Abstract

Background and Aims: Several non-invasive markers have been proposed to assess liver damage in NAFLD. We measured by ultrasound (US) the perihepatic adipose tissue thickness (PATT), i.e. the thickness of the fat between the abdominal muscular layer and the hepatic surface, in addition to waist circumference, BMI, biochemistry and serum adipokines, to predict the severity of liver damage in NAFLD. Methods: 63 NAFLD patients and 45 controls were studied. PATT and US steatosis score were assessed in all patients. Histology was obtained in those with an US steatosis score ≥ 2. Results: PATT was 13.5±4.1 mm in NAFLD vs 8.0±4.1 in controls (p<0.001). A PATT value of 11.2 mm seems to represent a cut-off below which NAFLD is unlikely. Test sensitivity, specificity and the area under the ROC curve were 100%, 50% and 75%, respectively, suggesting a good discrimination between patients with non-NASH and those with NASH or borderline NASH. In addition, PATT strongly correlated with waist circumference (p<0.001). Both PATT and waist circumference correlated with US steatosis, HOMA-IR, TNF-α, IL-6 and leptin. Based on a multiple logistic regression analysis, waist circumferences ≥ 110, 113 and 122 cm were associated with a probability ≥ 50% of abnormal HOMA-IR, TNF-α and leptin values, respectively. Conclusion: PATT and waist circumference could represent non-invasive markers predicting the severity of liver damage in NAFLD.

Key Words

Ultrasound – fatty liver – perihepatic adipose tissue thickness – adipokines – metabolic syndrome.

Introduction

NAFLD is a clinicopathological entity with histological features resembling alcohol-induced liver injury, though occurring in patients with little or no history of alcohol consumption [1,2]. Its prevalence ranges from 3% to 30% in the general population worldwide, and rises markedly in patients with diabetes, dyslipidaemia and, especially, obesity [3-5].

Although the pathogenetic hallmark of NAFLD is insulin-resistance, little is known about factors favouring the progression of liver damage from simple steatosis to steatohepatitis (NASH), cirrhosis and even hepatocellular carcinoma. A number of models have been proposed to provide non-invasive indices of liver damage in NAFLD [6], including biochemical [7-9], ultrasonographic [10] and anthropometric [8, 9, 11] markers, as well as the quantification of apoptosis [12] and liver breath tests [13]. Moreover, the measurement of the subcutaneous tissue thickness seems useful in differentiating NAFLD from other chronic hepatic disorders [10].

The aim of the present study was two-fold: first, to investigate whether the measurement of the perihepatic adipose tissue thickness, i.e. the thickness of the adipose tissue comprised between the abdominal muscular layer and the hepatic surface, could be a predictor of fatty liver of metabolic origin and could distinguish between patients with and without NASH; second, to examine whether other non-invasive markers, including serum adipokines which have been shown to have a predictive value for hepatic fibrosis in NAFLD [14-16], could contribute in the assessment of the severity of liver damage in NAFLD.

Methods

This is a cross-sectional study performed in a total of 108 subjects including 63 patients with NAFLD and 45 controls. NAFLD patients were both outpatients referred to our liver clinic because of elevated liver tests and/or because US had shown fatty liver, and in-patients admitted to the First Medical Division of the University of Padua Medical School for reasons other than NAFLD, in whom fatty liver
was accidentally discovered. The latter group also included six patients with morbid obesity undergoing bariatric surgery. Inclusion criteria were: age between 18 and 75 years, bright liver on US, daily alcohol intake < 20 g, and exclusion of any other known cause of liver disease: viral (presence of HBsAg or anti-HCV antibodies), autoimmune, genetic or drug-induced. Patients with major systemic conditions were also excluded from the study, as were pregnant women. Most healthy controls included hospital staff members as well as their relatives and friends. All had a normal liver scan on US and a daily alcohol intake within the same limit as in the NAFLD group. All subjects had a complete work-up including a detailed medical history, a general physical examination, laboratory tests and a liver US scan. BMI, waist circumference and systolic and diastolic blood pressure were also measured. Obesity was defined as a BMI > 30 kg/m².

The metabolic syndrome was diagnosed according to Adult Treatment Panel III criteria [17]. Laboratory investigations included serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), total and conjugated bilirubin, blood sugar, insulin, Homeostasis Model Assessment of Insulin Resistance (HOMA-IR), glycated haemoglobin (HbA1c), lipid pattern (total cholesterol, HDL, LDL, triglycerides), iron studies (iron and ferritin) and C-reactive protein (CRP). We also measured the serum levels of TNF-α, IL-6 and TGF-β1 (Quantikine Immunoassay Kit, R&D Systems, Minneapolis, MN, USA), leptin (ACTIVE Human Leptin IRMA DSL-23100i RIA Kit, Diagnostic Systems Laboratories, Webster, TX, USA) and adiponectin (Human Adiponectin RIA Kit, Linco Research, St. Charles, MO, USA).

Each patient had a liver US performed by the same operator (NV) using a Toshiba Aplio XV scanner equipped with a broadband 5-2 MHz curved-array probe to assess the presence of liver steatosis (“bright liver”), which was defined as diffuse hyperechogenicity of liver tissue with hepato-renal contrast, deep attenuation of US beam and blurring of vascular structures [18]. Where present, steatosis was graded using a semi-quantitative scale of 1 (mild) to 3 (severe). Moreover, in all subjects we measured by US the thickness of the adipose tissue comprised between the abdominal muscular layer and the surface of the liver (peripheral adipose tissue thickness, PATT), as a possible non-invasive marker of visceral fat accumulation. We used a standard approach with the patient lying in the supine position with the right arm placed above the head. The probe was placed between the ribs along the mid-axillary line and the ultrasound beam was aimed towards the right branch of the portal vein. We measured the echogenic tissue between the hyperechogenic line of the deeper surface of the abdominal muscle and the liver surface. This assessment represents a modification of the measurement of the subcutaneous tissue thickness by Riley et al. who measured the distance between the skin surface and the hepatic surface, thus including the muscular layer [10].

Thirty-five out of the 63 NAFLD patients (56%) were eligible for liver biopsy, based on the presence of steatosis of at least score 2 on US. Of these, two had a clotting disorder (due to warfarin treatment for atrial fibrillation) that represented a contraindication to the procedure. Thus, a total of 33 NAFLD patients underwent liver biopsy. Each sample was evaluated by the same pathologist, according to the classification by Brunt et al [19], in order to quantify the score of steatosis, necro-inflammation (grading) and fibrosis (staging). NASH index, defined as the sum of these three scores, was then calculated. NASH was diagnosed when a grading of 1 or more, or a staging of 1 or more were present. Hepatic iron accumulation was measured by means of the Perls’ stain and assessed on a semi-quantitative scale (0-4).

In a subsequent analysis of liver biopsies, liver damage was assessed by means of the NAFLD activity score which is the sum of steatosis, lobular inflammation and hepatocellular ballooning scores, according to Kleiner et al [20]. This scoring system addresses the full spectrum of lesions of NAFLD and allows a diagnostic categorisation into NASH, borderline or not NASH. It was therefore used to evaluate the predictive value of PATT in two groups of patients: those with NASH or borderline NASH vs those without NASH.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. A written informed consent was obtained from all subjects participating in the study.

Statistical analysis
Data analysis was performed using the software package R [21]. Data were expressed as means ± standard deviation (SD) for numeric variables, and as percentage for the qualitative ones. Student’s t test was used for mean comparison of quantitative variables. For comparison between proportions, the χ² test was used. Linear regression analysis was performed to evaluate possible associations between variables, whereas a multinomial logistic regression analysis (fitted by the maximum likelihood method) was used to create a mathematical model capable of predicting the probability of abnormal levels of adipokines and HOMA-IR for a given waist circumference value. In addition, we performed a receiver operating characteristic (ROC) curve analysis to evaluate PATT ability to discriminate with respect to the diagnosis of NASH or borderline NASH vs non-NASH. The cut-off value of PATT associated with the optimal combination of sensitivity and specificity was then determined and the area under the ROC curve (AUROC) calculated. A value of p<0.05 was considered statistically significant.

Results
Table I reports the anthropometric and clinical data in NAFLD patients and controls. The two groups were comparable in terms of mean age and gender distribution. Patients with NAFLD showed a significantly higher BMI and waist circumference. The prevalence of obesity, hypertension and dyslipidemia was significantly higher in NAFLD than in controls, whereas type 2 diabetes/IGT (impaired glucose
tolerance) was also more frequent in NAFLD, although not significantly so. Moreover, the prevalence of the metabolic syndrome was significantly more elevated in patients with NAFLD than in controls.

Biochemical data are shown in Table II. The indices of cytolysis and cholestasis, except bilirubin, were all significantly higher in NAFLD, as compared to controls. Mean ALT values were only slightly above the upper limit of normal in the NAFLD patients, whereas the other liver function tests were, on average, within the normal range in this group.

Figure 1 shows the levels of TNF-α, IL-6 and leptin in NAFLD patients and controls. Significant differences were found between the two groups as regards TNF-α (9.1±5.0 ng/L vs 6.3±2.4 ng/L, p=0.004), IL-6 (4.3±4.1 ng/L vs 2.5±2.0 ng/L, p=0.02) in both genders, and leptin in males (16.7±13.5 μg/L vs 4.5±4.5 μg/L, p=0.0005) but not in females (27.6±13.4 μg/L vs 20.3±12.2 μg/L). Adiponectin was significantly lower in NAFLD than in controls: 9.1±4.1 μg/mL vs 16.2±9.4 μg/mL (p=0.0004). TGF-β showed no significant differences between the two groups (11±32 μg/L vs 113±29 μg/L).

Regarding US steatosis, almost half of NAFLD patients had mild steatosis (44%), approximately one third had moderate steatosis (32%) and one quarter had severe steatosis (24%). Mean PATT was 13.5±4.1 mm in NAFLD and 8.0±4.1 mm in controls; this difference was highly significant (p=0.000002) (Fig. 2). A cut-off value of PATT of 11.8 mm had the highest sensitivity (100%) and specificity (50%) for prediction of NASH (see methods), with an AUROCC of 75%. When this analysis was applied to individual histological features, the results for sensitivity, specificity and AUROCC were respectively: steatosis (75%, 43%, 43%), grading (80%, 50%, 60%) and staging (75%, 43%, 57%). The values of sensitivity, specificity and AUROCC were 86%, 67% and 82% respectively when the same cut-off of PATT was used as a predictor of US steatosis.

Concerning the histological findings, a total of 33 samples were available. Two patients had simple steatosis

### Table I. Anthropometric and clinical features of NAFLD patients and controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>NAFLD</th>
<th>Controls</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>63</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>55</td>
<td>50</td>
<td>19</td>
</tr>
<tr>
<td>BMI (Kg/m², 20-25)</td>
<td>30.4</td>
<td>6.9</td>
<td>25.2</td>
</tr>
<tr>
<td>Waist (cm, &lt;102 M, &lt;88 F)</td>
<td>106 M</td>
<td>11</td>
<td>92</td>
</tr>
<tr>
<td>F</td>
<td>113</td>
<td>17</td>
<td>87</td>
</tr>
<tr>
<td>Obesity (%)</td>
<td>51</td>
<td>16</td>
<td>0.0004</td>
</tr>
<tr>
<td>Type 2 DM/IGT (%)</td>
<td>44</td>
<td>29</td>
<td>n.s.</td>
</tr>
<tr>
<td>Dyslipidemia (%)</td>
<td>52</td>
<td>16</td>
<td>0.0002</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>65</td>
<td>44</td>
<td>0.05</td>
</tr>
<tr>
<td>Metabolic syndrome (%)</td>
<td>49</td>
<td>16</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

BMI = body mass index, Type 2 DM/IGT = type 2 diabetes mellitus/impaired glucose tolerance

### Table II. Biochemical data of NAFLD patients and controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>NAFLD</th>
<th>Controls</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (IU/L, 10-50)</td>
<td>56</td>
<td>42</td>
<td>25</td>
</tr>
<tr>
<td>AST (IU/L, 10-45)</td>
<td>40</td>
<td>28</td>
<td>25</td>
</tr>
<tr>
<td>ALP (IU/L, 56-119)</td>
<td>89</td>
<td>55</td>
<td>71</td>
</tr>
<tr>
<td>GGT (IU/L, 3-65)</td>
<td>63</td>
<td>52</td>
<td>27</td>
</tr>
<tr>
<td>Total bilirubin (μmol/L, 1.7-17)</td>
<td>12.8</td>
<td>5.7</td>
<td>13.9</td>
</tr>
<tr>
<td>Blood sugar (mmol/L, 3.7-5.6)</td>
<td>6.4</td>
<td>2.2</td>
<td>5.7</td>
</tr>
<tr>
<td>Insulin (mU/L, 6-24)</td>
<td>13.9</td>
<td>10.8</td>
<td>6.4</td>
</tr>
<tr>
<td>HOMA-IR (mmolxmiU/L, 0.75-2.25)</td>
<td>2.39</td>
<td>3.04</td>
<td>0.72</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.7</td>
<td>1.9</td>
<td>6.0</td>
</tr>
<tr>
<td>Cholesterol (mmol/L, &lt;5.18)</td>
<td>5.11</td>
<td>1.06</td>
<td>4.55</td>
</tr>
<tr>
<td>HDL (mmol/L, &gt;1.04 M, &gt;1.30 F)</td>
<td>1.20</td>
<td>0.43</td>
<td>1.41</td>
</tr>
<tr>
<td>F</td>
<td>1.48</td>
<td>0.29</td>
<td>1.57</td>
</tr>
<tr>
<td>Abnormal HDL (%)</td>
<td>33</td>
<td>7</td>
<td>0.002</td>
</tr>
<tr>
<td>LDL (mmol/L, &lt;4.11)</td>
<td>3.21</td>
<td>0.77</td>
<td>3.00</td>
</tr>
<tr>
<td>Triglycerides (mmol/L, &lt;1.69)</td>
<td>1.93</td>
<td>1.29</td>
<td>1.07</td>
</tr>
<tr>
<td>Iron (μmol/L, 9-30.4)</td>
<td>17.3</td>
<td>6.7</td>
<td>15.2</td>
</tr>
<tr>
<td>Ferritin (µg/mL, 31-300)</td>
<td>215</td>
<td>159</td>
<td>122</td>
</tr>
<tr>
<td>CRP (mg/L, 0-6)</td>
<td>7.59</td>
<td>10.09</td>
<td>4.65</td>
</tr>
</tbody>
</table>

HOMA-IR = Homeostasis Model Assessment of Insulin Resistance, CRP = C-Reactive Protein.

![Fig 1. TNF-α, IL-6, leptin and adiponectin serum levels in NAFLD patients and controls. TNF-α and IL-6 concentrations are expressed in ng/L. Leptin and adiponectin concentrations are expressed in ug/L. Normal ranges: TNF-α 0-8.1 ng/L; IL-6 0-5.9 ng/L; leptin 2-15 ug/L (males) and 2-25 ug/L (females); adiponectin 11-20 ug/L. * p=0.004, ** p=0.02, *** p≤0.0005.](image-url)
PATT, and of waist circumference could be proposed as non-invasive markers to evaluate liver injury in patients with NAFLD. Both markers correlated with the degree of US steatosis, insulin resistance and the indices of inflammation and fibrosis. In addition, PATT exhibited a good predictive value in discriminating between patients with and without NASH, whereas waist circumference was associated with the degree of histological steatosis and, beyond certain thresholds, was predictive of high levels of pro-fibrogenic cytokines.

In their study, Riley et al [10] did not evaluate the thickness of the subcutaneous abdominal tissues in a control group. They measured the distance between the skin surface and the hepatic surface, and suggested that a cut-off value of 20 mm could discriminate NAFLD from other chronic liver disorders. This cut-off is somewhat higher than what we found, but it is important to note that the measurement by Riley et al [10] also comprised the muscular layer. We preferred not to include this layer in our measurement as it has no metabolic role in relation to the development of NAFLD. Also, in our opinion, PATT represents a better marker for the assessment of the intra-abdominal adipose tissue than the traditional US measurement of the distance between the abdominal wall and the aorta, which is difficult to obtain and is often associated with artefacts caused by intestinal gases. Moreover, based on the ROC curve analysis, PATT appeared a good marker for the prediction of the severity of liver disease.

Though hepatic US is a simple and inexpensive test for the detection of steatosis, this technique is liable to a subjective interpretation of the images, accounting for a high inter- and intra-observer variability. It also cannot effectively differentiate between steatohepatitis and simple steatosis, and its diagnostic performance (conventional grey-scale ultrasonography) ranges from good to poor [22]. Thus, PATT, and of waist circumference could be proposed as non-invasive markers to evaluate liver injury in patients with NAFLD. Both markers correlated with the degree of US steatosis, insulin resistance and the indices of inflammation and fibrosis. In addition, PATT exhibited a good predictive value in discriminating between patients with and without NASH, whereas waist circumference was associated with the degree of histological steatosis and, beyond certain thresholds, was predictive of high levels of pro-fibrogenic cytokines.

Discussion

The results of our study show that the measurement of PERIHEPATIC ADIPOSE TISSUE THICKNESS (PATT) in NAFLD patients and controls. The boxplot displays the 25th, median and 75th percentiles and the minimum and maximum levels as horizontal lines outside the box. Extremely abnormal values are indicated as circles outside the box. Asterisk denotes a significant (p=0.000002) difference.

27 had non-alcoholic steatohepatitis (NASH; 82%), and 4 had cirrhosis (12%). Median steatosis score was 2, median grading was 1 and median staging was 2, reflecting, on average, a moderate liver damage, as confirmed by the NASH index (median=5). Median Perls’ stain score was 1.

Using the Spearman’s correlation index, the following significant correlations were found: PATT correlated positively with US steatosis, waist circumference, HOMA-IR, TNF-α, IL-6 and leptin; a negative trend was observed between PATT and adiponectin levels but it did not reach statistical significance. Waist circumference correlated with US steatosis, TNF-α, IL-6, leptin and CRP, and ALT with both US and histological steatosis. Adiponectin correlated negatively with US steatosis, insulin and HOMA-IR, and positively with HDL. The other adipokines were positively correlated with HOMA-IR and BMI. In addition, IL-6 correlated positively with US steatosis, necro-inflammation and fibrosis.

Figure 3 shows the sigmoid curves obtained plotting waist circumference against HOMA-IR, TNF-α and leptin by multiple logistic regression analysis: a value of 113 cm for waist circumference was identified as the threshold corresponding to a probability of 50% of observing an abnormal TNF-α value. Similarly, the 50% probability of an abnormal concentration of leptin for both men and women was set at 122 cm, whereas a 50% probability of an increased HOMA-IR corresponded to 110 cm. In other words, according to this mathematical model, people with a moderate to severe abdominal obesity have a much higher probability of having abnormal values of HOMA-IR and of these two pro-fibrogenic cytokines, which, in turn, are associated with insulin resistance.

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although US has relatively high sensitivity (82-94%) and specificity (66-95%) in detecting fatty liver, it does not seem to be a perfect gold standard test for NAFLD as it may lead to an incorrect diagnosis in 10-30% of cases [23].

PATT showed a positive correlation with TNF-α, IL-6, leptin and also with HOMA-IR. This finding could reflect, at least in part, the role of adipose tissue as an endocrine organ, able to secrete bioactive peptides which favour the accumulation of fat in the liver and are associated with insulin resistance, inflammation and fibrosis.

It is noticeable that our cohort of NAFLD patients had, overall, a rather mild degree of liver injury. In fact, three quarters of them had a mild to moderate US steatosis, thus confirming data by Angelico et al. who found a severe US steatosis in only one-third of patients with bright liver [24]. Moreover, 49% of our NAFLD patients had normal transaminase levels and mean ALT values were only slightly above the upper limit of normal. Theoretically, therefore, the relationship between a number of non-invasive markers with the degree of liver damage might have been even greater, had we investigated a cohort of patients with more severe fatty liver disease.

As expected, NAFLD patients presented a significantly higher prevalence of obesity, dyslipidaemia, hypertension and the metabolic syndrome compared to controls [3-5, 25]. A number of reports have shown that abdominal obesity represents the key feature of the metabolic syndrome [17]. In our study waist circumference not only correlated with PATT, but also with increased levels of pro-inflammatory and pro-fibrogenic cytokines, insulin resistance and CRP, suggesting an association between adipokines imbalance, central obesity and liver damage.

BMI and waist circumference (or waist-to-hip ratio) have been reported to be independent predictors of hepatic fibrosis in middle-aged adults [6, 8, 11, 26-28], and even in paediatric patients [29] with NAFLD. However, neither anthropometric variable can distinguish between visceral from subcutaneous abdominal adipose tissue. If anything, the assessment of the fat distribution pattern usually requires CT scans or magnetic resonance imaging that are substantially more expensive than US and are not suitable for routine evaluation [30, 31].

Recent reports also suggest an association between NAFLD, the metabolic syndrome and an increased risk of cardiovascular disease, raising the possibility that NAFLD may play a role as an early mediator of cardiovascular disease [25, 32]. Therefore, the simple measurement of waist circumference might represent a non-invasive marker with predictive value for both cardiovascular disorders and NAFLD. With respect to the latter disorder, the probability curves plotting waist circumference against HOMA-IR, TNF-α and leptin, further support this finding.

Histological results matched the echographic ones, and showed that NAFLD patients had a mild to moderate degree of steatosis, necro-inflammation and fibrosis.

Our findings might be deemed as the result of a referral bias (82% NASH on biopsy), thus limiting the analysis and conclusions regarding predictors of advanced liver disease (i.e. cirrhosis) or of NASH versus simple steatosis. However, these figures are similar to those reported in a multicentre clinical study on NAFLD in Italy (80% of the cases had histological steatosis with a mild degree of inflammation and fibrosis) [33], and are consistent with the histological characteristics described in several longitudinal studies assessing the progression of fibrosis in NAFLD [34-38].

Another limitation of the study is the limited number of hepatic biopsies available, based on the US score. Hence, before advocating the clinical usefulness of PATT in patients with NAFLD, our findings need to be confirmed in larger samples, especially considering that no established treatment exists to prevent the development of moderate-severe fibrosis in this condition [39-43]. However, had we chosen to select patients on the basis of the presence of abnormal liver function tests, some 50% would not have been subjected to liver biopsy.

In conclusion, the measurements of PATT and waist circumference could represent non-invasive markers predicting liver damage in NAFLD. A PATT value of around 12 mm seems to represent a cut-off below which NAFLD is unlikely to exist. Whether the combination of these two variables may be regarded as a new easy-to-perform panel test requires confirmation in large-scale studies and different populations. If this is the case, the panel test could be recommended to clinicians as a useful tool to identify patients at risk for NASH who may benefit from liver biopsy and, conversely, could be used to avoid this invasive procedure in others, either before or during pharmacological interventions or lifestyle modifications.

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Conflicts of interest

None to declare.

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