Nonalcoholic Fatty Liver Disease (NAFLD) has become a major concern both in the developed and developing world due to sedentary lifestyle and excess consumption of processed energy-rich foods. The parallel rapid growth of overweight and obese individuals and of type 2 diabetes is striking, and led to the coining of the term 'diabesity'. It has also been shown conclusively that the most prevalent form of insulin resistance is found among patients with an excess of abdominal fat. In the West, about 40% of the NAFLD patients have associated Metabolic Syndrome (MS), however, the same has not been studied in the Indian population; a country which is estimated to house 79.4 million diabetics by 2030. Non-alcoholic steatohepatitis (NASH), a type of NAFLD, is also now being recognized as one of the commonest causes of cryptogenic cirrhosis.

It is difficult to establish the association of NAFLD or cirrhosis with MS because the body fat distribution in Indians is different from the West, especially with reference to the abdominal obesity pattern and this poses problems in estimating the prevalence of MS as per NCEP-ATP-III. However there is a paucity of literature in this area from developing countries of the Asia Pacific region. We studied the biochemical and clinical profile of NAFLD patients and the association of NAFLD with metabolic syndrome in the Indian patients.

Adipokines, the hormones secreted by the adipose tissue, such as adiponectin, leptin, resistin, TNF-α and interleukin-6 have an important role in the development of insulin resistance and NAFLD. Co-ordinated action of liver, adipose tissue and muscles is essential for normal energy homeostasis. The coordinated action requires communication between these organs and here adipokines, plays an important role. Any discord between adipose tissue-liver-muscle axis leads to aberrant distribution and utilization of lipids and carbohydrates are manifested as glucose intolerance, dyslipidemia, lipid accumulation in liver, muscle, arterial walls etc. Adiponectin is an insulin sensitizing hormone with anti-inflammatory action. It is found to be protective against steatosis and inflammation of liver. Hypoadiponectinemia is a feature of NAFLD and adiponectin level correlates negatively with the hepatic fat and insulin resistance. Resistin is an adipokine associated with insulin resistance and has pro-inflammatory action. Leptin, plays a role in appetite regulation and adaptation to reduced energy availability. Leptin is directly involved in hepatic
fibrogenesis through hepatic stellate cell activation. However, the role of leptin in NAFLD is debatable. TNF-α is an adipokine that impairs insulin signaling and is pro-inflammatory and might play an important role in the pathogenesis of NAFLD. Our studies have earlier shown that serum TNF-α level are raised in patients with NASH and they are significantly reduced after treatment with pentoxyfylline.

There is limited data where all these adipokines have been evaluated together in a given characterized population. Moreover, it is presumed that Indians differ from the population in the West genotypically, phenotypically as well as in relation to their dietary habits and lifestyle which may make them more vulnerable to metabolic syndrome. However, there is no detailed study of serum adipokines in the Indian patients with NAFLD. This was another area we studied: the association of NAFLD with adipokines especially adiponectin.

The region of chromosome 3 (3q27) that contains the adiponectin structural gene (APM1) has also been found to contain a quantitative trait locus (QTL) with a strong influence on phenotypes of the metabolic syndrome. Adiponectin is the most abundant adipokine. Mutations in the gene are known to cause loss or gain of function. We investigated the pattern and frequency of mutations in adiponectin gene in NAFLD patients of Indian origin. We PCR amplified and sequenced the DNA from NAFLD patients and control subjects and analyzed the exons 2, 3 and significant parts of intron 2 of adiponectin. We also analyzed two regions ahead of exon 1 (where we expected some regulatory elements) of adiponectin. Exon 2 and 3 of adiponectin are translated while exon 1 is untranslated.

SUMMERY OF RESULTS:

NAFLD is not a single disease, but a spectrum of overlapping clinical conditions. It is likely that several genetic defects modulated by an individual's lifestyle and environment play a role. More over, there are several deficiencies in the current nomenclature definition and diagnostic and management which are addressed in the first chapter of synthesis.
Seventy six biopsy proven NASH patients, of which 60 were men, with a mean age 40.05 years (range 18-66 years) and 100 apparently healthy controls with out any evidence of fatty liver on ultrasound scan were studied. More than 60% of NAFLD patients had raised ALT a marker of liver inflammation; while others had normal. Thus ALT values were deceptive in a significant proportion of NAFLD patients. About one fifth had Metabolic Syndrome as per ATP III criteria, compared to one tenth in the control group who had normal liver on ultrasound. When a modified ATP III criterion was used about 40% of the patients and one tenth of the controls had MS. About a third of the non-diabetic patients were insulin resistant with HOMA-IR cut-off set at 3. Patients were obese compared to the controls: mean BMI (25.2 vs. 22.7, p<0.01) and waist circumferences (92.9 vs. 80.8 cm, p<0.01). Stage 1 fibrosis was seen in 30 (39.5%), stage 2 in 10 (13.2%), stage 3 in 6 (7.9%) and stage 4 in 13 (17.1%) patients.

The components of MS and measures of obesity were analyzed using ROC curves to identify which of the components of MS help in discriminating an NAFLD patient from control. We analyzed only male patients as the number of female patients was small. Among the variables, the waist circumference emerged as a dominant predictor of NAFLD in men (men: AUC=0.87, p<0.0001) compared to FBS (AUC=0.64), serum triglycerides (AUC=0.6), hypertension (AUC=0.56) and BMI (AUC=0.72).

We analyzed adipokine profile using ELISA in 56 patients and 18 apparently healthy control subjects with no evidence of fatty liver on ultrasound scan. We thus grouped our study subjects into three categories: (i) fatty liver on ultrasound with persistently normal ALT for at least 6 months (fatty liver), (ii) fatty liver with persistently raised ALT for at least 6 months (NASH) and, and (iii) cryptogenic cirrhosis (when known causes for cirrhosis other than NAFLD were excluded). We had 10, 30 and 16 male patients in the fatty liver, NASH and cirrhosis groups respectively and 18 men in control group. Liver biopsy was done in 25 of the 30 patients who had a fatty liver and raised ALT.

In patients with NASH, adiponectin levels were lower than controls (5.4 ±3 μg/ml vs. 7.2 ±2.9 μg/ml, p=0.037). Lean NAFLD patients had adiponectin levels lower
than overweight patients (3 ± 1 μg/ml vs. 6.7 ± 3.8 μg/ml respectively, p=0.003). Serum resistin levels were higher in NAFLD patients (3.7 ± 3 ng/ml) than controls (2.1 ± 1.7 ng/ml, p=0.007). This difference was significant even when cirrhotic patients were excluded (3.4 ± 2.7 ng/ml, p=0.036). Serum leptin levels were raised in cryptogenic cirrhosis in comparison with ‘fatty liver’, NASH and controls (median=8.5, range: 0.5-44 pg/ml; median=5.0, range: 1.4-7.4 pg/ml; median=3.4 pg/ml, range: 1-19.7; median=3.6, range: 2.1-7.7 pg/mL respectively). Serum TNF-α was significantly elevated in cryptogenic cirrhosis and NASH with reference to fatty liver or controls (cirrhosis median 49.9 (5.6-300), NASH median 31 (9.9-288), ‘fatty liver’ median 21.5 (4.8-242), controls 0.65 (0.1-10.8) pg/ml, p<0.001). Serum TNF-α showed a positive correlation with blood pressure, serum insulin, HOMA-IR and AST in patients.

We analyzed single nucleotide polymorphisms by direct sequencing in A3 region (exon 2 and intron 2) in 20 apparently healthy controls with out fatty liver on ultrasound scan (10 both strands and 10 one strand) and 11 biopsy proven NAFLD patients (one strand in 10, both strands in one) and in A2 region (exon 3) we analyzed in 14 controls (both strands in 10 and one strand in 4) and 10 patients (one strand in 10, both strands in one). In S7’ region we amplified and sequencing was attempted in 10 controls and 18 patients (both strands). In P1 region (where certain regulatory elements are expected) we analyzed 10 controls and 20 patients (both strands in all cases). There were corrupt areas in sequences which were ignored. The known single nucleotide polymorphisms identified in the regions (A3, A2, S7’, P1) through an NCBI database search.

We identified single nucleotide polymorphisms rs 2241766, rs 3821799, rs 28973162 and +349 in A3 region. In our study SNP rs 2241766 was associated with NASH patients compared to controls. The association was nearly significant (p=0.064). But, we didn’t observe polymorphism at +276. We got an indication that rs 3821799 in A3 region could be significant in NAFLD. We had only one patient sequence in which this region could be read. Locations of other SNPs were examined for polymorphism but were not observed. In A2 region we searched for known SNPs rs 62625753, rs 17366743, rs 28973168, rs 4686440 and rs 28973167
but we were not able to detect any polymorphism. We were not able to sequence the
S7' amplicons obtained after electrophoretic separation and purification as there was
homopolymer region in this DNA sequence which was not amenable to ordinary
sequencing method. We analyzed P1 region for SNPs in 60 sequences (30 forward
and 30 reverse). We observed no polymorphism with reference to the two known
SNPs rs35088921 and rs62620185 reported in the database. We did not identify any
novel mutation/SNPs in this region in any of the sequences analyzed. Here in our
study allelic equilibrium of the observed variations was not evaluated because of the
small sample size and weakness of the sequencing data. Similarly, we were unable
to evaluate the association of the SNPs observed and their significance to NAFLD,
liver biopsy findings, severity and progression of the disease because of the small
sample size.

CONCLUSIONS:

- Insulin resistance and obesity were associated in a proportion of Indian
patients with NAFLD but the association with MS in Indian patients was not
strong enough. About one fifth had Metabolic Syndrome as per ATP III
criteria, compared to one tenth in the control group who had normal liver on
ultrasound. About 60% of NAFLD patients had raised ALT, a marker of
liver inflammation while others had normal; thus ALT values need to be
taken with caution.

- Waist circumference was the most dominant predictor of NAFLD among all
five ATP-III defining criteria for MS. Thus waist circumference could be
used as a simple but effective screening method for obesity and associated
NAFLD in population studies or in rural Indian settings where resources,
access to biochemical tests and ultrasonography are limited.

- Serum adiponectin was significantly decreased while serum resistin was
increased in NAFLD patients compared to apparently healthy controls who
had normal liver on ultrasonography. Serum leptin did not show significant
difference between patients and controls. It is possible that adiponectin and
resistin has some role in the pathogenesis of NAFLD.
• Serum adiponectin was paradoxically decreased in lean NAFLD compared to obese. This paradoxical decrease of serum adiponectin in lean NAFLD suggests a different new etiology for this subset.

• Certain SNPs (rs 2241766 T/G and rs 3821799) could be important in NAFLD but validity of our study is limited because of its cross-sectional nature, preventing any causal inference, and the extremely small number of subjects and therefore this could be considered as a small pilot screening study attempting to associate SNPs in adiponectin gene with NAFLD. The finding that SNPs in adiponectin gene are associated with NAFLD supports the observation that plasma adiponectin levels are altered in NAFLD. This underscores the possible role of adiponectin in the pathogenesis of NAFLD. Large population studies are needed to evaluate the possible usefulness of adiponectin SNPs as a genetic marker in NAFLD.