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Evaluation of Serum Cytokeratin 18m30 Levels in Cases of Non Alcoholic Fatty Liver Disease

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ABSTRACT:BACKGROUND Cytokeratin (CK) 18 M30 antigen has been proposed as a diagnostic marker of non alcoholic fatty liver disease (NAFLD). Liver biopsy is the gold standard for the diagnosis and staging of the disease. A simple,noninvasive test that accurately distinguishes NASH from NAFL as well as determines the disease severity is urgently needed.Recently, it was found that determination of cytokeratin-18 M30 in the serum, predicts and correlates with stages of NAFLD, supporting its usefulness in clinical practice.

AIMTo evaluate the levels of serum Cytokeratin18M30 in the various stages of non alcoholic fatty liver disease – nonalcoholic fatty liver (steatosis) and non alcoholic steatohepatitis(NASH) diagnosed by ultrasound and transient elastography (fibroscan) and compare it with apparently healthy controls .

PATIENTS AND METHODS85 patients were divided into 3 groups: group I: including 35 patients with NAFL, group II: including 25 patients with NASH, and group III: including 25 healthy individuals as controls. Diagnosis of NASH and its differentiation from NAFL was done by ultrasound and fibroscan. CK-18 M30 level in serum was measured using ELISA.

RESULTS CK-18 M30 was significantly elevated in NASH group in comparison to NAFL and controls, with mean ± SD: 497.2 ± 173.6, 179.8 ± 17.4 and 76.3± 9.56, respectively, and p value: <0.001. The (ROC) curve diagnostic performance of CK18 M30 in diagnosis of NASH shows: cutoff value of >553.81 U/L, with specificity of 92.9% and sensitivity of 81.3%.

CONCLUSION Cytokeratin 18M30 is a useful diagnostic , screening and staging marker in the diagnosis of non alcoholic fatty liver and NASH .

Key words – NASH - non alcoholic steatohepatitis, NAFL- non alcoholic fatty liver CK - cytokeratin

I. INTRODUCTION

Non alcoholic fatty liver disease is a commonly occurring chronic liver disease which includes a spectrum of conditions of varying severity characterized by increased deposition of fat in the liver caused by factors other than significant alcohol consumption[(1)].It is the most common chronic liver disease occurring globally especially in the developed countries [(2)].The prevalence of the disease is not known exactly, but 9 – 37% of the general population is affected by the disease[(3,4)]. The prevalence and incidence is expected to rise, as the global obesity incidence grows in developing countries, with the trend as seen in western lifestyle [(5,6)].

The disease may affect any age group with the highest rate of incidence in the 40 to 49 years. It is the most common disease affecting the liver in the pediatric age group. It affects children mainly between ages 2 to 19[(7)].

It comprises a broadspectrum of pathological conditions involving the liver with varying clinical prognoses. The clinically mild end of the spectrum, is the simple accumulation of triglycerides inside the hepatocytes (simple fatty liver or steatosis)[(8)].It progresses to the more severe non-alcoholicsteatohepatitis (NASH). In 10% of cases it may progress to cirrhosis in the next 10 years [(9)].

NAFLD includes the following stages,

1. Hepatocellular steatosis (simple fatty liver)
2. Steatohepatitis
3. Fibrosis
4. Cirrhosis

Hepatic fibrosis and cirrhosis are potentially more deleterious,

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End stage liver failure or even hepatocellular carcinoma [10,11]constitute the most severe end of the spectrum.

It includes a wide range of disease pathology, ranging from simple steatosis (simple deposition of fat in the hepatocytes, which is mostly stable) to non-alcoholic steatohepatitis (cellular ballooning, necro-apoptosis, inflammation and fibrosis, which is more severe) and progresses to cirrhosis[12].Cirrhosis is clinically the most severe end of the spectrum. In a minority of cases it may worsen resulting in end stage liver failure or hepatocellular carcinoma (2 to 3%). Due to the rapid increase in prevalence, NAFLD related cirrhosis (cryptogenic cirrhosis) has become a common indication for liver transplantation.

Liver transplantation is the only treatment for NASH related cirrhosis [13] and end stage liver failure.It has been proven that NASH related cirrhosis is the second leading cause for liver transplant next to hepatitis C virus related liver disease. With increasing industrialization and the global epidemic of NAFLD, cirrhosis caused by NASH is likely to become the leading cause of liver transplantation by 2030.

There has been a rapid increase in the incidence as well as prevalence of the disease and a dramatic rise in obesity and type 2 diabetes has been observed.[14] The estimated prevalence ranges between 20 and 30% in the general population and increasing upto 90% in morbidly obese individuals. Non alcoholic steatohepatitis (NASH), the clinically more severe form of the disease, is less common, and affects about 2–3% of the general population, and up to 74% of the morbidly obese[15].

NAFLD occurs in about 94% of obese individuals (BMI >30 kg/m^2), 67% of the individuals with overweight (BMI >25 kg/m^2), and 25% of the normal individuals .

It is closely linked to type 2 diabetes, and fatty liver occurs in 70% of type 2 diabetics screened by ultrasonography. It represents the hepatic manifestation of the metabolic syndrome[16].

The etiological conditions associated with non alcoholic fatty liver are metabolic syndrome,Type 2 diabetes mellitus associated with insulin resistance, obesity (especially truncal/central obesity),hyperlipidemia (especially hyper triglyceridemia), hypertension and polycystic ovarian disease. Genetic / metabolic conditions include Lipodystrophies , Wilson's disease, Abetalipoproteinemia, Galactosemia, Heriditary fructose intolerance , Systemic carnitine deficiency and Tyrosinemi, Malnutrition conditions including t,otal parenteral nutrition,kwashiorkor and celiac disease.

Insulin resistance is the core mechanism in NAFLD in type 2 diabetes mellitus . Hyperinsulinemia and insulin resistance can be noticed by the action of insulin on the target tissues like adipose tissue (continual lipolysis and reduced uptake of free fatty acids), and liver (reduced glycogenesis).

When hepatocytes accumulate fat, they disrupt the intermediate filaments in the cytoplasm composed of cytokeratin. This results in the formation of Mallory bodies.A Mallory body is made up of of abnormally phosphorylated and cross linked keratins such as cytokeratin18.

The hepatocytes with Mallory bodies are more prone for apoptosis.[17] So these keratins including cytokeratin 18 are released into the peripheral blood during apoptosis [18]. So, levels of cytokeratin 18 in the serum are progressively elevated in non alcoholic fatty liver (NAFL) and non alcoholic steatohepatitis (NASH).

Mild to moderate increase in serum aminotransferase levels is commonly found in NAFLD patients(mean range 100 – 200 U/ L). Liver enzymes may be normal in a large percentage of patients, and normal levels of aminotransferases do not exclude presence of advanced disease.

Earlier studies have proved that the elevations in alanine aminotransferase(ALT) and aspartate aminotransferase (AST) are typically mild when present in NAFL and elevated two to three times the upper limit of normal in NASH patients. The AST/ALT ratiois usually < 1 in patients who have either absence or minimal fibrosis, and this ratio may be > 1 with the progression of fibrosis and development of cirrhosis [19].

Majority of NAFLD patients are without any symptoms. Most of them are diagnosed,when a liver imaging is done for other unrelated symptoms, i.e., when clinical assessment is done for metabolic syndrome.In asymptomatic patients, ultrasound is a non-invasive and easily available tool for diagnosing NAFLD. The specificity and sensitivity of ultrasound was found to be about 88% to 95% and 60% to 94%.But, the sensitivity diminishes in milder fatty infiltration. When there is ≥ 30% infiltration of fat, the sensitivity is 80% but it is 55% when hepatic fat content is 10% to 19% [20]. Moreover, the ultrasonography sensitivity for the detection of steatosis progressively decreases as the BMI increases. Another major drawback of ultrasound is the significant intra- and inter-observer variability.

Transient elastography also called fibroscan , is based on measuring shear wave

velocity.It measures the degree of liver stiffness and thus the degree of fibrosis in the liver.The probe of the fibroscan device is placed in an intercostal space over the right lobe of the liver. A 50 MHz elastic shear wave is transmitted from a small transducer at the end of the ultrasound probe into the liver.There is also another transducer in the probe, which can detect the speed of the shear wave (in meters/ sec) as the wave is transmitted through the liver.The speed of the shear wave is a measure of the stiffness of the liver, which is expressed in kilopascals (kpa).The whole process is known as liver ultrasound elastography.

Advantages of fibroscan are it's non-invasive, point of care testing, no pain, sedation is not required, quick test and is completed in 5 to 7 minutes and no side effects. Disadvantages of fibroscan are it cannot be used in patients with ascites, large amount of fat in the chest wall and morbid obesity[21]

Fibroscan can detect and quantify steatosis if the Controlled Attenuation Parameter (CAP) probe is used[22].It correlates with the decrease in amplitude of ultrasound waves as they propogate through the liver fat. It is based on the fact that fat affects ultrasound propogation. Hence, the more the steatosis, the higher, the CAP result will be.

Liver biopsy is currently the gold standard to make a definite and accurate diagnosis of the disease. It is useful to differentiate NAFLD from other differential diagnosis,staging fibrosis, grading activity, for follow up , and assessing the response to treatment. But, liver biopsy is unreliable in early cases and is an invasive procedure which can cause bleeding complications[23]. It is a costly technique too .Sampling error[24] and inter and intra observer variation are other disadvantages So a simple and noninvasive test has to be developed and validated to identify NAFLD. This test should be reproducible and reliable, that is both diagnostic and highly accurate to differentiate NASH from NAFL and for grading and staging of the disease.It should help to noninvasively monitor progression of the disease and response to treatment.

Cytokeratins are a family of intracytoplasmic ,fibrous, structural proteins that are the predominant components of intermediate filaments of epithelial cells[25,26] Cytokeratin 18 amounts to about 5 % of total amount of proteins in the hepatic cells and epithelial cells. Keratins like cytokeratin are released into peripheral blood during apoptosis .Levels of cytokeratin 18 in the serum are progressively elevated in non alcoholic fatty liver (NAFL) and non alcoholicsteatohepatitis (NASH)[27].So in our study levels of Cytokeratin 18 M30 a noninvasive biomarker is assessed in various stages of NAFLD.

AIM OF THE STUDY

To evaluate the levels of serum cytokeratin18M30 in the various stages of non alcoholic fatty liver disease – simple fatty liver (steatosis) and non alcoholicsteatohepatitis(NASH) diagnosed by ultrasound and transient elastography (fibroscan) and compare it with apparently healthy controls .

II. MATERIALS &METHODS

This is a cross sectional case control study and was conducted after getting ethical committee approval. The study comprised of a total number of 85 subjects including 35 patients of simple fatty liver and 25 patients of NASH diagnosed by ultrasonography in the Medical gastroenterology departments and Medical departments (outpatient and inpatients) and 25 normal controls in Rajiv Gandhi Government General Hospital, Chennai.

INCLUSION CRITERIA

Cases of NAFLD including non alcoholic fatty liver (NAFL)and non alcoholicsteatohepatitis (NASH) diagnosed by ultrasonography and transient elastography (fibroscan).

EXCLUSION CRITERIA

- Patients with history of alcohol consumption (> 30 g /day for males and 20 g / day for females)
- Viral hepatitis
- Autoimmune liver diseases
- Hepatocellular carcinoma patients.
- Congenital liver diseases

Cases were selected based findings of fatty liver and fibrosis (steatohepatitis) in ultrasonography and transient elastography.

They were divided into two groups-

1. Non alcoholicfatty liver (NAFL)
2. Non alcoholic fatty liver (NASH).

Non alcoholic simple fatty liver was diagnosed based on the findings of fatty liver (steatosis) – grade 1, 2 or 3 by ultrasonography with normal findings on fibroscan (< 7 kpa).

NASH was diagnosed based on, ultrasonographic findings of fatty liver and fibroscan values of > 7 kpa and elevated ALT and AST levels (twice or thrice normal) or AST/ALT ratio > 1.

In our Hepatology department fibroscan values are interpreted as follows: <7 kpa - Normal , 7 to 9 kpa - Early fibrosis, 9 to 13 kpa - Advanced fibrosis, >13 kpa - cirrhosis

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A control group with normal ultrasonographic findings with normal fibroscan findings was also included in the study.

SAMPLE COLLECTION:

Blood was collected after 8-12 hrs of overnight fasting. About 5mL of venous blood was collected from antecubital vein after aseptic precautions. after fulfilling selection criteria. 3mL of blood was collected in plain vacutainer tube for serum cytokeratin18M30 estimation and 2 mL in EDTA tube for fasting plasma glucose estimation. Fasting plasma glucose was estimated within three hours. Serum was separated by centrifugation at 3000 rpm for 15 mts and stored at -20℃ for further analysis.

ESTIMATION OF SERUM CYTOKERATIN 18M30

Serum cytokeratin 18 M30 was estimated by Quantitative sandwich enzyme immunoassay. Kit from Cusabio.

Reference range: Healthy : < 150 U/L,Slightly elevated : 150 – 200 U/L (indication for mild fibrosis as in NAFL),Elevated : > 200 U/L (substantial fibrosis as in NASH).

Estmation of plasma glucose was done in ERBA 640 automated analyser.

Method: Glucose Oxidase - Peroxidase Method (GOD – POD) End point assay.

ANTHROPOMETRIC MEASUREMENTS

Height is measured in meters,weight is measured in kilograms and Body mass index is calculated.

ULTRASOUND ABDOMEN: Ultrasound abdomen has been performed for all patients to find out the echogenic pattern characteristic of visceral fatty liver.The patients are graded as having grade 1, grade II or grade III fatty liver with (or) without hepatomegaly.

TRANSIENT ELASTOGRAPHY (FIBROSCAN):Fibroscan was performed for those patients diagnosed as fatty liver on ultrasound and those patients with elevated AST and ALT levels.This was performed with a special probe which was placed in the right upper quadrant of the abdomen.This measured the fibrosis of the liver (stiffness of the liver) in kilopascals.

III. STATISTICAL ANALYSIS

Data was analyzed using SPSS software version 16.0 and P value less than 0.05 was considered as statistically significant.

- The association of BMI,Total cholesterol, Triglycerides ,HDL, and Uric acid, with cytokeratin 18M30 were studied by Pearsons correlation method.
- One way ANOVA was done to compare more than two variables in the same group & between two group . It was carried out to compare Cytokeratin 18 M30 between NAFL& NASH patients.
- Receiver operating characteristics curve analysis was done to assess the utility of cytokeratin 18M30 in the diagnosis of NASH.
- Serum Cytokeratin 18M30 levels between the groups were analyzed by regression analysis and scatter diagram plotted.
- Plasma glucose was compared between study groups by Student's t-test.

IV. RESULTS OF THE STUDY

Table:1 COMPARISON OF AGE BETWEEN NAFL AND NASH

Age	NAFL	NASH
20 – 30	2	0
30-40	5	2
40 –50	9	8
50 – 60	11	10
60 – 70	8	5

There were 9 ,11 and 8 patients of NAFL in the fourth ,fifth and sixth decades.
There were 2,8 and 10 patients of NASH in the fourth,fifth and sixth decades

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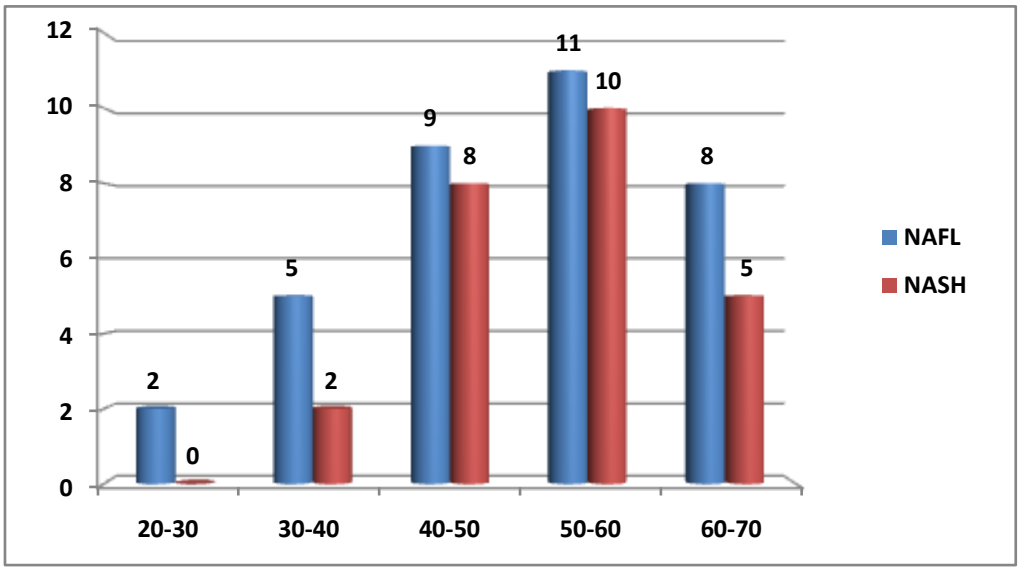
BAR DIAGRAM SHOWING COMPARISON OF AGE BETWEEN NAFL and NASH



Occurrence of NASH and NAFL is much higher in 40-50, 50-60 and 60-70 age group compared to 20 -30 and 30-40 age group.

Table 2 : BMI IN NAFL and NASH COMPARED TO HEALTHY CONTROLS.

	No	Mean	Standard Deviation	Standard Error of Mean	p value
NASH	25	28.98	2.56	.512	0.004
NAFL	35	25.81	1.67	.282	
CONTROLS	25	24.22	1.08	.217	

Table 3 :COMPARISON OF CYTOKERATIN18M30 IN NAFL AND NASH WITH HEALTHY CONTROLS - ANOVA

	No.	MEAN	STANDARD DEVIATION	STANDARD ERROR OF MEAN	p value
CONTROLS	25	76.30	9.56	1.91	< 0.01
NAFL	35	179.84	17.45	2.94	
NASH	25	497.22	173.67	34.73	

Using anova test,TABLE – 3 shows Cytokeratin 18M30 in various stages of NAFLD including NAFL and NASH compared to healthy controls.There was statistically significant difference in CK 18M30 levels between the three groups with the highest being in NASH compared to NAFL and controls. A highly significant p value of <0.01 was obtained .

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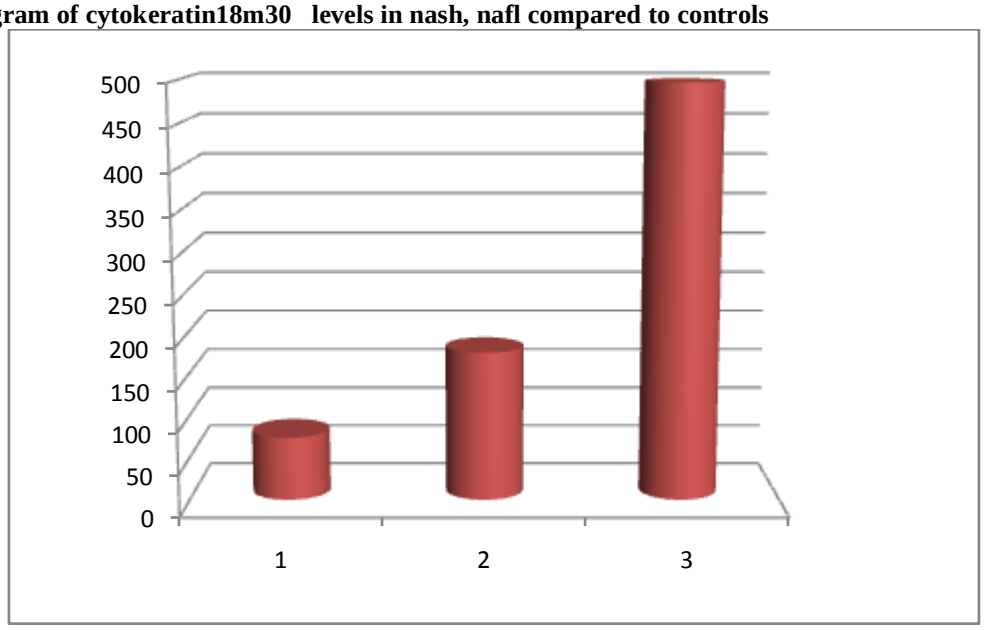
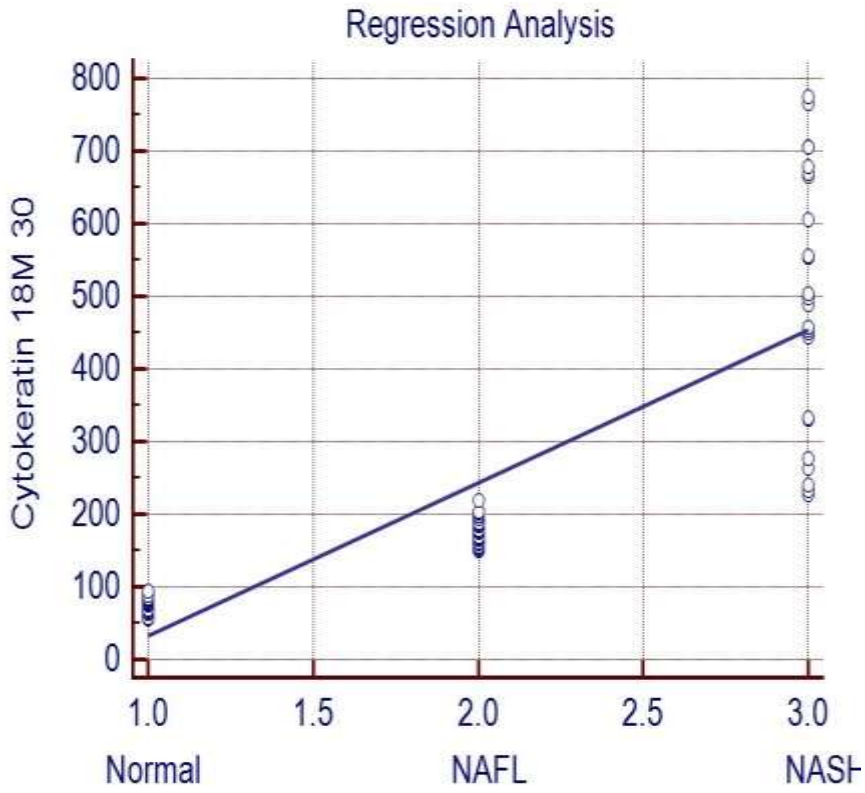
Bar diagram of cytokeratin18m30 levels in nash, nafl compared to controls



1.Controls 2.NAFL 3. NASH
Cytokeratin 18M30 levels are greatly elevated in NASH compared to NAFL and controls.

REGRESSION ANALYSIS – SCATTER PLOT OF CYTOKERATIN 18M30 in NASH, NAFL AND CONTROLS



Scatter plot shows linear plot with positive gradient of cytokeratin 18M30.It also shows highly elevated levels of cytokeratin 18M30 in NASH compared to NAFL and controls.

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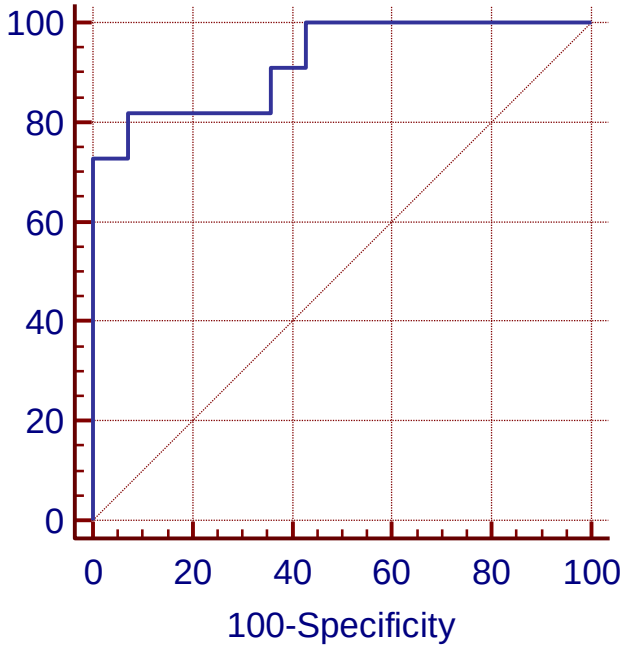
ROC (RECEIVER OPERATING CHARACTERISTICS) CURVE FOR CYTOKERATIN 18M30 IN NASH



ROC curve plotted between cytokeratin 18M30 levels and fibroscan in NASH shows specificity of 92.9 %, and, sensitivity of 81.8%, at a cut off point of 553.87U/L. Area under the curve (AUROC) is 0.922, with a highly significant p value of < 0.01(95% CI of 0.742 to 0.990).

TABLE 4 : COMPARISON OF PLASMA GLUCOSE LEVELS between NAFL, NASH AND CONTROLS

	No	Mean	Standard Deviation	Standard error of Mean	p value
CONTROLS	25	84.20	11.46	2.29	0.003
NAFL	35	104.86	10.57	1.78	
NASH	25	111.48	15.95	3.19	

The mean fasting blood plasma glucose was 84.20 (+/- 11.46) in the control group,104.86 (+/- 10.57) in the NAFL group and 111.48 (+/- 17.122) in the NASH group with a significant p value of 0.003.There was stastistically significant difference in FBS levels between NASH and NAFL group, and between NASH and control groups.

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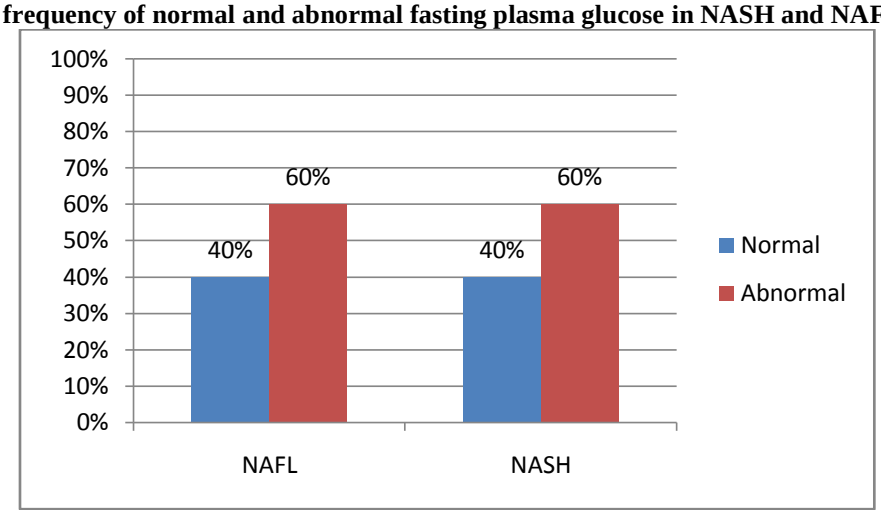
Figure shows frequency of normal and abnormal fasting plasma glucose in NASH and NAFL groups



Poor glycemic control was seen in 60% NAFL and 60% NASH cases.

V. DISCUSSION

Non alcoholic fatty liver disease is the most common chronic liver disease especially in the developed and industrialized countries[28]. NAFLD encompasses non alcoholic fatty liver (NAFL)[29] ,NASH and NAFLD – related cirrhosis[30]. In 10% of cases NASH may progress to cirrhosis in 10 years[31]. Once cirrhosis has occurred, 45% patients will develop serious complications such as variceal bleeding, ascites, and end stage liver failure. The most severe end of the spectrum is hepatocellular carcinoma.

Patients with NASH may revert to simple fatty liver or even normal and the severity of the degree of steatosis may come down if the condition is diagnosed early and therapeutic interventions are started early. The gold standard for confirming the diagnosis, assessing the severity of fibrosis, grading the extent of liver damage and to follow up the response to therapy in NAFLD is liver biopsy.[32] But this is an invasive procedure which needs expertise in the technique[33], and produces pain and bleeding complications in patients. The ultrasonography is able to pick up steatosis but not in very early cases .Transient elastography (fibroscan) can diagnose NASH and fibrosis, but is not easily available.

So, when the disease is diagnosed at a very initial stage using a simple and easily available biomarker and lifestyle modification and lipid lowering agents instituted early, the progress of the disease can be curtailed . This study aimed at evaluating serum levels of Cytokeratin 18 M30 as a noninvasive biomarker in various stages of NAFLD. In this study we evaluated serum Cytokeratin 18 M30 levels using sandwich enzyme immunoassay . The mean age in NASH group was 49.36 (+/-10.34) and in NAFL group was 58.40 (+/-8.46) .This is similar to the conclusions by Koehler EM[34] that NAFLD mainly affects the middle aged and the elderly,because, the risk factors for its development, tend to increase with advancing age .In Indian studies mean age was reported to be 42.90 (+/- 10.54) by Roli Agarwal[35].

The mean BMI was greatly increased in NASH and NAFL groups when compared to the control group. Ruhl et al concluded that prevalence of NAFLD increases with increase in BMI[36].Prevalence studies have proved that the occurrence of the disease is about 60 – 70 % obese patients and it is closely linked with central adiposity.

In our study the CK 18 M 30 levels were found to be significantly higher in the NASH group with a mean of 497.22 (+/- 173.67) IU/ L and in the NAFL group it was 179.84 (+/-17.45) IU/L compared to the control group where the mean was 76.30 (+/- 9.569) IU/l. A highly recommended significant p value of <0.01was obtained. There is statistically significant difference in CK 18M30 levels between the three groups with the highest being in NASH compared to NAFL and controls. . Hence, CK 18M30 levels can discriminate NASH from non alcoholic fatty liver, as well as normal individuals. This is similar to the study by Feldstein et al who proved that, CK18 M30 levels were increased in biopsy proven NASH patients[37] compared to NAFL and normal controls.

Wieckowska et al said that, CK 18 M30 levels could serve as a marker of hepatic inflammation. This is explained by the hepatocyte apoptosis which plays a key role in the pathogenesis of NAFLD and progression of NAFL

to NASH[38].Hepatocyte apoptosis is mediated by caspase 3 which cleaves the cytokeratin 18 of NAFLD patients and is released into the sera. The magnitude of apoptosis correlates with the degree of liver damage and fibrosis. In this study, CK 18 M30 levels also increased with increasing grades of fibrosis detected by fibroscan.So, CK 18 M30 can noninvasively grade the severity of NASH.

In this study,the regression analysis and scatter plot shows positive prediction of CK 18M30 in the diagnosis of NASH and NAFL.

In this study, the ROC curve shows diagnostic performance of CK 18M30 with a high specificity of 92.9 % and moderate sensitivity of 81.8% at a cut off point of553.87U/L with AUROC of 0.922 (p value of<0.01) in NASH .**It** may be considered that, CK 18M30 is a highly specific and moderately sensitive marker in the diagnosis of NASH. Younossi et al proved that, " CK 18M30 is an independent predictor of NASH and CK 18M30 has sufficient to excellent, diagnostic accuracy, for the detection and exclusion of NASH in the setting of NAFLD[39]"

In this study, the fasting plasma glucose levels was increased in 60% of NAFL patients as well as NASH. The known cases of type 2 diabetes was 56% of NAFL patients and 67% of NASH patients. In this study, there was a statistically positive significant correlation between CK 18M30 levels and fasting plasma glucose levels (r = 0.334). Gupte.P. et al studied that NAFLD is commonly associated with type 2 diabetes mellitus[40].Since insulin resistance is the main underlying mechanism in NAFLD, insulin sensitisers may be used in treatment. Assessment of the serum levels of CK 18 M30 can be a useful screening, diagnostic and staging biomarker for non alcoholic fatty liver and NASH, based on this study.

VI. CONCLUSION

From this study we conclude that,

1 .Cytokeratin 18M30 is a promising screening, diagnostic and staging biomarker for non alcoholic fatty liver and NASH.

2 Cytokeratin 18M30 can be used as a simple, and non invasive biomarker instead of liver biopsy.

3. Cytokeratin 18 M30 positively correlates with increasing grades of fibrosis in NASH.

LIMITATIONS

1. The sample size for NASH is very small.
2. There are no reference values for cytokeratin 18M30 for NAFL and NASH in our population.
3. Follow up after instituting therapeutic management could not be done.

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