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Liver transcriptional profile of atherosclerosis-related genes in human nonalcoholic fatty liver disease

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ABSTRACT

Objectives and design: Epidemiological studies have suggested a role of nonalcoholic fatty liver disease (NAFLD) in the development of cardiovascular disease. We evaluated liver mRNA expression of 84 genes encoding proteins involved in the atherosclerosis pathway in patients with NAFLD proven through biopsy in a case-control design, and examined the putative role of the histological disease severity in the molecular events associated with the atherogenic profile.
Results: Nonalcoholic steatohepatitis (NASH), when compared with simple steatosis (SS), significantly increases the expression of *TGFB1* (6.8, $p < 0.005$), angiotensin I-converting enzyme (*ACE*) (2.1, $p < 0.007$), *LAMA1* (2.1, $p < 0.007$), *SERPINB2* (2.1, $p < 0.007$), *CSF2* (2.5, $p < 0.002$), *IL1A* (2.5, $p < 0.005$), *IL3* (2.1, $p < 0.007$), *IL4* (2.1, $p < 0.007$), *LIF* (2.1, $p < 0.007$), and *MMP1* (2.1, $p < 0.007$), and decreases the transcript levels of genes involved in the negative regulation of cell-death pathways. A post hoc analysis of liver biopsies of NASH patients who were treated with enalapril monotherapy because of arterial hypertension showed a significant association with lower fibrosis scores in comparison with untreated patients. *BIRC3*, a severe hypoxia-activated gene, was significantly increased in SS (8.2, $p < 0.004$), when compared with the controls. NASH, but not SS, was also associated with a significant increase in platelet abundance of *TGFB1* mRNA. Systems biology analysis revealed highly scored pathways involved in the regulation of programmed cell death, angiogenesis, and immune system, in which *TGFB1* was mostly involved.
Conclusion: NASH, but not SS, may increase atherosclerotic and cardiovascular risk by local overexpression of mediators of atherogenesis, endothelial damage, and regulators of blood pressure; this observation may have therapeutic implications, because ACE inhibitors may improve both cardiovascular outcomes and liver fibrosis. Hepatocyte hypoxia seems to have an important role in the molecular events activated by liver steatosis.

1. Introduction

Epidemiological studies have suggested a possible role of nonalcoholic fatty liver disease (NAFLD) in the development of cardiovascular disease [1]. In fact, in patients with metabolic syndrome (MS), NAFLD is associated with an increased cardiovascular risk and independently predicts the risk of future cardiovascular events [2]. In a recent meta-analysis, we showed that NAFLD patients carry an increase of 13% of carotid intima-media thickness [3]. We also observed that patients with NAFLD not only have increased circulating levels of biomarkers of atherosclerosis, such as soluble intercellular adhesion molecule 1 (sICAM-1), plasminogen activator inhibitor (PAI-1), and soluble CD40 ligand (sCD40L), but the affected liver can even participate in the local expression of these

Abbreviations: ACE, angiotensin I-converting enzyme; *BIRC3*, baculoviral IAP repeat-containing 3; CRP, C-reactive protein; *CSF2*, colony-stimulating factor 2; *IL1A*, interleukin 1 alpha; *IL3*, interleukin 3; *IL4*, interleukin 4; *IL6*, interleukin; sICAM-1, intercellular adhesion molecule 1; *LAMA1*, laminin alpha 1; LIF, leukemia inhibitory factor; *MMP1*, matrix metallopeptidase 1 (interstitial collagenase); MS, metabolic syndrome; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PAI-1, plasminogen activator inhibitor; *PPARG*, peroxisome proliferator-activated receptor gamma; *SELPLG*, selectin P ligand; *SERPINB2*, serpin peptidase inhibitor, clade B (ovalbumin), member 2; SS, simple steatosis; sCD40L, soluble CD40 ligand; *TGFB1*, transforming growth factor beta 1.

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proteins [4]. Interestingly, cross-sectional studies showed that cardiovascular complications in patients with NAFLD increase with the histological severity of the disease [2], suggesting a putative role of hepatic necroinflammation in the systemic atherogenic phenotype.

Overall, the current body of evidence raises the possibility that NAFLD is not merely associated with the cardiometabolic risk factors, but is an independent causal factor that promotes by itself a systemic proatherogenic and inflammatory state. In particular, it was suggested that nonalcoholic steatohepatitis (NASH), the clinical form of NAFLD, which is associated with either liver inflammation and/or fibrosis, can contribute to a more atherogenic risk profile over the more benign stage of steatosis alone or simple steatosis (SS). This observation is supported by several independent human case-control studies that have demonstrated that NASH, but not SS, is associated with changes in the hepatic messenger RNA (mRNA) or protein expression levels of molecular mediators of atherosclerosis, such as PAI-1 [5], monocyte chemoattractant protein (MCP-1) [6], interleukin 6 (IL6) [7], and sICAM1 [4]. Nevertheless, the evidence is inconclusive and is still unknown whether SS is just a benign disorder or the trigger event in a cascade of dysregulated molecular pathways.

We agree with the current hypothesis that an abnormal intrahepatic accumulation of triglycerides along with a global disruption of the metabolic homeostasis may play a causative role in the development of the proatherogenic state associated with the MS phenotype [1]. Hence, to gain insights into the molecular events occurring in the liver that may explain how NAFLD participates in the systemic phenotype associated with atherogenesis and cardiovascular disease, two sequential approaches are proposed. First, the liver mRNA gene expression signature of 84 genes encoding proteins involved in the atherosclerosis pathway in patients with NAFLD proven through biopsy in a case-control design was evaluated, and further the putative role of the histological severity of NAFLD in the molecular events associated with the atherogenic profile was examined. Second, a systems biology approach was used to integrate a large set of functional association data and identity common pathways in the liver-related proatherogenic phenotype. Finally, as platelets are involved in the systemic atherosclerotic process, we examined NAFLD patients and control subjects for differences in the abundance of platelet mRNA of the most significantly dysregulated liver transcript.

2. Methods and study design

2.1. Selection of patients and controls

The mRNA expression of the 84 genes in 12 liver samples of NAFLD patients (6 SS and 6 NASH) and 6 control subjects was analyzed in a cross-sectional case-control study involving untreated patients with NAFLD proven through biopsy. We studied platelet-circulating mRNA expression of a target gene in additional 84 individuals; see the following description.

In addition, 66 patients, who were diagnosed for NASH and prescribed either enalapril (an ACE inhibitor) monotherapy ($n = 36$) as an antihypertensive medication or metformin monotherapy ($n = 30$) for type 2 diabetes mellitus, were included to compare fibrosis and grading scores with a subset of 56 NASH patients who did not receive any concomitant medication by the time of liver biopsy (details given later). All the patients mentioned in the enalapril, metformin, and untreated group were participating in an unsponsored observational study about the natural history of NAFLD and genetic factors associated with the disease, and were included in a post hoc analysis. This analysis was performed because when compared with SS patients, the abundance of the liver transcript of angiotensin-converting enzyme (ACE) was found to be increased in NASH patients.

Control subjects were selected from patients attended by the Liver Unit, whose age and sex matched the NAFLD patients. In addition to the standard health assessment, a careful ultrasonographic (US) examination of the liver was performed in all the control individuals. For examining the gene expression, control liver specimens were obtained by percutaneous liver biopsy. Details about patients and controls selection can be seen in Supplementary material.

2.2. Physical, anthropometric, biochemical, and cardiovascular evaluation

Health examinations included anthropometric measurements, a questionnaire on health-related behaviors, and biochemical determinations, including C-reactive protein (CRP). Determination of a 10-year risk of developing coronary heart disease outcomes (myocardial infarction and coronary death) was carried out using Framingham risk scoring.

Complete details about the inclusion and exclusion criteria, physical, anthropometric, and biochemical evaluation in all the studied groups are shown in Table 1 and Supplementary material; patients with alcohol consumption above 20 or 30 g of intake daily for women and men, respectively, were not included in the study.

All the investigations performed in this study were conducted in accordance with the guidelines of the 1975 Declaration of Helsinki. Written consent from individuals was obtained in accordance with the procedures approved by the Ethical Committe of the authors' institution.

2.3. Liver biopsy and histopathological evaluation

The degree of steatosis was assessed according to the system developed by Kleiner et al., based on the percentage of hepatocytes containing macrovesicular fat droplets [8]. NASH was defined as steatosis plus mixed inflammatory-cell infiltration, hepatocyte ballooning and necrosis, glycogen nuclei, Mallory's hyaline, and any stage of fibrosis, including absence of fibrosis [8]; details can be seen in Supplementary material.

2.4. Evaluation of liver gene expression by quantitative PCR (qPCR)

We evaluated the hepatic expression profile of 84 genes encoding proteins involved in the atherosclerosis pathway using the pre-designed Human Atherosclerosis RT^2 Profiler^{TM} PCR Array (SABiosciences, Frederick MD, USA), according to the manufacturer's instructions; a complete list of the gene expression assay can be found in Appendix Table 2. Genes on PCR array were selected by the manufacturer based on previous knowledge about published association with the atherosclerosis pathway and specific information gathered from multiple accessible databases and text mining relevant literature. The array included genes involved in the processes of blood coagulation and circulation as well as genes involved in cell-adhesion and lipid transport and metabolism, stress response, cell growth and proliferation, and apoptosis. Details about calculation of the threshold cycle (Ct) values for all the genes on the PCR array can be seen in Supplementary material.

2.5. Platelet isolation and evaluation of mRNA expression

Evaluation of mRNA expression was performed on platelets of 44 NAFLD patients and 40 control subjects whose clinical and biochemical characteristics are shown in Table 1; neither patients nor the control individuals were prescribed any medication. Briefly, blood (8 ml) was drawn from the cubical vein in a plastic syringe

Table 1
Clinical and biochemical characteristics of the whole population according to disease status: control subjects, simple steatosis (SS) and non-alcoholic steatohepatitis (NASH) patients.

Variables	Control subjects	SS	NASH
(A) Patients included in the analysis of liver mRNA expression of 84 atherosclerosis-related genes.			
Number of subjects	6	6	6
Female/male (*n*)	3/3	3/3	3/3
Age (years)	45.7 ± 5.1	46.7 ± 4.7	48.7 ± 2.3
Smoking habit, cigarettes/day	0	10.0 ± 10	0
BMI (kg/m$^{2)}$	24.0 ± 1.8	33.1 ± 1.5	30.6 ± 1.6
Waist circumference (cm)	86.5± 2.8	114.5 ± 2.5	107.5 ± 0.5
Waist/hip ratio	0.85 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
SABP (mmHg)	120.0 ± 3.0	125.0 ± 2.7	123.3 ± 3.3
DABP (mmHg)	75.8 ± 2.9	80.2 ± 3.0	76.7 ± 3.3
Cardiovascular risk[a] (%)	1.0 ± 0.9	4.4 ± 0.9	4.6 ± 0.6
Fasting plasma glucose (mmol/l)	4.40 ± 0.42	5.18 ± 0.74	6.03 ± 0.43
Fasting plasma insulin (pmol/l)	41.0 ± 16.0	186.1 ± 88.2	95.1 ± 2.1
HOMA-IR index	1.1 ± 0.1	5.8 ± 2.3	3.7 ± 0.3
Total cholesterol (mmol/l)	4.78 ± 0.57	6.33 ± 0.70	5.28 ± 0.61
HDL-cholesterol (mmol/l)	2.12 ± 0.13	1.52 ± 0.21	1.16 ± 0.08
LDL-cholesterol (mmol/l)	2.66 ± 0.42	4.00 ± 1.00	3.13 ± 0.49
Triglycerides (mmol/l)	1.02 ± 0.07	2.16 ± 0.06	2.28 ± 0.17
Uric acid (mmol/l)	167 ± 6	351 ± 5	280 ± 3
ALT (U/l)	64.7 ± 16.7	60.0 ± 13.6	76.0 ± 13.0
AST (U/l)	44.0 ± 12.7	37.0 ± 0.6	39.7 ± 4.0
GGT (U/l)	100.3 ± 18.9	90.0 ± 21	84.3 ± 38.3
AP (U/l)	121.5 ± 46.5	318.0 ± 148.0	221.0 ± 48.1
Histological features			
Degree of steatosis (%)	0	66.7 ± 13.3	53.3 ± 14.5
Necroinflammatory activity	0	0	1 ± 0.0
Fibrosis stage	0	0	1.7 ± 0.4

Variables	Control subjects	SS	NASH	*p* value[*]	*p* value[#]
(B) Patients included in the analysis of platelet abundance of *TGFβ1* mRNA					
Number of subjects	40	24	20	–	–
Female/male (%)	72.5/27.5	75.0/25.0	79.0/21	NS	NS
Age (years)	50.2 ± 2.5	53.8 ± 3.7	55.4 ± 2.1	NS	NS
Smoking habit, cigarettes/day	1.8 ± 0.7	6.5 ± 5	2.0 ± 1.6	NS	NS
BMI (kg/m^2)	24.4 ± 0.6	31.7 ± 1.2	33.5 ± 1.3	1×10^{-6}	NS
Waist circumference (cm)	84.0 ± 2.4	103.2 ± 2.7	111.8 ± 3.1	1×10^{-6}	NS
Waist/hip ratio	0.85 ± 0.01	0.95 ± 0.01	0.97 ± 0.05	2×10^{-4}	NS
SABP (mmHg)	108.2 ± 1.6	124.4 ± 4.6	132.7 ± 3.9	6×10^{-5}	NS
DABP (mmHg)	66.5 ± 1.0	76.9 ± 2.8	78.8 ± 2.3	4×10^{-4}	NS
Cardiovascular risk[a] (%)	1.8 ± 1.0	2.2 ± 0.8	6.7 ± 2.2	0.02	0.03
C-RP (μg/l)	21000 ± 22000	63000 ± 5000	110000 ± 21000	0.03	0.04
Fasting plasma glucose (mmol/l)	4.1 ± 0.07	5.40 ± 0.31	6.79 ± 0.70	1×10^{-6}	NS
Fasting plasma insulin (pmol/l)	50.7 ± 6.3	93.8 ± 18.1	112.5 ± 13.9	7×10^{-5}	NS
HOMA-IR index	1.3 ± 0.2	3.4 ± 0.8	4.5 ± 0.7	1×10^{-6}	0.05
Total cholesterol (mmol/l)	5.26 ± 0.16	5.59 ± 0.28	5.79 ± 0.20	0.05	NS
HDL-cholesterol (mmol/l)	1.42 ± 0.05	1.29 ± 0.07	1.24 ± 0.08	NS	NS
LDL-cholesterol (mmol/l)	3.29 ± 0.13	3.64 ± 0.34	3.43 ± 0.18	NS	NS
Triglycerides (mmol/l)	1.19 ± 0.11	1.39 ± 0.15	2.59 ± 0.33	1×10^{-4}	0.006
Uric acid (mmol/l)	173 ± 6	327 ± 54	345 ± 18	1×10^{-6}	NS
Leukocyte count, (cells/μl)	6183 ± 266.3	6900 ± 520	8217 ± 1146	0.03	NS
ALT (U/l)	15.6 ± 1.0	41.1 ± 8.4	51.3 ± 6.2	1×10^{-6}	NS
AST (U/l)	17.2 ± 0.9	35.1 ± 5.3	39.5 ± 3.2	1×10^{-6}	NS
GGT (U/l)	21.8 ± 1.9	33.8 ± 4.8	63.2 ± 8.6	1×10^{-6}	0.009
AP (U/l)	122.5 ± 4.7	234.3 ± 17.8	263.2 ± 17.9	1×10^{-6}	NS

Variables	Enalapril group	Untreated group	*p* value
(C) NASH patients included in the post-hoc analysis of the effect of angiotensin-converting enzyme *(ACE)* inhibition on liver fibrosis scores			
Number of subjects	36	56	
Female/male (%)	64.9/35.1	77.5/22.5	NS
Age (years)	57.5 ± 2.7	53.9 ± 1.3	NS
Smoking habit, cigarettes/day	7.7 ± 3.8	1.2 ± 0.6	NS
BMI (kg/m$^{2)}$	33.1 ± 1.4	34.3 ± 0.9	NS
Waist circumference (cm)	107.7 ± 3.0	102.3 ± 1.7	NS
Waist/hip ratio	1.0 ± 0.02	0.9 ± 0.01	NS
SABP (mmHg)	134.3 ± 3.9	126.0 ± 3.0	NS
DABP (mmHg)	81.1 ± 2.0	76.4 ± 1.8	NS
Cardiovascular risk[a] (%)	8.7 ± 2.1	3.2 ± 0.9	0.03
C-RP (μg/l)	89,000 ± 11,000	77,000 ± 8000	NS
Fasting plasma glucose (mmol/l)	7.32 ± 0.59	7.02 ± 0.38	NS
Fasting plasma insulin (pmol/l)	81.3 ± 10.4	131.3 ± 13.9	0.03
HOMA-IR index	3.4 ± 0.4	5.6 ± 0.7	0.03
Total cholesterol (mmol/l)	5.47 ± 0.25	5.45 ± 0.16	NS
HDL-cholesterol (mmol/l)	1.40 ± 0.10	1.25 ± 0.05	NS

Table 1
(*Continued*).

Variables	Enalapril group	Untreated group	*p* value
LDL-cholesterol (mmol/l)	3.13 ± 0.22	3.32 ± 0.13	NS
Triglycerides (mmol/l)	2.88 ± 0.43	2.24 ± 0.18	NS
Uric acid (mmol/l)	446 ± 113	286 ± 24	NS
Leukocyte count (cells/μl)	7203.3 ± 753.9	7451.5 ± 453.3	NS
ALT (U/l)	55.9 ± 6.4	60.0 ± 4.9	NS
AST (U/l)	50.1 ± 5.26	44.2 ± 3.9	NS
GGT (U/l)	109.8 ± 21.4	78.2 ± 11.1	0.04
AP (U/l)	291.6 ± 32.8	230.5 ± 14.3	NS
Histological features			
Degree of steatosis (%)	49.7 ± 3.6	60.0 ± 3.5	0.04
Necroinflammatory activity	1.5 ± 0.14	1.6 ± 0.08	NS
NAS score	5.0 ± 0.5	6.2 ± 0.37	NS

Results are expressed as mean ± SE. All measurements are in SI units. P value stands for statistical significance using Mann-Whitney *U* test. NS: non significant. BMI: body mass index; SABP and DABP: systolic and diastolic arterial blood pressure; HOMA-IR: homeostatic model assessment-insulin resistance.

[a] Risk for developing coronary heart disease outcomes using Framingham risk scoring. ALT and AST, serum alanine and aspartate aminotransferase; GGT, gamma-glutamyl-transferase; AP, alkaline phosphatase; C-RP, C reactive protein.

[*] Indicates comparisons between NASH vs. control subjects.

[#] Comparisons between NASH vs. simple steatosis.

with 1 ml of acid citrate dextrose anticoagulant after at least 8 h or overnight fast; details can be found in Supplementary material.

2.6. Systems biology: pathway-based association analysis

Based on the results on the liver mRNA expression profile of the 84 genes encoding proteins involved in the atherosclerosis pathway, we performed a functional association analysis that included protein and genetic interactions, pathways, coexpression, colocalization, and protein domain similarity using the bioinformatic resource GenMANIA [9]; details are given in Supplementary material.

2.7. Statistical analysis

Details are given in Supplementary material.

3. Results

A heat plot of liver gene expression comparing all genes present in the SS, NASH, and control liver is shown in Fig. 1. The genes are grouped in a hierarchical tree according to their downregulation or upregulation in each subgroup of the subjects. Some gene expression levels clearly clustered the different subgroups of the subjects.

3.1. NASH, but not SS, significantly increases the liver expression of genes associated with atherosclerotic risk

A comparison of mRNA gene expression between the liver samples of NASH patients and control liver revealed a significant increase in the *TGFβ1* transcript level (3.8 fold, $p < 0.008$) and a decrease in the *PPARγ* (−3.2 fold, $p < 0.006$) and *SELPLG* (−3.8 fold, $p < 0.0004$) transcript levels. As our goal was to contrast the hypothesis of a differential gene expression in SS and NASH to further examine the distinctive role of the histological severity of NAFLD in the atherogenic phenotype, we performed a targeted analysis in SS vs. NASH. The fold changes of all significantly overexpressed and underexpressed liver mRNAs in NASH vs. SS are shown in Fig. 2. Across the panel of 84 atherosclerosis-related gene mRNAs screened in the array, 10 transcripts were induced 2-fold or more in the NASH samples when compared with the SS samples as follows: three genes associated either with atherosclerotic (*SERPINB2*, *alias PAI2*, and *TGFB1*) or cardiovascular risk (*ACE*), four genes involved in inflammation and cytokine signaling (*CSF2*, *IL1A*, *IL3*, and *IL4*), one gene involved in cell proliferation/growth (LIF), one gene involved in extracellular matrix remodeling (*MMP1*), and one gene involved in cell adhesion-cell-matrix glycoconjugate (*LAMA1*). Among the genes whose mRNA expressions were downregulated in the NASH samples when compared with the SS samples, the most significant ones were four genes involved in the signaling cascade of TNFα-induced necrotic cell death, such as TNFα-induced protein 3-*TNFAIP3*, *NFkB*, *CFLAR*, and *FAS*.

In addition, we examined the liver gene expression profile of the 84 atherosclerosis-related gene mRNAs in the control liver in comparison with SS and observed that abnormal liver triglyceride accumulation promotes overexpression of the inhibitor of apoptosis protein 1 (*BIRC3*); details are given in Supplementary material.

3.2. NASH is associated with changes in platelet abundance of TGFβ1 mRNA

Although TGFβ1 is produced virtually in all cells, the blood platelets contain 40–100 times as much TGFβ1 as in other cells [10]. Moreover, recent studies have provided insight into platelet functions in inflammation and atherosclerosis [11]. Hence, based on both our finding about a significant overexpression of *TGFβ1* in NASH liver tissue (6.78 fold when compared with SS) and the key role that *TGFβ1* plays in atherosclerosis development, we decided to examine the level of platelet mRNA expression of *TGFβ1* in NAFLD patients and control subjects. Hence, 84 individuals, 40 control subjects, and 44 NAFLD patients were included; the NAFLD patients showed all the characteristics of the MS (Table 1). NASH patients showed significantly higher HOMA-IR index, plasma triglycerides, GGT and CRP levels, and cardiovascular risk as measured by the Framingham score when compared with SS patients.

The abundance of platelet *TGFβ1* mRNA in control patients was similar to that observed in patients with SS ($n = 24$; Fig. 3). Interestingly, NASH patients ($n = 20$) had significantly higher platelet mRNA levels of *TGFβ1* when compared with either the control subjects or SS patients (Fig. 3). Multiple regression analysis showed that circulating platelet *TGFB1* mRNA levels were also significantly associated with HOMA-IR (β: 0.4, SE: ±0.15, $p < 0.009$).

3.3. ACE inhibition is significantly associated with lower fibrosis scores in NASH patients

We observed that *ACE* transcript was significantly upregulated in NASH liver, and despite local hepatic expression of *ACE* being previously reported [12], its upregulation in human NASH is a novel feature.

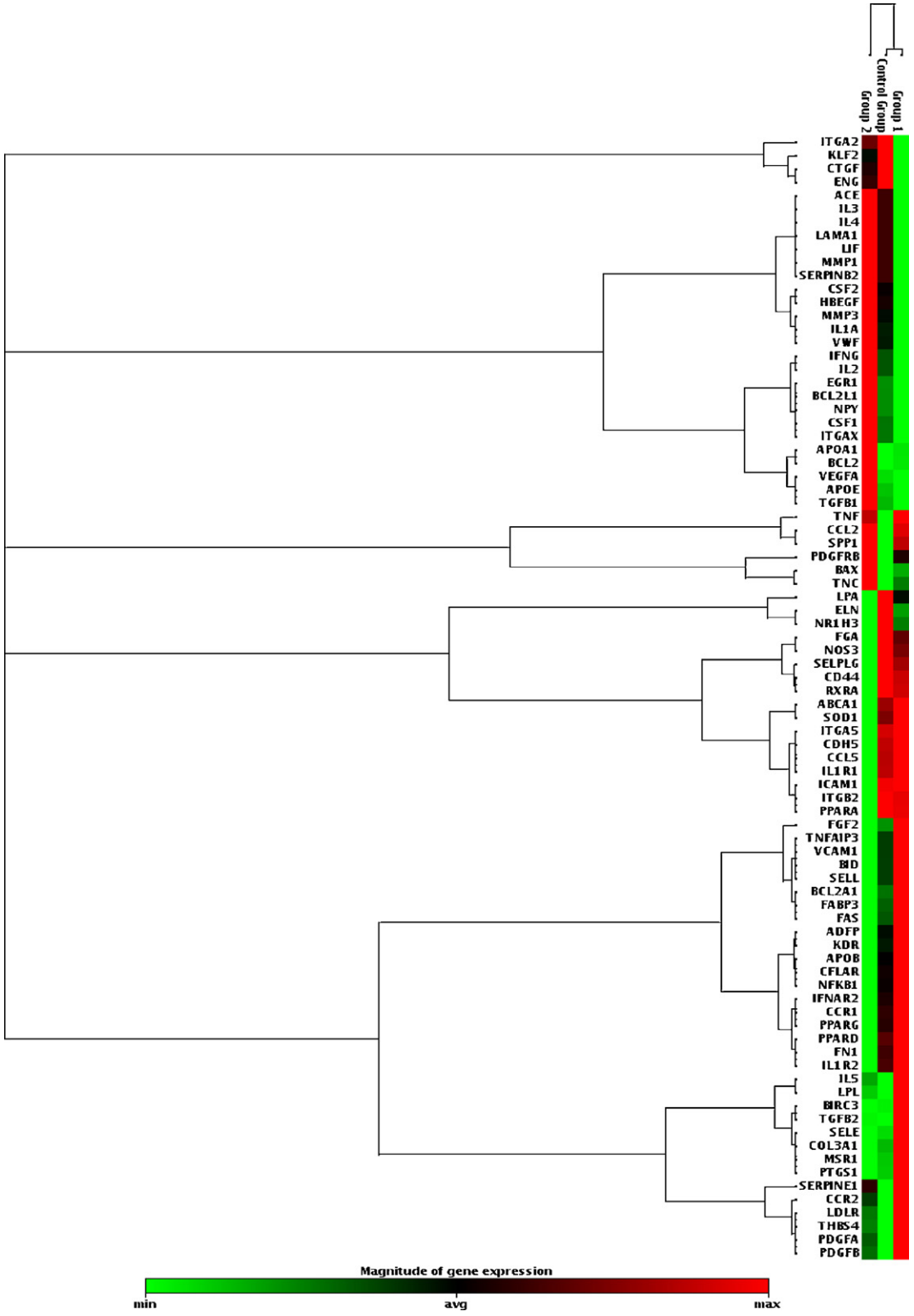

Fig. 1. Hierarchical cluster analysis of 84 atherosclerosis pathway – focused genes. Each column represents change in gene expression from the three histological groups: control liver, SS (group 1), and NASH (group 2). Upregulated mRNAs are shown in red; downregulated mRNAs are in green; and no changes in black. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

As the renin-angiotensin system (RAS) is involved in hepatic fibrosis [13], in an effort to evaluate a potential role of ACE in the NASH physiopathology and to examine the potential beneficial effect of ACE inhibition on liver histological endpoints, such as fibrosis and necroinflammation scores, we evaluated a subset of patients with NASH proven by liver biopsy, who were prescribed long-term treatment (24 months) with enalapril, an ACE inhibitor, (up to 10 mg daily) because of arterial hypertension ($n = 36$), in comparison with a subset of NASH patients who did not receive any concomitant medication by the time of liver biopsy ($n = 56$). NASH

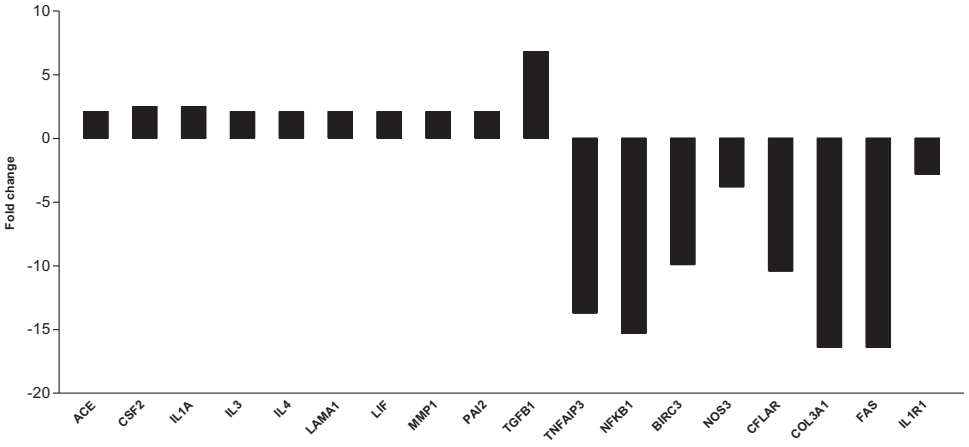

Fig. 2. Changes in liver gene expression for atherosclerosis-related genes observed in NASH versus SS. Changes are expressed as fold change with a significant p value <0.01.

patients treated with enalapril significantly differed from untreated patients in cardiovascular risk as measured by Framingham score, HOMA-IR index, and fasting insulin and GGT levels (Table 1).

In the univariate analysis, fibrosis scores were inversely associated with enalapril treatment (Spearman R: −0.34, $p < 0.001$) (Fig. 4). Interestingly, using ANCOVA for a multinomial ordinal distribution with probit as a link function, we showed that enalapril treatment was significantly associated with low fibrosis scores (χ^2: 6.8, $p < 0.01$), adjusted by log HOMA-IR index (χ^2: 6.2, $p < 0.02$) and sex (χ^2: 4.6, $p < 0.04$). Furthermore, addition of arterial blood pressure to the model did not alter this result (data not shown). Necroinflammation scores were only significantly associated with HOMA-IR (χ^2: 7.4, $p < 0.007$) independent of sex, enalapril treatment, and age. Conversely, in another group of NASH patients ($n = 30$) who were prescribed metformin (for more than 12 months, up to 1500 mg daily) because of concomitant compensated type 2 diabetes, differences in the fibrosis scores were not seen when compared with 56 patients who did not receive any concomitant medication.

3.4. Analysis of biological networks in NASH

There were highly scored pathways involved in the regulation of programmed cell death, cytokine and signal transduction, regulation of angiogenesis and immune system, and foam cell differentiation (Appendix Table 2). Interestingly, *TGFB1* is involved in most of the biological process predicted *in silico*, and *ACE* participates in two important nodes of connectivity: lymphocyte and mononuclear cell proliferation and regulation of systemic arterial blood pressure mediated by a chemical signal and vasodilatation. Details are shown in Supplementary material and Appendix Fig. 2.

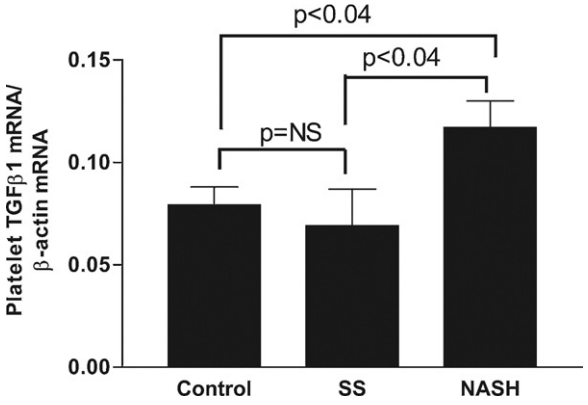

Fig. 3. Platelet mRNA expression in the whole population according to disease status: control (control), simple steatosis (SS), and nonalcoholic steatohepatitis (NASH) subjects. Results are expressed as mean ± SE of the *TGFβ1* mRNA/*β-actin* mRNA abundance ratio.

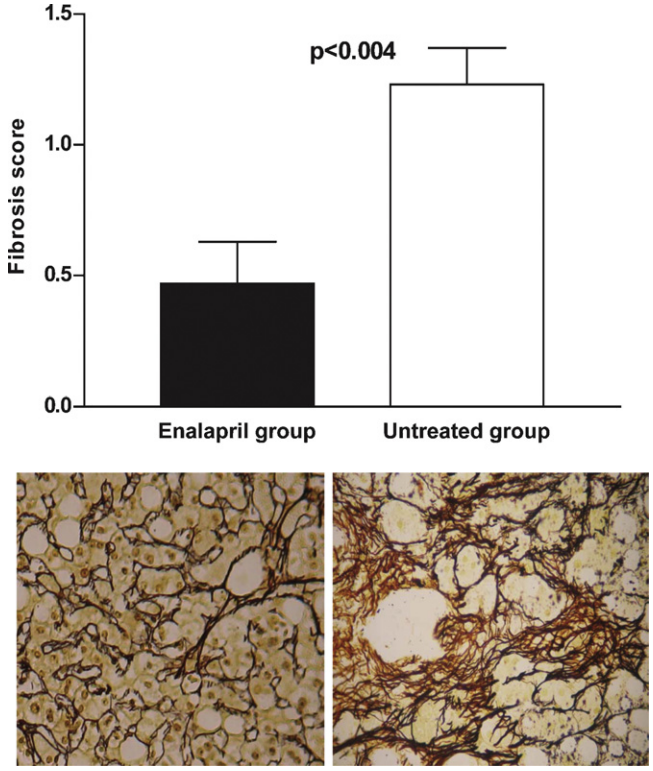

Fig. 4. Quantitative evaluation of fibrosis scores in NASH patients included in the post hoc analysis of the effect of angiotensin-converting enzyme *(ACE)* inhibition. (Panel A). The data are presented as mean ± SE. Examples of reticulin silver staining of liver sections (Panel B). The photomicrographs show that ACE inhibition is associated with lower fibrosis scores in NASH patients. Original magnification 400×.

4. Discussion

Several epidemiological studies were conducted to examine the association of NAFLD and cardiovascular disease; however, there is limited evidence of liver gene expression or transcriptomics, which support the molecular events associated with the liver involvement in the proatherogenic profile. It seems that the quantitative

differences in the abundance of specific transcripts partially account for the phenotypic differences among the individuals [14]. Hence, in this study we evaluated the expression of 84 genes encoding proteins involved in the atherosclerosis pathway in the liver of patients suffering from the histological disease severity spectrum of NAFLD, namely, SS and NASH, to contrast the hypothesis that cardiovascular risk is greater among patients with hepatic necroinflammation owing to a particular molecular signature; patients with normal liver were also included in the analysis.

The main findings of our study can be summarized as follows: (1) NASH presents a distinct panel of regulatory genes which are dys-regulated compared to the control and SS subjects, (2)-*BIRC3*, a severe hypoxia-activated gene, is increased in SS, suggesting that hepatocyte hypoxia may be associated with liver steatosis, (3) NASH, but not SS, is associated with an increased platelet abundance of *TGFβ1* mRNA, (4) NASH, but not SS, is associated with the regulation of genes in the liver and platelets which are associated with the atherosclerotic risk and, as such, may contribute to the pro-atherogenic state, (5) ACE inhibition may play a role in ameliorating liver fibrosis in NASH.

Here we provide evidence that 18 genes showed a statistically significant difference in expression when NASH was compared with SS, including 10 genes that were upregulated and 8 genes that were downregulated. Overall, the genes whose transcripts were downregulated were mostly related to the negative regulation of cell-death pathways, which is an expected finding, because hepatocyte apoptosis is a hallmark feature of NASH and disease severity [15]. For example, the liver transcriptional activity of *TNFAIP3*, a key player in the negative feedback regulation of NF-kappa B signaling in response to multiple stimuli, was significantly decreased in NASH samples.

Interestingly, genes upregulated in NASH include some highly expressed in human atherosclerotic plaques, such as *ACE* [16], *CSF2* [17], *IL1A* [18], *LIF* [19], *MMP1* [20], and *TGFβ1* [21]. Similarly, *IL4*, another transcript upregulated in the liver of NASH patients, has proinflammatory and proatherogenic effect in the vascular tissues regulating the expression of MCP-1 and decreasing nitric oxide bioavailability through activation of NADPH oxidase in the endothelial cells [22]. These findings strongly support the hypothesis about the proatherogenic role of hepatic necroinflammation in patients with NAFLD.

TGFβ1, the most upregulated gene in NASH samples (6.8 fold when compared with SS), plays a pivotal role in the regulation of vasculogenesis and angiogenesis, and in promoting apoptosis in endothelial cells. Hence, we decided to explore whether the increase observed in the liver of NASH patients mirrors the systemic phenomena observed in patients with increased occurrence of coronary artery disease [23]. The transcript level of *TGFβ1* in the circulating platelets was measured, one of the major reservoirs of this gene, and a significant increase in NASH but not in SS patients was observed. This observation might suggest an increased risk of NASH patients suffering from thrombogenic events [24], as *TGFβ1* may promote thrombogenesis [25].

To obtain a clear insight into the molecular events that accompany the steatosis alone state, we examined the transcript levels of the 84 genes in comparison with their expression in the control liver. The most remarkable finding was 8.2-fold upregulation of *BIRC3* transcript, also known as the inhibitor of apoptosis protein 1, whose basic function is antagonizing cell death and regulating the cell cycle. BIRC3 protein is coexpressed with *SMAC* (second mitochondria-derived activator of caspase, also known as DIABLO) and with most of the members of the caspase family (caspase 1, 3, and 7; Appendix Fig. 1). This highly activated pathway that involves many mitochondrial apoptogenic proteins observed in SS suggests that the molecular events associated with liver damage start very early in the pathophysiology of NAFLD.

A previous human study that investigated gene expression in simple steatosis by high-throughput technology in patients undergoing laparoscopic gastric bypass surgery showed interesting results about the differential profile between patients with extremely low or high liver fat content [26]. In agreement with our findings, the authors found several genes involved in inflammation, extracellular matrix formation and remodeling, and coagulation to be altered in subjects with high liver fat content although they did not show histologically detectable inflammation.

Perhaps an important mechanism to highlight is that *BIRC3* is induced by hypoxia, a phenomenon previously described to play a role in the fatty liver – associated events and the associated mitochondrial dysfunction [27]. This observation not only suggests that hypoxia might be one of the molecular switches between normal to steatotic liver, but also shows that the molecular events occurring in fatty liver mirror the systemic events associated with the MS-associated cardiovascular disease. This is supported by previous studies in humans and rodents that have demonstrated that tissue hypoxia is an essential feature of chronic inflammation and insulin resistance [28]. In addition, hypoxia inducible pathways have been associated with lesion progression and regulation of human intraplaque angiogenesis in human atherosclerotic plaques [29]. A recent paper on the hypoxia effect on cholesterol removal from macrophages also supports these findings [30].

To better understand the potential functional interactions between the products of genes that were differentially expressed in the liver of NASH patients relative to the controls or SS, the differentially expressed genes were analyzed *in silico*. We observed multiple interconnected networks between the gene products, and unsurprisingly, *TGFβ1* was mostly involved in many pathways, including immune-system regulation, signal transduction, apoptosis, and issue remodeling. In addition, the most remarkable gene involved in both cardiovascular pathophysiology and immune-system regulation was *ACE* that produces the physiologically active peptide angiotensin II. Interestingly, the local RAS is upregulated during liver injury and contributes to oxidative stress, recruitment of inflammatory cells, and fibrogenesis [31]. Based on these findings, we performed a post hoc analysis of liver biopsies of NASH patients who were treated with enalapril monotherapy because of arterial hypertension, and observed that when compared with the NASH untreated patients, enalapril was significantly associated with lower fibrosis scores, even after adjusting for HOMA-IR index, sex, and age. We suggest that this post hoc analysis can be considered as hypothesis-generating and might be regarded as proof of principles when deciding the future combination clinical therapeutic trials in NASH, particularly because no current assayed monotherapy has showed significant improvement in liver fibrosis scores, including amelioration of inflammation and insulin resistance [32].

The present study data provide the first profile of liver gene expression of atherosclerotic related pathways in NAFLD. Nevertheless, the cross-sectional design of the study is a limitation, and future prospective studies should determine the extent to which liver transcript profiles can predict the development of cardiovascular disease or whether modification of lifestyle or therapeutic intervention leads to changes in the transcript expression and eventually revert the phenotype, which have been suggested by previous experimental work [33].

In conclusion, beyond demonstrating that altered transcriptional regulation of proatherogenic genes occurs in the liver of patients suffering from NASH, a key finding of this study is that NASH, but not SS, is associated with the activation of molecular events that not only mirror the systemic MS profile characterized by inflammation, atherogenesis, and cardiovascular disease, but may also be responsible for the local production of mediators or modifiers of circulatory homeostasis. This finding strongly suggests the

necessity for an early therapeutic intervention in NASH patients, not only for ameliorating the liver injury, but also for improving the systemic proatherogenic state.

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Competing interests

The authors have no conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2011.05.014.

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