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Research Article

Serum levels of cytokines and adipokines in patients with non-alcoholic steatohepatitis and type 2 diabetes mellitus

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Abstract. Background: Non-alcoholic steatohepatitis (NASH) – the severe form of non-alcoholic fatty liver disease (NAFLD) – diagnosed by liver biopsy, was associated with the risk of rapid progression of cardiovascular disease, in several large longitudinal studies. Although the pathogenesis of NASH remains poorly understood, proinflammatory cytokines seem to play an important role in the process of NASH. The aim of this study was to assess the chronic systemic inflammatory profile (i.e., proinflammatory cytokines IL-6, IL-1β, and TNF-α), hs-CRP, and the anti-inflammatory profile (i.e., serum adiponectin) in patients with type 2 diabetes mellitus (DMT2) and NASH. Materials and methods: A total of 117 participants (32 patients with NASH (diagnosed by liver biopsy) and DMT2; 45 patients with DMT2 only; and 40 controls) were included in the present study. The serum levels of a panel of markers of chronic systemic inflammation (tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), and adiponectin) were measured by the enzyme-linked immunosorbent assay (ELISA) method. Results: Patients with NASH and DMT2, in comparison with patients with DMT2 only, exhibited significantly higher serum levels of IL-6 (134.83 ± 57.18 pg/ml vs. 55.68 ± 18.29 pg/ml, p = 0.001), IL-1β (73.45 ± 15.11 pg/ml vs. 22.07 ± 9.17 pg/ml, p = 0.001), and TNF-α (60.88 ± 12.31 pg/ml vs. 18.81 ± 7.25 pg/ml, p = 0.021). In contrast to the increase of proinflammatory cytokine concentrations, the serum level of adiponectin in patients with NASH and DMT2 was found lower than in patients with DMT2 only (3950.5 ± 954 ng/ml vs. 6745 ± 1122 ng/ml, p = 0.002), and controls. Moreover, regarding the hs-CRP, this parameter of chronic systemic inflammation was found significantly elevated in patients with NASH and DMT2 (8.4 ± 5.9 mg/l) compared to patients with DMT2 only (4.72 ± 3.6 mg/l) and controls (2.11 ± 1 mg/l) (p=0.001). The results of correlation analyses revealed a number of significant interactions between the proinflammatory cytokines, antiinflammatory adipocytokines, and hs-CRP, with multiple clinical and biochemical patient characteristics. Conclusions: Overall, this study's evidence indicates that the serum levels of IL-6, IL-1β, TNF-α, and hs-CRP are significantly increased in patients with NASH and DMT2, and the serum levels of adiponectin are decreased in this population.

Key Words: non-alcoholic fatty liver disease (NAFLD); non-alcoholic steatohepatitis (NASH); type 2 diabetes mellitus (DMT2); cardiometabolic risk; IL-6; IL-1β; TNF-α; adiponectin.

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Introduction

In many Western countries, type 2 diabetes mellitus (DMT2) represents a growing public health issue, being strongly associated with non-alcoholic fatty liver disease (NAFLD). The spectrum of NAFLD ranges from simple steatosis to non-alcoholic steatohepatitis (NASH), and its pathological evolution may lead to advanced fibrosis, cirrhosis, and in some cases, to hepatocellular carcinoma.

While overnutrition and obesity are primarily involved in the emergence of fatty liver disease, it remains unclear why 10% of all people with fatty liver develop the 'inflammatory' phenotype (NASH). It is increasingly acknowledged that the cell-synthesized soluble mediators of the immune system (cytokines, chemokines) and fat cells (adipokines, also known as adipocytokines) are involved in NAFLD, its progression, and in the regulation of insulin-resistance (Marra & Tacke 2014).

To date, a significant number of studies centered on the serum levels of cytokines and adipocytokines, in patients with NASH, were conducted on diabetes-free Asian and Western subjects. In contrast, fewer studies have focused their attention on diabetic patients with NASH. Besides, most often, those works present conflicting evidence regarding cytokines, their role, and their possible correlations with various clinical and biochemical parameters. More specifically, limited research has addressed the link between proinflammatory cytokines and the severity of liver disease, as represented by NASH (Jarrar et al 2008; Kern et al 2001; Moschen et al 2011).

Several studies have suggested that proinflammatory cytokines such as IL-6, IL-1β, and TNF-α are involved in the pathogenesis

of NAFLD and its progression to NASH, through their metabolic and antiinflammatory activity (Carter-Kent et al 2008; Jarrar et al 2008). Adiponectin is an antiinflammatory cytokine, primarily secreted by adipocytes, and it features anti-inflammatory, antilipogenic, and antiatherogenic properties. The adiponectin secretion is reduced in cases of obesity, insulin resistance, and type 2 diabetes mellitus (Musso et al 2005; Tilg & Moschen 2008). To date, limited research to address the levels of pro- and anti-inflammatory cytokines, in patients with NASH and DMT2, is available.

Based on these premises, the present study has sought to examine the links between the pro- and anti-inflammatory cytokines and components of cardiometabolic risk, in patients with NASH and DMT2, in Romania. The findings of this study will contribute to supplement and enrich the literature with new empirical data on the serum levels of markers of chronic systemic inflammation, in patients with DMT2 and NASH.

Materials and methods

Subjects

This study was conducted on 117 subjects (68 males and 49 females) divided in 3 groups, as follows: 32 patients with DMT2 and NASH (group I), 45 patients with DMT2 only, and without liver disease (group II), and 40 sex-and-age-matched controls (group III; subjects from this group had no history of DMT2, NAFLD or other liver diseases). All the healthy volunteers forming the control group exhibited normal liver function tests, no history of liver disease, negative serology for viral hepatitis, no history of diabetes or abnormal results of the oral glucose tolerance test. The patients from groups I and II were recruited from the Medical Clinic IV, Cluj-Napoca, Romania, while the healthy controls were selected for participation from the CF Cluj-Napoca Polyclinic, Traffic Safety Service.

This research was approved by the Board of Ethics of "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, and conducted in accordance with the Helsinki Declaration. Prior to the study, all participants have signed an informed consent form.

The following conditions or diseases represented exclusion criteria from this study: positive serology for viral hepatitis, history of liver disease, alcohol consumption (> 20 g/day), autoimmune hepatitis, hemochromatosis, hepatotoxins or medication known to be steatogenic, as well as other rare liver diseases and malignancies.

Methodology

The diagnosis of NASH was established by liver function tests, ultrasound, and liver biopsy, with the characteristic features of the pattern for hepatic steatosis. Each liver histologic specimen was fixed in formalin, stained with hematoxylin and eosin, as well as with Masson's trichrome. Grading and staging of NASH followed the criteria described and used in previous studies (i.e., Dixon et al 2001).

The determination of steatosis in NASH was completed based on two criteria, namely the presence of lobular inflammation with neutrophils or mononuclear cells, and the presence of ballooning cells or perisinusoidal or pericellular fibrosis, respectively.

The diagnosis of DMT2 was established on the basis of the American Diabetes Association (ADA) definition criteria (ADA, 2010; ADA, 2014), in due observance of participants' fasting.

For all participants included in the study were determined the fasting plasma glucose, glycated hemoglobin (HbA1c), fasting insulinemia and the degree of insulin resistance, measured by the HOMA-IR index (Homeostasis Model Assessment-Insulin Resistance). HOMA-IR index was determined based on the following formula: fasting blood glucose (mg/dl) × fasting insulinemia (μU/ml)/405, as described in previous studies (cf. Matthews et al 1985).

All participants provided a medical history and underwent a detailed clinical examination. The following parameters were recorded: age, sex, anthropometric indicators (weight, height, waist circumference), traditional cardiovascular risk factors, namely the family history, the presence of hypertension, dyslipidemia, diabetes, hyperuricemia, the current medication (antidiabetic, antihypertensive, lipid-lowering, other), and the food survey.

To assess the prevalence of metabolic syndrome, we followed the definition criteria from the 2009 Consensus (Alberti et al 2009).

The UKPDS risk engine (http://www.dtu.ox.ac.uk/riskengine) was used to assess the UKPDS (United Kingdom Prospective Diabetes Study) cardiovascular risk score in people with DMT2, relative to mortality by coronary and cerebrovascular causes. The estimation of UKPDS score includes the following cardiovascular risk factors: age, the presence of DMT2, sex, ethnicity, smoking/non-smoking status, the presence or absence of atrial fibrillation, HbA1c, total cholesterol, HDL cholesterol, and the systolic blood pressure. This software for estimating cardiovascular risk is particularly relevant to individuals with diabetes, and allows the risk estimation, with 95% confidence interval prediction, regarding four categories of clinical endpoints: coronary morbidity, coronary mortality, cerebrovascular morbidity, and cerebrovascular mortality, over a 10-year period. Thus, depending on the value of the UKPDS risk score, the patients with DMT2 can be classified into three different risk categories, as follows: low risk patients (UKPDS score < 15%), moderate risk patients (UKPDS score between 15-30%), and high risk patients (UKPDS score > 30%).

For all study participants, blood samples were collected in the morning, à jeun, after 12 hours of digestive rest. Blood samples were collected for the following determinations: liver function tests (transaminases, AST, ALT, gamma GT, alkaline phosphatase), and the panel of the lipid profile (total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides).

For the chronic systemic inflammation were determined the following parameters: high sensitivity C-reactive protein (hs-CRP) with normal values in the range of 1-3 mg/l; serum fibrinogen with normal values in the range of 150-300 mg/dl; and the pro-inflammatory cytokines, namely IL-6, IL-1β, and TNF-α, wich were determined using the ELISA Sandwich Kit, as follows: Human IL-6 DuoSet ELISA, Human IL-1β DuoSet ELISA, and Human TNF-α DuoSet ELISA. The serum levels of adiponectin were determined using the Human Total Adiponectin/Acrp30 Quantikine ELISA Kit. All determinations using ultrasensitive kits were performed in dual sample, following the instructions of the manufacturing company.

As specified above, determination of IL-6, IL-1β, TNF-α were performed using the ELISA kits, which are highly sensitive and capable to detect serum levels between 7-1000 pg/ml, for IL-6 and TNF-α, and levels between 3.90 to 250 ng/ml, for adiponectin. The normal values of serum IL-6 range between 2-25 pg/ml; the normal values of TNF-α range between 3-33 pg/ml; for IL-1β, between 2-17 pg/ml; and the normal values

Table 1. Demographic, clinical, and biochemical characteristics of patients and controls

Variable	Group I (DMT2 + NASH)	Group II (DMT2)	Group III (Controls)	p
N (subjects)	32	45	40	0.93
Age (years)	56.0±4.6	58.5±5.1	57.75±2.2	0.67
Male (%)	18 (56.2%)	25 (55.5%)	23 (57.5%)	0.89
Female (%)	14 (43.7%)	20 (44.4%)	17 (42.5%)	0.99
Smokers (%)	5 (15.6%)	10 (22.2%)	16 (40%)	0.03
Non-smokers (%)	27 (84.3%)	35 (77.7%)	24 (60%)	0.035
Sedentary (%)	17 (53.1%)	26 (57.7%)	12 (30%)	0.02
WC male (cm)	112.6±9.8	106.2±3.1	94.0±4.5	0.021
WC female (cm)	98.7±3.5	92.1±4.3	85.3±5.2	0.025
SBP (mmHg)	141.5±35.4	135.6±4.7	128.1±2.2	0.035
DBP (mmHg)	87.2±6.1	82.0±2.8	79.1±2.4	0.037
Hypertension (%)	23 (71.8%)	24 (53.3%)	8 (20%)	0.001
BMI < 25 kg/m²	4 (12.5%)	13 (28.8%)	17 (42.5%)	0.004
BMI 25-29.9 kg/m²	15 (46.8%)	18 (40%)	21 (52.5%)	0.006
BMI > 30 kg/m²	13 (40.6%)	14 (31.1%)	2 (5%)	0.003
Duration of diabetes (years)	14.2±5.1	13.3±3.1	–	0.067
AST (UI/L)	59±3.9	31±1.7	28±4.1	0.024
ALT (UI/L)	62±6.3	35±3.1	33±2.5	0.017
Gamma GT (UI/L)	68±4.5	45±5.2	39±3.8	0.014
High sensitivity C-Reactive Protein (mg/l)	8.4±5.9	4.72±3.6	2.11±1	0.001
Total Cholesterol (mg/dl)	244±20	229±18	190±14	0.006
LDL Cholesterol (mg/dl)	137.5±9.3	125.4±12.9	113.1±33	0.003
HDL Cholesterol (mg/dl)	36.9±4.2	41.8±4.7	49.6±14.3	0.002
TG/HDL *	6.47 (3.12-10.08)	4.16 (2.37-7.13)	3.05 (2.1-4.7)	0.001
Triglycerides (mg/dl)	239±41.5	174.3±22.9	151.3 ±66.5	0.005
Fasting plasma glucose (mg/dl)	148±22.5	129±14.7	97.5±9.4	0.017
HbA1c (%)	7.85±6.9	6.95±3.1	5.8±1.5	0.024
HOMA-IR *	6,9 (2,7;12,4)	3,45 (2,01;5,85)	1,32 (1,15;2,2)	0,001
Fibrinogen (mg/dl)	412.2±69.9	290.3±70.1	250.3± 51.2	0.020
UKPDS score (10-year risk)				
CAD (%)	39.04	23.8	-	0.031
Fatal CAD (%)	12.4	5.9	-	0.017
Stroke (%)	25.6	16.4	-	0.025
Fatal stroke (%)	3.8	1.9	-	0.029

of adiponectin range between 7500-12000 ng/ml, respectively. All determinations were performed in duplicate sample, according to the instructions provided by the manufacturing company.

Statistical analysis

Data is reported as mean ± standard deviation for the variables with normal distribution, as median (quartiles 1 and 3) for the variables with non-normal distribution, and as frequency (%) for the categorical (nominal) variables. To examine the normality of distribution for the quantitative variables, we used the Kolmogorov-Smirnov test. Differences between the means for continuous quantitative variables were assessed using the Student's t-test. For the variables with non-Gaussian distribution, were used non-parametric tests (Mann-Whitney U test). To compare the differences between three or more groups, we used the one-way ANOVA test for quantitative variables with normal distribution, and for those with non-normal distribution or ordinal, we used the Kruskal-Wallis test. Correlation analysis was applied to examine the interactions between the study

Table 2. The serum levels of IL-6, IL-1β, TNF-α, and adiponectin in all three groups

Cytokine	Group I (DMT2 + NASH)	Group II (DMT2)	Group III (Controls)	p
IL-6 (pg/ml)	134.83±57.18	55.68±18.29	11.54 (1.1-21.4)	0.001
IL-1β (pg/ml)	73.45±15.11	22.07±9.17	9.62 (1.2-18.7)	0.001
TNF-α (pg/ml)	60.88±12.31	18.81±7.25	14.7 (2.3-22.6)	0.021
Adiponectin (ng/ml)	3950.5±954	6745±1122	9687±1456	0.002

variables. To that end, we used the Pearson and Spearman correlation tests. A value of $p < 0.05$ was considered statistically significant.

Results

The characteristics and the laboratory data pertaining to patients and controls are displayed in Table 1. Data is presented as mean ± standard deviation for variables with normal distribution; as median * (quartiles 1 and 3) for variables with non-normal distribution; and as frequency (%) for categorical variables; WC = waist circumference; CAD = coronary artery disease; BMI = body mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure; TG = triglycerides.

The average age for the entire cohort (patients and controls) was 57±4 years, and 56.41% of participants were men. There were no significant differences regarding the number of subjects, age, and sex among all three groups included in the study.

The serum levels of aminotranferases were higher in patients from group I compared to patients from group II, and controls, while the fasting blood glucose was significantly higher in patients from group I as compared with those from group II and controls (group III).

The patients with DMT2 and NASH exhibited significantly increased levels of atherogenic dyslipidemia, compared to patients with DMT2 only, and controls (Table 1).

In group I, 70% of patients displayed hypertension, and 43.3% exhibited a BMI index > 30 kg/m2. The proportion of obese patients (BMI > 30 kg/m2) was 40.6% in group I; 31.1% in group II; and 5% in the control group (group III), with statistically significant differences between groups ($p = 0.02$ for group I vs. group II; and $p = 0.003$ for group I vs. controls).

The average duration of diabetes was similar in the first two groups ($p = 0.067$), but the levels of HbA1c were increased in patients with DMT2 and NASH compared to patients with DMT2 only ($p=0.031$).

As displayed in Table 1, the values for SBP/DBP were outside the range of optimal values recommended for the patients with DMT2.

UKPDS cardiovascular risk score

In our population, the UKPDS risk score for coronary artery disease (CAD) in patients from group I was 39.04% vs. 23.8% in patients from group II ($p = 0.031$). The risk of mortality from CAD was 12.4% in group I vs. 5.9% in group II ($p = 0.017$). The risk of stroke was 25.6% in group I vs. 16.4% in group II ($p = 0.025$), and the risk of mortality from stroke was 3.8% in group I vs. 1.9% in group II ($p = 0.029$). A UKPDS cardiovascular risk score > 30% (i.e., 39.04%) in group I, suggests that patients with DMT2 and NASH are at high risk to developing CAD in the next 10 years, compared to patients with DMT2 only, classified as moderate risk patients.

The proinflammatory cytokines (IL-6, IL-1β, TNF-α) and the antiinflammatory cytokines (adiponectin)

Since IL-6, IL-1β, and TNF-α in group III displayed values with non-normal distribution, this data is expressed as median (quartiles 1 and 3).

As shown in Table 2, the serum level of IL-6 exhibits an increase of more than 11 times the normal value in group I, and an increase of almost 5 times the normal value in group II vs. controls ($p = 0.001$), thus indicating the state of chronic systemic inflammation. Similar increases were found also for IL-1β (group I vs. group II vs. controls, $p = 0.001$) and TNF-α (group I vs. group II vs. controls, $p = 0.021$), with values which increased the most in group I vs. group II. As for the serum level of adiponectin, it reached significantly lower concentrations in group I vs. group II vs. controls ($p = 0.002$).

In group I, results of correlation analyses indicate positive relationships between the serum level of IL-6 and clinical parameters (WC, BMI, duration of diabetes, hypertension) and laboratory parameters (TG, LDL cholesterol, ALT, HbA1c, and HOMA-IR index). In addition, the serum level of IL-6 was found positively correlated with the UKPDS cardiovascular risk score, and negatively correlated with HDL cholesterol (Table 3). In group II, the analyses reveal correlations similar to those found in group I, except for ALT (Table 3).

As for the serum level of IL-1β, the results show that in group I it was positively correlated with the following parameters: BMI, WC, ALT, AST, gamma GT, LDL cholesterol, hypertension, and HOMA-IR index; no significant correlation was found between the serum level of IL-1β and the UKPDS cardiovascular risk score. Regarding patients from group II, correlation analyses indicate significant relationships between the serum levels of IL-1β and BMI, WC, and LDL cholesterol (Table 3).

In a similar way, in group I we found a number of positive correlations between the serum level of TNF-α and the following parameters: BMI, WC, hypertension, AST, ALT, gamma GT, HbA1c, duration of diabetes, and HOMA-IR index, respectively. However, the serum level of TNF-α did not correlate with triglycerides, HDL cholesterol, LDL cholesterol, fasting plasma glucose, and the UKPDS cardiovascular risk score. In group II, the serum level of TNF-α was positively correlated with BMI, WC, hypertension, HbA1c, and the duration of diabetes (Table 3). In addition, as displayed in Table 3, results of correlation analyses indicate positive links between the serum level of TNF-α and BMI and WC, in the control group.

With respect to adiponectin, in group I we found negative correlations between the serum level of adiponectin and the following parameters: BMI, WC, HbA1c, LDL cholesterol, UKPDS

Table 3. Results of correlation analyses between IL-6, IL-1β, TNF-α, adiponectin, hs-CRP and clinical and biochemical variables

Cytokine/ Adipokine/ hs-CRP	Variable	Group I (DMT2 + NASH)		Group II (DMT2)		Group III (Controls)	
IL-6	BMI (kg/m^2)	r = 0.345	p = 0.002	r = 0.310	p = 0.014	r = 0.107	p = 0.118
	WC (cm)	r = 0.379	p = 0.001	r = 0.293	p = 0.016	r = 0.112	p = 0.210
	ALT (UI/L)	r = 0.290	p = 0.015	r = 0.134	p = 0.150	r = 0.09	p = 0.199
	Triglycerides (mg/dl)	r = 0.258	p = 0.024	r = 0.189	p = 0.019	r = 0.130	p = 0.103
	HDL Cholesterol (mg/dl)	r = -0.279	p = 0.030	r = -0.250	p = 0.017	r = -0.117	p = 0.145
	LDL Cholesterol (mg/dl)	r = 0.258	p = 0.001	r = 0.258	p = 0.001	r = 0.258	p = 0.091
	Hypertension (SBP / DBP) (mmHg)	r = 0.207	p = 0.022	r = 0.235	p = 0.020	r = 0.101	p = 0.105
	HbA1c (%)	r = 0.289	p = 0.005	r = 0.192	p = 0.014	r = 0.077	p = 0.16
	HOMA-IR	r = 0.355	p = 0.001	r = 0.231	p = 0.008	r = 0.02	p = 0.93
	Duration of diabetes (years)	r = 0.42	p = 0.003	r = 0.26	p = 0.002	r = 0.16	p = 0.11
	UKPDS score	r = 0.411	p = 0.001	r = 0.305	p =0.005	–	–
IL-1β	BMI (kg/m^2)	r = 0.321	p = 0.008	r = 0.298	p = 0.011	r = 0.097	p = 0.119
	WC (cm)	r = 0.298	p = 0.009	r = 0.286	p = 0.003	r = 0.113	p = 0.120
	AST (UI/L)	r = 0.321	p = 0.004	r = 0.127	p = 0.211	r = 0.101	p = 0.222
	ALT (UI/L)	r = 0.224	p = 0.027	r = 0.097	p = 0.152	r = 0.05	p = 0.280
	Gamma GT (UI/L)	r = 0.245	p = 0.024	r = 0.013	p = 0.677	r = 0.008	p = 0.496
	LDL Cholesterol (mg/dl)	r = 0.289	p = 0.003	r = 0241	p = 0.005	r = 0.111	p = 0.145
	Hypertension (SBP / DBP) (mmHg)	r = 0.341	p = 0.002	r = 0.118	p = 0.276	r = 0.091	p = 0.378
	HOMA-IR	r = 0.229	p = 0.006	r = 0.118	p = 0. 143	r = 0.134	p = 0.155
TNF-α	BMI (kg/m^2)	r = 0.489	p =0.001	r = 0.278	p = 0.006	r = 0.378	p = 0.023
	WC (cm)	r = 0.211	p =0.037	r = 0.432	p = 0.001	r = 0.55	p = 0.011
	AST (UI/L)	r = 0.461	p =0.018	r = 0.0 2	p = 0.510	r = 0.02	p = 0.461
	ALT (UI/L)	r = 0.544	p =0.021	r = 0.07	p = 0.487	r = 0.06	p = 0.302
	Gamma GT (UI/L)	r = 0.531	p =0.027	r = 0.01	p = 0.699	r = 0.06	p = 0.497
	Hypertension (SBP / DBP) (mmHg)	r = 0.467	p = 0.005	r = 0.345	p = 0.006	r = 0.098	p = 0.114
	HbA1c (%)	r = 0.162	p = 0.034	r = 0.142	p = 0.041	r = 0.07	p = 0.187
	HOMA-IR	r = 0.601	p = 0.001	r = 0.11	p = 0.142	r = 0.08	p = 0.131
	Duration of diabetes (years)	r =0.405	p = 0.002	r = 0.345	p = 0.005	r = 0.098	p = 0.172
Adiponectin	BMI (kg/m^2)	r = -0.288	p = 0.008	r = -0.366	p = 0.007	r = -0.21	p = 0.179
	WC (cm)	r = -0.65	p = 0.001	r = -0.56	p = 0.001	r = -0.43	p = 0.122
	LDL Cholesterol (mg/dl)	r = -0.45	p = 0.001	r = -0.03	p = 0.501	r = -0. 76	p = 0.092
	HbA1c (%)	r = -0.55	p = 0.001	r = -0.29	p = 0.011	r = -0.10	p = 0.102
	HOMA-IR	r = -0.76	p = 0.001	r = -0.21	p = 0.171	r = -0.06	p = 0.164
	UKPDS score	r = -0.65	p = 0.001	r = -0.13	p = 0.189	r = -0.13	p = 0.114
hs-CRP	Age (years)	r = 0.22	p = 0.033	r = 0.19	p = 0.040	r = 0.18	p = 0.145
	BMI (kg/m^2)	r = 0.28	p = 0.024	r = 0.22	p = 0.026	r = 0.17	p = 0.131
	WC (cm)	r = 0.30	p = 0.019	r = 0.20	p = 0.029	r = 0.35	p = 0.126
	AST (UI/L)	r = 0.33	p = 0.024	r = 0.29	p = 0.163	r = 0.32	p = 0.198
	ALT (UI/L)	r = 0.21	p = 0.033	r = 0.29	p = 0.133	r = 0.27	p = 0.233
	HbA1c (%)	r = 0.292	p = 0.019	r = 0.251	p = 0.025	r = 0.283	p = 0.166
	UKPDS score	r = 0.216	p = 0.025	r = 0.176	p = 0.037	–	–

cardiovascular risk score, and HOMA-IR index, respectively. In group II, negative correlations between the serum level of adiponectin were found only with BMI, WC, and HbA1c (Table 3). Finally, correlation analyses indicate, in group I, a number of positive associations between the serum level of hs-CRP and the following clinical and laboratory parameters: age, BMI, WC, AST, ALT, HbA1c, and UKPDS cardiovascular risk score. These correlations were maintained also in group II, except for AST and ALT (Table 3).

Discussion

In the current literature, the level of cytokines and adipocytokines in patients with NASH has been examined in a number of studies conducted on non-diabetic Asian and Western subjects. Only few investigations have focused on diabetic patients with NASH. Besides, the available works often present conflicting evidence regarding cytokines, their role, and their possible correlations with various clinical and biochemical parameters. In the same vein, very few studies have addressed the link between proinflammatory cytokines and the severity of liver disease as represented by NAFLD.

Given the evidence from our study, the profile of patients with DMT2 and biopsy-proven NASH features the following traits: increased prevalence of morbid obesity compared to patients with DMT2 and controls, poor control of diabetes, an increase in transaminases, gamma GT levels, and insulin resistance; and an increased cardiovascular risk (UKPDS score) over a 10-year span, both in terms of morbidity and mortality from coronary and cerebrovascular causes.

A major proinflammatory cytokine, IL-6 is one of the first proteins synthesized in acute phase, and plays an important role in the transition from the acute to chronic inflammation. This cytokine is involved in obesity, in the development of insulin resistance, and in DMT2 (Kern et al 2001).

Elevated levels of IL-6 were previously reported in animal models of fatty liver disease (Cai et al 2005) as well as in patients with NAFLD (Diehl et al 2005).

Our results identified positive correlations between the expression of serum IL-6, in patients with NASH and DMT2, and a number of parameters such as WC, BMI, duration of diabetes, hypertension, TG, LDL cholesterol, ALT, HbA1c, and HOMA-IR index; similar correlations with these parameters, except for ALT, were found also in patients with DMT2 only. Moreover, in this study we found that the level of IL-6 was significantly increased, nearly 12 times the normal value in group I vs. controls, and 2.4 times higher in group I vs. group II, respectively.

As for IL-1β, this cytokine is associated with the inflammatory syndrome; it induces the occurrence of fever and hypotension as well as the secretion of other proinflammatory cytokines such as IL-6. The IL-1β plays an important role in the transformation process of hepatic simple steatosis into NASH. For example, recent studies conducted on animal models, reported that the lack of IL-1α or IL-1β inhibited the transformation of steatosis into steatohepatitis and liver fibrosis, in hypercholesterolemic mice (Kamari et al 2011).

In our study, the serum levels of IL-1β were 7.6 times higher in group I vs. controls, and 3.3 times higher in group I vs. patients from group II. Results of correlation analyses indicate that in patients from group I, the serum levels of IL-1β were positively associated with transaminases (ALT, AST), gamma GT, hypertension, and HOMA-IR index. Noteworthy, our evidence indicates that these correlations have occurred only in patients with DMT2 and NASH, and were not previously reported in the literature.

Given that the patients with NASH and DMT2 from this study exhibited an increased cardiovascular risk (cf. Table 1) and elevated serum levels of IL-1β (cf. Table 2) – a biomarker of chronic systemic inflammation, it is likely that this segment of patients might benefit in their treatment from an antiinflammatory, IL-1β specific therapy.

Previous research has hypothesized that TNF-α plays a role in the pathogenesis of IR-related diseases, including NAFLD and NASH. In addition, studies have reported associations between TNF-α and elevated levels of oxidative stress, hepatocyte apoptosis, and elevated serum levels of free fatty acids (Tracey & Cerami, 1993; Cortez-Pinto et al 2006). Adding to this body of empirical evidence, our study found an increased serum concentration of TNF-α, namely 3.23 times higher in patients from group I compared to those from group II, and 4.14 times higher in patients from group I vs. controls.

Unlike IL-6, IL-1β, and TNF-α, adiponectin is an antiinflammatory cytokine, primarily secreted by adipocytes. It improves hepatic and peripheral insulin sensitivity, and has antiinflammatory, antilipogenic, and antiatherogenic properties. The adiponectin secretion is decreased in cases of obesity, insulin resistance, type 2 diabetes mellitus, and other conditions associated with the metabolic syndrome. Several studies have reported hypoadiponectinemia in patients with NASH, and its correlation with elevated serum levels of TNF-α (Hui et al 2004; Musso et al 2005; Tilg & Moschen, 2008).

In our study, we identified significantly lower concentrations of serum adiponectin in patients from group I compared to patients from group II, and controls. To our knowledge, no other works have reported on the levels of adiponectin in patients with NASH and DMT2; instead, other studies conducted on patients with NASH, but without DMT2, reported that the serum levels of adiponectin were significantly lower in patients with NASH, being associated with more extensive necroinflammation (Hui et al 2004; Musso et al 2005).

Our findings indicate that in patients from group I, the serum level of adiponectin was negatively correlated with multiple parameters, but the correlations between adiponectin and LDL cholesterol, UKPDS cardiovascular risk score, and HOMA-IR index were unique to patients with DMT2 and NASH .

The high sensitive C-reactive protein (hs-CRP) is one of the acute-phase proteins, and its synthesis is directly dependent on the secretion of IL-6 and direct interaction between IL-6 and IL-1β (Gabay & Kuschner, 1999).

A significant body of past research shows that elevated serum levels of hs-CRP are a strong predictor of future cardiovascular events, while the insulin resistance syndrome is associated with increased serum levels of hs-CRP and visceral obesity (Koenig et al 1999; Danesh et al 2000; Ridker et al 2002; Yudkin et al 1999).

The results of our study revealed a significantly elevated serum concentration of hs-CRP in patients from group I, nearly 4 times higher vs. controls, and almost 2 times higher vs. patients from group II.

Moreover, in groups I and II, our evidence highlights positive correlations between the serum levels of hs-CRP and multiple parameters including age, BMI, WC, HbA1c, and the UKPDS cardiovascular risk score, common to both groups; however, unique to patients with DMT2 and NASH (group I), were the correlations between the serum level of hs-CRP and AST and ALT, differentiating thus the two groups.

Besides the contributions of this study, its limitations should also be mentioned. First, the cross-sectional design of this research does not allow for longitudinal observation of the evolution in time of cytokines and adipokines, in patients with NASH and DMT2. Another limitation refers to the small sample size of the study. Future studies should consider adopting a longitudinal perspective in addition to cross-sectional design, and target larger samples of subjects, to advance the research efforts centered on this specific segment of population.

Conclusions

Overall, with regards to the patients with NASH and DMT2, the findings of this study reveal increments in serum levels of several proinflammatory cytokines such as IL-6, IL-1β, and TNF-α; and decreased levels of serum adiponectin. In addition, the serum concentration of hs-CRP was found elevated in this group of patients. Our results indicate the presence of multiple correlations between the serum levels of cytokines and adipokines, and a set of clinical and biochemical patient characteristics. These observations call for further investigation that should focus on the mechanisms contributing to the development of both diseases (i.e., NASH and DMT2), and on the use of these biomarkers of chronic systemic inflammation as a potential antiinflammatory therapeutic strategy for this population.

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