

Plasma metabolomic profile in nonalcoholic fatty liver disease

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Abstract

The plasma profile of subjects with nonalcoholic fatty liver disease (NAFLD), steatosis, and steatohepatitis (NASH) was examined using an untargeted global metabolomic analysis to identify specific disease-related patterns and to identify potential noninvasive biomarkers. Plasma samples were obtained after an overnight fast from histologically confirmed nondiabetic subjects with hepatic steatosis ($n = 11$) or NASH ($n = 24$) and were compared with healthy, age- and sex-matched controls ($n = 25$). Subjects with NAFLD were obese, were insulin resistant, and had higher plasma concentrations of homocysteine and total cysteine and lower plasma concentrations of total glutathione. Metabolomic analysis showed markedly higher levels of glycocholate, taurocholate, and glycochenodeoxycholate in subjects with NAFLD. Plasma concentrations of long-chain fatty acids were lower and concentrations of free carnitine, butyrylcarnitine, and methylbutyrylcarnitine were higher in NASH. Several glutamyl dipeptides were higher whereas cysteine-glutathione levels were lower in NASH and steatosis. Other changes included higher branched-chain amino acids, phosphocholine, carbohydrates (glucose, mannose), lactate, pyruvate, and several unknown metabolites. Random forest analysis and recursive partitioning of the metabolomic data could separate healthy subjects from NAFLD with an error rate of approximately 8% and separate NASH from healthy controls with an error rate of 4%. Hepatic steatosis and steatohepatitis could not be separated using the metabolomic profile. Plasma metabolomic analysis revealed marked changes in bile salts and in biochemicals related to glutathione in subjects with NAFLD. Statistical analysis identified a panel of biomarkers that could effectively separate healthy controls from NAFLD and healthy controls from NASH. These biomarkers can potentially be used to follow response to therapeutic interventions.

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of metabolic abnormalities ranging from a simple accumulation of triglycerides in the hepatocytes (hepatic steatosis) to hepatic steatosis with inflammation (steatohepatitis), fibrosis, and cirrhosis [1,2]. Although hepatic steatosis is related to a number of clinical disorders and has been studied in several different animal models, NAFLD in humans is characterized by obesity, insulin resistance, and associated

metabolic perturbations [1,2]. Data from studies in humans and animal models have suggested a paradigm for the pathogenesis of nonalcoholic steatohepatitis (NASH) involving alterations in hepatic lipid metabolism, increased generation of reactive oxygen species and consequently oxidative stress, changes in mitochondrial function, DNA damage, and release of various cytokines resulting in progression of disease [3,4]. Although such mechanisms have been inferred from data in animals and from indirect evidence in humans, no single biomarker or a cluster of metabolites in the plasma has been related to the progression of hepatic insult. Several diagnostic techniques have been developed to document impaired mitochondrial function, evidence of inflammation, or indices of hepatic fibrosis [3].

Metabolomics, the quantification of small molecules in plasma and tissue fluids, allows for a comprehensive view of

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the changes in several metabolic and signaling pathways and their interactions [5–8]. Recent developments in high-throughput analysis, curation, and robust statistical analysis have allowed investigators to understand the changes in the cellular and tissue metabolism based upon the metabolomic data. In the present study, we have examined the plasma metabolome of a biopsy-proven group of nondiabetic subjects with steatosis and steatohepatitis and compared it with a group of healthy controls. Studies were done after a defined period of fasting.

The goals of the present study were (1) to describe the changes in plasma metabolome in subjects with hepatic steatosis and in those with NASH as compared with the controls; (2) to identify biomarkers, if any, associated with steatosis and NASH; and (3) by using appropriate statistical methods, to identify the metabolomic patterns that could discriminate steatosis and NASH from healthy controls.

2. Materials and methods

Metabolomic analyses were performed on 35 nondiabetic subjects with histologically confirmed hepatic steatosis ($n = 11$) and steatohepatitis (NASH; $n = 24$) and on 25 nondiabetic healthy controls. Subjects with NAFLD were recruited from the metabolic clinics of the Cleveland Clinic and MetroHealth Medical Center in Cleveland, OH. Hepatic ultrasonography was performed on all control subjects by the same investigator (SD) to confirm absence of steatosis. Patient demographics and clinical characteristics are displayed in Table 1. Subjects with other forms of chronic liver disease were excluded by screening for autoimmune liver

disease (autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis), for metabolic liver disease (hemochromatosis, Wilson disease), for α -1 antitrypsin deficiency, and for viral hepatitis (hepatitis B and hepatitis C). Subjects with history of daily alcohol intake (women ≥ 20 g, men ≥ 30 g), intravenous drug use, or history of bowel surgery were also excluded. None of the subjects were on any medications known to cause hepatic steatosis or were taking vitamin supplements.

Liver biopsies were reviewed in a blinded manner by the same pathologist and given an NAFLD activity score (0–8) based upon the guidelines of the NASH Clinical Research Network [9]. Subjects with a diagnosis of indeterminate NASH were excluded. *Hepatic steatosis* was defined as a NASH activity score less than or equal to 3 in the absence of ballooning degeneration. *Steatohepatitis* (NASH) was defined as a NASH activity score greater than or equal to 4 and a histologic diagnosis of NASH by the pathologist.

Written informed consent was obtained from all participants after fully explaining the procedure to them. The study protocol was approved by the Institutional Review Boards at the Cleveland Clinic and MetroHealth Medical Center. Subjects reported to the General Clinical Research Center after an overnight fast of at least 10 hours. After obtaining the height, weight, and vital signs, an indwelling cannula was placed in a superficial vein of the forearm. After a 30-minute rest period, 3 venous blood samples were obtained 5 minutes apart for the measurements of serum glucose and insulin concentrations. An additional blood sample (5 mL) was collected into an EDTA-containing tube for metabolomic analysis. Blood samples were centrifuged at 4°C, and the plasma was stored at -80°C .

Table 1
Demographic and clinical laboratory analysis

	Control	Steatosis	NASH	P^a
n	25	11	24	
Age (y)	42.6 (9.2)	43.5 (10.7)	43.6 (12.6)	NS
Sex				
Male	7	6	8	
Female	18	5	16	
Body mass index (kg/m^2)	24.5 (2.6)	34.0 (4.0)*	34.8 (4.7)*	.00
Insulin (pmol/L)	52.5 (21.3)	142.9 (62.3)*	183.9 (103.6)*	.00
Glucose (mmol/L)	4.7 (0.5)	4.9 (0.6)	5.3 (0.9)*	.035
Insulin resistance (HOMA)	0.96 (0.4)	2.6 (1.1)*	3.26 (1.6)*	.00
ALT (IU/L)	17.6 (5.0)	44.4 (30.0)	84.6 (58.6)*	.00
AST (IU/L)	22.8 (5.7)	31.4 (15.4)	63.4 (46.7)*	.0001
Triglycerides (mg/dL)	78.5 (33.6)	154.4 (70.4)*	161.4 (82.8)*	.002
HDL (mg/dL)	60.9 (20.2)	40.4 (9.9)*	44.1 (10.1)*	.0005
LDL (mg/dL)	118.7 (21.2)	156.0 (43.7)*	127.9 (34.1)	.02
Glutathione ($\mu\text{mol}/\text{L}$)	7.9 (2.7)	5.7 (2.7)*	5.2 (1.9)*	.001
Homocysteine ($\mu\text{mol}/\text{L}$)	7.3 (2.0)	8.8 (2.6)	9.1 (1.9)*	.01
Cysteine ($\mu\text{mol}/\text{L}$)	361.4 (59.2)	410.6 (58.0)	432.4 (63.9)*	.001

Data are mean (SD). ALT indicates alanine aminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol.

^a One-way analysis of variance.

* Significantly different compared with controls. $P < .05$ Tukey pairwise comparison.

2.1. Analytical methods

The concentration of glucose in the serum was measured using the glucose oxidase method (Yellow Springs Instruments, Yellow Springs, OH); serum insulin concentrations were measured by double-antibody radioimmunoassay (Linco Research, St Charles, MO). The total concentration of homocysteine, cysteine, and glutathione in plasma was determined by high-performance liquid chromatography (Agilent 1100 series HPLC; Agilent Technologies; Wilmington, DE), as described by Garcia and Apitz-Castro [10]. Plasma lipid profile and hepatic function panel were measured using standard laboratory methods in the clinical laboratory of the hospital.

Insulin resistance was estimated by the homeostasis model assessment (HOMA) calculator V2.2 (<http://www.dtu.ox.ac.uk/home>). The HOMA model is a structural computer model of the glucose-insulin feedback system in the homeostatic (overnight-fasted) state that allows deduction of insulin sensitivity from the concurrent measurements of glucose and insulin levels in the fasting state [11].

2.2. Metabolomic analysis

The global, unbiased metabolic profiling platform was based on a combination of 3 independent platforms: ultra-high-performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) optimized for basic species, UHPLC/MS/MS optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). This platform was described in detail in a previous publication [8]. The major components of the process are summarized as follows:

Sample extraction: One hundred microliters of each plasma sample was thawed on ice and extracted using an automated MicroLab STAR system (Hamilton, Salt Lake City, UT) in 400 μ L of methanol containing the recovery standards.

GC/MS and UHPLC/MS/MS analysis: UHPLC/MS was carried out using a Waters Acquity UHPLC (Waters, Milford, MA) coupled to an LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with an electrospray ionization source. Two separate UHPLC/MS injections were performed on each sample: one optimized for positive ions and one for negative ions. Derivatized samples for GC/MS were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole MS operated at unit mass resolving power. Chromatographic separation followed by full-scan mass spectra was carried out to record retention time, molecular weight (m/z), and MS/MS of all detectable ions presented in the samples.

Metabolite identification: Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular

weight (m/z), preferred adducts, and in-source fragments as well as their associated MS/MS spectra. This library allowed the rapid identification of metabolites in the experimental samples with high confidence.

Data imputation and statistical analysis: The samples were analyzed over the course of 2 days. After the data were corrected for minor variations resulting from instrument interday tuning differences, the missing values for a given metabolite were imputed with the observed minimum detection value on the assumption that they were below the limits of detection. For the convenience of data visualization, the raw area counts for each biochemical were rescaled by dividing each sample value by the median value for the specific biochemical.

Statistical analysis of the data was performed using JMP (SAS, Cary, NC; <http://www.jmp.com>), a commercial software package, and “R” (<http://cran.r-project.org/>), which is a freely available open-source software package. A log transform was applied to the observed relative concentrations for each biochemical because, in general, the variance increased as a function of a biochemical’s average response. Welch t tests were performed to compare data obtained from experimental groups. Multiple comparisons were accounted for with the false discovery rate method, and each false discovery rate was estimated using q values.

Random forest (RF) analysis was performed on untransformed data using R. Random forest is a supervised classification technique based on an ensemble of decision trees. For a given decision tree, a subset of samples is selected to build the tree; and then the remaining samples are predicted from this tree. This process is repeated thousands of times to produce a forest. The final classification is determined by computing the frequencies (“votes”) of predictions for each group over the whole forest. This method is unbiased because the prediction for each sample is based on trees built from a subset of samples not including it; thus, the prediction accuracy is an unbiased estimate of predicting a new data set. To see which variables contribute the most to the separation, an “importance” measure is computed. We used the “mean decrease accuracy” as this metric. This value is determined by randomly permuting a variable and then running the values through the trees and reassessing the prediction accuracy. If a variable is not important, then this procedure will have little change in the accuracy (permuting random noise will give random noise), whereas if a variable is important, the accuracy will drop after such a permutation.

3. Results

The demographics and clinical laboratory data are displayed in Table 1. The subjects were matched for age and sex. As anticipated, the subjects with NAFLD were

obese and had significantly higher body mass index; plasma alanine aminotransferase, aspartate aminotransferase, and insulin levels; and insulin resistance (HOMA). The plasma levels of triglycerides and low-density lipoprotein cholesterol were higher, whereas the concentration of high-density lipoprotein cholesterol was lower in subjects with NAFLD.

The plasma concentration of glutathione was significantly lower in subjects with steatosis and NASH when compared with controls. The concentrations of homocysteine and total cysteine in the plasma were significantly higher in subjects with NASH. A significant positive correlation between the plasma levels of homocysteine and cysteine was observed in healthy controls ($r^2 = 0.63$, $P < .0001$) but not in NAFLD.

3.1. Metabolomic analysis

Using LC/MS and GC/MS analysis, 437 distinct metabolites were identified in the plasma sample. Of these, 228 biochemicals matched a named structure in our reference library. The remaining 209 represent distinct chemical entities, but they do not match a named biochemical in the reference library. The changes in the metabolites between the experimental groups were calculated by the ratio of their group means. The statistical significance of the changes was analyzed by Welch t test, with $P < .05$ deemed to be significant. The list of known metabolites along with their relative change among the steatosis and NASH subjects is displayed in the supplemental table. Unknown metabolites found to be significantly different between NAFLD and controls are also displayed.

3.2. Bile acids

As shown in Fig. 1, there was a 4-fold increase in the plasma concentration of glycocholate and taurocholate and a 2-fold increase in glycochenodeoxycholate in subjects with NASH as compared with controls. These bile acids were also higher in the steatosis group compared with controls; however, only taurocholate met the statistical significance cutoff of $P < .05$.

3.3. Glutathione metabolism

Consistent with decreased plasma glutathione in subjects with steatosis and NASH (Table 1), the concentration of cysteine-glutathione disulfide, a product of glutathione and cysteine conjugate, was significantly lower in subjects with steatosis and NASH (Fig. 2). In addition, several glutamyl dipeptides—glutamyl valine, glutamyl leucine, glutamyl phenylalanine, and glutamyl tyrosine—were higher in both NASH and steatosis. The increase was of similar magnitude in both groups.

3.4. Lipids

Subtle differences in lipid profiles were found between subjects with NAFLD and the healthy controls. Several free fatty acids—eicosapentaenoate (C20:5n3), docosahex-

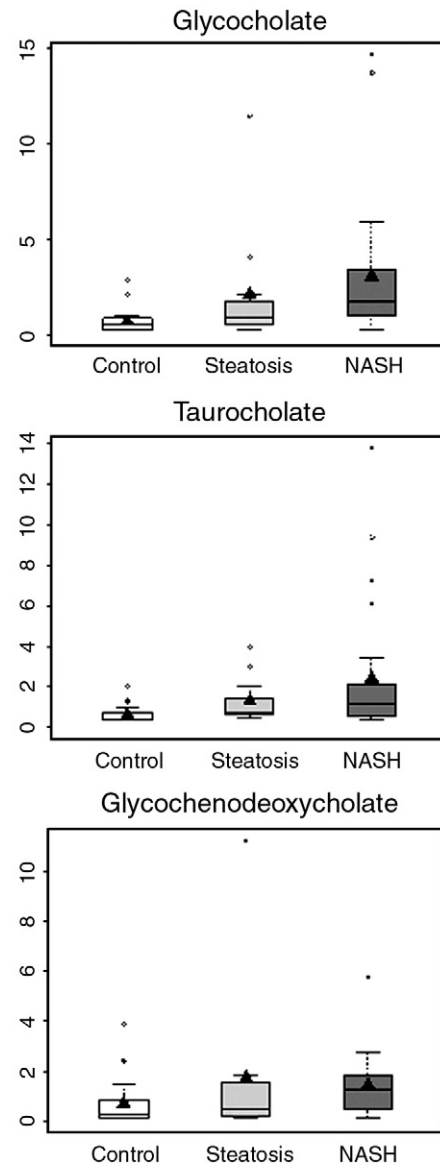


Fig. 1. Box plots of plasma levels of bile salts in healthy controls and subjects with steatosis and NASH. Median scaled values are presented on the y-axis. Only bile salts that were significantly different ($P < .05$) between controls and NASH are shown. Others are presented in supplemental tables.

aenoate (C22:6n3), 10-undecenoate (C11:1n1), and arachidonate (C20:4n6)—were significantly lower in individuals with NASH as compared with controls (Supplemental data). In contrast, only caprate (C10:0) and 10-undecenoate (C11:1n1) were significantly lower in subjects with steatosis as compared with controls. Only linolenate (C18:3n3 or 6) and undecanoate (C11:0) were significantly higher in subjects with steatosis when compared with those with NASH. There were no other differences in the fatty acids profile among steatosis and NASH subjects.

Metabolomic analysis could quantify 14 species of carnitine in the plasma. Free carnitine and butyrylcarnitine levels were significantly elevated in both steatosis and NASH compared with controls (Fig. 3). In addition,

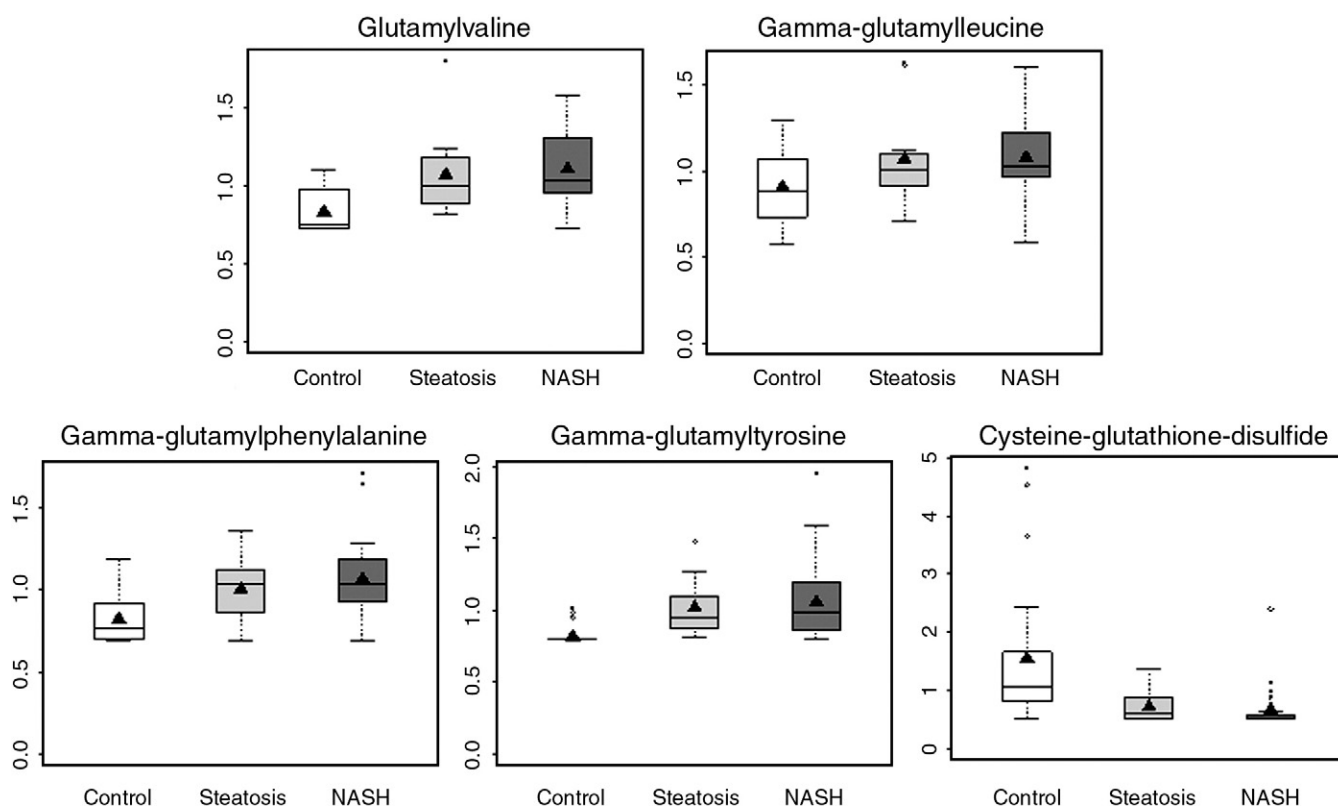


Fig. 2. Glutathione metabolism is up-regulated in subjects with NAFLD. The plasma levels (box and whisker plots) of glutamyl amino acids. All are significantly different ($P < .05$) in NASH and steatosis compared with controls except γ -glutamylleucine, which is significantly higher in NASH only.

propionylcarnitine and 2-methylbutyrylcarnitine levels were significantly higher in subjects with NASH only.

Significant differences in the levels of lysophosphocholines were observed between individuals with NASH and controls. Specifically, the concentration of glycerophosphocholine, 1-oleoylglycerophosphocholine, 1-linoleoylglycerophosphocholine, and 1-arachidonoylglycerophosphocholine were significantly lower in NASH when compared with controls. Only 1-oleoylglycerophosphocholine was significantly lower in subjects with steatosis.

3.5. Carbohydrates

Glucose and pyruvate were significantly higher in subjects with NASH. Mannose and lactate levels were higher in both steatosis and NASH. In addition, erythronate levels were higher in NAFLD subjects.

3.6. Amino acids

Among the essential amino acids, phenylalanine, and branched-chain amino acids, leucine, isoleucine, and valine were higher in subjects with NASH as compared with controls (Fig. 4). Glutamate, aspartate, and tyrosine were also elevated in individuals with NASH. In contrast to subjects with NASH, only glutamate, lysine, tyrosine, and isoleucine were significantly higher in subjects with steatosis compared with controls. There were no significant differences in amino acid levels among subjects with steatosis and NASH.

3.7. Others

A number of unnamed biochemicals in the plasma were significantly higher in NAFLD subjects (Supplemental data), in particular x-11546 and x-11529, which were almost 3-fold higher in NASH compared with controls.

3.8. NASH vs steatosis

As detailed in the supplemental table, plasma levels of very few metabolites were significantly different in subjects with steatosis and NASH. These include glutamate, creatine, pyruvate, and unknown X-01911_200, which were significantly lower, and undecenoate (C11:0) and linolenate (α or γ), which were significantly higher in subjects with steatosis when compared with those with NASH.

3.9. Random forest analysis

An RF analysis of the plasma biochemical profile data was performed to test the ability of the metabolomic data to correctly classify the samples into their respective groups (Table 2). For the healthy group, 92% of the subjects (23 of 25) could be correctly separated from the individuals with NAFLD (steatosis and steatohepatitis), with a class error of 0.08. Among the NAFLD subjects, the error rate was high for subjects in the NASH and steatosis groups, suggesting that the metabolic profile of the NASH and steatosis group was not distinguishable based on this data set. The

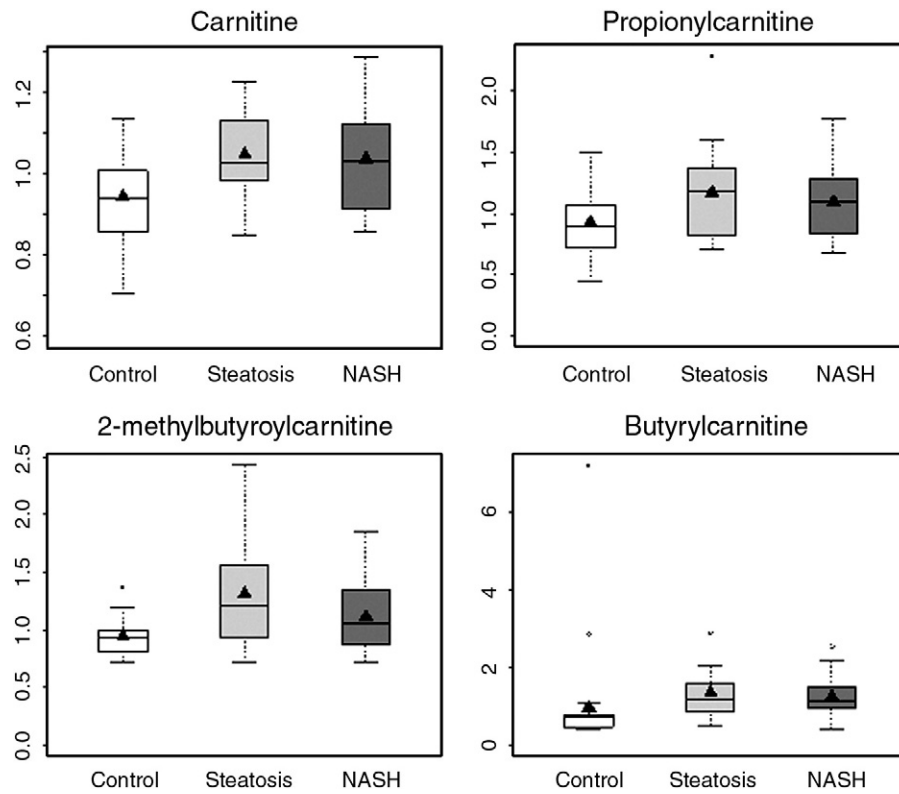


Fig. 3. Box and whisker plots of plasma concentration of carnitine and acylcarnitines in subjects with NAFLD and healthy controls (carnitine and butyrylcarnitine: $P < .05$ NASH vs controls and steatosis vs controls; propionylcarnitine and 2-methylbutyrylcarnitine: $P < .05$ NASH vs controls, not significant for steatosis vs controls).

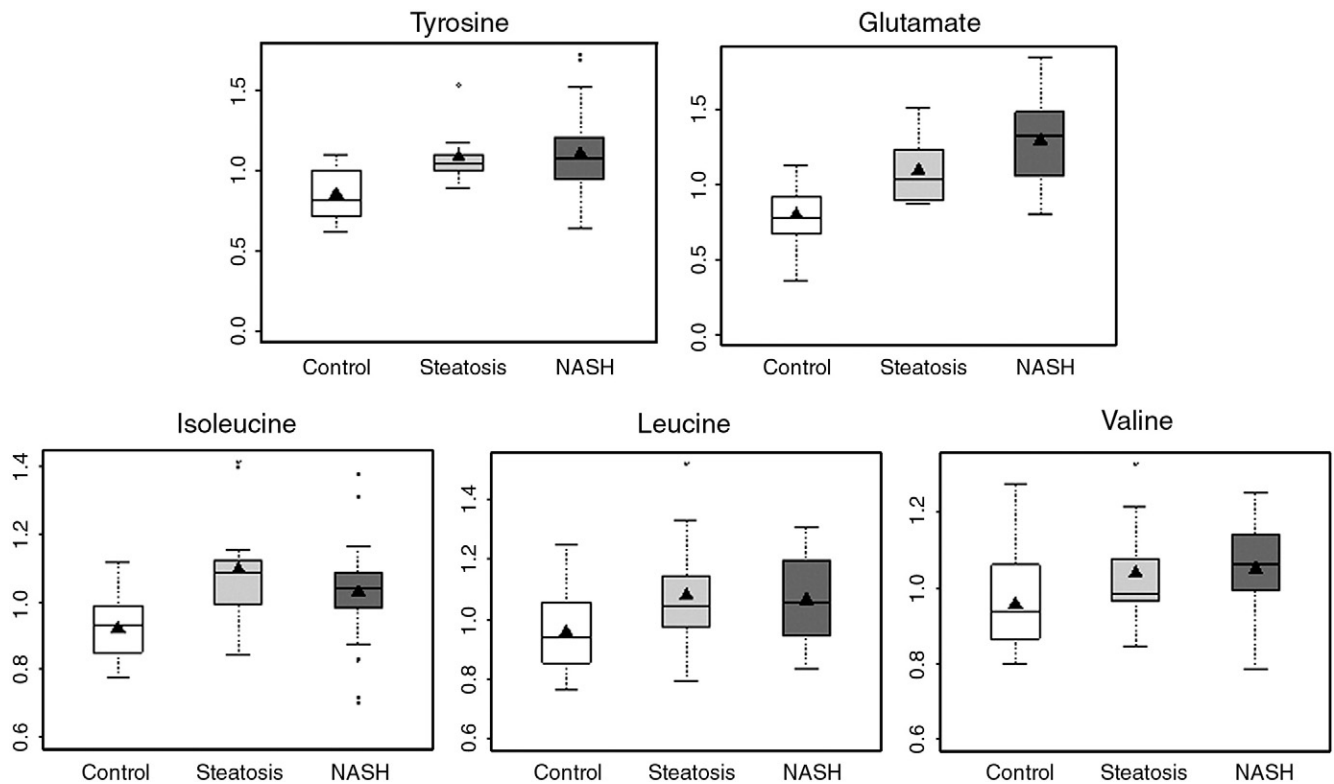


Fig. 4. Box and whisker plots of plasma concentration of branched-chain amino acids, tyrosine, and glutamate in healthy controls and subjects with steatosis and NASH (NASH vs controls: $P < .05$ for all; steatosis vs controls: $P < .05$ for glutamate, tyrosine, and isoleucine).

Table 2

Confusion matrix of the sample by RF analysis

	Healthy	NASH	Steatosis	Class error
Healthy	23	0	2	0.08
NASH	2	11	11	0.54
Steatosis	1	6	4	0.64

Rows represent the actual groups; columns list the predicted groupings by metabolomic analysis.

metabolites that most effectively separated the groups are shown in the importance plot (Fig. 5).

Because the steatosis group was small (not powered enough), we also performed an RF analysis between subjects with NASH only and healthy controls. Twenty-three of 24 subjects with NASH could be separated from healthy controls, with an error rate of 4.1%. The metabolites that most effectively separated the group are shown in the importance plot (Fig. 6).

4. Discussion

In the present study, we performed metabolomic analysis of the plasma to obtain a comprehensive view of changes in several metabolic pathways in patients with NAFLD to identify disease-related patterns and to identify biochemical perturbations. The data revealed significant changes in certain key pathways, specifically bile acids, glutathione metabolism, and lipid and amino acid metabolism. The changes in the plasma metabolome were more evident in subjects with NASH

than in individuals with hepatic steatosis. It should be underscored that the plasma metabolome, during the steady state, represents the sum of changes in several tissues and organs and may not reflect any specific organ system. This is particularly true in the case of NAFLD where, as a result of insulin resistance and changes in hormones and cytokines, a number of organ systems, that is, adipose tissue and skeletal muscle, in addition to the liver, may be affected [1,2].

The subjects in the present study were carefully selected to minimize confounding variables, and the plasma samples were obtained at rest after an overnight fast. Only biopsy-proven subjects with steatosis and steatohepatitis were included. We excluded subjects with “indeterminate” diagnosis. All subjects had normal plasma levels of B₁₂ and folate. Other causes of liver disease were excluded by appropriate laboratory investigations.

4.1. Bile acids

The concentrations of glycocholate, taurocholate, and glycochenodeoxycholate were markedly higher in subjects with NASH. Taurocholate and glycochenodeoxycholate were also significantly higher in subjects with steatosis when compared with controls. Direct measurement of bile acids confirmed these findings (unpublished data). To our knowledge, the higher concentration of bile salts in NASH and steatosis subjects has not been reported previously; and the precise mechanism that is responsible for this increase in concentration remains speculative. It could be the consequence of a higher bile acid pool due to a higher rate of bile acid synthesis, be the result of increased peroxisomal and

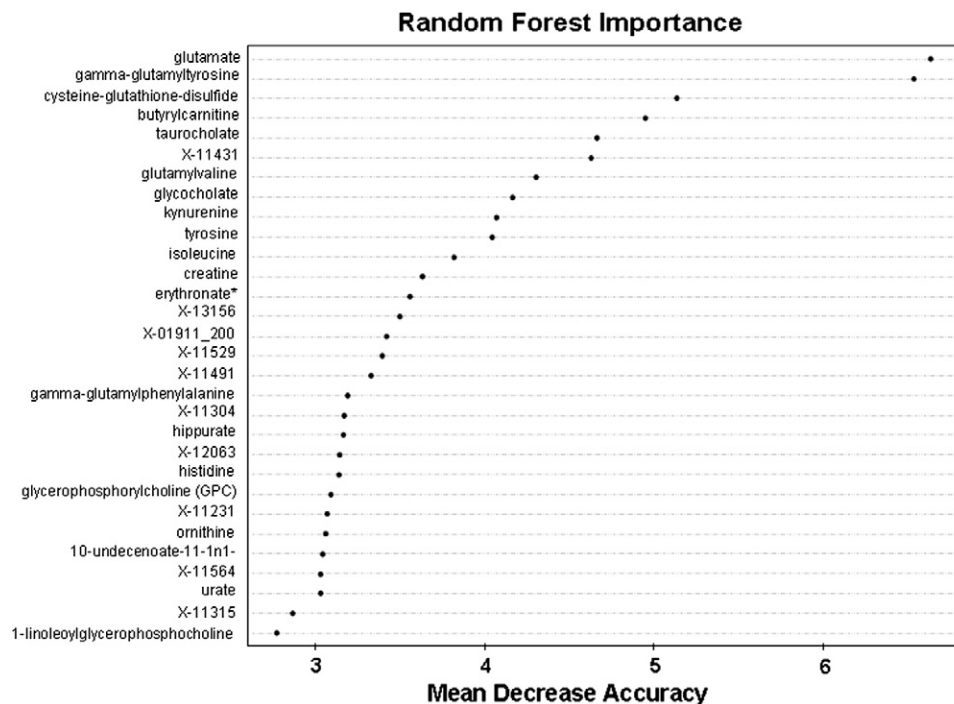


Fig. 5. Random forest importance plot for all subjects.

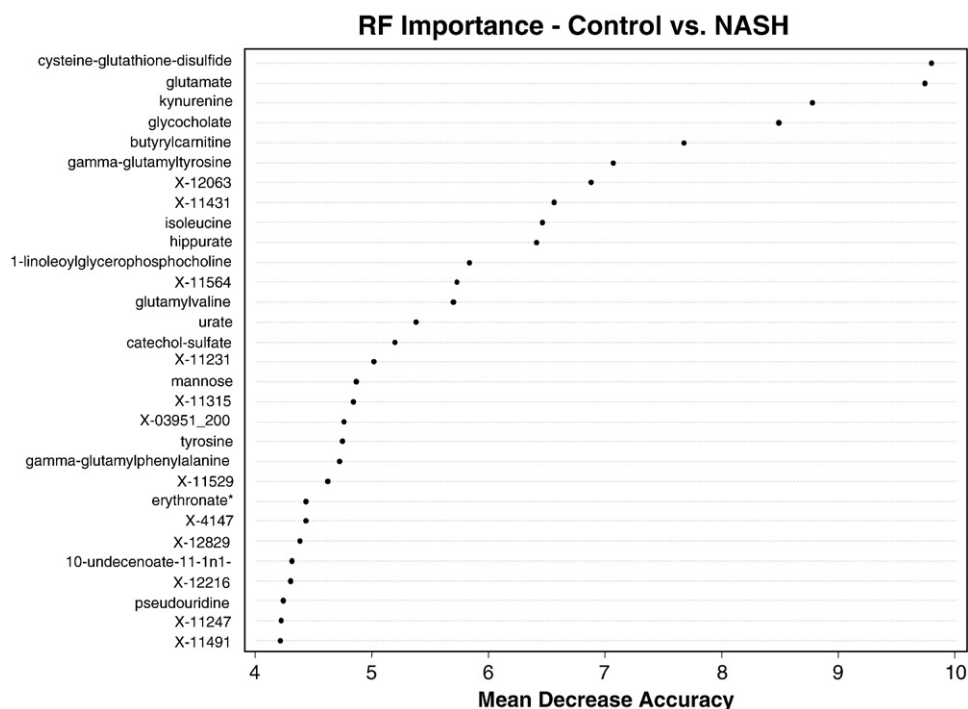


Fig. 6. Random forest importance plot for controls vs NASH.

microsomal metabolism, be caused by hepatocellular injury, or possibly be an adaptive response to the accumulation of triglycerides in the liver. A higher concentration of bile acids has been previously reported in subjects with hyperlipidemia [12]. The healthy liver is very efficient at capturing and removing bile acids from hepatic-portal circulation. When liver function is compromised, more bile acids appear in the circulation because the liver is not adequately removing them. Thus, the concentration of bile acids in the serum has been suggested as an indicator of early liver dysfunction and is considered more sensitive than most traditional assays [13,14]. It is plausible that the accumulation of triglycerides in the liver or increased fatty acid oxidation compromises liver function, resulting in its inefficiency in bile acid uptake from the circulation.

The higher plasma levels of bile acids could also be related to the higher insulin resistance in subjects with NAFLD. The interaction between insulin, hepatic insulin receptors, and bile acids is complex [15–20]. Experimentally induced diabetes in animal models has been shown to result in higher plasma bile acids that decreased after insulin therapy [15,16]. Insulin is a suppressor of sterol 12 α hydroxylase (CYP8B) in the liver, the key enzyme for the synthesis of cholic acid [17]. Liver-specific disruption of insulin receptors and therefore hepatic insulin resistance in mice, however, resulted in lower synthesis of bile acid and decreased expression of the bile acid synthetic enzyme CYP7b1 [18]. The data in the transgenic mice cannot be easily reconciled with the other studies cited above [15–17,19,20].

Irrespective of the mechanism of the increase, a higher concentration of bile acids in individuals with NAFLD could modulate lipid homeostasis through activation of the farnesoid X receptor [21–23], resulting in changes in very low-density lipoprotein cholesterol metabolism and hepatic β -oxidation. Only future studies will delineate the role of bile acids in the alteration in hepatic metabolism observed in subjects with NAFLD.

4.2. Oxidative stress

Metabolomic analysis revealed significantly higher concentrations of γ -glutamyl peptides in subjects with both steatosis and steatohepatitis (Fig. 2), and lower concentrations of cysteine-glutathione disulfide. Targeted analysis showed that the plasma concentration of total glutathione was significantly lower in individuals with NAFLD (Table 1). Glutathione is the major antioxidant in the liver, which is also the primary source of plasma glutathione. A linear correlation between plasma and hepatic glutathione concentration has been reported in liver disease [24], and a lower hepatic glutathione concentration has been reported in nonalcoholic steatosis [25]. After its extrusion from hepatocytes, glutathione is degraded by glutamyl transpeptidase to form cystenyl glycine and glutamyl amino acid [26]. The lower plasma concentration of glutathione and a higher concentration of glutamyl peptides suggest a high rate of hepatic glutathione turnover in subjects with NAFLD as a result of oxidant stress. The latter may be the consequence of increased influx of fatty acid and higher rate of β -oxidation

in the livers of individuals with NAFLD [27–29]. The higher plasma glutamate and cysteine concentration in subjects with NAFLD as compared with controls may also be the consequence of higher rate of turnover of glutathione, similar to that seen in the insulin-deficient state [30]. Studies using tracer isotopic measurements of glutathione kinetics will be required to confirm these observations.

4.3. Lipid metabolism

The plasma concentrations of total carnitines were significantly higher in subjects with both steatosis and NASH. Among the 14 species of carnitines measured, short-chain acylcarnitines (C3, C4, and C5) were significantly higher in individuals with NASH. Only butyrylcarnitine (C5) was significantly elevated in steatosis. Interestingly, we did not find any significant change in most of the fatty acids measured except eicosapentaenoate (20:5n3), docosahexaenoate (22:6n3), and 10-undecenoate (11:1n1), which were lower in subjects with NASH. These changes in plasma long-chain fatty acids are similar to those described by targeted lipidomic analysis of hepatic tissue in subjects with NAFLD [31]. These observed changes may be caused by altered metabolism in the liver, the skeletal muscle, and the contribution of gut microbes. In particular, changes in branched-chain amino acid metabolism in the skeletal muscle may have resulted in higher levels of short-chain acylcarnitines [32]. Further studies will be required to determine if increase dietary intake of these specific polyunsaturated fatty acids can improve the outcome for individuals with NASH.

4.4. Amino acids

Several essential amino acids—leucine, isoleucine, valine, and phenylalanine—were elevated in subjects with NASH and not steatosis. The increase in essential amino acids suggests a higher rate of whole-body protein turnover. In addition, the lack of increase in essential amino acids in individuals with steatosis suggests that changes in protein turnover may be a late event in the progression of steatosis to NASH and may be modulated by other factors such as cytokines and inflammation, in addition to insulin resistance [33–36].

Changes in nonessential amino acids—aspartate and glutamate—may be due to increased anaplerosis of amino acids into the tricarboxylic acid cycle (TCA) cycle, resulting in an increased cataplerosis to ensure the required removal of the resulting carbon skeletons of these amino acids from the cycle. The higher levels of glutamate in the plasma of subjects with NASH, in addition to higher glutathione turnover, could also be due to increased transamination of amino acids being degraded in the liver and skeletal muscle.

4.5. Steatosis vs steatohepatitis

Plasma concentrations of only a few biochemicals were significantly different between subjects with steatosis and steatohepatitis. The physiologic significance of these

changes is uncertain. If one considers steatosis and steatohepatitis as part of a continuum, the lack of any significant difference between the 2 groups in the metabolomic profile is not surprising.

4.6. RF and principal component analysis

To assess the ability to classify subjects as healthy, with steatosis, or with NASH, RF analysis was performed using the entirety of the metabolomic data. An excellent separation of the healthy subjects and NAFLD subjects was achieved. However, the steatosis and NASH subjects were not readily distinguishable (Table 2). This is consistent with the result from Welch *t* test. Many metabolites were deemed to be statistically significant when either the steatosis group or the NASH group was compared with the healthy control group. Only a few metabolites were significantly different between the steatosis and the NASH groups. It is worth noting that the number of subjects in the steatosis group was rather limited (*n* = 11), and the statistical significance is impacted by the group size. In future studies, we are planning to increase the size of the groups to assess if significant differences in plasma metabolic profiles between steatosis and NASH can be detected. As shown in Fig. 6, a panel of markers that provided the most contribution to the separation of the healthy group and NASH group was discovered. Not surprisingly, these markers matched with the metabolites identified by the Welch *t* tests and described in this manuscript: glutathione metabolites and bile acids, amino acids, etc. These markers can be potentially used as diagnostic markers for NAFLD and for the assessment of therapeutic intervention in patients with NASH.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.metabol.2010.03.006](https://doi.org/10.1016/j.metabol.2010.03.006).

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