Serum aminotransferases in nonalcoholic fatty liver disease are a signature of liver metabolic perturbations at the amino acid and Krebs cycle level[1,2]

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ABSTRACT

Background: Extensive epidemiologic studies have shown that cardiovascular disease and the metabolic syndrome (MetS) are associated with serum concentrations of liver enzymes; however, fundamental characteristics of this relation are currently unknown.

Objective: We aimed to explore the role of liver aminotransferases in nonalcoholic fatty liver disease (NAFLD) and MetS.

Design: Liver gene- and protein-expression changes of aminotransferases, including their corresponding isoforms, were evaluated in a case-control study of patients with NAFLD (n = 42), which was proven through a biopsy (control subjects: n = 10). We also carried out a serum targeted metabolite profiling to the glycolysis, gluconeogenesis, and Krebs cycle (n = 48) and an exploration by the next-generation sequencing of aminotransferase genes (n = 96). An in vitro study to provide a biological explanation of changes in the transcriptional level and enzymatic activity of aminotransferases was included.

Results: Fatty liver was associated with a deregulated liver expression of aminotransferases, which was unrelated to the disease severity. Metabolite profiling showed that serum aminotransferase concentrations are a signature of liver metabolic perturbations, particularly at the amino acid metabolism and Krebs cycle level. A significant and positive association between systolic hypertension and liver expression levels of glutamic-oxaloacetic transaminase 2 (*GOT2*) messenger RNA (Spearman R = 0.42, P = 0.03) was observed. The rs6993 located in the 3′ untranslated region of the *GOT2* locus was significantly associated with features of the MetS, including arterial hypertension [P = 0.028; OR: 2.285 (95% CI: 1.024, 5.09); adjusted by NAFLD severity] and plasma lipid concentrations.

Conclusions: In the context of an abnormal hepatic triglyceride accumulation, circulating aminotransferases rise as a consequence of the need for increased reactions of transamination to cope with the liver metabolic derangement that is associated with greater gluconeogenesis and insulin resistance. Hence, to maintain homeostasis, the liver upregulates these enzymes, leading to changes in the amounts of amino acids released into the circulation. *Am J Clin Nutr* 2016; 103:422–34.

Keywords: fatty liver, gene expression, liver injury, metabolomics, transaminases

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD)[10] is a chronic liver disease that affects adults and children in most parts of the world (1). NAFLD has emerged as a contributor to cardiovascular disease (CVD) morbidity and mortality (2) and overall non–liver-related causes of death (3). NAFLD is regarded as the hepatic manifestation of the metabolic syndrome (MetS) because it has long been associated with all of their components (4–8).

In recent decades, plasma concentrations of liver enzymes that are ordinarily involved in the intermediary metabolism of glucose and amino acids, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) [also known as glutamate-pyruvate transaminase (GPT) and glutamic-oxaloacetic transaminase (GOT), respectively] have been used as surrogate indicators of NAFLD severity (9, 10). However, this concept is being largely revised because it was shown that aminotransferase

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[2] Supplemental Material, Supplemental Figures 1–4, and Supplemental Tables 1–5 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

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[10] Abbreviations used: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CVD, cardiovascular disease; GOT, glutamic-oxaloacetic transaminase; GPT, glutamate-pyruvate transaminase; MetS, metabolic syndrome; mRNA, messenger RNA; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NGS, next-generation sequencing; NNLH, near-normal liver histology; TCA, tricarboxylic acid; UTR, untranslated region.

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concentrations are poorly informative of NAFLD progression (11), which ranges from a mild disease stage called simple steatosis or nonalcoholic fatty liver (NAFL) to a disease stage characterized by liver cell injury, inflammatory lobular infiltrate, hepatocellular ballooning, and variable fibrosis known as nonalcoholic steatohepatitis (NASH) (12).

Several large epidemiologic studies showed that CVD, including coronary heart disease (13), the atherothrombotic risk profile (14), overall risk of CVD (15), and type 2 diabetes (16), visceral fat accumulation (17), and obstructive sleep apnea (18) are associated with serum concentrations of liver enzymes. Collectively, these observations led to the suggestion that hepatic steatosis might be the underlying cause of elevated circulating concentrations of aminotransferases. However, it remains unclear whether abnormal concentrations of these enzymes are exclusively explained by the leaking of the intracellular hepatocyte content into the circulation owing to a hepatocyte injury or whether elevations of aminotransferases toward the upper-normal threshold reflect high levels of hepatic transamination in response to increased NAFLD-associated metabolic demands (19, 20). In the latter scenario, the induction of liver gene expression might potentially occur; however, it is currently unknown whether human NAFLD is associated with an induction of aminotransferase activity. In this study, we explored the changes of liver gene and protein expression of aminotransferases, including their corresponding isoforms, in a case-control study of patients with NAFLD, which was proven through a biopsy. In addition, because of the role of aminotransferases in hepatic metabolism of amino acid and glucose, we carried out a serum-targeted metabolite profiling to the glycolysis, gluconeogenesis, and tricarboxylic acid (TCA) cycle. Finally, we performed an exploration of genetic variation by the next-generation sequencing (NGS) in genes encoding for transaminases to study its contribution to the disease biology. In the current study, we applied these strategies to test the hypothesis that, in the framework of metabolic derangements associated with fatty liver and MetS, serum concentrations of transaminases reflect the changes in the hepatic metabolism to adapt to a stressful metabolic environment.

METHODS

Study design and patient selection

Serum and DNA samples from healthy individuals and those diagnosed with NAFLD as well as liver biopsies from all the patients were obtained (after the receipt of the written informed consent of subjects) according to institutional review board–approved protocols (protocols IDIM 0104/04, IDIM 2212/08, 104/HGAZ/09, 89/100, and 1204/2012). All investigations that were performed in this study were conducted in accordance with the guidelines of the 1975 Declaration of Helsinki.

In this case-control study, case participants and control subjects were selected during the same study period from the same population of patients who were attending the Liver Unit, and all subjects shared the same demographic characteristics. The recruitment of participants and the collection of biospecimens was initiated in January 2006 and ended in December 2014.

During the study period, we included all subjects, either cases or controls, who were willing to participate, who met the eligibility criteria, and in whom the biological specimens (liver tissue, serum sample, and DNA extracted from white blood cells) were available to conduct the molecular, metabolomics, or genetic studies.

Cases (patients with NAFLD) were included in the study if there was histopathologic evidence of NAFLD, either NAFL or NASH, on the basis of a liver biopsy conducted within the study period. Exclusion criteria were secondary causes of steatosis, including alcohol abuse ($\geq$30 g alcohol/d for men and $\geq$20 g alcohol/d for women), total parenteral nutrition, hepatitis B and hepatitis C virus infections, and the use of drugs known to precipitate steatosis. With the use of a standard clinical and laboratory evaluation, as well as liver-biopsy features when applicable, autoimmune liver disease, metabolic liver disease, Wilson disease, and α-1-antitrypsin deficiency were likewise ruled out in all patients. The different proportion of patients with NAFL and NASH included in each substudy was explained by the availability of the biological sample required for the molecular studies, including RNA for gene expression or the amount of serum for metabolomic profiling.

For obvious ethical reasons, control subjects were not exposed to the potential risk of a liver biopsy; hence, in explorations that involved molecular studies requiring liver tissue, patients with near-normal liver histology (NNLH) were included. Patients with NNLH were referred to the Liver Unit by primary care providers or medical specialists from other disciplines because they had abnormal liver enzymes, either aminotransferases or γ-glutamyltransferase or alkaline phosphatase; NNLH subjects were asymptomatic patients. In NNLH subjects, a liver biopsy was justified because of the presence of persistently mildly elevated serum liver enzyme activity for $>$6 mo. In all NNLH subjects, all causes of common liver disease were ruled out, and these patients were included in the study if they did not present either histologic evidence of fatty change or necrotic-inflammatory activity and had either minimal changes or mild cholestasis as the histologic diagnosis. During the follow-up period, none of the NNLH patients developed autoimmune disease or chronic hepatitis.

Healthy subjects enrolled in the targeted-metabolomics study and in the NGS study were selected from individuals whose age and sex matched those of the NAFLD patients in whom, in addition to the standard health assessment, a careful ultrasonographic examination of the liver was performed to exclude fatty liver infiltration. Controls were included at any study phase if they did not show features of MetS and did not have fatty liver at the liver ultrasound.

Physical, anthropometric, and biochemical evaluation and biochemical determinations

Anthropometric measurements and blood samples were obtained from each patient at the time of the liver biopsy and before any intervention; details are provided in **Supplemental Material**.

Liver biopsy, histopathologic evaluation, and immunohistochemistry

A liver biopsy was performed before any intervention with ultrasound guidance and with a the use of a modified 1.4-mm-diameter

Menghini needle (Hepafix; Braun) under local anesthesia in an outpatient setting. Immunostaining for aminotrasferases was performed on liver specimens of NAFLD patients and controls that were previously included in paraffin. Details are provided in Supplemental Material.

RNA preparation and real-time reverse transcriptase–polymerase chain reaction for quantitative assessment of messenger RNA expression

Total RNA was prepared from liver tissue with the use of a phenol-extraction step method with an additional deoxyribonuclease digestion. Complete details are provided in Supplemental Material; primer sequences are shown in **Supplemental Table 1**.

Serum targeted–TCA cycle metabolite profiling

Serum-targeted–TCA cycle metabolite profiling from both NAFLD patients and controls was obtained with the use of an HPLC–mass spectrometry method (details are provided in Supplemental Material). Metabolomic profiling was performed at the Northwest Metabolomics Research Center Core facility (University of Washington), and liquid-chromatography conditions as well as a complete list of the explored metabolites are provided in **Supplemental Tables 2** and **3**, respectively.

Functional enrichment analysis

To understand the biological meaning of the observed metabolic changes, we performed a functional enrichment analysis with the use of the MetaboAnalyst 2.0 program (http://www.metaboanalyst.ca/MetaboAnalyst/) (21). The analysis was based on several libraries that contain ~6300 groups of biologically meaningful metabolite sets that were collected primarily via human studies.

NGS of transaminase genes

The genetic analysis was performed on genomic DNA extracted from white blood cells (22) and was quantified with the use of a Qubit DNA high-sensitivity assay kit (Thermo Fisher Scientific). NGS technology was used to screen for the presence of genetic variation in aminotransferase loci that covered all protein-coding exons (*GPT*: 3.09 kb, 11 exons; *GPT2*: 46.09 kb, 12 exons; *GOT1*: 33.90 kb, 9 exons; and *GOT2*: 27.00 kb, 10 exons) in addition to 5′ and 3′ untranslated regions (UTRs), which were examined with the use of semiconductor technology offered in the Ion Torrent PGM system (Life Technologies) with a 316 chip. Complete details, including information about the variant calling, estimation of quality control, data analysis, and prediction of a variant or mutation effect are provided in Supplemental Material.

Cell culture study

Huh7 human hepatoma cells were treated with increasing concentrations (50, 100, or 250 μmol/L) of a free fatty acid (palmitic acid) to explore changes in the transcriptional level and enzymatic activity of aminotransferases. Complete details are provided in Supplemental Material.

Statistical analysis

Quantitative data were expressed as means ± SDs unless otherwise indicated. Because statistically significant differences in SDs were observed between the groups in most variables, and the distribution was significantly skewed in most cases, we chose to be conservative and assessed the differences between the groups with the use of nonparametric Mann-Whitney *U* or Kruskal-Wallis tests. For a multiple regression, ordinal multinomial, logistic, or ANCOVA analysis, we adjusted for covariables such as BMI, HOMA-IR, or even aminotransferase concentrations, which were normally distributed through log transformation. Univariate correlations were obtained via the Spearman rank correlation test. The CSS/Statistica program package version 6.0 (StatSoft) was used in the analyses.

RESULTS

As expected, compared with control subjects, patients with NAFLD showed most of the risk factors of the MetS, including elevated BMI, waist-hip ratio, fasting glucose, insulin and HOMA indexes, and CVD risk factors. Clinical and biochemical characteristics of patients with NAFLD and controls are shown in **Tables 1–3**.

Liver transcriptional activity of aminotransferases is significantly upregulated in NAFLD

Although 2 isoforms of human ALT exist, namely ALT1 and ALT2 (protein names), in circulation, the enzymatic activity of isoforms is measured indistinguishably as a whole. The gene that encodes for the cytosolic ALT1, also known as *GPT* or *GPT1*, is located in chromosome 8 (8q24.3), whereas ALT2 is encoded by a different gene (*GPT2*), which is located in chromosome 16 (16q12.1). Likewise, AST (protein name) and GOT exist as cytoplasmic and the mitochondrial forms GOT1 and GOT2, respectively, which are also measured indistinctly in the circulation. The gene that encodes for the soluble GOT1 is located in chromosome 10 (10q24.2), whereas the gene that encodes for the mitochondrial GOT2 is located in chromosome 16 (16q21). In this study, we will refer to aminotransferase genes with the use of their official gene symbols, including their related isoforms, *GPT*, *GPT2*, *GOT1*, and *GOT2*.

On the basis of the available evidence indicating that isoforms 1 and 2 of aminotransferases have not only different tissue expression patterns but also cellular localization as recently reviewed (23), we tested the hypothesis of whether the liver transcriptional activity of aminotransferases is deregulated in human NAFLD. For this purpose, we conducted a study that included 52 individuals of whom 42 subjects were patients with NAFLD, including 18 patients with NAFL and 24 patients with NASH, and 10 patients had NNLH (Table 1). Subjects with NNLH had abnormal aminotransferase concentrations without any evidence of fatty liver. Notably, we observed that fatty liver was associated with significantly greater *GPT*, *GPT2*, and *GOT1* mRNA levels than in subjects with NNLH (**Figure 1**), and the differences between groups remained significant after adjustment for BMI; *GOT2* mRNA was expressed at a very low level compared with that of the other transcripts explored in this study.

We next examined the pattern of liver transcript levels of aminotransferases according to the NAFLD histologic features,

TABLE 1
Physical, anthropometric, biochemical, and cardiovascular evaluation of patients with NAFLD and subjects with NNLH enrolled in the gene- and protein-expression analysis[1]

Variable	Group					
	NNLH	NAFL	NASH	P^2	P^3	P^4
Subjects, *n*	10	18	24	—	—	—
Women:men, %	60:40	50:50	60:40	NS	NS	NS
Age, y	44 ± 12[5]	55 ± 9	52 ± 9.6	<0.01	NS	<0.04
Physical activity, h/wk	0.7 ± 2	0.7 ± 1.5	1.9 ± 12.6	NS	NS	NS
Obesity and central obesity						
BMI, kg/m^2	25 ± 2.3	31 ± 5	33 ± 5	<0.003	NS	<0.0006
Waist circumference, cm	90 ± 9	109 ± 13	108 ± 14	<0.01	NS	<0.01
Waist:hip ratio	0.9 ± 0.05	0.97 ± 0.05	0.97 ± 0.08	<0.04	NS	<0.04
Body fat content, %	26 ± 3	38 ± 4.5	41 ± 3	<0.04	NS	<0.001
Peripheral insulin resistance						
Fasting plasma glucose, mg/dL	90 ± 14	99 ± 16	123 ± 60	NS	<0.01	<0.003
Fasting plasma insulin, mg/dL	5.5 ± 0.7	12 ± 4	15 ± 7	<0.001	NS	<0.004
HOMA-IR index	1.2 ± 0.3	3 ± 1.1	3.6 ± 1.8	<0.002	NS	<0.001
CVD risk factors						
SABP, mm Hg	100 ± 11	126 ± 13	129 ± 13	<0.02	NS	<0.01
DABP, mm Hg	62.5 ± 9.5	77 ± 11	83 ± 12.5	NS	NS	<0.02
Leukocyte count, cells/mm^3	8500 ± 3905	8280 ± 2298	8315 ± 2704	NS	NS	NS
Total cholesterol, mg/dL	193 ± 44	185 ± 42	226 ± 46	NS	NS	NS
HDL cholesterol, mg/dL	75 ± 20	51 ± 17	48 ± 11	<0.07	NS	<0.01
LDL cholesterol, mg/dL	90 ± 47	109 ± 31	141 ± 35	NS	<0.02	NS
Triglycerides, mg/dL	193 ± 200	143 ± 64	201 ± 132	NS	NS	<0.06
Uric acid, mg/dL	3.4 ± 0.8	5.3 ± 1.2	5.2 ± 1.5	<0.03	NS	<0.03
Liver phenotype						
ALT, U/L	99.7 ± 127	44 ± 32	74 ± 58	NS	<0.009	NS
AST, U/L	69.7 ± 60	30 ± 12	51 ± 28	<0.07	<0.003	NS
GGT, U/L	46 ± 26	45 ± 24	61 ± 37	<0.07	<0.06	NS
AP, U/L	216 ± 147	235 ± 150	189 ± 106	NS	NS	NS
CK-18 fragment	—	106 ± 98	397 ± 370	—	NS	—
Histologic features						
Degree of steatosis, %	0 ± 0	59 ± 27	58 ± 18	<0.0009	NS	<0.0005
Lobular inflammation, 0–3	—	1.0 ± 0.6	1.07 ± 0.5	—	NS	—
Portal inflammation, 0–2	—	0.0 ± 0.0	1.4 ± 0.6	—	<0.0002	—
Hepatocellular ballooning, 0–2	—	0.0 ± 0.0	0.93 ± 0.3	—	<0.00002	—
Fibrosis stage	—	0.0 ± 0.0	1.4 ± 0.6	—	<0.000006	—
NAS	—	3.3 ± 1.1	5.8 ± 1.1	—	<0.00002	—

[1]*P* values were calculated with the use of the Mann-Whitney *U* test with the exception of the women:men ratio for which the *P* value was calculated with the use of the chi-square test. ALT, serum alanine aminotransferase; AP, alkaline phosphatase; AST, serum aspartate aminotransferase; CK-18, cytokeratin-18; CVD, cardiovascular disease; DABP, diastolic arterial blood pressure; GGT, γ-glutamyl-transferase; NAFL, nonalcoholic fatty liver or simple steatosis; NAFLD, nonalcoholic fatty liver disease; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis; NNLH, near-normal liver histology and abnormal liver enzymes; SABP, systolic arterial blood pressure.
[2]For comparisons between NAFL and NNLH groups.
[3]For comparisons between NAFL and NASH groups.
[4]For comparisons between NASH and NNLH groups.
[5]Mean ± SD (all such values).

including those associated with potential liver injury. We observed that the abundance of *GPT*, *GPT2*, *GOT1*, and *GOT2* mRNAs did not differ between patients with an absence or presence of ballooning or patients with an absence or presence of lobular inflammation (**Table 4**). Similar results were observed when patients were classified according to the NASH activity score. We observed that the liver expression of *GPT* mRNA was significantly larger in patients with advanced fibrosis (Table 4) than in patients with mild fibrosis; fibrosis scores were significantly correlated with liver *GPT* mRNA levels (Spearman $R = 0.34$, $P = 0.03$). Accordingly, we observed that ALT (Spearman $R = 0.20$, $P = 0.0007$) and AST (Spearman $R = 0.30$, $P = 0.000001$) concentrations in circulation were significantly associated with liver fibrosis; however, neither ALT nor AST concentrations were associated with lobular inflammation.

When we explored the association between the abundance of aminotransferase transcripts and features of the MetS, we only observed a significant and positive association between systolic hypertension and liver expression levels of *GOT2* mRNA (Spearman $R = 0.42$, $P = 0.03$). In addition, in patients with arterial hypertension, significantly higher amounts of liver *GOT2* mRNA were obtained (Table 4).

Note that the liver transcriptional activity of aminotransferases showed not only a significant degree of correlation between

TABLE 2
Physical, anthropometric, biochemical, and cardiovascular evaluation of patients with NAFLD and healthy controls enrolled in the targeted metabolomics study[1]

Variable	Group					
	Control	NAFL	NASH	P^2	P^3	P^4
Subjects, *n*	16	16	16	—	—	—
Women:men, %	50:50	50:50	59:41	NS	NS	NS
Age, y	47.5 ± 13[5]	52 ± 8	51.7 ± 9	NS	NS	NS
Physical activity, h/wk	2.3 ± 2.2	0.23 ± 0.6	0.5 ± 0.9	<0.01	NS	<0.04
Obesity and central obesity						
BMI, kg/m^2	25 ± 2.5	30 ± 4.3	32 ± 7	<0.0008	NS	<0.001
Waist circumference, cm	85 ± 13	102 ± 9	107 ± 14	<0.0005	NS	<0.0002
Waist:hip ratio	0.87 ± 0.1	0.94 ± 0.06	0.96 ± 0.07	<0.01	NS	<0.02
Body fat content, %	27 ± 6	36 ± 10	35 ± 6	<0.07	NS	<0.008
Peripheral insulin resistance						
Fasting plasma glucose, mg/dL	74 ± 7	97 ± 15	98 ± 22	<0.00004	NS	<0.0004
Fasting plasma insulin, mg/dL	6 ± 2	10 ± 6	14 ± 7	<0.05	NS	<0.001
HOMA-IR index	1.1 ± 0.4	2.4 ± 1.4	3.5 ± 2	<0.001	NS	<0.0003
CVD risk factors						
SABP, mm Hg	113 ± 8.5	126 ± 17	127 ± 14	<0.03	NS	<0.01
DABP, mm Hg	69 ± 7.6	79 ± 9	81.5 ± 10	<0.01	NS	<0.008
Leukocyte count, cells/mm^3	5907 ± 1592	6043 ± 1243	6000 ± 1473	NS	NS	NS
Total cholesterol, mg/dL	221 ± 45	212 ± 48	228 ± 45	NS	NS	NS
HDL cholesterol, mg/dL	55 ± 12	57 ± 29	48 ± 10	NS	NS	NS
LDL cholesterol, mg/dL	143 ± 34	120 ± 40	133 ± 52	NS	NS	NS
Triglycerides, mg/dL	117 ± 68	161 ± 76	198 ± 117	<0.07	NS	<0.010
Uric acid, mg/dL	3 ± 0.6	4.8 ± 1.4	4.9 ± 2.4	<0.0002	NS	<0.002
Liver phenotype						
ALT, U/L	22 ± 8.5	53 ± 37	92 ± 84	<0.0004	<0.04	<0.000006
AST, U/L	19.5 ± 4.6	34 ± 14	58 ± 42	<0.0006	<0.03	<0.000002
GGT, U/L	39 ± 42	74 ± 48	71 ± 72	<0.02	NS	<0.02
AP, U/L	139.5 ± 57	237 ± 117	222 ± 127	<0.001	NS	<0.007
CK-18 fragment	—	262 ± 320	293 ± 278	NS	NS	NS
Histologic features						
Degree of steatosis, %	—	38 ± 14	61 ± 18	—	<0.001	—
Lobular inflammation, 0–3	—	0.64 ± 0.67	1.09 ± 0.7	—	NS	—
Portal inflammation, 0–2	—	0.0 ± 0.0	1.65 ± 0.6	—	<0.000001	—
Hepatocellular ballooning, 0–2	—	0.0 ± 0.0	0.82 ± 0.6	—	<0.01	—
Fibrosis stage	—	0.0 ± 0.0	1.06 ± 1.34	—	<0.02	—
NAS	—	2.3 ± 1.1	5.75 ± 1.7	—	<0.0001	—

[1]*P* values were calculated with the use of the Mann-Whitney *U* test with the exception of the women:men ratio, for which the *P* value was calculated with the use of the chi-square test. ALT, serum alanine aminotransferase; AP, alkaline phosphatase; AST, serum aspartate aminotransferase; CK-18, cytokeratin-18; CVD, cardiovascular disease; DABP, diastolic arterial blood pressure; GGT, γ-glutamyl-transferase; NAFL, nonalcoholic fatty liver or simple steatosis; NAFLD, nonalcoholic fatty liver disease; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis; SABP, systolic arterial blood pressure.
[2]For comparisons between NAFL and control groups.
[3]For comparisons between NAFL and NASH groups.
[4]For comparisons between NASH and control groups.
[5]Mean ± SD (all such values).

isoforms (*GPT* and *GPT2* mRNAs: Spearman $R = 0.45$, $P = 0.005$) but also between genes of different function (*GPT2* and *GOT1* mRNAs: Spearman $R = 0.51$, $P = 0.001$).

Liver protein-expression levels of aminotransferases: GPT2 and GOT2 (the mitochondrial isoforms of GPT and GOT, respectively) are significantly lower in NAFLD

Liver protein immunostaining patterns of GPT, GPT2, GOT1 and GOT2 were evaluated according to the disease status and NAFLD histologic features. The comparison between immunostaining scores of patients with NAFLD and those of subjects with NNLH showed no significant differences with the exception of a significant reduction of liver GPT2 and GOT2 protein levels in patients with NAFLD (**Figure 2**).

We observed that the immunostaining was confined to the hepatocytes, which showed a primary cytoplasmic staining pattern (Figure 2) Aminotransferase immunostaining, irrespective of the disease condition (i.e., either NNLH or NAFLD), was pronounced at the edge of the hepatocytes (Figure 2, insets), which was a finding that was particularly noticeable for GOT1 protein.

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TABLE 3
Physical, anthropometric, biochemical, and cardiovascular evaluation of patients and controls enrolled in the next-generation sequencing study[1]

Variable	Group					
	Control	NAFL	NASH	P^2	P^3	P^4
Subjects, *n*	32	32	32	—	—	—
Women:men, %	50:50	64:36	60:40	NS	NS	NS
Age, y	48 ± 13[5]	52 ± 9.7	51 ± 11	NS	NS	NS
Physical activity, h/wk	1.9 ± 2.5	3.3 ± 0.9	0.9 ± 1.9	NS	NS	NS
Obesity and central obesity						
BMI, kg/m^2	23 ± 2.5	32 ± 5	36 ± 5.6	0.000001	0.002	0.000001
Waist circumference, cm	81 ± 10	102 ± 8.5	112 ± 14	0.000001	0.001	0.000001
Waist:hip ratio	0.83 ± 0.09	0.93 ± 0.05	0.95 ± 0.09	0.000005	NS	0.000001
Body fat content, %	30 ± 8	36 ± 8.5	38 ± 8	NS	NS	0.04
Peripheral insulin resistance						
Fasting plasma glucose, mg/dL	81 ± 7	96 ± 19	127 ± 49	0.0002	0.001	0.000001
Fasting plasma insulin, mg/dL	5.5 ± 2.5	12 ± 6.5	21 ± 14	0.000001	0.0005	0.000001
HOMA-IR index	1.1 ± 0.5	3 ± 2	6.2 ± 4.6	0.000001	0.00007	0.000001
CVD risk factors						
SABP, mm Hg	117 ± 9.5	125 ± 12	133 ± 16	0.03	NS	0.001
DABP, mm Hg	73 ± 8.6	78.5 ± 8.2	79 ± 15	0.04	NS	0.01
Leukocyte count, cells/mm^3	5033 ± 1073	6500 ± 1570	7900 ± 2285	NS	NS	0.006
Total cholesterol, mg/dL	219 ± 47	210 ± 59	208 ± 46	NS	NS	NS
HDL cholesterol, mg/dL	57 ± 15	51 ± 29	47 ± 13	NS	NS	0.09
LDL cholesterol, mg/dL	145 ± 36	127 ± 58	121 ± 42	NS	NS	NS
Triglycerides, mg/dL	90.6 ± 29	150.6 ± 350	222 ± 142	0.01	0.07	0.007
Uric acid, mg/dL	3.3 ± 1	4.7 ± 2.8	5.2 ± 2.5	0.09	NS	0.006
Liver phenotype						
ALT, U/L	21 ± 4.5	68 ± 99	57.5 ± 35	0.008	NS	0.0002
AST, U/L	22 ± 4	38 ± 19	49 ± 33	0.001	0.04	0.0002
GGT, U/L	34 ± 24	50.5 ± 31	86.5 ± 82	NS	0.05	0.01
AP, U/L	159 ± 62	222 ± 89	269 ± 92	0.01	0.05	0.008
CK-18 fragment	—	235 ± 200	355 ± 284	—	0.06	—
Histologic features						
Degree of steatosis, %	—	48 ± 23	60 ± 20	—	0.04	—
Lobular inflammation, 0–3	—	0.53 ± 0.52	1.4 ± 0.5	—	0.0006	—
Portal inflammation, 0–2	—	0.0 ± 0.0	1.7 ± 0.7	—	0.000001	—
Hepatocellular ballooning, 0–2	—	0.0 ± 0.0	0.7 ± 0.6	—	0.005	—
Fibrosis stage	—	0.1 ± 0.5	2 ± 1.2	—	0.000001	—
NAS	—	2.4 ± 1	6.4 ± 1.6	—	0.00002	—

[1]*P* values were calculated with the use of the Mann-Whitney *U* test with the exception of the women:men ratio, for which the *P* value was calculated with the use of the chi-square test. ALT, serum alanine aminotransferase; AP, alkaline phosphatase; AST, serum aspartate aminotransferase; CK-18, cytokeratin-18; CVD, cardiovascular disease; DABP, diastolic arterial blood pressure; GGT, γ-glutamyl-transferase; NAFL, nonalcoholic fatty liver or simple steatosis; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis; SABP, systolic arterial blood pressure.
[2]For comparisons between NAFL and control groups.
[3]For comparisons between NAFL and NASH groups.
[4]For comparisons between NASH and control groups.
[5]Mean ± SD (all such values).

In vitro exposure of liver cells to palmitic acid is associated with induction of aminotransferase gene expression and release into the extracellular compartment of liver enzymes

We examined the relative abundance of aminotransferase mRNAs in an in vitro model of steatosis. A quantitative measurement of fat accumulation in Huh7 cells showed that the degree of positive staining for intracellular lipid droplets was nearly proportional to the concentration of palmitic acid (**Supplemental Figure 1**); in all experimental conditions, cell viability was >85% compared with for untreated cells (100% viability). We observed that the treatment of cells with increasing concentrations of palmitic acid led to a significant upregulation of *GPT* and *GOT* isoforms compared with that of control cells (**Figure 3**). These differences were still significant for *GPT2* and *GOT1* mRNAs at the lowest concentration of palmitic acid (50 μmol/L), which suggested that steatosis, even at moderate degree, is associated with the induction of aminotransferases. Although the enzymatic activity of both ALT and AST was significantly lower in the cell lysates, it was significantly and primarily increased in the cell culture medium (Figure 3).

The trend analysis, as shown in Figure 3, suggested that there was a significant concentration-dependent effect of palmitic

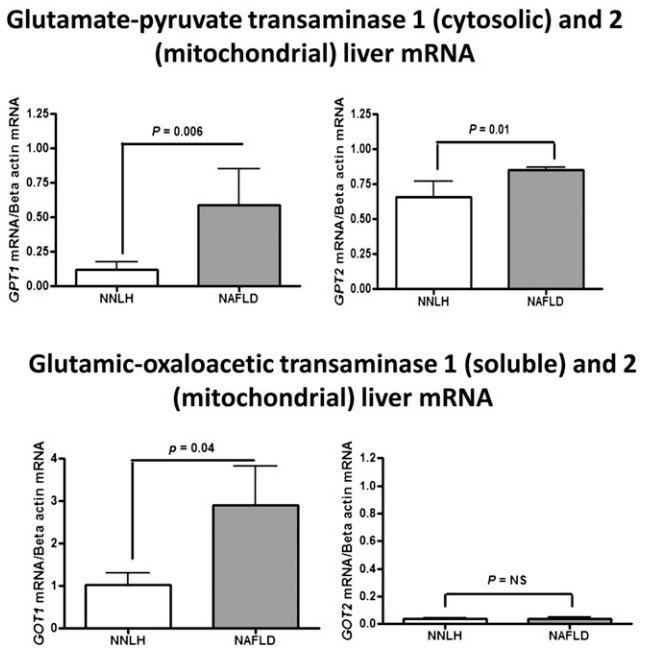

FIGURE 1 Exploration of mean ± SD changes in gene-expression levels of aminotransferases *GPT* and *GOT* isoforms (*1* and *2*) according to disease status. Bars show the liver abundance of *GPT1*, *GPT2*, *GOT1*, and *GOT2* mRNA evaluated with the use of a reverse transcriptase-quantitative polymerase chain reaction (patients with NAFLD: $n = 42$; subjects with NNLH: $n = 10$). In each sample, the abundance of target genes was normalized to the amount of β-actin to carry out comparisons between the groups. Differences between groups were evaluated with the use of an ANCOVA with BMI as a covariate and log-transformed variables. *P* values indicated above each bar allow for a comparison of NAFLD with NNLH groups. *GOT*, glutamic-oxaloacetic transaminase; *GPT*, glutamate-pyruvate transaminase; mRNA, messenger RNA; NAFLD, nonalcoholic fatty liver disease; NNLH, near-normal liver histology.

acid either for aminotransferase gene expression or enzymatic activity.

Targeted profiling of TCA cycle–related metabolites and their association with aminotransferase serum concentration

With the use of a targeted metabolomic approach, we investigated the association between serum ALT and AST concentrations and serum concentrations of metabolites, including TCA-cycle components, amino acids, urea cycle intermediates, glucose, and fatty acid metabolism. The study cohort included 48 individuals, of whom 32 patients had NAFLD and 16 subjects were healthy controls (Table 2).

We observed that both ALT and AST concentrations were positively correlated with metabolites involved in gluconeogenesis, methionine metabolism, transmethylation reactions as methyl-donor groups, intermediate products of transamination reactions, lactic acid, and cystamine (**Table 5**). Moreover, circulating concentrations of essential amino acids or derivatives, including L-glutamic acid and 2-hydroxyglutarate, were significantly and positively correlated with AST serum concentrations (Table 5), which indicated that the greater concentration in the corresponding aminotransferase was further supported by the fact that the enzyme catalyzed the conversion between the 2 molecules.

In addition, we showed that the L-alanine:pyruvate ratio ($R = 0.36$, $P = 0.01$) and 2-hydroxyglutarate concentrations ($R = 0.41$, $P = 0.004$) were significantly and positively correlated with the presence of NAFLD as a disease trait. Likewise, L-glutamic acid ($R = 0.30$, $P = 0.03$) and 2-hydroxyglutarate ($R = 0.32$, $P = 0.028$) concentrations and the L-alanine:pyruvate ratio ($R = 0.32$, $P = 0.02$) were significantly correlated with the NAFLD severity (data not shown for the remaining metabolites). In each of these cases, the aminotransferase directly mediated the conversion of one metabolite to the other. In contrast, aminotransferase concentrations showed inverse and significant correlations with direct metabolic intermediates of the TCA cycle such as fumaric acid, citraconic acid, and taurine (Table 5).

Through the exploration of the association between metabolite serum concentrations and the liver abundance of aminotransferase transcripts, we observed a common pattern that was shared with ALT and AST serum concentrations. More specifically, the liver expression of *GPT*, *GPT2* and *GOT1* mRNAs was correlated with metabolites involved in gluconeogenesis, taurine, and components of alanine and aspartate metabolism (**Table 6**). Note that there was a negative and significant correlation between the abundance of aminotransferase transcripts and the concentration of enzyme cofactors including pyridoxal 5′-phosphate (the necessary coenzyme of transamination reactions).

Targeted profiling of TCA cycle–related metabolites and their association with MetS-related phenotypes

In line with the findings reported, we observed that metabolites that were significantly associated with serum concentrations of aminotransaminases were also associated with BMI and HOMA-IR (**Supplemental Table 4**). Moreover, fumaric acid (or maleic acid) and taurine were inversely correlated, whereas cystamine, L-kynurenine and N2,N2-dimethylguanosine were positively correlated with BMI and HOMA-IR (Supplemental Table 4). Only taurine ($\beta \pm$ SE: -0.38 ± 0.18; $P = 0.035$) and L-kynurenine ($\beta \pm$ SE: 0.50 ± 0.17; $P = 0.006$) remained significantly associated with BMI after adjustment for aminotransferase concentrations and HOMA-IR. In addition, the L-alanine:pyruvate ratio was significantly correlated with BMI ($R = 0.37$, $P = 0.01$) even after adjustment for HOMA-IR and aminotransferase concentrations ($\beta \pm$ SE: 0.42 ± 0.18; $P = 0.02$). The glutaric or oxaloacetate ratio was significantly associated with both systolic and diastolic hypertension (Supplemental Table 4). However, after adjustment for BMI, HOMA-IR, and aminotransaminase concentrations, the association remained significant ($P = 0.05$) with diastolic hypertension only. An additional pathway and metabolite enrichment analysis is shown in Supplemental Material and **Supplemental Figure 2**.

NGS of liver enzyme genes: a *GOT2* 3′ UTR variant, the rs6993, is associated with MetS features

To better understand the potential role of genetic variation in liver enzyme genes that contribute to NAFLD and MetS-associated phenotypes, we followed the NGS approach, which was applied to all the exons and regulatory regions of aminotransferase loci. The study cohort comprised 96 subjects, including 64 patients with NAFLD and 32 healthy controls. Participant characteristics are shown in Table 3.

TABLE 4
Liver gene-expression changes of alanine and aspartate aminotransferase isoforms and their association with histologic- and metabolic syndrome–related clinical variables[1]

	GPT mRNA	*GPT2* mRNA	*GOT1* mRNA	*GOT2* mRNA
Hepatocellular ballooning				
None, 0 (n = 18)	0.33 ± 0.4	1.01 ± 0.14	5.8 ± 9	0.02 ± 0.02
Mild or marked, 1–2 (n = 24)	0.29 ± 0.4	0.89 ± 0.17	2.7 ± 4	0.07 ± 0.09
P	NS	0.05	NS	NS
Lobular inflammation				
None, 0 (n = 12)	0.03 ± 0.03	0.96 ± 0.17	4.5 ± 8	0.05 ± 0.07
Mild or marked, 1–3 (n = 30)	0.04 ± 0.05	0.95 ± 0.16	3.9 ± 4.6	0.02 ± 0.008
P	NS	NS	NS	NS
Liver fibrosis				
None or mild, 1 (n = 31)	0.21 ± 0.3	0.95 ± 0.17	4.2 ± 7.0	0.04 ± 0.07
Advanced, 2–4 (n = 10)	0.53 ± 0.4	0.90 ± 0.19	2.0 ± 2.0	0.05 ± 0.02
P	<0.01	NS	NS	NS
NAS				
<5 (n = 18)	0.033 ± 0.4	1.00 ± 0.14	6.2 ± 9.0	0.02 ± 0.02
≥5 (n = 24)	0.29 ± 9.35	0.91 ± 0.17	2.5 ± 1.2	0.07 ± 0.09
P	NS	NS	0.06	0.05
Metabolic features: fasting plasma glucose concentrations[2]				
≤100 mg/dL (n = 22)	0.32 ± 0.35	0.96 ± 0.17	4.5 ± 7.7	0.023 ± 0.02
>100 mg/dL (n = 20)	0.19 ± 0.29	0.92 ± 0.16	2.3 ± 3.7	0.05 ± 0.09
P	NS	NS	NS	NS
Cardiovascular disease risk factors: arterial hypertension				
SABP <135 mm Hg (n = 33)	0.27 ± 0.34	0.95 ± 0.14	3.09 ± 4.1	0.02 ± 0.02
SABP ≥35 mm Hg (n = 9)	0.26 ± 0.37	0.93 ± 0.26	8.27 ± 12.4	0.08 ± 0.05
P	NS	NS	NS	0.03

[1]All values are means ± SDs. The liver expression of *GPT*, *GPT2*, *GOT1*, and *GOT2* was normalized to the β-actin reference gene. Differences between groups were assessed with the use of the nonparametric Mann-Whitney test. *GOT*, glutamic-oxaloacetic transaminase; *GPT*, glutamate-pyruvate transaminase; mRNA, messenger RNA; NAS, nonalcoholic fatty liver disease activity score; SABP, systolic arterial blood pressure.

[2]The American Diabetes Association has recently established a cutoff of ≥100 mg/dL. Concentrations greater than this threshold indicate either prediabetes (impaired fasting glucose) or diabetes.

We generated >2 million reads with an average length of 145 bases of quality equal to or exceeding a quality filter score of 20 and coverage, on average, >34 times. Overall, 18 different SNPs were detected in the entire population; complete details are shown in **Supplemental Table 5**.

Notably, the rs6993 A/G, which was predicted to have regulatory functions because it is located in the 3′ UTR region of the *GOT2* locus, was significantly associated with MetS features, including arterial hypertension [P = 0.028; OR: 2.285 (95% CI: 1.024, 5.09); adjusted by NAFLD severity] and plasma lipid concentrations (**Figure 4**). Furthermore, individuals with the risk GG genotype had significantly higher γ-glutamyl-transferase concentrations (Figure 4). However, we could not assign any cause-effect relation to this variant because the rs6993 is in strong linkage disequilibrium with ≥5 other SNPs in the *GOT2* locus (**Supplemental Figure 3**).

DISCUSSION

In this study, we aimed to explore the role of ALT and AST liver enzymes in NAFLD and the MetS. For this purpose, we performed an integrative approach that included the exploration of changes at both transcriptional and protein levels, the targeted profiling of TCA cycle–related metabolites, and an analysis of genetic variants with the use of NGS. The use of these strategies was intended to make a novel connection between biological and physiologic processes that would not be otherwise interpreted on the basis of the current and classic view that aminotransferase concentrations are merely a consequence of an intracellular leak out of enzymes into the peripheral circulation associated with hepatocyte injury. The classic view that links serum aminotransferase concentrations with liver damage has far overshadowed the role of ALT and AST isoforms in the regulation of liver metabolism.

Liver expression of aminotransferases is deregulated in NAFLD

We showed that the liver transcriptional activity of aminotransferases was deregulated in patients with NAFLD, which provided evidence of significantly higher transcript levels associated with fatty liver transformation that in controls. In additional, patients with NAFLD showed a marked reduction in liver protein expression of the mitochondrial GPT2 and GOT2 aminotransferase isoforms.

To explain the simultaneous upregulation in gene expression and downregulation in protein amounts, we performed an in vitro study in which Huh7 cells were exposed to incremental amounts of palmitic acid. We showed that, although the gene expression of all aminotransferase isoforms was induced in response to a fat overload, the intracellular enzymatic activity, which is a surrogate of protein expression, was significantly diminished. The

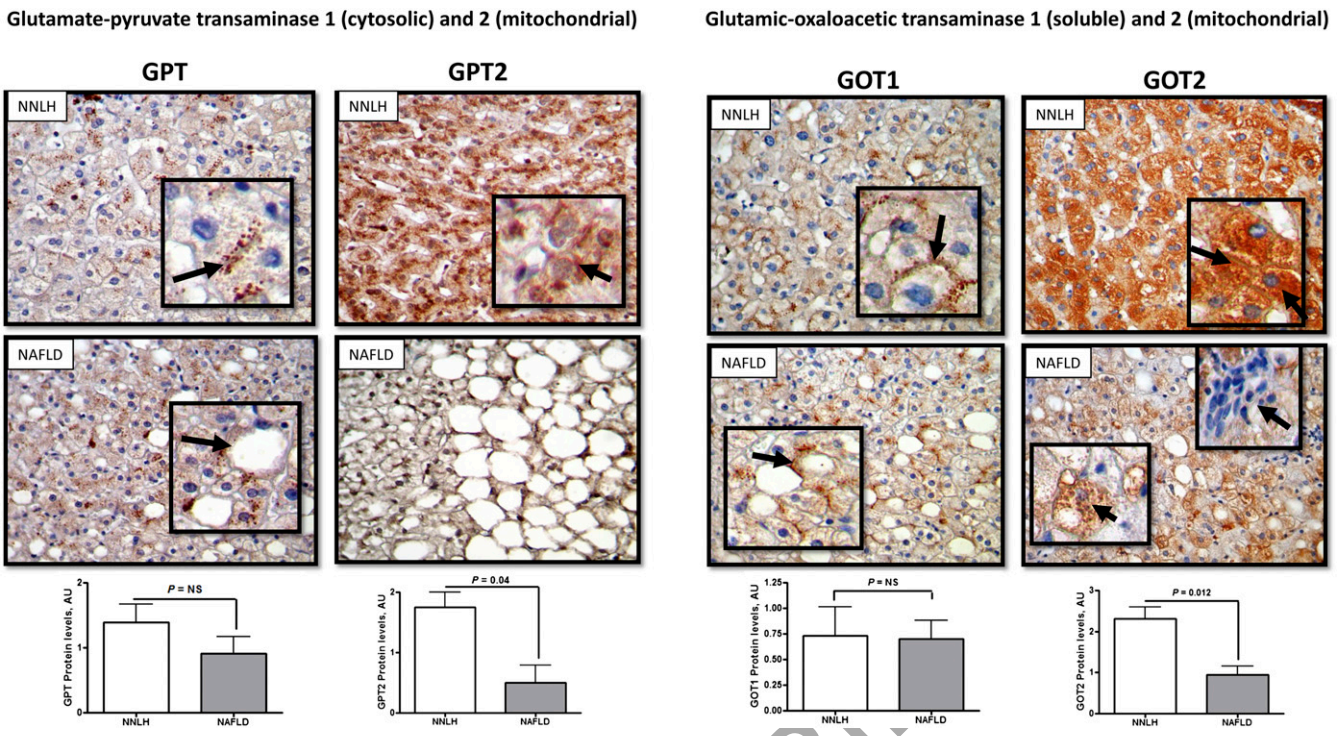

FIGURE 2 Exploration of changes in protein-expression levels of aminotransferases GPT and GOT isoforms (1 and 2) according to the disease status. Protein expression was explored with the use of immunohistochemistry. Upper panels show representative liver expression patterns of GPT, GPT2, GOT1, and GOT2 evaluated with the use of immunohistochemistry according to disease status (NNLH and NAFLD). Black arrows in inserts indicate the immunoreactivity in hepatocyte cytoplasm or surrounding the lipid droplets showing a granular pattern. The GOT2 insert depicts lobular inflammatory infiltrate showing negative immunoreactivity. Aminotransferase immunoreactivity was examined with the use of light microscopy of liver sections; counterstaining was performed with hematoxylin. Original magnification: 400×. Bars (means ± SDs) in bottom panels show scores on the liver protein expression of GPT, GPT2, GOT1, and GOT2 evaluated with the use of immunohistochemistry according to disease status (NNLH and NAFLD; $n = 6$/group). Differences between groups were evaluated with the use of the Mann-Whitney U test; P values indicated above each bar allow for a comparison of NAFLD with NNLH groups. GOT, glutamic-oxaloacetic transaminase; GPT, glutamate-pyruvate transaminase; NAFLD, nonalcoholic fatty liver disease; NNLH, near-normal liver histology.

liver enzymes ALT and AST were secreted at high concentrations into the culture media in the experimental conditions in which cells were exposed to palmitic acid. Therefore, it is plausible to suggest that the decrease in the intracellular level of aminotransferases is explained by an incremented release into the extracellular compartment. Hence, the observed opposite direction between gene and protein levels would have reflected the dynamics of the flowing of aminotransferases between compartments. The final and largest reservoir of aminotransferases is the circulation compartment; therefore, it is reasonable to assume that both gene and protein expressions of aminotransferases were greater in NAFLD than in controls in response to the metabolic overload.

Because NAFLD was previously associated with diminished liver mitochondrial biogenesis (24), this association could provide an alternative explanation of the marked reduction in the immunostaining scores of the mitochondrial (GPT2 and GOT2) aminotransferase isoforms observed in our patients.

Overall, our findings were consistent with previous experimental results; e.g., Jadhao et al. (25) reported an induction of *GPT2* gene expression by 2-fold in the liver of obese (*ob/ob*) mice. Likewise, Liu et al. (26) showed, in a mice model of NAFLD, that increased serum concentrations of ALT were associated with the induction of hepatic expression of ALT isoforms. Furthermore, it was shown in vitro that metabolic stress is associated with the induction of liver *GPT2* expression (27). Finally, evidence from human studies has suggested that aminotransferases are induced by metabolic insults such as hyperalimentation (28) or a fast-food diet (29).

Liver enzymes in circulation mirror perturbations in liver metabolism of amino acid and Krebs cycle

Metabolite profiling provided compelling evidence that aminotransferase serum concentrations seem to be a robust signature of liver metabolic perturbations, particularly at the amino acid and TCA cycle levels. The relation between aminotransferase liver transcripts and serum metabolite concentrations involved in pathways of gluconeogenesis, taurine metabolism, and alanine and aspartate metabolism components further supports the role of aminotransferases in the fine tuning of liver metabolism. In support of our findings, Sunny et al. (30) showed that NAFLD is associated with impaired hepatic mitochondrial metabolism, including mitochondrial anaplerosis that is increased by almost 50% and, thus, contributes to elevated gluconeogenesis rates, all of which account for the attendant increase in hepatic energy demands. Newgard et al. (31) showed that several essential (leucine, isoleucine, valine, and phenylalanine) and nonessential amino acids (alanine, tyrosine, glutamate/glutamine, aspartate/asparagine, and arginine) were significantly elevated in obese patients compared with in lean controls. The authors further speculated that a high rate of flux through branched-chain amino

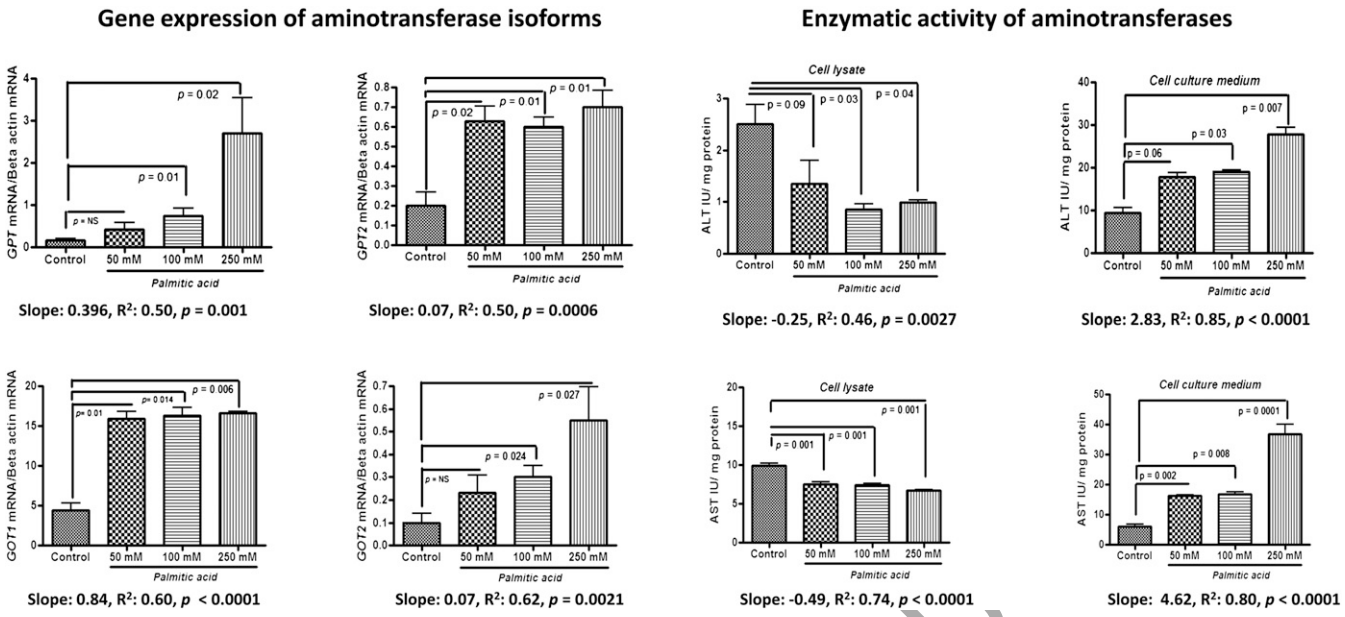

FIGURE 3 Induction of aminotransferase gene expression in an in vitro model of steatosis. Huh7 human hepatoma cells were exposed to increasing concentrations (50, 100, or 250 μmol/L) of palmitic acid for 24 h to assess the mean ± SD mRNA expression and enzymatic activity of liver transaminases (n = 4/group); control cells (n = 4) were treated with an equivalent concentration (volume:volume) of vehicle. mRNA levels were measured with the use of a quantitative polymerase chain reaction and normalized by β-actin concentrations. The enzymatic activity of ALT and AST was measured in cell culture medium (cell-free culture supernatant fluid) and cell lysates and was normalized by the protein content. The liver abundance of *GPT*, *GPT2*, *GOT1*, and *GOT2* mRNAs was significantly upregulated during incremental amounts of lipid accumulation, whereas the ALT and AST enzymatic activity showed a significant reduction in cell lysates but significantly larger levels in the cell-free culture supernatant fluid, which was the major reservoir. The mean value of each experimental condition was compared with that of the control untreated cells with the use of an ANOVA with repeated measurements. Differences between groups were evaluated with the use of a Neuman-Keuls test; the level of significance was set at $P = 0.05$. An ANOVA posttest for a linear trend was performed, and the slope, R^2, and P values are shown under the graphs of vertical bars for each variable. ALT, alanine aminotransferase; AST, aspartate aminotransferase; *GOT1*, glutamic-oxaloacetic transaminase 1, soluble; *GOT2*, glutamic-oxaloacetic transaminase 2, mitochondrial; *GPT*, cytosolic alanine aminotransaminase 1; *GPT2*, cytosolic alanine aminotransaminase 2; mRNA, messenger RNA.

acid catabolic pathways and the accumulation of glutamate may increase the transamination of pyruvate to alanine (31). On the basis of these findings and with the use of systems biology, we predicted a relation in these metabolomics changes, in particular the glutamine cycle pathway and liver transaminases (19).

Furthermore, we observed a consistent association between cystamine serum concentrations (decarboxycystine) and aminotransferases as well as a significant association with taurine concentrations. Both cystamine and taurine have been implicated as antioxidants (32); therefore, it is plausible to speculate that aminotransferase concentrations also reflect hepatocellular changes in response to oxidative stress, which is a feature of NAFLD (30).

Liver transaminases and CVD: GOT2 and arterial hypertension

We observed that the liver abundance of *GOT2* mRNA was associated with arterial hypertension, which is a finding that supports the involvement of NAFLD in CVD (4, 5, 8). GOT (AST) is involved in the transamination of L-cysteinesulfinate to develop *b*-sulfinylpyruvate, which decomposes spontaneously to pyruvate and sulfur dioxide. It has previously been shown that sulfur dioxide, which is the product of GOT transamination, regulates vascular function. In the extant literature, sulfur dioxide has been associated with the development of CVD (33), including arterial hypertension and vascular collagen remodeling (34), thereby suggesting that GOT could be a rate-limiting enzyme in the production of sulfur dioxide (35). To our knowledge, the novelty of our data lies in the involvement of liver GOT2 in the CVD phenotype.

Finally, we showed a significant association between the *GOT2* rs6993 and MetS features. Of note, the exploration of associations of this variant in the Genetic Association of Complex Diseases and Disorders database showed a consistent and robust association with lipid concentrations (36) and CVD (37).

To our knowledge, this is the first patient-oriented study on NAFLD that evaluated the changes in gene and protein expressions of aminotransferases as well as the association between transcript and serum concentrations of liver enzymes with serum TCA cycle–related metabolites. A potential limitation of our study was the observational cross-sectional nature, which cannot prove causality. In addition, although differences in the liver abundance of aminotransferase mRNAs remained significant after adjustment for BMI, a note of caution should be added because the control group showed significantly lower BMI than that of the NAFLD patients. A previous study showed that the liver triglyceride content and increased free fatty acid flux from adipose tissue are both the major determinants of plasma elevation of transaminases (38). Nevertheless, although the potential role of adipose tissue in driving the observed molecular changes could not be directly addressed in our study, the fact that fatty liver (regardless of its pathogenic mechanisms, including direct lipotoxicity by the overflowing of free fatty acids from dysfunctional adipose tissue) is associated with the gene expression and induction of aminotransferases was shown. Moreover,

TABLE 5
Profiling of circulating TCA cycle–related metabolites according to serum ALT and AST aminotransferase concentrations[1]

Metabolite	HMDB[2]	Spearman rank correlation test	*P*
ALT serum concentration			
Direct metabolic intermediates of the TCA cycle			
Fumaric acid or maleic acid	HMDB00134	−0.45	0.001
Citraconic acid (methylmaleate)	HMDB00634	−0.48	0.0006
Gluconeogenesis, glycolysis, and glucose metabolism			
D-Glucose	HMDB00122	0.30	0.034
Glucose 6-phosphate	HMDB01401	−0.34	0.01
Taurine and hypotaurine metabolism			
Taurine	HMDB00251	−0.36	0.011
Cystamine (decarboxycystine)	ChemSpider ID2812[3]	0.40	0.004
Methionine metabolism, transmethylation reactions, methyl donor groups for DNA			
L-Methionine	HMDB00696	0.34	0.01
L-Methyladenosine	HMDB03331	0.33	0.019
N2,N2-Dimethylguanosine	HMDB04824	0.30	0.036
Intermediate products of transamination reactions			
L-Kynurenine	HMDB00684	0.33	0.02
L-Lactic acid	HMDB00190	0.30	0.034
Alanine:pyruvate ratio	—	0.34	0.018
AST serum concentration			
Metabolic intermediates of the TCA cycle			
Fumaric acid or maleic acid	HMDB00134	−0.54	0.000073
Citraconic acid (methylmaleate)	HMDB00634	−0.53	0.0001
Gluconeogenesis, glycolysis, and glucose metabolism			
D-Glucose	HMDB00122	0.30	0.034
Glucose 6-phosphate	HMDB01401	−0.31	0.03
Amino acids and amino acid biosynthesis			
L-Lysine	HMDB00182	0.32	0024
L-Glutamic acid (glutamate)	HMDB00148	0.32	0.026
L-Arginine	HMDB00517	0.31	0.033
L-Tyrosine	HMDB00158	0.28	0.04
2-Hydroxyglutarate	HMDB59655	0.29	0.04
Taurine and hypotaurine metabolism			
Taurine	HMDB00251	−0.40	0.005
Cystamine (decarboxycystine)	HMDB02991	0.38	0.006
Methionine metabolism, transmethylation reactions, methyl donor groups for DNA			
L-Methionine	HMDB00696	0.39	0.05
L-Homocysteine	HMDB00742	0.30	0.03
L-Methyladenosine	HMDB03331	0.35	0.013
N2,N2-Dimethylguanosine	HMDB04824	0.38	0.008
Intermediate products of transamination reactions			
L-Lactic acid	HMDB00190	0.28	0.048
L-Kynurenine	HMDB00684	0.34	0.015
L-Alanine:pyruvate ratio	—	0.36	0.010

[1]ALT, alanine aminotransferase; AST, aspartate aminotransferase; HMDB, Human Metabolome Database; TCA, tricarboxylic acid.
[2]Available from: http://www.hmdb.ca/.
[3]ChemSpider, http://www.chemspider.com.

results from the Framingham Heart Study robustly showed that the association between aminotransferases and cardiometabolic risk factors is beyond visceral adipose tissue (39).

In conclusion, our results were intended to introduce a new hypothesis into the biological role of liver aminotransferases in the regulation of systemic metabolic functioning. In addition, we aimed to connect the epidemiologic observations reporting abnormal concentrations of liver enzymes in all intermediate phenotypes of the MetS with perturbed liver gene expression and circulating metabolite profiling. Therefore, in view of our clinical findings and data yielded by previous experimental studies, it is reasonable to suggest that, rather than a simple biomarker of hepatocyte injury, aminotransferases are important contributors to liver metabolic functioning. Hence, ALT and AST concentrations might be regarded as sensors of global metabolic deregulation, including mitochondrial energetic control. The larger concentrations in ALT and AST in the big circulation compartment observed in patients with NAFLD, along with the liver

TABLE 6
Serum profiling of TCA cycle–related metabolites associated with liver transcript levels of aminotransferases[1]

Metabolite/HMDB[2]	Biofunction/pathway	Spearman rank correlation test	*P*
Liver expression levels of *GPT* mRNA			
Glucose 6-phosphate/HMDB01401	Gluconeogenesis, glycolysis, nucleotide sugars metabolism	−0.90	0.03
Pyridoxal 5′-phosphate (vitamin B-6)/HMDB01491	Component of vitamin B-6 metabolism/enzyme cofactor	−0.90	0.037
Cystamine (decarboxycystine)/—	Taurine metabolism	0.90	0.037
Uridine/HMDB00296	Component of pyrimidine metabolism	0.90	0.037
Glucuronic acid/HMDB00127	Inositol metabolism	0.90	0.037
Liver expression levels of *GPT2* mRNA			
Pyroglutamic acid (glutiminic acid)/HMDB00267	Component of glutathione metabolism/essential amino acid	−0.82	0.041
Biotin/HMDB00030	Component of biotin metabolism/essential vitamins	−0.82	0.04
Liver expression levels of *GOT1* mRNA			
Taurine/HMDB00251	Essential amino acid, bile acid biosynthesis, taurine, and hypotaurine metabolism	0.90	0.037
Carnitine/HMDB00062	Component of alanine and aspartate metabolism/component of fatty acid metabolism/essential amino acid	0.90	0.037
Pantothenic acid (vitamin B-5)/HMDB00210	Component of pantothenate and CoA biosynthesis/essential vitamins	0.90	0.037

[1]*GOT*, glutamic-oxaloacetic transaminase; *GPT*, glutamate-pyruvate transaminase; HMDB, Human Metabolome Database; mRNA, messenger RNA; TCA, tricarboxylic acid.
[2]Available from: http://www.hmdb.ca.

gene- and protein-expression data, suggest a greater synthesis and release of liver aminotransferases as an adaptation of the liver to energetic demands in the context of MetS development (**Supplemental Figure 4**). However, note that the serum concentrations of metabolites and correlations observed in our study could also be the result of contribution by other organs and tissues because aminotransferases are expressed almost ubiquitously. This observation may be particularly significant in relation to the MetS.

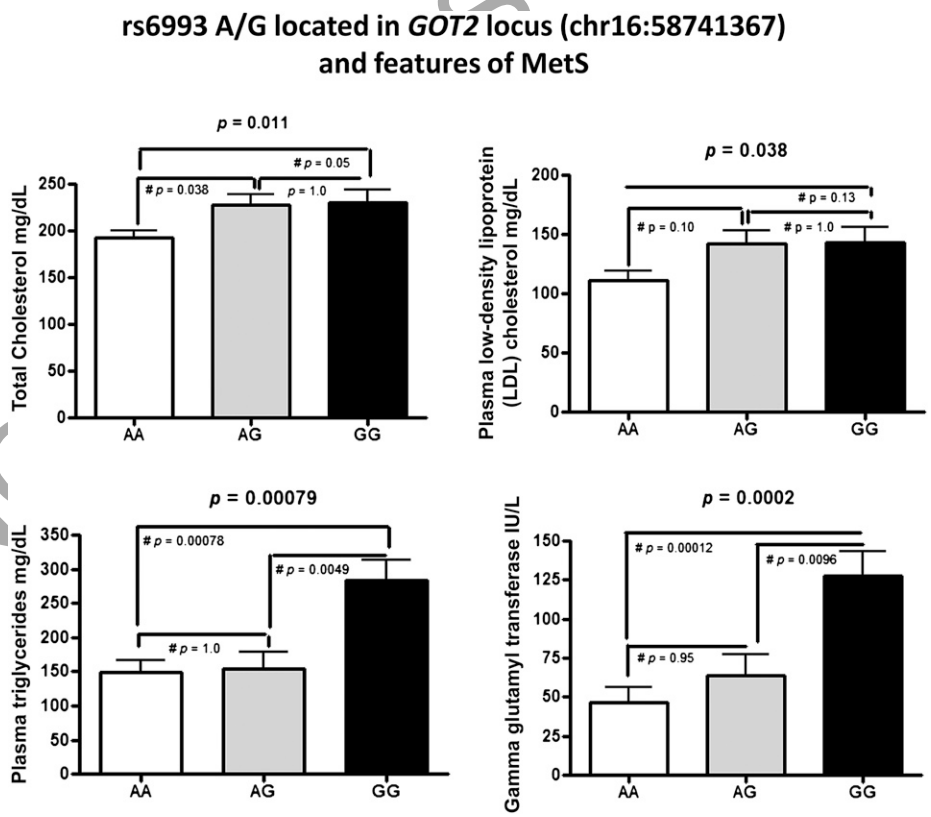

FIGURE 4 Lipid profile and γ-glutamyl-transferase activity according to *GOT2* rs6993-3′ UTR variant genotypes. *P* values pertain to differences between genotypes evaluated with the use of an ANOVA and log-transformed variables. Values are means ± SDs. $^{\#}P$ values for multiple post-hoc group comparisons evaluated with the use of the Bonferroni test. Genotypes counts were as follows: AA, $n = 45$; AG, $n = 36$; and GG, $n = 15$. *GOT2*, glutamic-oxaloacetic transaminase 2; MetS, metabolic syndrome; UTR, untranslated region.

The authors' responsibilities were as follows—SS, HD, CR, and CJP: analyzed the NGS data; SS and CJP: designed the research (project conception, development of overall research plan, and study oversight), analyzed the overall results data, performed the statistical analysis, wrote the manuscript, and had primary responsibility for the final content of the manuscript; SS and GOC: performed liver biopsies and collected biological samples; RS: performed gene expression experiments; TFG and CJP: performed the NGS; GG and JSM: performed the histopathologic evaluation and immunohistochemistry; and IS and DF: performed the in vitro study. None of the authors reported a conflict of interest related to the study.

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