Investigation of the relationship between genetic and environmental risk factors associated with obesity and insulin resistance in South African patients with non-alcoholic fatty liver disease (NAFLD)

By

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Thesis presented in partial fulfilment of the requirements for the degree of Master of Medical Science (MMedSc Pathology) at Stellenbosch University South Africa

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October 2012
Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: ..................  Date:  ..................
SUMMARY

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in the world. The disease spectrum of NAFLD extends from steatosis (types 1,2) to non-alcoholic steatohepatitis (NASH) with inflammation (types 3,4). The aims of the study were 1) to analytically validate high-throughput real time polymerase chain reaction (RT-PCR) assays for three selected single nucleotide polymorphisms (SNPs), FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G), and 2) to perform genotype-phenotype association studies in relation to biochemical abnormalities, disease severity and age of onset.

A total of 119 patients with fatty liver identified on ultrasound, including 88 histologically confirmed NAFLD patients, and 166 control individuals were genotyped for the three selected SNPs. RT-PCR validated against direct sequencing as the gold standard was used for detection of genetic variation. All three SNPs were in Hardy Weinberg equilibrium in the study population, except for a deviation in genotype distribution detected for PPARγ rs1801282 in the NAFLD patient subgroup (p<0.001). After adjustment for age and gender, the risk-associated FTO rs9939609 A-allele was detected at a significantly higher frequency in the Caucasian compared with Coloured patients (p=0.005). The opposite was detected for the risk-associated TNF-α rs1800629 A-allele, which occurred at a significantly higher frequency in the Coloured compared with Caucasian NAFLD patients (p=0.034).

The onset of fatty liver disease symptoms was on average 5 years younger in the presence of each risk-associated TNF-α rs1800629 A-allele (p=0.028). When considered in the context of an inferred genotype risk score ranging from 0-6, disease onset occurred on average 3 years earlier (p=0.008) in the presence of each risk-associated FTO A-allele, TNF-α A-allele or PPARγ C-allele. After adjustment for age, gender and race, no differences in genotype distribution or allele frequencies were observed between histologically confirmed NAFLD (types 1,2) and NASH (types 3,4) patients, while the minor allele frequency for the TNF-α rs1800629 was significantly higher in the total NAFLD (types 1-4) (p=0.047) as well as NASH subgroup (NAFLD types 3,4) (p=0.030) compared with obese patients without a histologically confirmed NAFLD diagnosis. A significant correlation was furthermore observed between the number of TNF-α rs1800629 A-alleles and increasing CRP levels (p=0.029), with a favourable
reduced effect in the presence of low- to moderate alcohol intake. The average waist circumference of physically active NAFLD patients was 12% lower than in physically inactive patients (p=0.004).

In view of the results presented in this study, the inclusion of the selected SNPs, and in particular the pro-inflammatory TNF-α rs1800629 polymorphism, may be considered as part of a comprehensive cardiovascular risk evaluation of NAFLD patients. Ultimately, early detection of patients with fatty liver disease symptoms and effective intervention based on the underlying disease mechanism may prevent progression from NAFLD to NASH, shown to be an independent risk factor for cardiovascular diseases.
Opsomming

Nie-alkoholie lewervervetting (NALV) is die mees algemene kroniese lewersiekte in die wêreld. Die siektespektrum van NALV strek van steatose (vervette lewer tipos 1,2) tot steatohepatitis met inflammasie (NASH tipos 3,4). Die doel van die studie was 1) om analities die hoë omset polimerase kettingreaksie (RT-PKR) metode te valideer vir die geselekteerde enkel nukleotied polimorfismes (ENPs) FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) en PPARγ rs1801282 (Pro12Ala, 34 C>G), en 2) om genotipe-fenotipe assosiasie studies uit te voer ten opsigte van relevante biochemiese abnormaliteite, graad van die siekte en aanvangsouderdom.

’n Totaal van 119 pasiënte met vervette lewers is geïdentifiseer met behulp van ultraklank, insluited 88 histologies-bevestigde NALV pasiënte, en 166 kontrole individue. Hierdie pasiënte is gegenotipeer vir die 3 geselekteerde ENP’s. RT-PKR gevalideer met direkte DNA volgorde bepaling as die goue standaard, is gebruik vir opsporing van genetiese variasie. Al die ENP’s was in Hardy Weinberg ekwilibrium in die studie populasie, behalwe vir ’n afwyking in genotipe verspreiding waargeneem vir PPARγ in die NALV subgroep (p<0.001). Nadat aanpassings gemaak is vir ouderdom en geslag, is die risiko-geassosieerde FTO rs9939609 A-alleel waargeneem teen ’n betekenisvol hoër frekwensie in die Kaukasiese pasiënte in vergelyking met Kleurling pasiënte (p=0.005). Die teenoorgestelde is waargeneem vir die risiko-geassosieerde TNF-α rs1800629 A-alleel wat voorgekom het teen ’n betekenisvol hoër frekwensie in die Kleurling NALV pasiënte, in vergelyking met Kaukasiese NALV pasiënte (p=0.034).

Die aanvang van NALV was gemiddeld 5 jaar vroeër in die teenwoordigheid van elke risiko-geassosieerde TNF-α rs1800629 A-alleel (p=0.028). Met inagneming van ’n genotipe risiko telling tussen 0–6, het aanvang van siekte gemiddeld 3 jaar vroeër voorgekom (p=0.008) in die teenwoordigheid van elke toenemende risiko-geassosieerde FTO A-alleel, TNF-α A-alleel en PPARγ C-alleel. Nadat aanpassings gemaak is vir ouderdom, geslag en ras, is geen verskille waargeneem in genotipe verspreiding of alleel frekwensies tussen histologies bevestigde NALV (tipes 1,2) en NASH (tipes 3,4) pasiënte nie, terwyl die minor alleel telling vir die TNF-α rs1800629 betekenisvol hoër was in die totale NALV (tipes 1–4) (p=0.047) asook die NASH subgroep (NALV tipes 3,4) (p=0.03) in vergelyking met vetsugtige pasiënte sonder ’n histologies bevestigde diagnose. ’n Statisties betekenisvolle korrelasie is verder waargeneem tussen die aantal
TNF-α rs1800629 A-allele en toenemende CRP vlakke (p=0.029), met n gunstige verlaagde effek in die teenwoordigheid van lae alcohol gebruik. Die gemiddelde middellyf-omtrek van fisies aktiewe NALV pasiënte was 12% minder as fisies onaktiewe pasiente (p=0.004).

Na aanleiding van die resultate van hierdie studie behoort insluiting van geselekteerde ENP’s, en in besonder die pro-inflammatoriese TNF-α rs1800629 polimorfisme, as deel van ’n omvattende kardiovaskulêre risiko evaluasie oorweeg te word. Aan die einde van die dag mag vroeë identifikasie van NALV pasiente en effektieve intervensie gebasseer op die onderliggende siekte meganisme, vordering tot NASH verhoed wat getoon is om ’n onafhanklike risiko faktor vir kardiovaskulêre siekte te wees.
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“It always seems impossible until it’s done - Nelson Mandela”
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LIST OF ABBREVIATIONS AND SYMBOLS

3’ 3-prime
5’ 5-prime
$^{32}\text{P}$ radioactive isotope of Phosphorus
$\alpha$ alpha
$\beta$ beta
© Copyright
°C degrees Celsius
= equal to
$\gamma$ gamma
> greater than
$\geq$ greater than or equal to
$\mu g/l$ microgram per litre
$\mu l$ micro litre
- minus
% percentage
+ plus
± plus-minus
® registered trademark
< less than
$\leq$ less than or equal to

A
A  Adenine
A(Ala) Alanine
ABI Applied Biosystems
ALD Alcoholic liver disease
ALP Alkaline Phosphatase
ALT Alanine Transaminase
ApoC3 Apolipoprotein class III
Apo E Apolipoprotein E (allele 2, E2; allele 4, E4)
APRI Aspartate aminotransferase-to platelet ratio
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<th>Adenosine 5'-Triphosphate</th>
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<tr>
<td><strong>Bp</strong></td>
<td>base pair</td>
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<td>Cytosine</td>
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<tr>
<td><strong>CRP</strong></td>
<td>C - reactive protein</td>
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<td>Acronym</td>
<td>Definition</td>
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<td>Fatty Liver Disease</td>
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<td>FRET</td>
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<td>Insulin resistance</td>
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<td>K</td>
<td>Kilo</td>
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<tr>
<td>k</td>
<td>kilo</td>
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<tr>
<td>kb</td>
<td>kilo-bases</td>
</tr>
<tr>
<td>KCNJ11</td>
<td>Potassium inwardly-rectifying channel subfamily J</td>
</tr>
<tr>
<td>kD</td>
<td>kilo-Dalton</td>
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<tr>
<td>Symbol</td>
<td>Term</td>
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<td>--------</td>
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<tr>
<td>L</td>
<td>Litre</td>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<tr>
<td>LDL-C</td>
<td>Low-density lipoprotein cholesterol</td>
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<tr>
<td>M</td>
<td>Molar</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
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<tr>
<td>MGB</td>
<td>Minor Groove Binder</td>
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<tr>
<td>MgCl$_2$</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>MIM</td>
<td>Mendelian Inheritance in Man</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
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<tr>
<td>mm</td>
<td>millimeter</td>
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<tr>
<td>mM</td>
<td>milli-molar</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>N</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaCl</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Non-Alcoholic-Fatty-Liver-Disease</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non-Alcoholic-Steatohepatitis</td>
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<td>Non-Alcoholic-Steatohepatitis</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
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<tr>
<td>NFKB1</td>
<td>Nuclear factor NF-kappa-B1 subunit</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>ng/µl</td>
<td>nanogram per micro litre</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-Steroidal Anti-Inflammatory Drug</td>
</tr>
<tr>
<td>NTC</td>
<td>Non-Template Control</td>
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<tr>
<td>NF-κβ</td>
<td>Nuclear Factor κβ</td>
</tr>
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P
p short arm of chromosome
P Phosphorous
PNPLA3 Patatin-like phospholipase domain containing protein 3
P (Pro) Proline
PCR Polymerase Chain Reaction
pmol picomole
PPARγ Peroxisome Proliferator Receptor gamma

Q
q long arm of chromosome
QUICKI Quantitative Insulin Sensitivity Check Index

R
Refseq Reference sequence
RFLP Restriction Fragment Length Polymorphism
RNA Ribonucleic Acid
rpm revolutions per minute
ROS Reactive Oxygen Species
RT-PCR Real-Time Polymerase Chain Reaction

S
SNP(s) Single Nucleotide Polymorphism(s)

T
T (Thr) Threonine
T Thymine
Tₐ Annealing temperature
Taq *Thermus aquaticus* Polymerase enzyme
TBE Tris-Borate-EDTA buffer
TCF7L7 Transcription factor 7-like 2
TE Tris-EDTA buffer
Tₘ Melting Temperature
TM Trademark
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>V</td>
<td>Volts</td>
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<tr>
<td>v</td>
<td>volume</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
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<tr>
<td>v/v</td>
<td>volume per volume</td>
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<tr>
<td>W</td>
<td>Weight</td>
</tr>
<tr>
<td>WC</td>
<td>waist circumference</td>
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<tr>
<td>w/v</td>
<td>weight per volume</td>
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<tr>
<td>X</td>
<td>times</td>
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<tr>
<td>x</td>
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<tr>
<td>x g</td>
<td>times gravity</td>
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CHAPTER I

Review of Literature
1.1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a clinical histo-pathological disease with histological features that resemble alcohol-induced liver disease, but manifests when fat is deposited (steatosis) in the liver without excessive alcohol consumption. The histological spectrum of NAFLD ranges from fat that accumulates in hepatocytes without inflammation (hepatic steatosis) or severe fibrosis to complex hepatic steatosis, with concomitant inflammation (steatohepatitis).

NAFLD is well known to progress to serious liver related cardiovascular diseases (carotid artery wall thickening, vasodilation), especially in type 2 diabetic individuals (Targher et al. 2007; Neuschwander-Tetri et al. 2003). Obesity, diabetes and insulin resistance (IR) are the main contributing risk factors for more advanced forms of liver diseases. According to Marchesini et al. (2003) 70-80% of individuals with NAFLD have insulin resistance, obesity and/or diabetes as confirmed by Ratziu et al. (2010).

NAFLD is the most common cause of liver disease in westernised countries, affecting almost 33% of the general population and up to 75% of specific sub-groups such as obese individuals. The clinical significance of NAFLD is emphasised by the fact that a considerable proportion of patients (20%-30%) progress to non-alcoholic steatohepatitis NASH (Petta et al. 2009). The pathogenesis of NAFLD has not been fully elucidated, as many risk factors contribute to the development or predisposition of NAFLD. The most extensively supported theory implicates insulin resistance (IR) as the key biological mechanism involved in the development of hepatic steatosis.

Other risk factors contributing to the pathogenesis of NAFLD are the metabolic syndrome (visceral obesity, hypertension, dyslipidaemia and elevated plasma glucose concentration), oxidative stress, cell injury, cytokines/adipokines, fibrogenesis, hepatic iron accumulation, environmental carcinogens and a genetic predisposition.

The clinical characteristics of NAFLD have recently been described for the first time in the South African population (Kruger et al. 2010). The study population included 111 patients with liver biopsy-confirmed NAFLD stage 1 and 2, of which 37% had NASH and 17% advanced fibrosis (stage 3 and 4). Insulin resistance was identified as a universal factor in these NAFLD patients studied in the Western Cape area of South Africa. Patients with NASH showed significantly higher mean serum cholesterol and triglyceride...
levels compared with those with fatty liver only. The aspartate aminotransferase to platelet ratio index (APRI) was furthermore found to be significantly increased in NAFLD patients with advanced fibrosis compared to those with no/mild fibrosis (Kruger et al. 2011). Use of APRI in a new algorithm for detection of advanced fibrosis was therefore proposed, to ultimately replace invasive histopathologic diagnosis based on liver biopsy. Recently, a significant increase in alanine transaminase (ALT) levels was detected in a subgroup of South African patients with NASH who tested positive for the iron-related H63D (Histidine position 63) and C282Y (Cysteine position 282) mutations in the High Iron Fe haemochromatosis (HFE) gene (Fisher, 2011). This finding raised the possibility that assessment of APRI in conjunction with clinically useful genetic risk factors may facilitate the prevention of cumulative risk in NAFLD patients. Deleterious mutations in the HFE gene are associated with alterations in inflammatory responses, oxidative stress, impairment of glucose metabolism and in its most severe form and loss of iron homeostasis, the preventable genetic disorder hereditary haemochromatosis (HH). Since the implementation of a routine diagnostic service for HH more than 10 years ago, the use of genetic testing in conjunction with serum iron studies have largely replaced the need for liver biopsies to diagnose HH in the local population (Kotze et al. 2004, 2009). HFE genotyping combined with measurement of ferritin, glucose and serum iron studies also facilitates discrimination between NAFLD and HH, which is complicated in the presence of diabetes shown to be an independent risk factor for development of hepatic fibrosis in HH patients (Wood et al. 2012). Effective management of type II diabetes, found to be directly related to alterations in iron status, inflammation and oxidative stress in patients with HH or NAFLD, is important to reduce the risk of liver injury and fibrosis in both conditions. Increased iron load associated with the -308 TNF-α variant warrants further studies to determine possible interaction with the HFE gene (Fargion et al. 2001a; Krayenbuehl et al. 2006) in relation to insulin resistance and inflammation in patients with NASH.

The close association between the prevalence of insulin resistance and the occurrence of steatohepatitis in NAFLD patients may have a genetic basis (Tilg and Moschen, 2011). Aller et al. (2010) reported that more than 80% of NAFLD patients with a functional single nucleotide polymorphism (SNP) at nucleotide position -308 in the promoter region of the tumour necrosis factor-alpha (TNF-α) gene exhibit inflammatory liver disease (NASH), whereas in the not detected (wild-type) group only 19.7% of
patients developed NASH. Weight-reduction can be considered an effective anti-inflammatory strategy since a marked reduction of TNF-α expression in hepatic and adipose tissue were observed after extensive weight loss (Moschen et al. 2010). This could be the first step of implementing an intervention strategy based on the detection of clinically useful polymorphisms in the TNF-α gene associated with the development of NASH or other typical NASH associated factors, such as insulin resistance. mRNA expression levels of the TNF-α gene was found to correlate significantly with the presence of NASH in South African NAFLD patients (Kruger, 2008), which support the role of TNF-α in the disease process.

Insulin resistance and type 2 diabetes develop when there is an imbalance between conditions promoting excessive fatty acid synthesis (e.g. dietary, genetic, hormonal, and exercise) combined with deficient fatty acid oxidation (Wood, 2004). Peroxisome proliferator-activated receptor γ (PPARγ), an important transcription factor, is a critical regulator of adipogenesis, insulin sensitivity and lipid metabolism (Yen et al. 1997; Gurnell et al. 2003). The missense mutation at codon 12 of the PPARγ gene results in a reduction of PPARγ activity. This risk associated allele occurs at a high frequency (~85% in Caucasians) in the general population with a modest effect translating into a large population-attributable risk (Lohmueller et al. 2003). PPARγ is the molecular target for insulin sensitizing agents like the thiazolidinedione’s, a class of anti-diabetic drugs improving insulin sensitivity (Gawrieh et al. 2012). In a meta-analysis study of more than 3000 individuals, Altshuler et al. (2000) concluded that the proline variant increases the risk of diabetes 1.25 fold and accounts for to the development of type 2 diabetes in up to 25% of the general population. The interaction between the PPARγ variant and smoking indicated that both factors exhibited a synergistic effect on the development of NAFLD (Yang et al. 2012). The interaction between these two independent risk factors underlines the importance of genetic and environmental factors contributing to the development and progression of NAFLD (Day, 2006).

Enhanced oxidative stress is associated with reactive oxygenated species (ROS) induced lipid peroxidation causing liver cell necrosis, apoptosis and immunological dysfunction that could progress to hepatic fibrogenesis (Byrne, 2010). Voluminous fatty acids and not enough fatty acid oxidation seem to be critical to the development of insulin resistance and eventually type 2 diabetes. There is also broad agreement that
long-chain $\omega$-3 fatty acids in the diet are highly beneficial, by opposing the actions of insulin through various mechanisms that may directly suppress fatty acid synthesis and promote fatty acid oxidation (Clarke, 2001). Genetic variation involved in fatty acid metabolism (PPAR$\gamma$ gene) and inflammation (TNF-$\alpha$ gene) are important in this context. Information on gene-gene and gene-environment interactions is needed in a translational context to understand the health impact and implications of SNPs in metabolic associated disorders such as NAFLD.

The common form of obesity is a multifactorial trait thought to develop from an intricate interplay of genetic and environmental factors. Therefore, the occurrence of gene-gene and gene-environment interactions make it difficult to clearly elucidate the role of specific genetic variants in obesity related risk (Marti et al. 2004). Genome wide association study’s (GWAS) for type 2 diabetes susceptibility discovered the fat mass and obesity-associated gene (FTO) (Frayling et al. 2007). Simultaneously other research studies reported that the FTO gene was strongly associated with obesity related traits (Dina et al. 2007; Scuteri et al. 2007). FTO is highly expressed in the hypothalamus, a region in the brain involved in the regulation of energy homeostasis. FTO risk variants appear to promote the development of obesity by increasing energy intake through its influence on appetite-regulating regions of the hypothalamus. Variation in the FTO gene (intron 1) is associated with obesity in both children and adults.

To the best of our knowledge FTO variation in individuals with NAFLD has never been genotyped before. Since obesity and type 2 diabetes are key contributors to the metabolic syndrome and possible parallel risk factors for the development of more advanced forms of liver disease, it is highly likely that genetic variation in FTO could be associated with the risk of developing NASH.

The underlying mechanism and pathogenesis of NAFLD remains elusive. At present, treatment is focused on managing underlying metabolic risk factors as there is no pharmacotherapy available for effective treatment of this condition. Lifestyle intervention to achieve weight loss and increase exercise is persistently associated with improved liver histology (Cheung and Sanyal, 2009). Family members of children with NAFLD are considered at high risk for NAFLD. Data provided by Schwimmer et al. (2009) suggest that familial factors are a major determinant for the development of NAFLD. Risk factors vary between populations depending on modifier genes and lifestyle related exposures.
The influence of low-to-moderate alcohol consumption in patients with NAFLD remains a controversial subject. In a study performed by Cotrim et al. (2009), 132 patients undergoing liver biopsy during bariatric surgery were divided into three groups: 1) alcohol intake greater than 20 g/day and less than 40 g/day; 2) alcohol intake less than 20 g/day, and 3) no alcohol intake. The presence of insulin resistance was similar in the groups with moderate and no alcohol consumption (81.3 and 78.7%) but significantly less in the light consumption group (54%, \( P<0.05 \)). According to Dunn et al. (2012) moderate alcohol consumption is associated with a decreased prevalence of steatohepatitis and a reduced risk of coronary heart disease (CHD) mortality with metabolic related risk factors in patients with NAFLD. Addressing the challenge of understanding the underlying mechanisms or disease processes provides an opportunity to translate new genetic knowledge into therapeutic intervention strategies.

Therefore examining the complex relations between genes and environment in the development and progression of NAFLD will give us a better understanding of potentially modifiable risk factors underlying the disease phenotype. Figure 1 shows the histological spectrum of NAFLD ranging from fat that accumulates in hepatocytes without inflammation (hepatic steatosis) or the development of complex hepatic steatosis to severe fibrosis (scarring), with concomitant inflammation (steatohepatitis).

![Histological spectrum of NAFLD](image)

**Figure 1.** Histological changes/stages of liver disease
A central feature in the pathogenesis of NAFLD, both histologically and metabolically, relate to the accumulation of triacylglycerol in the liver (Donnelly et al. 2005). Plasma non-esterified fatty acids that flow to the liver in the fasted state, delivers the bulk of fatty acids to the liver. Newly (de novo) synthesised fatty acids (lipogenesis) derived from acetyl-co-enzyme A deliver the remaining Free fatty acid (FFA) pool to the liver in the form of very low density lipoproteins (VLDLs). Hepatic steatosis is the clinical manifestation of excessive triglyceride accumulation in the liver. The overall distribution of hepatic steatosis in an urbanized American population revealed that 45% of Hispanics, followed by 33% European-Americans and 24% African-Americans developed hepatic steatosis (Browning et al. 2004).

The prevalence of hepatic steatosis in diabetic individuals as reported by Williamson et al. (2011) was 56.9%. This was considerably lower when compared to a large Italian population study of type 2 diabetic patients, in which 85.3% of patients presented hepatic steatosis (Targher et al. 2007).

1.2. NAFLD and Insulin Resistance

Insulin resistance (IR) is a patho-physiological condition, where the naturally produced hormone insulin becomes less effective at reducing blood glucose levels. The plasma glucose concentration is regulated by numerous hormones, insulin being the most important one. When glucose levels are high insulin is released into the blood stream to stimulate the following actions:

• Glucose uptake
• Inhibition of lipolysis
• Inhibition of fatty acid oxidation
• Inhibition of glycogen breakdown

The opposite apply when glucagon is released. Blood glucose levels rise and fall to meet the body’s needs. The liver plays a central role in regulating blood glucose levels. Glucose is released by the liver from the breakdown of glycogen or from intermediates of carbohydrate, protein and fat metabolism. The liver receives glucose-rich blood from the digestive tract by means of the portal vein. Blood glucose levels beyond the normal range cause adverse health effects. Risk factors that contribute to insulin resistance are grouped as genetic risk factors for example PPARγ mutation, Insulin receptor mutations,
physiological factors (age, obesity, hypertension, and hypertriglyceridemia), pathology abnormalities (metabolic syndrome, liver pathologies, infections, haemochromatosis, and hypercortisolism) and environmental risk factors (diet and lifestyle). Insulin resistance plays a key role in the development of hepatic steatosis and potentially steatohepatitis (Cullan and Redlich, 1997; Willner et al. 2001; Chitturi et al. 2002; Williamson et al. 2011).

NASH is more prevalent in patients with metabolic disorders as opposed to other associations such as drug toxicity, lipodystrophy and inherited disorders (Chitturi et al. 2001). Peripheral insulin resistance is frequently observed in obese and type 2 diabetic patients with NAFLD, however it also has been reported in patients with NASH who are not obese or diabetic (Marchesini et al. 2001; Kim et al. 2004).

Insulin resistance is a significant predictor of NAFLD and metabolic associated disorders (hypertriglyceridemia and hyperuricemia). Regardless of the strong association, not all patients with NASH exhibit insulin resistance. This suggests that NASH could be a heterogeneous disorder with more than one attributed cause. The genetic origin for insulin resistance associated with NASH remains elusive.

Excessive abdominal fat accumulation both visceral and intrahepatic correlates with abnormalities in glucose and lipid metabolism. FFA levels and intrahepatic fat are associated with hepatic IR (Gastaldelli et al. 2007). The inability of insulin in non-diabetic NAFLD patients to suppress endogenous glucose production was demonstrated by Seppala-lindroos et al. (2002). This study revealed that the amount of insulin required for normalized glycaemic control correlated with liver fat content in NAFLD patients (Bugianesi et al. 2005; Kelley et al. 2003; Seppala-lindroos et al. 2002).

Alterations in the transcriptional activity of the transcription factor peroxisome proliferator-activated receptor gamma co-activator 1α (PPARγ-1α) showed a strong association with the insulin resistance phenotype and the manifestation of NAFLD (Sookoian et al. 2010). A study conducted by Carulli et al. (2009) revealed that interleukin 6 (IL-6) polymorphisms could be used as markers for insulin resistance and inflammation. Despite recent advances in the understanding of metabolic and inflammatory pathways the complex interplay between genetic and environmental factors
involved in NAFLD is not yet fully understood. A study by Petersen et al. (2010) found that APOC3 (apolipoprotein class III) were associated with NAFLD and IR.

Treating NAFLD patients with rosiglitazone improved their insulin sensitivity and reduced the inflammation rate (Ryan et al. 2003). Thiazolidinedione anti-diabetic drug treatment significantly improved steatosis and necro-inflammation in patients with NASH and insulin resistance (Gastaldelli et al. 2009). Thiazolidinedione’s activate PPARγ by binding to its receptor; this causes up-regulation of PPARγ transcription factors that decreases IR. This gives emphasis to the beneficial effects of glucose-sensitizing medications in NAFLD patients.

Patients exhibiting NAFLD with glucose intolerance were significantly more insulin resistant when compared to glucose intolerant patients without a fatty liver (Kelley et al. 2003; Facchini et al. 2002). Lipid metabolism is altered by the resistance to the action of insulin. This enhances peripheral lipolysis, triglyceride synthesis and hepatic uptake of free fatty acids (Sanyal et al. 2001). The accumulation of triglycerides in hepatocytes cause a preferential shift from carbohydrates to free fatty acid beta-oxidation, an occurrence observed in insulin resistant individuals. This may explain the increased FFA levels observed in NAFLD patients with type 2 diabetes as opposed to type 2 diabetics without NAFLD (Kelley et al. 2003).

Undoubted epidemiological, biochemical, and therapeutic evidence support the principle that the primary pathophysiological derangement in most patients with NAFLD is the molecular pathways leading to insulin resistance at a cellular level. Genetic studies are warranted to further investigate the role of gene-environment interactions in this context.

1.3. NAFLD and the Metabolic Syndrome

The metabolic syndrome is a constellation of interconnected metabolic risk factors that contribute to the co-occurrence of both cardiovascular disease (CVD) and type-2 diabetes. Individuals with metabolic syndrome have a 2-3 fold increase risk of developing coronary heart disease (CHD; Kurl et al. 2006) with a considerable greater risk of developing diabetes (Sattar et al. 2003; Hanley et al. 2005a). Low grade inflammation and the occurrence of thrombosis are strongly associated with the metabolic syndrome (Grundy et al. 2005).
The international diabetes federation (IDF) consensus worldwide definition of the metabolic syndrome (Anderson et al, 2001; Bonora et al, 1998; Carr et al. 2004, Nakamura et al. 1994) define a person as having this condition when they have central obesity (waist circumference ≥ 94 cm for European men and ≥ 80 cm for European women, the values differ with respect to other ethnicities) with the addition of any two of the following factors:

- Raised triglyceride levels (≥1.7 mmol/L);
- Reduced high density lipoprotein (HDL) cholesterol levels (<1.03 mmo/L in males and <1.29 mmo/L in females);
- Hypertension (systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥ 85 mmHg;
- Elevated fasting plasma glucose levels (≥5.6 mmol/L).

The IDF consensus group has highlighted additional metabolic criteria that appear to be related to the metabolic syndrome. This could be included in research studies to augment the predictive power of additional factors in CVD and diabetes. The platinum standard definition of the IDF consensus group includes the following:

- Body fat distribution determined by adipose tissue biomarkers: leptin, adiponectin and Liver fat content;
- Atherogenic dyslipidemia determined by apolipoprotein B and small Low-density lipoprotein particles;
- Dysglycaemia determined by the oral glucose tolerant test (OGTT);
- Insulin resistance determined by fasting/pro-insulin, homeostasis model assessment HOMA-IR, insulin resistance by Berman minimal model, elevated free fatty acids;
- Vascular dysregulation (endothelial dysfunction and/or microalbuminuria);
- Pro-inflammatory state (elevated C-reactive protein, increased inflammatory cytokines; (eg.TNF-α) and decrease in adiponectin plasma levels);
- Prothrombotic state (fibrinolytic factors and/or clotting factors);
- Hormonal factors (pituitary-adrenal axis).

The pathogenesis of the metabolic syndrome with each of its components is complex and not fully understood; however central obesity and insulin resistance are the major
causative factors of the disease. NAFLD is considered the hepatic manifestation of the metabolic syndrome (Wild et al. 2004; Dunstan et al. 2002).

In a study done by Marchesini et al. (2003) the prevalence of metabolic syndrome increased dramatically from 18% in normal-weight individuals to 67% in obese subjects as assessed by body mass index (BMI). Insulin resistance as assessed by the HOMA-IR model was significantly associated with metabolic syndrome (odds ratio 2.5, CI 95% and P<0.001 significance). The metabolic syndrome was significantly more prevalent in patients with NASH (88%) compared to those with a fatty liver alone (53%). Logistic regression analysis demonstrated that the presence of metabolic syndrome imposed a high risk of NASH development among NAFLD subjects (odds ratio 3.2, CI 95% and P<0.03 significance) (Marchesini et al. 2003).

The frequency of obesity as a component of the metabolic syndrome in patients with NASH has been reported in about 69% to 100% of patients (Bacon et al. 1994; Pinto et al. 1996; Sattar et al. 2003; Seppa-Lindroos et al. 2002; Targer et al. 2007). In addition the frequency of type 2 diabetes mellitus as a complication of obesity has been reported in 34% to 75% of patients with NASH (Hamaguchi et al. 2005; Mulcahy, 2003; Ong et al. 2005; Pinto et al. 1996). Insulin resistance unifies NAFLD and the metabolic syndrome by the clustering of metabolic related risk factors and alterations observed in insulin resistant pathways concomitant with metabolic syndrome. This may provide the molecular and biochemical basis for the link with NAFLD and insulin resistance. Insulin resistance contributes to steatosis by enhancing free fatty acid efflux from adipose tissue to the liver causing abnormalities of lipid storage (Lewis et al. 2002). Lipid peroxidation in insulin resistance subjects may activate inflammatory cytokines, promoting the progression of steatosis to nonalcoholic steatohepatitis translating into liver fibrosis (Angelico et al. 2005).

1.3.1. Clinical diagnosis of NAFLD
Most patients exhibiting NAFLD are asymptomatic; however right upper abdominal discomfort and fatigue urge patients to seek medical attention (Bacon et al. 1994). The most common presentation of NAFLD is elevated liver enzymes (aminotransferases) detected by routine laboratory screening methods while serum ferritin is an independent predictor of histological severe fibrosis in patients with NAFLD (Kowdley et al. 2012). Liver markers that present as a consequence of liver injury are aspartate-
aminotransferases (AST) and alanine-aminotransferase (ALT) (Mulhall et al. 2002). It is important to note that normal serum aminotransferases values do not exclude the presence of abnormal liver histological features as illustrated by Mofrad et al. (2003). Other laboratory tests include cholestatic enzymes (Alkaline Phosphatases) and gamma glutamyl transferase (GGT), cholesterol, triglycerides and determination of insulin resistance using the homeostasis model assessment (HOMA) or the quantitative insulin sensitivity check index (QUICKI).

Features of metabolic syndrome with the exclusion of alcohol consumption form part of the initial diagnostic process of NAFLD. The definitive diagnosis of NAFLD is through liver biopsy, a technique not applicable for large epidemiological studies (Hanley et al. 2005b). Modern imaging systems that include ultrasound, magnetic resonance imaging, computerized tomography and spectroscopy are regularly used as a diagnostic tool to screen for NAFLD. However, these techniques can only accurately identify steatosis and cirrhosis while fibrosis and necro-inflammation may remain undetected by these instruments (Aubè et al. 2007; Friedrich et al. 2010).

A combination of different screening methods and laboratory tests could be used to develop a specific algorithm to identify patients with NAFLD/NASH and to potentially avoid the need of liver biopsies for definitive diagnoses. One example is the aspartate aminotransferase to platelet ratio index (APRI) that was evaluated and implemented as a marker for advanced fibrosis in South African NAFLD/NASH patients to avoid the need of liver biopsies (Kruger et al. 2011).

1.3.2. Genetic testing for NAFLD

Excessive alcohol consumption may translate into alcoholic liver disease (ALD) in parallel with metabolic syndrome related NAFLD; both of which are recognised as the most common causes of liver disease worldwide. Since the majority of heavy drinkers with concomitant obesity/insulin resistance develop steatosis and only a minority progress to develop steatohepatitis, fibrosis and/or cirrhosis, subtle inter-individual genetic variation interacting with environmental factors could determine the disease phenotype and progression to NAFLD. Upon completion of the human genome project in 2003 new insights into the role of genetic contributing factors relating to inter-individual variability translated into better patient care, with particular focus on the mechanistic pathogenesis of complex diseases such as NAFLD (Anstee et al. 2011). Understanding
the pathogenesis and genetic modifiers of progressive NAFLD combined with biochemical and immunological markers of liver injury could provide a new model to approach risk management of NAFLD patients. Kotze et al. (2009) established such an approach termed pathology supported genetic testing (PSGT) for incorporation of low-penetrance functional SNPs that are not sufficient to explain the phenotype into an intervention plan for complex diseases such as CVD, HH and NAFLD.

This approach to risk management requires selection of candidate genes based on both disease association and mechanism. Since selection of only a limited number of known candidate genes could underscore the potential role of other genes that may influence disease susceptibility, it is important to view any genetic findings as one component of multiple factors that may contribute to obesity and insulin resistance in NAFLD, as the focus of the current study. For this reason functional SNPs in the FTO, TNF-α and PPARγ genes were selected for analyses based on existing knowledge reported in the scientific literature.

1.4. Fat mass and obesity associated gene (FTO_MIM ID* 612938)

The FTO gene was first discovered by Peters et al. (1999) through exon trapping. It is widely expressed in a variety of human tissues, including the brain, pancreatic islets and the liver. Ubiquitous expression of FTO was found in all human embryo and adult tissues examined in a study by Boissel et al. (2009). The molecular mass of the 502 amino acid FTO protein is 58 kD. Bioinformatic analysis of FTO showed that FTO shared mutual sequence motifs with iron- and 2-oxoglutarate (2OG)-dependent oxygenases, highly expressed in the brain. FTO is localised to the nucleus, catalysing 2OG to produce succinate, formaldehyde and carbon dioxide, by-products of nucleic acid de-methylation (Gerken et al. 2007). FTO mRNA is mostly abundant in the brains of mice, particularly in the hypothalamic nuclei, the region that regulates feeding and fasting. This could explain the association between variation in the FTO gene and obesity.

Molecular Genetics

The T to A base change (rs9939609) in the first intron of the FTO gene is located on chromosome 16 q12.2. It causes an increase in gene transcription, especially in the region where the sequence is strongly conserved as demonstrated by in vitro primer extension studies (Frayling et al. 2007). The fat mass and obesity associated protein
coded by the FTO gene is also known as alpha-ketoglutarate-dependent dioxygenase FTO. The conserved amino acid sequence of the transcribed FTO protein is highly homologous with AlkB (alkylation) an enzyme that oxidatively de-methylates DNA (Gerken et al. 2007). Although there is a 50% chance of passing on the faulty gene in a family, variation in the FTO gene does not cause obesity in the absence of other genetic and environmental factors that may lead to cumulative risk.

Molecular genetic research led to the identification of numerous obesity and diabetic related genes involved in the predisposition of pancreatic B-cell function. FTO is one of the most common and extensively studied loci associated with both obesity and type 2 diabetes. Other genes include [Insulin receptor substrate-1(IRS1), Ectonucleotide pyrophosphate/phosphodiesterase-1 (ENPP1) and Gastric inhibitory polypeptide receptor (GIPR)] (Meyre, 2012). Genome wide association studies of type 2 diabetes confirmed FTO as a diabetes susceptibility locus (Frayling et al. 2007; Scott et al. 2007; Zeggini et al. 2007).

The strong association between the FTO intronic variant (rs9939609) and type 2 diabetes observed in 3757 type 2 diabetic patients in the UK (OR 1.09-1.23, p= 9 x 10^{-6}) was obliterated after the adjustment for BMI (OR 0.96-1.10, p=0.44) (Frayling et al. 2007). However, more recently Hertel et al. (2011) reported that the association of FTO with type 2 diabetes was independent of its effect on BMI. A meta-analysis of 20 686 non-diabetic individuals prospectively followed for more than 10 years revealed that 3 153 patients developed type 2 diabetes and that this incident risk was strongly associated with the FTO polymorphism (OR 1.10-1.22, p= 3.2 x 10^{-8}). After adjustment of age, sex and further adjustment of BMI during the follow up, the association of rs9939609 with the incident risk of type 2 diabetes was attenuated. The FTO minor allele increases BMI by 0.26 kg/m^2 in South Asians and 0.39 kg/m^2 in white Europeans. The heterogeneity may be due to the fact that BMI (kg/m^2) in Asians does not represent the same adiposity phenotype as in individuals of European decent (Li et al. 2012).

Spontaneous cortical activity in lean individuals is increased through insulin; however in obese individuals this effect is absent. Cerebrocortical insulin resistance prevents normal body weight regulation, because insulin is needed as an adiposity- and satiety signal (Tschritter et al. 2007; Shimaoka et al. 2010). The FTO genotypic effect on cerebrocortical insulin sensitivity is similar to the effect of increased weight; this implies
that variation in FTO contributes to the pathogenesis of obesity. The effect of the risk genotype on overall weight, detected in children from age 7 years upwards, has been estimated to be approximately increased by 3 kilograms (Freyling et al. 2007).

The expression of FTO mRNA in pancreatic beta- and alpha cells, liver cells, skeletal muscle cells and adipose tissue are modulated by type 2 diabetes status (pathogenesis), blood glucose levels, oxidation rate of glucose and treatment with the hypoglycaemic drug rosiglitazone (Bravard et al. 2011; Kirkpatrick et al. 2010). A significant increase in both FTO mRNA and protein levels were observed in muscle cells of type 2 diabetic patients when compared with lean control subjects. This confirms that the overexpression of FTO in myotubes and its role in oxidative metabolism, lipogenesis and oxidative stress, a constellation of metabolic defects is linked to type 2 diabetes. However gene expression studies have demonstrated that FTO expression in human islets cells are not associated with BMI (Kirkpatrick et al. 2010). Age may contribute to the heterogeneity of the association between FTO and BMI (kg/m$^2$) independent of sex (Hardy et al. 2009; Speliotes et al. 2010).

FTO mRNA is linked to the expression of several other genes involved in gluconeogenesis in the liver. TNF, NFkB1 (mRNA in subcutaneous adipose tissue), KCNJ11 (mRNA in beta cells); all are involved in glucose regulation. Transcription-factor-7-like-2 (TCF7L2), a major contributing factor of type 2 diabetes risk, binds to the FTO promoter region and may cause overexpression of FTO (Zhou et al. 2012).

FTO acts as a transcriptional co-activator, enhancing transactivation of CCAAT/enhancer binding proteins translating to unmethylated and methylated inhibited promoters. This epigenetic regulatory process was demonstrated by Wu et al. (2010). The role of FTO in mechanisms of nucleic acid repair and epigenetic regulation support the notion that FTO may be a pleiotropic factor contributing to obesity and type 2 diabetes (Huang et al. 2011).

**Disease Association**

The FTO A-allele was shown to be significantly associated with higher BMI, higher body fat percentage, and higher waist circumference (Dina et al. 2007; Zhou et al. 2012). It also positively correlated with other symptoms of the metabolic syndrome, including hypertension, higher fasting insulin, glucose, triglycerides and lower HDL-cholesterol.
levels. The risk of being overweight increased between 1.27 and 1.35 fold with each additional risk allele (Frayling et al. 2007). The association with type 2 diabetes has been replicated in several populations and its effect is mediated through increased BMI and adiposity (Li et al. 2012).

The occurrence of two copies of the risk-associated A-allele is the strongest common genetic predictor of obesity known to date. The A-allele is associated with an approximately 30% increase in the risk of developing obesity in the general population as a consequence of increased energy intake, especially fat intake, and impaired satiety responsiveness (Frayling et al. 2007). The average weight gain by an adult with the A-allele is 1.2 kg. The T allele is protective against overeating by promoting responsiveness to internal signals of satiety. Table 1.1 summarises examples of medical conditions associated with genetic variation in the FTO gene.

Table 1.1. A summary of the disorders that have been associated with FTO gene variation

<table>
<thead>
<tr>
<th>Associated Disorder</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer's disease</td>
<td>Keller et al. 2011</td>
</tr>
<tr>
<td>Breast and prostate cancer</td>
<td>Kaklamani et al. 2011; Lewis et al. 2010</td>
</tr>
<tr>
<td>Cerebrocortical insulin resistance</td>
<td>Tschritter et al. 2007</td>
</tr>
<tr>
<td>Endometrial cancer</td>
<td>Lurie et al. 2011</td>
</tr>
<tr>
<td>Osteoporosis phenotypes</td>
<td>Guo et al. 2011</td>
</tr>
<tr>
<td>Peripheral insulin resistance</td>
<td>Shimaoka et al. 2010</td>
</tr>
<tr>
<td>Structural brain atrophy</td>
<td>Ho et al. 2010</td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td>Meyre, 2012</td>
</tr>
</tbody>
</table>

Obesity is related to personality addictive disorders such as alcohol dependence (Barry et al. 2009). Low to moderate alcohol consumption may protect obese individuals as well as diabetic or cardiovascular patients (O’Keefe et al. 2007). The protective mechanism is possibly through the induction of microsomal ethanol-oxidizing systems and both the inhibition or secretion of ghrelin, an amino acid produced in the fundus (stomach), responsible for food seeking behaviour (Badaoui et al. 2008). Sobczyk-Kopciol et al.
Chapter I

Review of Literature

(2010) reported an inverse association of the obesity predisposing FTO polymorphism (rs9939609) with alcohol consumption and the risk for alcohol dependence.

**Population genetics**

The frequencies of the FTO rs9939609 risk associated A allele for obesity in Western and Central Europeans is 46%, in Yorubans 51% and in Chinese individuals 16% (Ho et al. 2010). The minor allele (risk allele) frequency of FTO was lower in East-Asians (12-20%) compared to South-Asians (30-33%). Interestingly, the effect of the variant on obesity related risk factors and type 2 diabetes were similar in both groups (Li et al. 2012). In white Europeans approximately 66% carry at least one risk allele and 18% carry both risk alleles. Despite the differences in genetic background, the effect of FTO on obesity related traits in East and South Asians were similar to white Europeans.

**Combined effects**

Lifestyle factors modify the genetic risk of obesity associated with variation in the FTO gene, particularly in individuals who are both inactive and have a high calorie intake. Homozygous individuals (AA) were found to be on average two BMI (kg/m²) units heavier compared to physically active homozygous individuals with the same allele (Andreasen et al. 2008). In a study by Speakmen et al. (2008) adults with the AT and AA genotypes were found to consume between 500 and 1250 kilo Joules (kJ) more each day than those carrying the protective TT genotype (equivalent to between 125 and 280 kilo calories (kC) per day more intake). Results from several studies have shown that high physical activity could reduce the effect of FTO on the risk of obesity. A long term intervention diet study revealed that a diet rich in monosaturated and polyunsaturated fat (Mediterranean diet) especially in AA genotype subjects reduced body weight. This supports the notion of a conventional low-fat diet being beneficial for the A allele carriers (Razquin et al. 2010). In a similar manner, diets with different macronutrient composition (i.e. fat, carbohydrate, and protein) and fibre content could also influence appetite and satiety and thereby influence the risk. Weight reduction is associated with improved plasma glucose and insulin levels in diabetic patients (Shai et al. 2008).

An interaction between FTO and the apolipoprotein E (ApoE) gene known to be strongly associated with Alzheimer’s disease was reported by Keller et al. (2011). Those individuals who test positive for both the FTO AA genotype and the E4 allele of the ApoE gene had the highest risk for development of Alzheimer’s disease. The finding that the
FTO AA-genotype increases the risk of dementia in particular Alzheimer's disease, independently of physical activity, BMI, diabetes and CVD is in line with its association with reduced brain volume and impaired verbal fluency in cognitively healthy individuals (Benedict et al. 2011; Ho et al. 2010).

Common SNPs such as FTO are influenced by other parameters including lifestyle choices, gender and ethnicity. Their combined effects play an important role in fat deposition, obesity prevalence and incidence. The FTO risk allele could be a predictive factor for the development of obesity and subsequently type 2 diabetes, and these risk factors combined may also contribute to the pathogenesis of NAFLD. To our knowledge the FTO rs9939609, intron 1 T>A has not been studied in a South African population and have not been investigated in patients with NAFLD.

1.5. Tumour necrosis factor alpha (TNF-α_MIM ID*191160)

Tumor necrosis factor alpha (TNF-α), a multifunctional pro-inflammatory cytokine secreted mainly by macrophages, plays a key role in immune function. Its effect on inflammation, apoptotic cell death, cellular proliferation, tumorgenesis, lipid metabolism, coagulation, insulin resistance and endothelial function makes it one of the most extensively studied genes to date.

TNF was originally identified in Mus musculus serum after a Mycobacterium bovis strain injection (Pennica et al. 1984; Shirai et al. 1985.) It is also known as cachexin and was firstly recognised for its ability to lyse tumors. The biologically active form of TNF-α binds to either tumor necrosis factor receptor 1 (TNFR1) or tumor necrosis factor receptor 2 (TNFR2) initiating the immune response (Zhang, 2004).

Molecular genetics

The TNF gene maps to chromosome 6p21.3 and contains 4 exons which span 3 kilobases. The last exon codes for more than 80% of the functional protein. TNF is mainly produced as a 212-amino acid transmembrane protein arranged in stable homotrimers (Tang et al. 1996). Proteolytic cleavage by the metalloprotease TNF alpha converting enzyme yields the soluble form of the homotrimeric cytokine (Black et al. 1997). The region spanning the TNF cluster in the human major histocompatibility complex has been implicated in the susceptibility to numerous diseases, including diabetes (Stechova et al. 2012). The nucleotide change (mutation) of guanine to adenine
at position -308, also known as rs1800629 at the TNF-α promoter region forms the three common genotypes: not detected (GG), heterozygous (GA) and homozygotes (AA).

TNF is employed against a variety of pathogens (defence against infection), each relating to a specific pattern of risk or benefit. This could favour the diversity of genetic and environmental elements controlling or influencing TNF production. The relationship between genetic variation of TNF-α and anthropometric variables associated with insulin resistance and diabetes were demonstrated as early as 1999 by Zinman et al. (1999).

TNF-α is an important cytokine regulating factor in insulin pathways, stimulating the release of free fatty acids from adipocytes. Several studies have implicated TNF-α gene variation, in the development of NASH. The levels of TNF-α was reported higher in steatohepatitis subjects when compared to healthy controls. Functional SNPs are associated with an increased risk of genetic susceptibility to NAFLD. A frequency of 0.31 was reported in carriers of the -238 TNF-α polymorphism compared to 0.15 in the control group. Patients positive for both the -238 and -308 TNF-α polymorphisms exhibited higher insulin resistance, a higher prevalence of impaired glucose tolerance, and a lower number of associated risk factors for steatosis (Wang et al. 2011).

The synthesis of pro-inflammatory cytokines such as TNF-α is an early and important marker of liver injury, triggering the production of many immune-modulatory cytokines resulting in the recruitment of inflammatory cells to initiate fibrogenesis. The depletion of critical important cytokines impairs the liver’s ability to regenerate itself, emphasizing the importance of cytokines in liver regeneration (Cressman et al. 1996).

Chronic low grade inflammation has been implicated in both obese and diabetic patients. Enhanced TNF-α expression observed in adipose tissue of obese individuals reduced synergistically with weight loss (Tilg and Moschen, 2010). Fatty acids and TNF-α molecules stimulate the inhibitory phosphorylation of serine in the insulin receptor. This demonstrated the role of TNF-α in the pathogenesis of diabetes and/ or obesity. TNF-α antibody administration is beneficial in metabolic inflammation and has been demonstrated to improve steatosis (Barbuio et al. 2007). TNF-α expression in NASH/NAFLD patients significantly correlated with the degree of fibrosis (Manco et al. 2007; Wong et al. 2006). Ligands of the peroxisome proliferator receptor gamma
(PPARγ), pioglitazone, are able to suppress TNF-α function which positively affect fatty liver disease (Musso et al. 2012).

It is clear from the aforementioned that NAFLD/NASH has a heritable component, emphasizing that genetic components may predispose individuals to these traits. NAFLD is a syndrome overlapping with obesity related traits and insulin resistance; it seems likely that treating or managing these diseases could influence the genetic aspect of the syndrome.

**Disease association**

Overproduction of TNF-alpha has been associated with a variety of human diseases, including obesity, insulin resistance, hypertension, osteoporosis and cancer. The risk of acute kidney allograft rejection was 2.5 times higher among the A-allele carriers compared to the GG carriers in patients having transplant surgery (Pawlik et al. 2005). A meta-analysis of 2500 participants indicated that carriers of the A-allele were 1.46 times higher at risk of developing asthma compared to individuals without the risk allele (Aoki et al. 2006). The association between rs1800629 and the risk of chronic obstructive pulmonary disease was statistically significant in the Asian population (Zhang et al. 2011). It was proposed that TNF-α could be a modifying gene for diabetes; especially in type 2 diabetic patients from families with a history of both type 1 and type 2 diabetes. The -308 G>A TNF-α variant was associated with an insulin deficient phenotype in type 2 diabetic patients (Li et al. 2003).

Circulating TNF-α molecules derived from adipose tissue induces the activation of serine kinases, which stimulates the transcription of inflammatory genes. These genes transcribe for an inflammatory protein within tissues, including the liver and muscles, enhancing inflammation and insulin resistance (Kelly et al. 2011).

The significance of the TNF-alpha -308 A-allele in metabolic syndrome was demonstrated in a meta-analysis, indicating a 23% increased risk of developing obesity compared with controls as well as significantly higher systolic arterial blood pressure and plasma insulin levels (Sookoian et al. 2005). TNF promotes the inflammatory response, which in turn causes many of the clinical problems associated with autoimmune disorders commonly treated by using TNF inhibitors (e.g. infliximab/Remicade, adalimumab/Humira, etanercept/Enbrel) (Serio, 2003; Utz et al. 2003). Relevant
autoimmune disorders include rheumatoid arthritis (Guis et al. 2007), ankylosing spondylitis (Zhu and Yang, 2008), Crohn's disease (Han et al. 2010), psoriasis (Li et al. 2007) and refractory asthma (Aoki et al. 2006). Furthermore depressive symptoms improved in individuals with major depression when TNF-α levels were reduced (Cerri et al. 2010). According to Tilg and Moschen, (2011) more than 80% of individuals with the heterozygous rs1800629 TNF-α genotype (GA) exhibited inflammatory liver disease (NASH), compared to only 19.7% without the risk associated A-allele. The GA genotype group demonstrated more liver fibrosis, elevated TNF-α serum levels and a higher rate of insulin resistance, leading to the conclusion that variation of TNF-α may predispose individuals to develop NASH. Table 1.2 summarises examples of medical conditions associated with genetic variation in the TNF-α gene.

**Table 1.2.** A summary of disorders that have been associated with TNF-α gene variation

<table>
<thead>
<tr>
<th>Associated disorder</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allograft rejection</td>
<td>Pawlik et al. 2005</td>
</tr>
<tr>
<td>Asthma</td>
<td>Aoki et al. 2006</td>
</tr>
<tr>
<td>Ankylosing spondylitis</td>
<td>Zhu and Yang, 2008</td>
</tr>
<tr>
<td>Chronic obstructive pulmonary disease</td>
<td>Zhang et al. 2011</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>Ferreira et al. 2005; Han et al. 2010</td>
</tr>
<tr>
<td>Graves’ disease</td>
<td>Li et al. 2008</td>
</tr>
<tr>
<td>Heart disease</td>
<td>Antonicelli et al. 2005</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>Jeng et al. 2007</td>
</tr>
<tr>
<td>Leprosy</td>
<td>Vejbaesya et al. 2007</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>Bel et al. 2007</td>
</tr>
<tr>
<td>Major depression</td>
<td>Cerri et al. 2010</td>
</tr>
<tr>
<td>Non-alcoholic steatohepatitis</td>
<td>Tilg and Moschen, 2011</td>
</tr>
<tr>
<td>Psoriasis</td>
<td>Li et al. 2007</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>Guis et al. 2007</td>
</tr>
</tbody>
</table>
Population genetics

The frequency of the -308 TNFα (G>A) SNP varies between different ethnic groups. For instance, the minor allele frequency in Chinese individuals is 9%, French and Scandinavian 16%, Germans 18% and up to 24% in Australians (Feng et al. 2011). The overall frequency of the -308 polymorphism in a NAFLD population group studied by Wang et al. (2012) was 14.2% and in the control group 15.6%. The genotype distribution of the TNF-α -308 polymorphism in 244 South African women with cervical cancer was 71% (GG), 26% (GA) and 3% (AA) respectively (Govan et al. 2006). A study conducted by Stanczuk et al. (2003) in Zimbabwe reported that 72% (GG) of cervical cancer patients tested negative for TNF-α -308, 27% (GA) were heterozygous and 1% homozygous (AA) for the risk-associated allele.

Combined effects

Macrophages infiltrating adipose tissue, promote the production of cytokines such as TNF-α, interleukin 6 (IL-6) and monocyte chemo-attractant protein-1 (MCP-1). These cytokines contribute to the development of inflammation-induced insulin resistance and eventually type 2 diabetes (Gonzalez et al. 2005; Heilbronn and Campbell, 2008). Exercise improves both insulin sensitivity and inflammation status in obese individuals compared with lean individuals (Kelly et al. 2011). The combined advantage of managing both dietary carbohydrate intake (Low-glycaemic index food) and lifestyle factors (exercise) were demonstrated in obese individuals, suggesting potential reversal of the effects of obesity on inflammation and insulin resistance. Furthermore, lifestyle and diet intervention decreased circulating TNF-α, IL-6 and MCP-1 cytokines, collectively controlling hyperglycaemia in patients (Hartman et al. 2010).

Co-existence of the TNF-alpha -308 G>A and IL-6 -174G>C gene variations (A-C allelic combination) is a strong predictor for conversion of insulin resistance to type II diabetes. When considered separately, only the TNF-alpha -308 A-allele predicted the progression to type II diabetes, but the combined effect is much stronger due to gene-gene interaction (Haddy et al. 2005).

In NASH patients elevated hepatocyte apoptosis significantly correlated with disease severity, suggesting that apoptotic cells could be a powerful predictor of NASH (Zhang et al. 2010). TNF-α is not only involved in the pathogenesis of NAFLD, it also correlates to
other components of metabolic syndrome. Its effect on metabolic syndrome is marked through changes in lipid metabolism and insulin levels. TNF-α is a marker of systemic inflammation during cardiovascular events and a risk factor of metabolic syndrome which forms part of a constellation of risk factors contributing to the pathogenesis of NAFLD in South African patients (Kruger, 2008).

1.6. Peroxisome proliferator nuclear receptor gamma (PPARγ MIM ID* 601487)

Peroxisome proliferator nuclear receptors, such as peroxisome proliferator-activated receptor gamma (PPARγ), induce the proliferation of peroxisomes. These organelles contribute to the oxidation of fatty acids and control the expression of multiple targeted genes. PPARs serve as unique lipid sensors, activated by both fatty acids and metabolic by-products redirecting the metabolism. Although PPARγ is expressed throughout the body it is mainly expressed in liver and adipose tissue where it’s involved in adipocyte differentiation (Gouda et al. 2010).

Molecular genetics

Somatic cell hybridization and linkage analysis mapped the PPARγ gene to chromosome position 3p25. The gene contains 9 exons and spans 100 kb producing 2 protein isoforms, PPARG1 and PPARG2 with alternative mRNA splicing of the gene (Fajas et al. 1997; Greene et al. 1995). The unique ligand binding domain of PPARγ mark its mode for receptor interaction that translates into distinct pharmacological properties (e.g. Rosiglitazone and Thiazolidinedione anti-diabetic drugs). Modulators of PPARγ are selective for PPARγ insulin signalling and adipogenic signalling pathways. The C to G missense mutation at nucleotide position 34 of the PPARγ gene results in the substitution of an alanine for a proline at amino acid position 12 (CCG-Pro to GCG-Ala). The proline variant is associated with a reduction in PPARγ activity and differential lipid response to dietary fat intake (Gouda et al. 2010).

Type 2 diabetes develops after years of increased insulin resistance and eventual loss and failure of pancreatic beta-cells. The genetic constitution of an individual may determine who is likely to develop insulin resistance and type 2 diabetes or who, regardless of their diet or lack of exercise, may be resistant to the development of diabetes. PPARγ has been identified as a key factor in this process due to its critical role in regulation of adipogenesis, insulin sensitivity and lipid metabolism (Gouda et al.
The association between the Pro12Ala variant (rs1801282) and diabetes has been a major focus of several meta-analysis studies (Altshulder et al. 2000; Gouda et al. 2010; Ludovico et al. 2007; Vardarli, 2007). Insulin resistance and type 2 diabetes associated with this SNP often co-exist with CVD, obesity, dyslipidaemia, non-alcoholic hepatic steatosis and metabolic syndrome.

**Disease association**

PPARγ represents an attractive NAFLD candidate gene, because both hepatic lipid metabolism and insulin resistance are contributors to the pathogenic phenomena of NAFLD (Gawrieh et al. 2012). Moreover, numerous PPARγ agonists have been used in therapeutic intervention trials for individuals with NASH, emphasizing the role of PPARγ in liver related diseases (Sanyal et al. 2010).

A major contributor to end stage renal disease in developed countries is diabetic nephropathy and PPARγ has been linked to the risk of developing this condition (Liu et al. 2010). Enhanced oxidative stress, especially in hyperglycaemic conditions contributes to the development and progression of diabetic nephropathy (Forbes et al. 2008). A significant association between carriers of the Pro12 variant and diabetic nephropathy were made, with a possible synergistic interaction between smoking and the PPARγ Pro12 genotype (Liu et al. 2010). Experimental studies of PPARγ agonists showed a beneficial effect of PPARγ variants on ulcerative colitis. The Pro12Ala SNP influences PPARγ activity, especially in immunocytes and epithelial cells, as a result of this susceptibility to inflammatory bowel disease is altered (Zhang et al. 2012). Another study indicated a significant association between the Pro12Ala mutation and Crohn’s disease (Aoyagi et al. 2010). Cellular and immune dysfunction in combination with environmental factors influence the transcription of PPARγ therefore influencing the epithelial cells found in patients with ulcerative colitis. Genetic variation in PPARγ alters the susceptibility to hepatic steatosis, lobular inflammation and fibrosis in patients with histologically advanced NAFLD (Gawrieh et al. 2012).

The clinical outcome of patients may be affected by the genetic background of the individual. To determine whether low-penetrance mutations like the Pro12Ala could predispose to NAFLD all known contributing factors need to be considered in
conjunction with clinical co-factors. Table 1.3 summarises examples of medical conditions associated with genetic variation in the PPARγ gene.

**Table 1.3. A summary of the disorders that have been associated with PPARγ gene variation**

<table>
<thead>
<tr>
<th>Associated Disorder</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crohns disease</td>
<td>Aoyagi <em>et al.</em> 2010</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Gawrieh <em>et al.</em> 2012</td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>Lui <em>et al.</em> 2010</td>
</tr>
<tr>
<td>Non-alcoholic fatty liver disease</td>
<td>Gawrieh <em>et al.</em> 2012</td>
</tr>
<tr>
<td>Polycystic ovary syndrome</td>
<td>Christopoulos <em>et al.</em> 2010</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>Zhang <em>et al.</em> 2012</td>
</tr>
</tbody>
</table>

**Combined effects**

As previously mentioned, PPARγ controls the expression of several genes involved in adipocyte differentiation, lipid storage and insulin sensitivity. PPARγ is also activated by fatty acids, thus the effect of fatty acids on glucose metabolism might be mediated through the action of PPARγ and therefore could modify its association with fatty acids glucose levels and insulin resistance (Yionen *et al.* 2008).

Diets rich in polyunsaturated fatty acids (eicosapentaenoic acid) may up regulate the expression of PPARγ to a greater extent compared to diets high in saturated fatty acids, thereby mediating an individual's physiological response to lipids. An intervention study suggested that fish oil supplementation could affect insulin concentrations and serum triacylglycerol levels especially in PPARγ genotypic individuals (Lindi *et al.* 2003).

Carriers of the Ala12 variant eating a westernised diet have a lower risk of developing insulin resistance and diabetes than Pro/Pro homozygotes on the same diet (Hara *et al.* 2002). In the presence of this protective allele, intake of a significantly greater amount of polyunsaturated and monounsaturated fats than saturated fats exert a beneficial effect on weight loss. The possible mechanisms by which fatty acids beneficially mediate
glucose metabolism include lowering of triacylglycerol, free fatty acids levels and intramuscular lipids (Pighin et al. 2003).

Variation in PPARy is considered to be an important modifier of insulin resistance in Caucasian women with polycystic ovary syndrome (PCOS), a common endocrinopathy in women of reproductive age. PCOS is characterized by hyperandrogenism and chronic anovulation and is associated with hyperinsulinemia, cardiovascular risk and endothelial dysfunction (Chrisopoulos et al. 2010). Insulin sensitizing agents such as the thiazolidinediones, an agonist for the PPAR receptors, improved ovarian androgen biosynthesis and ovulatory function (Orio et al. 2003). Identification of the Pro12Ala polymorphism in women with PCOS is associated with an increased body mass index (BMI) and attenuation of insulin resistance. Non-diabetic Pro/Ala heterozygous Caucasian patients were found to be more insulin sensitive than Pro/Pro homozygotes with PCOS, as evidenced by a lower homeostasis model assessment index and lower levels of insulin at both the fasting and 2 hour time points during the oral glucose tolerance test (Hara et al. 2002).

One of the first studies to explore a gene-diet interaction with PPARy was a study conducted by Luan et al. (2001). Data from the study suggested that when the dietary polyunsaturated fat to saturated fat ratio is low, the mean BMI in Ala carriers is greater than in Pro homozygotes and when the ratio is high the opposite is true. PPARy ligands (fatty acids) might be more effective stimulators of adipogenesis especially in Pro12 individuals.

The need to include environmental data when assessing low penetrance mutations such as the Pro12Ala SNP was illustrated by Robitaille et al. (2003). They found the PPARy Ala12 variant to be associated with a higher BMI, waist circumference, fat mass and a greater accumulation of visceral and sub-cutaneous adipose tissue. However, when dietary fat and saturated fat was also taken into account, they found that these dietary variables were closely associated with components of the metabolic syndrome in Pro/Pro homozygotes, but not in Ala carriers. Pro12 individuals are considered to be hyper-responders, and are more sensitive and susceptible to environmental stimuli such as fat intake. This observation suggests a protective role in individuals that carry the Ala12 allele, emphasizing that not every one responds to a high saturated fat diet in the same way (inter-individual variability). The PPARy polymorphism modulates dietary fat
intake and components of metabolic syndrome contributes to the pathogenesis of NAFLD.

**Population genetics**

According to Paracchini *et al.* (2005) the frequency of PPARγ 34 C>G minor allele ranged from 2% to 18% in the general population. The 12Ala allele frequency in Caucasian control samples were 12.7% (median) and in East Asians 4.5% (median) (Gouda *et al.* 2010). It is important to denote the specific transcript variant used with its unique reference sequence to analyse and interpret the Pro12Ala results obtained. According to NCBI (National Centre for Biotechnology Information) the rs1801282 (Pro12Ala) variant accounts for an amino acid change at position 12. The protein coding missense mutation is situated in an exon (ENSP287820). However, depending on the specific transcript uploaded and used from NCBI, the risk variant (Pro12Ala) is situated in a synonymous coding region.

The progressive increase in the prevalence of insulin resistance and its related clinical manifestations (type 2 diabetes, obesity etc.) have led to the identification of NAFLD and its more severe and progressive subtype, NASH, as an emerging disease involving a high percentage of individuals maintaining a western type of lifestyle. Recent studies are focused on new molecular mediators that may be implicated in the progression of the disease, which may identify new molecular targets that could potentially become the object of therapeutic interventions. However, further research needs to identify the different pathways that lead to inert disease (simple fatty liver) or to progressive disease (NASH) and validating the current evidence to establish the most appropriate pharmacological and non-pharmacological therapeutic approach in treating individuals with liver related diseases.
1.7. Aims and Objectives of this study

The long-term objective of the study is to develop a pathology supported gene-based intervention program for improved clinical management of patients with NAFLD/NASH. The specific aims were:

- Analytical validation of high-throughput real-time polymerase chain reaction (RT-PCR) assays (for selected SNPs in the FTO, TNF-a and PPARg genes) against direct sequencing as the gold standard

- Comparison of genotype distribution and allele frequencies between NAFLD patient subgroups and controls

- Correlation of clinical and biochemical parameters of insulin resistance with genotype and environmental factors assessed in NAFLD patients

- Determination of the potential impact of risk factors incorporated into a genotype risk score on age of onset in fatty liver disease patients in relation to NAFLD disease severity

Ultimately, these findings will be incorporated into a comprehensive genetic screening assay for patients at increased risk of metabolic associated disorders (e.g. insulin resistance, CVD) and liver disease to be performed in conjunction with a medical and lifestyle assessment to facilitate the prevention of cumulative risk.
CHAPTER II

Research

Methodology
2.1. Study Population

Study participants were recruited at the Gastroenterology Unit of the Department of Internal Medicine, (Tygerberg Academic Hospital University of Stellenbosch) and from clinicians working at other academic or private hospitals. Participants who fulfilled the criteria for fatty liver disease were grouped as steatosis (fatty liver types 1,2) and steatohepatitis with inflammation (NASH types 3,4).

Inclusion criteria

- Voluntary written informed consenting NAFLD patients.
- Histologically and ultrasound confirmed features of NAFLD.

Exclusion criteria

- Females with a daily intake of alcohol above 20g and males with a daily alcohol intake above 30g.
- Patients with a secondary attributable cause for fatty liver disease;
  - Nutritional (rapid weight loss, starvation, intestinal bypass surgery)
  - Drugs (Glucocorticoids, Estrogens etc.)
  - Toxins
  - Metabolic (Weber-Christian disease, Wolman’s disease, Acute fatty liver of pregnancy, Reye’s disease)
- Any other histological or blood results suggestive of liver disease;
  - Increased bilirubin
  - Hypoalbuminemia
  - Elevated liver enzymes
  - Hepatitis

A questionnaire was used to indicate alcohol consumption and other personal information (including lifestyle, medications and dietary elements, family history, and clinical characteristics). Liver function tests including ALT and AST were used to diagnose underlying liver disease. Insulin resistance was determined for each patient by
using the homeostasis model assessment (HOMA) of insulin resistance (HOMA-IR) 
(fasting insulin $\mu$U/ml $\times$ [fasting glucose {mmol/l}/22.5]). In addition lipid status was 
assessed by measuring the total cholesterol, LDL cholesterol, HDL cholesterol and 
triglycerides using standard methods.

DNA samples of 119 patients with fatty liver on ultrasound, of which 88 had 
histologically-confirmed NAFLD (47 Coloured, 35 Caucasian, 5 Black and 1 Indian), 
were obtained as an extension of the parent study performed by Kruger et al. (2010). For 
the purpose of analytical validation of the SNP assays the 31 patients with fatty liver on 
ultrasound only were grouped with the histologically confirmed patients into the “NAFLD 
group”. For the purpose of genotype-phenotype correlations the 31 patients with fatty 
 liver on ultrasound only was excluded from the NAFLD patients (types 1-4) for 
comparative analysis and was grouped as “obese”. Within this group 23 patients were 
Coloured, 6 patients Caucasian and 2 patients Black. Hereafter we refer to the 31 
patients with fatty liver on ultrasound (not histologically confirmed) as the obese patient 
group. Reasons for not confirming fatty liver on histology include insufficient clinical 
indication for liver biopsy and exclusion of NAFLD after liver biopsy.

A specialized data extraction tool was used to retrieve information from an online genetic 
database (http://gknowmix.org) whereby Caucasian control individuals (n=166) for this 
study was selected. Any familial related individuals in the database were excluded using 
their family index number. Individuals with a medical history of insulin resistance, 
diabetes or NAFLD were also excluded from the control group.

**Ethical Approval**

Ethical approval for the research project on NAFLD individuals has been granted in 2004 
by the University of Stellenbosch, under project number N04/02/033 and thereafter 
annually approved until December 2012. In addition, ethical approval has been granted 
for the inclusion of control samples recruited as part of project N09/08/224 focused on 
individuals with and without features of the metabolic syndrome.

2.2. DNA Extraction

2.2.1. DNA extraction from Whole Blood using the QIAamp® DNA Blood Midi/Maxi 
kit supplied by QIAGEN (Spin protocol)
Chapter II

Research Methodology

The QIAamp DNA whole blood protocol was used to extract and purify DNA from 2 ml of whole blood that has been treated with EDTA.

QIAGEN protease stock solution (200 µl) was added with 2 ml of whole blood into the bottom of a 15 ml centrifuge tube and mixed briefly by vigorous shaking (vortexing) to increase the overall efficacy of the reaction. The protease enzyme will conduct proteolysis (protein catabolism) in the blood sample, lysing the cells to release their DNA from the nucleus into the solution. Buffer AL (2.4 ml) was added to the 15 ml centrifuge tube and mixed thoroughly by inverting the tube 15 times, followed by additional vigorous shaking of 1 min to ensure adequate lysis of the sample and to yield a homogenous solution. The homogenous solution was then air incubated on a dry block at 70 °C for 10 min to ensure for a maximum DNA yield after lysis.

Upon removal from the dry block 2 ml of ethanol (96%-100%) was added to the homogenised sample in the 15 ml centrifuge tube and mixed by inverting the tube 10 times, followed by additional vigorous shaking (vortexing). In order to ensure efficient binding of the lysate to the QIAamp® Midi spin column, it is essential to mix the sample thoroughly after the addition of ethanol.

One half (~3 ml) of the solution volume was carefully transferred on to the QIAamp® Midi spin column and placed back in to the 15 ml centrifuge tube and centrifuged at 1850 x g (3000 rpm) for 3 min. When centrifugation was completed the filtrate was discarded and the remainder (~3 ml) of the solution were transferred on to the QIAamp® Midi spin column and centrifuged at 1850 x g (3000 rpm) for 3 min. The QIAamp® Midi spin column was removed after centrifugation and the remaining filtrate discarded. Complete removal of the filtrate is necessary to prevent the nozzle of the QIAamp® Midi spin column from being submerged in the filtrate, which could reduce the washing efficacy.

Each column was properly sealed to avoid cross-contamination during centrifugation. The column was placed back into the 15 ml centrifuge tube and 2 ml of the wash buffer AW1 was added on to the column membrane and centrifuged at 4500 x g (5000 rpm) for 1 min. Thereafter wash buffer AW2 (2 ml) was added on to the QIAamp® Midi spin column and centrifuged at 4500 x g (5000 rpm) for 15 min. The increased centrifugation time is essential to remove all traces of Buffer AW2 from the column before elution, as
the residual ethanol in the elute may cause inhibition of PCR reactions leading to false-negative results.

The QIAamp® Midi spin column was then placed in a clean 15 ml centrifuge collection tube and the collection tube containing the filtrate was discarded. Nuclease free water (300 µl), equilibrated to room temperature (15-25 °C) was added directly onto the membrane of the column and incubated at room temperature for 5 min and centrifuged at 4500 x g (5000 rpm) for 2 min. To obtain maximum concentration, the eluted (300 µl) sample was reloaded onto the membrane of the QIAamp® Midi spin column and incubated at room temperature (15-25 °C) for 5 min followed by centrifugation at 4500 x g (5000 rpm) for 2 min. The DNA solution was placed on a shaker at room temperature overnight to ensure proper homogenization of the sample. Thereafter it was stored at 4 °C for frequent use and the remaining DNA solution stored in aliquots at – 20°C for long term use.

2.2.2. DNA extraction from Saliva samples using the Oragene®-DNA/ saliva Kit

The following step-by-step protocol was used to purify DNA from 500 µl of Oragene®-DNA/saliva sample.

The Oragene®-DNA/saliva sample was mixed thoroughly by inverting the vial several times followed by gentle shaking for a few seconds. This was to ensure that the viscous saliva sample was properly mixed with the Oragene®-DNA solution. The sample was incubated at 50 °C in a water bath for a minimum of 1 hour to ensure that the DNA was adequately released and the nucleases permanently inactivated. The mixed Oragene®-DNA/saliva sample (500 µl) was transferred to a 1.5 ml microcentrifuge tube. A volume of 20 µl of Oragene®-DNA purifier (OG-L2P) was added to the microcentrifuge tube and mixed by vortexing for 10 seconds.

The sample was incubated on ice for 10 min to allow for the precipitation of impurities and inhibitors. After incubation the vial was centrifuged at room temperature (15-25°C) for 5 min at 13 000 rpm (15000 x g), resulting in separation of the DNA containing supernatant from the precipitated impurities.

Glycogen was added to the supernatant to make the pellet more easily visible. The supernatant was carefully transferred with a pipet tip into a fresh microcentrifuge tube
and the pellet containing the impurities was discarded. An equal amount (500 µl) of 95-
100% ethanol at room temperature (15-25°C) was added to the supernatant and mixed
gently by inverting the tube 10 times. Thereafter, it was incubated for 10 min at room
temperature (15-25°C) to allow the DNA to fully precipitate.

The tube was loaded into a microcentrifuge in a known orientation (ex. hinge of the tube
pointing away from the centre of the rotor in order to visualize the tiny pellet at the tip of
the tube below the hinge) and centrifuged at room temperature for 2 min at 13000 rpm
(15000 x g). The supernatant was carefully removed with a pipet tip and discarded
without disturbing the DNA pellet. A volume of 250 µl ethanol (70%) was added onto the
DNA pellet and incubated at room temperature (15-25°C) for 1 min. Upon removal of the
70% ethanol, 100 µl of DNA buffer (Nuclease Free Water) was added to reconstitute the
DNA pellet, followed by vortexing for at least 5 seconds. The freshly extracted DNA from
the Oragene®-DNA/saliva sample was incubated overnight on a shaker at room
temperature (15-25°C) to ensure homogenization and stored at 4°C for frequent use.

2.3. DNA Quantification

A NanoDrop® ND-1000 spectrophotometer (NanoDrop®, Techonologies, USA) with the
v3.5.2 software package was used to determine the concentration and purity of the DNA
samples.

The instrument was turned on followed by selection of the Nucleic Acid analysis option,
thereafter cleaning of the pedestal and loading of a Nuclease Free Water sample onto
the pedestal to initialize the instrument. A volume of 2 µl RNase Free water was placed
on the pedestal to blank (containing all the components of your solution except what is to
be tested for i.e. DNA) the instrument before making any measurements. Upon
completion of the blanking process, all the samples were measured and stored. The
pedestal was cleaned between each measurement with 70% ethanol followed by double
distilled water. The ratio absorbance reading at 230 nm, 260 nm and 280 nm was used
to assess the purity of DNA. Purity values for all samples were within the acceptable
range (260/280 absorbance ratio: 1.6-1.9; 260/230 absorbance ratio : >1.9) indicating
the absence of contaminants such as proteins, salts or phenols in the sample. Nuclease
free water was used in the dilution of all the DNA samples to obtain a final concentration
of 10 ng/µl, as required for Real-Time Polymerase Chain Reaction instruments.
2.4. Polymerase Chain Reaction Amplification

2.4.1. Oligonucleotide Primers

Oligonucleotide primers were specifically designed to detect genetic variation in the FTO (rs9939609, intron 1 T>A), PPARγ (rs1801282, 34 C>G, P12A) and TNF-α (rs1800629, -308 G>A) genes using the LightCycler® Probe Design Software 2.0 (Version 1.0. R.36) package. The genomic reference sequences for FTO (NG_012969.1) PPARγ (NG_011749.1) and TNF-α (NG_007462.1) were obtained from the national centre for biotechnology information (NCBI, www.ncbi.nlm.nih.gov) database. The primers used in the conventional polymerase chain reaction (PCR) method and direct DNA sequencing reactions are summarized in table 2.1.
Table 2.1. Particulars of the primers used in the conventional PCR experiment and direct DNA sequencing.

<table>
<thead>
<tr>
<th>Gene</th>
<th>*SNP</th>
<th>Primer</th>
<th>Nucleotide Sequence (5’ to 3’)</th>
<th>G/C Content (%)</th>
<th>T_M (°C)</th>
<th>T_A (°C)</th>
<th>SNP Position</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTO</td>
<td>rs9939609</td>
<td>Forward</td>
<td>CTGGCTCTTTGAATGAAATAGGA</td>
<td>40.9</td>
<td>52.4</td>
<td>53.9</td>
<td>Intron 1 T&gt;A</td>
<td>275</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CTTAAAGTTGATGGCTTCAGGG</td>
<td>40.9</td>
<td>51.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPARγ</td>
<td>rs1801282</td>
<td>Forward</td>
<td>CTCCTAATAGGACAGTGCCA</td>
<td>50.0</td>
<td>53.5</td>
<td>55</td>
<td>P12A C&gt;G</td>
<td>734</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GCTGAGAGTACTTCAAGAG</td>
<td>50.0</td>
<td>52.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>rs1800629</td>
<td>Forward</td>
<td>CCAGCATTATGAGTCTCCG</td>
<td>52.6</td>
<td>52.7</td>
<td>56</td>
<td>-308 G&gt;A</td>
<td>742</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GCTTCTCTCCCTCTTAGC</td>
<td>55.6</td>
<td>51.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Single nucleotide polymorphism
2.4.2. PCR Amplification Mixture and Thermal Cycling Conditions.

Polymerase chain reaction amplification of the various amplicons under investigation was performed with the Promega GoTaq® Flexi DNA polymerase PCR kit. Applied Biosystems® thermal cyclers (2700, 2720 and 9700) were used to amplify the specific region of interest in 25 µl reactions. The reaction mixture consisted of 100 ng/µl template DNA, 5 x Colourless Go Taq® flexi buffer (Promega), 0.2 mM dNTP mix (dATP, dTTP, dGTP, dCTP) from Fermentas, 1.5 mM MgCl₂ (Promega), 0.24 µM of each primer (Forward and Reverse) (WhiteHead Scientific) and 1.25 U Go Taq® DNA Polymerase (Promega). The Promega PCR reagent mixture was used for all the primer sets under investigation.

The specific PCR conditions (initial denaturation, denaturation, annealing, extension and final extension) used to amplify each gene (FTO, PPARγ and TNF-α) are summarised in tables 2.2- 2.4.

**Table 2.2.** Thermal Cycling Conditions for amplification of FTO.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Cycles</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>1</td>
<td>95</td>
<td>02:00</td>
</tr>
<tr>
<td>Denaturation</td>
<td>30</td>
<td>95</td>
<td>00:30</td>
</tr>
<tr>
<td>Annealing</td>
<td></td>
<td>53.9</td>
<td>00:30</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td>72</td>
<td>00:30</td>
</tr>
<tr>
<td>Final Extension</td>
<td>1</td>
<td>72</td>
<td>05:00</td>
</tr>
</tbody>
</table>
Table 2.3. Thermal Cycling Conditions for amplification of PPARγ.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Cycles</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>1</td>
<td>95</td>
<td>02:00</td>
</tr>
<tr>
<td>Denaturation</td>
<td></td>
<td>95</td>
<td>00:30</td>
</tr>
<tr>
<td>Annealing</td>
<td>35</td>
<td>55</td>
<td>00:30</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td>72</td>
<td>00:30</td>
</tr>
<tr>
<td>Final Extension</td>
<td>1</td>
<td>72</td>
<td>05:00</td>
</tr>
</tbody>
</table>

Table 2.4. Thermal Cycling Conditions for amplification of TNF-α.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Cycles</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>1</td>
<td>95</td>
<td>02:00</td>
</tr>
<tr>
<td>Denaturation</td>
<td></td>
<td>95</td>
<td>00:30</td>
</tr>
<tr>
<td>Annealing</td>
<td>40</td>
<td>56</td>
<td>00:30</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td>72</td>
<td>00:30</td>
</tr>
<tr>
<td>Final Extension</td>
<td>1</td>
<td>72</td>
<td>05:00</td>
</tr>
</tbody>
</table>

2.5. Gel Electrophoresis

PCR products were electrophoresed on a 2.5% (w/v) horizontal agarose gel to determine successful amplification. The gel mixture consisted of 2.5g Seakem® LE agarose, 100 ml Tris-borate/EDTA (1xTBE) (90 mM Tris-HCl (pH 8.0), 90 mM boric acid (H₃BO₃), 1 mM EDTA (Ethylenediaminetetraacetic Acid) at a pH of 8.0 and 70 µl of 0.01% (v/v) Ethidium Bromide (EtBr) that was added to the electrophoretic buffer (1x TBE). The PCR product (5 µl) and Ficoll Orange G loading buffer (5 µl) [consisting of 0.02% (w/v) ficoll, 10 mM EDTA at pH 7.0] was thoroughly mixed on Para-film and loaded into the wells of the
agarose gel. A 100bp molecular size marker from Promega, Wisconsin USA verified amplification of the correct fragment size. Electrophoresis of the PCR products was conducted at 80 V for one hour in 1 x TBE buffer solution. Visualization of the PCR products was accomplished using an ultraviolet light trans-illuminator (GibcoBRL Life Technologies, California, USA).

2.6. DNA Sequencing Analysis

PCR products obtained for FTO (T>A), PPARγ (C>G) and TNF-α (G>A) were sent to the Central DNA Sequencing facility of Stellenbosch University for automated sequencing as well as post-PCR clean up. FinchTV Version 1.4.0 (developed by Geospiza Research Team) was used to analyze the ABI™ files received from the DNA sequencing facility to ultimately generate electropherograms. The application of the software was to view the nucleotide sequences of each gene (FTO, PPARγ, TNF-α) and compare it directly to the reference sequence as obtained from the NCBI (National Centre for Biotechnology Information) database for detection of SNPs.

The automated sequencing technique consists of a reaction mixture which includes a DNA template, nucleotides (dNTPs and ddNTPs), an enzyme and a single primer (forward or reverse). Four distinct colours of fluorescence are used to differentiate between the four ddNTP (ddATP, ddCTP, ddGTP and ddTTP) molecules. The sequential addition of the bases to the 3’-end of the primer sustain the elongation process (5’-3’ direction) until the incorporation of a ddNTP. The extension reaction requires a hydroxyl group (OH⁻) on the third carbon of the molecule, but ddNTP molecules carry a hydrogen atom instead therefore the extension reaction is terminated.

Upon completion of the sequencing reaction (either forward or reverse primer) that is almost identical to PCR (both forward and reverse primers), gel electrophoreses follow. The automated sequencer will detect the colour of the last base (fluorescently labelled) through excitation of a laser beam at a specific wavelength (colour dependant). Based on the wavelength of light detected by the spectrograph, the light will be separated by a specialized charge coupled camera. All the data captured by the sequencer, are represented in sequentially determined colour peaks in the form of an analysable electropherogram.
2.7. Real-Time Polymerase Chain Reaction (RT-PCR) Amplification

2.7.1. Applied Biosystems® TaqMan® SNP Genotyping Assays

The ABI™ TaqMan® SNP Genotyping 5' nuclease technology Assays are standardized assays that use two allele-specific TaqMan® Minor Groove Binder (MGB) probes (VIC® and FAM™ dye-labelled) and a specific PCR primer pair to detect the single nucleotide polymorphism (SNP) of interest. The specific probes and primers uniquely align with the template DNA, to provide unmatched specificity. The pre-designed assays in this study were used for end-point genotyping by allelic discrimination analysis for SNPs. Two different real-time PCR instruments were used i.e. the ABI™ 7900 HT fast real-time PCR machine and the Corbett Rotor-Gene™ 6000. The assay employed in this study were FTO intron 1 T>A (rs9939609, C_30090620_10), PPARγ Pro12Ala, 34 C>G (rs1801282, C_1129864_10) and TNF-α -308 G>A (rs1800629, C_7514879_10). As required by real-time PCR instruments, the 40x TaqMan® 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™ TaqMan® SNP genotyping assays was used to genotype large sample quantities obtained during the course of this study. The reaction volume (10 µl) consisted of; 10ng/µl template specific DNA (2.0 µl), 5 µl of universal TaqMan® genotyping master mix (P/N 4371355), 20x TaqMan® SNP genotyping assay (0.5 µl) and 2.5 µl SABAX (nuclease-free) water. The amplification run was carried out with a three step thermal cycling program starting with 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 and finally followed by an end-point genotyping allelic discrimination scan.

2.7.3. Corbett Rotor-Gene™ 6000/ QIAGEN Rotor-Gene Q

The Rotor-Gene™ 6000 series Multiplexing System, 5-Plex HRM model (Corbett Research, Australia) was used with the compatible ABI™ TaqMan® SNP genotyping assays (C_30090620_10, C_1129864_10 and C_7514879_10) for mutation detection. Allelic discrimination and scatter plot analysis data of the fluorescence in each channel was obtained during the RT-PCR experiment. A total volume of 10µl per individual
reaction used for allelic discrimination consisted of: 5µl TaqMan® universal genotyping PCR master mix (P/N 4371355), 10ng/µl template specific DNA (2.0 µl), 20x TaqMan® SNP genotyping assay (0.5 µl) and 2.5 µl nuclease-free water. The specific thermal cycling program used for amplification was as follows: 45 cycle repeat of the initial hold step for 10 minutes at 95°C, denaturation for 15 seconds at 92°C, followed by an annealing and final extension step for 1 minute at 60°C set to acquire (VIC®/ yellow and FAM™/ green dye) at the end of each cycling step.

2.8. Statistical Analysis

Population frequencies of the selected SNPs in the FTO, TNFα and PPARγ genes studied were estimated from allele counts. Hardy-Weinberg equilibrium was assessed using the exact test. We calculated a risk score for each individual by counting their risk alleles (FTO T>A, TNF-α G>A, PPARγ C>G i.e. A-A-C). We also inferred frequencies of 3-SNP allelic combinations, using the R haplo stats package. Prior to inclusion of the genotyping results in the statistical model used for genotype-phenotype association studies, potential confounders were identified in the data set and adjusted for in order to test for both single independent and combined allelic effects incorporated into the genotype risk score. For descriptive purposes, cross tabulation and frequency tables were used to denote occurrences of various qualitative attributes (such as gender, ethnicity, diagnostic groups, etc.) whereas the median and interquartile range were used to describe quantitative phenotypes (insulin, HOMA-IR, etc.). Log regression models were used to compare pairs of groups, while linear regression models were used to compare quantitative characteristics between diagnostic and genotypic groups and scores. These models were adjusted for possible confounders by including the confounders as factors. Log transformations were used when the distributions of quantitative traits were not symmetric. All p-values and effect sizes were derived from these models. All statistical analyses were done by a qualified biostatistician using functions from R software and R packages genetics and R haplo.stats, freely available from http://www.r-project.org.
CHAPTER III

Results
3.1. Characteristics of the study population

Functional SNPs in the FTO (rs9939609, intron 1 T>A), TNF-α (rs1800629, -308 G>A) and PPARγ (rs1801282, Pro12Ala C>G) genes (Table 3.1) were evaluated in 119 patients and 166 control individuals to evaluate the relationship between genetic and environmental risk factors as may be reflected by biochemical abnormalities implicated in the development or severity of NAFLD. The clinical and biochemical characteristics of the study population are summarised in tables 3.2 and 3.3, respectively. Based on the assessment of clinical usefulness performed as part of the literature review, the SNPs selected for analysis were considered suitable for genotype-phenotype association studies in view of their role in inflammation, obesity, insulin resistance and type II diabetes. The risk-associated alleles as described in this context are underlined in table 3.1.

Table 3.1. Single nucleotide polymorphisms studied and their metabolic associations.

The risk-associated alleles as previously described in relation to insulin resistance are underlined.

<table>
<thead>
<tr>
<th>Risk Area</th>
<th>Gene</th>
<th>Genetic Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obesity and metabolic syndrome features</td>
<td>FTO</td>
<td>rs9939609, Intron 1 T&gt;A</td>
</tr>
<tr>
<td>Inflammation and insulin resistance</td>
<td>TNF-α</td>
<td>rs1800629, -308 G&gt;A</td>
</tr>
<tr>
<td>Insulin resistance</td>
<td>PPARγ</td>
<td>rs1801282, 34 C&gt;G, P12A</td>
</tr>
</tbody>
</table>

DNA samples of the 166 Caucasian control individuals were used to perform analytical validation of the high-throughput RT-PCR genotyping method, against direct sequencing as the gold standard (Sections 3.2-3.5). Genotyping of the patient study group (n=119) was subsequently performed using the standardized RT-PCR assays for the three selected SNPs. The patients with a fatty liver (without liver biopsies) were grouped separately (n=31) from the 88 histologically confirmed NAFLD patients for comparative studies and included 23 Coloured, 6 Caucasian and 2 Black patients. Within the histologically confirmed NAFLD patient group 47 patients were Coloured, 35 Caucasian, 5 Black and 1 Indian.
Table 3.2. Description of clinical characteristics in the general Caucasian control individuals, obese patients with fatty liver on ultrasound and histologically confirmed NAFLD patients, subdivided into types 1,2 and 3,4 (NASH) [median; (inter quartile range)].

<table>
<thead>
<tr>
<th></th>
<th>General Caucasian Controls</th>
<th>Obese patients</th>
<th>All NAFLD Patients (types 1-4)</th>
<th>Caucasian NAFLD (types 1-4) Patients</th>
<th>Coloured NAFLD (types 1-4) Patients</th>
<th>NAFLD (types 1,2)</th>
<th>NASH (types 3,4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>166</td>
<td>31</td>
<td>88</td>
<td>35</td>
<td>47</td>
<td>45</td>
<td>43</td>
</tr>
<tr>
<td>Male, number</td>
<td>56 (34%)</td>
<td>7 (23%)</td>
<td>28 (32%)</td>
<td>18 (51%)</td>
<td>8 (17%)</td>
<td>16 (36%)</td>
<td>12 (28%)</td>
</tr>
<tr>
<td>Female, number</td>
<td>110 (66%)</td>
<td>24 (77%)</td>
<td>60 (68%)</td>
<td>17 (49%)</td>
<td>39 (83%)</td>
<td>29 (64%)</td>
<td>31 (72%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>48 (±12)</td>
<td>47 (±12)</td>
<td>53 (±11)</td>
<td>53 (±9)</td>
<td>54 (±11)</td>
<td>53 (±9)</td>
<td>53 (±12)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26 (23-29)</td>
<td>37 (33-43)</td>
<td>33 (30-38)</td>
<td>31 (30-34)</td>
<td>34 (31-39)</td>
<td>33 (31-38)</td>
<td>33 (30-38)</td>
</tr>
<tr>
<td>BMI class, number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Underweight (&lt;18.4kg/m²)</td>
<td>3 (2%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Normal (18.5-24.9 kg/m²)</td>
<td>64 (39%)</td>
<td>0</td>
<td>4 (5%)</td>
<td>1 (3%)</td>
<td>3 (6%)</td>
<td>2 (4%)</td>
<td>2 (5%)</td>
</tr>
<tr>
<td>Overweight (25-30 kg/m²)</td>
<td>61 (37%)</td>
<td>0</td>
<td>17 (19%)</td>
<td>9 (26%)</td>
<td>7 (15%)</td>
<td>8 (18%)</td>
<td>9 (21%)</td>
</tr>
<tr>
<td>Obese (&gt;30 kg/m²)</td>
<td>35 (21%)</td>
<td>31 (100%)</td>
<td>67 (76%)</td>
<td>25 (71%)</td>
<td>37 (79%)</td>
<td>35 (78%)</td>
<td>32 (74%)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>91 (81-99)</td>
<td>111 (102-126)</td>
<td>105 (100-114)</td>
<td>109 (103-113)</td>
<td>104 (98-115)</td>
<td>106 (99-112)</td>
<td>104 (100-115)</td>
</tr>
<tr>
<td>Alcohol consumption, yes</td>
<td>n/a</td>
<td>1 (3%)</td>
<td>13 (15%)</td>
<td>11 (32%)</td>
<td>1 (2%)</td>
<td>6 (14%)</td>
<td>7 (16%)</td>
</tr>
<tr>
<td>Physical activity, yes</td>
<td>115 (70%)</td>
<td>2 (7%)</td>
<td>13 (15%)</td>
<td>8 (24%)</td>
<td>3 (6%)</td>
<td>6 (14%)</td>
<td>7 (17%)</td>
</tr>
<tr>
<td>Smoking, yes</td>
<td>8 (5%)</td>
<td>3 (10%)</td>
<td>21 (24%)</td>
<td>9 (26%)</td>
<td>12 (26%)</td>
<td>13 (29%)</td>
<td>8 (19%)</td>
</tr>
<tr>
<td>Heart Disease, yes</td>
<td>n/a</td>
<td>2 (6%)</td>
<td>7 (8%)</td>
<td>5 (14%)</td>
<td>1 (2%)</td>
<td>3 (7%)</td>
<td>4 (9%)</td>
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<td>Family history, yes</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Diabetes</td>
<td>30 (18%)</td>
<td>18 (75%)</td>
<td>49 (62%)</td>
<td>14 (52%)</td>
<td>33 (72%)</td>
<td>22 (56%)</td>
<td>27 (68%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>n/a</td>
<td>13 (54%)</td>
<td>43 (55%)</td>
<td>10 (37%)</td>
<td>31 (69%)</td>
<td>24 (62%)</td>
<td>19 (49%)</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>n/a</td>
<td>7 (30%)</td>
<td>38 (49%)</td>
<td>15 (58%)</td>
<td>22 (48%)</td>
<td>21 (55%)</td>
<td>17 (42%)</td>
</tr>
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</table>

n/a, not available
Table 3.3 Description of biochemical characteristics in the general Caucasian control individuals, obese patients with fatty liver on ultrasound and histologically confirmed NAFLD patients, subdivided into types 1, 2 and 3, 4 (NASH) [median, (inter quartile range)].

<table>
<thead>
<tr>
<th></th>
<th>General Caucasian Controls</th>
<th>Obese Patients</th>
<th>All NAFLD Patients (types 1-4)</th>
<th>Caucasian NAFLD (types 1-4)</th>
<th>Coloured NAFLD (types 1-4)</th>
<th>NAFLD (types 1,2)</th>
<th>NASH (types 3,4)</th>
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<tr>
<td>n</td>
<td>166</td>
<td>31</td>
<td>88</td>
<td>35</td>
<td>47</td>
<td>45</td>
<td>43</td>
</tr>
<tr>
<td>ALT (units/L)</td>
<td>35 (29-51)</td>
<td>47 (32-68)</td>
<td>57 (45-91)</td>
<td>38 (30-56)</td>
<td>38 (28-62)</td>
<td>51 (38-70)</td>
<td></td>
</tr>
<tr>
<td>AST (units/L)</td>
<td>32 (26-51)</td>
<td>34 (27-68)</td>
<td>42 (29-38)</td>
<td>31 (32-50)</td>
<td>33 (24-47)</td>
<td>35 (29-61)</td>
<td></td>
</tr>
<tr>
<td>AST/ALT ratio</td>
<td>0.79 (0.68-0.95)</td>
<td>0.77 (0.65-0.94)</td>
<td>0.71 (0.63-0.84)</td>
<td>0.78 (0.65-0.99)</td>
<td>0.79 (0.66-0.98)</td>
<td>0.71 (0.64-0.91)</td>
<td></td>
</tr>
<tr>
<td>C-Reactive Protein (mg/l)</td>
<td>6.8 (5.3-11.5)</td>
<td>7.8 (3.4-10.7)</td>
<td>7.8 (3.4-10.5)</td>
<td>6.6 (3.4-10.7)</td>
<td>8.2 (3.8-12)</td>
<td>6.6 (3.3-11)</td>
<td></td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>5.0 (4.7-5.2)</td>
<td>5.6 (5.3-7.1)</td>
<td>6.6 (5.3-8.7)</td>
<td>6.4 (5.3-7.4)</td>
<td>8.0 (5.6-9.2)</td>
<td>5.8 (5.1-8.2)</td>
<td>7.8 (5.9-9)</td>
</tr>
<tr>
<td>Insulin concentration (mmol/l)</td>
<td>23 (12-31)</td>
<td>20 (10-28)</td>
<td>19 (10-26)</td>
<td>22 (13-30)</td>
<td>19 (9.8-25)</td>
<td>21 (12-33)</td>
<td></td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.3 (0.2-1.3)</td>
<td>1.1 (0.7-1.5)</td>
<td>1.1 (0.7-1.4)</td>
<td>1.2 (0.8-1.7)</td>
<td>1.0 (0.6-1.4)</td>
<td>1.3 (0.8-2)</td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.3 (5.0-5.6)</td>
<td>7.1 (6.0-8.6)</td>
<td>7.1 (6.0-8.9)</td>
<td>6.3 (5.4-8.4)</td>
<td>7.5 (6.5-9.0)</td>
<td>6.5 (5.6-7.4)</td>
<td>7.9 (6.5-9)</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/l)</td>
<td>1.4 (1.2-1.6)</td>
<td>1.3 (1.0-1.6)</td>
<td>1.2 (1.1-1.4)</td>
<td>1.2 (1.0-1.3)</td>
<td>1.3 (1.1-1.5)</td>
<td>1.2 (1.1-1.4)</td>
<td>1.2 (1.1-2)</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/l)</td>
<td>3.5 (2.9-4.3)</td>
<td>3.6 (3.1-4.0)</td>
<td>3.5 (2.7-4.1)</td>
<td>3.8 (2.5-4.2)</td>
<td>3.4 (2.8-4.1)</td>
<td>3.5 (2.7-4.1)</td>
<td>3.4 (3-4.1)</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>5.4 (4.8-6.4)</td>
<td>5.3 (4.8-6.3)</td>
<td>5.6 (4.8-6.5)</td>
<td>5.7 (4.8-6.2)</td>
<td>5.6 (4.7-6.6)</td>
<td>5.6 (4.7-6.2)</td>
<td>5.7 (5-6.6)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.9 (0.7-1.3)</td>
<td>1.4 (1.1-2.1)</td>
<td>1.8 (1.3-2.8)</td>
<td>2.0 (1.4-3.0)</td>
<td>1.8 (1.4-2.5)</td>
<td>1.6 (1.3-2.4)</td>
<td>1.9 (1.4-3)</td>
</tr>
<tr>
<td>Tf Saturation (%)</td>
<td>30 (23-37)</td>
<td>17 (13-21)</td>
<td>19 (12-26)</td>
<td>23 (16-27)</td>
<td>19 (12-25)</td>
<td>15 (11-26)</td>
<td>20 (15-27)</td>
</tr>
</tbody>
</table>
3.2. Conventional Sequencing- Agarose Gels and Electropherograms

The specific amplicons obtained for the FTO (T>A), TNF-α (G>A) and PPARγ (G>C) mutations using conventional primers visualized with ethidium bromide in a 2.5 % agarose gel are presented in figures 3.2.1, 3.2.5, 3.2.9 respectively. The sequencing results of each genotype in relation to the risk-associated allele when not detected (ND), heterozygous (HET) or homozygous (HOM) are presented in electropherograms for one of the control samples in figures 3.1.2 (FTO-ND), 3.2.3 (FTO-HET), 3.2.4 (FTO-HOM), 3.2.6 (TNFα-ND), 3.2.7 (TNFα-HET), 3.2.8 (TNFα-HOM), 3.2.10 (PPARγ-ND), 3.2.11 (PPARγ-HET) and 3.2.12 (PPARγ-HOM). Forward (sense) and reverse (anti-sense) sequencing were carried out for each genotype to emphasize the analytical validity. Only the forward sequence is shown as the reverse sequence continually substantiated the result obtained.

Figure 3.2.1. A 2.5% (w/v) agarose gel depicting the PCR amplicons synthesised with the FTO (rs9939609) primer set visualized with 0.0001% (v/v) Ethidium Bromide. Lane 1 contains a 100-bp DNA ladder. Lanes 2 to 4 contains amplicons of 275bp and lane 5 contains the NTC (non-template control) PCR reaction product.

Figure 3.2.2. Electropherogram depicting the forward (sense) sequence reaction of a PCR amplicon obtained with the FTO primer set. The red arrow indicates the FTO (intron 1 T>A) nucleotide (126) mutation position. The “T” highlighted position resembles the TT genotype where the risk-associated allele was not detected.
Figure 3.2.3. Electropherogram depicting the forward (sense) sequence reaction of a PCR amplicon obtained with the FTO primer set. The red arrow indicates the FTO (intron 1 T>A) nucleotide (126) mutation position. The “W” highlighted position resembles a heterozygous TA genotype.

Figure 3.2.4. Electropherogram depicting the forward (sense) sequence reaction of a PCR amplicon obtained with the FTO primer set. The red arrow indicates the FTO (intron 1 T>A) nucleotide (126) mutation position. The “A” highlighted position resembles a risk-associated homozygous AA genotype.
Figure 3.2.5. A 2.5% (w/v) agarose gel depicting the PCR amplicons synthesised with the TNF-α (rs1800629) primer set visualized with 0.0001% (v/v) Ethidium Bromide. Lane 1 contains a 100-bp DNA ladder. Lanes 2 to 7 contains amplicons of 742bp and lane 8 contains the NTC (non-template control) PCR reaction product.

Figure 3.2.6. Electropherogram depicting the forward (sense) sequence reaction of a PCR amplicon obtained with the TNF-α primer set. The red arrow indicates the TNF-α (-308 G>A) nucleotide (348) mutation position. The “G” highlighted position resembles the GG genotype where the risk-associated allele was not detected.

Figure 3.2.7. Electropherogram depicting the forward (sense) sequence reaction of a PCR amplicon obtained with the TNF-α primer set. The red arrow indicates the TNF-α (-308 G>A) nucleotide (345) mutation position. The “R” highlighted position resembles a heterozygous GA genotype.
Figure 3.2.8. Electropherogram depicting the forward (sense) sequence reaction of a PCR amplicon obtained with the TNF-α primer set. The red arrow indicates the TNF-α (-308 G>A) nucleotide (346) mutation position. The “A” highlighted position resembles a risk-associated homozygous AA genotype.

Figure 3.2.9. A 2.5% (w/v) agarose gel depicting the PCR amplicons synthesised with the PPARγ (rs1801282) primer set visualized with 0.0001% (v/v) Ethidium Bromide. Lane 1 contains a 100-bp DNA ladder. Lanes 2 to 4 contain amplicons of 734bp and lane 5 contains the NTC (non-template control) PCR reaction product.

Figure 3.2.10. Electropherogram depicting the forward (sense) sequence reaction of a PCR amplicon obtained with the PPARγ primer set. The red arrow indicates the PPARγ
(34 C>G, P12A) nucleotide (217) mutation position. The “G” highlighted position resembles the GG genotype where the risk-associated allele was not detected.

**Figure 3.2.11.** PCR amplicon obtained with the PPARγ primer set. The red arrow indicates the PPARγ (34 C>G, P12A) nucleotide (217) mutation position. The “S” highlighted position resembles a heterozygous CG genotype.

**Figure 3.2.12.** Electropherogram depicting the forward (sense) sequence reaction of a PCR amplicon obtained with the PPARγ primer set. The red arrow indicates the PPARγ (34 C>G, P12A) nucleotide (220) mutation position. The “C” highlighted position resembles a risk associated homozygous CC genotype.
3.3. RT-PCR Genotyping with the ABI™ 7900HT.

In this investigation the ABI™ 7900HT Real-Time PCR instrument was used with ABI™ TaqMan® SNP Genotyping assays to determine the specific genotypes of the various patient and control samples screened in this study. The ABI™ SDS version 2.3 software package (Foster City, California, USA) was used as the user interface of the instrument. Analytical validation of the assays were achieved using positive control samples with known genotypes determined by conventional PCR and DNA sequencing prior to the RT-PCR run and 12 non-template controls (NTC, consisting of nuclease-free water as a replacement for DNA) in each 96 well plate reaction run.

Genotyping was achieved in three distinct stages. The first was an amplification run during which amplicons were exponentially synthesized and the fluorescence data plotted as a graph (ΔRn-unit of fluorescence vs. cycles) using the standard curve (AQ) setting of the instrument. The second stage was a post-amplification scan determining the allelic discrimination using the SDS software package. The software presents the results on an allelic discrimination scatterplot by contrasting the fluorescence data obtained from the VIC® and FAM™ reporter dyes. Upon signal normalization and multi component analysis the data obtained from a single well is plotted as a single data-point on the scatterplot. The allelic discrimination analysis displays the results as an allele Y (assay identification and allele) on the y-axis and allele X (assay identification and alternate allele) on the x-axis of the graph. Analytical validation and verification of the genotyping results obtained concluded the final stage of the process.

All three assays (FTO rs9939609 intron 1 T>A, TNF-α rs1800629 -308 G>A and PPARγ rs1801282 34 C>G P12A) employed on the ABI™ 7900HT were successful in the mutation screening, yielding clear amplification of the polymorphic target sequence and precise genotyping of each individual sample. Internal control samples loaded on each plate verified the DNA sequence data obtained and clear NTC clustering per plate corresponds to the absence of contamination during the analytical procedure, ensuring the credibility of the results generated during the study. Due to the bulk of genotyping results generated from this study only one sample batch of each assay is illustrated in this section to exemplify the results obtained through the ABI™ 7900HT RT-PCR genotyping system.
The investigation of FTO (intron 1 T>A) yielded the amplification graph presented in figure 3.3.1. The graph signifies successful amplification and fluorescence acquired by the ABI™ TaqMan® SNP genotyping assay. The allelic discrimination scatterplot of FTO is depicted in figure 3.3.2 grouping the genotypes of each sample screened with black dots representing NTCs, blue dots matching the risk associated not detected, green dots equivalent to heterozygotes and red dots indicating the homozygotes.

The investigation of TNF-α (~308 G>A) yielded the amplification graph presented in figure 3.3.3. The graph signifies successful amplification and fluorescence acquired by the ABI™ TaqMan® SNP genotyping assay. The allelic discrimination scatterplot of TNF-α is depicted in figure 3.3.4 grouping the genotypes of each sample screened with black dots representing NTCs, blue dots matching absence of the risk allele, green dots equivalent to heterozygotes and red dots signifying the less prevalent homozygotes.

The investigation of PPARγ (P12A, 34 C>G) yielded the amplification graph presented in figure 3.3.5. The graph signifies successful amplification and fluorescence acquired by the ABI™ TaqMan® SNP Genotyping assay. The allelic discrimination scatterplot of PPARγ is depicted in figure 3.3.6 grouping the genotypes of each sample screened with black dots representing NTCs, green dots equivalent to heterozygotes and red dots indicating the homozygotes.
Figure 3.3.1. Distinctive amplification attained using the ABI™ TaqMan® assay for FTO (ΔRn vs numeral cycles). (Abbreviation: ΔRn = unit of fluorescence)

Figure 3.3.2. Typical allelic discrimination analysis using the ABI™ TaqMan® assay for FTO [Allele Y (C_30090620_10-T) vs Allele X (C_30090620_10-A)]. Black = NTC (Non template control), Blue = Not detected TT, Green = Heterozygous TA, Red = Homozygous AA.
Figure 3.3.3 Distinctive amplification attained using the ABI™ TaqMan® assay for TNF-α (∆Rn vs. numeral cycles). (Abbreviation: ∆Rn = unit of fluorescence).

Figure 3.3.4 Typical allelic discrimination analysis using the ABI™ TaqMan® assay for TNF-α [Allele Y (C_7514879_10-G) vs. Allele X (C_7514879_10-A)]. Black = NTC (Non template control), Blue = Not detected GG, Green = Heterozygous GA, Red = Homozygous AA.
Figure 3.3.5. Distinctive amplification attained using the ABI™ TaqMan® assay for PPAR-γ (ΔRn vs numeral cycles). (Abbreviation: ΔRn = unit of fluorescence)

Figure 3.3.6. Typical allelic discrimination analysis using the ABI™ TaqMan® assay for PPARγ [Allele Y (C_1129864_10-G) vs Allele X (C_1129864_10-C)]. Black = NTC (Negative template control), Green=Heterozygous CG, Red = Homozygous CC, Blue= Not detected GG (absent)
3.4 RT-PCR genotyping with the Corbett Rotor-Gene™ 6000/ QIAGEN Rotor-Gene Q

In this study, the Corbett Rotor-Gene™ 6000 series multiplexing system (5-Plex HRM model) with the ABI™ TaqMan® SNP genotyping assays were employed using allelic discrimination and scatterplot analysis for amplification and genotyping of the patient and control samples. Allelic discrimination analysis was accomplished using the real time kinetic data from multiple (2 or more) channels simultaneously to genotype the samples. The ABI™ TaqMan® SNP genotyping assays consisted of unlabelled primers specifically designed to amplify the target sequence and dual labelled TaqMan® MGB (Minor Groove Binding) probes, VIC® and FAM™ used for allelic discrimination detection.

The dual labelled probes have unique fluorophores acting as fluorescent dyes (VIC® and FAM™) which are allele specific to ultimately differentiate between the different alleles of each SNP under investigation. The yellow channel sensor was encaged for detection of the VIC® fluorescence probe with excitation wavelength of 530 nm and emission wavelength of 555 nm and the green sensor channel was employed for the detection of the FAM™ fluorescence probe with excitation wavelength 470 nm and emission wavelength 510 nm by the Rotor-Gene™ fluorometer.

A scatterplot analysis was performed using multiple channels (green and yellow) to genotype the patient and control samples. The genotypes were determined on the basis of regions defined on the scatterplot and the relative expression of the two channels after normalization to accentuate the differences in expression between the samples.

All three assays (FTO intron 1 T>A, TNF-α -308 G>A and PPARγ 34 C>G, P12A) employed on the Corbett Rotor-Gene™ 6000 series were successful in the mutation screening process, yielding clear amplification of the polymorphic target sequence and precise genotyping of each individual sample. Internal control samples were included in each run to verify the DNA sequence data obtained. To quantify the data generated during each run a threshold was set to exclude small changes of fluorescence, due to probe degradation or other effects (NTCs) which do not show true amplification but rather a steady line. No amplification was acquired with the non-template controls correspond to the absence of contamination during the analytical procedure, ensuring the credibility of the results generated during the study.
Due to the bulk of genotyping results generated from this study only one sample batch of each assay is illustrated in this section to exemplify the results obtained through the Corbett Rotor-Gene™ 6000 series multiplexing system. Analytical validation and verification of the genotyping results obtained during the investigation concluded the final phase of the process.

The allelic discrimination analysis for the FTO (intron 1 T>A, rs9939609) assay is presented in figure 3.4.1 with the legend and genotypes illustrated by table 3.4.1. The lines without intermitted circles represent the (T) allele, which is detected by FAM™-labelled probes, while the lines with the intermitted circles signifies the homozygote (A) allele detected by the VIC®-labelled probes. Amplification of both alleles simultaneously indicative of significant fluorescence of both dyes represents the heterozygous (T/A) genotype. A threshold level (>0.1) was established as a discriminatory parameter during analysis to indicate the presence of allele specific fluorescence above the threshold setting.

**Figure 3.4.1.** Allelic discrimination analysis of FTO (rs9939609, Intron 1 T>A) using the ABI™ TaqMan® (C_30090620_10) genotyping assay. (Normalized fluorescence vs. number of cycles)
In addition to allelic discrimination analysis, scatterplot analysis was also performed to further validate the results of the study. Scatterplot analysis was applied using two channels simultaneously. Genotype acquisition is based on specific regions found on the scatterplot as well as relative expression of amplification of the green (FAM™) and yellow (VIC®) channels respectively. Upon normalization the different fold increases of each channel and log transformation accentuated the differences in expression of each individual sample.

The scatterplot analysis for the FTO (Intron 1 T>A) assay is presented in figure 3.4.2 with the legend and genotypes depicted in table 3.4.1. Genotype determinations were based on the dual VIC® and FAM™ Minor Groove Binding (MGB) probes.
Table 3.4.1. Legend for figure 3.3.1 and 3.3.2, specifying genotypes of the samples based on Allelic discrimination data and Scatterplot analysis.

<table>
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<tr>
<th>No.</th>
<th>Colour</th>
<th>Name</th>
<th>Genotype</th>
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<th>VIC®</th>
</tr>
</thead>
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<td>Reaction</td>
<td>Reaction</td>
</tr>
<tr>
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<td>Reaction</td>
<td>Reaction</td>
</tr>
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</tbody>
</table>

Figure 3.4.2. Genotypes grouped by scatterplot analysis (FAM™ fluorescence vs. VIC® fluorescence) of the ABI™ TaqMan® (C_30090620_10) FTO assay. The legend is presented in table 3.4.1.
The allelic discrimination analysis for the TNF-α (rs1800629, -308 G>A) assay is presented in figure 3.4.3 with the legend and genotypes illustrated by table 3.4.2. The lines without intermitted circles represent the (G) allele, which is detected by FAM™-labelled probes, while the lines with the intermitted circles signifies the homozygote (A) allele detected by the VIC®-labelled probes. Amplification of both alleles simultaneously indicative of significant fluorescence of both dyes represents the heterozygous (G/A) genotype. A threshold level (>0.1) was established as a discriminatory parameter during analysis to indicate the presence of allele specific fluorescence above the threshold setting.

![Figure 3.4.3](image)

**Figure 3.4.3.** Allelic discrimination analysis of TNF-α (rs1800629, -308 G>A) using the ABI™ TaqMan® (C_7514879_10) genotyping assay. (Normalized fluorescence vs. number of cycles)

The scatterplot analysis for the TNF-α (rs1800629, -308 G>A) assay is presented in figure 3.4.4 with the legend and genotypes depicted in table 3.4.2. Genotype determinations were based on the dual VIC® and FAM™ Minor Groove Binding (MGB) probes.
Table 3.4.2. Legend for figure 3.3.3 and 3.3.4, specifying genotypes of the samples based on Allelic discrimination data and Scatterplot analysis.

<table>
<thead>
<tr>
<th>No.</th>
<th>Colour</th>
<th>Name</th>
<th>Genotype</th>
<th>FAM™</th>
<th>VIC®</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Red</td>
<td>sample</td>
<td>Heterozygous</td>
<td>Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>2</td>
<td>Yellow</td>
<td>sample</td>
<td>Heterozygous</td>
<td>Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>3</td>
<td>Blue</td>
<td>sample</td>
<td>Not detected</td>
<td>Reaction</td>
<td>No Reaction</td>
</tr>
<tr>
<td>4</td>
<td>Magenta</td>
<td>sample</td>
<td>Heterozygous</td>
<td>Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>5</td>
<td>Magenta</td>
<td>sample</td>
<td>Not detected</td>
<td>Reaction</td>
<td>No Reaction</td>
</tr>
<tr>
<td>6</td>
<td>Blue</td>
<td>sample</td>
<td>Heterozygous</td>
<td>Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>7</td>
<td>Green</td>
<td>sample</td>
<td>Not detected</td>
<td>Reaction</td>
<td>No Reaction</td>
</tr>
<tr>
<td>8</td>
<td>Pink</td>
<td>sample</td>
<td>Not detected</td>
<td>Reaction</td>
<td>No Reaction</td>
</tr>
<tr>
<td>9</td>
<td>Green</td>
<td>sample</td>
<td>Not detected</td>
<td>Reaction</td>
<td>No Reaction</td>
</tr>
<tr>
<td>10</td>
<td>Pink</td>
<td>sample</td>
<td>Not detected</td>
<td>Reaction</td>
<td>No Reaction</td>
</tr>
<tr>
<td>11</td>
<td>Black</td>
<td>sample</td>
<td>Heterozygous</td>
<td>Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>12</td>
<td>Green</td>
<td>sample</td>
<td>Heterozygous</td>
<td>Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>13</td>
<td>Green</td>
<td>sample</td>
<td>Heterozygous</td>
<td>Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>14</td>
<td>Green</td>
<td>sample</td>
<td>Heterozygous</td>
<td>Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>15</td>
<td>Magenta</td>
<td>sample</td>
<td>Not detected</td>
<td>Reaction</td>
<td>No Reaction</td>
</tr>
<tr>
<td>16</td>
<td>Magenta</td>
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<td>Not detected</td>
<td>Reaction</td>
<td>No Reaction</td>
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<tr>
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<td>sample</td>
<td>Not detected</td>
<td>Reaction</td>
<td>No Reaction</td>
</tr>
<tr>
<td>18</td>
<td>Red</td>
<td>sample</td>
<td>Not detected</td>
<td>Reaction</td>
<td>No Reaction</td>
</tr>
<tr>
<td>19</td>
<td>Brown</td>
<td>sample</td>
<td>Not detected</td>
<td>Reaction</td>
<td>No Reaction</td>
</tr>
<tr>
<td>20</td>
<td>Green</td>
<td>blank</td>
<td>None</td>
<td>No Reaction</td>
<td>No Reaction</td>
</tr>
</tbody>
</table>

**Figure 3.4.4.** Genotypes grouped by scatterplot analysis (FAM™ fluorescence vs. VIC® fluorescence) of the ABI™ TaqMan® (C_7514879_10) TNF-α assay. The legend is presented in table 3.4.2.
The allelic discrimination analysis for the PPARγ (rs1801282, 34 C>G, P12A) assay is presented in figure 3.4.5 with the legend and genotypes illustrated by table 3.4.3. The lines without intermitted circles represent the (C) allele, which is detected by FAM™-labelled probes, while the lines with the intermitted circles signifies the homozygous (G) allele detected by the VIC®-labeled probes. Amplification of both alleles simultaneously indicates significant fluorescence of both dyes and represents the heterozygous (C/G) genotype. A threshold level (>0.05) was established as a discriminatory parameter during analysis to indicate the presence of allele specific fluorescence above the threshold setting.

![Figure 3.4.5. Allelic discrimination analysis of PPARγ (rs1801282, 34 C>G, P12A) using the ABI™ TaqMan® (C_1129864_10) genotyping assay. (Normalized fluorescence vs. number of cycles)](image)

The scatterplot analysis for the PPARγ (rs1801282, 34 C>G, P12A) assay is presented in figure 3.4.6 with the legend and genotypes depicted in table 3.4.3. Genotype determinations were based on the dual VIC® and FAM™ Minor Groove Binding (MGB) probes.
Table 3.4.3. Legend for figure 3.3.5 and 3.3.6, specifying genotypes of the samples based on Allelic discrimination data and Scatterplot analysis.

<table>
<thead>
<tr>
<th>No.</th>
<th>Colour</th>
<th>Name</th>
<th>Genotype</th>
<th>FAM™</th>
<th>VIC®</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>sample</td>
<td>Heterozygous</td>
<td>Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>sample</td>
<td>Mutant</td>
<td>No Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>sample</td>
<td>Mutant</td>
<td>No Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>sample</td>
<td>Mutant</td>
<td>No Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>sample</td>
<td>Not Reaction</td>
<td>Reaction</td>
<td>No Reaction</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>sample</td>
<td>Mutant</td>
<td>No Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>sample</td>
<td>Mutant</td>
<td>No Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>sample</td>
<td>Mutant</td>
<td>No Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>sample</td>
<td>Mutant</td>
<td>No Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>sample</td>
<td>Mutant</td>
<td>No Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>sample</td>
<td>Heterozygous</td>
<td>Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>sample</td>
<td>Mutant</td>
<td>No Reaction</td>
<td>Reaction</td>
</tr>
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<td>sample</td>
<td>Mutant</td>
<td>No Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>sample</td>
<td>Mutant</td>
<td>No Reaction</td>
<td>Reaction</td>
</tr>
<tr>
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<td></td>
<td>sample</td>
<td>Mutant</td>
<td>No Reaction</td>
<td>Reaction</td>
</tr>
<tr>
<td>16</td>
<td></td>
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<td>sample</td>
<td>Heterozygous</td>
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<td></td>
<td>blank</td>
<td>None</td>
<td>No Reaction</td>
<td>No Reaction</td>
</tr>
</tbody>
</table>

Figure 3.4.6. Genotypes grouped by scatterplot analysis (FAM™ fluorescence vs. VIC® fluorescence) of the ABI™ TaqMan® (C_1129864_10) PPARγ assay. The legend is presented in table 3.4.3.
3.5 Genotype distribution in the general control study population

The genotype distribution observed for FTO rs9939609 (intron 1 T>A) among the 166 control samples achieved through application of RT-PCR is summarized in figure 3.5.1.

**Figure 3.5.1.** Genotype distribution of 166 samples obtained using the ABI™ TaqMan® FTO assay. Among the 166 control samples, the risk-associated FTO A-allele was excluded in 63 (38%) individuals (not detected TT), one copy detected in 70 (42.2%) individuals (heterozygous TA) and two copies of the risk-associated allele detected in 33 (19.9%) individuals (homozygous AA).
The genotype distribution observed for TNF-α rs1800629 (-308 G>A) among the 166 control samples achieved through application of RT-PCR is summarized in figure 3.5.2.

![Figure 3.5.2](image-url)

**Figure 3.5.2.** Genotype distribution of 166 samples obtained using the ABI™ TaqMan® TNF-α assay. Among the 166 control samples, the risk-associated TNF-α A-allele was excluded in 119 (71.7%) individuals (not detected GG), one copy detected in 43 (25.9%) individuals (heterozygous GA) and two copies of the risk-associated allele detected in 4 (2.4%) individuals (homozygous AA).
The genotype distribution observed for PPARγ rs1801282 (Pro12Ala, 34 C>G) among the 166 control samples achieved through application of RT-PCR is summarized in figure 3.5.3.

**Figure 3.5.3.** Genotype distribution of 166 samples obtained using the ABI™ TaqMan® PPARγ assay. Among the 166 control samples, the risk-associated PPARγ C-allele was excluded in 5 (3.0%) individuals (not detected GG), one copy detected in 39 (23.5%) individuals (heterozygous GC) and two copies of the risk-associated allele detected in 122 (73.5%) individuals (homozygous CC).
3.6. Comparative analysis of Allelic and Genotype Distribution

Following the optimisation of the RT-PCR assays for the FTO rs9939609, TNF-α rs1800629 and PPARγ rs1801282 SNPs, a total of 119 patients (31 with fatty liver on ultrasound and 88 histologically confirmed NAFLD) were genotyped using the ABI™ 7900HT and Corbett Rotor-Gene™. Among the 166 general Caucasian control individuals all three SNPs were in Hardy Weinberg equilibrium (p>0.05). However, in the NAFLD patient group subdivided into Coloured (p=0.005) and Caucasian (p=0.043) patients, significant deviation was observed for PPARγ rs1801282 (table 3.6.1) in these groups and the total group of 88 histologically confirmed NAFLD patients (p<0.001). This is ascribed to over-representation of the PPARγ rs1801282 GG genotype, a finding unrelated to the specific allelic risk implication.

Table 3.6.1. P-values of Hardy Weinberg Equilibrium testing for the FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G) single nucleotide polymorphisms in the study groups.

<table>
<thead>
<tr>
<th>Mutations</th>
<th>General Caucasian Controls</th>
<th>All NAFLD patients (types 1-4)</th>
<th>Obese</th>
<th>Caucasian NAFLD (types 1-4) patients</th>
<th>Coloured NAFLD (types 1-4) patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTO rs9939609</td>
<td>0.109</td>
<td>0.077</td>
<td>0.718</td>
<td>0.095</td>
<td>0.495</td>
</tr>
<tr>
<td>TNF-α rs1800629</td>
<td>1.000</td>
<td>0.633</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>PPARγ rs1801282</td>
<td>0.360</td>
<td>&lt;0.001</td>
<td>1.000</td>
<td>0.043</td>
<td>0.005</td>
</tr>
</tbody>
</table>

In table 3.6.2 the FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G) minor allele frequencies and genotype distributions are compared between Coloured and Caucasian NAFLD patients single and combined, and Caucasian control samples. P-values are given in table 3.6.3. A control group for the coloured population was not available for this study.

Since the allelic distribution of most SNPs vary considerably between ethnic groups, and due to possible age and gender effects, statistical comparisons between study groups were made within the same ethnic group or after adjustment for ethnicity, age and gender.
Table 3.6.2. Genotype distribution and minor allele counts and frequencies in the Caucasian control individuals and patient subgroups genotyped for the FTO rs9939609 (intron 1 T>A), TNF-α s1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G) single nucleotide polymorphisms.

<table>
<thead>
<tr>
<th>SNP</th>
<th>General Caucasian Controls</th>
<th>Obese</th>
<th>All NAFLD Patients (types 1-4)</th>
<th>Caucasian NAFLD Patients (types 1-4)</th>
<th>Coloured NAFLD Patients (types 1-4)</th>
<th>NAFLD (types 1,2)</th>
<th>NASH (types 3,4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTO Typed</td>
<td>166</td>
<td>31</td>
<td>88</td>
<td>35</td>
<td>47</td>
<td>45</td>
<td>43</td>
</tr>
<tr>
<td>T/T</td>
<td>63 (0.38)</td>
<td>10 (0.32)</td>
<td>29 (0.33)</td>
<td>7 (0.20)</td>
<td>21 (0.45)</td>
<td>17 (0.38)</td>
<td>12 (0.28)</td>
</tr>
<tr>
<td>T/A</td>
<td>70 (0.42)</td>
<td>14 (0.45)</td>
<td>50 (0.57)</td>
<td>23 (0.66)</td>
<td>23 (0.49)</td>
<td>23 (0.51)</td>
<td>27 (0.63)</td>
</tr>
<tr>
<td>A/A</td>
<td>33 (0.20)</td>
<td>7 (0.23)</td>
<td>9 (0.10)</td>
<td>5 (0.14)</td>
<td>3 (0.06)</td>
<td>5 (0.11)</td>
<td>4 (0.09)</td>
</tr>
<tr>
<td>A</td>
<td>136 (0.41)</td>
<td>28 (0.45)</td>
<td>68 (0.39)</td>
<td>33 (0.47)</td>
<td>29 (0.31)</td>
<td>33 (0.37)</td>
<td>35 (0.41)</td>
</tr>
<tr>
<td>TNF-α Typed</td>
<td>166</td>
<td>31</td>
<td>88</td>
<td>35</td>
<td>47</td>
<td>45</td>
<td>43</td>
</tr>
<tr>
<td>G/G</td>
<td>119 (0.72)</td>
<td>25 (0.81)</td>
<td>67 (0.76)</td>
<td>29 (0.83)</td>
<td>33 (0.70)</td>
<td>34 (0.76)</td>
<td>33 (0.77)</td>
</tr>
<tr>
<td>G/A</td>
<td>43 (0.26)</td>
<td>6 (0.19)</td>
<td>19 (0.22)</td>
<td>6 (0.17)</td>
<td>13 (0.28)</td>
<td>11 (0.24)</td>
<td>8 (0.19)</td>
</tr>
<tr>
<td>A/A</td>
<td>4 (0.02)</td>
<td>0 (0.00)</td>
<td>2 (0.02)</td>
<td>0 (0.00)</td>
<td>1 (0.02)</td>
<td>0 (0.00)</td>
<td>2 (0.05)</td>
</tr>
<tr>
<td>A</td>
<td>51 (0.15)</td>
<td>6 (0.10)</td>
<td>23 (0.13)</td>
<td>6 (0.09)</td>
<td>15 (0.16)</td>
<td>11 (0.12)</td>
<td>12 (0.14)</td>
</tr>
<tr>
<td>PPARγ Typed</td>
<td>166</td>
<td>31</td>
<td>88</td>
<td>35</td>
<td>47</td>
<td>45</td>
<td>43</td>
</tr>
<tr>
<td>C/C</td>
<td>122 (0.73)</td>
<td>30 (0.97)</td>
<td>78 (0.89)</td>
<td>29 (0.83)</td>
<td>43 (0.91)</td>
<td>38 (0.84)</td>
<td>40 (0.93)</td>
</tr>
<tr>
<td>C/G</td>
<td>39 (0.23)</td>
<td>1 (0.03)</td>
<td>6 (0.07)</td>
<td>4 (0.11)</td>
<td>2 (0.04)</td>
<td>4 (0.09)</td>
<td>2 (0.05)</td>
</tr>
<tr>
<td>G/G</td>
<td>5 (0.03)</td>
<td>0 (0.00)</td>
<td>4 (0.05)</td>
<td>2 (0.06)</td>
<td>2 (0.04)</td>
<td>3 (0.07)</td>
<td>1 (0.02)</td>
</tr>
<tr>
<td>G</td>
<td>49 (0.15)</td>
<td>1 (0.02)</td>
<td>14 (0.08)</td>
<td>8 (0.11)</td>
<td>6 (0.06)</td>
<td>10 (0.11)</td>
<td>4 (0.05)</td>
</tr>
</tbody>
</table>
The differences in minor alleles of the three selected SNPs between the general Caucasian controls (21% obese) compared with NAFLD Caucasian patients (71% obese) (shown in table 3.6.2 shaded areas), were not statistically significant. The observed frequency of the risk-associated TNF-α A-allele (9%) was lower in the 35 NAFLD patients (AA genotype not detected) than the 166 controls (15%) but the difference did not reach statistical significance (p=0.981). When a sub-analysis was done comparing the frequency of the risk associated TNF-α A allele Caucasian NAFLD patients (n=35) with only those Caucasian controls (n=67) with normal BMI (<25 kg/m2), this difference was also not significant (p=0.129). Further investigation was focused on genotype-phenotype comparisons between patient subgroups (table 3.6.3) as the identification of genetic risk factors that could help to identify patients at high risk of progression from fatty liver disease to the more severe NASH (NAFLD types 3,4) subtype, represents the most important aspect of this study.

Table 3.6.3 P-values for comparing the genotype and minor allele frequencies for the FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G) single nucleotide polymorphisms between patient subgroups after adjusting for age, gender and ethnicity (unless comparison is within an ethnic group).

<table>
<thead>
<tr>
<th>Typed</th>
<th>NAFLD (types 1-4) patients vs. Obese</th>
<th>NAFLD (types1-4) Coloured patients vs. NAFLD (types 1-4) Caucasian patients</th>
<th>Obese vs. NASH (types 3,4) patients</th>
<th>NAFLD (types 1,2) patients vs. NASH (types 3,4) patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td>0.377</td>
<td><strong>0.018</strong></td>
<td>0.401</td>
<td>0.694</td>
</tr>
<tr>
<td>Allele (A)</td>
<td>0.307</td>
<td><strong>0.005</strong></td>
<td>0.513</td>
<td>0.690</td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td>0.123</td>
<td>0.102</td>
<td>0.073</td>
<td>0.179</td>
</tr>
<tr>
<td>Allele (A)</td>
<td><strong>0.047</strong></td>
<td><strong>0.034</strong></td>
<td><strong>0.030</strong></td>
<td><strong>0.300</strong></td>
</tr>
<tr>
<td>PPARγ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td>0.387</td>
<td>0.431</td>
<td>0.513</td>
<td>0.352</td>
</tr>
<tr>
<td>Allele (C)</td>
<td>0.263</td>
<td>0.288</td>
<td>0.528</td>
<td>0.152</td>
</tr>
</tbody>
</table>

After adjustment for age and gender, both the genotype distribution (p=0.018) and allele frequencies (p=0.005) of FTO rs9939609 was found to differ significantly between the Coloured and Caucasian NAFLD patients (table 3.6.3). The risk-associated FTO
rs9939609 A-allele was detected at a significantly higher frequency in the Caucasian than Coloured patients, while the opposite was detected for the risk-associated TNF-α rs1800629 A-allele that occurred at a significantly higher frequency in the Coloured compared with Caucasian NAFLD patients (p=0.034). No difference in genotype or allelic distribution was observed for PPARγ rs1801282, despite the deviation in Hardy Weinberg equilibrium found in NAFLD patients only (not controls). After adjustment for age, gender and race, no differences in genotype distribution or allele frequencies were observed between histologically confirmed NAFLD (types 1,2) and NASH (types 3,4) patients, while the minor allele count for the TNF-α rs1800629 was significantly higher in NAFLD patients (types 1-4) (p=0.047) and NASH (NAFLD types 3,4) (p=0.03) compared with obese patients without a histologically confirmed diagnosis. This finding was in accordance with the expectation of increased disease severity ranging in our study population from obese to NAFLD (types 1,2) to NASH (NAFLD types 3,4).

3.7. Allelic effects and risk score assessment

According to the literature review performed (chapter 1) the minor A-alleles of FTO rs9939609 (intron 1 T>A) and TNF-α rs1800629 (-308 G>A), and the common C-allele of PPARγ rs1801282 (Pro12Ala, 34 C>G), are the risk associated alleles (underlined) for obesity and/or insulin resistance. The inferred frequencies for seven allelic combinations (the eighth, A-A-G was not detected) are summarised in table 3.7.1 and depicted in figure 3.7.1. The allelic combination A-A-C was significantly more frequent in NAFLD types 1,2 patients than in types 3,4 (NASH) patients, while T-A-C was the risk combination for NASH types 3,4 compared to the reference combination T-G-G, after adjustment for age, gender and race. However, the allelic combinations A-A-C and T-A-C had the same effects within the obese, NAFLD types 1,2 and NASH types 3,4 groups after adjustment for age, gender, race, smoking, alcohol consumption and physical activity.
Table 3.7.1. Inferred frequencies of allelic combinations for the FTO rs9939609 (intron 1 T>A), TNF-α s1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G) single nucleotide polymorphisms.

<table>
<thead>
<tr>
<th>Allele combination</th>
<th>Obese</th>
<th>NAFLD (types 1,2)</th>
<th>NASH (types 3,4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typed</td>
<td>31</td>
<td>45</td>
<td>43</td>
</tr>
<tr>
<td>A-A-C</td>
<td>0.05</td>
<td>0.11</td>
<td>0.02</td>
</tr>
<tr>
<td>A-G-C</td>
<td>0.38</td>
<td>0.21</td>
<td>0.37</td>
</tr>
<tr>
<td>A-G-G</td>
<td>0.02</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>T-A-C</td>
<td>0.05</td>
<td>0.02</td>
<td>0.11</td>
</tr>
<tr>
<td>T-A-G</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>T-G-C</td>
<td>0.50</td>
<td>0.55</td>
<td>0.45</td>
</tr>
<tr>
<td>T-G-G</td>
<td>0.00</td>
<td>0.06</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Figure 3.7.1. Frequencies of the allelic combinations for FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G) in the obese, NAFLD (types 1,2) and NASH (types 3,4) patient groups.

A risk score was calculated for each individual by counting the risk associated FTO rs9939609 A-alleles, the TNF-α rs1800629 A-alleles, and the PPARγ rs1801282 C-alleles. A minimum risk score of 0 and a maximum of 6 could therefore be obtained for each individual. The frequencies of the risk score of the three patient groups subdivided into obese, NAFLD (types 1,2) and NASH (NAFLD types 3,4) are summarised in table 3.7.2 and depicted in figure 3.7.2. No patient had all three risk homozygotes, which
would correspond to the highest risk score of 6. A similar risk score was observed in the obese (most individuals had a risk score of 3, 42%), NAFLD types 1, 2 (most had a score of 2, 36%) and NASH types 3, 4 (most had a score of 3, 53%) groups (table 3.7.2). No significant association with an increasing risk score was detected between the obese patients, NAFLD types 1,2 and NASH types 3,4 groups after adjusting for age, gender, race, smoking, alcohol consumption and physical activity (p=0.454).

Table 3.7.2. Counts and frequencies of calculated risk allele score of FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G) combined.

<table>
<thead>
<tr>
<th>Risk Score</th>
<th>Obese</th>
<th>NAFLD (types 1,2)</th>
<th>NASH (types 3,4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 (0%)</td>
<td>1 (2%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>1</td>
<td>0 (0%)</td>
<td>3 (7%)</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>2</td>
<td>9 (29%)</td>
<td>16 (36%)</td>
<td>9 (21%)</td>
</tr>
<tr>
<td>3</td>
<td>13 (42%)</td>
<td>13 (29%)</td>
<td>23 (53%)</td>
</tr>
<tr>
<td>4</td>
<td>7 (23%)</td>
<td>10 (22%)</td>
<td>9 (21%)</td>
</tr>
<tr>
<td>5</td>
<td>2 (6%)</td>
<td>2 (4%)</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>6</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

Figure 3.7.2. The frequencies of the calculated risk score (0-6) for FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G) compared between the three patient groups classified according to disease
severity from obese to NAFLD types 1, 2 and 3, 4 (NASH).

In table 3.7.3 age, gender, race and environmental factors including smoking, drinking and physical activity were compared between the three patients groups and also compared in relation to genetic variation in the FTO (rs9939609, intron 1 T>A), TNF-α (rs1800629, -308 G>A) and PPARγ (rs1801282, 34 C>G) genes. Unadjusted p-values are shown in table 3.7.3, to assess the need for adjustment of possible confounding factors when genotype-phenotype association studies are performed.

Table 3.7.3. Unadjusted P-values for difference in environmental factors between obese and NAFLD types 1,2 and 3,4 (NASH), as well as the association between environmental and genetic risk factors in the combined group.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patient groups</th>
<th>FTO</th>
<th>TNFα</th>
<th>PPARγ</th>
<th>Risk Score</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.036</td>
<td>0.288</td>
<td>0.028</td>
<td>0.185</td>
<td>0.008</td>
<td>0.057</td>
</tr>
<tr>
<td>Gender</td>
<td>0.477</td>
<td>0.848</td>
<td>0.288</td>
<td>0.756</td>
<td>0.505</td>
<td>0.822</td>
</tr>
<tr>
<td>Race</td>
<td>0.306</td>
<td>0.056</td>
<td>0.163</td>
<td>0.687</td>
<td>0.788</td>
<td>0.003</td>
</tr>
<tr>
<td>Drinker</td>
<td>0.226</td>
<td>0.274</td>
<td>0.617</td>
<td>0.158</td>
<td>0.526</td>
<td>0.020</td>
</tr>
<tr>
<td>Smoker</td>
<td>0.145</td>
<td>0.616</td>
<td>0.542</td>
<td>0.001</td>
<td>0.022</td>
<td>0.009</td>
</tr>
<tr>
<td>Physical activity</td>
<td>0.501</td>
<td>0.582</td>
<td>0.797</td>
<td>0.063</td>
<td>0.683</td>
<td>0.093</td>
</tr>
</tbody>
</table>

Two important age effects were noted. Histologically confirmed NAFLD patients were, on average, 6 years older at entry into the study (also documented as the age of disease diagnosis) than the obese patient group (p=0.036). Age of onset of fatty liver disease was on average 5 years younger in the presence of each risk-associated TNF-α rs1800629 A-allele (p=0.028) among the 119 patients included in this study. This age effect was not observed on further assessment of the histologically confirmed NAFLD group only, after exclusion of the obese group (p=0.230). When considered in the context of FTO rs9939609 and PPARγ rs1801282 as part of the risk score (table 3.7.2), a 3 year earlier onset of fatty liver disease symptoms was observed with each of the three risk-associated (p=0.008) alleles detected. No gender effect was observed (table 3.7.3), while the race difference could be ascribed to a frequency of 8.8% for the allelic...
combination T-A-C in Coloureds, while absent in Caucasians. Among alcohol users, none had the A-A-C allelic combination (risk-associated genotype in the context of insulin resistance according to the literature review), while it was detected in 6.3% of non-users (p=0.020). The allelic combinations T-G-G (11.1% vs. 2.5%) and A-G-G (9.8% versus none) were much more prevalent in smokers compared to non-smokers (p=0.009). The effect of smoking was mainly ascribed to the PPARγ rs1801282 C allele detected at a frequency of 79% (38/48) in smokers, compared with 97% (184/188) in non-smokers (p=0.001). All 4 patients with the PPARγ rs1801282 GG genotype, were smokers, whereas all the non-smokers were either heterozygous or homozygous for the risk-associated C-allele. Of smokers, 17% (4) had the PPARγ rs1801282 GG genotype. While none of the non-smokers had it. These findings have no obvious clinical implication, but confirm the need to adjust for multiple variables when genotype-phenotype correlations are assessed in the study population. No potential confounding effect for physical activity was detected.

In table 3.7.4 clinical and biochemical factors were summarized for obese patients with fatty liver on ultrasound alone and histologically confirmed NAFLD (types 1,2) and NASH (NAFLD types 3,4) patients. A significant joint effect of age, gender and race was detected for BMI (kg/m$^2$) (p=<0.009), HbA1c levels (p=0.010) and HDL-cholesterol (p=0.004) in the combined patient group. BMI was found to be significantly higher in both Coloured NAFLD patients (on average 4 kg/m$^2$) and Black NAFLD patients (on average 8 kg/m$^2$) compared to the Caucasian NAFLD patients (p=0.009) after adjusting for age, gender and race. Race also significantly affected HbA1c levels, which were found to be increased by 1.32%, on average, in Coloured NAFLD patients compared with Caucasian NAFLD patients (p=0.010). HDL-cholesterol was significantly affected by gender (p=0.004); on average female patients had a 0.22 mmol/l higher HDL-cholesterol level compared to male patients.

The test of difference between the obese, NAFLD (types 1,2) and NASH (NAFLD types 3,4) after adjustment for age gender and race did not show a difference in BMI and waist circumference between the histologically confirmed NAFLD (types 1,2) and NASH (NAFLD types 3,4) patients. However, both NAFLD patient subgroups had a lower BMI (on average 4.4 kg/m$^2$ for NASH and 4.2 kg/m$^2$ for NAFLD types 1,2) (p=0.015) and
waist circumference (on average 10 cm for NASH and 12 cm for NAFLD types 1.2) (p=0.002) compared to the obese patient group (table 3.7.4).

Significantly higher levels of ALT (on average 23.4 units/L higher) (p=0.031), glucose (2.7 mmol/l) (p=0.005), HbA1c (1.54%) (p=0.003), HOMA-IR (1.13 units) (p<0.001) and triglyceride (1.33 mmol/l) (p=0.017) were observed in the NASH patient subgroup (NAFLD types 3,4) compared with the obese patient subgroup, after adjustment for age, gender and race (table 3.7.4).
Table 3.7.4. Description (median, interquartile range) and p-values for comparison of clinical and biochemical factors in obese patients with fatty liver on ultrasound alone and histologically confirmed NAFLD (types 1,2) and NASH (NAFLD types 3,4) patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Obese (N=31)</th>
<th>NAFLD (types 1,2, N=45)</th>
<th>NASH (types 3,4, N=43)</th>
<th>Joint effect of age, gender and race</th>
<th>P-values for difference between groups, adjusted for age, gender and race</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>31</td>
<td>45</td>
<td>43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>37 (33-43)</td>
<td>33 (31-38)</td>
<td>33 (30-38)</td>
<td>0.009</td>
<td>0.015</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>111 (102-126)</td>
<td>106 (99-112)</td>
<td>104 (100-115)</td>
<td>0.345</td>
<td>0.002</td>
</tr>
<tr>
<td>ALT (units/L)</td>
<td>35 (29-51)</td>
<td>38 (28-62)</td>
<td>51 (38-70)</td>
<td>0.066</td>
<td>0.031</td>
</tr>
<tr>
<td>AST (units/L)</td>
<td>32 (26-51)</td>
<td>33 (23.5-47)</td>
<td>35 (29-60.5)</td>
<td>0.068</td>
<td>0.078</td>
</tr>
<tr>
<td>AST/ALT ratio</td>
<td>0.79 (0.68-0.95)</td>
<td>0.79 (0.66-0.98)</td>
<td>0.71 (0.64-0.91)</td>
<td>0.477</td>
<td>0.420</td>
</tr>
<tr>
<td>C-Reactive Protein (mg/l)</td>
<td>6.8 (5.3-11.5)</td>
<td>8.2 (3.8-11.5)</td>
<td>6.6 (3.3-10.7)</td>
<td>0.183</td>
<td>0.424</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.6 (5.3-7.1)</td>
<td>5.8 (5.1-8.2)</td>
<td>7.8 (5.9-9.2)</td>
<td>0.155</td>
<td>0.005</td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>7.1 (6.0-8.6)</td>
<td>6.5 (5.6-7.4)</td>
<td>7.9 (6.5-9.4)</td>
<td>0.010</td>
<td>0.003</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.3 (0.2-1.3)</td>
<td>1.0 (0.6-1.4)</td>
<td>1.3 (0.8-2.1)</td>
<td>0.583</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin (mmol/l);</td>
<td>23 (12-31)</td>
<td>18.8 (9.8-24.7)</td>
<td>21.3 (12.0-33.2)</td>
<td>0.690</td>
<td>0.949</td>
</tr>
<tr>
<td>Tf Saturation (%)</td>
<td>17 (13-21)</td>
<td>15 (11-26)</td>
<td>20 (15-27)</td>
<td>0.592</td>
<td>0.224</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>5.3 (4.8-6.3)</td>
<td>5.6 (4.7-6.2)</td>
<td>5.7 (4.8-6.6)</td>
<td>0.315</td>
<td>0.270</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/l)</td>
<td>3.6 (3.1-4.0)</td>
<td>3.5 (2.7-4.1)</td>
<td>3.4 (2.8-4.1)</td>
<td>0.241</td>
<td>0.949</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/l)</td>
<td>1.3 (1.0-1.6)</td>
<td>1.2 (1.1-1.4)</td>
<td>1.2 (1.1-1.5)</td>
<td>0.004</td>
<td>0.421</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.4 (1.1-2.1)</td>
<td>1.6 (1.3-2.4)</td>
<td>1.9 (1.4-3.1)</td>
<td>0.478</td>
<td>0.017</td>
</tr>
</tbody>
</table>
Table 3.7.5 shows the P-values for difference in clinical characteristics between patient groups [obese versus NAFLD (types 1,2) versus NASH (types 3,4) (first column)] and association with genetic factors, adjusted for age, gender, race, smoking, alcohol consumption and physical activity in the combined patient group. When the results presented in table 3.7.4 (last column) were re-assessed in the patient groups in relation to BMI and waist circumference after adjustment for smoking, alcohol consumption and physical activity (in addition to previous adjustment age, gender and race but excluding possible genetic effects), only the difference in waist circumference between the patient subgroups remained significant. This finding was not caused due to the effect of any of these environmental factors, but resulted from reduced statistical power mainly due to the fact that information on these variables were not provided for four patients. Waist circumference was on average 11.3 cm smaller in NAFLD (types 1,2) and 8.9 cm smaller in NASH (NAFLD types 3,4) than obese patients.

Table 3.7.6 shows P-values for association between clinical and biochemical characteristics and also environmental factors in the 119 obese, NAFLD (types 1,2) and NASH (types 3,4) patients after adjusting for age, gender and race. The average waist circumference of physically inactive patients was 12% increased compared to the active patients (p=0.004). Patients who reported low-moderate alcohol intake (below the cut-off level for diagnosis of NAFLD) had on average 70% reduced CRP levels compared with patients who abstain from alcohol drinking (p=0.038).

Tables 3.7.7, 3.7.8 and 3.7.9 contains p-values for tests of interaction between environmental factors (BMI and waist circumference considered a reflection of the diet, physical activity, smoking and alcohol consumption) and genetic factors [FTO rs9939609 (intron 1 T>A) (table 3.7.7), TNF-α rs1800629 (-308 G>A) (table 3.7.8) and PPARγ rs1801282 (Pro12Ala, 34 C>G) (table 3.7.9)] on clinical and biochemical characteristics in the total group of 119 obese, NAFLD (types 1,2) and NASH (types 3,4) patients, after adjusting for age, gender and race. The nature of the detected significant interactions is described next with the interaction between smoking and each of the FTO rs9939609 and TNF-α rs1800629 alleles on BMI as follows: the allelic effects in non-smokers were not significant, while in smokers each risk-associated FTO rs9939609 A-allele and TNF-α rs1800629 A-allele detected reduced BMI (kg/m²) by 13% and 27%, respectively,
compared with patients with two copies of the major SNP alleles (p=0.010). The interaction between smoking and FTO rs9939609 in relation to transferrin saturation was that the rate of change was not significant in smokers (lower for each risk allele) nor in non-smokers (higher), but the difference between the effects was significant (p=0.039). In patients who reported low-moderate alcohol intake, each risk-associated FTO A-allele increased LDL-cholesterol by 54%, compared to having inherited no A-allele (p=0.002), while there was no allelic effect in those who did not report drinking.

Significant interactions were detected between TNF-α rs1800629 and BMI or waist circumference on CRP levels; a significant 6% increase in CRP levels was detected for each extra kg/m² in BMI in the absence of the risk-associated A-allele (GG genotype), while CRP levels are reduced by 11% (GA genotype) or 26% (AA genotype), respectively for each additional kg/m² in BMI, compared to patients with the GG genotype (p=0.005). For each extra cm in waist circumference, CRP levels were 10% (GA genotype) or 21% (AA genotype) reduced compared to patients with the GG genotype, for whom waist circumference did not show a significant effect (p=0.003).

From the data presented (p-values) in table 3.7.9, triglyceride levels of patients homozygous for the PPARγ rs1801282 risk-associated C allele decreased by 3% with each added unit of BMI (1 kg/m²) and increased by 6% with each added BMI unit in GG homozygote patients (p=0.013). There was no effect in PPARγ rs1801282 CG heterozygotes. No allelic effect for PPARγ rs1801282 was detected in smokers nor non-smoking on LDL-cholesterol levels, only the difference between those effects was significant (p=0.046). The same was observed for PPARγ rs1801282 in relation to the effect of alcohol consumption on AST levels; neither drinking nor abstaining was associated with a significant effect and only the difference between those effects was significant (p=0.048). Abstaining from alcohol was not associated with a significant allelic effect of PPARγ rs1801282 on insulin, while alcohol drinkers showed an increase of 175% with each G-allele (decrease of 175% with each C-allele). Since this estimate was based on data from 1 drinker (AST 67) and three non-drinkers (average AST 29) the results should be interpreted with caution.
Table 3.7.5 P-values for testing of association between clinical characteristics and patient groups for obese versus NAFLD (types 1,2) versus NASH (types 3,4) (first column) and genetic factors, after adjusting for age, gender, race, smoking, alcohol consumption and physical activity in the combined patient group.

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Groups</th>
<th>FTO</th>
<th>TNFα</th>
<th>PPARγ</th>
<th>Risk score</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>0.139</td>
<td>0.659</td>
<td>0.346</td>
<td>0.020</td>
<td>0.060</td>
<td>0.027</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>0.006</td>
<td>0.381</td>
<td>0.043</td>
<td>0.175</td>
<td>0.021</td>
<td>0.063</td>
</tr>
<tr>
<td>ALT (units/L)</td>
<td>0.042</td>
<td>0.246</td>
<td>0.823</td>
<td>0.993</td>
<td>0.360</td>
<td>0.378</td>
</tr>
<tr>
<td>AST (units/L)</td>
<td>0.065</td>
<td>0.136</td>
<td>0.495</td>
<td>0.776</td>
<td>0.130</td>
<td>0.015</td>
</tr>
<tr>
<td>AST/ALT ratio</td>
<td>0.610</td>
<td>0.870</td>
<td>0.536</td>
<td>0.683</td>
<td>0.529</td>
<td>0.977</td>
</tr>
<tr>
<td>C-Reactive Protein (mg/l)</td>
<td>0.913</td>
<td>0.717</td>
<td>0.029</td>
<td>0.598</td>
<td>0.296</td>
<td>0.257</td>
</tr>
<tr>
<td>Glucose, fasting (mmol/l)</td>
<td>0.008</td>
<td>0.468</td>
<td>0.744</td>
<td>0.599</td>
<td>0.676</td>
<td>0.970</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>&lt;0.001</td>
<td>0.348</td>
<td>0.196</td>
<td>0.121</td>
<td>0.476</td>
<td>0.183</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>0.007</td>
<td>0.486</td>
<td>0.857</td>
<td>0.786</td>
<td>0.659</td>
<td>0.398</td>
</tr>
<tr>
<td>Insulin concentration (mmol/l)</td>
<td>0.930</td>
<td>0.635</td>
<td>0.731</td>
<td>0.165</td>
<td>0.387</td>
<td>0.406</td>
</tr>
<tr>
<td>Tf Saturation (%)</td>
<td>0.284</td>
<td>0.769</td>
<td>0.960</td>
<td>0.072</td>
<td>0.326</td>
<td>0.191</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>0.339</td>
<td>0.930</td>
<td>0.627</td>
<td>0.345</td>
<td>0.519</td>
<td>0.889</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/l)</td>
<td>0.879</td>
<td>0.591</td>
<td>0.392</td>
<td>0.457</td>
<td>0.659</td>
<td>0.232</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/l)</td>
<td>0.384</td>
<td>0.897</td>
<td>0.812</td>
<td>0.695</td>
<td>0.878</td>
<td>0.437</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.052</td>
<td>0.617</td>
<td>0.670</td>
<td>0.553</td>
<td>0.399</td>
<td>0.844</td>
</tr>
</tbody>
</table>
Table 3.7.6. P-values for testing of association of clinical and biochemical characteristics with environmental factors, (including BMI and WC as a reflection of the diet) in obese, NAFLD (types 1,2) and NASH (NAFLD types 3,4) patients after adjusting for age, gender and race.

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>BMI</th>
<th>Waist circumference</th>
<th>Physical activity</th>
<th>Smoking</th>
<th>Drinking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass index (BMI) (kg/m²)</td>
<td>-</td>
<td>-</td>
<td>0.065</td>
<td>0.098</td>
<td>0.089</td>
</tr>
<tr>
<td>Waist circumference (WC) (cm)</td>
<td>-</td>
<td>-</td>
<td><strong>0.004</strong></td>
<td>0.765</td>
<td>0.203</td>
</tr>
<tr>
<td>ALT (units/L)</td>
<td><strong>0.003</strong></td>
<td>0.083</td>
<td>0.172</td>
<td>0.511</td>
<td>0.332</td>
</tr>
<tr>
<td>AST (units/L)</td>
<td><strong>0.003</strong></td>
<td>0.102</td>
<td>0.074</td>
<td>0.856</td>
<td>0.512</td>
</tr>
<tr>
<td>AST/ALT ratio</td>
<td>0.469</td>
<td>0.499</td>
<td>0.824</td>
<td>0.152</td>
<td>0.464</td>
</tr>
<tr>
<td>C-Reactive Protein (mg/l)</td>
<td>0.625</td>
<td>0.952</td>
<td>0.675</td>
<td>0.283</td>
<td><strong>0.038</strong></td>
</tr>
<tr>
<td>Glucose, fasting (mmol/l)</td>
<td><strong>0.014</strong></td>
<td>0.147</td>
<td>0.248</td>
<td><strong>0.013</strong></td>
<td>0.452</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.631</td>
<td>0.540</td>
<td>0.754</td>
<td>0.462</td>
<td>0.078</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td><strong>0.006</strong></td>
<td>0.215</td>
<td>0.339</td>
<td>0.171</td>
<td>0.829</td>
</tr>
<tr>
<td>Insulin concentration (mmol/l)</td>
<td>0.263</td>
<td>0.136</td>
<td>0.875</td>
<td>0.759</td>
<td>0.349</td>
</tr>
<tr>
<td>Tf Saturation (%)</td>
<td><strong>0.001</strong></td>
<td><strong>0.043</strong></td>
<td>0.958</td>
<td>0.124</td>
<td>0.862</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td><strong>0.032</strong></td>
<td>0.180</td>
<td>0.723</td>
<td>0.842</td>
<td>0.385</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/l)</td>
<td>0.107</td>
<td>0.287</td>
<td>0.411</td>
<td>0.785</td>
<td>0.516</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/l)</td>
<td>0.325</td>
<td>0.711</td>
<td>0.968</td>
<td>0.166</td>
<td>0.708</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td><strong>0.009</strong></td>
<td>0.110</td>
<td>0.999</td>
<td>0.581</td>
<td>0.917</td>
</tr>
</tbody>
</table>
Table 3.7.7. P-values for testing of interaction between the FTO rs9939609 A-allele and environmental factors on clinical characteristics in 119 obese, NAFLD (types 1,2) and NASH (NAFLD types 3,4) patients after adjusting for age, gender and race.

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>BMI (kg/m(^2))</th>
<th>Waist circumference (cm)</th>
<th>Physical activity</th>
<th>Smoking</th>
<th>Drinking</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m(^2))</td>
<td>-</td>
<td>-</td>
<td>0.550</td>
<td><strong>0.010</strong></td>
<td>0.302</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>-</td>
<td>-</td>
<td>0.753</td>
<td>0.051</td>
<td>0.368</td>
</tr>
<tr>
<td>ALT (units/L)</td>
<td>0.147</td>
<td>0.407</td>
<td>0.749</td>
<td>0.593</td>
<td>0.715</td>
</tr>
<tr>
<td>AST (units/L)</td>
<td>0.569</td>
<td>0.985</td>
<td>0.578</td>
<td>0.435</td>
<td>0.561</td>
</tr>
<tr>
<td>AST/ALT ratio</td>
<td>0.096</td>
<td>0.108</td>
<td>0.808</td>
<td>0.838</td>
<td>0.827</td>
</tr>
<tr>
<td>C-Reactive Protein (mg/l)</td>
<td>0.476</td>
<td>0.278</td>
<td>0.506</td>
<td>0.460</td>
<td>0.897</td>
</tr>
<tr>
<td>Glucose, fasting (mmol/l)</td>
<td>0.298</td>
<td>0.549</td>
<td>0.847</td>
<td>0.075</td>
<td>0.307</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.938</td>
<td>0.766</td>
<td>0.380</td>
<td>0.808</td>
<td>0.448</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>0.840</td>
<td>0.440</td>
<td>0.362</td>
<td>0.728</td>
<td>0.405</td>
</tr>
<tr>
<td>Insulin concentration (mmol/l)</td>
<td>0.938</td>
<td>0.594</td>
<td>0.548</td>
<td>0.444</td>
<td>0.274</td>
</tr>
<tr>
<td>Tf Saturation (%)</td>
<td>0.239</td>
<td>0.877</td>
<td>0.218</td>
<td><strong>0.039</strong></td>
<td>0.950</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>0.332</td>
<td>0.764</td>
<td>0.570</td>
<td>0.922</td>
<td>0.063</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/l)</td>
<td>0.243</td>
<td>0.690</td>
<td>0.704</td>
<td>0.487</td>
<td><strong>0.002</strong></td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/l)</td>
<td>0.112</td>
<td>0.080</td>
<td>0.897</td>
<td>0.841</td>
<td>0.053</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.689</td>
<td>0.745</td>
<td>0.497</td>
<td>0.750</td>
<td>0.539</td>
</tr>
</tbody>
</table>
Table 3.7.8. P-values for testing of interaction between TNF-α rs1800629 (-308 G>A) and environmental factors on clinical characteristics in 119 obese, NAFLD (types 1,2) and NASH (NAFLD types 3,4) patients after adjusting for age, gender and race.

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>BMI</th>
<th>Waist circumference</th>
<th>Physical activity</th>
<th>Smoking</th>
<th>Drinking</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>-</td>
<td>-</td>
<td>0.293</td>
<td>0.010</td>
<td>0.280</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>-</td>
<td>-</td>
<td>0.630</td>
<td>0.051</td>
<td>0.208</td>
</tr>
<tr>
<td>ALT (units/L)</td>
<td>0.924</td>
<td>0.645</td>
<td>0.650</td>
<td>0.427</td>
<td>0.402</td>
</tr>
<tr>
<td>AST (units/L)</td>
<td>0.910</td>
<td>0.682</td>
<td>0.247</td>
<td>0.630</td>
<td>0.484</td>
</tr>
<tr>
<td>AST/ALT ratio</td>
<td>1.000</td>
<td>0.824</td>
<td>0.379</td>
<td>0.487</td>
<td>0.665</td>
</tr>
<tr>
<td>C-Reactive Protein (mg/l)</td>
<td>0.003</td>
<td>0.005</td>
<td>n/a</td>
<td>0.778</td>
<td>0.359</td>
</tr>
<tr>
<td>Glucose, fasting (mmol/l)</td>
<td>0.266</td>
<td>0.213</td>
<td>0.932</td>
<td>0.848</td>
<td>0.252</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.574</td>
<td>0.112</td>
<td>0.151</td>
<td>0.137</td>
<td>0.526</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>0.242</td>
<td>0.106</td>
<td>0.312</td>
<td>0.731</td>
<td>0.854</td>
</tr>
<tr>
<td>Insulin concentration (mmol/l)</td>
<td>0.855</td>
<td>0.310</td>
<td>*n/a</td>
<td>0.476</td>
<td>0.947</td>
</tr>
<tr>
<td>Tf Saturation (%)</td>
<td>0.267</td>
<td>0.163</td>
<td>*n/a</td>
<td>0.197</td>
<td>0.545</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>0.732</td>
<td>0.889</td>
<td>0.603</td>
<td>0.158</td>
<td>0.737</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/l)</td>
<td>0.855</td>
<td>0.430</td>
<td>0.754</td>
<td>0.198</td>
<td>0.096</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/l)</td>
<td>0.139</td>
<td>0.167</td>
<td>0.143</td>
<td>0.655</td>
<td>0.330</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.712</td>
<td>0.348</td>
<td>0.611</td>
<td>0.272</td>
<td>0.543</td>
</tr>
</tbody>
</table>

n/a, not available
Table 3.7.9. P-values for the interaction between PPARγ rs1801282 (Pro12Ala, 34 C>G) and environmental factors on clinical characteristics in 119 obese, NAFLD (types 1,2) and NASH (NAFLD types 3,4) patients after adjusting for age, gender and race.

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>BMI</th>
<th>Waist circumference</th>
<th>Physical activity</th>
<th>Smoking</th>
<th>Drinking</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>-</td>
<td>-</td>
<td>0.713</td>
<td>0.704</td>
<td>0.548</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>-</td>
<td>-</td>
<td>0.742</td>
<td>0.425</td>
<td>0.215</td>
</tr>
<tr>
<td>ALT (units/L)</td>
<td>0.726</td>
<td>0.766</td>
<td>0.672</td>
<td>0.803</td>
<td>0.100</td>
</tr>
<tr>
<td>AST (units/L)</td>
<td>0.745</td>
<td>0.675</td>
<td>0.337</td>
<td>0.846</td>
<td><strong>0.048</strong></td>
</tr>
<tr>
<td>AST/ALT ratio</td>
<td>0.285</td>
<td>0.196</td>
<td>0.514</td>
<td>0.463</td>
<td>0.972</td>
</tr>
<tr>
<td>C-Reactive Protein (mg/l)</td>
<td>0.795</td>
<td>0.819</td>
<td>0.645</td>
<td>*n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Glucose, fasting (mmol/l)</td>
<td>0.955</td>
<td>0.996</td>
<td>0.729</td>
<td>0.821</td>
<td>0.640</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.544</td>
<td>0.208</td>
<td>0.743</td>
<td>0.672</td>
<td>0.236</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>0.191</td>
<td>0.827</td>
<td>0.555</td>
<td>0.123</td>
<td>0.960</td>
</tr>
<tr>
<td>Insulin concentration (mmol/l)</td>
<td>0.676</td>
<td>0.289</td>
<td>0.876</td>
<td>0.782</td>
<td><strong>0.032</strong></td>
</tr>
<tr>
<td>Tf Saturation (%)</td>
<td>0.286</td>
<td>0.951</td>
<td>0.425</td>
<td>0.396</td>
<td>0.838</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>0.377</td>
<td>0.899</td>
<td>0.076</td>
<td>0.346</td>
<td>0.090</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/l)</td>
<td>0.860</td>
<td>0.715</td>
<td>0.119</td>
<td><strong>0.046</strong></td>
<td>0.117</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/l)</td>
<td>0.093</td>
<td>0.287</td>
<td>0.279</td>
<td>0.245</td>
<td>0.315</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td><strong>0.013</strong></td>
<td>0.374</td>
<td>0.167</td>
<td>0.829</td>
<td>0.109</td>
</tr>
</tbody>
</table>

n/a, not available
Age of onset of fatty liver disease was on average 5 years younger in the presence of each risk-associated TNF-α rs1800629 A-allele \((p=0.028)\) and 3 years younger when incorporated as part of the genotype risk score tested in the total group of 119 patients included in this study. Extended statistical analysis to verify these age effects in relation to alcohol intake and smoking was subsequently undertaken. Single effects were assessed after adjusting for multiple variables, as well as combined effects to determine the impact of cumulative risk.

**Table 3.7.10.** P-values for assessment of genetic association with age of onset of fatty liver disease symptoms in the combined patient group \((n=119)\) according to sets of factors including the presence or absence of alcohol consumption or smoking.

<table>
<thead>
<tr>
<th>Variables tested</th>
<th>Adjusted for</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol</td>
<td>Race, gender</td>
<td>0.095</td>
</tr>
<tr>
<td>TNFα rs1800629</td>
<td>Race, gender, alcohol</td>
<td><strong>0.015</strong></td>
</tr>
<tr>
<td>Genotype risk score</td>
<td>Race, gender, alcohol</td>
<td><strong>0.020</strong></td>
</tr>
<tr>
<td>TNFα, race, gender, alcohol*</td>
<td>-</td>
<td><strong>0.008</strong></td>
</tr>
<tr>
<td>Risk score, race, gender, alcohol*</td>
<td>-</td>
<td><strong>0.009</strong></td>
</tr>
<tr>
<td>Race, gender, alcohol*</td>
<td>-</td>
<td>0.059</td>
</tr>
<tr>
<td>Alcohol, risk score</td>
<td>Race, gender</td>
<td>0.639</td>
</tr>
<tr>
<td>Smoker</td>
<td>Race, gender</td>
<td>0.882</td>
</tr>
<tr>
<td>TNFα rs1800629</td>
<td>Race, gender, smoker</td>
<td>0.766</td>
</tr>
<tr>
<td>Genotype risk score</td>
<td>Race, gender, smoker</td>
<td>0.418</td>
</tr>
<tr>
<td>TNFα, race, gender, smoker*</td>
<td>-</td>
<td>0.477</td>
</tr>
<tr>
<td>Risk score, race, gender, smoker*</td>
<td>-</td>
<td>0.412</td>
</tr>
<tr>
<td>Race, gender, smoker*</td>
<td>-</td>
<td>0.363</td>
</tr>
<tr>
<td>Smoker, risk score</td>
<td>Race, gender</td>
<td>0.595</td>
</tr>
</tbody>
</table>

*combined effects of multiple variables were tested to determine cumulative risk

Further analysis showed that each TNF-α rs1800629 risk-associated A-allele subtracts on average 5 years from age in the patient study group, after adjusting for race, gender and alcohol consumption \((5.5\text{ years}, p=0.015)\). When these factors were combined to assess cumulative risk, the association becomes even more significant \((p=0.008)\). For each extra risk allele incorporated in the genotype risk score \((0-6)\) for FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34
C>G), 2.6 years are subtracted (figure 3.7.3) after adjusting for race, gender and alcohol consumption (p=0.020). Alcohol intake or smoking had no effect on age of onset of fatty liver disease symptoms with or without adjusting for confounders.

Figure 3.7.3. Box plots of age of onset of fatty liver disease symptoms in the patient study group (n=119) and a genotype risk score for FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G). The risk-associated alleles as previously described in the literature in relation to obesity and/or insulin resistance are underlined.

The association between age of onset of fatty liver disease symptoms and allelic combination for FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G) in NAFLD types 1,2, NAFLD types 3,4 (NASH) and obese patients was significant (p=0.039) after adjustment for gender. The modelled effect of allelic combination on age of diagnoses is illustrated in figure 3.7.4.
Figure 3.7.4. Modelled ages for onset of fatty liver disease symptoms or NAFLD diagnosis for all the allelic combinations of FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G) for males and females.

Gender had an independently significant effect in the joint model of gender and allelic combinations (p=0.023). After the adjustment for allelic combination, females were 5.2 years younger at onset compared to male patients.

There was no independent significant association between age of diagnosis and PPARγ rs1801282 (Pro12Ala, 34 C>G) (p=0.113) even when adjusted for gender (p=0.127). However the combination of age and gender was significant (p=0.033) and with each risk associated PPARγ rs1801282 C-allele age of onset is reduced by 3.7 years. The largest effect on earlier age of onset of fatty liver disease symptoms was exerted by TNF-α rs1800629 (-308 G>A), while FTO rs9939609 (intron 1 T>A) was not associated with earlier age of onset of disease symptoms or diagnosis.
CHAPTER IV

Discussion
Functional SNPs in the FTO (rs9939609, intron 1 T>A), TNF-α (rs1800629, -308 G>A) and PPARγ (rs1801282, Pro12Ala, 34 C>G) genes were studied in 166 control individuals and 119 NAFLD patients subdivided into obese patients with fatty liver identified on ultrasound alone (n=31) and histologically confirmed NAFLD patients (n=88). An important objective of the study was to identify genetic determinants of disease progression from fatty liver disease to NASH, with a particular focus on the role of insulin resistance and inflammation in the disease process. Insulin resistance has previously been identified as a central feature of NAFLD in the South African population, while obesity was excluded as an independent determinant of disease severity at the clinical level by Kruger et al. (2010). This parent study provided a clinically well-characterised study population for possible confirmation of these findings at the DNA level. Functional SNPs in the FTO (rs9939609, intron 1 T>A), TNF-α (rs1800629, -308 G>A) and PPARγ (rs1801282, Pro12Ala, 34 C>G) genes were selected for evaluation in this context, in view of their proven role in inflammation, obesity, insulin resistance and/or type II diabetes (Frayling et al. 2007 Gouda et al. 2010; Namvaran et al. 2011; Prakash et al. 2012; Sobati et al. 2012; Zhou et al. 2012a).

The genetic data provided in this study indeed confirmed the central role of insulin resistance in the disease process, while obesity was excluded as an independent risk factor for development of NAFLD and/or progression to NASH. The genetic contribution of the TNF-α SNP (rs1800629, -308 G>A) was shown to be of particular importance in relation to NAFLD. Due to the invasiveness of histopathologic diagnosis based on liver biopsy the identification of predictive genetic risk factors represents an important area of research to facilitate the prevention of cumulative risk that may lead the development of CVD or hepatic fibrosis. Kruger et al. (2011) have demonstrated the clinical usefulness of APRI as a non-invasive marker for liver fibrosis to avoid liver biopsy in South African patients with NAFLD. However, the undefined nature of hereditary factors impacting on disease development and severity in the ethnically diverse South African population warrants genetic studies to identify the underlying disease mechanism(s) in clinically well characterised NAFLD patients.

A critical step for implementation of genetic tests is to understand where the additional information provided by this technology fits into the context of the current clinico-pathological prognostication of NAFLD. This study was therefore modelled on a
pathology supported genetic testing (PSGT) approach recently described in the context of CVD, to identify patient subgroups requiring different treatment strategies (Kotze and van Rensburg, 2012). Genetic testing for CVD risk factors provides a prototype for complex conditions that frequently share disease mechanisms due in part to overlap between genetic risk factors. NAFLD is the hepatic manifestation of the metabolic syndrome and is an established CVD risk factor (Neuschwander-Tetri et al. 2003; Targher et al. 2007). An important consideration was therefore to extend the CVD multigene test developed in South Africa (Kotze et al. 2003, Kotze and Thiart 2003) by adding a genetic component related to obesity, insulin resistance and inflammation for CVD risk evaluation in patients with symptoms of fatty liver disease. Functional SNPs in the FTO (rs9939609, intron 1 T>A), TNF-α (rs1800629, -308 G>A) and PPARγ (rs1801282, Pro12Ala C>G) genes were selected for this purpose as discussed below in relation to the specific aims defined for this study: 1) to perform analytical validation of high-throughput RT-PCR assays for the selected SNPs against direct sequencing as the gold standard; 2) compare the genotype distribution and allele frequencies between NAFLD patient subgroups and controls; 3) correlate clinical and biochemical parameters of insulin resistance with genotype and environmental factors assessed in NAFLD patients; and 4) determine the potential impact of risk factors incorporated into a genotype risk score on age of onset in fatty liver disease patients in relation to NAFLD disease severity.

4.1. Analytical validation of high-throughput RT-PCR

DNA samples of the 166 Caucasian control individuals were used to perform analytical validation of the high-throughput real time polymerase chain reaction (RT-PCR) genotyping method, against direct sequencing as the gold standard. Analytical validation of the RT-PCR screening method used was accomplished by comparing pre-sequenced positive control samples and including them as internal control samples during each run to corroborate the results generated during the screening process.

Methods currently available to genotype an individual’s DNA include conventional or real time PCR using DNA fragment analysis or allele specific oligonucleotide probes, conventional DNA sequencing, next generation DNA sequencing, nucleic acid hybridization (microarrays/beads), restriction fragment length polymorphism (RFLP) analysis, terminal or amplified (t-FLP or AFLP) and multiplex ligation-dependant probe
amplification. PCR amplification using fluorescence resonance energy transfer probes was applied successfully in this study to genotype DNA samples of the study population for FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282, (Pro12Ala, 34 C>G). The probes used amplified the specific mutation sites of interest and the results obtained were corroborated by direct DNA sequencing. Comparison of PCR-based mutation screening methods against direct sequencing as the gold standard has proven to be a dependable approach for analytical validation of new genotyping assays. In this study conventional PCR amplification was used in conjunction with DNA sequencing to first identify positive and negative controls for the FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 G>C) SNPs used as internal controls in this study. High-throughput RT-PCR provided a rapid method for simultaneous analysis of large numbers of DNA samples in this study.

The principle of RT-PCR is nearly identical to that of conventional PCR except that the reaction mixtures differ. The “real time” reaction mixture used included a fluorescence resonance energy transfer probe that binds to the PCR product in a specific head to tail manner. The fluorescence data were collected after each amplification cycle. This allowed for optimisation of individual samples when compared to positive and negative controls resulting in an increased specificity and sensitivity of the individual assays performed in the study population.

The exponential phase of amplification (starting DNA and amplification product) is the most useful type of quantification and this forms the basis of RT-PCR. The two common methods for detecting RT-PCR products are the intercalating of fluorescent dyes with double stranded DNA; and specific DNA probes that are labelled with a reporter dye (fluorescent) detected after hybridization of the probe with its complimentary DNA. RT-PCR using TaqMan® assay in its simplest form is DNA binding to a dye such as SYBR Green that intercalates with the double stranded DNA. Fluorescence of the dye is detected after excitation; however this binding process of SYBR Green is non-specific. Therefore, in order to detect a specific amplicon an oligonucleotide probe labelled with a specific reporter molecule was added in the reaction of SYBR Green. When the probe binds to its target sequence a signal was generated that is directly proportional to the number of molecules. To detect the fluorescence as it proceeds specific thermal cycler such as the Applied Biosystems® 7900HT (Brown, 2003) was used.
The availability of high quality DNA extracted from blood or saliva was essential to successfully genotype (allelic discrimination) the individuals included in this study. The quality or quantity of many samples from the original database of NAFLD patients was not sufficient and therefore only 88 histologically confirmed NAFLD patients could be included in this study. Even though peripheral blood is the preferred source of DNA the extraction procedure required to obtain a sample is invasive. A study done by Abraham et al. (2012) investigated the feasibility of using saliva extracted DNA in comparison to blood-extracted DNA. Their findings suggested that the total DNA yield in saliva were lower (mean 24µg) compared to blood (mean 210 µg) after adjusting for the volume difference. Protein contamination and the rate of DNA fragmentation measured higher in saliva compared to blood. However, when genotyping using TaqMan® assays as we did during the allelic discrimination phase of this project, genotype call rates and concordance were the same for all the samples assessed. Saliva sample collection is less invasive for the patient and more convenient for the clinician and this could positively affect participant recruitment for genetic studies and be a viable alternative DNA source for high throughput genotyping techniques. Within this study an optimal quantity and quality of DNA was obtained, irrespective of the extraction protocol used (blood or saliva).

4.2. Comparison of genotype distribution and allele frequencies between study groups

Following the optimisation of the RT-PCR assays for the FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G) SNPs in 166 Caucasian control individuals, a total of 119 patients were genotyped using the ABI™ 7900HT and Corbett Rotor-Gene™. Thirty-one patients with a fatty liver detected on ultrasound alone were grouped separately for comparative studies from the 88 histologically confirmed NAFLD patients, including 47 Coloured, 35 Caucasian, 5 Black and 1 Indian patient. Within the non-histologically confirmed obese patient group 23 patients were Coloured, 6 Caucasian and 2 Black. Since genotype distribution and allele frequencies of most SNPs vary considerably between ethnic groups, statistical comparisons were made within the same ethnic group or after adjustment for ethnicity.

The prevalence of NAFLD/NASH varies greatly among different population groups and
raised the question whether this could partly be ascribed to differences in allele frequencies of disease-associated SNPs across ethnic groups. The incidence of non-alcoholic hypertransaminasemia in Japanese individuals was 31 cases per 1000 person-years (Suzuki et al. 2005) and 29 cases per 100 000 person years in Europeans (Hamaguchi et al. 2005). The prevalence of NAFLD in Korean liver transplant donors was 51% (Lee et al. 2007) and 20% in American donors (Marcos et al. 2000). The prevalence of steatohepatitis and fibrosis in lean autopsied Canadian individuals was 3% and 7%, respectively (Wanless and Lentz, 1990). Autopsied cases from Greece revealed steatosis in 31% and NASH in 40% of traffic accident deaths, after exclusion of other liver diseases (Zois et al. 2010). There is convincing evidence substantiating that genetic factors account for considerable variability in the natural history of NAFLD. A family clustering study conducted by Wilner et al. (2001) has shown that one fifth of patients with NASH have a similarly affected first-degree relative. In a study by Struben et al. (2000), coexistence of NASH and cryptogenic cirrhosis was observed in seven out of eight families studied. To fully elucidate an accurate global incidence rate for NAFLD further research is needed. A variety of diagnostic tools have been used to assess the prevalence of NAFLD in the general population, liver biopsies being the gold standard method for NASH diagnosis and staging.

For this study a control group for the coloured population was not available for study. All three SNPs tested in the 166 South African Caucasian control individuals were in Hardy Weinberg equilibrium, while in the NAFLD patient group subdivided into Coloured (p=0.005) and Caucasian (p=0.043) patients a significant deviation was observed for PPARγ rs1801282. This was caused by over-representation of the PPARγ rs1801282 GG genotype, an observation that is unrelated to increased or decreased disease risk related to the G- or C-allele. PPARγ rs1801282 (Pro12Ala, 34 C>G) groupings were found to be in Hardy Weinberg equilibrium in the 31 obese patients, but since this group includes both Caucasian and Coloured individuals this finding may not be reliable. Hardy Weinberg equilibrium is a mathematical statement that describes genetic balance (allele and genotype frequencies) within a population. The genetic characteristics tend to remain constant unless outside forces (mutations, immigration, disease state etc.) act to change it.

Comparison of the FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and
PPARγ rs1801282 (Pro12Ala, 34 C>G) minor allele frequencies and genotype distribution between the different study groups did not show significant differences between the general Caucasian controls (21% obese) and Caucasian NAFLD patients (71% obese). Although the risk-associated TNF-α A-allele detected in 9% of the 35 NAFLD patients appeared to be lower than the 15% detected in the 166 controls this difference did not reach statistical significance (p=0.981), as was also confirmed in a sub-analysis of 67 Caucasian controls with normal BMI (<25 kg/m²).

The allele frequencies and genotype distribution of FTO rs9939609 (intron 1 T>A), (38% TT, 42% TA, 20% AA) in the Caucasian controls were found to be similar to a study of 4291 Caucasian individuals conducted by Lurie et al. (2011), (36% TT, 47% TA, 17% AA). A significant difference in genotype distribution (p=0.018) and allele frequencies (p=0.005) for the FTO rs9939609 T>A SNP was identified in NAFLD Coloured patients compared with NAFLD Caucasian patients. The minor A-allele was detected at a lower frequency in the South African Coloured NAFLD patients compared to the Caucasian NAFLD patients. The number of Black NAFLD patients included in the study was too small to determine allele frequencies with accuracy. Lombard et al. (2012) reported the genotype distribution of FTO rs9939609 (intron 1 T>A) in Black South African adolescents from the Birth to Twenty cohort to be 20% TT, 53% TA and 27% AA, with a minor allele frequency of 47%. These authors identified a stronger association between FTO rs17817449 and obesity in the Black population studied, compared with FTO rs9939609 analysed in our study. The minor A-allele of FTO rs9939609 was detected at a similar frequency in the NAFLD (types 3,4) group (41%) compared to the NASH (NAFLD types 1,2) patient group (37%), with the lowest overall frequency in the Coloured NAFLD patient group.

The FTO rs9939609 A-allele was reported as the risk allele (Li et al. 2012) and is considered a predictive factor for the development of obesity and subsequently type II diabetes. Failure to confirm the role of this SNP on disease development or progression in the context of obesity in our study population corresponds with earlier findings in NAFLD patients at the clinical level (Kruger et al. 2010). This may partly be ascribed to the fact that the non-histologically confirmed obese patients with fatty liver detected on ultrasound only was preselected as being obese. To our knowledge, the FTO rs9939609 has not previously been studied in South African NAFLD patients, and the allele
frequencies and genotype distribution are also reported for the first time in patients with fatty liver disease.

In the case of TNF-α rs180629 (G>A) the allele frequencies and genotype distribution (72% GG, 26% GA, 2% AA) in the general Caucasian control samples were found to be similar to those previously reported (72% GG, 27% GA, 1% AA) by Paskulin et al. (2011). In a study performed by Joffe et al. (2011) in the South African Caucasian population comparison of genotype distribution (p=0.345) between normal-weight (55% GG, 43% GA, 2% AA) and obese (58% GG, 40% GA, 1% AA) individuals or allele frequencies (p=0.453) did not reveal any statistically significant differences for TNF-α rs180629. After adjustment for age, gender and race the minor allele count for the TNF-α rs1800629 A-allele was significantly higher in NAFLD patients (types 1-4) (p=0.047) and NASH (NAFLD types 3,4) (p=0.03) compared with obese patients without a histologically confirmed diagnosis (see table 3.6.3). This finding is in accordance with the expectation of increased disease severity ranging in our study population from obese to NAFLD (types 1,2) to NASH (NAFLD types 3,4) and may be related to an increased risk of inflammation known to be associated with NASH (Argo et al. 2011).

The risk-associated A-allele of TNF-α rs1800629 was present at a significantly higher frequency in Coloured NAFLD patients (16%) compared with Caucasian NAFLD patients (9%) (p=0.034). This finding may be related to different disease patterns of NAFLD seen among ethnic groups. In a diet intervention study performed in the South African Black population the frequency of the TNF-α rs1800629 A-allele ranged between 14% to 20% in normal weight and obese individuals, respectively (Joffe et al. 2011). The A-allele frequency in Chinese individuals was reported to be 9%, French and Scandinavian 16%, Germans 18% and in Australians 24% (Feng et al. 2011). The overall frequency of the -308 TNF-α rs1800629 G>A polymorphism as reported by Wang et al. (2012) within a NAFLD population group were similar or slightly lower (14.2%) compared to the control group (15.6%). This trend was also observed in our study, as 26% of the Caucasian control samples tested positive for the TNF-α rs1800629 polymorphism and 22% of the NAFLD types 1-4 patient group. The genotype distribution of TNF-α rs1800629 were very similar in the Caucasian control samples and NAFLD Caucasian patient samples. A similar TNF-α rs1800629 genotype distribution in 244 South African women with cervical cancer (71% GG, 26% GA and 3% AA) was also reported by Govan et al. (2006).
Homozygosity of for the TNF-α AA genotype is relatively rare in the general population ranging from 1% as reported by Stanczuk et al. (2003) in Zimbabwean individuals to 5% in several other population groups.

The allele frequencies and genotype distribution (73% CC, 23% CG, 3% GG) of PPARγ rs1801282 (Pro12Ala, 34 C>G) in the Caucasian control samples were comparable to those observed by Mook-Kanamori et al. (2009) (77% CC, 21% CG, 2% GG). Although the difference was not statistically significant, the risk-associated C-allele of PPARγ rs1801282 was found to be more prevalent in the NASH (types 3,4) patient group (95%) compared to the NAFLD (types 1,2) patient group (89%) as would be expected in relation to disease severity. The PPARγ rs1801282 (Pro12Ala, 34 C>G) C-allele occurred at a higher frequency in the Coloured NAFLD patients (91%) compared to the Caucasian NAFLD patients (83%), but after adjustment for age this difference did not reach statistical significance.

The PPARγ rs1801282 (Pro12Ala, -34 C>G) SNP is located in exon 2 of the PPARγ gene, consisting of 9 exons. The SNP arises from a C to G substitution at nucleotide position 34 of the NM_015869.4 PPARG transcript- (http://www.ncbi.nlm.nih.gov/gene/?term=N015869.4%20). However, the position of the variant may change depending on the specified transcript. For example, one of the PPARγ splice variants relates to a SNP that is located in an intronic region, while in others the SNP site is allocated to an exonic region (Pro40Ala). Therefore, it is very important to specify the splice variant from which the sequence surrounding the Pro12Ala was obtained and to provide the correct SNP identity number (whether rs1801282 or rs1805192) when designing the genotype assay for functional PPARγ rs1801282.

There is controversy regarding the role of PPARγ rs1801282 (Pro12Ala, 34 C>G) in type 2 diabetes, obesity and insulin resistance. This is mostly ascribed to the fact that in different population groups this SNP acts in the background of other polymorphisms that may vary in frequency between ethnic groups and/or exerts its effect in combination with different environmental exposures to precipitate certain phenotypes. For example, 12Ala (GG genotype) has been associated with decreased BMI, lower insulin, higher insulin sensitivity, and higher HDL-cholesterol (Huguenin and Rosa, 2010). In contrast, Passaro et al. (2011) associated the variant with increased BMI (kg/m²) and body fat mass.
Gouda et al. (2010) showed that in women the 12Ala variant was associated with type 2 diabetes. Oh et al. (2000), on the other hand, did not find any link between Pro12Ala rs1801282 C>G and either diabetes or obesity. The frequency of the Pro12Ala polymorphism varies between cohorts from different European countries (Helwig et al. 2007). The association between insulin resistance and PPARγ rs1801282 may be dependent on age as reported by Li et al. (2003). Hasstedt et al. (2001) suggested that the variant acts in a recessive manner affecting phenotypes commonly associated with insulin resistance syndrome, and this may explain the inconsistent associations reported in previous studies.

The risk-associated PPARγ rs1801282 C-allele contributes to insulin resistance (Namvaran et al. 2011; Sanghera et al. 2010) and as NAFLD is considered the hepatic manifestation of the metabolic syndrome, individuals homozygote for C-allele may be at a higher risk for developing NAFLD or to progress to NASH. The role of PPARγ in the development of NAFLD is still not conclusive but point to the direction that NAFLD patients are more insulin resistant and have a higher risk allele frequency compared to the general population. Since the PPARγ CC genotype is associated with an increased risk of type II diabetes it is feasible that evolutionary pressure due to environmental hardship could have influenced the survival advantage and spread of this genotype in previous generations. Pirie et al. (2010) conducted a study of genetic variation of PPARγ in South African subjects of Zulu descent presenting with type 2 diabetes, but the allelic distribution did not differ between subjects with or without type 2 diabetes. We also did not find any statistically significant differences in genotype distribution or allele frequencies between the study groups, despite the fact that the PPARγ rs1801282 allelic distribution deviated from Hardy Weinberg equilibrium only in South African NAFLD patients.

### 4.3. Correlation of clinical and biochemical parameters of insulin resistance and inflammation with genotype and environmental factors assessed in NAFLD patients

Subtle genetic variation of FTO rs9939609 T>A, TNF-α rs1800629 G>A and PPARγ interacting with environmental factors such as smoking, physical activity and alcohol consumption combined with clinical (BMI, waist circumference) and biochemical parameters (lipid profile, CRP etc.) may contribute to the disease process as may be
reflected by age at diagnosis/onset of disease symptoms and progression from mild NAFLD types 1,2 to the more severe NAFLD types 3,4 (NASH). In order to investigate these aspects, potential confounding factors was taken into account for comparative analyses.

In this study a significant effect of age, gender and race was detected for BMI (p<0.009), HbA1c levels (p=0.010) and HDL-cholesterol (p=0.004) among obese and NAFLD patients subdivided into types 1,2 and types 3,4 (NASH). BMI kg/m$^2$ was found to be significantly higher in both Coloured NAFLD patients and Black NAFLD patients compared to the Caucasian NAFLD patients (p=0.009), after adjusting for age, gender and race. The impact of obesity varies greatly, even among individuals with a similar BMI, it is also a rather crude method of describing the total adiposity because other factors such as genetics, gender, age, masculinity, ethnicity and body fat distribution (visceral or abdominal) that influence BMI (Stommel et al. 2010). Obesity in NAFLD may also vary greatly among NAFLD patients with a similar BMI (Lomonaco et al. 2012). Female patients and older individuals have a higher adiposity for any given BMI compared to younger male subjects, and obesity early in life significantly predisposes individuals to future metabolic related diseases such as CVD and type 2 diabetes (Cusi, 2012).

The average plasma glucose concentration over a prolonged period of time may be reflected by means of measuring HbA1c levels. In this study race was found to significantly affect HbA1c levels, with higher levels detected in Coloured NAFLD patients compared with Caucasian NAFLD patients (p=0.010). Since HDL-cholesterol is significantly affected by gender (Whakabayashi, 2012) the finding that female patients had on average a 0.22 mmol/l higher HDL-cholesterol level compared to male patients (p=0.004) could be expected.

The importance of race as a risk factor for NAFLD was demonstrated by reports that the prevalence of NAFLD, hepatic steatosis and concentrated aminotransferase levels are the highest in Hispanic individuals followed by Whites, with the lowest rate in African Americans (Kalwitz et al. 2008; Williams et al. 2011). The finding of ethnicity as an independent risk factor for advanced liver disease in NAFLD patients may explain the protective role African Americans have against advanced liver disease. The true explanation for ethnic differences in the prevalence of NAFLD in still not fully understood.
Differences in visceral adiposity and other characteristics such as adiponectin levels in African Americans may be affected by unidentified genetic and environmental factors (Wagenknecht et al. 2009). The heritability component of hepatic steatosis is based on large family cohort studies is 0.27 (Speliotes et al. 2011). However, this clustering could simply be a reflection of the heritability of other risk factors affecting NAFLD such as obesity and insulin resistance. Two studies examining ethnic differences in the prevalence of NAFLD strongly suggest that susceptibility to NAFLD rather than to its risk factors may have a genetic component (Browning et al. 2004; Caldwell et al. 2002).

The prevalence of NAFLD-related fibrosis consistently increases with age as reported in a study by Firth et al. (2009). The 351 histologically confirmed NAFLD patients divided into older, middle aged and younger groups showed a significant association between the prevalence of NAFLD and fibrosis with increasing age. Older patients have more NAFLD related risk factors, such as hypertension, obesity, insulin resistance and hyperlipidaemia, which could explain the association between age and the prevalence of NAFLD in elderly individuals (Vernon et al. 2011). Advanced age is considered an independent risk factor for hepatic steatosis (Ascha et al. 2010; Lee et al. 2007). It is important to note that younger NAFLD patients with unexplained liver enzyme elevations were associated with hepatic steatosis and higher ALT activity. Since the age range of both our control samples and patient sample groups were above 45 years (median), age as an independent risk factor is unlikely to affect the comparative analyses performed between subgroups in our study group; however, where possible we have adjusted for age.

The idea of gender influencing NAFLD susceptibility lacks empirical support. Initially NAFLD was thought to be more common in females (Fan et al. 2005; Singh et al. 2004), but a study of 26 527 Asian subjects revealed that the prevalence of NAFLD was higher in men (32%) than in women (16%) (Chen et al. 2008). Within our study more females was present in both the general Caucasian control group (66%) and NAFLD types 1,2, NAFLD types 3,4 and NASH patient (88%) groups compared to the males in each group. Some studies reported that male gender is associated with elevated aminotransferase levels, hepatic fibrosis, histologically confirmed NASH and overall mortality (Papatheodoridis et al. 2007; Kallwitz et al. 2008). The association between female gender and NAFLD or fibrosis has only been reported in a few studies (Hashimoto et al. ...
2005; Ong et al. 2005); however one study reported that female gender is an independent risk factor for NASH especially in patients with the metabolic syndrome (Sorrentino et al. 2004). Female gender may be an important risk factor for earlier development of metabolic syndrome features that may cumulate in NAFLD as females had a significantly earlier age of onset of disease symptoms which correlated significantly with presence of the risk-associated A-C allelic combination of the TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G) SNPs.

The test of difference between the obese, NAFLD (types 1,2) and NASH (NAFLD types 3,4) after adjustment for age gender and race did not show a difference in BMI and waist circumference between the histologically confirmed NAFLD (types 1,2) and NASH (NAFLD types 3,4) patients. However, both NAFLD patient subgroups had a lower BMI (on average 4.4 kg/m² for NASH and 4.2 kg/m² for NAFLD types 1.2) (p=0.015) and waist circumference (on average 10 cm for NASH and 12 cm for NAFLD types 1.2) (p=0.002) compared to the obese patient group. This finding is in accordance with the selection of patients with BMI above 30 kg/m² for the obese patient group, in order to further investigate the role of obesity in the development/progression of NAFLD. Significantly higher levels of ALT (p=0.031) (on average 23.4 units/L higher), glucose (2.7 mmol/l higher) (p=0.005), HbA1c (1.54 % higher) (p=0.003), HOMA-IR (1.13 units higher) (p<0.001) and triglycerides (p=0.017) (1.33 mmol/l higher) were observed in the NASH patient subgroup (NAFLD types 3, 4) compared with the obese patient subgroup, after adjustment for age, gender and race. HbA1c (p=0.003), HOMA-IR (p<0.001) and triglyceride levels (p=0.017) were also significantly higher in NASH (types 3, 4) compared to NAFLD (types 1,2) patients.

NAFLD is the hepatic manifestation of the metabolic syndrome characterised by elevated triglyceride levels, for this reason we expected to find the highest triglyceride levels in the NASH types 3,4 patient group. A statistically significant difference was earlier observed in the mean serum triglyceride levels when South African patients with NAFLD (types 1,2) were compared with NASH (NAFLD types 3,4) patients, while these levels did not differ significantly between patients with no/mild fibrosis and those with advanced fibrosis (Kruger et al. 2010). These findings implicated dyslipidemia in the pathogenesis of NASH, while apparently unrelated to the risk of advanced fibrosis that was not investigated in the current study. Even though virtually all the South African
patients studied by Kruger et al. (2010) were either overweight or obese, the presence of marked obesity did not predict the presence of more advanced liver disease. These findings from the parent study suggest that in addition to obesity or insulin resistance as a consequence of high BMI, secondary hits involving gene-environment interactions may be required to develop NASH and advanced fibrosis after the first signs of fatty liver becomes evident on ultrasound. The results presented in this study provide supporting evidence for these earlier clinical findings now also at the DNA level.

After further adjustment for smoking, alcohol consumption and physical activity the significant differences in waist circumference and biochemical parameters for ALT (p=0.042), glucose (p=0.008), HOMA-IR (p<0.001) and HbA1c (p=0.007) levels detected in NASH types 3,4 patients compared to the obese and/or NAFLD (types 1, 2) patients remained significant, while the previous associations reported for BMI and triglyceride levels disappeared. This finding was mainly ascribed to reduced statistical power with inclusion of three more variables adjusted for in the statistical model and small sample size. After adjustment for all 6 variables, a statistically significant association between the risk-associated TNF-α rs1800629 A-allele and CRP levels (p=0.029) was furthermore observed due to a favourable effect of low- to moderate alcohol intake on CRP levels. This biochemical marker of inflammation increased significantly (25.7 mg/l) with each risk-associated A-allele detected among fatty liver disease patients. The favourable effect of low-moderate alcohol intake on CRP-levels in patients with fatty liver disease is an important observation. Chronic systemic low grade inflammation increases with the degree of obesity and insulin resistance, especially if coronary artery disease is also present (Wannamethee et al. 2004). Patients who reported low-moderate alcohol intake had on average 70% reduced CRP levels compared with patients who abstained from alcohol drinking (p=0.038).

The grade of hepatic necro-inflammation correlates with the categories of steatohepatitis as mild, moderate or severe. It is well known that serum TNF-α levels correlate with metabolic associated risk factors such as BMI and percentage body fat. Tsukui et al. (2000) reported that exercise and TNF-α rs1800629 independently correlates with changes in HbA1c and fasting insulin levels. The finding that CRP levels are significantly higher in NASH patients compared to the less severe obese/NAFLD (types 1,2) patient groups, while BMI and waist circumference were lower, is ascribed to the fact that the
obese group included only patients with BMI above 30 kg/m\(^2\) due to the selecting criteria used for this patient subgroup. Furthermore, the finding that each PPAR\(\gamma\) C-allele is associated with a BMI reduction of 3.45 kg/m\(^2\) on average \((p=0.020)\), may be explained in the same manner.

The elevated C-reactive protein levels for each added TNF-\(\alpha\) A-allele is contradictive to the reduced waist circumference finding with each additive A-allele in the selected patient group. Therefore the reduced waist circumference (6 cm less in the presence of each risk-associated A-allele) that seems to be associated with the risk-associated TNF-\(\alpha\) rs1800629 A-allele cannot be ascribed to the gene effect but rather to the how the patients were selected for this study and other confounders such as smoking shown to be associated with reduced BMI and waist circumference. Patients of the same age, gender and race who smoke furthermore had on average 20% lower glucose levels than non-smokers. This unexpected finding, as well as the observation that a kg/m\(^2\) unit increase in BMI decreases ALT, AST, glucose, HbA1c, transferrin saturation, total cholesterol and triglycerides with between 1-3%, excluded obesity as an independent risk factor for progression from fatty liver disease to NASH as previously reported in a clinical assessment performed in the parent study \((\text{Kruger et al. 2010})\). In view of the fact that NAFLD is a syndrome overlapping with obesity related traits and insulin resistance, it nevertheless seems likely that treating or managing both these conditions would influence the genetic predisposition to fatty liver disease. In a study performed by Timpson \textit{et al.} (2011) the role of FTO rs9939609 in adiposity supported the causal effect of BMI on circulating CRP levels.

The test of interaction between environmental factors (BMI and waist circumference considered a reflection of the diet, physical activity, smoking and alcohol consumption) and SNPs FTO rs9939609 (intron 1 T>A), TNF-\(\alpha\) rs1800629 (-308 G>A) and PPAR\(\gamma\) rs1801282 (Pro12Ala, 34 C>G) on clinical and biochemical characteristics in the total group of 119 obese, NAFLD types 1,2 and NAFLD types 3,4 (NASH) patients, after adjusting for age, gender and race, revealed that physically active subjects were leaner compared to the inactive patients. Increased physically activity are therefore likely to reduce the influence of genetic factors to develop obesity. The complex relationship between physical activity and the degree of genetic influence on BMI and waist circumference was in accordance with the Mustelin \textit{et al.} (2009) study. The average
waist circumference of physically inactive NAFLD patients was 12% higher compared to the active NAFLD patients (p=0.004).

Smoking had a significant effect on BMI in relation to the FTO rs9939609 and TNF-α rs1800629 SNPs analysed in South African NAFLD patients. For each risk-associated FTO rs9939609 A-allele and TNF-α rs1800629 A-allele detected among smokers, BMI was significantly reduced by 13% and 27%, respectively, compared with patients with two copies of the major SNP alleles (p=0.010). Obesity is related to personality addictive disorders such as alcohol dependence (Barry et al. 2009). Poor decision making is a core symptom of addictive behaviours such as smoking and alcohol abuse as addicts tend to choose the action that brings them immediate relieve of their craving even when these actions lead to deleterious outcome. This could explain why individuals who smoke have a reduced waist circumference and BMI despite the presence of a genetic predisposition for obesity. Instead of eating they smoke to bring immediate reward for their emotional state (Davies and Carter, 2009; Bechara and Martin, 2004). When the potential allelic effect of FTO rs9939609 was investigated on transferrin saturation levels, it was shown that the rate of change was not significant in smokers (lower for each risk allele) nor in non-smokers (higher), but the difference between the effects was significant. The potential implications of this finding is not clear but needs to be evaluated with caution due to small sample size.

In patients who reported low-moderate alcohol intake, each FTO A-allele increased LDL-cholesterol by 54%, compared to having inherited no A-allele (p=0.002), while there was no allelic effect in those who abstain from alcohol consumption. A similar marginally significant effect was observed for reduced HDL-cholesterol (p=0.053), which together with total cholesterol (p=0.063) and triglycerides determine LDL-cholesterol levels. A study conducted by Zhou et al. (2012) confirmed that FTO rs9939609 positively correlates with features of the metabolic syndrome, including hypertension, higher fasting insulin, glucose, triglycerides and lower HDL-cholesterol levels. Our findings may imply that the deleterious effect of the risk-associated A-allele of the FTO rs9939609 SNP may become clinically relevant with alcohol intake.

Increased TNF-α levels favour the development of insulin resistance and impaired glucose tolerance (Hui et al. 2004). TNF-α is a marker of systemic inflammation during cardiovascular events and a risk factor of metabolic syndrome which forms part of a
constellation of risk factors contributing to the pathogenesis of NAFLD in South African patients (Kruger, 2008). After adjustment for age, smoking and alcohol consumption, AA homozygotes (risk-associated genotype) for the TNF-α rs1800629 polymorphism studied by Lakka et al. (2006) had higher CRP levels compared to other genotypes and these levels decreased with exercise. In this study opposite effects were detected between TNF-α rs1800629 and CRP levels when considered in relation to BMI, with decreased CRP levels in the presence of the risk-associated A-allele. This finding demonstrates that a proven or expected phenotypic effect of low-penetrance genetic risk factors such as TNF-α rs1800629 could be masked or overridden by clinical characteristics possibly due in part to other, unidentified genetic risk factors that may be more relevant in a specific clinical context. While a significant 6% increase in CRP was observed for each extra kg/m$^2$ in BMI in the absence of the risk-associated A-allele (GG genotype) as can be expected, CRP levels were however reduced by 11% (GA genotype) and 26% (AA genotype), respectively, for each extra kg/m$^2$ in BMI in patients with the risk allele, compared to patients with the GG genotype ($p=0.005$). For each extra cm of waist circumference, CRP levels were 10% (GA genotype) or 21% (AA genotype) reduced, compared to patients with GG, for whom waist circumference did not show a significant effect ($p=0.003$). These findings are clearly contradictor and highlight the complexity of utilising genetic information in risk management of complex disorders. For this reason, the PSGT approach has been developed in South Africa that takes family history, current health status and lifestyle risk factors into account for clinical decision-making (Kotze and van Rensburg, 2012).

The above-mentioned approach is also relevant to the observations made in relation to triglyceride levels and PPARγ rs1801282. In patients homozygous for the PPARγ rs1801282 risk-associated C allele triglycerides decreased by 3% with each added unit of BMI and increased by 6% with each added BMI unit in GG homozygote patients ($p=0.013$). Abstaining from alcohol was not associated with a significant allelic effect of PPARγ rs1801282 on insulin, while alcohol drinkers showed an increase of 175% with each G-allele (decrease of 175% with each C-allele). Since this finding was based on data from 1 drinker (AST 67 units/L) and three non-drinkers (average AST 29 units/L) these results as well - as the above-mentioned apparent interactions - should be interpreted with caution due to relatively small patient numbers in the subgroups available for gene-environment interaction studies in relation to biochemical parameters.
Discussion

Activation of PPARγ receptors using pioglitazone and rosiglitazone (anti-diabetic drugs) reduced alcohol consumption according to Stopponi et al. (2011). These findings provide new information about the role of PPARγ receptors as a potential candidate treatment for alcoholism and warrant further investigation of the above-mentioned insulin-lowering effect of alcohol in the presence of the risk-associated PPARγ rs1801282 C-allele, even though the results were obtained in a small number of patients. Further research is warranted to determine the benefit of using PPARγ receptors as the target sites for the treatment of alcoholism especially in alcoholic fatty liver disease patients (Purohit et al. 2009).

4.4 Impact of risk factors incorporated into a genotype risk score on age of onset in fatty liver disease patients in relation to NAFLD disease severity.

While genetic testing could help clinicians to diagnose HH or rare liver diseases that may be caused by high penetrance mutations, multiple low penetrance genetic variations (e.g. SNPs) influencing phenotypic expression are considered important contributors to the overall risk of disease development in NAFLD patients. An individual’s level of risk and age of onset of disease depends on a monogenic genetic predisposition, rare variants and/or a complex interaction between modifiable environmental factors and SNPs in modifier genes (Krawczyk et al. 2010).

A genotype risk score was calculated for each patient included in this study by counting the risk associated FTO rs9939609 A-allele, the TNF-α rs1800629 A-allele, and the PPARγ rs1801282 C-allele. A minimum risk score of 0 and a maximum of 6 were obtained for each individual. None of the patients in the obese, NAFLD (types 1,2) or NASH (NAFLD types 3,4) subgroups were awarded the highest risk score of 6. A similar risk score of 2-3 was observed in these patient subgroups. No significant association with an increasing risk score was detected between the obese patients, NAFLD types 1,2 and NASH types 3,4 groups after adjusting for age, gender, race, smoking, alcohol consumption and physical activity. When genotype-phenotype association studies are performed, careful consideration of possible confounding factors is required to prevent the documentation of false associations (Laurie et al. 2010). This possibility was considered in relation with the finding that the allelic combination T-G-G was associated with a 5.93 kg/m² increased BMI compared to the T-G-C allelic combination and a 9.09 kg/m² increased BMI compared to the risk-associated A-A-C allelic combination.
adjusted for age, gender, race, smoking, alcohol consumption and physical activity (p=0.027). The most likely explanation for unexpected findings like these in the present study was the selection criteria applied for the obese group based on BMI, while the NAFLD types1-4 patients were included in the study based on liver histology to make the diagnosis of NAFLD. Insulin resistance and inflammation are the key drivers in NAFLD disease severity and genetic risk factors underlying these metabolic abnormalities may mask or override the effect of the FTO rs9939609 SNP included in this study.

Histologically confirmed NAFLD patients were, on average, 6 years older at entry into the study (also documented as the age of disease diagnosis) than the obese patient group (p=0.036). Age of onset of fatty liver disease was on average 5 years younger in the presence of each risk-associated TNF-α rs1800629 A-allele (p=0.028) among the 119 patients included in this study. This age effect was not observed on further assessment of the histologically confirmed NAFLD group only, after exclusion of the obese group, probably due to reduced statistical power in the smaller sample size. Several studies have implicated TNF-α gene variation in the development of NASH. The levels of TNF-α was reported higher in steatohepatitis subjects when compared to healthy controls (Barbuio et al. 2007; Wang et al. 2011; Musso et al. 2012). When the effect of the TNF-α rs1800629 A-allele on age of disease onset was considered in the context of FTO rs9939609 and PPARγ rs1801282 as part of a risk score, a 3 year onset of fatty liver disease symptoms was observed with each of the three risk-associated alleles detected (p=0.008).

Differences in environmental factors and other variables among study groups necessitate the need for adjustment of these factors when performing comparative analyses. A frequency of 8.8% for the allelic combination T-A-C was detected in the Coloured population while absent in Caucasians, which confirmed the need to consider the potential effect of population substructures and ethnic differences when comparing allelic distribution of SNPs in relation to phenotypic expression. Differences in alcohol consumption and smoking between the study groups were also identified as confounders that needed to be adjusted for in comparative studies. The interaction between the PPARγ variant and smoking described by Yang et al. (2012) indicated that the presence of both factors exhibited a synergistic effect on the development of NAFLD. In this study the PPARγ rs1801282 C allele was detected at a frequency of 79% in
smokers, compared with 97% in non-smokers, while 17% of patients with the GG genotype were smokers and all the non-smokers were either heterozygous or homozygous for the risk-associated C-allele. Although the observations of these statistical analyses to identify potential confounders have no obvious clinical implications, it confirmed the need to adjust for multiple variables in our study population during genotype-phenotype correlation studies.

As mentioned previously the age of onset of fatty liver disease was on average 5 years younger in the presence of each risk-associated TNF-α rs1800629 A-allele (p=0.028) and 3 years younger when incorporated as part of the genotype risk score tested in the total group of 119 patients included in this study. Extended statistical analysis was performed to verify these age effects in relation to alcohol intake and smoking. Single effects were assessed after adjusting for multiple variables, as well as combined effects to determine the potential impact of cumulative risk. The data obtained confirmed that each extra TNF-α rs1800629 risk-associated A-allele subtracts on average 5 years from age of onset in the patient study group, after adjusting for race, gender and alcohol consumption (5.5 years, p=0.015). When these factors were combined to assess cumulative risk, the association becomes even more significant (p=0.008). For each extra risk allele incorporated in the genotype risk score (0-6) for FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G) 2.6 years are subtracted, even after adjusting for race, gender and alcohol use (2.6 years, p=0.020). When these factors were combined the association was much stronger (p=0.009). Alcohol intake or smoking had no independent effect on age of onset of fatty liver disease symptoms, which demonstrated the significance of the risk-associated TNF-α rs1800629 A-allele and even more so as part of the genotype risk score.

The association between age of onset of fatty liver disease symptoms and allelic combination for FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G) in NAFLD types 1,2, NAFLD types 3,4 (NASH) and obese patients was also statistically significant (p=0.039). The modelled ages in relation to all the allelic combinations for FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G) showed that gender had an independent significant effect in the joint model of gender and allelic combinations (p=0.023). After the adjustment for allelic combination, females were 5.2
years younger at onset compared to male patients.

While no independent significant association between age of onset of fatty liver disease and PPARγ rs1801282 (Pro12Ala, 34 C>G) (p=0.113) was detected, even when adjusted for gender, the combination of age and gender was significant (p=0.033). With each risk associated PPARγ rs1801282 C-allele age of onset was reduced by 3.7 years. An independent effect on age of onset for development of fatty liver disease symptoms was not observed for FTO rs9939609 (intron 1 T>A), which corresponds with previous exclusion of obesity as a significant risk factor in the NAFLD disease process (Kruger et al. 2010). The association between FTO rs9939609 and insulin resistance reported in the literature therefore appears to be secondary to obesity as the major phenotype associated with the FTO gene effect.

To date GWAS studies demonstrated increased risk of type 2 diabetes associated with SNPs in approximately 40 genes (McCarthy, 2010). Linder et al. (2012) included nine of these SNPs selected from the TCF7L2, KCNJ11, HHEX, SLC30A8, WFS1, KCNQ1, MTNR1B, FTO and PPARG genes, to develop a weighted genotype risk score. These authors found that the diabetes risk genotype score contributed significantly to prediction of impaired glucose tolerance and impaired fasting glycemia due to increased genetic load. This effect was not present in male, lean and insulin sensitive individuals that may indicate a protective role of beneficial environmental factors on the genetic risk (Linder et al. 2012). It also seems likely that in these cases the gene effect becomes clinically relevant in the presence of a second hit exerted by gender-related risk or insulin resistance. When the genotype risk score was reduced to 7 SNPs to reflect mainly a disruption of insulin secretion, after omitting the FTO (rs9939609) and PPARG (rs1801282) risk-associated alleles, the association with prediabetes was not abolished but rather strengthened. This finding raised the possibility that the genetic risk for prediabetes is mediated mainly through the genetic variation affecting insulin secretion. In the statistical model adjusted for insulin sensitivity, BMI was negatively associated with impaired glucose tolerance as a clinical outcome parameter. The association between BMI and impaired glucose tolerance was only positive when insulin sensitivity was omitted from the covariates. This finding is very relevant to our observation that NASH (types 3,4) patients have lower BMI and waist circumference than the less severe NAFLD (types 1,2) patients. Linder et al. (2012) suggested that once controlling for insulin sensitivity, a higher BMI may become protective against impaired glucose
tolerance and that this phenomenon can possibly be explained by a statistical
dissociation of BMI into metabolically harmful and metabolically non-harmful BMI by
adjustment for insulin sensitivity. Over-representation of obese individuals with normal
insulin sensitivity could therefore lead to biased results during association studies.
Notably, the HOMA-IR levels of the obese patients (0.3) in our study population was
markedly lower than in NAFLD (types 1,2) (1.0) and NASH (types 3,4) (1.3) patients.

Since the effect of the 9-SNP score was found to be independent of well-known
environmental risk factors such as age, body weight and insulin resistance, Linder et al.
(2012) concluded that early transition from normal glucose tolerance to impaired glucose
tolerance is significantly affected by genetically determined risk. Data reported by Meigs
et al. (2008) who genotyped SNPs at 18 diabetes-associated loci in more than 2000
participants of the Framingham Offspring Study, have furthermore shown that although
individual per-allele effects are small, individuals with more risk alleles are at greater risk
than those with fewer alleles irrespective of how many and which genes are considered.
The most important research question was whether detection of the SNPs included in
the statistical model allows better prediction of risk than knowledge of common
phenotypic risk factors alone. Gender-adjusted odds ratio for diabetes was found to be
1.12 per risk allele and resulted in risk reclassification of, at most, 4% of the subjects
after further adjustment for age, family history, BMI, fasting glucose level, systolic blood
pressure, HDL-cholesterol level and triglycerides. The marginal ability of the genotype
score to discriminate risk when relevant biochemical abnormalities are already present
support the use of a PSGT approach to determine gene expression, if any, and
response to treatment in order to monitor the effectiveness of the intervention strategy.
Notably, lifestyle intervention was found to attenuate the significantly increased risk for
developing type II diabetes and lower probability to return to normal glucose regulation,
found to be associated with an extended weighted genotype risk score of 34 SNPs
(Hivert et al. 2011). This extended genotype risk score was evaluated in more than 2000
participants from five ethic groups who had been randomised to placebo, metformin, or a
lifestyle intervention.

Extended genotype-phenotype association studies are required to identify the most
important SNPs implicated in insulin resistance and inflammation known to confer
increased risk for development of multi-factorial diseases such as type 2 diabetes and
NAFLD. Assessment in the presence and absence of relevant environmental triggers may determine the relative contribution to biochemical abnormalities and disease severity and development of effective intervention programs targeted at the cause of the disease. Incorporation of an increasing number of clinically validated biomarkers into a genotype risk score combined with clinical indicators and lifestyle risk factors may therefore facilitate the prevention of cumulative risk underlying the development of insulin resistance, type 2 diabetes, NAFLD, CVD and liver fibrosis.

4.5 Clinical application

Genomic based personalized medicine has the potential to enhance individualised treatment and disease prevention of common chronic diseases such as NAFLD and CVD. Genetic testing applications used as a diagnostic tool in clinical practice is rising rapidly (Pagon, 2006). However, only a few genome based discoveries have translated into evidence-based applications for individualized preventative medicine. According to Lenfant, (2003) most clinically derived research findings are “lost in translation”. To enhance translational research, a multidisciplinary approach in basic science is needed. Due to the limitations of genetic testing recognised early on through extensive research performed in the genetically diverse South African population a new pathology supported genetic testing (PSGT) approach was developed by Kotze and van Rensburg (2012) to facilitate the prevention of cumulative risk in multi-factorial diseases.

The FTO rs9939609, TNF-α rs1800629 and PPARγ rs1801282 SNPs evaluated in this study for their clinical usefulness in patients with NAFLD are well recognised risk factors for the development and progression of type 2 diabetes and metabolic syndrome. These pathogenic factors include obesity, low-grade chronic inflammation, and insulin resistance. The metabolic risk profile of insulin resistant NAFLD patients is greater when compared to non-diabetic patients. Hyperglycaemic NAFLD patients have a higher risk of developing fibrosis and cirrhosis (Smith and Adams, 2011). Insulin resistance unifies NAFLD with metabolic syndrome through the clustering of metabolic related factors such as glucose intolerance and/or diabetes, increased triglycerides, high cholesterol and obesity. A steatotic liver contributes to the development of insulin resistance, because of impaired insulin clearance from the portal vein. The ability to predict complications associated with insulin resistance will be valuable to treat and clinically manage NAFLD patients. Identification of the underlying disease mechanism is very important as insulin
resistance can also occur in patients without overt glucose alterations, where the ability of insulin to inhibit glucose is reduced and glucose utilization is stimulated.

Chronic low-grade systemic inflammation as reflected by elevated serum markers (e.g. TNF-α) are continuously present in obese individuals. NASH is the more advanced form of liver disease and a marked component of the pro-inflammatory state. Several studies have implicated variation in the TNF-α gene in the development of NASH (Manco et al. 2007; Stechové et al. 2012; Wang et al. 2011). Fatty acids and TNF-α molecules stimulate the inhibitory phosphorylation pathway of serine in the insulin receptor. This finding correlates with the genetic link of the TNF-α gene with insulin resistance and inflammation in the development of NASH. The association of TNF-α with hepatic insulin resistance could possibly clarify the connection between insulin resistance and the liver’s role in the development of cardiovascular related diseases.

The susceptibility to develop progressive NASH is influenced by genetic variation as genes involved in inflammation, oxidative stress and fibrogenesis have been implicated with the severity of liver disease (Aller et al. 2010; Valentini et al. 2002). The heritable component of NAFLD and NASH has also been confirmed in this study, especially in relation to the TNF-α rs1800629 -308 G>A polymorphism alone or even more so as part of a 6-allele risk score. Since the clinical characteristics of NAFLD include obesity and insulin resistance it seems probable that treating NAFLD patients with anti-inflammation agents such as pioglitazone may be an attractive treatment option. Pharmacogenetic studies are warranted to compare the effectiveness of this intervention strategy between patients with and without the TNF-α rs1800629 -308 G>A SNP.

In relation to the PPARγ rs1801282 (Pro12Ala, 34 C>G) SNP, a significant association with HbA1c levels were demonstrated in diabetic patients with the minor G-allele treated with rosiglitazone, with a significant decrease in HOMA-IR levels compared to those with the risk-associated PPARγ C-allele (Kang et al. 2005). It therefore seems likely that NAFLD patients with the minor allele will benefit most from this treatment but again, pharmacogenetic studies are needed in the South African population to investigate this treatment option for a subgroup of patients with insulin resistance further. A variety of ligands can bind to PPARγ, including fatty acids, thiazolidinediones and non-steroidal anti-inflammatory drugs such as ibuprofen.
PPARγ is a thrifty transcription factor gene co-ordinating adipogenesis and glucose homeostasis. Variation in the PPARγ gene is associated with insulin sensitivity, inflammation and adipogenesis. PPARγ represents an attractive NAFLD candidate gene, because both hepatic lipid metabolism and insulin resistance are contributors to the pathogenic phenomena of NAFLD (Gawrieh et al. 2012). Moreover, numerous PPARγ agonists have been used in therapeutic intervention trials for individuals with NASH, emphasizing the role of PPARγ in liver related diseases (Sanyal et al. 2010). When there is an imbalance between lipid influx and lipid removal, accumulation of lipids in the liver occurs. More than half of the triglycerides found in the liver of NAFLD patients originate from plasma albumin bounded-fatty acids and 15% derived directly from the diet (Tailleux et al. 2012). Under conditions of insulin resistance the hepatic fatty acid flux and uptake are enhanced and contributes to the pathogenesis of type 2 diabetes.

Over expression of PPARγ is recognised as a feature of the steatotic liver and attributed to the activation of lipogenic genes involved in steatotic development (Gavrilova et al. 2003). Specific targeted deletion of PPARγ in the hepatocytes of mice protected them against diet induced hepatic steatosis, suggesting a pro-steatotic role of PPARγ (Morán-Salvador et al. 2011). In contrast, over expression of PPARγ improved hepatic steatosis inflammation and fibrosis in the liver (Wu et al. 2010). The heterogenic effects of PPARγ modulating NAFLD features could be due to its action on different metabolic pathways and tissues (Tailleux et al. 2012).

Studies performed on children with NAFLD have shown that children develop the full spectrum of NAFLD (Lavine et al. 2004). Obese or overweight children with NAFLD typically have elevated fasting serum triglyceride levels. Tominaga et al. (1995) conducted a study in Japan with 810 children aged 4-12 years and demonstrated that the presence of sonographically detectable NAFLD was 2.6% and its presence correlated with obesity.

To facilitate the prevention of cumulative risk related to low-penetrance mutations such as those studied in the FTO, PPARγ and TNF-α genes a pathway approach to validation and incorporation of an increasing number of clinically useful SNPs into a risk score is needed. Intervention strategies based on a mechanistic understanding of the disease and elimination of known modifiable risk factors may facilitate prevention of disease.
progression from insulin resistance to NASH. Development of innovative approaches to risk management of complex multifactorial diseases, which could be applied in a clinical context (combining the clinical relevant information, genetic testing and biochemical profiles), is becoming increasingly important for both preventative and therapeutic interventions.

4.6 Gene Based Intervention Strategy
Development of health guidelines by combining evidence-based medical and lifestyle advice with genetic information based on the functional effects and associated disease mechanisms or pathways affected by genetic variation is an important consideration for clinical application. When the knowledge gained in this study is combined with consistent information from the literature and a patient’s family history, pathology and environmental risk, it may be possible to counteract the deleterious effects of FTO rs9939609 intron 1 T>A, TNF-α rs1800629 -308 G>A and PPARγ rs1801282 34 C>G. Potential actionable information in relation to these SNPs is provided below, for possible future application in a clinical implementation study.

FTO rs9939609 (intron 1 T>A)
Since increased expression of FTO was found to be associated with an increased body mass (Li et al. 2012), it is reasonable to assume that intervention strategies that may reduce gene expression would be beneficial for NAFLD patients to reduce the risk of obesity and associated medical conditions. Recent studies have shown that FTO expression is regulated by glucose and the availability of essential amino acids (Cheung et al. 2012), which may explain why individuals with the FTO rs1558902 variant (occurring in the same gene region as FTO rs9939609) had a greater reduction in weight and improved fat distribution in response to a high-protein diet. An opposite genetic effect was observed on changes in fat distribution in response to a low-protein diet (Zhang et al. 2012). FTO A- allele carriers must also ensure that their fat intake does not exceed 30% of total energy, as the increase in BMI associated with the FTO A-allele appears to be restricted to individuals with a high fat intake (Frayling et al. 2007). A Mediterranean diet may be particularly beneficial for FTO A-allele carriers (Razquin et al. 2010). Although Kruger et al. (2008) reported that the presence of obesity did not predict the presence of more advanced liver disease in South African NAFLD patients, a healthy body weight should always be an important clinical goal and failure to detect a gene
effect relating to obesity underscores the importance of a combining evidence-based clinical health guidelines with genetic information using the PSGT approach.

Spontaneous cortical activity in lean individuals is increased through insulin, while in obese individuals this effect was found to be absent. Cerebrocortical insulin resistance prevents normal body weight regulation, because insulin is needed as an adiposity- and satiety signal (Tschritter et al. 2007; Shimaoka et al. 2010). The genotype effect on cerebrocortical insulin sensitivity is similar to the effect of increased weight; this implies that variation in the FTO gene contributes to the pathogenesis of obesity. In this study the A-allele of FTO rs9939609 increased LDL-cholesterol on average by 54%, compared to having inherited no A-allele (p=0.002). A similar effect was observed in relation to decreased for HDL-cholesterol (p=0.053), which together with total cholesterol (p=0.063) and triglycerides determine LDL-cholesterol levels. FTO rs9939609 A-allele carriers were shown to loose most weight on a low fat hypocaloric diet causing metabolic improvements through decreased total and LDL cholesterol and CRP levels observed in this genetic subgroup (de Luis et al. 2012). A study conducted by Zhou et al. (2012) confirmed that FTO rs9939609 T>A positively correlates with features of the metabolic syndrome, including hypertension, higher fasting insulin, glucose, triglycerides and lower HDL-cholesterol levels.

Intervention strategies targeted at components of metabolic syndrome is therefore advisable irrespective of mutation status, while avoidance of weight gain should be strongly advised in individuals with the FTO A-allele. It was shown that individuals meeting exercise recommendations of at least 60 minutes moderate to vigorous physical activity per day could overcome the effect of FTO rs9939609 T>A on obesity related traits (Ruiz et al. 2010). The importance of exercise was confirmed in this study as the average waist circumference of physically inactive NAFLD patients was 12% higher compared to the active patients (p=0.004), irrespective of mutation status. The finding that FTO has a demethylation function in mRNA expression indicated a novel, reversible epigenetic regulatory mechanism (Guifang et al. 2011).

**TNF-α rs1800629 (-308 G>A)**

In patients with the pro-inflammatory TNF-α SNP medical and nutritional therapy needs to be targeted at regulation of inflammation. Fat accumulation needs to be avoided as it
is accompanied by low-grade systemic inflammation which results in resistance to weight loss, which is particularly relevant to NAFLD patients. The data generated in this study demonstrated a statistically significant association between the risk-associated TNF-α rs1800629 A-allele and CRP levels (p=0.029). This biochemical marker of inflammation increased significantly (25.7 mg/l) with each risk-associated A-allele detected among fatty liver disease patients. Notably, this association was revealed only after adjusting also for alcohol intake due to the significant effect of low-moderate alcohol intake (below the level for diagnosis of NAFLD) to reduce CRP levels. Patients who reported low-moderate alcohol intake had on average 70% reduced CRP levels compared with patients who abstain from alcohol drinking (p=0.038) therefore moderate alcohol consumption may be beneficial in the context of inflammation; however it must be considered in conjunction with other medical and lifestyle factors. Chronic systemic low grade inflammation reflected by CRP increases with the degree of obesity and insulin resistance, especially if coronary artery disease is also present (Wannamethee et al. 2004).

Tsukui et al. (2000) reported that exercise and TNF-α rs1800629 independently correlates with changes in HbA1c and fasting insulin levels. It is therefore advisable for individuals with TNF-α rs1800629 (-304 G>A) to consume foods with a low glycemic index to reduce the risk of obesity, insulin resistance and diabetes (Willet et al. 2002) and to include omega-3 fatty acids in the diet on a regular basis, since it has anti-inflammatory effects (Simopoulos, 2002). Rapidly digested and absorbed carbohydrates should also be avoided since it appears to exacerbate the pro-inflammatory process especially in overweight individuals prone to insulin resistance (Radulian et al. 2009). According to Joffe et al. (2010) the TNF-α rs1800629 (-308 G>A) SNP modifies the relationship between dietary fat intake and total cholesterol concentrations in Caucasian South African women. When intake of saturated fatty acids are increased, serum total cholesterol levels decreased in individuals with the TNF-α GG genotype and increased in individuals with the GA or AA TNF-α genotypes. This confirms a diet-genotype interaction which suggests that individuals with the risk associated TNF-α rs1800629 A-allele are more responsive to changes in dietary fat intake compared with the G-allele carriers and would benefit from a diet intervention strategy. Adequate intake of foods high in antioxidants especially vitamin E, glutamine (e.g. raw spinach, parsley), arginine (e.g. dairy products, meat, peanuts), vitamin C (60mg/day) and vitamin B
supplementation (Aeberli et al. 2006; Majewicz et al. 2005) may also be beneficial for TNF-α rs1800629 G>A individuals since it enhances immune function and reduces inflammation. Several studies have reported higher TNF-α concentrations associated with CVD and other diseases such as chronic obstructive pulmonary disease in smokers compared to non-smokers (Tappia et al. 1995; Bennet et al. 2006; Jang et al. 2007; Gingo et al. 2008). A combined effect for smoking and the TNF-α SNP was not observed in our study population where insulin resistance was identified as the most important risk factor that may mask less prominent environmental risk factors. Smoking should always be discouraged irrespective of genetic test results and demonstration of a significant interaction with genetic risk factors would reinforce the importance of smoking cessation.

**PPARγ rs1801282 (Pro12Ala, 34 C>G)**

Given the potential role of PPARγ as a nutrient sensor of fatty acids, dietary fat interaction with genetic variation in the gene is likely to contribute to the pathogenesis of the metabolic syndrome, insulin resistance and NAFLD. Individuals with the PPARγ rs1801282 SNP are considered hyper-responders as they are more sensitive to environmental stimuli such as fat intake (Robitaille et al. 2003). Intervention strategies targeted at avoidance of weight gain by increased activity and calorie restriction, and the use of medication where indicated to lower blood fatty acids would be beneficial for individuals with the PPARγ rs1801282 SNP (Bruss et al. 2010; Eakin et al. 2010; Guo et al. 2002). Replacement of saturated fat with polyunsaturated and monounsaturated fats has a beneficial effect on weight loss. When the dietary polyunsaturated fat to saturated fat ratio is low, the mean BMI in G-allele carriers is greater than in individuals with the risk-associate C-allele and when the ratio is high the opposite is true (Luan et al. 2002). A regular dietary intake of foods rich in omega-3 fatty acids should be encouraged as it improves insulin sensitivity, promotes glucose control, and reduces inflammation (Kuda et al. 2009; Simopoulos, 2002). Since PPARγ rs1801282 C>G is associated with insulin resistance a diet high in plant fats and foods with a low glycemic index (GI≤55) would be beneficial to reduce insulin resistance in these individuals (Gilbertson et al. 2003; Fowler, 2007). The interaction detected between the PPARγ SNP and smoking indicated that both factors exhibited a synergistic effect on the development of NAFLD (Yang et al. 2012). The interaction between these two independent risk factors supports the idea of genetic and environmental factors contributing to the development and progression of
NAFLD (Day, 2006). NAFLD patients (types 1,2) are at a high risk to progress to NASH especially in the presence of relevant genetic and environmental factors such as smoking. Simultaneous analysis of the SNPs with associated modifiable risk factors may increase the clinical usefulness for improved risk profiling of NAFLD patients.

Metabolic syndrome was found to be significantly more prevalent in patients with NASH (88%) compared to those with a fatty liver alone (53%) (Marchesini et al. 2003). Interindividual variability in response to dietary changes is greatly determined by genetic and environmental factors. The phenotypic response to diet is affected by genetic factors with external factors contributing to the clinical variation observed. To increase the clinical usefulness of SNPs the results should be interpreted in the context of clinical indicators and lifestyle risk factors (Ordovas and Corella, 2005). Considering all the known risk factors relevant to the disease and genes tested would contribute to improved health and disease management.

4.7. Potential modifiers of NAFLD

When considering the NAFLD disease process, genetic modifiers should be considered in the context of relevant environmental factors, clinical co-factors and use of medication.

Alcohol consumption

Alcohol abuse is common in the United States where a chronic relapse prevalence of 8.5% was reported for individuals who participated in a 12 month intervention program (Grant et al. 2004). Predictors of future addiction include both genetic (family history of alcoholism) and environmental factors. A meta-analysis of 10 000 twin pairs revealed that the overall heritability of addictive substances was 40%-70% with alcoholism about 50% (Goldman et al. 2005). This accentuates that both genetic and environmental factors are equally important to fully elucidate the pathogenesis of complex polygenic diseases (McCarthy and Hirschhorn, 2008). According to Dunn et al. (2012) the prevalence of steatohepatitis in patients with NAFLD decreased with moderate alcohol consumption. In the United States as many as 50% of adults regularly consume a modest amount of alcohol and within our study patients who reported low-moderate alcohol intake had on average 70% reduced CRP levels compared with patients who abstain from alcohol drinking.
The proposed protective mechanism with moderate alcohol consumption is possibly through the action of insulin resistance and it has shown to improve the metabolic risk factors for NAFLD (Dixon et al. 2001; Davies et al. 2002; Howard et al. 2004). In heavy drinkers obesity increases the risk of steatosis by twofold (Vernon et al. 2011).

A meta-analysis of more than 34 prospective studies demonstrated a survival benefit with moderate alcohol consumption, especially in diabetic patients. Moderate alcohol consumption not only improves the prevalence of steatohepatitis, but also modifies cardiovascular risk (Di-Castelnuovo et al. 2006). Data provided by an atherosclerosis study showed that moderate alcohol consumption lowered the risk of a cardiovascular event by 38% in non-drinkers who began to consume alcohol (10g of alcohol/day) (Valmadrid et al. 1999). Even though moderate alcohol consumption is beneficial in some settings, even a miniscule amount of alcohol could aggravate diseases or conditions such as breast cancer or hepatitis C (Dunn et al. 2012). More research is needed regarding the role of moderate alcohol consumption in the prevention or treatment of NASH. We could not evaluate the effect of high alcohol consumption in this study in relation to disease severity, because patients were pre-selected on the basis of alcohol consumption for the non-alcoholic form of fatty liver disease (NAFLD). However low to moderate alcohol consumption was higher in the Caucasian NAFLD patients (32%) compared to the Coloured NAFLD patients (2%).

**Iron metabolism**

Liver disease progression is influenced by both alcohol consumption and impaired iron homeostasis. Hyperferritinaemia is frequently observed in both the metabolic syndrome and NAFLD patients (Wrede et al. 2006; Zelber-Sagi et al. 2007). The hepatic iron overload syndrome characterised by hyperferritinaemia unrelated to hemochromatosis is frequently associated with various components of the metabolic syndrome and insulin resistant pathways (Mendler et al. 1999) Hence the term *insulin-resistance associated hepatic iron overload syndrome*. Mild hepatic iron accumulation in NAFLD involves both the hepatocytes and sinusoidal Kupffer cells.

Low-penetrance HFE gene mutations H63D and C282Y are responsible for the majority of hereditary hemochromatosis (HH) cases in the Caucasian population. Since NAFLD and HFE mutations are relatively common many studies focused on the association between these two conditions. Iron overload and the presence of HFE mutations with the
more severe forms of liver disease (NASH) has been established (Fisher, 2011). The features of hereditary iron overload include cardiomyopathy, cirrhosis of the liver, diabetes and skin pigmentation. The heart may be especially sensitive to the toxic effects of iron overload (Sullivan, 1990). Hemochromatosis can easily be treated by phlebotomy once diagnosed; therefore this condition is considered a preventable form of heart disease amongst other equally important health risks. The risk of cardiovascular death in postmenopausal women with at least one copy of the C282Y mutation is stronger in women who are hypertensive or current smokers, with a nearly 20-fold increased risk when both risk factors are present, compared with non-smokers, non-hypertensives, and non-mutation carriers (Roest et al. 1999). This finding again emphasised the importance of multiple risk factor assessment when low-expression mutations such as those implicated in multifactorial diseases such as NAFLD are analysed.

A marked increase of pro-inflammatory cytokines such as TNF-α has been observed in NAFLD and obese patients. TNF-α is an important regulator of iron homeostasis through its stimulatory effects on ferritin formation (Aigner et al. 2008). Iron overload contribute to the progression of NAFLD through oxidative stress and fibrogenesis within the liver (Tsuchiya et al. 2009).

The mechanisms involved in hepatic iron accumulation in NAFLD patients have not yet been fully elucidated and may include many genetic factors (including HFE mutations), iron regulatory pathways (Kupffer cells) and metabolic factors (insulin resistance) (Sumida et al. 2009).

The appropriateness of applying the information generated from this and other studies is determined by the individuals risk profile, family history, age of onset of disease and the mutations correlated with the disease. Kotze et al. (2009) established the PSGT approach for incorporation of low-penetrance functional SNPs that are not sufficient to explain the phenotype into an intervention plan for complex diseases such as HH and CVD. This approach may in future also be used in patients with NAFLD to identify molecular targets for risk reduction within the context of the medical history and relevant lifestyle factors.
Insulin-sensitizers

Thiazolidinedione (TZD) therapy was evaluated and post treatment histologically showed improved steatosis, hepatocellular ballooning, and inflammation with TZD treatment. The risk of fibrosis progression was significantly reduced with TZDs treatment (Musso et al. 2012). Insulin sensitizing agents such as the thiazolidinedione's, a agonist for the PPAR receptors, improved ovarian androgen biosynthesis and ovulatory function (Orio et al. 2003). Non-steroidal anti-inflammatory drugs (NSAID) have the ability to activate PPARγ receptors. Epidemiological studies have shown that NSAID could reduce the risk of Alzheimer's disease by as much as 80%; these effects are attributed to the ability of these drugs to stimulate PPARγ activity and inhibit inflammatory processes in the brain (Heneka et al. 2011). Metformin has anorexigenic and weight loss properties which increases insulin sensitivity. This is an important consideration for diabetic NAFLD individuals with the FTO rs9939609 T>A mutation, since the FTO A-allele is significantly associated with higher BMI, higher body fat percentage, and higher waist circumference (Dina et al. 2007; Zhou et al. 2012b). It also positively correlated with other symptoms of the metabolic syndrome, including hypertension, higher fasting insulin, glucose, triglycerides and lower HDL-cholesterol levels. Metformin significantly reduces body weight, waist circumference and HOMA-IR (Sofer et al. 2011).

Lipid-lowering drugs

Statins has been shown to improve ALT and radiological steatosis in hyperlipidaemic NAFLD patients and have a cardiovascular benefit (Musso et al. 2012). Ezetimibe reduces histological ballooning and fibrosis by connecting non-estrified cholesterol accumulation to hepatocyte apoptosis, liver injury and NASH development (Nelson et al. 2009). The modulation of insulin sensitivity, systemic inflammation, hepatic lipogenesis and fibrogenesis could be achieved using angiotensin blockers. Hypertensive NASH, telimisartin an angiotensin receptor blocker with PPARγ regulating activity improved steatosis, necroinflammation, fibrosis and HOMA-IR (Fabbrini et al. 2010). Combining a diet intervention program with lipid-lowering drugs (gene based medical intervention) may improve the health outcomes of NAFLD and NASH individuals in some genetically predisposed patients.
Anti-inflammatories

The anti-TNF-α agent pentoxifylline improved histological steatosis and lobular inflammation in NAFLD individuals (Musso et al. 2012). TNF-α antibody administration is beneficial in metabolic inflammation and has been demonstrated to improve steatosis (Barbuio et al. 2007). TNF-α expression in NASH/NAFLD patients significantly correlates with the degree of fibrosis (Manco et al. 2007; Wong et al. 2006). Ligands of the peroxisome proliferator receptor gamma (PPARγ), pioglitazone, are able to suppress TNF-α function which positively affected fatty liver disease (Musso et al. 2012).

For individuals not responding to lifestyle intervention strategies such as the more severe form of NAFLD types 3,4 (NASH), pharmacological treatment may be considered in conjunction with a lifestyle intervention strategy. Currently no specific pharmacological treatments are available. However among the available agents TZDs, statins and metformin have consistently been used, but their impact on liver histology is still unknown.
Chapter V

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As a result of this study high throughput genetic assays for the FTO rs9939609 (intron 1 T>A), TNF-α rs1800629 (-308 G>A) and PPARγ rs1801282 (Pro12Ala, 34 C>G) SNPs implicated in obesity, inflammation and insulin resistance was successfully optimised. The clinical significance of the rare risk-associated A-allele of TNF-α rs1800629 (-308 G>A) and common risk-associated C-allele of PPARγ rs1801282 (Pro12Ala, 34 C>G) has furthermore been firmly established in South African patients with the hepatic manifestation of the metabolic syndrome. The TNF-α rs1800629 and PPARγ rs1801282 A-C allelic combination underlying inflammation and insulin resistance was the most important determinant for earlier development of NAFLD in patients with features of the metabolic syndrome. The well-established risk of obesity associated with the A-allele of FTO rs9939609 (intron 1 T>A), either independently or as part of the inferred allelic combination A-A-C for the FTO (rs9939609, intron 1 T>A), TNF-α (rs1800629, -308 G>A) and PPARγ (rs1801282, Pro12Ala C>G) SNPs, did not significantly contribute to development of NAFLD or progression to NASH in the South African cohort studied. This finding provided genetic confirmation of earlier findings by Kruger et al. (2010), which excluded obesity as an independent risk factor for progression from NAFLD (types 1,2) to NASH (NAFLD types 3,4), prior to genotyping of South African patients that was performed for the first time in this study. Although FTO rs9939609 (intron 1 T>A) has previously been associated with insulin resistance and type II diabetes, this effect can be entirely explained by its impact on BMI and no fatness-independent allelic effects on a wide range of inflammatory markers could also be observed in healthy individuals (Zimmermann et al. 2011).

The significant correlation observed between the number of TNF-α rs1800629 A-alleles and increasing CRP levels (p=0.029) and the favourable effect of low- to moderate alcohol intake on CRP levels in our study population are important observations. This information combined with the finding that the average waist circumference of physically active NAFLD patients was 12% lower than in physically inactive patients (p=0.004) may be incorporated in future preventative strategies based partly on genetic knowledge. This study provided evidence at the DNA level that the TNF-α rs1800629 A-allele increases the risk of disease progression from NAFLD (types 1,2) to NASH (types 3,4); age of onset of fatty liver disease was on average 5 years younger in the presence of each risk-associated TNF-α rs1800629 A-allele (p=0.028). Our findings are in agreement with earlier RNA expression studies in the parent study, demonstrating differential gene
expression between South African NAFLD (types 1,2) and NASH (types 3,4) patients (Kruger, 2008).

5.1. Ethical Considerations

The information obtained from genetic testing may not only relate to the tested individual, but also his/her close family members. Therefore information should be kept strictly confidential and all patients referred for genetic testing should preferably receive both pre- and post-test counselling by registered genetic counsellors. However, this is most relevant to genetic testing of high-penetrance mutations to advise on family screening for (presymptomatic) disease diagnosis aimed at disease prevention and targeted treatment of high-risk individuals.

Due to the increasing use of genetic testing to direct treatment, it is now accepted internationally that because of the small number of registered genetic counsellors and the diversity of the types of genetic tests that are available, clinicians will increasingly request genetic tests for their patients to guide treatment. Genetic testing of low-penetrance mutations is best performed in the context of the family history and/or testing of high penetrance mutations where indicated due to biochemical abnormalities or early disease onset, to prevent misinterpretation of complex genetic information. The following should therefore be explained where relevant to each individual before specimens are collected for laboratory testing, possibly as an extension of the CVD multigene test (Kotze et al. 2003, Kotze and Thiart 2003), as summarised in the ethically approved study protocol:

- The genetic test will only screen for specific genetic alterations expected to provide useful information in relation to treatment/diet intervention.
- 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.
- Early detection or pre-clinical diagnosis of treatable or preventable genetic diseases is beneficial.
• 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.
• The genetic material is stored for reference purposes or to perform follow-up testing and may be stored and included in a genetic database for research related to the test requested, unless declined.
• The genetic material may be tested at different laboratories or using different mutation detection methods as part of a quality control process.
• 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.
• 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.

5.2. Study Limitations
The control population consisted only of Caucasian samples; no Coloured control samples were available in this study for comparative analysis. Blood biochemistry was also not done for all the Caucasian control samples. For the general Caucasian control population individuals who reported a diagnosis of NAFLD, insulin resistance or diabetes were excluded, but the population was not sonographically or histologically confirmed to be NAFLD free. Some of the fatty liver patients (obese group with BMI >30 kg/m²) identified on ultrasound alone did not fulfil the criteria for a liver biopsy and there are some uncertainty regarding their disease diagnosis.

Not all genetic variations that potentially could contribute to the pathogenesis of NAFLD were analysed. Three SNPs were selected for this study to determine the appropriateness of the study population for extended SNP analysis to construct a more comprehensive genotype score. DNA availability from samples of the parent study was insufficient or of poor quality which reduced the sample size for statistical analysis.
5.3. Future prospects

Determination of the genetic basis of differences in response to environmental exposures such as alcohol consumption, smoking, physical inactivity, diet and certain drugs could provide a valuable adjunct to current clinical practice. Linking genes to disease in the context of known environmental triggering or protective factors is fundamental for implementation of preventive strategies. Genetic variation in low penetrance genes such as FTO (rs9939609 intron 1 T>A), TNF-α (rs1800629 -308 G>A) and PPARγ (rs1801282 Pro12Ala C>G) should always be correlated with the appropriate clinical and biochemical parameters to assess gene expression and response to treatment, while also taking family history and the presence and absence of known environmental risk factors affecting the genes into account. Improvement of biochemical abnormalities after elimination of modifiable risk factors could provide a measure of the effectiveness of the intervention program.

It is important to identify both genetic and environmental components contributing to multi-factorial diseases such as NAFLD, as a single SNP is not sufficient to cause disease. Further study is required to further clarify the role of FTO in the relationship between obesity and insulin resistance in NAFLD patients. This should include analysis of additional SNPs in the FTO gene as well as the transcription-factor-7-like-2 (TCF7L2) gene (also known as TCF4), described as a transcription factor influencing the transcription of FTO and several other genes. TCF7L2 binds to the FTO promoter region causing the overexpression of FTO (Zhou et al. 2012). TCF7L2 have consistently been associated with type 2 diabetes, irrespective of ethnic descent, making it one of the most important loci known today to predispose individuals to type 2 diabetes.

TCF7L2 SNP rs7903146 increases type 2 diabetes susceptibility in the general population through β-cell dysfunction and impaired incretin action (Musso et al. 2009). This functional SNP modulates postprandial hepatocyte apoptosis, combined with the unfavourable adipokine and lipoprotein profile induced by fat ingestion in NASH individuals. Although the severity of steatosis and fibrosis seems to be directly associated with the TCF7L2 polymorphism, its association with necro-inflammation
appears to be mediated by adiponectin and oxidized-LDL. In this context, it is noteworthy that PPARγ agonists, currently proposed for the treatment of NAFLD, promote adipocyte differentiation by reducing β-catenin levels and inhibiting TCF activation (Gustafson et al. 2006). TCF7L2 predisposes individuals to NAFLD and to metabolic related disorders involving β-cell function, hepatic insulin sensitivity, glucose and lipoprotein homeostasis (Musso et al. 2009). Prospectively, interventions toward restoring hepatic insulin sensitivity and β-cell function may potentially direct more tailored intervention strategies.

Other genetic determinants for further study in the context of dyslipidaemia contributing to NAFLD are the apolipoprotein E (ApoE2 or ApoE4), apolipoprotein C3 (ApoC3) and patatin-like phospholipase domain-containing 3 (PNPLA3) genes (He et al. 2010). Studies have reported smaller LDL particle size in ApoE2 allele carriers compared to larger LDL particle size in ApoE4 individuals (Moreno et al. 2004). Furthermore a strong correlation between presence of the I148M SNP in the PNPLA3 gene and elevated serum ALT and AST levels with liver inflammation were found. Genetic variation in the ApoC3 gene alone, the PNPLA3 gene alone, and the combined risk-associated genotypes, accounted for 11.0%, 6.5% and 13.1% of the variance in the risk of NAFLD, respectively (Petersen et al. 2010).

In the last decade several biomarkers have been investigated to noninvasively distinguish between NAFLD and its subgroup NASH (Lockman et al. 2010). Recent studies have shown that biomarkers associated with NAFLD and liver disease present from a young age and may help to discriminate between NAFLD patients at increased risk of coronary artery disease as opposed to the development of advanced liver disease (NASH) (Valentini et al. 2010). The metabolic syndrome is an independent cardiovascular risk marker for diabetes and coronary artery disease (Lee et al. 2007). This was substantiated in a study that reported a high prevalence of coronary artery disease in South African Africans with the metabolic syndrome and insulin resistance (Ntyintyane et al. 2006). According to Shimada et al. (2007), 90% of early stage NASH can be predicted by combining HOMA-IR with other biochemical markers such as adiponectin and HbA1c. Since NASH may progress to cirrhosis, clinicians assessing obese patients must be able to differentiate NASH from simple steatosis. Sorrentino et al. (2010) suggested that a higher HOMA-IR level is independently associated with a more progressive stage of fibrosis and also an independent predictive factor for fibrosis.
worsening. HbA1c could possibly be used as a predictive factor or marker for postprandial glucose metabolism in NAFLD patients with normal fasting glucose levels.

In addition to standard metabolic indicators, LDL particle size and the aspartate aminotransferase to platelet ratio index (APRI) can be valuable biomarkers to distinguish between NAFLD patients at increased risk of coronary artery disease or advanced liver disease, respectively. APRI was found to be significantly higher in South African NAFLD patients with advanced fibrosis compared with patients with no or mild fibrosis (p<0.01) (Kruger et al. 2011).

The development of a more comprehensive risk score that combines both lifestyle (diet, exercise score) and genetic (PNPLA3, TCF7L2, APOC3 HFE etc.) factors may be beneficial in risk management of patients with NAFLD. However the aforementioned genes in conjunction with other biochemical factors needs to be considered in a holistic approach do determine the clinical relevance of the risk score in future treatment of multi-factorial diseases. The information gathered from the current study may be considered for implemented as part of a comprehensive genetic screen for disease subtyping, which includes the analysis of gene mutations associated with dyslipidemia, iron overload, oxidative stress and inflammation in conjunction with a gene based nutritional and pharmacological intervention program. This approach may in future prove to improve the sensitivity and specificity of biomarkers such as APRI. To fully integrate all the information a systematic approach is needed. The Gkwnomix™ platform aims to combine all the relevant information to facilitate the prevention of cumulative risk based on disease subtyping to identify pathways for targeted treatment. The clinical, lifestyle and biochemical information could be captured in a database that could be used for future studies.

Even though these findings are very promising, its potential application for disease prevention and improved clinical management of NAFLD remains to be investigated in the diverse South African population. More research is needed in African Black populations to develop an appropriate intervention strategy, since ethnicity influence the prevalence of NAFLD the disease outcome may differ within this ethnic group. As obesity and diabetes mellitus type 2 is becoming more prevalent in South African children, it is important to extend this study to children with or at risk of NAFLD as well.
While the potential impact of an increasing genotype risk score and allelic combinations were assessed on disease progression from obese, NAFLD (types 1, 2) and NASH (types 3,4) after adjustment for several variables, development of a model that evaluates the cumulative effect of allelic variation in the presence and absence of known environmental risk factors such as smoking, alcohol consumption and physical activity resided outside the scope of this study. Further studies are therefore warranted to include an increasing number of clinically useful SNPs for evaluation as well as modifiable environmental risk factors to develop a genotype “activity” score or weighted score for more accurate prediction of phenotypic expression. Use of a pathway-based approach to analyse the influence of modifiable environmental risk factors in the presence of low-penetrance genetic risk factors implicated in insulin resistance as a central feature of NAFLD, may eventually enable the development of a risk assessment model incorporating multiple variables that collectively are sufficient to cause biochemical and metabolic abnormalities underlying disease progression from NAFLD (types 1, 2) to NASH.
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