High Serum Levels of Endothelial Adhesion Molecules E-selectin, ICAM-1 and VCAM in Fatty Liver Patients

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ABSTRACT
This search aims to find a correlation between adhesion molecules and fatty liver patients. Raised serum levels of endothelial-leukocyte adhesion molecule-1 (E-selectin), intercellular adhesion molecule-1 (ICAM-1) and circulating vascular cell adhesion molecule-1 (VCAM-1) have been observed mainly in patients with infections, inflammatory and fatty liver disease. Non-Alcoholic Fatty Liver Disease (NAFLD) was diagnosed by persistently elevated ALT and ultrasonographic bright liver with no other liver disease. A study was carried out to analyze whether fatty liver patients with increasing cholesterol and triglycerides is related to the serum levels of E-selectin, ICAM-1 and VCAM-1. Ninety serum samples from NAFLD patients were classified to four groups. The results showed that ALT and AST were significantly higher in fatty liver patients with high TG and cholesterol (group 1), compared to controls (92.16±31.197 vs. 33.56±8.322, p<0.001) for AST and (76.8±28.872 vs. 29.32±6.479, p<0.001) for ALT. While no significant difference was observed between ALT levels of fatty liver (with normal TG and cholesterol) and controls (32.36±7.745 vs. 29.32±6.479, p = 0.05). Positive E-selectin samples were found in 88% of patients in group (1), 90% in group (2), 100% in group (3) and 32% in group (4). These values were significantly higher than those in healthy individuals (p<0.0001). High ICAM-1 level was found in 80% in-group (1), 75% in-group (2), 100% in-group (3) and 40% in-group (4). Level of ICAM-1 in group (1) was extremely significant compared to group (2) (465.44±56.501 vs. 407.48±32.686 p<0.0001 ES). The values of VCAM-1 in group (1) were significantly higher than those in healthy individuals (p<0.0001) and positive VCAM-1 samples were found in 72% in group (1), 35% in group (2), 40% in group (3) and 0% in group (4). In conclusion, there was a significant increase in circulating levels of ICAM-1, VCAM-1 and E-selectin in NAFLD compared to healthy control subjects and it may be used to comprehensively using the ability of circulating VCAM-1, E-selectin and ICAM-1 to predict fatty liver disease and evaluated the relationship between circulating adhesion molecules and fatty liver.

Key words: Fatty liver, E-selectin, NAFLD, adhesion molecule

INTRODUCTION
The endothelium is involved in several homeostatic mechanisms, such as the maintenance of a non-thrombotic surface, the metabolism of lipoproteins and in immune response (Sampietro et al., 2005). Several conditions may induce activation of endothelial cells, which leads to the appearance of Adhesion Molecules (AMs) on the cell surface.
Adhesion Molecules (AMs) are proteins expressed on a variety of cells, which mediate the interaction between endothelial cells with lymphocytes, monocytes, and leucocytes (Madan et al., 2004). ICAM-1 and VCAM-1 are two members of the Ig-like supergene family of adhesion molecules (molecular weight 90-110 KDa range), play key roles in promoting migration of immunological cells from the circulation to target site in the inflammatory state (Papayianni et al., 2002; Bruno et al., 2005).

Several factors, cytokine, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) activate and up-regulate the expression of AMs on the cell surface where they support the interaction of leucocytes and endothelial cells which play a fundamental role in many pathophysiological processes (Song et al., 2010). In addition to being expressed on the cell surface, soluble forms of adhesion molecules have been detected in circulating blood and have been shown to retain their functional ability (Brevetti et al., 2006).

Raised serum levels of vascular cell AMs (E-selectin, ICAM-1 and VCAM-1) have been observed mainly in patients with infections, inflammatory, neoplastic diseases and fatty liver disease (Musso et al., 2008a). In addition, these molecules are considered as markers of atherosclerosis (Hwang et al., 1997) and specifically of ischemic heart disease (Ghalsas et al., 1997). In these clinical settings, measuring serum levels of AMs may be useful to detect the subclinical activity and prognosis of these diseases.

Nonalcoholic Fatty Liver Disease (NAFLD) is the most common chronic disease in Western countries (Bloomgarden, 2005) and is emerging as a marker of early atherosclerosis (Musso et al., 2008a).

NAFLD was diagnosed by persistently (>6 months) elevated aminotransferase (ALT)>80 units L⁻¹ in men and >20 units L⁻¹ in women, based on recently proposed cutoff values, which increase the sensitivity for detection of NAFLD (Prati et al., 2002; Chang et al., 2007) and ultrasonographic bright liver with no other liver disease.

**MATERIALS AND METHODS**

The study was conducted on 90 patients and 25 healthy subjects served as control, the 115 serum samples were collected (90 serum samples from NAFLD patients and 25 controls) from Gastroenterology Center, Mansoura University. Samples are centrifuged at 1500 rpm for 10 min and stored at -20°C till the time of analysis.

**Exclusion criteria:** Fatty liver and control subjects were excluded from the study if they met one of the following criteria: human immunodeficiency virus infection or other causes of immunodeficiency, hepatitis B or C virus infection, cirrhosis, consumption of drugs with effect on immunity (steroids, immunosuppressants) or chronic disease such as diabetes mellitus. Patients with diabetes mellitus, autoimmune disease or malignancies were excluded in order to avoid effects of these combed conditions on cytokine production.

**Quantification of soluble adhesion molecules:** Serum levels of circulating ICAM-1, VCAM-1 and E-selectin were determined by a commercially available sandwich ELISA technique (RayBiotech, Inc, for VCAM and E-Selectin and R and D systems, Inc Minneapolis for ICAM-1).

The procedures were performed according to the manufactures' instructions. Briefly, a conjugated monoclonal antibody against ICAM-1, VCAM-1 and E-selectin was added to microtitre plates coated with a murine monoclonal IgG antibody recognizing a different epitope of the
corresponding molecule. After incubation with samples or standards in appropriate dilution, the color reaction was developed with tetramethylenediamine (TMB) and the plates were read on an automated multiscanner at 450 nm. All measurements were performed in duplicate.

**Analysis of serum markers:** Routine biochemical determinations as levels of liver enzymes, (including ALT and AST) and albumin were performed in sera samples by standard automated methods using an automatic analyzer (Hitachi 902 autoanalyzer S.N. 1048008).

**Statistical methods:** Data were analyzed with standard program of SPSS, Echo Soft Corporation, USA, 1995 statistical package. Unpaired Student t test was applied to the data conforming to normal distribution. Data are expressed as Mean±SD. Unpaired t test were used for between-group analysis at baseline. A value of p<0.0001 regards as extremely significant, p<0.001 very significant and p<0.05 regards as significant.

**RESULTS**

**Clinical and biochemical analysis:** The study was conducted on 90 NAFLD patients and 25 healthy subjects served as control, from Gastroenterology Center, Mansoura University. The patients were classified to four groups: group (1) contain twenty-five samples with high both TG and cholesterol group (2) contain twenty samples with abnormal cholesterol and normal TG, group (3) contain twenty samples with abnormal TG and normal cholesterol and group (4) contain twenty-five samples with normal both TG and cholesterol.

The results showed that, the fatty liver patients with normal and high both triglycerides and cholesterol· and healthy control had a mean age (±SD) of 42.48±5.987, 50.96±7.919 and 38.72±7.84 years, respectively. Serum ALT and AST were significantly higher in fatty liver patients with high TG and cholesterol, compared to controls (92.16±31.197 vs. 33.56±8.322, p<0.001) for AST and (76.8±28.872 vs. 29.32±6.479, p<0.001) for ALT indicating very significant relationship. No significant was observed between ALT levels of fatty liver (with normal TG and cholesterol) and controls (32.36±7.745 vs. 29.32±6.479, p = 0.05 NS) (Table 1). On the other hand, ALT and AST levels of fatty liver patients with high either TG or cholesterol had moderate significant compared to control (for ALT p<0.05 S, for AST p<0.0001 and p<0.01 for AST) for high TG and high cholesterol, respectively. While p>0.05 NS, for ALT of high cholesterol compared to control indicating not significant.

Serum albumin levels of fatty liver patients with abnormal both TG and cholesterol was lower compared to that of controls (3.376±0.3711 vs. 3.992±0.2448 p>0.05 NS), indicating no significant.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 25)</th>
<th>Normal TG and Chol (n = 25)</th>
<th>Normal TG and abn Chol (n = 20)</th>
<th>Abnor TG and nor Chol (n = 20)</th>
<th>Abnormal TG and Chol (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>38.72±7.84</td>
<td>42.48±5.99</td>
<td>44.26±6.62</td>
<td>42.9±5.64</td>
<td>50.96±7.92</td>
</tr>
<tr>
<td>Sex M/F</td>
<td>14/11</td>
<td>12/25</td>
<td>10/20</td>
<td>6/20</td>
<td>16/25</td>
</tr>
<tr>
<td>Alb (mg%)</td>
<td>3.99±0.24</td>
<td>3.8±0.22</td>
<td>3.87±0.23</td>
<td>3.68±0.25</td>
<td>3.376±0.37ns</td>
</tr>
<tr>
<td>ALT (IU L⁻¹)</td>
<td>29.32±6.48</td>
<td>32.36±7.75</td>
<td>38.8±10.38</td>
<td>43.7±11.5***</td>
<td>76.8±28.87***</td>
</tr>
<tr>
<td>AST (IU L⁻¹)</td>
<td>33.56±8.32</td>
<td>38.36±12.58</td>
<td>49.7±17.1***</td>
<td>61.2±17.17***</td>
<td>92.16±31.19***</td>
</tr>
</tbody>
</table>

ns: Not significant, ***p<0.001: Extremely significant.
Table 2: Prevalence of E-selectin, ICAM-1 and VCAM-1 in serum of fatty liver patients and healthy controls

<table>
<thead>
<tr>
<th>Group</th>
<th>No</th>
<th>E-selectin positive=183.416</th>
<th>ICAM-1 positive=377.792</th>
<th>VCAM-1 positive=1075.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Range (Mean±SD)</td>
<td>+Ve/-Ve</td>
<td>Range (Mean±SD)</td>
</tr>
<tr>
<td>Healthy control</td>
<td>25</td>
<td>59-182 (109.64±36.89)</td>
<td>0/25</td>
<td>189-372 (280.38±48.76)</td>
</tr>
<tr>
<td>Fatty with high</td>
<td>25</td>
<td>168-300 (228.6±38.50***</td>
<td>22/3</td>
<td>365-548 (465.4±56.5***</td>
</tr>
<tr>
<td>TG and chol</td>
<td>20</td>
<td>193-250 (239.38±38.50***</td>
<td>20/0</td>
<td>399-512 (465.4±56.5***</td>
</tr>
<tr>
<td>Fatty with normal</td>
<td>20</td>
<td>168-245 (226.2±20.52***</td>
<td>18/2</td>
<td>296-459 (457.5±38.21***</td>
</tr>
<tr>
<td>chol and high TG</td>
<td>20</td>
<td>112-230 (214.75±20.91***</td>
<td>8/17</td>
<td>296-459 (396.7±47.05***</td>
</tr>
<tr>
<td>both TG and chol</td>
<td>25</td>
<td>194.16±32.82***</td>
<td>0/25</td>
<td>396-620 (407.48±32.69***</td>
</tr>
</tbody>
</table>

***p<0.001: Extremely significant

Evaluation of E-selectin, ICAM-1 and VCAM-1 levels in serum of fatty liver patients and controls

E-selectin: As shown in Table 2, the level of E-selectin, in group (1) ranged from 168-300 and mean 239.38 with standard deviation (SD) equal to 38.585, from 168-245 with mean 214.75 and SD 20.913 for group (2), ranged from 193 - 250 with mean 228.6 and SD 20.519 for group (3) and 112-230 with mean 194.16 and SD = 32.823 for group (4). These values were significantly higher than those in healthy individuals that had E-selectin ranged from 59-182 and mean 109.64 with SD 36.898 (p<0.0001) indicating extremely significant.

Cut off value for the E-selectin in the patients with NAFLD were arbitrarily defined as 183.416 which was 2 SD above the mean of the healthy individuals. Therefore, Serum E-selectin above 183.416 was defined as positive. Our result showed that the positive E-selectin samples were found in 22 out of 25 (88%) patients in group (1), 18 out of 20 (90%) in group (2), 20 out of 20 (100%) in group (3) and 8 out of 25 (32%) in group (4). The specificity of E-selectin was calculated according to the healthy individuals and found to be 100% and the overall accuracy of the test equal to 94, 95.5, 100 and 66% for the four groups respectively as shown in Table 2. Level of E-selectin in fatty liver with high both TG and cholesterol was extremely significant compared to fatty liver patients with normal both TG and cholesterol (239.38±38.585 vs. 194.16±32.822, p<0.0001). The mean serum E-selectin level of male fatty liver patients was nearly equal to those of female patients and the mean difference was not statically significant (237.87±37.232 vs. 247.86±42.698, p = 0.5605).

ICAM-1: The serum level of ICAM-1, in group (1) ranged from 365-548 and mean 465.44 with SD equal to 56.501, from 296-459 with mean 396.7 and SD 47.05 for group (2), from 399-512 and mean value 457.5 with SD 38.209 for group (3) and 290 - 436 with mean 407.48 and SD = 32.686 for group (4). These values were significantly higher than those in healthy individuals that had ICAM-1 ranged from 189-372 and average value 280.28 with SD 48.756 (p<0.0001) (Table 2).

The Cut off value for the ICAM-1 in the patients with NAFLD were arbitrarily defined as 377.792. Therefore, serum ICAM above 377.792 was defined as positive. Our result showed that the positive ICAM-1 samples were found in 20 out of 25 (80%) patients in group (1), 15 out of 20 (75%) in group (2), 20 out of 20 (100%) in group (3) and 20 out of 25 (80%) in group (4). The
specificity of ICAM was calculated and found to be 100% and the overall accuracy of the test were 90, 88.9, 100 and 90% for the four groups respectively as shown in Table 2. Level of ICAM-1 in fatty liver with high TG and cholesterol was extremely significant compared to fatty liver patients with normal both TG and cholesterol (465.44±56.501 vs. 407.48±32.686, p<0.0001 ES). The mean serum ICAM-1 level of male fatty liver patients was equal to those of female patients and the mean difference was not statistically significant (479.67±54.037 vs. 441.63±57.862, p = 0.1178).

VCAM-1: The level of VCAM-1, in group (1) ranged from 140-1450 and mean 1118.8 with SD 287.63, from 750-1200 with mean 978.25 and SD 145.58 for group (2), from 650-1250 and mean 1021.35 with SD 163.01 for group (3) and 588-1050 with mean 908.92 and SD = 149.38 for group (4). These values were significantly higher than those in healthy individuals that have VCAM-1 ranged from 58-988 with mean 588.96 and SD 244.27 (p<0.0001) (Table 2).

The cut off value for the VCAM-1 in the patients with NAFLD was 1075.5. Therefore, serums VCAM-1 above 1075.5 were defined as positive. Our result showed that the positive VCAM-1 samples were found in 18 out of 25 (72%) patients in group (1), 7 out of 20 (35%) in group (2), 8 out of 20 (40%) in group (3) and 0 out of 25 (0%) in group (4). The specificity of VCAM-1 was calculated and found to be 100% and the overall accuracy of the test were 86, 71.1, 73.3 and 50% for four groups respectively as shown in Table 2. Level of VCAM-1 in fatty liver with high TG and cholesterol was very significant compared to fatty liver patients with normal both TG and cholesterol (1118.8±287.63 vs. 906.92±149.38, p = 0.0020). The mean serum VCAM-1 level of male fatty liver patients was equal to those of female patients and the mean difference was not statically significant (1112.4±22.72 vs 1112.9±22.99, p = 0.999). Overall accuracy of ELISA for E-selection ICAM-1 and VCAM-1 is shown in Fig. 1.

DISCUSSION

The vascular endothelium regulates the adhesion of leucocytes to the vascular compartment by ensuring the presence of inflammatory AMs on the surface of the endothelial cell (Gimbrone, 1995). AMs, such as E-selectin, ICAM-1 and VCAM-1, mediate the attachment of certain leucocytes to the endothelial surface and may be important in controlling the extravasation of leucocytes from the circulation to sites of inflammation (Bevilacqua, 1993).
The accumulated data imply that selectin mediate initial rolling of leukocytes along the endothelium and that VCAM-1 and ICAM-1 play important roles in the firm attachment and transendothelial migration of leukocytes. Results of immuno-histochemical studies show different levels of expression of these molecules that reflect their unique structural and functional characteristics (Poston et al., 1992; Van der Wal et al., 1992; Davies et al., 1993; Wood et al., 1993; O’Brien et al., 1993; O’Brien et al., 1996). The origins of circulating VCAM-1, E-selectin and ICAM-1 are unclear, but they may arise from shedding or proteolytic cleavage from endothelial cells (Rothlein et al., 1991; Pigott et al., 1992).

Endothelial AMs may be detected in a soluble form in the serum, usually reflecting higher expression on the cell membrane, and elevated circulating concentrations of the soluble forms of VCAM-1, ICAM-1 and E-selectin have been increased in inflammatory processes (septic shock, Vasculitis, Atherosclerosis) and were observed in subjects with atherosclerosis and those at increased risk due to diabetes and hyperlipidaemia (Ceriello et al., 1996; Hwang et al., 1997; Bannan et al., 1998; Doo et al., 2004).

Non-Alcoholic Fatty Liver Disease (NAFLD) refers to a histological spectrum of liver damage from simple steatosis to advanced fibrosis and cirrhosis in individuals without a relevant alcohol consumption (Matteoni et al., 1999; Angula, 2002) and it has been recognized as a major cause of liver-related morbidity and mortality (Pagano et al., 2005). The prevalence of NAFLD has risen rapidly in parallel with the dramatic rise in obesity and diabetes (Charlton, 2004) since it has been documented in up to 10 to 15% of normal individuals and 70 to 80% of obese individuals (Duvnjak et al., 2007).

In fatty liver patients, analysis of the serum levels of endothelial AMs has been focused on subjects with high both TG and cholesterol, although fatty liver with high TG or cholesterol only exhibited significantly higher serum levels of these three AMs compared to healthy control.

In the present study, analysis of the serum levels of fatty liver patients with abnormal both TG and cholesterol exhibited significantly higher levels of these three AMs compared to healthy control group (p<0.0001). Serum levels of E-selectin and ICAM-1 were higher in fatty liver patients with high TG value, with sensitivity 100% and overall accuracy 100% in both levels while the levels were decreased in case of fatty liver patients with high cholesterol value only since for E-selectin, the sensitivity and over all accuracy were reduced to 90 and 95.5% respectively and for ICAM-1, sensitivity and overall accuracy were 75 and 88.9%, respectively. Significantly higher serum levels of E-selectin and ICAM-1 in fatty liver patients suggesting endothelial and for immune activation (Sacanella et al., 1999). Raised levels of E-selectin always reflect endothelial activation due to its unique origin, whereas increased levels of ICAM-1 could reflect endothelial or immune activation (T-lymphocyte activation) Musso et al. (2005) found that soluble AMs levels were higher in NAFLD than in insulin resistance without fatty liver and fatty liver predicted increased E-selectin and ICAM-1 levels and his findings therefore suggested that NAFLD may be an early marker of endothelial dysfunction, independently of insulin resistance and traditional risk factors (Schindhelm et al., 2007; Musso et al., 2008b).

On the other hand, the sensitivity and overall accuracy of VCAM-1 levels detection in patients with high TG was 40% and 73.35, respectively and it was 35 and 71.1% in case of fatty liver patients with high cholesterol and normal TG.

High serum levels of E-selectin and ICAM-1 compared to VCAM-1 levels has already been observed in patients with asthma (Montefort et al., 1994) or ischemic heart disease (Hwang et al., 1997) VCAM-1 is a member of the immunoglobulin superfamily which bindsa
heterodimeric integrin receptor expressed on monocyte and lymphocytes but not neutrophiles. This targeted expression of AMs explains the selective inhibition of monocyte adhesion by antibodies directed against VCAM-1 (Bochner et al., 1991). VCAM-1 is therefore key to the selectivity of monocyte recruitment in early atherogenesis (Williams et al., 2004).

The present study showed a significant increase in circulating levels of ICAM-1, E-selectin and VCAM-1 in fatty liver patients compared with control subjects and this is in accordance with previous report (Musso et al., 2008b).

Mechanism (s) linking NAFLD to endothelial dysfunction are unclear, but impaired lipoprotein metabolism and oxidized LDL accumulation are potential candidates (Musso et al., 2003; Musso et al., 2005, 2006, 2007).

Giron-Gonzalez et al. (2005) reported that endothelial activation plays an active role in modifications of the circulatory status of cirrhotic patients, then de novo expression of ICAM-1 and VCAM-1 on endothelial cells mediates the transmigration of inflammatory cells, which induce inflammation and tissue damage in liver. Up-regulated ICAM-1 or VCAM-1 expression in chronic liver disease has been reported in several studies, suggesting that they may play roles in the pathogenesis of chronic hepatitis, cirrhosis or fatty liver (Capra et al., 2000; Abdalla et al., 2002; Ho et al., 2004; Musso et al., 2008a) and elevated circulating concentrations of the soluble forms of VCAM-1, ICAM-1 and E-selectin have been observed in subjects with atherosclerosis and those at increased risk due to diabetes and hyperlipidaemia (Hwang et al., 1997; Ceriello et al., 1996; Doo et al., 2004).

The selectin family of cell AMs is generally thought to promote inflammatory reactions by facilitating leukocyte recruitment. High levels of soluble E-selectin have been reported in acute and chronic inflammatory disorders (Tacke et al., 2003).

In day to day clinical practice, a diagnosis of NAFLD would simply require chronically elevated liver enzyme levels, and an ultrasonographic bright liver and exclusion of viral infection and of exposure to hepatotoxins (including alcohol) by interview of patients and relatives (Musso et al., 2008a).

The endothelium interacts continuously with plasma lipids and lipoproteins with considerable potential for adverse consequences in terms of endothelial activation, dysfunction and ultimately initiation of atherogenesis. It is, therefore not surprising that the role of diet-related lipoprotein particles in endothelial activation and induction of adhesion molecules expression has received considerable attention in recent years (Williams et al., 2004).

Serum ALT and AST were significantly higher in fatty liver patients with high both TG and cholesterol, compared to controls (p<0.001) for AST and (p<0.001) for ALT indicating very significant relationship, whereas no difference was observed between ALT levels of fatty liver (with normal TG and cholesterol) and controls (p = 0.05) indicating no significant and that ALT and AST for fatty liver patients were correlated with the high level of TG and cholesterol in fatty liver patients and this result was in accordance with some studies as Wyszomirska et al. (2008) which concluded that NAFLD was recognized as one of the most frequent reason of liver tests elevation without clinical symptoms.

The development of NAFLD is strongly associated with metabolic syndrome reflected by the fact that approximately 90% of the patients with NAFLD have more than one feature of metabolic syndrome and about 33% have three or more criteria (Marchesini et al., 2003).

A high level of TG (>150 mg dL⁻¹) is one of the diagnostic criteria of the metabolic syndrome according to World Health Organization (WHO) and American Association of Clinical Endocrinologists (AACE) (Valdez et al., 2009).
In the hypercholesterolaemic rabbit, the earliest indicator of endothelial activation, which precedes infiltration of the area with monocytes, is the increased expression of VCAM-1 on the endothelial surface (Li et al., 1993).

The expression of AMs such as ICAM-1, VCAM-1 and E-selectin was high in the chronic hepatitis, or cirrhosis was reported in several studies and these adhesion molecules may play roles in the pathogenesis of liver fibrosis. In our study very less expression of these molecules was found in the normal liver, but in fatty liver, there were high expressions of them in the sera of patients.

In conclusion the study showed a significant increase in circulating levels of ICAM-1, VCAM-1 and E-selectin in NAFLD compared to healthy control subjects and it point out the important role of using the ability of circulating VCAM-1, E-selectin and ICAM-1 to predict fatty liver disease and evaluate the relationship between circulating adhesion molecules and fatty liver.

REFERENCES


