Fibroscan versus simple noninvasive screening tools in predicting fibrosis in high-risk nonalcoholic fatty liver disease patients from Western India

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Abstract

Background The aim of the study was to determine the efficacy of Fibroscan versus noninvasive markers, i.e. nonalcoholic fatty liver disease (NAFLD) fibrosis score (NFS); Aspartate-aminotransferase (AST)/platelet ratio (APRI); and AST/Alanine-aminotransferase (AAR) as a screening tool in NAFLD patients with high risk of liver fibrosis.

Methods This is a single-center study carried out in patients attending the outpatient department for dyspepsia and diagnosed with fatty liver on ultrasound. Liver biopsy was advised in diabetics, metabolic syndrome, body mass index >30 kg/m², raised transaminases and hypothyroidism. Fibroscan, APRI, AAR and NFS were calculated. Area under the curve (AUROC), negative (NPV) and positive predictive values (PPV) were calculated for each diagnostic test.

Results Of the 1500 patients screened, 110 with the above-described risk factors underwent liver biopsy (stage 3/4 fibrosis = 38). Diabetes predicted severe fibrosis (stage 3/4). Sensitivity, specificity, PPV, NPV and AUROC for Fibroscan at value 12 kPa were 0.9, 0.8, 0.70, 0.93 and 0.91 respectively for predicting stage 3/4 fibrosis. With increase in severity of liver fibrosis there was stepwise increase in Fibroscan values (P=0.000038, Kruskal-Wallis test). Sensitivity, specificity, PPV and NPV for AAR and NFS at cutoff of 1.5 and 0.676 were 0.8, 1.0, 1.0 and 0.92 and 0.8, 1.0, 1.0 and 0.92 respectively.

Conclusion Fibroscan, NFS and AAR are simple noninvasive markers of fibrosis that can be utilized as screening tools in patients with high risk for fibrosis to determine the need for biopsy. The cutoff of Fibroscan for stage 3/4 fibrosis was 12 kPa.

Keywords Liver stiffness measurement, tissue elastography, nonalcoholic fatty liver disease, diabetes, liver fibrosis

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Introduction

The term nonalcoholic fatty liver disease (NAFLD) characterizes a condition of excess fat accumulation in the liver in the absence of significant amounts of alcohol consumption, usually defined as less than 20 g of ethanol per day. NAFLD is a common disorder worldwide with prevalence ranging from 10-30% in various countries [1]. The disease spectrum includes a benign steatosis with low probability of progression and a small number of patients, steatohepatitis (15%), with its course characterized by rapid progression to fibrosis and cirrhosis. Detection of this small fraction is of utmost importance for aggressive management. Ultrasound detects fatty liver with a good sensitivity, but does not differentiate simple steatosis from steatohepatitis. Serum transaminases have low value in predicting steatohepatitis. The only gold standard test for detection of this fraction with increased risk of progression is liver biopsy. However, application of liver biopsy to all patients with NAFLD is impractical and should be advised in patients with diabetes, obesity, metabolic syndrome or raised liver enzymes [2]. Even when there is high risk of fibrosis such as in diabetic patients, the population is still large enough to merit carrying out an invasive procedure to rule out fibrosis. Therefore, an easy, rapid, accurate, and noninvasive screening test is needed to select the small fraction of NAFLD patients for liver biopsy. Fibroscan (transient elastography) measures liver stiffness through estimation of velocity of propagation of a shear wave through liver tissue [3]. The value depends on the

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viscoelastic properties of the liver. We carried out this study to
determine the efficacy of Fibroscan, NAFLD fibrosis score
(NFS), aspartate aminotransferase (AST) / platelet ratio (APRI),
and AST / alanine aminotransferase (AAR) as screening tools
in patients with high risk for disease progression, thereby
reducing the need for liver biopsies.

Patients and methods

Study design

This is a single-center study carried out at Lokmanya Tilak
Municipal General Hospital between December 2011 and
December 2012. An informed consent was obtained from all
patients. The institutional Ethics Committee clearance was taken.

Patients

Eligible were 18 to 80 years of age patients attending the
outpatient department (OPD) of our tertiary care center (non-
referral patients) for dyspepsia and who were diagnosed with
fatty liver on ultrasound (hyperechoic liver where the echo-
texture of the liver was brighter than the kidney, and had
blurred vascular margins and deep attenuation of ultrasound
signal). Of these, patients with any one of the following were
selected for liver biopsy: diabetes (fasting blood sugar levels
>126 g/dL); metabolic syndrome (diagnosed on the basis of
NCEP-ATP III criteria); body mass index >30 kg/m$^2$; serum
AST/alanine aminotransferase (ALT) greater than the upper
limit of normal (40 IU/mL); and hypothyroidism (serum
thyroid stimulating hormone [TSH] >5.5 IU/mL).

Exclusion criteria were: history of alcohol intake greater
than 20 g per day (during previous 5 years); hepatitis B surface
antigen (HBsAg) reactive; presence of antibody against
hepatitis C (HCV); human immunodeficiency virus reactive;
active hepatitis; biliary obstruction on ultrasonography;
cirrhosis diagnosed at any time in the past; tuberculosis;
malabsorption; chronic drug use; pregnancy; and those
with any cardio-respiratory comorbidities. a1-Antitrypsin
deficiency and hemochromatosis are rarely seen in Indian
patients and thus were not investigated in our patients.
Besides, patients who fulfilled inclusion criteria but did not
consent were excluded.

Screening and evaluation

We performed complete physical examination, height,
weight, waist circumference, complete blood count, serum
transaminases, prothrombin time, serum creatinine, fasting
and 2-h postprandial blood sugar levels, complete lipid
profile, anti-nuclear antibody, HBsAg, anti-HCV, HIV,
serum ceruloplasmin, thyroid function tests, serum ferritin,
utrasound and upper gastrointestinal endoscopy. Eligible

Patients with any of the risk factors described above were
advised to undergo liver biopsy. APRI, AAR and NFS were
calculated. NFS was calculated as per the following formula:

\[-1.675+0.037 X \text{age (years)} + 0.094 X \text{body mass index} (\text{BMI}, \text{kg/m}^2) + 1.13 X \text{impaired fasting glucose/diabetes} \text{yes} = 1, \text{no} = 0 + 0.99 X \text{AST/ALT ratio} - 0.013 X \text{platelet} (\times 10^9 /L) - 0.66 X \text{Albumin} (\text{g/dL}).\]

Patients who consented underwent liver biopsies with 16G needle and a specimen of
minimum 2 cm length was obtained. All liver biopsies were
assessed by a senior histopathologist and were graded and
staged according to Brunt criteria [4].

Liver stiffness measurements

Fibroscan (M probe, Echosens, Paris) was carried out by
an experienced examiner in all patients (with at least 6 h of
fasting) in left lateral position and the median liver stiffness
of the 10 successful measurements fulfilling the criteria (success
rate of greater than 60% and interquartile range /median ratio
of <30%) were noted (in kPa). Fibroscan measurements were
not limited to patients who underwent liver biopsy but were
carried out in all NAFLD patients.

Statistical analysis

Based on the presence or absence of significant fibrosis
on biopsy the patients were subclassified into patients with
Stage 0/1 or 2 fibrosis and patients with Stage 3/4 fibrosis.
The difference in the demographic, physical and serologic
investigations between the two groups was calculated using
Student’s t test. The median liver stiffness values (with 95%
confidence intervals) were obtained for each group with
fibrosis. Kruskal–Wallis test was applied to determine the
association of liver stiffness measurement, NFS, APRI and
AAR values with fibrosis staging. Sensitivity, specificity,
positive predictive value (PPV), negative predictive value
(NPV), and area under the curve (AUROC) were calculated
for liver stiffness measurement values.

Results

Fifteen hundred consecutive patients presented at
the OPD were screened. Of these only 295 patients
(population=2,00,000, Confidence level-95%, margin of error
5.6%) with fatty liver underwent the baseline evaluation as
described. One hundred and ten patients in total underwent
liver biopsy (Fig. 1) as eight patients who were advised
had significantly higher BMI,

Clinical characteristics of the patients who were compared to those who were not
advised liver biopsy is shown in Table 1. The patients who
were advised liver biopsy had significantly higher BMI,
abdominal girth, serum triglycerides, and serum cholesterol
levels. However, there was no difference in demographic
characteristics, alcohol intake, hypertension, hemogram and liver size on ultrasound.

The indications for liver biopsy in the study group are described in Table 2. Diabetes and raised transaminases were the major reasons for liver biopsy. Seventy two patients had fibrosis belonging to stage 1 and 2, while rest had severe fibrosis or cirrhosis (Table 2). The difference between the two groups was significant only in terms of diabetes. Only 18 patients with diabetes had raised liver enzymes. The cutoff used in our study for transaminases was the traditional cutoff of 40 IU/mL. Twenty six of 38 patients (71%) with stage 3/4 fibrosis had diabetes, though only 18 of 54 patients with diabetes had raised liver enzymes stressing the fact that significant liver fibrosis may be present in diabetics without raised liver enzymes. Twenty six patients with BMI greater than 30 kg/m$^2$ had metabolic syndrome. The patients with higher age and higher levels of triglycerides, cholesterol and abdominal girth had higher level of fibrosis. Eight of 12 with hypothyroidism had metabolic syndrome.

The biopsy sample was considered adequate in all cases. The median cumulative sample size was 2.4 cm (min 2.0 cm and max 3.2 cm). The histopathologist had examined a minimum of 12 portal tracts. Twenty eight patients had only steatosis with or without portal/lobular inflammation, while rest had either ballooning, Mallory-Denk bodies or fibrosis - changes consistent with nonalcoholic steatohepatitis (NASH). The number of patients with stage 0 (no fibrosis), stage 1 (perivenular/central fibrosis), stage 2 (perivenular with periportal fibrosis), stage 3 (bridging fibrosis) and stage 4 (cirrhosis) fibrosis were 31, 29, 12, 29 and 9 respectively.

Fibroscan was carried out successfully in all NAFLD patients. However, obese patients required an increased number of readings to achieve its criteria for successful reading of 60%. Getting a proper window was difficult in obese patients, though we managed to get successful readings in all of them. The median Fibroscan value in patients in whom a biopsy was not advised was 4.6 kPa. The difference in liver stiffness measurements between this group and the ones having undergone liver biopsy showed a statistical significant difference (P<0.001). None of these individuals had a value above 12 kPa. Median Fibroscan values for stages 0/1, 2, 3 and 4 were 8, 9.1, 12 and 20 kPa respectively (Fig. 2). There was a statistically significant

![Figure 1 Flow chart of the study](OPD, outpatient department; NAFLD, nonalcoholic fatty liver disease)

Table 1 Baseline characteristics of patients screened for fatty liver

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Advised liver biopsy high-risk group (n=118)</th>
<th>No liver biopsy advised low-risk group (n=177)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>42.37±3.2</td>
<td>41.6 ± 3.8</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (F:M)</td>
<td>2.3:1</td>
<td>1.8:1</td>
<td>NS</td>
</tr>
<tr>
<td>History of hypertension</td>
<td>20</td>
<td>29</td>
<td>NS</td>
</tr>
<tr>
<td>Alcohol intake (&lt;20 g)</td>
<td>32</td>
<td>48</td>
<td>NS</td>
</tr>
<tr>
<td>Mean weight (kg)</td>
<td>68.27±1.2</td>
<td>66.48±1.7</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>29.15±0.56</td>
<td>25.14±0.54</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Abdominal girth (cm)</td>
<td>85.2±0.89</td>
<td>81±1.01</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.2±0.17</td>
<td>13.16±0.15</td>
<td>NS</td>
</tr>
<tr>
<td>Platelet count (&lt;10$^9$/µL)</td>
<td>243±8.2</td>
<td>316±8.5</td>
<td>NS</td>
</tr>
<tr>
<td>Serum albumin (g/dL)</td>
<td>3.33±0.03</td>
<td>3.8±0.03</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Triglyceride levels</td>
<td>220±11.8</td>
<td>109±4.1</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Cholesterol levels</td>
<td>223±7.28</td>
<td>126±8.47</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Liver size on ultrasound</td>
<td>15.36±0.15</td>
<td>12.7±0.12</td>
<td>NS</td>
</tr>
<tr>
<td>Liver biopsy</td>
<td>110</td>
<td>Nil</td>
<td>-</td>
</tr>
</tbody>
</table>

The values are expressed as mean ± SEM (Student's t test and Fischer exact tests applied)
difference in liver stiffness measurements in patients with stage 0/1/2 fibrosis as compared to stage 3/4 fibrosis (P<0.05). With increase in severity of liver fibrosis there was stepwise increase in Fibroscan values (P=0.000038 by Kruskal-Wallis test). The AUROC for Fibroscan for detecting stage 3/4 fibrosis in NAFLD was 0.91. The sensitivity, specificity, PPV and NPV of Fibroscan at 12 kPa for stage 3/4 fibrosis were 0.9, 0.8, 0.72 and 0.93 respectively (Table 3).

The correlation of APRI, AAR and NFS in these patients according to histological severity was comparable to Fibroscan. Median values of APRI, AAR and NFS for stage 0/1 fibrosis were 0.48, 0.67 and -1.97; for stage 2 fibrosis were 1.0, 1.1 and -1.2; for stage 3 fibrosis were 1.62, 1.4 and 2.03; and for stage 4 fibrosis were 1.64, 1.9 and 5.5 respectively. The sensitivity, specificity, PPV, NPV for APRI at cutoff of 1.0 were 0.7, 0.8, 0.6 and 0.84; for AAR at cutoff of 1.6 were 0.8, 1.0, 1.0 and 0.92; and for NFS at cutoff of 0.676 were 0.8, 1.0, 1.0 and 0.92 respectively. At a low cutoff of -1.455 for NFS, the sensitivity, specificity, PPV and NPV are as 1.0, 0.69, 0.62 and 1.0 respectively (Fig. 3, Table 3).

We have compared the patients undergoing liver biopsy for raised transaminases to those undergoing liver biopsy with normal transaminases (Table 4). There seems to be no statistical significant difference in the Fibroscan, APRI, AAR and NFS values and proportion of patients with Brunt stage 3/4 fibrosis between the two groups. The demographic profile was also similar between the two groups except for the presence of diabetes, which was more often the cause of liver biopsy in patients with normal transaminases (P<0.05).

### Discussion

The results of the present study conducted on patients from Western India with dyspepsia and no hepatic complaints (Yellow discolouration of jaundice in past, abdominal distention, hematemesis, Malena, altered sensorium) showed that liver stiffness measurement with Fibroscan is an effective method for screening patients with NAFLD. As per the current recommendations all patients with diabetes, raised liver enzymes, obesity or metabolic syndrome are to be considered for liver biopsy [2]. Recent studies have also implicated hypothyroidism as a risk factor for disease progression in NAFLD [5]. Although they are predictors of severe underlying fibrosis, they themselves constitute a large proportion of patients in India with a growing epidemic of diabetes and obesity. Even presence of these factors should not in itself merit liver biopsy. None of the risk factors besides diabetes is suggestive of advanced fibrosis in these patients. Raised enzymes, hypothyroidism, metabolic syndrome, and obesity were equivalent among patients with stages 0/1/2 and 3/4 fibrosis. Although patients with stage 3/4 were older than those with stage 0/1/2 fibrosis, the difference between them was not significant. Patients who have normal transaminases can even have significant fibrosis, as the proportion of patients with significant fibrosis and normal transaminases was equal to the proportion of patients with raised transaminases and significant fibrosis. BMI, abdominal girth, triglyceride levels, cholesterol levels were higher in patients with stage 3/4 fibrosis, but again the difference between the two groups was not significant. So, none of these risk factors in itself can predict severe fibrosis.

Liver biopsy is an invasive procedure with complications like pain, bleeding, pneumothorax, hemothorax, bile

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**Table 2** Indications of liver biopsy in our study

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number of patients who underwent liver biopsy (n=110)</th>
<th>Stage 0/1/2 fibrosis (n=72)</th>
<th>Stage 3/4 fibrosis (n=38)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>History of diabetes</td>
<td>54</td>
<td>28</td>
<td>26</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>12</td>
<td>6</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>BMI &gt;30 (kg/m²)</td>
<td>34</td>
<td>22</td>
<td>12</td>
<td>NS</td>
</tr>
<tr>
<td>ALT &gt;40 (IU/mL)</td>
<td>64</td>
<td>44</td>
<td>20</td>
<td>NS</td>
</tr>
<tr>
<td>AST &gt;40 (IU/mL)</td>
<td>50</td>
<td>36</td>
<td>14</td>
<td>NS</td>
</tr>
<tr>
<td>Metabolic syndrome</td>
<td>36</td>
<td>20</td>
<td>12</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fischer exact test applied

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**Table 3** Fibroscan versus NAFLD fibrosis score (NFS), APRI and AST to ALT ratio (AAR) in patients with NAFLD to predict stage 3 or 4 fibrosis

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroscan</td>
<td>0.9</td>
<td>0.8</td>
<td>0.72</td>
<td>0.93</td>
</tr>
<tr>
<td>NFS (cut off: 0.676)</td>
<td>0.82</td>
<td>1.0</td>
<td>1.0</td>
<td>0.92</td>
</tr>
<tr>
<td>NFS (cut off: 1.455)</td>
<td>1.00</td>
<td>0.69</td>
<td>0.62</td>
<td>1.0</td>
</tr>
<tr>
<td>APRI (cut off: 1.0)</td>
<td>0.7</td>
<td>0.8</td>
<td>0.6</td>
<td>0.84</td>
</tr>
<tr>
<td>AST/ALT (cut off: 1.6)</td>
<td>0.8</td>
<td>1.0</td>
<td>1.0</td>
<td>0.92</td>
</tr>
</tbody>
</table>

PPV, positive predictive value; NPV, negative predictive value; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NAFLD, non alcoholic fatty liver disease

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**Table 4** Comparison of NAFLD patients with and without raised transaminases (liver biopsy proven)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients with raised transaminases (n=64) (Mean±SEM)</th>
<th>Patients with normal transaminases (n=46) (Mean±SEM)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (in years)</td>
<td>41.44±3.6</td>
<td>42.8±2.5</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (F:M)</td>
<td>46:18</td>
<td>34:12</td>
<td>NS</td>
</tr>
<tr>
<td>History of diabetes</td>
<td>N=18</td>
<td>N=36</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>N=4</td>
<td>N=8</td>
<td>NS</td>
</tr>
<tr>
<td>BMI &gt;30 (kg/m²)</td>
<td>N=19</td>
<td>N=15</td>
<td>NS</td>
</tr>
<tr>
<td>Fibroscan</td>
<td>8.2±4.9</td>
<td>7.6±3.9</td>
<td>NS</td>
</tr>
<tr>
<td>AST/ALT ratio</td>
<td>1.3±0.4</td>
<td>1.1±0.3</td>
<td>NS</td>
</tr>
<tr>
<td>APRI</td>
<td>1.4±0.6</td>
<td>1.28±0.45</td>
<td>NS</td>
</tr>
<tr>
<td>NFS</td>
<td>1.69±0.8</td>
<td>1.56±0.8</td>
<td>NS</td>
</tr>
<tr>
<td>Brunt stage 3/4 fibrosis</td>
<td>N=20</td>
<td>N=18</td>
<td>NS</td>
</tr>
</tbody>
</table>

The value expressed at each place is in Mean±SEM

SEMs, standard error of mean; APRI, AST/ALT ratio index; NFS, NAFLD fibrosis score
peritonitis, hemobilia, puncture of the kidney and intestine, infections, anxiety, and even death. There are intra- and interobserver discrepancies. It measures only 1/50000 of liver tissue. However, liver biopsy remains gold standard for diagnosis, it cannot be applied to all patients [6].

Liver stiffness measurements carried out in these high-risk patients can further narrow down the number of patients who actually require biopsy evaluation. As per our present study, at the cutoff value of 12 kPa, with an NPV of 93%, we can safely select patients for liver biopsies. There is only 7% chance of a higher stage of fibrosis if values are lower than 12 kPa. Thus, Fibroscan could be carried out at a predetermined interval to screen patients with NAFLD and to subject them to liver biopsy once they cross a cutoff of 12 kPa. There is a stepwise increase in Fibroscan values with increase in fibrosis (P<0.0001). However, a future prospective study is required to characterize the significance of serial Fibroscan measurements over time and to subject patients to liver biopsy if there is significant rise in Fibroscan values compared to previous ones.

Liver stiffness measurement using Fibroscan is reproducible and independent of the operator and explores a volume of liver parenchyma which can be approximated to a cylinder of 1 cm in diameter and 4 cm in length. This volume is 100 times larger than the biopsy specimen volume and is thus much more representative of the entire hepatic parenchyma. It can be carried out on OPD basis and the patients do not experience pain. It is rapid as well as noninvasive and patient friendly. However, acute hepatitis and liver congestion as in cardiac failure can cause false high scores and they need to be ruled out before carrying out Fibroscan [3].

During these years, a variety of other noninvasive markers have been developed for prediction of fibrosis in HCV and NAFLD. However, in a developing country like ours, where cost is a major issue, tests like Fibrotest [7], FibroMeter [8], and European Liver Fibrosis panel [9] are difficult to carry out. Their availability is also a concern at most places. Fibroscan is only available at specialized private centers owing to its high cost.

Tests like APRI, AAR and NFS can be easily carried out without the need for specialized centers. We have also evaluated these tests in our patients with high risk for fibrosis. AAR and NFS at their cutoff of 1.6 and 0.676 had a specificity and PPV of 100%. These tests applied to our high-risk patients can predict fibrosis and these patients can thus be subjected to liver biopsy and treatment. NFS at a low cutoff of -1.455 had an NPV of 100% and liver biopsy can be avoided in such patients. This finding is in accordance to the study by Paul Angulo et al [10]. APRI index on the other hand has low sensitivity and specificity.

There are various studies substantiating the use of Fibroscan in NAFLD. In a study by Wong et al [11] 246 patients underwent liver stiffness measurement by Fibroscan. The AUROC values of transient elastography for F3 or higher and F4 disease were 0.93 and 0.95, respectively, significantly higher.
than those of AAR, APRI and NFS. In a study carried out by Yoneda et al [12], AUROC for F1, F2, F3, and F4 fibrosis were 0.881, 0.876, 0.914, and 0.997, respectively. Musso et al [13] showed that for NASH with advanced fibrosis, pooled AUROC, sensitivity and specificity of NFS and Fibroscan were 0.85, 0.90, 0.97 and 0.94, 0.94 and 0.95. Another study from India by Sarin et al showed similar efficacy of Fibroscan in NAFLD in patients from Northern India.

In conclusion, high BMI, metabolic syndrome, raised liver enzymes, age, altered lipid profile by themselves do not predict underlying severe fibrosis. Fibroscan, AST/ALT ratio and NFS can effectively screen these patients and can be applied to all patients before subjecting them to liver biopsy thereby reducing the number of overall biopsies required. Since Fibroscan is available only at specialized centers in India, the above markers can be safely used as alternative options.

Acknowledgment

We acknowledge the assistance of the Dean of LTMMC & LTMGH, Department of Biochemistry, Radiology and Pathology and the participation of the patients; without their contribution we would have been unable to carry out this study.

References