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Identification of Noninvasive Biomarkers for Alcohol-Induced Liver Disease Using Urinary Metabolomics and the *Ppara*-null Mouse

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Abstract

Alcohol-induced liver disease (ALD) is a leading cause of non-accident-related deaths in the United States. Although liver damage caused by ALD is reversible when discovered at the earlier stages, current risk assessment tools are relatively non-specific. Identification of an early specific signature of ALD would aid in therapeutic intervention and recovery. In this study the metabolic changes associated with alcohol-induced liver disease were examined using alcohol-fed male *Ppara*-null mouse as a model of ALD. Principal components analysis of the mass spectrometry-based urinary metabolic profile showed that alcohol-treated wild-type and *Ppara*-null mice could be distinguished from control animals without information on history of alcohol consumption. The urinary excretion of ethyl-sulfate, ethyl- β -D-glucuronide, 4-hydroxyphenylacetic acid, and 4-hydroxyphenylacetic acid sulfate was elevated and that of the 2-hydroxyphenylacetic acid, adipic acid, and pimelic acid was depleted during alcohol treatment in both wild-type and the *Ppara*-null mice albeit to different extents. However, indole-3-lactic acid was exclusively elevated by alcohol exposure in *Ppara*-null mice. The elevation of indole-3-lactic acid is mechanistically related to the molecular events associated with development of ALD in alcohol-treated *Ppara*-null mice. This study demonstrated the ability of metabolomics approach to identify early, noninvasive biomarkers of ALD pathogenesis in *Ppara*-null mouse model.

Keywords

Alcohol-induced liver disease; PPAR α ; *Ppara*-null mouse; steatosis; metabolomics; UPLC-ESI-QTOF-MS; multivariate data analysis; biomarker; indole-3-lactic acid

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Supporting Information Available: This material is available free of charge via the Internet at <http://pubs.acs.org>.

1. Introduction

Excessive alcohol consumption is the third most common cause of lifestyle-associated mortality in the United States and in 2003 more than half of these deaths were attributed to alcohol-induced liver disease (ALD) ¹. Chronic alcohol consumption can lead to steatosis (fatty liver) due in part to alterations in lipid metabolism ² and, without intervention, may progress to advanced, irreversible stages of ALD including fibrosis and cirrhosis ³. Since the initial stages of ALD are reversible ⁴, an early and reliable tool to assess ALD risk would be helpful for intervention. Current diagnosis includes biochemical assays for enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), along with patient history and other clinical symptoms ^{4, 5}. However, the elevated activity of these enzymes is not strictly limited to ALD and other liver disorders may present similarly ^{6, 7}. Additionally, the initial stages of ALD can be largely asymptomatic ⁴ and cases are frequently reported only at an advanced, irreversible stage. Presently, an early, reliable, and noninvasive ALD-specific risk assessment tool remains elusive.

Since the earliest observable change in ALD pathogenesis is the deposition of free fatty acids in the liver, substantial effort to understand ALD pathogenesis has been devoted to identify pathways involved in fatty acid metabolism. The nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR α) ⁸ plays a crucial role in the catabolism of fatty acids in the liver. Recently, studies involving knockout mice fed an alcohol-containing liquid diet, reported that PPAR α activity protects against ALD in the mouse ⁹. The marked similarities of the liver pathology of the alcohol-treated *Ppara*-null mice to hallmarks of the human disease makes it an excellent model for studying system level (epigenomic, transcriptomic, proteomic, and metabolomic) changes associated with ALD.

Metabolomics is a rapidly evolving field that aims to identify and quantify the concentration changes of all the metabolites due to endogenous or exogenous perturbations. Since the production of a particular metabolite is the end result of a cascade of interactions involving numerous biological molecules (including DNA, RNA, and proteins), they together, i.e., the metabolome, represent the closest molecular level description of the physiological state. Thus, in principle, any physiological perturbation is expected to be associated with characteristic changes in the metabolome. This approach to understand systems biology has yielded promising results in a number of recent studies including radiation biodosimetry, pharmacometabolomics, and cancer ^{10–13}. Hence, the application of metabolomics to understand the effects of ALD represents a powerful means not only to identify the earliest biomarkers, but also to unravel the molecular mechanism of its pathogenesis ^{14, 15}.

This study combines the power of metabolomics along with the well-characterized *Ppara*-null mouse model to search for biomarkers for ALD. Wild-type control mice were studied in parallel with the *Ppara*-null mice in order to differentiate pathways specifically associated with ALD development from those related to the general effects of alcohol consumption. A combination of ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS) and chemometrics were used to identify urinary biomarkers associated with ALD. A discussion of the newly identified biomarkers and implicated biochemical pathways is presented.

2. Materials and Methods

2.1. Chemicals

All compounds were obtained from Sigma-Aldrich (St. Louis, MO) and were of the highest grade available. HPLC grade solvents were purchased from Fisher Scientific (Hampton, NH).

2.2. Animals and Treatments

Male 6-to-8-week-old wild-type and *Ppara*-null (129Sv background, four animals per group) mice were fed ad libitum a 4% alcohol-containing liquid diet (Lieber-DeCarli Diet, Dyets, Inc., Bethlehem, PA). Control animals were fed ad libitum an isocaloric diet supplemented with maltose dextran (Dyets, Inc.). All animal studies were approved by the Georgetown University Animal Care and Use Committee.

2.3. Histology

After one month on the alcohol diet, mice were euthanized, serum collected and portions of liver harvested for histology. Livers were formalin-fixed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin.

2.4. Biochemistry

The serum AST and ALT activities were measured using VetSpec™ kits (Catachem Inc, Bridgeport, CT) following the manufacturer's instruction. Liver and serum triglycerides were estimated using ELISA kit from Wako (Richmond, VA).

2.5. Urine Collection

After biochemical and histological evaluation of the model and ensuring that all animals were tolerating the liquid diet, mice were transferred to urinary metabolomics protocol at two months. Urine samples were collected monthly from mice placed in Nalgene metabolic cages (Tecniplast USA, Inc., Exton, PA) over 24 hours and stored at -80°C in glass vials until analyzed. All the mice were acclimated to the metabolic cages by placing them in the metabolic cages before the actual sample collection.

2.6. Preparation of Urine Samples and UPLC-ESI-QTOFMS Analysis

One volume of urine was added to one volume of 50% aqueous acetonitrile containing internal standards (50 μM 4-nitrobenzoic acid and 1 μM debrisoquine) in a Sirroco™ protein precipitation plate (Waters Corp., Milford, MA) and vortexed briefly. The deproteinized extracts were collected into 96-well collection plates, under vacuum, according to the manufacturer's instructions. A 5 μL aliquot of supernatant was injected into a Waters UPLC-ESI-QTOFMS system (Milford, MA). An Acquity UPLC BEH C18 column (Waters Corp.) was used to separate urinary constituents. The mobile phase was comprised of 0.1% aqueous formic acid (A) and acetonitrile containing 0.1% formic acid (B). A 0.5 ml/min flow rate was maintained during a 10-min run. The QTOF Premier mass spectrometer was operated in electrospray ionization positive (ESI+) and negative (ESI-) mode. Capillary voltage and cone voltage were maintained at 3 kV and 20 V, respectively. Source temperature and desolvation temperature were set at 120 $^{\circ}\text{C}$ and 350 $^{\circ}\text{C}$, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (600 L/h), and argon was used as collision gas. Sulfadimethoxine was used as the lock mass (m/z 311.0814⁺) for accurate mass calibration in real time. As for MS/MS fragmentation of target ions, collision energy ranging from 10 to 40 eV was applied. All urine samples were analyzed in a randomized fashion to avoid complications due to artifacts related to injection order and

changes in instrument efficiency. Mass chromatograms and mass spectral data were acquired using MassLynx software (Waters Corp.) in centroid format.

2.7. Multivariate Data Analysis

Centroided and integrated raw mass spectrometric data were processed using MarkerLynx software (Waters Corp., Milford, MA). The intensity of each ion was normalized with respect to the total ion count (TIC) to generate a data matrix that consisted of the retention time, m/z value, and the normalized peak area. The multivariate data matrix was analyzed by SIMCA-P+12 software (Umetrics, Kinnelon, NJ). The unsupervised segregation of control and alcohol-treated animals was checked by principal components analysis (PCA) using Pareto-scaled data¹⁶. The supervised orthogonal projection to latent structures (OPLS) model was used to concentrate group discrimination into the first component with remaining unrelated variation contained in subsequent components. The magnitude of the parameter $p(\text{corr})[1]$ obtained from the OPLS analysis correlates with the group discriminating power of a variable. Since it was observed that ions with $p(\text{corr})[1] > 0.8$ or $p(\text{corr})[1] < -0.8$ showed statistically significant ($P < 0.05$) difference in abundance between control and alcohol-treated animals a list of ions showing considerable group discriminating power ($-0.8 > p(\text{corr})[1]$ or $p(\text{corr})[1] > 0.8$) was generated from the loading S-plot for metabolic pathway analysis. However, only the ions that were consistently attenuated on alcohol treatment throughout the study, at least in case of one genotype, were selected for further identification and quantitation.

2.8. Metabolic Pathway Analysis

MassTRIX (<http://metabolomics.helmholtz-muenchen.de/masstrix/>), a web-based tool designed to assign ions of interest from a metabolomics experiment to annotated pathways¹⁷ without any systematic identification¹⁸, was used to identify the affected metabolic pathways. The masses of the ions that are significantly elevated ($p(\text{corr})[1] > 0.8$) or depleted ($p(\text{corr})[1] < -0.8$) on alcohol treatment were used to identify affected pathways using the KEGG (<http://www.genome.jp/kegg/>) database (including HMDB, Lipidmaps, and updated KEGG). A mass error of 5 ppm in the respective ionization modes and the possibility of formation of Na^+ -adducts in the electrosprayer (ESI+ mode) was also taken into account.

2.9. Identification of Urinary Biomarkers

Elemental compositions were derived considering a mass error less than 5 ppm following the Seven Golden Rules¹⁹. Metabolomics databases were also searched to find out possible candidates for these ions^{20, 21}. Finally, identities of the ions were confirmed by comparison of retention time and fragmentation pattern with authentic standards. Sulfate conjugates were confirmed by the disappearance of the peak corresponding to the metabolite following treatment of the urine samples with sulfatase enzyme (Sigma-Aldrich, St. Louis, MO). Briefly, urine samples and the standards were incubated with 40 U/ml of the enzyme solution in 200 mM sodium acetate buffer (pH 5.0) overnight at 37°C. The enzyme and other particulates were precipitated with 50% aqueous acetonitrile, and the supernatant was analyzed by UPLC-ESI-QTOFMS. 4-Nitrocatechol sulfate was used as a positive control for the sulfatase activity. Acid hydrolysis was carried out by heating the urine samples with 6M HCl at 100°C for 1 hour under refluxing condition.

2.10. Quantitation of Urinary Metabolites

Quantitation of urinary metabolites was carried out using an Acquity® UPLC system coupled with a XEVO™ triple-quadrupole tandem mass spectrometer (Waters Corp.) by multiple reaction monitoring (MRM). The following MRM transitions were monitored for

the respective compounds: indole-3-lactic acid (206→118; ESI+), indole-3-pyruvic acid (204→130; ESI+), tryptophan (205→118; ESI+), 2-hydroxyphenylacetic acid (151→107; ESI−), 4-hydroxyphenylacetic acid (151→107; ESI−), adipic acid (147→101; ESI+), pimelic acid (159→97; ESI−) and creatinine (114→86). Standard calibration plots for quantitation were generated using authentic standards. Deproteinized urine samples containing 0.5 μM debrisoquine were analyzed in the same fashion as that of authentic compounds. The quantitative abundances were calculated from the normalized (with respect to internal standard) peak area with the help of the calibration plot.

2.11. Statistics

All values are presented as mean ± standard error of the mean (SEM). One-way ANOVA with Bonferroni's correction for multiple comparisons were performed using GraphPad Prism 4 software and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Animal Monitoring and Liver Histology

There was no significant difference in the body weight of wild-type and *Ppara*-null mice either in control or alcohol-treated groups (data not shown). Although not statistically significant, liver histology (Figure 1A) and triglyceride measurements (Figure 1B) showed a clear trend indicating increase in hepatic fat deposition in the *Ppara*-null mice after one month of alcohol treatment. The alcohol-fed wild-type animals showed no such increase. In addition, there were no significant changes in the serum ALT, AST, and triglyceride levels after one month of alcohol treatment (see supplementary Figure S1).

3.2. Metabolomic Analysis

PCA analysis of metabolomic data showed distinct segregation of control and alcohol-treated mice (Figure 1C and supplementary Figure S2A) at 2 months. PCA analysis including samples from all the time points (2 to 6 months) resulted in separate clustering of the control and alcohol-treated wild-type animals indicating consistent underlying differences in the metabolic pattern arising from chronic alcohol consumption (supplementary Figure S2B). However, similar analysis for the *Ppara*-null animals (supplementary Figure S2C) showed that samples after 4 months of alcohol treatment occupied completely different metabolomic space. Moreover, the distance between the wild-type and *Ppara*-null genotype clusters was found to increase on chronic alcohol treatment (Figure 1D) further indicating the underlying difference in their metabolic response. The supervised OPLS model was used to enhance biomarker discovery efforts. The ions that showed significant difference in abundance between the control and treated animals ($-0.8 > p(\text{corr})[1]$ or $p(\text{corr})[1] > 0.8$) and contributed to the observed separation were selected from the respective S-plots for wild-type (Figure 1E, ESI+ and Figure S2B, ESI−) and *Ppara*-null animals (Figure 1F, ESI+ and Figure S2D, ESI−) as potential markers. The lists of discriminating ions at different time points were further screened to identify the ions that consistently contributed to the separation of control and alcohol-treated mice. Table 1 and 2 contain lists of such ions that were found to be significantly depleted or elevated in the urine of the wild-type and *Ppara*-null mice, respectively, during the course of alcohol treatment. It is interesting to note that almost all the markers were elevated or depleted from the beginning of sample collection (after 2 months of alcohol treatment) in case of the wild-type animals. However, many markers were found to either arrive late (3–4 months) or be minimally attenuated during the early course of alcohol exposure in case of the *Ppara*-null animals. This parallels the observation that the *Ppara*-null mice clustered separately after 4 months of alcohol treatment in the PCA analysis. Table 1 and 2 show that ions such as N1, N4, N4a, N18, N18a, N19, N21, P5, P5a, P6 and P7 were more abundant in the wild-type

urine compared to their *Ppara*-null counterparts. Ions such as N5, N5a, N6, N6a, N6b, N7, N7a, N15, N16, N16a, N17, N20, P4 and P9 were prevalent in the urine of the *Ppara*-null animals. Moreover, many of the ions were found to be exclusively present only in wild-type (N8, N10, N12, N13, N24, N25, N26, N27, P3, P6, P7; Table 1) or *Ppara*-null animals (N28, N29, P2, P10, P11; Table 2). Hence, these ions presumably represent the metabolic pathways that are differentially affected in the wild-type and *Ppara*-null animals on alcohol treatment.

3.3. Metabolic Pathway Analysis

Although mass measurement of ions with high accuracy (<5 ppm) is helpful to arrive at possible elemental compositions, mass accuracy alone is not always sufficient for unequivocal identification^{19, 22}. In order to identify possible pathways that were affected by the alcohol treatment, ions contributing to the separation of control and alcohol-treated animals were analyzed using MassTRIX. Metabolites related to the tryptophan metabolism were significantly upregulated following chronic alcohol exposure (Figure 2A). The number of upregulated metabolites potentially belonging to this pathway gradually decreased with time in wild-type animals while the corresponding number of metabolites increased in *Ppara*-null animals. In addition, tyrosine, phenylalanine, lysine, arginine and proline metabolic pathways were also indicated to be differentially affected in wild-type and *Ppara*-null animals due to alcohol treatment (data not shown). However, since assignment of the identity of the metabolites through MassTRIX is only tentative, detailed characterization of important marker ions were carried out by comparing with authentic standards.

3.4. Identification and Quantitation of Metabolites

Ion N1 was identified as ethyl sulfate ($C_2H_6O_3S$) by MSMS fragmentation. The presence of the sulfate group was further confirmed by the disappearance of this peak after treatment with sulfatase (supplementary Figure S5A and B). The increase in the urinary abundance of ethyl sulfate was observed at 2 months in alcohol-treated wild-type ($P < 0.001$) but not in *Ppara*-null animals (Figure 2B and 2C, respectively). Moreover, the increase in abundance of this metabolite in the urine of alcohol-treated *Ppara*-null mice was significantly lower than wild-type counterparts at 2 ($P < 0.001$) and 3 ($P < 0.001$) months of alcohol treatment. A significant ($P < 0.001$) elevation in urinary concentration of this metabolite was observed after 4 months of alcohol treatment in the *Ppara*-null mice (Figure 2C). However, the elevation in the excretion of ethyl sulfate in the *Ppara*-null mice (4-fold) was significantly ($P < 0.01$) lower than that observed in wild-type mice (7-fold).

Ions N2, P1, and P1a, all of which eluted at the retention time of 0.6 min, disappeared after acid hydrolysis of the urine sample (supplementary Figure S6). Subsequently, these were identified as the deprotonated form, Na^+ -adduct, and in-source fragment of Na^+ -adduct (with a neutral loss of $HCOOH$) of the glucuronide metabolite, respectively, of ethyl- β -D-glucuronide ($C_8H_{14}O_7$). A number of other co-eluting ions (N2a, N2b, N2c, N2d, P1b, P1c, P1d and P1e) were identified as either fragments or adducts of ethyl- β -D-glucuronide. Figures 2D and 2E show that the increase in the urinary concentration of ethyl- β -D-glucuronide in the *Ppara*-null mice was 25% of that found in the wild-type mice after 3 months of alcohol consumption ($P < 0.001$). As observed in case of ethyl sulfate, the excretion of ethyl glucuronide increased markedly after 4 months in the *Ppara*-null mice ($P < 0.01$ at 6 months), albeit only to a lower level compared to wild-type mice ($P < 0.001$ at 6 months).

The analysis of the metabolic signatures in the ESI- mode (Table 1 and 2) showed that the abundance of three ions (N3, N4a and N5) with $m/z = 151.04$ and empirical formula $C_8H_8O_6S$ were attenuated by alcohol consumption. N5 was identified as 2-

hydroxyphenylacetic acid. MRM-based quantitation showed that the basal level of this metabolite in the control *Ppara*-null animals (Figure 3B) was 4-fold higher than that in the control wild-type (Figure 3A) animals ($P < 0.001$). It was found to decrease by 50% in the wild-type mice on alcohol treatment (Figure 3A). Alcohol-treated *Ppara*-null mice also showed a continuous depletion in the urinary excretion of this metabolite leading to a 3-fold decrease ($P < 0.01$) after 6 months of alcohol treatment (Figure 3B). N3 was identified as 4-hydroxyphenylacetic acid. Chronic alcohol treatment was found to result in elevation of this metabolite in urine from both wild-type (Figure 3C) and *Ppara*-null (Figure 3D) mice. However, *Ppara*-null animals showed insignificant elevation of this metabolite in the early period (up to 3 months) of the alcohol treatment. The urinary excretion of 4-hydroxyphenylacetic acid was found to increase by 25-fold and 20-fold in the wild type ($P < 0.001$) and *Ppara*-null ($P < 0.01$) mice after 6 months of alcohol treatment. Fragmentation pattern and sulfatase assay (see supplementary Figure S5C and D) established the identity of N4a as the in-source fragmentation product of 4-hydroxyphenylacetic acid sulfate ($C_8H_8O_6S$; N4). Alcohol treatment resulted in early elevation in the excretion of this metabolite by 4-fold ($P < 0.001$) in the wild-type animals (Figure 3E). The *Ppara*-null animals showed only 1.5-fold ($P < 0.05$) elevation of this metabolite after 2 months of alcohol treatment. Although 3-fold ($P < 0.001$) elevation was observed after 4 months, the overall excretion of this metabolite was significantly ($P < 0.05$) lower in the *Ppara*-null mice compared to wild-type animals.

N6 and N7 were identified as pimelic acid ($C_7H_{12}O_4$) and adipic acid ($C_6H_{10}O_4$), respectively. The abundance of adipic acid and pimelic acid was found to be 4-fold ($P < 0.001$) and 3-fold ($P < 0.01$) higher in the urine of the control *Ppara*-null animals (Figure 4B and D) compared to wild-type (Figure 4A and C) counterparts. Alcohol treatment of wild-type mice resulted in a 2-fold and 3-fold decrease in the urinary concentration of adipic acid (Figure 4A) and pimelic acid (Figure 4C), respectively, throughout the duration of treatment. However, the urinary excretion of these dicarboxylic acids was found to decrease minimally or insignificantly up to 3 months of alcohol treatment in *Ppara*-null animals (Figure 4B and 4D). Nevertheless, on continued alcohol consumption, urinary excretion of adipic acid and pimelic acid was found to decrease, respectively, by 2-fold ($P < 0.01$, Figure 4B) and 5-fold ($P < 0.05$, Figure 4D) on average after 4 months in the *Ppara*-null animals, which is more than the decrease observed in the wild-type animals.

Markers P2 and N29 were elevated exclusively in the *Ppara*-null mice and identified as the protonated and deprotonated forms of indole-3-lactic acid ($C_{11}H_{11}NO_3$), respectively. This tryptophan metabolite (Figure 5B) showed a 1.5-fold increase in the urinary excretion in the *Ppara*-null animals after two months of alcohol exposure. This was increased further to 3-fold ($P < 0.001$) by 3 months with continued alcohol consumption. The urinary concentration of this metabolite in the alcohol-treated *Ppara*-null animals remained 2.5-fold higher ($P < 0.01$) than that in the control animals during the rest of the study. Moreover, the urinary excretion of this marker in the alcohol-treated *Ppara*-null mice was significantly higher ($P < 0.05$) than their wild-type counterparts throughout the experiment. However, the urinary concentration of its precursors (supplementary Figure S4), i.e. indole-3-pyruvic acid and tryptophan were found to be unaffected by alcohol consumption in wild-type as well as in *Ppara*-null mice.

3.5. Discriminatory Power of the Identified Metabolites

PCA analysis of the variation in the urinary excretion (creatinine normalized concentration) of the identified markers (Figure 5C) showed segregation of the animals according to alcohol exposure and genotype at 2 months. The four group of animals distinctly populated four quadrants of the scores scatter plot (Figure 5D) according to the alcohol treatment and genotype after 3 months of alcohol treatment. The separation along the vertical component reflected alcohol exposure and that along the horizontal component reflected the inherent

ALD susceptibility due to difference in PPAR α expression. Interestingly, exclusion of ethyl sulfate and ethyl- β -D-glucuronide did not change the clustering pattern (Figure 5E and 5F).

4. Discussion

Alcohol-treated *Ppara*-null mice in this study had considerable deposition of lipid after one month indicating the onset of steatosis, and confirming that *Ppara*-null animals served as a model for early stages of ALD development. Mass spectrometry-based metabolomic analysis revealed that alcohol exposure generates a distinct metabolic phenotype such that alcohol-treated mice are distinguishable from control mice. However, the metabolomic response to the alcohol exposure was found to be significantly different in the *Ppara*-null mice compared to the wild-type. This was evident from the differences in the onset time as well as the abundances of biomarker metabolites, and was also reflected in the difference in the PCA segregation pattern of the wild-type and *Ppara*-null animals. These general observations demonstrate that the metabolic traits closely represent the observed phenotype.

PPAR α is a key transcriptional regulator of many genes involved in fatty acid β -oxidation. It activates the tryptophan-quinolinic acid-NAD $^{+}$ pathway by down-regulating α -amino- β -carboxymuconate- ϵ -semialdehyde decarboxylase²³. Therefore, in the *Ppara*-null mice, NAD $^{+}$ production is reduced thus decreasing fatty acid catabolism and predisposing these mice towards fat deposition in the liver. Chronic alcohol consumption further complicates this situation. Alcohol is initially converted into acetaldehyde (a reactive electrophile) in the liver by the action of alcohol dehydrogenase²⁴. Aldehyde dehydrogenase further oxidizes acetaldehyde to acetic acid which can take part either in the Krebs cycle or in fatty acid synthesis²⁴. Both of these reactions consume NAD $^{+}$ and produce NADH. Thus, chronic alcohol consumption leads to a marked decrease in the redox ratio (NAD $^{+}$ /NADH)²⁵. Such a shift in the redox balance significantly impairs fatty acid catabolism and results in fat deposition in the liver (Figure 6). However, a minor amount of alcohol is also shunted through urine as sulfate and glucuronide conjugates by the action of the respective phase II enzymes in the liver²⁶. This not only reduces the concentration of free alcohol and its conversion to acetaldehyde and acetic acid, but also prevents the drop in NAD $^{+}$ levels. The present results showed that in contrast to the wild-type mice, *Ppara*-null animals had very low glucuronide and sulfate conjugates of alcohol in urine during the initial period of alcohol treatment. Although there was an increase in activities of these pathways after 4 months, they were still lower than that in the wild-type. The inefficiency of the *Ppara*-null animals to excrete alcohol would result in the increase in acetaldehyde production and a decrease in NAD $^{+}$ levels. Thus, the lower activity of these phase II metabolic pathways in the *Ppara*-null animals may contribute to the initiation of ALD pathogenesis in *Ppara*-null animals. Such difference in the phase II metabolic fluxes was also evident from the difference in 4-hydroxyphenyl acetic acid sulfate excretion of wild-type and *Ppara*-null mice. In case of humans, some of these enzymes were shown to be regulated by PPAR α ^{27, 28}. However, a recent study suggested that altered host-gut microbiome interaction may also modulate these enzymatic activities²⁹. The differential excretion of 2-hydroxyphenyl acetic acid and 4-hydroxyphenyl acetic acid, which are potential microbial metabolites, are also indicative of a change in the host-microbiome interactions.

Although β -oxidation is the dominant route for fatty acid catabolism, a minor fraction is also metabolized through the ω -oxidation pathway to small chain dicarboxylic acids like adipic acid and pimelic acid. The down-regulation of the genes related to fatty acid β -oxidation, can elevate fatty acid catabolism through this route. Thus, the basal levels of these dicarboxylic acid were found to be much higher in the control *Ppara*-null animals due to the activation of the ω -oxidation pathway compared to their wild-type counterparts. However, this pathway involves consumption of the NAD $^{+}$ during the conversion of the hydroxyacid

to the dicarboxylic acid. Therefore, chronic alcohol consumption reduces the ω -oxidation activity in all alcohol-treated animals due to the decrease in the NAD^+ level. This results in the decrease in the concentration of adipic acid and pimelic acid. An earlier human study showed that, in contrast to alcoholic men, alcoholic women showed no elevation in the urinary dicarboxylic acids and this was implicated in their increased susceptibility to alcohol-induced liver damage³⁰. Thus, the fact that the decrease in the urinary excretion of these acids was more prominent in the *Ppara*-null animals may be associated with the higher susceptibility of these mice towards ALD development.

The MassTRIX analysis indicated that chronic alcohol consumption affected the tryptophan metabolism more in the *Ppara*-null mice compared to the wild-type. Subsequently, indole-3-lactic acid was found to be exclusively elevated in the *Ppara*-null animals that developed ALD. It was notable that this elevation was statistically significant not only compared to the control *Ppara*-null mice but also compared to the alcohol-treated wild-type animals throughout the study. Indole-3-lactic acid is a terminal tryptophan metabolite that is produced by reduction of indole-3-pyruvic acid. Tryptophan is an essential amino acid that is metabolized in the liver. Typically, NAD^+ , tryptamine, and serotonin are the major metabolic products of tryptophan³¹. A minor pathway converts tryptophan to the indole-3-pyruvic acid through the action of tryptophan aminotransferase^{31, 32}. Tryptophan-2,3-dioxygenase oxidizes tryptophan to N-formyl kynurenine which is either catabolized to acetyl-CoA or converted to NAD^+ . Loss of $\text{PPAR}\alpha$ results in decreased conversion of tryptophan into NAD^+ ²³. In addition, chronic alcohol consumption was shown to decrease the activity of tryptophan-2,3-dioxygenase³³. This unused tryptophan gets shunted through the aminotransferase pathway to indole-3-pyruvic acid. The huge build up of NADH due to oxidation of alcohol helps readily to reduce the indole-3-pyruvic acid to indole-3-lactic acid in order to restore the redox balance. This drives the equilibrium forward to convert the unused tryptophan finally into indole-3-lactic acid. Chronic alcohol treatment of the *Ppara*-null animals results in severe impairment of fatty acid catabolism leading to the deposition of free fatty acids. Therefore, both the development of alcoholic fatty liver (steatosis) and the rise of indole-3-lactic acid in the *Ppara*-null animals likely represent a shift in the redox balance due to chronic alcohol consumption (Figure 6).

In spite of considerable differences in their genomes, the mouse is an excellent model for elucidating the molecular events leading to the development of human diseases. The observed similarities between the histopathology of the human and mouse ALD as well as the attenuation of the $\text{PPAR}\alpha$ expression levels during chronic alcohol consumption^{9, 34, 35} suggest that this metabolite may serve as a potential biomarker for the early stages of the ALD pathogenesis in humans subject to further experiments. Moreover, it was interesting to note that the identified endogenous biomarkers (indole-3-lactic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid sulfate, 2-hydroxyphenylacetic acid, adipic acid, and pimelic acid) could collectively distinguish the phenotypes without any information about the status of alcohol consumption as well as irrespective of the inclusion of the phase II alcohol metabolites (ethyl-sulfate, ethyl- β -D-glucuronide). These alcohol metabolites are currently used to detect recent alcohol consumption but have a detection limit of less than 3 days^{26, 36} and unavailability of information on the drinking history of the patients often cause problem in attributing the observed liver damage to ALD. Thus a combined targeted profiling of the endogenous biomarkers may offer an opportunity not only to detect ALD onset but also to identify ALD susceptibility without the requirement of knowledge of drinking history of the subjects and, thus, invites further studies in animal as well as human subjects. These biomarkers may be particularly useful for understanding the physiological effects of $\text{PPAR}\alpha$ and potential impact of allelic variants^{37–39} in the receptor in humans on ALD.

Future metabolomic studies in human populations with ALD will be needed to validate the biomarkers found in the mouse model. Indeed, the human urinary metabolome can be a rich, noninvasive source of disease biomarkers. However, it should be noted that factors such as the presence of other diseases, nutritional status, lifestyle, and xenobiotic, and environmental exposures, should be taken into account when attempting to establish a urinary metabolite as an ALD biomarker. This is especially important in humans where many of these factors cannot be strictly controlled.

5. Conclusions

The power of mass spectrometry-based metabolomics to capture and elucidate metabolic changes during chronic alcohol consumption has been demonstrated in this study. The identified metabolites were found to encompass a variety of pathways related to alcohol, amino acid, and fatty acid metabolism. The simultaneous use of wild-type and *Ppara*-null animals helped to distinguish the metabolic processes associated with alcohol-induced liver damage from the non-specific effects of chronic alcohol consumption. Finally, a tryptophan metabolite, indole-3-lactic acid, was found to be exclusively elevated in the alcohol-treated *Ppara*-null mice and mechanistically associated with the molecular events leading to ALD. The collective ability of these identified biomarkers to predict not only the ongoing liver damage but also the ALD susceptibility may contribute to the simplification and cost reduction of screening and diagnostic procedure subject to further investigation and validation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALD	alcohol-induced liver disease
PPARα	peroxisome proliferator-activated receptor alpha
<i>Ppara</i>-null	peroxisome proliferator-activated receptor alpha knockout mouse model
UPLC-ESI-QTOF-MS	ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry
ESI+	electrospray ionization in positive mode
ESI-	electrospray ionization in negative mode
MRM	multiple reaction monitoring
PCA	principal components analysis
OPLS	orthogonal projection to latent structures
NAD⁺	oxidized nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide

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Figure 1. Histology, biochemistry and multivariate data analysis. (A) Liver histology (HE staining) of wild-type (WT, left panel) and peroxisome proliferator-activated receptor alpha knockout (*Ppara*-null, right panel) mice after one month of control (upper panel) and 4% alcohol containing liquid diet (lower panel) showing significant increase in fat deposition in the alcohol-treated *Ppara*-null animals. (B) Liver triglyceride levels in the control (empty triangles) and alcohol-treated (empty diamonds) *Ppara*-null mice after one month. The triglyceride levels in wild-type mice are represented by filled triangles and diamonds, respectively. (C) Scores scatter plot from principal components analysis (PCA) showing unsupervised segregation of urinary metabolome (recorded in positive electrospray ionization mode) of control and alcohol-treated *Ppara*-null mice after two months. (D) PCA scores scatter plot showing increase in the difference between wild-type and *Ppara*-null metabolome due to chronic alcohol treatment (at 6 months). The solid triangles, empty triangles, solid diamonds and empty diamonds represent the control wild-type, control *Ppara*-null, alcohol-treated wild-type and alcohol-treated *Ppara*-null mice, respectively. (E) Loading S-plots from the supervised orthogonal projection to latent structures (OPLS) analysis of the positive ionization mode metabolic signatures (at 6 months) for the selection of candidate markers of chronic alcohol exposure in wild-type and (F) *Ppara*-null mice. Each triangle represents an ion characterized by unique mass and retention time. The p(corr)

[1] values represent the interclass difference and $w(1)$ values indicate the relative abundance of the ions. The ions that contribute highly to the interclass metabolomic difference were selected as candidate biomarkers. Some representative candidates have been highlighted (solid box) in the plots (see also Table 1 and Table 2). S-plots show P1, P1a, P4, P5, P5a, P8 were present in both wild-type and *Ppara*-null mice as common biomarkers for alcohol exposure. Differential metabolic response was characterized by biomarkers that were exclusively present in *Ppara*-null (P2 and P10) or wild-type (P3 and P6) mice.

Figure 2.

Effect of alcohol treatment on tryptophan and phase II alcohol metabolism. (A) Variation in the number of putative metabolites elevated during alcohol treatment in tryptophan metabolism detected in ESI+ mode predicted by MassTRIX analysis. The solid and dotted lines represent the response of the wild-type and *Ppara*-null animals, respectively. (B) Variation in the urinary abundance of ethyl sulfate in the control (dotted line) and alcohol-treated (solid line) wild-type and (C) *Ppara*-null mice. (D) Variation in the urinary abundance of ethyl- β -D-glucuronide in the control (dotted line) and alcohol-treated (solid line) wild-type and (E) *Ppara*-null mice. One-way ANOVA with Bonferroni's correction for multiple comparisons was used to estimate statistical significance of the variation of the metabolites on alcohol-treatment. One-way ANOVA with Bonferroni's correction for multiple comparisons was used to estimate statistical significance of the variation of the metabolites on alcohol-treatment. The *P* values for the elevation of these metabolites on alcohol treatment of the wild-type animals were calculated to be $P < 0.05$ throughout the course of experiment. *, significantly different ($P < 0.05$) from alcohol-treated *Ppara*-null mice; #, significantly different ($P < 0.05$) from control *Ppara*-null mice.

Figure 3.

Effect of alcohol treatment on phenylalanine and tyrosine metabolism. The dotted and solid lines represent the variation in the concentration of these metabolites in the urine of control and alcohol-treated mice, respectively. (A) Decrease in urinary excretion of 2-hydroxyphenylacetate in the wild-type and (B) *Ppara*-null mice during chronic alcohol treatment. (C) Increase in the urinary excretion of 4-hydroxyphenylacetic acid in the wild-type and (D) *Ppara*-null mice on alcohol treatment. (E) Chronic alcohol treatment increases the abundance of 4-hydroxyphenylacetic acid sulfate in the urine of wild-type and (F) *Ppara*-null mice during alcohol treatment. One-way ANOVA with Bonferroni's correction for multiple comparisons was used to estimate statistical significance of the variation of the metabolites on alcohol-treatment. The *P* values for the elevation of 4-hydroxyphenylacetic acid and 4-hydroxyphenylacetic acid sulfate on alcohol treatment of the wild-type animals were calculated to be $P < 0.05$ throughout the course of experiment (except for 4-hydroxyphenylacetic acid at 2 months). ‡, significantly different ($P < 0.05$) from the control *Ppara*-null mice (for control wild-type mice); *, significantly different ($P < 0.05$) from alcohol-treated *Ppara*-null mice; #, significantly different ($P < 0.05$) from control *Ppara*-null mice.

Figure 4.

Effect of alcohol treatment on urinary excretion dicarboxylic acids. The dotted and solid lines represent the variation in the concentration of these metabolites in the urine of control and alcohol-treated mice, respectively. (A) Decrease in the urinary excretion of adipic acid in the wild-type and (B) *Ppara*-null mice due to chronic alcohol treatment. (C) Decrease in the urinary excretion of pimelic acid in the wild-type and (D) *Ppara*-null mice during chronic alcohol treatment. One-way ANOVA with Bonferroni's correction for multiple comparisons was used to estimate statistical significance of the variation of the metabolites on alcohol-treatment. ‡, significantly different ($P < 0.05$) from the control *Ppara*-null mice (for control wild-type mice); *, significantly different ($P < 0.05$) from alcohol-treated *Ppara*-null mice; #, significantly different ($P < 0.05$) from control *Ppara*-null mice.

Figure 5.

Identification and assessment discriminatory power of non-invasive urinary ALD biomarkers. The dotted and solid lines represent the variation in the concentration of these metabolites in the urine of control and alcohol-treated mice, respectively. (A) Variation of the urinary excretion of indole-3-lactic acid in the control and alcohol-treated wild-type mice without any significant change due to alcohol treatment. (B) Elevation of urinary excretion of indole-3-lactic acid in alcohol-treated *Ppara*-null mice that develops alcohol-induced liver disease (ALD). One-way ANOVA with Bonferroni's correction for multiple comparisons was used to estimate statistical significance of the elevation of indole-3-lactic acid on alcohol-treatment. #, significantly different ($P < 0.05$) from control *Ppara*-null mice; *, significantly different ($P < 0.05$) from the alcohol-treated wild-type mice. (C) PCA scores scatter plot for the variation of the urinary excretion of the endogenous (indole-3-lactic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid sulfate, 2-hydroxyphenylacetic acid, adipic acid, and pimelic acid) and exogenous (ethyl-sulfate, ethyl- β -D-glucuronide) biomarkers to distinguish the observed phenotypes after 2 months and (D) 3 months of alcohol treatment. The solid triangles, empty triangles, solid diamonds, and empty diamonds represent the control wild-type, control *Ppara*-null, alcohol-treated wild-type, and alcohol-

treated *Ppara*-null mice, respectively. The vertical separation in the scatter plots correlates with alcohol exposure status (in the direction of the vertical arrow). The horizontal separation in the scatter plots correlates with *Ppara*-null expression that determines the susceptibility towards ALD with the horizontal arrow indicating decrease in PPAR α expression and increase in ALD susceptibility. The ANOVA for the corresponding OPLS-DA plot showed that the *P* values for segregation of the animals according to alcohol exposure and PPAR α expression to be $P < 0.002$ and $P < 0.0001$ at two and three months, respectively. (E) The scores scatter plot for the PCA of the variation of urinary concentration of the endogenous metabolites shows their collective discriminatory power to identify the observed phenotypes after 2 months and (D) 3 months of alcohol treatment. The ANOVA for the corresponding OPLS-DA plot showed that the *P* values for segregation of the animals according to alcohol exposure and ALD susceptibility to be $P < 0.002$ and $P < 0.00005$ at two and three months, respectively.

Figure 6.

Proposed mechanism behind the origin of indole-3-lactic as a biomarker of alcohol-induced liver disease in the alcohol-treated *Ppara*-null. The deletion of *PPAR α* gene impairs conversion of tryptophan to NAD⁺ through quinolinic acid and fatty acid β -oxidation. Oxidation of alcohol to acetaldehyde and acetic acid further decreases the NAD⁺/NADH ratio leading to free fatty acid (FFA) accumulation in the liver (steatosis). The build-up of NADH also drives the reduction indole-3-pyruvic acid to indole-3-lactic acid leading to an increase in the urinary excretion of this compound.

Table 1

List of marker ions for chronic alcohol exposure in the wild-type mice.

Marker	RT	m/z	w(l)	p(corr)[l]	Trend	Identity	Onset
N1	0.4	124.992	0.28	0.92	↑ ^a	Ethyl sulfate	Early ^b
N2	0.6	221.066	0.25	0.95	↑	Ethyl-β-D-glucuronide	Early
N2a	0.6	203.057	0.05	0.97	↑	(N2 - H ₂ O)	Early
N2b	0.6	443.141	0.04	0.93	↑	(2N2 + H ⁺)	Early
N2c	0.6	465.124	0.03	0.98	↑	(N2b + Na ⁺ - H ⁺)	Early
N2d	0.6	341.017	0.05	0.99	↑	(N1 + 2HCOOH + CO)	Early
N3	2.5	151.04	0.01	0.77	↑	4-Hydroxy phenylacetic acid	Early
N4	1.9	230.997	0.3	0.99	↑	4-Hydroxy phenylacetic acid sulfate	Early
N4a	1.9	151.04	0.06	0.99	↑	(N4-SO ₃)	Early
N5	3.0	151.04	-0.03	-0.93	↓ ^c	2-Hydroxy phenylacetic acid	Early
N5a	3.0	107.049	-0.03	-0.94	↓	(N5 - CO ₂)	Early
N6	1.9	145.05	-0.07	-0.93	↓	Adipic acid	Early
N6a	1.9	101.06	-0.03	-0.92	↓	(N6 - CO ₂)	Early
N6b	1.9	167.032	-0.04	-0.7	↓	(N6 + Na ⁺ - H ⁺)	Early
N7	2.7	159.066	-0.04	-0.75	↓	Pimelic acid	Early
N7a	2.7	181.048	-0.03	-0.89	↓	(N7 + Na ⁺ - H ⁺)	Early
N8	2.1	204.03	-0.10	-0.91	↓	-	Early
N8a	2.1	158.082	-0.20	-0.92	↓	(N8 - HCOOH)	Early
N8b	2.1	339.154	-0.06	-0.94	↓	(2N8a + Na ⁺)	Early
N9	5.0	201.113	-0.08	-0.91	↓	-	Early
N9a	5.0	223.095	-0.04	-0.93	↓	(N9 + Na ⁺ - H ⁺)	Early
N10	2	156.066	-0.05	-0.96	↓	-	Early
N11	0.3	166.018	0.24	0.97	↑	-	Early
N12	5.1	291.144	0.06	0.87	↑	-	Early
N13	4.8	155.107	-0.04	-0.93	↓	-	Early
N13a	4.8	221.079	-0.03	-0.96	↓	(N13 + CO ₂ + Na ⁺ - H ⁺)	Early
N14	2.2	276.054	-0.03	-0.92	↓	-	Early
N15	2.4	113.059	-0.03	-0.95	↓	-	Early

Marker	RT	m/z	w(1)	p(corr)[1]	Trend	Identity	Onset
N16	3.2	191.032	-0.02	-0.65	↑	-	Early
N16a	3.2	127.076	-0.03	-0.94	↓	(N16 - HCOOH - H ₂ O)	Early
N17	3.2	193.046	-0.02	-0.87	↓	-	Early
N18	3.4	231.077	-0.08	-0.90	↓	-	Early
N18a	3.4	253.059	-0.03	-0.99	↓	(N18 + Na ⁺ - H ⁺)	Early
N19	0.4	240.018	-0.03	-0.95	↑	-	Early
N20	1.6	186.077	0.03	0.74	↑	-	Early
N21	5.4	317.16	0.05	0.89	↑	-	Early
N22	1.0	196.028	0.03	0.8	↑	-	Early
N23	3.2	260.149	0.01	0.77	↑	-	Early
N24	4.6	277.129	0.04	0.92	↑	-	Early
N25	5.5	329.162	0.04	0.91	↑	-	Early
N26	1.6	158.081	0.05	0.87	↑	-	Early
N27	2.8	222.026	-0.03	-0.91	↓	-	Early
N30	0.9	145.05	0.04	0.80	↑	-	Early
N30a	0.9	127.04	0.02	0.89	↑	N30-H ₂ O	Early
N30b	0.9	101.06	0.01	0.75	↑	N30-CO ₂	Early
P1e	0.6	245.063	0.20	0.88	↑	Ethyl-β-D-glucuronide + Na ⁺	Early
P1a	0.6	199.022	0.10	0.97	↑	(P1 - HCOOH)	Early
P1b	0.6	159.03	0.06	0.72	↑	(P1 - CO ₂ - H ₂ O - Na ⁺ + H ⁺)	Early
P1c	0.6	467.139	0.06	0.83	↑	(P1 + N2 + H ⁺)	Early
P1d	0.6	705.177	0.04	0.86	↑	(3N2 + 3H ⁺ + K ⁺)	Early
P1e	0.6	141.019	0.06	0.73	↑	(P1b - H ₂ O)	Early
P3	2.0	144.084	-0.11	-0.98	↓	-	Early
P4	1.6	290.16	-0.03	-0.93	↓	-	Early
P5	3.9	220.064	-0.08	-0.95	↓	-	Early
P5a	3.9	145.99	-0.04	-0.93	↓	(P5 - HCOOH - CO)	Early
P6	0.3	198.088	0.05	0.89	↑	-	Early
P7	3.8	240.158	-0.05	-0.91	↓	-	Early
P8	1.4	272.112	0.13	0.94	↑	-	Early
P9	0.5	162.061	0.06	0.72	↑	-	Early

Note. RT, retention time in minutes; m/z , value of the mass-to-charge ratio of the ion; $w(1)$, modeled weight coefficient for the ion; $p(cor)[1]$, modeled correlation coefficient for the ion; the ions designated with 'N' and 'P' represents those identified in negative and positive electrospray ionization mode, respectively.

Table 2List of marker ions for chronic alcohol exposure in the *Ppara*-null mice.

Marker	RT	m/z	w(1)	p(corr)[1]	Trend	Identity	Onset
N1	0.4	124.992	0.21	0.99	↑	Ethyl sulfate	Early
N2	0.6	221.066	0.26	0.97	↑	Ethyl-β-D-glucuronide	Early
N2a	0.6	203.057	0.04	0.98	↑	(N2 - H ₂ O)	Early
N2b	0.6	443.142	0.04	0.97	↑	(2N2 + H ⁺)	Early
N2c	0.6	465.124	0.03	0.96	↑	(N2b + Na ⁺ - H ⁺)	Early
N2d	0.6	341.017	0.05	0.96	↑	(N2 + 2HCOOH + CO)	Early
N3	2.5	151.04	0.02	0.89	↑	4-Hydroxy phenylacetic acid	4 months
N4	1.9	230.997	0.23	0.97	↑	4-Hydroxy phenylacetic acid sulfate	Early
N4a	1.9	151.04	0.04	0.91	↑	(N4-SO ₃)	Early
N5	3.0	151.04	-0.05	-0.96	↓	2-Hydroxy phenylacetic acid	Early
N5a	3.0	107.049	-0.05	-0.97	↓	(N5 - CO ₂)	Early
N6	1.9	145.05	-0.11	-0.98	↓	Adipic acid	4 months
N6a	1.9	101.06	-0.05	-0.97	↓	(N6 - CO ₂)	4 months
N6b	1.9	167.032	-0.06	-0.94	↓	(N6 + Na ⁺ - H ⁺)	4 months
N7	2.7	159.066	-0.05	-0.76	↓	Pimelic acid	Early
N7a	2.7	181.048	-0.03	-0.96	↓	(N7 + Na ⁺ - H ⁺)	Early
N9a	5.0	223.095	-0.04	-0.83	↓	(N9 + Na ⁺ - H ⁺)	Early
N11	0.3	166.018	0.36	0.93	↑	-	3 months
N14	2.2	276.054	-0.06	-0.91	↓	-	Early
N15	2.4	113.059	-0.04	-0.96	↓	-	Early
N16	3.2	191.032	-0.04	-0.98	↓	-	3 months
N16a	3.2	127.076	-0.06	-0.88	↓	(N16 - HCOOH - H ₂ O)	Early
N17	3.2	193.046	-0.04	-0.94	↓	-	Early
N18	3.4	231.077	-0.05	-0.93	↓	-	3 months
N18a	3.4	253.059	-0.02	-0.91	↓	(N18 + Na ⁺ - H ⁺)	5 months
N19	0.4	240.018	-0.02	-0.78	↓	-	3 months
N20	1.6	186.077	0.10	0.94	↑	-	3 month
N21	5.4	317.16	0.03	0.92	↑	-	5 months

Marker	RT	m/z	w(1)	p(corr)[1]	Trend	Identity	Onset
N22	1.0	196.028	0.04	0.98	↑	-	4 months
N23	3.2	260.149	0.03	0.97	↑	-	4 months
N28	1.1	142.05	0.05	0.87	↑	-	3 months
N29	3.7	204.066	0.06	0.85	↑	Indole-3-lactic acid	3 months
N30	0.9	145.05	0.03	0.99	↑	-	Early
N30a	0.9	127.04	0.04	0.99	↑	N30-H ₂ O	Early
N30b	0.9	101.06	0.09	0.99	↑	N30-CO ₂	Early
P1	0.6	245.063	0.2	0.92	↑	Ethyl-β-D-glucuronide + Na ⁺	Early
P1a	0.6	199.022	0.13	0.99	↑	(P1 - HCOOH)	Early
P1b	0.6	159.03	0.10	0.97	↑	(P1 - CO ₂ - H ₂ O - Na ⁺ + H ⁺)	Early
P1c	0.6	467.138	0.07	0.97	↑	(P1 + N2 + H ⁺)	4 months
P1d	0.6	705.178	0.05	0.96	↑	(3N2 + 3H ⁺ + K ⁺)	4 months
P1e	0.6	141.019	0.10	0.99	↑	(P1b - H ₂ O)	Early
P2	3.7	206.082	0.05	0.86	↑	Indole-3-lactic acid	3 months
P2a	3.7	188.071	0.03	0.82	↑	(P2 - H ₂ O)	3 months
P2b	3.7	160.076	0.02	0.89	↑	(P2 - HCOOH)	3 months
P2c	3.7	449.102	0.02	0.81	↑	(2P2 - 2H ⁺ + K ⁺)	3 months
P4	1.6	290.16	-0.06	-0.95	↓	-	3 months
P5	3.9	220.064	-0.07	-0.94	↓	-	4 months
P5a	3.9	145.991	-0.03	-0.94	↓	(P6 - HCOOH-CO)	4 months
P8	1.4	272.112	0.11	0.97	↑	-	Early
P9	0.5	162.061	0.10	0.86	↑	-	3 months
P10	1.1	166.048	0.05	0.93	↑	-	4 months
P11	0.4	172.062	0.04	0.94	↑	-	4 months