Non Alcoholic Fatty Liver Disease – A cross-sectional study with special emphasis on the role of TNF alpha and TNF alpha gene polymorphisms in disease progression

A dissertation submitted in part fulfillment of DM (Gastroenterology) examination of the Tamil Nadu Dr. MGR Medical University, Chennai to be held in August 2010.

CERTIFICATE

This is to certify that this dissertation entitled Non Alcoholic Fatty Liver Disease – A cross-sectional study with special emphasis on the role of TNF alpha and TNF alpha gene polymorphisms in disease progression is a bonafide work done by Dr. Sudipta Dhar Chowdhury in partial fulfillment of the rules and regulations for D.M. (Gastroenterology) examination of Tamil Nadu Dr. MGR Medical University, to be held in August 2010.

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INTRODUCTION

Non alcoholic fatty liver disease (NAFLD) comprises of a disease spectrum which includes variable degrees of simple steatosis (fatty liver), non alcoholic steatohepatitis (NASH) and cirrhosis[1]. Though fatty liver is a commonly reported problem, a lot of people actually have a more advanced liver disease (NASH or NASH with fibrosis). These advanced forms of the disease may not be detected by simple ultrasound. NAFLD affects both sexes and both obese and non obese individuals. The prevalence of NAFLD amongst the adult population in India has been estimated to be approximately 5 – 28%. [2] Despite the high prevalence of the disease in Indians, there are a few biopsy based studies from India which correlated the clinical, biochemical and histological features of patients with NAFLD. Also, the pathogenesis of NAFLD and the mechanisms responsible for liver injury and disease progression remain incompletely understood, but are of significant biomedical importance.[3]. Insulin resistance has been found to be present universally in patients with NAFLD [4]. But, studies indicate that there is more to the pathogenesis of NASH than Insulin resistance. [5]

Most studies have shown that development of NAFLD requires a baseline of steatosis but, progression requires a 'second hit' producing necroinflamation and fibrosis. The interaction of cytokines with oxidative stress and lipid peroxidation has been considered to be one of the key mediators of the second hit. [6] A lot of theories have been propounded regarding the mechanism of disease progression viz. insulin resistance, oxidative stress and abnormal cytokine production. Abnormal cytokine production has been variously attributed to: (a) abnormal macrophage function, (b) an effect of oxidative stress through nuclear translocation of the transcription factor

nuclear factor kB, (c) direct release by adipose tissue (of tumour necrosis factor α), (d) polymorphism in the cytokine gene leading to cytokine over production and (e) endotoxin production in the small bowel.

Tumor necrosis factor alpha (TNF α) has been implicated as one of the key cytokines involved in the pathogenesis of NAFLD. [7]However, this has not been conclusively proven. Increased levels of serum TNF alpha have been reported in patients with NASH [6, 8] and variations have been noted in the production rates of cytokines among individuals.[9] Some of these inter-individual differences in cytokine production; may be related to polymorphisms in the cytokine genes themselves, or to polymorphisms in genes that regulate cytokine gene transcription.

Two polymorphisms in the TNF-α promoter region that affect TNF-α production have been identified: one at position -308 (TNF2 allele) [10] and another at position -238 (TNFA allele). [11] Experimental evidence suggest that the -308 and -238 variants are associated with high TNF production and the severity of some diseases. Valenti et al have reported that Italian NAFLD patients have a high frequency of the - 238A allele [12] But, in a similar study conducted on a Japanese population with NAFLD which evaluated six polymorphisms (including -238 and -308) no significant difference in the allele frequencies was noted between the cases and controls.[13] Thus, population differences exist in the TNF α promoter polymorphism. No data exists as to the role of the cytokines and their genetic polymorphism among Indian patients with NASH. Identification of such a genetic risk factor among Indians may enable us to identify patients at risk and formulate treatment strategies for them. This was the chief reason for embarking on the study.

AIM

1. To study the clinical, biochemical and the pathological features of patients with NAFLD
2. To compare the anthropological measurements with patients without NAFLD as defined by ultrasound.
3. To look for variations in TNF alpha in patients with NAFLD.
4. To evaluate for the presence of two specific genetic polymorphisms in the TNF alpha gene; this may be of importance in inflammation and fibrosis in NAFLD.

REVIEW OF LITERATURE

Non alcoholic fatty liver disease is a broad term used to encompass an entire spectrum of liver disease ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), which can eventually lead to nonalcoholic, noncholestatic cirrhosis and probably hepatocellular carcinoma. [14] The term non alcoholic steatohepatitis was first coined by Ludwig et al in 1980. They described a series of twenty non alcoholic patients mostly women who were obese and had liver disease that mimicked alcoholic hepatitis histologically and may progress to cirrhosis. [15] Many other synonymous terms were used by different authors such as fatty liver hepatitis, non alcoholic steatonecrosis, non alcoholic fatty hepatitis to describe this disease. [16, 17, 18]

DEFINITION:

Based on studies that reported that the normal content of liver fat is 5% of the weight, The American Association for the Study of Liver Diseases (AASLD) has defined NAFLD as fat accumulation exceeding 5 – 10% by weight. [19] To overcome the problems associated with liver biopsy, the Asia Pacific Association for the Study of Liver has proposed an operational definition of NAFLD based on hepatic imaging, supported by appropriate exclusion criteria. [20] The operational definition defined fatty liver as per the Japanese standard i.e. diffusely increased echogenicity (“bright”) liver with liver echogenicity greater than kidney, with vascular blurring, and deep attenuation of ultrasound signal [21]. NAFLD is highly likely provided that other causes of liver disease particularly significant alcohol intake (more than 140 g/wk in men, or 70 g/wk in women) and medication use have been rigorously excluded. [20]

PREVALENCE:

With the dramatic increase in obesity, NAFLD is becoming the most common liver disease globally. [14] In absence of large scale epidemiological studies determining the true prevalence of NAFLD is difficult. Based on data obtained from people undergoing master health check-ups, ultrasonography for non-liver-related causes, healthy relatives of hospitalized patients, and railway employees and their families the estimated prevalence of NAFLD in India is 5 – 28%. [2] The high prevalence of visceral adiposity among Asian Indians make them more susceptible to the development of insulin resistance and its complications. [22]

GENDER:

Early studies emphasized that NAFLD was more common in women. [23] Recent studies have shown a more equal distribution of NAFLD among both the sexes. Amarapurkar et al reported a higher prevalence amongst males. [24] Dusseja et al also reported a higher frequency of NAFLD among males, though they attributed it to areferral bias. [25]

Age:

NAFLD can be found in all age groups; however the prevalence appears to increase with age. Children with NAFLD may on follow up develop end-stage liver disease with the consequent need for liver transplantation. [26] Though no large scale study has been done among children in India, the growing prevalence of obesity amongst school children makes them particularly at risk for fatty liver disease.

RISK FACTORS FOR NAFLD:

Obesity:

Worldwide, obesity remains the most important risk factor for NAFLD. [27] In a review of several cross sectional and case control studies Jakobsen et al have shown that

people with NAFLD have higher waist circumference (WC) or BMI than those without NAFLD, and have reported significant associations between abdominal obesity and NAFLD. [27] Amongst the Caucasian population obesity is defined by a BMI of ≥ 30 kg/m^2. Using this criterion, only 2 – 3 % of Asians are classified obese. [28] But, amongst Asians the health risks associated with obesity occur at a much lower body mass index (BMI). The International Diabetes Institute has therefore recommended different ranges of BMI for the Asia-Pacific region based on risk factors and morbidities. Amongst Asians, the cut-off for overweight and obesity is ≥ 23 kg/ m^2 and ≥ 25 kg/ m^2 respectively. [29]

The distribution of fat may be more important than the total adipose mass. Central obesity is a correlate of visceral adiposity and is more closely linked to insulin resistance, the central event in NASH. [30] In a Japanese survey of 2500 men, subdivided into four groups by BMI and waist/height measurements the odds ratio for fatty liver was most influenced by central obesity. [31] Amarapurkar et al also found a high prevalence of NAFLD amongst individuals having a BMI ≥ 25 kg/ m^2 and a waist circumference more than 90 cm in male and more than 80 cm in female. [24]

Diabetes Mellitus:

In addition to obesity, type 2 diabetes mellitus may be a particularly important risk factor for NAFLD. Non-alcoholic fatty liver disease is the most common chronic liver disease seen in patients with T2DM. Epidemiologic studies have demonstrated that T2DM occurs in 21% to 45% of patients with NAFLD, and approximately an additional 30% have a family history in a first-degree relative. [32, 33, 34] Though among Indians Duseja et al reported that only 12% had diabetes mellitus, with another 14% having impaired glucose tolerance.[25]

Polycystic ovary syndrome:

The classic syndrome originally was described by Stein and Leventhal as the association of amenorrhea with polycystic ovaries, and variably, hirsutism and obesity [35] It is now recognized that PCOS represents a spectrum of disease characterized primarily by the following features cutaneous hyperandrogenism (eg, hirsutism, severe acne, and/or pattern alopecia), menstrual irregularity (eg, oligo- or amenorrhea, or irregular bleeding), polycystic ovary, obesity and insulin resistance. PCOS in women has been associated with NAFLD. [36]

Metabolic Syndrome and Insulin resistance:

The term metabolic syndrome refers to a cluster of cardiovascular risk factors associated with insulin resistance. When the appropriately revised ATP III criteria for Asians are used, the prevalence rate of the metabolic syndrome in Asians is comparable to those in Western populations. [37] The risk of fatty liver; increases in proportion to the increase in the number of components of metabolic syndrome. [37] Duseja et al found reduced insulin sensitivity in Indian patients with NAFLD. [25] Insulin resistance enhances lipolysis and increases delivery of adipose-derived FFAs to the liver. In the liver different cell types express insulin receptors and within each cell, insulin/insulin receptor interactions trigger diverse signaling cascades. Hyperinsulinemia apparently triggers mechanisms that desensitize hepatocytes to insulin effects that normally suppress postprandial gluconeogenesis, resulting in postprandial hyperglycemia that characterizes the prediabetic state. On the other hand, these same hepatocytes retain sensitivity to insulin effects that promote lipogenesis, causing the cells to become fatty. [38]

Hepatic stellate cells also retain sensitivity to insulin and the transcription factor FoxO1 plays a crucial role in the transdifferentiation and proliferation of HSCs in liver

fibrosis. FoxO1 inhibits proliferation via cell cycle arrest at the G1 phase. Hyperinsulinemia inactivates FoxO1 in HSCs, resulting in HSC activation and may result in the fibrosis in nonalcoholic fatty liver disease. [39]

PATHOGENESIS OF NAFLD:

Ingestion of dietary fatty acids and lipolysis in peripheral adipose tissues generate free fatty acids (FFAs), which are ultimately taken up by hepatocytes. Lipid metabolism within the liver balances three primary mechanisms:

(1) Hepatocyte uptake and de novo synthesis of free fatty acids (FFAs),

(2) Disposal of FFAs via oxidation or de novo triglyceride synthesis, and

(3) Export of triglycerides from hepatocytes as very low density lipoproteins (VLDL).[40]

In 1998 CP Day et al proposed that NAFLD resulted from <u>two hits</u> to the liver. [41]

<u>The First hit</u>: In NAFLD, the normal equilibrium in hepatocyte lipid metabolism is disrupted. De - novo lipogenesis is markedly increased in NAFLD relative to rate of fatty acid oxidation. [42] This stimulates increased triglyceride synthesis to dispose of the excess FFAs. When the rate of triglyceride synthesis overwhelms the capacity for VLDL synthesis/export, triglycerides accumulate within hepatocytes, resulting in steatosis.[38] Thus the 'first hit' involves an imbalance in fatty acid metabolism that leads accumulation of triglyceride within hepatocytes (i.e.,steatosis) the hallmark of NAFLD. [41]

<u>Second hit</u>: FFAs may elicit hepatotoxicity and stimulate progression from NAFL to NASH via several mechanisms.

a. Direct cytotoxicity: FFAs can be directly cytotoxic[43] and Feldstein et al have demonstrated that simply incubating HepG2 cells in fatty acid-containing medium increased lysosomal permeability evoked nuclear

factor-kappa B (NF-kB) activation and proinflammatory cytokine production. [44]

b. Oxidative stress: The oxidation of FFAs generates reactive oxygen species (ROS) and reducing equivalents, such as nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH). This promotes redox stress and interferes with normal functioning of cellular organelles, such as the endoplasmic reticulum and mitochondria. [45] Oxidation of FFA in hepatic microsomes appears particularly likely to generate excessive ROS. Sustained overreliance on mitochondrial fatty acid oxidation may also generate ROS that damage the mitochondria themselves. Also there is dysregulated cytokine production that results from efforts to compensate for altered lipid homeostasis. All this constitute the second hit. [41]

PROGRESSION FROM NASH TO CIRRHOSIS:

Most individuals with simple hepatic steatosis (NAFL) will not develop cirrhosis. However, if NASH occurs the risk of progressing to cirrhosis increases substantially with perhaps 30 to 50% of individuals demonstrating advanced fibrosis/cirrhosis within a decade. [46] Elucidating the mechanisms by which steatohepatitis progresses to fibrosis and cirrhosis is an essential component in understanding the natural history of NAFLD. Jou et al have proposed hepatocyte death as the 'third hit' in the pathogenesis of NASH. [38] This according to them is probably the most significant hit in NAFLD pathogenesis because this event drives progression from NASH to cirrhosis. [38]

In models of chronic liver injury, sustained inflammation and hepatocyte death leads to increased stellate cell activation and decreased stellate cell apoptosis. Hepatic

stellate cells are the major mediators of liver fibrosis. Upon activation, these cells release factors ultimately leading to deposition of extracellular matrix (ECM). Experimental studies have also suggested a key role of certain cytokines in fibrogenesis.[7] In an animal model of NASH, it was found that enhancement of the TNF-α/TNFR mediated signalling pathway via activation of Kupffer cells in an autocrine or paracrine manner may be critically involved in the pathogenesis of liver fibrosis. [47]

ROLE OF ADIPOCYTOKINES IN NAFLD:

Adipose tissue itself is increasingly being recognized as a metabolically active endocrine organ that has the capability of secreting a myriad of proteins and cytokines that regulate energy balance by modulating the flux of substrates through lipid and glucose metabolic pathways. [38] Certain adipose-derived factors e.g., TNF-α, resistin, angiotensinogen, plasminogen activator inhibitor (PAI)-1, antagonize the lipogenic actions of insulin, resulting in increased release of FFAs from adipose depots. Other factors that are produced by adipose tissue (e.g., leptin, adiponectin, visfatin) function as insulin sensitizers and thus, enhance storage of FFA in adipose triglyceride depots, reducing release of FFA into the circulation. [48] Studies support the importance of unbalanced adipocytokine production in NAFLD pathogenesis. [49]

TNF α and NAFLD:

Tumor necrosis factor (formerly known as cachexin or cachectin) is a cytokine involved in systemic inflammation and is a member of a group of cytokines. TNF- α is produced by macrophages, lymphoid cells, mast cells, endothelial cells, cardiac myocytes, fibroblasts, neuronal tissue, and hepatocytes. Adipose tissue is also an important source of this proinflammatory factor. [38] Human TNF-alpha is a non-

glycosylated protein comprised of 157 amino acids (molecular weight ~ 17 kDalton). [50]

TNF-α levels correlate with the degree of insulin resistance [51] and acute infusion of TNF-α inhibits insulin-stimulated glucose disposal [52]. The intracellular and molecular mechanisms responsible for TNF-α-induced insulin resistance and lipid overloading of liver cells have been increasingly elucidated and appear to involve both activation of stress-related protein kinases, such as Jun N-terminal kinase (JNK), as well as the inhibitor kappa beta kinase beta (IKKβ)/NF-κB pathway [53]

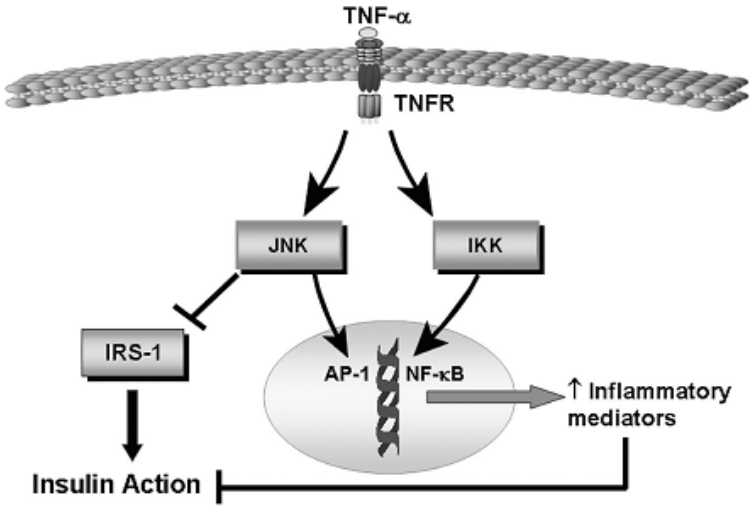

Figure 1. Molecular pathways of TNF-α-induced insulin resistance.

JNK activation may impair insulin action by phosphorylation of insulin receptor substrate (IRS)-1 at serine (Ser) sites. [54, 55, 56] IKKβ activation leads to NF-κB translocation to the nucleus, resulting in a feed forward loop that promotes the synthesis of TNF-α and other mediators of inflammation that then may cause insulin resistance. [57] Selective low-grade activation of the IKKβ/NF-κB pathway in liver cells results in a state of subacute chronic inflammation with increased production of

cytokines, such as TNF-α and interleukin (IL)-6, and both hepatic and systemic insulin resistance. [58]

TNF-α and other inflammatory cytokines may also have a role in the progression from fatty liver to NASH. A cytokine imbalance, in particular, an increase in the TNF-α/adiponectin ratio may play an important role in the development of NASH. [3] Crespo J et al in a study have shown that gene expression of TNF-α and TNF receptors is increased in the liver of patients with NASH as compared with both normal liver and fatty liver, and the expression is higher in those patients with more severe NASH. [59] Some studies have noted that circulating adiponectin levels are significantly lower and TNF-α levels are significantly higher in patients with NASH as compared with controls. [60,61] Moreover serum levels of soluble TNF receptors were found to be elevated in patients with NASH. [62] The molecular mechanisms linking TNF-α to liver damage in NAFLD is shown in figure 2.

Interindividual difference exists in the levels of cytokines and its been found that the production rates of the various cytokines is different amongst individuals. [9] Some of these inter-individual differences in cytokine production may be related to polymorphisms in the cytokine genes themselves, or to polymorphisms in genes that regulate cytokine gene transcription. Recent studies have described extensive polymorphisms within the TNF- α promoter region at positions -1031, -863, -857, -308, and -238 and interest has been generated in the relationships between these TNF gene polymorphisms and susceptibility of individuals to both autoimmune and infectious diseases. [63, 64, 65]

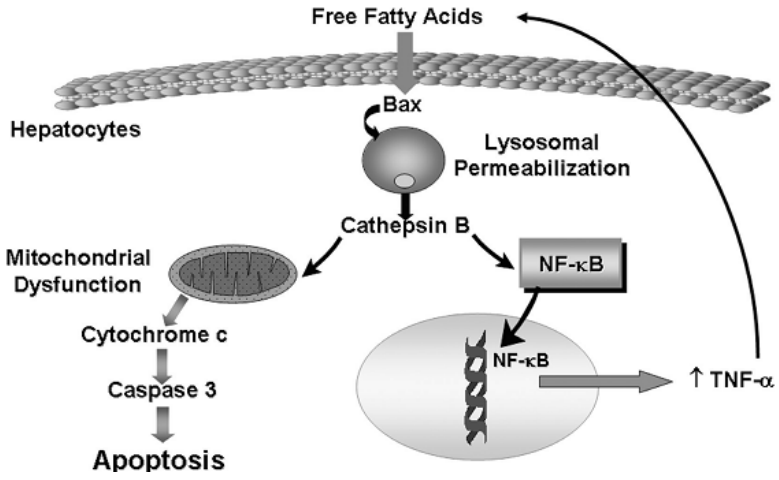

Figure 2. Molecular mechanisms linking TNF-α to liver damage in NAFLD.

Polymorphisms in the TNF- α promoter region one at position -308 (called TNF2 allele) [10] and another at position -238 (TNFA allele) [11] have been identified. Studies with the TNF- α promoter regions have shown that the TNF2 allele leads to increased constitutive and inducible expression of TNF α compared with the wild type (TNF-1). [66, 10] Conflicting data have been reported with TNF A allele [67,68] but most investigators believe that TNFA allele also increases the release of this cytokine. In India studies have been conducted looking at the role of TNF α polymorphisms in other diseases. Gupta et al conducted a study on patients with oral cancer and did not find any significant change at the -238 promoter site. [69] Another study by Singhal et al on patients with viral hepatitis also did not find any polymorphism at -238 site. [70]

Valenti et al in their study found that a significantly higher prevalence of TNFA (-238 polymorphism) but not of TNF2 (-308 polymorphism) allele was present in patients with NAFLD. Patients positive for either of the 2 TNF-α polymorphisms had more severe insulin resistance and a reduced pancreatic β-cell function compared with those who were negative. Insulin resistance was present in more than half of those positive for the -238 polymorphism despite a normal BMI. [12] K. Tokushige et al in their study on Japanese patients with NAFLD

found that the mean soluble TNF receptor titres were higher in patients who carried the -1031C allele, but the patients had a lower frequencies of polymorphism at -238 and -308. [13] This suggests that ethnic difference exists in the frequencies of TNF alpha promoter polymorphisms. Confirming a role of TNF α and its genetic polymorphism amongst Indians may help in identification of newer therapeutic targets.

CLINICAL PRESENTATION:

Most patients with NAFLD are asymptomatic and the diagnosis of fatty liver is often made when abdominal imaging is performed for evaluation of abnormal liver tests or for other indications. [71] Fatigue, malaise, and vague right upper abdominal discomfort bring some patients to medical attention. [72] Duseja et al in their study found that malaise, fatigue and right upper quadrant discomfort were common presentations among Indian patients with NAFLD. A number of patients come to medical attention on having had an incidental detection of raised ALT either during workup for another illness, during organ donation, or during an executive checkup. [25] Most studies have reported a higher BMI among patients with NAFLD. [25, 73] Features of advanced liver disease are unusual. [71]

DIAGNOSIS:

Biochemical tests:

NAFLD is identified as the principal underlying cause of abnormal liver tests in persons without excessive alcohol use or viral hepatitis. [71]

SGOT and SGPT:

SGPT concentration is the most commonly used variable for assessment of liver disease. [74, 75] Prati et al suggested that the upper limit of normal for ALT and AST

amongst patients with NAFLD should be decreased to 30 U/L for men and 19 U/L for women. [76]

The serum levels of SGOT and SGPT are elevated in patients with NAFLD. However, liver aminotransferase levels are seldom higher than 5 times the upper limit of normal, and typically fluctuate with normal levels seen in more than two-thirds of NASH patients at any give time.[77,78] Mofrad and colleagues demonstrated that the entire histological spectrum of NAFLD can be seen inpatients with normal ALT values. [79] Angulo et al showed that an SGOT/SGPT ratio of > 1 was predictive of liver fibrosis. [80]

Other tests for liver function:

Serum ALP, GGT, or both are usually mildly elevated in many patients with NAFLD. However, their utility for the diagnosis of NASH is poor. Hypoalbuminemia, prolonged prothrombin time, and hyperbilirubinemia may be seen with cirrhotic NASH, but are not present until decompensated disease arises. [81]

Imaging studies:

Several imaging techniques have been advocated as noninvasive diagnostic tests for NAFLD.

Ultrasonography:

Ultrasonography (US) is currently the preferred method for screening asymptomatic patients with elevated liver enzymes and suspected NAFLD. Ultrasonographic findings of fatty liver include hepatomegaly, diffuse increase in echogenicity of the liver parenchyma, and vascular blunting. Studies have demonstrated that the sensitivity, specificity, and PPV of ultrasound to detect steatosis is as high as 80 to 100%. [82] But, ultrasound has major limitations as its operator dependent and is and

subject to significant intra and interobserver variability. The sensitivity and specificity also decreases if the degree of hepatic steatosis is < 30% [83] and in patients with morbid obesity in whom sensitivity lower than 40% has been. [84] But, the most important limitation of US is the inability to diagnose NASH and hepatic fibrosis.

Computerized tomography and magnetic resonance imaging:

Both computerized tomographic (CT) scanning and magnetic resonance imaging (MRI) are sensitive techniques for quantification of steatosis. More recently, localized proton magnetic resonance spectroscopy (MRS), has been shown to be a noninvasive method that is highly accurate in measuring hepatic triglyceride content (HTGC). HTGC obtained byMRS closely coincides with biopsy derived triglyceride concentrations. [85]

Transient Elastography:

Transient elastography is a technique for measuring tissue elasticity based on ultrasound technology. It has been applied for the noninvasive assessment of hepatic fibrosis. The use of transient elastography in the assessment of hepatic fibrosis amongst patients with hepatitis C virus infection has shown promising results. [86] Foucher J, et al in a prospective study from France in more than 1000 male subjects undergoing transient elastography showed that the only independent risk factor for failure of the procedure was a BMI >28 with an odds ratio close to 10.[87] Considering that a large number of patients with NAFLD have a high BMI, the chances of failure of transient elastography to detect hepatic fibrosis in this population may be low.

Liver Biopsy:

A liver biopsy is probably the only reliable way to distinguish between a fatty liver, and steatohepatitis. Biopsy is also the only reliable way to evaluate the amount of fibrosis, a key marker of disease progression, in subjects with NASH. There is, however, considerable controversy about the value and need for a liver biopsy in subjects with suspected NAFLD. This controversy stems from the invasive nature and risks of a biopsy, absence of approved therapy for NASH and lack of consensus that the histological findings guide therapy. Gaidos et al using a decision tree model tracked the potential outcomes of NAFLD between a liver biopsy directed approach versus no initial liver biopsy. The baseline probabilities were determined by literature review and expert opinion. An initial liver biopsy strategy was projected to have a lower mortality compared with the no initial liver biopsy group and fewer transplant eligible patients after 5 years. [88]

The principal histological features of NASH include the presence of macrovesicular fatty changes of hepatocytes with displacement of the nucleus to the edge of the cell, ballooning degeneration of hepatocytes, and a mixed lobular inflammation. [89] The presence of ballooning is the most important diagnostic criteria distinguishing steatohepatitis from simple steatosis. Features such as perisinusoidal, pericellular fibrosis, Mallory hyaline, megamitochondria, acidophil bodies, glycogenated nuclei, can be present but are not always required to establish the diagnosis of NASH. [90] Liver fibrosis appears to be one of the most important prognostic factors in patients with NAFLD,as the presence of fibrosis suggests a more advanced and severe liver injury. [91]

A number of systems have been proposed for assessing the severity of fatty liver disease. One such scheme is the Brunt system. [92] One criticism of the Brunt score

has been the incorporation of fatty change, ballooning and inflammation into an overall grade. Thus implying that these three histological features increase in parallel to each other, which is not necessarily the case. [93] In an attempt to address some of these problems a modified histological scoring system has been devised by a group of North American pathologists working under the auspices of the Non-alcoholic Steatohepatitis Clinical Research Network.[94] The NAFLD Activity Score (NAS) (0–8) is the sum of scores for steatosis, lobular inflammation and hepatocellular ballooning.

Steatosis (0–3)

0 = <5% hepatocytes involved
1 = 5–33% hepatocytes involved
2 = 33–66% hepatocytes involved
3 = >66% hepatocytes involved

Lobular Inflammation (0–3)

0 = none
1 = <2 foci per · 200 field
2 = 2–4 foci per · 200 field
3 = >4 foci per · 200 field

Hepatocyte ballooning (0–2)

0 = none
1 = few ballooned cells
2 = many cells/prominent ballooning

Fibrosis stage

1 Perisinusoidal or periportal
1A Mild, zone 3, perisinusoidal
1B Moderate, zone 3, perisinusoidal
1C Portal/periportal fibrosis only
2 Perisinusoidal and portal/periportal fibrosis
3 Bridging fibrosis
4 Cirrhosis

Liver biopsy has its limitiations, being an invasive procedure with potential for complications its not suitable as a screening test. [81] Needle biopsy of the liver is also subject to sampling variability. [95] Another important limitation of liver biopsy relates to the fact that histological analysis remains subjective, and thus prone to intra- and interobserver variability. [96]

Panel Markers:

To improve the accuracy of noninvasive diagnosis and the stage of fibrosis several groups have created panels using different combinations of a series of clinical and biochemical markers to generate various scoring systems.

The HAIR (Hypertension, ALT, Insulin Resistance) score was described by Dixon and colleagues in a group of 105 severely obese patients undergoing gastric bypass surgery. The score was designed for prediction of NASH diagnosis and used a combination of presence of hypertension, elevated ALT (>40 U/L), and insulin resistance (defined as an insulin resistance index above 5). The presence of at least 2 parameters predicted NASH with high sensitivity and specificity. [97]

Palekar et al used a combination of 6 different variables including age (>50 years), female gender, AST (>45 U/L), body mass index (BMI >30 kg/m2), and AST/ALT ratio (>1), and serum hyaluronic acid (HA; >55 mg/L) to differentiate NASH from simple steatosis. The AUC for this model was 0.76. The presence of 3 or more of these factors had a sensitivity and specificity for NASH diagnosis of 74% and 66%, respectively. [98] Gholam et al using logistic regression analysis proposed a simplified model using only AST and diagnosis of diabetes and were able to separate NASH from fatty liver with or without nonspecific inflammation in bariatric surgery patients with similar accuracy as the panels described in previous studies. [99] None of these panels yet has been independently validated in different populations in a prospective fashion.

Other workers have focussed on models to predict fibrosis. Ratziu and colleagues combined 4 clinical variables to generate the BAAT score, including BMI (>28 kg/m2), age (>50 years), ALT (>2XN), and serum triglycerides (>1.7 mmol/L). Each variable received a score of 0 or 1. Although a total score of 0 had sensitivity close to 100% and a specificity of 47%, a high total score of 4 gave a sensitivity of 14% and a specificity of 100% for detection of septal fibrosis. [100]

Ratziu's group also tested the utility of FibroTest-FibroSURE for prediction of liver fibrosis in patients with NAFLD. [101] This proprietary panel has been extensively studied in chronic hepatitis C and combines 5 biochemical markers including α2-macroglobulin, apolipoprotein A1, haptoglobulin, total bilirubin, and GGT. The score is computed by entering patients age and sex along with the 5 components into a proprietary program. The test yielded an AUC for the diagnosis of advanced fibrosis of 0.86. A cutoff value of 0.3 had a 90% NPV for advanced fibrosis, whereas a cutoff value of 0.7 had a 73% positive predictive value (PPV) for advanced fibrosis. The

most frequent causes of FibroTest (FT) failure include Gilbert's syndrome, cholestasis, and acute inflammation, which result in increases in bilirubin and haptoglobulin, respectively and also abnormal apolipoprotein A1 concentration. Angulo and colleagues developed a NAFLD fibrosis score to separate patients with or without advanced fibrosis in a large cohort of biopsy-proven NAFLD patients. An algorithm was constructed using 6 readily available laboratory and clinical variables including age, hyperglycemia, BMI, platelet count, albumin, and AST/ALT ratio. The AUC for this model was 0.88 and 0.82 in the estimation and validation group, respectively. Using this curve they generated two (high and low) cutoff values that allow the diagnoses of advanced fibrosis with high accuracy (NPV between 88 and 93%, PPV between 82 and 90%). However, similar to the FT study in which 33% of cases the presence or absence of advanced fibrosis could not be predicted, there were 25% of indeterminate cases in this series. [102]

These studies suggest that either a combination of clinical and biochemical markers or specific markers of fibrosis may be used for noninvasive staging of NAFLD patients. However, to date, all of these studies have been tested in a cross-sectional fashion and the role of these biomarkers for monitoring disease progression, response to therapy, and prognosis remains completely unknown.

MANAGEMENT:

Lifestyle modifications:

Dietary intervention and exercise, though lacking appeal and often limited by patient compliance, remain first line therapy for this disease In a study by Huang et al 23 patients with biopsy-proven NASH underwent nutritional counseling for 1 year. The diet was designed so patients would obtain 40 to 45% of their calories from carbohydrates, 35 to 40% from fat, and 15 to 20% from protein. A liver biopsy

repeated 12 months later showed showed improvement in histologic features, in 9 of 15 patients which was associated with a greater degree of weight loss. [103]

Pharmacotherapy:

As lifestyle changes are difficult to sustain long-term, other modalities such as pharmacotherapeutic or surgical approaches have been investigated in the treatment of NASH.

Metformin:

Marchesini et al treated 20 biopsy-proven NASH patients with metformin 500 mg 3 times per day for 4 months. Significant improvements were seen in insulin resistance and aminotransferase levels, with 50% of subjects normalizing their transaminases. Liver volume, as measured by ultrasound, decreased by 20%.[104] A more recent Turkish study of 36 biopsy-proven NASH patients treated with metformin 850 mg twice per day plus a calorie-restricted diet versus diet alone for 6 months showed no significant differences in necroinflammatory activity or fibrosis between the two groupson repeat biopsy. [105]

Thiazolidinedione:

The thiazolidinediones (TZDs) are a class of diabetic medications that have been extensively studied in the treatment of NASH. These medications include pioglitazone and rosiglitazone, which act as peroxisomal proliferator activated receptor-γ (PPAR-γ) agonists leading to increased fatty acid oxidation and decreased fatty acid synthesis within hepatocytes. The resultant improved insulin sensitivity in both hepatocytes and skeletal muscle is one mechanism of action that may explain TZDs usefulness in NASH patient populations. [106] The FLIRT trial was a randomized placebo control trial of rosiglitazone in NASH. There was a marked antisteatogenic effect of RSG together with a significant reduction in transaminase

values. However, there was no clear improvement in necroinflammatory lesions and liver fibrosis. [107] The study was then continued as an open label extension trial (FLIRT 2) to determine whether prolonged therapy with rosiglitazone is associated with further histological improvement, and, in particular, if fibrosis regression can be obtained. The study demonstrated that despite a maintained effect on insulin sensitivity and transaminase levels no further improvement in liver histology could be obtained. [108]

The side effects of the TZDs include weight gain has been universally noted, usually on the order of 2 to 5 kg, and this increase does not always return to baseline on medication discontinuation. In addition, lower extremity edema is seen in up to 5% of patients chronically taking a TZD. These medications are contraindicated in congestive heart failure.

Statins:

HMG CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase inhibitors, also known as statins, have played a critical role in decresing mortality in patients with diabetes abd coronary arthery disease. Rallidis et al conducted a pilot study using Pravastatin in 4 patients with NAFLD, comparing histological changes after 6 months of therapy. Three patients had improvement in grade of inflammation, while one had improvement in degree of steatosis. [109]

Ekstedt M et al in a recent study showed that despite initial higher Body Mass Indexes and rates of diabetes, those patients receiving statins had a significant reduction in hepatic steatosis when compared with those who did not, with an overall low rate of fibrosis progression. [110]

But there is also concern regarding the use of statins in patients with liver disease. Hepatic injuries associated with statins include (1) asymptomatic elevations in

aminotransferases(common), (2) clinically significant acute liver injury(very rare), (3) fulminant hepatic failure (profoundly rare).[111] Lewis et al in a prospective, randomized, double-blind, placebo-controlled,multicenter trial conducted to determine the safety and efficacy of high-dose pravastatin in hypercholesterolemic patients with well-compensated chronic liver disease did not find any statistically significant changes in serum ALT in patients treated with pravastin versus placebo.[112]

Ursodeoxycholic acid:

Ursodeoxycholic acid (UDCA) is a naturally occurring bile acid. Laurin et al in a pilot study comparing UDCA to clofibrate in biopsy proven NASH, showed a significant improvement in both ALT levels and histology in UDCA treated patients. [113] Subsequently LIndor et al conducted a randomized controlled trial comparing UDCA with placebo. Comparable improvements in the liver histology scores were noted in both the groups. This study highlighted that spontaneous improvement in the liver histology was seen in NASH. [114]

Vitamin E:

Antioxidants intuitively are intriguing as a potential therapy. Pathophysiologically, oxidative stress appears to be a part of mitochondrial and endoplasmic reticulum stress. As such vitamin E has been evaluated as a possible treatment for NASH. Lavine et al in their study on children with NASH found that administration of vitamin E for a mean of 5.2 months normalized serum aminotransferase and alkaline phosphatase levels. [115] In a pilot study by Kugelmas et al, evaluated the role of Vitamin E supplementation in adults with NASH. Sixteen biopsy-proven NASH patients were treated with lifestyle modification (diet and exercise) with or without vitamin E for a period of 12 weeks. Vitamin E supplementation provided no

statistically significant improvement in serum aminotransferase levels. [6] Currently a randomized, multicenter, double-masked, placebo-controlled trial of 96 weeks of treatment with metformin or vitamin E in children is underway. (TONIC trial). [116]

Orlistat:

Orlistat inhibits gastric and pancreatic lipase, which is needed to break down triglycerides into free fatty acids and has been shown to prevent 30% of dietary triglycerides from being absorbed. Zelber et al conducted a double blind placebo control trial of orlistat in patients with NAFLD. All patients participated in an identical behavioral weight loss program. Serum alanine transaminase (ALT) levels decreased significantly in both groups, with an almost 2-fold reduction in the orlistat group (48% vs 26.4%). There was also a statistically significant reversal of fatty liver by US only in the orlistat group ($P<.05$).[117]

Rimonabant:

The endocannabinoid (EC) system is involved in the regulation of food intake and body weight. In the setting of obesity, the EC system appears to be upregulated and as such, represents a novel target for medical therapy of NASH. The cannabinoid type I (CB1) receptors are found throughout the body and their activation leads to increased hepatic lipogenesis, fatty acid synthesis in adipocytes, and decreased adiponectin. Rimonabant is a selective CB1 receptor antagonist that has been shown to decrease hepatic lipogenesis and increase satiety, adiponectin levels, and glucose uptake, thereby improving insulin levels and lipid profiles. [118] One potential side effect that requires further evaluation is an association with psychiatric problems with rimonabant.

Pentoxyphylline:

Pentoxyphylline is a xanthine derivative which attenuates TNF release in a dose-dependent manner. Satapathy et al evaluated whether histological improvement occurred in patients of NASH with pentoxyphylline. Nine patients (mean age 31.6 +/- 7.2 years) with histologically proven NASH and with persistently elevated ALT (>1.5 times) were given pentoxyfylline at a dosage of 400 mg t.i.d. for 12 months. Significant reduction in the ALT, AST levels was observed. Steatosis and lobular inflammation each reduced in 55% and six (67%) patients down-staged on Brunt's staging (P = 0.009). Four out of six patients with baseline fibrosis had reduction in their fibrosis stage. [119] Lee et al randomized 20 patients to 3 months of treatment with a step 1 American Heart Association diet and daily exercise with Pentoxifylline or placebo. Body mass index (BMI), ALT and aspartate aminotransferase (AST) decreased significantly in both groups. No difference between the two groups in reduction of BMI (P = 0.897). There was significantly greater reduction in AST in the Pentoxifylline group (P = 0.038). [120] Thus in patients with NASH having elevated TNF alpha, pentoxyphylline may be a therapeutic option.

METHODOLOGY

The study was designed as a crossectional study. It was conducted at a tertiary care hospital in South India (Christian Medical College, Vellore, Tamil Nadu) between the period February 2008 to February 2010.

Study subjects:

Consecutive patients diagnosed to have fatty liver disease either during workup for another illness, during planned organ donation, during a medical checkup, or in the course of evaluation for chronic liver disease were evaluated for inclusion in the study.

Inclusion criteria:

a. Patients with fatty liver disease identified on ultrasound and who underwent a liver biopsy
b. Liver biopsy showing features of fatty liver disease.

Exclusion criteria:

Patients with:

a. History of alcohol use
b. Viral hepatitis (hepatitis B / C)
c. Wilson's, Autoimmune Hepatitis
d. Exposure to drugs known to cause hepatic steatosis.

An informed consent was obtained from all the patients.

Controls:

A group of consecutive healthy individuals matched with the cases for the age, sex and community were also selected for the study.

Inclusion criteria for controls:

1. Apparently healthy individuals
2. Ultrasound abdomen showing no evidence of fatty liver disease
3. Consent given for inclusion in the study.

Exclusion criteria for controls:

1. Anyone with a history of diabetes mellitus, hypertension, dyslipidemia, liver disease or coronary artery disease.
2. History of alcohol use.

ANALYSIS:

All the individuals were interviewed using a standard questionnaire as regards their symptoms, presence of risk factors for fatty liver disease, alcohol use and drug intake. Exercise history was obtained in all patients as well as controls. The individuals were divided into four groups based on their frequency of exercise. Anyone who performed aerobic exercise > 5 days per week was labeled as regular, 3-4 times per week as modest, 0-2 times per week as occasional and the last group was those who never exercised.

Anthropometric measures included body mass index (BMI Kg/m^2), waist circumference and waist hip ratio was calculated for all patients and the controls. For measuring weight, subject was instructed to stand still in the platform, with the body weight evenly distributed between both the feet. After removing heavy clothing weight was measured to the nearest of 0.1 kg. Height was measured using stadiometer with head held straight and corrected to the nearest of 0.1 cm. Body

mass index (BMI) was calculated by the following formula; weight (kg)/height (m_2). Waist circumference (WC) was measured mid-way between iliac crest and lowermost margin of the ribs, in quiet breathing. Hip circumference (HC) was measured at the maximum protruding part of buttocks at the level of the greater trochanter with the patient wearing minimal clothing and feet together. Waist Hip Ratio (WHR) was calculated as WC/HC.

Biochemical analysis:

Metabolic profile:

From the patients a fasting venous blood sample was obtained for metabolic profile. Estimations for total cholesterol (TC), serum triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) were performed by enzymatic method. Fasting blood sugar estimation was also done.

Fasting serum insulin was determined by radioimmunoassay and insulin resistance was calculated by HOMA-IR (homeostasis model assessment of insulin resistance). The value of HOMA-IR was calculated by using the equation (fasting insulin (μU/ml) X fasting glucose (mg/dl))/405 and depicted as HOMA-IR value.

Liver function test:

Estimation of total bilirubin, direct bilirubin, total protein, albumin, SGOT, SGPT and serum alkaline phosphatase was done in the patients.

Both the cases and controls underwent ultrasound examination. The examination was done by a single qualified radiologist who was blinded to the clinical history and laboratory findings of the patient. Fatty liver on ultrasound was defined as per the Japanese standard: diffusely increased echogenicity (“bright”) liver with liver echogenicity greater than kidney, with vascular blurring, and deep attenuation of

ultrasound signal[21]. Healthy individuals without ultrasound evidence of fatty liver were taken as controls.

HISTOPATHOLOGY:

Liver biopsy was one of the inclusion criteria and was done in all the patients either as ultrasound assisted percutaneous liver biopsy or transjugular liver biopsy. In one patient the biopsy was taken from the explant liver obtained at liver transplantation. The biopsy specimen was evaluated by a single pathologist who was blinded to the clinical history of the patients. The biopsy slides were graded as per a histological scoring system devised by the Non-alcoholic Steatohepatitis Clinical Research Network. [94]

The NAFLD Activity Score (NAS) (0–8) is the sum of scores for steatosis, lobular inflammation and hepatocellular ballooning

Steatosis (0–3)

0 = <5% hepatocytes involved
1 = 5–33% hepatocytes involved
2 = 33–66% hepatocytes involved
3 = >66% hepatocytes involved

Lobular Inflammation (0–3)

0 = none
1 = <2 foci per · 200 field
2 = 2–4 foci per · 200 field
3 = >4 foci per · 200 field

Hepatocyte ballooning (0–2)

0 = none
1 = few ballooned cells
2 = many cells/prominent ballooning

The degree of fibrosis was assessed

Fibrosis stage

1 Perisinusoidal or periportal
1A Mild, zone 3, perisinusoidal
1B Moderate, zone 3, perisinusoidal
1C Portal/periportal fibrosis only
2 Perisinusoidal and portal/periportal fibrosis
3 Bridging fibrosis
4 Cirrhosis

Patients who had evidence of macrovescisular steatosis, with or without inflammation or fibrosis were included in the study.

TNF α ASSAY:

For TNF-α assay a fasting sample of blood was collected from the cases in an empty vacutainer tube. The sample of blood was centrifuged at 3000 rpm and the serum was extracted. A total of 9 samples amongst the cases was damaged as a result of contamination with haemoglobin.

TNF- α assay was performed using Biosource (Biosource Europe S.A.- Belgium) TNF- α – EASIA. This is a solid phase Enzyme Amplified Sensitivity Immunoassay performed on microtiter plate using monoclonal antibodies (MAbs) directed against epitopes of TNF- α. Calibrators and samples react with capture monoclonal antibody

(MAb 1) coated on microtiter well and with a monoclonal antibody (MAb 2) labelled with horseradish peroxidise (HRP). After an incubation period allowing the formation of a sandwich: coated MAb - human TNF- α –Mab 2 – HRP, the microtiterplate was washed to remove unbound enzyme labelled antibody. Bound enzyme – labelled antibody is measured through a chromogenic reaction. Chromogenic solution (TMB) is added and incubated. The reaction was stopped with the addition of Stop solution and the microtiterplate was then read at the appropriate wavelength (450nm and 490 nm). The amount of substrate turnover was determined colourimetrically by measuring the absorbance, which is proportional to the TNF- α concentration. A calibration curve was plotted and TNF-α concentration in samples was determined by interpolation from the calibration curve. The detection limit, defined as the apparent concentration two standard deviations above the average OD at zero binding, was 0.7 pg/ml.

Genetic analysis:

For genetic analysis blood sample was collected from the patients and controls in a 9 ml EDTA tube. The sample was stored at -80^{o} Centigrade till DNA extraction.

DNA Extraction:

Genomic DNA was extracted from EDTA-preserved peripheral venous blood by QIAmp Mini kit method.

Principle: The QIAamp DNA Blood Mini Kit is used to isolate DNA from blood with fast spin-column. DNA binds specifically to the QIAamp silica-gel membrane while contaminants pass through. PCR inhibitors such as divalent cations and proteins are completely removed in two efficient wash steps, leaving pure nucleic acid to be eluted in either water or a buffer provided with the kit.

Procedure:

Optimized buffers lyse the samples, stabilize nucleic acids, and enhance selective DNA adsorption to the QIAamp membrane. Alcohol is then added and lysates loaded onto the QIAamp spin column. Wash buffers are used to remove impurities and pure, ready-to-use DNA is then eluted in water or low-salt buffer.

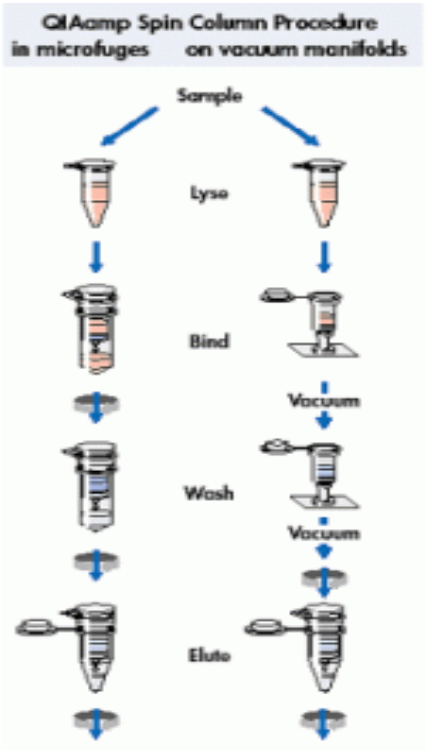

Fig 3: QIAamp Spin Column Procedure

PCR-RFLP:

Screening for both TNF polymorphisms was by done by PCR-RFLP as described by Melita A et al. [121]

TNFA -308: A single-base polymorphism at position -308 in the promoter region was screened using flanking PCR primers, the forward primer incorporating a base mismatch to create an Nco I recognition site (primers: forward S'AGG CAA TAG GTT TTG AGG GCC AT3', reverse 5'TCC TCC CTG CTC CGA TTC

CG3'). After digestion with Nco I (Fermentas), PCR products were sized using a 9% polyacrylamide gel and ethidium bromide staining (product size after digestion: allele I=87 and 20 bp, allele 2= 107 bp).

TNFA -238: A single-base polymorphism at position -238 in the promoter region was screened using flanking PCR primers. The reverse primer was incorporated in a base mismatch to create an *Ava* II recognition site (primers: forward S'GAA GCC CCT CCC AGT TCT AGT TC3', reverse S'CAC TCC CCA TCC TCC CTG GTC3'). After digestion with *Ava* II (Fermentas), PCR products were sized using a 14% polyacrylamide gel and ethidium staining (product size after digestion: allele 1=77, 63, 49 and 21 bp, allele 2=77, 70 and 63 bp).

STATISTICAL ANALYSES:

Data was analysed using STATA software (STATA CORP TX, USA). Continuous data was presented as mean with standard deviation. Data with a skewed distribution was represented as median with ranges.

Parametric data was compared using the student T test, and non parametric data was compared using Mann Whitney U test. To test the relationship between two sets of variables Pearson's coefficient of correlation was used.

RESULTS

Twenty nine consecutive patients who fulfilled the inclusion criteria were selected for the study. A total of 74 individuals were evaluated for inclusion as controls. Forty four of them were excluded, 22 had evidence of fatty liver on ultrasound, 10 of them had a history of alcohol use, 5 had history of diabetes mellitus, and 7 had hypertension. A total of 30 healthy individuals were included as controls.

AGE DISTRIBUTION:

The mean age of patients was 43.38 years (SD 8.8). The mean age of the controls was 42.6 years (SD 8.16)

SEX DISTRIBUTION: CONTROLS

Most of the patients were males. Out of a total of 29 patients, 22 (75.86%)) were males and 7 (24.14%)) were females. The average age of male patients was 42.05 (SD 8.15) years. The average age of the female patients was 47.57 (SD10.11).

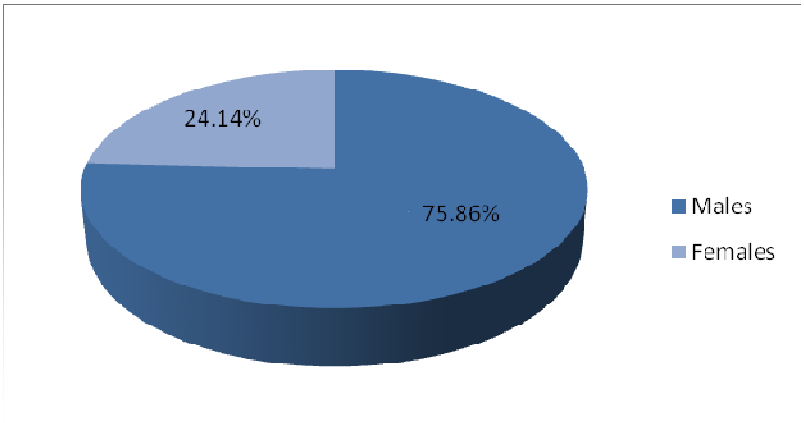

Fig 4: Sex distribution of the patients with NAFLD

Amongst the controls there were 7 females (SD 23.3%). The average age of the males was 42.9 (SD 8.3) years, and that of the females was 42.14 (SD 9.02).

GEOGRAPHIC DISTRIBUTION:

The patients were distributed according zones into north, east, north-east, west and south India. Most of the patients were from eastern India.

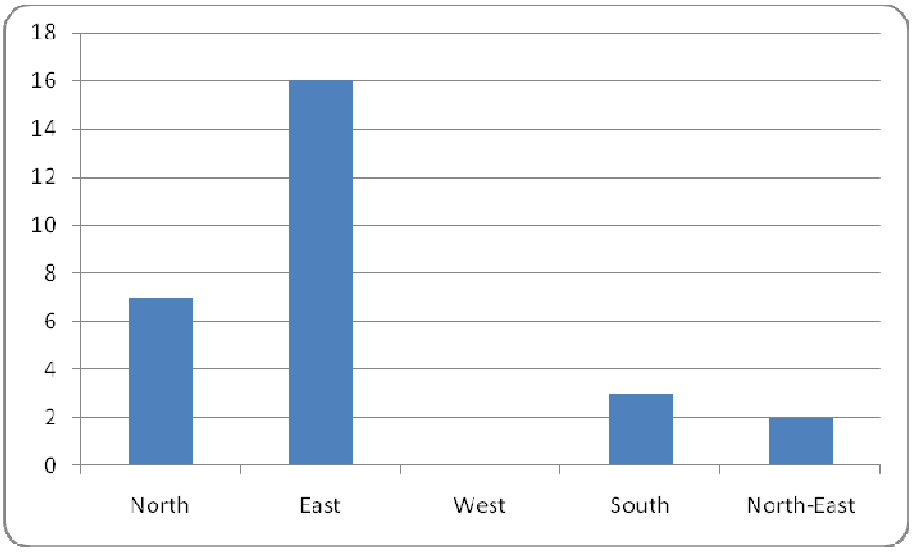

Fig 5: Zonal distribution of cases of NAFLD

Sixteen (55.15%) of the patients were from east India, 7 (24.14%) were from north, three (10.34%) from south, and 2 (6.9%) were from the north east. None of the patients were from the West.

CLINICAL PRESENTATION:

Six (20.7%) of the patients were detected to have fatty liver disease on biopsy while they were being investigated for chronic liver disease. Pedal edema was the commonest manifestation amongst those with chronic liver disease.

Symptoms	**No. Of patients**
Pedal edema	6
Abdominal distension	1
GI bleed	0
Hepatic encephalopathy	0

Table1: Presenting symptoms of the patients with chronic liver disease.

Amongst the rest (n=23) most were asymptomatic (n=14 (60.9%)) and or discovered to have fatty liver on ultrasound while being investigated for minor symptoms such as fatigue (n=9(39.01%)) or when they were incidentally detected to have raised ALT either during workup for another illness, during planned organ donation or during a medical check-up.

HISTORY OF COMORBIDITIES:

Among the patients, 4(13.8%) had history of diabetes mellitus but were not on any medications, 7 (24.13%) were hypertensive and 4 (13.8%) had history of dyslipidemia. None of the female patients had a history of polycystic ovarian disease. Presence of two risk factors was noted in only 3 subjects. As absence of co-morbidities was an inclusion criterion for controls none of the controls had any of them.

Co-morbidity	**Number**	**Percentage**
Diabetes mellitus	4	13.8%
Hypertension	7	24.13%
Dyslipidemia	4	13.8%

Table 2: Self reported frequency of comorbidities in patients with NAFLD.

FAMILY HISTORY:

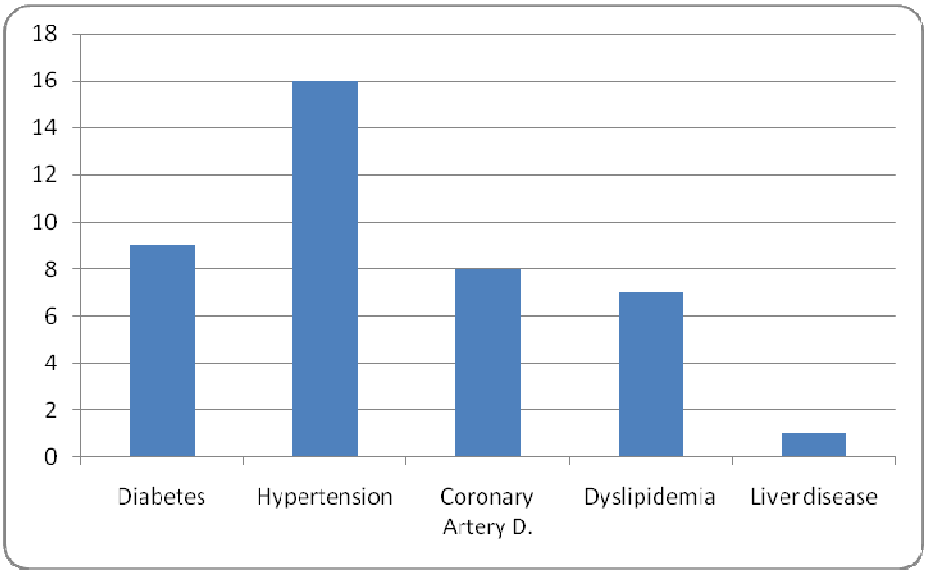

Fig 6: Family history of patients with NAFLD

Sixteen (55.2%) patients had a family history of hypertension. Nine (31.1%) had diabetes mellitus, 8 (27.6%) had coronary disease, and 7(24.1%) had dyslipidemia. Only one of the patients had a family history of liver disease, details of which were not available.

ADDICTIONS:

As alcohol use was a criteria of exclusion none of the patients had a history of alcohol use. Three of the patients were regular smokers and 7 patients had a history of consumption of chewing tobacco.

EXERCISE:

Patients were divided into four groups based on their frequency of exercise. Anyone who performed aerobic exercise > 5 days per week was labelled as regular, 3-4 times per week as modest, 0-2 times per week as occasional and the last group was those who never exercised. Most patients (n = 24 (82.7%)) never exercised, or did it

very infrequently. Only one patient did exercise every day of the week. Amongst the controls none had a history of regular exercise or manual labour.

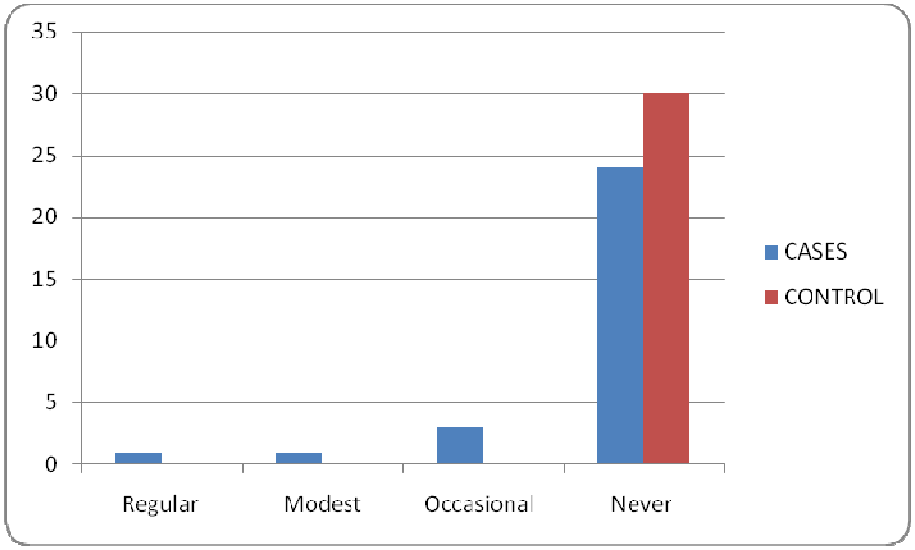

Fig 7: Excercise frequency amongst cases and controls

ANTHROPOMETRY:

One of the patients had a decompensated chronic liver disease so in him BMI was not calculated. In the rest, the average body mass index of patients was 25.18 kg/m^2 (SD 2.8). Fifteen (51%) of the patients had a BMI of ≥ 25. The mean BMI of the controls was 22.2 kg/ m^2 (SD 2.2). There was significant difference between the BMIs of the cases and controls (*P*< 0.001).

Parameter	Cases (Mean)+SD	Controls (Mean)	*p*
BMI	25.18 kg/m^2	22.2 kg/ m^2	< 0.001
WC	93.7 cms	77.5 cms	<0.001
WHR	0.99	0.92	<0.001

Table 3: Comparing BMI, waist circumference (WC) and waist hip ratio (WHR) between the cases and controls.

The average waist circumference (WC) in 28 patients was 93.7 cms (SD 11.21). Amongst the controls the average waist circumference was 77.5 cms (SD 8.7).

There was a significant difference between the two groups (P<0.001). There was also a significant difference in the waist to hip ratio (WHR) between the cases and controls (P<0.001).

PHYSICAL EXAMINATION:

Pedal edema was noted in 6 (20.6%) patients. Two (6.8%) of the patients had jaundice. Hepatomegaly was noted in 7 (24.14%) patients. One had ascites on physical examination. None had history of GI bleed or hepatic encephalopathy. Cardiovascular, respiratory and neurological examination was normal in all the patients.

BIOCHEMISTRY:

Fasting blood sugar (FBS):

FBS was determined in all the patients, the mean FBS was 104.31 (SD 20.4). Six (20.7%) patients had a fasting sugar > 126 mg/dl. Impaired glucose tolerance (FBS ≥ 100 mg/dl) was noted in 7 (24.1%) patients.

Serum Insulin:

The serum insulin estimation was done in 24 of the 29 study subjects. The mean S.insulin levels was 13.54 (SD 10.38). The HOMA – IR (homeostasis model of insulin resistance) was calculated using the equation (fasting insulin (μU/ml) X fasting glucose (mg/dl))/405) and depicted as HOMA-IR value. The mean HOMA-IR was 3.47 (SD 2.76). Duseja et al in a study done in PGI Chandigarh used a cut-off for HOMA-IR of >1.64 to define insulin resistance.[25] Using this as a cut-off we find that 20 (83.3%) out of 24 patients in whom HOMA – IR has been calculated are insulin resistant.

Lipids:

Parameters	Values
Cholestrol mg% (Mean (SD))	162.75 (50.2)
Triglycerides mg% (Median(range))	146 (50 -429)
HDL mg%(Mean (SD))	36.9 (18.4)
LDL mg%(Mean (SD))	95.4 (44.4)

Table 4: Lipid profile of patients with NAFLD

Thirteen (44.8%) of the patients had high triglycerides (> 150). Three (10.3%) of the patients had high LDL(> 160mg%).

Liver function test:

The median S. Bilirubin was 0.8 mg/dl with a range of 0.4 – 6.1 mg /dl. The mean SGOT was 61.86 IU (SD 34.84). The mean SGPT was 84.51 IU (SD 28.13).

LFT parameters	Values
Total Bilirubin (median)	0.8 mg% (range 0.4 – 6.1)
Direct Bilirubin (median)	0.3 mg%(range 0.1 – 4.6)
Total Protein (mean)	8.04 mg% (SD 0.77)
Albumin (mean)	4.4 mg% (SD 0.7)
SGOT (mean)	61.86 IU (SD 34.84)
SGPT (mean)	84.51 IU (SD 28.13).
S. Alkaline phosphatise (mean)	86.68 IU (SD 24.1)

Table 5: Liver function test in patients with NAFLD

ULTRASOUND:

Fatty liver was detected on ultrasound in 23 (79.3%) patients. None of the six patients with chronic liver disease had ultrasound evidence fatty liver. The average grade of fatty liver was 2. No space occupying lesion in the liver was noted in any of the patients. One of the patients had ascites on ultrasonography.

HISTOPATHOLOGY:

Liver biopsy was done in all the patients. In 23 patients the biopsy was done as ultrasound assisted percutaneous liver biopsy. There was no per procedural or post procedural complications. Five patients had a transjugular liver biopsy due to persistent coagulopathy. In one patient the biopsy was taken from the explant liver at cadaveric liver transplantation.

On an average approximately 13 – 14 portal tracts were available for examination. The biopsy was evaluated by a single pathologist who was blinded to the clinical history of the patients. The predominant histological feature was steatosis. The steatosis was diffuse and predominantly macrovescicular, though microvescicular steatosis was also noted. The mean steatosis grade as per NAS score was 2. [94] Lobular inflammation was seen in all except one case. The lobular inflammation consisted of a mixed infiltrate of lymphocytes, histiocytes and neutrophils. On average 1 – 2 foci of lobular inflamtion was noted. The mean lobular inflammation as per NAS score was 1.4 (SD 0.57). [94] Ballooning of hepatocytes was noted in 28 patients (96.5%). Mallory's hyaline was noted in 12 patients (41%). In most cases the Mallory's hyaline was not well formed. Bile ductular changes in the form of ductular proliferation were seen in 5 (17%) patients. Fourteen (48.2%) patients had evidence of fibrosis on liver biopsy. Various grades of fibrosis was noted in the patients. Six had evidence of stage 4 fibrosis and steatohepatitis.

FIBROSIS STAGE	Number (%)(n=14)
1A	0
1B	1(0.07%)
1C	5(35%)
2	0
3	2(14%)
4	6(42.8%)

Table 6: Different stages of fibrosis in patients with NAFLD

Comparison between patients with fibrosis and those without fibrosis:

As the presence of fibrosis suggests a more advanced and severe liver injury. [91]

A subgroup analyses was done comparing those with and those without fibrosis.

Parameter	**Fibrosis *n*= 14**	**No Fibrosis *n*=15**	***P* value**
Age mean (SD) yrs	47.7 (7.29)	39.2 (7.8)	0.006
BMI kg/m^2 * (mean (SD))	(n=13) 25.5 (2.8)	(n=15) 24.8 (2.2)	0.53
Waist Circumference cms ** (WC)	(n=13) 96 (11.2)	(n=15) 91.1 (8.7)	0.03
HOMA-IR *** (mean (SD))	(n=12) 4.15 (3.3)	(n=12) 2.73 (1.9)	0.25
Lipid profile			
Triglycerides mg% (mean (SD))	129.2 (96.4)	191.8 (84.9)	0.01
HDL mg% (mean (SD))	31.7 (10.15)	41.7 (23.15)	0.15
SGOT U/L (mean (SD))	71.9 (36.6)	54.26 (23.13)	0.35
SGPT U/L (mean (SD))	95.1(49.9)	73.14 (53.17)	0.25

Table 7: Comparison between patients with fibrosis and those without fibrosis. *BMI was calculated in 28 patients **Waist circumference was calculated in 28 patients *** HOMA-IR was calculated in 24 patients

There was a significant difference between the two groups in their mean age, waist circumference, and triglyceride concentrations.

TNF α IMMUNOASSAY:

TNF α immunoassay was done in twenty cases with NAFLD. The median TNF α level was 23.85 with a range of 0 – 495. There was no correlation between the TNFα levels and the BMI, degree of SGPT elevation, NAFLD activity score (NAS) or the degree of fibrosis.

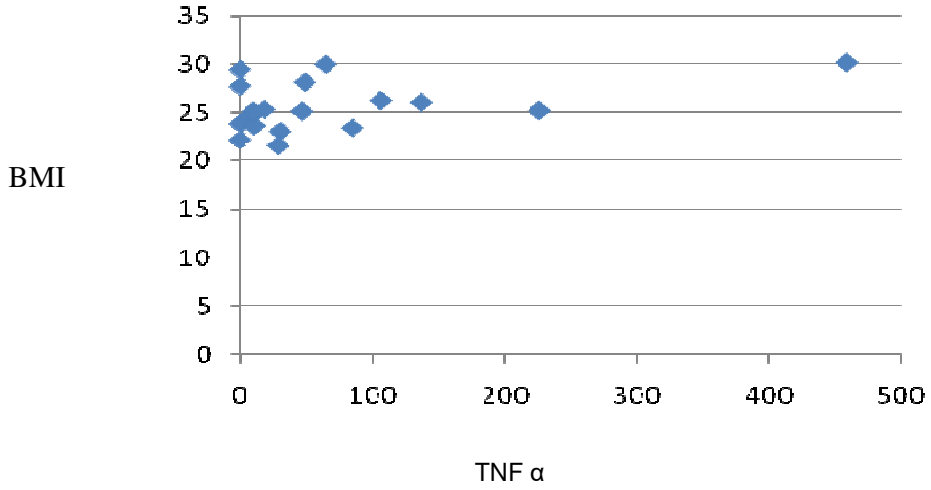

Fig 8: Scatter plot showing no correlation of TNFα with BMI. r = 0.42

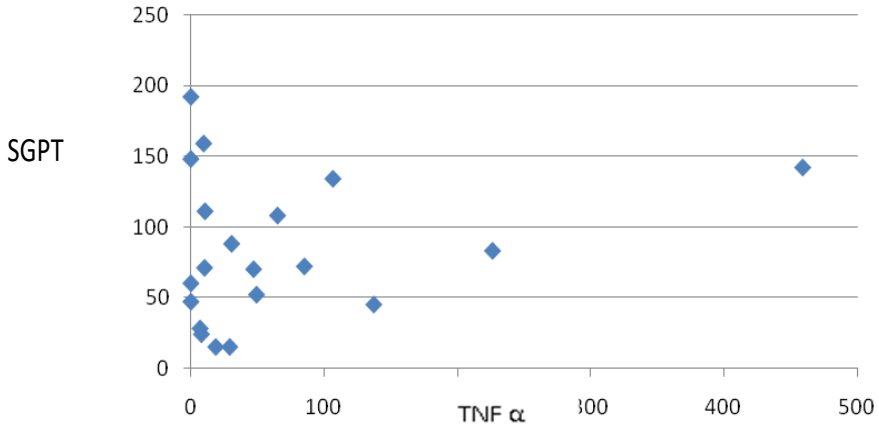

Fig 9: Scatter plot showing no correlation of TNFα with SGPT. r = 0.21

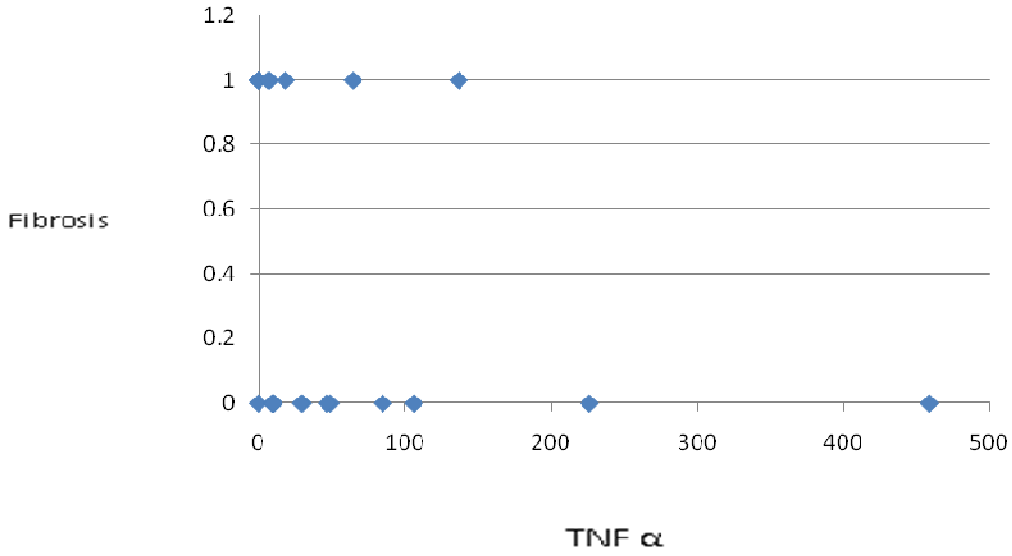

Fig 10: Scatter plot showing no correlation of TNFα with fibrosis. r =

INDIRECT MARKERS OF LIVER FIBROSIS:

Two scores were evaluated as non invasive predictors for the presence or absence of significant fibrosis.

AST/ ALT ratio:

A ROC curve was constructed using AST/ALT ratio to differentiate patients with histological evidence of fibrosis from those without fibrosis. The area under curve (AUC) was 0.88. Using a cut off of ≥ 0.72, the sensitivity and specificity for detection of fibrosis was 85.6% and 80.1% respectively.

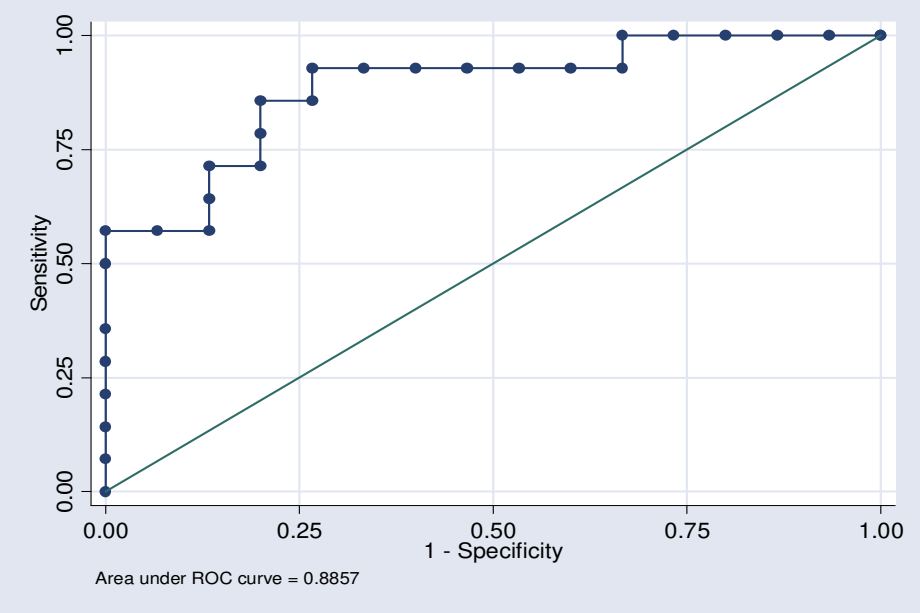

Fig 11: ROC curve using AST/ALT ratio to differentiate fibrosis from no fibrosis. (AUC = 0.88)

APRI:

The aspartate aminotransferase/platelet ratio index (APRI) as predictive model for liver fibrosis consists of objective and readily available laboratory variables.

Its calculated by the formula as per Wai et al:[122]

$$\text{APRI} = \frac{\text{AST level (/ULN)}}{\text{Platelet counts } (10^9/\text{L})} \times 100$$

ROC curve was constructed using APRI to differentiate patients with histological evidence of fibrosis from those without fibrosis. The area under curve (AUC) was 0.81. Using a cut off of > 1, the sensitivity and specificity for the detection of fibrosis was 78.5% and 87% respectively.

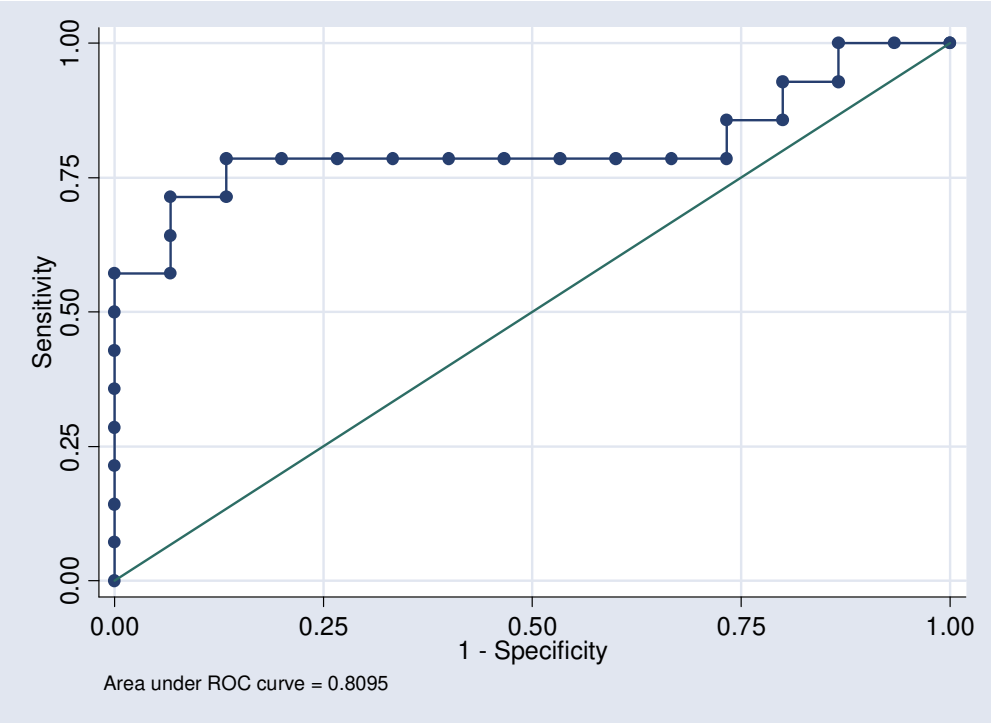

Fig 12: ROC curve using APRI to differentiate fibrosis from no fibrosis. (AUC =0.81).

TNF α POLYMORPHISMS:

Polymorphisms in the promoter region of the TNF α gene was evaluated using restriction fragment length polymorphism in both the cases and the controls. Two specific polymorphisms that was evaluated were -238 and -308. Of the 29 cases we were unable to amplify 4 samples for both -238 and -308. Of the 30 controls DNA for -238 could not be amplified for 2 samples. The frequency distribution of the two polymorphism is shown in tables below.

-238	G/G	A/G	AA	*p* value
NAFLD (n=25)	25(100%)	0	0	0.46
CONTROL(n=28)	26(92.9%)	2(7.1%)	0	

Table 8: Frequency distribution of -238 site polymorphism

-308	G/G	A/G	AA	*p* value
NAFLD (n=25)	22 (88%)	3(12%)	0	0.33
CONTROL(n=30)	29(96.67%)	1(3.33%)	0	

Table 9: Frequency distribution of -308 site poymorphism

There was no significant difference in the allele frequency between the cases and control in both loci.

DISCUSSION

The study is a cross sectional study of 29 patients with Non Alcoholic Fatty Liver Disease (NAFLD) conducted over a period of 24 months at Christian Medical College, Vellore. Numerous studies have been published on NAFLD since its initial description by Ludwig et al in 1980. Very few studies have been done in India on NAFLD, using liver biopsy. No study has yet examined the role of TNF α among Indian patients with NAFLD.

The patient population for the study was selected from patients attending the gastroenterology and liver clinics at CMC, Vellore. Consecutive patients diagnosed to have fatty liver disease either during workup for another illness, during planned organ donation, during a medical check-up, or in the course of evaluation for chronic liver disease were evaluated for inclusion in the study. This approach of selection of cases we felt reflected the scenario faced by a clinician.

Demography:

The mean age of the patients was 43.38 years (SD 8.8), with an age range of 30 – 60 years. Though NAFLD can affect people of any age, the prevalence of the disease seems to increase with age. [26] Amarapurkar et al in their study on the prevalence of NAFLD reported that the disease had its highest prevalence in the age group of 40 to 60 years. [24] Duseja et al also reported a mean age of 37.4 (SD 10.71) with an age distribution between 16 – 69 years in their patients with NAFLD [25]. This was similar to our observations.

In the current study majority of the patients were males (75.86%). Initial studies had shown a higher prevalence of the disease amongst women [23]. Though, recent studies have shown an almost equal prevalence in both sexes. Two studies from India have reported a higher prevalence of NAFLD amongst the males.[24,25] Duseja et

al had attributed this high male prevalence to the existence of a referral bias. As our study was biopsy based, so factors other than referral (viz. reluctance of females to undergo liver biopsy) may have affected the gender distribution.

In the present study the patients were divided into different geographic regions, viz. North, East, North-East, West and South. Majority of the patients were from Eastern India, (55.15%). This probably reflects the profile of patients visiting CMC for treatment.

CLINICAL PRESENTATION:

Most patients with NASH are asymptomatic although fatigue, malaise, and vague right upper abdominal discomfort bring some patients to medical attention. [72] In this study, many patients had an incidental detection of raised ALT either during workup for another illness, during planned organ donation, or during a medical check-up. Easy fatigability was the most common symptom reported by the patients. In a recent study it was found that autonomic symptoms are prevalent in NAFLD and is associated with objective measures of autonomic dysfunction. Fatigue in NAFLD is probably associated with autonomic dysfunction. [123]

COMORBIDITIES:

Diabetes mellitus has been associated with NAFLD. Amarapurkar et al reported diabetes mellitus in 22 % of their patients with NAFLD. [24] In our study we found that only 13.8% of the patients were known diabetics. But, on evaluation it was found that 20.7% of the patients were diabetics. Hypertension was noted in 24.13% of the patients. None of the patients reported obesity as a problem. Most of the patients were unaware of their lipid status. Only 13.8% of the patients had a history of dyslipidemia.

The low rates of reporting of the metabolic factors amongst patients with NAFLD are probably because of unawareness amongst the patients of the presence of metabolic risk factors. Bansal et al in a study among urban Indians showed that a significant proportion of the individuals are unaware of the existence of metabolic risk factors in them. [124]

PHYSICAL ACTIVITY:

Based on the degree of regular excercises performed by an individual, the patients were divided into four groups. Most of the individuals (n = 23 (79%)) never exercised. None of the controls participated in exercise. Zelber-Sagi et al have shown that patients with NAFLD engaged in less aerobic, resistance or other kinds of physical activity. [125] Also in Indians levels of physical activity are low [126] putting them at particularly high risk for the development of metabolic syndrome.

FAMILY HISTORY:

A number of patients (31%) had a family history diabetes mellitus. Previous studies have shown that approximately 30% have a family history of diabetes mellitus in a first-degree relative. [33]

ANTHROPOMETRY:

Amongst Asians, the cut-offs for overweight and obesity is ≥ 23 kg/ m^2 and ≥ 25 kg/ m^2 respectively. [29] Twenty four percent of patients had a BMI above 23 kg/ m^2 and 51% of patients had a BMI of ≥ 25 kg/ m^2. Dusseja et al in their study reported that 20% of their patients were overweight as per the Asian criteria and 51% were obese.[25] This is similar to our observations. Most studies have noted a high BMI in patients with NAFLD. Studies have shown that BMI is an independent predictor of the degree of fat infiltration, but not fibrosis. [80]

The average waist circumference in the 28 patients without ascites was 93.7 cms (SD 11.21). Studies have shown that central obesity is a correlate of visceral adiposity and is more closely linked to insulin resistance, the central event in NASH.[30]

There was a significant difference in the BMI and waist circumference of individuals with NAFLD when compared to the controls. Jakobsen et al in a review of several cross sectional and case control studies have shown that people with NAFLD have higher waist circumference (WC) or BMI than those without NAFLD, and have reported significant associations between abdominal obesity and NAFLD. [27]

PHYSICAL EXAMINATION:

Examination revealed that only a few patients had jaundice (6.8%) and pedal edema (20.6%). Hepatomegaly was noted in 24.14% patients and ascites in only one patient. Mark Anthony et al in their study noted that physical examination in patients with NAFLD is unremarkable .[127]

BIOCHEMISTRY:

Fasting blood sugar:

Epidemiologic studies have demonstrated that T2DM occurs in 21% to 45% of patients with NAFLD. [32, 33,] Diabetes mellitus was noted in 20.7% and impaired fasting glucose in 24.1%. Duseja et al report that diabetes mellitus is not a common association with NAFLD. In their study they found that only 12% of the patients had diabetes mellitus and 14% had impaired fasting glucose. [25] But, in the other Indian study by Amarapurkar et al diabetes mellitus was noted in 22% of the patients which is similar to our observations.

Dyslipidemia:

The average trigylceride concentrations in our patients with NAFLD is 161.5 mg%. Hypertriglyceridemia was seen in 44.8% patients. Duseja et al had noted hypertriglyceridemia in 53 % of the patients.[25] Thus a significant number of people with NAFLD have hypertriglyceridemia.

HOMA-IR:

Homeostatic model assessment (HOMA-IR) is a method for assessing β-cell function and insulin resistance (IR) from basal (fasting) glucose and insulin concentration. HOMA has been compared with a number of well-validated methods used to measure IR and β-cell function. There is good correlation between estimates of IR derived from HOMA and from the euglycemic clamp, which is considered the gold standard. [128] The mean HOMA-IR was 3.47 (SD 2.76). Different workers have used differing cut off of HOMA-IR to define insulin resistance. [25,129] Duseja et al used a cut off of > 1.64 to define insulin resistance. [25] Using the same cut-offs as Duseja et al we found that 83.3% fo our patients have insulin resistance. A previous study conducted by us comparing the HOMA-IR between patients with NAFLD and healthy young males in whom fatty liver was excluded using 1H NMR spectroscopy showed a significantly higher HOMA-IR in the patients with NAFLD as compared to healthy controls. [130]

SGOT and SGPT:

SGPT concentration is the most commonly used variable for assessment of liver disease. [74,75] Prati et al suggested that the upper limit of normal for SGPT and SGOT amongst patients with NAFLD should be decreased to 30 U/L for men and 19 U/L for women. [76] Both the SGOT and SGPT were elevated in our patients with NAFLD. The mean SGOT was 61.86 IU (SD 34.84), and mean SGPT was 84.51 IU (SD 28.13)

but, the degree of elevation was low. Powell et al noted that most patients with NAFLD, have mild-to-moderate elevations of serum aminotransferase levels, which are typically less than five times the upper limit of normal. [131]

TNF α Immunoassay:

TNF α immunoassay was done using EASIA in 20 cases. A wide dispersion was noted in the TNF α levels. No correlation was noted between the serum TNF alpha and the aminotransferase levels, the NAS score or the degree of fibrosis. This is in contrast to most western studies which have shown high levels of TNF alpha in patients with NAFLD. Louthan et al in a study on a paediatric population did not find any significant correlation of serum TNF α AND NAFLD. [133] The reasons for the discrepancy could be that TNF-*α* has a relatively short half-life and low circulating levels, which may not reflect the changes occurring in the liver tissue The sensitivities of the assay and the population in which it is being tested may also play a role. [134]

HISTOLOGY:

In this study liver biopsy was done in all patients. In patients with normal coagulation parameters ultrasound assisted percutaneous liver biopsy was done. In six of the patients liver biopsy was done using the transjugular route. The biopsy was staged using the system devised by NASH Clinical Research Network (CRN). [94]

Both macro and microvescicular steatosis were noted in our patient but, macrovescicular steatosis was the predominant form. In NAFLD, studies have noted that though there is both macro and microvescicular steatosis, fatty change is predominantly macrovescicular. [93] Lobular inflammation was noted in all except for one patient. The lobular inflammation consisted of a mixed infiltrate of lymphocytes, histiocytes and neutrophils. On an average there were about 1 – 2 foci of lobular

inflammation was noted. A mixed lobular inflammation has been reported in NAFLD by Yeh et al. [89] The most important diagnostic criterion for distinguishing steatohepatitis from simple steatosis is the presence of hepatocyte ballooning. [93] Ballooning was noted in 96.5% patients. Thus, majority of our patients had steatohepatitis. Long-term follow-up studies confirm that patients with steatohepatitis may have increased liver-related mortality compared with non-NASH patients. [135]

Liver fibrosis appears to be one of the most important prognostic factors, as the presence of fibrosis suggests a more advanced and severe liver injury. [91] Fibrosis was noted in 48.2% of our patients with NAFLD. This was notably higher than that reported by Madan et al. [136] This may be probably because of a different selection criteria used in the two studies. In this study we included cases detected to have fatty liver disease either during workup for another illness, during planned organ donation, during a medical checkup, or in the course of evaluation for chronic liver disease this we believe is the clinical spectrum seen by the clinicians in OPD.

As fibrosis is an important prognostic factor and representative of more advanced liver disease, we evaluated the difference in certain parameters between those with and those without liver fibrosis. Only three parameters were found to be significantly different between the two groups viz. the mean age, waist circumference, and triglyceride concentrations. Age > 45 and waist circumference have been noted in to be independent predictors of liver fibrosis in NAFLD. [80, 5] We noted that patients with fibrosis had lower triglyceride concentration that those without fibrosis this was different from the study by Ratziu V et al [100].

No difference was noted in the BMI of patients with and without liver fibrosis. Beymer et al also noted that there was no difference in BMI between patients with and without NASH or advanced fibrosis.[137]

Insulin resistance as determined by HOMA-IR in patients with fibrosis on histology was also no different from those without fibrosis. Insulin resistance did not appear to be linked to fibrosis and decreasing insulin resistance may not affect regression of advanced disease. This seems to be borne out by the results of the trial on Rosiglitazone (FLIRT 2 trial), in which improvement in insulin sensitivity did not improve the NASH score. [95] Similarly there was no difference in the SGOT and SGPT levels between those with and those without liver fibrosis. Studies have shown that the SGOT and SGPT do not correlate with the degree of liver damage in NASH. Mofrad et al in their study noted that the entire histological spectrum of NAFLD can be seen in individuals with normal ALT values. The histological spectrum in these individuals is not significantly different from those with elevated ALT levels, and a low normal ALT value does not guarantee freedom from underlying steatohepatitis with advanced fibrosis. [79]

NON INVASIVE MARKERS OF LIVER FIBROSIS:

As liver biopsy is an invasive test we evaluated the role of two non invasive markers to identify liver fibrosis in patients with NAFLD.

AST/ALT ratio:

An AST/ALT ratio of > 0.72 was found to be predictive of fibrosis with a sensitivity and specificity of 85.6% and 80.1% respectively. Williams et al had suggested that an AST/ALT ratio of > 1.0 may suggest they prescence of cirrhosis.[138] But, two other studies on patients with hepatitis C did not find the predictive accuracy of AST/ALT ratio to be high.[139,140]

AST to platelet ratio index:

This predictive model consists of objective and readily available laboratory variables.[122] In the current study using a cut off of ≥ 0.72 for APRI, the sensitivity

and specificity for detection of fibrosis was 78.5% and 87% respectively. Several studies have described the test characteristics of APRI in HCV infected patients. Loaeza et al in their study found that in patients with NAFLD, APRI values tend to increase with the degree of fibrosis.[141]

We find that both the tests are useful but, not 100% specific to predict fibrosis. We therefore feel that the AST/ALT ratio and APRI could be useful in this disease to counsel patients about their need for a liver biopsy.

TNF α POLYMORPHISM:

In the current study we evaluated the presence of two polymorphisms in the TNF α promoter region(-238, -308). In India, studies have been conducted looking at the role of TNF α polymorphisms in other diseases. Gupta et al conducted a study on patients with oral cancer and did not find any significant change at the -238 promoter site.[69] Another study by Singhal et al on patients with viral hepatitis also did not find any polymorphism at -238 site.[70] This is the first study in an Indian population looking at the presence of TNF α polymorphisms in patients with NAFLD. We found 2 heterozygotes at -238 amongst controls which is different from the two prior studies mentioned above. We did not find any difference in the allele frequency between the cases and controls for both loci. In a study conducted by Luca Valenti et al on an Italian population of patients with NAFLD the genotype distribution of the -238, but not of the 308, was significantly different between patients and controls ($P < 0.0001$). [12] In our study we found that polymorphisms in -238 do exist amongst Indians, and increasing the sample size may help us better understand the role of these two polymorphisms in patients with NAFLD.

CONCLUSION

1. In this study we found six (20.7%) NAFLD patients, presented with features of chronic liver disease. Amongst the rest, most were asymptomatic (60.9%) or had minor symptoms such as fatigue (39%). This may be a reflection of our method of recruitment and the also the nature of the site of our study. (tertiary care in center)
2. Amongst those with chronic liver disease pedal edema was the commonest manifestation.
3. The average BMI of our patients with NAFLD was 25.18 kg/m2. Using the Asian criteria, 51.7 % of our patients are obese.
4. In the NAFLD patients, diabetes mellitus was present in 20%, and hypertriglyceridemia in 44%.
5. Insulin resistance is present in patients with NAFLD. Using previously quoted cut off > 1.64 for HOMA-IR, 83.3% of our patients were insulin resistant.
6. Insulin resistance does not correlate with fibrosis and factors other than insulin resistance may be responsible for disease progression.
7. Using hepatocyte ballooning as the most important diagnostic criterion to distinguish NASH from simple steatosis, we found that almost all our patients had evidence of NASH.
8. A significant number (48.2%) of the patients have evidence of liver fibrosis.
9. An older age and a larger waist circumference is associated with an increased risk for fibrosis.
10. The SGOT and SGPT levels do not correlate with the liver fibrosis.
11. We found that simple laboratory test such as AST/ALT ratio and APRI were useful as indirect markers of fibrosis. Fibrosis could be predicted in ~ 80% of

individuals using these markers. These markers may be a useful guide to counsel patients regarding their need for a liver biopsy.

12. TNF alpha levels do not correlate with the histological activity or fibrosis. However, as the number of samples tested was small increasing the sample size may give a different result.
13. Polymorphisms in the TNF α promoter region (-238, -308) exist in Indians.
14. No significant difference is noted in the allele frequency between the cases and controls for both -238 and -308 loci in the TNF α promoter region.

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PROFORMA

Name: Age: Sex: Male/Female (1/2)

Hosp. No.:

Address:

Tel. / Mobile:

Presenting symptoms: Duration:

Jaundice:

GI bleed / Ascites / Edema:

Incidental detection of elevated transaminases:

Incidental detection of fatty liver:

Co morbidities:

Diabetes Mellitus: Y-1, N-2

Duration: Treatment: Insulin (1) / OHA (2) / Lifestyle modification (3)

Hypertension: Y-1, N-2 PCOD: Y-1, N-2

Dyslipidemia: Y-1, N-2, TG 1 / LDL 2 / Low HDL 3 / Cholestrol 4

Treatment: Drugs Y-1, N-2 Stalin 1 / Fibrates 2 / Others 3 Lifestyle: Y-1, N-2

Surgery: Y-1, N-2

Drug History: (Drugs taken within previous 6 months) Y-1, N-2

Allopathic:

Homeopathic:

Indigenous / Ayurvedic:

Family History: (first degree relatives) Y-1, N-2

Diabetes: Y-1, N-2 Hypertension: Y-1, N-2 CAD: Y-1, N-2

Hyperlipidemia: Y-1, N-2 Liver diseases: Y-1, N-2

Personal History:

Addictions:

1. Alcohol Y-1, N-2 Amount Type: Frequency: Duration:

2. Smoking Y-1, N-2 Type: No.: Duration:

3. Tobacco Y-1, N-2 Type: Duration:

Exercise: 1. Regular (>5 days per week) 2. Modest (3-4 days per week)

3. Occasionally (0-2 days per week) 4. Never

Physical Examination:

Body weight: Height: BMI: Waist: Hip: WHR:

Spiders: Y-1, N-2 Parmar erythema: Y-1, N-2 Clubbing: Y-1, N-2

BP: mm Hg Edema: Y-1, N-2 Liver span: cms Spleen (BCM): cms

Ascites: Y-1, N-2 Other findings:

CVS: Normal / abnormal CNS: Normal / Abnormal RS: Normal / abnormal

Abnormal (details):

Investigation:

Date: Hb: Platelet count:

Date: Lipid profile: Cholesterol TG LDL HDL

Date: LFT: TB DB Prot. Alb. AST ALT SAP

Date: AC Insulin HOMA

Date: HBsAg + / - Anti HCV + / -

Utrasound: Fatty liver: Y-1, N-2 Grade I / II / IIISize: Normal 1 / Enlarged 2 / Shrunken 3

CLD: Y-1, N-2 FF: Y-1, N-2 Collateral: Y-1, N-2 SOL: Y-1, N-2

Liver biopsy: Y-1, N-2

Steatosis (0–3)

0 = <5% hepatocytes involved
1 = 5–33% hepatocytes involved
2 = 33–66% hepatocytes involved
3 = >66% hepatocytes involved

Lobular Inflammation (0–3)

0 = none
1 = <2 foci per · 200 field
2 = 2–4 foci per · 200 field
3 = >4 foci per · 200 field

Hepatocyte ballooning (0–2)

0 = none
1 = few ballooned cells
2 = many cells/prominent ballooning

Fibrosis stage

1 Perisinusoidal or periportal
1A Mild, zone 3, perisinusoidal
1B Moderate, zone 3, perisinusoidal
1C Portal/periportal fibrosis only
2 Perisinusoidal and portal/periportal fibrosis
3 Bridging fibrosis
4 Cirrhosis

TNF- α ASSAY:

TNF-α – 238 / -308 POLYMORPHISM:

CONSENT FORM

1. **Informed consent document for patients with NAFLD:**

I understand that Dr. Sudipta is doing a study on patients with a condition that I suffer from. Diagnosis in my case was made after blood test, ultrasound and a liver biopsy. After the diagnosis was made he asked me permission to take sample of blood for genetic and biochemical studies. I am agreeable for this. I understand that my participation in the study may or may not have any impact on the future of treatment of my condition and that it has been taken purely for research purpose. I also understand that my diagnosis and the results obtained from the study would be kept strictly confidential and no part of it would be divulged without my permission.

Study Title: Non Alcoholic Fatty Liver Disease – A cross-sectional study with special emphasis on the role of TNF alpha and genetic polymorphisms in the TNF alpha gene in the disease progression

Study Number:

Subject's Initials: _____ Subject's Name: _____

Date of Birth / Age:_____

Please initial box

(Subject)

(i) I confirm that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask questions. [ ]

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. [ ]

(iii) I understand that the authors of the study and others working on the author's behalf, the Ethics Committee and the regulatory authorities will not need my

permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published. [ ]

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s) [ ]

(v) I agree to take part in the above study. [ ]

Signature (or Thumb impression) of the Subject/Legally Acceptable Representative:_____ Date: _____/_____/_____

Signatory's Name: _____

Signature of the Investigator: _____Date: _____/_____/_____ Study Investigator's Name: _____

Signature of the Witness: _____

Date:_____/_____/_____

Name of the Witness: _____

2. Informed consent document for controls:

I understand that Dr. Sudipta is doing a study on patients with a condition called Non alcoholic fatty liver disease. I also understand that I have no disease and my inclusion in the study is as part of a healthy control population. I have been explained about the need of obtaining a 9 ml sample of blood for the study, and that I have to undergo an ultrasound examination to rule out fatty liver. I am agreeable for this. I understand that my participation in the study is purely for research purpose and that my diagnosis and the results obtained from the study would be kept strictly confidential and no part of it would be divulged without my permission.

Study Title: Non Alcoholic Fatty Liver Disease – A cross-sectional study with special emphasis on the role of TNF alpha and genetic polymorphisms in the TNF alpha gene in the disease progression

Study Number:

Subject's Initials: _____ Subject's Name: _____

Date of Birth / Age:_____

Please initial box

(i) I confirm that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask questions. [ ]

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to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published. [ ]

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s) [ ]

(v) I agree to take part in the above study. [ ]

Signature (or Thumb impression) of the Subject/Legally Acceptable Representative:_____ Date: _____/_____/_____

Signatory's Name: _____

Signature of the Investigator: _____Date: _____/_____/_____ Study Investigator's Name: _____

Signature of the Witness: _____

Date:_____/_____/_____

Name of the Witness: _____