. 2004; 4: 337-348.

Yoneda M, Mawatari H, Fujita K, Iida H, Yonemitsu K, Kato S, Takahashi H, Kirikoshi H, Inamori M, Nozaki Y, Abe Y, Kubota K, Saito S, Iwasaki T, Terauchi Y, Togo S, Maeyama S, Nakajima A. High-sensitivity C-reactive protein is an independent clinical feature of non-alcoholic steatohepatitis (NASH) and also of the severity of fibrosis in NASH. J Gastroenterol. 2007; 42: 573-582.

Younossi Z. Current management of non-alcoholic fatty liver disease. Aliment Pharmacol Ther. 2008; 28: 2-12.

Zalavras CG, Giotopoulou S, Dokou E, Mitsis M, Ioannou HV, Tzolou A, Kolaitis N, Vartholomatos G. Lack of association between the C677T mutation in the 5,10-methylenetetrahydrofolate reductase gene and venous thromboembolism in Northwestern Greece. Int Angiol. 2002; 21: 268-271.

Zanzoni A, Soler-López M, Aloy P. A network medicine approach to human disease. FEBS Lett. 2009; 583 (11): 1759-1765.

Zehnder JL, Hiraki DD, Jones CD, Gross N, Grumet FC. Familial coagulation factor V deficiency caused by a novel 4 base pair insertion in the factor V gene: factor V Stanford. Thromb Haemost. 1999; 82: 1097-1099.

Zetterberg H, Regland B, Palmer M, Ricksten A, Palmqvist L, Rymo L, Arvanitis DA, Spandidos DA, Blennow K. Increased frequency of combined methylenetetrahydrofolate reductase C677T and A1298C mutated alleles in spontaneously aborted embryos. Europ J Hum Genet. 2002; 10: 113-118.

Zetterberg M, Tasa G, Palmer MS, Juronen E, Teesalu P, Blennow K, Zetterberg H. Apolipoprotein E polymorphisms in patients with primary open-angle glaucoma. Am J Ophthal. 2007; 143: 1059-1060.

Zhang AS, West AP Jr, Wyman AE, Bjorkman PJ, Enns CA. Interaction of hemojuvelin with neogenin results in iron accumulation in human embryonic kidney 293 cells. J Biol Chem. 2005; 280: 33885–33894.

Zhu J, Ren A, Hao L, Pei L, Liu J, Zhu H, Li S, Finnell RH, Li Z. Variable contribution of the MTHFR C677T polymorphism to non-syndromic cleft lip and palate risk in China. Am J Med Genet. 2006; 140A: 551-557.

Zivelin A, Mor-Cohen R, Kovalsky V, Kornbrot N, Conard J, Peyvandi F, Kyrle PA, Bertina R, Peyvandi F, Emmerich J, Seligsohn U. Prothrombin 20210G-A is an ancestral prothrombotic mutation that occurred in whites approximately 24,000 years ago. Blood. 2006; 107: 4666-4668.

Zivelin A, Rosenberg N, Faier S, Kornbrot N, Peretz H, Mannhalter C, Horellou MH, Seligsohn U. A single genetic origin for the common prothrombotic G20210A polymorphism in the prothrombin gene. Blood. 1998; 92: 1119-1124.

Zorai A, Harteveld CL, Rachdi R, Dellagi K, Abbes S, Delbini P, Giordano PC. Frequency and spectrum of hemochromatosis mutations in Tunisia. Hematol J. 2003; 4: 433-435.

Electronic References

National Centre for Biotechnology Information: Genes & Expression – Gene: APOE apolipoprotein E [Homo sapiens] [Internet]. [updated 2011 May 12]. Bethesda (MD): National Center for Biotechnology Information, U.S. National Library of Medicine; [cited 2011 May 15]. Available from:
http://www.ncbi.nlm.nih.gov/sites/entrez?Db=gene&Cmd=ShowDetailView&TermToSearch=348

NCBI: http://www.ncbi.nlm.nih.gov

NanoDrop® users manual: http://www.nanodrop.com/techsupport/nd-1000-usersmanual.pdf

Pathology Supported Genetic Testing: www.gknowmix.com

Appendix A

Dr FC Kruger
Durbanville Medi Clinic
Room 106
Wellington Road
Durbanville
7550

Dear Dr Kruger

"Non-alcoholic fatty liver disease (NAFLD): the genotype and phenotype expression in the South African setting."

ETHICS REFERENCE NO: N04/02/033

RE : PROGRESS REPORT

At a meeting of the Health Research Ethics Committee that was held on 17 November 2010, the progress report for the abovementioned project has been approved and the study has been granted an extension for a period of one year from this date.

Please remember to submit progress reports in good time for annual renewal in the standard HREC format.

Approval Date: 17 November 2010 Expiry Date: 17 November 2011

Yours faithfully

MRS MERTRUDE DAVIDS
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za
Fax: 021 931 3352

Appendix B

17 May 2011

MAILED

Dr MJ Kotze
Department of Pathology
6th Floor, Clinical building
Stellenbosch University
Tygerberg campus
7505

Dear Dr Kotze

"Development and application of a pathology supported genetic assay to assess the impact of hereditary factors on health outcomes in individuals subjected to a wellness screen."

ETHICS REFERENCE NO: N09/08/224

RE : PROGRESS REPORT

At a meeting of the Health Research Ethics Committee that was held on 16 May 2011, the progress report for the abovementioned project has been approved and the study has been granted an extension for a period of one year from this date.

Please remember to submit progress reports in good time for annual renewal in the standard HREC format.

Approval Date: 16 May 2011 Expiry Date: 16 May 2012

Yours faithfully

MRS MERTRUDE DAVIDS
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za
Fax: 021 931 3352