Metabolomic Profiling Distinction of Human Nonalcoholic Fatty Liver Disease Progression from a Common Rat Model

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Objective: Characteristic pathological changes define the progression of steatosis to nonalcoholic steatohepatitis (NASH) and are correlated to metabolic pathways. A common rodent model of NASH is the methionine and choline deficient (MCD) diet. The objective of this study was to perform full metabolomic analyses on liver samples to determine which pathways are altered most prominently in this condition in humans, and to compare these changes to rodent models of nonalcoholic fatty liver disease (NAFLD).

Methods: A principal component analysis for all 91 metabolites measured indicated that metabolome perturbation is greater and less varied for humans than for rodents.

Results: Metabolome changes in human and rat NAFLD were greatest for the amino acid and bile acid metabolite families (e.g., asparagine, citrulline, gamma-aminobutyric acid, lysine); although, in many cases, the trends were reversed when compared between species (cholic acid, betaine).

Conclusions: Overall, these results indicate that metabolites of specific pathways may be useful biomarkers for NASH progression, although these markers may not correspond to rodent NASH models. The MCD model may be useful when studying certain end points of NASH; however, the metabolomics results indicate important differences between humans and rodents in the biochemical pathogenesis of the disease.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by pathological changes, starting with simple fatty liver (steatosis) and progressing to severe hepatocellular damage and inflammation in a state termed nonalcoholic steatohepatitis (NASH), which can ultimately develop into cirrhosis or liver cancer (1). The worldwide prevalence of NAFLD is estimated to be 25%, with the North American prevalence at 24% (2). Prevalence varies with age and the presence of comorbidities such as obesity, diabetes, and hypertension (2). Multiple histopathologic changes occur within the liver during disease progression, including excess fat accumulation, necroinflammation, and fibrosis (3). These hallmarks can take years to develop in humans and are therefore difficult to model accurately in animal models. Nevertheless, because of the difficulty of obtaining human clinical samples, animal models provide critical information for understanding the molecular mechanisms responsible for histopathologic changes (4,5).

Rodent dietary models can imitate the important histological features of human NAFLD and are powerful tools in determining the effect of disease progression on human cellular functions such as drug metabolism and disposition (6,7). An ideal animal model should reflect both the pathological etiologies of human NAFLD and the histological changes during disease progression.

Methionine and choline deficiency (MCD) in rodents substantially disturbs the formation of phosphatidylcholine and the formation and export of very-low-density lipoprotein (4,8). The MCD diet also impairs fatty acid beta-oxidation, further contributing to the development of hepatic steatosis (9). The stepwise progression of liver damage...
in the MCD model has been well characterized in Wistar rats (10). Over the course of 5 weeks, fatty liver worsens from isolated microvesicular to diffuse macrovesicular steatosis with spotty necrosis (4). Finally, after 7 weeks, clusters of inflammatory cells accumulate, and fibrosis develops in the perivenular and centrilobular regions (4). Liver injury induced by MCD is accompanied by elevated serum transaminases and proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α; therefore, the model is consistent with respect to inflammatory responses (11). While the MCD diet exhibits similar hepatic characteristics to human NASH, it does not reflect the development of obesity or insulin resistance, which are recognized comorbidities of human NASH (6). The high-fat diet (HFD), while increasing free fatty acids and contributing to the development of steatosis, diabetes, and obesity, fails to produce an adequate amount of inflammation and hepatic fibrosis to be considered NASH after only 8 weeks on the diet (6).

Research has demonstrated that MCD rats have similar changes in mRNA and protein expression profiles of the xenobiotic transporters to those seen in human NASH (7,12,13). It is unknown whether the rat MCD model also reflects changes in the metabolome seen in human NAFLD progression. Detailed knowledge of the biochemical changes that occur during NAFLD progression in humans and the prominent animal models is needed to aid in early diagnosis and development of intervention strategies.

The purpose of the present study was to compare the metabolomic response to disease progression between human steatosis and NASH and rodent models of these liver pathologies. Elucidating the similarities and differences of the metabolome in rodent models compared to human NASH will help to determine the limitations of these models. While the high-fat and MCD diets accurately reflect pathological changes along the NAFLD spectrum, differences in the biochemical events leading to the pathology of NASH may limit their use as models in therapeutic studies.

Methods

Rodent liver samples

Male Sprague-Dawley rats (8 weeks old) weighing 200 to 250 g were purchased from Harlan Laboratories (Indianapolis, Indiana). All animals were acclimated in 12-hour light and dark cycles in a University of Arizona Association for Assessment and Accreditation of Laboratory Animal Care-certified animal facility for a week before initiation of experiments and allowed standard chow and water ad libitum. Housing and experimental procedures were in accordance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and approved by the University of Arizona Institutional Animal Care and Use Committee. Rats were fed with methionine and choline sufficient diet (D#518754) as controls as previously described (7,14). Alternatively, rats were fed HFD to recapitulate steatosis (high cholesterol, 18% butter fat, D#112280; n = 7) or methionine-choline deficient (MCD, D#518810; n = 9) diet (all diets from Dyets, Inc., Bethlehem, Pennsylvania) to recapitulate NASH. After 8 weeks on diet, unfasted rats were euthanized by CO₂ asphyxiation. The liver was immediately harvested, weighed, and frozen in liquid nitrogen.

Human liver samples

Frozen postmortem human liver samples were obtained previously from the NIH funded Liver Tissue Cell Distribution System coordinated through Virginia Commonwealth University (Richmond, Virginia) and the Universities of Minnesota (Minneapolis, Minnesota) and Pittsburgh (Pittsburgh, Pennsylvania), funded by NIH Contract #HHSN276201200017C. The detailed clinical information of these human liver samples was published previously, and fasting status is unknown (15). Histology scoring was performed using the NAFLD activity scoring system (16). The samples used in these metabolomics analyses were diagnosed as healthy (n = 17), steatosis (n = 4), NASH (fatty) (n = 14), or NASH (not fatty) (n = 23). Samples exhibiting more than 10% fat deposition in hepatocytes without inflammation and fibrosis were diagnosed as steatosis. Samples exhibiting more than 5% fatty deposition accompanying inflammation and fibrosis were diagnosed as NASH (fatty). NASH (not fatty) was diagnosed when less than 5% fatty infiltration of hepatocytes accompanied significant inflammation and fibrosis.

Analytical methods for liquid chromatography with tandem mass spectrometry

Fifty- to one hundred-milligram rat or human liver samples were homogenized in 10 times the tissue weight of ice-cold methanol solution with 0.1% formic acid for 18 to 20 seconds using a Polytron™ homogenizer over ice. Supernatants were centrifuged prior to being aliquoted, dried, and frozen in a 96-well plate as described previously (17). Samples were reconstituted in 90:10 (v:v) water:methanol prior to analysis. The internal standard d5-hippurate was added to the samples, which were then injected in a randomized fashion to a Thermo Scientific Accela ultra-high-performance liquid chromatography (UHPLC) system coupled to an Exactive™ Orbitrap Mass Spectrometer. The peaks were acquired in positive and negative ion mode (separate injections) with a mass accuracy within 5 ppm at 25 K resolution. Metabolite peak area under the curve (AUC) measurements for liquid chromatography and mass spectrometry (LC-MS) were calculated using Component Elucidator, a software package developed by Bristol-Myers Squibb (Princeton, New Jersey) as described previously (17). The potential biomarkers were identified according to chromatographic retention time, molecular weight (m/z), and MS/MS fragmentation. The metabolites were matched using the databases KEGG (http://www.genome.jp/kegg/), METLIN (http://metlin.scripps.edu/), HMDB (http://www.hmdb.ca/), and so on.

Bioinformatics and statistical analysis

Ninety-one metabolites were analyzed by the University of Arizona Statistical and Bioinformatics Consortium. Principal component analysis (PCA) was used as an unsupervised statistical method to study the differences of intergroup metabolites among all experimental groups. Hierarchical clustering analysis was performed on the metabolite data to reveal any clustering effects related to the stage of the disease and species. Peak AUC values from the LC-MS data were log transformed into a normal distribution approximation. After log transformation, a one-way analysis of variance (ANOVA) with Tukey honest significant difference testing was used for multiple comparisons of metabolites among the diagnosis groups and species to test for mean differences. The Benjamini-Hochberg procedure was used to adjust the reported P values to control for the rate of false discoveries. Although human metabolite analyses were performed using the four diagnosis categories, due to the lack of statistical differences between human NASH (fatty) and NASH (not fatty), these two categories were combined as human NASH. Significance was defined as adjusted P ≤ 0.05. The interpretation of the data described herein refers to the reported log-transformed values and the respective adjusted P values.
Results

The distribution of 91 rat and human metabolites is shown by PCA, which demonstrates any relationship or differences between rat and human metabolic profiles. The PCA plot (Figure 1) reflects the fact that the human and rat metabolites were predominantly separated by the first component (PC1), the x-axis, completely differentiating the two species as indicated by the lack of overlap between any rat and human sample. The NASH and control samples were unable to be completely separated by the second component (PC2), the y-axis. Diagnoses were not able to be differentiated on an axis orthogonal to species in this analysis. The variance explained by PC1 was 26.71%, and that explained by PC2 was 13.64%.

To explore the potentially significant biochemical differences in NAFLD progression by metabolic class, the metabolites were divided into functional categories: bile acids, amino acids, fatty acids and carnitine metabolites, and “other” metabolites.

Bile acid metabolomics

Select bile acids present in both humans and rats at detectable levels were chosen for this metabolomic comparison (18). Eleven bile acid metabolites in rat and human liver samples with NAFLD were quantified by LC-MS/MS. Further analysis revealed significant differences in the bile acid profile between rat MCD samples and human samples diagnosed as NASH (Table 1). The average log-transformed AUC data for cholic acid (CA) and deoxycholic acid (DCA) were significantly increased in rat NASH. Conversely, significant decreases in CA and DCA were observed in human NASH, though there were increases in glycochenodeoxycholic acid, taurocholic acid, and taurodeoxycholic acid. CA, DCA, glycochenodeoxycholic acid, glycodeoxycholic acid, glycuroursolic acid, and tauursodeoxycholic acid exhibited significant interspecies differences (P ≤ 0.05).

![Figure 1 Principal component analysis distinguished any correlated variables between PC1 and PC2 for the metabolite’s projections to the orthogonal coordinate transformation. The distribution of 91 metabolites in the plot indicates the relationship between rat high-fat diet (HFD) and methionine and choline deficiency (MCD) and human nonalcoholic fatty liver disease (NAFLD) progression. The human and rat metabolites were predominantly separated by the first component (PC1), and the diagnosis/model was mostly separated by the second component (PC2). [Color figure can be viewed at wileyonlinelibrary.com]](image)

| Bile acid | HFD | MCD | Adjusted P value | Steatosis | NASH | Adjusted P value | Interspecies adjusted P value |
|-----------|-----|-----|------------------|-----------|------|------------------|-------------------------------|
| CA        | 0.73 ± 0.51 | 1.06 ± 0.53 | 0.003a | −0.38 ± 0.16 | −1.01 ± 0.47 | 0.000b | 0.000b |
| DCA       | 0.76 ± 1.74 | 2.91 ± 1.56 | 0.008a | 0.53 ± 2.02 | −3.22 ± 1.79 | 0.000b | 0.000b |
| GCDCA     | −0.71 ± 0.97 | −0.82 ± 0.93 | 0.896 | −0.46 ± 0.81 | 0.70 ± 0.83 | 0.028b | 0.000b |
| GCA       | −0.70 ± 1.02 | −0.31 ± 1.24 | 0.954 | −0.34 ± 0.74 | 0.28 ± 0.79 | 0.716 | 0.117 |
| GDCA      | 0.76 ± 1.34 | 0.30 ± 0.92 | 0.954 | −0.07 ± 0.87 | −2.50 ± 1.11 | 0.000b | 0.000b |
| GUCa      | −1.05 ± 1.96 | 0.34 ± 1.31 | 0.954 | −1.08 ± 1.64 | −1.09 ± 1.38 | 0.105 | 0.015b |
| GUDCA     | −2.64 ± 1.54 | −0.71 ± 1.13 | 0.931 | −0.67 ± 1.60 | −0.60 ± 1.91 | 0.708 | 0.956 |
| TCA       | 0.67 ± 1.27 | 1.15 ± 0.84 | 0.102 | 0.03 ± 0.51 | 1.15 ± 0.94 | 0.001a | 0.999 |
| TDCA      | 1.12 ± 0.54 | 1.02 ± 1.06 | 0.102 | −0.27 ± 0.55 | 1.65 ± 0.83 | 0.000a | 0.090 |
| TUCA      | 1.44 ± 0.65 | 0.99 ± 0.77 | 0.094 | −0.81 ± 2.51 | −0.15 ± 1.21 | 0.992 | 0.018b |
| TUDCA     | −1.78 ± 1.72 | 0.16 ± 0.62 | 0.954 | −0.35 ± 0.99 | 0.37 ± 1.59 | 0.827 | 0.841 |

Bile acid metabolite values represent natural log-transformed area under the curve values relative to species-specific control samples ± the standard deviation. Test statistics calculated with one-way ANOVA with Tukey honest significant difference, with significance defined as adjusted P < 0.05.

aSignificant difference across samples for that species.

bSignificant difference in human NASH to rat MCD samples.

CA, cholic acid; DCA, deoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GUCa, glycoursocholic acid; GUDCA, glycoursodeoxycholic acid; HFD, high-fat diet; MCD, methionine and choline deficient; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; TCA, taurocholic acid; TDCA, taurodeoxycholic acid; TUCA, taoursocholic acid; TUDCA, taursodeoxycholic acid.
A heat map of a hierarchical cluster analysis represents the clustering of bile acid metabolites in human and rat samples within different NAFLD diagnosis categories for each species (Figure 2). However, bile acid metabolome alterations were only able to differentiate the dissimilarity between species at this higher level of analysis and showed no clustering effects for diagnosis in either humans or rats. Chenodeoxycholic acid and ursodeoxycholic acid were not able to be differentiated, as the peaks were superimposed, and were thus excluded from further analysis.

Amino acid metabolomics
Analysis of 19 amino acid metabolites (Table 2) in rat and human hepatic samples measured by LC-MS revealed that asparagine, citrulline, and lysine exhibited a significant increase in both human and rat NASH, while other changes in amino acid levels did not carry the trend across species. Alanine, gamma-aminobutyric acid, and z-ketoglutarate were decreased, while serine and threonine were increased in MCD rats compared to controls. In human NASH, histidine, methionine, and tryptophan were decreased, and isoleucine, phenylalanine, tyrosine, valine, and succinic acid were increased. Significant interspecies interaction was seen in alanine, asparagine, citrulline, histidine, z-ketoglutarate, methionine, phenylalanine, proline, serine, succinic acid, threonine, tryptophan, and valine.

The metabolism pathways of selected amino acid metabolites analyzed illustrate the important role in the citric acid and urea cycle pathways (Figure 3). All amino acids ultimately enter the citrate cycle or undergo glycolysis/glucogenesis for degradation or metabolism. The concurrent changes and contrasts observed in these metabolites in rat and human NAFLD are highlighted (Figure 3).

Fatty acids, carnitine, and lysophosphatidyl choline metabolomics
Metabolomics analysis identified 15 fatty acids (including carnitine) and 17-lysophosphatidyl choline. Fatty acid metabolites significantly differed between humans and rats for controls and NASH (Table 3). All highlighted metabolites in Table 3 were significantly altered in human NASH and revealed significant interspecies interaction. Arachidonic acid, choline, linoleic acid, and stearoyl-lyso-PE (18:0) were significantly decreased in human NASH, whereas stearoyl-lyso-PE was increased in MCD rats. Additionally, significant increases in the levels of arachidonoyl-lyso-PC (20:4), butyryl carnitine (C4), lauryl carnitine (C12), stearoyl carnitine (C18:0), and tetradecanoyl carnitine (C14) were observed in human samples during NAFLD progression. MCD rats also exhibited increased stearoyl carnitine (C18:0) levels.

Other metabolomics
The remaining metabolites analyzed from the liver samples were classified as other metabolites and included miscellaneous metabolites involved in energetics pathway processes and by-products as
TABLE 2 Amino acid metabolomics in hepatic samples of rats and humans

| Amino acid | HFD | MCD | Adjusted P value | Human | Steatosis | NASH | Adjusted P value | Interspecies adjusted P value |
|------------|-----|-----|-----------------|-------|----------|------|-----------------|-----------------------------|
| Alanine    | −0.34 ± 0.18 | −0.46 ± 0.38 | 0.024* | −0.07 ± 0.25 | −0.19 ± 0.28 | 0.117 | 0.035* |
| Asparagine | −0.12 ± 0.50 | 0.72 ± 0.32 | 0.010* | −0.87 ± 0.40 | 0.28 ± 0.26 | 0.006* | 0.000* |
| Citrulline  | 1.05 ± 0.69 | 2.19 ± 0.60 | 0.000* | −1.16 ± 2.32 | 2.86 ± 1.11 | 0.000* | 0.121* |
| GABA       | −0.16 ± 0.24 | −0.68 ± 0.47 | 0.022* | −0.58 ± 0.67 | −0.74 ± 0.52 | 0.000* | 0.755 |
| Glutamine  | −0.48 ± 0.22 | −0.10 ± 0.63 | 0.964 | 0.01 ± 0.42 | 0.09 ± 0.29 | 0.657 | 0.211 |
| Histidine  | −0.05 ± 0.13 | −0.11 ± 0.27 | 0.752 | −2.25 ± 0.46 | −2.6 ± 0.31 | 0.000* | 0.165 |
| Isoleucine | 0.34 ± 0.34 | 0.09 ± 0.45 | 0.964 | −0.49 ± 0.34 | 0.32 ± 0.38 | 0.022* | 0.000* |
| α-Ketoglutarate | 0.90 ± 0.58 | −2.68 ± 2.45 | 0.094 | −0.89 ± 3.37 | −0.50 ± 1.85 | 0.862 | 0.007* |
| Leucine    | 0.32 ± 0.39 | 0.10 ± 0.45 | 0.964 | −0.52 ± 0.42 | 0.25 ± 0.35 | 0.076 | 0.303 |
| Lysine     | 0.82 ± 0.64 | 0.73 ± 0.52 | 0.036* | −0.53 ± 0.37 | 0.44 ± 0.37 | 0.001a | 0.092 |
| Methionine | 0.47 ± 0.82 | 0.21 ± 0.85 | 0.964 | −0.44 ± 0.61 | −0.74 ± 0.80 | 0.016* | 0.005* |
| Phenylalanine | 0.08 ± 0.28 | −0.14 ± 0.40 | 0.848 | −0.47 ± 0.43 | 0.46 ± 0.38 | 0.001* | 0.000* |
| Proline    | 0.35 ± 0.35 | 0.35 ± 0.49 | 0.436 | −0.19 ± 0.10 | −0.20 ± 0.35 | 0.308 | 0.001* |
| Serine     | −1.12 ± 0.72 | 1.40 ± 0.44 | 0.004* | −0.30 ± 0.64 | −0.25 ± 0.34 | 0.099 | 0.000* |
| Succinic acid | −0.3 ± 0.3 | −0.48 ± 0.47 | 0.199 | −0.15 ± 0.29 | 0.43 ± 0.40 | 0.033a | 0.000* |
| Threonine  | −0.58 ± 0.30 | 0.98 ± 0.45 | 0.001* | −1.05 ± 0.28 | 0.14 ± 0.40 | 0.571 | 0.000* |
| Tryptophan | 0.01 ± 0.29 | −0.05 ± 0.49 | 0.998 | −1.65 ± 0.07 | −0.69 ± 0.35 | 0.038a | 0.000* |
| Tyrosine   | 0.23 ± 0.52 | 0.23 ± 0.53 | 0.796 | −0.55 ± 0.57 | 0.36 ± 0.38 | 0.021a | 0.413 |
| Valine     | 0.16 ± 0.25 | −0.35 ± 0.44 | 0.283 | −0.50 ± 0.35 | 0.40 ± 0.28 | 0.000a | 0.000b |

Amino acid metabolite values represent natural log-transformed area under the curve values relative to species-specific control samples ± standard deviation. Test statistics calculated with one-way ANOVA with Tukey honest significant difference, with significance defined as adjusted P ≤ 0.05.

*Significant difference across samples for that species.

**Significant difference in human NASH to rat MCD samples.

GABA, gamma-aminobutyric acid; HFD, high-fat diet; MCD, methionine and choline deficient; NASH, nonalcoholic steatohepatitis.

well as organic acids that were significantly different in subjects with NASH or MCD rats. These include 4-hydroxyproline, betaine, creatine, creatinine, glucose, and taurine (Table 4). Among these metabolites, all but creatine exhibited an interspecies interaction. 4-hydroxyproline was increased in human NASH but unchanged in MCD rats. Betaine was decreased in MCD rats but increased in NASH, while creatine was increased in MCD rats but decreased in human NASH. Glucose levels decreased in MCD rats and trended toward increase in NASH. Taurine was increased significantly in human NASH and trended toward an increase in MCD rats.

**Discussion**

The utility of NAFLD rodent models in the future is both extensive and selective: extensive in the need for appropriate mechanistic studies in animal models, but selective due to characteristics needed to be accurately reflected in the model. Rodent NAFLD models vary dramatically in their ability to reproduce both the clinical and histopathologic features of the disease (7). Both rats and mice fed an MCD diet develop histopathologic features associated with NASH but fail to represent the full spectrum of the clinical features of NASH such as insulin resistance (19).

Changes in metabolic pathways that occur from NAFLD progression can be ascertained by studying the metabolome. Moreover, perturbed levels of metabolites can reflect the degree of liver injury. The current study describes a metabolomic analysis of liver samples to compare the biochemical changes in both human NAFLD progression and in rodent models designed to reflect stages of the NAFLD spectrum: HFD representing steatosis and MCD representing NASH. Importantly, many significant changes were found in metabolic pathways in NAFLD that were more pronounced with progression of the disease, and we determined whether these trends were reflected in the rodent models.

A more detailed look at bile acid metabolomics in human NAFLD has been published previously, highlighting elevated levels of taurocholic acid and taurodeoxycholic acid in NASH and decreased levels of CA and glycineoxygenic acid (20). In this study, those changes were compared to the rodent HFD and MCD diet models, which conversely exhibited increased CA and no changes in the other bile acids measured. A major contributor to this discrepancy is the interspecies difference in bile acid composition. Targeted profiling research of hepatic bile acids in humans and rats has demonstrated that glycine-conjugated bile acids are the most abundant in the human liver, whereas tauro-bile acids are the predominant conjugated forms in MCD rats (18). This is coincident with our finding that the metabolic profile of hepatic bile acids was dominated by conjugated bile acids (Table 1). The interspecies differences in the hepatic bile acid pool composition may be a determinant in potential liver damage induced by the accumulation of specific bile acids in humans, which may be missing in rodent models (21,22). Among bile acids, hydrophobic bile acids are recognized as the main nuclear receptor signaling ligands and can also behave as toxic precursors that can lead to the direct activation of cell apoptosis and necrosis (23,24). Changes in bile acid
composition in humans and rodent models of disease must be interpreted cautiously and include analysis of nuclear receptor activation and regulation of rate-limiting enzymes due to well-defined differences in hydrophilic and hydrophobic bile acid pools and signaling between rodents and humans (25).

The liver is the main site for amino acid catabolism and would be a prime target in metabolomic remodeling in NAFLD. In the present study, almost all amino acids exhibited a significant change in NASH and had a significant interspecies interaction. A detailed investigation into branched-chain amino acid regulation in NASH

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**TABLE 3** Fatty acids, carnitine, and lysophosphatidyl choline metabolomics in hepatic samples of rats and humans

| Fatty acid                  | HFD       | MCD       | Adjusted P value | Steatosis | NASH      | Adjusted P value | Interspecies adjusted P value |
|-----------------------------|-----------|-----------|------------------|-----------|-----------|------------------|-------------------------------|
| Arachidonic acid            | −0.12 ± 0.57 | 1.02 ± 0.82 | 0.056            | −1.00 ± 0.51 | −0.92 ± 0.60 | 0.000a            | 0.000b                         |
| Arachidonoyl-lyso-PC (20:4) | 0.1 ± 0.31 | 0.19 ± 0.38 | 0.589            | −0.36 ± 0.13 | 0.59 ± 0.39 | 0.000a            | 0.011b                         |
| Butyryl carnitine (C4)      | 0.13 ± 0.84 | −2.00 ± 2.24 | 0.116            | 1.76 ± 0.92 | 1.75 ± 0.69 | 0.000a            | 0.000b                         |
| Choline                     | 0.79 ± 0.58 | 0.20 ± 0.70 | 0.878            | −0.11 ± 0.23 | −0.57 ± 0.27 | 0.000a            | 0.000b                         |
| Lauryl carnitine (C12)      | 1.62 ± 0.78 | −0.17 ± 1.10 | 1.000            | 2.25 ± 1.51 | 2.50 ± 0.81 | 0.000a            | 0.000b                         |
| Linoleic acid               | 0.29 ± 1.43 | 2.20 ± 1.88 | 0.110            | −1.43 ± 2.25 | −2.12 ± 1.45 | 0.001a            | 0.000b                         |
| Stearoyl carnitine (C18:0)  | 1.3 ± 0.48  | 1.37 ± 0.81 | 0.016a           | 0.36 ± 0.35 | 0.54 ± 0.65 | 0.020a            | 0.004b                         |
| Stearoyl-lyso-PE (18:0)     | 0.95 ± 0.52 | 1.39 ± 0.65 | 0.001a           | −0.54 ± 0.25 | −0.86 ± 0.45 | 0.000a            | 0.000b                         |
| Tetradecanoyl carnitine (C14)| 2.17 ± 0.55 | −0.32 ± 1.56 | 1.000            | 2.30 ± 0.80 | 1.48 ± 0.77 | 0.000a            | 0.000b                         |

Fatty acid metabolite values represent natural log-transformed area under the curve values relative to species-specific control samples ± standard deviation. Test statistics calculated with one-way ANOVA with Tukey honest significant difference, with significance defined as adjusted $P < 0.05$.

*Significant difference across samples for that species.

**Figure 3** Amino acid metabolism pathway analysis. Amino acid analysis was performed to show the change of amino acid metabolites between different species and diagnosis/model. The boxed amino acids were analyzed and matched by liquid chromatography and mass spectrometry and metabolomics software. Green means the log-transformed area under the curve significantly increased in rat methionine and choline deficiency and human nonalcoholic steatohepatitis (NASH). Red shows a significant decrease in rat methionine and choline deficiency and human NASH. Black shows no change regardless of species. Blue shows that the change trend comparing controls to NASH was different between humans and rats. The asterisk (*) represents the trend in rats, and the pound sign (#) represents the trend in humans. [Color figure can be viewed at wileyonlinelibrary.com]
has been published previously (26); however, it is worth noting that none of the altered branched-chain amino acids was changed in either HFD or MCD. Only asparagine, citrulline, gammaaminobutyric acid, and lysine exhibited the same trend across both species. Although each amino acid has its own unique catabolic pathway, nitrogen metabolism is mainly via urea formation and carbon conservation via gluconeogenesis and the citrate cycle (Figure 3). Asparagine, citrulline, and lysine contribute directly to the formation of acetyl-CoA, and disruption of these levels can have a direct impact on the metabolic and antioxidant function of the mitochondria (27).

The pathogenesis of NAFLD involves the accumulation of excess triglyceride (TG) and free fatty acids in the hepatocytes, which contributes to the hallmark inflammatory response (28). TG accumulation in hepatocytes arises from an imbalance between lipid uptake and removal (28). TG synthesis is stimulated to dispose of excess free fatty acids, which come from peripheral adipose tissue to the liver via the lipogenic pathway (29). Animal models such as the MCD diet exhibit TG and lipoperoxide accumulation as early as 2 to 5 days after starting the diet (30), and these changes in TG composition and levels are reflected in the results of the analysis described in Table 3. The absence of choline, an essential nutrient, induces steatosis and liver cell death in humans, and these data support decreased choline levels in human NASH (31). The current studies have shown the short-chain carnitines are significantly higher in individuals with NASH, consistent with our previous study (26), while long-chain fatty acid and phospholipids are lower in NASH. This is similar to previous studies that have performed target lipidomics analyses of hepatic tissue in subjects with NAFLD (32,33).

In the comparison of human and MCD rat model metabolomes, several endogenous metabolites in the “energetics metabolite” category in Table 4 were assessed. 4-Hydroxyproline, betaine, glucose, creatinine, creatine, and taurine were all significantly changed in human NASH samples. Taurine is an organic acid that conjugates bile acids and was significantly increased in human NASH. Recent clinical studies support the use of taurine administration to alleviate inflammation and lipid accumulation in NAFLD (34). Taurine increases in the rat models did not reach statistical significance. Betaine and creatine exhibited opposite changes between rat models and the human NAFLD samples. Overall, some of the similarities between human NASH and rodent MCD diet model metabolites may be developed as useful biomarkers, while others may not.

In summary, this comparison profiling analysis of human and rodent NAFLD provides a foundation for further investigation into diagnosis and monitoring. This analysis does have some limitations, including the inability to differentiate diagnoses in PCA, and that the human liver samples were obtained by the Liver Tissue Cell Distribution System, most likely from deceased donors, thus overrepresenting the more severe precirrhotic end of the NAFLD spectrum. Nevertheless, select essential amino acids could be potential biomarkers for NAFLD progression. The combination of metabolomic changes of bile acids, amino acids, and fatty acids lends insight into the physiological and biochemical response integral to NAFLD pathogenesis in both species. Furthermore, the dissimilarities noted between human NAFLD progression and rodent models suggest a limited utility of these models for comparing biochemical biomarkers. While they are appropriate for certain end points, such as pharmacokinetics or enzyme regulation, therapeutic intervention or the biochemical aspects of pathogenesis of NASH may prove to have important differences.

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