Serum and urinary metabolomics and outcomes in cirrhosis

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Abstract

Background
Cirrhosis can alter several metabolic pathways. Metabolomics could prognosticate outcomes like hepatic encephalopathy (HE), transplant, hospitalization and death.

Aim
Determine changes in serum and urine metabolomics in cirrhotics who develop outcomes.

Methods
Cirrhotic outpatients underwent data, serum/urine collection and were followed for 90 days. Demographics, cirrhosis details and medications were collected. Metabolomics was performed on urine/serum using GC/MS with subsequent bioinformatics analyses (ChemRICH, MetaMAPP and PLS-DA). Logistic regression adjusting for covariates (demographics, alcohol etiology, prior HE, PPI, SBP prophylaxis, rifaximin/lactulose) were performed and ROC curves comparing MELD to adjusted serum & urine metabolites were created.

Results
211 patients gave serum, of which 64 were hospitalized, 19 developed HE, 13 were transplanted and 11 died. 164 patients gave urine of which 56 were hospitalized, 18 developed HE, 12 were transplanted and 11 died. Metabolomics: Saturated fatty acids, amino acids and bioenergetics-related metabolites differentiated patients with/without outcomes. After regression, 232, 228, 284 and 229 serum metabolites were significant for hospitalization, HE, death and transplant. In urine 290, 284, 227 & 285 metabolites were significant for hospitalization, HE, death and transplant respectively. AUC was higher for serum metabolites vs MELD for HE (0.85 vs.0.76), death (0.99 vs.0.88), transplant (0.975 vs.0.94) and hospitalizations (0.84 vs.0.83). Similarly, urinary metabolite AUC was also higher than MELD for HE (0.87 vs.0.72), death (0.92 vs 0.86), transplant (0.99 vs.0.90) and hospitalizations (0.89 vs.0.84).
Conclusions
In this exploratory study, serum and metabolites focused on lipid, bioenergetics and amino acid metabolism are altered in cirrhotics who develop negative outcomes.

Introduction
The development of complications in cirrhosis can result in hospitalizations, death and need for liver transplant[1]. These complications such as hepatic encephalopathy (HE) are a major burden[2]. Currently, the prediction of outcomes is focused on clinical severity scores and the presence of complications[3]. Other biomarkers related to inflammation and microbiota have been reported but have not been studied across a breadth of outcomes[4–7]. In prior studies, metabolomics is helpful in predicting survival in decompensated cirrhosis, could differentiate between patients with without HE, and could be used to follow therapy withdrawal[8–11]. They are also able to provide pathophysiological insight into the development of these complications, including HE. However, the impact of blood and urine metabolites in those who experience a broader range of outcomes and their potential role in the prediction of these outcomes needs to be studied.

In this exploratory study, our aim was to determine the alterations in serum and urine metabolomics at baseline in patients who developed outcomes, and to study the additional impact of serum and urine metabolomics in the prediction of clinically relevant outcomes such as HE, hospitalizations, transplant and death over 90 days in outpatients with cirrhosis adjusted for clinical biomarkers.

Materials and methods
We recruited cirrhotic outpatients prospectively from GI and Hepatology Clinics at Virginia Commonwealth University and McGuire VA Medical Centers after informed consent. Cirrhosis was diagnosed using either liver biopsy, cirrhotic liver on imaging, frank decompensation (ascites, HE, prior variceal bleeding) or evidence of varices in patients with chronic liver disease. We excluded patients with an unclear diagnosis of cirrhosis, prior organ transplant, HIV infection, hepatocellular cancer (HCC), those unable to consent, unable to provide any sample or those who were not willing to allow follow-up reviews. We obtained written informed consent from all participants after IRB approval at Virginia Commonwealth University and McGuire VA Medical centers (approval numbers BAJAJ004, BAJAJ015, HM13191 and HM13466). The population was recruited between October 2015 and November 2016 and is representative of patients with cirrhosis found in this region of the world.

Patients were then followed for 90 days for evaluation of (a) hospitalizations (b) overt HE (c) transplant and (d) death. Only non-elective hospitalizations were considered. Overt HE was defined as grade ≥ 2 on West-Haven criteria[2]. These follow-ups were performed as part of a scheduled chart review at days 30 and 90 post-enrollment. If no follow-up was noted in the chart review and the patient was still alive and without transplant, they were called to inquire about hospitalizations and other outcomes that may have required interventions at other facilities.

Data collected were demographics, diabetes, etiology of cirrhosis (alcohol/not), MELD score, prior HE, use of PPI, lactulose, rifaximin and SBP prophylaxis. Fasting morning serum and urine samples were collected. These were adjusted for in the final analysis of the serum
and urine metabolites with respect to individual outcomes apart from MELD score, which was used as the clinical comparator. The biological MELD score was calculated without exception points apart from if the patient was on dialysis.

**Metabolomics analysis methods**

Serum and urine were analyzed for multi-variate metabolomics at the NIH West Coast Metabolomics Center using published GC-TOF MS techniques\[12\] (Supplementary methods).

**Statistical analysis methods**

The data was first transformed using the generalized log 10 transformation and then auto-scaled\[13\]. Using the statistical analysis website Metabox, we performed multivariate logistic regressions based on each single metabolite against each outcome, having age, gender, diabetes, alcoholic etiology, prior HE, PPI, SBP prophylaxis, rifaximin use as covariates\[14\]. We concluded statistical significance with \( p < 0.05 \) and regression coefficients were used as a measure of the effect size. Benjamini-Hochberg procedure was used to control the false discovery rate (FDR).

Two analyses were performed to determine the changes in metabolomics and their effect on outcomes.

*Analysis 1*: was to determine the inter-relationship and pathophysiology of the metabolites that were significantly associated with individual outcomes. We performed chemical similarity enrichment analysis (ChemRICH) to provide chemical classes significantly altered in patients who developed a particular outcome compared to the rest\[15\]. ChemRICH performed enrichment analysis based on the chemical structures that are not defined by the pathways, which can be inherently flawed and depended on the background databases. The \( p \)-values of the clusters were obtained by employing the Kolmogorov-Smirnov test. A \( p \)-value less than 0.05 indicates a statistically significant enriched compound cluster. In addition, the compound significance, the effect sizes and the altering directions were visualized by MetaMapp\[16\]. Individual metabolite VIP scores were calculated for each outcome and the top 20 metabolites by the VIP score for each outcome were further analyzed.

*Analysis 2*: was the comparison of the predictability of using urine and serum metabolites and using MELD respectively. We first adjusted the confounder effects on the urine and serum data. Then the adjusted urine and serum metabolites and the MELD score was used to build the partial least square–discriminant analysis (PLS-DA) for each of the outcomes. The predictions were made based on leave-one-out cross-validation procedure, and the area under the ROC curve (AUC) was used to access the predicting power of each PLS-DA models. These were compared statistically.

**Results**

We recruited 211 cirrhotic outpatients (Table 1) who gave serum, of which 164 patients provided urine samples. Of the 211 patients, at 90 days, 64 were hospitalized, 19 developed an HE episode, 13 were transplanted and 11 died. In those who gave urine (Table 1), 56 were hospitalized, 18 developed an HE episode, 12 were transplanted and 11 died. Of the people who did not provide urine, the majority were on dialysis (\( n = 35 \)), while the rest were not willing to provide it. Patients with an outcome (death, overt HE, transplant or hospitalization), largely had a higher MELD score, lactulose, SBP prophylaxis and prior HE compared to the rest. Age, gender, diabetes, rifaximin use and PPI were largely non-significant. Prior HE did not affect
transplant but there was a difference in gender between those did or did not get transplanted. Hospitalizations were due to liver-related causes in the majority (n = 43, HE, n = 19, renal/ metabolic causes, n = 13, ascites/anasarca n = 9, others n = 2) followed by infection (n = 13, SBP n = 4, Urinary tract infections n = 4, pneumonia n = 3 and C. difficile n = 2) and liver-

### Table 1. Clinical characteristics of patients who gave serum and urine and developed outcomes within 90 days.

| Patients who gave serum | All patients (n = 211) | Hospitalization | Overt HE | Death | Transplant |
|------------------------|-----------------------|-----------------|----------|-------|------------|
|                        | No (n = 147) | Yes (n = 62) | No (n = 192) | Yes (n = 19) | No (n = 200) | Yes (n = 11) | No (n = 198) | Yes (n = 13) |
| Age (median, IQR)      | 58.0 (7.5) | 58.0 (6.0) | 58.0 (10.25) | 58.0 (7.0) | 58.0 (9.0) | 58.0 (16.0) | 58.0 (6.75) | 58.0 (7.0) | 61.0 (7.0) |
| Male Gender            | 163 (77%) | 107 (73%) | 56 (90%) | 147 (77%) | 16 (84%) | 152 (67%) | 11 (100%) | 148 (67%) | 13 (100%) |
| p                      | 0.005     | 0.005     | <0.0001 | <0.0001 | 0.03     | <0.0001 | <0.0001 | <0.0001 | 0.04      |
| Diabetes               | 63 (30%)  | 47 (32%)  | 16 (25%) | 61 (32%) | 2 (11%)  | 62 (31%)  | 1 (9%)    | 58 (30%)  | 5 (39%)|
| Alcohol etiology       | 52 (25%)  | 33 (22%)  | 19 (31%) | 49 (25%) | 3 (16%)  | 51 (25%)  | 1 (9%)    | 49 (25%)  | 3 (23%)|
| MELD score (median, IQR) | 11.0 (7.0) | 9.0 (5.0) | 17.0 (9.25) | 10.0 (7.0) | 17.0 (8.0) | 10.0 (7.0) | 22.0 (10.0) | 10.0 (7.25) | 22.0 (8.0) |
| p                      | <0.0001  | <0.0001  | <0.0001 | <0.0001 | <0.0001 | <0.0001  | <0.0001  | <0.0001  | <0.0001  |
| Ascites                | 113 (54%) | 73 (49%)  | 40 (65%) | 98 (51%) | 15 (79%) | 102 (51%) | 11 (100%) | 101 (52%) | 12 (92%)|
| Prior variceal bleeding| 23 (11%)  | 15 (10%)  | 8 (13%)  | 19 (10%) | 1 (9%)   | 20 (10%)  | 3 (27%)   | 17 (9%)   | 6 (48%)|
| Prior HE               | 121 (57%) | 77 (52%)  | 44 (71%) | 105 (53%) | 16 (84%) | 112 (66%) | 9 (81%)   | 113 (57%) | 8 (62%)|
| p                      | 0.005     | 0.005     | <0.0001 | <0.0001 | 0.01     | <0.0001 | <0.0001 | <0.0001 | 0.005     |
| PPI use                | 97 (46%)  | 59 (40%)  | 38 (61%) | 87 (45%) | 10 (51%) | 90 (45%)  | 7 (64%)   | 89 (46%)  | 8 (62%)|
| Lactulose use          | 70 (33%)  | 28 (19%)  | 42 (72%) | 55 (30%) | 15 (79%) | 61 (30%)  | 9 (81%)   | 60 (31%)  | 10 (77%)|
| Rifaximin use          | 88 (42%)  | 60 (41%)  | 28 (45%) | 77 (40%) | 11 (56%) | 84 (42%)  | 4 (36%)   | 84 (43%)  | 4 (31%)|
| SBP prophylaxis        | 18 (9%)   | 7 (5%)    | 11 (18%) | 13 (7%)  | 5 (27%)  | 14 (7%)   | 4 (36%)   | 16 (9%)   | 2 (15%)|

Data in median (IQR) or in raw numbers (%); Significant p-values on Mann-Whitney test, Fisher exact or Chi-square test as appropriate are shown in each box in those who developed that outcome compared to those who did not.

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unrelated in the rest (n = 6, cardiovascular n = 3, pulmonary n = 2, other n = 1). Of the 13 renal/metabolic causes, 7 patients had acute kidney injury (AKI) without hepato-renal syndrome, 3 had hyponatremia, 1 had hypernatremia and 2 had hepato-renal syndrome. None of the patients developed HCC or variceal bleeding during the follow-up.

Metabolomics ChemRICH analysis (S1, S2, S3 and S4 Tables)

Hospitalizations (Fig 1). Serum metabolite clusters increased in patients who were hospitalized (blue) vs the rest (yellow dots), PLS-DA showing visual separation between those who were hospitalized (blue) vs those who were not (yellow dots) and Permutation test plots indicating the validation of the PLS-DA models with visual separation between hospitalized (green) versus not hospitalized (blue). B: Urine PCA/PLSDA and permutation plots showing visual separation between those who were hospitalized (blue) vs the rest (yellow dots), PLS-DA showing visual separation between those who were hospitalized (blue) vs those who were not (yellow dots) and Permutation test plots indicating the validation of the PLS-DA models with visual separation between hospitalized (green) versus not hospitalized (blue). C: ChemRICH analysis of serum. Red clusters associated with higher and blue one associated with lower outcomes. D. ChemRICH analysis of urine. Red clusters associated with higher outcomes.

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acidic, sulfur and aromatic), glutarates, hexuronates, malates, hexoses, sugar acids, citrates, indoles, saturated FA, dicarboxylates, purinones were associated with hospitalizations.

**HE (Fig 2).** Patients who developed HE demonstrated higher serum clusters related to pyridines and pyrimidines, sugar acids, sugar alcohols, amino acids, phenylacetates, disaccharides, monosaccharides, hexoses, uronic acid and glutarates while basic amino acids, saturated fatty acids, dicarboxylic acids, purinones and adipates were lower. In the urine, higher clusters related to pentoses, purines, disaccharides, phenylacetates, sugar alcohols, amino acids (Cyclic, basic, acidic, sulfur and aromatic), glutarates, hexuronates, malates, hexoses, sugar acids, citrates, indoles, saturated FA, dicarboxylates, purinones were associated with HE.

**Death (Fig 3).** In the serum, pyridines, sugar acids, sugar alcohols, acidic amino acids, phenylacetates, disaccharides, monosaccharides, hexoses, and glutarates were higher in those

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Fig 2. 90-day hepatic encephalopathy (HE) development. A: Serum PCA/PLS-DA and permutation plots PCA showing visual separation between those who developed HE (blue) vs the rest (yellow dots), PLS-DA showing visual separation between those who developed HE (blue) vs those who did not (yellow dots) and Permutation test plots indicating the validation of the PLS-DA models with visual separation between HE (green) versus no HE (blue). B: Urine PCA/PLS-DA and permutation plots PCA showing visual separation between those who developed HE (blue) vs the rest (yellow dots), PLS-DA showing visual separation between those who developed HE (blue) vs those who did not (yellow dots) and Permutation test plots indicating the validation of the PLS-DA models with visual separation between HE (green) versus no HE (blue). C: ChemRICH analysis of serum. Red clusters associated with higher and blue one associated with lower outcomes D. ChemRICH analysis of urine. Red clusters associated with higher outcomes.

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who died while saturated and unsaturated fatty acids, purine nucleosides, basic and sulfur amino acids, dicarboxylic acids, and adipates were lower. In the urine, metabolites related to saturated FAs, pentoses, Indoles, amino acids (basic, cyclic, acidic and basic), purines malates, disaccharides, dicarboxylic acids, hexuronic acids, hexoses, phenylacetate and citrates were associated with higher death.

**Transplant (Fig 4).** Patient who received a transplant demonstrated again a similar pattern to those who died with higher serum pyridines, sugar acids, sugar alcohols, phenylacetate, disaccharides, monosaccharides, hexoses, and glutarates and lower serum saturated fatty acids, purine nucleosides, basic, acidic and sulfur amino acids, dicarboxylic acids, and adipates in those who were not transplanted. In the urine again, there were higher metabolites related
to saturated FAs, pentoses, indoles, amino acids, purines, malates, disaccharides, dicarboxylic acids, hexuronic acids, hexoses, phenylacetate and citrates in those who got transplanted.

**Metabolomics.**  On logistic regression after adjustment for age, gender, diabetes, prior HE, medication use and controlling for FDR we found that for 90-day hospitalization there were 290 urinary and 232 serum metabolites that were significant, for HE there were 284 urinary and 228 serum metabolites while 284 serum and 227 urine metabolites were significant for death. 285 urine and 229 serum metabolites were significant for transplant. The specific metabolites are shown in S5 to S12 Tables.

**PCA and PLS-DA.**  Using the variables significant on logistic regression, the PCA and PLS-DA and permutation analyses showed visual separation between patients who developed HE, needed transplant, required hospitalization or died (Figs 1A/1B, 2A/2B, 3A/3B and 4A/4B) for urine and serum metabolites. MetaMaPP changes are shown in S1–S8 Figs for each fluid and specific outcomes.

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Table 2. Top 20 individual serum metabolites with the highest VIP scores for all outcomes.

| Death | VIP  | Overt HE | VIP  | Transplant | VIP  | Hospitalization |
|-------|------|----------|------|------------|------|-----------------|
| hypoxanthine mix spec with ornithine | 1.691674911 | sophorose | 1.761664709 | N-acetyl-D-tryptophan minor | 2.077881 | 2-deoxyerythritol NIST | 1.743146383 |
| oxoproline | 1.6136051 | leucine | 1.632356319 | oxoproline | 1.811711 | conduritol betal epoxide minor | 1.735224901 |
| 2-hydroxybutanoic acid | 1.547067628 | oxoproline | 1.600209198 | caprylic acid | 1.571073 | arabitol | 1.735079543 |
| aconitic acid | 1.532266306 | isoleucine | 1.582285313 | xylulose NIST | 1.525188 | 3-phenylactic acid | 1.582691715 |
| 1-deoxyerythritol | 1.479701386 | stearic acid | 1.543379415 | stearic acid | 1.506294 | valine | 1.564061841 |
| urea | 1.467688896 | glycine | 1.533971676 | ornithine | 1.436212 | tocopherol alpha | 1.558857206 |
| heptadecanoic acid NIST | 1.467584884 | valine | 1.474016441 | malic acid | 1.428341 | threitol 2 | 1.536478704 |
| palmitic acid | 1.465626513 | isocenonic acid | 1.45732143 | asparagine 2TMS minor | 1.41985 | alpha ketoglutaric acid | 1.507600006 |
| linoleic acid | 1.439986096 | heptadecanoic acid NIST | 1.44794942 | 2-deoxyerythritol NIST | 1.415952 | pseudo uridine | 1.490157196 |
| oleic acid | 1.419174514 | 3-hydroxybutanoic acid mix spec | 1.439422872 | dodecanol | 1.40343 | 2-deoxyerythritol | 1.415289639 |
| 3-hydroxybutanoic acid mix spec | 1.416205224 | palmitic acid | 1.439262932 | trans-4-hydroxyproline | 1.394916 | phenylethylamine | 1.406504891 |
| galacturonic acid 2 | 1.415715043 | linolenic acid | 1.415766801 | tyrosine minor | 1.394313 | ethanolamine | 1.404227462 |
| glucose 2 | 1.409341965 | linolenic acid | 1.441241309 | 2-hydroxybutanoic acid | 1.392622 | leucine | 1.40017147 |
| 1-methyladenosine | 1.39527319 | xylitol | 1.401049684 | isolinolenic acid NIST | 1.351142 | 2-ketoacidic acid | 1.378520717 |
| isoleucine | 1.388507228 | oleic acid | 1.389973739 | N-acetylglutamate | 1.336039 | N-acetyl-D-hexosamine | 1.376185515 |
| leucine | 1.3862066 | 2-hydroxybutanoic acid | 1.388619203 | glycine TMS1x | 1.332613 | inuloseo 2 | 1.351429174 |
| phthalic acid | 1.381706001 | glutamine | 1.381953827 | adipic acid | 1.332586 | maltose 1 | 1.341200867 |
| valine | 1.367678245 | 1-deoxyerythritol | 1.356001613 | succinic acid | 1.326415 | isoleucine | 1.331328379 |
| 1,5-anhydroglucitol | 1.360892249 | methylhexadecanoic acid | 1.34876304 | threitol 2 | 1.323956 | aspartic acid | 1.33037414 |
| glucaric acid mix spec | 1.360625469 | tocopherol alpha | 1.338038687 | glutamine | 1.296891 | 3-methoxystroine NIST | 1.328860561 |

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VIP results. Tables 2 and 3 show top 20 metabolites that was associated for each outcome in serum and urine. The entire dataset is in S13 and S14 Tables.

Serum ROC results (Fig 5). AUC for metabolites was 0.99 compared to 0.88 MELD score for death. Serum metabolites for overt HE had an AUC of 0.85 compared to MELD with an AUC of 0.75. AUC for transplants were higher in serum metabolites 0.97 vs 0.94 for MELD score. 90-day hospitalization AUCs were similarly higher with serum metabolites 0.84 vs 0.83 on MELD alone. Of these comparisons, prediction of death and overt HE was statistically higher for metabolites compared to MELD (p = 0.03 and p = 0.05 respectively).

Urine ROC results (Fig 5). 90-day death AUC for metabolites was 0.92 compared to 0.86 for MELD score. For overt HE episodes also the urine metabolites AUC was higher 0.87 vs MELD at 0.72. Urine metabolite prediction for transplants was 0.99 compared to 0.90 for MELD score. An AUC of 0.89 vs 0.84 for hospitalizations was seen with metabolites compared to MELD alone. Of these comparisons, prediction of transplant was statistically significant with metabolites (p = 0.001) with a trend towards better hospitalization prediction (p = 0.058). Therefore, serum and urine metabolites had higher AUC compared to MELD on all outcomes. In addition, serum metabolites were better predictors than urine for 90-day overt HE and death while the reverse was true for transplant and hospitalizations.

Discussion

The current exploratory study found that specific patterns of changes in serum and urine metabolites were associated with prediction of clinically relevant outcomes centered on hepatic encephalopathy, hospitalizations, death and transplant. Metabolites linked with changes in
Changes in metabolomics in cirrhosis are important to analyze due to the major role of the liver in several important metabolic processes\cite{11}. These span amino acid, lipid and energy metabolism that have the potential to create serum and urine biomarkers and provide pathophysiological insight into the disease process. Development of HE is clinically relevant and was the leading cause of hospitalization in our population\cite{2}. HE has emerged as the leading reason for readmissions and the potential pathophysiology and clinical prediction of this outcome is very important\cite{17, 18}. Saturated serum FAs (caprylic, arachidic, lauric, stearic etc.) were associated with lower while FAs belonging to the eicosanoid pathway (iso-linoleic and icosanoic acids) were associated with greater HE. This is interesting because rifaximin therapy, which is usually protective against overt HE, is associated with higher serum saturated medium and long chain fatty acids\cite{19}. Saturated fatty acids have also been associated with a lower liver injury due to alcohol, which may play a role in this relative protection\cite{20}. On the other hand, branched chain amino acids typically associated with lower ammonia production, and glutamine, that represents ammonia capture with glutamate were protective\cite{21, 22}. Benzoic acid was associated with lower HE development and is likely related to gut microbial changes associated with cirrhosis, which can influence hippurate formation\cite{23}. Also supporting the HE-related systemic milieu, there was higher inositol which is extruded from astrocytes after

| Table 3. Top 20 individual urine metabolites with the highest VIP scores for all outcomes. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Death | VIP | Overt HE | VIP | Transplant | VIP | Hospitalizations |
| 3-aminoisobutyric acid | 1.643070115 | xylitol | 1.872164501 | phosphoric acid.1 | 1.725698799 | glycine | 1.827212618 |
| 2,3-dihydroxybutanonic acid NIST | 1.614889928 | 5-methoxytryptamine | 1.867140102 | 2-hydroxyvaleric acid | 1.701237906 | 3-hydroxypropionic acid | 1.7560861 |
| 2-hydroxy-2-methylbutanoic acid | 1.610992622 | creatinine | 1.741549209 | butane-2,3-diol (NIST) | 1.650237473 | butane-2,3-diol (NIST) | 1.573148827 |
| 3-ureidopropionate | 1.605150109 | 2-hydroxy-2-methylbutanoic acid | 1.738767214 | serine minor | 1.630710133 | creatinine | 1.55118776 |
| 2-hydroxyvaleric acid | 1.54878599 | 2-hydroxyvaleric acid | 1.52753461 | leucine | 1.625056096 | 2,3-dihydroxybutanoic acid NIST | 1.545142682 |
| 5-methoxytryptamine | 1.528630586 | glutamine | 1.505993268 | adipic acid | 1.624222805 | fructose 2 | 1.542568903 |
| uracil | 1.482652188 | glycolic acid | 1.45153304 | creatinine | 1.61593923 | glycine | 1.530902016 |
| glycine TMS1x | 1.478569024 | dodecanol | 1.414469964 | oxalic acid | 1.560206429 | glycine TMS1x | 1.491973532 |
| proline | 1.451424687 | azelaic acid | 1.414292736 | 2-hydroxy-2-methylbutanoic acid | 1.498640399 | azelaic acid | 1.411987043 |
| glycine | 1.443983922 | histidine | 1.403092474 | glycine TMS1x | 1.46031286 | fructose 1 | 1.409805348 |
| xylitol | 1.399886017 | threitol 2 | 1.40223294 | indole-3-lactate | 1.460261311 | 2-hydroxy-2-methylbutanoic acid | 1.397753125 |
| 3-hydroxypropionic acid | 1.397233101 | UDP-glucuronic acid | 1.398986905 | 3-hydroxybutanoic acid | 1.443439445 | xylulose NIST | 1.391645123 |
| butane-2,3-diol (NIST) | 1.391963441 | N-methylalanine | 1.386943204 | isorhamnose | 1.420245818 | benzoic acid mix spec | 1.38144168 |
| pelargonic acid | 1.370941662 | glycolic TMS1x | 1.382226302 | benzoic acid mix spec | 1.408113448 | cyclohexylamine NIST | 1.342365615 |
| benzoic acid mix spec | 1.352670887 | 2,3-dihydroxybutanoic acid NIST | 1.380952853 | 3-ureidopropionate | 1.407285453 | threitol 2 | 1.32700058 |
| 2-deoxyerythritol NIST | 1.342240486 | butane-2,3-diol (NIST) | 1.367099666 | galacturonic acid | 1.386165728 | dodecanol | 1.304573934 |
| citric acid | 1.34097281 | shikimic acid | 1.349692331 | glycolic acid | 1.381902268 | uracil | 1.28514236 |
| shikimic acid | 1.332763509 | inulotriose 1 | 1.34653941 | dodecanol | 1.315481375 | stearic acid | 1.281632065 |

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ammonia influx, and aromatic amino acid metabolites such as phenylacetates, phenyl-lactates, indoles, and urea cycle intermediates that were higher in those who developed overt HE [24, 25]. Lactic acid and high free sugars and sugar alcohols were also associated with minimal HE in prior serum studies using nuclear magnetic resonance (NMR) [26]. Our findings extend
these using GC/MS spectroscopy and link these to outcome development in this exploratory experience. Since HE was the major cause of hospitalization, the majority of the metabolomic findings were similar in pattern and predictive capability for that outcome as well.

Using individual VIP analyses, similar changes were noted above using CHEMRICH. Urinary and serum metabolites that were consistently in the top twenty by VIP were related to hippurate metabolism (benzoic acid and glycine). Glycine is associated with several important metabolites that were also highly represented in urine and serum, which are creatinine, and metabolites required for glutathione formation. These were glycine, glutamic acid and 2-Hydroxybutyric acid, which are involved in glutathione formation, an important hepatoprotective metabolite[27]. There was also a contribution of urea cycle intermediates (ornithine, urea, aspartate) and products of ammonia metabolism such as glutamine and aspartate in the serum. In addition, butanoate or butyric acid metabolites and propionate metabolites, which are major short-chain fatty acids, were also found to be predictive of outcomes[28]. Lastly, again long-to medium-chain fatty acids and branched chain amino acids, valine, leucine and isoleucine, were consistently represented among the serum metabolites.

The prediction of outcomes in cirrhosis is challenging given the multiple competing factors related to prior complications, cirrhosis severity, etiology, demographics and medications[3]. We demonstrated in this initial experience that serum and urine metabolomics were complementary and tended to be better than the MELD score alone in predicting transplant, hospitalizations, death and HE. This is important because these could be prevented or anticipated if their occurrence can be more reliably predicted[29]. We found that the relative predictive capability of the urine was greater than serum for transplant and hospitalizations while serum was better than urine for HE and prediction of death. Prior studies have been performed in decompensated cirrhosis and plasma metabolomics by McPhail et al and Mindikoglu et al[30, 31]. They demonstrated excellent predictive capability for death using multiple metabolomic platforms in decompensated cirrhosis and focused in those with kidney dysfunction and hepatorenal syndrome. Our experience extends this by including compensated and decompensated cirrhosis, patients with and without pre-existing kidney disease, analyzing both serum and urine, and analyzing the pattern of change of metabolomics associated with other outcomes as well. Most of the complications showed a statistically significant or a trend towards better prediction with metabolites compared to MELD. These may expand the generalizability of these findings in a more general outpatient cirrhosis population once validated in other cohorts.

It is interesting that similar groups of serum and urinary metabolites could predict the major complications regardless of the specific outcome and remained better than MELD despite adjusting for clinical indices and several medications. The 90-day interval was chosen because this is the validity period of the MELD score and also to reflect prior studies on readmissions in this population[32]. The importance of this additive component to the MELD score using metabolomics reflects other potential biomarkers of disease severity such as minimal/covert HE, sarcopenia or microbiota that are not captured by MELD[7, 33–37]. Samples were collected from cirrhotic outpatients, who already have a skewed metabolic baseline. Therefore, despite controlling for clinically relevant variables and in this skewed background, we were able to define added value of these metabolites in both fluids with trends or with statistical significance. However, the need to develop better biomarkers is even greater in the more complex hospitalized cirrhotic patients[38], where better predictors for acute-on-chronic liver failure are needed. There is a call for hybrid clinical and biological markers for improving prognostication in this population[39].

We recognize that although it may improve prognostication, it is not viable to routinely perform metabolomics in the clinic. Therefore, these results are the initial experience and need to be further validated. However, the demonstration of these alterations, we believe may help
focus on specific metabolite patterns that could be narrowed down in the future to potentially add to our current clinical biomarkers. Our study is also limited using only the GC/MS platform and it is likely that use of NMR and lipidomics could have further improved the prognostication. Since many outcomes follow one another, we found similar groups of metabolites that were associated with these predictions. Therefore, our analysis also focused on HE and hospitalizations, which precede transplant and death. This could also reflect the altered metabolomic milieu in advancing stages of liver disease, rather than be focused on specific complications. Due to the relatively lower number of non-OHE hospitalizations, we did not perform a subgroup analysis of hospitalizations that were related to hepato-renal syndrome, hyponatremia, infections and liver-unrelated reasons. Not all patients provided urine, the majority due to dialysis, which skewed the potential prediction towards transplant in the serum providers since serum was collected from every patient. This may also be the reason for better differentiation between MELD score and transplant prediction in those who gave urine since this only included patients not on dialysis. Due to the relatively short follow-up, we did not perform a time-to-event and associated competing-risks analysis.

We conclude in this exploratory study, that there are major alterations in serum and urinary metabolomics focused on lipid, amino-acid and bioenergetic metabolism that are associated with development of overt HE, hospitalization, transplant and death over 90 days. Glycine metabolism intermediates, urea cycle intermediates, branched chain amino acids and long to medium chain fatty acids should form the focus of future directed metabolomic strategies. Further validation of these results is needed in larger multi-center studies to determine the utility of hybrid scores combining biomarkers with clinical variables for predicting outcomes in cirrhosis.

**Supporting information**

**S1 Methods.**

(DOCX)

**S1 Fig. MetaMapp in serum for hospitalizations.** Red nodes = ↑ in those with outcomes, Blue nodes = ↓ in those with outcomes, Yellow nodes = not significant, Red edges: KEGG similarity and Blue edges: Tanimoto chemical similarity.

(TIF)

**S2 Fig. MetaMapp in urine for hospitalizations.** Red nodes = ↑ in those with outcomes, Blue nodes = ↓ in those with outcomes, Yellow nodes = not significant, Red edges: KEGG similarity and Blue edges: Tanimoto chemical.

(TIF)

**S3 Fig. MetaMapp in serum for HE development.** Red nodes = ↑ in those with outcomes, Blue nodes = ↓ in those with outcomes, Yellow nodes = not significant, Red edges: KEGG similarity and Blue edges: Tanimoto chemical.

(TIF)

**S4 Fig. MetaMapp in urine for HE development.** Red nodes = ↑ in those with outcomes, Blue nodes = ↓ in those with outcomes, Yellow nodes = not significant, Red edges: KEGG similarity and Blue edges: Tanimoto chemical.

(TIF)

**S5 Fig. MetaMapp in serum for death.** Red nodes = ↑ in those with outcomes, Blue nodes = ↓ in those with outcomes, Yellow nodes = not significant, Red edges: KEGG similarity and Blue edges: Tanimoto chemical.
edges: Tanimoto chemical.

(S6 Fig. MetaMapp in urine for death. Red nodes = ↑ in those with outcomes, Blue nodes = ↓ in those with outcomes, Yellow nodes = not significant, Red edges: KEGG similarity and Blue edges: Tanimoto chemical.

(S7 Fig. MetaMapp in serum for transplant. Red nodes = ↑ in those with outcomes, Blue nodes = ↓ in those with outcomes, Yellow nodes = not significant, Red edges: KEGG similarity and Blue edges: Tanimoto chemical.

(S8 Fig. MetaMapp in urine for transplant. Red nodes = ↑ in those with outcomes, Blue nodes = ↓ in those with outcomes, Yellow nodes = not significant, Red edges: KEGG similarity and Blue edges: Tanimoto chemical.

(S1 Table. ChemRICH overt HE prediction.

(S2 Table. ChemRICH hospitalization prediction.

(S3 Table. ChemRICH death prediction.

(S4 Table. ChemRICH transplant prediction.

(S5 Table. Logistic regression of serum metabolites with 90-day hospitalizations.

(S6 Table. Logistic regression of urine metabolites with 90-day hospitalizations.

(S7 Table. Logistic regression serum 90 day HE development.

(S8 Table. Logistic regression urine 90 day HE development.

(S9 Table. Logistic regression serum 90 day death.

(S10 Table. Logistic regression urine 90 day death.

(S11 Table. Logistic regression serum 90 day transplant.

(S12 Table. Logistic regression urine 90 day transplant.

(S13 Table. Serum VIP scores for individual named metabolites for each complication arranged by VIP score.
S14 Table. Urine VIP scores for individual named metabolites for each complication arranged by VIP score.

(DOCX)

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script occurred while he was not part of CDS and instead the Principal Statistician at the University of Califor-
nia Davis. We have changed his affiliation to reflect this fact and he maintains his stated no conflict of
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