RESEARCH ARTICLE

TNF-α messenger ribonucleic acid (mRNA) in patients with nonalcoholic steatohepatitis

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ABSTRACT. ***Background and aim:*** **tumor necrosis factor (TNF)-α plays a significant role in the pathogenesis of nonalcoholic steatohepatitis (NASH). A few studies have confirmed high TNF-α plasma protein levels in patients with NASH compared to healthy volunteers. We herein aimed to revisit these findings using other molecular techniques.** ***Methods:*** **a cross-sectional evaluation of patients newly diagnosed with NASH. A quantitative assay for the measurement of TNF-α messenger ribonucleic acid (mRNA) was performed for NASH patients and controls using real-time reverse transcription polymerase chain reaction (RT-PCR).** ***Results:*** **in 39 patients with NASH (mean age 38.6 ± 9.4 years, range 28-60 years; 79% males), the mean TNF-α mRNA level was significantly higher than that found for controls (137.6 ± 102.3 ng/mL *versus* 83.5 ± 43.8 ng/mL, respectively; P = 0.012). A TNF-α mRNA cut-off of 100 ng/mL predicted NASH most optimally (AUC 0.685 ± 0.066, P = 0.01; with 66.7% sensitivity and 74.1% specificity). Serum TNF-α and soluble TNF-α receptor II (sTNFRII) levels were significantly higher in patients compared to controls using ELISA.** ***Conclusion:*** **high TNF-α mRNA levels, determined by RT-PCR, characterize patients with NASH.**

Key words: nonalcoholic steatohepatitis, tumor necrosis factor, RNA, PCR

"Fatty liver" or "hepatic steatosis" is an accumulation of lipids in the liver that had previously been associated with excessive consumption of alcohol. However, three decades ago, NAFLD or "nonalcoholic fatty liver disease" was identified after description of an alcoholic-type liver injury in obese persons or diabetics who did not consume alcohol [1]. NAFLD is defined as a hepatic lipid accumulation of more than 5% of the weight of the liver associated with an alcohol consumption of less than 10 g per day [2].

NAFLD constitutes a spectrum of diseases, ranging from simple hepatic steatosis, to nonalcoholic steatohepatitis (NASH) to hepatic fibrosis progressing to cirrhosis and often leading to hepatocellular necrosis [3].

The prevalence of NAFLD in developed countries is high. Estimates vary between 20 to 30%, and approximately 2-3% of the population has NASH [4]. The prevalence of NAFLD increases in parallel with weight or body mass index (BMI). The prevalence of steatosis in obese individuals (BMI>30) and individuals with morbid obesity (BMI>40) is estimated to be 65-75% and 85-90% respectively [4]. Furthermore, the prevalence of NASH increases with obesity, affecting 15-20% of obese individuals. Forty to 90% of NASH patients are obese, more than half have type II diabetes, and up to 80% have dyslipidemia [5].

NASH is considered to be the hepatic manifestation of metabolic syndrome [6, 7], and inflammation is considered to be the link between obesity and insulin resistance (IR). The most important inflammatory mediator is tumor necrosis factor alpha (TNF-α) [8]. TNF-α regulates inflammation, survival, metabolism and the production of other cytokines [9]. TNF-α antagonizes adiponectin, an anti-inflammatory adipocytokine [10], so TNF-α shifts the balance in favor of inflammation. TNF-α is elevated in NASH patients, and its concentration increases as obesity progresses to steatosis and then to NASH [11]. TNF-α release might be mediated by bacterial growth in the gut that leads to the activation of Kupffer cells as suggested in animal models [12, 13], and in patients with NASH [14]. The activation of TNF receptors by TNF-α-binding on hepatocytes leads to the recruitment of various adaptor proteins that activate downstream kinases and proteases [15]. The factors subsequently released from mitochondria, such as reactive oxygen species and cytochrome C oxidase, play a pivotal role in TNF-induced cell death [15, 16]. The involvement of TNF-α in hepatocyte injury is therefore central to the development of NASH. TNF-α exerts its effect by binding to two types of specific membrane receptors: type I, TNF-R55 and type II, TNF-R75

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[17]. These surface receptors constantly release extracellular components that are identified in serum as soluble receptors for TNF-α (sTNF-Rs), and the concentration of sTNF-R in serum reflects the activation rate of the complex TNF-α/TNF-R. In healthy individuals, the concentration of sTNF-RI is 2-3 ng/mL, and that of sTNF-RII is 2-6 ng/mL [18]. The concentration of the soluble receptors of TNF-α is increased in obesity [17]. One study showed that the concentration of sTNF-RII correlated positively with insulin resistance [19], while another study showed that the level of sTNF-RI was increased in NASH patients compared with patients with simple steatosis or controls [20]. Few studies have evaluated the clinical significance of TNF-α levels in patients with NASH. Elevated TNF-α levels in plasma obtained from patients with NASH has been documented [14, 21, 22]. Moreover, high TNF-α and soluble TNF-R levels were associated with the induction of fibrosis and inflammation and were found to correlate with the severity of hepatocellular injury in patients with NASH [11, 23-26]. However, studies assessed plasma TNF-α levels using enzyme-linked immunosorbent assay (ELISA) techniques, which cannot detect low levels of circulating TNF-α. A reverse transcription polymerase chain reaction (RT-PCR) test could potentially quantify TNF-α with a higher sensitivity than protein measurement; hence, we herein evaluate the role of such a technique in identifying NASH patients as compared to healthy controls.

DONORS AND METHODS

Patients

We conducted a cross-sectional evaluation of newly-diagnosed patients with NASH attending the St George Hospital University Medical Center, Beirut, Lebanon and the Montreal University Hospital Center, Quebec, Canada. The diagnosis of NASH was based on the following criteria: (1) intake of less than 20 g of ethanol per day, (2) a positive ultrasonography for steatosis, (3) biopsy-proven steatohepatitis; steatosis, inflammatory infiltrates, and ballooning degeneration with or without Mallory bodies or pericellular/perivenular fibrosis, and (4) appropriate exclusion of other liver diseases. Patients also had to have an increase in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of one to four times the upper limit of normal, with an AST/ALT ratio of <1. Patients with chronic inflammatory disease, active infection, who have had recent surgery or trauma, or a history of chronic drug use (nonsteroidal anti-inflammatory drugs, corticosteroids, high-dose estrogens, methotrexate, tetracycline, or amiodarone) were excluded. Healthy, non-obese (BMI<30) volunteers, who were evaluated for the absence of liver disease by means of ultrasound, AST and ALT levels, served as controls. The Institutional Review Board at each center approved the study protocol and written informed consent was obtained from each patient.

Laboratory evaluation

The laboratory evaluation in all patients included a blood cell count and measurement of total cholesterol, LDL, HDL, aspartate aminotransferase (AST), alanine aminotransferase (ALT), C-reactive protein (CRP), total cholesterol, total bilirubin, glucose and insulin.

Measurement of serum levels of TNF-α and soluble TNF-RII

Levels of TNF-α protein and sTNF-RII were measured in triplicate using a human sandwich ELISA (abcam, Beirut, Lebanon) according to the manufacturer's instructions, and the averages values were used in the statistical analyses.

TNF - quantitative assay:

A quantitative assay for the measurement of TNF-α messenger ribonucleic acid (mRNA) was performed for all patients and controls using the LightCycler® system (Roche Diagnostics, Laval, QC, Canada). Whole blood was sampled in EDTA-coated blood collection tubes. Total RNA was extracted from 6 mL of whole blood using the Qiagen RNA blood kit (Qiagen Canada Inc., Mississauga, ON, Canada) according to the manufacturer's protocol. All RNA samples were quantified by measuring 260/280 OD ratio. RNA integrity of each sample was checked by 1% agarose gel electrophoresis. Total RNA (150 ng) was reverse transcribed in a 20 μL volume reaction. Final concentrations were: 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mmol/L of each dNTP, 2.5 μM of random hexamer primers, 1000 kU/L of RNasin, and 10 000 kU/L of RT enzyme. Reverse transcription was performed at 42 °C for 60 minutes, followed by denaturation at 99°C for 5 minutes, and then maintained at 4 °C until amplification.

Quantitative PCR was performed using TNF-α-specific primers (Invitrogen Canada Inc., Burlington, ON, Canada) and SYBR Green I Detection with the LightCycler®. TNF-α-specific primers were designed to amplify a 279-bp product from bp 247 to bp 525 in the cDNA sequence (accession number: X01394) and from bp 864 (exon 1) to bp 2236 (exon 4) in the gene sequence (accession number: AY066019). Forward and reverse primer sequences were 5'-TCCTCAGCCTCTTCTCCTTC-3' and 5'-CCAGCTGGTTATCTCTCA-3', respectively. PCR amplifications were performed in a total volume of 20 μL in a LightCycler® capillary containing 4 μL sample of cDNA, 11.6 mM Tris-HCl, 23 mM KCl, 1.6 mmol/L $MgCl_2$, 2.08 mM DTT, 0.01 mM EDTA, 0.05% Nonidet, 5% glycerol, 200 μmol/L of each dNTP, 2.45 μmol/L of each primer, 1/100 000 of SYBR Green I (Sigma Aldrich, St. Louis, MO, USA), and 250 kU/L Taq Expand (Roche Diagnostics). PCR conditions were as follows: 1 minute of initial denaturation at 95 °C, followed by 35 cycles of annealing at 60° C for 5 seconds and elongation at 72° C for 10 seconds. Melting temperature curves were performed by increasing denaturation temperature from 72° C to 95° C at a rate of 0.2° C/second. Each sample was assayed in duplicate and the average value considered for analysis. All PCR products were visualized by 3% agarose gel electrophoresis and stained with ethidium bromide.

RESULTS

Comparison of clinical parameters between patients with NASH and those with simple steatosis.

Table 1 shows the comparison of the parameters analyzed between the NASH patients and healthy volunteers. There was no difference in age or gender between patients. In

Table 1
Clinical values for patients with NASH, and healthy volunteers.

Variables	NASH	Control	P-value
Gender (M/F)	30/9	28/9	
Age (years)	38.6 ± 9.4	41.9 ± 10.9	
AST (IU/L)	88.74 ± 17.189	23.79 ± 7.328	0.0001
ALT (IU/L)	115.75 ± 115.751	23.33 ± 23.333	0.0001
ALP (IU/L)	191.25 ± 44.22	159.00 ± 31.91	0.1343
CRP (mg/dL)	1.04 ± 0.999	0.28 ± 0.123	0.0001
t-cholesterol (mg/dL)	204.90 ± 35.544	191.38 ± 41.831	0.477
t-bilirubin (mg/dL)	0.67 ± 0.274	0.52 ± 0.227	0.215
Glucose (mg/dL)	99.12 ± 9.30	94.30 ± 8.55	0.193
Insulin (U/mL)	17.81 ± 11.454	9.68 ± 9.680	0.0129

Values expressed are means ± SD. AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; t-bilirubin: total bilirubin; CRP: C reactive protein.

laboratory evaluations, AST, ALT, CRP and insulin were found to be significantly elevated in the NASH patients compared to controls. There were no significant differences regarding other laboratory data between the two groups.

Levels of TNF-α mRNA

Thirty nine consecutive patients with NASH were recruited for this study (mean age 38.6 ± 9.4 years, range 28-60 years; 79% males). Thirty nine healthy volunteers served as controls (mean age 41.9 ± 10.9 years, range 25-63 years; 74% males). The mean TNF-α mRNA level was significantly higher in patients with NASH as compared to controls (137.6 ± 102.3 ng/mL *versus* 83.5 ± 43.8 ng/mL, respectively; P = 0.012 using the independent samples t-test). Using receiver operating characteristic (ROC) curve analysis, a TNF-α mRNA cut-off of 100 ng/mL most optimally predicted NASH (AUC 0.685 ± 0.066, P = 0.01; with 66.7% sensitivity and 74.1% specificity) (*figure 1*).

Serum levels of TNF-α and sTNF-RII

Next we evaluated circulating levels of TNF-α and sTNF-RII in the serum of NASH patients and healthy volunteers. We found that there was a significant increase in TNF-α levels in those patients with NASH compared to controls, with a P value of 0.0032. (*figure 2*). In addition, levels of sTNF-RII were significantly higher in NASH patients compared to control subjects (P = 0.0196) (*figure 3*).

Correlation between CRP levels, sTNF-RII and TNF-α mRNA

We evaluated the correlation between serum CRP and sTNF-RII, and TNF-α mRNA. No positive or significant correlation was found between CRP and TNF-α mRNA or between sTNF-RII and TNF-α mRNA, as shown in *figure 4*.

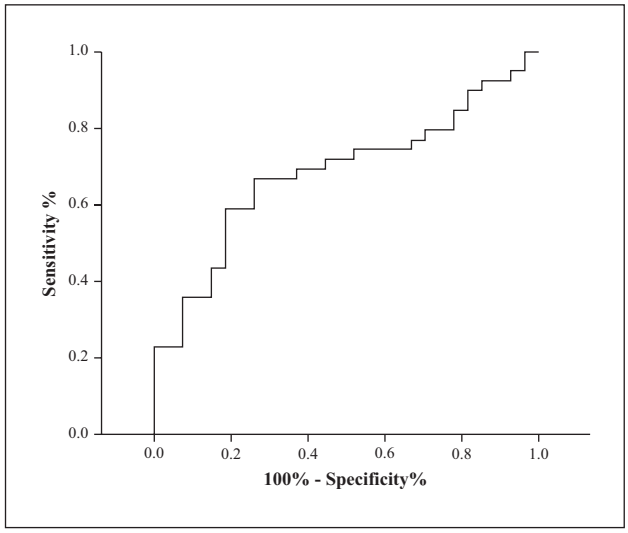

Figure 1
Receiver operating characteristics curve for the diagnosis of NASH by TNF-α mRNA levels. AUC 0.685 ± 0.066, P = 0.01.

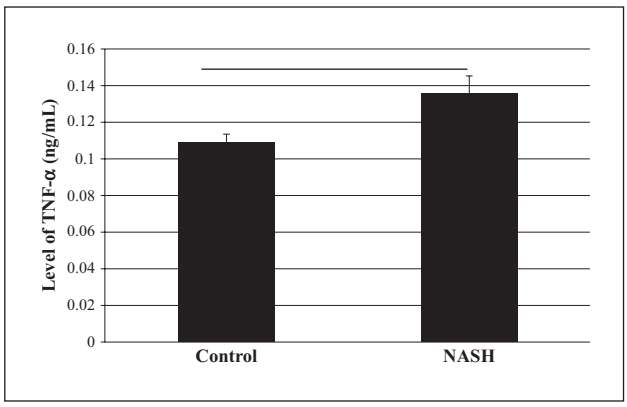

Figure 2
Levels of TNF- α in the serum of NASH patient in comparison with healthy controls.
TNF- α was measured by ELISA as described in the Materials and methods section. Data represent mean ± SEM of triplicates (ELISA) from 39 different donors/serum from NASH patients and healthy volunteers. TNF- α in NASH patients was significantly increased. Unpaired T-test; p = 0.025.

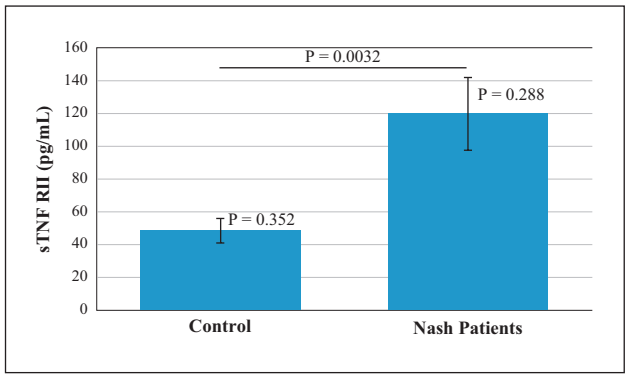

Figure 3
sTNF-RII concentrations in pg/mL in the serum of control subjects and patients with NASH. TNF- α was measured by ELISA as described in the Materials and methods section. Data represent mean ± SEM of triplicates (ELISA) from 39 different donors/serum of NASH patients. The levels of sTNF-RII were significantly increased in NASH patients compared with control subjects, with P = 0.0196 (101.18 ± 30 pg/mL in patients with NASH and 48.62 ± 30 in controls).

DISCUSSION

Our results confirm the findings of other studies in which TNF-α levels were significantly higher in patients with NASH compared to controls [14, 20-23, 27, 28]. TNF-α is known to play a major role in perpetuating inflammation in many autoimmune and inflammatory diseases [29-32]. It is reported to be implicated in the pathogenesis of liver fibrosis in the NASH model [12]. It also contributes to mitochondrial dysfunction by forming reactive nitrogen species (RNS) and reactive oxygen species [28, 33], which trigger initial inflammation in NASH along with hepatocyte injury [34]. TNF-α membrane receptors are cleaved by enzymatic activity to form its soluble receptors [35]. These receptors bind TNF-α, blocking its biological effects [36], giving the soluble form a therapeutic modality. In this study, sTNF-RII was significantly elevated in NASH patients although there was no correlation with the increased TNF mRNA levels or CRP levels, in contrast with other studies where levels of sTNF-RII correlated with CRP and disease activity [20, 30]. It was not surprising to find elevated level of sTNF-RII since it correlated with increased serum TNF- α and confirmed the enhanced activity of TNF itself in the pathogenesis of NASH. Further studies are needed to elucidate the exact relationship between TNF and its soluble form in NASH.

However, our findings, for the first time, relied on quantification of TNF-α mRNA through real time RT-PCR rather than the more conventional TNF-α protein assessment by ELISA. The technique is simple, can be performed with 2 to 6 mL of blood, and requires no post-PCR manipulation. Our findings also echo previous results that showed a high expression of TNF-α mRNA in the liver and in adipose tissue of patients with NASH [24, 37, 38]. This novel technique could be useful for prospective clinical studies aiming to evaluate the role of TNF-α in the pathophysiology of NASH, or as a biomarker for predicting progression of the disease or response to new, targeted therapies. Moreover, as the search for a diagnostic tool or system that could potentially replace the invasive liver biopsy is ongoing, the sensitivity and specificity of TNF-α mRNA measurement in patients with NASH warrants further evaluation.

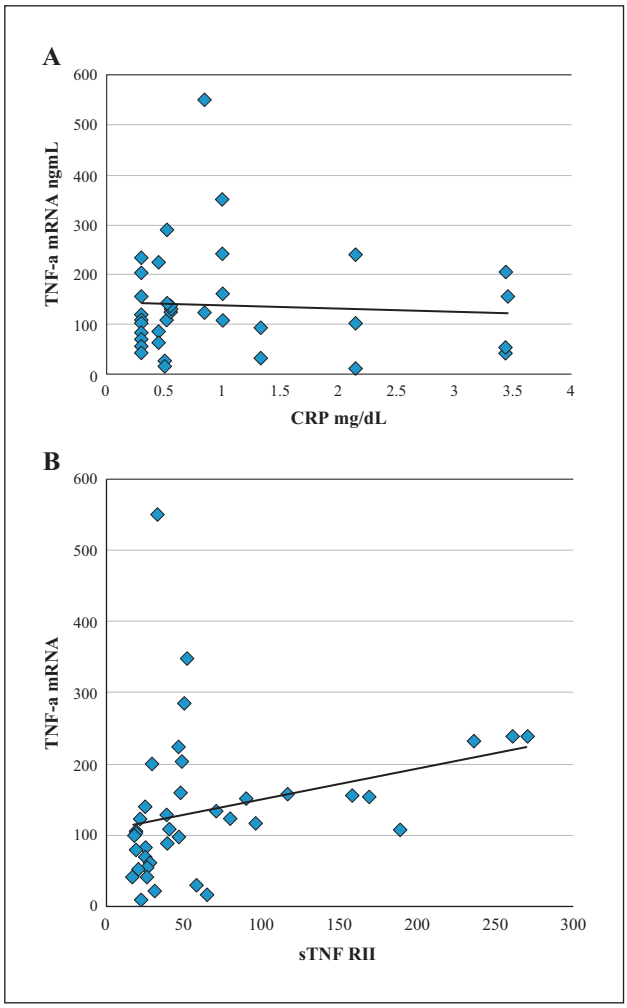

Figure 4
A) Correlation between circulating levels of TNF-alpha mRNA and CRP levels in non-alcoholic steatohepatitis (NASH) patients. **B)** Correlation between circulating levels of TNF-alpha mRNA and sTNF-RII levels in non-alcoholic steatohepatitis (NASH) patients. No significant correlation was found.

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