# A study of non-alcoholic fatty liver disease (NAFLD) in South African patients and analysis of selected candidate genes implicated in insulin resistance and fatty acid oxidation.

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Dissertation presented for the degree of




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# Declaration

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# SUMMARY

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in Western countries, extending from steatosis (FLD) to steatohepatitis (NASH). Differentiation between NASH and nonprogressive NAFLD is difficult on clinical grounds therefore a need exists to identify reliable biomarkers of disease progression.

The aims of the study were 1) to describe the disease profile of NAFLD/NASH in South African patients of the Western Cape, 2) to investigate the metabolic derangements associated with this condition, including insulin resistance, lipid abnormalities and liver fibrogenesis, and 3) to assess the possible involvement of candidate genes in relation to the disease phenotype in the patient cohort.

A total of 233 patients (73% female) were enrolled in this study, consisting of 69% Cape Coloured, 25% Caucasian, 5% Black and 1% Asian individuals. All subjects were obese or overweight based on the assessment of body mass index (BMI). Screening for NAFLD identified 182 patients (87%) with ultrasonographical evidence of fatty infiltration and/or hepatomegaly. Liver biopsies were performed on patients with persistently abnormal liver functions and/or hepatomegaly. NAFLD was confirmed histologically in 111 patients of whom 36% had NASH and 17% advanced liver fibrosis. None of the Black patients had advanced fibrosis.

Laboratory analyses to investigate metabolic derangements included determination of liver biochemistry, insulin resistance (HOMA-IR), serum lipogram, LDL(low-density lipoprotein) particle size and serum iron status. Patients with NASH had significantly higher mean serum cholesterol ($p<0.01$) and triglyceride ($p=0.03$) levels than those with fatty liver only. LDL particle size decreased significantly from fatty liver disease to NASH ($p=0.03$), while no difference was observed between no/mild fibrosis and advanced fibrosis ($p=0.44$). Small LDL particle size adds to the atherogenic profile in NASH characterised by significantly increased serum cholesterol and triglyceride levels, known to increase the risk of cardiovascular morbidity and mortality in patients with NAFLD. Failure to detect a significant association between LDL size and degree of fibrosis largely excluded coronary artery disease (CAD) as a disease complication in NAFLD patients with advanced fibrosis (stage 3 and 4).

Discrimination between NAFLD patients at increased risk of CAD as apposed to the development of advanced liver disease poses an important clinical question. Therefore, the aspartate aminotransferase to platelet ratio index (APRI) and HFE mutation status were also investigated in an attempt to identify non-invasive markers for advanced liver disease. APRI was found to be significantly higher in the advanced fibrosis group compared with patients with no or mild fibrosis ($p<0.01$). When compared to previously validated markers for advanced fibrosis such as the NASH Fibrosis score (NFS) and the AST/ALT ratio, APRI was more sensitive and specific for advanced fibrosis. The frequencies of HFE mutations C282Y and H63D did not differ significantly between patient groups with no, mild or advanced fibrosis and the genotype distribution was similar to that previously reported in the South African Caucasian and Coloured populations.

Gene expression analyses of adiponectin, manganese superoxide dismutase (MnSOD/SOD2), tumour necrosis factor alpha (TNF-$\alpha$) and the kappa-beta kinase gene (IKBK$\beta$/IKK$\beta$) were assessed in 80 liver biopsies of NAFLD patients subdivided into 4 clinical groups. Statistically significant differential gene expression profiles were demonstrated for TNF-$\alpha$ ($p=0.04$) and IKK$\beta$ ($p=0.02$) in patients with NASH, while no significant associations were observed in relation to stage of fibrosis.

In conclusion, this study is the first to describe the clinical characteristics of NAFLD in the South African population, albeit confined to the Western Cape region. In addition to standard metabolic indicators, LDL particle size and APRI proved to be valuable biomarkers to distinguish between NAFLD patients at increased risk of CAD or advanced liver disease, respectively, with important clinical implications for targeted treatment. Expression patterns of the TNF-$\alpha$ and IKK$\beta$ genes implicated in insulin resistance and inflammation showed the most significant association with NASH, in accordance with the important role of insulin resistance as a universal risk factor for development of CAD and advanced liver disease. The formulation of an algorithm based on the findings obtained in this study, integrated with existing knowledge in the field, made the utilisation of non-invasive biomarkers as an alternative to liver biopsy in risk management of NAFLD patients a possibility.

# OPSOMMING

Nie alkoholiese vetveranderinge van die lewer (NAFLD) is die algemeenste chroniese lewersiekte in die wêreld. Die siekte varieer van steatose alleenlik (FLD) tot steatohepatitis (NASH), die nie-benigne vorm van die siekte. Klinies is dit onmoontlik om tussen die toestande te onderskei. Daar is ‘n aanvraag na ‘n paneel van betroubare biomerkers vir beter voorspelling van die gevorderde fase van die siekte.

Die doelwitte van die studie was 1) om die siekte-profiel van NAFLD/NASH onder die verskilllende rassegroepe in die Wes-Kaap van Suid-Afrika te beskryf, 2) om die verskillende metaboliese verskynsels wat gepaardgaan met NAFLD/NASH, insluitende insulien weerstandigheid, lipied versteurings en lewer fibrogenese te beskryf, en 3) om die moontlike betrokkendheid van 4 kandidaatgene in verband met die die siekte fenotipe te ondersoek.

‘n Totaal van 233 pasiente (73% vrouens) is ingesluit in die studie waarvan 69% Kaapse Kleurlinge was, 25% Kaukasiers, 5% Swart en 1% Indiër. Al die betrokke deelnemers was obees of oorgewig soos bepaal deur hul liggaamsmassa indeks (BMI). Deur sifting met ultraklank vir vetveranderanderinge van die lewer of hepatomegalie is 182 (87%) pasiënte geidentifiseer. Lewerbiopsie is uitgevoer op die pasiënte met persisterende abnormale lewerfunksies of hepatomegalie. NAFLD is bevestig in 111 pasiënte waarvan 36% NASH gehad het en 17% gevorderde lewerfibrose.

Biochemiese analise vir metaboliese versteurings het ingesluit: insulien weerstandigheid, serum lipogram, LDL partikel grootte en serum yster studies. Die serum cholesterol ($p<0.01$) en trigliseried ($p=0.03$) waardes het betekenisvol verskil tussen pasiënte met FLD en NASH. Die verskil tussen pasiënte met geen/geringe fibrose en dié met gevorderde fibrose was nie betekenisvol nie ($p=0.44$). Die LDL partikel grootte het betekenisvol afgeneem van FLD na NASH ($p=0.03$) terwyl die partikel grootte nie verskil het tussen die groepe met geen/geringe fibrose en gevorderde fibrose nie ($p=0.44$). NASH word dus gekenmerk deur die teenwoordigheid van klein digte LDL wat kan lei tot aterosklerose en saam met hipertriglisedemie en hipercholesterolemie bydra tot die risiko vir kardiovaskulere morbiditeit en mortaliteit. Die gebrek aan ‘n assosiasie tussen LDL partikel grootte en die graad van fibrose sluit koronêre hartsiekte grootliks uit as ‘n komplikasie van NASH met gevorderde fibrose (stadium 3 en 4).

Die onderskeid tussen pasiënte met NAFLD met verhoogde risiko vir koronêre hartsiekte en gevorderde lewerfibrose skep ‘n belangrike kliniese probleem. Die aspartaat aminotransferase tot plaatjie verhouding (APRI) en HFE mutasies is ondersoek as moontlike nie-ingrypende merkers vir gevorderde lewersiekte in NASH. APRI was betekenisvol verhoog in die groep met gevorderde fibrose in vergeleke met die groep met min/matige fibrose (<0.01). In vergeleke met reeds gevalideerde merkers vir gevorderde fibrose soos NASH fibrose telling (NFS) en AST/ALT was APRI meer sensitief en spesifiek vir gevorderde fibrose. Die frekwensie van die HFE mutasies C282Y en H63D het nie betekenisvol verskil in die groepe met verskillende grade van fibrose nie en die genotipe verspreiding was dieselfde as wat voorheen beskryf is in die Suid-Afrikaanse Kaukasiese en Kleurling populasies.

Die geen ekspressie van adiponectin, manganese superoksied dismutasie (MnSOD), tumor nekrose faktor-$\alpha$ (TNF-$\alpha$) en die kappa-beta kinase (IKBK$\beta$/IKK$\beta$) gene in die lewer is ondersoek in 80 lewerbiopsies van NAFLD pasiënte onderverdeel in 4 kliniese groepe. Statisties betekenisvolle differensiële uitdrukking is waargeneem vir TNF-$\alpha$ (p=0.04) and IKK$\beta$ (p=0.02) in pasiënte met NASH, terwyl daar geen verskil waargeneem is tussen individue met verskillende grade van fibrose nie.

Ter samevatting, hierdie is die eerste studie waar kliniese eienskappe van NAFLD in die Suid-Afrikaanse bevolking beskryf word, alhoewel beperk tot die Wes-Kaapse bevolking. Bykomstig tot die standaard biochemiese merkers is gewys dat LDL partikel grootte en APRI statisties betekenisvol onderskei tussen NAFLD pasiënte met ‘n verhoogde risiko vir koronêre hartsiekte en gevorderde lewersirrose onderskeidelik, met gevolglike implikasies vir doelgerigte behandeling. Die TNF-$\alpha$ en IKK$\beta$ gene wat geïmpliseer word in insulien weerstandigheid en inflammasie toon die mees betekenisvolle assosiasie met NASH, wat in lyn is met insulien weerstandigheid as ‘n universiele risikofaktor vir beide koronêre hartsiekte en gevorderde lewersiekte. Die bevindinge van die studie gekombineer met gepubliseerde literatuur het gelei tot die samestelling van ‘n algoritme, wat van nie-ingrypende biomerkers gebruik maak as ‘n alternatief tot lewerbiopsie in die risikobestuur van pasiënte met NAFLD.

# DEDICATION

During my training in Gastroenterology at the Division of Gastroenterology, Tygerberg Hospital, many patients were referred with unexplained abnormal liver functions. I observed that the majority of these patients were either obese or diabetic and this sparked my interest in non-alcoholic fatty liver disease (NAFLD), which at that stage was not well known in South Africa. During this time a guest lecture was presented by Prof Pauline Hall at one of the weekly academic meetings of the local Faculty of Health Sciences, on the latest knowledge and her experience with NAFLD/NASH. Following the meeting she agreed to meet with me; a meeting that marked the start of a working relationship that lasted until her unfortunate death in 2006. At that time Prof Hall and her co-workers at the University of Cape Town were doing research on mice models of NASH. I seized the opportunity to collaborate with her on NAFLD/NASH in human subjects, resulting in this dissertation.

Prof Hall, a native from Adelaide, Australia, was appointed at the Division of Anatomical Pathology of the University of Cape Town. She played an important role in the stimulation and training of aspiring pathologists in the field of Gastroenterology and Hepatology in South Africa. She was also co-founder of the South African liver interest group that was instrumental in giving those of us interested in Hepatology exposure to some of the World's most respected Hepatologists. She was not only the external promoter for this dissertation, but also a source of great personal and professional inspiration to me. Her dedication to research in the field of NAFLD/NASH, her contribution to the field of Hepatology in South Africa and above all, her work ethic, was truly remarkable.

I dedicate this dissertation to the memory of Prof Pauline Hall.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACADM | Acyl-coenzyme A dehydrogenase |
| ACC1 | Acetyl CoA carboxylase |
| ACOX | Straight chain acyl-CoA oxidase |
| ADRP | Adipose differentiation-related protein |
| ALD | Alcoholic liver disease |
| ALP | Alkaline phosphatase |
| ALT | Alanine aminotransferase |
| APRI | Aspartate aminotransferase to platelet ratio index |
| ARB | Angiotensin receptor blocker |
| ASH | Alcoholic steatohepatitis |
| AST | Aspartate aminotransferase |
| AT I | Angiotensin II receptor type I |
| AT II | Angiotensin II receptor type II |
| ATP | Adenosine triphosphate |
| AMP | Adenosine monophosphate |
| AUC | Area under the curve |
| BCAT1 | Branched-chain aminotransferase 1 |
| BMI | Body mass index |
| BOX | Branched-chain acyl-CoA oxidase |
| CAD | Coronary artery disease |
| CCL2 | Chemokine (C-motif) ligand 2 |
| CD36 | CD36 molecule (throbospondin receptor) |
| CP | Crossing point |
| CRN | Clinical research network |
| CRP | C-reactive protein |
| CT | Computerised tomography |
| CTFG | Connective transforming growth factor |
| CTP1a | Carnitine palmitoyltransferase 1a |
| CYP2E1 | Cytochrome P4502E1 |
| DNA | Deoxyribonucleic acid |
| DGAT | Diacylglycerol o-acyltransferase 1 |

| | |
|---|---|
| ELF | European liver fibrosis |
| FABP4 | Fatty acid binding protein |
| FAS | Fatty acid synthase |
| FFA | Free fatty acids |
| GGE | Gradient gel electrophoresis |
| GGT | *y*-glutamyltranspeptidase |
| GNB3 | G-protein subunit 3 |
| HADHα | Hydroxyacylcoenzyme A dehydrogenase alpha |
| HBsAg | Hepatitis B surface antigen |
| HCC | Hepatocellular carcinoma |
| HCV | Hepatitis C virus |
| HDL-C | High density lipoprotein cholesterol |
| HH | Hereditary haemochromatosis |
| HOMA | Homeostasis model assessment |
| HOMA-IR | Homeostasis model assessment method for insulin resistance |
| HSL | Hormone sensitive lipase |
| IKKβ | Kappa-beta kinase |
| IKBKβ | Inhibitor of kappa light polypeptide gene enhancer in B cells, kinase beta |
| IL | Interleukin |
| IR | Insulin resistance |
| LCAD | Long chain acyl-CoA dehydrogenase |
| LDL | Low-density lipoprotein |
| LFT | Liver function test |
| LPS | Lipopolysaccharide |
| MnSOD | Manganese superoxide dismutase |
| MRI | Magnetic resonance imaging |
| NAFLD | Nonalcoholic fatty liver disease |
| NASH | Nonalcoholic steatohepatitis |
| NFS | Nash fibrosis score |
| NHANES | National Health and Nutritional Examination Survey |
| NIH | National Institute of Health |
| NPV | Negative predictive value |
| OELF | Original European liver fibrosis |

| | |
|---|---|
| PCR | Polymerase chain reaction |
| PLIN | Perilipin |
| PPAR | Peroxisome proliferator-activated receptor |
| PPV | Positive predictive value |
| QUICKI | Quantitative insulin sensitivity check index |
| RAS | Renin-angiotensin system |
| RNA | Ribonucleic acid |
| ROC | Receiver operating characteristic |
| ROS | Reactive oxidative species |
| SBBO | Small bowel bacterial overgrowth |
| SIClamp | Clamp derived index of insulin sensitivity |
| SOD | Superoxide dismutase |
| SOD2 | Superoxide dismutase 2 |
| SREBP-1c | Sterol regulatory element-binding protein 1c |
| SVR | Sustained virologic response rate |
| T2DM | Type 2 diabetes mellitus |
| TLR4 | Toll-like receptor 4 |
| TG | Triglyceride |
| TGF | Transforming growth factor |
| Th | T helper |
| TNF | Tumour necrosis factor |
| UCP2 | Uncoupling protein 2 |
| VLDL | Very low-density lipoprotein |

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# CHAPTER 1

# INTRODUCTION

## LITERATURE OVERVIEW

### 1.1 Definition of non-alcoholic fatty liver disease (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of liver disease ranging from hepatic steatosis through steatohepatitis to cirrhosis where the ingestion of significant amounts of alcohol has been excluded (Farrell et al. 2005). At present we use a corroborated consumption of less than 20 grams alcohol/day or less than 140 g/week.

Non-alcoholic steatohepatitis (NASH) is a subset of NAFLD and is currently defined as a constellation of findings that includes steatosis and necroinflammatory injury (Brunt 2001). Essential for the diagnosis is the pathologist's overall impression that the findings meet the criteria for steatohepatitis along with the clinician's assessment that the patient is drinking little or no alcohol. Excluding other causes of liver disease was once thought to be important, but NASH is now recognized to occur in the presence of other forms of liver disease.

### 1.2 History of NAFLD and non-alcoholic steatohepatitis (NASH)

Before 1980 all histological biopsies revealing significant steatosis with or without hepatitis were regarded as alcoholic fatty liver disease. This notion was challenged in a seminal paper in 1980 by Jurgens Ludwig, a Mayo Clinic pathologist (Neuschwander-Tetri, 2006, citing Ludwig et al. 1980). A constellation of histological abnormalities were described in patients who denied alcohol abuse yet had histological findings on liver biopsy strongly suggesting alcoholic liver disease. He later popularised the term "non-alcoholic steatohepatitis". Earlier papers and abstracts described similar patients. For example, Zelman (1952, cited by Neuschwander-Tetri 2006) described liver biopsy findings in 19 obese men that included steatosis and varying degrees of inflammation and fibrosis. Furthermore, Leevy (1962, cited by Neuschwander-Tetri 2006) described 270 patients with fatty liver, 64 of whom did not consume alcohol. Even though these early studies did not deliver quite the same message as Ludwig’s paper, they set the stage for the Ludwig series. This early study was relatively small, with only 20 patients included and had flaws as some of the patients may have had other causes of liver disease such as hepatitis C (testing was not possible at the time), hepatitis B (1 out of 9 tested had detectable HBsAg), and primary biliary cirrhosis (1 out of 5 tested had a positive antimitochondrial antibody titer). However, he convinced many in the field that NASH was truly unrelated to alcohol

consumption and could no longer be ignored (Neuschwander-Tertri 2006). This paper led to a wider recognition of NASH and sparked exponential growth in the field over the ensuing decades with the current level of awareness of NASH and research into its aetiology and treatment being attributed to this research.

Up until 1999, most clinicians believed NASH to be a benign disorder until the landmark paper by Matteoni et al. (1999) showed that NASH patients developed cirrhosis just like other patients with chronic liver diseases and that the mortality was similar. This paper even made a suggestion of possible development of hepatocellular carcinoma (HCC), which has subsequently been confirmed in subsequent studies.

## 1.3 Epidemiology

### 1.3.1 Risk factors

Initial studies characterised NASH by obesity, diabetes mellitus type 2 (T2DM), and affecting mainly females. However, these papers were influenced by referral bias on the characterisation of NASH patients. Later studies changed the common belief to also include patients who are lean, non-diabetic, and male (Brunt 2007; Falck-Ytter et al. 2001).

The most common underlying risk factor of the development of NASH is the presence of insulin resistance (Wanless et al. 1990; Willner et al. 2001; Dixon et al. 2001). Obesity, especially centripetal obesity combined with sedentary lifestyle is the most important risk factors for insulin resistance (Kral et al. 2001; Ruhl 2003), but genetic predisposition is recognised as the reason why even lean people can develop T2DM, a late complication of insulin resistance with aging. Dyslipidemia, typically hypertriglyceridemia, is associated with NAFLD. In most circumstances, this association is explained by the hypertriglyceridaemia associated with insulin resistance. However, other forms of hypertriglyceridaemia independently of insulin resistance can be associated with NAFLD. In addition to insulin resistance and dyslipidemia representing the main risk factors for NAFLD, other associated risk factors are shown below in Table 1.

**Table 1.1** Risk factors other than insulin resistance and dyslipidemia associated with the development of non-alcoholic fatty liver disease

| | |
|---|---|
| Drugs | Tamoxifen<br>Corticosteroids<br>Amiodarone<br>Estrogens<br>Calcium-channel blockers (true association uncertain)<br>Anti-retroviral treatment |
| Toxins | Extensive exposure to volatile hydrocarbons |
| Dietary Abnormalities | Carbohydrate excess (associated with total parenteral nutrition)<br>Protein deficiency<br>Rapid weight loss<br>Vitamin B12 deficiency<br>Choline deficiency |
| Altered small-bowel anatomy | Obesity surgery with blind loop of small bowel<br>Small-bowel diverticulae<br>Short gut |
| Metabolic Diseases | Hypobetalipoproteinemia<br>Abetalipoproteinemia<br>Wilson's disease<br>Lipodystrophies<br>Andersen's disease<br>Weber-Christian syndrome<br>Mauriac syndrome |
| Infections | Chronic hepatitis C (usually genotype 3)<br>Acquired Immuno Defeciency Syndrome<br>Bacillus cereus infection |

### 1.3.2 Prevalence of NAFLD in adults

It is estimated that 25%-37% of the US population have NAFLD and that 2%-5% have NASH. These estimates are based on studies by the National Health and Nutritional Examination Survey (NHANES) where abnormal serum alanine aminotransferase (ALT) levels were used as the inclusion criteria. Subsequently Colecchia and colleagues (2007a,b) evaluated the prevalence of NAFLD in 1055 subjects and its relationship to abnormal liver enzyme levels and found that 36% of the cohort had NAFLD, but only 8% had abnormal serum ALT. The prevalence of NAFLD was higher (68%) in subjects with abnormal ALT than in those with normal ALT (33%). Therefore even though elevated ALT levels are associated with NAFLD, they largely underestimate disease prevalence and cannot be viewed as a reliable marker of NAFLD. In studies where histological determinants were used, the prevalence of fatty liver was much higher than previous estimates using serum ALT as a surrogate marker. Patton et al.

(2007) evaluated the histological prevalence of NAFLD among 283 consecutive subjects with liver tissue available from autopsies. The overall prevalence of steatosis was 48%, with most (83%) representing mild grades; advanced fibrosis was present in 8% of subjects. Body mass index (BMI) equal or above 25 and abdominal fat layer equal or above 3 cm (central adiposity) were both associated with steatosis. In another autopsy series of 351 patients who died as inpatients and who underwent autopsy, steatohepatitis was identified in 2.7% of lean patients and 18.5% of markedly obese patients (Wanless and Lenz 1990).

The high estimates of NAFLD/NASH have been viewed with an element of scepticism as the disease burden continues to be under recognised by most healthcare providers. In South Africa, data is lacking on the prevalence in the local population.

### 1.3.3 Prevalence of NAFLD in children

A major contribution to current knowledge can be gained from studies performed in children with NAFLD. These studies have shown that children develop the full spectrum of NAFLD (Lavine and Schwimmer 2004). Whereas adults with NAFLD may have normal body weight, children are almost invariably overweight or obese and typically have elevated fasting serum triglycerides. The prevalence of NAFLD in children is unknown, but appears to be growing in parallel with the rise in obesity. One study in Japan of 810 children aged 4-12 years old demonstrated the presence of sonographically detectable NAFLD in 2.6% and its presence correlated with obesity (Tominaga et al. 1995).

### 1.3.4 NAFLD and ethnicity

Even though risk factors are highly prevalent in black individuals, there is an unexpected relative paucity of documented NAFLD**.** Puri and colleagues (2007a) prospectively defined the histological spectrum of NAFLD in 35 obese black subjects compared with a similar cohort of 71 whites undergoing bariatric surgery and liver biopsy. Blacks had less steatosis (37% vs 59%) even though they were significantly heavier. After correcting for age and BMI, blacks with steatosis had less severe lobular inflammation and portal fibrosis than whites, but portal inflammation was more common in blacks with steatosis (25% vs 5%). The underlying genetic or environmental factors need to be defined.

### 1.3.5 NAFLD and age

Non-alcoholic fatty liver disease (can occur at any age as seen in the paedriatric studies but is more common in adults. In paedriatric patients liver cirrhosis was not an uncommon finding, but most series report an age older than 45 years as an independent risk factor for advanced disease (Angulo et al. 1999).

## 1.4 Pathohistological findings

NAFLD is pathohistologically characterised by the presence of hepatocellular steatosis of which more than 5% of hepatocytes must contain macrovesicular fat. An occasional finding is a component of microvesicular steatosis along with macrovesicular steatosis. Histologically, the hepatocellular triglyceride accumulation in NAFLD is commonly a mixture of microvesicular and macrovesicular fat droplets. Microvesicular fat is identified as droplets smaller than the nucleus that do not displace the nucleus whereas macrovesicular fat displaces cell contents, including the nucleus, to the cell periphery. It is unknown whether the differences between the two patterns of fat accumulation are related to different pathophysiological processes or the rate at which fat accumulates.

Establishing the diagnosis of NASH and distinguishing it from simple steatosis relies on finding steatosis, characteristic inflammation, and evidence of cellular injury (Brunt 2001). See figure 1.1 and figure 1.2. The extent of necroinflammatory changes and fibrosis (if any) required to establish the diagnosis of NASH continues to be refined. The necroinflammatory changes noted in NASH is characterised by the presence of inflammatory cells characterised by a mix of mononuclear and polymorphonuclear cells. Hepatic ballooning is usually present but is not a diagnostic requirement. Mallory's hyaline is a ropy condensation of cytoskeletal elements often seen in conjunction with ballooning. Acidophil bodies can be present and these represent apoptotic hepatocytes. The fibrosis seen in NASH is pericellular "chicken-wire" fibrosis around hepatocytes in zone 3 (near a central vein).

Semi-quantitative liver histological scoring systems in use in contemporary studies of NAFLD include those proposed by Brunt et al. (1999) and the Pathology Committee of the NIH NASH CRN (Brunt et al. 1999; Kleiner et al. 2005). The Brunt classification has separate categories for the grade of hepatic necro-inflammation and stage of hepatic fibrosis (Brunt et al. 1999). After

assessing individual components within these categories (steatosis, ballooning, lobular and portal inflammation, hepatic fibrosis), liver biopsies are graded as mild, moderate, or severe steatohepatitis and staged as 1-4 (from perivenular, perisinusoidal fibrosis through to cirrhosis). The CRN classification draws on the Brunt scheme but differs from it by providing a single score (NAFLD activity score, NAS) to categorise liver biopsies into three groups: NASH, borderline NASH, and no NASH (Kleiner et al. 2005). The stage of fibrosis is evaluated separately, and broadly similar to the Brunt scheme, except that stage 1 is subdivided to three components to include portal/periportal hepatic fibrosis. The CRN scheme is applicable across the spectrum of NAFLD and has reasonable inter-rater agreement (kappa weighted scores of 0.84 for fibrosis, 0.79 for steatosis, 0.56 for hepatic injury, 0.45 for lobular inflammation, and 0.61 for diagnostic category). For this reason, the working party recommends that NASH now be incorporated into routine reporting of NAFLD, as well as for clinical trials.

While the framework of the CRN scheme appears reasonably straightforward, clarification has been sought with respect to patients with cirrhosis. In particular, ballooning hepatocytes are common in cirrhosis of any cause. Hui et al. (2004) proposed different categories for NASH-associated cirrhosis: definite, probable, possible, and cryptogenic. In this scheme, liver biopsies with cirrhosis showing steatosis and intralobular mixed inflammatory infiltrate would be marked 'definite', those with steatosis and lobular mononuclear infiltrates as 'probable', and cases of cirrhosis with either steatosis or intralobular mixed inflammation as 'possible NASH associated cirrhosis', respectively. The term 'cryptogenic' was reserved to designate cirrhosis in the absence of steatosis or necroinflammation, but occurring in persons with metabolic risk factors. Now that cryptogenic cirrhosis is generally regarded as the endstage of NASH (largely because of the high rate of metabolic comorbidity, such as diabetes, hypertension and obesity, but also because of a high rate of recurrence after liver transplantation), the working party was concerned that cases of cirrhosis due to other occult causes could be mislabelled in such cases. A rigorous search should therefore be made for secondary disorders, including viral hepatitis, surreptitious alcohol use, sclerosing cholangitis, and drug-induced liver disease.

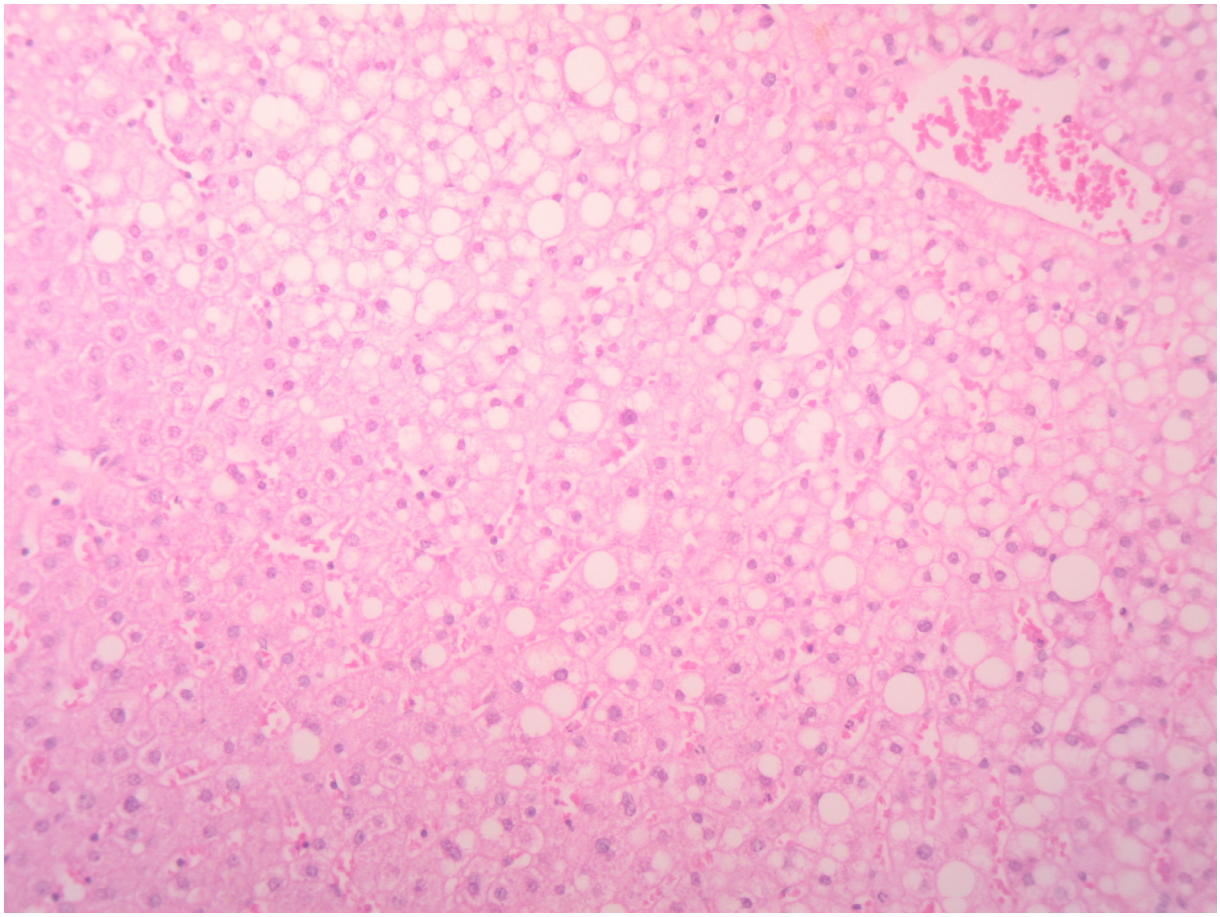


**Figure 1.1** Simple steatosis with minimal portal inflammation.

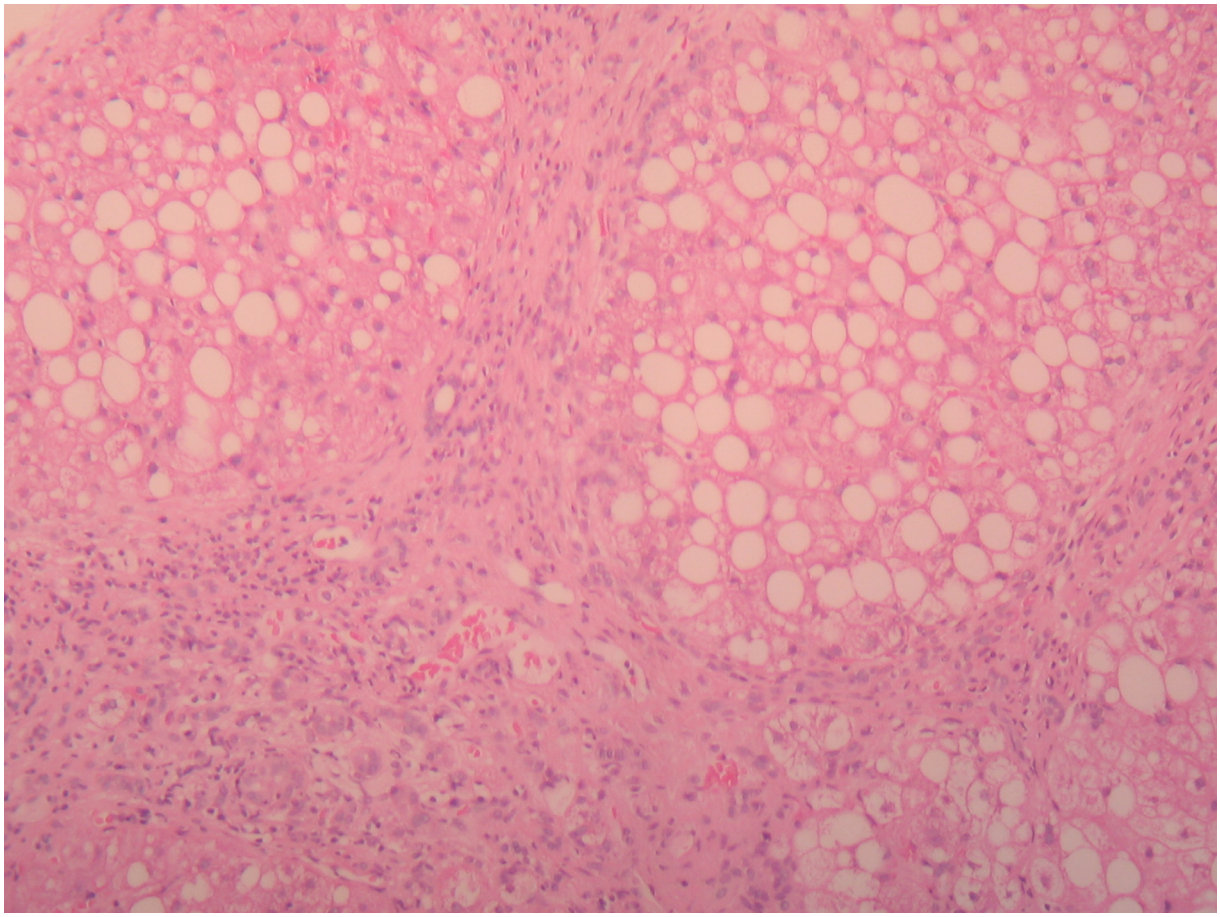


**Figure 1.2** Active cirrhosis. The portal tracts are markedly inflamed and there is ongoing damage of the interface of the parenchyma and fibrosis.

## 1.5 Clinical features

### 1.5.1 Symptoms

NASH is most commonly asymptomatic (Falck-Ytter et al. 2001). However, many patients present with vague upper abdominal or right hypochondrial pain. The symptoms can be difficult to discern from symptoms of cholelithiasis in some patients. The presence of NAFLD is often first suspected based on the results of imaging studies such as the finding of a diffusely echogenic liver on ultrasound.

Patients with alcoholic liver disease tend to be more symptomatic with similar degree of fatty liver (Pinto et al. 1996). Many patients with NASH note fatigue and poor exercise tolerance. These are probably part of the metabolic syndrome and not of NAFLD/NASH per se.

### 1.5.2 Signs

There are no specific physical findings typical of NAFLD/NASH. Hepatomegaly is present in up to 75% of patients (Falck-Ytter et al. 2001). This can be associated with tenderness in the epigastrium and/or right hypochondrial area. The clinical findings of insulin resistance can also be present namely centripetal obesity, hypertension, and possibly acanthosis nigricans. The findings of centripetal obesity can be very subtle and only detected with waist circumference. Atypical fat distribution of the body can also be a feature and must be distinguished from lipodystrophy that can also present with fatty liver (Garg 2004).

## 1.6 Diagnosis

The diagnosis of NAFLD/NASH is considered in the correct clinical setting, including features of metabolic syndrome, with the presence of fatty liver on ultrasound. This can be confirmed by liver biopsy, although the current practice of most clinicians is not to pursue the diagnostic impression further when the liver enzymes are normal. There are, however, potential pitfalls of this clinical algorithm. The diagnosis of NAFLD/NASH is dependant on an accurate history whereby significant alcohol consumption is excluded. How much alcohol constitutes enough to contribute to liver disease is controversial. The Dionysos study from Italy suggested that consumption of less than 30 grams of alcohol daily is not associated with adverse sequelae

(Bellentani and Teribelli 2001). This seems quite generous to many, and an upper limit of alcohol consumption for most studies of NAFLD is set at 20 grams daily or 140 grams weekly to exclude fully the possibility that alcohol could be playing a role. Certainly, when consumption reaches more than 60 grams daily, then the role of alcohol in the development of hepatic steatosis is likely. For an alcohol intake between 20 and 60 grams daily, the role of alcohol remains uncertain.

Quantifying alcohol consumption is at best a rough approximation in the clinical setting. Daily alcohol consumption is typically described as grams of ethanol consumed every day. A commonly used conversion is that 10 grams of alcohol is roughly the alcohol content of one beer, one glass of wine, or one measurement of distilled spirits. In reality, these drinks typically contain anywhere from 10 to 20 grams of ethanol, depending on the type of beer or wine. Furthermore, there are huge variations in the description of alcohol usage as to what constitutes a "glass of wine" and the volume of distilled spirits actually used to prepare a drink.

### 1.6.1 Laboratory tests.

#### 1.6.1.1 Aminotransferases

NASH is the most common cause of elevated aminotransferases once other causes of liver diseases have been excluded (Berasain et al. 2000). Data obtained from morbidly obese subjects undergoing bariatric surgery have demonstrated that the entire spectrum of NAFLD ranging from minor amounts of fat infiltration to aggressive NASH and cirrhosis are present even though each phase can present with normal aminotransferases (Luyckx et al. 1998; García-Monzón et al. 2000; Ruhl and Everhart 2003). Although aminotransferases lack specificity and sensitivity for the detection of NAFLD (Ruhl and Everhart 2003), better laboratory tests are not available. The AST/ALT ratio can help to distinguish NAFLD from alcoholic steatohepatitis (ASH). An AST greater than the ALT is highly suggestive of alcoholic liver disease (Sorbi et al. 1999). However, this is only true in the early stages of NAFLD/NASH because with the development of significant fibrosis or cirrhosis in patients with NASH, the AST can also exceed the ALT (Sorbi et al. 1999; Angulo et al. 1999).

#### 1.6.1.2 Cholestatic enzymes

Both GGT and ALP can be raised in NAFLD even though it is primarily a hepatocellular disease. Occasionally biliary tract abnormalities can be identified on biopsy (Luyckx et al. 1998). Studies in NAFLD with insulin-sensitizing agents were also found to cause improvement not only in aminotransferases but also in serum ALP and GGT (Neuschwander-Tetri et al. 2003a).

#### 1.6.1.3 Other laboratory tests

The role of other laboratory tests in the diagnosis of NAFLD is primarily to exclude other causes of liver disease. Concomitant diseases can also be identified such as Hepatis C. Autoimmune antibodies are sometimes found in low to moderate titers. However, high titers can provide an additional impetus to perform a liver biopsy to exclude Autoimmune Hepatitis. Wilson's disease must be fully excluded as an underlying disorder in children and adolescents with NAFLD by measuring the serum caeruloplasmin and 24 hour urinary copper.

#### 1.6.1.4 Insulin resistance and lipid abnormalities

The ability to estimate insulin resistance and thus predict the presence of complications associated with this disorder has become necessary in the management of patients with NAFLD. The presence of insulin resistance suggests that therapeutic options aimed at improving insulin sensitivity may be beneficial, whereas the occasional patient with NAFLD who has normal insulin sensitivity should be further evaluated for uncommon or unrecognised causes such as other metabolic abnormalities or covert alcohol abuse.

Unfortunately, how best to define and measure insulin resistance is a source of continued debate (Wallace and Matthews 2002). Despite potential problems, biochemical assessment of insulin resistance commonly relies on measuring serum insulin levels. Two methods, the homeostasis model assessment for insulin resistance (HOMA-IR) and the quantitative insulin sensitivity check index (QUICKI), use the fasting insulin level multiplied by the fasting glucose level (Mather et al. 2001). Although HOMA-IR has been widely used, Katz et al. (2000) suggest that the QUICKI is a reasonable estimate of the glucose clamp-derived index of insulin sensitivity (SIClamp) because the QUICKI is linearly related to the SIClamp (Katz et al. 2000;

Abbasi and Reaven 2002). This may be due to the fact that both the QUICKI and the SIClamp calculations use log-transformed values.

Despite the controversy between the two methods the choice of method to determine insulin resistance is still dependant on preference of the clinician or investigator relating to previous experience with both tests. A lower limit of normal QUICKI in the range of 0.357-0.382 is often reported with values less than this indicating IR (typically in the range of 0.25-0.35 for NASH patients). An upper limit of normal HOMA-IR is in the range of 1.0-1.5 with higher values signifying insulin resistance. A HOMA-IR of greater than 2 is regarded by most to correlate with insulin resistance. Obtaining postprandial insulin levels after an oral glucose challenge may prove to be an even better means of assessing insulin resistance (Ikai et al. 1995; Neuschwander-Tetri et al. 2003a), although further study is required.

High triglyceride levels are a feature of both the metabolic syndrome and NAFLD/NASH. In a study by Ryan et al. (2007), 56 249 Korean subjects were evaluated and BMI, waist circumference, and the concentration ratio of triglyceride/high-density lipoprotein cholesterol (TG/HDL-C) were significantly higher in subjects with steatosis and also higher in subjects with NASH. Odds ratios for the presence of NAFLD and NASH, with increasing quartiles of TG/HDL-C ratio, were increased 5- to 7-fold over controls, independent of age, BMI, and waist circumference. In the absence of insulin resistance, certain primary lipid abnormalities are associated with NAFLD/NASH.

### 1.6.2 Liver biopsy

The diagnosis of NASH can only be established with certainty by performing a liver biopsy (Bianchi 2001). Due to the invasive nature of this procedure and the potential complications as well as the cost involved, not all patients with suspected NAFLD/NASH are required to have a liver biopsy. However, in a country like South Africa, a percutaneous liver biopsy is competitively priced when compared to newer non-invasive modalities. From clinical experience, liver biopsy is considered safe when performed under ultrasound guidance with adequate procedural experience and where haematological abnormalities have been excluded. Furthermore, studies have shown at least a third of patients suspected of having NAFLD on clinical grounds have another cause for liver enzyme elevations when a biopsy was performed (Skelly et al. 2001). As

newer drug therapies are being developed the need for liver biopsy might increase (Sorbi et al. 2000; Skelly et al. 2001).

### 1.6.3 Imaging studies

Imaging studies often provide the first evidence that a patient has otherwise unsuspected NAFLD. Each modality has its strengths and weaknesses. Imaging can variably identify steatosis and cirrhosis. No imaging study can assess the necroinflammatory changes or fibrosis that distinguish NASH from less worrisome forms of NAFLD (Siegelman and Rosen 2001; Saadeh et al. 2002).

#### 1.6.3.1 Ultrasound

Ultrasound examination of the liver may be the least costly imaging method, but it lacks both specificity and sensitivity. Fat in the liver confers a "bright" appearance on ultrasound, but significant fibrosis without fat can have a similar appearance (Siegelman and Rosen 2001). Methods have been proposed to increase the sensitivity of ultrasound using quantitative techniques, but these have not been widely accepted or established in clinical practice. Despite these shortcomings, ultrasound remains a commonly used method of identifying NAFLD as a cause of unexplained liver enzyme elevations and can also be used as a screening tool.

#### 1.6.3.2 Computerised tomography

Computerised tomography (CT) imaging of the liver is more sensitive than ultrasound for detecting NAFLD, but at a greater cost (Siegelman and Rosen 2001). Like ultrasound, CT cannot identify necroinflammatory changes that signify the presence of NASH or fibrosis up to bridging fibrosis. Radiographic tissue density is estimated using Hounsfield units. The fatty liver has a lower density than normal and the presence of fat can be calculated by comparing the liver density to spleen or paraspinal muscle density. Commonly used formulas include either subtracting the liver density from the spleen density or calculating the liver-to-spleen ratio. A difference between liver and spleen of above 10 Hounsfield units indicates liver fat (Jacobs et al.1998), as does a liver-to-spleen ratio of less than 1.

#### 1.6.3.3 Magnetic resonance

Magnetic resonance (MR) imaging and MR spectroscopy are the most sensitive means of detecting NAFLD, with the trade off of also being the most costly (Siegelman and Rosen 2001). Different MR techniques have been proposed for optimising the detection of fat (Fishbein and Stevens 2001). Spectroscopy techniques have also proved useful in detecting disruption of ATP production in experimental studies (Nair et al. 2003).

#### 1.6.3.4 Nuclear medicine

Improvements in ultrasound, CT, and MRI have led to general abandonment of nuclear medicine studies as a means of detecting hepatic abnormalities in general. One very sensitive means of estimating liver fat content is the use of Xe washout (Lewis et al. 1989), a technique reported in the past but no longer in clinical use.

### 1.7. Progression of NAFLD

There are two hypotheses to explain progression of NAFLD to NASH. In the first hypothesis, simple fatty liver progresses to steatohepatitis and then to fibrosis and cirrhosis. The alternate hypothesis is that individuals prone to develop necroinflammatory injury do so as the fat accumulates. The limited long-term follow-up studies support the latter paradigm more than the former. The study reported by Matteoni et al. (1999) found that most people with just fat and no steatohepatitis continued to have just fat over time and typically did not progress to NASH and its sequelae. Based on these studies, 10-15% of people with NAFLD will have NASH and a subset, 20% to 33% of them, will be at risk of developing cirrhosis (Harrison et al. 2003a).

The factors that determine whether a patient with NAFLD also develops necroinflammatory changes and fibrosis are not known but are widely studied. The most likely role players include genetic risk factors, dietary composition, and concomitant forms of other liver disease (e.g. chronic hepatitis C). There also appears to be important racial and ethnic predispositions, but these remain to be characterized at this time (Caldwell et al. 2002; Browning et al. 2004a). With increasing recognition of NASH emerged a parallel recognition of its role in causing liver failure and cancer.

Approximately 2% of liver transplants were performed for a known diagnosis of NASH. The true fraction of the 4000-5000 liver transplants performed annually in the USA due to NASH is likely several several fold more if cryptogenic cirrhosis is included. About 10% of liver transplants are performed for cryptogenic cirrhosis and epidemiological data have implicated prior NASH as the most likely causative factor in most (Clark and Diehl 2003; Caldwell and Crespo 2004). It is also unknown how many patients thought to be dying of end-stage diabetes or other terminal complications of insulin resistance also have occult cirrhosis that contributes to their death.

**1.8 Predictors of fibrosis**

Appropriate management of patients with suspected NASH requires the ability to predict which patients with elevated liver enzymes or NAFLD detected by imaging are at risk for progressive disease and thus warrant more aggressive evaluation and treatment. One study of morbidly obese patients undergoing bariatric surgery (BMI above 35 kg/m2) found that hypertension, ALT elevation, and insulin resistance predicted the presence of NASH in patients with NAFLD (Dixon et al 2001). In fact, three-quarters of these morbidly obese patients with both hypertension and diabetes had NASH whereas only 7% with neither condition had NASH. Diabetes and hypertension were also predictive of advanced fibrosis. Another study established the importance of age; significant fibrosis (stage 3 or 4) was present in only 4% of NASH patients under the age of 45 yet it was present in 40% of those 45 years and older (Angulo et al. 1999). The available data can be summarized by observing that the greatest risk for significant fibrosis on a liver biopsy is the presence of obesity and diabetes in a patient over the age of 45 years with an AST:ALT ratio above 0.8 (Harrison et al. 2003a).

Several studies have attempted to develop clinical parameters that can reliably identify fibrosis in a cohort of patients with NAFLD. Many markers have been proposed, including hyaluronic acid (Kaneda et al. 2006), lipid peroxidation related antibodies (Albano et al. 2005), and “predictive panels” based on the multivariate analysis of clinical and biochemical parameters (Gholam et al. 2007). Several predictive panels have been proposed, including a relatively simple test, aspartate aminotransferase to platelet ratio index (APRI), requiring only two parameters (Wai et al. 2003). APRI has not been validated in patients with NAFLD. Another panel, the Fibrometer, is a little more complex and combines platelets, prothrombin index, AST, β2-macroglobulin, hyaluronate, urea and age (Cales et al. 2005).

The sensitivity and specificity of these tests have been evaluated only in a small number of studies. Nevertheless, the most widely used panel for fibrosis, the FibroTest, has been predominantly characterised in patients with chronic hepatitis C. Although this test can reliably distinguish the absence of fibrosis from hepatitis C virus–related cirrhosis, its ability to detect smaller changes in the stage of fibrosis has been debated (Ngo et al. 2006). A recent study reported good performance of the FibroTest in patients with NAFLD for detection of bridging fibrosis or cirrhosis. Nevertheless, large, multicenter studies are needed to independently evaluate the clinical value of the FibroTest in patients with NAFLD (Ratzui et al. 2006).

Two additional panels have been developed to assess hepatic fibrosis in NAFLD. First, the so-called “Simple Test” for NAFLD is a relatively easy-to-use panel that includes age, hyperglycemia, body mass index, platelet count, albumin, and AST/ALT (Angulo et al. 2007). In addition to this Simple Test, another panel for hepatic fibrosis is the Original European Liver Fibrosis (OELF) panel (Rosenberg et al. 2004). OELF parameters include age, hyaluronic acid, amino-terminal propeptide of type III collagen, and tissue inhibitor of matrix metalloproteinase 1. A simplified version of OELF is ELF, which does not include age, seems to perform well in patients with NAFLD (Guha et al. 2008). The OELF panel has been designed to stage hepatic fibrosis in several liver diseases, including NAFLD, where an ELF threshold of 1.8454 will have a perfect positive predictive value for any fibrosis. This information can be used for establishing a patient’s prognosis and for targeting these patients for aggressive lifestyle and diet modification programs or enrollment in a research protocol for NAFLD.

Another noninvasive approach is to estimate hepatic fibrosis by assessing elasticity. In recent years, a shear elasticity probe based on 1-dimensional transient elastography has been proposed for noninvasive diagnosis of liver fibrosis (Sandrin et al 2003). This technique uses both ultrasound (5 MHz) and low-frequency (50 Hz) elastic waves with a propagation velocity directly related to elasticity. This elastograph device is called FibroScan. Its main application is for patients with chronic hepatitis C (Nguyen-Khac and Capron 2006). At present, well-controlled studies of FibroScan in patients with NAFLD are lacking, although the observation that hepatic steatosis does not affect liver stiffness is encouraging (Kim et al. 2007).

### 1.9 The metabolic syndrome and NAFLD/NASH

NAFLD is now recognised as the hepatic manifestation of metabolic syndrome (Marchesini et al. 2001). Although there is not a complete consensus on the criteria required to define metabolic syndrome, the most popular definition includes the presence of central obesity, hypertension, hyperlipidemia and insulin resistance (Alberti and Zimmet 1998). Importantly, however, metabolic syndrome can occur in non-obese individuals and can still be associated with NAFLD. The separate components of the metabolic syndrome have also been independently linked to NAFLD.

#### 1.9.1 Central obesity

Almost 75% of obese patients have NAFLD and central obesity poses a particular risk of developing NAFLD (Marchesini and Marzocchi 2007). Although BMI is a measure of total body fat, athletes with significant muscle mass can have a high BMI but not be obese. Indeed, athletes with a high BMI but no evidence of central obesity or other components of metabolic syndrome might not be at risk of developing NAFLD. On the other hand, lean individuals with a low BMI and evidence of central obesity might be at greater risk of developing NAFLD than those with a high BMI but no evidence of central obesity.

#### 1.9.2 Type 2 diabetes mellitus

Patients with type 2 diabetes mellitus (T2DM) are at a high risk of developing NAFLD. In one study, NAFLD was detected by ultrasound in 62% of patients with newly diagnosed T2DM (Jimba et al. 2005). T2DM is also a risk factor for severe fibrosis in patients with NAFLD. In one study cohort, patients with NAFLD and T2DM had higher overall mortality and liver-related mortality than NAFLD patients without T2DM (Younossi et al. 2004).

#### 1.9.3 Hypertension, hypertriglyceridemia and mixed hyperlipidemia

NAFLD has been shown to be associated with hypertension in two Japanese studies (Akahoshi et al. 2001; Ikai et al. 1995) and one Italian study (Donati et al. 2004). Hypertriglyceridemia and mixed hyperlipidemia have also been independently linked to NAFLD (Assy et al. 2000).

### 1.9.4 The cardiovascular risk of patients with NAFLD/NASH

The cardiovascular risk is increased for patients with metabolic syndrome. This fact implies that patients with NAFLD/NASH will have an increased risk for cardiovascular heart disease. In the study by Patton and colleagues (2007), subjects with steatohepatitis were at increased risk for adverse cardiovascular outcomes. In a prospective, national, population-based sample, Ruhl and Everhart (2007) examined whether elevated serum ALT was associated with increased risk of all-cause and cardiac heart disease mortality. Data were analyzed from 14,950 adult participants in the third US NHNES, conducted from 1988-1994. The prevalence of elevated ALT was 5%. During the 12-year follow-up, the cumulative mortality was 14% from all causes and 4% from coronary heart disease. Participants with an elevated ALT level had a 40% higher risk of all-cause mortality; however, their risk of coronary heart disease mortality was not increased.

Patients with NAFLD have elevated CRP levels, increased aortic stiffness, and media intima thickness, indicating both functional and structural changes in large arteries. The CRP concentration, used as a marker of acute-phase reaction, was found to be a strong predictor of coronary heart disease in 183 patients with T2DM and/or NAFLD (Abe et al. 2007). NAFLD patients were at risk of developing coronary heart disease irrespective of whether they had diabetes mellitus. Furthermore, NAFLD patients with poorly controlled diabetes mellitus had an increased risk of developing coronary heart disease. In a multivariate analysis, Abouzari and colleagues (2007) documented an independent association between ischemic heart disease and NAFLD in 203 adults, showing a statistically significance (p-value) of less than 0.001. The predictive value of ALT levels for angiography-documented atherosclerosis was furthermore evaluated in 630 patients (Adibi et al. 2007). An elevated ALT/ AST ratio in women predicted atherosclerosis independent of the metabolic syndrome and serum CRP concentration. These studies indicate the need for intensified surveillance for coronary heart disease in patients with or at risk of NAFLD.

## 1.10 Pathophysiology

Obesity represents a low-grade generalized systemic inflammation as reflected by elevated serum markers such as C-reactive protein (CRP) and tumor necrosis factor-alpha (TNF-$\alpha$). NASH is a component of this generalised pro-inflammatory state. NASH is thus viewed as the

result of "two hits." The first "hit" is fat accumulation. The postulated sequence of events begins with insulin resistance, which is antecedent to the accumulation of hepatocellular lipid. High serum free fatty acid concentrations lead to increased fatty acid delivery to the liver and thus enhanced hepatic fatty acid uptake. Nakamuta et al. (2007) evaluated the expression of fatty acid metabolism-related genes in liver biopsy samples from patients with NAFLD and confirmed that increased *de novo* synthesis and uptake of fatty acids leads to further accumulation of fatty acids in hepatocytes. Mitochondrial fatty acid oxidation is decreased and peroxisomal and microsomal oxidation is complementally upregulated to decrease fatty acid accumulation. Retention of lipids in the hepatocyte triggers oxidative stress (the "second hit"), generating reactive oxygen species and cytokine release. Excess intracellular fatty acids, oxidative stress, energy depletion, and mitochondrial dysfunction then cause cellular injury in the steatotic liver. Antioxidant pathways are enhanced to neutralize reactive oxygen species overproduced by oxidation, and lipid droplet formation is enhanced. The relative efficacy of these injury and repair mechanisms governs the outcome.

### 1.10.1. Defective regulation of triglyceride metabolism

Histological similarities are widely reported between human NASH and murine steatohepatitis induced by a methionine/choline-deficient diet. The methionine/choline-deficient diet induces adaptive changes in hepatic lipid metabolism to deal with a dietary fat load. The liver still suppresses fatty acid synthesis and enhances fatty acid oxidation, but these changes are insufficient to prevent lipid accumulation, possibly due to impaired egress of triglycerides. Ishitobi et al. (2007) documented further that methionine/choline-deficient treatment caused triglyceride accumulation, increased oxidative stress, inhibited cell proliferation, and increased mRNA expression of CYP2E1 (cytochrome P4502E1), TNF-$\alpha$, and transforming growth factor-beta-1 (TGF-beta1) in hepatocytes. This model could be suitable for investigating the pathogenesis of NASH and also the effects of therapeutic drugs on NASH.

### 1.10.2. Organelle injury

Endoplasmic reticulum stress, an important trigger for liver injury, is increased in NASH. Endoplasmic reticulum stress promotes disease progression in NAFLD through the activation of apoptotic mechanisms. Puri et al. (2007b) noted a sex difference in the activation of endoplasmic reticulum stress pathways. Endoplasmic reticulum stress occurred over time in female mice with NAFLD. There was milder steatohepatitis in male mice, presumably due to their ability to effectively activate adaptive responses to counter the stress. This indicates that sex influences the expression of endoplasmic reticulum stress pathways and may modulate the ability of the liver to adapt and influence the phenotype of NAFLD.

### 1.10.3. Mitochondrial abnormalities

Mitochondrial dysfunction has been proposed to be one of the primary defects in NASH (Begriche et al. 2006). Seminal work by Shulman has identified muscle mitochondrial dysfunction among young lean children of diabetic patients long before the onset of diabetes. (Petersen et al. 2004; Lowell and Shulman 2005) Interestingly defects in insulin-stimulated muscle phosphate uptake have also been identified by this group, raising the question of whether impaired mitochondrial ATP synthesis could be caused by insufficient intracellular phosphate (Petersen et al. 2005). These studies raise a provocative possibility that genetic defects of muscle energy metabolism might not only predispose to insulin resistance and its sequelae, but they could also cause exercise intolerance beginning early in life that would predispose to a sedentary lifestyle. Evidence of muscle dysfunction and injury in NASH patients has been slowly emerging with the finding of extraocular muscle dysfunction (Al-Osaimi et al. 2005) and unexplained serum creatine kinase elevations in small series of NASH patients (Gogia and Neuschwander-Tetri 2006).

### 1.10.4. Oxidative stress

Haem oxygenase-1, an inducible enzyme, is cytoprotective against various cellular stresses (e.g. ischaemia, inflammation, or radiation). Inoue and colleagues (2007) reported that haem oxygenase-1 overexpression via genetic ablation prevents lipid accumulation and reduces activation of hepatic stellate cells. Thus, heme oxygenase-1 may protect against the development of NASH through the prevention of lipid accumulation as well as via its

antioxidative activity. Oxidized phospholipids accumulate in hepatocytes from patients with NAFLD and correlate with disease severity.

Platelet-activating factor acetylhydrolase, an enzyme produced and secreted by liver cells, is uniquely positioned to inactivate deleterious phospholipids. Platelet-activating factor acetylhydrolase expression was significantly lower in the livers of patients with NASH compared with those with simple steatosis (Radhakrishnan et al. 2007). Moreover, a negative correlation was observed between the extent of platelet-activating factor acetylhydrolase expression and the degree of lobular inflammation. Loss of this protective enzyme may be implicated in disease progression in NAFLD, which suggests a potential new therapeutic strategy.

### 1.10.5. Adipocytokines

Intra-abdominal fat accumulation is an independent predictor of NAFLD. Abdominal adipose tissue is a source of free fatty acids and inflammatory factors. Persistent TNF-$\alpha$ stimulation leads to adipogenesis and induction of oxidative stress. Adiponectin, an anti-inflammatory adipocytokine that opposes TNF-alpha activity in the liver, has antifibrotic properties, reduces hepatic stellate cell proliferation, inhibits the enlargement of mature adipocytes, reduces fat accumulation, and increases apoptosis (Takenaka et al. 2007; Jarrar et al. 2007). Serum levels of adiponectin are reduced in NASH and are inversely associated with necro-inflammatory activity and hepatic fibrosis. In NAFLD, leptin may act as a mediator of inflammation and fibrosis, and low adiponectin levels may contribute to the development of necro-inflammation and exacerbate oxidative stress. Takenaka et al. (2007) demonstrated that estradiol, which functions as a potent antioxidant in hepatocytes, could protect women against the progression of NAFLD by inhibiting lipogenesis in adipocytes via induction of adiponectin and suppression of TNF-$\alpha$. Theoretically, visfatin, an adipokine secreted by white adipose tissue, could also be involved in the pathogenesis of NASH. Jarrar et al. (2007) showed that among the spectrum of patients with NAFLD, patients with NASH had the lowest serum visfatin level when compared with patients with simple steatosis. Furthermore, in those patients with simple steatosis, serum visfatin negatively correlated with levels of the pro-inflammatory cytokine, TNF-$\alpha$. Additional assessment of this link is needed.

Expression of hepatic interleukin-6 (IL-6), a major pro-inflammatory cytokine, is increased in animal models of NAFLD. Up-regulation of IL-6 in the mouse liver results in systemic insulin

resistance and diabetes. IL-6 expression was markedly increased in the livers of patients with NASH as compared with patients with simple steatosis, correlating with the stage of fibrosis (Wieckowska et al. 2007b). Patients with diabetes had significantly higher IL-6 levels. These data implicate increased hepatic IL-6 production as a common link between NASH and diabetes.

### 1.10.6 Immune response

Hepatosteatosis is associated with increased levels of the pro-inflammatory T helper (Th) 1-associated cytokines TNF-$\alpha$ and IL-12. There is concomitant loss of hepatic natural killer T cells, which contributes to the observed imbalance in local hepatic cytokine production. Hepatosteatosis thus results in Kupffer cell and IL-12-dependent depletion of hepatic natural killer T cells and promotes a Th1-shifted hepatic cytokine phenotype (Kremer et al. 2007). These results suggest a pivotal and multifunctional role for Kupffer cell-derived IL-12 in the altered immune response in NAFLD. This could represent an additional mechanism by which steatosis renders the liver more susceptible to secondary injury.

### 1.10.7 The role of gastrointestinal flora

It has been suggested that small bowel bacterial overgrowth (SBBO) increases intestinal permeability, allows entry of gut-derived endotoxin into the portal circulation, stimulates pro-inflammatory cytokines (TNF-$\alpha$), and suppresses anti-inflammatory cytokines, culminating in hepatic injury. Chalamalasetty et al. (2007) reported that SBBO, diagnosed using quantitative jejunal aspirate culture, is uncommon in patients with NASH, and therefore it is unlikely to play a major role in the pathogenesis of this disorder.

### 1.10.8 Renin-angiotensin system

An exciting area of progress is in recognizing that the renin-angiotensin system (RAS) is a major modulator of insulin resistance. Uncontrolled clinical studies have shown that blocking the angiotensin II receptor type 1 (AT1) with losartan improves insulin sensitivity and may be beneficial for NASH. For example, in an uncontrolled study of five patients with impaired glucose tolerance, losartan 100 mg daily for 8 weeks caused the Homeostasis Model Assessment (HOMA) measure of insulin resistance to significantly improve from 5.3 to 3.7

(Zandbergen et al. 2006). Seven patients with NASH were similarly treated, but for 48 weeks, and were found to have improved serum AST, ALT, and serum markers of fibrosis (Yokohama et al. 2004). Liver biopsies were also improved (Yokohama et al. 2004) and exhibited decreased stellate cell activation (Yokohama 2006). These provocative early findings now require confirmation in placebo-controlled randomised clinical trials.

Studies in animals have provided some possible mechanistic insights into the role of RAS in insulin resistance, acknowledging the caveat that the RAS in rodents may not fully recapitulate human physiology. Rodents fed a high fructose, high fat diet develop insulin resistance and NAFLD (Ran et al. 2004). Recent work by this group showed that rats infused with angiotensin II by osmotic pump or fed a high fructose diet exhibited decreased insulin sensitivity and adiponectin levels (Ran et al. 2006). Low levels of adiponectin have been shown in patients with NASH (Aygun et al. 2006) and genetic deletion of adiponectin in mice causes insulin resistance (Nawrocki et al. 2006). In one study of rats, the lower adiponectin levels and impaired insulin sensitivity appeared to be mediated by the receptor AT1 because the AT1-specific ARB olmesartan improved NAFLD (Ran et al. 2004), insulin sensitivity and adiponectin levels (Ran et al. 2006).

Specifically examining the role of AT1 is important because the angiotensin II receptor type 2 (identified as AT2, not to be confused with AII which denotes the peptide angiotensin II) mediates important effects of angiotensin, often in ways that oppose AT1 signaling (Kaschina and Unger 2003). The studies in angiotensin treated or fructose fed rats described above specifically demonstrated that AT2 was not responsible for the insulin resistance and decreased adiponectin levels by the use of AT2 agonists and receptor blockers (Ran et al. 2006). On the other hand, seminal work by Hsieh has shown that while AT1 mediates hepatic insulin resistance in fructose fed rats, this effect of AT1 requires coactivation of AT2 (Hsieh et al. 2005; Hsieh 2005).

A study of the ARB telmisartan in fructose fed rats demonstrated that treated rats not only accumulated less fat in the liver, but that this particular ARB also increased energy expenditure and prevented weight gain (Sugimoto et al. 2006). Some have argued that many of the beneficial effects of telmisartan are due to its unique (among ARBs) PPARγ ligand effects, (Kurtz, 2006) while others believe that its effects are mediated primarily via its ARB properties (Sharma, 2006). Either way, telmisartan appears to be an agent particularly worthy of study in

patients with NASH to determine if these benefits in rodents translate into similar benefits in humans.

### 1.10.9 Mechanism of the observed alternative pattern of NAFLD in children

NAFLD in children is characterized by marked steatosis with portal-based fibrosis and little or no hepatocellular ballooning or Mallory hyaline (Type 2 NAFLD), which is distinct from the zone 3 injury pattern of classic (adult-type) steatohepatitis (Type 1 NAFLD). The question is raised whether diet is responsible. Tetri et al. (2007) characterized the hepatic and metabolic phenotypes of sedentary mice fed a diet identical in composition to commonly consumed American fast foods. The fast-food-diet mice gained significantly more weight than controls, serum ALT levels progressively increased, hepatic triglyceride content increased, and liver TNF-$\alpha$ mRNA increased indicating a progressively increasing inflammatory response. Liver procollagen mRNA was increased, suggesting an early fibrogenic response. Hepatic steatosis increased progressively in a distinctly zone 1 to zone 3 distribution pattern. Thus, paediatric "type 2" NAFLD, with evidence of an early profibrogenic response, was induced in sedentary young mice fed a high trans-fat diet and high-fructose corn syrup. This phenotype induced by major components of typical fast food was associated with impaired fasting glucose and impaired glucose tolerance, underscoring the clinical relevance of this model.

## 1.11 Treatment

Although many treatment modalities have been used in patients with NASH, none have been convincingly shown to be effective (Andersen et al. 1991; Ueno et al. 1997; Li et al. 2005; Dixon 2007; Kadayifici et al. 2007). Most of the current regimens have been tested in open label, uncontrolled trials that have been carried out over a relatively short period of time. In addition, most of these studies did not adhere to a strict histological end point.

The approach to the treatment of NAFLD/NASH entails addressing the underlying metabolic abnormalities. Invariably the treatment will involve loss of weight. Treatment of risk factors, specifically weight reduction, is the only proven effective therapy for NAFLD. Shields and Coyle (2007) in a prospective, randomized, placebo-controlled trial of 40 patients with NASH, documented that a decrease in BMI (via diet and exercise) was associated with an improvement in serum ALT levels as well as inflammatory grade and fibrosis stage on histology. Patients

losing more than 5% of body weight over 9 months improved insulin resistance and steatosis, but only those patients losing 9% or more body weight achieved improvement in insulin resistance, steatosis, and inflammation (NASH) (Harrison et al. 2007).

There are three main treatment approaches namely lifestyle changes, pharmacological therapy and surgery.

### 1.11.1 Weight loss through lifestyle changes

Lifestyle changes, including weight loss and exercise, have been shown to improve ALT levels (Andersen et al. 1991; Vajro et al. 1994) and, less frequently, liver histology (Ueno et al. 1997). To achieve sustained weight loss, comprehensive programs including diet, exercise and behaviour modification might be needed. Rapid weight loss has been associated with worsening liver histology (Andersen et al. 1991) and most experts therefore recommend that weight loss in obese patients with NAFLD should be gradual (less than 1.6 kg per week). The validity of this recommendation in the era of bariatric surgery, however, might no longer be substantiated.

### 1.11.2 Weight loss with anti-obesity medication

In a pilot study, orlistat (an enteric lipase inhibitor) has been shown to promote weight loss and to reduce aminotransferase levels, with improved liver histology in 9 out of 10 NAFLD patients (Harrison et al. 2004). Harrison et al. (2007) reported that orlistat, a reversible inhibitor of gastric and pancreatic lipase, did not enhance weight loss, nor did it enhance outcomes with respect to ALT levels or histopathology in patients with NASH. The effect of using sibutramine (a selective serotonin reuptake inhibitor) has been compared with orlistat (Sabuncu et al. 2003), and both treatment groups lost weight and improvements were demonstrated in both liver enzyme levels and in the extent of steatosis visible on ultrasound. Despite these encouraging findings, questions remain as to whether these medications can be tolerated long term by patients and whether sustained weight loss can be achieved (Li et al. 2005). Furthermore, difference in response to treatment and long-term success of weight loss can be ascribed partly to the genetic background of the individual. In a pharmacogenetic study with sibutramine, a relatively common polymorphism in the G-protein subunit-3 gene (GNB3) was associated with clinical outcome (Hauner et al. 2003)

### 1.11.3 Weight loss through antiobesity surgery

For morbidly obese patients (BMI above 35 kg/$m^2$), lifestyle modification might not be enough to achieve sustained weight loss. In these patients, bariatric surgery is now a viable option for achieving sustained weight loss. The risks of bariatric surgery vary depending on the procedure undertaken. At the moment, there is a trend towards performing less invasive procedures with lower morbidity and mortality (e.g. laparoscopic adjustable gastric band). In patients who undergo bariatric surgery, an improvement can be expected in the severity of their metabolic syndrome, in both their mental and physical health, and potentially in their life expectancy (Dixon 2007). The hepatic effects of bariatric surgery in NAFLD patients seem to be favourable. Both Roux-en-Y gastric bypass and gastroplasty have been shown to reduce hepatic steatosis and even fibrosis (Silverman et al. 1995; Clark et al. 2005). In obese NASH patients who underwent a laparoscopic adjustable gastric band procedure, regression of NASH was shown in paired liver biopsies (Dixon 2007). As already noted, there is evidence to suggest that rapid weight loss (more than 1.6 kg per week) achieved by following a rigid diet can lead to the development of inflammation and fibrosis. Although it is not entirely clear how rapid weight loss and worsening liver histology are linked, this phenomenon could result from the massive liberation of free fatty acids into the bloodstream during these drastic diets (Friis et al. 1987). This issue has become an important concern in relation to some types of bariatric procedures, such as biliopancreatic diversion and Roux-en-Y gastric bypass. Nevertheless, the available data suggest that in the setting of appropriate surgical skill, these procedures can be safe and may reverse NASH and fibrosis.

### 1.11.4 Pharmacological approaches that address the underlying metabolic disorder

#### 1.11.4.1 Drugs that improve insulin sensitivity

As previously noted, most NASH patients have insulin resistance. In fact, in patients with NAFLD who have type 2 diabetes mellitus, the level of insulin resistance has been correlated with the grade of steatosis and even fibrosis (Matteoni et al. 1999). Treatment strategies focused on improving insulin resistance by using thiazolidinediones and metformin have therefore been explored.

*Thiazolidinediones*

Among the thiazolidinediones, troglitazone was the first to be studied in NASH patients, showing some biochemical improvement (Caldwell et al. 2001). However, troglitazone was subsequently withdrawn due to hepatotoxicity. The potential of two newer types of thiazolidinedione – rosiglitazone and pioglitazone – to treat patients with NASH has since been assessed. Both rosiglitazone and pioglitazone have been shown to improve liver enzyme levels and liver histology (Neuschwander-Tetri et al. 2003b; Promrat et al. 2004; Belfort et al. 2006). Although histologic improvement occurred with pioglitazone treatment, a significant reduction in fibrosis was not seen (Belfort et al. 2006). It also appears that the improvement generated by rosiglitazone and pioglitazone is short lasting, and liver enzyme levels become abnormal again once these medications have been discontinued. There remains concern associated with thiazolidinedione treatment as these drugs have the potential for toxicity (Reynaert et al.2005) and patients tend to gain weight on treatment. Furthermore, because these studies were conducted with relatively small numbers of individuals and over short time periods, well-controlled clinical trials are needed to establish their efficacy.

*Metformin*

Another anti-diabetic medication, metformin, has been used to treat NAFLD. The lack of weight gain associated with metformin treatment might provide an advantage in the typically obese NAFLD population. Although metformin has been shown to improve liver enzyme levels (Uygun et al. 2004), extensive data on histological improvements are currently lacking.

*Angiotensin II type 1 receptor blocker*

Kudo et al. (2007) evaluated the effect of an antihypertensive agent, the angiotensin II type 1 receptor blocker telmisartan, in a mouse model of NASH. This agent, which is also a peroxisome-proliferator activated receptor-gamma agonist, increased serum adiponectin levels and attenuated the progression of NASH, suggesting that it may represent a potential therapeutic approach.

### 1.11.4.2 Drugs that prevent fat accumulation in the liver

Song et al. (2007) investigated the effects of betaine, a naturally occurring choline metabolite, on fat accumulation in the liver in a mouse model of NAFLD. Betaine significantly attenuated steatosis. This change was associated with increased activation of hepatic AMP-activated

protein kinase, which subsequently attenuated lipogenic capability. Furthermore, betaine pretreatment reduced triglyceride accumulation in liver cells. Betaine may serve as a potential therapeutic tool to attenuate hepatic steatosis.

#### 1.11.4.3 Anti-oxidants

*Vitamins E and C*

The theory that NASH develops as a result of oxidative stress on the fatty liver has prompted interest in the therapeutic role of antioxidants in NAFLD. Therapy with Vitamin E has been studied alone and in combination with Vitamin C (Hasegawa et al. 2001; Kugelmas et al. 2003; Harrison et al. 2003b; Ersoz et al. 2005), but the findings have been inconsistent and future studies are needed to assess the efficacy of antioxidants in combination with other medications or regimens.

#### 1.11.4.4 Drugs that inhibit the cytokine response

Buranawuti et al. (2007) determined the efficacy and safety of pentoxifylline, a TNF-$\alpha$ inhibitor, versus placebo in patients with NAFLD. Although the mean serum ALT and serum fasting glucose levels decreased significantly from baseline in patients who received pentoxifylline, there was no significant improvement in BMI, insulin resistance, or serum TNF-$\alpha$. Adiponectin levels were not significantly different between the groups.

### 1.12 NAFLD/NASH and hepatitis C

NASH was once considered a diagnosis of exclusion, implying that the diagnosis could not be reached unless all other causes of liver disease were excluded. As the histological criteria have been refined and underlying pathophysiological mechanisms better understood, the coexistence of NASH with other causes of liver disease is increasingly recognised. For example, 5.5% of biopsies with steatohepatitis were found to have concomitant other diseases in one series (Brunt et al. 2003). Chronic hepatitis C was the most common; conversely, NASH was found in about 4% of biopsies obtained for hepatitis C. Another series identified alpha1-antitrypsin deficiency as the most common concomitant disease process in NAFLD, occurring in about 8% of biopsies (Sanyal et al. 2003).

### 1.13 The role of genetic risk factors in NAFLD/NASH

Patients with NAFLD are usually obese and have other conditions associated with the metabolic syndrome such as and insulin resistance. These patients typically do not consume excessive alcohol. It is clear, however, that whereas the majority of patients with these risk factors develop hepatic steatosis, only a minority develop more advanced liver disease - steatohepatitis, fibrosis, cirrhosis, and HCC. These observations have led to the obvious question: which factors determine whether a patient with the metabolic syndrome to develop advanced NAFLD?

A role for genetic factors in NAFLD is suggested by two reports of family clustering. Struben et al. (2000) reported the co-existence of NASH and/or cryptogenic cirrhosis in seven of eight kindreds studied, while Willner et al. (2001) found that 18% of 90 patients with NASH had an affected first degree relative. Clearly, this clustering could simply be a reflection of the well established heritability of the risk factors for NAFLD - obesity and insulin resistance. However, two studies examining ethnic differences in the prevalence of NAFLD and NAFLD-related 'cryptogenic' cirrhosis strongly suggest that susceptibility to NAFLD rather than to its risk factors may have a genetic component (Browning et al. 2004a; Caldwell et al. 2002). In the most recent study from Dallas, Texas, the prevalence of cryptogenic cirrhosis in Hispanic and African-Americans was three-fold higher and four-fold lower, respectively, compared with European-American patients despite a similar prevalence of type 2 diabetes mellitus (Browning et al. 2004b).

To perform family linkage studies in subjects with NAFLD is complex and therefore have resulted in most of the relevant genetic information thus far coming from classical case--control, candidate gene, and allele association studies. Accordingly, these results must be interpreted with caution (Daly and Day 2001).

#### 1.13.1 Genes influencing oxidative stress

The principal class of genes that influences the oxidant load in patients with obesity, insulin resistance, and the metabolic syndrome are those encoding proteins involved in the oxidation of free fatty acids (FFA). The role of FFA oxidation in the pathogenesis of NAFLD is complex. On the one hand, appropriate fat oxidation is required to prevent fat accumulation in the liver, while on the other; excessive fatty acid oxidation leads to oxidative stress (Day 2002a; Sanyal et al.

2001; Miele et al. 2003).

Children with inherited defects in mitochondrial ß-oxidation develop steatosis but they do not get NASH, strongly suggesting that intact mitochondrial oxidation of FFA is required for progression to inflammation and fibrosis. With respect to peroxisomal and microsomal fat oxidation, as both are capable of generating ROS, it might be predicted that 'gain-of-function' polymorphisms in genes encoding proteins involved in these processes would predispose to NASH. However, these pathways play a role in limiting mitochondrial overload during times of excessive FFA supply and therefore it may be that 'loss-of function' polymorphisms effecting these pathways would predispose to NASH. This latter hypothesis is supported by a study showing that mice lacking the gene encoding fatty acyl-CoA oxidase, the initial enzyme of the peroxisomal ß-oxidation system, develop severe microvesicular NASH (Fan et al. 1998). Similar difficulties apply to interpreting a preliminary report that a mutation (*PPARA 3*) in the gene encoding PPAR$\alpha$ is associated with NASH (Merriman et al. 2001). PPAR*s* regulates the transcription of a variety of genes encoding enzymes involved in mitochondrial, peroxisomal ß-oxidation, and microsomal *y*-oxidation of fatty acids (Berger and Moller 2002). Functional data on PPAR$\alpha$ mutations are somewhat contradictory at present (Sapone et al. 2000). The fact that adiponectin activates PPARs and protects against steatosis (Yamauchi et al. 2002), suggests that any *PPAR$\alpha$* mutation associated with NASH should be associated either with loss-of-function or reduced gene expression.

As with ALD, other genes that may influence the magnitude and effect of oxidative stress in NAFLD include the *HFE* gene and the gene encoding SOD2/MnSOD.

### 1.13.2. Genes influencing the response to endotoxin

Evidence supporting a role for endotoxin-mediated cytokine release in the pathogenesis of ALD and NAFLD, together with the identification of promoter polymorphisms in genes encoding endotoxin receptors, has recently suggested an alternative set of 'candidates' to explain genetic susceptibility to advanced fatty liver diseases. CD14, a lipopolysaccharide (LPS) receptor on monocytes, macrophages, and neutrophils, has no intracellular domain but enhances signalling through another LPS receptor, toll-like receptor-4 (TLR4). A C/T polymorphism is present at nucleotide position 159 in the CD14 promoter, with the TT genotype associated with increased levels of soluble and membrane CD14 (Baldini et al. 1999). A preliminary study in NASH has

reported an association with the CD14 polymorphism but not with the TLR4 polymorphism (Day 2002). With respect to the SOD2/MnSOD polymorphism, there has been one preliminary report of an association with the severity of fibrosis in patients with NAFLD (Saksena et al. 2003).

### 1.13.3 Genes influencing the release or effect of cytokines

The first association between a cytokine gene polymorphism and ALD was reported between alcoholic hepatitis and a polymorphism at nucleotide position -238 in the TNF-α promoter region (Grove et al. 1997). This polymorphism has subsequently also been associated with NASH (Valenti et al. 2002). The functional significance of the polymorphism is, however, unclear and the associations may well be either spurious or due to linkage disequilibrium with another true 'disease-associated" (functional) polymorphism on chromosome 6, although the ALD association has recently been confirmed in a study from Spain (Pastor 2005). An association with ALD has also been reported for a promoter polymorphism in the interleukin-10 (IL-10) gene. IL-10 is the classical anti-inflammatory cytokine which inhibits: (i) the activation of CD4+ T-helper cells, (ii) the function of cytotoxic CD8+ T cells and macrophages, (iii) class II HLA/B7 expression on antigen-presenting cells, and (iv) hepatic stellate cell collagen synthesis. A variant C to A substitution at nucleotide position -627 in the IL-10 promoter has been associated with decreased reporter gene transcription, decreased IL-10 secretion by peripheral blood monocytes and an increased response to £\-interferon in patients with chronic hepatitis C - all consistent with the polymorphism being associated with lower IL-10 production. A strong association between possession of the A allele and ALD has been reported from a study of over 500 heavy drinkers with and without advanced liver disease (Grove et al. 2000). This is consistent with low IL-10 favouring inflammatory and immune-mediated mechanisms of disease as well as hepatic stellate cell collagen production.

### 1.13.4 Genes influencing the severity of fibrosis

Genes encoding proteins involved in fibrogenesis or fibrinolysis in the liver are clearly candidates for a role in ALD and NAFLD-related fibrosis. Obvious candidates would include the polymorphic genes encoding transforming growth factor (TGF)-ß1, CTGF, matrix metalloproteinase 3, PPAR*y*, and various fibrogenic adipocytokines including angiotensin II. The only study thus far in this regard is a recent report that obese patients inheriting both high TGFß1 and angiotensinogen producing polymorphisms may be more susceptible to advanced

fibrosis (Dixon et al. 2003). Although this relatively small study awaits replication, it does suggest that 'fibrosis genes' may be important in determining susceptibility to the more advanced forms of fatty liver disease.

### 1.14 Genetic predisposition for haemochromatosis in patients with NAFLD

Hereditary haemochromatosis (HH) is a disease regarded as the most common autosomal recessive disorder in Caucasians from Northern European decent. HH is characterized by increased iron deposition in different organs of which iron deposition in the liver leads to liver cirrhosis and hepatocellular carcinoma (HHC). Several studies have implicated heterozygous HFE mutations in the exacerbation of other chronic liver diseases to progress to liver cirrhosis. This phenomenon can possibly be explained by the concept that excessive iron accumulation in the liver leads to increased production of reactive oxygen species (ROS). In a liver already compromised by another disease, the increased production of ROS can cause further insult and therefore more severe disease.

The presence and role of HFE mutations in patients with NASH and the development of advanced fibrosis remains controversial. The majority of the studies were limited by sample size and referral bias. A recent, large multicentre study from the US and Canada found that the prevalence of HFE mutations was not increased among Caucasians, however the presence of heterozygous C282Y mutation status in Caucasians was associated with advanced liver fibrosis (Nelson et al. 2007). An initial study from Australia showed that 31% of 51 patients with NASH possessed at least one copy of the *C282Y HFE* mutation compared with only 13% of controls (George et al. 1998). Most recently, however, an Italian study of 263 consecutive patients with NAFLD has reported a prevalence of the *C282Y* and *H63D* mutations identical to the locally matched population (blood donors) (Bugianesi et al. 2004). Furthermore, among the NAFLD patients, liver iron content was no different in patients with and without the mutations and the severity of fibrosis was unrelated either to liver iron content or to HFE genotype.
The frequency of the C282Y mutation in the HFE gene was found to be increased in Italians patients from Northern Italy (Valenti et al. 2003). This finding suggests that heterozygosity for C282Y HFE mutation confers susceptibility to NAFLD. The study also concluded that carriers of the C282Y mutation in the HFE gene develop NAFLD in the presence of less severe metabolic abnormalities. In this study they also found that in the presence of increased serum iron the

release of insulin was decreased, proposing it as the mechanism involved for the increased susceptibility to NAFLD.

### 1.15 The significance of gene expression analysis in NAFLD/NASH

Despite the high prevalence of NAFLD, little is known about the pathogenesis of this condition in relation to gene expression profiling in human liver tissue. Greco et al. (2008) used Affimetrix Gene Chips to determine the expression patterns of 17,601 genes in human liver of NAFLD patients with extreme steatosis without inflammation compared with patients showing low liver fat content. Genes involved in hepatic glucose and lipid metabolism, insulin signalling, inflammation, coagulation, cell adhesion, and ceramide metabolism were found to be significantly associated with liver fat content. Up-regulation of several genes involved in key metabolic processes (Table 1.2) was validated by using real-time polymerase chain reaction (PCR) technology in subjects with high liver content. This data demonstrated that simple steatosis is characterised by multiple changes in gene expression related to various metabolic pathways.

**Table 1.2** Up-regulated genes implicated in steatosis in NAFLD

| Gene | Gene Symbol | Metabolic risk area |
|---|---|---|
| Perilipin | PLIN | Lipid metabolism |
| Acyl-Coenzyme A dehydrogenase | ACADM | Lipid metabolism |
| Fatty acid binding protein | FABP4 | Fatty acid transport |
| CD36 molecule (throbospondin receptor) | CD36 | Fatty acid transport |
| Branched-chain aminotransferase 1 | BCAT1 | Amino acid catabolism |
| Chemokine (C-motif) ligand 2 | CCL2 | Inflammation |

The significance of genes involved in fatty acid metabolism was highlighted by the changes found in expression patterns that could contribute to increased fatty acid synthesis (Kohijma et al. 2007). Real-time PCR analysis of 26 NAFLD and 10 normal liver samples for differential expression of 20 different genes revealed that although fatty acids accumulated in hepatocytes, their de novo synthesis and uptake were up-regulated in association with increased expression of acetyl-CoA carboxylase (ACC1), fatty acid synthase (FAS), sterol regulatory element-binding protein 1c (SREBP-1c) and adipose differentiation-related protein (ADRP). The finding that seven fatty acid oxidation related genes, long-chain acyl-CoA dehydrogenase (LCAD), long-chain L-3-hydroxyacylcoenzyme A dehydrogenase alpha (HADH$\alpha$), uncoupling protein 2 (UCP2), straight-chain acyl-CoA oxidase (ACOX), branched-chain acyl-CoA oxidase (BOX),

cytochrome P450 2E1 (CYP2E1) and CYP4A11, were all over-expressed showed that oxidation is enhanced in NAFLD. The expression of carnitine palmitoyltransferase 1a (CTP1a) and peroxisome proliferator-activated receptor-alpha (PPAR$\alpha$) was reduced.

The following insights regarding the pathogenesis of NAFLD were obtained based on the findings of Kohijma et al. (2007).

- increased de novo synthesis and uptake of fatty acids lead to further fatty acid accumulation in hepatocytes
- mitochondrial fatty acid oxidation is decreased or fully activated
- in order to complement the function of mitochondria (beta-oxidation), peroxisomal (beta-oxidation) and microsomal (omega-oxidation) oxidation is up-regulated to decrease fatty acid accumulation
- antioxidant pathways including superoxide dismutase (SOD) and catalase are enhanced to neutralize ROS overproduced during mitochondrial, peroxisomal, and microsomal oxidation
- lipid droplet formation is enhanced due to increased diacylglycerol O-acyltransferase 1 (DGAT) expression and decreased hormone-sensitive lipase (HSL) expression.

The significance of genes involved in the transition from steatosis to hepatosteatosis was highlighted in the study of Browning and Horton (2004). Insulin resistance and oxidative stress play an important role in the development and progression of NAFLD and analysis of genes involved in these processes formed an important aspect of the present study. Adiponectin was selected as one of four genes included for expression analysis in South African NAFLD patients, due to its role in the transport of fatty acids into the mitochondria where $\beta$-oxidation occurs. Suppression of hepatic fatty acid synthesis is necessary to counteract the effects of high serum insulin levels. Mangenese superoxide dismutase (MnSOD) is important in this context, as enzyme deficiency increase mitochondrial oxidative stress and aggrevate age-dependent vascular dysfunction (Wenzel et al. 2008). These effects are related to a reduction in free bioavailable nitric oxide that is inactivated by an age-dependent increase in superoxide formation.

Low serum adiponectin levels are a characteristic of NASH and distinguish this condition from simple steatosis (Hui et al. 2004). However, gene expression in human liver was shown to be significantly increased in patients with NASH compared with normal liver (Uribe et al. 2008). In

the same study, mRNA expression levels of the interleukin-6 gene was significantly lower in NASH compared with normal livers. Another area of particular interest in the context of NAFLD relates to the IKKβ pathway, which is crucial for cytokine induced insulin resistance mediated via NF-κβ activation (Yuan et al. 2001). TNFα is liberated by the adipose tissues of obese persons and could worsen hepatic insulin resistance via activation of IKKβ.

**1.16 Aims of the study**

The overall objective of the present study was to study the clinical, metabolic and genetic contribution to the development and progression of NASH/NAFLD. The disease profile of NAFLD has not previously been described in the genetically distinct population groups of South Africa. The findings generated from this study would therefore be unique in the knowledge gained. The specific aims were as follows:

1. Describe the phenotype of NAFLD/NASH in South African patients of the Western Cape.

The Western Cape has a potpourri of different ethnic groups. The Cape Coloured population, a population of mixed ancestry, represents the majority group in the Western Cape and has a unique genetic background. The need exists to define NAFLD/NASH in the local population and compare the findings with existing disease profiles elsewhere in the world. At the start of this project, there was little awareness of the disease entity NAFLD/NASH in South Africa. By defining NAFLD/NASH in the genetically distinct population groups of South Africa, awareness of this important disease will be increased.

2. To investigate the metabolic derangements associated with NAFLD/NASH, including insulin resistance, lipid abnormalities and liver fibrogenesis.

An attempt has been made to accurately describe the metabolic derangements, specifically insulin resistance and dyslipidemia, in the different population groups studied and to determine the relationship of these derangements with the different stages of NAFLD/NASH. An important goal was to evaluate these metabolic derangements in combination with markers for fibrosis for use in an algorithm for prediction of advanced disease.

3. To assess the possible involvement of candidate genes in relation to the NAFLD/NASH phenotype in patients from the Western Cape region.

Candidate genes implicated in the pathogenesis of NAFLD/NASH have been identified from the literature, for investigation of their potential role in disease progression in the local population. The role of HFE gene mutations in the development of NASH and advanced fibrosis were investigated, as well as gene expression of adiponectin, TNF-alpha, MnSOD and IKK$\beta$.

# CHAPTER 2

# SUBJECTS AND METHODS

## 2.1 Ethical Approval

Ethical approval for this study has been granted by the Ethics Review Committee of the University of Stellenbosch (project registration number N04/02/033).

## 2.2 Study population

The study population included patients who attended the clinics at the Gastroenterology and Hepatology Division of the Department of Internal Medicine at Tygerberg Hospital as well as Louis Leipoldt and Durbanville Medi-clinics. A total of 233 overweight/obese patients were screened for NAFLD by ultrasonography. A questionnaire was used to denote personal details, family history and clinical characteristics.

Subjects were grouped according to those who fulfilled the criteria for NASH and fatty liver disease. Fatty liver disease is defined as steatosis only or steatosis with inflammation but not fulfilling the criteria for NASH. The subjects were also grouped according to the severity of fibrosis namely advanced fibrosis (grade 3 and 4) versus no/mild fibrosis (grade 0,1 and 2) according to Kleiner et al. (2005).

*Exclusion criteria*
History of significant alcohol usage (> than 20 g/d).
Histology and/or blood investigations suggestive of another liver disease.
Patients with a secondary cause for fatty liver disease.
*Inclusion criteria*
Histology confirming NAFLD/NASH
Patients who provided written informed consent for participation in the study

## 2.3 Methods

Blood was collected from all the patients screened for NAFLD after obtaining informed consent. Laboratory analysis was performed to identify metabolic derangements in NAFLD/NASH and to exclude other liver diseases. Liver biopsies were performed on those individuals who also had abnormal liver functions, hepatomegaly or any features associated with advanced disease. Blood samples and liver tissue were furthermore used for genetic analysis, following DNA and

RNA extraction. After collection the liver tissue samples were immediately snap frozen in liquid nitrogen and stored at –70°C until use.

### 2.3.1 Anthropometric parameters

Height and weight measurements were recorded from which BMI was calculated as follows: weight in kg/ height in $m^2$.

### 2.3.2 Liver Biopsies

The liver biopsies were performed percutaneously under ultrasound guidance. This procedure was performed on the patients with fatty infiltration on ultrasound and who had persistently abnormal liver functions and/or hepatomegaly.

### 2.3.3 Histological analysis

All of the biopsy samples were histologically analysed by the same two pathologists according to the criteria of the National Institute of Health Non Alcoholic Steatohepatitis Clinical Research Network (NIH NASH CRN) (Kleiner et al. 2005).

### 2.3.4 Biochemical parameters

Liver functions, full blood count, fasting serum glucose, serum insulin, lipograms and serum iron studies were determined after an overnight fasting period using standard laboratory techniques. Additional blood tests were performed where necessary to exclude other liver diseases. Insulin resistance was determined by using the homeostasis model assessment (HOMA) formula = insulin x glucose/ 22.5. Known diabetics were excluded from HOMA-IR calculations.

### 2.3.5 LDL particle size analysis

Blood was collected from 108 NAFLD patients for LDL particle size determination. For comparative analysis, LDL size was also determined in 34 patients (used as controls) without the metabolic syndrome (diagnosed other forms of liver disease or with normal livers). The LDL phenotype was determined by non-denaturing gradient gel electrophoresis (GGE). The plasma

was stained with Sudan Black in ethylene glycol for direct visualization in a 2-8 g% polyacrylamide mini-gel run at 4°C overnight, using previously obtained large and small LDL species for calibration.

### 2.3.6 APRI as a non-invasive marker for advanced liver disease

One hundred and eleven patients with histologically confirmed NAFLD were included in this part of the study to assess the usefulness of APRI as a non-invasive marker for advanced liver disease. The same two pathologists reported on the histology of each sample. The samples were specially stained to exclude iron and copper overload. The samples were staged and graded according to the NASH National Institute of Health Chronic Research Network criteria. The samples were classified into 4 histologically defined groups namely: Fatty Liver disease not fulfilling the criteria for NASH (FLD), NASH, No or mild fibrosis (stage 1 and 2) and advanced fibrosis (stage 3 and 4). For each group ALT, AST/ALT ratio, APRI and NFS were performed and compared as to predictive of advanced NAFLD. APRI was calculated by using the formula: (AST/Upper limit of normal x 100) /platelet count. NFS was calculated according to Angulo et al. (2007): - 1.675 + 0.037 x age (years) + 0.094 x BMI ($kg/m^2$) + 1.13 x IFG/diabetes (yes =1, no = 0) + 0.99 x AST/ALT ratio – 0.013 x platelets (x $10^9$/L) – 0.66 x albumin (g/dL).

### 2.3.7 HFE mutation analysis

Blood samples were analysed for HFE gene mutations C292Y and H63D in 56 NAFLD patients, 18 with advanced fibrosis, 18 with mild fibrosis and 20 with no fibrosis on their liver histology. This study cohort were selected equally from the Caucasian population (n=28) and Coloured population of mixed ancestry (n=28) and was age-matched. All of the patients had routine blood investigations and anthropometric evaluations performed at the time of enrolment, which included serum iron studies HFE mutation analysis was performed using a polymerase chain reaction (PCR) based method (Feder et al. 1996). Allele frequencies and genotype distribution were compared between patients with no/mild fibrosis and advanced fibrosis in relation to previous findings in different ethnic groups in the general South African population (de Villiers et al. 1999). DNA samples of individuals with and without these HFE mutations were included as positive and negative controls, respectively.

### 2.2.8 Analysis of gene expression in liver tissue

The liver tissue samples obtained at the time of liver biopsy were used for gene expression analysis in 80 patients with NAFLD. mRNA expression levels were determined for the adiponectin, IKKβ, TNF-α and MnSOD genes due to their role in insulin resistance, inflammation, hepatic acid metabolism and oxidation (Maeda et al. 2002; Saksena et al. 2003; Hui et al. 2004; Hotamisligil et al. 1996).

It is acknowledged that the four genes included for expression analysis in the present study represent only a fraction of those previously implicated in NAFLD/NASH. It was not feasible to perform microarray analysis including thousands of genes in order to define a molecular signature of specific relevance to the South African population. Therefore, gene expression analysis performed in the South African patient cohort aimed to determine whether similar trends were observed for the selected genes when compared with previous studies. If confirmed, significant findings reported elsewhere could be extrapolated to the South African population and generalised across population groups in an evidence-based manner.

*Isolation and purification of total RNA*

Liver biopsy specimens were obtained and immediately snap frozen in liquid nitrogen and then stored at -70°C. Total RNA was extracted from liver samples according to the protocol of the RNeasy total RNA reagent set (Qiagen). The amount of RNA was measured spectrophotometrically by the absorbance at 260 nm. One microgram of RNA was incubated for 15 minutes at room temperature with DNase I (1 U/µg; Invitrogen), followed by thermal inactivation of the enzyme (65°C for 10 min) in the presence of 2.5 mmol/L EDTA and a rapid cooling down step to 4°C. The purity of the RNA was estimated by the ratio of the absorbance at 260/280 ($A_{260/280}$). The RNA was stored at -80°C until use.

*Reverse transcription*

The reverse transcription reaction was carried out in a total volume of 20 µL of 1x reverse transcriptase buffer containing 10 mM dithiothreitol, 500 µM deoxynucleotide triphosphates, 3 µM oligo$(dT)_{15}$, 60 units of RNasin, and 200 U of Superscript RNase $H^-$ (Invitrogen). To this mixture 1 µg of total RNA treated with DNase I was added. The reaction was allowed to proceed

for 60 min at 42 °C, followed by 5 min of heating at 95 °C and rapid cooling on ice. The cDNA was stored at -20 °C until use.

*Preparation of cDNA calibrators*

cDNA calibrators were prepared by PCR amplification run to saturation (35 PCR cycles) with the appropriate primers. The resulting cDNAs were purified by column chromatography (high pure product purification reagent set; Roche Diagnostics) and eluted with Tris-EDTA (pH 8.0) buffer. The samples showed a unique band in agarose electrophoresis. The amount of DNA was determined by Pico Green fluorescence (Molecular Probes).

*TaqMan real-time RT-PCR*

The primers and probes used in the analysis of Adiponectin, MnSOD, Tumor Necrosis Factor Alpha (TNF-alpha), Inhibitor of Kappa Light Polypeptide Gene Enhancer in B-cells, Kinase beta (IKBKB) and the housekeeping gene, Hypoxanthine guanine phosphoribosyl-transferase (HPRT), gene expression are given in Table 2.1. The primers were designed using specific primer analysis software (Oligo 4.0) and these sequences were analyzed by FASTA in the EMBL database (http://www.embl-heidelberg.de/). The probes used in the study were obtained from the prevalidated probes for quantification of gene-expression levels by real-time PCR obtained from the Universal Probe Library Resource of Roche Diagnostics (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp).

Quantitative real-time RT-PCR assays were performed with the LightCycler DNA Master Hybridisation Probe kit (Roche Diagnostics). The final reaction mixture (20 µl) consisted of 1,5 µl of cDNA (diluted 1:10), 4 µl of the LightCycler RT-PCR mixture, 0.4 µl of the LightCycler RT-PCR enzyme mixture, and 5 mM $MgCl_2$. In the final optimized format, the concentrations of the primers and probes were as follows: for the Adiponectin and MnSOD genes, 0.2 µM each probe with 0.4 µM each primer; for the TNFalpha gene, 0.4 µM probe and 0.4 µM each primer; and for the IKKβ assay, 0.4 µM probe and 0.3 µM each primer. The thermal cycling conditions consisted of 95°C for 10min, followed by amplification for 45 cycles, at 95°C for 0 min, and annealing-extension at 60°C for 60 s.

After PCR, a melting curve was constructed by increasing the temperature from 55 °C to 95°C with a temperature transition rate of 0.1 °C/s. All sequences were amplified in duplicate from all

the patient samples. To ensure that the correct product was amplified in the reaction, all samples were separated by 2% agarose gel electrophoresis. The LightCycler apparatus measured the fluorescence of each sample in every cycle at the end of the annealing step. The Second Derivative Maximum Method was used to determine the crossing point (Cp) automatically for the individual samples. This was achieved by a software algorithm (Ver. 3.5) that identifies the first turning point of the fluorescence curve, corresponding to the first maximum of the second derivative curve, which serves as the Cp. The LightCycler software constructed the calibration curve by plotting the Cp vs the logarithm of the number of copies for each calibrator. The numbers of copies in unknown samples were calculated by comparing their Cps with the calibration curve. To correct for differences in both RNA quality and quantity between samples, data were normalized using the ratio of the target cDNA concentration to that of HPRT. A test result was considered positive if all positive and negative control reactions gave the expected values.

**Table 2.1:** Primer sets designed across the exon-intron boundaries of the adiponectin, TNF-$\alpha$, MnSOD, IKK-$\beta$ and HPRT genes.

| Gene Name | Forward Primer | Reverse Primer |
|---|---|---|
| Adiponectin | 5'-CCTGGTGAGAAGGGTGAGAA-3' | 5'-CACCGATGTCTCCCTTAGGA-3' |
| TNF-alpha | 5'-CAGCCTCTTCTCCTTCCTGA-3' | 5'-GCCAGAGGGCTGATTAGAGA-3' |
| MnSOD | 5'-CGTCACCGAGGAGAAGTACC-3' | 5'-CTGATTTGGACAAGCAGCAA-3' |
| IKK$\beta$ | 5'-CTGAGCCAGCCAAGAAGAGT-3' | 5'-GCAGGGTGCAGAGGTTATGT-3' |
| HPRT | 5'-TTGTTGGATATGCCCTTGACT-3' | 5'-CCGCTGTCTTTTAGGCTTTG-3' |

### 2.4 Statistical analysis

For descriptive purposes, cross tabulation and frequency tables were used to describe occurrences of various attributes (gender, ethnicity, etc.) of the patient sample. Comparisons between different groupings of the study cohort for measurements (BMI, HOMA-IR, etc) were done using one-way ANOVA. Possible deviations from the assumptions were always checked, and highlighted in cases where it caused a problem. The non-parametric Mann-Whitney U test was then used. Receiver Operating Characteristic (ROC) analysis was used to determine optimal cut-off points for diagnosis. In the comparative study of non-invasive markers the area under the curve (AUC), sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were reported.

Mutation frequency and genotype distribution were compared between groups following estimation from allele counts and use of chi-square statistics. For comparison of mRNA expression patterns between ethnic groups and the different histopathological groups log transformed values were used. Two-way analysis of variance (ANOVA) was performed to determine possible differences between ethnic groups and between the different patient groups. Since ethnic differences did not have a significant effect on gene expression patterns, one-way ANOVAs were used to compare different groupings. For comparison of percentages of overexpression between groups cross-tabulation and the chi-square test was used. A 5% significance level was used as guideline for determining significant differences.

# CHAPTER 3

# RESULTS

### 3.1 Demographics and clinical characteristics of NAFLD/NASH

The disease profile of non-alcoholic fatty liver disease (NAFLD), the most prevalent chronic liver disease in Western countries, has not yet been described in South Africa. For this demographic study 233 overweight/obese individuals were screened by ultrasound to identify patients with fatty liver/hepatomegaly. The majority of these patients (73%) were female. The ethnic distribution of this patient cohort was 69% Cape Coloured, 25% Caucasian, 5% Black and 1% Asian.

A total of 182 patients (87%) had ultrasonographical evidence of fatty infiltration and/or hepatomegaly. Of this group of patients with fatty liver on ultrasonography and who fulfilled the criteria for liver biopsy, 127 patients consented to the procedure. Figure 3.1 displays histological findings of this group that received liver biopsies. NAFLD was confirmed in 111 patients. Of the patients with NAFLD 46 (36%) had NASH. Sixty-three (54%) of the patients had no fibrosis on histology, while 20 (17%) had advanced liver fibrosis (stage 3 to 4) (Figure 3.2).

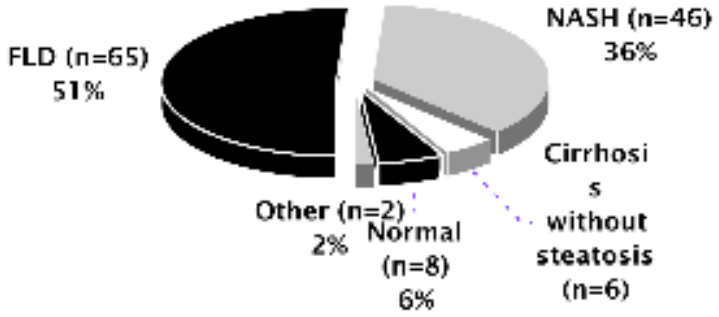



**FIgure 3.1** Histological findings in the group of 127 patients that received liver biopsies.

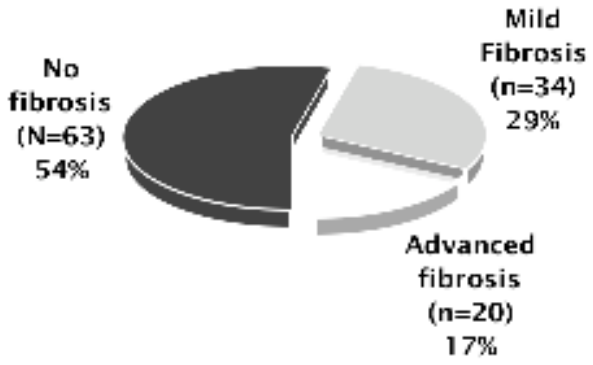



**Figure 3.2** Fibrosis grading based on the NASH NIH CRN criteria.

Figure 3.3 illustrates the disease distribution in males and females affected by NAFLD with reference to NASH versus FLD and advanced fibrosis versus no/mild fibrosis.

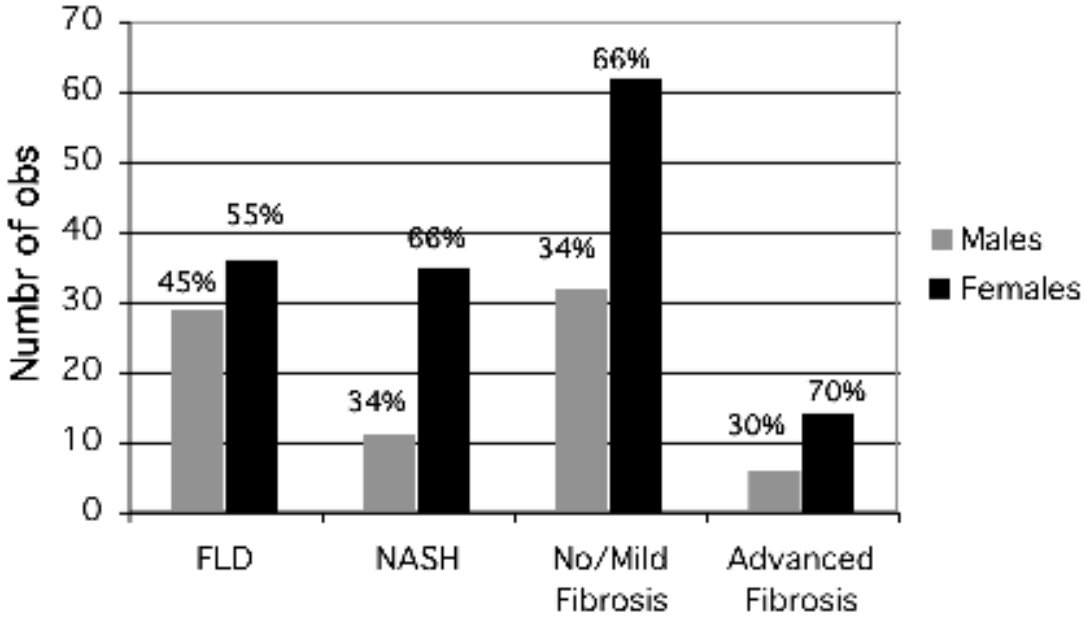



**Figure 3.3** Gender and association with NAFLD

Figure 3.4 illustrates the association of the mean age of the patients who presented with either NAFL/NASH as well as no/mild fibrosis and advanced fibrosis. The mean age (with 95% confidence interval in brackets) for the group with fatty liver disease was 50 years (48-53) compared with 54 years (51-58) for NASH (p=0.1). The mean age for the group with no/mild fibrosis was 51 years (49-53) and for the group with advanced fibrosis 57 years (49-65) (p=0.06).

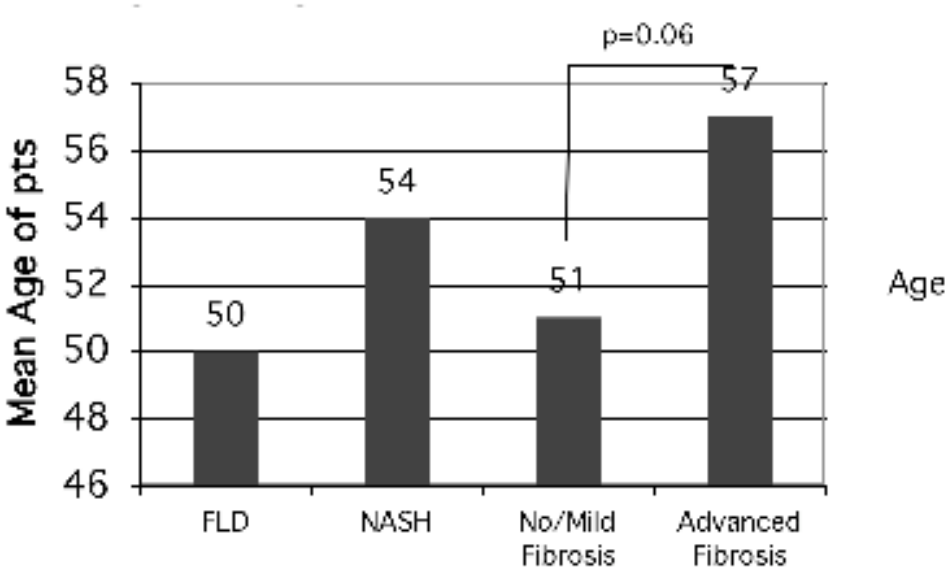



**Figure 3.4** Age and association with NAFLD

Figure 3.5 illustrates the difference between the different South African population groups as to their presentation with NASH versus fatty liver disease and grading of fibrosis. The Indian population group was excluded from the analysis as there was one patient in this group.

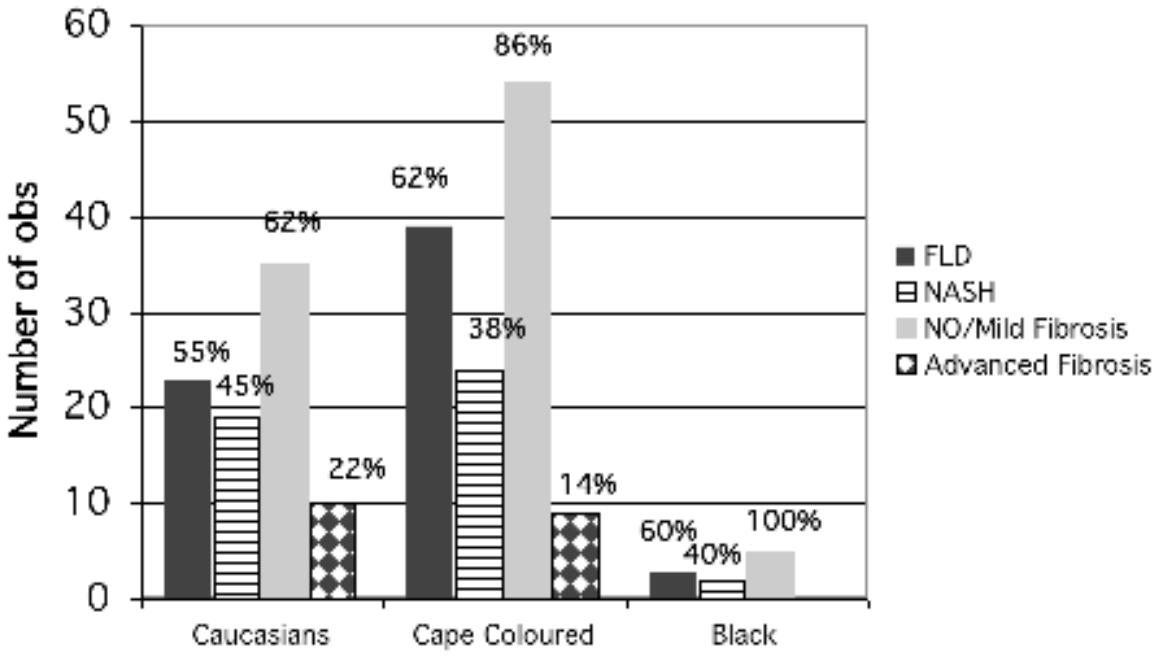



**Figure 3.5** Ethnical distribution of the study population in relation to NAFLD/NASH and stage of fibrosis.

Figure 3.6 illustrates the prevalence of Type II diabetes in the different patient groups with NAFLD. Table 3.1 summarises the mean total cholesterol level (mg/dl), serum triglyceride level (mg/dl), BMI ($kg/m^2$) and HOMA-IR for each patient group subdivided according to disease status.

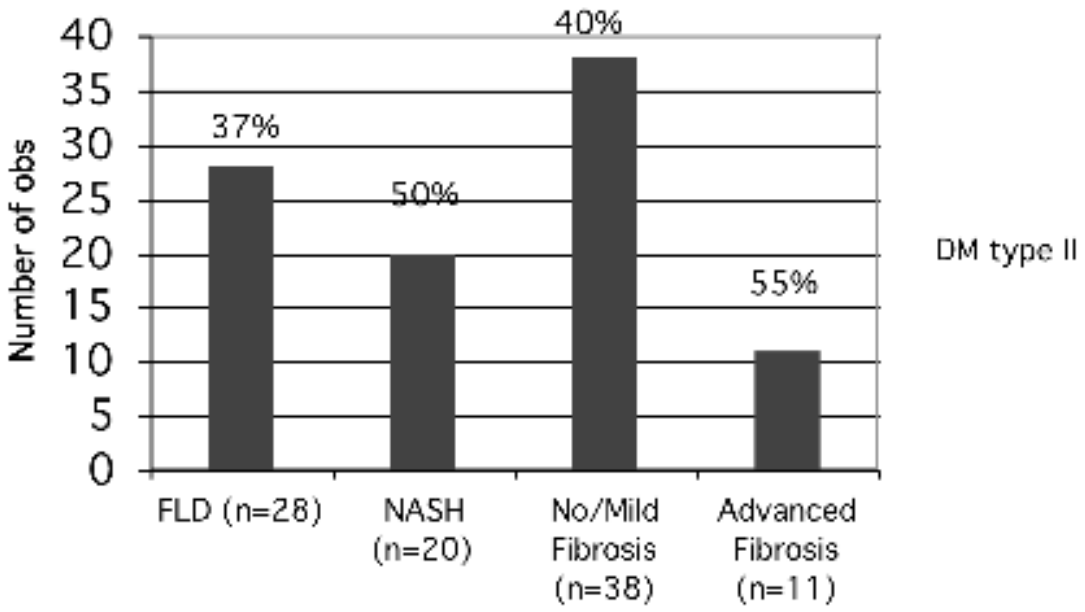



**Figure 3.6** Diabetes Mellitus and association with NAFLD/NASH.

**Table 3.1** Comparison of biochemical and anthropometric parameters of the study population.

| | **FLD** | **95%CI** | **NASH** | **95%CI** | **p-value** | **No/Mild Fibrosis** | **95%CI** | **Advanced Fibrosis** | **95%CI** | **p-value** |
|---|---|---|---|---|---|---|---|---|---|---|
| TC (mg/dl) | 5.4 | 5.1-5.8 | 6.1 | 5.7-6.5 | ≤0.01 | 5.7 | 5.4-6.0 | 5.8 | 5.1-6.4 | 0.95 |
| TG (mg/dl) | 1.9 | 1.3-2.6 | 3.1 | 2.3-3.9 | 0.03 | 2.3 | 1.2-2.9 | 2.9 | 1.7-4.2 | 0.38 |
| BMI ($kg/m^2$) | 36 | 34-38 | 34 | 32-36 | 0.17 | 36 | 34-37 | 31 | 28-34 | 0.01 |
| HOMA-IR | 5.7 | 2.8-8.6 | 6.1 | 4.3-8 | 0.8 | 5.8 | 3.5-8.1 | 11 | 5.3-16.7 | 0.09 |

TC, total cholesterol; TG, triglycerides, BMI, body mass index, HOMA-IR, homeostasis model assessment for insulin resistance

### 3.2 LDL size and the association with NAFLD

Low-density lipoprotein (LDL) particle size was compared among 108 NAFLD patients with FLD and NASH, as well as between patients with different stages of fibrosis, thereby indirectly determining their risk for cardiovascular disease. Table 3.1 describes the clinical characteristics of patients aged 50 years and older that were evaluated for LDL size in mean values. The mean age was significantly lower in patients with FLD than with NASH (p=0.02). Notably, the mean BMI was significantly lower in patients with advanced fibrosis compared with the other groups (p=0.01). The mean HOMA-IR for patients with advanced fibrosis were almost twice that of the group with no/mild fibrosis, with a strong trend toward statistical significance (p=0.09). The serum triglyceride level differed significantly in the NASH group compared to the FLD group (p=0.03). For the other groups the comparisons did not show statistically significant differences.

**Table 3.2** Clinical characteristics of the study cohort including 108 patients with NAFLD/NASH analysed for LDL particle size.

| Mean | FLD | NASH | P-value | No/Mild Fibrosis | Advanced fibrosis | P-value |
|---|---|---|---|---|---|---|
| Age | 50 | 54 | 0.02 | 51 | 57 | 0.06 |
| BMI | 36 | 34 | 1.7 | 36 | 31 | 0.01 |
| HOMA-IR | 5.7 | 6.1 | 0.8 | 5.8 | 11 | 0.09 |
| TG | 1.9 | 3.1 | 0.03 | 2.3 | 2.9 | 0.37 |

TG, triglycerides, BMI, body mass index, HOMA-IR, homeostasis model assessment for insulin resistance

The mean LDL size for the group with FLD was 2.5 (CI: 2.2-2.8) compared to 2.18 (CI: 1.9-2.5) in the groups with NASH (Figure 3.7). Mean LDL particle size differed significantly (p=0.03) from the control group with other liver diseases showing a value of 2.9 (CI: 2.5-3.2). Vertical bars denote 0.95 confidence intervals in Figure 3.7.

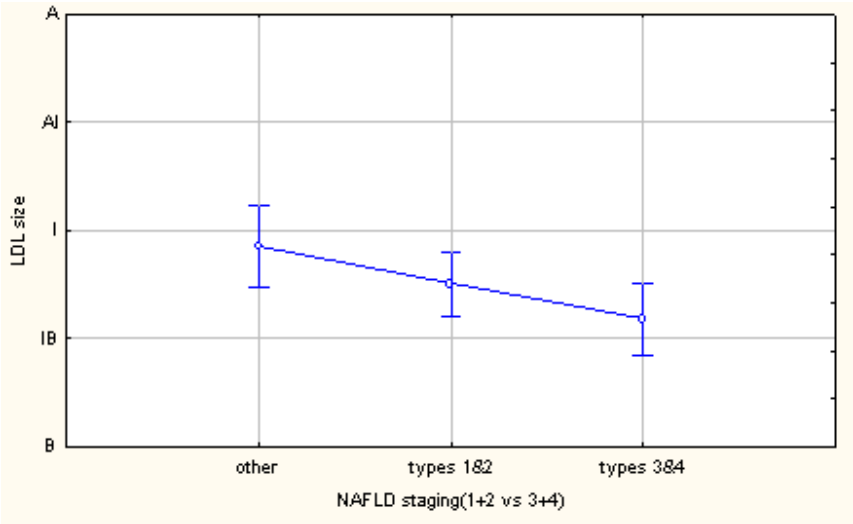



**Figure 3.7** LDL particle size in NAFLD patients and controls (other diseases) according to histological activity grading.

Figure 3.8 illustrates the mean LDL size for the group with no/mild fibrosis (2.4, CI: 2.1-2.6) compared with patient group with advanced fibrosis (2.6, CI: 2.1-3.1). The difference observed did not reach statistical significance (P=0.44).

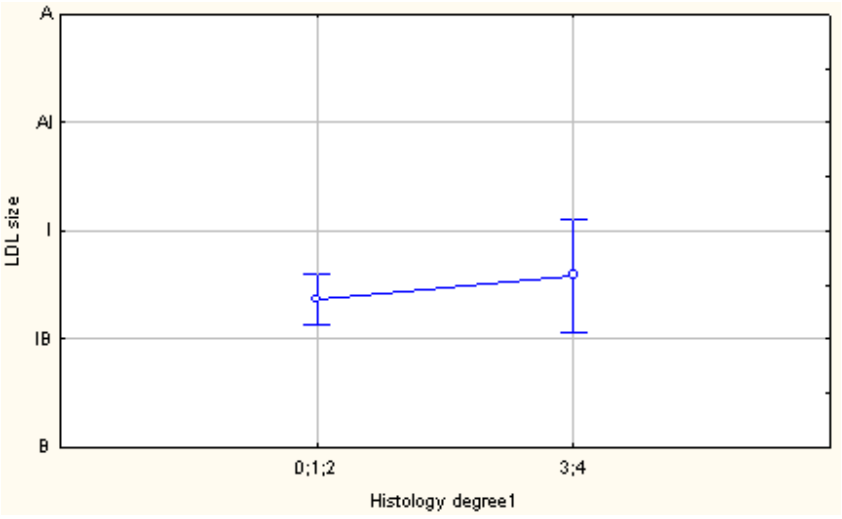



**Figure 3.8** LDL size according to fibrosis staging.

### 3.3 Assessment of APRI as a non-invasive marker for advanced liver disease

Aspartate aminotransferase to platelet ratio index (APRI) is a simple calculation of two laboratory variables, namely AST and platelets. Due to the fact that this score can easily be used at the bedside or in an outpatient setting its use as a non-invasive marker of advanced fibrosis was assessed in subjects with NAFLD. It is anticipated that by proving superior sensitivity and specificity of APRI compared to AST/ALT ratio and comparable sensitivity and specificity to NFS, APRI could be used as part of a simple, user friendly and reliable algorithm to predict advanced fibrosis in subjects with NAFLD and thereby avoiding liver biopsies for patients with no or minimal fibrosis.

The mean age of the cohort of 94 NAFLD patients included for APIR assessment was 52 years (CI: 50-54yr) with a mean BMI of 35 kg/m$^2$ (CI: 34-36). Nearly half of the patients (43%) were type II diabetics. The mean HOMA-IR of the non-diabetic patients was 7 (CI:4-9). None of the patients had decompensated liver disease, 41% had NASH and 17% had advanced fibrosis. The mean AST/ALT ratio for the patient groups illustrated in Figure 3.9. The ROC was 0.61 with an AST/ALT ratio of 0.8, with a sensitivity of 58% and specificity of 62% (Figure 3.10).

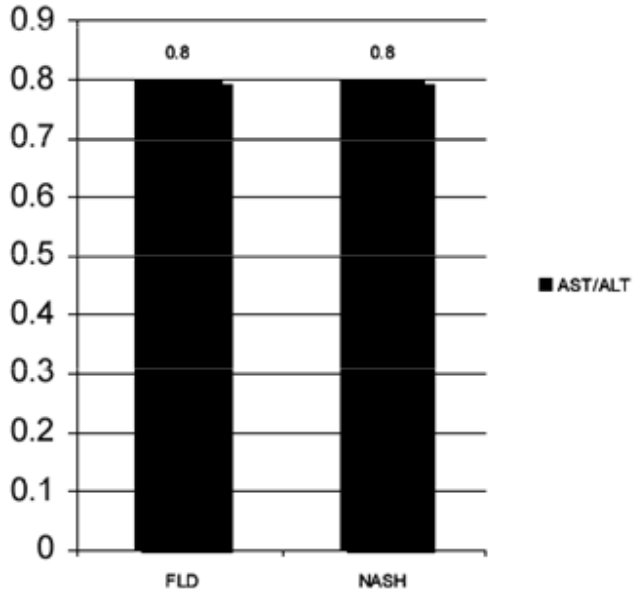



**Figure 3.9** AST/ALT ratio and association with severity of NAFLD.

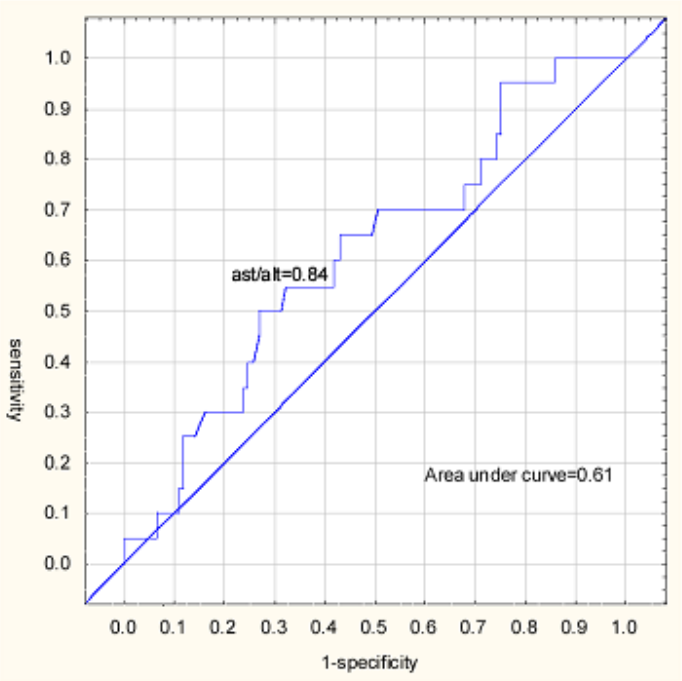



**Figure 3.10** ROC for AST/ALT ratio and advanced fibrosis in NAFLD

The mean APRI for the different patient groups is illustrated in Figure 3.11. The APRI was significantly higher in the advanced fibrosis group compared with patients with no or mild fibrosis ($P<0.01$).

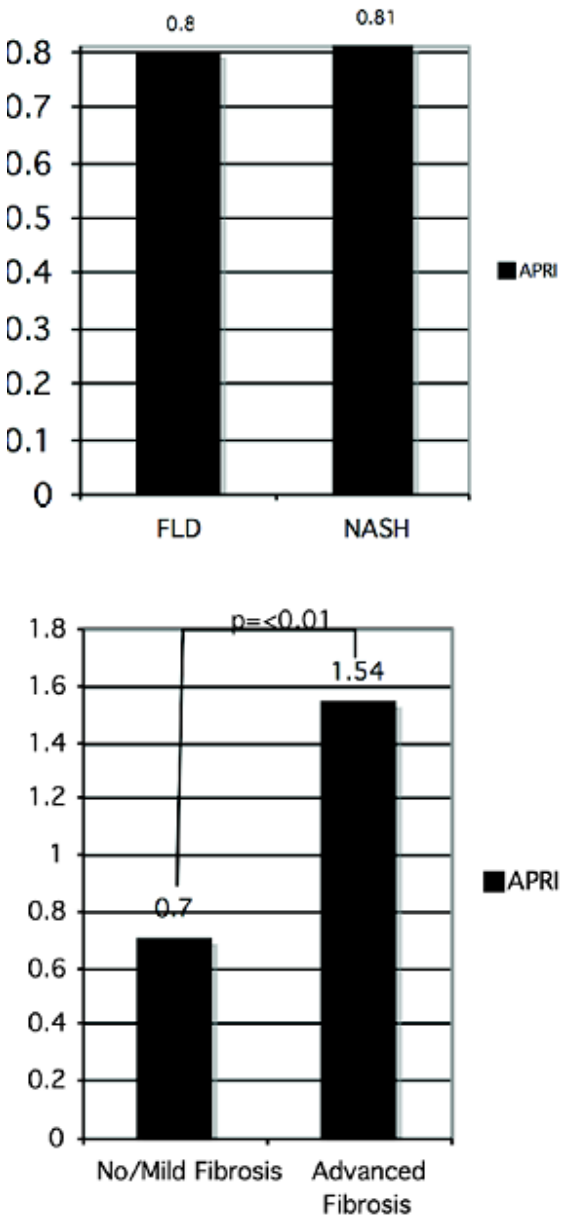



**Figure 3.11** APRI and association with staging (top) and grading (bottom) of NAFLD.

The ROC for APRI is illustrated in Figure 3.12. The ROC for APRI was 0.85 with a cut-off of 0.98 giving a sensitivity of 75% and a specificity of 86%.

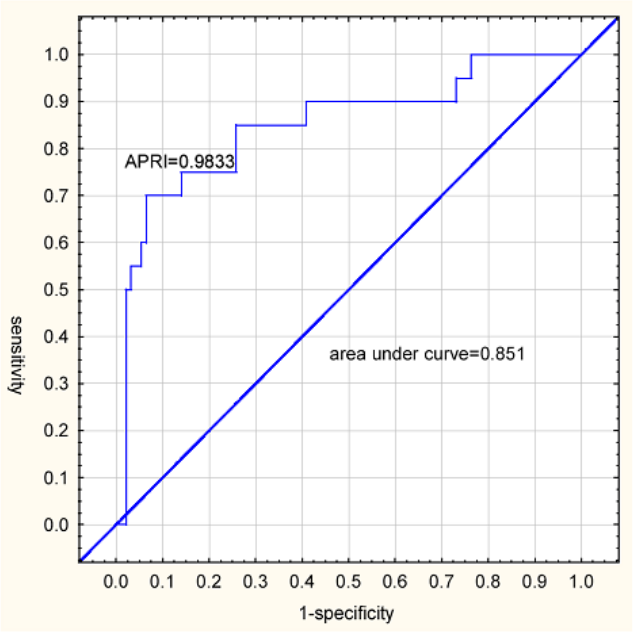
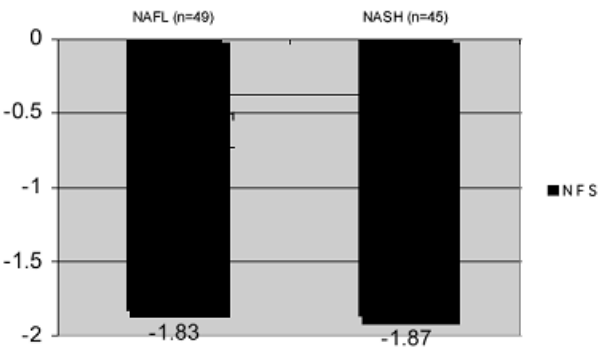



**Figure 3.12** RUC for APRI and advanced fibrosis in NAFLD.

The mean NASH fibrosis score (NFS) for the different groups is illustrated in Figure 3.13A and B, showing a significantly lower NFS in the advanced fibrosis group.

A




B

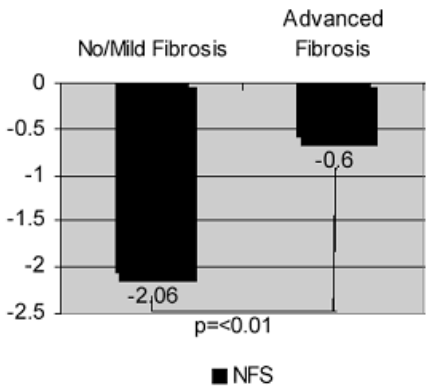



**Figure 3.13** NASH fibrosis score in relation to severity of NAFLD (A) and grading of NAFLD (B).

The ROC for NFS is illustrated in Figure 3.14. The AUC for NFS was 0.77 given at a cut-off of -1.31. The sensitivity and specificity for NFS was 76% and 69% respectively.

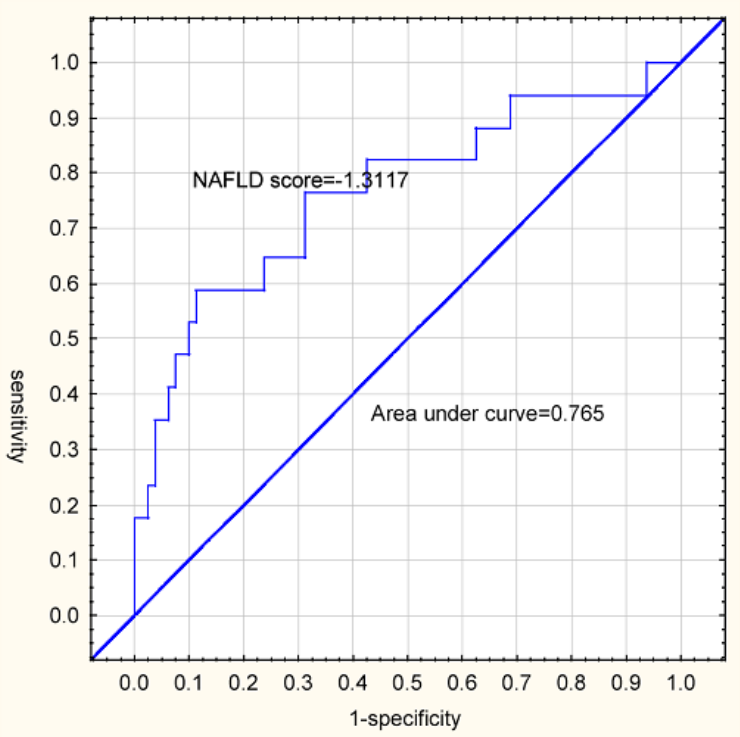



**Figure 3.14** The receiver operator curve for NASH fibrosis score and advanced fibrosis in NAFLD.

A comparison of the sensitivity, specificity, positive and negative predictive values for APRI and NFS is provided in Table 1. The positive predictive value for APRI was 54% as opposed to 34% for NFS. The negative predictive value was 93% for APRI and 94% for NFS.

**Table 3.3** Sensitivity, specificity, positive and negative predictive value of APRI and NASH fibrosis score.

| | **Value** | **Sensitivity** | **Specificity** | **PPV** | **NPV** |
|---|---|---|---|---|---|
| APRI | 0.98 | 75% | 86% | 54% | 94% |
| NFS | -1.31 | 76% | 69% | 34% | 93% |

APRI, aspartate aminotransferase to platelet ratio index; NFS, NASH fibrosis score, PPV, positive predictive value; NPV, negative predictive value

This study confirmed the usefulness of the APRI for the detection of advanced fibrosis in subjects with NAFLD and also favourably compared with NFS as a tool to predict advanced fibrosis. However, it is simpler to use APRI than NFS and also an inexpensive tool that can be

used in an outpatients setting and at the bedside. The positive predictive values of these two tests were low. As a result of these findings the algorithm shown in Figure 3.15 is proposed for the prediction and management of advanced fibrosis in NAFLD patients. This algorithm needs to be validated in future studies.

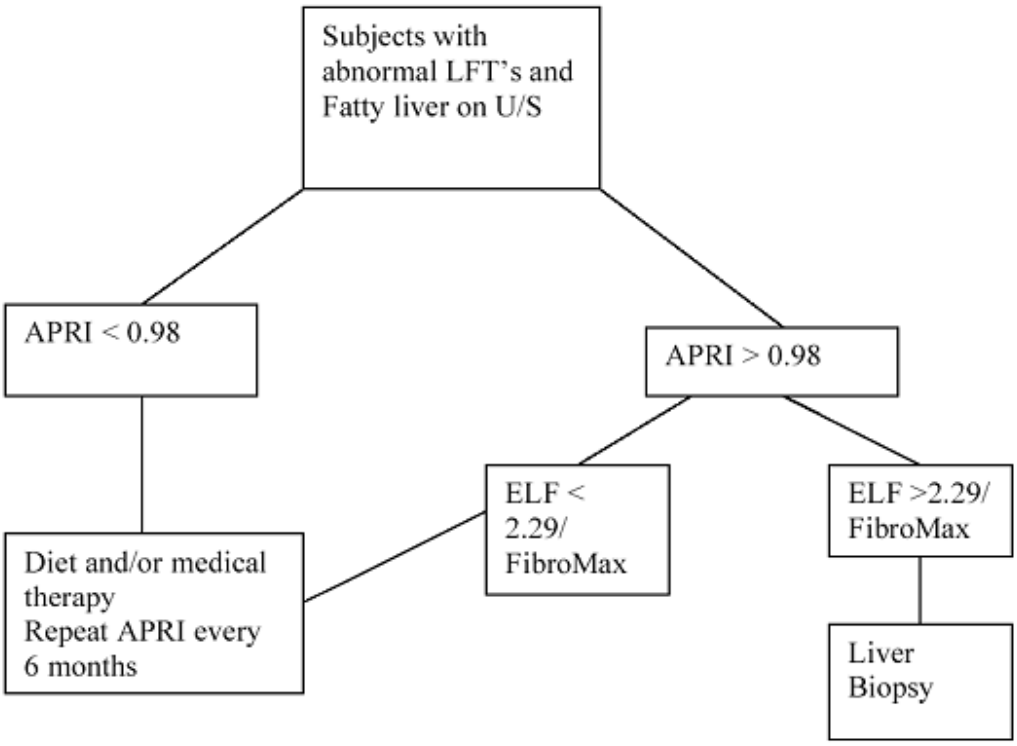



**Figure 3.15** Proposed algorithm for the prediction and management of advanced fibrosis in NAFLD patients.

### 3.4 Family history and risk of NAFLD

A role for genetic factors in NAFLD in the local population is supported by family clustering observed in two families severely affected by NAFLD. In Family 1 the indication for referral was cryptogenic cirrhosis and in Family 2 a diagnosis of haemochromatosis was indicated.

*Family 1*

In the family illustrated in Figure 3.16, the index patient (II 10) referred with cryptogenic cirrhosis also suffered from type II diabetes and dyslipidaemia. A four-vessel cardiac bypass was performed in 2000 and during this hospitalisation he was diagnosed with ascites. A liver biopsy

revealed liver cirrhosis despite a non-significant alcohol history. This patient died at the age of 69 years due to liver cirrhosis.

On taking his family history (Figure 3.16), it became evident that four of nine siblings (generation II) had been diagnosed with cryptogenic liver cirrhosis. All four brothers had central obesity while none had a significant history of excessive alcohol intake. In generation III six family members were diagnosed with insulin resistance, while the two sons of the index patient (III 15, III 17) also had NAFLD. Generation IV has two members already diagnosed with insulin resistance, the youngest being 18 years of age.

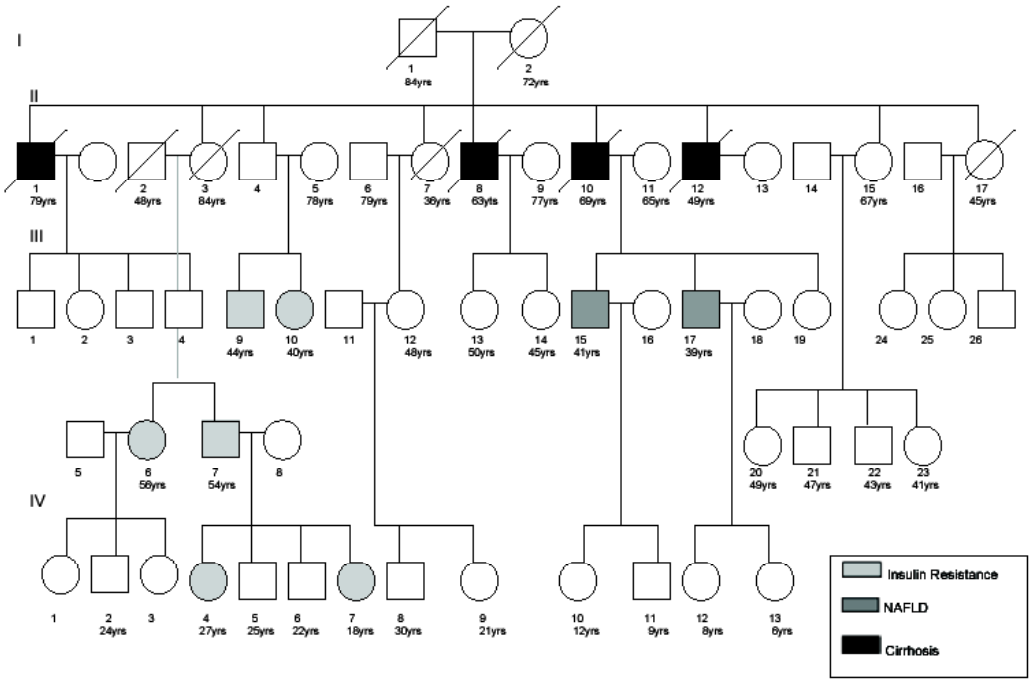



**Figure 3.16** Pedigree of a family with NAFLD with multiple affected members. The index case (II10) diagnosed with ascites and liver cirrhosis has two sons with NAFLD and three of his brothers were also diagnosed with liver cirrhosis.

*Family 2*

The index patient (II 3) aged 56 years was referred with liver cirrhosis secondary to hereditary haemochromatosis (HH). The genetic analysis of the HFE gene revealed that he was a carrier

for mutation H63D (heterozygote, +/-), which alone could not explain the high serum ferritin (768 ug/L) and transferrin saturation (96%) levels. Although these iron levels were initially ascribed to HH due to the HFE mutation and a possible second undefined mutation in the HFE or other iron-related gene, a diagnosis of HH was excluded by liver biopsy. Liver histology was indicative of NASH with cirrhosis and special staining for hepatic iron was negative. The family pedigree is shown in Figure 3.17, while the serum iron parameters for ferritin and transferrin saturation relevant to HH is provided in Table 3.4.

Documentation of the family history revealed that the sister of the index patient (II 2, 60 years) was also diagnosed with NASH and stage 2 fibrosis. Mutation analysis indicated that she is a compound heterozygote for mutations H63D and C282Y with elevated ferritin levels (514 ug/L) in the presence of normal transferrin saturation levels (27%). One of the brothers of the index case (II 5, 55 years) with NAFLD was shown to be a compound heterozygote for mutations C282Y and H63D with a similar serum iron profile (ferritin 399 ug/L, transferrin saturation 27%). Three brothers in generation III were furthermore diagnosed with NAFLD. Member III 4 is homozygous for HFE H63D mutation. As indicated in Table 3.4, his serum ferritin was 272 ug/L, which is within the normal range for males, and the transferrin saturation was also normal. His phenotype is typically that of the metabolic syndrome. All siblings of generation II have been diagnosed with type II diabetes. These findings are in accordance with the notion that HFE mutation carriers could develop NAFLD in the presence of less severe metabolic abnormalities and prompted the association study discussed in section 3.5.

The term “carrier” usually refers to unaffected individuals (as two copies of the defective HFE gene is required for development of HH). However, since one or two copies of the faulty gene may contribute to the development or severity of NFLD in the context of the current study, this term is used in Figure 3.17 to denote the presence of one or more HFE mutations.

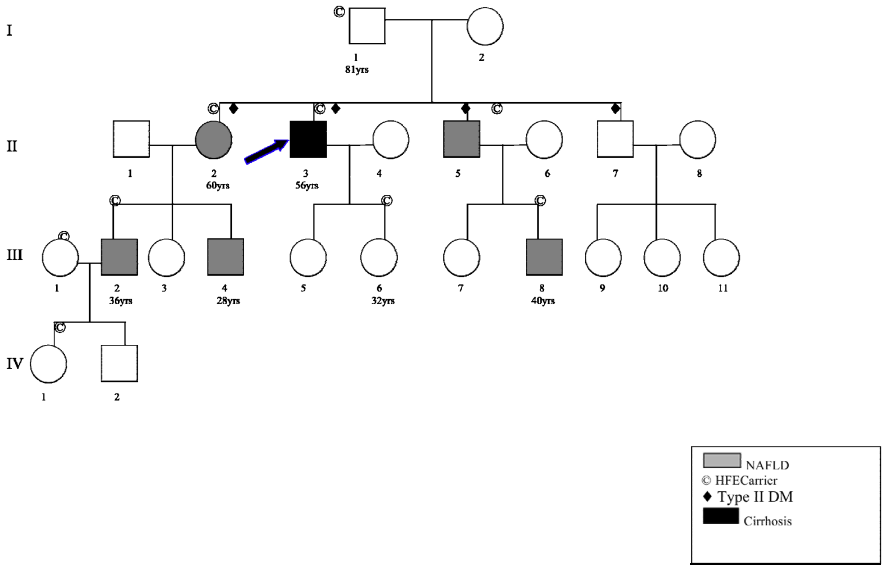



**Figure 3.17** Pedigree of a family with NAFLD with multiple affected members. The index case (II3) was heterozygous for mutation H63D in the HFE gene. HFE mutation "carriers" are indicated by small circles and indicate the presence of HFE mutations, either one copy (heterozygote), two copies (homozygote) or one copy each of the C282Y and H63D mutations (compound heterozygote).

**Table 3.4 Clinical characteristics, HFE mutation detection and serum iron status in Family 2**

| Subject | Gender | Age | NAFLD | HFE mutation(s) | Ferritin ug/L | Tf saturation |
|---|---|---|---|---|---|---|
| I-1 | Female | 81 | ND | H63D+/- | 213 | 46 |
| II-2 | Female | 60 | Yes | H63D+/-;C28Y+/- | 514 | 27 |
| II-3* | Male | 57 | Yes | H63D+/- | 768 | 96 |
| II-4 | Female | 57 | No | None | ND | ND |
| II-5 | Male | 55 | Yes | H63D+/-;C28Y+/- | 399 | 27,3 |
| III-1 | Female | 32 | No | H63D+/- | ND | ND |
| III-2 | Male | 36 | Yes | C28Y+/- | 114 | 41 |
| III-3 | Female | 29 | No | None | 11 | 17 |
| III-4 | Male | 28 | Yes | H63D+/+ | 272 | 43 |
| III-5 | Female | 35 | No | None | 27 | 23 |
| III-6 | Female | 32 | No | H63D+/- | 212 | 69 |

*Index case
Reference range for ferritin: 20-300µg/L; For Tf saturation in male: 20-55%; For Tf saturation in female: 16-50%
ND, not done

Co-existence of NAFLD and HFE gene mutation(s) is evident from the family pedigree and Table 3.4, including clinical and biochemical data from patients available for further study.

### 3.5 HFE gene mutation association study in NAFLD/NASH

The potential role of HFE gene mutations implicated in hereditary haemochromatosis (HH) in the development of advanced fibrosis has not previously been studied in unrelated South African patients with NAFLD. The clinical characteristics of the patient cohort, randomly selected to include an equal number of NAFLD patients from the Caucasian and Coloured populations of South Africa for HFE mutation screening, is summarized in Table 3.5. Mean age, gender composition, BMI, iron parameters relevant to the HH phenotype, and proportion of patients with advanced fibrosis are provided. Since these values did not differ significantly between the two ethnic groups studied, numbers were pooled for comparative analysis in NAFLD patients subdivided according to disease severity.

**Table 3.5** Clinical characteristics of NAFLD patients subjected to HFE mutation analysis (mean values are provided with standard deviation in brackets).

| | **Caucasians (n=28)** | **Mixed Ancestry (n=28)** |
|---|---|---|
| Mean age (years) | 53 (Std: 11.8) | 55 (Std: 9.6) |
| Gender (% female) | 58% | 85% |
| BMI (kg/m2) | 31 (Std: 5.2) | 35 (Std: 5.4) |
| Ferritin (ug/L) | 16 (Std: 7.0) | 15 (Std: 6.1) |
| Transferrin saturation (%) | 24 (Std: 12) | 21 (Std: 11.1) |
| Advanced fibrosis (%) | 18% | 12% |

The prevalence of the C282Y and H63D mutations (allele frequency and genotype distribution) were first determined in Caucasian and Coloured patients with NAFLD (Table 3.5), for comparison with occurrence in the general population for these two ethnic groups in South Africa. The mutation frequencies for both H63D and C282Y were similar to that previously described by de Villiers et al. (1999) in the South African population.

**Table 3.6** Comparison of genotype distribution and allele frequencies of HFE gene mutations in the Caucasian and Coloured patient groups (numbers observed are indicated with percentages in brackets).

| HFE mutations | Caucasians with NAFLD (n=28) | Caucasian controls* (n=102) | Coloured with NAFLD (n=28) | Coloured controls* (n=156) |
|---|---|---|---|---|
| **C282Y** | | | | |
| Wild type | 24 (86%) | 85 (83%) | 27 (96%) | 151 (97%) |
| Heterozygous | 4 (14%) | 15 (15%) | 1 (4%) | 4 (2.6%) |
| Homozygous | 0 | 2 (2%) | 0 | 1 (0.1%) |
| Normal G allele | 52 | 185 | 55 | 306 |
| Mutant A allele | 4 | 19 | 1 | 6 |
| **H63D** | | | | |
| Wild type | 20 (71%) | 77 (75%) | 24 (86%) | 135 (86%) |
| Heterozygous | 8 (29%) | 25 (25% | 3 (12%) | 19 (12%) |
| Homozygous | 0 | 0 | 1 (4%) | 2 (1.3%) |
| Normal C allele | 48 (86%) | 179 (88%) | 51 (91%) | 289 (93%) |
| Mutant G allele | 8 (14%) | 25 (12%) | 5 (9%) | 23 (7%) |

From de Villiers et al. (1999)

The presence of mutations H63D and C282Y were compared between NAFLD patients with no, mild and advanced fibrosis (Table 3.7). None of these comparisons showed statistically significant differences. There was also no statistically difference in mutation frequency between patients with fatty liver (36%) and NASH (30%) for the two mutations combined or individually (data not shown) .

**Table 3.7** Comparison of HFE mutation frequency in 56 NAFLD patients according to grading of fibrosis (the combined numbers of mutations observed in the different groups are indicated with percentages in brackets).

| HFE mutation | No fibrosis (n=20) | Mild fibrosis (n=18) | Advanced fibrosis (n=18) |
|---|---|---|---|
| **C282Y** | | | |
| Wild type | 0 | 0 | 0 |
| Heterozygous | 4 | 0 | 1 |
| Homozygous | 0 | 0 | 0 |
| **H63D** | | | |
| Wild type | 0 | 0 | 0 |
| Heterozygous | 3 | 3 | 5 |
| Homozygous | 0 | 0 | 1 |
| **Total mutations** | 7 (35%) | 3 (17%) | 8 (44%) |

### 3.6 Gene expression in liver tissue of patients with NAFLD/NASH

Gene expression studies were performed in 80 patients with NAFLD who provided informed consent for liver biopsy, including 48 (60%) of mixed ancestry (Coloured population), 28 Caucasians (35%) and 4 blacks (5%). m-RNA expression levels of the adiponectin, tumour necrosis factor-alpha (TNF-$\alpha$), manganese superoxide dismutase (MnSOD/SOD2), and inhibitor of Kappa light polypeptide gene enhancer in B-cells, kinase beta (IKBK$\beta$/IKK$\beta$) genes were analysed. The objective was to investigate the potential role of these genes in (1) the development from fatty liver to NASH and/or (2) the degree of disease severity as reflected by stage of fibrosis (0,1,2 vs 3,4). No statistically significant differences were observed between ethnic groups relating to the results provided below, therefore patients from different population groups were pooled for expression analysis.

Compared to mRNA expression levels in a commercially available normal liver for adiponectin (0.0364), MnSOD (0.15), TNF-$\alpha$ (<0.001) and IKBK$\beta$ (0.0368), over-expression was observed in all patient subgroups for three of the genes (Table 3.8). Only in the case of the MnSOD gene the majority of NAFLD patients showed lower expression levels compared with the normal liver. No statistically significant differences in percentage of over- or under-expression compared to the normal liver were, however, observed for any of the genes between patients with fatty liver and NASH or between patients with no/mild fibrosis and severe fibrosis. Due to ethical constrainsts, normal liver samples of an extended control group representative of the South African population were not available to further investigate these findings.

**Table 3.8** Proportion of patients within different histopathological groups with increased mRNA expression above the values obtained in a commercially available normal liver (provided under normal values) for adiponectin, MnSOD, TNF-$\alpha$ and IKBK$\beta$.

| | **Normal Values** | **FLD (n=42)** | **NASH (n=38)** | **No/mild fibrosis (n=69)** | **Severe fibrosis (n=11)** |
|---|---|---|---|---|---|
| Adiponectin | 0.0364 | 27 (64%) | 26 (68%) | 45 (65%) | 8 (73% |
| MnSOD | 0.15 | 14 (33%) | 19 (50%) | 28 (41%) | 5 (45%) |
| TNF-$\alpha$ | <0.001 | 36 (86%) | 31 (82%) | 57 (83% | 10 (90%) |
| IKK$\beta$ | 0.0368 | 40 (95%) | 36 (95%) | 65 (94%) | 11 (100%) |

Mean mRNA expression levels (expressed in log transformed values) are compared in Table 3.9. The mean mRNA expression levels provided in Table 3.9 is expressed in log values for the different patient groups. There was no difference in gene expression for adiponectin and

MnSOD. However, statistically significant differences in gene expression levels were observed between patients with fatty livers and NASH for IKBKβ (p=0.02) (Figure 3.18) and TNF-α (p=0.04) (Figure 3.19). Expression levels were significantly lower in the patients with NASH compared with the FLD group. Notably, none of the four genes studied in the South African patient cohort appears to be involved in the transition from no/mild to advanced fibrosis in NAFLD/NASH.

**Table 3.9** Comparison of m-RNA expression levels of the adiponectin, MnSOD, TNF-α and IKBKβ genes (mean values are given in log statistics) in 83 NAFLD patients subdivided into those with FLD or NASH (77 patients) and stage of fibrosis (83 patients).

| **Adiponectin gene** | | | | |
|---|---|---|---|---|
| **Disease severity** | **Number** | **Mean mRNA expression level** | **Std deviation** | **P-value** |
| FLD (types 1,2) | 42 | 0.2005 | 3.2922 | 0.93 |
| NASH (types 3,4) | 38 | 0.1337 | 3.0409 | |
| No/mild fibrosis (0,1,2) | 69 | 0.1909 | 3.2579 | 0.88 |
| Advanced fibrosis (3,4) | 11 | 0.0300 | 2.5424 | |
| **Manganese superoxide dismutase (MnSOD/SOD2)** | | | | |
| **Disease severity** | **Number** | **Mean mRNA expression level** | **Std deviation** | **P-value** |
| FLD (types 1,2) | 42 | -1.890 | 2.9199 | 0.22 |
| NASH (types 3,4) | 38 | -1.1003 | 2.7245 | |
| No/mild fibrosis (0,1,2) | 69 | -1.4689 | 2.8775 | 0.72 |
| Advanced fibrosis (3,4) | 11 | -1.8044 | 2.6939 | |
| **Tumour necrosis factor alpha (TNF-α)** | | | | |
| **Disease severity** | **Number** | **Mean mRNA expression level** | **Std deviation** | **P-value** |
| FLD (types 1,2) | 42 | 0.7909 | 2.2341 | 0.04 |
| NASH (types 3,4) | 38 | -0.1674 | 1.7617 | |
| No/mild fibrosis (0,1,2) | 69 | 0.2490 | 2.1118 | 0.35 |
| Advanced fibrosis (3,4) | 11 | 0.8794 | 1.7543 | |
| **Kappa-beta kinase gene (IKBKβ/IKKβ)** | | | | |
| **Disease severity** | **Number** | **Mean mRNA expression level** | **Std deviation** | **P-value** |
| FLD (types 1,2) | 42 | -0.0745 | 0.6904 | 0.02 |
| NASH (types 3,4) | 38 | -0.4523 | 0.6960 | |
| No/mild fibrosis (0,1,2) | 69 | -0.2774 | 0.7128 | 0.46 |
| Advanced fibrosis (3,4) | 11 | -0.1064 | 0.7408 | |

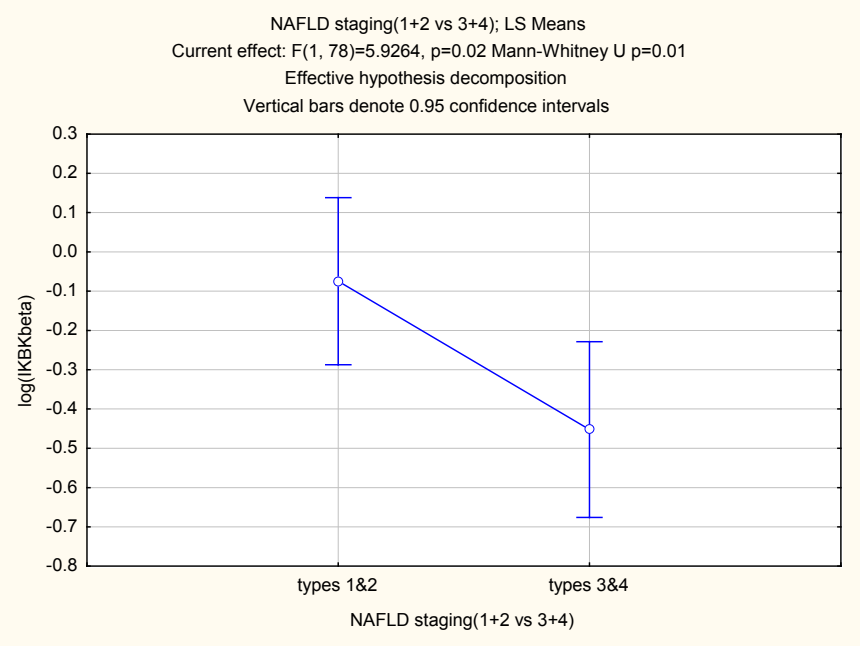



**Figure 3.18** Comparison of expression levels of the IKBKβ gene in 42 patients with FLD and 38 with NASH.

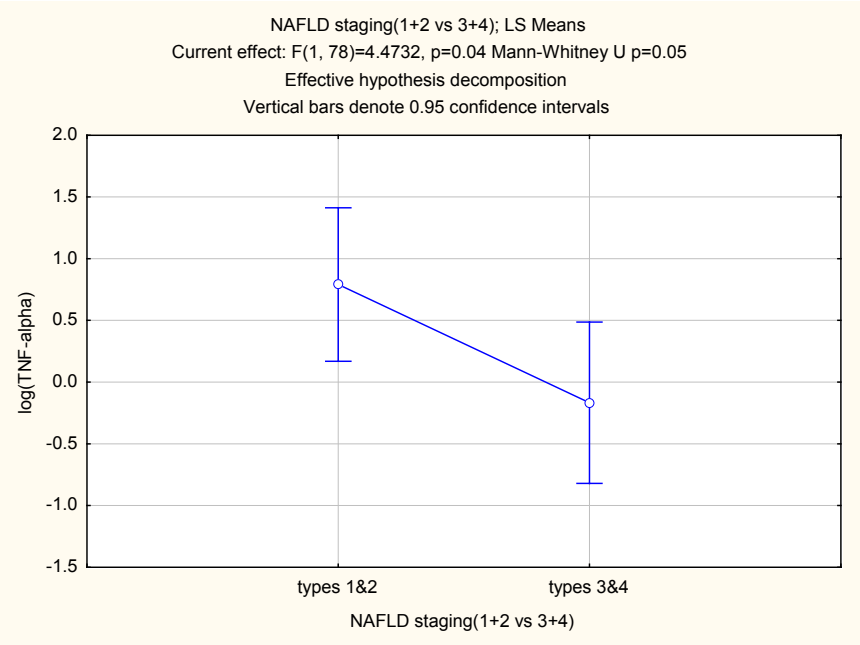



**Figure 3.19** Comparison of expression levels of the TNF-α gene in 42 patients with FLD and 38 with NASH.

# CHAPTER 4

# DISCUSSION AND CONCLUSIONS

### 4.1 Non-alcoholic fatty liver disease (NAFLD) in the South African context

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in the world today. Prevalence rates reported for different populations throughout the world ranges from 17-33%. Although the prevalence of NAFLD remains largely unknown in the South African population, there is great concern about the impact of this fast-growing disease in this part of the world. At initiation of the present study, there was virtually no awareness of the existence or clinical consequences of NAFLD in the genetically distinct populations of South Africa.

Non alcoholic steatohepatitis (NASH) is the non-benign form of the disease and is present in one third to fifty percent of cases. The estimated prevalence is 5.7-17% and approximately 20% of patients with NASH will develop cirrhosis over 8 years. Patients with liver cirrhosis secondary to NASH are also at risk to develop hepatocellular carcinoma. To date, there are no published data on demographics, clinical characteristics, and genetic studies performed in South African patients with NAFLD/NASH.

The objective of this study therefore was to describe the clinical and biochemical phenotype of NAFLD/NASH in South African patients of the Western Cape and to assess the possible involvement of candidate genes in disease progression and clinical outcome.

### 4.2 Clinical characteristics and metabolic derangements in NAFLD/NASH

The disease profile of NAFLD/NASH is described for the first time in South African patients of the Western Cape region. The majority of the inhabitants of this geographic area are Cape Coloured individuals of mixed ancestry. This ethnic group also represented the majority (69%) of the initial study cohort including 233 patients. For the rest of the population groups studied 25 % were Caucasian, 5% black (African) and 1 % Indian.

In the USA ethnicity was shown to be an independent risk factor for advanced disease. According to these reports, African Americans appear to be protected against advanced disease. Our findings suggest the same, as the incidence of obesity and type II diabetes is high in the South African black population. However, this ethnic group was underrepresented in the study and warrants further studies to draw comparisons with international studies. The Coloured population of the Western Cape is the predominant ethic group in this geographical area and

also represented the majority of patients in the study cohort. This population of mixed ancestry is also severely affected by insulin resistance and the metabolic syndrome. The phenotypical presentation of NAFLD in the Cape Coloured patient cohort was similar to the Caucasian subjects included in the study.

The clinical and biochemical characteristics of the study population were described in terms of histological features and presence of fibrosis in relation to gender, age, ethnicity, body mass index (BMI), insulin resistance (HOMA-IR) and type II diabetes. Almost all patients with NAFLD are overweight or obese, a feature that is also characteristic of the patients enrolled in the present study. The distribution of fat in the body is more important than total fat mass and visceral fat rather than total fat has been associated with hepatic steatosis as well as peripheral and hepatic insulin resistance. Of 111 South African patients with liver biopsy-confirmed NAFLD included in the study, 37% had NASH and 17% had advanced fibrosis (stage 3 and 4). These findings are similar to observations reported elsewhere in the world. The majority of the patients in the study were female (73%). Gender was not associated with the severity of NAFLD in the South African patients studied.

Insulin resistance is the common pathogenic factor in the majority of patients with NAFLD and is regarded the main factor in the pathogenesis of this condition. Insulin resistance is also associated with severe inflammation and more extensive fibrosis. Diabetes is responsible for more severe disease and is an independent risk factor for hepatic fibrosis. The South African patients not diagnosed with type II diabetes had a markedly increased mean HOMA-IR. This finding confirmed the pathogenic role of insulin resistance in subjects with NAFLD. Although insulin resistance was not predictive of NASH in the present study, there was a strong tendency towards predicting advanced fibrosis. There was a statistically significantly difference in the mean serum triglycerides and total cholesterol levels when patients with fatty liver were compared with NASH, but these levels did not differ significantly between patients with no/mild fibrosis and those with advanced fibrosis. These findings implicated dyslipidemia in the pathogenesis of NASH, while apparently unrelated to the risk of advanced fibrosis.

Even though all the patients in the study were either overweight or obese, the presence of marked obesity did not predict the presence of more advanced liver disease. These findings suggest that obesity and dyslipidemia associated with cardiovascular disease (CVD) risk are important factors in the progression from fatty liver to NASH, while secondary hits involving

gene-environment interactions may be required to develop advanced fibrosis, with insulin resistance as a universal factor.

In light of the above-mentioned findings in the local population, it was realised that in parallel to the candidate gene approach designed for this study, extended analysis is required of biochemical markers that may distinguish more specifically between NAFLD patients at increased risk of CVD versus severe liver disease. Identification of metabolic risk areas beyond current knowledge and assessment of genes relevant to these processes (through literature study and/or laboratory analysis), may lead to avoidance of liver biopsy in a greater proportion of patients through development of individualised risk management programs. The potential application of LDL particle size determination, aspartate aminotransferase to platelet ratio index (APRI), and genetic variation related to iron metabolism, inflammation, insulin resistance and fatty acid oxidation, was investigated towards this goal in the following two overlapping patient groups:

1) Patients with fatty liver versus NASH (increased risk of CVD)
2) Patients with no/mild versus severe fibrosis (increased risk of severe liver disease)

Ultimately, the development of a pathology supported gene-based intervention program would improve clinical management of patients with NAFLD/NASH and metabolic syndrome, based on the information obtained in this study integrated with existing knowledge in the field.

### 4.3 Assessment of LDL particle size as a biomarker for cardiovascular risk

NAFLD is considered to be an important risk factor for the development of CVD due to a complex interaction between genetic and environmental risk factors. Recent studies have shown that fatty liver on ultrasonography is a marker for severe CVD complications. From clinical experience, fatty liver disease is detected on routine ultrasonography before patients are diagnosed with insulin resistance or type T2DM. Therefore, fatty liver has become a marker for insulin resistance and the development of T2DM.

Small dense LDL is a feature of insulin resistance and is associated with an increased CVD risk. This association was investigated in the context of South African patients with NAFLD, by comparison of mean LDL particle size between patients with fatty liver and NASH, in relation to control individuals made up of patients with normal liver tissue or diseases other than

NAFLD/NASH. LDL particle size was also compared between patients with no/mild fibrosis and advanced disease. LDL particle size decreased significantly from the control group to NASH. The opposite effect was observed from no/mild fibrosis to advanced fibrosis in the South African study cohort.

These results support the concept of an increased risk of coronary artery disease associated with NAFLD, especially in patients with NASH, while apparently unrelated to development of severe liver disease. It seems likely that fat metabolism and export in the liver may be under genetic control in patients with NASH, as the predominance of small dense LDL is characteristic of a genetically influenced lipoprotein phenotype. Consideration of the role of genetic determinants of LDL particle size, such as apolipoprotein E (ApoE) genotype, may therefore be of particular relevance in this context. Most studies reported smaller LDL particle size in individuals with the ApoE2 allele compared with larger LDL particles in ApoE4 carriers, however this effect appears to be diet related (Moreno et al. 2004).

Lipid and lipoprotein response to diet varies considerably between individuals. ApoE is a major genetic determinant of LDL particle size that is largely dependent on environmental factors such as dietary fat composition. In healthy subjects a Mediterranean diet enriched in monounsaturated fatty acids (MUFA) was shown to increase LDL size in comparison with a low-fat, high-carbohydrate (CHO) diet (Moreno et al. 2004). However, this effect was dependent of the ApoE genotype. Individuals with the ApoE3/4 genotype displayed a significant increase in LDL particle size on the low-fat CHO diet, while a significant decrease was observed in individuals with the E3/3 genotype. Replacement of the CHO diet by a MUFA diet increased LDL particle size in ApoE3/3 individuals, while it decreased in ApoE3/4 individuals. Based on these findings the authors concluded that an individualised approach is required when dietary recommendations are made as apposed to current generilisation without knowing more about those being targeted. A practical application of this knowledge could be to replace a diet high in saturated fat, known to contribute to the development of CVD, with a CHO diet in ApoE4 carriers and with a MUFA diet in patients with the ApoE3/3 and E2/3 genotypes.

In light of the significance of LDL particle size as a risk factor for CVD in NAFLD patients, ApoE genotyping may provide a valuable adjunct to patient management with specific relevance to dietary intervention. The ApoE gene functions in a relatively uniform way in all populations, despite differences in genetic background, lifestyle and nutrition patterns (Hallman et al. 1991).

The Apo E4 allele was shown to be associated with significantly increased serum cholesterol levels in the local population (Kotze et al. 1993) and occurs at a frequency of 30-40% across ethnic groups. In a meta-analysis performed by Song et al. (2004) it was shown that carriers of the Apo E4 allele had a 42% higher risk for coronary heart disease compared with individuals with the neutral E3/3 genotype. Weight control is particularly important in carriers of the ApoE4 allele due to its effect on fasting insulin and glucose levels (Elosua et al. 2003). Based on these findings, the functional ApoE polymorphism was included in a multi-gene CVD assay applied for genetic testing of patients at increased risk of CVD and related disorders (Kotze and Thiart 2003; Kotze et al. 2006). Due to the findings relating to dyslipidaemia, obesity and insulin resistance in the South African study cohort, a diagnosis of NAFLD or metabolic syndrome is regarded an important indication for referral of chronic disease risk management using the multi-gene CVD assay, which is performed in conjunction with a medical and lifestyle/nutrition assessment (Kotze and Badenhorst 2005).

**4.4 Assessment of APRI as a non-invasive biomarker for severe liver disease**

The assessment of patients with NAFLD/NASH for advanced disease by liver biopsy is currently regarded as the gold standard (Bianchi 2001). However, liver biopsy is an invasive procedure with potential complications. Sampling error of liver biopsy can furthermore result in substantial misdiagnosis and staging inaccuracies. The major concern regarding liver biopsies is the lack of resources mainly due to the large number of subjects affected by NAFLD. Approximately 60-90 % of the NAFLD affected subjects will have a benign form of the disease not requiring biopsy. Improved methods are therefore required to identify patients at increased risk of severe liver disease without the need to perform a liver biopsy in all patients.

Due to the increased need for non-invasive tests to diagnose advanced liver disease numerous test panels have been developed. Only four of these panels have been evaluated in NAFLD (Wieckowska et al.2007). The group that investigated the BAAT score replaced this test by the Fibro Test. The FibroTest combines five biochemical markers, namely β2-macroglobulin, apolipoproteien A1, haptoglobulin, total bilirubin and GGT. Age and sex, together with the aforementioned markers, are entered into a computer program using an undisclosed formula. The AUC for the Fibro Test as predictive of advanced fibrosis is 0.87. Unfortunately, the Fibro Test is fairly expensive and not widely available. The European Liver Fibrosis Study Group recently examined a panel of ECM-related components from which an algorithm was developed

with an AUC for severe fibrosis of 0.8718. This panel is also not readily available and will be fairly expensive, especially in developing countries. The NASH fibrosis score (NFS) is an algorithm of six readily available laboratory and clinical variables including age, hyperglycemia, BMI, platelet count, albumin, and AST/ALT ratio (Angulo et al. 2007). By applying this model almost 75% of the 733 patients in this study could have avoided liver biopsy. Guha et al. (2008) determined in their study that the addition of established simple markers to the ELF panel augmented the diagnostic performance (Guha et al. 2008). Wai et al. (2003) validated the APRI score in patients with Hepatitis C. APRI is a simple calculation of two laboratory variables, namely AST and platelets. This score can easily be used at the bedside or in an outpatient setting.

In the present study we attempted to validate APRI as a non-invasive marker of advanced fibrosis in subjects with NAFLD. By proving superior sensitivity and specificity of APRI compared to AST/ALT ratio and comparable sensitivity and specificity to NFS, the use of APRI is proposed as part of a simple, user friendly and reliable algorithm to predict advanced fibrosis in subjects with NAFLD and thereby avoiding liver biopsies for patients with no or minimal fibrosis. The present study confirmed that ALT could neither differentiate between the stage of disease nor the grade of fibrosis. An AST/ALT ratio of more than 0.8 is one of the indicators of advanced disease. In the South African study cohort there was a strong tendency towards subjects with advanced fibrosis having a higher ratio. However, the ROC curve for AST/ALT ratio and advanced fibrosis was only 0.62, indicating that the positive and negative predictive values were too low to make it a very useful tool. APRI is a simple and inexpensive calculation making use of the AST value and platelet count. This formula has been validated in patients with hepatitis C but not in patients with NAFLD. This study showed that the APRI for South African patients with advanced fibrosis differed significantly from the other patients with less severe disease. The ROC curve for an APRI of 0.98 and detection of advanced fibrosis was 0.85, with positive and negative predictive values of 54% and 94% respectively.

Based on the above-mentioned findings, APRI can be considered statistically superior to AST/ALT ratio for the prediction of advanced fibrosis and has now been validated for use in patients with NAFLD for the first time. The NFS was validated by Angulo et al. (2007) for use in patients with NAFLD. In this study we had similar results by showing that subjects with advanced fibrosis had a NFS significantly different from those subjects without advanced fibrosis. The ROC curve for the NFS of -1.31 and prediction of advanced fibrosis was 0.765.

The positive and negative predictive values were 34% and 93 % respectively.

This study confirmed the usefulness of the APRI for the detection of advanced fibrosis in subjects with NAFLD and also favourably compared with NFS as a tool to predict advanced fibrosis. It is, however, easier to use APRI than NFS and it is also an inexpensive tool that can be used in an outpatient setting and at the bedside. The positive predictive values of these two tests were low. According to a recent article by Guha et al. (2008), the addition of the ELF panel to the NFS increased the positive predictive value for advanced fibrosis. Therefore, an algorithm was proposed for application in general practice (see results section).

**4.5 Familial clustering and genetic predisposition for haemochromatosis**

Interethnic variation provides evidence to support genetic susceptibility towards NAFLD. In this respect, it is noteworthy that the most common mutations causing hereditary haemochromatosis (HH) in the Caucasian population is virtually absent in the South African Black population (de Villiers et al. 1999), where HH is virtually absent. African Iron Overload related to mutations in the ferroportin gene and brewing of beer in iron containers, on the other hand, is a known cause of hemosiderosis in the Black population.

HH is characterized by increased iron deposition in different organs of which iron deposition in the liver leads to liver cirrhosis and hepatocellular carcinoma. In this study, the HFE gene was selected as one of the candidate genes studied in the context of NAFLD/NASH due to its role in oxidative stress. The significance of genetic factors in NAFLD in the local population is supported by family clustering observed in two Caucasians families severely affected by NAFLD/NASH. In one of these families co-inheritance of NAFLD and HFE gene mutations were observed in all affected patients, implying that the presence of a deleterious mutation in the HFE gene (single copy or two copies of the faulty genes) contributed to disease development or severity in this South African family.

Further investigation of HFE mutation status in 56 unrelated NAFLD patients demonstrated similar genotype distribution and allele frequencies for mutations C28Y and H63D compared with the general population, as previously reported in the South African Caucasian and Coloured population groups (de Villiers et al. 1999). Mutations frequencies were also determined in NAFLD patients grouped according to the grade of fibrosis on their liver histology.

Although these groups were matched in terms of mean age, BMI, serum iron ferritin and percentage transferrin saturation, there were no significant difference in mutation frequency (single or combined) between the groups with no, mild and advanced fibrosis. Mutation frequencies did also not differ between patients with fatty liver and NASH.

These above-mentioned findings reflect the difficulties in implicating HFE gene mutations in the pathogenesis of advanced fibrosis for patients with NAFLD, especially when the sample size is relatively small. Association does not necessarily equate to causality and is largely dependent on presence or absence of other known risk factors and genetic background of the population.

Several studies have implicated heterozygous HFE mutations in the exacerbation of chronic liver disease to progress to liver cirrhosis. This phenomenon can possibly be explained by the concept that excessive iron accumulation in the liver leads to increased production of reactive oxygen species (ROS). In a liver already compromised by another disease, the increased production of ROS can cause further insult and therefore more severe disease. Valenti et al. (2003) provided convincing evidence that carriers of the C282Y mutation may develop NAFLD in the presence of less severe metabolic abnormalities. In the presence of increased serum iron the release of insulin is decreased, thereby providing a mechanism for increased susceptibility to NAFLD.

Based on the findings in this study, it seems highly likely that carrier status or inheritance of two copies of a faulty HFE gene could contribute to disease development or severity in South African NAFLD patients, although not in all families. The combined effects of different genetic and environmental risk factors could explain the disease phenotype, but the factors involved could differ between families. In the South African family with multiple affected cases it seems likely that HFE gene mutations may underlie the familial clustering of NAFLD due to its role in oxidative stress and risk of diabetes, despite normal iron parameters in the majority of cases. Co-existence of NAFLD and HFE mutations was demonstrated in all affected family members subjected to mutation analysis. Mutation-positive family members without NAFLD most likely lack other risk factors required for disease development (including age effects) in the presence of one or more HFE gene mutations. Knowledge of a genetic predisposition for HH in NAFLD patients would nevertheless affect clinical management, as iron levels needs to monitored on a regular basis and phlebotomy treatment implemented to keep levels within the normal range, if necessary.

HH provides a classical example of how advances in molecular technology have led to the replacement of liver biopsy as the diagnostic method of choice for this low-penetrance genetic disease. Identification of the causative HFE gene in 1996 by Feder and co-workers paved the way to universal use of DNA testing in conjunction with serum iron status to make a diagnosis of HH, without the need for an invasive liver biopsy. Today, liver biopsy is only performed in molecularly uncharacterised patients with the HH phenotype.

### 4.6 Expression analysis of candidate genes implicated in insulin resistance and fatty acid oxidation

There is convincing evidence that genetic factors account for considerable variability in the natural history of NAFLD. Family clustering studies have shown that about one fifth of patients with NASH have a similarly affected first-degree relative (Wilner et al. 2001). In a study by Struben et al. (2000), coexistence of NASH and cryptogenic cirrhosis was observed in seven out of eight families studied. In the present study, two Caucasian families were identified with family clustering of NAFLD/NASH. HFE gene mutations H63D and C282Y appear to contribute to disease severity in the family discussed above, while the other family demonstrated insulin resistance and dyslipidaemia as a universal finding in affected cases. This family was not only affected by liver disease but also by coronary artery disease. Increased risk of CVD correlates with the findings in the South African study cohort that demonstrated insulin resistance by means of HOMA-IR values and presence of diabetes as well as an increased risk for CVD through the presence of small dense LDL in their peripheral blood.

Insulin resistance has been implicated as a universal factor in the pathogenesis of NAFLD, with fatty acid oxidation as an important contributor to disease development and progression. However, the role of genetic risk factors in these processes has not previously been studied in South African patients with NAFLD. In this study, expression levels of the adiponectin, TNF-$\alpha$, MnSOD and IKK$\beta$ genes were determined in 80 NAFLD patients who provided informed consent for liver biopsy. Very limited information on expression levels for these genes are available in the literature.

Compared to mRNA expression levels in a commercially available normal liver for these four genes, over-expression was observed in all patient subgroups except for the MnSOD gene

where the majority of NAFLD patients showed lower expression levels compared with the normal liver. Unfortunately, normal liver samples of an extended control group were not available to further investigate these findings. No statistically significant differences in percentage of over- or under-expression compared to the normal liver were, however, observed for any of the genes between patients with fatty liver and NASH or between patients with no/mild fibrosis and severe fibrosis.

Statistically significant differential expression of these TNF-$\alpha$ and IKK$\beta$ genes was observed in patients with NASH as opposed to subjects with fatty liver disease. No differences in mRNA expression levels were observed between patients with no/mild fibrosis and those with advanced fibrosis. Racial differences did not show a significant effect on gene expression levels for the four genes studied.

The gene expression patterns observed for TNF-$\alpha$ and IKK$\beta$ South African patients confirmed the genetic link with insulin resistance and inflammation in the development of NASH. Association with hepatic insulin resistance could possibly explain the connection between insulin resistance and the liver's role in the development of coronary artery disease. Increased TNF-$\alpha$ levels favour the development of insulin resistance and impaired glucose tolerance. The IKK$\beta$ pathway is crucial for cytokine induced insulin resistance. These actions are mediated via NF-$\kappa\beta$ activation (Yuan et al. 2001). Kaser et al. (2005) reported decreased expression of adiponectin and AdipoR2 in serum of patients with NASH compared with patients with simple steatosis. Uribe et al. reported significantly higher mRNA adiponectin levels in patients with NASH compared wit normal livers. In this present study adiponectin was also overexpressed compared with the normal liver sample analysed in the majority of patients with NAFLD, but showed similar expression levels patterns across the groups subdivided according to disease stage and severity. Hui et al. (2004) has shown that reduced serum adiponectin levels may be related to extensive necroinflammation and may therefore contribute to the development of necroinflammation in NAFLD.

Adipocytes secrete various proteins called adipocytokines. These adipocytokines have a profound effect on insulin sensitivity. Adiponectin is the predominant protein synthesized from adipose tissue. Adiponectin has anti-atherogenic, anti-inflammatory and insulin sensitizing properties. Plasma levels of adiponectin are markedly reduced in visceral obesity and states of insulin resistance such as NASH, T2DM diabetes and atherosclerosis (Hui et al. 2004; Kubota

et al. 2002; Kumada et al. 2003). Adiponectin induces its anti-inflammatory properties through induction of other mediators such as IL-10 and IL-1 receptor antagonist in various cell types and suppression of IL-6 and interferon γ (Kumada et al. 2004; Wulster-Radcliffe et al. 2004; Wolf et al. 2004). Adiponectin also counteracts pro-inflammatory cytokines such as TNF-α. Recently adiponectin receptors have been cloned namely AdipoR1 and AdipoR2. AdipoR1 is primarily expressed in adipose tissue and has a wide distribution throughout the body. AdipoR2 is primarily expressed in liver tissue (Yamauchi et al. 2003). T-cadherin has also recently been discovered and is also a receptor for adiponectin with wider distribution (Hug et al. 2004). In serum samples of patients with NASH, the expression levels of adiponectin and AdipoR2 are significantly decreased compared to patients with simple steatosis (Kaser et al. 2005).

TNF-α plays a major role in the pathogenesis of NAFLD, as increased TNF-α levels favor the development of insulin resistance and impaired glucose tolerance. TNF-α may be produced by Kupffer cells in response to gut derived endotoxin, but also by hepatocytes in response to an increased supply of free fatty acids (FFA), or by adipose tissue macrophages (Day 2006; Farrell et al. 2006). A promoter polymorphism of the TNF-α gene associated with increased cytokine expression was suggested to affect susceptibility towards NAFLD and to be associated with higher insulin resistance indices and a higher prevalence of impaired glucose tolerance. The prevalence of the -238 TNF-α polymorphism was significantly higher in subjects with nonalcoholic fatty liver than in controls (31% vs. 15%), and patients positive for the -238 and -308 TNF-α polymorphisms had higher insulin resistance indices, a higher prevalence of impaired glucose tolerance, and a lower number of associated risk factors for steatosis (Valenti et al 2002). A meta-analysis performed by Sookoian et al. (2005) furthermore indicated that individuals with the TNF-α -308A allele have a 23% increased risk of developing obesity compared with controls and showed significantly higher systolic arterial blood pressure and plasma insulin levels.

TNF-α induces insulin resistance by down regulation of the insulin receptor through both the JNK and IKKβ pathways (Tilg and Hotamisligil 2006). The IKKβ pathway is crucial for cytokine induced insulin resistance. These actions are mediated via NF-κβ activation (Yuan et al. 2001). In two studies in mice the relationship between IKKβ expression in the liver was associated with insulin resistance (Cai et al. 2005; Arkan et al. 2005). In the first study chronic subacute inflammation in the liver was created by selective hepatocellular activation of NF-κβ leading to

low level expression of IKKβ and a diabetic phenotype mice with moderate systemic insulin resistance (Cai et al. 2005). In the second study liver specific deletion of IKKβ in mice on a high fat diet or inter-crossed with the *ob/ob* model of genetic obesity resulted in insulin sensitivity in the liver and insulin resistance in muscle and fat tissue (Arkan et al. 2005).

Superoxide dismutase catalyzes the conversion of two molecules of superoxide anion, a highly unstable ROS, into hydrogen peroxide and molecular oxygen, a more stable ROS that is neutralized by the action of catalase. Eukariotes contain two forms of superoxide dismutase, a copper–zinc dependent cytosolic form and a manganese-containing version located in mitochondria, critical organelles for the production of ROS within the cell (Pessayre et al. 2002). A functional polymorphism (T to C) in the first exon of the MnSOD gene determines the substitution of valine with alanine in the mitochondrial targeting region of the enzyme. The resulting structural change brought about by the presence of alanine is associated with an increased localization of the active MnSOD within the mitochondrial matrix, as elegantly confirmed in a recent study (Sutton et al. 2003). This localization pattern may increase the ability of MnSOD to process superoxide anion produced in the mitochondria. In the study by Namikawa et al. (2004) a statistically significant difference in allele frequencies of the MnSOD polymorphism was observed between NASH patients and the control group. This group of patients had an increased frequency of genotypes predisposing not only to fatty liver, but also to oxidative stress, one of the factors believed to be involved in the causation of more severe injury and the development of NASH (Namikawa et al. 2004). Failure to detect a significant difference in mRNA expression levels for MnSOD between South African patients subdivided according to severity of liver disease warrants further studies to assess the role of individual functional polymorphisms in the gene.

### 4.7 Conclusions

The description of the phenotypic features of NAFLD/NASH in the local population, in conjunction with genetic alterations associated with disease-related metabolic pathways that may interact with diet and other environmental factors, has greatly enhanced our understanding of the disease pathogenesis and, accordingly, the ability to design an effective management strategy and tailored therapies. Determination of LDL particle size and APRI in NAFLD patients provided valuable information to distinguish between South African patients at higher risk of CVD as opposed to advanced liver disease.

The important role of obesity and dyslipidemia in the progression from fatty liver to NASH were confirmed in the local population, with insulin resistance as a universal factor relating to both CVD risk and development of advanced liver disease. Analysis of candidate genes implicated in insulin resistance and inflammation, TNF-$\alpha$ and IKK$\beta$, provided additional support for the important role of these biological processes in progression from fatty liver to NASH. These findings paved the way for an integrated approach of pathology supported gene-based intervention, which may in future include the analysis of functional polymorphisms in genes implicated in relevant metabolic risk areas.

**4.8 Future directions**

Family studies and inter-ethnic variations in susceptibility suggest that genetic factors may be important in determining disease risk. Although no genetic associations with advanced NAFLD have been replicated in large studies, preliminary data suggest that polymorphisms in the genes encoding microsomal triglyceride transfer protein, phosphatidylethanolamine, superoxide dismutase 2, the CD14 endotoxin receptor, TNF-$\alpha$, TGF$\beta$ and angiotensinogen may be associated with an increased risk of steatohepatitis and/or fibrosis (De Alwis and Day 2008). With the advent of high throughput gene analyses and the reduced cost of whole genome wide scans it seems likely that genes contributing to inherited susceptibility to this common disease will be identified and implemented as part of clinical management in the near future.

We know today that patients with NAFLD are not only at risk of developing advanced fibrosis, liver cirrhosis with decompensation, and hepatocellular carcinoma, but they also have a high likelihood of developing CVD and cancers associated with the metabolic syndrome. In a community based study performed in Olmsted County, Minnesota, NAFLD patients followed up for 16 years were found to have liver disease as the third leading cause of death with cancer and CVD in the first and second place (Adams et al. 2005). NAFLD has been confirmed as an independent risk factor for cardiovascular morbidity. Two studies showed that ALT predicts an increased risk for cardiovascular events (Ioannou et al. 2005; Schindheim et al. 2007). There is also evidence to show that NAFLD is independently associated with endothelial dysfunction, carotid atherosclerosis, coronary artery disease and increased risk of cardiovascular events (Targher et al. 2007; Mirbagheri et al. 2007; Hamaguchi et al. 2007). In another study, NAFLD was associated with cardiac fat accumulation and abnormal cardiac energy metabolism

(Perseghini et al. 2008). It is postulated that hepatic steatosis leads to hepatic insulin resistance that contributes to systemic inflammation. Coupled with systemic insulin resistance and dyslipidemia, this contributes to cardiovascular morbidity and mortality. In a study by Peterson et al. (2005) improvement in fatty liver leads to improvement of global insulin resistance.

Patients with fatty liver on ultrasound and/or asymptomatic abnormal liver functions are often referred for specialist investigation. Fatty liver on ultrasound is not only a liver disease, but a marker for the metabolic syndrome. It is therefore important to know when liver biopsies need to be performed on these patients and which patients are at increased risk for CVD and cancer. Incorporation of genetic testing in conjunction with assessment of metabolic indicators and lifestyle risk factors may lead to improved patient management in this context. APRI was shown to be a simple, user-friendly tool that is highly sensitive to detect advanced fibrosis in patients with NAFLD. On the other hand, LDL particle size highlighted the importance of dyslipdaemia in the progression from FLD to NASH. As a result of this study, an algorithm has been formulated to guide clinicians as to the ideal time to perform a liver biopsy in patients with NAFLD. It proposes to 1) identify the range of metabolic and genetic risk factors that could cause or contribute to disease development or progression, and (2) intervene effectively at an individual basis to prevent cumulative effects that may result in CVD and/or advanced liver disease. The clinical utility of this algorithm illustrated in figure 4.1 needs to be validated in future studies.

In light of the fact that CVD and cancer are the leading causes of death in patients with NAFLD, the incorporation of genetic screening for more directed management of the metabolic syndrome is warranted. It is proposed that the multi-gene CVD assay developed in South Africa (Kotze et al. 2003, Kotze and Thiart 2003) be applied in risk management of NAFLD patients, possibly after including additional clinically-useful polymorphisms in genes such as TNF-$\alpha$ implicated in NAFLD/NASH (De Alwis and Day 2008) as part of a comprehensive dysmetabolic genescreen. It is important to note that the current CVD assay includes analysis of the most common haemochromatosis mutation, C282Y, due to its link with premature cardiovascular death (Tuomainen et al. 1999; Roest et al. 1999). Knowledge of increased risk of oxidative stress, as a consequence of mutation(s) in the HFE gene, could be a value adjunct to clinical management of NAFLD patients.

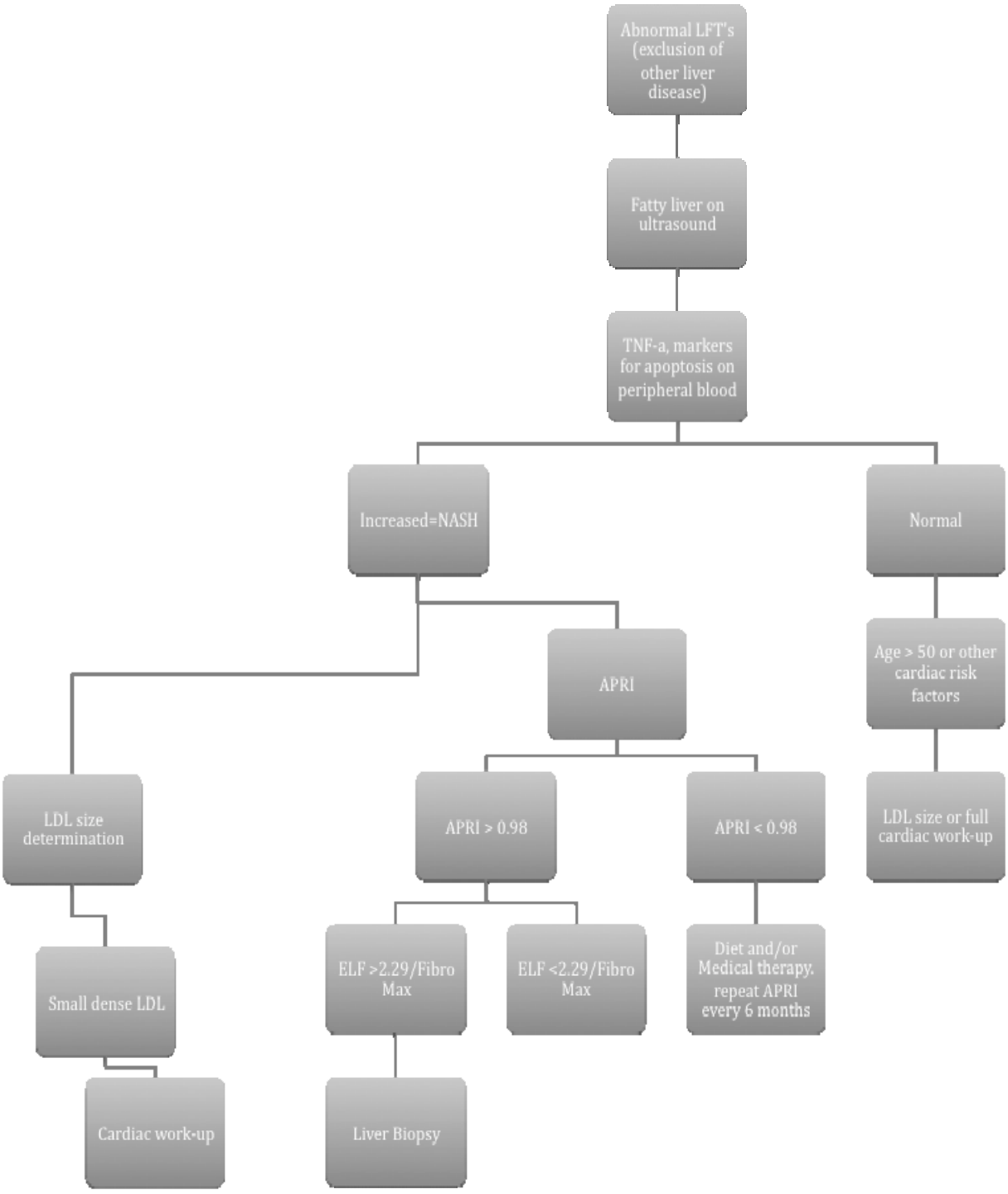



**Figure 4.1** Proposed algorithm for clinical management of NAFLD/NASH.

Detection of a genetic predisposition for HH, or carrier status of C282Y may be beneficial as iron levels could then be monitored on a regular basis as part of a preventative treatment program. Since haemochromatosis can be easily treated by phlebotomy once diagnosed, this condition is considered a preventable form of heart disease, some forms of cancer, and other equally health risks associated with iron overload.

Determination of the genetic basis of difference in response to environmental exposures such as smoking, inactivity, diet and certain drugs could provide a valuable adjunct to current clinical practice. The detection of genetic variation in high network genes of low penetrance (e.g. TNF-$\alpha$) needs to be interpreted together with a medical and lifestyle assessment, to identify a combination of risk factors that could cause or contribute to disease development if left untreated. This approach is based on the knowledge that a single genetic risk factor, even as severe as heterozygous familial hypercholesterolaemia (Kotze et al. 1993), is not sufficient to cause CVD or related disorders. NAFLD patients with a family history of CVD and/or cancer (or metabolic syndrome) may benefit most from genetic testing. The aim would be to prevent an accumulative effect that may lead to a CVD event or related complications in NAFLD patients. Furthermore, genetic variation in certain key genes may prove valuable as targets for treatment.

In future, it may be possible to target treatment to genetic subgroups likely to benefit most from certain medications. In patients with NASH, TNF-$\alpha$ inhibitors such as pentoxifylline effectively achieved significant clinical and biochemical improvement with reduction in HOMA-IR (Satapathy et al. 2004). This effect appears to be mediated through suppression of TNF-$\alpha$. Notably, it has been shown that fish oil suppresses TNF-$\alpha$ production and has variable anti-inflammatory effects on disease. Grimble et al. (2002) have shown that the ability of fish oil to suppress TNF-$\alpha$ production by peripheral blood mononuclear cells in healthy men is associated with the –308G$\rightarrow$A polymorphism, which is associated with a 2-fold increase in TNF-$\alpha$ levels. In a study of 5220 genes to identify potential targets for therapeutic intervention, Younossi et al. (2005) implicated several genes differentially expressed in NASH, relating to lipid metabolism, liver regeneration, apoptosis, and the detoxification process.

Ultimately, the implementation of a pathology supported gene-based intervention program, based on the information obtained in this study integrated with existing knowledge in the field, would improve clinical management of patients with NAFLD/NASH and metabolic syndrome.

# CHAPTER 5

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## ADDENDUM

**NAFLD DATA SHEET**

PATIENT DEMOGRAPHICS: ENTRY DATE:…….

STUDY NUMBER ..... FOLDER NUMBER……..
SUBJECT INITIALS ..... D.O.B..... AGE.....
RACE ..... SEX.....

PATIENT CHARACTERISTIC:

HEIGHT..... WEIGHT..... BMI..... WAIST/HIP RATIO:.....
SMOKING: Y/N NO. OF CIGARETTES/DAY:..... DURATION:.....
ALCOHOL: Y/N UNITS/WEEK:..... DURATION:.....
EXERCISE OCCUPATION.....
STRUCTURED.....
DIET: MEAT/WEEK.....
FRUIT/VEG/WEEK.....
TEA/COFFEE: CUPS/DAY.....
SPOONS OF SUGAR/CUP.....
WATER INTAKE/DAY.....

PATIENT HISTORY:
CO-MORBID DISEASE: .....
.....
CONCOMMITANT MEDS: .....
.....
FAMILY HISTORY OF DIABETES, HYPERTENSION, LIVER DISEASE & ISCHAEMIC HEART DISEASE:
.....
.....
PREVIOUS SURGICAL HISTORY:.....
CONTRACEPTION/HRT ..... DURATION.....
MINERAL SUPPLEMENTS: Y/N TYPE:..... DURATION.....
BLOOD TRANSFUSION: Y/N DATE .....

PATIENT SYPMTOMS:

| | | | |
|---|---|---|---|
| UPPER ABD. PAIN: | Y/N | JAUNDICE: | Y/N |
| MALAISE: | Y/N | LETHARGY: | Y/N |
| POLYURIA: | Y/N | POLYPHAGIA: | Y/N |
| DEPRESSION: | Y/N | | |

SPECIAL INVESTIGATIONS:
LIVER FUNCTIONS: DATE:.....
ALT:..... AST:..... RATIO:..... ALP:.....
GGT:..... ALB:..... BILI:.....
ULTRASOUND: DATE: .....
FINDINGS:.....
.....
HISTOLOGY: DATE:..... STA NO:.....
FINDINGS: ACTIVITY:.....
FIBROSIS:.....
CIRRHOSIS.....