Baseline Urine Metabolic Phenotype in Patients With Severe Alcoholic Hepatitis and Its Association With Outcome

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Severe alcoholic hepatitis (SAH) has a high mortality rate, and corticosteroid therapy is effective in 60% patients. This study aimed to investigate a baseline metabolic phenotype that could help stratify patients not likely to respond to steroid therapy and to have an unfavorable outcome. Baseline urine metabolome was studied in patients with SAH using ultra-high performance liquid chromatography and high-resolution mass spectrometry. Patients were categorized as responders (Rs, n = 52) and nonresponders (NRs, n = 8) at day 7 according to the Lille score. Multivariate projection analysis identified metabolites in the discovery cohort (n = 60) and assessed these in a validation cohort of 80 patients (60 Rs, 20 NRs). A total of 212 features were annotated by using metabolomic/biochemical/spectral databases for metabolite identification. After a stringent selection procedure, a total of nine urinary metabolites linked to mitochondrial functions significantly discriminated nonresponders, most importantly by increased acetyl-L-carnitine (12-fold), octanoylcarnitine (4-fold), decanoylcarnitine (4-fold), and alpha-ketoglutaric acid (2-fold) levels. Additionally, urinary acetyl-L-carnitine and 3-hydroxysebasic acid discriminated nonsurvivors (P < 0.01). These urinary metabolites significantly correlated to severity indices and mortality (r > 0.3; P < 0.01) and were associated with nonresponse (odds ratio >3.0; P < 0.001). In the validation cohort, baseline urinary acetyl-L-carnitine documented an area under the receiver operating curve of 0.96 (0.85-0.99) for nonresponse prediction and a hazard ratio of 3.5 (1.5-8.3) for the prediction of mortality in patients with SAH. Acetyl-L-carnitine at a level of >2,500 ng/mL reliably segregated survivors from nonsurvivors (P < 0.01, log-rank test) in our study cohort. Conclusion: Urinary metabolome signatures related to mitochondrial functions can predict pretherapy steroid response and disease outcome in patients with SAH. (Hepatology Communications 2018;2:628-643)

Alcoholic hepatitis is a common ailment and is associated with systemic inflammatory response syndrome, organ failure, and short-term mortality of up to 50%.(1) The pathophysiology of severe alcoholic hepatitis (SAH), however, is poorly understood because of the lack of appropriate animal models and limited translational studies. (2,3) Severity of SAH is assessed based on histologic features, although many noninvasive scoring systems, such as Maddrey’s discriminant function (MDF) ≥32 and the Model for
End-Stage Liver Disease (MELD) score has been developed for prognostication of SAH. It is important to identify patients with SAH at a high risk of mortality before considering specific therapies. Corticosteroid therapy, although controversial, remains the only option to improve the morbidity and short-term mortality in SAH. While the precise mechanisms of action of steroids in SAH are unknown, inhibition of inflammatory reactions and immune-mediated hepatic destruction play a dominant role. However, the anti-anabolic effects of steroids may suppress hepatic regeneration and healing. Corticosteroid therapy can prove deleterious in patients with clinical manifestations similar to patients with SAH (10%-30%). Further, continuing corticosteroids in the nonresponsive patients could result in predisposition to secondary bacterial infections, spontaneous bacterial peritonitis, and increased mortality. Early identification of nonresponders to corticosteroid therapy, which may be around 40%, is therefore essential. A Lille score of ≥0.45 is used to define steroid nonresponse at day 7. However, waiting for 7 days leads to unnecessary exposure to steroids in the eventual steroid-nonresponsive patients. Thus, there is an urgent need of identifying novel indicators for differentiating nonresponders from responders prior to the start of therapy. Severity and progression of alcoholic hepatitis also needs to have better markers, preferably noninvasive ones.

Urine as a biofluid has gained importance for the identification of putative biomarkers because it is mostly sterile in nature, less complex, easy to obtain in large volume, and largely free from interfering proteins or lipids. In addition, ease of urine sample processing makes it a favored biofluid for identifying altered metabolic pathways associated with disease/therapy. Metabolomics is a powerful technology that allows assessment of global metabolic profiles in biofluids. In order to explore new indicators of steroid nonresponse, we studied the urine metabolome profile at baseline before corticosteroid therapy. We also investigated whether urinary metabolites correlate with disease severity and mortality. Finally, we developed an approach integrating urine metabolomics and liver transcriptomics in order to explore the possible links between urine metabolites and liver genes and enhance our understanding of SAH pathophysiology.

Patients and Methods

Patients with SAH seen between 2013 and 2015 at the Department of Hepatology, Institute of Liver and Biliary Science, New Delhi, India, and confirmed to have MDF ≥32, recent onset of jaundice, chronic alcohol abuse, and liver biochemistry and histologic features of SAH (n = 180) were screened for corticosteroid therapy. All 180 patients underwent transjugular liver biopsy, and a minimum of 10 portal spaces were analyzed before characterization of a patient as SAH. Patients with hepatocellular carcinoma (n = 10), portal vein thrombosis (n = 15), or recent variceal bleed (n = 12) were excluded from analysis. In addition, patients with hepatitis B virus, hepatitis C virus, and human immunodeficiency virus infection were excluded. The remaining 140 patients with SAH were enrolled in the study, and written informed consent was obtained from every patient. The study was approved by the institutional ethics committee.

Baseline demographic profiles were recorded and early morning fasting urine samples were collected before start of prednisolone at 40 mg/day. Patients were characterized as responders (Rs) or nonresponders (NRs) at day 7 using the Lille score. At baseline, none of the enrolled patients with SAH documented high serum creatinine level, suggesting normal
functioning of the kidneys. Further, serum creatinine-based estimation of glomerular filtration rate was found to be >90 mL/minute/1.73 m² in all patients, confirming the absence of kidney injury in these patients. The laboratory staff performing the experiments was unaware of the clinical details. Patients were managed according to the standard of care, including intensive care monitoring, high calorie diet (35-40 cal/kg/day), intravenous albumin, and broad-spectrum antibiotics. Severity of liver disease was assessed by MDF, Child-Pugh, and MELD scores at the initial presentation, and steroid responsiveness was assessed by the Lille score during follow-up. Among the 140 patients, the first 60 patients (enrolled during 2013) formed the discovery cohort and the subsequent 80 patients (enrolled in 2014 and 2015) constituted the validation cohort.

URINE METABOLOMICS

Urine metabolomics was performed in the discovery cohort. About 20 mL of early morning urine sample was aliquoted and stored at –80°C. Urine samples were centrifuged at 1,430 g for 5 minutes, diluted at 1:5 in 5% acetonitrile:95% water, spiked with internal standards at known concentrations, and subjected to reverse-phase chromatography on an ultra-high performance liquid chromatographic system followed by high-resolution mass spectrometry (MS) as detailed in the Supporting Methods.

MEASUREMENTS OF URINARY ACETYL-L-CARNITINE

The determination of acetyl-L-carnitine concentrations in the urine samples was performed using the acetyl-L-carnitine detection kit (cat. no. CEO400Ge) in both the discovery and the validation cohort (details in the Supporting Methods).

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical tests were two-tailed with \( P < 0.05 \). Statistical analyses were performed using SPSS version 20. Baseline clinical parameters were represented as median (range) or proportions.

Metabolomics and Pathway Analysis

To analyze the metabolomics data, filtered features of the XCMS peak tables were normalized using mass spectrum total useful signal (MSTUS) normalization, which works on the variation in urine volume and diuresis and is much more effective than creatinine normalization. This normalization method has been introduced into the Metaboanalyst 3.0 (www.metaboanalyst.ca) server and into SIMCA P12 software (Umetrix, Sweden) for multivariate projection analyses, such as principal component analysis and partial least square discriminant analysis. A three-step statistical filtering of the metabolites was carried out, as detailed in Supporting Fig. S1 and the Supporting Methods. Pathway enrichment patterns were analyzed using Metaboanalyst, a web-based tool designed for untargeted metabolomics data and pathway analysis.

Prediction of Nonresponse to Corticosteroids and Mortality

Receiver operating characteristic (ROC) curves for predicting nonresponse to corticosteroid with metabolites or other variables were generated by computing sensitivity and specificity at each observed cutoff for the variable of interest. Area under the ROC (AUROC) curve was calculated for each variable of interest. Data on time to death were estimated with the Kaplan–Meier method and were compared between groups by the log-rank test, with hazard ratios and 95% confidence limits estimated by the Cox model.

Gene–Metabolite Integration Analysis

We recently published results of high-throughput transcriptomics in liver and corresponding peripheral blood mononuclear cells (PBMCs) of 32 patients with SAH before they received corticosteroid therapy (i.e., under baseline conditions). These patients were subsequently classified as NR (n = 16) or R (n = 16) after 7 days of corticosteroid therapy, according to the Lille score. In brief, genes overexpressed in the liver compared to the PBMCs were found to be liver specific (henceforth, liver-specific genes), while genes underexpressed in the liver compared to the PBMCs were considered mainly related to immune cell functions (henceforth, immune-cell-related genes). Because patients who had transcriptomic results were also enrolled in the present metabolomics study, we combined “omics” data sets using the following strategy: first, among the sets of liver-specific genes and immune-cell-related genes, we identified genes that were differentially expressed between NRs and Rs; second, we used hierarchical clustering to identify gene...
clusters that accounted for differences between NRs and Rs, according to the method by Li et al.\(^\text{26}\) We used Gene Set Enrichment Analysis (http://software.broadinstitute.org/gsea/index.jsp)\(^\text{27}\) to query the open source databases of Kyoto Encyclopedia of Genes and Genomes (http://www.genome.ad.jp/kegg/),\(^\text{28}\) REACTOME (https://reactome.org/), and Gene Ontology (http://www.geneontology.org), with the aim to functionally characterize gene clusters. Gene sets or pathways were considered as relevant when they included at least five genes and \(P < 0.05\) and the false discovery rate was \(<0.05\). Next, for patients with results of both transcriptomics and metabolomics, as described,\(^\text{26}\) a mean value was calculated for each cluster intensity and each cluster intensity was regressed against each value of the validated metabolites, using stepwise linear regression and Spearman correlation.

**Results**

**PATIENTS**

Patient characteristics at enrollment for the discovery and validation cohorts according to their response to corticosteroid therapy are shown in Table 1. The number of NRs was 8 (13\%) and 20 (25\%) in the discovery and validation cohort, respectively (Table 1).
each cohort, the clinical profile, including age, proportion of males, and indices of severity of liver disease, was similar in NRs and Rs (Table 1). However, in each cohort, the baseline leukocyte and platelet counts were significantly higher in NRs than Rs. The 90-day mortality was also higher in NRs in both cohorts (Table 1).

CHARACTERIZATION OF THE URINE METABOLOME IN THE DISCOVERY COHORT

In this untargeted urine metabolome profiling approach, 4,472 features were detected in positive and negative electrospray ionization conditions. We were able to annotate and validate a total of 212 (~5%) features from the negative and positive ionization modes (Supporting Table S1). We considered a feature only if it matched any two of the following validation criteria: (a) m/z matching structure, (b) retention times matching standard metabolite, (c) tandem (MS/MS) matching standard metabolite, (d) interpretation of MS-MS spectrum, or (e) interpretation of MS spectrum (Supporting Methods; Supporting Table S1). Functional annotation of metabolites identified diverse subclasses (e.g., alkaloid derivatives, amino acid derivatives, benzyl alcohols, primary and secondary bile acids, fatty acids and derivatives, steroids, sugar alcohols), each being enriched with more than five distinct metabolites (Supporting Table S1).

BASELINE URINE METABOLOME ROBUSTLY DISTINGUISHES NRs IN THE DISCOVERY COHORT

Partial least square discriminating analysis clearly separated NRs from Rs (Fig. 1A) and was validated by 100 permutation tests (Fig. 1B; principal component analyses in Supporting Fig. S2). A total of 29/212 (13.6%) urinary metabolites with variable importance on projection scores >1 were identified (Supporting Table S2). The up-regulated metabolites in NRs were linked to the energy metabolism/trichloroacetic acid cycle; D-glutamine/D-glutamate metabolism; alanine, aspartate, and glutamate metabolism; lysine biosynthesis; and vitamin B6 metabolism (P < 0.05; pathway impact >0.05) (see Supporting Methods; Fig. 1C). The down-regulated metabolites were significantly enriched in beta-alanine metabolism and phenylalanine metabolism (Fig. 1D). In the cohort, nine metabolites (4.2%) significantly differed between NRs and Rs before and after MSTUS normalization and fulfilled each of the following criteria: change >1.5-fold; P < 0.05; variable importance on projection >1; and Benjamini–Hochberg q correction <0.05. Accordingly, these nine metabolites were considered to be the most reliable metabolites. Of these, seven were increased in NRs, including acetyl-L-carnitine, octanoylcarnitine, decanoylcarnitine, decenedioic acid, alpha-ketoglutaric acid, histidylproline diketopiperazine, and Gly-Ala-Pro-Thr (tetra peptide), and two were decreased: glycerol-3-phosphate and N-acetylneuraminic acid (Fig. 1E; Table 2; Supporting Table S2).

BASELINE URINE METABOLITES CORRELATE WITH OUTCOMES IN THE DISCOVERY COHORT

In the discovery cohort, higher levels of acetyl-L-carnitine, octanoylcarnitine, and alpha-ketoglutaric acid corresponded to a higher risk of nonresponse to corticosteroids (Table 3). In addition, there was a positive correlation between the levels of each of these three metabolites and each of the severity scores (MELD, MDF, Child-Pugh) and the 1-month mortality rate (Table 3; Supporting Table S3). Because acetyl-L-carnitine was associated with the highest values of AUROC (Table 3) and odds ratio for predicting NRs in the discovery cohort, we validated these results using enzyme-linked immunosorbent assay (ELISA) measurement of acetyl-L-carnitine in the validation cohort. In the discovery cohort, we found that acetyl-L-carnitine levels assessed by MS significantly correlated with levels measured using the ELISA technique (regression coefficients (r) = 0.838; P < 0.001; Supporting Fig. S3). Levels of acetyl-L-carnitine (ELISA) were significantly increased in NRs in both the discovery and validation cohorts (Fig. 2A).

URINE ACETYL-L-CARNITINE LEVELS CORRELATE WITH OUTCOME

In the discovery cohort, higher acetyl-L-carnitine levels and higher MDF were independent predictors of death (Supporting Table S4). In the entire cohort (discovery plus validation), higher acetyl-L-carnitine levels, higher total leukocyte count, and higher MDF were independent predictors of death (Fig. 2B). Interestingly, in the entire cohort, acetyl-L-carnitine documented the highest AUROC of 0.96 (95% confidence
FIG. 1. Baseline urine metabolome in severe alcoholic hepatitis patients (responders and nonresponders) according to their 7-day response to corticosteroid therapy. (A) PLS-DA plot documenting clear differences between NRs (n = 8) and Rs (n = 52). (B) Internal cross-validation plot (Q2) for baseline urine metabolites of NRs versus Rs. (C) Up-regulated metabolite pathway enrichment (bubble plot analysis) based on the HMDB database in NRs. (D) Down-regulated pathway enrichment (bubble plot analysis) based on the HMDB database in NRs. (E) Key mitochondrial metabolites significantly altered in NRs versus Rs after normalization (**P < 0.01, ***P < 0.001). Abbreviations: CoA, coenzyme A; Comp, Component; Do, day zero (baseline); HMDB, Human Metabolome Database; PLS-DA, partial least square discriminating analysis plot; t, matrix consisting of n row vectors; TCA, trichloroacetic acid. Data is represented as Mean and SD for the metabolites.
### TABLE 2. NINE METABOLITES THAT DIFFERENTIATE RESPONDERS FROM NONRESPONDERS AFTER A STRINGENT SELECTION PROCESS OPERATED IN THE DISCOVERY COHORT*

| Metabolites Label | RT (Minutes) | m/z Attribution | Subclass | Before MSTUS Normalization | After MSTUS Normalization | P value | VIP | Median R | NR | NR/R |
|-------------------|--------------|------------------|----------|----------------------------|----------------------------|---------|-----|----------|-----|-------|
| Acetyl-L-carnitine  | M198         | 1.3              | 204.12   | [(M+H)+] Fatty acid esters |                            | 7.62E+07 | 4.44E+09 | 58.25 | 0.00 | 1.38 |
| Gin Ala Pro Thr    | M191         | 5.78             | 416.21   | [(M+H)+] Amino acids, peptides, and analogues | 1.52E+07 | 5.95E+08 | 39.19 | 0.00 | 1.22 |
| Octanoylcarnitine  | M159         | 7.92             | 288.22   | [(M+H)+] Fatty acid esters | 1.24E+07 | 3.31E+08 | 26.64 | 0.00 | 1.19 |
| Decanoylcarnitine  | M171         | 9.02             | 316.25   | [(M+H)+] Fatty acid esters | 1.40E+07 | 2.68E+08 | 18.17 | 0.00 | 1.18 |
| Decenedioic acid   | M90          | 7.35             | 201.11   | [(M+H)+] Fatty acids and conjugates | 1.34E+07 | 2.19E+08 | 16.36 | 0.00 | 1.02 |
| C12H15O2N4/ histidylproline diketopiperazine | M136 | 6.15            | 247.12   | [(M-H)]- Piperazino piperidines | 5.63E+04 | 6.78E+05 | 12.03 | 0.00 | 1.44 |
| Alpha-ketoglutaric acid | M26 | 1.1              | 145.01   | [M-H]– Gamma keto acid and derivatives | 3.49E+08 | 3.14E+09 | 9.01  | 0.00 | 1.20 |
| Glycerol 3-phosphate | M45         | 0.89             | 171.01   | HMDB02520 Glycerophosphates | 1.77E+05 | 2.27E+05 | 1.28  | 0.02 | 1.00 |
| N-Acetylneuraminic acid | M169 | 0.91             | 308.1    | [(M-H)-] Sugar acid and derivatives | 4.48E+05 | 7.15E+05 | 1.60  | 0.03 | 1.02 |

*To be selected, metabolites should be significantly different between NRs and Rs before and after MSTUS normalization and after univariate and multivariate analysis, exhibit a VIP score of >1, have a fold-change > (for up-regulated) or < (for down-regulated) 1.5, with P < 0.05. Abbreviations: HMDB, Human Metabolome Database; M+H, Mono isotopic ion state in positive mode; M–H, Mono isotopic ion state in negative mode; RT, Retention Time; VIP, variable important in projection.

### TABLE 3. ASSESSMENT OF ABILITY OF TOP NINE METABOLITES TO PREDICT THE RESPONSE TO CORTICOSTEROIDS AND CORRELATION OF THESE METABOLITES WITH MORTALITY AND SEVERITY SCORES IN THE DISCOVERY COHORT

| Test Result Variable(s) | AUROC Curves | OR for Nonresponse Determination | Predictive Values | Spearman Correlation Coefficient |
|-------------------------|--------------|---------------------------------|-------------------|----------------------------------|
|                         |              | Area                             | Sig               | LB                               | UB | OR                  | P value | PPV | NPV | Mortality | MELD score | MDF | CP score |
| Acetyl-L-carnitine      | 0.909        | 0.000                           | 0.816             | 0.99                            |    | 15.3                | 0.0001  | 100 | 90  | 0.458**   | 0.340**    | 0.317** | 0.302**  |
| Octanoylcarnitine       | 0.868        | 0.001                           | 0.743             | 0.992                           |    | 5.1                 | 0.0010  | 87  | 60  | 0.412**   | 0.532**    | 0.732** | 0.283**  |
| Alpha ketoglutaric acid | 0.798        | 0.007                           | 0.68              | 0.916                           |    | 4.1                 | 0.0010  | 92  | 70  | 0.482**   | 0.416**    | 0.351** | 0.390**  |
| Decanoylcarnitine       | 0.767        | 0.016                           | 0.616             | 0.918                           |    | 3.4                 | 0.0210  | 85  | 67  | 0.148     | 0.017      | 0.094   | 0.064    |
| Gln Ala Pro Thr (tetra-peptide) | 0.72    | 0.047                           | 0.523             | 0.917                           |    | 3.0                 | 0.0410  | 75  | 70  | 0.171     | 0.144      | 0.146   | 0.251    |
| Decenedioic acid        | 0.742        | 0.029                           | 0.526             | 0.958                           |    | 1.6                 | 0.0450  | 85  | 80  | 0.134     | 0.061      | -0.013  | 0.045    |
| C12H15O2N4/ histidylproline diketopiperazine | 0.663 | 0.139                           | 0.431             | 0.896                           |    | 1.2                 | 0.0310  | 86  | 65  | 0.218     | -0.32**    | -0.334** | -0.308*  |
| Glycerol 3-phosphate    | 0.227        | 0.014                           | 0.109             | 0.345                           |    | 2.0                 | 0.0040  | 85  | 75  | -0.455**  | -0.483**   | -0.455** | -0.506** |
| N-Acetylneuraminic acid | 0.244        | 0.021                           | 0.109             | 0.379                           |    | 3.6                 | 0.0170  | 80  | 90  | -0.351**  | -0.351**   | -0.321*  | -0.318*  |

* = P < 0.05, ** = P < 0.01, and *** = P < 0.001. Abbreviations: CI, confidence interval; CP, Child-Pugh; LB, lower bound; NPV, negative predictive value; OR, odds ratio; PPV, positive predictive value; Sig, Significance at P < 0.05; UB, upper bound.
FIG. 2. Performance evaluation of baseline predictors of nonresponse and mortality. (A) Acetyl-L-carnitine measurements in urine (ELISA) in 8 NRs (3,350 ng/mL) and 2 Rs (936 ng/mL) in the discovery cohort and validated (3,293 ng/mL) in 20 NRs and 60 Rs (1,566 ng/mL; *** 𝑃 < 0.001). (B) Cox proportional analysis of Acetyl-L-carnitine in comparison to other clinical factors. Hazard ratio of Acetyl-L-carnitine was significantly higher than any other clinical factors compared in multivariate analysis. (C) AUROC was significantly higher with acetyl-L-carnitine than with CTP, MELD, MDF, or TLC for predicting nonresponse. (D) Kaplan–Meier curve analysis documented differences between nonsurvivors and survivors based on the cut-off point of acetyl-L-carnitine (2,500 ng/mL) in urine samples of patients with SAH. Abbreviations: ALCAR, acetyl-L-carnitine; b, standardize; CI, confidence interval; CTP, Child-Turcotte-Pugh; HR, hazard ratio; Sig, Significance at 𝑃 < 0.05; TLC, thin-layer chromatography.
Baseline hepatic transcriptome in patients with SAH (NRs and Rs). (A) The 1,340 genes that were up-regulated at baseline in liver compared to corresponding PBMCs were analyzed for their expression in NRs versus Rs. We found 403 DEGs between NRs and Rs: 12 genes were up-regulated in NRs (cluster 1); 391 genes were down-regulated in NRs (cluster 2). The 322 genes that were down-regulated in liver versus PBMCs were also analyzed for their expression in NRs versus Rs. There were 118 DEGs between NRs and Rs: 89 genes up-regulated in NRs (cluster 3); 29 genes down-regulated in NRs (cluster 4). Average intensities for gene clusters 1-4 are provided for NRs versus Rs. (B) GSEA of genes included in clusters 2-4. Cluster 1 did not show any significant enrichment. Abbreviations: DEG, differentially expressed gene; FDR, false discovery rate; GSEA, gene set enrichment analysis.
| Model  | Metabolite ID | Metabolite ID                      | Unstandardized Coefficients | Standardized Coefficients | Sig. | HMDB       | KEGG | Pathway                   | Biofunctions                        |
|--------|---------------|-----------------------------------|-----------------------------|---------------------------|------|------------|------|---------------------------|-------------------------------------|
|        |               |                                   | B                           | 95% CI of B (Lower Bound) | Beta | 95% CI of B (Upper Bound) |       |                           |                                     |
|        |               |                                   | 5.748                       | 5.747                     | 5.749 | 0.661      | 0.000 | HMDB33840                 | Nutrient                            |
|        |               |                                   | -1.015E-05                  | 0.000                     | 0.000 | 0.000      | 0.001 | HMD800491 C03465          | Valine, leucine, and isoleucine      |
|        |               |                                   | 1.474                       | 1.473                     | 1.475 | 0.356      | 0.000 | HMD802064 C02714          | Arginine and proline metabolism     |
|        |               |                                   | 0.000                       | 0.000                     | 0.000 | 0.004      | 0.001 | HMD800562 C00791          | Arginine and proline metabolism     |
|        |               |                                   | 0.001                       | 0.0001                    | 0.01  | 0.001      | 0.000 | HMD800177 C00135          | Beta-glutamime metabolism            |
|        | M16           | 3-Methyl-2-oxovaleric acid         | -0.928                      | -0.927                    | -0.929 | -0.103     | 0.000 | HMD811723 C01586          | NA                                  |
|        |               |                                   | 0.056                       | 0.055                     | 0.057 | 0.092      | 0.000 | HMD800424 C00135          | NA                                  |
|        |               |                                   | 0.079                       | 0.077                     | 0.080 | 0.053      | 0.000 | -                         | NA                                  |
|        |               |                                   | 0.021                       | 0.020                     | 0.022 | 0.003      | 0.000 | HMD800212 C01074          | Aminoacidias                         |
|        | M18           | N-acetylputrescine                 | 1.547                       | 1.546                     | 1.548 | 0.202      | 0.000 | HMD840529                 | NA                                  |
|        | M29           | Creatinine                         | -0.928                      | -0.927                    | -0.929 | -0.103     | 0.000 | C16359                    | Caffeine metabolism                  |
|        | M32           | L-histidine                        | 0.056                       | 0.055                     | 0.057 | 0.092      | 0.000 | C01586                    | Phenylalanine                        |
|        | M49           | Iso valeric acid isomer            | 0.000                       | 0.000                     | 0.000 | 0.004      | 0.001 | HMD800399 C16359          | NA                                  |
|        | M57           | 1-Methylvaleric acid               | -0.928                      | -0.927                    | -0.929 | -0.103     | 0.000 | C01586                    | Phenylalanine                        |
|        | M83           | 2-Methylthiopurine acid            | 0.056                       | 0.055                     | 0.057 | 0.092      | 0.000 | C01586                    | NA                                  |
|        | M116          | 3-Hydroxysebacic acid              | 0.079                       | 0.077                     | 0.080 | 0.053      | 0.000 | -                         | NA                                  |
|        | M134          | C11 H2O 04 N2/ Glutamine derivative| 0.021                       | 0.020                     | 0.022 | 0.003      | 0.000 | HMD800212 C01074          | Aminoacidias                         |
|        | M141          | N-acetylglactosaminyl acetate      | 0.000                       | 0.000                     | 0.000 | 0.000      | 0.000 | HMD800212 C01074          | Aminoacidias                         |
|        | M98           | Acetyl-L-carnitine                 | -0.207                      | -0.206                    | -0.300 | -0.186     | 0.000 | HMD800201 C02571          | Mitochondrial beta-oxidation of short chain saturated fatty acids |
|        | M159          | Octanoylcarnitine                  | -0.137                      | -0.136                    | -0.138 | -0.158     | 0.000 | HMD800791 C02838          | Mitochondrial beta-oxidation of short chain saturated fatty acids |
|        | M171          | Decanoylcarnitine                  | -0.014                      | -0.0130                   | -0.0150 | -0.027     | 0.000 | HMD800651 C02838          | Mitochondrial beta-oxidation of short chain saturated fatty acids |
|        | M174          | Valproic acid glucuronide          | 1.267                       | 1.266                     | 1.269 | 1.232      | 1.203 | HMD800901 C03033          | Valproic acid metabolism pathway     |
|        | M211          | Glycochenodeoxycholic acid 3-glucuronide or isomer | 0.155 | 0.153 | 0.156 | 0.574 | 0.000 | HMD802579 C03033          | Bile secretion                        |
| Model | Metabolite ID | Metabolite ID | Unstandardized Coefficients | Standardized Coefficient | Beta | Sig. | HMDB | KEGG | Pathway | Biofunctions |
|-------|---------------|---------------|-----------------------------|--------------------------|------|------|------|------|---------|--------------|
|       |               | 3-Hydroxy-3- | -0.323 | -0.321 | -0.324 | -0.342 | 0.000 | HMB00754 | - | NA | NA |
|       | M7            | methylbutyric | acid (3-hydroxyisovaleric acid) |               |       |       |      |      |         |              |
|       | M8            | 2-Hydroxy-3- | 0.055 | 0.052 | 0.057 | 0.052 | 0.000 | HMB001488 | 000253 | NA | Fuel or energy source |
|       |               | methylbutyric | acid |               |       |       |      |      |         |              |
|       | M10           | Nicotinic acid | 0.275 | 0.274 | 0.277 | 0.141 | 0.000 | HMB001488 | 000253 | Nicotinate and niacinamide metabolism |
|       | M11           | 4-Pyridoxolactone | 0.453 | 0.452 | 0.455 | 0.243 | 0.000 | HMB003454 | 000971 | Vitamin B6 metabolism |
|       | M75           | Methylhippuric | acid | -0.001 | -0.0001 | -0.01 | 0.000 | 000971 | - | NA | Essential vitamins |
|       | M108          | Hexose | 0.032 | 0.031 | 0.033 | 0.017 | 0.000 | HMB12326 | C15923 | Ascorbate and aldarae metabolism |
|       | M129          | Tigrlycarnitine/2-ethylacrylylcarnitine | 1.714 | 1.712 | 1.715 | 0.935 | 0.000 | HMB02366 | - | NA | Lipid catabolism, fatty acid transport, energy production |
|       | M130          | N-Acetyl-Di-tryptophan | 0.007 | 0.006 | 0.008 | 0.009 | 0.001 | HMB13713 | - | NA | NA |
|       | M146          | Glu-Leu | 1.042 | 1.041 | 1.043 | 0.323 | 0.000 | HMB18823 | - | NA | NA |
|       | M153          | Isovalerylglucuronide | -0.415 | -0.413 | -0.416 | -0.118 | 0.000 | HMB02091 | 030333 | - | Waste products |
|       | M154          | 4-Hydroxyphenylacetilglutamine | -0.235 | -0.234 | -0.236 | -0.284 | 0.000 | HMB006061 | 005595 | Tyrosine metabolism |
|       | M195          | Phe Try Asp | -0.047 | -0.045 | -0.048 | -0.066 | 0.000 | - | - | NA | Steroid hormone biosynthesis |
|       | M202          | Androsterone glucuronide | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | HMB02829 | C11135 | Waste products |
|       | M210          | Glycochenodeoxycholic acid 3-glucuronide or isomer | -0.541 | -0.540 | -0.542 | -0.480 | 0.000 | HMB02579 | 030333 | Bile secretion |
|       | M212          | Glycochenodeoxycholic acid 3-glucuronide or isomer | 0.000 | 0.000 | 0.000 | -0.044 | 0.000 | HMB02579 | 030333 | Bile secretion |
|       | M14           | D-1-Piperidine-2-carboxylic acid | -0.279 | -0.278 | -0.280 | -0.071 | 0.000 | HMB01084 | 004092 | Lysine degradation |
|       | M20           | Isoleucine/Leucine | 1.330 | 1.329 | 1.332 | 0.127 | 0.000 | HMB00172 | 000407 | Protein synthesis, amino acid biosynthesis |
|       | M31           | 2,5-Dihydroxybenzoic acid | 5.453 | 5.452 | 5.454 | 0.781 | 0.000 | HMB00152 | 000628 | Tyrosine metabolism |
|       | M52           | Acetyl-(Leu/Ile) | -0.476 | -0.475 | -0.478 | -0.036 | 0.000 | - | - | NA | NA |
|       | M56           | 8-Hydroxy-7-methylguanine | -0.889 | -0.887 | -0.890 | -0.184 | 0.000 | HMB006037 | - | NA | NA |
|       | M86           | C9h10n2o3/Pyridylacetylglycine | 2.768 | 2.766 | 2.769 | 0.348 | 0.000 | HMB59723 | - | NA | NA |
|       | M112          | C6h14o6/Mannitol or isomers | 4.511 | 4.509 | 4.512 | 0.561 | 0.000 | HMB00765 | 000392 | Phosphotransferase system |
| Model | Metabolite ID | Metabolite ID | Unstandardized Coefficients | Standardized Coefficient | Beta | Sig. | HMDB | KEGG | Pathway | Biologs |
|-------|---------------|---------------|-----------------------------|--------------------------|------|------|------|------|---------|--------|
|       |               |               | B | 95% CI of B (Lower Bound) | 95% CI of B (Upper Bound) | Beta | Sig. |       |       |         |        |
| M117  | Pantothenic acid | -0.677 | -0.676 | -0.678 | -0.196 | 0.000 | HMDB00210 | C00864 | beta-Alanine metabolism |
|       | Isovalerylcarnitine | -0.171 | -0.170 | -0.172 | -0.098 | 0.000 | HMDB00688 | - | NA |
|       | Decanoylcarnitine | 0.107 | 0.106 | 0.108 | 0.214 | 0.000 | HMDB00651 | - | NA |
|       | Galactosylhydroxlysine | 0.744 | 0.743 | 0.745 | 0.321 | 0.000 | HMDB00600 | C05547 | NA |
|       | Gin Ala Pro Thr (tetra peptide) | 0.091 | 0.090 | 0.092 | 0.375 | 0.000 | - | - | NA |
|       | Glycocholic acid | 3.035 | 3.034 | 3.036 | 0.680 | 0.000 | HMDB00138 | C01921 | Primary bile acid biosynthesis |
|       | Glycocholic acid | 0.280 | 0.279 | 0.282 | 0.918 | 0.004 | HMDB02497 | - | NA |
|       | Glycocholic acid | 0.001 | 0.0001 | 0.01 | 0.242 | 0.001 | HMDB02579 | C03033 | NA |
|       | N-Acetylputrescine | -0.134 | -0.132 | -0.135 | -0.194 | 0.000 | HMDB02064 | C02714 | Arginine and proline metabolism |
|       | 4-Hydroxybenzoic acid | -0.117 | -0.116 | -0.118 | -0.173 | 0.000 | HMDB00500 | C00156 | Ubiquinone biosynthesis |
|       | 3-Methylcrotonyl glycine | 0.026 | 0.025 | 0.027 | 0.029 | 0.000 | HMDB00459 | - | NA |
|       | L-Fucose | 0.242 | 0.240 | 0.243 | 0.215 | 0.000 | HMDB00174 | C01019 | Fructose and mannose metabolism |
|       | 8-Hydroxy-7-methylguanine | -6.775E-06 | 0.000 | 0.000 | 0.000 | 0.000 | HMDB06037 | - | NA |
|       | Di-Tryptophan | 0.000 | 0.000 | 0.000 | 0.001 | 0.002 | HMDB03396 | - | NA |
|       | C8h14o4/Manitol or isomers | -1.883 | -1.882 | -1.884 | -1.388 | 0.000 | HMDB00765 | C00392 | Fructose and mannose metabolism |
|       | Tigliylcarnitine/2-ethylacrylylcarnitine | 0.217 | 0.215 | 0.218 | 0.287 | 0.000 | HMDB02366 | - | NA |
|       | 3-Hydroxydecanedioic acid | 0.032 | 0.031 | 0.033 | 0.431 | 0.000 | HMDB00413 | - | Beta oxidation of FFA |
|       | Norepinephrine sulfate | -0.169 | -0.168 | -0.170 | -0.306 | 0.000 | HMDB02062 | - | NA |
|       | Ophthalmic acid | -0.018 | -0.017 | -0.019 | -0.012 | 0.000 | HMDB05765 | - | NA |
|       | Acylcarnitine of a dicarboxylic acid (C8h14o4) | 0.006 | 0.004 | 0.006 | 0.028 | 0.000 | - | - | NA |
interval, 0.89–0.96) for predicting a response to corticosteroids when compared to other clinical factors (Fig. 2C). Further, based on the AUROC of acetyl-L-carnitine of 0.96 and a likelihood ratio of 5.6, a cutoff for the prediction of nonresponse was determined at 2,500 ng/mL and was used to assess survival. In the entire cohort, survival was significantly lower among patients with acetyl-L-carnitine levels above 2,500 ng/mL than among those with levels below 2,500 ng/mL (log-rank test < 0.01; Fig. 2D).

BASELINE HEPATIC TRANSCRIPTOME CORROBORATES WITH URINARY METABOLOME SIGNATURES

We assessed whether changes in baseline urine metabolome in patients with SAH were linked to alterations in basal hepatic gene expression. We used results of hepatic and PBMC transcriptomics of 32 patients with SAH before any treatment. At baseline, there were 1,662 differentially expressed genes between liver and PBMCs. Of these, 1,340 were overexpressed in the liver (liver-specific genes) and 322 underexpressed in the liver (immune-cell-related genes; see Patients and Methods). Of the 1,340 liver-specific genes, 403 were differentially expressed between NRs and Rs (Fig. 3A). Among these, very few (12 genes, “cluster 1”) had higher expression in NRs than Rs, while the remaining 391 genes (“cluster 2”) had lower expression in NRs than Rs (Fig. 3A; Supporting Table S5). Functional annotation of cluster 1 did not show any significant feature; in contrast, genes in cluster 2 were related to protein synthesis and tissue homeostasis (Fig. 3B; Supporting Table S6). Of the 322 immune-cell-related genes, 118 genes differentially expressed between NRs and Rs. Of these, 89 had higher expression in NRs than Rs (“cluster 3”), while the remaining 29 genes (“cluster 4”) had lower expression in NRs than Rs (Fig. 3A; Supporting Table S5). Functional annotation of cluster 3 did not show any significant feature; in contrast, genes in cluster 4 were related to positive immune cell regulation and cell adhesion (Fig. 3B; Supporting Table S6). The results for both transcriptomics and metabolomics were available in 16 Rs and 8 NRs. In these patients, we tested the association between the two “omics” and found that gene cluster intensities significantly regressed against 54 metabolites (Table 4) irrespective of their response status. Sixteen metabolites regressed against cluster 1 intensities, 15 metabolites against

| Table 4. Continued |
|-------------------|
| Model | Metabolite ID | Unstandardized Coefficients | Standardized Coefficients |
|       |             | B          | 95% CI of B (Upper Bound) | 95% CI of B (Lower Bound) | Beta | Sig. | HMDB | KEGG Pathway | Biofunctions |
|-------|--------------|------------|--------------------------|--------------------------|------|------|------|-------------|--------------|
| M173  | C12 H15 O10/Glucuronide of a dicarboxylic acid | 5.551 5.550 5.552 | 1.641 0.000 0.000 | 0.013 0.000 0.000 | 1.641 | 0.000 | NA | NA | |
| M182  | C11 H17 O10/Glucuronide of a dicarboxylic acid | 0.008 0.007 0.009 | 0.013 0.000 0.000 | 0.013 0.000 0.000 | 0.013 | 0.000 | NA | NA | |
| M190  | Glucuronide of C14 H23 O2 | 0.053 0.052 0.054 | 0.226 0.000 0.000 | 0.226 0.000 0.000 | 0.226 | 0.000 | NA | NA | |

For more information on the strategy used for identification of gene clusters and on the composition of these clusters, see Patients and Methods and Results sections; Fig. 3; Supporting Table S5. For more information on the strategy used for identification of gene clusters and on the composition of these clusters; see Patients and Methods and Results sections; Fig. 3; Supporting Table S5. Abbreviations: CI, confidence interval; CoA, coenzyme A; FFA, free fatty acid; HMDB, Human Metabolome Database; KEGG, Kyoto Encyclopedia of Genes and Genomes; NA, not applicable; Sig, significance at P < 0.05. Significance at P < 0.05.
cluster 2 intensities, 15 metabolites against cluster 3 intensities, and 15 metabolites against cluster 4 intensities. Some metabolites (e.g., decanoylcarnitine) regressed against different gene clusters. Top metabolites predicting poor outcome (see Table 3) were among metabolites that regressed against clusters exhibiting a “prominence” of NRs over Rs. Counterintuitively, the regression of acetyl-L-carnitine, octanoylcarnitine, and decanoylcarnitine levels against cluster 1 intensities exhibited negative regression coefficients (i.e., negative $\beta$ values for unstandardized coefficients; see Table 4). Accordingly, we examined the influence of being Rs or NRs on the direction of the association between metabolites and cluster 1 intensity (using Spearman correlation). In Rs, there was a significant negative correlation of each metabolite with cluster 1 intensity ($r_x$ values were $-0.75$, $-0.64$, and $-0.70$, for acetyl-L-carnitine, octanoylcarnitine, and decanoylcarnitine, respectively). In contrast, in NRs, metabolites either were correlated positively ($r_x$ was 0.98 for acetyl-L-carnitine) or did not correlate with cluster intensity ($r_x$ value was similarly 0.07 with octanoylcarnitine and decanoylcarnitine). The differences in the direction of the association between metabolites and cluster 1 intensity in NR versus R may explain the low value of regression (Table 4) and correlation (Supporting Fig. S4A) coefficients observed in the whole group. Together, these results suggest that the counterintuitive negative correlation of metabolites versus cluster 1 intensity found in the whole population may reflect the negative correlation observed in Rs. For the whole patient group, the coefficient values (i.e., standardized coefficient beta for regression in Table 4; $r_x$ in Supporting Fig. S4A) were relatively low, suggesting that the strength of the association between metabolite levels and cluster 1 intensity was weak; hence, results should be interpreted with caution. More interestingly, cluster 3 intensities positively correlated with decanoylcarnitine and Gln-Ala-Pro-Thr (tetra peptide) levels (Table 4; Supporting Fig. S4B).

Discussion

Noninvasive and easy to access methods for early identification of corticosteroid nonresponse or mortality for patients with SAH are not available. To address this issue, we investigated 140 patients with SAH who were divided into two cohorts; the first (discovery cohort) enrolled 60 patients (13% NRs) and the second (for validation) enrolled 80 patients (25% NRs). We have no clear explanation for finding that the proportion of NRs was lower in our discovery cohort than in our validation cohort. The only difference between the two cohorts was related to the period of enrollment; patients in the first cohort were enrolled during 2013, and those of the validation cohort were enrolled in 2014 and 2015. The response to steroid in our population of SAH was slightly higher with fewer NRs compared to Western countries (35%).

The reasons for these differences between countries are unclear but may involve differences in genetic and environmental factors between Indian and Western patients that contribute to differences in the response to corticosteroids. Further studies are needed.

We investigated baseline urinary metabolites (i.e., before corticosteroid therapy) in the discovery cohort. Using a high-resolution MS-based, untargeted, metabolomics approach, we could annotate 212 metabolites that were enriched in energy metabolism pathways, bile acid biosynthesis, amino acid biosynthesis, and others. Our novel observations demonstrated that baseline urinary metabolome can be used to identify patients with SAH who are unlikely to respond to corticosteroid therapy or die within a month.

Our technique for urine metabolome analysis was carefully designed. Although the preparation of urine samples for analysis is simple and the concentration of many metabolites is amplified by bladder storage, the biological interpretation of data is complicated by a variation in diuresis from subject to subject. Various normalization methods have been used and published to address this issue, including the traditional use of urinary creatinine concentration, osmolality, total useful MS signal, and specific gravity, as well as a combination of creatinine concentration and normalization of the MS signal and the determination of the total concentration of chemically labeled metabolites by using liquid chromatography-ultraviolet. However, many studies do not use normalization procedures, and there is still no consensus on this point. We employed a MSTUS normalization strategy, which uses the total intensity of metabolites that are common to all samples and which is easy to implement and was found to perform better than creatinine normalization. For the selection of metabolites of interest, we chose to take into account metabolites that had concentration differences between Rs and NRs that were statistically significant with or without MSTUS normalization in order to improve result reliability.

In our discovery cohort, the baseline urine excretion of acetyl-L-carnitine, octanoylcarnitine, decanoylcarnitine, decenedioic acid, and alpha-ketoglutaric acid was
significantly higher among NRs. This suggests a marked derangement of energy biosynthesis and beta-oxidation of fatty acids in NRs, consistent with results showing that SAH is associated with an alteration in the trichloroacetic acid cycle and beta-oxidation of fatty acids.\(^{(33)}\)

Because urine acetyl-L-carnitine levels measured with MS significantly correlated with levels measured with an ELISA technique in our discovery cohort, we used this technique in a validation cohort of 80 patients. In the validation cohort, higher levels of acetyl-L-carnitine significantly predicted the nonresponse to steroids and mortality. These findings were confirmed when results obtained in the discovery and validation cohorts were pooled.

Integration of data sets obtained with high-throughput omics approaches can provide new insights into the pathophysiology of liver diseases. In this study, we explored the hypothesis that changes in baseline urine metabolome in patients with SAH could be associated with alterations in basal hepatic gene expression. For this, we identified four hepatic gene clusters that differentiated NRs from Rs at baseline. Two clusters were composed of liver-specific genes, and the other two included genes related to immune cell functions. Using stepwise linear regression, we found that 54 metabolites significantly regressed against gene clusters, suggesting a link between alterations in gene expression within the liver and changes in urine metabolome composition. It is noteworthy that metabolites found to strongly predict poor outcome were among metabolites that were associated with intensity of clusters, including genes overexpressed in NRs. Cluster 1, which was up-regulated in NRs, negatively regressed with acetyl-L-carnitine, octanoylcarnitine, and decanoylcarnitine (stepwise regression; Table 4). This surprising negative regression may be related to the fact that the correlation was strongly negative in Rs but was either positive or nonsignificant in NRs. Of note for the whole group of patients, the values of coefficients (i.e., standardized coefficient beta for regression [Table 4]; \(r\) for correlation [Supporting Fig. 4A]) were relatively low, suggesting that the strength of the association between metabolite levels and cluster 1 intensity was weak; hence, results should be interpreted with caution. More interestingly, cluster 3 (which is up-regulated in NRs) positively correlated with decanoylcarnitine and Gln-Ala-Pro-Thr (tetra peptide). Genes included in cluster 3 were related to GPCR signaling activity consistent with enhanced GPCR signals in liver of NRs. Cells exhibiting increased GPCR signaling could be immune cells (infiltrating and/or resident) or progenitors.\(^{(36)}\) Our finding that metabolites correlated with cluster 3 suggests that these metabolites are markers of crucial pathophysiological mechanisms that develop in the liver of NRs.

Our study has a limitation of being monocentric. Future multicentric studies should be performed to integrate data sets obtained with metabolomics and transcriptomics in large series of patients with SAH. To conclude, baseline urine metabolome clearly discriminates corticosteroid Rs from NRs. In particular, baseline acetyl-L-carnitine can be used as a marker for early assessment of corticosteroid nonresponse and clinical outcome. Our study affirms that integration of metabolomics and liver transcriptomics substantially improves understanding the pathophysiology of alcoholic hepatitis.

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Supporting Information
Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1176/full.