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RESEARCH ARTICLE

Expression of Cytokine Signaling Genes in Morbidly Obese Patients with Non-Alcoholic Steatohepatitis and Hepatic Fibrosis

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Abstract
Background White adipose tissue (WAT) from visceral adiposity plays an important role in the pathogenesis of non-alcoholic steatohepatitis (NASH). Development of NASH and its progression to fibrosis is partially due to cytokines and adipokines produced by WAT. The aim of this study was to assess the association of hepatic fibrosis and NASH by evaluating the intrinsic differences in the inflammatory cytokine signaling in the visceral adipose tissue obtained from morbidly obese patients.
Methods We used targeted microarrays representing human genes involved in the inflammatory and fibrogenic reactions to profile visceral adipose samples of 15 well-matched NASH patients with and without fibrosis. Additionally, visceral adipose samples were subjected to real-time polymerase chain reaction profiling of 84 inflammations related genes.
Results Eight genes (*CCL2*, *CCL4*, *CCL18*, *CCR1*, *IL10RB*, *IL15RA*, and *LTB*) were differentially expressed in NASH with fibrosis. Additionally, an overlapping but distinct list of the differentially expressed genes were found in NASH with type II diabetes (DM; *IL8*, *BLR1*, *IL2RA*, *CD40LG*, *IL1RN*, *IL15RA*, and *CCL4*) as compared to NASH without DM.
Conclusions Inflammatory cytokines are differentially expressed in the adipose tissue of NASH with fibrosis, as well in NASH with DM. These findings point at the interaction of adipose inflammatory cytokines, DM, hepatic fibrosis in NASH, and its progression to cirrhosis and end-stage liver disease.

Keywords NASH · Fibrosis · Morbid obesity

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome (MS) and is commonly found in patients undergoing bariatric surgery [1, 2]. There is increasing data that weight loss with bariatric surgery can lead to improvement of a number of complications of MS. [3]. The role of obesity in the development of NAFLD, and its progressive type, non-alcoholic steatohepatitis (NASH), is due, in part, to the changes in the milieu of the cytokines and adipokines produced by white adipose tissue (WAT). Although once considered to be an inert storehouse for fat, the current view of WAT is of a complex and active endocrine organ [4–6] producing several signaling molecules that are integral to the promotion or suppression of inflammation. These include adiponectin, leptin, resistin, apelin, vaspin, visfatin, interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-18 (IL-18), complement component 3 (C3), tumor necrosis factor alpha (TNF-α), and plasminogen activator inhibitor type 1 (PAI-1) [7]. Many of these factors have previously been studied in patients with NAFLD.

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From the spectrum of NAFLD, it has been shown that only the NASH subtype of NAFLD can progress to cirrhosis [8, 9]. Liver fibrosis is a progenitor and the key histologic feature of cirrhosis [10]. The relationship between NASH and hepatic fibrosis is complex, with some investigators proposing to use serum hepatic fibrosis markers, particularly type IV collagen, for diagnosing NASH [11]. Connection between NASH and fibrosis may reside in the role of inflammatory processes induced by a variety of mechanisms. In NAFLD, inflammation is caused by several factors including oxidative stress, which could potentially promote the progression of disease from simple or bland steatosis to NASH [12, 13]. Furthermore, inflammation stimulates transdifferentiation of hepatic stellate cells into fibrogenic myofibroblast-like cells [14]. Pro-inflammatory cytokines produced by WAT, including TNFα, IL-6, and IL-1, have been implicated in the development of both NASH and NASH-related fibrosis [15, 16].

One explanation for why only some of the patients with NASH develop fibrosis may be the differences seen in the level of the inflammatory cytokines produced by WAT. In addition to the contribution of WAT in the development of NASH and associated fibrosis, obesity has also been shown to adversely affect the outcome of patients undergoing orthotopic liver transplantation (OLT). Finally, the presence of significant hepatic steatosis in donor livers has been associated with primary non-function post-transplantation. All of these issues point out to the potential role of visceral obesity, adipocytokines (produced by WAT) in the development of NASH, its progression, and possibly their contribution to adverse outcomes post-OLT. To investigate this possibility, we used microarrays of oligonucleotide probes for genes encoding inflammatory cytokines as well as real-time polymerase chain reaction (PCR) profiling of 84 inflammations-related genes and profiled these genes in WAT of NASH patients with fibrosis and without it.

Materials and Methods

Patient Population

This study included 15 patients, all of whom had histological-proven NASH. In establishing the diagnosis of NASH, patients with other liver diseases and excessive alcohol use (>10 g/day). Cohorts of NASH patients with fibrosis (F+) and without fibrosis (F−) were matched for age, race, gender, body mass index, and insulin resistance (Table 1). Additionally, NASH patients were divided into those with and without type II diabetes (D+ and D−) according to the previous diagnosis and/or relevant medications (Table 2). This study was approved by our Institutional Review Boards.

For each patient, snap-frozen omental adipose tissue samples from the time of bariatric surgery were available. These samples had been snap-frozen in liquid nitrogen, stored at −80, and used for gene expression assays. At the time of sample collection, each patient had a liver biopsy read using our NASH pathologic protocol. NASH was defined when steatosis with lobular inflammation was accompanied by hepatocellular ballooning. Fibrosis was assessed with the Masson trichrome stain. Portal fibrosis and intralobular pericellular fibrosis were graded as 0 = none; 1 = mild; 2 = moderate; or 3 = marked. Biopsies of the F− group of patients were scored 0 for both kinds of fibrosis; biopsies of the F+ group of patients had mild to moderate per cellular or portal fibrosis, or both. In addition to the sample collection and liver biopsy, a number of pertinent clinical and laboratory data were collected.

Table 1 Clinico-demographic and laboratory data for cohorts of NASH with concomitant fibrosis (F+) and NASH without fibrosis (F−)

	NASH F− (*N*=7; mean±SD or %)	NASH F+ (*N*=8; mean±SD or %)	*P* value
Age (years)	42.9±13.6	49±7	NS
Female (%)	85.7% (6)	75% (6)	NS
Caucasian (%)	86% (6)	87.5% (7)	NS
Body mass index (BMI)	47.6±5.3	47.8±5.7	NS
Hip to waist ratio	1.05±0.1	1.04±0.1	NS
AST level (IU/L)	17.4±6	28±10.7	NS
ALT level (IU/L)	23±8	32±16.7	NS
AST/ALT	0.79±0.2	0.79±0.22	NS
Type 2 DM (%)	42.86% (3)	50% (4)	NS
Fasting serum triglyceride (mg/dL)	165.5±84.7	191.3±134.6	NS
Fasting serum cholesterol (mg/dL)	177.7±26.3	209.1±42.4	NS
Fasting serum glucose (mg/dL)	93.9±19.2	129.3±45.7	NS

N/A non-applicable, *NS* not significant (*P*>0.05)

Table 2 Clinico-demographic and laboratory data for cohorts of NASH with concomitant diabetes (D+) and NASH without diabetes (D−)

	NASH D− (*N*=8; mean±SD or %)	NASH D+ (*N*=7; mean±SD or %)	*P* value
Age (years)	49.5±10.9	42.4±9.9	NS
Female (%)	75% (6)	85.7% (6)	NS
Caucasian (%)	87.5% (7)	85.7% (6)	NS
Body mass index	44.2±2.9	51.3±4.9	NS
AST level (IU/L)	21±8.9	25.4±11.6	NS
ALT level (IU/L)	27±13.4	34.7±16.5	NS
AST/ALT	0.81±0.2	0.76±0.21	NS
Fibrosis (%)	50% (4)	57.1% (4)	NS
Fasting serum triglyceride (mg/dL)	149.3±86.4	205.1±133.2	0.04
Fasting serum cholesterol (mg/dL)	209.7±46.8	188.7±29.9	<0.001
Fasting serum glucose (mg/dL)	97.7±15.1	125.4±51.5	NS

NS not significant ($P>0.05$)

Gene Expression Using Microarrays

Omental tissue samples were homogenized using BaroCycler (Boston Biomedical, Inc.) RNA was extracted using the RNeasy® lipid tissue mini kit (QIAGEN) according to the manufacturer's recommendations. Synthesized cRNAs were labeled by biotinylation and purified using the TrueLabeling-AMP™ 2.0 kit and ArrayGrade™ cRNA cleanup kit (Superarray Biosciences). A total of 4 μg of each target cRNA was hybridized for 16 h on a thermoshaker to a GEArray® express Human Inflammatory Cytokines & Receptors microarray (Superarray Biosciences) using the manufacturer's HybPlate protocol. Each sample was run in duplicate. Images from the arrays were captured immediately after washing using a CCD scanner (Kodak). Rigorous quality controls were observed at all levels of the microarray experiment. These include electrophoresis and spectrophotometrical quantification of extracted RNA and amplified cRNA, parallel processing of incubations and hybridizations, and visual verification of the presence of all detected spots. Microarrays were analyzed using the GEArray® expression analysis suite (Superarray Biosciences) after background correction and normalization to a set of housekeeping genes. Duplicate microarrays had an average correlation coefficient of 0.89 (*P* value between 1.78×10^{-46} and 4.66×10^{-21}). A list of the cytokine-signaling-related (CSR) genes profiled in microarray experiments is given in Electronic Supplementary Material Table 1 (http://www.gmu.edu/ departments/mmb/baranova/pages/focus-fibrosis-adipose.htm).

Table 3 Clinico-demographic and laboratory data for cohorts of NASH with concomitant diabetes and fibrosis (F+D+) and NASH without diabetes or fibrosis (F−D−)

	NASH F−D− (*N*=4; mean±SD or %)	NASH F+D+ (*N*=4; mean±SD or %)	*P* value
Age (years)	46.48±14.6	45.58±6.7	NS
Female (%)	75% (3)	75% (3)	N/A
Caucasian (%)	100% (4)	100% (4)	N/A
Body mass index	45.4±2.5	52.5±3.1	NS
Hip to waist ratio	1.03±0.05	1±0	0.001
AST level (IU/L)	15±5.0	29±14.1	<0.001
ALT level (IU/L)	19.75±5.6	40.3±19.5	<0.001
AST/ALT	0.8±0.3	0.75±0.3	NS
Fasting serum triglyceride (mg/dL)	145.3±30.7	219.8±155.9	NS
Fasting serum cholesterol (mg/dL)	197±21.7	199.3±27.2	NS
Fasting serum glucose (mg/dL)	88.75±11.1	144±58.5	NS

N/A non-applicable, *NS* not significant ($P>0.05$)

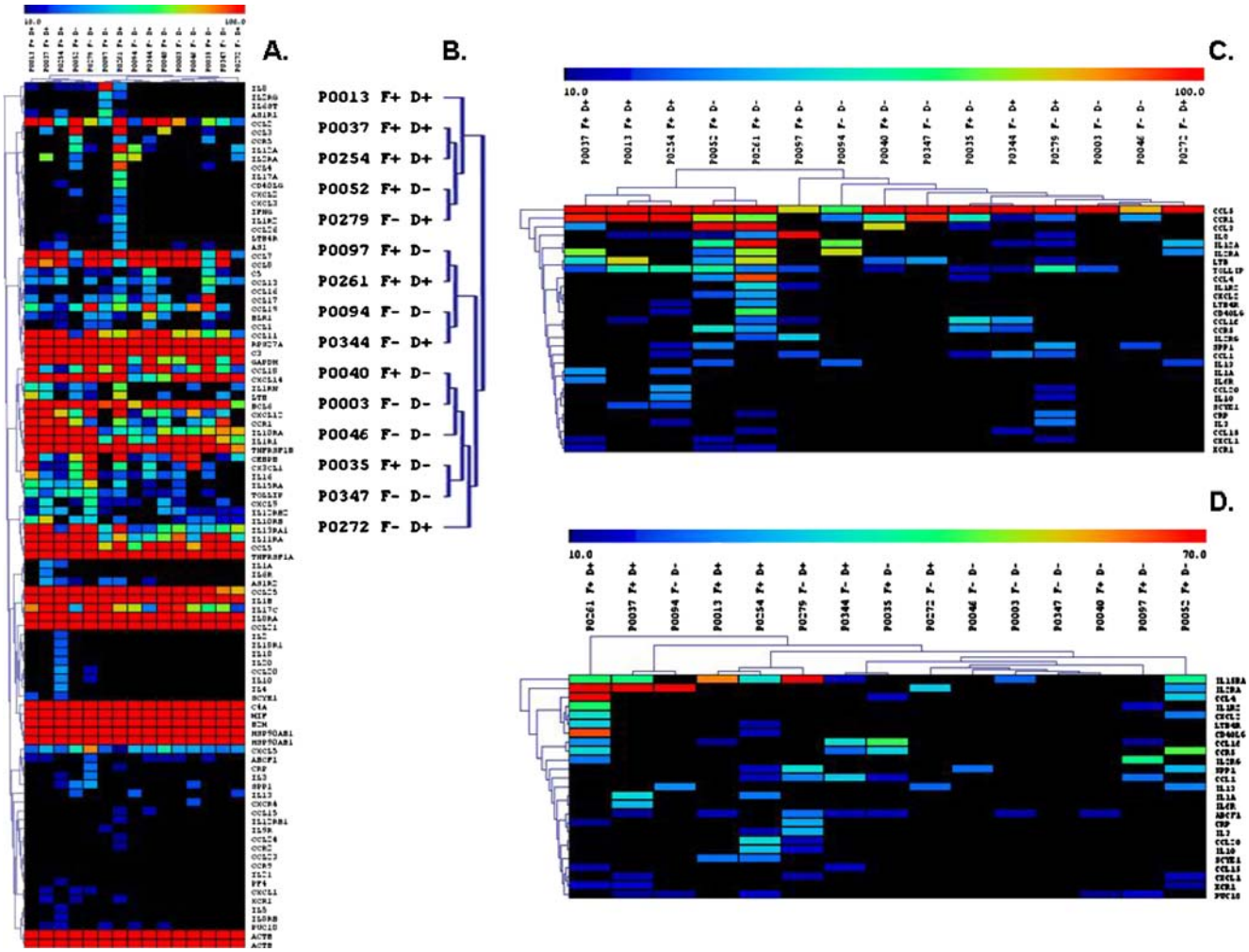

Fig. 1 Patient sample clustering by gene expression pattern evaluated on microarrays. **a** Hierarchical trees of patient samples were constructed using Pearson correlation and average linkage. **b** Detail of clustering by sample. Presence of the hepatic fibrosis marked by letter "*F+*", presence of diabetes by "*D+*". **c** Patient sample clustering by subset of genes significantly different by their expression in the adipose samples of the patients with NASH-related fibrosis when compared to F− NASH. **d** Patient clustering by subset of genes significantly different by their expression in the insulin resistant patients as compared to the patients without prior diagnosis of diabetes type II. In **c** and **d**, only genes yielding Mann–Whitney significance value of <0.05 have been used for clustering

Gene Expression Using Real-Time PCR

Additionally, adipose samples of six patients with NASH and fibrosis and six patients with NASH without fibrosis were profiled for expression of 84 inflammation-related genes along with five housekeeping control genes and three RNA quality controls. One microgram aliquots of extracted RNA samples were used for first-strand cDNA synthesis using the RT^2 first strand kit (Superarray Biosciences) according to the manufacturer's specifications. The resulting cDNAs were used as templates for Human Inflammatory Cytokines & Receptors RT^2 Profiler™ PCR arrays (Superarray Biosciences) and quantified using iCycler™ RT-PCR machine (BioRad). List of the CSR genes profiled in real-time PCR experiments given in Electronic Supplementary Material Table 2 (http://www.gmu.edu/departments/mmb/baranova/pages/focus-fibrosis-adipose.htm).

Results

Clinical and demographic data for patients are summarized in Tables 1, 2, and 3. Patients were subdivided into the following groups: NASH with fibrosis (F+, *N*=8); NASH without fibrosis (F−, *N*=7); NASH with concomitant diabetes (D+, *N*=7); NASH without diabetes (D−, *N*=8); NASH with both diabetes and fibrosis (F+D+, *N*=4); and NASH without diabetes or fibrosis (F−D−, *N*=4). Matching of the cohorts sampled for adipose tissue was supported by comparisons via non-parametric Mann–Whitney test.

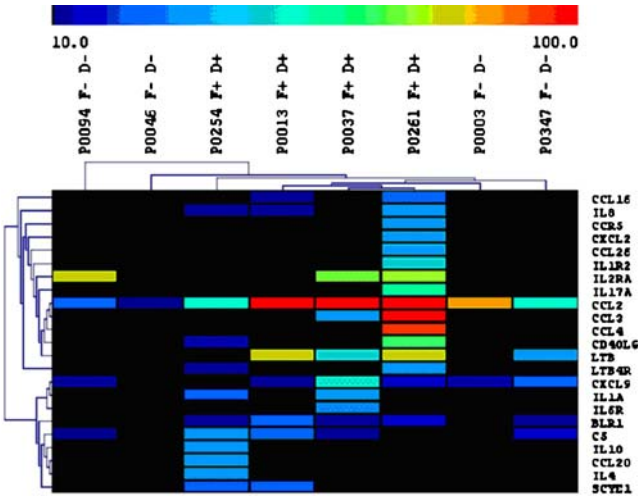

Fig. 2 Patient clustering by subset of genes significantly different in D+F+ NASH patients and D−F− NASH patients: Genes with Mann–Whitney significance values of <0.05 and a GE ratio with an absolute value >1.4 in a comparison between D+F+ NASH patients vs. D−F− NASH were used to generate hierarchical clustering

Gene Expression Profiling by Microarrays

Adipose-specific expression of the CSR genes (N=131) in each of the groups was assessed by microarray analysis (Electronic Supplementary Material Table 1). In order to be registered as "expressed," a gene had to have a gene expression (GE) value of at least 20% of the adjusted mean of the background-corrected and normalized spot intensity value of the entire microarray. The smallest number of CSR genes (N=36) were detected in the samples from patients classified as F−D− NASH. The D− NASH and F− NASH patients expressed 40 and 41 genes, respectively. The D+ NASH and F+ NASH patients expressed 49 and 50 genes, respectively. The largest number of CSR gene mRNAs was seen in the F+D+ NASH cohort, with 51 genes expressed. A summary of the genes expressed in each cohort of the patients is in Electronic Supplementary Material Table 3. The increase of the number of inflammation-related genes detected in visceral adipose tissue of the patients with NASH complicated by diabetes or by fibrosis may be indicative of the participation of adipose tissue inflammation in the pathogenesis of these disorders.

Hierarchical clustering of samples by Pearson correlation and average linkage using entire microarrays shows a general segregation of fibrotic and non-fibrotic patients as well as patients with and without diabetes (Fig. 1). Clustering of the genes differentially expressed in F+ and F− NASH patients by Mann–Whitney test (P<0.05) reflected a general increase in a number of transcripts involved in inflammation that can be seen in the fibrotic group; furthermore, among the fibrotic group, D+ and D− patients also showed a tendency to segregate (Fig. 1c). Genes differentially expressed in D+ and D− NASH patients by Mann–Whitney test (P<0.05) also clustered in a similar way (Fig. 1d). An increase in the overall levels of cytokine-signaling-related mRNA was especially pronounced when clustering was performed according to the genes differentially expressed between F+D+ and F−D− cohorts (Fig. 2).

Genes Differentially Expressed in Visceral Adipose Tissue of NASH Patients with and Without Hepatic Fibrosis

Of the 113 genes tested using the microarray (Electronic Supplementary Material Table 1), ten genes were identified as showing significant differences in mRNA abundance in a comparison of NASH patients with and without fibrosis

Table 4 Genes differentially expressed between F+ and F− NASH patients

Symbol	GE in F− NASH, mean	75% Confidence interval	GE in F+ NASH, mean	75% Confidence interval	GE ratio, microarray	*P* value	Fold change, RT-PCR
CCL18	41.06	10.01	96.5	26	2.35	<0.05	1.27
CCL2	51.41	10.86	142.45	32.82	2.77	<0.01	1.38
CCL3	10	NA	42.51	15.3	4.25	<0.001	−1.05
CCL4	10	NA	22.18	8.14	2.22	<0.001	1.11
CCR1	29.19	9.05	71.38	18.8	2.45	<0.01	1.05
IL-10RB	16.24	3.03	26.78	6.78	1.65	<0.001	1.46
IL-15RA	16.97	5.6	24.31	5.13	1.43	<0.001	NA
LTB	12.39	2.04	25.81	6.76	2.08	<0.001	1.18
TNFRSF1B	191.65	41.64	315.41	67.96	1.65	<0.05	NA
TOLLIP	14.30	3.81	25.13	4.59	1.76	<0.001	−1.11

Genes with GE intensity values ≤10 were set to the common background levels, as they were undetectable in the particular group of the patients by microarrays

NA not applicable

regardless of DM status (F+D± NASH patients vs. F−D± NASH patients; Table 4).

A gene was considered significant if it met the following rigorous criteria: the GE ratio had an absolute value >1.4; the comparison yielded a P value <0.05 according to a Mann–Whitney test; 75% confidence intervals for both sides of the comparison did not overlap; and at least one side of the comparison had a GE value of at least 20% of the adjusted mean of the background-corrected and normalized spot intensity value of the entire microarray.

A total of eight of these ten genes were also represented on the real-time PCR array. Of these eight genes, the expression levels of six genes were registered as changing in the same direction on both microarray and real-time PCR array (Table 4). Additionally, two genes were found to be differentially expressed using the real-time PCR array only, as their expression levels have not exceeded background signal threshold of microarray. By real-time PCR, *CCL16* displayed a downregulation of 3.52-fold in F+D± vs. F−D± NASH patient adipose tissue samples (P<0.05), while *CCR9* showed lower expression from the F+ D± NASH patients samples by 3.91-fold (P<0.05).

Genes Differentially Expressed in Visceral Adipose Tissue of NASH Patients with and Without Type II Diabetes

A microarray comparison of samples from diabetic and non-diabetic NASH patients identified seven genes that met the criteria for significance (see above; Table 5). Two of these genes, *CCL4* and *IL-15RA*, were also identified as differentially expressed in the analysis of fibrotic and non-fibrotic NASH (Table 4). Four of these seven genes were present on real-time PCR arrays; expression levels for all four of these genes were directionally consistent between the two platforms. Additionally, expression of *CCR9* gene not detected by microarrays showed a 3.09-fold lower expression (P<0.05) in the adipose from F±D+ NASH patients as compared to that from F±D− NASH patients.

Discussion

Obesity is a pro-inflammatory state, partially due to the secretion of inflammatory adipokines and cytokines from WAT [4–6]. The role of inflammation in the pathogenesis of both NASH and liver fibrosis has been previously shown [12–15]. Recent evidence points to the visceral adipose tissue as the primary source of circulating inflammatory cytokine levels in obese subjects with NAFLD [5, 6]. Interestingly, an expansion of adipose tissue in the obese individuals elevates the release of pro-inflammatory substances TNF-alpha, IL-6, and IL-8 per gram of adipose tissue [15], while weight loss reduces production of pro-inflammatory cytokines by adipose tissue [16]. Nevertheless, in a subset of obese individuals, adipose tissue remains relatively inert, sustaining low levels of cytokine secretion and not causing downstream damage to end-organs such as the liver [17]. Inflammation has also been implicated as an important etiological factor in the development of insulin resistance as well as clinically overt type 2 diabetes mellitus [18].

In this study, we hypothesized that the development of the hepatic fibrosis in some, but not all patients with NASH, may be explained by intrinsic differences in the cytokine signaling in the visceral adipose. These differences in cytokines may contribute to the development of NASH-related cirrhosis, end-stage liver disease leading to liver transplantation. Additionally, visceral obesity, WAT, and associated cytokines may be partly responsible for the adverse outcomes of obese patients' post-OLT. To investigate this hypothesis, targeted microarrays representing

Table 5 Genes differentially expressed in D+ and D− NASH patients

Symbol	GE D− NASH, mean	75% Confidence interval	GE D+ NASH, mean	75% Confidence interval	GE ratio, microarray	P value	Fold change, RT-PCR
IL8	17.77	8.8	12.64	1.84	−1.41	<0.001	−2.08
BLR1	14.10	2.45	20.06	5.6	1.42	<0.001	NA
IL2RA	15.21	4.88	21.85	6.85	1.44	<0.001	NA
CD40LG	10	NA	14.41	4.5	1.44	<0.001	1.52
IL-1RN	15.02	2.85	24.07	5.73	1.6	0.001	1.89
IL-15RA	16.19	3.28	26.26	7.02	1.62	<0.01	NA
CCL4	11.9	1.66	21.75	9.31	1.83	<0.001	1.78

Genes with GE intensity values ≤10 were set to the common background levels, as they were undetectable in the particular group of the patients by microarrays

NA not applicable

human genes involved in the inflammatory and fibrotic reactions (see Electronic Supplementary Material Table 1 for complete list of genes) were employed to profile visceral adipose samples of the well-matched cohorts of the NASH patients with and without concomitant fibrosis.

Our results show that adipose tissue samples of NASH patients with liver fibrosis and/or type II diabetes display a marked increase of mRNAs for inflammatory cytokines and their receptors. In general, clustering analysis of differentially expressed genes pointed at substantial overlap in the types of overexpressed mRNAs between adipose of diabetic patients with NASH and those NASH patients who had hepatic fibrosis.

An important example of a cytokine encoding gene that shows increased expression in the visceral adipose of NASH patients with fibrosis is *CCL2* (MCP1), previously associated with obesity [19], type II diabetes [20], and hepatic fibrosis [21]. The higher levels of *CCL2* production by macrophages embedded in adipose tissue as compared to pro-inflammatory M1 macrophages are thought to play a role in the development of insulin resistance [22]. However, in this study, the comparison of *CCL2* mRNA levels were shown to be related to fibrosis rather than to diabetes. Interestingly, in NASH patients with fibrosis, the levels of RNAs were increased both for *CCL2* as well as for its receptor, which could cause a local increase in pro-inflammatory signaling. Additionally, excessive *CCL2* produced by adipose may be delivered to the liver by portal vein and exert a direct fibrogenic effect.

Another interesting finding of our study was the exclusive overexpression of mRNA for CD40 ligand (CD40LG) in the diabetic patients with NASH and fibrosis. In fact, the expression level of CD40 ligand mRNA was below the detection threshold in all other groups, including NASH patients with diabetes, but not fibrosis. CD40LG has been previously implicated in the pathogenesis of metabolic syndrome in relation to heart disease and diabetes [23]. Interestingly, CD40LG has been also shown to induce apoptosis in hepatocytes and biliary epithelial cells as well as to stimulate NF-κB signaling [24]. Our findings support further investigation into the possibility of the using soluble CD40LG as a component for the predictive diagnostic marker for fibrosis in patients with NASH.

It is currently unclear whether the increased production of pro-inflammatory cytokines by WAT reflects an enhancement of the cytokine expression all over the body or it represents an isolated, tissue-specific phenomenon. Recent immunohistochemical study of the IL-6 production in the biopsies of the NAFLD patients showed a marked increase in the NASH livers as compared to the liver of patients with simple steatosis [25]. Moreover, a positive correlation was observed between hepatocyte IL-6 expression, stage of fibrosis, and the degree of systemic insulin resistance [25]. On the other hand, a sheer increase in the adipose tissue mass due to obesity may also result in substantial exposure of the liver to the pro-inflammatory cytokines which are promptly delivered to the hepatic tissue by portal veins. A comparative study of adipokine concentrations revealed that IL-6 levels in the portal vein of bariatric patients are approximately 50% greater than that in the arterial blood [26], suggesting a mechanistic link between visceral fat and hepatic exposure to the factors that may be involved in the pathogenesis of NASH. Whether local or systemic production of pro-inflammatory cytokines prevails must be further investigated.

An unavoidable weakness of this study is its relatively small sample size. Two factors contributed to the difficulty in procuring samples suitable for this study. First, our study design required control for as many potential confounders as possible, including age, ethnicity, body mass index, and others (Tables 1, 2, and 3). This led to a very tightly controlled study, but to a decrease in the number of patients available for inclusion in each group. The second factor leading to a decrease in the number of potential cases was the requirement to include NASH patients without fibrosis. Since many NASH patients have some degree of portal or pericellular fibrosis, this requirement led to exclusion of a large number of patients. Another weakness of this study was unavailability of the HOMA scores for most of the patients and the reliance on historical data for type 2 diabetes. The diabetes-specific clustering performed in our study, however, did serve as a proof of principle, while warranting further research into adipose-specific determinants of diabetes type II.

In summary, our data provide information regarding the expression levels of many pro- and anti-inflammatory molecules in the visceral adipose tissue of NASH patients. Analysis of the patterns of the mRNAs expressed in the visceral adipose showed some difference when NASH without fibrosis were compared to NASH with hepatic fibrosis. Additionally, several changes in the expression of inflammatory molecules seen in the adipose tissue of NASH patients were specific to diabetes. These data may offer an insight into NASH-related fibrogenesis and NASH-related cirrhosis. Nevertheless, further study will be necessary to understand the particular molecular mechanisms induced by the inflammatory cytokines from adipose tissue and how they relate to the progression of NASH. It will also be important to assess improvement in the pattern of gene expression after medical or surgical weight loss intervention for obese or morbidly obese patients.

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