Study of Risk Factors Involved in the Progression of Non Alcoholic Fatty Liver Disease in Egyptian Patients

Elsayed A. Wasfy1, Nadia M. Elwan1, Shreif L. Bayomi2, Thanaa F. El- Sheikh3, Sahar A. El-yamani1 and Boshra E. Talha1

Tropical Medicine1, Pathology2 and Biochemistry3 Departments, Tanta University, Tanta, Egypt

*nadiaelwan@yahoo.com

Abstract: Nonalcoholic fatty liver disease (NAFLD) includes hepatic steatosis, nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis. NAFLD has also the potential to progress to hepatocellular carcinoma (HCC) or liver failure. NAFLD is linked to obesity, insulin resistance, hyperlipidaemia and genetic factors. Our objective was to study the risk factors that involved in the progression of non alcoholic fatty liver disease. Subjects and methods: Thirty-three patients and ten healthy controls were included in our study. Patients were classified into 3 groups. Group I included 12 patients with simple liver steatosis. Group II included 11 patients with NASH. Group III included 10 patients with cirrhosis most probably a late sequel of NASH. Results: BMI, fasting blood glucose, insulin and HOMA-IR were significantly higher in patients with fatty liver, NASH and cirrhosis, also, NASH patients showed a significant high serum triglycerides and ALT. All previous parameters were significantly increased with the increased severity of histopathological score in patients with fatty liver and NASH. Serum AST levels and AST / ALT ratio were significantly increased in NASH and cirrhotic patients as compared to patients with steatosis alone and controls. Mitochondrial ATP levels in patients with fatty liver and NASH showed a statistically significant decrease. Also patients with NASH showed a statistically significant decrease when compared to patients with fatty liver. Finally, patients with fatty liver and NASH showed a significant decrease in mitochondrial ATP with increased BMI and histopathological score. Conclusion: Increased BMI, hyperglycemia, hypertriglyceridaemia, insulin resistance and depletion of mitochondrial ATP in hepatocytes can be considered risk factors involved in the development and progression of fatty liver to NASH and cirrhosis.

Key words: BMI, insulin resistance, mitochondrial ATP, NAFLD

1. Introduction:

Nonalcoholic fatty liver disease (NAFLD) is a significant health problem and affects 70 million adults in the United States. NAFLD occurs in up to 53% of obese children. NAFLD is defined as an excess of fat in the liver in which at least 5% of hepatocytes display lipid droplets that exceed 5% - 10% of liver weight in patients who do not consume significant amounts of alcohol.

Non alcoholic fatty liver disease refers to a spectrum of diseases of the liver ranging from steatosis (i.e., fatty infiltration of the liver), nonalcoholic steatohepatitis (NASH) (i.e., steatosis with inflammation and hepatocyte necrosis) to cirrhosis. Nonalcoholic steatohepatitis (NASH) is defined as necroinflammatory disorder with fatty infiltration of hepatocytes, this term is employed when steatohepatitis occurs in individuals whose alcohol consumption is nil or negligible i.e less than 20g ethanol/day in women and less than 40g in men.

Nonalcoholic fatty liver disease, NAFLD is now considered a metabolic pathway to advanced liver disease; cirrhosis and hepatocellular carcinoma but hepatic steatosis without concomitant inflammation or fibrosis usually considered a benign condition. Two types of NASH exist: primary NASH (which is associated with metabolic syndrome–related conditions, such as insulin resistance, obesity, type -2 diabetes, and hyperlipidemia) and secondary NASH (which occurs after obesity-related intestinal surgery, rapid weight loss in the obese, total parenteral nutrition, treatment with drugs such as amiodarone or corticosteroids, lipodystrophy, or Wilson’s disease). Many aspects of the disease are common to both presentations.

Hepatic steatosis can be reversible or progress to NASH depending on the cessation or persistence of the underlying provocative cause respectively.
The first step of the pathophysiology of nonalcoholic steatohepatitis is the lipid accumulation in the liver causing steatosis. This increases the sensitivity of the liver to injury, inflammation and fibrosis.\(^{(8)}\)

The second step involves the cytokines and other factors causing oxidative stress and lipid peroxidation, in time leading to steatohepatitis.\(^{(9)}\)

Our objective were studying the risk factors such as obesity, insulin resistance, type-2 diabetes, hyperlipidemia and mitochondrial ATP, that involved in the progression of non alcoholic fatty liver disease.

2. Subjects and methods:

All patients were selected from inpatient and outpatient clinics of Tropical Medicine and Infectious Diseases Department and Surgery Department of Tanta University Hospital in the period from August 2004 to January 2008.

This study was conducted on thirty-three patients and ten healthy controls, subjects were divided into the following groups:-

**Group I:** Included (12) patients with steatosis.

**Group II:** Included (11) patients with NASH.

**Group III:** Included (10) patients with cirrhosis most probably a late sequel of NASH;

Diagnosis of this group was based on history of fatty liver and absence of history of any chronic liver disease, drugs causing NAFLD, abdominal ultrasonography, negative viral markers for HBV and HCV and negative laboratory tests of autoimmune hepatitis, primary biliary cirrhosis, Wilson's disease, alcoholic liver diseases, and haemochromatosis.

**Group IV:** Included (10) normal healthy individuals as a control group.

All patients were subjected to full history and clinical examination, body mass index (BMI), was calculated by the weight in kilograms divided by the square of the height in meters. (BMI; kg/m\(^2\)), ultrasonography, urine and stool analysis, biochemical tests including complete blood count, lipid profile, liver function tests. Two-hour a 75-g oral glucose tolerance test (OGTT) was done for patients not known to have diabetes mellitus and measurement of level of fasting insulin and glucose in the blood during the test, insulin resistance was calculated using the homeostasis assessment model (HOMA-IR) ) on the basis of fasting glucose and fasting insulin. HOMA-IR was calculated using the following equation: -

\[\text{Fasting glucose (mg/dl) } \times \text{ fasting insulin (µU/ml)} \]  
\[ /405.\]

A HOMA-IR greater than 2.0 is considered to indicate the presence of insulin resistance.

Serum in sulin level was measured using kit IMMULITE 2000 Insulin which is a solid-phase, two-site chemiluminescent immunometric assay.

Serological tests for HBV; HBsAg and HBCAb and HCV; HCV Ab.

Liver biopsy was done for group I and group II. Liver biopsies of controls were obtained intraoperatively from surgery department from individuals admitted for cholecystectomy. Each liver biopsy was examined for steatosis, inflammation, fibrosis and mitochondrial ATP level was estimated.

Different histological parameters were evaluated including steatosis, lobular inflammation, ballooning degeneration, and pericellular fibrosis, portal/ septal fibrosis.

**Histological grading of steatosis was done according to kleiner et al. (2005)\(^{(10)}\):**

The main histological features commonly described in NALFD/NASH including:- Steatosis, inflammation (portal and lobular), hepatocyte ballooning and fibrosis.

Histological criteria of NASH were based on steatosis (≥5 % of lobular hepatocytes affected) and two of the following three: lobular inflammation, ballooning degeneration, and pericellular fibrosis.

The main histological features were scored according to the scoring system for NAFLD, recently developed by Kleiner et al.\(^{(10)}\); NAS-II (NASH activity score) (NASH clinical research network revision).

**NASH activity score;** is defined as the sum of the scores for steatosis (0–3), lobular inflammation (0 - 3) and ballooning degeneration (0–2).

Scores therefore ranged from 0 to 8. Cases with NAS- II of 0 to 2 were considered not diagnostic of NASH.

Finally, ATP concentration of mitochondrial suspension was measured by luminometer using commercial kits.

3. Results

BMI, fasting blood glucose and insulin and HOMA-IR were significantly higher in patients with fatty liver, NASH and cirrhosis (Fig.1,2,3,4) also, NASH patients showed a significant high serum triglycerides (Table,1) and ALT. Histological examination of group I and group II is illustrated in (Table 2, 3)

According to score of Kleiner, et al, (2005)\(^{(10)}\); NAS-II (NASH activity score) (NASH clinical research network revision), the results were as follows:

**Group I:** 41.7% of patients had S1; steatosis
< 33%, 50% had S2; steatosis 33%-66% and 8.3% had S3; steatosis > 66%. All fatty infiltration was macrovesicular except one patient had mixed macrovesicular and microvesicular.

**Group II:** 18.2% of patients had score 3, 54.5% of patients had score 5, 18.2% of patients had score 7 and 9.1% of patients had score 8.

Fibrosis was scored as 0 (no fibrosis) in 18.2% of patients, 1 (Perisinusoidal fibrosis) in 27.3% of patients, 2 (Perisinusoidal and portal / periportal fibrosis) in 18.2% of patients and 3 (Bridging fibrosis) in 36.3% of patients.

All previous parameters were significantly increased with increase severity of histopathological score in patients with fatty liver and NASH. (Table, 4, 5).

Serum AST levels and AST / ALT ratio were significantly increased in NASH and cirrhotic patients as compared to patients with steatosis alone and controls.

Mitochondrial ATP levels in patients with fatty liver and NASH showed a statistically significant decrease. Also patients with NASH showed a statistically significant decrease when compared to patients with fatty liver (Table 6). Finally, patients with fatty liver and NASH showed a significant decrease in mitochondrial ATP with increase BMI and histopathological score. (Table 7).

![Fig. (1) BMI (kg/m²) of the studied groups.](image1)

![Fig. (2) Fasting blood glucose (mg/dl) in the studied groups.](image2)

![Fig. (3) Fasting blood insulin (µ IU/ml) in the studied groups.](image3)

![Fig. (4) HOMA-IR in the studied groups.](image4)
Table (1): Results of lipid profile in the studied groups

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Control Group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=12)</td>
<td>(n=11)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td></td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>Range</td>
<td>range</td>
<td>range</td>
<td>range</td>
<td></td>
</tr>
<tr>
<td><strong>Cholesterol (mg/dl)</strong></td>
<td>147-196</td>
<td>160.5±27.4</td>
<td>133-201</td>
<td>163.9±27.2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>163.9±27.2</td>
<td>106-188</td>
<td>161.4±23.6</td>
<td>117-148</td>
<td>164.8±21.5</td>
</tr>
<tr>
<td><strong>HDL (mg/dl)</strong></td>
<td>43-51</td>
<td>45.58±3.15</td>
<td>48-52</td>
<td>46.18±4.85</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40-53</td>
<td>49.9±5.74</td>
<td>43-64</td>
<td>50.5±6.4</td>
</tr>
<tr>
<td><strong>Triglyceride (mg/dl)</strong></td>
<td>126-240</td>
<td>201±18.43</td>
<td>138-261</td>
<td>*214±43.9</td>
<td>&lt;0.05 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt;0.05 a</td>
</tr>
</tbody>
</table>

a: GII vs GIII, GIV.  
P is significant < 0.05  * significant.

Table (2) : Histopathological score in group I and group II

<table>
<thead>
<tr>
<th></th>
<th>Histopathological score in group I (n=12)</th>
<th>Histopathological score in group II(n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 1 2 3 -</td>
<td>3 4 5 6 7 8</td>
</tr>
<tr>
<td>Number of patients</td>
<td>0 5 6 1 -</td>
<td>2 0 6 0 2 1</td>
</tr>
<tr>
<td>%</td>
<td>0 41.7% 50% 8.3% -</td>
<td>18.2% 54.5% 0 18.2% 9.1%</td>
</tr>
</tbody>
</table>

Table (3): Liver fibrosis score in group II

<table>
<thead>
<tr>
<th></th>
<th>Score of liver fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 1 2 3 4</td>
</tr>
<tr>
<td>Number of patients</td>
<td>2 3 2 4 0</td>
</tr>
<tr>
<td>%</td>
<td>18.2% 27.3% 18.2% 36.3% 0</td>
</tr>
</tbody>
</table>

0: None  
1: Perisinusoidal or periportal  
2: Perisinusoidal and portal / periportal  
3: Bridging fibrosis  
4: cirrhosis
Table (4): Correlation between body mass index (BMI) (kg/m²) and histopathological score of group I and group II

<table>
<thead>
<tr>
<th></th>
<th>BMI (kg/m²)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group I (n=12)</td>
<td>r</td>
<td>P</td>
<td>Group II (n=11)</td>
</tr>
<tr>
<td>NAS-II (NASH activity score)</td>
<td>0.619</td>
<td>0.0308*</td>
<td>0.653</td>
<td>0.026*</td>
</tr>
<tr>
<td>Fibrosis score</td>
<td>-</td>
<td>-</td>
<td>0.717</td>
<td>0.011*</td>
</tr>
</tbody>
</table>

Table (5): Correlation between fasting blood glucose (mg/dl), fasting blood insulin (µIU/L) & HOMA-IR and histopathological and fibrosis scores of group I and group II

<table>
<thead>
<tr>
<th></th>
<th>Histopathological score (NAS-II)</th>
<th>Fibrosis score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group I (n=12)</td>
<td>Group II (n=11)</td>
</tr>
<tr>
<td></td>
<td>r</td>
<td>P</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>0.741</td>
<td>0.004*</td>
</tr>
<tr>
<td>Fasting blood insulin (µIU/L)</td>
<td>0.574</td>
<td>0.043*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.853</td>
<td>0.0004*</td>
</tr>
</tbody>
</table>

*significant

NAS-II: (NASH activity score-II)

Table (6): Results of mitochondrial ATP (n mol/mg protein) in group I, group II and group IV

<table>
<thead>
<tr>
<th></th>
<th>Group I (n=12)</th>
<th>Group II (n=11)</th>
<th>Control group (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>13.7-21.8</td>
<td>14.3-18.1</td>
<td>19.2-23.2</td>
</tr>
<tr>
<td>Mean ±SD</td>
<td>17.76 ± 2.25*</td>
<td>14.25 ± 1.49*</td>
<td>22.14 ± 1.67*</td>
</tr>
<tr>
<td>Significance</td>
<td>*S (P-value &lt; 0.05) a,b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ATP: Adenosine triphosphate

*significant

a: GI,GII vs IV

b: GII vs GI
Table (7): Correlation between mitochondrial ATP (n mol /mg protein) and BMI in patients of Group I & Group II

<table>
<thead>
<tr>
<th></th>
<th>Mitochondrial ATP (n mol /mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group I (n=12)</td>
</tr>
<tr>
<td></td>
<td>r</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>-0.634</td>
</tr>
<tr>
<td></td>
<td>Group II (n=11)</td>
</tr>
<tr>
<td></td>
<td>r</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>-0.141</td>
</tr>
</tbody>
</table>

4. Discussion:

In our study, patients with fatty liver, NASH and cirrhosis showed a significant higher BMI as compared to control group. This finding agrees with Wanless and Lentz (1990) (11), Abhasnee Sobhonslidsuk et al, (2007) (12) and Fabbrini E et al, (2009) (13) who reported that, obesity was found in 40%-100% of fatty liver. Fatty liver has been documented in up to 70% to 80% of obese individuals, also Clark and Diehl (2003) (14), who reported that patients with steatosis and NASH had elevated BMI.

Patients with NASH and steatosis alone showed increased serum triglycerides with significant high serum triglycerides in NASH patients as compared to control group and patients with cirrhosis.

Our findings were in agreement with Ender Sern et al, (2002) (15) and Joong-Won; et al, (2007) (16) who reported that serum triglyceride did not show significant differences between steatosis and NASH groups and triglyceride, was significantly associated with NASH.

Fasting blood glucose increased in patients with fatty liver, NASH and cirrhosis and showed a significant high level in patients with NASH as compared to control group. This finding agreed with James and Day, et al, (1998) (17) who stated that up to one third of patients have diabetes or fasting hyperglycemia at the time of diagnosis of NASH.

Bookman et al, (2006) (18) reported that fasting serum glucose showed significant increase in NASH patients. This finding is supported by data showing the profibrogenic role of hyperglycemia in experimental animals. (Paradis, et al, 2001). (19)

Fasting blood insulin showed statistically significant increase in all patients as compared to control group. Our findings were in agreement with Bookmann, et al (2006) (18) who reported that the patients with steatosis and NASH had high levels of fasting blood insulin. This hyperinsulinaemia basically results from compensatory hypersecretion by beta-cells in fatty liver and NASH patients and from reduced insulin breakdown in liver as a result of cirrhosis. (Marchesini, et al, 2003). (20)

Insulin resistance was significantly increased across all patients as compared to control group, and the difference between NASH patients and fatty liver alone was statistically significant. These findings are consistent with many published studies done by Marchesini, et al (2003); (20) Bookman, et al (2006) (18), they stated that Insulin resistance was higher in both NASH and fatty liver patients than healthy controls and subjects with NASH had more severe insulin resistance when compared to those with simple fatty liver.

The patients with steatosis and NASH had varying degrees of steatosis, parenchymal inflammation and fibrosis. A significant positive correlation had been found between BMI and histopathological score in patients with fatty liver alone and histopathological and fibrosis score of patients with NASH.

This finding agreed with Abhasnee Sobhonslidsuk, et al, (2007) (21); they reported increased BMI was positively correlated with the grades of parenchymal inflammation and stages of fibrosis in patients with NASH. Other study by Luyckx, et al (2000) (22) concluded that, the grade of steatosis correlates with the severity of obesity and obesity also correlates with the stages of fibrosis in NASH.

The fasting serum glucose and insulin level in patients with fatty liver and NASH were positively correlated with the histopathological score. These results were in agreement with Ender Sern, et al, (2002) (23) who found that serum insulin levels correlated with severity of steatosis and inflammation in NASH patients, Klein, et al, (2004) (24) who reported that hyperinsulinemia was positively correlated with severity of histopathology of fatty liver and NASH and Pierre, et al, (2007) (25) who stated that hyperglycemia was strongly associated with the presence of NASH.
The fasting serum glucose and insulin level in our study were not significantly correlated with the fibrosis score of NASH patients. These results were in agreement with Bookmann, et al (2006) (18) who reported that fasting blood glucose levels were not significantly correlated with any stage of fibrosis and Chow, et al, (2007) (26) who stated that high serum glucose was not significantly correlated with hepatic fibrosis in NASH.

Our work, showed significant positive correlation between HOMA-IR levels and histopathological and fibrosis scores in patients with fatty liver and NASH respectively.

This finding was in accordance with many different studies. Bookman (2006) (18) reported a significant positive correlation between liver histopathology in patients with fatty liver alone and NASH and insulin resistance, also Rector, et al, (2008) (27) who mentioned that insulin resistance associated with the exacerbation of NAFLD.

Our results agreed with Cortez-Pinto, et al (1999) (28) who found that; a greater association of severity of histopathology with triglyceride values in NASH. These findings could suggest that hypertriglyceridemia may be considered as a risk factor for development of steatosis and progression to NASH.

Mitochondrial ATP levels in patients with fatty liver and NASH were significantly decreased when compared to the control group. Also patients with NASH showed a statistically significant decrease when compared to patients with fatty liver. These results were in accordance with many different studies. Cortez-Pinto, et al, (1999) (28) demonstrated that patients with NASH have decreased mitochondrial ATP levels and Fan, et al, (2005) (29) reported that, mitochondrial ATP levels were significantly reduced in rats with NAFLD compared with the control group. Finally, Serviddio, et al, (2008) (30) reported that, the mitochondrial ATP content was significantly lower in NASH livers in a rodent model.

This may be explained by mitochondrial injury as a one cause of reduced hepatocellular ATP stores in NASH. This is supported by Dominique Pessayre, et al, (2002) (31) that, identified crystalline structures of uncertain composition coupled with the mitochondrial matrix in patients with NASH, these ultrastructural mitochondrial lesions, decreased activity of respiratory chain complexes, and impaired ability to synthesize ATP. These data were in agreement with our work as they reported that mitochondrial ATP levels negatively correlated with severity of obesity in NAFLD.

These findings could be explained by the following, in obesity hepatocytes induce uncoupling protein-2 (UCP-2) mRNA and protein expression. Thus, when confronted with an abundant substrate supply i.e free fatty acids (FFA), hepatocytes activate pathways that are not efficiently coupled to ATP synthesis i.e uncoupling of oxidative phosphorylation (Skulachev, et al, 1996) (32). Additionally, there is growing evidence that uncoupling protein -2 (UCP-2) may be a tumor necrosis factor- α (TNF- α) inducible gene that involved in pathogenesis of NASH. (Gimeno, et al, 1997). (33)

In our study, an insignificant negative correlation was found between mitochondrial ATP levels in fatty liver and NASH patients and fasting serum glucose, fasting serum insulin and HOMA-IR. i.e mitochondrial ATP was decreasing with increasing these parameters but the relation was not statistically significant. This could be supported to some extent with significant correlation between these parameters and obesity that significantly associated with hepatic ATP depletion. (Fan, et al, 2005). (29)

Patients with fatty liver and NASH showed a significant negative correlation between mitochondrial ATP levels and histopathological score. This finding may be explained by ATP depletion that may predispose to hepatocellular injury because ATP is critical for maintaining cellular integrity (Chavin, et al, 1999)(34) and the patients had factors lead to increased free fatty acids and reactive oxygen species (ROS) oxidize accumulated unsaturated fatty acids, causing lipid peroxidation, which releases reactive aldehyde that increase hepatic fibrogenesis in two ways. First, these lipid peroxidation products enhance the hepatic production of transforming growth factor -β1 (TGF- β1), which activates hepatic stellate cells into collagen-secreting myofibroblasts. Second, lipid peroxidation products also directly enhance collagen production by hepatic stellate cells.

ROS also increase the synthesis of several cytokines in the liver, particularly TNF- α, which can cause both apoptosis and necrosis .Finally, ROS-associated lipid peroxidation and cytokines may be involved in the inflammatory cell infiltrate, because, lipid peroxidation products, TGF-β1, and interleukin-8 are chemoattractants for neutrophils. (Pessayre, et al, 2001). (35)

These data suggest that, mitochondrial dysfunction and decreased mitochondrial ATP levels are involved in progression of simple fatty liver to NASH and cirrhosis. This is supported by, the fact that ATP is critical for maintaining cellular integrity so its depletion may predispose to hepatocellular injury and necrosis.
5. Conclusion: Nonalcoholic fatty liver disease, NAFLD is now considered a metabolic pathway to advanced liver disease; cirrhosis and hepatocellular carcinoma. Increased BMI, hyperglycemia, hypertriglyceridaemia, insulin resistance and depletion of mitochondrial ATP in hepatocytes can be considered risk factors involved in the development and progression of fatty liver to NASH and cirrhosis.

Corresponding author
Nadia M. Elwan
Tropical Medicine Department, Tanta University, Tanta, Egypt
*nadiaelwan@yahoo.com

6. References:


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