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Interleukin-32 Contributes to Human Nonalcoholic Fatty Liver Disease and Insulin Resistance

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Nonalcoholic fatty liver disease (NAFLD) is a metabolic disorder due to increased accumulation of fat in the liver and in many cases to enhanced inflammation. Although the contribution of inflammation in the pathogenesis of NAFLD is well established, the cytokines that are involved and how they influence liver transformation are still poorly characterized. In addition, with other modifiers, inflammation influences NAFLD progression to liver cirrhosis and hepatocellular carcinoma, demonstrating the need to find new molecular targets with potential future therapeutic applications. We investigated gene signatures in 38 liver biopsies from patients with NAFLD and obesity who had received bariatric surgery and compared these to 10 control patients who had received a cholecystectomy, using DNA microarray technology. A subset of differentially expressed genes was then validated on a larger cohort of 103 patients who had received bariatric surgery for obesity; data were thoroughly analyzed in terms of correlations with NAFLD pathophysiological parameters. Finally, the impact of a specific cytokine, interleukin-32 (*IL32*), was addressed on primary human hepatocytes (PHHs). Transcript analysis revealed an up-regulation of proinflammatory cytokines *IL32*, chemokine (C-X-C motif) ligand 9 (CXCL9), and CXCL10 and of ubiquitin D (UBD), whereas down-regulation of insulin-like growth factor-binding protein 2 (IGFBP2) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) was reported in patients with NAFLD. Moreover, *IL32*, which is the major deregulated gene, correlated with body mass index (BMI), waist circumference, NAFLD activity score (NAS), aminotransferases (alanine aminotransferase [ALAT] and aspartate aminotransferase [ASAT]), and homeostasis model assessment of insulin resistance (HOMA-IR) index in patients. Consistent with an instrumental role in the pathophysiology of NAFLD, treatment of control human hepatocytes with recombinant *IL32* leads to insulin resistance, a hallmark metabolic deregulation in NAFLD hepatocytes. *Conclusion: IL32* has a critical role in the pathogenesis of NAFLD and could be considered as a therapeutic target in patients. (*Hepatology Communications* 2019;3:1205-1220).

Nonalcoholic fatty liver disease (NAFLD) is a pathologic condition characterized by enhanced accumulation of lipids in the liver and is considered by many as a common cause for liver cirrhosis development and progression to hepatocellular carcinoma.[1,2] NAFLD can be dissociated in two pathologic entities; liver steatosis, which represents the majority of cases of NAFLD (~80%), is

Abbreviations: AKT, protein kinase B; ALAT, alanine aminotransferase; ALP, alkaline phosphatase; ASAT, aspartate aminotransferase; BMI, body mass index; Cont, control; CXCL, chemokine (C-X-C motif) ligand; DE, differentially expressed; FAT10, F adjacent transcript 10; FC, fold change; GGT, gamma-glutamyl transferase; HCV, hepatitis C virus; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; HPRT1, hypoxanthine phosphoribosyltransferase 1; hsCRP, highly sensitive C-reactive protein; IFN, interferon; IGFBP2, insulin-like growth factor-binding protein 2; IL32, interleukin-32; LDL, low-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis; Ob_NAFLD, obese and nonalcoholic fatty liver disease; Ob_NASH, obese and nonalcoholic steatohepatitis; Ob_NL, obese and normal liver; Ob_ST, obese and hepatic steatosis; p-AKT, phosphorylated protein kinase B; PHH, primary human hepatocyte; PPAR, peroxisome proliferator-activated receptor; PRKCE, *protein kinase C epsilon gene; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; TNF, tumor necrosis factor; UBD, ubiquitin D; usCRP, ultrasensitive C-reactive protein.*

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**These authors contributed equally to this work.*

defined by the accumulation of triglycerides in the liver, is often benign, and is associated with good prognosis and nonalcoholic steatohepatitis (NASH), which is associated with liver steatosis, inflammation, ballooning of hepatocytes, and liver fibrosis.[3] A subset of patients with NASH (~30%) develops cirrhosis within 10 years, and NASH has become the second leading etiology among adults awaiting liver transplantation in the United States.[3-5] Recently, it has been shown that liver fibrosis is the most important histologic feature to predict the severity of disease and long-term outcome in patients with NAFLD.[6,7] It is worth mentioning that the presence of isolated steatosis does not preclude the progression to NASH.[1] Originally, it was thought that hepatic steatosis was the first event in NAFLD followed by a second hit in which inflammation and fibrosis take place. This hypothesis has been challenged, and NASH is now viewed as a condition in which multiple pathogenic events occur simultaneously to promote liver injury.[8] One major contributing factor to NAFLD is obesity,[9] and weight loss is an indication for NAFLD prevention. However, patients without obesity are also susceptible to develop NAFLD but tend to have less severe disease and may have a better prognosis than patients with obesity.[10] Importantly, high values of fatty liver index have been associated with enhanced insulin resistance in a large European population of middle-aged subjects without diabetes.[11] Moreover, the presence of metabolic syndrome was correlated with a high risk of NASH and particularly with a high risk of severe fibrosis among patients with NAFLD after correction for sex, age, and body mass index (BMI).[12] In the last decades, many findings have contributed to identify molecules that can be targeted to stop the progression of NAFLD into cirrhosis and liver cancer. For now, a number of emerging molecular targets have been identified to potentially treat NASH. These targets include transcription factors, such as the nuclear receptors farnesoid X receptor, pregnane X receptor, peroxisome proliferator-activated receptor alpha/beta/gamma (PPARα/β/γ), and nuclear erythroid 2 p45-related factor 2, and several proinflammatory chemokines with the aim being to reduce

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inflammation, fibrogenesis, and oxidative stress and to enhance fatty acid oxidation and insulin sensitivity.[13] Many of the molecules targeting either transcription factors or cytokines are currently tested in preclinical and clinical studies, and a treatment for NAFLD is eagerly awaited. Given the heterogeneity of NAFLD in patients, the treatment of NAFLD will most likely be customized to each patient based on the stage of NAFLD, and additional targets and predictive biomarkers are necessary to improve patient care.

In order to search for new molecular targets involved in NAFLD pathogenesis, we used DNA microarray chip technology to identify genes differentially expressed in liver biopsies from patients with NAFLD and obesity who had received bariatric surgery but who did not have diabetes compared to liver biopsies from patients without obesity who had received noninflammatory cholecystectomy. Surprisingly, the proportion of patients with obesity exhibiting no signs of NAFLD at histology (Ob_NL) was almost equal to that of patients with obesity with histologic hallmarks of NAFLD. We found that interleukin-32 (*IL32*) expression, a proinflammatory cytokine, was robustly induced in liver samples from patients with NAFLD compared to Ob_NL and control patients without obesity. Interestingly, liver *IL32* expression was significantly correlated with the NAFLD activity score (NAS), a system scoring for histolopathologic features of NAFLD developed by the NASH Clinical Research Network (CRN) and validated by the National Institute of Diabetes and Digestive and Kidney Diseases.[14] *IL32* also correlated with the homeostasis model assessment of insulin resistance (HOMA-IR) index in these patients, and treatment of primary human hepatocytes (PHHs) with recombinant *IL32* clearly impaired insulin signaling in these cells. Altogether, this study indicates that *IL32* along with other differentially expressed genes may play an important role in the pathogenesis of NAFLD and fatty liver-associated insulin resistance.

Patients and Methods

PATIENT RECRUITMENT

In order to recruit patients with NAFLD but without diabetes, patients who were morbidly obese and undergoing bariatric surgery were enrolled prospectively in the study in the Department of Hepatodigestive and Endocrine Surgery at Strasbourg University Hospital. Inclusion criteria included the following: adult men/women who were morbidly obese (BMI ≥40 kg/m^2) with histologic features of NAFLD and, as the control group, adult men/women without obesity who had received surgery for noninflammatory gallstone disease. All patients had no other illness or malignancy. Exclusion criteria included viral (hepatitis B virus [HBV] or hepatitis C virus [HCV]) or autoimmune hepatitis, diabetes, alcohol consumption >20 g/day for women and >30 g/day for men, genetic hematochromatosis with the homozygote C282Y mutation, infection or inflammation at the moment of biopsy, and impaired coagulation status with platelets <60,000/mm^3 and/or prothrombin rate <50%. Liver biopsies were performed for all patients who had received surgery, and histopathologic analysis of the biopsies allowed the classification of patients into four histopathologic entities as follows: controls (Cont, n = 19; mean age ± SEM, 49.5 ± 4.2 years), obese with a histologically "normal" liver without signs of steatosis (Ob_NL, n = 47; 41.5 ± 1.8 years), obese with hepatic steatosis (Ob_ST, n = 44; 40.9 ± 2.0 years), and obese with NASH (Ob_NASH, n = 12; 45.3 ± 3.5 years). The last two groups can be assimilated in the pathologic condition referred to as Ob_NAFLD, corresponding to patients with NAFLD (n = 56; 41.8 ± 1.8 years). Written informed consent was provided by all patients. This study was approved by the ethical committee board of Strasbourg University Hospital (approval number ID-RCB: 2007-A 00437-46).

BIOCHEMICAL ANALYSES

Blood samples were drawn from all fasted patients before induction of anesthesia in patients undergoing either cholecystectomy (lean control subjects) or bariatric surgery for patients with obesity (either sleeve gastrectomy or Roux-en-Y gastric bypass surgery). Blood samples were collected in vacutainer tubes and transported immediately to the clinical biochemistry laboratory where they were centrifuged and processed for serum measurements of standard and specialized clinical chemistry parameters (glucose, aspartate aminotransferase [ASAT], alanine aminotransferase [ALAT], gamma-glutamyl transferase [GGT], alkaline phosphatase [ALP], total bilirubin, triglycerides, free fatty acids, total cholesterol, high-density

lipoprotein [HDL]-cholesterol, low-density lipoprotein [LDL]-cholesterol, iron, ultrasensitive C-reactive protein [usCRP] using an ADVIA 2400 clinical chemistry analyzer (Siemens Healthcare SAS, France), following the manufacturer's instructions. Circulating insulin levels were quantified by an immunochemistry luminescence method using a COBAS 6000 analyzer (Roche Diagnostics SA, Switzerland). Ferritinemia was measured by a chemiluminescence immunoassay on an ADVIA Centaur CP analyzer (Siemens Healthcare SAS).

GENE EXPRESSION ANALYSIS USING MICROARRAY AND QUANTITATIVE REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION

We used gene expression microarray technology and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) to study genes that were differentially expressed in liver biopsies obtained from patients who were lean and had received a cholecystectomy (n = 10) and from patients with obesity who had received bariatric surgery. The analysis was carried out in 10 patients randomly chosen from each histopathologic group (8 patients in the NASH group; total, 38 patients). Agilent gene expression microarray chips (Reference SurePrint G3 Human GE 8x60K, Agilent Corp., France) were used to analyze genes that were differentially expressed (DE) in the liver of the four histopathologic groups (n = 10/group, 8 patients in the NASH group). To identify DE genes, we used the fold-change rank ordering statistics method,[15] which associates an *f* value with genes. The *f* values for down-regulated genes are small (near zero), while those associated with up-regulated genes are high (near 1). We first compared samples from Ob_NL, Ob_ST, and Ob_NASH to the Cont samples. These comparisons were performed in two steps as follows: first, comparison of groups using all data samples at a time; second, use of bootstrap analysis to select genes that were least dependent as possible to patients. In the bootstrap analysis, subsets of samples were selected from the groups in the comparison. This was repeated 100 times, and results were finally combined for each gene. The threshold *f* values 0.995 and 0.005 were used to select the up- and down-regulated genes, respectively. The lists were combined, and unique known genes were hierarchically clustered in a heatmap in which overexpressed genes are represented in red and down-regulated genes in blue, using Cluster 3.0 and Java Treeview software (version 1.1.6r2; http://jtreeview.sourceforge.net/). A microarray correlated genes diagram was drawn using Cytoscape 3.7.1 software (http://cytoscape.org).

For a subset of genes intercorrelated in microarray analysis, we confirmed the DE using qRT-PCR on all complementary DNA samples (n = 122) obtained from liver biopsy-extracted RNA, using a QIAGEN RNeasy Lipid Tissue Mini Kit (ID 74804).

PRIMARY HEPATOCYTE CULTURE AND WESTERN BLOTTING

PHHs were isolated and cultured as described.[16] Cells were plated 1 day before incubation with human recombinant *IL32* (1 ng/mL) (CliniScience, France) for 24 hours. PHHs were then treated with human recombinant insulin (100 nM) (Sigma-Aldrich, France) for different time points (0, 5, 10, 20, 30, and 60 minutes). Nontreated PHHs served as controls. Cells were lysed in Lysis Buffer 6 (R&D Systems), and protein concentration was determined using the Bradford assay (Bio-Rad), following the manufacturer's instructions. Cell lysates were run on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were saturated with phosphate-buffered saline containing 5% bovine serum albumin and 0.1% tween. For protein detection, polyclonal rabbit pan-protein kinase B (AKT) antibody (ab8805; Abcam, France), polyclonal rabbit anti-phosphorylated (phospho-)AKT (Thr-308) antibody (ab38449; Abcam), and monoclonal anti-β-actin antibody (ab8226; Abcam) were used. *IL32* protein was quantified in liver extracts with rabbit anti-*IL32* antibody (ab37158; Abcam). Protein expression was quantified using ImageJ software.

STATISTICAL ANALYSIS

We used analysis of variance for comparison among the four histopathologic groups analyzed, followed by Tukey's multiple comparison test when the distribution was normal and Kruskal-Wallis test

followed by Dunn's multiple comparison test when the distribution was not normal. Statistical significance was achieved at $P < 0.05$. Generated graphs and statistical regression analysis were performed using GraphPad Prism 6.0. Heatmaps for Spearman's correlations were generated using Mev_4_8 version 10.2 (http://mev.tm4.org).

Results

ANTROPOMORPHIC DATA AND BIOCHEMICAL PARAMETERS IN PATIENTS WITH OBESITY AND BARIATRIC SURGERY AND PATIENTS WITHOUT OBESITY WHO HAD RECEIVED A CHOLECYSTECTOMY

Patients with NAFLD are usually histologically separated into patients with steatosis and patients with NASH. Patients were stratified in four main groups based on their histopathologic profile. The first group (Cont) represented the control arm of the study, corresponding to patients without obesity who had a noninflammatory cholecystectomy (n = 19). The second group (Ob_NL) was represented by patients with obesity who underwent bariatric surgery with a histologically "normal" liver (i.e., no signs of hepatic steatosis, n = 47). The third group Ob_ST (n = 44) and the fourth group Ob_NASH (n = 12) corresponded to patients with obesity who had received bariatric surgery and had histopathologic signs of NAFLD, defined by a proportion of more than 5% of hepatocytes that exhibit the presence of cytoplasmic lipid droplets with no signs of inflammation or with inflammation, respectively.[17] Representative histopathologic pictures of liver biopsy sections stained with hematoxylin and eosin of the four groups are shown in Fig. 1A. BMI and waist circumference were increased in patients with obesity (Ob_NL and Ob_NAFLD) compared to the Cont group ($P < 0.001$; Table 1). Compared to the Cont group, fasting serum glucose levels were not changed and were slightly increased in the Ob_NL group and the Ob_NAFLD group ($P < 0.05$; Table 1), respectively. In addition, serum insulin levels and the HOMA-IR index were significantly increased in the Ob_NAFLD group compared to Cont ($P < 0.001$) and Ob_NL ($P < 0.01$) groups, indicating that patients with obesity with NAFLD were insulin resistant (Table 1). The serum lipid profile indicated that total and LDL-cholesterol levels were not changed among Ob_Cont, Ob_NL, and Ob_NAFLD, while HDL-cholesterol was decreased in Ob_NAFLD compared with Cont ($P < 0.001$). In addition, compared with the Cont and Ob_NL groups, total triglycerides were increased in Ob_NAFLD ($P < 0.05$ and $P < 0.01$, respectively). Hepatic enzymes ALAT ($P < 0.001$), ASAT ($P < 0.001$), and GGT ($P < 0.05$) were increased in Ob_NAFLD patients compared to Ob_NL and Cont patients (Table 1). ALP, total bilirubin, iron, and transferrin saturation capacity were not changed among the three groups. Ferritin was increased in the NAFLD group compared to Ob_NL ($P < 0.05$) but not Cont (Table 1). Data for Ob_ST and Ob_NASH are also presented separately in Table 1, and significant statistical differences with Cont and Ob_NL are also shown for Ob_ST and Ob_NASH.

Several groups have studied CRP status in individuals with obesity, yet the association with insulin resistance remains elusive. In this study, usCRP was significantly increased in the serum of patients with obesity and bariatric surgery (i.e., Ob_NL, Ob_ST and Ob_NASH patients; Fig. 1B) compared to control lean patients and correlated positively with BMI and waist circumference in patients (Fig. 1C,D, respectively). This result was not surprising given that obesity is characterized by metabolic low-grade and chronic inflammation, yet the values of usCRP were not as high as in the case of an acute inflammatory response. However, usCRP was not correlated with glucose, insulin, or HOMA-IR index in our cohort of patients (Fig. 1E-G, respectively). In summary, the inflammatory acute-phase response protein usCRP was not correlated with insulin resistance in our cohort of patients.

GENE SIGNATURE OF Ob_NAFLD PATIENTS REVEALED INVOLVEMENT OF SPECIFIC INFLAMMATORY CYTOKINES

To better understand the molecular changes that occur in the liver of patients with obesity in the context of NAFLD, we performed microarray analysis in 10 patients randomly chosen from each group (8 patients in the NASH group). We identified 846 genes that were significantly DE in patients with

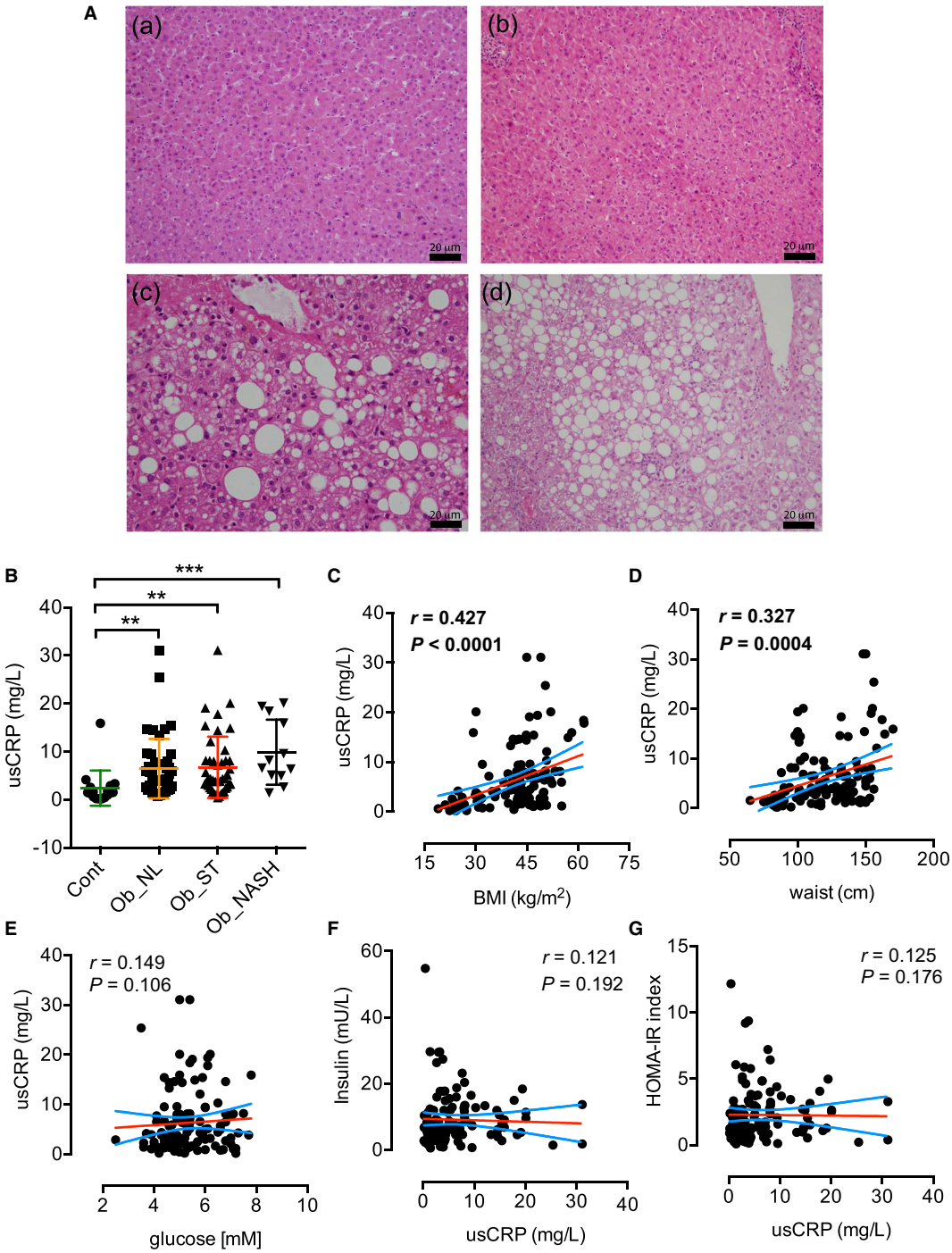

FIG. 1. Histology and usCRP measurement in patients who received bariatric surgery compared with patients who underwent cholecystectomy. (A) Histopathologic representative pictures of hematoxylin and eosin staining of liver sample paraffin-embedded sections from (a) control patients who received a cholecystectomy, (b) Ob_NL, (c) Ob_ST, and (d) Ob_NASH patients who received bariatric surgery (scale bar, 20 micrometers). (B) usCRP immunoanalytic measurement in patients with obesity who received bariatric surgery, with NAFLD (Ob_ST and Ob_NASH) and without NAFLD (Ob_NL), compared with control patients who received a cholecystectomy; usCRP was significantly correlated to BMI (C) and waist circumference (D) but not with glucose (E), insulin (F), and HOMA-IR index (G). Results are expressed as mean ± SEM; $^{**}P < 0.01$, $^{***}P < 0.001$.

TABLE 1. ANTROPOMORPHIC AND BIOCHEMICAL ANALYSES FROM PATIENTS WITH OBESITY WHO RECEIVED BARIATRIC SURGERY AND THEIR CONTROLS WITHOUT OBESITY

	Cont	Ob_NL	Ob_ST	Ob_NASH	Ob_NAFLD
n	19	47	44	12	56
Age (years)	49.5 ± 4.2	41.5 ± 1.8	40.9 ± 2.0	45.3 ± 3.5	41.8 ± 1.8
BMI (kg/m^2)	24.7 ± 0.6	42.6 ± 0.9§	45.3 ± 1.0§	46.2 ± 2.6§	45.5 ± 1.0§
Waist (cm)	84.1 ± 2.4	121.4 ± 2.9§	131.8 ± 3.2§	129.6 ± 5.6§	131.3 ± 2.7§
Glucose (mM)	5.0 ± 0.2	5.1 ± 0.1	5.7 ± 0.1*/†	5.8 ± 0.3	5.7 ± 0.1*/†
Insulin (mU/L)	5.3 ± 0.8	7.5 ± 0.9	11.7 ± 1.4†/*	10.6 ± 2.3*	11.4 ± 1.2‡/†
HOMA-IR index	1.2 ± 0.2	1.8 ± 0.2	3.1 ± 0.4‡/†	2.7 ± 0.5*	2.98 ± 0.3‡/†
Total chol (mmol/L)	4.4 ± 0.3	4.1 ± 0.2	4.1 ± 0.1	4.0 ± 0.2	4.1 ± 0.1
LDL-chol (mmol/L)	2.6 ± 0.2	2.5 ± 0.1	2.4 ± 0.1	2.2 ± 0.2	2.4 ± 0.1
HDL-chol (mmol/L)	1.2 ± 0.1	1.0 ± 0.04†	0.8 ± 0.03‡/*	0.9 ± 0.05	0.8 ± 0.02‡/*
TG (mmol/L)	1.4 ± 0.2	1.4 ± 0.1	2.1 ± 0.2*/*	1.9 ± 0.2	2.06 ± 0.1†/†
FFA (mmol/L)	0.7 ± 0.1	0.8 ± 0.04	0.8 ± 0.04	0.7 ± 0.07	0.8 ± 0.04
ALAT (U/L)	24.7 ± 6.0	29.8 ± 3.4	54.6 ± 4.5§/§	66.9 ± 20.8†/*	57.2 ± 5.6§/§
ASAT (U/L)	24.6 ± 4.0	27.3 ± 2.3	39.2 ± 2.9‡/†	49.5 ± 14.3*	41.4 ± 3.8§/‡
GGT (U/L)	26.9 ± 6.2	39.2 ± 7.0	40.4 ± 3.7*	68.9 ± 23.3*	46.5 ± 5.8†/*
ALP (U/L)	54.5 ± 4.8	68.3 ± 5.9	58.1 ± 2.4	69.5 ± 13.0	60.6 ± 3.4
Total bilirubin (μmol/L)	9.2 ± 1.2	9.8 ± 0.6	8.8 ± 0.5	14.2 ± 4.7	9.9 ± 1.1
Iron (μmol/L)	14.7 ± 1.6	11.9 ± 0.8	13.6 ± 0.7	14.0 ± 1.6	13.7 ± 0.7
Ferritin (μg/L)	139.3 ± 30.9	111.5 ± 17.9	179.2 ± 20.3^{ns}/*	142.7 ± 31.5	171.4 ± 17.3^{ns}/*
TSC (%)	30.3 ± 3.3	25.3 ± 1.9	28.5 ± 1.6	27.0 ± 2.7	28.2 ± 1.4

Symbols before the slash (/) represent comparison with the control group; those after the slash (/) represent comparison with the Ob_NL group. Results are expressed as mean ± SEM.
*$P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$, $^{\S}P < 0.0001$.
Abbreviations: Chol, cholesterol; FFA, free fatty acid; ns, not significant; TSC, transferrin saturation capacity.

obesity compared to control samples (fold change and *f* values are presented in Supporting Table S1). Of these, 595 genes were DE in the Ob_NL group, 395 genes in the Ob_ST group, and 243 genes in the Ob_NASH group. To identify DE genes that may be more relevant biologically, we set a fold-change threshold of 2 for up-regulated genes and 0.5 (−2-fold) for down-regulated genes (i.e., 0.5 ≥ fold change ≥2). Hence, we identified 144 DE genes in patients with obesity (Ob_NL, Ob_ST, or Ob_NASH). The list of DE genes (with 0.5 ≥ fold change ≥2) in each group is summarized in Fig. 2C and Supporting Table S2. Hierarchical clustering of the identified genes was represented in a heatmap in which genes are classified in rows and patients in columns (Fig. 2A; Supporting Fig. S2 for clarity). As expected, Cont patients were clustered together except 1 Ob_NL patient clustered with this group. Ob_NASH and Ob_ST patients clustered mainly together (except 1 Ob_NL clustering with this group), forming a fairly homogeneous molecular group named NAFLD that could be easily distinguished from the Cont patients who were lean (Fig. 2A). However, using this approach the NAFLD group was not separated into two clear independent groups, in line with the difficulty to histopathologically classify patients with fatty liver disease in either the steatosis or NASH entity. Indeed, 2/8 Ob_NASH patients clustered with the Ob_ST group (Fig. 2A). Venn diagrams (http://bioinfogp.cnb.csic.es/tools/venny/) presented show the number of genes specifically DE in each group and the ones that are commonly DE among the three groups.

Functional gene enrichment analysis of up-regulated genes in the Ob_NASH patients showed that inflammation-associated pathways and those associated with the adaptive and innate immune response, such as the pathogenesis multiple sclerosis pathway (Fisher's exact test, $P = 3.9E–06$) in which the proinflammatory chemokine (C-X-C motif) ligand 9 (*CXCL9*) and *CXCL10* genes are annotated or the dendritic cell maturation pathway (Fisher's exact test, $P = 4.7E–04$) in which a gene encoding a less well-characterized inflammatory cytokine, namely *IL32*, is annotated, were also enriched (Fig. 2D). This analysis supports the notion that the up-regulation of the inflammatory

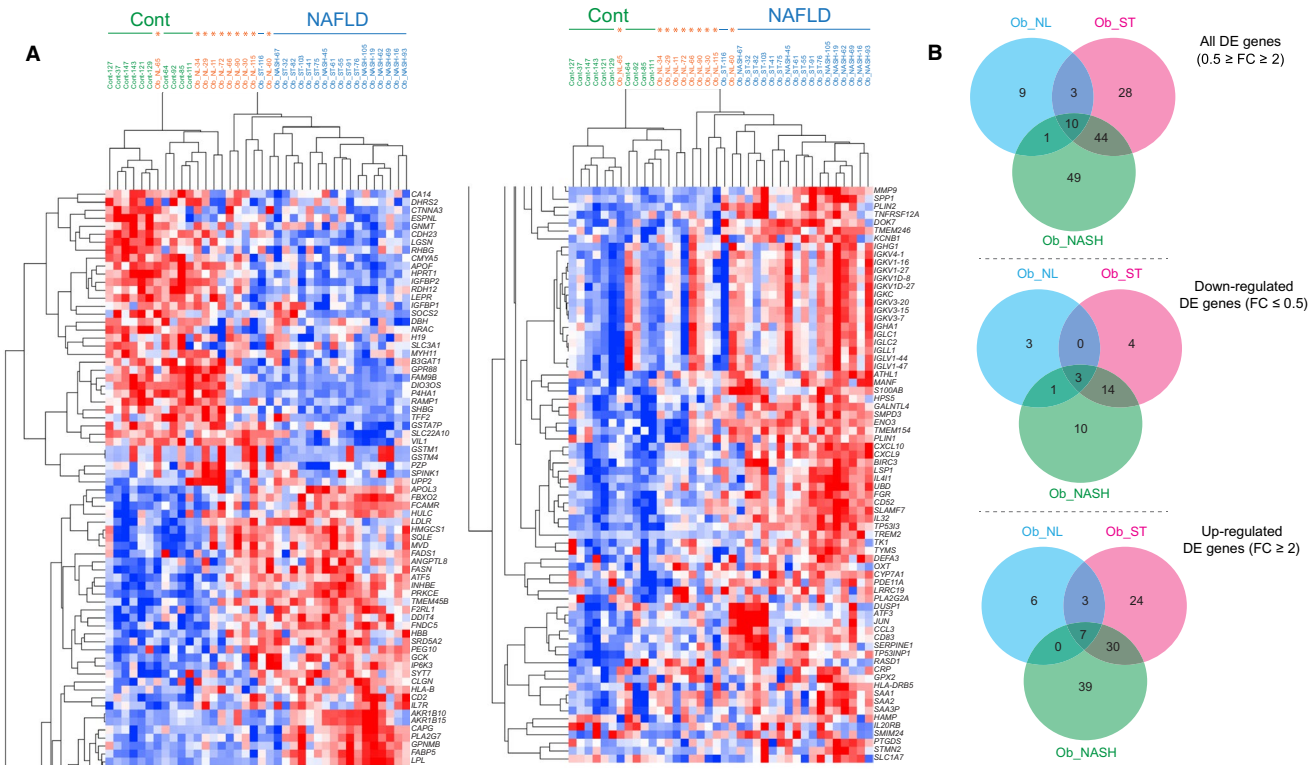

FIG. 2. Representation and pathway analysis of DE gene expression dataset. (A) Hierarchical cluster analysis of microarray DE genes in liver biopsies from patients with obesity who had bariatric surgery and control patients without obesity who had a cholecystectomy. DE genes are represented with FC ≥2 and ≤0.5. Overexpressed genes are in red, and down-regulated genes are in blue. Rows and columns represent genes and patients, respectively. (B) Venn representation of the DE genes. The number of genes commonly DE between the Ob_NL, Ob_ST, and OB_NASH groups are indicated as well as those specific to each group. (C) List of DE genes in the three groups of patients Ob_NL, Ob_ST, and Ob_NASH compared to the control group (0.5 ≥ FC ≥ 2). (D) Functional analysis (IPA) of differentially regulated genes between patients with steatohepatitis and their controls. Regarding up-regulated genes, the immune response and inflammatory pathways involving the proinflammatory genes *CXCL9*, *CXC10*, and *IL32* are among the most significantly enriched pathways. *IL32* is particularly enriched in the dendritic cell maturation pathway. For down-regulated genes, *IGFBP2* is annotated in the IGF-1 signaling pathway, the most significantly enriched pathway. *HPRT1* is annotated in the second and fourth most enriched nucleotide salvage pathways. The dashed line indicates the Fisher exact test *P* value threshold set at 0.05. (E) Heatmap of IPA disease and biological function comparison analysis between Ob_NL, Ob_ST, and Ob_NASH liver samples. The intensity of the orange color indicates the functions that are overrepresented in a given condition based on genes that are DE, whereas the intensity of the blue color reflects the functions that are underrepresented. The activation *z* score represents the bias in gene regulation that predicts that a biological function is overrepresented or underrepresented in a given condition. (F) Gene-to-gene correlation between microarray DE genes. Positive correlations ≥0.6 are indicated with red lines and negative correlations ≤−0.6 are indicated with blue lines. Abbreviations: FC, fold change; IPA, Ingenuity Pathway Analysis.

genes *CXCL9*, *CXCL10*, and *IL32* may play important roles in the pathogenesis of NASH. Interestingly, in Ob_ST patients, both *CXCL9* and *CXCL10* were annotated in the pathogenesis of the multiple sclerosis pathway, which is the second most enriched pathway (Fisher's exact test, P = 3.1E–06), whereas *IL32* was not annotated in the most significant enriched pathways (Fisher's exact test, P = 0.02), suggesting that *IL32* might play a more prominent role in NASH than in steatosis. The full list of enriched pathways and their annotated genes in the obese NAFLD bariatric groups (Ob_ST and Ob_NASH) regarding both down- and up-regulated genes is summarized in Supporting Table S3. Moreover, a comparison using the Ingenuity Pathway Analysis bioinformatics tool (http://qiagenbioinformatics.com/products/ingenuity-pathway-analysis)

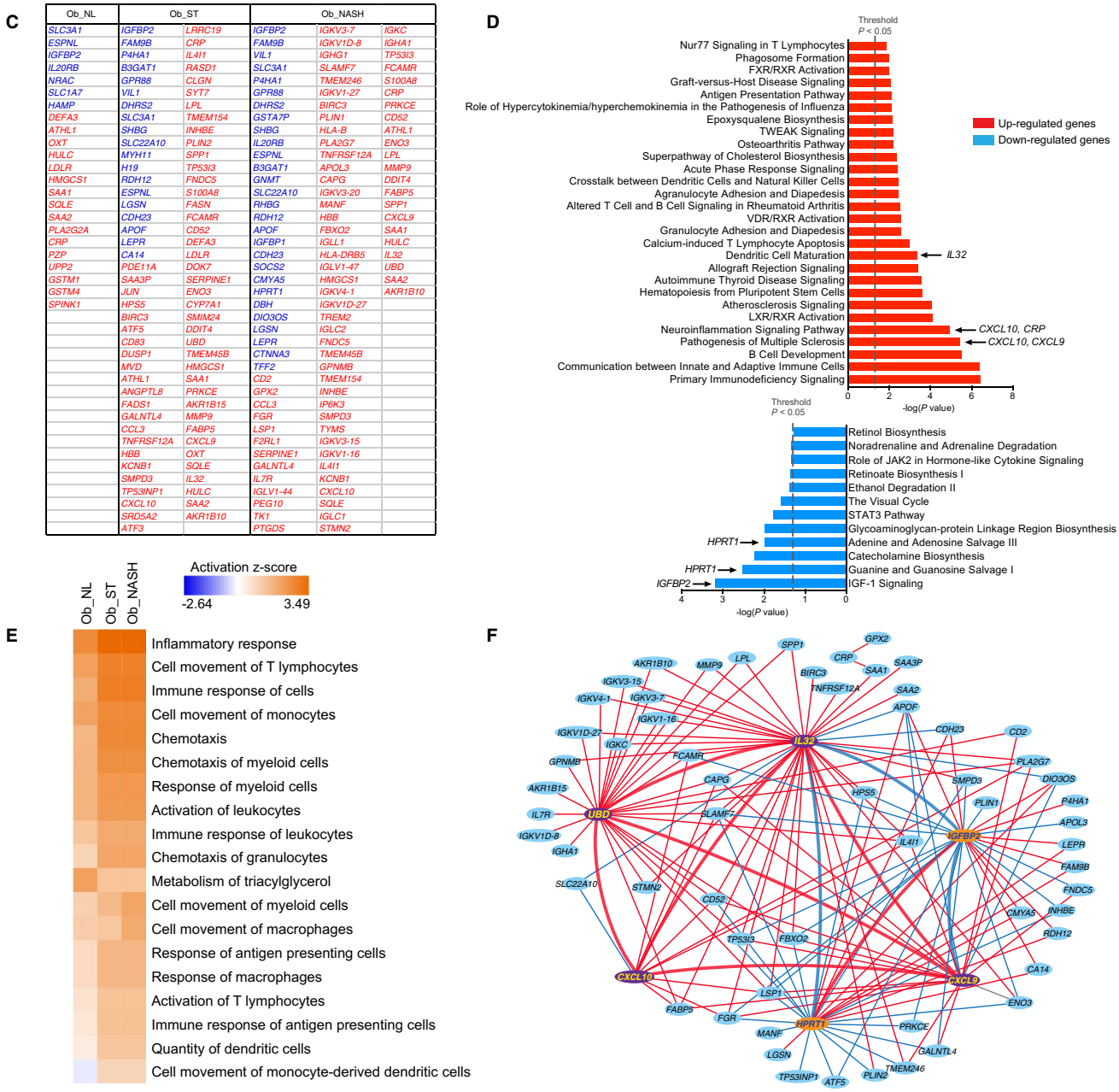

FIG. 2. (Continued)

showed that several biological functions linked to the inflammatory response, chemotaxis, and cell movement of immune cells are predicted to be activated in Ob_ST liver samples, and some of them even more in Ob_NASH patients as is the case for the movement of myeloid cells and macrophages (Fig. 2E). This analysis also revealed that the metabolism of triglycerides is predicted to be much less activated in liver samples of Ob_NASH patients compared to Ob_NL patients, in line with the observed decrease in the expression of the fatty acid oxidation-inducing gene *PPARα* (Supporting Fig. S3) in Ob_NAFLD samples and not in Ob_NL samples.

To address possible crosstalk between the different genes found in our molecular analysis, we applied Pearson's correlation analysis. Six genes were highly intercorrelated, including *IL32*, *CXCL9*, *CXCL10*, ubiquitin D (*UBD*)/F adjacent transcript 10

(*FAT10*), insulin-like growth factor-binding protein 2 (*IGFBP2*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*). The last three genes are: i) *UBD*, a tumor necrosis factor alpha (TNFα)-inducible gene mediating nuclear factor kappa B (NF-κB) activation[18] and highly up-regulated in HCC[19]; ii) *IGFBP2*, a leptin-regulated gene with antidiabetic effects[20]; and iii) *HPRT1*, the gene encoding the enzyme hypoxanthine phosphoribosyltransferase 1, which plays a central role in the generation of purine nucleotides through the purine salvage pathway. *HPRT1* deficiency has been shown to cause Lesch-Nyhan syndrome with hyperuricemia and gout[21] (Fig. 2F). These genes form a hexagram in which the proinflammatory genes (*IL32*, *CXCL9,* and *CXCL10*) and *UBD* are positively intercorrelated. *IGFBP2* and *HPRT1* were positively intercorrelated and negatively correlated with *IL32* (Fig. 2F). Altogether, our analysis showed that the up-regulation of specific proinflammatory cytokines, particularly *IL32*, is part of the molecular signature of metabolic-induced liver disease.

LARGE-SCALE ANALYSIS OF INFLAMMATORY GENE EXPRESSION AND POSSIBLE LINK WITH INSULIN RESISTANCE

To validate our microarray data, we used qRT-PCR to assess the relative expression of *IL32*, *CXCL9*, *CXCL10*, *UBD/FAT10*, *IGFBP2*, and *HPRT1* in the entire patient cohort (n = 122). Consistently, compared to control patients, *IL32* expression was increased 2.5-fold in the NAFLD group ($P < 0.001$) while a moderate but not statistically significant increase in *IL32* expression was observed in Ob_NL patients (1.6-fold; Fig. 3A). The expressions of *CXCL9* and *CXCL10* were also significantly increased in the Ob_NAFLD group (1.9-fold and 1.9-fold; $P < 0.001$ and $P < 0.05$, respectively), while the change in expression was significant only for *CXCL9* and not for *CXCL10* in the Ob_NL group (1.9-fold and 1.1-fold, respectively; Fig. 3C,D). The decreased expression of *IGFBP2* and *HPRT1* was confirmed in all patients with obesity with a more pronounced decrease in the NAFLD group (0.5-fold, $P < 0.001$ and 0.7-fold, $P < 0.01$, respectively; Fig. 3E,F). Finally, the expression of *UBD/FAT10* was significantly increased in patients with NAFLD, and this elevation was more pronounced in patients with NASH (3.3-fold, $P < 0.01$; Fig. 4B). Importantly, consistent with gene expression analysis, *IL32* protein level was increased in liver samples of patients with NAFLD (Fig. 5A).

IL32 GENE EXPRESSION LEVELS CORRELATE WITH NAS

In order to understand the biological relevance of the change in gene expression of the above-mentioned genes, we calculated NAS[14] and the fibrosis score (METAVIR) for all patients in the study. We found a significant increase in NAS in Ob_ST and Ob_NASH patients but not in Ob_NL patients (Fig. 4A); however, the fibrosis score was increased in Ob_NASH patients compared to Ob_ST and Ob_NL patients (Fig. 4B). NAS was significantly and positively correlated with waist circumference and BMI and not with the age of patients, whereas the fibrosis score was positively correlated with age but not with waist circumference and BMI (Fig. 4C). Furthermore, we analyzed the potential correlation of NAS and fibrosis score with classical biochemical parameters. NAS correlated significantly and positively with glucose, insulin, HOMA-IR, triglyceride levels, ALAT, ASAT, GGT, ferritin, and usCRP and negatively with HDL-cholesterol (Fig. 4D). Fibrosis score correlated with ferritin and NAS score (Fig. 4D), suggesting that hepatic steatosis contributes to fibrosis development. We then correlated the expression levels of the six genes analyzed by qRT-PCR with age and with clinical, histologic, and biological parameters, such as waist circumference, BMI, HOMA-IR, NAS, fibrosis score, and usCRP. Only *IGFBP2* transcript levels correlated positively with age. *IL32* correlated positively with waist circumference, BMI, HOMA-IR, NAS, and usCRP. *CXCL9, CXCL10,* and *UBD* positively correlated with HOMA-IR and NAS, suggesting that these genes contribute to hepatic steatosis and insulin resistance. *IGFBP2* and *HPRT1* negatively correlated with waist circumference, BMI, HOMA-IR, NAS, and usCRP. None of the genes correlated with the fibrosis score in our cohort of patients (Fig. 4E).

In line with our previous correlation on microarray data, *IL32* expression was positively correlated with *CXCL9*, *CXCL10,* and *UBD/FAT10* and negatively correlated with *HPRT1* (Fig. 4F). *CXCL9* was positively correlated with *CXCL10,* and *UBD* was correlated with both *CXCL9* and *CXCL10* (Fig. 4F). Altogether, our

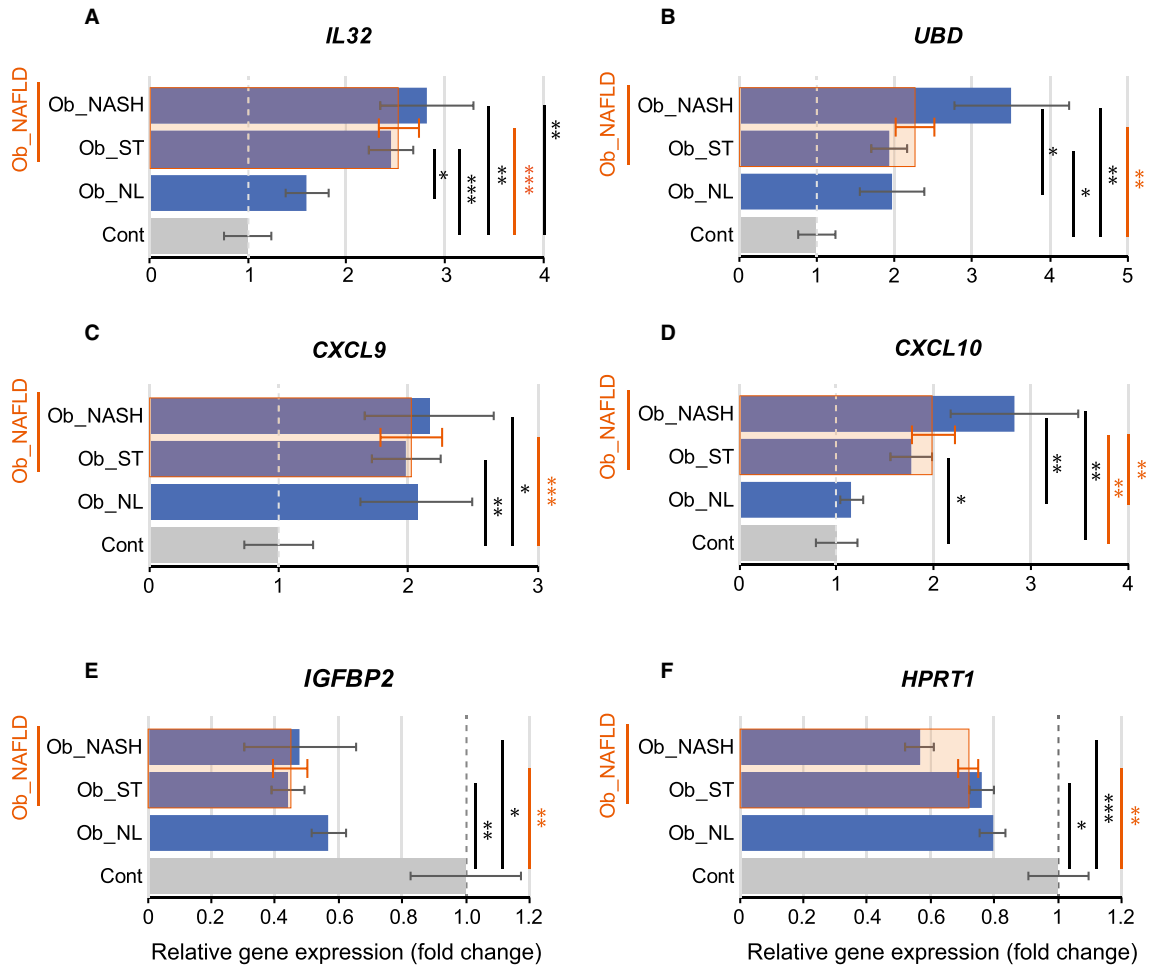

FIG. 3. qRT-PCR expression profile of the most intercorrelated genes found in microarray analysis. (A) *IL32*, (B) *UBD*, (C) *CXCL9*, and (D) *CXCL10* were significantly up-regulated in patients with NAFLD, whereas (E) *IGFBP2* and (F) *HPRT1* were significantly down-regulated compared to controls. Results are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

large-scale analysis confirmed the critical role of inflammatory cytokines, in particular *IL32*, in liver injury in NAFLD and particularly in patients with NASH. As such, these analyses further support our conclusion that increased *IL32*, *CXCL9*, *CXCL10*, and *UBD* levels and decreased *IGFBP2* and *HPRT1* expression represent a molecular signature of insulin resistance and liver injury in patients with obesity with NAFLD.

IL32 SUPPLEMENTATION PROMOTES INSULIN RESISTANCE IN PHHs

Following the observation that *IL32* protein levels were increased in patients with NASH (Fig. 5A), we aimed to address the direct impact of *IL32* on hepatocytes. We treated PHH cells with recombinant *IL32*. PHHs were treated with recombinant *IL32* (1 ng/mL) for 24 hours before a time-course exposition to insulin (100 nM) during 5, 10, 20, 30, and 60 minutes. The ratio of AKT phosphorylation (p-AKT^{T308})/AKT was determined in *IL32*-treated and untreated PHHs after insulin addition as an indicator of insulin signaling pathway activation. As expected, in the control condition (without *IL32* treatment), treatment of PHHs with insulin induced an increase in p-AKT/AKT ratio with time, with a maximum increase at 60 minutes (Fig. 5B). Pretreatment of PHHs with *IL32* yielded a robust reduction in p-AKT/AKT ratio 30 minutes after insulin addition, demonstrating that *IL32* contributes to insulin resistance and blunts insulin receptor signaling (Fig. 5B).

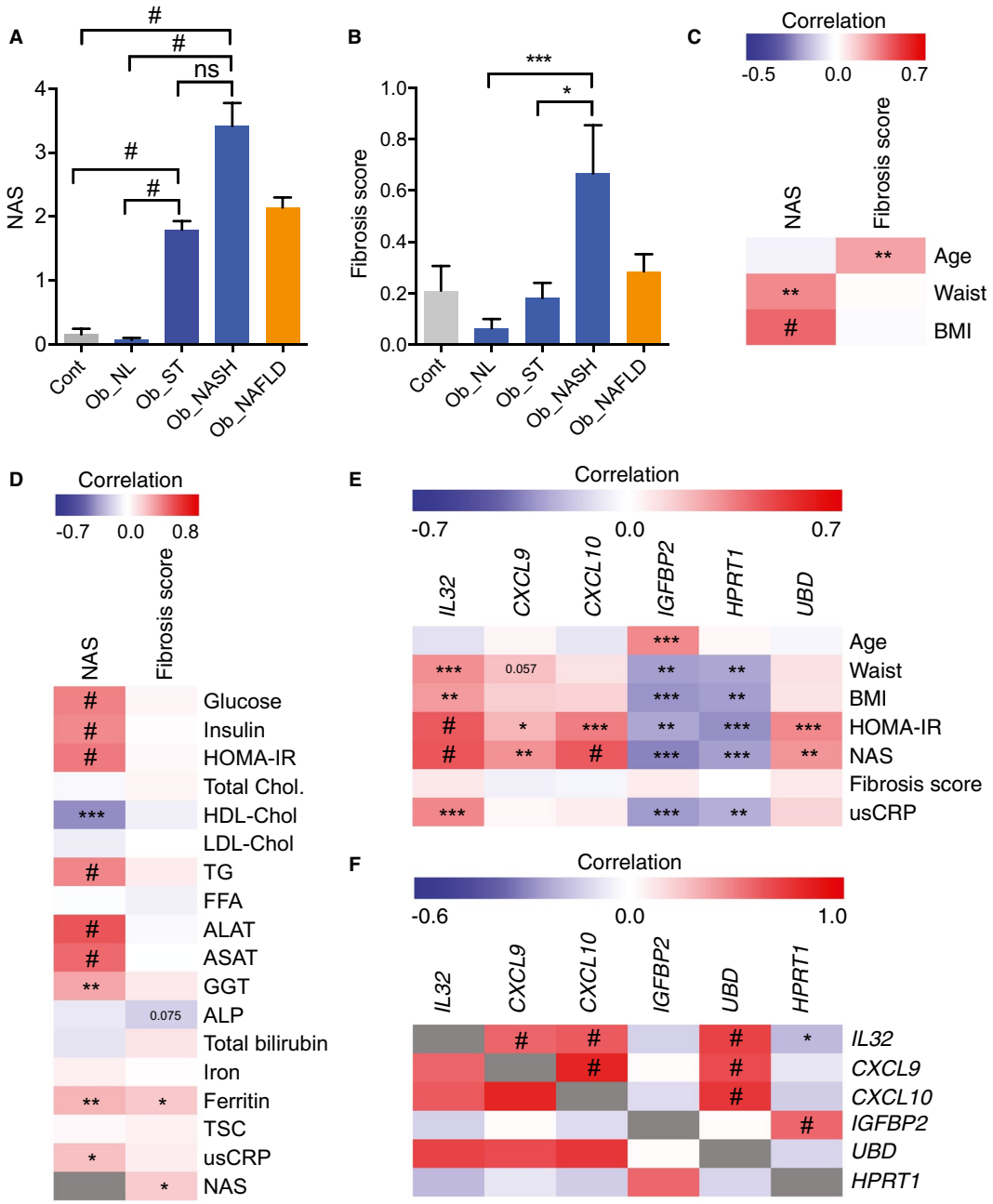

FIG. 4. NAFLD activity score, fibrosis score and correlation with biochemical parameters and NAFLD molecular signature. (A) NAS variation in control patients without obesity who received a cholecystectomy and in patients who received bariatric surgery in Ob_NL, Ob_ST, Ob_NASH, and Ob_NAFLD (Ob_ST + Ob_NASH) groups. (B) Fibrosis score variation in Ob_NL, Ob_ST, Ob_NASH, and Ob_NAFLD compared to controls. (C) Heatmap of the Spearman correlation of NAS and fibrosis score relative to age, waist circumference, and BMI in our cohort of patients. (D) Heatmap of the Spearman correlation of NAS and fibrosis score relative to biochemical parameters in our cohort of patients. (E) Heatmap of the Spearman correlation of the DE genes *IL32*, *CXCL9*, *CXCL10*, *IGFBP2*, *HPRT1*, and *UBD* in liver samples relative to age, waist circumference, BMI, HOMA-IR index, NAS, fibrosis score, and usCRP in our cohort of patients. (F) Heatmap of gene-to-gene correlation analysis of the qRT-PCR profile for the most intercorrelated genes found by microarray analysis. *IL32* correlated positively and significantly with *CXCL9*, *CXCL10*, and *UBD* and correlated negatively with *HPRT1*. The down-regulated genes *IGFBP2* and *HPRT1* were strongly and positively intercorrelated. The intensities of the red and blue color reflect the degree of positive and negative correlation, respectively. The gray color is the correlation of each parameter to itself ($r = 1$). Results are expressed as mean ± SEM; #$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Abbreviations: FFA, free fatty acid; TG, triglyceride; TSC, transferrin saturation capacity.

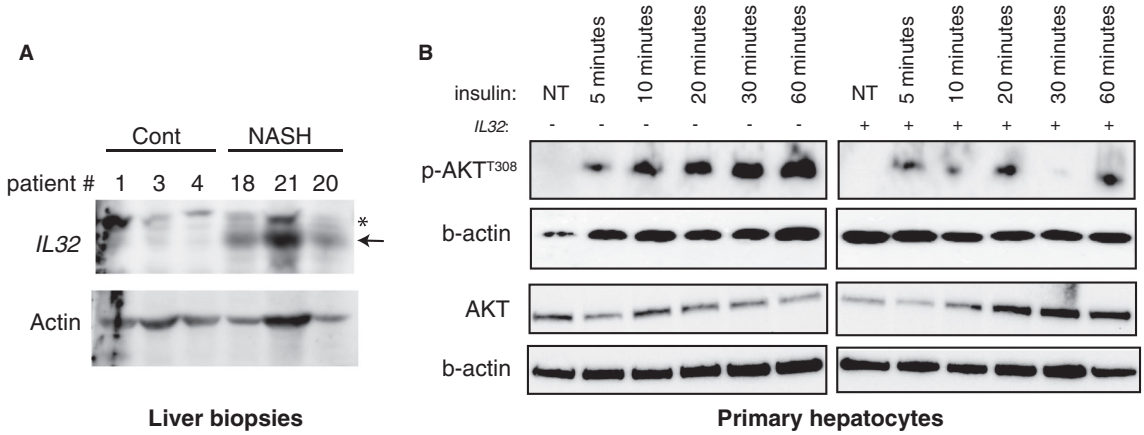

FIG. 5. *IL32* protein expression in liver biopsies from Cont and Ob_NASH patients and effect of recombinant *IL32* on insulin signaling in PHHs. (A) Immunoblots of protein extracts from patients with NASH and control liver biopsies showing an increase in *IL32* expression in NASH. (B) Recombinant *IL32* treatment induces decreased insulin signaling in protein lysates from PHHs as demonstrated by the robust decrease in AKT^{T308} phosphorylation after insulin stimulation. Abbreviations: Cont, controls; NT, nontreated; *, non specific.

Discussion

Here, we identified *IL32*, a proinflammatory cytokine known to play a role in host defense, inflammatory diseases, and cancer (reviewed in Joosten et al.[22]), as a novel factor clearly associated with NAFLD and insulin resistance in patients with obesity who underwent bariatric surgery. *IL32* expression was significantly increased in the liver of patients with NAFLD, and this elevation was more pronounced in patients with NASH. Similar to *IL32*, the expression of two other proinflammatory chemokines were enhanced, namely *CXCL9* and *CXCL10*. Furthermore, we observed a positive correlation between liver expression of *IL32* and *CXCL9* and *CXCL10*, supporting solid crosstalk among these cytokines in NASH pathophysiology. The expression of *CXCL9* and *CXCL10* has been reported to be increased in the liver and serum of patients infected with HCV[23] as was the expression of *IL32* in patients infected with HCV[24] and HBV.[25] Increased intrahepatic transcript level of *CXCL10* (also known as interferon γ [IFNγ]-induced protein 10) has been associated with intralobular inflammation, and *CXCL9* (also known as monokine induced by IFNγ) was shown to correlate with the grade of liver inflammation.[26] Furthermore, *CXCL10* has been recently shown to mediate macrophage-associated inflammation in a murine model of NASH.[27] These two cytokines are notoriously known to be activated by IFNγ,[28-30] a molecule with immune regulatory functions secreted by T helper 1 (Th1) inflammatory cells in the context of virus-induced and metabolic inflammation.[31-35]

How is *IL32* produced in the context of NAFLD and particularly in NASH? It is well established that myeloid and lymphoid cells infiltrate adipose tissue and liver as a result of excessive nutrient load.[34,36] One possibility is that *IL32* can be produced following the activation of Th1 cells. Indeed, the activation of these cells is known to produce IFNγ and TNFα in the context of obesity and oxidative stress-induced liver inflammation, which translates into the promotion of the classical M1 inflammatory-dominated response.[34,35] *IL32* was initially discovered as a protein that is produced following the activation of both natural killer cells and T cells.[37] Later, *IL32* was shown to stimulate the production of several proinflammatory cytokines, including TNFα, and to activate typical cytokine signaling pathways of NF-κB.[38] Reversely, TNFα and IFNγ were shown to activate *IL32* production in various pathologic contexts,[38-40] underpinning the presence of an autoperpetuating loop between *IL32* and TNFα to sustain inflammation. *UBD/FAT10* was also significantly correlated with *IL32*, *CXCL9*, and *CXCL10* in our cohort of patients. UBD is an

IFNγ- and TNFα-inducible ubiquitin-like protein with a putative role in immune response.[41,42] An IFN sequence response element is present on the promoter of the *UBD/FAT10* gene, which responds to synergistic stimulation by TNFα and IFNγ to induce Mallory-Denk body (MDB)-like aggresomes.[43] It was reported that *UBD* deletion prevents the formation of MDBs through the maintenance of the physiological 26S proteasome.[44] Overexpression of both *IL32* and *UBD* in the liver of patients with NAFLD and obesity strongly suggests that these two molecules participate in the immune response and hence contribute to alterations in cell proteostasis; this ultimately leads to liver injury in patients with NAFLD, especially in patients with NASH in whom *IL32* and *UBD* up-regulation was more pronounced.

Our analysis showed that recombinant *IL32* caused a significant decrease in insulin signaling in PHHs, underscoring a potential causal role in insulin resistance. Whether this effect is direct or indirect needs further clarification. Mechanistically, *IL32*-mediated insulin resistance could be due to the feed-forward loop involving *IL32* and TNFα. The latter has been shown to inhibit insulin signaling through c-Jun N-terminal kinase 1-mediated Ser^{307} phosphorylation of insulin receptor substrate 1 (IRS1).[45] The second possibility is that *IL32* could promote protein kinase C epsilon (PKCε) (encoded by the PKCε gene (*PRKCE*)) activation, which is known to suppress insulin signaling through reduced insulin-stimulated IRS2 tyrosine phosphorylation.[46] In line with this, our microarray data set showed a ~3-fold increase in *PRKCE* transcripts in the liver of patients with NAFLD compared to controls and a positive correlation between liver *IL32* expression and *PRKCE* (r = 0.607).

Most interestingly, the expression of *IL32*, *CXCL9*, *CXCL10,* and *UBD* correlated with NAS and the HOMA-IR index, while *IGFBP2* and *HPRT1* correlated negatively with these parameters. NAS was primarily designed by the NASH CRN as a system of scoring histopathologic features of NAFLD during clinical trials.[14] Although early studies have used threshold values of NAS ≥5 as a surrogate for the histologic diagnosis of NASH, a recent study showed that NAS <4 is not a synonym of a benign histology.[47] This was indeed our case as only 2 patients with NASH had NAS ≥5 while 10 patients with NASH had NAS ≤5. Consistent with results from the study by Brunt et al.[47] in which higher values of NAS were associated with increased levels of ALAT and ASAT, we observed a significant and positive correlation of NAS with hepatic aminotransferases, a hallmark for the severity of liver injury. The fact that the NAFLD molecular signature (increased *IL32*, *CXCL9*, *CXCL10*, and *UBD* and decreased *IGFBP2* and *HPRT1*) was correlated with NAS as well as aminotransferases (e.g., *IL32* correlates with ALAT and ASAT; P = 0.002 and P = 0.006, respectively) strongly suggests that these genes contribute to the pathogenesis of NASH. The reason why this molecular signature was not correlated with fibrosis lies in the possibility that the fibrosis was not extensive enough in our cohort of patients, or due to the lower number of patients with NASH (n = 12) compared to those with steatosis (n = 44) in whom few patients had a fibrosis score of 1, or simply that these genes are not associated with fibrosis in the context of metabolic-induced liver injury. Nevertheless, the expression of these genes remains to be verified in liver biopsies of patients with NAFLD in whom a Fibroscan or Fibrotest has revealed a high fibrosis score compared to patients with NAFLD with a low fibrosis score. Obviously, much effort is needed to understand how these genes interact and the signaling pathway network activated in order to achieve insulin resistance and liver injury.

Finally, our analysis reported a positive correlation between usCRP and BMI and between usCRP and waist circumference but did not reveal any correlation between usCRP with HOMA-IR. In line with this, whether usCRP is correlated to insulin resistance remains controversial in the literature. Indeed, Hossain et al.[48] reported a positive correlation between highly sensitive CRP (hsCRP) and HOMA-IR (P = 0.03) in 140 patients with prediabetes, yet these patients were not obese (BMI, 26.04 ± 4.51 kg/m^2). Another study showed that hsCRP correlated with BMI and waist to hip ratio but not with HOMA-IR in West Africans while it was correlated with all three parameters in an African-American population.[49] Finally, Namburi et al.[50] showed that hsCRP was correlated with BMI and insulin but not with HOMA-IR in children and adolescents with obesity. Although, we observed a positive correlation between liver *IL32* expression and usCRP, it seems that these two proteins may share common inflammatory pathways but not a pathway interfering with the insulin signaling

pathway. Altogether, we believe that although usCRP is associated with obesity and NAFLD, it is not a reliable marker of insulin resistance in patients with obesity who are prediabetic.

In summary, our large-scale analysis identifies new protagonists in the pathogenesis of NAFLD. Mainly, we showed a robust increase in liver expression of proinflammatory *IL32* that was significantly correlated with NAS, aminotransferases, and insulin resistance in patients with obesity who underwent bariatric surgery. Moreover, *IL32* supplementation on PHHs impairs insulin signaling, supporting a causal effect of *IL32* in the pathogenesis of liver insulin resistance. Nevertheless, molecular mechanisms governing the interplay between these proteins to promote liver injury, insulin resistance, and hepatic liver disease progression remain to be dissected. Given that *IL32* has been shown to be involved in hepatitis-induced liver damage, it is plausible that activation of the TNFα–*IL32*–IFNγ–UBD axis may be a general mechanism leading to liver injury. We propose that inhibition of *IL32*, for example, using neutralizing antibodies in patients with NAFLD, may prove efficient in the treatment of metabolic liver disease.

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