METABOLISM CLINICAL AND EXPERIMENTAL 60 (2011) 1001–1011

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Lipid in the livers of adolescents with nonalcoholic steatohepatitis: combined effects of pathways on steatosis

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ARTICLE INFO

Article history:
Received 22 July 2010
Accepted 10 October 2010

ABSTRACT

Fatty liver is a prerequisite for the development of nonalcoholic steatohepatitis (NASH). The homeostasis of hepatic lipid is determined by the dynamic balance of multiple pathways introducing lipids into or removing lipids from hepatocytes. We aim to study the different contributions of major lipid pathways to fat deposition in NASH livers. Expression of the lipid metabolism–related genes was analyzed by microarray and quantitative real-time polymerase chain reaction analysis. The expression levels of genes responsible for the rate-limiting steps of fatty acid uptake (*CD36*, *FABPpm*, *SLC27A2*, and *SLC27A5*), de novo synthesis (*ACACB*), oxidation (*CPT-1*), and very low-density lipoprotein (VLDL) secretion (ApoB) were used to evaluate the relative activity of each pathway. The expression levels for CD36 and CPT-1 were confirmed by Western blot analysis. Fatty acid uptake pathways were up-regulated to a higher degree than other pathways. The de novo synthesis pathway was also up-regulated more than both VLDL secretion and fatty acid oxidation pathways. In contrast to other NASH livers, one NASH liver exhibited lower *ApoB* and *CPT-1* expression levels than normal controls. The increased fatty acid uptake and de novo synthesis were the most common causes for steatosis in NASH patients. In a rare case, impaired VLDL secretion and fatty acid oxidation contributed to the development of steatosis. Our study promises a simple method for the determination of why hepatic steatosis occurs in individual patients. This method may allow specific targeting of therapeutic treatments in individual patients.

Author contributions: L Zhu: conceived and designed the experiments, performed the experiments, analyzed the data, and wrote the first draft. S Baker: conceived and designed the experiments, performed the experiments, analyzed the data, and revised the draft. W Liu: performed the experiments, analyzed the data, and revised the draft. M-H Tao: analyzed the data and revised the draft. R Patel: performed the experiments and revised the draft. N Nowak: performed the experiments and revised the draft. R Baker: conceived and designed the experiments, performed the experiments, analyzed the data, and revised the draft.

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doi:10.1016/j.metabol.2010.10.003

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of abnormal liver enzymes in the US population [1]. The advanced form of NAFLD with inflammation or fibrosis is termed *nonalcoholic steatohepatitis* (NASH). According to the current "2-hit" hypothesis [2], steatosis is a prerequisite for NASH patients to develop inflammation and fibrosis. Regarding the etiology for steatosis, studies focusing on 1 or 2 pathways of hepatic lipid metabolism have led to conflicting conclusions [3-9].

Homeostasis of hepatic lipids depends on the dynamic balance of several pathways including fatty acid uptake, de novo synthesis, oxidation, and very low-density lipoprotein (VLDL) secretion (reviewed in Goldberg and Ginsberg [10], Fabbrini et al [11], and Lavoie et al [12]). Therefore, to understand the mechanism for excess lipid accumulation in liver, all of the major pathways for lipid metabolism should be studied in parallel. Because a convenient quantitative proteomic method is not available, our first choice was a well-characterized NASH microarray data set [13], complemented by quantitative real-time polymerase chain reaction (qRT-PCR). Here, the expression levels of the lipid metabolism related genes were examined to test our hypothesis that fatty livers are of varied etiology. We found that several abnormalities in different lipid metabolism pathways collaboratively contributed to the development of steatosis.

2. Methods

2.1. Patients

This study was approved by the Institutional Review Board of the State University of New York at Buffalo. Only children and adolescents were included in this study to ensure that our patients were not sustained alcohol users. All our adolescent patients claimed that they were not regular drinkers of alcoholic beverages. We were assured by their parents that these adolescent patients had no access to alcoholic beverages. Even if our patients did ingest some alcohol, it could not be consistent long-term alcohol use simply because of their age. We considered other possible sources of alcohol (aspartame, fruit), but a 3-day food record before any biopsy failed to identify a significant source of alcohol. All NASH patients included in this study had a body mass index (BMI) greater than the 95th percentile for age and exhibited significant insulin resistance (IR). Insulin resistance was calculated based on the homeostasis model assessment (HOMA) method [14]. Liver biopsies were obtained, with prior written consent, from parents of patients suspected of having NASH as part of regular medical care. Patients signed an assent to the research. Diagnosis of NASH was based on hepatic fat infiltration, inflammation, and fibrosis as revealed by liver biopsy, following the criteria of Kleiner et al [15]. The clinical information for the patients and normal controls (NCs) subjected to qRT-PCR is listed in Table 1. Information for patients and NCs subjected to microarray analysis was described previously [13].

Table 1 – Characteristics of NASH patients and NCs

	RT-PCR		Western blot	
	NASH	NC[a]	NASH	Non-NASH control[e]
Sex (female-male)	12:15	2:4	4:5	3:1
Age (y)	9-18	1-19	11-18	4-12
BMI	37.4 ± 1.6[b]	18.4 ± 1.3[c]	32.7 ± 1.6[b]	20.6 ± 2.9[c]
Fasting insulin (mU/mL)	23.3 ± 2.6	NA	20.7 ± 5.1	<2
Fasting glucose (mmol/L)	5.4 ± 0.2	NA	5.7 ± 0.4	4.7 ± 0.2
IR (HOMA)[d]	5.6 ± 0.7	NA	5.7 ± 1.7	<1

NA indicates not available.
[a] Normal healthy liver intended for transplantation; no liver disease reported.
[b] Patients are all greater than the 95th percentile of the population.
[c] Normal controls are all less than the 80th percentile of the population.
[d] IR (HOMA) for healthy subjects is around 1.
[e] Liver biopsies from patients with hepatitis C, autoimmune hepatitis, gall stone, and cystic fibrosis, respectively. They were free from steatosis.

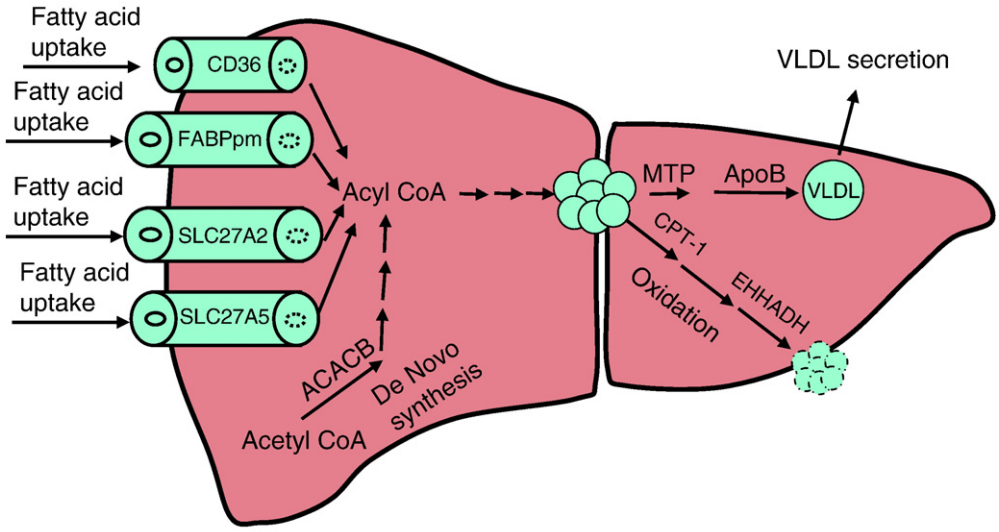
2.2. RNA extraction and microarray hybridization

Liver biopsies were stored in RNAlater before total RNA was extracted with RNeasy and treated with RNase-free DNase I set (Qiagen, Valencia, CA). RNA samples obtained from Admet were also treated with RNase free DNase before downstream experiments. Quality of the RNA samples was ensured with Bioanalyzer (Agilent Technologies, Santa Clara, CA) before downstream biotin labeling and hybridization to the CodeLink Human Whole Genome Bioarray (GE Health Care–Amersham Biosciences, Piscataway, NJ) following the manufacturer's manual. The original microarray data have been uploaded to the Gene Expression Omnibus Web site: http://www.ncbi.nlm.nih.gov/geo/index.cgi. The accession numbers are GSM435821 to GSM435827 for NASH liver data sets and GSM435828 and GSM435833 to GSM435835 for NC data sets.

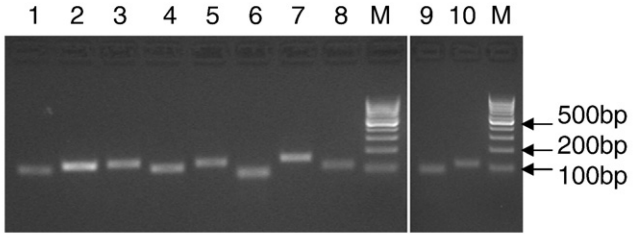
2.3. Quantitative RT-PCR

Selected genes (Fig. 1A) were analyzed by qRT-PCR. Primers (Table 2) were designed with the assistance of Primer 3 [16] and BLAST [17] search. Complementary DNA was synthesized with the iScript complementary DNA (cDNA) synthesis kit (Bio-Rad Laboratories, Hercules, CA). Quantitative RT-PCR was performed on an iCycler iQ real-time detection system (Bio-Rad Laboratories) using Sybergreen (iQ SYBR Green Supermix, Bio-Rad Laboratories) for real-time monitoring. For normalization purposes, GAPD RNA levels were analyzed in parallel with the genes of interest. The presence of a single specific PCR product was verified by melting curve analysis (data not shown) and confirmed on agarose gels (Fig. 1B).

The concentration of messenger RNA ([mRNA]) is represented by the following equation: $[mRNA] = M/E^{Ct}$, where constant M is an arbitrary threshold, E is the efficiency of PCR, and Ct is the threshold cycle. All PCR reactions had efficiencies around 1.9, as determined experimentally with



A Major pathways of lipid metabolism in liver, with genes of interest indicated



B Gel electrophoresis analysis of the PCR products targeting lipid metabolism pathways

Fig. 1 – Genes targeted in qRT-PCR. A, Schematic representation of the major pathways introducing lipid into or removing lipid from liver. Four fatty acid transporters—CD36, FABPpm, SLC27A2, and SLC27A5—are known to mediate fatty acid uptake into liver; ACACB catalyzes the rate-limiting step in fatty acid de novo synthesis; microsomal triglyceride transfer protein (MTP) is a key protein in the secretion of VLDL, the secretion rate of which is determined by the availability of ApoB. The rate limiting step for fatty acid oxidation in mitochondria is the translocation of fatty acid into mitochondria by CPT-1, which delivers fatty acids to a myriad of enzymes including enoyl-CoA:hydratase 3-hydroxyacyl-CoA dehydrogenase (EHHADH). B, Agarose gel electrophoresis of the qRT-PCR products. The end products of qRT-PCR were analyzed on a 3% agarose gel. The expected sizes are: (1) GAPD, 110 base pairs (bp); (2) CD36, 133 bp; (3) FABPpm, 142 bp; (4) SLC27A2, 116 bp; (5) CPT-1, 132 bp; (6) EHHADH, 77 bp; (7) MTP, 148 bp; (8) ApoB, 107 bp; (9) SLC27A5, 82 bp; and (10) ACACB, 107 bp. M is the GeneRuler 100-bp DNA Ladder, 100 to 1000 bp (Fermentas Life Sciences, Glen Burnie, MD).

4-times serial diluted samples. The relative mRNA concentration of each target gene was calculated as the mRNA concentration of target gene normalized against that of GAPD, as represented by the following equation: $[\text{mRNA}]_{\text{target}}/[\text{mRNA}]_{\text{GAPD}} = E_{\text{GAPD}}^{\text{Ct.GAPD}}/E_{\text{target}}^{\text{Ct.target}}$.

2.4. *Western blot analysis*

Liver biopsies stored in RNAlater were homogenized in phosphate-buffered saline and then boiled for 5 minute in sodium dodecyl sulfate polyacrylamide gel electrophoresis loading buffer. Samples (15 μg total protein each) were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels. After blotting onto nitrocellulose membranes, CD36 [18,19] (cat#sc-9154, Santa Cruz Biotechnology, Santa Cruz, CA), carnitine palmitoyltransferase I (CPT-1) [20] (cat#sc-98834, Santa Cruz Biotechnology), and β-actin [13] (Clone C4; MP Biomedicals, Cleveland, OH) were probed. The results were visualized using the SuperSignal West Dura Extended Duration Substrate (Invitrogen, Carlsbad, CA) and recorded with an image reader (LAS-1000; Fujifilm, Edison, NJ).

2.5. *Statistical analysis*

Characteristics and expression levels of genes in liver of NASH cases and controls were compared using the nonparametric test (Mann-Whitney U test) and the Student t test. All statistical tests were based on 2-sided probability and a significant level of $P \leq .05$. Statistical analyses were conducting using SAS, Version 9.2 (SAS Institute, Cary, NC).

Table 2 – Primer pairs for qRT-PCR analysis

Symbol	Description	Sequence	
GAPD	Glyceraldehyde-3-phosphate dehydrogenase	AGCCTCAAGATCATCAGCAATG	(Forward)
		ATGGACTGTGGTCATGAGTCCTT	(Reverse)
CD36	CD36 molecule (thrombospondin receptor)	GCCAAGGAAAATGTAACCCAGG	(Forward)
		CCACAGCCAGATTGAGAA	(Reverse
FABPpm	Plasma membrane fatty acid binding protein (aspartate aminotransferase 2)	ATCCCACGGGAGTGGACCCG	(Forward)
		CGCACAGCCCAGGCATCCTT	(Reverse
SLC27A2	Solute carrier family 27 (fatty acid transporter), member 2	GTATTGTGGCTGGTGCTACTC	(Forward)
		CCGAAGCAGTTCACCGATA	(Reverse
SLC27A5	Solute carrier family 27 (fatty acid transporter), member 5	AGAAGGCAACATGGGCTTAG	(Forward)
		GGACAGCATTCGGAGGAG	(Reverse
ACACB	Acetyl-CoA carboxylase β	TTGTGATGGTGACCCCCGAGGACCTTAAG	(Forward)
		CGGGGATTCTCTTGGCAATGTCCACAATC	(Reverse)
CPT-1	Carnitine palmitoyltransferase I	CTGGACTTCATTCCTGGAAAAAGAAG	(Forward)
		CGATCTTGGCGTACATCGTTGTCATC	(Reverse)
EHHADH	Enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase	CCACGCAGAGGCTCAAGTT	(Forward)
		GGAGAAGCTGGGTTCCTCTT	(Reverse)
MTP	Microsomal triglyceride transfer protein	CTCTGCTTCATTTCCTCATATTCAGCTTC	(Forward)
		CGGTAGCCCACGCTGTCTTGCAGTTTTCC	(Reverse)
ApoB	Apolipoprotein B	CAACCCTGAGGGCAAAGCCTTGCTG	(Forward)
		CCTGCTTCCCTTCTGGAATGGCC	(Reverse)

3. Results

3.1. *Gene expression for hepatic fatty acid uptake*

Fatty acid uptake by hepatocytes depends on both the concentration of plasma fatty acid and the capacity of the uptake transporter systems [21]. The very elevated plasma fatty acid concentration [22] found in NASH patients suggests that the uptake transporter systems are saturated; therefore, the capacity (quantity) of the fatty acid transporters is the rate-limiting factor in fatty acid uptake.

Four different fatty acid transporters were reported on the plasma membrane of hepatocytes. They are CD36 (also known as *fatty acid translocase*) [23], plasma membrane–associated fatty acid binding protein (FABPpm) [24], and solute carrier family 27 member 2 (SLC27A2) [25] and member 5 (SLC27A5) [26]. From the transcriptome data generated on the Codelink whole genome microarray, increased signals were observed in NASH livers for CD36 (NASH/NC=5.76, P = .004), FABPpm (NASH/NC = 4.27, P = .005), SLC27A2 (NASH/NC = 2.27, P = .015), and SLC27A5 (NASH/NC = 1.72, P = .08), although the latter did not achieve statistical significance (Table 3). This increase was observed in the context of similar gene transcription levels for most of the 45 000 targeted sequences as represented by several housekeeping genes (Table 3).

To validate these results, cDNA was prepared from a different but overlapping set of NASH and normal livers for qRT-PCR analysis (Fig. 2A-D). Increased expression of CD36, FABPpm, SLC27A2, and SLC27A5 in NASH livers was confirmed. The differences are statistically significant for all of these genes.

3.2. *Elevated expression of genes for other lipid metabolism pathways*

De novo synthesis is another important source of lipids in liver, with the rate-limiting reaction being the carboxylation of acetyl–coenzyme A (CoA), catalyzed by acetyl-CoA carboxylase β (ACACB). Because microarray analysis did not give good signals for this gene, ACACB expression level was examined by qRT-PCR (Fig. 2E). A significant difference was observed between NASH livers and NCs (NASH/NC = 3.8, P = .002). This result was confirmed with a different primer pair for ACACB in a similarly conducted qRT-PCR analysis (data not shown). Many other genes involved in lipid synthesis (Table 3), including fatty acid synthase gene, stearoyl-CoA desaturase 1 gene, and several acyltransferase genes, also exhibited significantly elevated expression in NASH livers.

Similarly, elevated expression was observed for genes involved in fatty acid oxidation (Table 3 and Fig. 2F, G), although statistical significance was not achieved for CPT-1, the rate-limiting factor for fatty acid oxidation in mitochondria.

The VLDL secretion pathway was also elevated in NASH livers at the gene expression level (Table 3 and Fig. 2H, I). Statistical significance was achieved for apolipoprotein B (ApoB) in qRT-PCR analysis, but not in microarray analysis.

The finding that all 4 hepatic lipid metabolism pathways were more or less up-regulated was intriguing, but there were 2 immediate major concerns. First, were the gene expression patterns consistent among NASH patients? Second, a comparison of the extent to which each pathway was up-regulated relative to the other lipid metabolism pathways had to be made to understand the molecular basis of the fatty livers.

3.3. *Lipid metabolism in NASH patient P112*

In examining the gene expression patterns of individual NASH livers and NCs, NASH liver P112 stood out because the gene expression pattern was different from all other NASH livers. Whereas other NASH livers exhibited high expression levels in all of the 4 hepatic fatty acid transporters, NASH liver P112 only had a high level in CD36. FABPpm, SLC27A2, and SLC27A5 exhibited similar expression levels as NCs (Fig. 3A-D). Similarly, the ACACB expression level in P112 was not higher than that in

Table 3 – Microarray analysis of gene expression between NASH livers and NCs [a]

	GenBank accession no.	Gene description	NASH liver (n = 7)	Normal liver (n = 4)	NASH/normal [b]	P value [c]
Housekeeping	NM_000194.1	Hypoxanthine phosphoribosyltransferase (HPRT)	2.99 ± 0.62	3.21 ± 0.62	0.93	.804
	NM_000402.2	Glucose-6-phosphate dehydrogenase (G6PD)	1.58 ± 0.10	1.53 ± 0.27	1.03	.875
	NM_003194.2	TATA box binding protein (TBP)	7.24 ± 0.44	8.70 ± 1.33	0.83	.364
	NM_004048.2	β-2-microglobulin (B2M)	167.17 ± 32.92	194.66 ± 16.32	0.86	.475
	NM_012423.2	Ribosomal protein L13a (RPL13A)	63.13 ± 19.19	80.55 ± 15.34	0.78	.497
	NM_001101.2	Actin, β (ACTB)	18.19 ± 2.91	16.07 ± 4.88	1.13	.723
	NM_030773.1	Tubulin, β1 (TUBB1)	0.30 ± 0.08	0.31 ± 0.07	0.96	.915
Fatty acid uptake	NM_003645.2	Solute carrier family 27 (fatty acid transporter), member 2	22.18 ± 3.52	9.79 ± 2.20	2.27	.015
	NM_012254.1	Solute carrier family 27 (fatty acid transporter), member 5	19.12 ± 2.12	11.13 ± 3.11	1.72	.080
	NM_002080.1	FABPpm; mitochondrial aspartate aminotransferase	42.05 ± 7.57	9.85 ± 3.05	4.27	.005
	NM_000072.1	CD36 antigen	16.55 ± 3.10	2.87 ± 1.63	5.76	.004
Lipid synthesis	NM_004104.3	Fatty acid synthase (FASN)	10.50 ± 2.34	1.80 ± 0.65	5.83	.009
	NM_005063.3	Stearoyl-CoA desaturase 1 (SCD1)	34.59 ± 10.41	3.80 ± 1.34	9.11	.025
	NM_003578.2	Acyl-CoA:cholesterol acyltransferase 2	1.43 ± 0.26	0.87 ± 0.21	1.65	.130
	NM_178176.2	Acyl-CoA:monoacylglycerol acyltransferase 3 (MGAT3)	4.61 ± 0.45	2.00 ± 0.17	2.31	.001
	NM_025098.2	Acyl-CoA:monoacylglycerol acyltransferase 2 (MGAT2)	2.19 ± 0.34	0.55 ± 0.13	3.96	.002
Fatty acid oxidation	NM_000098.1	Carnitine palmitoyltransferase II (CPT2)	12.12 ± 1.23	3.49 ± 2.52	3.47	.034
	NM_000387.2	Carnitine/acylcarnitine translocase	15.74 ± 2.62	3.65 ± 0.99	4.32	.003
	NM_001608.1	Long chain acyl-CoA dehydrogenase (ACADL)	8.61 ± 1.48	2.50 ± 0.08	3.45	.006
	NM_001966.1	Enoyl-CoA:hydratase 3-hydroxyacyl-CoA dehydrogenase (EHHADH)	37.93 ± 5.45	5.47 ± 1.43	6.93	.001
	NM_006111.1	Mitochondrial 3-oxoacyl-CoA thiolase	49.23 ± 10.43	21.29 ± 9.95	2.31	.088
	NM_006117.1	Peroxisomal D3,D2-enoyl-CoA isomerase (PECI)	95.83 ± 13.01	20.28 ± 3.02	4.73	.001
	NM_003500.1	Branched chain acyl-CoA oxidase	140.15 ± 18.30	73.41 ± 20.30	1.91	.043
	NM_001752.1	Catalase (CAT), mRNA	12.27 ± 2.16	0.97 ± 0.39	12.70	.002
VLDL secretion	NM_000253.1	Microsomal triglyceride transfer protein (MTP)	46.19 ± 7.43	16.86 ± 4.24	2.74	.008
	NM_000384.1	Apolipoprotein B-100	13.86 ± 3.19	7.62 ± 2.55	1.82	.161
	NM_001645.2	Apolipoprotein C-I (APOC1)	614.75 ± 82.47	301.14 ± 61.67	2.04	.014
	NM_000483.3	Apolipoprotein C-II (APOC2)	501.93 ± 30.57	288.10 ± 49.95	1.74	.013
	NM_000040.1	Apolipoprotein C-III (APOC3)	625.93 ± 126.08	312.83 ± 67.97	2.00	.058
	NM_001646.1	Apolipoprotein C-IV (APOC4)	57.96 ± 6.55	15.61 ± 5.89	3.71	.001
	NM_000041.1	Apolipoprotein E (APOE)	353.83 ± 49.92	258.38 ± 53.30	1.37	.229

[a] The gene expression levels (sample mean ± standard error) shown were median normalized.
[b] Fold difference of gene expression levels (sample mean) between NASH liver tissues and normal liver controls.
[c] Two-tailed student *t* test.

NCs (Fig. 3E). The more striking observation was that the expression of CPT-1 (Fig. 3F) and ApoB (Fig. 3G) in P112 was negligible compared with NCs, unlike all other NASH livers.

3.4. *Lipid metabolism in all NASH livers except NASH liver P112*

Because all major lipid metabolism pathways were elevated in NASH livers compared with NCs, the relative increase among all these pathways was compared to reveal their relative contributions to steatosis. For this purpose, genes whose products govern the rate-limiting step in each pathway were compared among NASH livers and NCs.

The ratio of the gene expressions for CD36 vs CPT-1 (CD36/CPT-1) was used to evaluate the relative up-regulation of the fatty acid uptake pathway (CD36) vs the fatty acid oxidation pathway (CD36 uptake/oxidation) in each individual subject. Calculated from the qRT-PCR results, the CD36 uptake to oxidation ratio was much greater in NASH patients than that in NCs (Fig. 4A, NASH/NC = 13.0, $P < .001$). Similar results were obtained for CD36/ApoB (Fig. 4B, NASH/NC = 15.9, $P < .001$), indicating that the up-regulation of CD36-mediated fatty acid uptake is far greater than that of fatty acid oxidation and VLDL secretion.

The ratio of the gene expression for FABPpm vs CPT-1 was greater in NASH livers than in NCs (Fig. 4C). However this increase was small and did not achieve statistical significance (NASH/NC = 1.8, $P = .13$). The ratio of the gene expression for FABPpm vs ApoB was also modestly increased in NASH livers without statistical significance (Fig. 4D, NASH/NC = 1.7, $P = .12$).

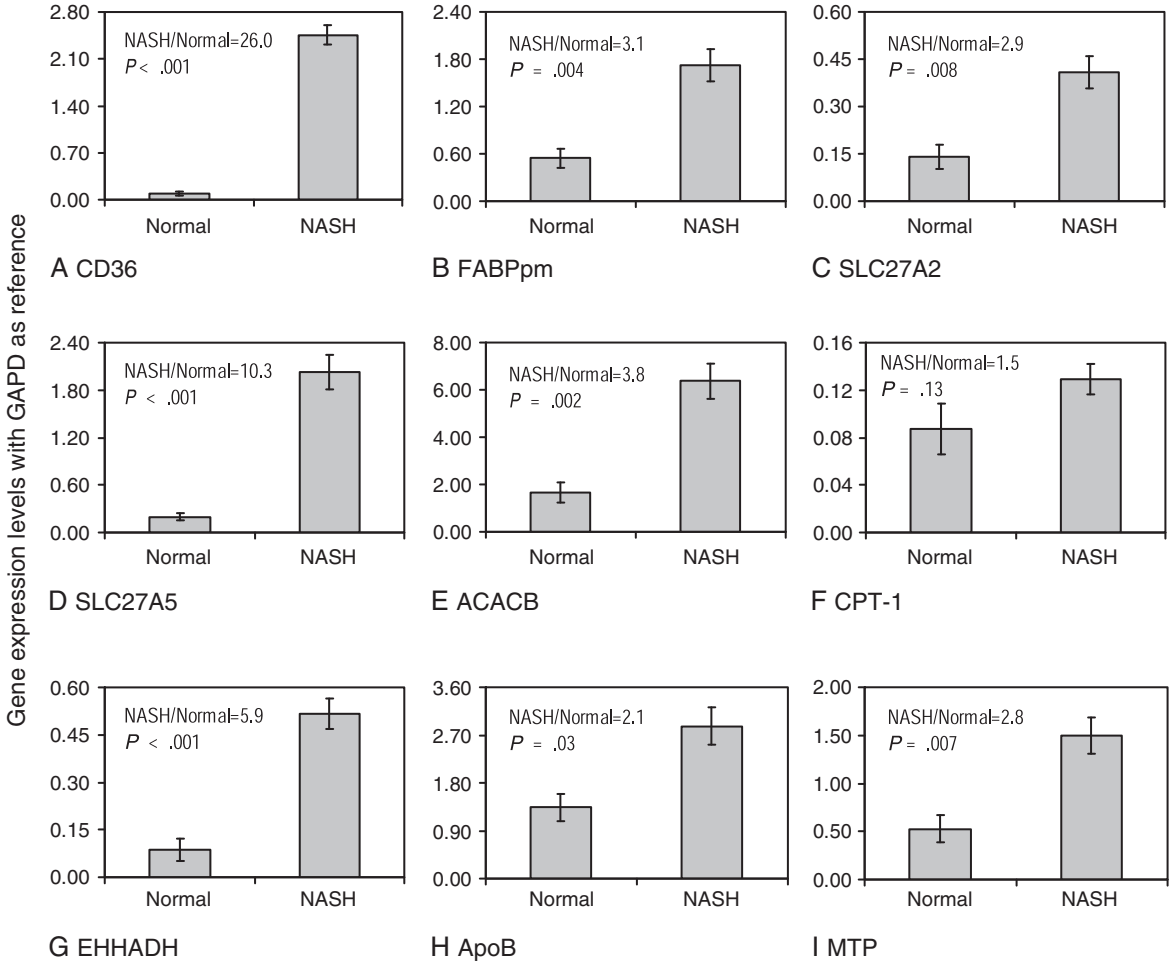


Fig. 2 – Quantitative RT-PCR analysis of the expression of genes involved in hepatic lipid metabolism. Gene expression levels of (A) CD36, (B) FABPpm, (C) SLC27A2, (D) SLC27A5, (E) ACACB, (F) CPT-1, (G) EHHADH, (H) ApoB, and (I) MTP were analyzed by qRT-PCR as described in "Methods." Specific primer pairs are specified in Table 2. The cDNAs prepared from NASH patient samples (n = 27) and NC samples (n = 6) were analyzed in duplicate. The gene expression level of each sample was normalized with that of GAPD. Sample means of the gene expression levels were plotted with error bars indicating the standard errors. The fold differences of gene expression between NASH livers and NCs (NASH/normal) and the P values of Student t tests are indicated. Statistically significant increases in gene expression were observed in NASH livers for all genes tested except for (F) CPT-1.

Similar results were obtained when examining fatty uptake pathways mediated by SLC27A2 (Fig. 4E, F) and SLC27A5 (Fig. 4G, H).

To evaluate the role of fatty acid de novo synthesis in steatosis, the relative expression level of ACACB vs CPT-1 was examined and found to be significantly higher in NASH livers (Fig. 4I, NASH/NC = 2.8, P = .03), as was the relative expression level of ACACB vs ApoB (Fig. 4J, NASH/NC = 2.4, P = .005). However, the up-regulation of the ACACB expression level in NASH livers was much lower than that of CD36 (compare with Fig. 4A and B).

3.5. *Elevated protein expression of CD36 and CPT-1 in NASH livers*

To examine the expression of CD36 and CPT-1 at the protein level, Western blot analyses were performed with the lysates made from NASH liver biopsies (NASH, n = 9; control, n = 4). As normal healthy liver tissue from adolescent is not available, non-NASH liver biopsies free from steatosis were used as controls. Blots were probed with antibodies specific for CD36 and CPT-1, respectively. A separate blot was also probed for β-actin as a loading controls. Whereas all samples had similar signals for actin, NASH patients exhibited higher expression of CD36 and CPT-1 (Fig. 5A). Quantitation of the results with National Institutes of Health Image software indicated that there were significant increases in CD36 and CPT-1 proteins in NASH livers: for CD36, NASH/NC = 6.7, P = .001; for CPT-1, NASH/NC = 3.3, P = .001 (Fig. 5B).

4. Discussion

4.1. *Elevated fatty acid uptake and de novo synthesis are common in NASH livers*

Most of the lipid metabolism activity in liver can be grouped into 4 major pathways: (1) fatty acid uptake, (2) de novo fatty

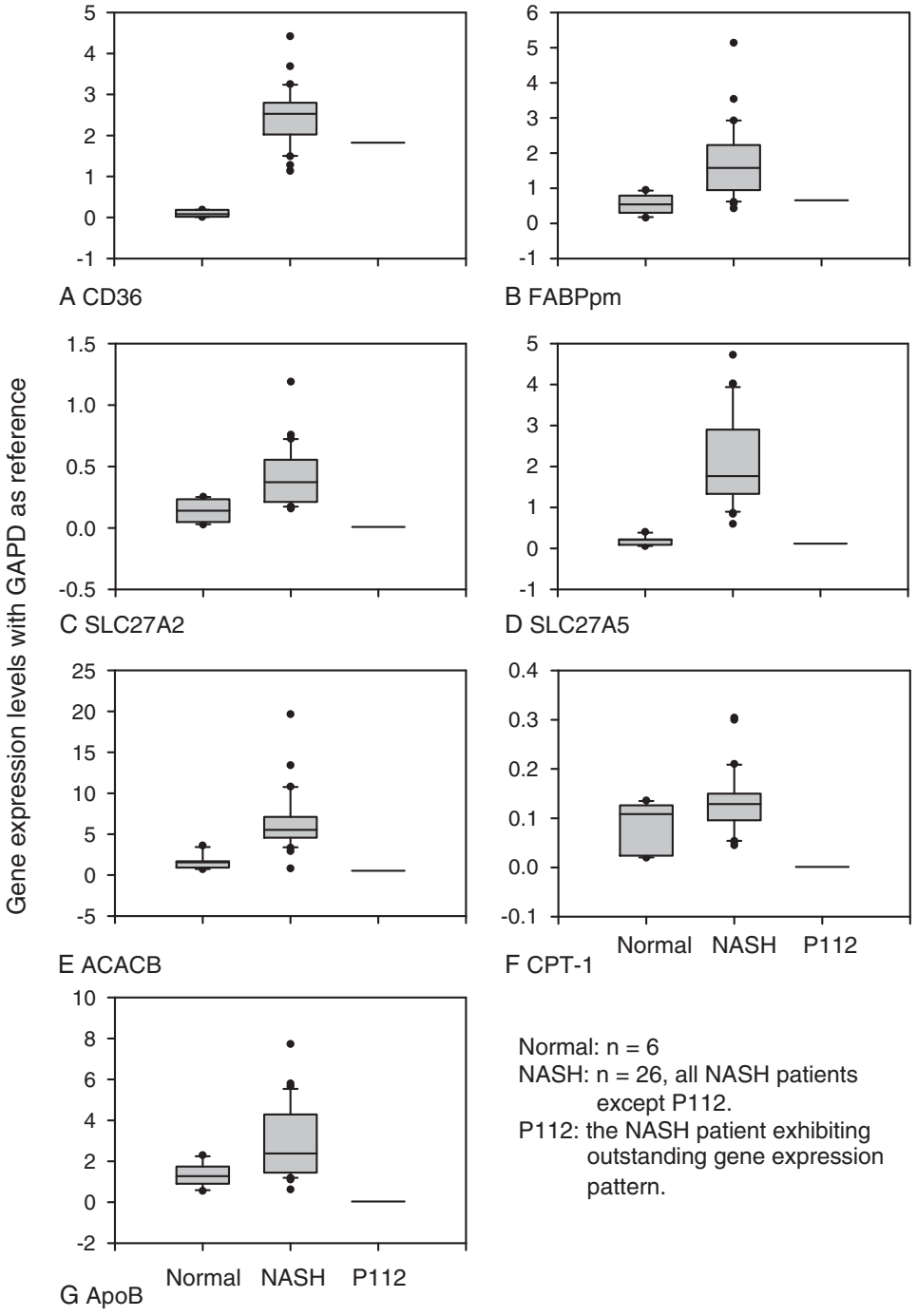
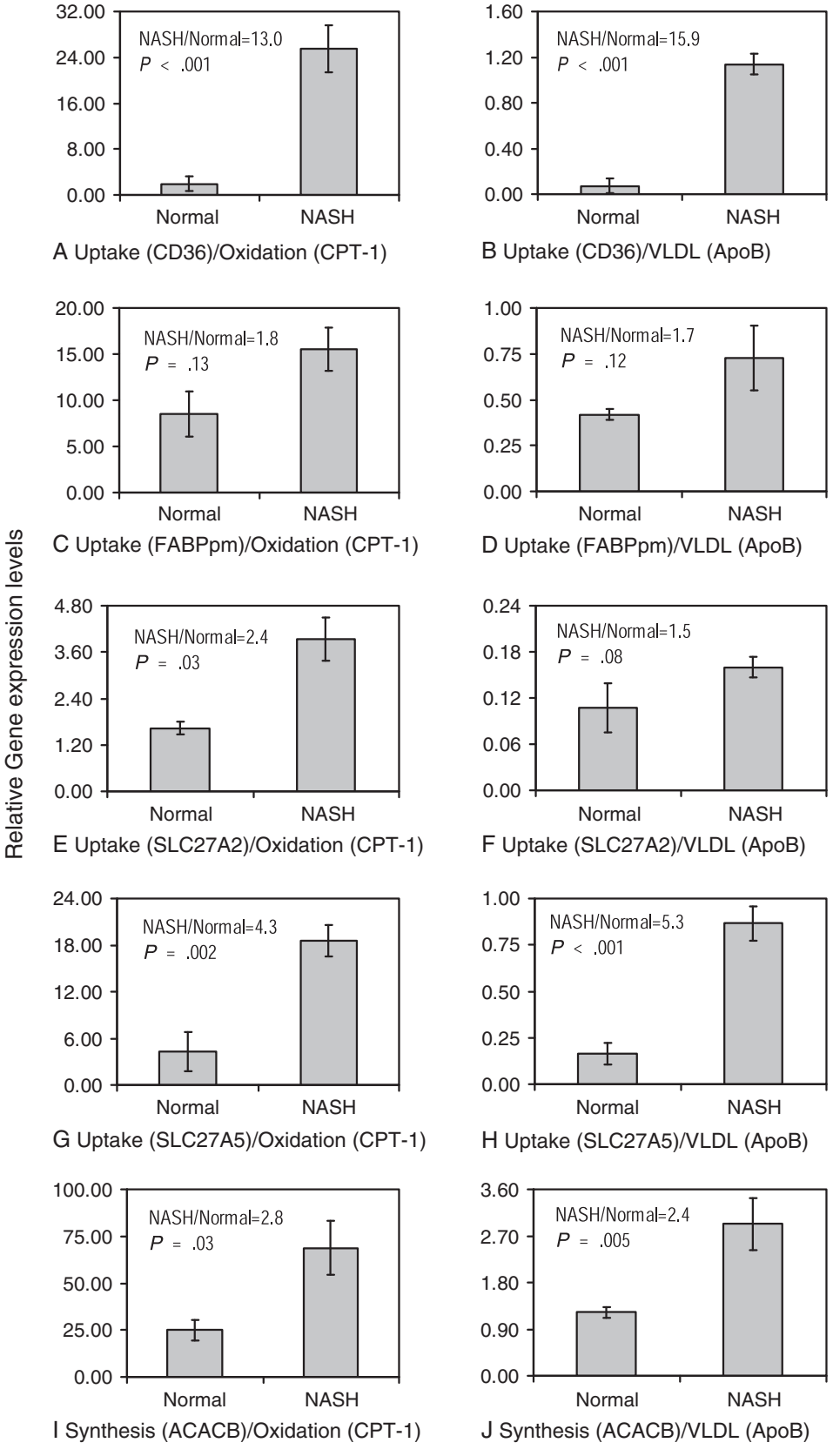


Fig. 3 – Two different expression patterns for lipid metabolism–related genes in NASH livers. Most of the NASH livers (26 of 27) share a similar gene expression pattern with increased gene expression for all genes examined; the other liver (P112) exhibited a very different pattern with several genes down-regulated. The gene expression levels of (A) CD36, (B) FABPpm, (C) SLC27A2, (D) SLC27A5, (E) ACACB, (F) CPT-1, and (G) ApoB were compared among NCs and 2 groups of NASH livers. P112 was similar to other NASH livers in that CD36 expression was increased, but the expression of all other genes examined differed from other NASH livers. Note that the CPT-1 and ApoB gene expression levels in P112 are negligible comparing to the means of the NCs. These data suggested that the down-regulated pathways for fatty acid oxidation (represented by the rate-limiting CPT-1) and VLDL secretion (represented by the rate-limiting ApoB), together with the up-regulated fatty acid uptake (through CD36), caused the development of steatosis in P112.

acid synthesis, (3) oxidation of fatty acids, and (4) secretion of VLDL. Abnormalities in any of these pathways may impair the dynamic balance of the homeostasis of lipids in hepatocytes, causing abnormal lipid depletion or accumulation. To study major lipid metabolism pathways in NASH livers, we examined these pathways at the same time, taking advantage of the

high throughput microarray technique complemented by qRT-PCR assays.

In general, NASH livers exhibited elevated activity in all lipid metabolism pathways. In the liver, 4 independent fatty acid transporters were reported: CD36, FABPpm, SLC27A2, and SLC27A5. Increased expression of all these transporters was observed, with CD36 exhibiting the highest increase. However, elevated gene expression was also observed for genes involved



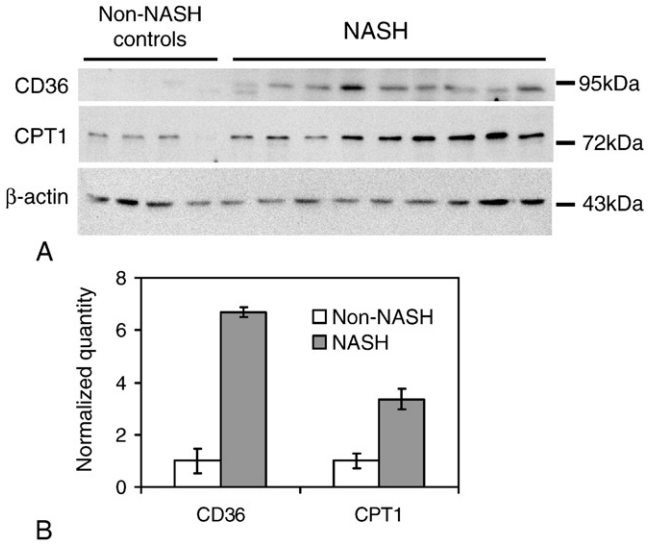


Fig. 5 – Elevated expression of CD36 and CPT-1 proteins in NASH livers. A, Western blot analyses were performed with lysates prepared from liver biopsies of NASH and non-NASH patients. The non-NASH controls were of normal BMI and free from steatosis. The blots were probed for CD36, CPT-1, and ß-actin as these proteins. Whereas similar signals for actin were detected for all samples, the NASH livers exhibited stronger signals for both CD36 and CPT-1. B, The Western blot results were quantitated with National Institutes of Health Image software. The normalized quantities of the CD36 and CPT-1 signals were the densities of the CD36 and CPT-1 bands divided by those of the ß-actin bands, respectively. The mean values for NASH and non-NASH patients were plotted with error bars representing the standard error of the means.

in fatty acid de novo synthesis, fatty acid oxidation, and VLDL secretion. By comparing the gene expression levels of the genes governing the rate-limiting step in each pathway, it became clear that the up-regulation of fatty acid uptake (through CD36) is more pronounced than other lipid metabolism pathways, suggesting a major role for CD36 in the development of steatosis. Our data also showed that fatty acid uptake mediated by SLC27A5, SLC27A2, and FABPpm, respectively, was up-regulated to a greater degree than the pathways of fatty acid oxidation or VLDL secretion, suggesting that these fatty acid uptake transporters also contribute to the development of fatty livers, albeit with a smaller contribution compared with CD36. The elevated expression for CD36 and CPT-1 at the protein level was also observed in NASH livers (NASH, n = 9; control, n = 4), consistent with the elevated mRNA levels for these genes. It is noteworthy that the increase in the protein level of CD36 in NASH livers was higher than that of CPT-1, consistent with the mRNA results.

The elevated gene transcriptions of these fatty acid transporters (Table 3 and Fig. 2) were consistent with the previous studies where individual transporters were examined [25,26]. Our new finding is that increases in the expression of fatty acid transporters were greater than those of the fatty acid oxidation and VLDL secretion pathways.

Our data also revealed that fatty acid de novo synthesis was up-regulated. Our results are consistent with those previously reported by several groups that the expression of genes involved in de novo fatty acid synthesis is increased in NASH livers [6,7]. Furthermore, we demonstrated that this increase in genes involved in de novo synthesis is greater than the increase in genes involved in fatty acid oxidation and VLDL secretion. The elevated de novo synthesis could be explained by the elevated insulin levels in our NASH patients [10]. Our previous work suggests that alcohol metabolism also contributes to the elevated lipid synthesis in NASH livers [13].

Lipid can also be introduced into liver through lipoprotein receptors including low-density lipoprotein (LDL) receptor-related proteins and LDL receptor. A recent study with nonobese patients carrying apolipoprotein C3 gene variants (C482T, T455C, or both) [27] implicated elevated activity of the LDL receptor in steatosis. These mutations are

Fig. 4 – Comparison of the gene expression levels among lipid metabolism pathways. To compare different lipid metabolism pathways, genes whose products govern the rate-limiting step in each lipid metabolism pathway were considered. Four fatty acid transporters (CD36, FABPpm, SLC27A2, and SLC27A5) mediate the rate-limiting steps of fatty acid uptake. ACACB, CPT-1, and ApoB were the protein factors mediating the rate-limiting step for de novo fatty acid synthesis, fatty acid oxidation, and VLDL secretion, respectively. A, Comparison between fatty acid uptake (CD36) and fatty acid oxidation (CPT-1) pathways. The ratio of the gene expression levels for CD36 and CPT-1 was calculated for each NASH liver (n = 26, excluding P112) and NC (n = 6). This ratio is significantly greater in NASH liver than in NC liver, indicating that the up-regulation of fatty acid uptake is greater than the up-regulation of fatty acid oxidation. B, Comparison between fatty acid uptake (CD36) and VLDL secretion (ApoB) pathways. The ratio of the gene expression levels for CD36 and ApoB was calculated for each NASH liver and NC. This ratio is significantly greater in NASH livers than in NCs, indicating that the up-regulation of fatty acid uptake is greater than the up-regulation of VLDL secretion. C, Comparison between fatty acid uptake (FABPpm) and fatty acid oxidation (CPT-1) pathways. D, Comparison between fatty acid uptake (FABPpm) and VLDL secretion (ApoB) pathways. E, Comparison between fatty acid uptake (SLC27A2) and fatty acid oxidation (CPT-1) pathways. F, Comparison between fatty acid uptake (SLC27A2) and VLDL secretion (ApoB) pathways. G, Comparison between fatty acid uptake (SLC27A5) and fatty acid oxidation (CPT-1) pathways. H, Comparison between fatty acid uptake (SLC27A5) and VLDL secretion (ApoB) pathways. I, Comparison between de novo fatty acid synthesis (ACACB) and fatty acid oxidation (CPT-1) pathways. J, Comparison between de novo fatty acid synthesis (ACACB) and VLDL secretion (ApoB) pathways. Note that the expression levels were always greater in fatty acid uptake pathways and de novo synthesis pathway than in fatty acid oxidation pathway and VLDL secretion pathway, although statistical significance was not achieved in panels C and F.

associated with higher levels of fasting plasma apolipoprotein C3 concentration and NAFLD. It is known that excess apolipoprotein C3 could inhibit lipoprotein lipase leading to hypertriglyceridemia, which leads to steatosis through increased activity of the LDL receptor. However, examination of our microarray data indicated that none of these receptors were significantly changed in NASH livers at the mRNA level (data not shown).

Therefore, we conclude that elevated fatty acid uptake and de novo synthesis are the common pathways leading to steatosis in NASH patients and that the CD36 mediated fatty acid uptake is the dominant cause.

Many of the genes studied here are regulated by the transcriptional factors peroxisome proliferator-activated receptors and sterol regulatory element binding proteins. However, examination of the microarray data indicated that none of these transcriptional factors were differentially expressed in NASH livers compared with NCs (data not shown). Although this finding needs to be confirmed, a different mechanism may cause the elevated gene expression in the NASH livers.

4.2. *NASH livers commonly exhibit elevated fatty acid oxidation and VLDL secretion*

Nonalcoholic steatohepatitis livers also featured elevated fatty acid oxidation and VLDL secretion, which consume/remove lipid from liver. The enhanced activities of fatty acid oxidation and VLDL secretion pathways indicated that the NASH livers tried but failed to reduce the lipid load toward a normal level.

It is argued that impaired fatty acid oxidation could lead to steatosis [8,9]. Blocking fatty acid oxidation by inhibiting CPT-1 induces steatosis in a mouse model [28]. Yet our data suggested an opposite scenario for NASH patients. Our microarray data suggested a significant increase of the CPT-1 expression in NASH livers. Moderate increase of CPT-1 was also observed by qRT-PCR, although it was not statistically significant. Nevertheless, Western blot analysis suggested that CPT-1 protein level is higher in NASH livers. Overall, fatty acid oxidation seemed to be increased in mitochondria and peroxisomes in our NASH patients, albeit to a level insufficient to compensate for the import/synthesis of fatty acids. Our data are consistent with the report that obese *ob/ob* mice exhibit elevated fatty acid oxidation [29]. However, it is noteworthy that all our NASH patients, being children and adolescents, have relatively mild fibrosis compared with adult NASH patients. Therefore, our conclusion may not apply to adult NASH patients, as more severe fibrosis is likely associated with more extensive damage in mitochondria and decreased activity of mitochondria oxidation.

Charlton et al [5] reported that all 7 NASH patients in their study groups had a consistent lower level of expression of ApoB than normal and obese (non-NASH) subjects. Their results suggested that impaired VLDL secretion is a universal phenomenon in NASH. However, a recent study by Fujita et al [30] led to the opposite conclusion that the VLDL secretion by NASH liver is significantly higher than that of NCs. As ApoB is the critical protein component of VLDL and the availability of ApoB is rate limiting in the assembly and secretion of VLDL, our result that NASH livers exhibited elevated ApoB expression is in harmony with the report of Fujita et al.

4.3. *Atypical lipid metabolism in NASH patient P112*

In examining the gene expression patterns of individual subjects, one NASH liver (P112) did not fit into the general picture described above. P112 had elevated expression of CD36. However, this subject did not exhibit elevated gene expression for other fatty acid transporters. Neither did this subject exhibit elevated expression in genes responsible for fatty acid de novo synthesis, fatty acid oxidation, or VLDL secretion. Instead, a much lower expression (lower than both NCs and NASH livers) was observed for ApoB gene and CPT-1, indicating impaired VLDL secretion and fatty acid oxidation. Apparently, the suppressed VLDL secretion and fatty acid oxidation could lead to lipid accumulation in hepatocytes and worsen the situation caused by elevated activity of CD36. Although rare (1 of 27 NASH subjects), this particular patient is very important because the patient represents a different gene expression pattern and suggests a distinct etiology for steatosis.

The interesting mRNA expression level of P112 prompted us to revisit the pathologic data for this patient and other NASH patients. However, the BMI, serum markers for lipid, glucose, and steatosis and fibrosis stages of this patient are similar to other NASH patients. High levels of VLDL were found in many of the NASH patients including P112 (data not shown). We were surprised that the serum VLDL level of P112 was not lower, as the liver VLDL pathway was down-regulated, at least at the mRNA level. Possible explanations for this inconsistency are as follows: (1) VLDL is also produced in the intestine, and so VLDL production by the intestine might compensate for a decrease in production by the liver; and (2) the serum level may also be affected by the consumption of VLDL, which could also be down-regulated. Further study with a larger study group is needed to determine the prevalence of NASH livers with suppressed fatty acid oxidation and VLDL secretion.

One concern about the strategy of our study is whether the "rate-limiting" steps at normal physiologic condition were still rate limiting in NASH livers. The fact that many genes were activated in one pathway suggested that many steps are rate limiting in NASH livers. Nevertheless, the "rate-limiting" enzymes showed a profound increase among all the enzymes in the same pathways. Likely, these enzymes are those of the rate-limiting steps and are at least good representations of the pathways.

In summary, comparison of the major pathways for lipid metabolism in NASH livers suggested that up-regulated fatty acid uptake and de novo synthesis are the accompanying causes for lipid accumulation in most NASH livers. Evidence is also provided to show that impaired VLDL secretion together with suppressed fatty acid oxidation could contribute to the development of steatosis. This is the first evidence at the molecular level that more than one pathway leading to steatosis is dysregulated in NASH patients. Our study promises a simple method for the accurate identification of the cause for steatosis in individual patients. This method may

allow us to specifically target therapeutic treatments for individual patients.

Acknowledgment

This work was supported by an unrestricted grant from the Peter and Tommy Fund, Buffalo, NY. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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