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Altered Hepatic Gene Expression in Nonalcoholic Fatty Liver Disease Is Associated With Lower Hepatic n-3 and n-6 Polyunsaturated Fatty Acids

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In nonalcoholic fatty liver disease, hepatic gene expression and fatty acid (FA) composition have been reported independently, but a comprehensive gene expression profiling in relation to FA composition is lacking. The aim was to assess this relationship. In a cross-sectional study, hepatic gene expression (Illumina Microarray) was first compared among 20 patients with simple steatosis (SS), 19 with nonalcoholic steatohepatitis (NASH), and 24 healthy controls. The FA composition in hepatic total lipids was compared between SS and NASH, and associations between gene expression and FAs were examined. Gene expression differed mainly between healthy controls and patients (SS and NASH), including genes related to unsaturated FA metabolism. Twenty-two genes were differentially expressed between NASH and SS; most of them correlated with disease severity and related more to cancer progression than to lipid metabolism. Biologically active long-chain polyunsaturated FAs (PUFAs; eicosapentaenoic acid + docosahexaenoic acid, arachidonic acid) in hepatic total lipids were lower in NASH than in SS. This may be related to overexpression of FADS1, FADS2, and PNPLA3. The degree and direction of correlations between PUFAs and gene expression were different among SS and NASH, which may suggest that low PUFA content in NASH modulates gene expression in a different way compared with SS or, alternatively, that gene expression influences PUFA content differently depending on disease severity (SS versus NASH). *Conclusion:* Well-defined subjects with either healthy liver, SS, or NASH showed distinct hepatic gene expression profiles including genes involved in unsaturated FA metabolism. In patients with NASH, hepatic PUFAs were lower and associations with gene expression were different compared to SS. (HEPATOLOGY 2015;61:1565-1578)

Nonalcoholic fatty liver disease (NAFLD) is associated with excessive energy and fat intake, insulin resistance, and/or altered lipid metabolism.[1] About 10%-15% of patients have nonalcoholic steatohepatitis (NASH), which can progress to cirrhosis and hepatocellular carcinoma (HCC).[2,3] Altered hepatic gene expression has been reported in NAFLD, including genes involved in lipid metabolism, apoptosis, liver generation, fibrosis, alcohol and drug metabolism, and detoxification.[4-8] In addition, gene expression differs between NAFLD patients with and those without fibrosis[9] and between simple steatosis (SS) and NASH, where main differences occurred for transcription factors, oxidative stress response,

Abbreviations: AA, arachidonic acid; ANOVA, analysis of variance; BMI, body mass index; DEG, differentially expressed gene; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; HC, healthy control; HCC, hepatocellular carcinoma; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PUFA, polyunsaturated fatty acid; SS, simple steatosis.

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fibrosis, apoptosis, insulin signaling, and metabolism-related genes.[5,6,10,11]

Hepatic gene expression can be altered in response to the type of fatty acids (FAs) consumed, especially the relative amount of long-chain n-6 and n-3 polyunsaturated FAs (PUFAs).[12,13] These PUFAs can modulate lipogenesis, insulin resistance, and inflammation, mainly through interaction with transcription factors.[12] Reduced n-3 and n-6 PUFAs in hepatic total lipids and triacylglycerols were reported in patients with NAFLD, especially those with NASH, compared to controls,[14-16] consistent with low dietary PUFA intakes.[17,18] Improvement of hepatic steatosis with n-3 PUFA supplementation has also been described,[19] suggesting that low n-3 PUFA intake may contribute to NAFLD pathogenesis. In addition, dysregulation of gene expression and oxidative stress observed in NALFD could contribute to PUFA depletion in the liver.[15,20] Although associations between hepatic total lipid PUFAs and the expression of single genes have been described,[20,21] there are no studies broadly examining the association between FA composition and global gene expression, in particular related to lipid metabolism, oxidative stress, inflammation, and fibrosis in these patients. Thus, the aim of the present study was to (1) compare hepatic gene expression among well-characterized groups of participants with healthy liver, SS, or NASH and (2) examine in SS and NASH the relationship between long-chain PUFAs in hepatic total lipids and expression levels of genes that might contribute to the disease phenotype. Oxidative stress and diet were also assessed.

Patients and Methods

Between March 2007 and November 2011, patients and healthy controls (HCs) were recruited from the liver clinic or the Multiorgan Transplant Program, respectively, at the University Health Network, Toronto, Canada. The study was approved by the local Research Ethics Board, was registered (NCT02148471, www.clinicaltrials.gov), and followed the guidelines of the 1975 Declaration of Helsinki and its revisions. All participants provided informed written consent. No organs were obtained from executed prisoners or other institutionalized persons.

Patients were approached when a liver biopsy for suspected NAFLD was scheduled; HCs were approached during their assessments for a live donor liver transplant. These participants underwent transient elastography, computed tomography, and/or magnetic resonance imaging. Inclusion criteria were as follows: male or female; ≥18 years; for HCs, presence of a normal liver (no steatosis or cirrhosis) on imaging and/or histology; and for NAFLD patients, a diagnostic liver biopsy. Exclusion criteria were as follows: alcohol consumption >20 g/day; any other liver disease; use of medications that may cause steatohepatitis, ursodeoxycholic acid or any experimental drug, antioxidants, or PUFA supplements in the 6 months prior to entry; pregnancy or breast-feeding; for NAFLD patients, anticipated need for liver transplantation within 1 year, complications of liver disease, or any reasons contraindicating a biopsy; and, for HCs, any reason excluding them from liver donation. Liver tissue was collected during percutaneous needle biopsy (NAFLD) or as a wedge biopsy during hepatectomy (HC) and divided in three parts to be stored in 10% formalin for histology; in RNA*later* (Qiagen, Hilden, Germany) for gene expression analysis; or at -80 °C for FA analysis. Participants provided one fasting blood sample and completed a 7-day food record and activity log. Anthropometric and clinical data were collected.

Liver Histology. Samples were stained with hematoxylin and eosin for morphologic evaluation and Prussian blue to rule out iron loading. A single pathologist reviewed the slides (S.E.F.). Either SS or NASH was diagnosed according to Brunt et al.,[22] and the NAFLD activity score was assessed.[23]

Hepatic Gene Expression. Total RNA was extracted from liver biopsies using the *mir*Vana miRNA Isolation kit (Life Technologies Corp., Carlsbad, CA); RNA concentration, purity, and quality were assessed spectrophotometrically and with an Agilent BioAnalyzer (Santa Clara, CA) (Supporting Information). Two hundred nanograms of RNA were used for analysis with the Whole Genome Gene DASL HT Assay (Illumina, Inc., San Diego, CA) and the Illumina Human HT-12 V4 BeadChip, which covers 29,285 probes of the >47,000 annotated transcripts from the National Center for Biotechnology Information Reference Sequence (RefSeq)

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database (Release 38, November 2009) (Supporting Information).[24] Data were checked for overall quality using R (v2.15.1) with the lumi Bioconductor package.[25] Nine outliers (four NASH, five SS), which fell below the threshold for one or more of the quality metrics calculated with this package, were excluded from further analysis. The data were imported in GeneSpring v12.5 (Agilent) and normalized using a quantile normalization followed by a "per probe" median-centered normalization. All analyses and visualizations were performed on log2-transformed data. Data were first filtered to remove the probes that showed no signal, and only 22,461 probes that were in the upper eightieth percentile of the distribution of intensities in at least 80% of the samples in any of the three groups were retained.

Differentially expressed genes (DEGs) were identified by one-way analysis of variance (ANOVA) with a Benjamini-Hochberg false-discovery rate $q < 0.05$ and Tukey's honestly significant difference *post hoc* test. Expression profiles were clustered for visualization using a Pearson centered correlation as a distance metric with average linkage rules in the tree-building algorithm. Differentially expressed genes were filtered for at least two-fold up- or down-regulation. A functional annotation and gene ontology enrichment analysis was performed using the DAVID Bioinformatics Resources 6.7.[26,27] In addition, gene lists were created for NAFLD-relevant processes: fibrosis, chronic inflammatory response, cellular response to oxidative stress, lipogenesis, beta-oxidation, and long-chain and unsaturated FA metabolism (Supporting Table S1). Due to limited liver tissue, confirmation of the results by real-time polymerase chain reaction was not possible. Several studies have previously shown consistency between differential gene expression measured by microarray and polymerase chain reaction, and confirmation is no longer considered essential.[28,29]

Hepatic Oxidative Stress and Fatty Acids. Liver tissue was homogenized in phosphate-buffered saline with butylated hydroxytoluene as antioxidant. Commercial kits were used to assess antioxidant power (TA02; Oxford Biomedical Research, Oxford, MI) and lipid peroxidation (Bioxytech LPO-586; Oxis International, Portland, OR).

Total lipids from 250 μL of the same homogenate were extracted in chloroform/methanol[30] and analyzed using gas chromatography (Supporting Information).[14,30,31] The relative amount of single FA was calculated as a percent of total lipids. Due to the small amount of tissue available for NAFLD, a separation into lipid subfraction was not possible. As patients with NAFLD have higher tri- and diacylglycerols and lower phosphatidylcholine than HCs, and these lipid subfractions can have different FA composition,[15,16] we compared hepatic FA only between NASH and SS patients, who are both characterized by triacylglycerol accumulation in the liver.

Blood Biochemistry and Nutritional Assessment. Plasma and serum were collected after an 8-hour fast. Liver function tests, lipid profile, glucose, insulin, hemoglobin A1c, and C-peptide were measured using standard procedures (Supporting Information). The homeostasis model of assessment for insulin resistance was calculated.[32] Erythrocytes were separated from ethylene diamine tetraacetic acid plasma; lipids were extracted and FA was measured by gas chromatography (Supporting Information). Body mass index (BMI = weight [kg]/height [m]^2) and waist circumference were measured. Diet was assessed by 7-day food records, using Food Portion Visual (Nutrition Consulting Enterprises, Framingham, MA) for portion size estimation and Food Processor SQL (ESHA Research, Salem, OR) for nutrient analysis. Participants recorded duration and intensity of physical activity for one week.[33]

Statistical Analysis. Gene expression analysis is described above. For sensitivity analysis of BMI influence on gene expression, a nested ANOVA where disease groups were compared within BMI categories (BMI <25 kg/m^2, normal weight; 25 $\leq$ BMI < 30 kg/m^2, overweight; BMI $\geq$30 kg/m^2, obese) was used. Other results are presented as mean $\pm$ SD, median (interquartile range), or percent of cases. Continuous variables were compared among groups by ANOVA with Tukey's *post hoc* test, *t* test, or Wilcoxon's test, depending on the distribution (Shapiro-Wilks test). The Fisher's exact test was applied for categorical variables. A principal component analysis was conducted to reduce the dimensionality of the data set. It was performed separately for five gene sets: fibrosis, chronic inflammation/response to oxidative stress, lipogenesis/beta-oxidation, long-chain/unsaturated FA metabolism, and genes that differed between NASH and SS. The relations between genes, FAs, and clinical parameters were examined by Spearman's correlation. In order to test the significance of differences in relationships of genes and FAs between SS and NASH, analysis of covariance was employed (SAS Enterprise Guide 4.3; SAS Institute, Inc., Cary, NC). $P < 0.05$ was considered significant.

Results

Clinical Characteristics and Oxidative Stress. Data from 63 participants were included in the study (20 SS, 19 NASH, 24 HCs) (Supporting Fig. S1). A subgroup of patients had hepatic (n = 28) and erythrocyte (n =

Table 1. Demography, Clinical Data, and Oxidative Stress Measurements

	n	HC	n	SS	n	NASH
Age (years)	24	37.2 ± 10.8	20	44.7 ± 9.1	19	43.5 ± 12.7
Male sex, % (n)	24	45.9% (11)	20	70.0% (14)	19	47.4% (9)
Ethnicity, % (n)						
Caucasian	20	85.0% (17)	13	53.9% (7)	16	68.8% (11)
Asian		10.0% (2)		30.8% (4)		25.0% (4)
Other		5.0% (1)		15.4% (2)		6.3% (1)
BMI (kg/m^2)	24	26.1 (5.6)	19	28.6 (4.9)[a]	18	31.9 (3.8)[A,b]
Waist	22	88.95 ± 10.96	19	99.73 ± 9.97	16	104 ± 10.4[a]
Smoking, % (n)	24	0.0% (0)	20	10.0% (2)	19	21.1% (4)[a]
Cigarettes/day	24	0.0 (0.0)	20	0.0 (0.0)	19	0.0 (0.0)
Alcohol	24	0.00 (2.80)	20	0.00 (1.93)	19	1.00 (4.00)
Steatosis (% of hepatocytes)	17	0.0 (0.0)	20	35.0 (40.0)[A]	19	40.0 (45.0)[A]
Steatosis grading, % of patients (n), 0/1/2/3	17	100.0/0/0/0% (17/0/0/0)	20	0/50.0/35.0/15.0% (0/10/7/3)	19	0/31.6/36.8/31.6% (0/6/7/6)
Fibrosis stage, % of patients (n), 0/1/2/3/4	16	62.5/37.5/0/0/0% (10/6/0/0/0)	20	85/15/0/0/0% (17/3/0/0/0)	19	21/26/11/21/21% (4/5/2/4/4)
NAFLD activity score (0-8)	7	0.00 (0.00)	19	2.00 (1.00)[A]	19	5.00 (2.00)[A,B]
Liver antioxidant power (μmol uric acid equivalents/g tissue)	24	36.8 (25.8)	20	32.3 (31.1)	18	30.0 (31.9)
Liver lipid peroxidation (μmol malondialdehyde/g tissue)	24	0.08 (0.20)	20	0.20 (0.43)	17	0.19 (1.18)

Values given are mean ± SD, median (interquartile range), or percent of valid cases.

Superscript letters show statistically significant difference from healthy controls (a, A) and from SS (b, B). $^{a,b}P < 0.05$, $^{A,B}P < 0.01$. We used ANOVA with Tukey's *post hoc* test for normally distributed data, Wilcoxon test for data with skewed distribution, and Fisher's exact test for categorical variables.

Steatosis grading according to Brunt et al.[22]: 0, <5% of hepatocytes involved; 1, 5%-33%; 2, 33%-66%; 3, >66%. The NAFLD activity score is according to Kleiner et al.[23]

16) FAs measured. The groups did not differ in age, sex, race/ethnicity, or alcohol intake; but the NASH group had more smokers than did the HCs (Table 1). Patients, especially those with NASH, had higher BMI and waist circumference than HCs. Liver histology showed increasing disease severity from SS to NASH (Table 1). Hepatic oxidative stress measures did not differ among the groups (Table 1). Liver transaminases, fasting insulin, C-peptide, triacylglycerols, and the homeostasis model of assessment for insulin resistance were higher in patients compared with HCs and, except for triacylglycerols, also higher in NASH than in SS (Supporting Table S2). Energy intake was higher in HCs (2780 ± 1169 kcal/day) than in SS (1673 ± 724 kcal/day) and NASH (1892 ± 656 kcal/day) patients ($P < 0.05$). The macronutrient distribution (percent of energy from protein, carbohydrates, fat, saturated, monounsaturated fat, PUFAs) and physical activity were not different (Supporting Table S3).

Hepatic Gene Expression Differed Mainly Between Patients and HCs, With 22 Genes Clearly Distinguishing NASH From SS. A total of 10,350 probes varied significantly among HCs and patients with SS or NASH, with a reasonable, albeit not perfect, separation of the samples into groups (Fig. 1); 822 probes corresponding to 732 unique genes passed the two-fold change filtering. Most differences were found between HCs and NASH (556 genes), while 530 genes were differentially expressed between HCs and SS; most genes were down-regulated compared with HCs (NASH 56%, SS 66%). Genes shared by these two comparisons (NASH versus HC/SS versus HC) varied in the same direction but with a different fold (Supporting Table S4a-c).

Twenty-two genes differed between NASH and SS (Table 2), including genes involved in FA desaturation (*ACOT1*), ether lipid metabolism (*ENPP2*), inflammatory pathways, detoxification, growth and development processes, and apoptosis (Supporting Table S5). The highest fold-changes were seen for *AKR1B10* (positive) and *HLA-DRB5* (negative). The functional annotation analysis showed that in both SS and NASH the down-regulated genes compared to HCs were enriched for terms related to cytokine signaling, apoptosis, response to exogenous or endogenous stimuli, cell migration, and angiogenesis (Supporting Tables S6 and S7). No enrichments were found for up-regulated genes or for DEGs between NASH and SS. As several processes related to NAFLD are not considered as such in the existing databases, we constructed focused gene sets of specific interest, which were used for more detailed analysis in relation to FA composition. Most genes in the fibrosis, inflammatory response, and oxidative stress sets were underrepresented in SS and NASH versus HC (Table 3). Thirty-three DEGs belonged to the gene set for lipogenesis, one to beta-oxidation, and seven to long-chain and unsaturated FA metabolism.

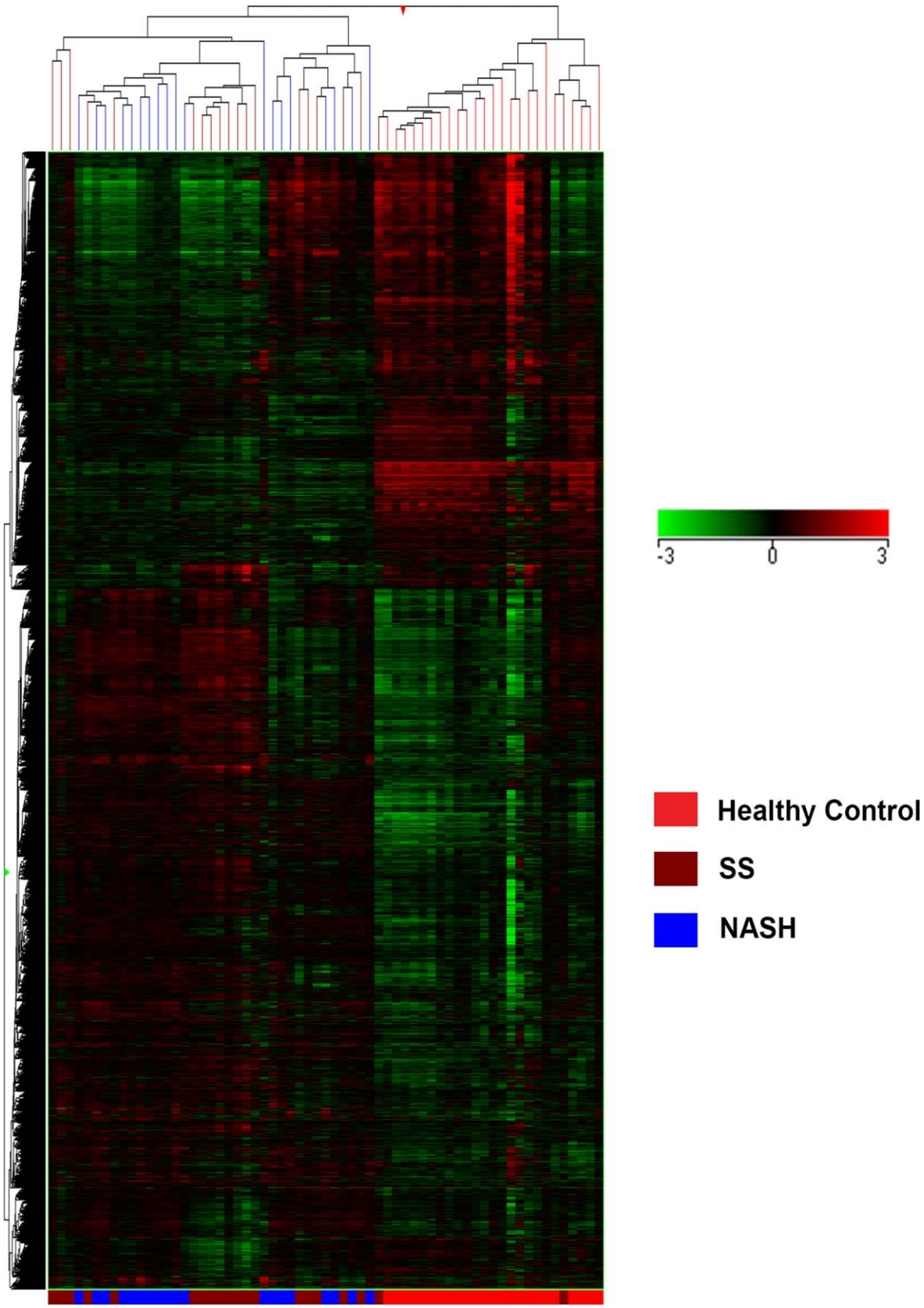

Fig. 1. Heat map illustrating unsupervised hierarchical clustering of hepatic gene expression in patients with SS or NASH and HCs. Hepatic gene expression was measured using the Whole Genome Gene DASL HT Assay and the Illumina Human HT-12 V4 BeadChip. An ANOVA with Tukey's *post hoc* test showed 10,350 significantly varying probes among NASH, SS, and HCs. The probes were clustered for visualization. Rows represent genes, and columns represent hybridized samples. The intensity of the color symbolizes the ratio between each value and the average expression of each gene across all samples. Red indicates higher and green indicates lower mRNA abundance in a sample. The color-coded legend underneath shows the groups, whereby red is HC, brown is SS, and blue is NASH. The heat map shows reasonable but not perfect separation of the three groups based on hepatic gene expression profile.

Table 2. Differentially Expressed Genes Between Patients With Simple Steatosis and Nonalcoholic Steatohepatitis

Entrez Gene ID	Gene Symbol	Fold-change			q Value	Definition
		NASH vs. SS	NASH vs. HC	SS vs. HC		
57016	*AKR1B10*	6.20	9.95	–	4.84E-11	Aldo-keto reductase family 1, member B10 (aldose reductase)
1917	*EEF1A2*	3.40	4.88	–	8.49E-08	Eukaryotic translation elongation factor 1 alpha 2
641371	*ACOT1*	2.99	–	−2.73	3.80E-02	Acyl-coenzyme A thioesterase 1
343071	*PRAMEF10*	2.57	–	–	7.01E-03	PRAME family member 10
80201	*HKDC1*	2.49	–	–	1.56E-03	Hexokinase domain containing 1
11075	*STMN2*	2.45	2.05	–	1.74E-03	Stathmin-like 2
2562	*GABRB3*	2.43	–	–	3.90E-04	Gamma-aminobutyric acid A receptor, beta 3, transcript variant 1
6696	*SPP1*	2.36	–	–	5.51E-04	Secreted phosphoprotein 1, transcript variant 1
10537	*UBD*	2.26	2.23	–	2.15E-05	Ubiquitin D
23089	*PEG10*	2.20	4.43	2.01	7.75E-11	Paternally expressed 10, transcript variant 1
2326	*FMO1*	2.19	5.07	2.31	3.29E-13	Flavin containing monooxygenase 1
5168	*ENPP2*	2.10	–	–	6.87E-05	Ectonucleotide pyrophosphatase/phosphodiesterase 2, transcript variant 2
51473	*DCDC2*	2.07	2.08	–	2.35E-03	Doublecortin domain containing 2
2191	*FAP*	2.04	–	–	1.16E-03	Fibroblast activation protein alpha
302	*ANXA2*	2.02	–	−2.19	2.79E-05	Annexin A2, transcript variant 2
677818	*SNORA36B*	−2.06	−2.22	–	2.60E-02	Small nucleolar RNA, H/ACA box 36B, small nucleolar RNA
4489	*MT1A*	−2.18	−5.06	−2.32	4.55E-08	Metallothionein 1A
692225	*SNORD94*	−2.25	–	2.06	1.20E-02	Small nucleolar RNA, C/D box 94, small nucleolar RNA
4099	*MAG*	−2.46	–	–	2.27E-03	Myelin-associated glycoprotein, transcript variant 2
4490	*MT1B*	−2.52	−3.26	–	1.24E-03	Metallothionein 1B
64150	*DIO3OS*	−2.53	−3.28	–	1.82E-06	DIO3 opposite strand (non-protein coding), noncoding RNA
3127	*HLA-DRB5*	−4.43	–	–	3.59E-02	Major histocompatibility complex, class II, DR beta 5

Gene expression levels are given as fold-changes between two groups. – not significant in two-group comparison (*post hoc* $P \geq 0.05$) and/or less than two-fold up- or down-regulation. The *q* value is the Benjamini-Hochberg corrected *P* value.

Except for *ACOT1* none of these genes differed between SS and NASH.

A sensitivity analysis was performed for the DEGs in these five sets as well as for those that differed between SS and NASH (in total 90 probes representing 78 unique genes). The BMI group was a significant predictor (ANOVA *F* test <0.05) only for five genes (*DIO3OS*, *SPP1*, *PLAUR*, *GABRB3*, *SERPINE1*) (Supporting Table S8). After including BMI in the model, disease state was still a significant predictor for all genes, except for *GABRB3*, and the direction of the fold-changes observed within BMI groups was the same as in the original analysis. In line with the ANOVA results, principal component analysis showed a clear separation between HCs and patients for genes related to fibrosis, inflammatory response/oxidative stress, lipogenesis/beta-oxidation, and long-chain/unsaturated FA metabolism, whereas for the 22 DEGs between NASH and SS, the SS patients grouped with the HCs (Fig. 2).

The Extent of Gene Deregulation Between NASH and SS Is Associated With Disease Severity. In the NAFLD patients (SS+NASH combined), most DEGs between NASH and SS were strongly correlated with NAFLD activity score and liver enzymes and, to a lesser extent, with the homeostasis model of assessment for insulin resistance and BMI (Supporting Table S9).

Correlations of PUFAs With Gene Expression Show Different Patterns in SS and NASH. Hepatic total FA profiles were compared only between SS and NASH. Hepatic n-3 and n-6 PUFAs, especially the biologically active long-chain PUFAs, i.e., eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) and arachidonic acid (AA), and the AA precursor dihomo-γ-linolenic acid, were lower in NASH compared to SS (Table 4). This did not correspond to the erythrocyte FA profiles (Table 4) or the dietary PUFA intakes, which were similar among groups (Supporting Table S3). Correlations between the expression levels of all DEGs and PUFAs (Supporting Table S10) showed different patterns depending on the group examined (SS, NASH, or both combined = NAFLD) (Fig. 3). Functional annotation analysis of genes correlated with EPA+DHA showed enrichment of the peroxisome proliferator-activated receptor signaling pathway (five genes, 21.7-fold enrichment, false discovery rate 0.044) in NAFLD and of cytokine-cytokine receptor interaction (eight genes, 11.1-fold enrichment, false discovery rate 8.77E-04) in NASH. The same analysis for AA showed no significant results. Correlated genes were then filtered for the gene sets described above (Table 5). The results differed between SS, NASH, and both combined (NAFLD). When differences between SS and NASH were tested in analysis of covariance models the interaction term for gene

Table 3. Genes Related to Biological Processes Relevant for NAFLD That Are Differentially Expressed Among Healthy Controls and Patients With Simple Steatosis or Nonalcoholic Steatohepatitis

		Fold-change			
Entrez Gene ID	Symbol	NASH vs. HC	SS vs. HC	*q* Value	Definition
Fibrosis					
3569	*IL6*	−8.96	−7.25	7.64E-11	Interleukin-6 (interferon-beta2)
4609	*MYC*	−7.43	−8.14	4.96E-17	v-myc myelocytomatosis viral oncogene homolog (avian)
7057	*THBS1*	−3.50	−4.46	1.10E-15	Thrombospondin 1
7043	*TGFB3*	−3.42	−3.91	1.35E-08	Transforming growth factor-beta3
6347	*CCL2*	−3.23	−3.57	6.12E-13	Chemokine (C-C motif) ligand 2
3553	*IL1B*	−3.17	−3.57	4.71E-09	Interleukin-1beta
5054	*SERPINE1*	−2.86	−3.28	6.16E-09	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
3586	*IL10*	−2.29	–	2.46E-08	Interleukin-10
3725	*JUN*	−2.28	−3.04	1.61E-13	Jun oncogene
6348	*CCL3*	−2.26	−2.00	1.77E-04	Chemokine (C-C motif) ligand 3
5265	*SERPINA1*	−2.05	−2.40	3.50E-07	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1, transcript variant 2
7852	*CXCR4*	−2.00	−2.59	1.82E-06	Chemokine (C-X-C motif) receptor 4, transcript variant 2
4318	*MMP9*	–	−2.31	1.21E-04	Matrix metallopeptidase 9 (gelatinase B, 92-kDa gelatinase, 92-kDa type IV collagenase)
Chronic inflammatory response					
7057	*THBS1*	−3.50	−4.46	1.10E-15	Thrombospondin 1
3557	*IL1RN*	−3.58	−4.44	4.53E-09	Interleukin-1 receptor antagonist, transcript variant 1
3557	*IL1RN*	−3.00	−2.90	6.30E-08	Interleukin-1 receptor antagonist, transcript variant 4
6279	*S100A8*	−2.98	−2.26	5.99E-06	S100 calcium binding protein A8
3586	*IL10*	−2.29	–	2.46E-08	Interleukin-10
Cellular response to oxidative stress					
3569	*IL6*	−8.96	−7.25	7.64E-11	interleukin-6 (interferon-beta2)
2353	*FOS*	−7.39	−6.82	4.09E-14	v-fos FBJ murine osteosarcoma viral oncogene homolog
9314	*KLF4*	−3.66	−3.24	3.77E-09	Kruppel-like factor 4 (gut)
4929	*NR4A2*	−3.35	−3.06	1.99E-09	Nuclear receptor subfamily 4, group A, member 2, transcript variant 1
2308	*FOXO1*	−2.18	−2.02	6.46E-10	Forkhead box O1
983	*CDC2*	2.09	–	1.30E-10	Cell division cycle 2, G_1 to S and G_2 to M, transcript variant 1
3934	*LCN2*	–	−2.13	2.15E-02	Lipocalin 2
5311	*PKD2*	–	2.07	2.46E-05	Polycystic kidney disease 2 (autosomal dominant)
Lipogenesis					
150094	*SIK1*	−5.29	−5.54	5.06E-12	Salt-inducible kinase 1
5743	*PTGS2*	−4.50	−3.79	2.54E-11	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
3491	*CYR61*	−3.54	−4.69	2.23E-14	Cysteine-rich, angiogenic inducer, 61
84803	*AGPAT9*	−3.17	−2.24	5.95E-17	1-Acylglycerol-3-phosphate O-acyltransferase 9
552	*AVPR1A*	−3.09	−3.32	6.75E-07	Arginine vasopressin receptor 1A
6279	*S100A8*	−2.98	−2.26	5.99E-06	S100 calcium binding protein A8
5329	*PLAUR*	−2.97	−3.72	9.19E-10	Plasminogen activator, urokinase receptor, transcript variant 2
55500	*ETNK1*	−2.58	−2.18	1.78E-10	Ethanolamine kinase 1, transcript variant 2
5329	*PLAUR*	−2.44	−2.34	1.46E-12	Plasminogen activator, urokinase receptor, transcript variant 1
9023	*CH25H*	−2.05	−2.14	9.81E-05	Cholesterol 25-hydroxylase
220	*ALDH1A3*	−2.03	−2.06	1.47E-04	Aldehyde dehydrogenase 1 family, member A3
23600	*AMACR*	2.00	–	3.26E-06	Alpha-methylacyl-coenzyme A racemase, transcript variant 1
2678	*GGT1*	2.01	–	1.67E-06	Gamma-glutamyltransferase 1, transcript variant 1
116285	*ACSM1*	2.03	–	8.62E-04	Acyl-coenzyme A synthetase medium-chain family member 1
9110	*MTMR4*	2.05	2.16	4.60E-09	Myotubularin related protein 4
3357	*HTR2B*	2.07	–	1.08E-05	5-Hydroxytryptamine (serotonin) receptor 2B
84962	*JUB*	2.10	–	2.57E-06	Jub, ajuba homolog (*Xenopus laevis*), transcript variant 1
23600	*AMACR*	2.13	–	1.33E-06	Alpha-methylacyl-coenzyme A racemase, transcript variant 2
3172	*HNF4A*	2.20	–	8.00E-05	Hepatocyte nuclear factor 4, alpha, transcript variant 3
55304	*SPTLC3*	2.37	–	3.65E-08	Serine palmitoyltransferase, long chain base subunit 3
80339	*PNPLA3*	2.38	–	2.27E-06	Patatin-like phospholipase domain containing 3
672	*BRCA1*	2.39	2.43	1.50E-07	Breast cancer 1, early onset, transcript variant BRCA1-delta14-17
672	*BRCA1*	2.45	2.46	4.41E-07	Breast cancer 1, early onset, transcript variant BRCA1-delta11b
56848	*SPHK2*	2.46	2.18	1.00E-05	Sphingosine kinase 2
3992	*FADS1*	2.49	2.09	9.61E-06	Fatty acid desaturase 1
80168	*MOGAT2*	2.57	2.65	4.14E-10	Monoacylglycerol O-acyltransferase 2
84962	*JUB*	2.72	–	1.21E-08	Jub, ajuba homolog (*Xenopus laevis*), transcript variant 2
9415	*FADS2*	2.81	–	1.07E-04	Fatty acid desaturase 2
57678	*GPAM*	2.82	2.40	3.46E-07	Glycerol-3-phosphate acyltransferase, mitochondrial, nuclear gene encoding mitochondrial protein

TABLE 3. *Continued*

Entrez Gene ID	Symbol	Fold-change		q Value	Definition
		NASH vs. HC	SS vs. HC		
2645	*GCK*	4.35	4.57	1.83E-06	Glucokinase (hexokinase 4), transcript variant 3
1581	*CYP7A1*	8.34	6.53	3.59E-12	Cytochrome P450, family 7, subfamily A, polypeptide 1
5359	*PLSCR1*	–	−2.15	7.23E-05	Phospholipid scramblase 1
3949	*LDLR*	–	−2.10	3.51E-12	Low-density lipoprotein receptor (familial hypercholesterolemia)
8613	*PPAP2B*	–	−2.03	6.48E-04	Phosphatidic acid phosphatase type 2B, transcript variant 1
1594	*CYP27B1*	–	−2.01	4.56E-08	Cytochrome P450, family 27, subfamily B, polypeptide 1, nuclear gene encoding mitochondrial protein
55902	*ACSS2*	–	2.07	1.54E-06	Acyl-coenzyme A synthetase short-chain family member 2, transcript variant 2
116255	*MOGAT1*	–	2.36	8.48E-06	Monoacylglycerol O-acyltransferase 1
Beta-oxidation					
8660	*IRS2*	−2.14	−2.15	7.03E-05	Insulin receptor substrate 2
Long-chain and unsaturated fatty acid metabolism					
5743	*PTGS2*	−4.50	−3.79	2.54E-11	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
552	*AVPR1A*	−3.09	−3.32	6.75E-07	Arginine vasopressin receptor 1A
7132	*TNFRSF1A*	−2.16	–	6.90E-11	Tumor necrosis factor receptor superfamily, member 1A
2678	*GGT1*	2.01	–	1.67E-06	Gamma-glutamyltransferase 1, transcript variant 1
3992	*FADS1*	2.49	2.09	9.61E-06	Fatty acid desaturase 1
9415	*FADS2*	2.81	–	1.07E-04	Fatty acid desaturase 2
641371	*ACOT1**	–	−2.73	3.80E-02	Acyl-coenzyme A thioesterase 1

Gene expression levels are given as fold-changes between two groups. – not significant in two-group comparison (*post hoc* $P \geq 0.05$) and/or less than two-fold up- or down-regulation. The *q* value is the Benjamini-Hochberg corrected *P* value.

*The only gene differentially expressed between SS and NASH related to the processes above was acyl-coenzyme A thioesterase 1 (*ACOT1*) (2.99-fold up-regulated in NASH versus SS).

expression and diagnosis was significant for AA in six genes: *CYP7A1*, *CXCR4* ($P < 0.01$), *JUB*, *MT1A*, *GGT1*, and *MTMR4* ($P < 0.05$) (Supporting Fig. S2). The same was true for *GGT1* in relation to EPA+DHA.

Discussion

Well-defined subjects with either healthy liver, SS, or NASH showed distinct hepatic gene expression profiles. Only a small number of genes were differentially expressed between SS and NASH, and they were significantly correlated with disease severity. In patients with NASH, hepatic long-chain PUFAs were lower and their associations with gene expression were different compared to patients with SS.

Our results on global gene expression are in line with other publications showing dysregulation of lipogenesis,[4,11,21] apoptosis, and oxidative stress-related genes[10] in NAFLD. Some observations like up-regulation of *ACSL4*[4] and changes in the transcription factors peroxisome proliferator-activated receptor-α and SREBP-1c[21] could not be reproduced. While others reported overexpression of genes related to wound healing and cell migration in NASH compared to SS and controls,[11] these genes were down-regulated in both NASH and SS versus HCs in our study. These discrepancies could be explained by differences in technology, bioinformatics, and the selected threshold of two-fold up- or down-regulation. In addition, our participants' characteristics were different from those in previous studies where all NAFLD patients were morbidly obese[4,5,21] and various control groups were used.[4,5,11,20,21] We included a true HC group of living liver donors, and our participants' mean BMI ranged from slightly overweight in HCs to overall mildly obese in NASH. This difference in BMI among the groups did not seem to influence our results, as demonstrated in our sensitivity analysis. However, the different liver biopsy protocols between NAFLD and HC (percutaneous liver biopsy versus hepatectomy) may have altered hepatic gene expression due to the effect of surgery.[34] We could not perform percutaneous liver biopsies in HC prior to surgery for ethical reasons.

Our study detected only 22 DEGs between SS and NASH, which may be due to the relatively mild disease in the NASH group (median NAFLD activity score = 5; 47% of patients no or mild fibrosis; oxidative stress not detectable). Similar to others,[5,6,11] we found an overexpression of genes associated with cancer progression and proliferation in NASH versus SS, including *AKR1B10*, *EEF1A2*, *SPP1* (osteopontin), and *PEG10*.[24] The *AKR1B10* gene was the most up-regulated in NASH versus SS and HC, and the expression correlated with NAFLD activity score ($r = 0.672$, $P < 0.0001$). This is in line with previous studies

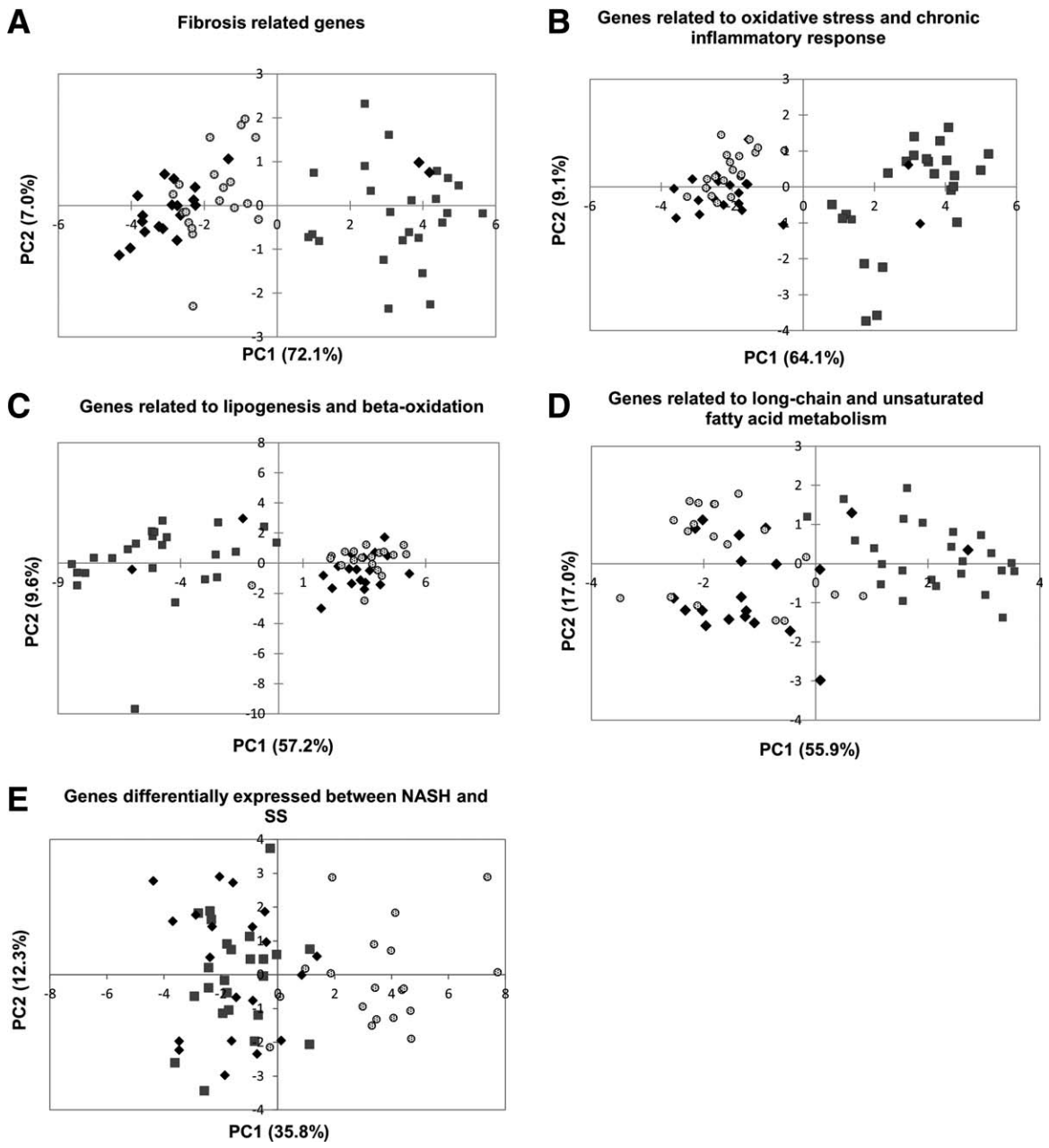

Fig. 2. Principal component analysis for hepatic gene expression in patients with SS or NASH and HCs. Points represent values for individual participants (HC, gray squares; SS, black diamonds; NASH, gray circles). Gene sets included (1) 13 genes related to fibrosis, (2) 13 genes related to chronic inflammation and cellular response to oxidative stress, (3) 38 genes related to lipogenesis and one gene related to beta-oxidation, and (4) seven genes related to long-chain and unsaturated fatty acid metabolism. Gene sets for (1) to (4) were derived from the literature (Supporting Table S1), and differentially expressed genes corresponding to these sets as listed in Table 2 were included in the principal component analysis. The gene sets show a clear differentiation between patients (SS and NASH) and HCs. (5) This analysis included the 22 genes that were differentially expressed between NASH and SS. Here, the patients with SS are grouped with HCs, and both are different from NASH. Abbreviation: PC, principal component.

identifying this gene as a potential biomarker for NASH and progression to HCC.[5,6,10,11] It is also interesting to note that *AKR1B10* inhibitors were reported to have antitumor effects, supporting its role in cancer progression.[35]

Due to the percutaneous liver biopsy technique performed in NAFLD, only very small samples were available for the FA composition. This prevented us from analyzing all lipid subfractions. Therefore, only hepatic total lipid FA composition was measured. Because patients with NAFLD have higher proportions of triacylglycerols and lower phospholipids in the liver due to steatosis,[15,16] results from total lipid FA composition can be skewed if compared to HCs who have

Table 4. Fatty Acid Profiles in Hepatic and Red Blood Cell Total Lipids in HCs and Patients With SS or NASH

	Total lipids (%)			
	Hepatic Fatty Acids		Red Blood Cell Fatty Acids	
Fatty Acid	SS (n = 13)	NASH (n = 15)	SS (n = 7)	NASH (n = 9)
Total SATs	40.51 ± 5.27	42.46 ± 4.31	40.53 ± 2.52	40.96 ± 3.69
Total MUFAs	33.31 ± 6.79	35.94 ± 4.97	19.74 ± 1.23	19.31 ± 1.52
Total PUFAs	24.06 ± 3.37	19.48 ± 4.34 **	36.72 ± 2.61	36.81 ± 4.56
Total n6 PUFAs	20.71 ± 2.67	17.11 ± 3.91 **	30.32 ± 1.43	31.07 ± 4.07
Total n3 PUFAs	3.32 ± 1.40	2.33 ± 0.65 *	5.87 (3.41)	5.40 (0.99)
Total n6/n3	7.20 ± 2.70	7.52 ± 1.35	5.16 ± 1.48	5.94 ± 1.71
Total trans fatty acids	2.31 (1.52)	1.80 (1.94)	3.01 ± 0.89	2.92 ± 0.84
16:0 (palmitic acid)	30.19 (6.01)	31.32 (1.99)	23.74 ± 1.54	23.98 ± 2.45
18:0 (stearic acid)	8.86 ± 2.35	7.64 ± 1.60	12.97 ± 1.61	13.64 ± 1.52
16:1n7 (palmitoleic acid)	2.63 ± 0.83	3.16 ± 0.77	0.55 ± 0.21	0.98 ± 0.17
18:1n9 (oleic acid)	27.13 ± 6.23	29.51 ± 4.81	14.59 ± 1.41	14.71 ± 1.21
18:2n6 (linoleic acid)	15.41 ± 2.69	13.45 ± 2.90	13.62 ± 1.05	14.02 ± 1.71
18:3n6 (γ-linolenic acid)	0.30 ± 0.21	0.24 ± 0.22	0.50 (0.77)	0.24 (0.57)
18:3n3 (α-linolenic acid)	0.86 ± 0.31	0.94 ± 0.27	0.27 ± 0.08	0.29 ± 0.04
20:3n6 (dihomo-γ-linolenic acid)	0.80 ± 0.26	0.57 ± 0.31 *	1.52 ± 0.25	1.72 ± 0.29
20:4n6 (AA)	3.99 ± 1.75	2.60 ± 1.13 *	10.75 (3.22)	12.52 (3.87)
20:5n3 (EPA)	0.17 (0.36)	0.12 (0.25)	0.76 (0.48)	0.49 (0.18)
22:6n3 (DHA)	1.73 (1.81)	1.13 (0.67)	3.19 (2.89)	2.77 (1.15)
EPA+DHA	2.20 ± 1.33	1.24 ± 0.62 *	4.05 (3.32)	3.16 (1.03)
AA/LA	0.27 ± 0.13	0.20 ± 0.08	0.83 ± 0.16	0.82 ± 0.12
(EPA+DHA)/ALA	2.41 (2.05)	1.42 (1.14)	17.54 (19.1)	11.30 (4.47)
Δ5 desaturase index 20:4n6 / 20:3n6	4.86 ± 1.05	4.05 ± 1.11	6.73 ± 1.80	6.27 ± 1.39
Δ6 desaturase index 18:3n6 / 18:2n6	0.02 ± 0.01	0.02 ± 0.01	0.04 (0.07)	0.02 (0.04)

Values are median (interquartile range) or mean ± SD. Significant differences between SS and NASH: *$P < 0.05$, **$P < 0.01$. The unpaired *t* test was used for normally distributed data and Wilcoxon's test for data with skewed distribution.

Abbreviations: ALA, α-linolenic acid; LA, linolenic acid; MUFA, monounsaturated fatty acid; SAT, saturated fatty acid.

no steatosis. Since FAs bound in these subfractions have distinct biological roles,[16] they may also be of different significance for hepatic gene expression. Therefore, we focused our FA analysis on SS and NASH, where steatosis is comparable (Table 1). We found lower n-3 and n-6 PUFAs, especially AA and EPA+DHA, in NASH versus SS; but dietary intake according to food records and erythrocyte FA profiles[36] was similar between groups. The lower hepatic PUFA content was not associated with increased PUFA utilization through eicosanoid production or lipid peroxidation[15] as cyclooxygenase-2 (*COX2* = *PTGS2*), a key enzyme in prostaglandin synthesis, was down-regulated similarly in NASH and SS compared to HCs and hepatic antioxidant power and lipid peroxidation were not different among the groups. Changes in FA metabolism, especially impaired conversion of essential FAs to their long-chain PUFA products,[14,15] would be another explanation for lower AA and EPA+DHA in NASH. However, our results did not support this as the genes for elongases (*ELOVL*) and desaturases (*FADS1*, *FADS2*) involved in this conversion were not different between NASH and SS. In fact, *FADS1* and *FADS2* were up-regulated in NASH and, to a smaller extent, in SS versus HCs, as reported.[20] This seems contradictory but can be explained if *FADS1* and *FADS2* are up-regulated by low levels of long-chain PUFAs, low protein expression, or impaired catalytic activity of Δ5 and Δ6 desaturases. This would be consistent with some of the negative estimates of correlation coefficients found between EPA+DHA and *FADS2* expression, which was significant in SS+NASH combined. Another reason could be genetic variations in the *FADS1/2* gene cluster, which were reported to influence transcription[37] and to be associated with lower indices for Δ5/Δ6 desaturase activity.[38] Based on animal and *in vitro* experiments, the dysregulation of PUFA desaturation may directly contribute to NASH development, and manipulation of the pathway could be a potential treatment target.[20]

Another DEG of interest is *PNPLA3*, coding for adiponutrin, a triacylglycerol hydrolase. A genetic variant, $PNPLA3^{I148M}$, predisposes to steatosis, fibrosis, and progression to HCC,[39] especially in obesity.[40] The $PNPLA3^{I148M}$ variant was reported to increase hepatic triacylglycerol content and change hepatic FA composition, reducing n-6 PUFAs.[39] The effects of

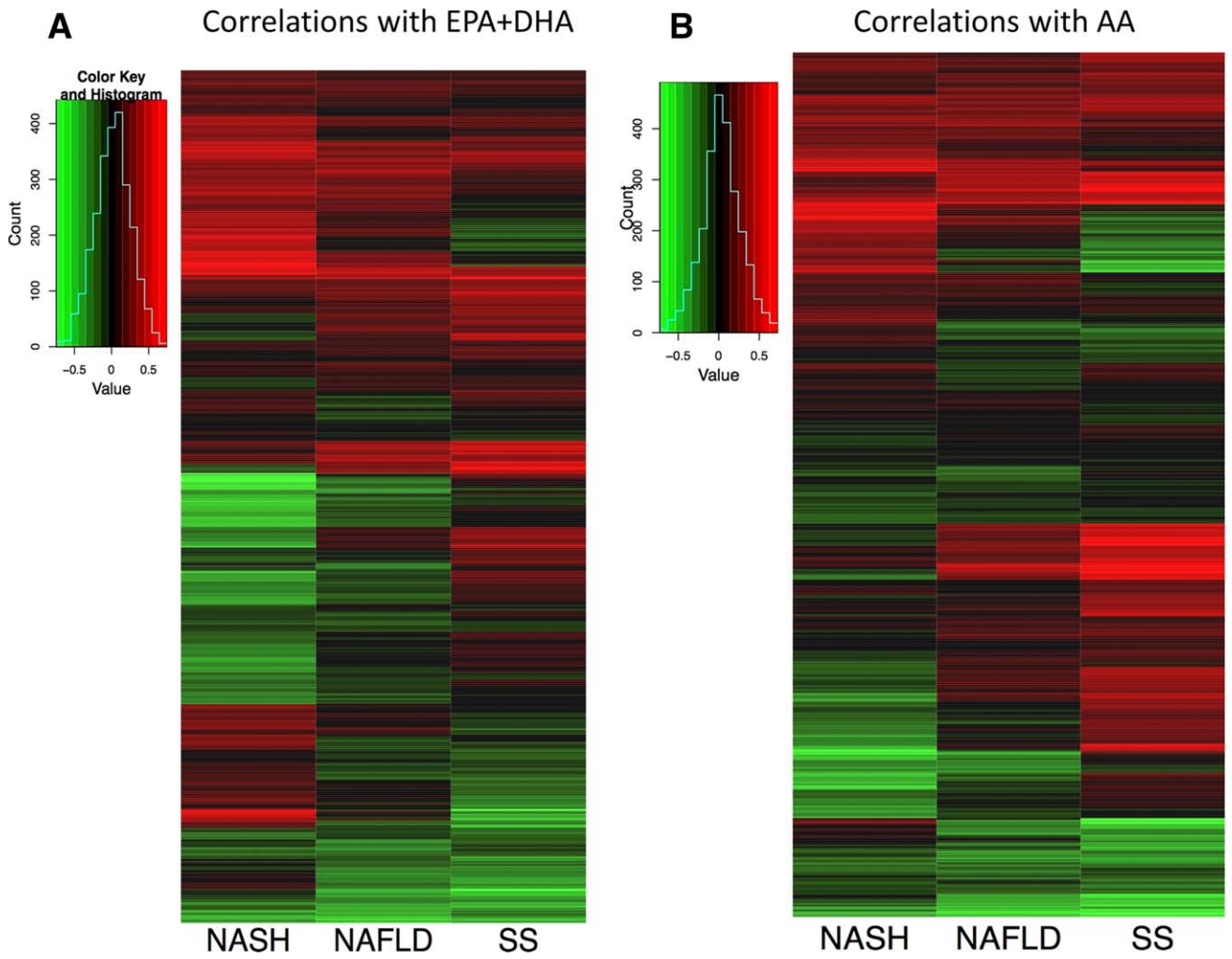

Fig. 3. Correlations between gene expression and long-chain PUFAs in the liver. The heat maps illustrate correlations between hepatic gene expression and (1) n-3 index (EPA + DHA) as well as (2) AA in hepatic total lipids in patients with SS or NASH or in both groups combined (NAFLD). All 822 probes that were differentially expressed among SS, NASH, and HCs were included; the heat maps were generated using unsupervised hierarchical clustering. Genes are listed vertically. The color intensity corresponds to the correlation coefficient (red = positive correlation, green = negative correlation). The two heat maps show a clear differentiation between SS and NASH as well as the whole group of NAFLD patients combined based on the correlation coefficients between mRNA levels and FA abundance in hepatic total lipids, which means, for many genes, the relationship differs depending on the patient group examined.

PNPLA^{I148M} can be modulated by a high n-6/n-3 PUFA ratio in the diet and can contribute to NAFLD as shown in obese youth carrying the risk allele.[40] A PUFA-gene interaction was also observed in a large epidemiological study, where a significant association between *PNPLA3*^{I148M} and plasma triacylglycerols was found only in overweight participants having a low ratio of n-6/n-3 PUFAs in their diet.[41] In our study, genotyping was not available, but *PNPLA3* was upregulated in NASH (+2.38) and SS (+1.7; $P < 0.05$) versus HCs; and expression levels were negatively correlated with AA in NAFLD (SS+NASH), supporting an interrelation between *PNPLA3*, n-6 PUFA, and NAFLD pathogenesis. Genotyping for the *PNPLA3*^{I148M} allele may be of interest in future studies as it can predispose to NAFLD and may modulate the response to PUFA supplementation.

Other genes had different associations with PUFAs in NASH compared with SS. For example, *CYP7A1*, crucial for bile acid synthesis and cholesterol catabolism,[24] was correlated with AA positively in NASH but negatively in SS ($P = 0.055$). These different associations between genes related to lipid metabolism and PUFA content in SS versus NASH could explain some of the conflicting results seen in n-3 PUFA supplementation studies where steatosis improved on imaging (no distinction between SS and NASH),[19] while no effect was seen on liver biopsy proven NASH.[42]

The strengths of this study include the true HC group confirmed by histology and the measurements of

Table 5. Spearman Correlations for Hepatic Gene Expression and Hepatic Long-Chain PUFAs in Patients With SS or NASH

	Spearman's rho					
	EPA+DHA			AA		
Gene Symbol	NAFLD (SS+NASH)	SS	NASH	NAFLD (SS+NASH)	SS	NASH
Fibrosis						
MMP9	−0.448 *	−0.385	−0.418	−0.275	−0.137	−0.334
CXCR4	−0.322	0.132	−0.664 **	−0.269	0.253	−0.617 *
CCL2	0.227	−0.044	0.543 *	0.297	0.225	0.452
Cellular response to oxidative stress						
CDC2	−0.470 *	−0.533	−0.064	−0.489 **	−0.549	−0.159
FOS	0.248	0.538	0.146	0.365	0.621 *	0.159
Lipogenesis						
GCK	−0.393 *	−0.396	−0.343	−0.510 **	−0.533	−0.377
JUB	−0.411 *	−0.462	0.025	−0.407 *	−0.599 *	0.164
PNPLA3	−0.301	−0.159	−0.121	−0.378 *	−0.363	−0.238
FADS2	−0.409 *	−0.516	−0.200	−0.327	−0.412	−0.181
GGT1	−0.166	−0.533	0.211	−0.211	−0.676 *	0.120
CYP7A1	−0.096	−0.379	0.514	−0.155	−0.549	0.547 *
MTMR4	−0.007	−0.264	0.043	−0.032	−0.604 *	0.206
ACSM1	0.265	0.692 *	0.293	0.044	0.418	0.130
CH25H	0.284	−0.055	0.550 *	0.236	0.247	0.254
SIK1	0.380 *	0.516	0.318	0.238	0.429	0.046
Long-chain and unsaturated fatty acid metabolism						
FADS2	−0.409 *	−0.516	−0.200	−0.327	−0.412	−0.181
GGT1	−0.166	−0.533	0.211	−0.211	−0.676 *	0.120
Different between SS and NASH						
PEG10	−0.501 **	−0.357	−0.354	−0.525 **	−0.549	−0.345
EEF1A2	−0.365	−0.280	0.093	−0.427 *	−0.313	0.005
MT1A	0.452 *	0.401	0.075	0.447 *	0.560 (*)	−0.046
DIO3OS	0.419 *	0.324	0.068	0.455 *	0.621 *	0.063

Significant correlations: $*P < 0.05$, $**P < 0.01$, $(*)$ $P = 0.05$.

No gene related to chronic inflammatory response or beta-oxidation was significantly correlated with EPA+DHA or AA.

both gene expression and FA in the same liver samples. Potential limitations include the use of tissue from hepatectomy, which may have influenced the gene expression in HCs[34] and the small amount of tissue available in NAFLD, which did not allow for a more detailed lipid analysis. Future studies could separate different cell types from the whole tissue as the cellular composition influences gene expression[43] and perform proteomic analysis to examine gene transcription.[5] Furthermore, the cross-sectional nature of our study did not allow us to establish causal relationships, especially since hepatic gene expression and FA composition are subject to reciprocal regulation. Further studies are needed to clarify this relationship and determine which changes in hepatic gene expression can predict the development of NASH and disease progression and which genes respond to specific PUFA supplementation. Due to the different relationships between genes and PUFAs in SS and NASH, these two groups should be analyzed separately.

In conclusion, hepatic gene expression differed between NAFLD and HCs, including genes that may influence FA composition. A small number of DEGs distinguished NASH from SS, but more of them related to HCC than to lipid metabolism. Hepatic long-chain n-3 and n-6 PUFAs, especially EPA, DHA, and AA, were lower in NASH compared with SS. This may be due to dysregulation of *FADS1/2* and *PNPLA3* as PUFA intake and oxidative stress are similar between groups. The associations between genes and PUFAs were different in NASH compared with SS. As a result, responses to specific PUFA supplementation may be different in NASH versus SS. In order to establish causal relationships between PUFAs and gene expression, intervention studies with n-3 and/or n-6 PUFAs are required in well-characterized patients. In addition, genotyping for *PNPLA3* may be considered.

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Supporting Information

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