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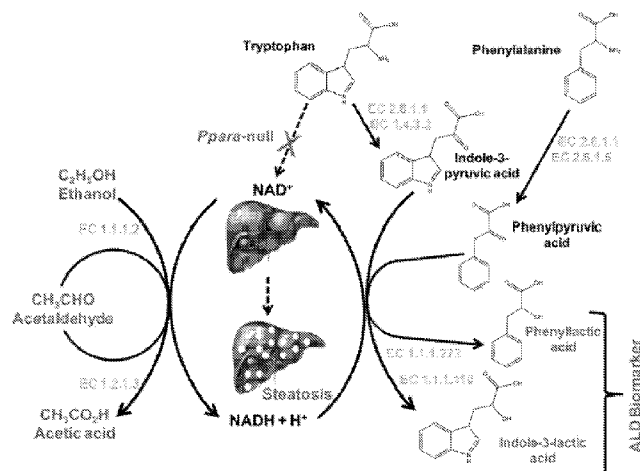
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(54) **Title:** IDENTIFICATION OF EARLY NONINVASIVE BIOMARKERS FOR ALCOHOLIC LIVER DISEASE USING URINARY METABOLOMICS AND THE PPARA-NULL MOUSE

Figure 13



(57) **Abstract:** The invention provides novel biomarkers for alcoholic liver disease.

IDENTIFICATION OF EARLY NONINVASIVE BIOMARKERS FOR ALCOHOLIC LIVER DISEASE USING URINARY METABOLOMICS AND THE PPARA-NUL MOUSEI**RELATED APPLICATIONS**

This application claims the benefit of U.S. Provisional Application Number 61/507,573 filed July 13, 2011, the entire contents of which is hereby incorporated by reference in its entirety.

**STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER
FEDERALLY SPONSORED RESEARCH**

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BACKGROUND OF THE INVENTION

Genetic composition is an intrinsic property that interacts with extrinsic factors such as food, environment, life-style, and xenobiotics to define the human phenotype. Unlike these extrinsic factors that can be controlled or manipulated to a certain degree, the genetic trait inherently predisposes individuals or groups of individuals to particular phenotypes. The etiology and pathogenesis of diseases and disorders are often dependent on genetic variation. It is therefore not surprising that identification of genetic background-independent diagnostic and predictive molecular signatures associated with diseases and disorders remains quite challenging to translational research.

Alcoholism is a common problem worldwide. According to the World Health Organization almost 4% of all deaths worldwide are attributed to alcohol. This rate of death is greater than deaths caused by HIV/AIDS, violence, or tuberculosis. Alcohol consumption is on the rise in developing as well as in developed countries, including the United States. However, epidemiological studies have revealed a significant variation in the propensity to, severity, and outcome of alcoholism depending on genetic background and composition.

Polymorphisms in genes related to alcohol metabolism was shown to affect incidence and physiological impacts of alcoholism. Apart from the neuropsychiatric effects, a significant portion of alcohol-related deaths are due to the development of alcoholic liver disease (ALD) and it is a leading cause for lifestyle-associated deaths in the United States. In 2003, around half of all liver disease-related deaths were attributed to alcohol consumption. Genetic background and polymorphism were shown to significantly affect the development and outcome of alcoholic liver disease as well.

ALD pathogenesis can be characterized into three stages: steatosis, alcoholic hepatitis, and fibrosis/cirrhosis. Approximately 90% of alcoholics develop fatty liver (steatosis) that resolves upon abstinence from alcohol. However, steatosis is not a benign condition, and with continuation of heavy drinking, the risk of developing cirrhosis was found to increase up to 37%. Cirrhosis is a fatal condition with overall 5-year survival rate as low as 35%. Patients with liver cirrhosis have also been found to be at increased risk of developing liver cancer, which also adds to the mortality rate. However, at earlier stages (steatosis), ALD is often asymptomatic but reversible and patients can recover completely. Detection of the disease at this stage is of key importance to improve the therapeutic outcome, quality of life, and reduce the mortality as well as healthcare burden.

Due to the heterogeneous influence of genetic background on disease onset, progress, and clinical symptoms, the most prudent strategy to prevent fatal consequences is to routinely monitor alcoholics for ALD onset. Currently, the most commonly used diagnostic tools for assessment of liver damage (liver enzyme levels) are nonspecific to etiology. The only confirmatory diagnosis of the disease is a liver biopsy. Unfortunately, the invasiveness and complications associated with biopsy precludes it as a viable tool for routine screening and diagnosis of large number of patients. Therefore, an early, noninvasive, high-throughput genetic background-independent ALD-specific biomarker is highly warranted.

SUMMARY OF THE INVENTION

As described below, this invention provides novel biomarkers for alcoholic liver disease (ALD).

In one aspect, the invention provides methods for identifying a subject as having or having a propensity to develop ALD. In embodiments, the methods involve detecting the level of a biomarker selected from the group consisting of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, and P11 in a sample obtained from the subject. In embodiments, the methods involve comparing the level of the biomarker to a reference. In embodiments, the methods involve identifying the subject as having or having a propensity to develop ALD when the level of the biomarker is increased relative to the reference.

In one aspect, the invention provides methods for identifying ALD in a subject. In embodiments, the methods involve detecting the level of a biomarker selected from the group consisting of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, and P11 in a sample obtained from the subject. In embodiments, the methods involve comparing the level of the biomarker to a reference. In embodiments, the methods involve identifying ALD in the subject when the level of the biomarker is increased relative to the reference.

In one aspect, the invention provides methods for identifying a subject as having or having a propensity to develop steatosis. In embodiments, the methods involve detecting the level of a biomarker selected from the group consisting of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, and P11 in a sample obtained from the subject. In embodiments, the methods involve comparing the level of the biomarker to a reference. In embodiments, the methods involve identifying the subject as having or having a propensity to develop steatosis when the level of the biomarker is increased relative to the reference.

In one aspect, the invention provides methods for identifying steatosis in a subject. In embodiments, the methods involve detecting the level of a biomarker selected from the group consisting of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, and P11 in a sample obtained from the subject. In embodiments, the methods involve comparing the level of the biomarker to a reference. In embodiments, the methods involve identifying steatosis in the subject when the level of the biomarker is increased relative to the reference.

In one aspect, the invention provides methods for characterizing the stage of ALD in a subject. In embodiments, the methods involve detecting the level of a biomarker selected from the group consisting of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, and P11 in a sample obtained from the subject. In embodiments, the methods involve comparing the level of the biomarker to a reference. In embodiments, the methods involve identifying the subject as having a later stage of ALD when there is an increase in the level of the biomarker relative to the reference.

In one aspect, the invention provides methods for determining the prognosis of ALD in a subject. In embodiments, the methods involve detecting the level of a biomarker selected from the group consisting of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, and P11 in a sample obtained from the subject. In embodiments, the methods involve comparing the level of the biomarker to a reference. In embodiments, the methods involve identifying the subject as having a poor prognosis when there is an increase in the level of the biomarker relative to the reference.

In one aspect, the invention provides methods for characterizing the degree of lipid accumulation during the early stage of ALD in a subject. In embodiments, the methods involve detecting the level of a biomarker selected from the group consisting of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, and P11 in a sample obtained from the subject. In embodiments, the methods involve comparing the level of the biomarker to a reference. In embodiments, the methods involve identifying the subject as having a higher level of lipid accumulation during the early stage of ALD when there is an increase in the level of the biomarker relative to the reference.

In any of the above aspects, the level of the biomarker is increased 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, 14, 14.5, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95-fold or more relative to the reference.

In any of the above aspects, the reference can be the level of the biomarker in a control. In embodiments, the control is a normal sample. In embodiments, the control is a control sample as described herein (*e.g.*, standardized control curve).

In one aspect, the invention provides methods for monitoring ALD therapy in a subject. In embodiments, the methods involve detecting the level of a biomarker selected from the group consisting of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, and P11 in a sample obtained from the subject. In embodiments, the methods involve comparing the level of the biomarker to a reference. In embodiments, the methods involve identifying the therapy as effective when there is a decrease in the level of the biomarker relative to the reference.

In embodiments, the reference is the level of the biomarker in a control. In related embodiments, the control is a normal sample. In related embodiments, the control is a control sample as described herein (*e.g.*, standardized control curve). In related embodiments, the control is a sample obtained from the subject prior to therapy or at an earlier time point during therapy.

In one aspect, the invention provides methods for detecting an agent's therapeutic efficacy in a subject having ALD. In embodiments, the methods involve detecting an alteration in the level of a biomarker selected from the group consisting of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, and P11 in a sample obtained from the subject. In embodiments, the methods involve comparing the level of the biomarker to a reference. In embodiments, the methods involve identifying the agent as having therapeutic efficacy in the subject when there is a decrease in the level. In embodiments, the methods involve identifying the agent as lacking therapeutic efficacy in the subject when there maintenance or increase in the level.

In embodiments, the reference is the level of the biomarker in a control. In related embodiments, the control is a normal sample. In related embodiments, the control is a control sample as described herein (*e.g.*, standardized control curve). In related embodiments, the control is a sample obtained from the subject prior to therapy or at an earlier time point during therapy.

In any of the above aspects, the biomarker can be phenyllactic acid.

In any of the above aspects, the biomarker can be phenyllactic acid in combination with indole-3-lactic acid.

In any of the above aspects, the biomarker can be i) phenyllactic acid or phenyllactic acid in combination with indole-3-lactic acid, and ii) one or more of N8; N9; N23; P7; and P11. In embodiments, the biomarker can further comprise one or more biomarkers that are well known in the art.

In any of the above aspects, the subject can be human.

In any of the above aspects, the sample can be a biological fluid (*e.g.*, blood, blood serum, plasma, saliva, or urine). In embodiments, the sample is a urine sample.

In any of the above aspects, the biomarker level is detected using any method well known in the art. In embodiments, the biomarker level is detected using one or more of the methods described herein. In embodiments, the biomarker level is detected using chromatography, mass spectrometry, spectroscopy, or immunoassay. In related embodiments, the chromatography is ultra performance liquid chromatography (UPLC). In related embodiments, the mass spectrometry is electrospray ionization quadruple time-of-flight mass spectrometry (ESI-QTF-MS). In related embodiments, the spectroscopy is NMR spectroscopy. In related embodiments, the immunoassay is ELISA.

In one aspect, the invention provides kits for aiding the diagnosis of ALD or steatosis.

In embodiments, the kits contain at least one reagent capable of detecting or capturing phenyllactic acid, indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof. In embodiments, the reagent is an antibody that specifically binds to phenyllactic acid, indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof. In embodiments, the kit further contains directions for using the reagent to analyze the level of phenyllactic acid, indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof.

In embodiments, the kits contain an adsorbent that retains phenyllactic acid, indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof. In related embodiments, the kits further contain directions for contacting a test sample with the adsorbent and detecting phenyllactic acid, indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof retained by the adsorbent.

In embodiments, the reagents and/or adsorbents are provided on a solid support (*e.g.*, chip, microtiter plate, bead, resin, and the like).

In embodiments, the kits contain washing solution(s) or instructions for making a washing solution, in which the combination of the reagent/adsorbent and the washing solution allows capture of the biomarkers on the reagent/adsorbent.

In embodiments, the kits include biomarker samples (*e.g.*, phenyllactic acid and indole-3-lactic acid), which can be used as standard(s) for calibration as may be desired.

In embodiments, the kits contain a container that houses the components of the kit (*e.g.*, boxes, ampoules, bottles, vials, tubes, bags, pouches, blister-packs, and the like). In related embodiments, the containers are made of plastic, glass, laminated paper, metal foil, and the like.

Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The objects and advantages of the invention will be realized and attained by means of the elements and combinations disclosed herein, including those pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention as claimed. The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments of the invention and, together with the description, serve to explain the principles of the invention.

Definitions

To facilitate an understanding of the present invention, a number of terms and phrases are defined below.

As used herein, the singular forms “a”, “an”, and “the” include plural forms unless the context clearly dictates otherwise. Thus, for example, reference to “a biomarker” includes reference to more than one biomarker.

Unless specifically stated or obvious from context, as used herein, the term “or” is understood to be inclusive.

The term “including” is used herein to mean, and is used interchangeably with, the phrase “including but not limited to.”

As used herein, the terms “comprises,” “comprising,” “containing,” “having” and the like can have the meaning ascribed to them in U.S. Patent law and can mean “includes,” “including,” and the like; “consisting essentially of” or “consists essentially” likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

A “biomarker” as used herein generally refers to a molecule that is differentially present in a sample taken from a subject of one phenotypic status (*e.g.*, having a disease) as compared with another phenotypic status (*e.g.*, not having the disease). A biomarker is differentially present between different phenotypic statuses if the mean or median level of the biomarker in a first phenotypic status relative to a second phenotypic status is calculated to represent statistically significant differences. Common tests for statistical significance include, among others, t-test, ANOVA, Kruskal-Wallis, Wilcoxon, Mann-Whitney and odds ratio. Biomarkers, alone or in combination, provide measures of relative likelihood that a subject belongs to a phenotypic status of interest. As such, biomarkers can find use as markers for, for example, disease (diagnostics), therapeutic effectiveness of a drug (theranostics), and of drug toxicity.

As used herein, the term “phenyllactic acid” refers to compound having the CAS number 828-01-3 (DL-3-phenyllactic acid), 7326-19-4 (D-(+)-3-Phenyllactic acid), or 20312-36-1 (L-(–)-3-Phenyllactic acid), including pharmaceutically acceptable salts, solvates, hydrates, geometrical isomers, tautomers, optical isomers, isotopic derivatives, polymorphs, prodrugs, or *N*-oxides thereof.

As used herein, the term “indole-3-lactic acid” refers to a compound having the CAS number 832-97-3, including a pharmaceutically acceptable salt, solvate, hydrate, geometrical isomer, tautomer, optical isomer, isotopic derivative, polymorph, prodrug, or *N*-oxide thereof.

By “agent” is meant any small molecule chemical compound, antibody, nucleic acid molecule, or polypeptide, or fragments thereof.

As used herein, the term “alcoholic liver disease” (ALD) refers to the spectrum of clinical pathologic changes in the liver caused by ethanol intake. ALD pathologies include fatty liver (steatosis), alcoholic hepatitis, and alcoholic cirrhosis.

The term “subject” or “patient” refers to an animal which is the object of treatment, observation, or experiment. By way of example only, a subject includes, but is not limited to, a mammal, including, but not limited to, a human or a non-human mammal, such as a non-human primate, murine, bovine, equine, canine, ovine, or feline.

As used herein, the terms “prevent,” “preventing,” “prevention,” “prophylactic treatment,” and the like, refer to reducing the probability of developing a disease or condition in a subject, who does not have, but is at risk of or susceptible to developing a disease or condition, *e.g.*, ALD.

As used herein, the terms “treat,” “treating,” “treatment,” and the like refer to reducing or ameliorating a disease or condition, *e.g.*, ALD, and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disease or condition does not require that the disease, condition, or symptoms associated therewith be completely eliminated.

By “alteration” or “change” is meant an increase or decrease. An alteration may be by as little as 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, or by 40%, 50%, 60%, or even by as much as 70%, 75%, 80%, 90%, or 100%.

As used herein, the term “sample” includes a biologic sample such as any tissue, cell, fluid, or other material derived from an organism.

By “reference” is meant a standard of comparison. For example, the phenyllactic acid and/or indole-3-lactic acid level present in a patient sample may be compared to the level of the compound(s) in a corresponding healthy cell or tissue or in a diseased cell or tissue (*e.g.*, a cell or tissue derived from a subject having ALD).

By “periodic” is meant at regular intervals. Periodic patient monitoring includes, for example, a schedule of tests that are administered daily, bi-weekly, bi-monthly, monthly, bi-annually, or annually.

As used herein, the terms “determining”, “assessing”, “assaying”, “measuring” and “detecting” refer to both quantitative and qualitative determinations, and as such, the term “determining” is used interchangeably herein with “assaying,” “measuring,” and the like. Where a quantitative determination is intended, the phrase “determining an amount” of an analyte and the like is used. Where a qualitative and/or quantitative determination is intended, the phrase “determining a level” of an analyte or “detecting” an analyte is used.

Unless specifically stated or obvious from context, as used herein, the term “about” is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

Any compounds, compositions, or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

DESCRIPTION OF THE DRAWINGS

Figures 1A-1D show the histological and biochemical properties of mice after alcohol administration. Figure 1A includes the 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). Figures 1B-1D includes graphs showing the alanine aminotransferase (ALT) (Figure 1B), aspartate aminotransferase (Figure 1C), and triglyceride (Figure 1D) levels in the serum of control (filled triangles) and alcohol-treated (filled diamonds) wild-type mice on the B6 background after one month of alcohol consumption. The corresponding levels in their *Ppara*-null counterparts are represented by empty triangles and diamonds, respectively.

Figures 2A-2F show biochemical and multivariate data analysis results of mice after alcohol administration. Figure 2A includes a graph showing the liver triglyceride levels in control (filled triangles) and alcohol-treated (filled diamonds) wild-type mice on the B6 background after one month. The triglyceride levels in their *Ppara*-null counterparts are represented by empty triangles and diamonds, respectively. Figure 2B includes a scatter plot from unsupervised Principal components analysis (PCA) of the urinary metabolomic signature showing segregation of metabolic fingerprints of control (solid diamonds) and alcohol-treated (empty diamonds) *Ppara*-null mice on the B6 background at two months. Figure 2C includes a scatter plot from PCA of the urinary metabolomic signature showing distinct metabolic phenotypes (metabotype) associated with chronic alcohol treatment of wild-type and *Ppara*-null mice on the B6 background 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. Figure 2D includes a PCA scores scatter plot showing distinct metabotype associated with the mice on the B6 (boxes) and 129S (squares) background throughout the duration of the study irrespective of alcohol exposure status. Figure 2E includes a PCA scores scatter plot illustrating the underlying genetic background-related differences in metabolic traits of the alcohol-treated *Ppara*-null mice on the B6 or 129S background at 6 months. Figure 2F includes a loadings S-plot from the supervised orthogonal projection to latent structures (OPLS) analysis of the metabolic signatures (at six months) for the selection of candidate markers of chronic alcohol exposure in *Ppara*-null mice on the B6 background. Each triangle represents an ion characterized by unique mass and retention time. The $p(\text{corr})[1]$ values represent the interclass difference and $w(1)$ values indicate the relative abundance of the ions. Ions shown in boxes represent the potential markers that showed consistent contribution to the interclass difference during alcohol exposure (see Table 2). Those in the upper right and lower left quadrants respectively represent ions with elevated and depleted abundance in the urine of the alcohol-treated B6 *Ppara*-null mice. Representative results from the positive electrospray ionization (ESI+) mode metabolomic analysis are shown here. Negative electrospray ionization (ESI-) mode also showed similar patterns (data not shown).

Figures 3A-3C show the results from metabolomic analysis of mice after alcohol administration. Figure 3A includes a PCA scores scatter plot showing the unsupervised segregation of the urinary metabolomes of the control (solid triangles) and alcohol-treated

(solid diamonds) B6 wild-type mice after two months of alcohol consumption. Figure 3B includes a PCA scores scatter plot showing underlying differences in the metabolic responses of the wild-type mice on the B6 (solid diamonds) and 129S (filled circles) background to chronic alcohol consumption after six months. Figure 3C includes a Loadings S-plot from the supervised orthogonal projection to latent structures (OPLS) analysis of the metabolic signatures (at six months) for the selection of candidate markers of chronic alcohol exposure in wild-type mice on B6 background. Those in the upper right and lower left quadrants respectively represent ions with elevated and depleted abundance in the urine of the alcohol-treated B6 wild-type mice. Representative results from the positive electrospray ionization (ESI+) mode metabolomic analysis are shown here. Negative electrospray ionization (ESI-) mode also showed similar patterns (data not shown).

Figures 4A-4F include graphs showing the effect of chronic alcohol consumption on tryptophan, phenylalanine, and alcohol metabolism in B6 mice. Variation in the number of putative metabolites, predicted by MassTRIX analysis, elevated in (Figure 4A) tryptophan metabolism detected in ESI+ mode and (Figure 4B) phenylalanine metabolism detected in ESI- mode, during alcohol treatment. The solid and dotted lines represent the response of the wild-type and *Ppara*-null mice, respectively. Variation in the urinary abundance of ethyl- β -D-glucuronide in the control (dotted line) and alcohol-treated (solid line) wild-type (Figure 4C) and *Ppara*-null mice (Figure 4D). Variation in the urinary excretion of *N*-acetylglycine in the control (dotted line) and alcohol-treated (solid line) wild-type (Figure 4E) and *Ppara*-null mice (Figure 4F). 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 within same background. #, significantly different ($P < 0.05$) from control mice of same genotype and *, significantly different ($P < 0.05$) from alcohol-treated *Ppara*-null mice.

Figures 5A-5F include graphs showing the effect of chronic alcohol consumption on *N*-acetylglycine and *N*-hexanoylglycine in 129S and B6 mice. Variation in the urinary excretion of *N*-acetylglycine in wild-type (Figure 5A) and *Ppara*-null mice on the 129S background (Figure 5B). Variation in the urinary excretion of *N*-hexanoylglycine in wild-type (Figure 5C) and *Ppara*-null mice on the B6 background (Figure 5D) as well as that in the wild-type (Figure 5E) and *Ppara*-null mice on the 129S background (Figure 5F). Solid

and dotted lines respectively represent the variation of the metabolite in control and alcohol diet-fed mice of the respective genotype and background. 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 within same background. ‡, significant difference ($P < 0.05$) between the wild-type and *Ppara*-null mice on control diet; *, significant difference ($P < 0.05$) between the wild-type and *Ppara*-null mice on alcohol diet; #, significantly different ($P < 0.05$) from control mice of same genotype.

Figures 6A-6D include graphs showing the genetic background-dependent changes in taurine excretion. Depletion of urinary taurine excretion in wild-type (Figure 6A) and *Ppara*-null mice on the B6 background (Figure 6B) in response to chronic alcohol consumption. Elevation in urinary taurine excretion in wild-type (Figure 6C) and *Ppara*-null mice on the 129S background (Figure 6D) in response to chronic alcohol consumption. Dotted and solid lines represent the variation in the urinary excretion of taurine in control and alcohol diet-fed mice of the respective genotypes and backgrounds. 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 within same background. ‡, significantly different ($P < 0.05$) from the *Ppara*-null mice of same treatment group; #, significantly different ($P < 0.05$) from control mice of same genotype and *, significantly different ($P < 0.05$) from alcohol-treated wild-type mice.

Figures 7A-7F include graphs showing the genetic background-dependent changes in the urinary excretion of aromatic amino acid metabolites. Lower urinary excretion of 2-hydroxyphenylacetic acid in wild-type (Figure 7A) and *Ppara*-null mice on the B6 background (Figure 7B). Variation in the urinary excretion of xanthurenic acid in wild-type (Figure 7C) and *Ppara*-null mice on the B6 background (Figure 7D) as well as that in the wild-type (Figure 7E) and *Ppara*-null mice on the 129S background (Figure 7F). Dotted and solid lines respectively represent the variation in the urinary excretion of the metabolite in control diet and alcoholic diet-treated mice of the respective genotype and background. 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 within same background. #, significantly different ($P < 0.05$) from control mice of same genotype.

Figures 8A-8D include graphs showing the genetic background-dependent changes in 4-hydroxyphenylacetic acid excretion. Variation in the urinary excretion of 4-hydroxyphenylacetic acid in wild-type (Figure 8A) and *Ppara*-null mice on the B6 background (Figure 8B). Variation in the urinary abundance of 4-hydroxyphenylacetic acid sulfate in wild-type (Figure 8C) and *Ppara*-null mice on the B6 background (Figure 8D). Solid and dotted lines, respectively, represent the variation of the metabolite in control and alcohol diet-fed mice of the respective genotype and background. 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 within same background. ‡, significant difference ($P < 0.05$) between the wild-type and *Ppara*-null mice on control diet; * significant difference ($P < 0.05$) between the wild-type and *Ppara*-null mice on alcohol diet; #, significantly different ($P < 0.05$) from control mice of same genotype.

Figures 9A-9F include graphs showing the genetic background-dependent changes in pimelic acid and suberic acid excretion. Variation urinary excretion of pimelic acid in wild-type (Figure 9A) and *Ppara*-null mice on the B6 background (Figure 9B). Variation in the excretion of suberic acid in wild-type (Figure 9C) and *Ppara*-null mice on the B6 background (Figure 9D) as well as that in the wild-type (Figure 9E) and *Ppara*-null mice on the 129S background (Figure 9F). Solid and dotted lines respectively represent the variation of the metabolite in control and alcohol diet-fed mice of the respective genotype and background. 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 within same background. ‡, significant difference ($P < 0.05$) between the wild-type and *Ppara*-null mice on control diet; * significant difference ($P < 0.05$) between the wild-type and *Ppara*-null mice on alcohol diet; #, significantly different ($P < 0.05$) from control mice of same genotype.

Figures 10A-10F include graphs showing the genetic background-independent elevation of phenylalanine and tryptophan metabolites in mouse model of ALD. Variation in the urinary excretion of indole-3-lactic acid in wild-type (Figure 10A) and *Ppara*-null mice on the B6 background (Figure 10B). Variation in the urinary excretion of phenyllactic acid in wild-type (Figure 10C) and *Ppara*-null mice on the B6 background (Figure 10D) as well as that in the wild-type (Figure 10E) and *Ppara*-null mice on the 129S background (Figure 10F). Dotted and solid lines respectively represent the variation in the urinary excretion of the

metabolite in control diet and alcoholic diet-treated mice of the respective genotype and background. 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 within same background. ‡, significantly different ($P < 0.05$) from the *Ppara*-null mice of same treatment group; #, significantly different ($P < 0.05$) from control mice of same genotype and *, significantly different ($P < 0.05$) from alcohol-treated wild-type mice.

Figures 11A-11D include graphs showing the genetic background-dependent changes in tryptophan and 3-indolepyruvic acid excretion. Variation urinary excretion of tryptophan and 3-indolepyruvic acid in wild-type (Figures 11A and 11C) and *Ppara*-null mice on B6 background (Figures 11B and 11D). Solid and dotted lines respectively represent the variation of the metabolite in control and alcohol diet-fed mice of the respective genotype and background. * significant difference ($P < 0.05$) between the wild-type and *Ppara*-null mice on the alcohol diet; #, significantly different ($P < 0.05$) from control mice of same genotype.

Figures 12A-12D include graphs showing the genetic background-dependent changes in tryptophan and 3-indolepyruvic acid excretion. Variation in the excretion of phenylalanine in wild-type (Figure 12A) and *Ppara*-null mice on B6 background (Figure 12B) as well as that in the wild-type (Figure 12C) and *Ppara*-null mice on 129S background (Figure 12D). Solid and dotted lines respectively represent the variation of the metabolite in control and alcohol diet-fed mice of the respective genotype and background. * significant difference ($P < 0.05$) between the wild-type and *Ppara*-null mice on alcohol diet; #, significantly different ($P < 0.05$) from control mice of same genotype.

Figure 13 is a proposed schematic of the biochemical mechanism behind elevation of genetic background-independent biomarkers of alcoholic liver disease in the alcohol-treated *Ppara*-null mice. Consumption of NAD^+ for the oxidation of alcohol along with concomitant impairment of NAD^+ biosynthesis leads in a marked shift in the redox balance and increase in NADH/NAD^+ ratio. This results in significant impairment of fatty acid β -oxidation in *Ppara*-null mice leading to steatosis. The increase in aspartate aminotransferase activity (EC 2.6.1.1) associated with concurrent liver damage may help the deamination of phenylalanine and tryptophan to produce α -keto acids; phenylpyruvic acid and indole-3-pyruvic acid, respectively. The increase in NADH/NAD^+ ratio subsequently drives the reduction α -keto

acid intermediates to the corresponding α -hydroxy acids, *i.e.*, phenyllactic acid and indole-3-lactic acid.

DETAILED DESCRIPTION OF THE INVENTION

This invention is based, at least in part, on the discovery that phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof are biomarkers for alcoholic liver disease (ALD), including steatosis. Accordingly, the invention provides for methods and kits that are useful in the diagnosis, treatment, and prevention of ALD, as well as for characterizing ALD to determine subject prognosis and aid in treatment selection. The invention further provides methods and kits for monitoring a patient identified as having ALD.

The nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR α) is a key regulator of the genes involved in lipid metabolism (Martin *et al.*, *Hepatology* 45:767-77 (2007); and Rakhshandehroo *et al.*, *PPAR Res* 2007:26839 (2007)). Earlier, chronic alcohol treatment of the peroxisome proliferator-activated receptor alpha knockout (*Ppara*-null) mouse has been shown to result in development of liver pathology remarkably similar to the early stages of the human ALD while the wild-type animals remained protected from such liver damage (Nakajima *et al.*, *Hepatology* 40:972-80 (2004)). Recently, mass spectrometry-based metabolomic analysis has revealed changes in the urinary excretion of metabolites in the alcohol-fed *Ppara*-null mice on the 129/SvJ (129S) background (Manna *et al.*, *J. Proteome Res.* 9:4176-88 (2010)). The use of ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS) platform in identification of these metabolites makes them amenable to high-throughput analysis. In addition, several other studies have also demonstrated that global analysis using mass spectrometry (Loftus *et al.*, *J Proteome Res.* 10:705-13 (2011); and Bradford *et al.*, *Toxicol. Appl. Pharmacol.* 232:236-43 (2008)) as well as NMR (Fernando *et al.*, *Alcohol Clin. Exp. Res.* 34 :1937-47 (2010)) can aid in identification of changes in metabolic profile associated with alcohol exposure and liver damage. While these metabolites hold potential to serve as high-throughput noninvasive biomarkers for the early stage of the disease, their dependence on genetic background has yet to be tested. Earlier studies have shown that the difference in genetic background of the 129S and C57BL/6 (B6) mice results

in considerable differences between them with respect to intrinsic physiological functions (Nguyen *et al.*, *Learn Mem.* 7:170-9 (2000)) as well as the biochemical response and outcome of xenobiotic insults (Syn *et al.*, *Liver Int.* 29:1262-72 (2009); and Liu *et al.*, *Toxicol. Appl. Pharmacol.* 176:1-9 (2001)). Since the metabolome is the final outcome of intertwined interactions between genes, transcripts, proteins, and metabolites, it is highly susceptible to variation in genetic background. However, etiologically and pathologically identical diseases may have some conserved metabolic fingerprint that result from the underlying molecular events. As described in detail below, the urinary metabolomic changes in wild-type and *Ppara*-null mice of B6 and 129S background were investigated in order to identify genetic background-independent conserved ALD biomarkers. A combination of UPLC-ESI-QTOFMS and chemometrics showed that although there were considerable differences in the metabolomic responses of the B6 and 129S mice to chronic alcohol exposure, ALD pathogenesis was associated with conserved changes in the urinary metabolome.

Reversed-phase gradient UPLC-ESI-QTOF-MS analysis revealed that urinary excretion of a number of metabolites, such as, ethylsulfate, 4-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid sulfate, adipic acid, pimelic acid, xanthurenic acid, and taurine were background-dependent. Elevation of ethyl- β -D-glucuronide and *N*-acetylglycine was found to be common signature of the metabolomic response to alcohol exposure in wild-type as well as in *Ppara*-null mice of both strains. However, increased excretion of indole-3-lactic acid and phenyllactic acid was found to be a conserved feature exclusively associated with the alcohol-treated *Ppara*-null mouse on both backgrounds that develop liver pathologies similar to the early stages of human ALD. These markers reflected the biochemical events associated with early stages of ALD pathogenesis. The results identify indole-3-lactic acid and phenyllactic acid as conserved and pathology-specific high-throughput noninvasive biomarkers for early stages of ALD.

Additional conserved molecules associated with alcohol-treated *Ppara*-null mouse on both backgrounds included markers N8, N9, N23, P7, and P11.

Diagnostics and Diagnostic Assays

The present invention features diagnostic assays for the detection of alcoholic liver disease (ALD), including steatosis. In embodiments, the level of a biomarker(s) is measured in a subject sample and used to characterize ALD or the propensity to develop ALD. In embodiments, the biomarker is phenyllactic acid; phenyllactic acid in combination with indole-3-lactic acid; N8, N9, N23, P7, P11, or a combination thereof.

Biological samples include tissue samples (*e.g.*, cell samples, biopsy samples, and the like) and bodily fluids, including, but not limited to, blood, blood serum, plasma, saliva, and urine. Samples can optionally be treated to enrich for the biomarker(s) using enrichment and separation methods well known in the art.

Elevated levels of the biomarker(s) are considered a positive indicator of ALD. In general, an increase in the levels of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof is indicative of ALD or the propensity to develop ALD. The increase in biomarker levels may be by at least about 10%, 25%, 50%, 75%, 90% or more. The increase in biomarker levels may be by at least about 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, 14, 14.5, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95-fold or more.

In embodiments, multiple biomarkers are measured (*e.g.*, one or more of phenyllactic acid; phenyllactic acid in combination with indole-3-lactic acid; N8, N9, N23, P7, P11; or one or more of i) phenyllactic acid; phenyllactic acid in combination with indole-3-lactic acid; N8, N9, N23, P7, and P11, and ii) one or more additional ALD biomarkers that are known in the art). The use of multiple biomarkers increases the predictive value of the test and provides greater utility in diagnosis, toxicology, patient stratification and patient monitoring. The process called “Pattern recognition” detects the patterns formed by multiple biomarkers greatly improves the sensitivity and specificity of the diagnostic assay for predictive medicine. Subtle variations in data from clinical samples indicate that certain patterns of biomarkers can predict phenotypes such as the presence or absence of a certain disease, a particular stage of disease-progression, or a positive or adverse response to drug treatments.

Detection of an alteration relative to a reference sample (*e.g.*, normal sample) can be used as a diagnostic indicator of ALD or steatosis.

In embodiments, the invention provides methods for identifying a subject as having or having a propensity to develop ALD or steatosis. In related embodiments, the methods involve detecting the level of a biomarker selected from the group consisting of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, and P11 in a sample obtained from the subject. In embodiments, the methods involve comparing the level of the biomarker to a reference. In embodiments, the methods involve identifying the subject as having or having a propensity to develop ALD or steatosis when the level of the biomarker is increased relative to the reference.

In embodiments, the invention provides methods for identifying ALD or steatosis in a subject. In related embodiments, the methods involve detecting the level of a biomarker selected from the group consisting of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, and P11 in a sample obtained from the subject. In embodiments, the methods involve comparing the level of the biomarker to a reference. In embodiments, the methods involve identifying ALD or steatosis in the subject when the level of the biomarker is increased relative to the reference.

In embodiments, the invention provides methods for characterizing the stage of ALD in a subject. In related embodiments, the methods involve detecting the level of a biomarker selected from the group consisting of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, and P11 in a sample obtained from the subject. In embodiments, the methods involve comparing the level of the biomarker to a reference. In embodiments, the methods involve identifying the subject as having a later stage of ALD when there is an increase in the level of the biomarker relative to the reference.

In embodiments, the invention provides methods for determining the prognosis of ALD in a subject. In related embodiments, the methods involve detecting the level of a biomarker selected from the group consisting of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, and P11 in a sample obtained from the subject. In embodiments, the methods involve comparing the level of the biomarker to a reference. In embodiments, the methods involve identifying the subject as having a poor prognosis when there is an increase in the level of the biomarker relative to the reference.

In embodiments, the invention provides methods for characterizing the degree of lipid accumulation during the early stage of ALD in a subject. In related embodiments, the methods involve detecting the level of a biomarker selected from the group consisting of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, and P11 in a sample obtained from the subject. In embodiments, the methods involve comparing the level of the biomarker to a reference. In embodiments, the methods involve identifying the subject as having a higher level of lipid accumulation during the early stage of ALD when there is an increase in the level of the biomarker relative to the reference.

In embodiments, the invention provides methods for monitoring ALD therapy in a subject. In related embodiments, the methods involve detecting the level of a biomarker selected from the group consisting of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, and P11 in a sample obtained from the subject. In embodiments, the methods involve comparing the level of the biomarker to a reference. In embodiments, the methods involve identifying the therapy as effective when there is a decrease in the level of the biomarker relative to the reference.

In embodiments, the invention provides methods for detecting an agent's therapeutic efficacy in a subject having ALD. In related embodiments, the methods involve detecting an alteration in the level of a biomarker selected from the group consisting of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, and P11 in a sample obtained from the subject. In embodiments, the methods involve comparing the level of the biomarker to a reference (*e.g.*, a patient sample taken at an earlier time point or prior to treatment). In embodiments, the methods involve identifying the agent as having therapeutic efficacy in the subject when there is a decrease in the level. In embodiments, the methods involve identifying the agent as lacking therapeutic efficacy in the subject when there maintenance or increase in the level.

In embodiments, the level of the biomarker(s) is measured on at least two different occasions and an alteration in the levels as compared to normal reference levels over time is used as an indicator of ALD, including steatosis. The level of the biomarker(s) in a sample from a subject (*e.g.*, bodily fluids such as blood, blood serum, plasma, saliva, and urine) of a subject having ALD or the propensity to develop such a condition may be altered by as little as 10%, 20%, 30%, or 40%, or by as much as 50%, 60%, 70%, 80%, or 90% or more relative

to the level of such biomarker(s) in a normal control. In embodiments, a subject sample is collected prior to the onset of symptoms of ALD. In embodiments, a subject sample is collected after the onset of symptoms of ALD. In embodiments, a subject sample is collected while the subject is undergoing treatment for ALD.

The diagnostic methods described herein can be used individually or in combination with any other diagnostic method described herein or well known in the art for a more accurate diagnosis of the presence or severity of ALD.

The diagnostic methods described herein can also be used to monitor and manage ALD, including steatosis.

As indicated above, the invention provides methods for aiding an ALD diagnosis using phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof as specified herein. These biomarker(s) can be used alone, in combination with other biomarkers in any set, or with entirely different markers in aiding ALD diagnosis. The markers are differentially present in samples of an ALD patient and a normal subject in whom ALD is undetectable. Therefore, detection of one or more of these biomarkers in a person would provide useful information regarding the probability that the person may have ALD or regarding the stage of ALD progression.

The detection of the biomarker(s) is then correlated with a probable diagnosis of ALD. In embodiments, the detection of the mere presence of a biomarker, without quantifying the amount thereof, is useful and can be correlated with a probable diagnosis of ALD. The measurement of biomarkers may also involve quantifying the markers to correlate the detection of markers with a probable diagnosis of ALD. Thus, if the amount of the biomarkers detected in a subject being tested is different compared to a control amount (*e.g.*, higher than the control), then the subject being tested has a higher probability of having ALD.

The correlation may take into account the amount of the biomarker(s) in the sample compared to a control amount of biomarker(s) (*e.g.*, in normal subjects or in non-ALD subjects such as where ALD is undetectable). A control can be, *e.g.*, the average or median amount of the biomarker(s) present in comparable samples of normal subjects in normal subjects or in non-ALD subjects such as where ALD is undetectable. The control amount is measured under the same or substantially similar experimental conditions as in measuring the

test amount. As a result, the control can be employed as a reference standard, where the normal (non-ALD) phenotype is known, and each result can be compared to that standard (*e.g.*, a standardized curve for use), rather than re-running a control.

In some embodiments, the control is derived from the patient and provides a reference level of the patient prior to, during, or after treatment for ALD.

Accordingly, a biomarker profile may be obtained from a subject sample and compared to a reference biomarker profile obtained from a reference population, so that it is possible to classify the subject as belonging to or not belonging to the reference population. The correlation may take into account the presence or absence of the biomarkers in a test sample and the frequency of detection of the same biomarkers in a control. The correlation may take into account both of such factors to facilitate determination of ALD status.

In certain embodiments of the methods of qualifying ALD status, the methods further comprise managing subject treatment based on the status. The invention also provides for such methods where the biomarker(s) are measured again after subject management. In these cases, the methods are used to monitor the status of ALD, *e.g.*, response to ALD treatment, including improvement, maintenance, or progression of the disease.

A biomarker, individually, can be useful in aiding in the determination of ALD status. First, the selected biomarker is detected in a subject sample using well known methods, including, but not limited to, the methods described herein. Then, the result is compared with a control that distinguishes ALD status from non-ALD status. As is well understood in the art, the techniques can be adjusted to increase sensitivity or specificity of the diagnostic assay depending on the preference of the diagnostician.

While an individual biomarker is a useful diagnostic marker, in some instances, a combination of biomarkers provides greater predictive value than single markers alone. The detection of a plurality of biomarkers (or absence thereof, as the case may be) in a sample can increase the percentage of true positive and true negative diagnoses and decrease the percentage of false positive or false negative diagnoses. Thus, in embodiments, methods of the present invention comprise the measurement of more than one biomarker.

Detection of Biomarkers

Any suitable method can be used to detect the biomarker(s). Successful practice of the invention can be achieved with one or a combination of methods that can detect and, in embodiments, quantify the biomarker(s).

Detection of the biomarkers can be conducted in the same or different samples, the same or separate assays, and may be conducted in the same or different reaction mixtures. Where the biomarkers are assayed in different samples, the samples are usually obtained from the subject during the same procedure (*e.g.*, blood draw, urine collection, tissue extraction, and the like) or with only a relative short time intervening so as to avoid an incorrect result due to passage of time. Where the biomarkers are detected in separate assays, the samples assayed are can be derived from the same or different samples obtained from the subject to be tested.

Phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof can be detected using one or more methods well known in the art, including, without limit, mass spectrometry, chromatography, spectroscopy (*e.g.*, NMR), elemental analysis, conventional chemical methods, immunoassays, and the like.

In embodiments, the biomarker(s) are detected using mass spectrometry. Mass spectrometry-based methods exploit the differences in mass of biomarkers to facilitate detection. Mass spectrometry can be combined with other assays, *e.g.*, resolving the analyte in a sample by one or two passes through liquid or gas chromatography followed by mass spectrometry analysis. Methods for preparing a biological sample for analysis by mass spectrometry are well known in the art. Suitable mass spectrometers for use include, without limit, electrospray ionization mass spectrometry (ESI-MS), ESIMS/MS, ESI-MS/(MS)_n (*n* is an integer greater than zero), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), electron impact ionization mass spectrometry (EI-MS), chemical ionization mass spectrometry (CI-MS), surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), desorption/ionization on silicon (DIOS), secondary ion mass spectrometry (SIMS), quadrupole time-of-flight (Q-TOF), atmospheric pressure chemical ionization mass spectrometry (APCI-MS), APCI-MS/MS, APCI(MS)₁₁, atmospheric pressure

photoionization mass spectrometry (APPI-MS), APPI-MS/MS, APPI-(MS), quadrupole, fourier transform mass spectrometry (FTMS), ion trap, and hybrids of these methods, *e.g.*, electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS) and two-dimensional gas chromatography electron impact ionization mass spectrometry (GCxGC-EI-MS).

The methods may be performed in an automated (Villanueva, *et al.*, *Nature Protocols* (2006) 1(2):880-891) or semi-automated format. This can be accomplished, for example with MS operably linked to a liquid chromatography device (LC-MS/MS or LC-MS) or gas chromatography device (GC-MS or GC-MS/MS). Methods for performing MS are known in the field and have been disclosed, for example, in US Patent Application Publication Nos: 20050023454 and 20050035286; US Patent No. 5,800,979; and the references disclosed therein.

Samples are collected on a collection layer. They may then be analyzed by a spectroscopic method based on matrix-assisted laser desorption/ionization (MALDI), electrospray ionization (ESI), and the like.

Other techniques for improving the mass accuracy and sensitivity of the MALDI-TOF MS can be used to analyze the analytes obtained on the collection membrane. These include the use of delayed ion extraction, energy reflectors and ion-trap modules. In addition, post source decay and MS--MS analysis are useful to provide further structural analysis. With ESI, the sample is in the liquid phase and the analysis can be by ion-trap, TOF, single quadrupole or multi-quadrupole mass spectrometers. The use of such devices (other than a single quadrupole) allows MS--MS or MSⁿ analysis to be performed. Tandem mass spectrometry allows multiple reactions to be monitored at the same time.

Capillary infusion may be employed to introduce the marker to a desired MS implementation, for instance, because it can efficiently introduce small quantities of a sample into a mass spectrometer without destroying the vacuum. Capillary columns are routinely used to interface the ionization source of a MS with other separation techniques including gas chromatography (GC) and liquid chromatography (LC). GC and LC can serve to separate a solution into its different components prior to mass analysis. Such techniques are readily combined with MS, for instance. One variation of the technique is that high performance

liquid chromatography (HPLC) can now be directly coupled to mass spectrometer for integrated sample separation/and mass spectrometer analysis.

Quadrupole mass analyzers may also be employed as needed to practice the invention. Fourier-transform ion cyclotron resonance (FTMS) can also be used for some invention embodiments. It offers high resolution and the ability of tandem MS experiments. FTMS is based on the principle of a charged particle orbiting in the presence of a magnetic field. Coupled to ESI and MALDI, FTMS offers high accuracy with errors as low as 0.001%.

In embodiments, the diagnostic methods of the invention may further comprise identifying significant peaks from combined spectra. The methods may also further comprise searching for outlier spectra. In other embodiments, the methods of the invention further comprise determining distant dependent K-nearest neighbors.

In embodiments, an ion mobility spectrometer can be used to detect and characterize the biomarker(s). The principle of ion mobility spectrometry is based on different mobility of ions. Specifically, ions of a sample produced by ionization move at different rates, due to their difference in, *e.g.*, mass, charge, or shape, through a tube under the influence of an electric field. The ions (typically in the form of a current) are registered at the detector which can then be used to identify a biomarker or other substances in a sample. One advantage of ion mobility spectrometry is that it can operate at atmospheric pressure.

In embodiments, the procedure is electrospray ionization quadrupole mass spectrometry with time of flight (TOF) analysis, known as UPLC-ESI-QTOFMS. UPLC-ESI-QTOFMS is well known in the art (see, *e.g.*, Manna *et al.*, *J. Proteome Res.* 9:4176-88 (2010)) and methods for using UPLC-ESI-QTOFMS are described in detail herein.

In embodiments, detection of the biomarker(s) involves chemical methods well known in the art. In embodiments, the chemical method is chemical extraction. In embodiments, the chemical method is chemical derivitization.

In embodiments, detection of the biomarker(s) involves use of chromatography methods that are well known in the art. Such chromatography methods include, without limit, column chromatography, ion exchange chromatography, hydrophobic (reverse phase)

liquid chromatography, or other chromatography, such as thinlayer, gas, or liquid chromatography (*e.g.*, high-performance liquid chromatography), or any combination thereof.

In embodiments, detection of the biomarker(s) involves use of spectroscopy methods that are well known in the art. Such chromatography methods include, without limit, NMR, IR, and the like.

In embodiments, detection of the biomarker(s) involves elemental analysis methods that are well known in the art. Such elemental analysis methods include, without limit, combustion analysis, gravimetry, atomic spectroscopy, and the like.

In embodiments, detection of the biomarker(s) involves use of immunoassays. In embodiments, the immunoassays involve the use of antibodies. Suitable immunoassays include, without limit, ELISA, flow chamber adhesion, colorimetric assays (*e.g.*, antibody based colorimetric assays), biochip (*e.g.*, antibody based biochip), and the like.

Analytes (*e.g.*, biomarkers) can be detected by a variety of detection methods selected from, for example, a gas phase ion spectrometry method, an optical method, an electrochemical method, atomic force microscopy and a radio frequency method. In one embodiment, mass spectrometry, and in particular, SELDI, is used. Optical methods include, for example, detection of fluorescence, luminescence, chemiluminescence, absorbance, reflectance, transmittance, birefringence or refractive index (*e.g.*, surface plasmon resonance, ellipsometry, a resonant mirror method, a grating coupler waveguide method or interferometry). Optical methods include microscopy (both confocal and non-confocal), imaging methods and non-imaging methods. Immunoassays in various formats (*e.g.*, ELISA) are popular methods for detection of analytes captured on a solid phase. Electrochemical methods include voltametry and amperometry methods. Radio frequency methods include multipolar resonance spectroscopy.

Other variations of the assays described herein to provide for different assay formats for detection of the biomarker(s) will be readily apparent to the one of ordinary skill in the art upon reading the present disclosure.

Diagnostic Kits

The invention provides kits for diagnosing or monitoring ALD (including steatosis), or for selecting a treatment for ALD.

In embodiments, the kits include one or more reagents capable of detecting and/or capturing phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof. In related embodiments, the reagent is an antibody or a mass spectrometry probe.

In embodiments, the kits include an adsorbent that retains phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof. In related embodiments, the kit further contains directions for contacting a test sample with the adsorbent and detecting phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof retained by the adsorbent.

In embodiments, the reagents and/or adsorbents are provided on a solid support (*e.g.*, chip, microtiter plate, bead, resin, and the like).

In embodiments, the kits include washing solution(s) or instructions for making a washing solution, in which the combination of the reagent/adsorbent and the washing solution allows capture of the biomarkers on the reagent/adsorbent.

In embodiments, the kits include phenyllactic acid, indole-3-lactic acid, N8, N9, N23, P7, and/or P11 samples, which can be used as standard(s) for calibration as may be desired.

In embodiments, the kit contains a container(s) that houses the components of the kit (*e.g.*, reagent, adsorbent, solid support, and the like). Such containers can be boxes, ampoules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable container forms known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, and the like.

In embodiments, the kits further contain directions for using the kit in any of the methods described herein (*e.g.*, diagnosing ALD, monitoring ALD, characterizing ALD, selecting a treatment for ALD, and the like). In embodiments, the instructions include at least

one of the following: description of the reagents, supports, and/or adsorbents; warnings; indications; counter-indications; animal study data; clinical study data; and/or references. The instructions may be printed directly on the container (when present), or as a label applied to the container, or as a separate sheet, pamphlet, card, or folder supplied in or with the container.

Subject Monitoring

The disease state or treatment of a subject having ALD (including steatosis), or a propensity to develop such a condition can be monitored using the methods and biomarkers of the invention. In embodiments, methods and biomarkers of the invention are used by a clinician to identify subjects as having or not having ALD. For example, a general practitioner may use the methods delineated herein to screen patients for the presence of ALD. In embodiments, the expression of biomarker(s) present in a patient sample, *e.g.*, bodily fluid such as blood, blood serum, plasma, saliva, and urine, is monitored. Such monitoring may be useful, for example, in assessing the efficacy of a particular drug in a subject or in assessing disease progression. Therapeutics that decrease the expression of a biomarker of the invention (*e.g.*, phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof) are taken as particularly useful in the invention.

In embodiments, the biomarker(s) are monitored prior to administering therapy. These results provide a baseline that describes the level of the biomarker(s) prior to treatment.

In embodiments, the biomarker(s) are monitored periodically. In embodiments, the biomarker(s) are monitored periodically throughout treatment. A therapy is identified as efficacious when a diagnostic assay of the invention detects a decrease in marker levels relative to the baseline level of marker prior to treatment.

Types of Biological Samples

The level of phenyllactic acid, phenyllactic acid in combination with indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof is measured in different types of samples. In embodiment, the level of the biomarker(s) is measured in a biologic sample. Suitable biologic samples include, without limit, a tissue sample (*e.g.*, from a biopsy) and biological fluids (*e.g.*, blood, blood serum, plasma, saliva, urine, or any other biological fluid useful in the methods of the invention). In embodiments, the sample is a urine sample derived from the patient.

EXAMPLES

It should be appreciated that the invention should not be construed to be limited to the examples that are now described; rather, the invention should be construed to include any and all applications provided herein and all equivalent variations within the skill of the ordinary artisan.

Example 1: Histology and Biochemistry

Liver histology (see Figure 1A) showed an increase in steatosis in livers of the alcohol-treated B6 *Ppara*-null mice compared to their counterparts on control diet, whereas no such change was observed in the wild-type mice. Subsequent triglyceride measurements (Figure 2A) showed a clear although not statistically significant trend indicating increase in fat deposition in livers of B6 *Ppara*-null mice after one month of alcohol treatment. No such increase was observed in alcohol-fed wild-type mice on B6 background. In addition, there were no significant changes in the serum ALT, AST, and triglyceride levels of these mice after one month of alcohol treatment (see Figure 1A-1D). These observations were similar to those reported earlier for alcohol treatment of wild-type and *Ppara*-null mice on the 129S background (Nakajima *et al.*, *Hepatology* 40:972-80 (2004); and Manna *et al.*, *J. Proteome Res.* 9:4176-88 (2010)).

Example 2: Metabolomic Analysis

Unsupervised principal components analysis (PCA) of the metabolic signature showed that alcohol exposed wild-type and *Ppara*-null mice generated distinct metabolotypes (metabolic phenotypes). The urinary metabolome of control and alcohol-treated *Ppara*-null (Figure 2B) as well as wild-type (Figure 3A) mice on the B6 background were found to segregate well after two months. Metabolomic analysis of B6 mice after six months of alcohol treatment also showed clear segregation of the mice according to their genotype and alcohol exposure status indicating distinct metabolotypes associated with alcohol treatment of the wild-type and *Ppara*-null mice (Figure 2C). However, the metabolic fingerprints of the B6 and 129S mice were different irrespective of their genotype and alcohol exposure status (Figure 2D). After six months of alcohol treatment, both wild-type and *Ppara*-null mice on the B6 background had a different metabolotype than their 129S counterparts (Figure 2E, Figure 3C). Nevertheless, during the course of alcohol treatment, the urinary metabolome of mice from both backgrounds showed a shift in the same direction indicating some underlying similarity in the metabolic response to alcohol exposure (Figure 2D, arrow).

The supervised OPLS model was used to identify metabolic changes associated with alcohol exposure and to explore background-independent responses. A summary of the results are provided in Tables 1-4 below.

Table 1: List of Markers Ions for Chronic Alcohol exposure in the wild-type mice on B6 background.

Marker	RT ^b	m/z	w(1)	p(corr)[1]	Trend	129S	Identity
N1 ^a	0.6	221.066	0.18	0.79	↑	Y ^c	Ethyl-β-D-glucuronide
N1a	0.6	203.056	0.04	0.82	↑	Y	(N1-H ₂ O)
N1b	0.6	341.017	0.06	0.87	↑	Y	(N1+2HCO ₂ H+CO)
N2	0.9	116.035	0.02	0.81	↑	Y	N-acetylglutamine
N4	0.4	187.073	0.03	0.93	↑	Y	
N5	1.4	235.081	0.04	0.91	↑	Y	
N6	0.9	145.05	0.06	0.90	↑	Y	
N7	1.5	142.05	0.03	0.91	↑	Y	
N8	1.6	186.077	0.03	0.86	↑	Y	
N9	0.4	166.018	0.17	0.95	↑	Y	
N10	1.3	209.006	-0.04	-0.99	↓	Y	
N11	1.3	141.019	-0.06	-0.99	↓	Y	
N12	1.4	168.03	-0.03	-0.98	↓	Y	
N13	1.4	198.04	-0.07	-0.97	↓	Y	
N14	0.7	133.05	-0.03	-0.96	↓	Y	

N15	5.0	172.088	-0.06	-0.95	↓	Y	
N16	0.3	196.03	-0.05	-0.90	↓	Y	
N18	2.3	188.035	-0.02	-0.84	↓	Y	
N19	0.3	135.03	-0.06	-0.93	↓	Y	
N19	3.5	172.097	0.15	0.79	↑	N	N-hexanoylglycine
N20	1.5	142.05	-0.03	-0.91	↓	N	
N21	2.3	279.1	0.04	0.90	↑	Op ^d	
N22	0.3	124.007	-0.13	-0.96	↓	Op	Taurine
P1	0.6	245.063	0.10	0.88	↑	Y	Ethyl-β-D-glucuronide + Na ⁺
P1a	0.6	199.023	0.06	0.88	↑	Y	(P1-HCO ₂ H)
P1b	0.6	181.013	0.02	0.76	↑	Y	(P1-HCO ₂ H-H ₂ O)
P1c	0.6	159.03	0.04	0.95	↑	Y	(P1-HCO ₂ H-H ₂ O-Na ⁺ +H ⁺)
P3	0.5	162.06	0.04	0.70	↑	Y	
P4	1.4	272.112	0.03	0.81	↑	Y	
P5	2.5	122.028	0.03	0.93	↑	Y	
P6	2.5	272.094	0.04	0.94	↑	Y	
P7	1.5	188.092	0.03	0.86	↑	Y	
P8	2.8	232.029	-0.03	-0.92	↓	Y	
P9	0.3	176.102	-0.04	-0.99	↓	Y	
P10	1.4	182.045	-0.03	-0.97	↓	Y	
P12	2.8	137.03	-0.04	-0.95	↓	N	
P13	2.8	307.062	-0.02	-0.74	↓	N	
P14	2.5	188.075	-0.09	-0.87	↓	N	
P15	4.7	340.258	-0.10	-0.92	↓	N	
P16	4.1	185.115	-0.08	-0.96	↓	N	
P17	4.0	475.348	-0.09	-0.88	↓	N	
P18	0.3	133.062	-0.06	-0.94	↓	N	
P19	0.3	104.107	-0.06	-0.90	↓	N	
P20	0.8	175.049	-0.09	-0.86	↓	N	
P21	2.0	206.045	0.11	0.80	↑	N	Xanthurenic acid

^a 'N' indicates marker detected in negative mode and 'P' indicates marker detected in positive mode.

^b RT (retention time) in minutes.

^c 'Y' indicates marker also detected in 129S mice and 'N' no significant change in 129S mice.

^d trend in 129S mice is opposite to that observed in B6 mice.

Table 2: List of Markers Ions for Chronic Alcohol exposure in the Ppara-null mice on B6 background.

Marker	RT _b	m/z	w(1)	p(corr)[1]	Trend	129S	Identity
N1 ^a	0.6	221.066	0.16	0.83	↑	Y ^c	Ethyl-β-D-glucuronide
N1a	0.6	203.056	0.03	0.86	↑	Y	(N1-H ₂ O)
N1b	0.6	341.017	0.06	0.89	↑	Y	(N1+2HCO ₂ H+CO)
N2	0.9	116.035	0.03	0.91	↑	Y	N-acetylglycine

N3	3.7	204.066	0.09	0.91	↑	Y	Indole-3-lactic acid
N5	1.4	235.081	0.04	0.69	↑	Y	
N6	0.9	145.05	0.07	0.94	↑	Y	
N8	1.6	186.077	0.05	0.87	↑	Y	
N9	0.4	166.018	0.22	0.86	↑	Y	
N10	1.3	209.006	-0.03	-0.87	↓	Y	
N11	1.3	141.019	-0.05	-0.90	↓	Y	
N12	1.4	168.03	-0.03	-0.87	↓	Y	
N13	1.4	198.04	-0.06	-0.93	↓	Y	
N15	5.0	172.088	-0.06	-0.95	↓	Y	
N16	0.3	196.03	-0.06	-0.80	↓	Y	
N17	3.3	165.055	0.06	0.83	↑	Y	Phenyllactic acid
N23	1.1	142.05	0.12	0.83	↑	Y	
N21	2.3	279.1	0.04	0.92	↑	Op ^d	
N22	0.3	124.007	-0.10	-0.82	↓	Op	Taurine
N24	2.9	260.054	-0.05	-0.89	↓	N	
N25	4.1	216.124	-0.12	-0.98	↓	N	
N26	3.2	170.082	-0.13	-0.97	↓	N	
N27	3.2	126.091	-0.04	-0.96	↓	N	N26-CO2
N28	2.0	188.092	0.15	0.95	↑	N	
P1	0.6	245.063	0.10	0.92	↑	Y	Ethyl-β-D-glucuronide + Na ⁺
P1a	0.6	199.023	0.05	0.86	↑	Y	(P1-HCO ₂ H)
P1b	0.6	181.013	0.02	0.86	↑	Y	(P1-HCO ₂ H-H ₂ O)
P1c	0.6	159.03	0.04	0.93	↑	Y	(P1-HCO ₂ H-H ₂ O-Na ⁺ +H ⁺)
P2	3.7	206.081	0.06	0.95	↑	Y	Indole-3-lactic acid
P2a	3.7	188.071	0.03	0.94	↑	Y	P2-H ₂ O
P2b	3.7	160.076	0.03	0.94	↑	Y	P2-HCO ₂ H
P3	0.5	162.06	0.04	0.95	↑	Y	
P4	1.4	272.112	0.04	0.98	↑	Y	
P5	2.5	122.028	0.03	0.94	↑	Y	
P6	2.5	272.094	0.04	0.93	↑	Y	
P7	1.5	188.092	0.05	0.88	↑	Y	
P8	2.8	232.029	-0.03	-0.92	↓	N	
P9	0.3	176.102	-0.03	-0.88	↓	Y	
P10	1.4	182.045	-0.03	-0.70	↓	Y	
P11	4.1	373.147	0.04	0.97	↑	Y	
P13	2.8	307.062	-0.04	-0.94	↓	N	
P14	2.5	188.075	-0.08	-0.92	↓	N	
P15	4.7	340.258	-0.08	-0.89	↓	N	
P16	4.1	185.115	-0.07	-0.90	↓	N	
P17	4.0	475.348	-0.09	-0.88	↓	N	
P21	2.0	206.045	0.13	0.76	↑	N	Xanthurenic acid
P22	1.9	126.091	0.03	0.97	↑	N	
P23	1.9	210.074	0.10	0.86	↑	N	
P24	4.7	359.231	-0.04	-0.95	↓	N	

P25	2.8	137.03	-0.04	-0.92	↓	N	
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^a 'N' indicates marker detected in negative mode and 'P' indicates marker detected in positive mode.

^b RT (retention time) in minutes.

^c 'Y' indicates marker also detected in 129S mice and 'N' no significant change in 129S mice.

^d Trend in 129S mice is opposite to that observed in B6 mice.

Table 3: List of markers contributing to the differences in the metabolic response of wild-type mice of B6 and 129S background to chronic alcohol exposure.

Marker ^a	RT ^b	m/z	B6 trend	129S trend	Identity
N19	3.5	172.097	↑	N ^c	N-hexanoylglycine
N20	1.5	142.05	↓	N	
N21	2.3	279.1	↑	↓	
N22	0.3	124.007	↓	↑	Taurine
P12	2.8	137.03	↓	N	
P13	2.8	307.062	↓	N	
P14	2.5	188.075	↓	N	
P15	4.7	340.258	↓	N	
P16	4.1	185.115	↓	N	
P17	4.0	475.348	↓	N	
P18	0.3	133.062	↓	N	
P19	0.3	104.107	↓	N	
P20	0.8	175.049	↓	N	
P21	2.0	206.045	↑	N	Xanthurenic acid
N29	2.5	151.04	N	↑	4-Hydroxyphenylacetic acid
N30	1.9	230.997	N	↑	4-Hydroxyphenylacetic acid sulfate
N30a	1.9	151.04	N	↑	(N4-SO ₃)
N31	3.0	151.04	N	↓	2-Hydroxyphenylacetic acid
N31a	3.0	107.049	N	↓	(N5 - CO ₂)
N32	1.9	145.05	N	↓	Adipic acid
N32a	1.9	101.06	N	↓	(N6 - CO ₂)
N32b	1.9	167.032	N	↓	(N6 + Na ⁺ - H ⁺)
N33	2.7	159.066	N	↓	Pimelic acid
N33a	2.7	181.048	N	↓	(N7 + Na ⁺ - H ⁺)
N34	2.1	204.03	N	↓	
N34a	2.1	158.082	N	↓	(N8 - HCOOH)
N34b	2.1	339.154	N	↓	(2N8a + Na ⁺)
N35	5.0	201.113	N	↓	
N36a	5.0	223.095	N	↓	(N9 + Na ⁺ - H ⁺)
N37	2	156.066	N	↓	
N38	5.1	291.144	N	↑	
N39	4.8	155.107	N	↓	

N39a	4.8	221.079	N	↓	(N13 + CO ₂ + Na ⁺ - H ⁺)
N40	2.2	276.054	N	↓	
N41	2.4	113.059	N	↓	
N42	3.2	191.032	N	↓	
N42a	3.2	127.076	N	↓	(N16 - HCOOH - H ₂ O)
N43	3.2	193.046	N	↓	
N44	3.4	231.077	N	↓	
N44a	3.4	253.059	N	↓	(N18 + Na ⁺ - H ⁺)
N45	0.4	240.018	N	↓	
N46	5.4	317.16	N	↑	
N47	1.0	196.028	N	↑	
N48	3.2	260.149	N	↑	
N49	4.6	277.129	N	↑	
N50	5.5	329.162	N	↑	
N51	1.6	158.081	N	↑	
N52	2.8	222.026	N	↓	
N53	0.4	124.992	N	↑	Ethyl sulfate
P27	2.0	144.084	N	↓	
P28	1.6	290.16	N	↓	
P29	3.9	220.064	N	↓	
P29a	3.9	145.99	N	↓	(P5 - HCOOH - CO)
P30	0.3	198.088	N	↑	
P31	3.8	240.158	N	↓	

^a 'N' indicates marker detected in negative mode and 'P' indicates marker detected in positive mode.

^b RT (retention time) in minutes.

^c 'Y' indicates marker also detected in 129S mice and 'N' no significant change in 129S mice.

Table 4: List of markers contributing to the differences in the metabolic response of *Ppara*-null mice of B6 and 129S background to chronic alcohol exposure.

Marker ^a	RT ^b	m/z	B6 trend	129S trend	Identity
N21	2.3	279.1	↑	↓	
N22	0.3	124.007	↓	↑	Taurine
N24	2.9	260.054	↓	N	
N25	4.1	216.124	↓	N	
N26	3.2	170.082	↓	N	
N27	3.2	126.091	↓	N	
N28	2.0	188.092	↑	N	
P8	2.8	232.029	↓	N	
P13	2.8	307.062	↓	N	
P14	2.5	188.075	↓	N	
P15	4.7	340.258	↓	N	
P16	4.1	185.115	↓	N	

P17	4.0	475.348	↓	N	
P21	2.0	206.045	↑	N	Xanthurenic acid
P22	1.9	126.091	↑	N	
P23	1.9	210.074	↑	N	
P24	4.7	359.231	↓	N	
P25	2.8	137.03	↓	N	
N29	2.5	151.04	N	↑	4-Hydroxyphenylacetic acid
N30	1.9	230.997	N	↑	4-Hydroxyphenylacetic acid sulfate
N30a	1.9	151.04	N	↑	(N4-SO ₃)
N31	3.0	151.04	N	↓	2-Hydroxyphenylacetic acid
N31a	3.0	107.049	N	↓	(N5 - CO ₂)
N32	1.9	145.05	N	↓	Adipic acid
N32a	1.9	101.06	N	↓	(N6 - CO ₂)
N32b	1.9	167.032	N	↓	(N6 + Na ⁺ - H ⁺)
N33	2.7	159.066	N	↓	Pimelic acid
N33a	2.7	181.048	N	↓	(N7 + Na ⁺ - H ⁺)
N36a	5.0	223.095	N	↓	(N9 + Na ⁺ - H ⁺)
N40	2.2	276.054	N	↓	
N41	2.4	113.059	N	↓	
N42	3.2	191.032	N	↓	
N42a	3.2	127.076	N	↓	(N16 - HCOOH - H ₂ O)
N43	3.2	193.046	N	↓	
N44	3.4	231.077	N	↓	
N44a	3.4	253.059	N	↓	(N18 + Na ⁺ - H ⁺)
N45	0.4	240.018	N	↑	
N46	5.4	317.16	N	↑	
N47	1.0	196.028	N	↑	
N48	3.2	260.149	N	↑	
N53	0.4	124.992	N	↑	Ethyl sulfate
P28	1.6	290.16	N	↓	
P29	3.9	220.064	N	↓	
P29a	3.9	145.991	N	↓	
P32	1.1	166.048	N	↑	
P33	0.4	172.062	N	↑	

^a 'N' indicates marker detected in negative mode and 'P' indicates marker detected in positive mode.

^b RT (retention time) in minutes.

^c 'Y' indicates marker also detected in 129S mice and 'N' no significant change in 129S mice.

The loadings S-plot for OPLS analysis of control and alcohol-treated *Ppara*-null (Figure 2F) and wild-type mice (Figure 3D) on the B6 background at six months were used to select ions that contributed significantly ($p(\text{corr})[1] < -0.8$ or $p(\text{corr})[1] > 0.8$) to the separation of the metabolome in response to alcohol treatment. The trend plots for time-dependent changes in the relative abundance of these ions were examined to screen only

those with consistent contribution to the separation of the metabolome during the course of the study. For example, ions P1 and P4 were elevated, while P14 and P16 were depleted in both *Ppara*-null (Figure 2F, Table 2) and wild-type mice (Figure 3C, Table 1) on the B6 background. Ions P2 and P23 were elevated, while P24 and P25 depleted (Figure 2F, Table 2) exclusively in *Ppara*-null mice on B6 background. These ions represent metabolic changes associated with ALD pathogenesis in alcohol-treated B6 *Ppara*-null mice. A list of ions involved in the metabolic response to alcohol exposure in wild-type and *Ppara*-null mice of B6 background is presented in Tables 1 and 2, respectively. These were compared to the list of ions contributing to the separation of the metabolome in the 129S mouse (Manna *et al.*) to detect potential biomarkers of alcohol exposure that are independent of genetic background. In keeping with the consistent differences in their overall metabolomic signature, a large number of markers present in the B6 mice were absent in 129S mice and vice versa (see Tables 3 and 4). However, ions such as P1, N1, and N2 were found to be markers of alcohol exposure in both wild-type and *Ppara*-null mice irrespective of their backgrounds. Table 1 and Table 2 list such ions that were detected and represent the background-independent metabolic signature of alcohol exposure in the wild-type and *Ppara*-null mice, respectively. Interestingly, ions such as P2 and N17 were found to be consistently elevated exclusively in the alcohol-treated *Ppara*-null mice irrespective of the background. These ions represent genetic background-independent metabolic responses associated with ALD pathogenesis.

Example 3: Metabolic Pathway Analysis

The ions contributing to the difference in the metabolic responses to alcohol exposure in wild-type and *Ppara*-null mice of B6 background were analyzed using MassTRIX in order to identify possible metabolic pathways affected by the alcohol treatment. The positive mode ionization data showed that metabolites related to tryptophan metabolism were upregulated following chronic alcohol exposure (Figure 4A). The number of potentially elevated tryptophan metabolites in *Ppara*-null mice gradually increased compared to that in the wild-type mice during the course of treatment. Analysis of the negative mode ionization data revealed that potential phenylalanine metabolites were also elevated in response to alcohol exposure in the wild-type mice (Figure 4B). However, while the number of elevated metabolites belonging to this pathway decreased with time in wild-type mice, the

corresponding number of metabolites increased in *Ppara*-null mice. It is noteworthy that these observations with respect to the difference between the wild-type and *Ppara*-null mice are similar to that found in mice of the 129S background (Manna *et al.*) and indicated that modulations in the tryptophan and phenylalanine metabolic pathway may bear some genetic background-independent signature of alcohol exposure in *Ppara*-null mice.

Example 4: Identification and Quantitation of Metabolites

Ion N1 was identified as the deprotonated form of ethyl- β -D-glucuronide ($C_8H_{14}O_7$) by comparison with an authentic standard. The urinary abundance of this metabolite in the control mice was negligible (Figures 4C and 4D). Similar to the 129S mice, the urinary abundance of the alcohol metabolite increased in alcohol-treated mice of the B6 background after 3 months of alcohol treatment. Unlike the 129S mice, there was no significant difference between alcohol-treated wild-type and *Ppara*-null mice of the B6 background with respect to excretion of this metabolite, except for a 5-fold higher abundance in the urine of *Ppara*-null mice compared to their wild-type counterparts at 3 months ($p < 0.0005$). However, unlike the 129S mice, the B6 mice did not show any elevation in the excretion of ethylsulfate on alcohol-treatment (data not shown).

The ion N2 was identified as *N*-acetylglycine by comparison with an authentic standard. This ion was elevated in both wild-type and *Ppara*-null mice of B6 as well as 129S background. The wild-type B6 mice showed a gradual elevation of this metabolite in the urine reaching 5-fold by four months ($p < 0.05$) in response to alcohol treatment (Figure 4E). The *Ppara*-null B6 mice showed a 2-fold elevation in the urinary excretion of this metabolite until four months and an increased to 3-fold by six months ($p < 0.005$) in response to alcohol treatment (Figure 4F). Urinary *N*-acetylglycine excretion in the wild-type 129S mice (Figure 5A) on control diet was negligible. The alcohol-treated wild-type 129S mice showed a 8-to-10-fold increase in the excretion of this metabolite starting from 2 months of alcohol treatment ($p < 0.05$) (Figure 5A). Alcohol-treated *Ppara*-null mice also showed increased excretion of this metabolite reaching a 15-fold elevation after five months ($p < 0.0005$) (Figure 5B). The excretion of this metabolite in the alcohol-treated *Ppara*-null mice of the 129S background was 12- and 7-fold higher after five ($p < 0.0005$) and six ($p < 0.005$) months of treatment, respectively, compared to their wild-type counterparts.

Comparison of retention time and fragmentation pattern of N19 with authentic standard identified the ion as *N*-hexanoylglycine. The urinary excretion of this metabolite in control B6 *Ppara*-null mice (Figure 5C) was 2-to-5-fold higher ($p < 0.05$) than the wild-type counterparts (Figure 5D). The alcohol-treated *Ppara*-null B6 mice also showed 3-to-5-fold higher ($p < 0.05$) urinary excretion of the metabolite than their wild-type counterparts. The urinary excretion of this metabolite was found to increase only marginally (not statistically significant) after alcohol treatment of wild-type mice (Figure 5C). However, alcohol-treated *Ppara*-null mice showed 1.5-fold elevation ($p < 0.05$) of the metabolite at four and five months compared to the mice on control diet (Figure 5D). The urinary excretion of this metabolite in the control and alcohol-treated *Ppara*-null 129S mice (Figure 5E) was also found to be 6-to-70 ($p < 0.05$, except at three and six months) and 3-to-10-fold higher compared to their wild-type counterparts (Figure 5F). The alcohol-treated *Ppara*-null B6 mice also showed a nine-fold elevation ($p < 0.005$) in urinary excretion of the metabolite than the wild-type counterparts after five months. Alcohol treatment was found to result in marginal elevation in excretion of this metabolite except a 3-fold elevation ($p < 0.005$) in the *Ppara*-null mice at six months (Figures 5E and 5F).

N22, which was identified as taurine, showed the opposite trend of modulation in urinary excretion in case of B6 and 129S mice in response to alcohol treatment. Taurine excretion in the urine of B6 mice tended to decrease after alcohol treatment (Figures 6A and 6B). In addition, it was noted that the taurine concentrations in the control as well as alcohol treated wild-type B6 mice were 2- to 3-fold ($p < 0.05$) lower than in their *Ppara*-null counterparts. On the other hand, wild-type 129S mice showed approximately 2-fold increase in taurine excretion following alcohol-treatment (Figure 6C). The alcohol-treated *Ppara*-null 129S mice showed 1.5-fold increase in urinary excretion after two months ($p < 0.005$) which increased to 5-fold at three months followed by a steady 2-fold elevation 4 month onwards ($p < 0.005$) (Figure 6D). There was no significant difference in taurine excretion between the wild-type and *Ppara*-null 129S mice on control diet. However, the alcohol-treated *Ppara*-null mice showed 1.5-fold ($p < 0.05$) elevation in taurine excretion compared to their wild-type counterparts.

Excretion of 2-hydroxyphenylacetic acid was found to be depleted 3- and 2- fold after four ($p < 0.0005$) and five ($p < 0.005$) months of alcohol treatment, respectively, in the urine of alcohol-treated wild-type B6 mice compared to the mice on control diet (Figures 7A and

7B). The urinary excretion of this metabolite was also found to decrease by 3- and 2- fold after three ($p < 0.005$) and four ($p < 0.005$) months of alcohol treatment, respectively, in the urine of alcohol-treated wild-type B6 mice compared to control mice.

MRM-based quantitation revealed that there was no consistent elevation in the excretion of 4-hydroxyphenylacetic acid in the urine of alcohol-treated wild-type B6 mice (Figure 8A) except for a 4- and 3.5-fold elevation at four ($p < 0.005$) and five ($p < 0.0005$) months, respectively. However, unlike that in case of the 129S mice, there was no effect on the urinary excretion of this metabolite on alcohol treatment of *Ppara*-null B6 mice (Figure 8B). In addition, although the urinary abundance of its sulfate conjugate, 4-hydroxyphenylacetic acid sulfate, was unaffected by alcohol treatment in B6 mice, the urinary abundance of the sulfate conjugate in the wild-type mice was generally higher than that in the *Ppara*-null mice (Figures 8C and 8D).

Ion P21 was identified as xanthurenic acid by comparison with an authentic standard. This metabolite was found to be elevated by 1.5-fold ($p < 0.05$) in the urine of alcohol-treated *Ppara*-null B6 mice while the alcohol-treated wild-type B6 mice showed a 2-fold ($p < 0.0005$) depletion compared to their respective counterparts on control-diet (Figures 7C and 7D). The urinary excretion of this metabolite was also elevated in response to alcohol treatment in the *Ppara*-null B6 mice by 2.5- and 2-fold at four ($p < 0.005$) and six ($p < 0.05$) months, respectively (Figure 7D). However, the wild-type B6 mice did not show any significant increase in the urinary excretion of the metabolite except for a 1.5-fold ($p < 0.05$) elevation at four months (Figure 7C). The alcohol treatment was found to cause only marginal elevation in the abundance of this metabolite in the urine of 129S mice (Figure 7F). The elevation of this metabolite in response to alcohol treatment was insignificant except for a 1.5-fold increase in both wild-type ($p < 0.05$) and *Ppara*-null ($p < 0.005$) mice at four months (Figures 7E and 7F).

The urinary excretion of pimelic acid (Figures 9A and 9B) as well as adipic acid (data not shown) in B6 mice were found to be unaffected by alcohol treatment. Suberic acid excretion in wild-type B6 mice was also unaffected by alcohol treatment (Figures 9C and 9D). The *Ppara*-null B6 mice showed only marginal but statistically insignificant decrease in the urinary excretion of suberic acid in response to alcohol treatment. The urinary excretion of suberic acid was also unaffected in 129S mice on alcohol treatment. However, control

129S *Ppara*-null mice were found to have 3-fold elevated excretion of suberic acid ($p < 0.05$) compared to their wild-type counterparts (Figures 9E and 9F). Similarly, alcohol-treated 129S *Ppara*-null mice were found to have 6- to 12-fold elevated excretion of suberic acid ($p < 0.05$, except at four and five months) compared to their wild-type counterparts.

The urinary excretion of indole-3-lactic acid (P2) in the wild-type B6 mice was unaffected by alcohol-treatment (Figure 10A). However, it was significantly elevated in *Ppara*-null mice in response to chronic alcohol treatment (Figure 10B). The urinary excretion of indole-3-lactic acid was elevated by 1.5-, 2-, and 2-fold after two ($p < 0.005$), five ($p < 0.0005$) and six ($p < 0.0005$) months of alcohol treatment, respectively. The indole-3-lactic levels in the alcohol-treated *Ppara*-null urines were also 2-fold higher ($p < 0.05$) than that in the alcohol-treated wild-type mice during this period. However, no significant change in the urinary excretion of tryptophan (Figures 11A and 11B) or indole-3-pyruvic acid (Figures 11C and 11D) was detected in the alcohol-treated *Ppara*-null mice compared to the control or alcohol-treated wild-type mice.

The ion N17 (retention time = 3.3 min, $m/z = 165.055$ -) was identified as phenyllactic acid by comparing the retention time and fragmentation pattern with authentic standard. Chronic alcohol treatment of B6 *Ppara*-null mice were found to elevate the urinary excretion of this metabolite by 4-, 5- and 3.5-fold after four, five ($p < 0.05$), and six ($p < 0.005$) months of alcohol treatment compared to the control mice (Figure 10D). The B6 wild-type mice on the other hand, did not show any significant increase in the abundance except a 2.5-fold elevation ($p < 0.0005$) at three months (Figure 10C). Interestingly, this metabolite was also found to increase in the urine of alcohol-treated 129S *Ppara*-null mice (Figure 10F). The urinary excretion of this metabolite was elevated by 2-fold after three and five months ($p < 0.05$) and 3-fold after six months ($p < 0.005$) of alcohol-treatment in *Ppara*-null mice compared to control mice (Figures 10E and 10F). The abundance of this metabolite in the urine of alcohol-treated 129S *Ppara*-null mice was also 3- and 4.5-fold higher than alcohol-treated wild-type mice after five ($p < 0.005$) and six ($p < 0.0005$) months of alcohol treatment, respectively (Figures 10E and 10F). However, the excretion of phenylalanine was found to be unaffected in the alcohol-treated *Ppara*-null mice of both B6 (Figure 12B) and 129S (Figure 12D) background compared to the control mice (Figures 12A and 12C).

These results show that, unlike that observed in 129S mice as previously described in Manna *et al.*, there was no significant change in the urinary excretion of 2-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid sulfate, pimelic acid, and adipic acid on alcohol treatment of *Ppara*-null mice on B6 background. In addition, modulation of taurine excretion was opposite in alcohol-treated B6 and 129S mice. However, the change in excretion of ethyl- β -D-glucuronide, phenyllactic acid, and indole-3-lactic acid due to chronic alcohol exposure was similar between mice of B6 and 129S background.

Both histology and liver triglyceride measurements revealed an increase in lipid deposition in the alcohol-treated *Ppara*-null mice after one month indicating the onset of steatosis. This suggested that, similar to the mice of 129S background (Nakajima *et al.* and Manna *et al.*), alcohol-treated B6 *Ppara*-null mice also served as a model for early stages of ALD development. However, no statistically significant increase in liver enzyme activities that are commonly used to assess liver damage could be detected at this point. On the other hand, mass spectrometry-based metabolomic analysis was able to clearly segregate the control mice from their alcohol-treated counterparts from the start of the metabolomics study (two months of alcohol treatment). It should be noted that apart from the presence of alcohol metabolites, changes in the abundances of endogenous metabolites contributed significantly to the segregation of the metabolome. Additionally, the metabolomic signature also revealed distinct metabolic responses to alcohol treatment in wild-type and *Ppara*-null B6 mice. However, it is interesting and important to note that B6 and 129S mice have intrinsic differences in their urinary metabolomes. The fact that such differences existed despite maintaining the mice under near-identical conditions (food, cages, temperature, and humidity), and were not overshadowed by gene deletion (*PPAR α* knockout) and/or xenobiotic (alcohol) exposure, testifies to the importance of genetic background in defining the metabotype.

In keeping with the differences in genetic background of the B6 and 129S mice, a number of metabolites were differentially modulated in response to alcohol. In fact, alcohol metabolism itself was different between these two strains of mice. Unlike the 129S mice, the B6 mice showed no elevation in ethylsulfate. Although ethyl- β -D-glucuronide was elevated in B6 mice, no significant difference between the wild-type and *Ppara*-null mice, similar to that in case of 129S mice (Manna *et al.*), was observed with respect to the excretion of the

glucuronide conjugate. However, *N*-acetylglycine was significantly elevated in the *Ppara*-null mice of both B6 and 129S background in response to alcohol treatment. Earlier studies have reported elevation of *N*-acetylglycine on alcohol intake in mice (Bradford *et al.*, *Toxicol. Appl. Pharmacol.* 232:236-43 (2008)). Alcohol is either oxidized in liver to acetic acid via acetaldehyde or converted to phase II metabolites, such as ethylsulfate and ethylglucuronide. Apart from its participation in numerous metabolic pathways, such as glycolysis, Krebs cycle, fatty acid biosynthesis, and amino acid metabolism in the form of acetyl-CoA, acetic acid can also undergo conjugation, presumably by glycine transferases, to be converted and excreted as *N*-acetylglycine leading to an increase in the urinary abundance of the metabolite in alcohol-treated mice.

Taurine is a sulfur-containing amino acid that is produced via oxidation of cysteine. Alcohol consumption is known to result in the formation of reactive oxygen species (ROS) causing increase in oxidative stress (Albano *et al.*, *Proc. Nutr. Soc.* 65:278-90 (2006); and Das *et al.*, *Life Sci.* 81:177-87 (2007)). Here, taurine was found to be elevated in the urine of alcohol-treated 129S *Ppara*-null mice. On the other hand, B6 mice showed a marginal decrease in taurine excretion on alcohol treatment. An earlier study demonstrated that ROS generation was significantly higher and the expression of antioxidant response genes, such as glutathione synthetase (GSS) and superoxide dismutases (SOD), was significantly lower in the liver of 129S mice compared to the B6 mice in response to high-fat diet although strains developed comparable liver pathologies (Syn *et al.*, *Liver Int.* 29:1262-72 (2009)). Thus, increased taurine excretion in the 129S mice might be a result of higher ROS production in the 129S mice. In addition, the fact that alcohol-treated 129S *Ppara*-null mice showed a significantly higher elevation in taurine levels compared to the wild-type mice is a manifestation of elevated ROS production in alcohol-treated *Ppara*-null mice as reported earlier (Rakhshandehroo *et al.*, *PPAR Res* 2007:26839 (2007)). On the other hand, the inherent elevated expression of anti-oxidant response genes in B6 mice (Syn *et al.*) might be responsible for the reduction in ROS levels and cysteine oxidation. Thus, taurine excretion appears to be a metabolomic signature of the background-dependent variation in defense mechanism against alcohol-induced oxidative stress.

Although they could be produced via phenylalanine and tyrosine metabolism, 2-hydroxyphenylacetic acid and 4-hydroxyphenylacetic acid levels were found to be influenced by gut microbes in a recent study using germ-free mice (Wikoff *et al.*, *Proc. Natl.*

Acad. Sci. USA 106:3698-703 (2009)). Thus, the observed differences between the B6 and 129S mice with respect to the excretion of these metabolites in response to chronic alcohol treatment might be a reflection of characteristic differences in the gut microbiome. The difference in the gut microbial metabolism in B6 and 129S is also evident from the differential modulation of a number of phase II metabolites. The gut microbiome has been shown to play a significant role in influencing the abundance of glycine-, taurine-, glucuronide-, and sulfate-conjugates (Wikoff *et al.* and Nicholson *et al.*, *Nat. Rev. Drug Discov.* 2:668-76 (2003)). The present results show that the excretion of *N*-hexanoylglycine, ethyl sulfate, and 4-hydroxyphenylacetic acid sulfate was different not only between the two strains, but also between wild-type and *Ppara*-null mice of the same background. These results are in line with the growing body of evidence about the role of the metagenome in defining biochemical traits and controlling pathophysiological processes.

MassTRIX analysis showed that tryptophan metabolism was differentially affected in *Ppara*-null mice on chronic alcohol exposure compared to their wild-type counterparts on both B6 and 129S backgrounds. A number of genes involved in tryptophan metabolism are regulated by PPAR α (Cariello *et al.*, *Toxicol. Sci.* 88:250-64 (2005)). Tryptophan is metabolized to 3-hydroxy-L-kynurenine in three steps with intermediate formation of L-formylkynurenine and L-kynurenine. This metabolite may either undergo hydrolysis to produce 3-hydroxy anthranilate or deamination to produce 4-(2-amino-3-hydroxyphenyl)-2,4-dioxobutanoate. 3-Hydroxyanthranilate is then oxidized to 2-amino-3-carboxymuconate semialdehyde which is converted to either NAD⁺ through quinolinic acid or acetyl Co-A. Earlier studies have shown that the activity of the first enzyme (ACMSD) involved in the catabolic process is regulated by PPAR α (Shin *et al.*, *Mol. Pharmacol.* 70:1281-90 (2006)). In wild-type mice, suppression of this gene by PPAR α promotes NAD⁺ biosynthesis (Shin *et al.*). On the other hand, upregulation of this gene in *Ppara*-null mice results in activation of the tryptophan catabolic pathway and impairs NAD⁺ biosynthesis. Impairment of NAD⁺ biosynthesis through quinolinic acid results in redistribution of fluxes through tryptophan metabolism, which is evident from the significant elevation of xanthurenic acid in B6 *Ppara*-null mice. Presumably, the build-up of 3-hydroxykynurenine as a result of impaired NAD⁺ biosynthesis increases the production of 4-(2-Amino-3-hydroxyphenyl)-2,4-dioxobutanoate, which undergoes spontaneous dehydration and cyclization to xanthurenic acid. However, 129S *Ppara*-null mice showed insignificant increase in the xanthurenic acid levels reflecting

genetic background-dependent mechanism of redistribution of fluxes through tryptophan metabolic pathways.

NAD⁺ is an essential cofactor for the oxidation of fatty acid through both β - and ω -oxidation pathways. The impairment of NAD⁺ biosynthesis leaves *Ppara*-null mice more prone to fat deposition in the liver compared to their wild-type counterparts. Alcohol is oxidized stepwise by, mainly, alcohol dehydrogenase (EC 1.1.1.2) and aldehyde dehydrogenase (EC 1.2.1.3) to acetaldehyde and acetic acid, respectively (Figure 13). Both of these reactions are associated with concomitant reduction of NAD⁺ to NADH. Thus, chronic alcohol consumption leads to a drastic shift in the redox balance in *Ppara*-null mice and impairs fatty acid oxidation leading to fat deposition or steatosis as shown earlier in the 129S *Ppara*-null mice (Nakajima *et al.*) and indicated by increasing hepatic triglyceride content in B6 *Ppara*-null mice.

Manna *et al.* previously reported that elevated urinary excretion of indole-3-lactic acid was found to be associated with development of steatosis in 129S *Ppara*-null mice. As discussed, this metabolite was found to be elevated in the urine of alcohol-treated B6 *Ppara*-null mice as well. Indole-3-lactic acid is an α -hydroxy acid and the reduction product of deaminated tryptophan. The deamination of tryptophan is typically carried out by L-amino acid oxidase (EC 1.4.3.2). Earlier studies have shown that microbial as well as mammalian aspartate aminotransferases (EC 2.6.1.1) can also catalyze this reaction, albeit with low efficiency (Recasens *et al.*, *Biochemistry* 19:4583-9 (1980); and Yagi *et al.*, *FEBS Lett.* 100:81-4 (1979)). However, aspartate aminotransferase is known to be elevated during alcohol-induced liver damage in the *Ppara*-null mice (Nakajima *et al.*). Thus, the unused tryptophan, resulting from impairment of NAD⁺ biosynthesis in *Ppara*-null mice (Shin *et al.*) and decrease in activity of tryptophan-2,3-dioxygenase during chronic alcohol consumption (Morland, J., *Biochem. Pharmacol.* 23:21-35 (1974)), could be converted to indole-3-pyruvic acid by the action of aspartate aminotransferase and/or L-amino acid oxidase (Figure 13). This α -keto acid intermediate would readily be reduced to indole-3-lactic acid due to build-up of NADH during chronic alcohol consumption. Thus, the increase in indole-3-lactic acid production appears to be related to the same biochemical events, *i.e.*, impairment of NAD⁺ biosynthesis and an increase in the NADH/NAD⁺ ratio that leads to steatosis as well as an increase in aminotransferase activity that is associated with concurrent liver damage in both 129S and B6 mice. Although the enzymes responsible for interconversion of indole-3-lactic

acid and indole-3-pyruvic acid, *i.e.*, indolelactate dehydrogenase (EC 1.1.1.110) or (R)-4-hydroxyphenyllactate dehydrogenase (EC 1.1.1.222), have been characterized in microbes (Jean *et al.*, *Can. J. Microbiol.* 14 :429-35 (1968)), no mouse equivalent has been reported. However, the conversion of tryptophan to indole-3-lactate by the action of aminotranseferases and NADH-dependent aromatic lactate dehydrogenases, as proposed here (Figure 13), has already been documented in the protozoa *Leishmania donovani* (Leelayoova *et al.*, *J. Protozool.* 39:350-8 (1992)). Thus the observed elevation in indole-3-lactic acid excretion during ALD development in alcohol-treated *Ppara*-null mice suggests the existence of similar and hitherto unknown metabolic machinery in mammals.

In addition, another α -hydroxy acid, namely, phenyllactic acid was also found to be elevated in the urine of both B6 and 129S *Ppara*-null mice in response to chronic alcohol consumption. This is also the reduction product of corresponding deaminated aromatic amino acid, phenylalanine. Apart from tyrosine aminotransferase (EC 2.6.1.5), aspartate aminotransferase can also catalyze the deamination of phenylalanine to phenylpyruvic acid (Yagi *et al.*, *FEBS Lett.* 100:81-4 (1979); and Owen *et al.*, *Biochem. J.* 143:541-53 (1974)). However, the shift in the redox balance in the alcohol-treated *Ppara*-null mice would drive the reduction of this intermediate to phenyllactate by the action of (R)-4-hydroxyphenyllactate dehydrogenase (EC 1.1.1.222) (Bode *et al.*, *Biochem. Physiol. Pflanz.* 181:189-198 (1986)) as shown in Figure 13.

The exclusive elevation of α -hydroxy acids in the alcohol-treated *Ppara*-null mice strongly indicates to the existence of some common enzymatic pathway linked to process of alcohol-induced liver injury and steatosis. The fact that these were found to be elevated in a genetic background-independent manner further strengthens the proposed mechanistic association between the biochemical events.

The findings described herein have great clinical significance as response to chronic alcohol consumption widely varies between different populations and even within populations in humans. These results indicate that metabolomic analysis can help to capture a wide-angle view of varying biochemical responses to alcohol. The observation of conserved metabolic signatures of chronic alcohol consumption in *Ppara*-null mice, *e.g.*, elevation of indole-3-lactic acid and phenyllactic acid, was remarkable in consideration of the widespread genetic background-related differences in the metabolic response. In addition, existing

literature suggests that the production of these metabolites may be a simultaneous reflection of the elevation in liver enzymes as well change in the redox balance associated with ALD pathogenesis. One of the major drawbacks of the liver enzyme assays that are frequently used to assess liver damage is their non-specificity. The proposed mechanistic relation between the elevation of these urinary metabolites and the biochemical events associated with steatosis caused by chronic alcohol consumption in these mice consolidates their candidature as biomarkers for use in noninvasive diagnostic tests for early stages of ALD.

Moreover, the results described herein also identified markers N8, N9, N23, P7, and P11 as additional background independent markers for alcohol-treated *Ppara*-null mouse. Accordingly, the results described herein demonstrate the ability of metabolomics to simultaneously capture genetic background-dependent variations in metabolism and reveal genetic background-independent metabolic signatures of steatosis, thus offering a promising opportunity to identify robust early non-invasive biomarkers of ALD to improve the therapeutic outcome and quality of life in patients.

The results reported herein were obtained using the following methods and materials.

Chemicals

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

Animals and Treatments

Male 6- to 8-week-old *Ppara*-null mice on B6 (C57BL/6N-*Ppara*^{tm1Gonz}/N) and 129S (129S4/SvJae-*Ppara*^{tm1Gonz}/N) background as well as their wild-type counterparts (four mice per group) were fed ad libitum a 4% alcohol-containing liquid diet (Lieber-DeCarli Diet, Dyets, Inc., Bethlehem, PA). Control mice were fed ad libitum an isocaloric diet supplemented with maltose dextran (Dyets, Inc.). Mice were euthanized after one month on the alcohol diet, serum collected, and portions of liver harvested for histology. After biochemical and histological evaluation of the model and ensuring that all mice were tolerating the liquid diet, mice were transferred to the urinary metabolomics protocol at two months. Urine samples were collected monthly from mice placed individually in Nalgene metabolic cages (Tecniplast USA, Inc., Exton, PA) over 24 hours and stored at -80°C in glass

vials until analyzed. All mice were acclimated to the metabolic cages by placing them in the metabolic cages before the actual sample collection. All animal studies were approved by the Georgetown University Animal Care and Use Committee.

Histology

Liver tissues were immediately formalin-fixed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (HE) following standard protocol. The HE-stained liver sections were examined under an Olympus BX41 microscope.

Biochemistry

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

Preparation of Urine Samples and UPLC-ESI-QTOFMS Analysis

Urine samples were deproteinized using SirrocoTM protein precipitation plates (Waters Corp., Milford, MA) after addition of one volume of 50% aqueous acetonitrile containing internal standards (50 μ M 4-nitrobenzoic acid and 1 μ M debrisoquine) and deproteinized extracts were collected into 96-well collection plates according to the manufacturer's instructions. A 5 μ L aliquot of supernatant was injected into a Waters UPLC-ESI-QTOFMS system (Milford, MA). An Acquity UPLC BEH C18 column (1.7 μ m, 2.1x50mm, 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). The gradient elution was performed over 10 minutes at a flow rate of 0.5 ml using: 2% B for 0.5 min, 2-20% B in 4 min, 20-95% B in 8 min, 95-99% B in 8.1 min, holding at 99%B up to 9.0 min, bringing back to 2% at 9.1 min and holding at 2% till end. Column temperature was maintained at 40°C throughout the run. The QTOF Premier mass spectrometer was operated in electrospray ionization positive (ESI+) and negative (ESI-) mode using sulfadimethoxine as the lock mass for accurate mass calibration in real time. Capillary and cone voltages were maintained at 3 kV and 20 V, respectively. Source and desolvation temperatures were set, respectively, at 120 °C and 350 °C. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (600 L/h), and argon was used as collision gas. Collision energy ranging from

10 to 40 eV was applied for MS/MS fragmentation of target ions. All urine samples were analyzed in a randomized fashion to avoid complications due to artifacts related to injection order and changes in instrument efficiency. MassLynx software (Waters Corp.) was used to acquire the chromatogram and mass spectrometric data in centroid format.

Multivariate Data Analysis

Centroided and integrated raw mass spectrometric data were processed using MarkerLynx software (Waters Corp., Milford, MA). The individual ion intensities were 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. This data matrix was analyzed by SIMCA-P+12 software (Umetrics, Kinnelon, NJ). Principal components analysis (PCA) of the Pareto-scaled data (Pearson, K. *Philosophical Magazine*, 2:559-572 (1901)) was performed to check unsupervised segregation of the metabolome. 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. 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 loadings 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 of the respective background, were selected for further identification and quantitation.

Metabolic Pathway Analysis

The effect of alcohol treatment on the potential metabolic pathways was analyzed with the help of the MassTRIX website (Suhre *et al.*, *Nucleic Acids Res.* 36:W481-4 (2008)) (<http://metabolomics.helmholtz-muenchen.de/masstrix/>) using the above list of ions that contributed consistently to the separation of the metabolome as described earlier (Manna *et al.*, *J. Proteome Res.* 9:4176-88 (2010)).

Identification of Urinary Biomarkers

Seven Golden Rules (Kind *et al.*, *BMC Bioinformatics* 8:105 (2007)) was used to calculate elemental compositions considering a mass error less than 5 ppm. Possible candidates for the ions were also searched using metabolomics databases (Cui *et al.*, *Nat. Biotechnol.* 26:162-4 (2008); and Smith *et al.*, *Ther. Drug Monit.* 27:747-51 (2005)). 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) as described in Manna *et al.*

Quantitation of Urinary Metabolites

An Acquity[®] UPLC system coupled with a XEVO triple-quadrupole tandem mass spectrometer (Waters Corp.) was used to quantitate urinary metabolites by multiple reaction monitoring (MRM). A number of MRM transitions were chosen to detect each compound according to their fragmentation patterns using authentic standards. Standard compounds were mixed together to optimize the condition for separation and detection of the metabolites from a complex mixture such as urine. Debrisoquine (0.5 μ M) was used as the internal standard. Finally, following MRM transitions were monitored for the respective compounds: debrisoquine (176 $\rightarrow$ 134; ESI+), phenylalanine (166 $\rightarrow$ 120; ESI+), phenyllactic acid (165 $\rightarrow$ 103; ESI-), suberic acid (173 $\rightarrow$ 111; ESI-), *N*-hexanoylglycine (174 $\rightarrow$ 76; ESI+), indole-3-lactic acid (206 $\rightarrow$ 118; ESI+), indole-3-pyruvic acid (204 $\rightarrow$ 130; ESI+), tryptophan (205 $\rightarrow$ 118; ESI+), xanthurenic acid (206 $\rightarrow$ 160, ESI-), *N*-acetylglycine (116 $\rightarrow$ 74, ESI-), taurine (124 $\rightarrow$ 80, ESI-), 2-hydroxyphenylacetic acid (151 $\rightarrow$ 107; ESI-), 4-hydroxyphenylacetic acid (151 $\rightarrow$ 107; ESI-), adipic acid (147 $\rightarrow$ 101; ESI+), pimelic acid (159 $\rightarrow$ 97; ESI-) and creatinine (114 $\rightarrow$ 86; ESI+). An Acquity UPLC BEH C18 column (1.7 μ m, 2.1x50mm, Waters Corp., Milford, MA) was used to separate mixture of authentic standards as well urine samples. The mobile phase was comprised of 0.1% aqueous formic acid (A) and acetonitrile containing 0.1% formic acid (B). The gradient elution was performed over 6 minutes at a flow rate of 0.3 ml using: 1-99% B in 4 min, holding at 99%B up to 5.0 min, bringing back to 1% at 5.5 min and holding at 1% till end. Column temperature was maintained at 40°C throughout the run. The area under the peak for each metabolite was divided by that for the internal standard to calculate response and serial dilution was

performed to generate a standard calibration plot of response vs. concentration. Serially diluted urine samples containing 0.5 μ M debrisoquine were analyzed in the same fashion as that of authentic compounds. The quantitative abundances were calculated from the response using the linear range of detection of the calibration plot. All analyses were performed using TargetLynx software (Waters Corp., Milford, MA)

Statistics

All values are presented as mean $\pm$ 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.

Other Embodiments

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

Incorporation by Reference

All patents, publications, and CAS numbers mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent and publication was specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. A method for identifying a subject as having or having a propensity to develop alcoholic liver disease (ALD), the method comprising
 - (a) detecting the level of a biomarker selected from the group consisting of phenyllactic acid; phenyllactic acid in combination with indole-3-lactic acid; N8; N9; N23; P7; and P11 in a sample obtained from the subject; and
 - (b) comparing the level of the biomarker to a reference.
2. The method of claim 1, wherein the subject is identified as having or having a propensity to develop ALD when the level of the biomarker is increased relative to the reference.
3. A method for identifying alcoholic liver disease (ALD) in a subject, the method comprising
 - (a) detecting the level of a biomarker selected from the group consisting of phenyllactic acid; phenyllactic acid in combination with indole-3-lactic acid; N8; N9; N23; P7; and P11 in a sample obtained from the subject; and
 - (b) comparing the level of the biomarker to a reference.
4. The method of claim 3, wherein ALD is identified in the subject when the level of the biomarker is increased relative to the reference.
5. A method for identifying a subject as having or having a propensity to develop steatosis, the method comprising
 - (a) detecting the level of a biomarker selected from the group consisting of phenyllactic acid; phenyllactic acid in combination with indole-3-lactic acid; N8; N9; N23; P7; and P11 in a sample obtained from the subject; and
 - (b) comparing the level of the biomarker to a reference.

6. The method of claim 5, wherein the subject is identified as having or having a propensity to develop steatosis when the level of the biomarker is increased relative to the reference.
7. A method for identifying steatosis in a subject, the method comprising
 - (a) detecting the level of a biomarker selected from the group consisting of phenyllactic acid; phenyllactic acid in combination with indole-3-lactic acid; N8; N9; N23; P7; and P11 in a sample obtained from the subject; and
 - (b) comparing the level of the biomarker to a reference.
8. The method of claim 7, wherein steatosis is identified in the subject when the level of the biomarker is increased relative to the reference.
9. A method for characterizing the stage of alcoholic liver disease (ALD) in a subject, the method comprising
 - (a) detecting the level of a biomarker selected from the group consisting of phenyllactic acid; phenyllactic acid in combination with indole-3-lactic acid; N8; N9; N23; P7; and P11 in a sample obtained from the subject; and
 - (b) comparing the level of the biomarker to a reference.
10. The method of claim 9, wherein an increase in the level of the biomarker relative to the reference identifies the subject as having a later stage of ALD.
11. A method for determining the prognosis of alcoholic liver disease (ALD) in a subject, the method comprising
 - (a) detecting the level of a biomarker selected from the group consisting of phenyllactic acid; phenyllactic acid in combination with indole-3-lactic acid; N8; N9; N23; P7; and P11 in a sample obtained from the subject; and
 - (b) comparing the level of the biomarker to a reference.
12. The method of claim 11, wherein an increase in the level of the biomarker relative to the reference identifies the subject as having a poor prognosis.

13. A method for characterizing the degree of lipid accumulation during the early stage of alcoholic liver disease (ALD) in a subject, the method comprising
 - (a) detecting the level of a biomarker selected from the group consisting of phenyllactic acid; phenyllactic acid in combination with indole-3-lactic acid; N8; N9; N23; P7; and P11 in a sample obtained from the subject; and
 - (b) comparing the level of the biomarker to a reference.
14. The method of claim 13, wherein an increase in the level of the biomarker relative to the reference identifies the subject as having a higher level of lipid accumulation during the early stage of ALD.
15. The method of any one of claims 1 to 14, wherein the level of the biomarker is increased 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15-fold or more relative to the reference.
16. The method of any one of claims 1 to 15, wherein the reference is the level of the biomarker in a control.
17. A method for monitoring alcoholic liver disease (ALD) therapy in a subject, the method comprising
 - (a) detecting the level of a biomarker selected from the group consisting of phenyllactic acid; phenyllactic acid in combination with indole-3-lactic acid; N8; N9; N23; P7; and P11 in a sample obtained from the subject; and
 - (b) comparing the level of the biomarker to a reference.
18. The method of claim 17, wherein a therapy that reduces the level of the biomarker is identified as effective.
19. The method of claim 17 or 18, wherein the reference is the level of the biomarker in a control.

20. The method of claim 19, wherein the control is a sample obtained from the subject prior to therapy or at an earlier time point during therapy.
21. A method for detecting an agent's therapeutic efficacy in a subject having alcoholic liver disease (ALD), the method comprising
 - (a) detecting an alteration in the level of a biomarker selected from the group consisting of phenyllactic acid; phenyllactic acid in combination with indole-3-lactic acid; N8; N9; N23; P7; and P11 in a sample obtained from the subject following treatment; and
 - (b) comparing the level of the biomarker to a reference.
22. The method of claim 21, wherein the reference is the level of the biomarker in a control.
23. The method of claim 22, wherein the control is a sample obtained from the subject prior to treatment or at an earlier time point during treatment.
24. The method of any one of claims 21 to 23, wherein (i) a maintenance or increase in the level indicates that the agent lacks efficacy in the subject, and (ii) a decrease in the level indicates that the agent has therapeutic efficacy in the subject.
25. The method of any one of claims 1 to 24, wherein the biomarker is phenyllactic acid.
26. The method of any one of claims 1 to 24, wherein the biomarker is phenyllactic acid in combination with indole-3-lactic acid.
27. The method of any one of claims 1 to 26, wherein the subject is human.
28. The method of any one of claims 1 to 27, wherein the sample is a biological fluid selected from the group consisting of blood, blood serum, plasma, saliva, and urine.
29. The method of claim 28, wherein the sample is a urine sample.

30. The method of any one of claims 1 to 29, wherein the level is detected by chromatography, mass spectrometry, spectroscopy, or immunoassay.
31. The method of claim 30, wherein the chromatography is ultra performance liquid chromatography (UPLC).
32. The method of claim 30, wherein the mass spectrometry is electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-QTF-MS).
33. The method of claim 30, wherein the spectroscopy is NMR spectroscopy.
34. The method of claim 30, wherein the immunoassay is ELISA
35. A kit for aiding the diagnosis of ALD or steatosis, the kit comprising at least one reagent capable of detecting or capturing phenyllactic acid, indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof.
36. The kit of claim 35, wherein the reagent is an antibody that specifically binds to phenyllactic acid, indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof.
37. The kit of claim 35 or 36, wherein the kit further comprises directions for using the reagent to analyze the level of phenyllactic acid, indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof.
38. A kit for aiding the diagnosis of ALD or steatosis, the kit comprising an adsorbent that retains phenyllactic acid, indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof.
39. The kit of claim 37, wherein the kit further comprises directions for contacting a test sample with the adsorbent and detecting phenyllactic acid, indole-3-lactic acid, N8, N9, N23, P7, P11, or a combination thereof retained by the adsorbent.

Figure 1A

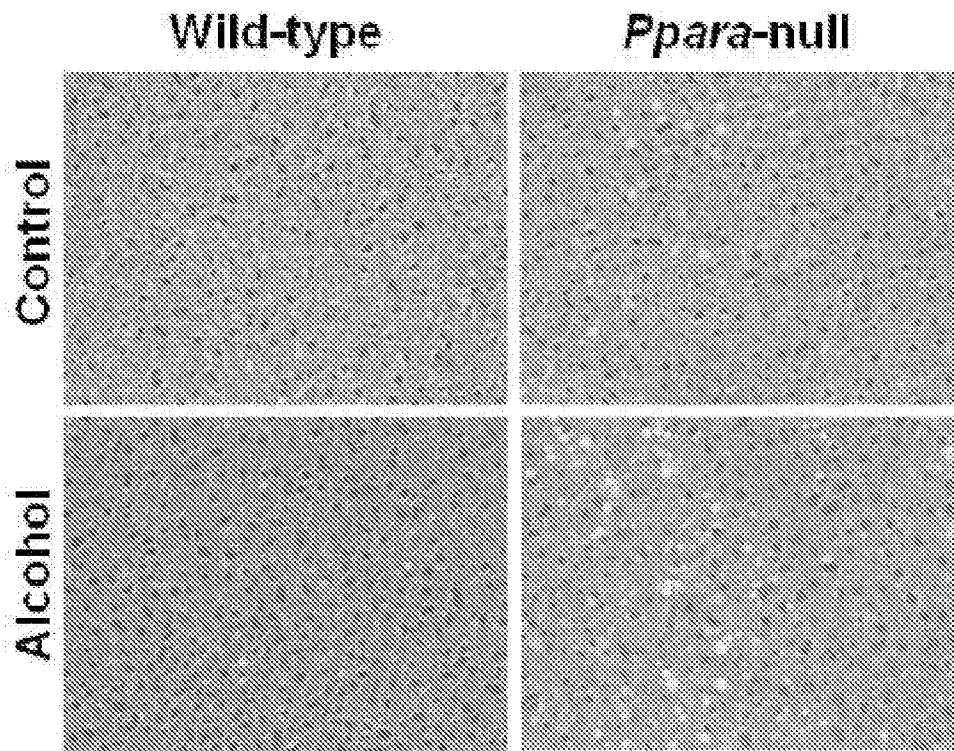


Figure 1B

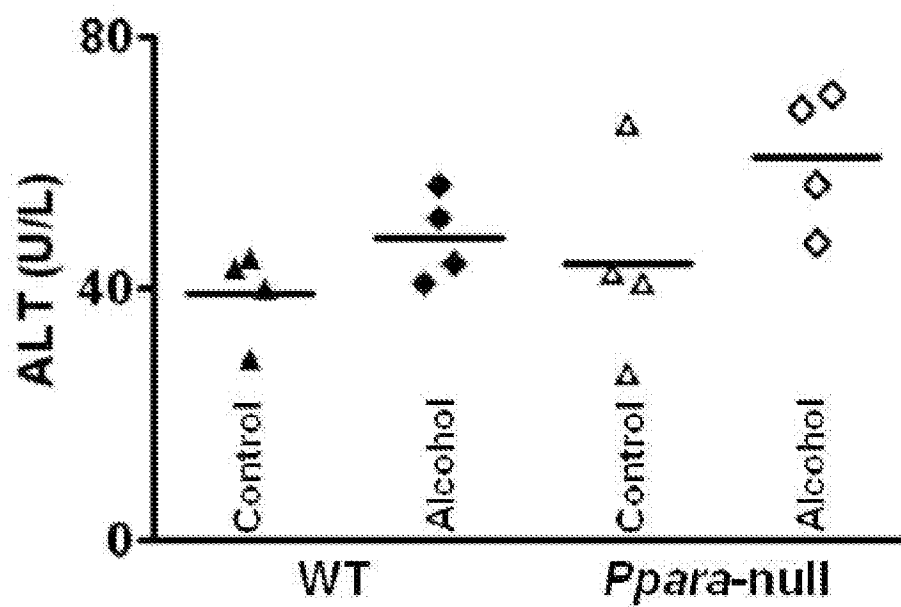


Figure 1C

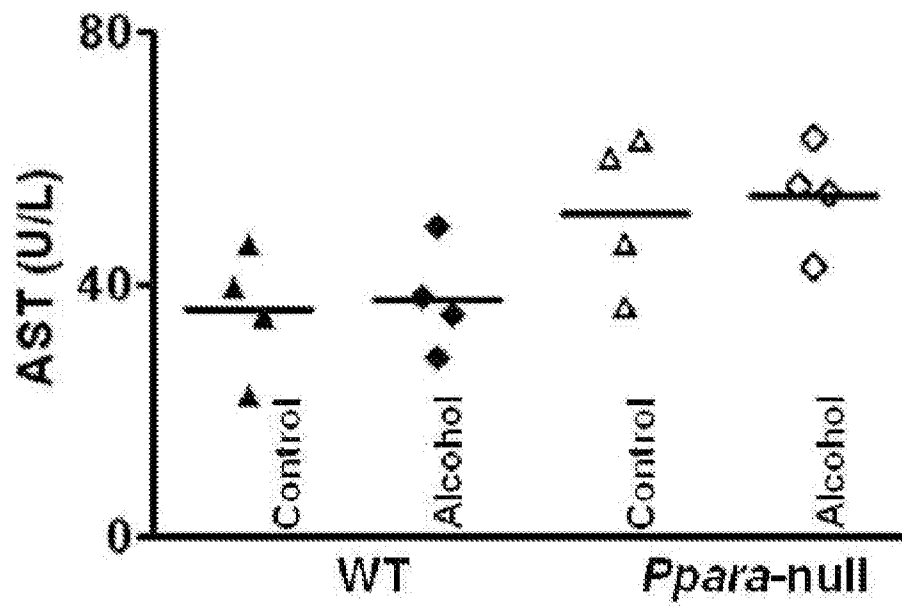


Figure 1D

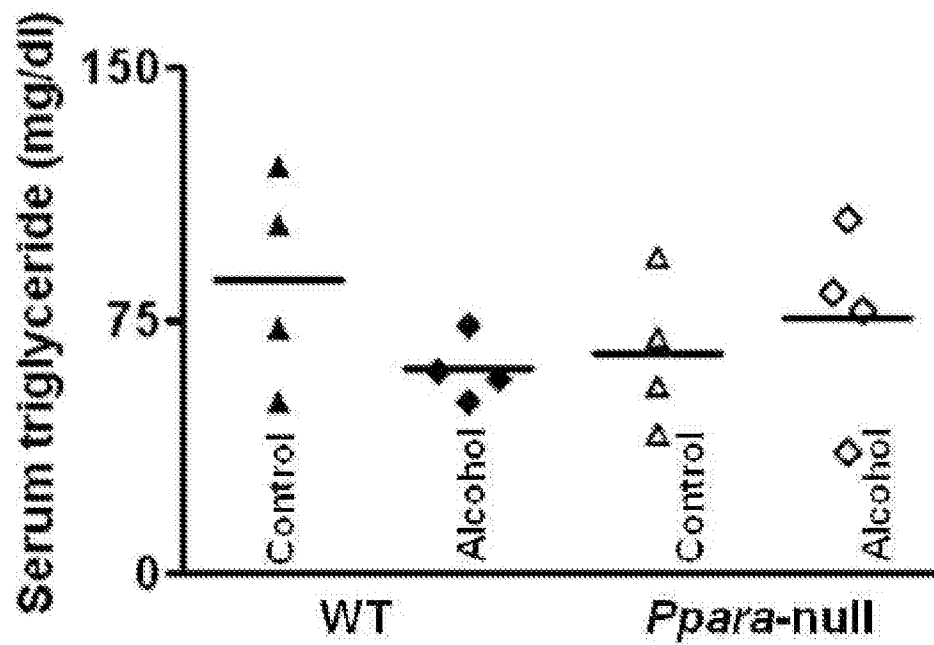


Figure 2A

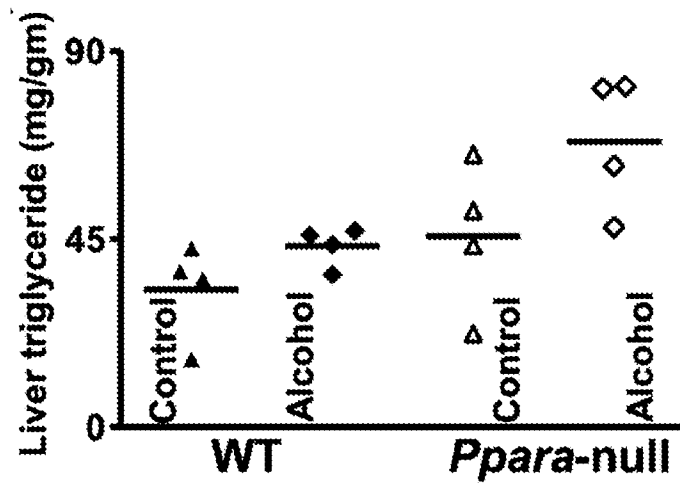


Figure 2B

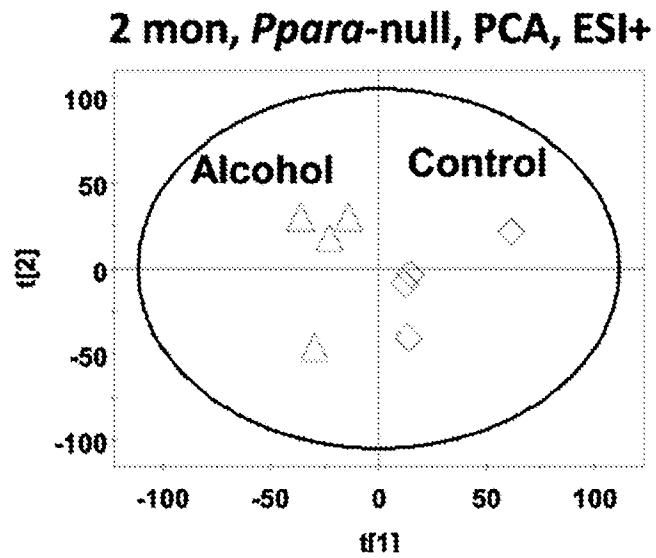


Figure 2C

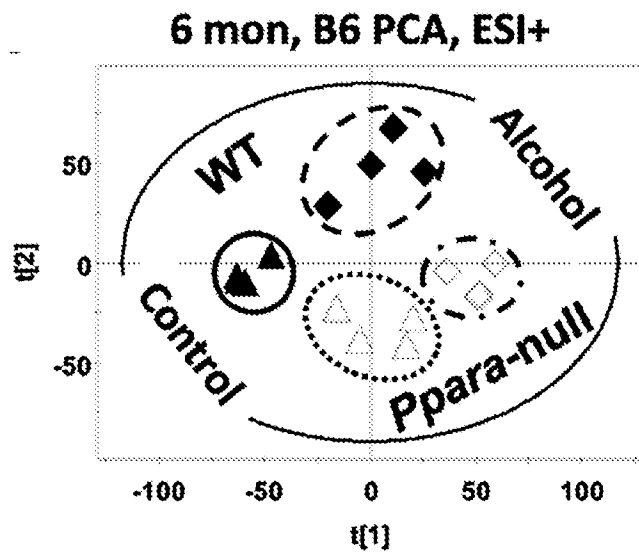


Figure 2D

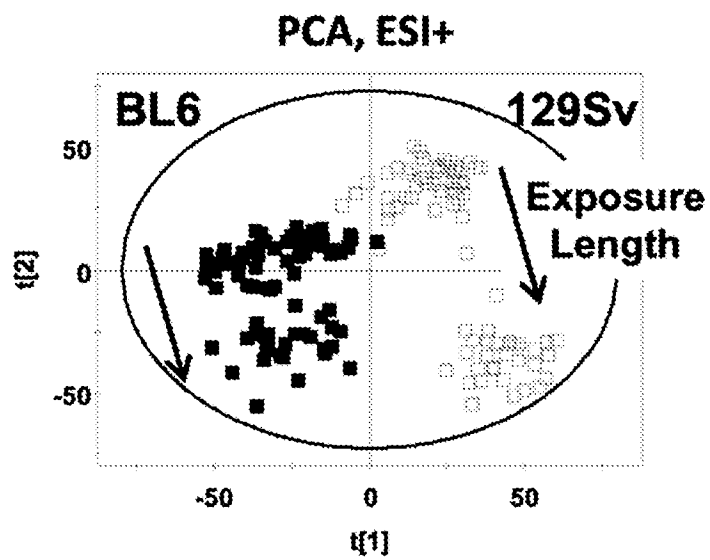


Figure 2E

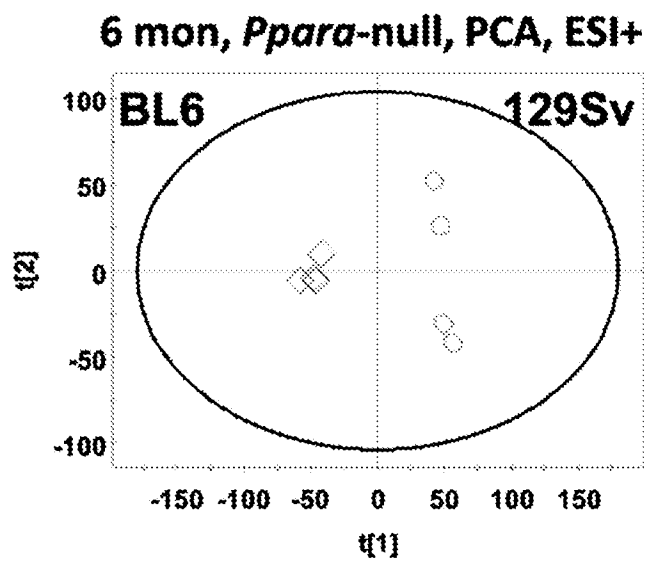


Figure 2F

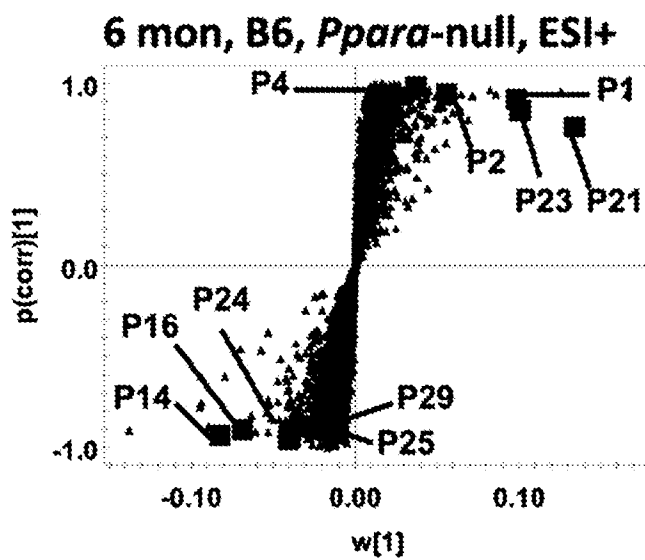


Figure 3A

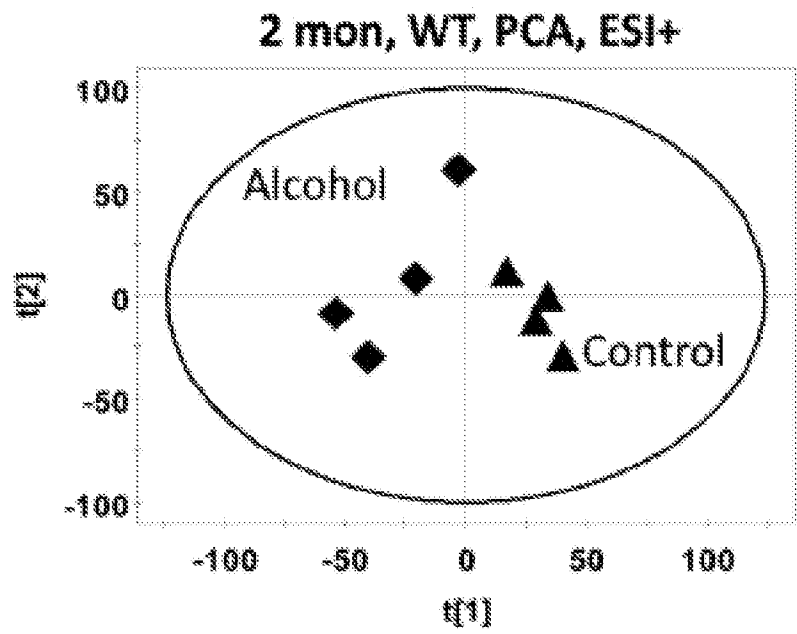


Figure 3B

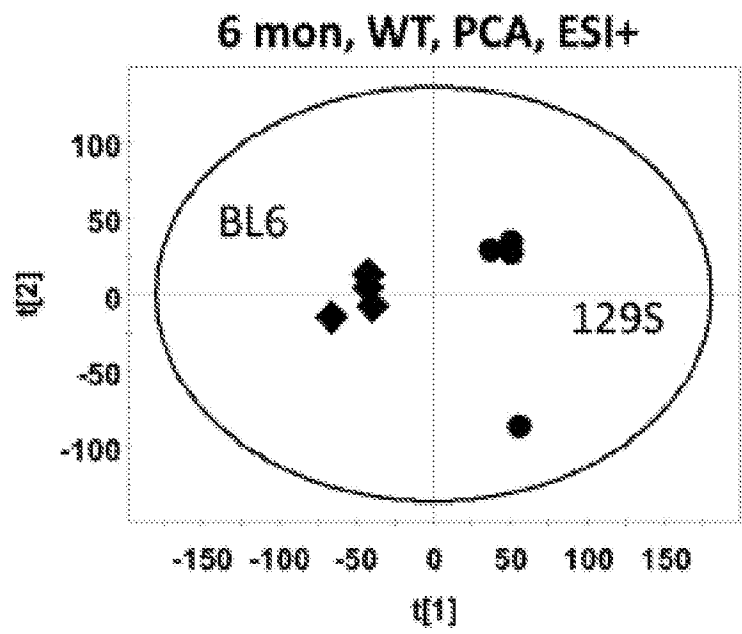


Figure 3C

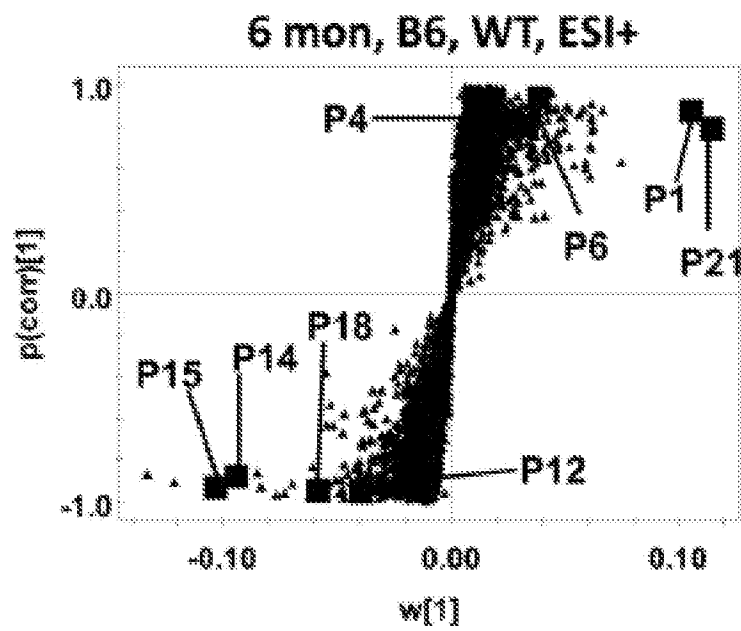


Figure 4A

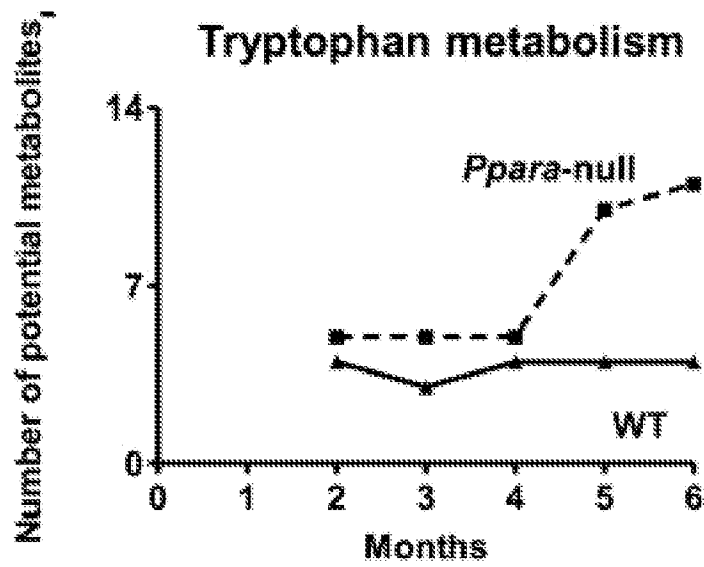


Figure 4B

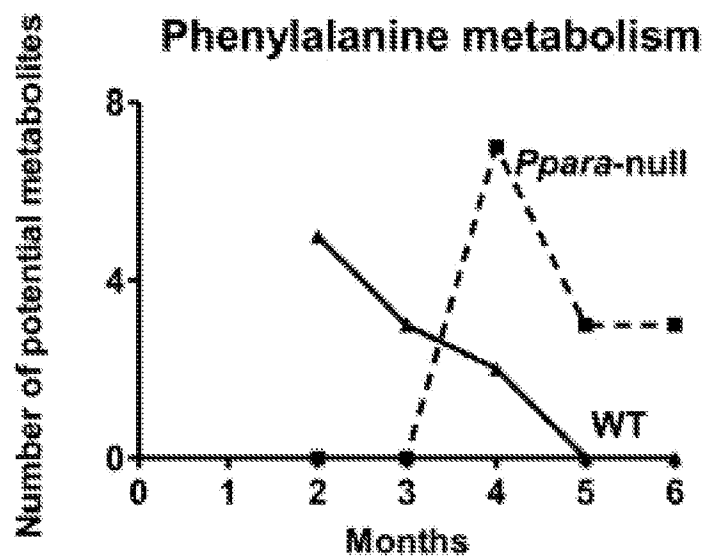


Figure 4C

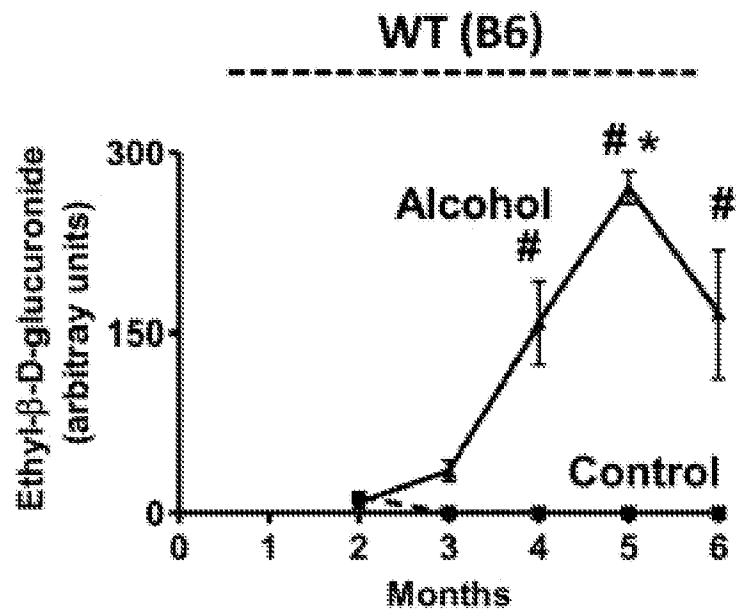


Figure 4D

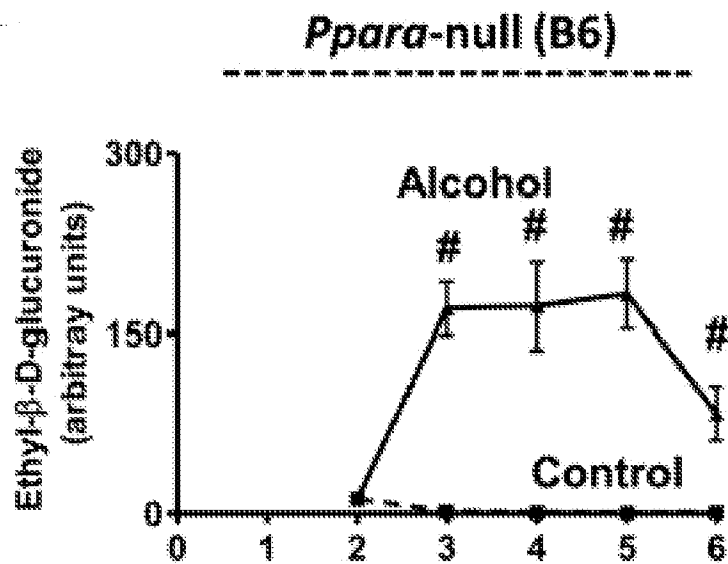


Figure 4E

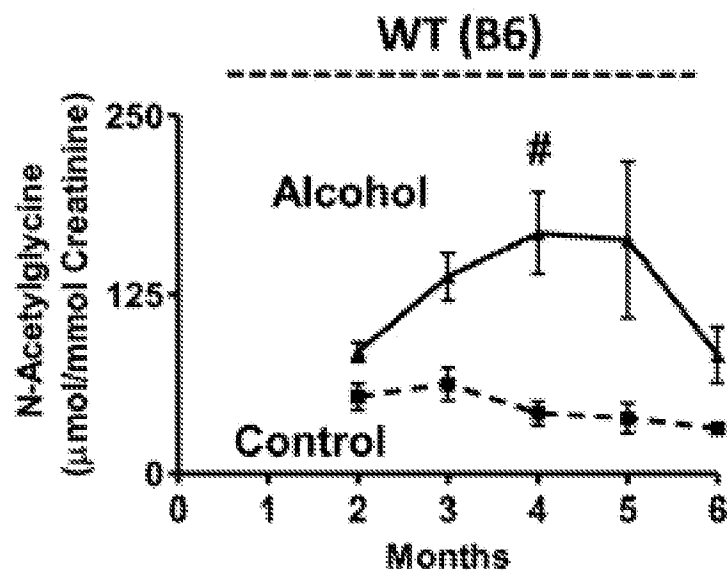


Figure 4F

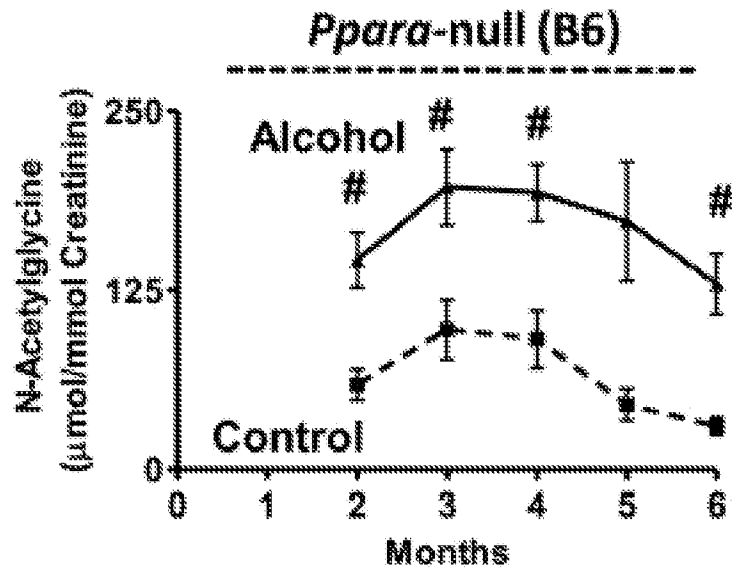


Figure 5A

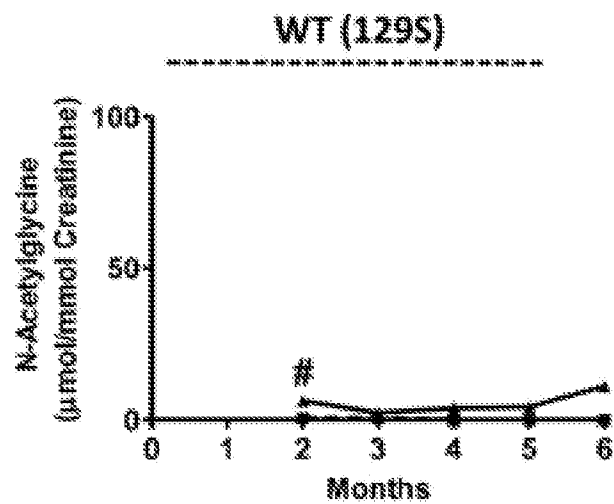


Figure 5B

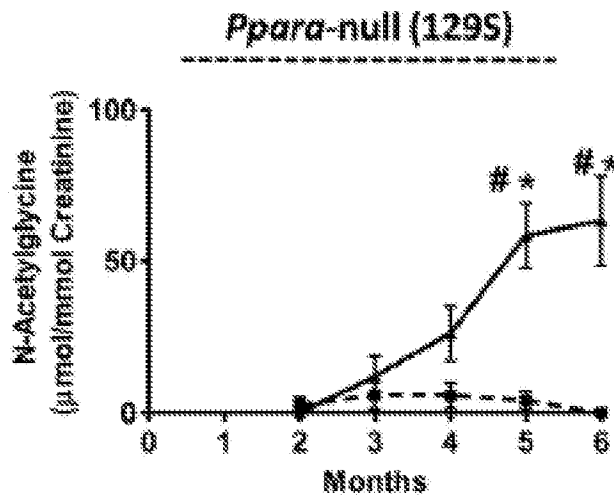


Figure 5C

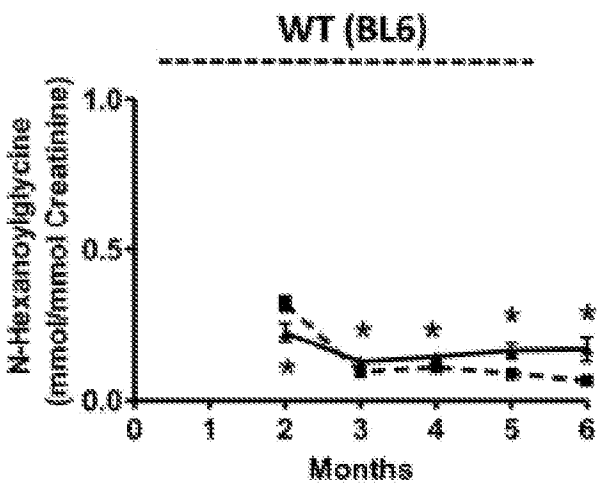


Figure 5D

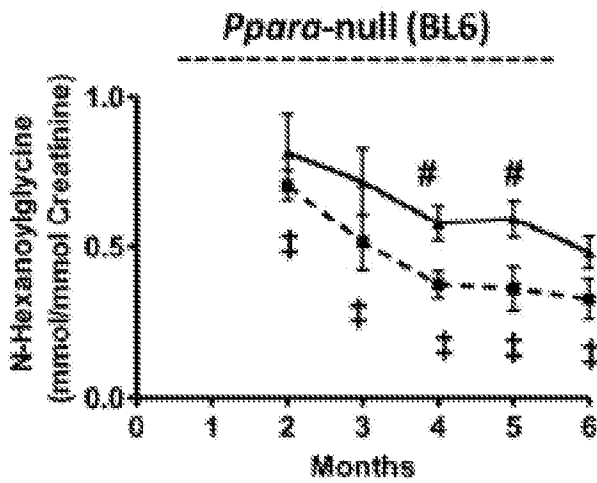


Figure 5E

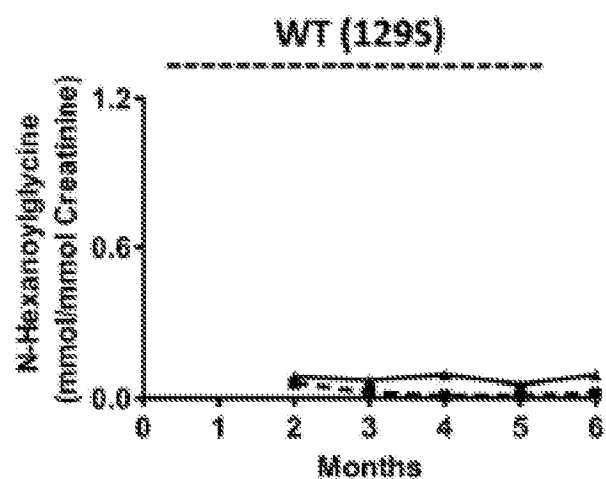


Figure 5F

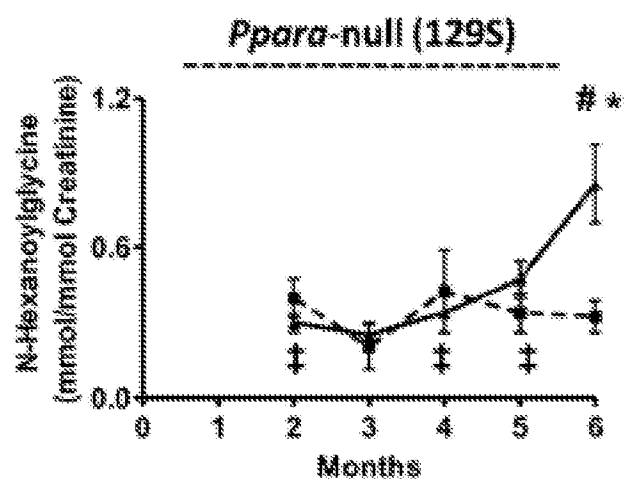


Figure 6A

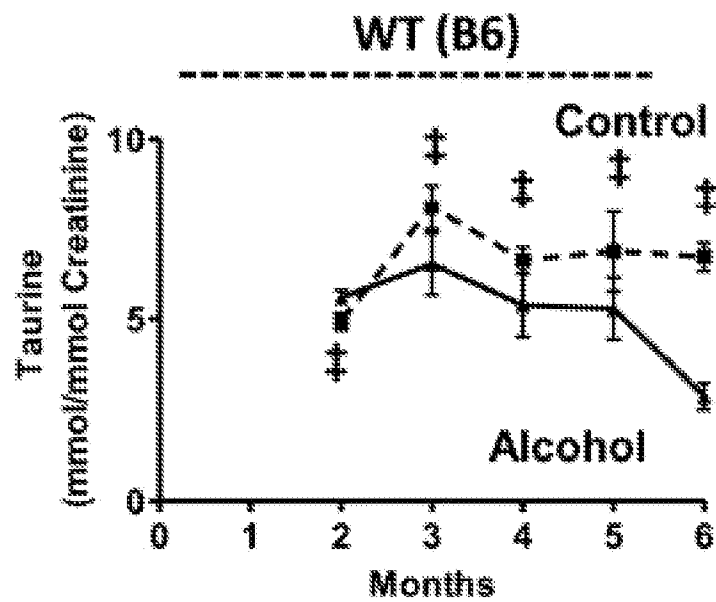


Figure 6B

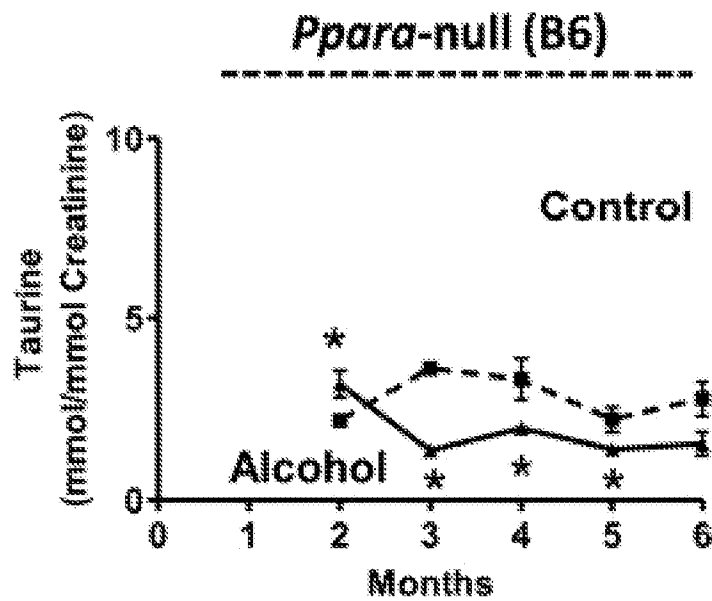


Figure 6C

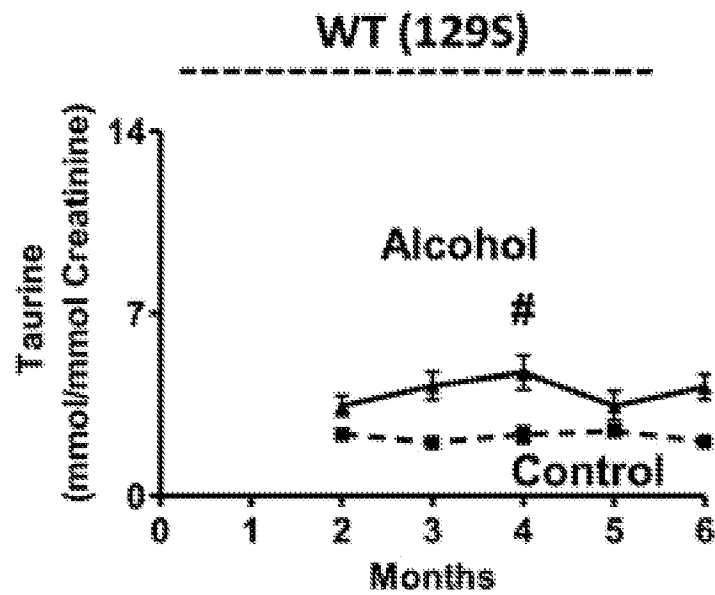


Figure 6D

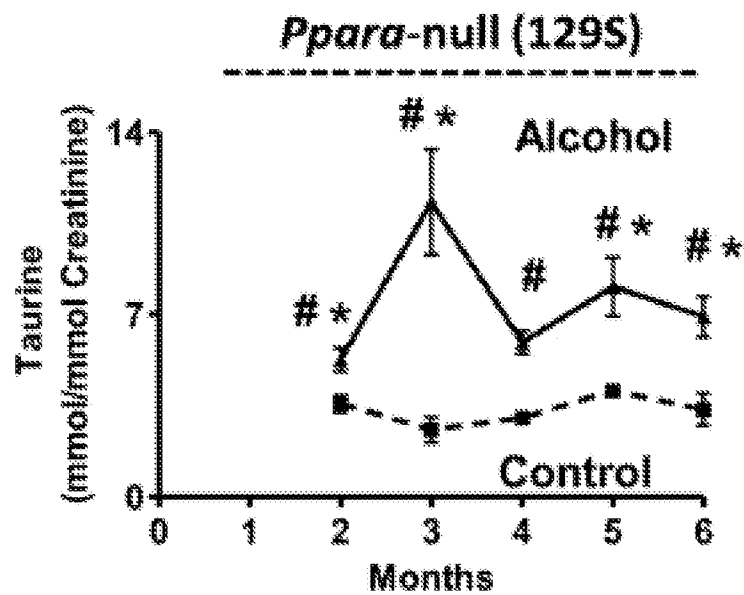


Figure 7A

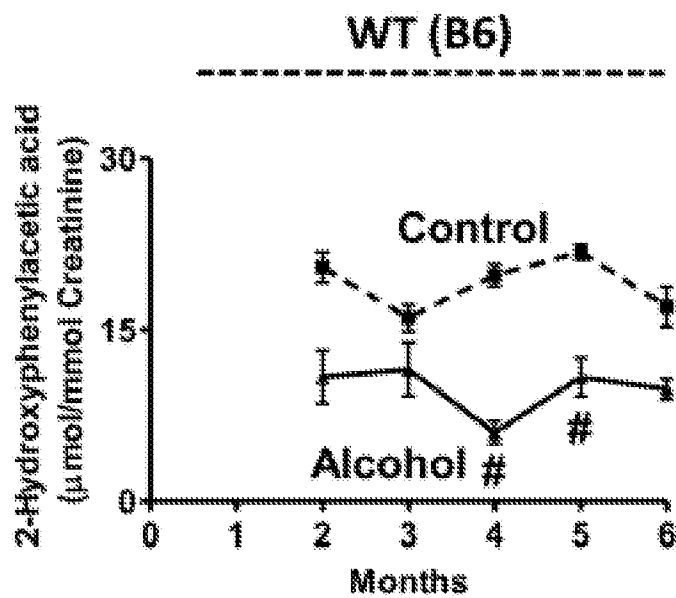


Figure 7B

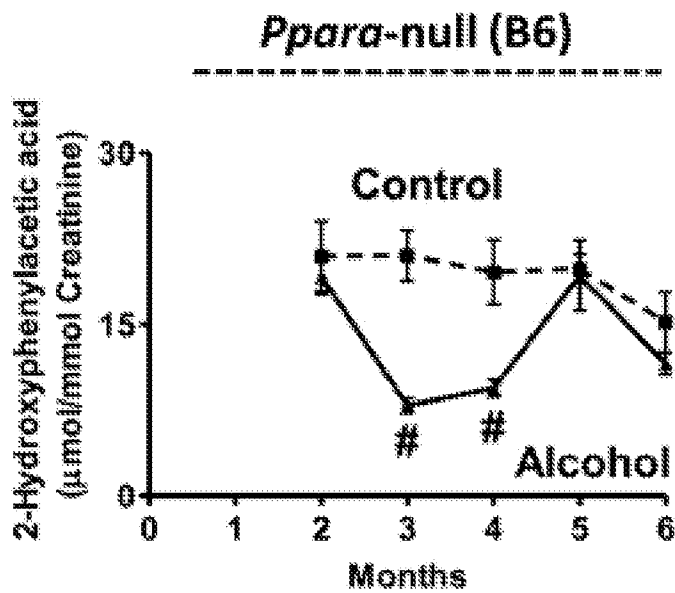


Figure 7C

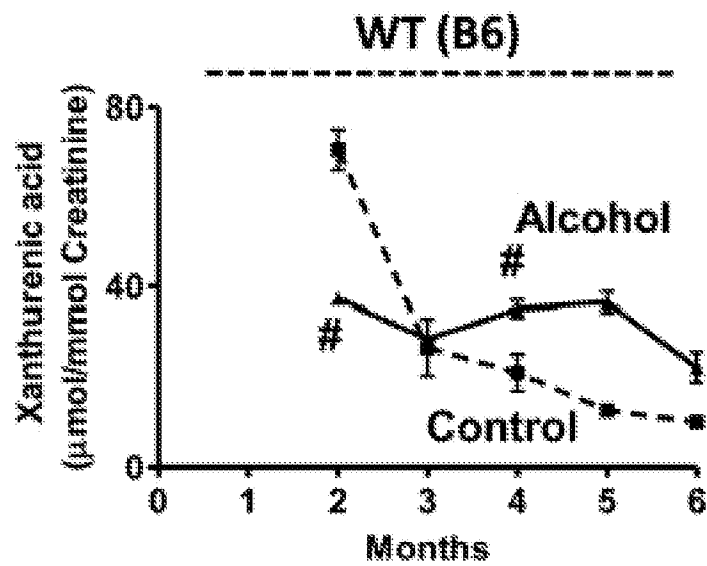


Figure 7D

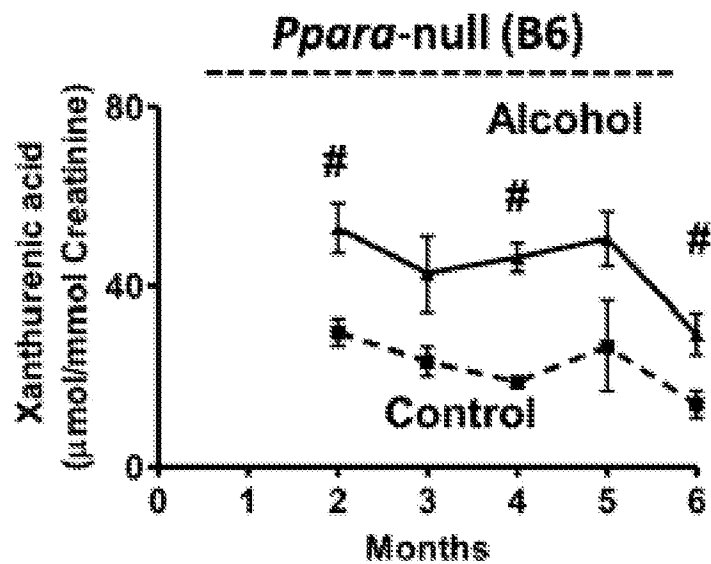


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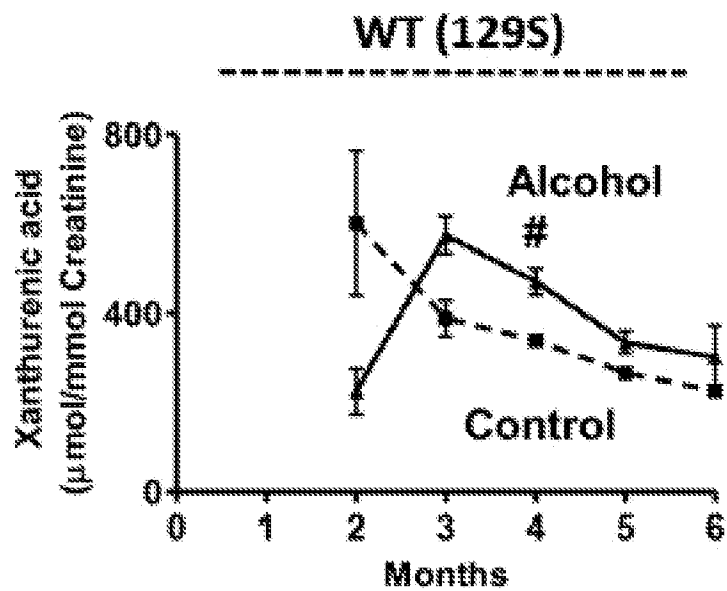


Figure 7F

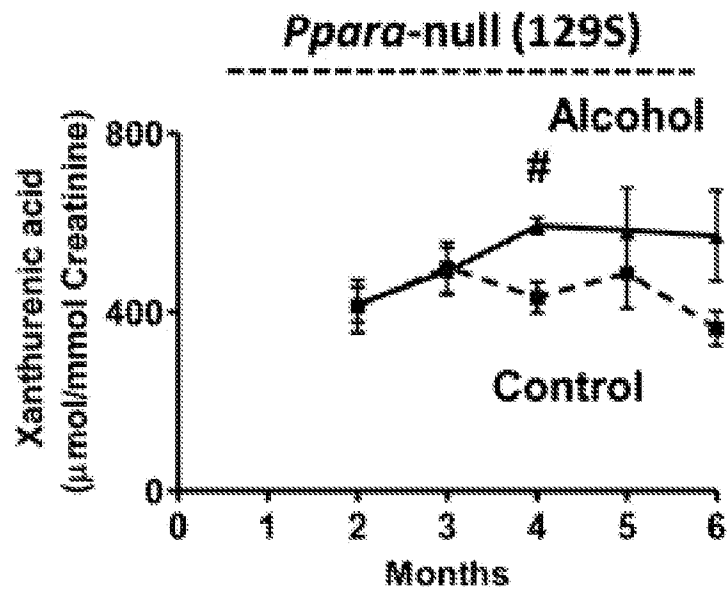


Figure 8A

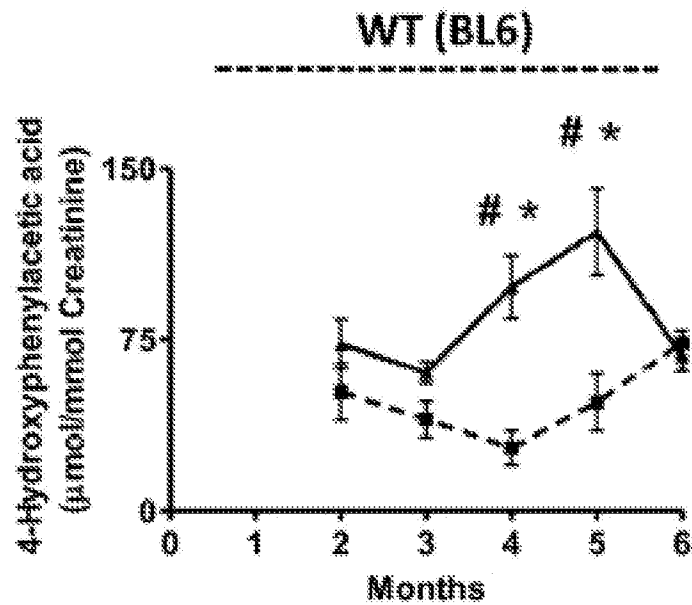


Figure 8B

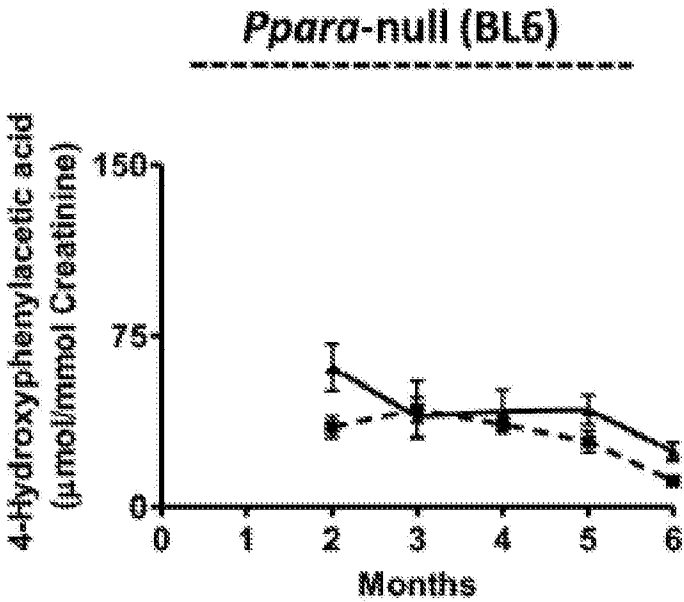


Figure 8C

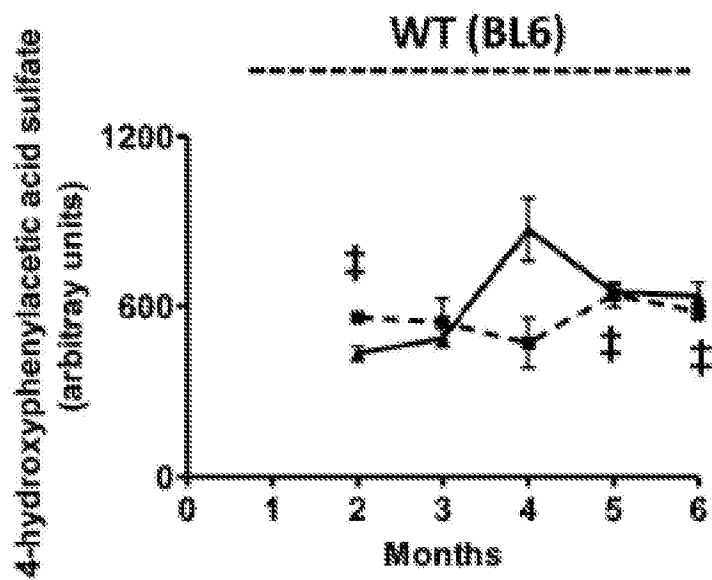


Figure 8D

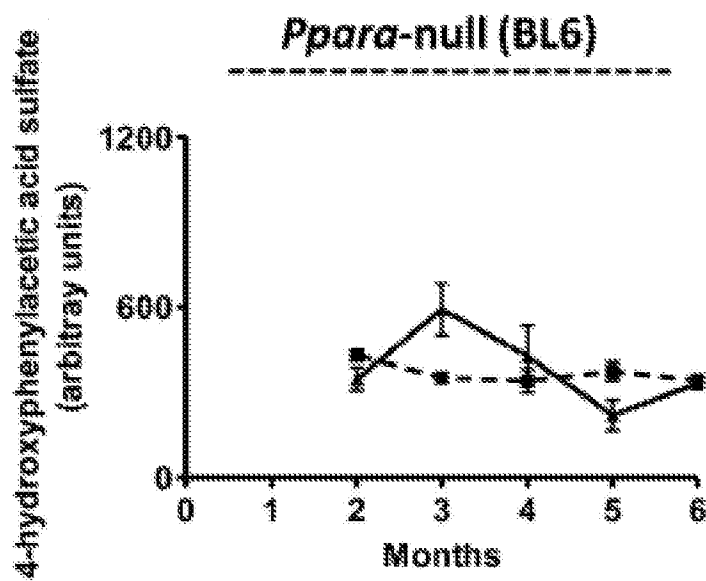


Figure 9A

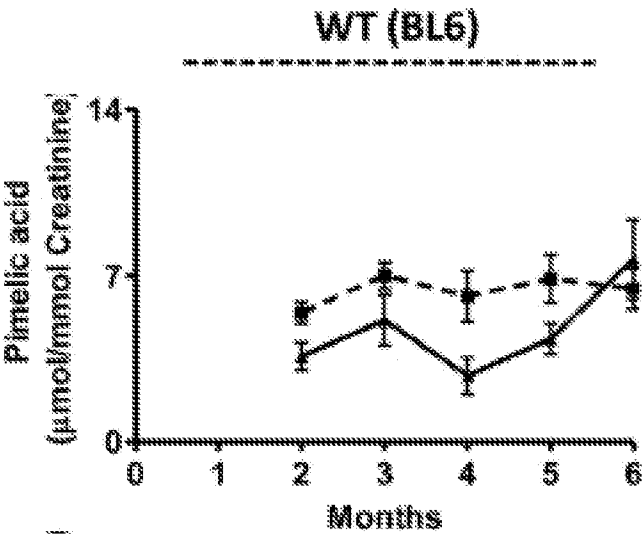


Figure 9B

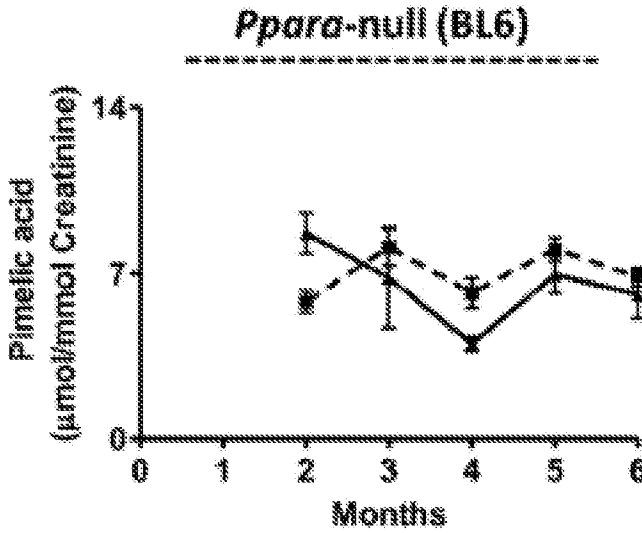


Figure 9C

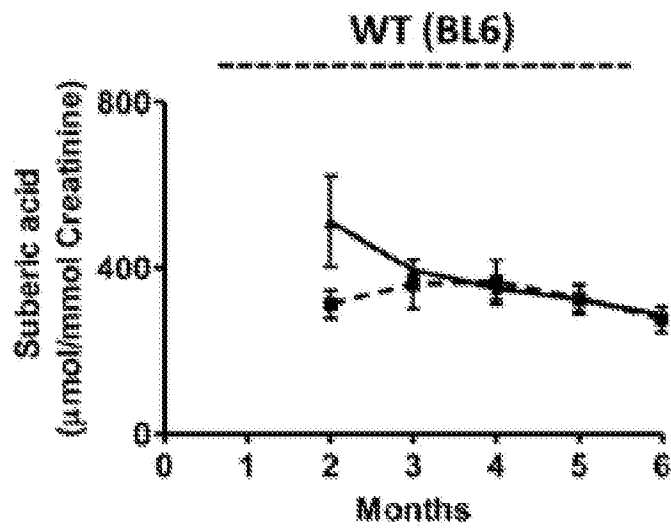


Figure 9D

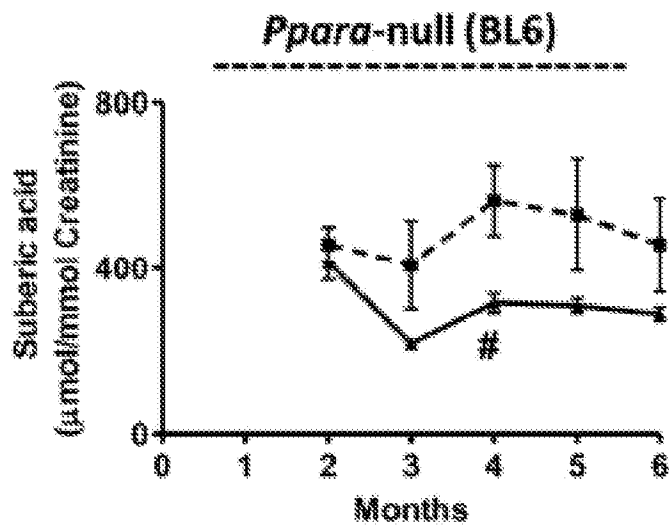


Figure 9E

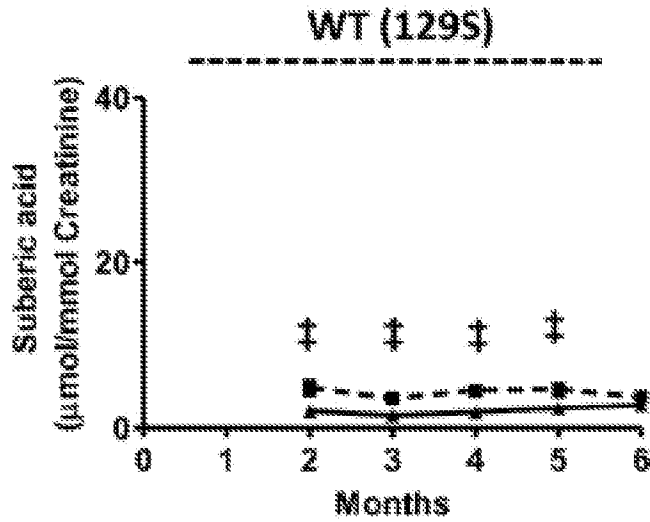


Figure 9F

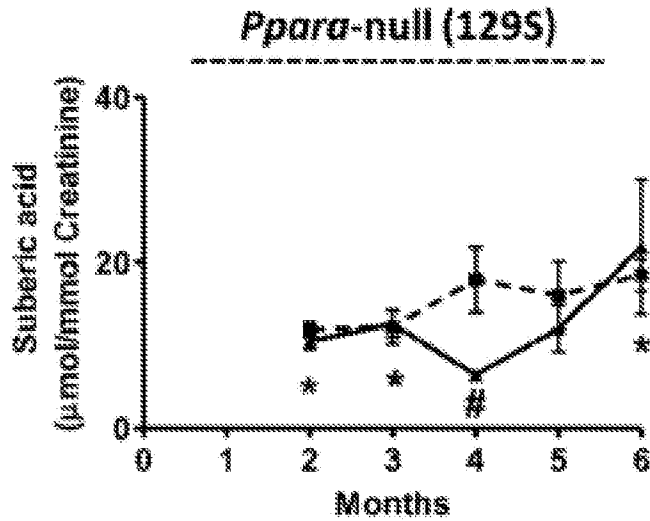


Figure 10A

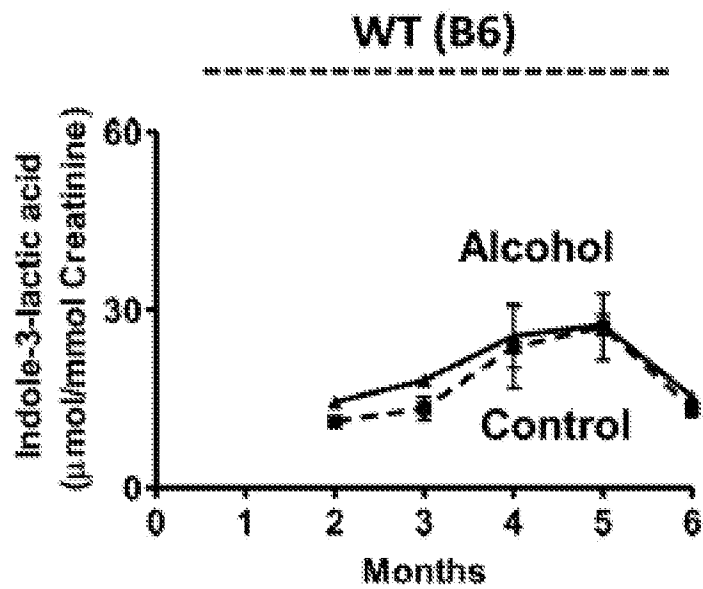


Figure 10B

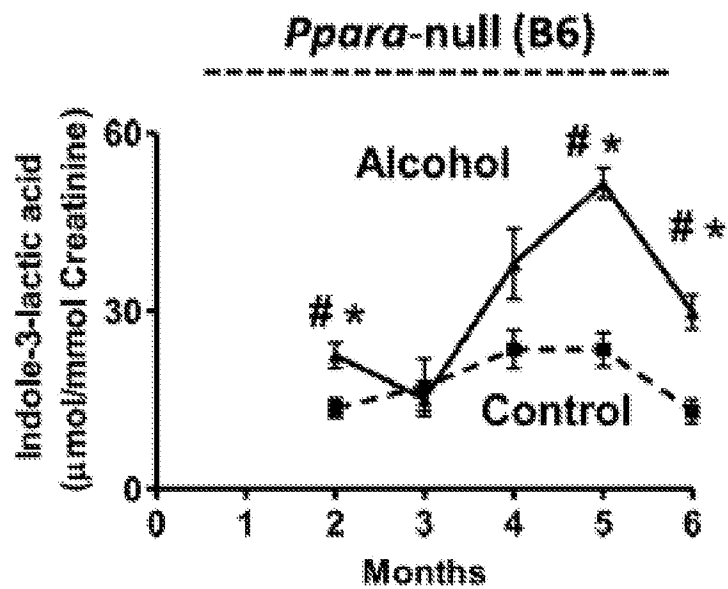


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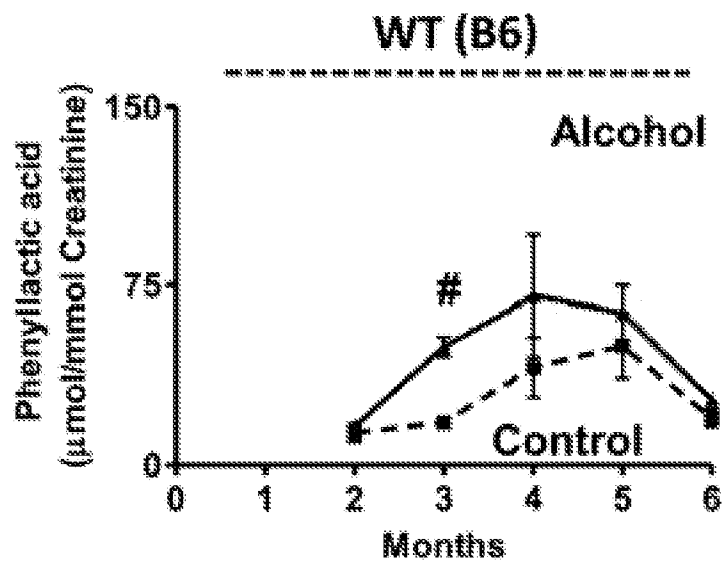


Figure 10D

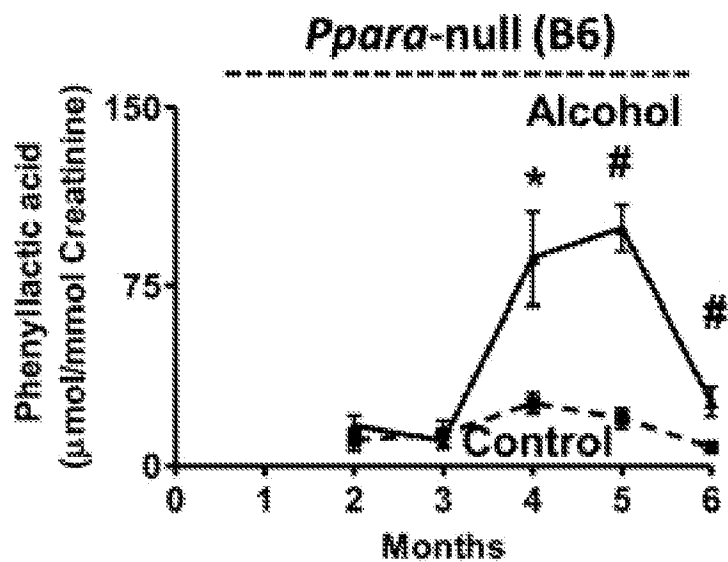


Figure 10E

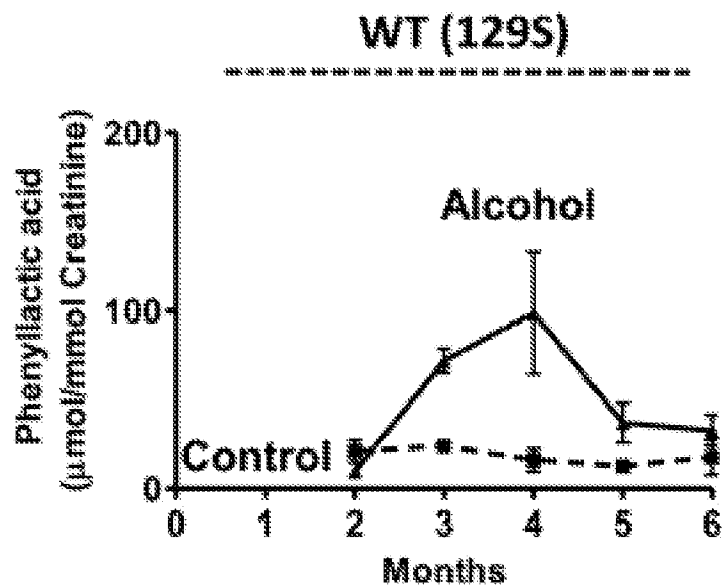


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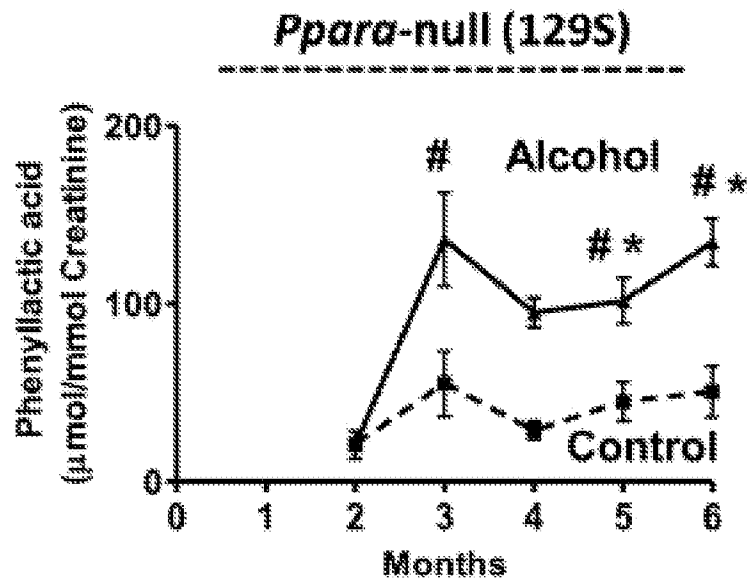


Figure 11A

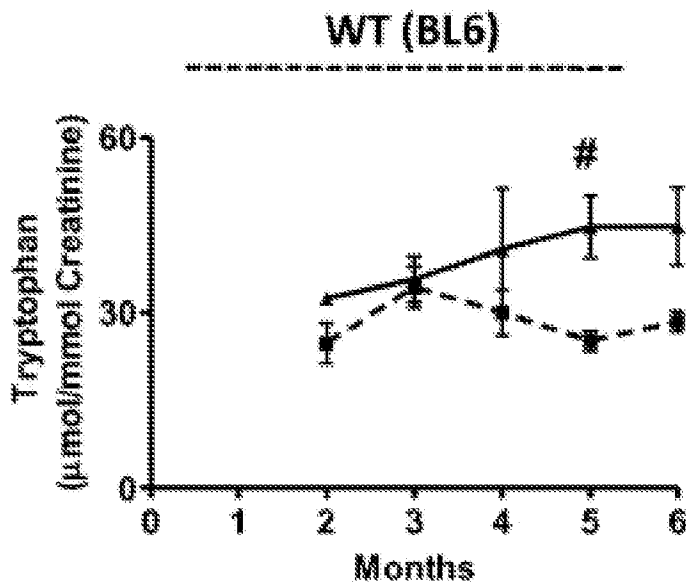


Figure 11B

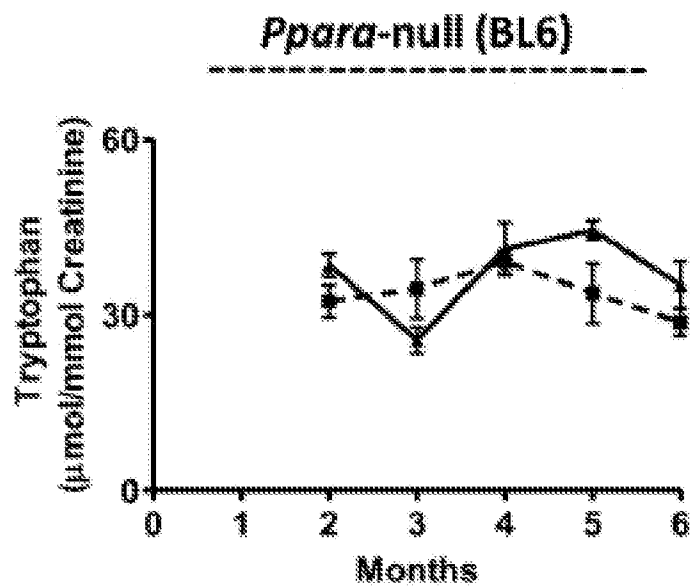


Figure 11C

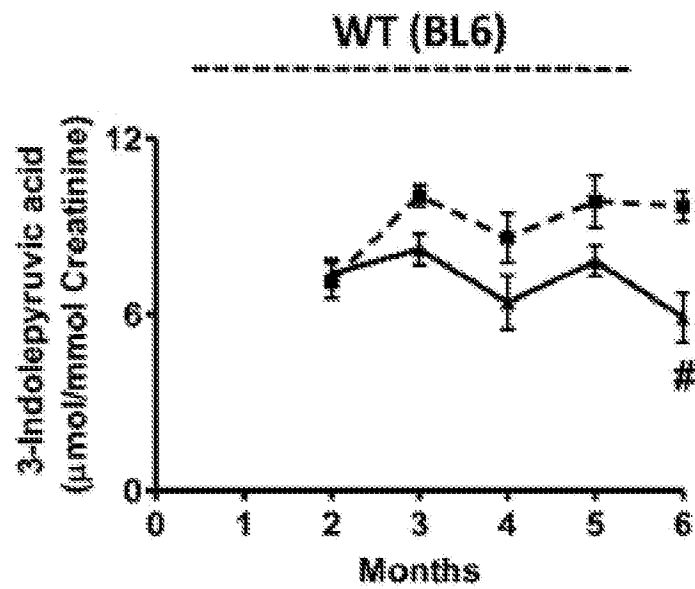


Figure 11D

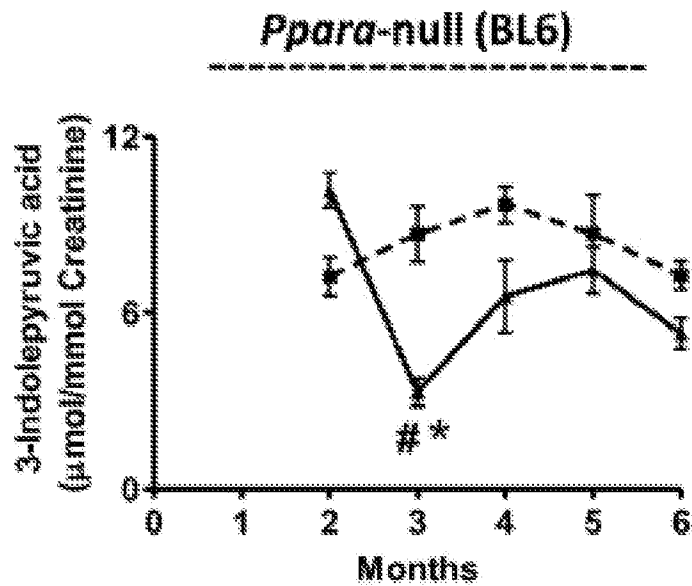


Figure 12A

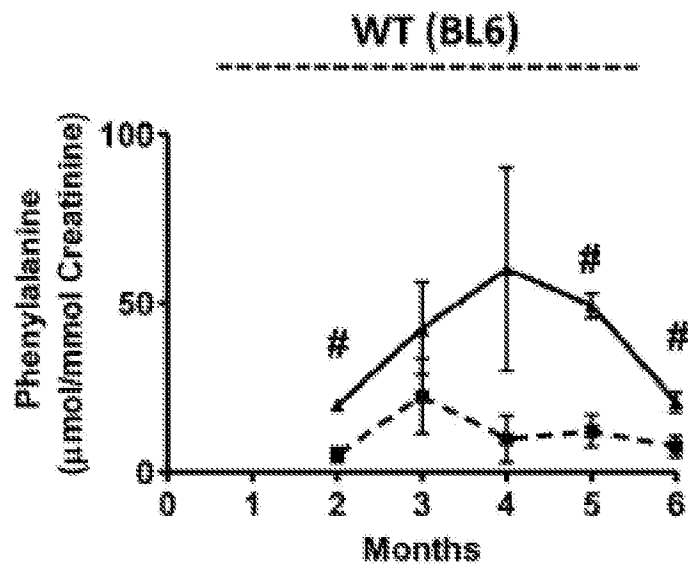


Figure 12B

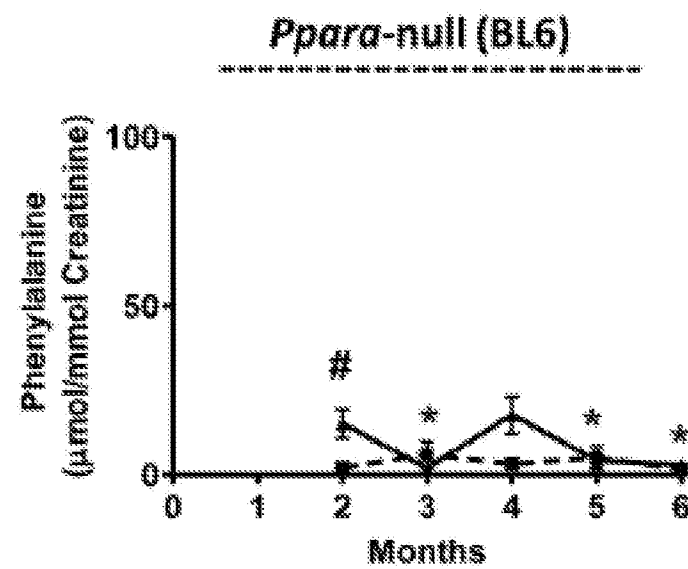


Figure 12C

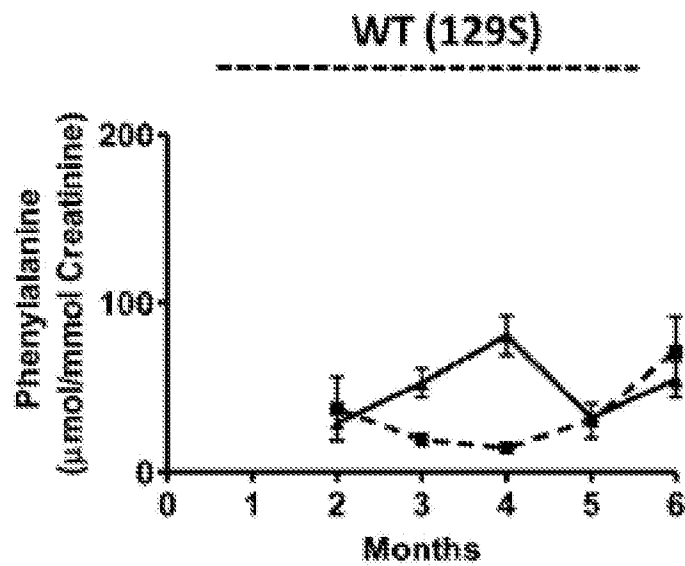


Figure 12D

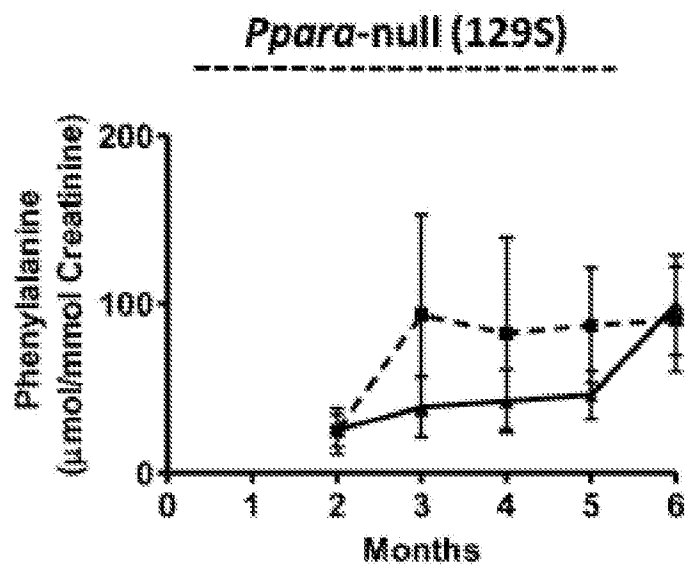
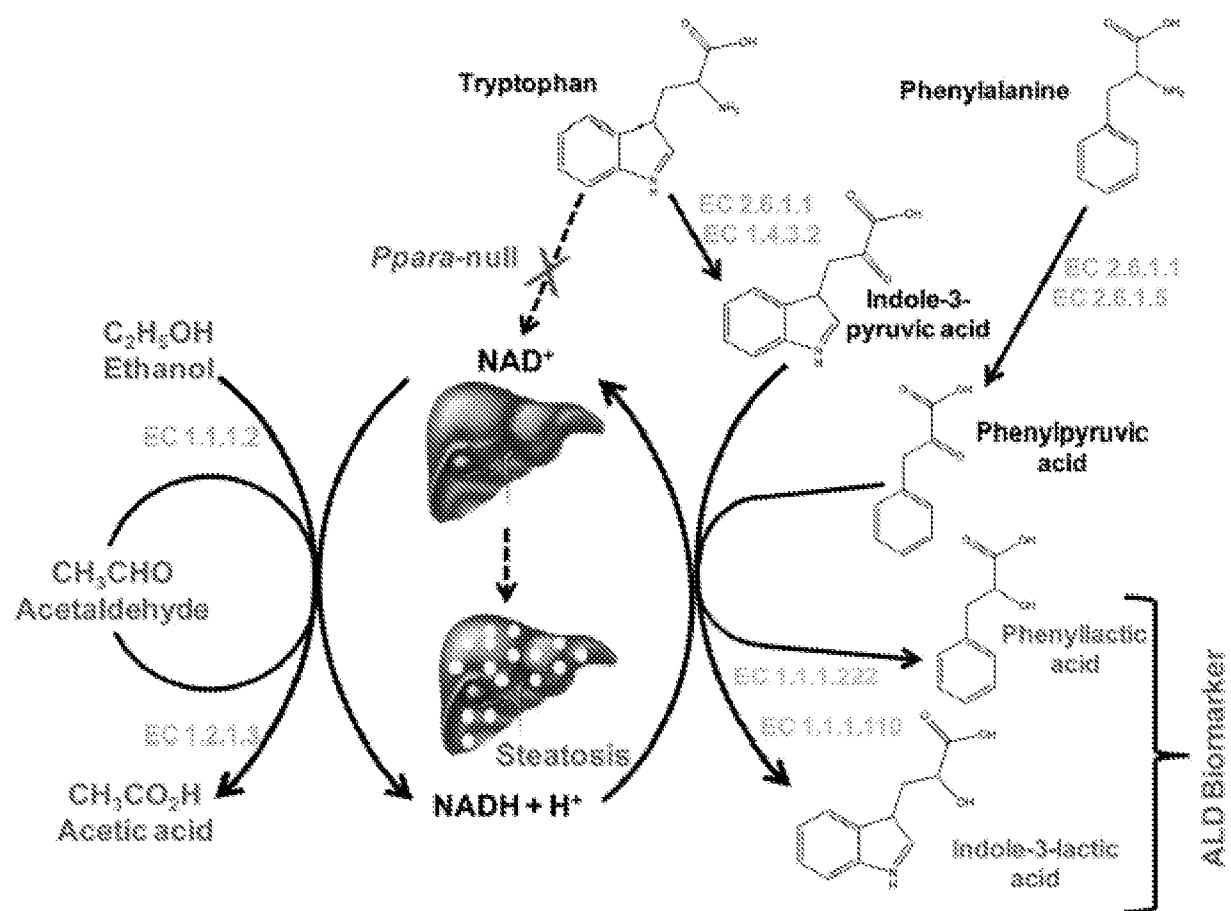


Figure 13



INTERNATIONAL SEARCH REPORT

International application No

PCT/US2012/046705

A. CLASSIFICATION OF SUBJECT MATTER
 INV. G01N33/50 G01N33/53
 ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	MANNA SOUMEN K ET AL: "UPLC-MS-based Urine Metabolomics Reveals Indole-3-lactic Acid and Phenyllactic Acid as Conserved Biomarkers for Alcohol-induced Liver Disease in the Ppara-null Mouse Model", JOURNAL OF PROTEOME RESEARCH, vol. 10, no. 9, September 2011 (2011-09), pages 4120-4133, XP002683391, ISSN: 1535-3893 the whole document ----- -/--	1-39



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

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INTERNATIONAL SEARCH REPORT

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