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(57) Abstract: The invention includes a method of identifying a subject suspected of sleep deprivation. The method comprises testing a set of biomarkers in a test sample and comparing the test level with the baseline level. The invention also includes a method of identifying a subject suspected of having cognitive impairment as a result of sleep deprivation.



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TITLE OF THE INVENTION

Biomarkers of Sleep Deprivation and Cognitive Impairment

5 CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Application No. 62/101,047, filed January 8, 2015, which application is incorporated herein by reference in its entirety.

10 STATEMENT REGARDING FEDERALLY SPONSORED
RESEARCH OR DEVELOPMENT

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20 BACKGROUND OF THE INVENTION

Sleep duration has decreased in modern society, with potential implications for increased incidence of disease. Chronic restriction of sleep duration results in weight gain (Spaeth AM *et al.*, 2013, *Sleep* 36:981–990; Markwald RR *et al.*, 2013, *Proc Natl Acad Sci USA* 110:5695–5700) and has been linked to common clinical phenotypes such as
25 obesity (Singh MD *et al.*, 2005, *J Clin Sleep Med* 1:357–363; Di Milia L *et al.*, 2013, *Sleep Medicine* 14:319–323), as well as metabolic (Schmid SM *et al.*, 2014, *Lancet Diabetes Endocrinol* 14:70012-9) and cardiovascular (Ayas NT *et al.*, 2003, *Arch Intern Med* 163:205–209) diseases. The mechanisms underlying such effects of sleep restriction are not understood, but an intriguing possibility is that sleep loss alters metabolic function, which
30 could impact many physiological systems. Indeed, links between sleep loss and metabolism have been identified, with sleep-deprived humans displaying perturbations in carbohydrate metabolism and endocrine function (Spiegel K *et al.*, 1999, *Lancet* 354:1435–1439), and

circadian metabolic processes (Möller-Levet CS *et al.*, 2013, *Proc Natl Acad Sci USA* 110:E1132–41). Recent work suggests that sleep drives metabolite clearance within the brain (Xie L *et al.*, 2013, *Science* 342:373–377) and thus may act as a reparative process at the metabolic level. However, the systemic factors that drive metabolic perturbations during sleep loss and the basis of downstream pathophysiology are largely unknown.

The human body requires on average 6-9 hours of sleep per day for normal cognitive function. Sleep loss impairs the ability to process information and make decisions. As such, sleep deprivation results in cognitive impairment.

Therefore, there is a need in the art for a method to determine sleep deprivation and a method to determine cognitive impairment in an individual for the purpose of public safety and optimal health. The present invention satisfies this need.

SUMMARY OF THE INVENTION

The present invention includes a method of identifying and treating a subject suspected of having sleep deprivation. The method comprises the following steps: determining the test level of a set of biomarkers in a test sample obtained from a subject, where the set of biomarkers are one or more metabolites selected from the metabolites in Table 1; and comparing the test level of the set of biomarkers in the test sample with a baseline level of the set of biomarkers. If the test level of the set of biomarkers in the test sample is significantly different from the baseline level of the set of biomarkers, then the subject is suspected to have sleep deprivation and treatment is initiated. In one embodiment, the subject is a human. In another embodiment, the test sample is a blood sample. In certain embodiments, the set of biomarkers comprises two metabolites selected from Table 1. In other embodiments, the set of biomarkers comprises three metabolites selected from Table 1. In yet other embodiments, the set of biomarkers comprises four metabolites selected from Table 1. In yet other embodiments, the two metabolites for detecting sleep deprivation of a subject are oxalic acid and DG 36:3.

The baseline level of the biomarkers is determined in a sample obtained from the subject when the subject is not sleep-deprived. In one embodiment, the subject has slept between about 6 hours to about 10 hours prior to determining the baseline level. In another embodiment, the subject has slept for about 7 hours prior to determining the baseline level. In yet another embodiment, the subject has slept for about 8 hours prior to determining the

baseline level. In yet another embodiment, the subject has slept for about 9 hours prior to determining the baseline level.

In one embodiment, when the test level of the set of biomarkers is elevated, compared to the baseline level, the subject is suspected of having sleep deprivation. In
5 another embodiment, when the test level of the set of biomarkers is reduced, compared to the baseline level, the subject is suspected of having sleep deprivation.

The present invention also relates to a method of identifying and treating a subject suspected of having cognitive impairment as a result of sleep deprivation, the method comprises: a) obtaining a test sample from the subject; and b) determining the
10 presence of a set of biomarkers in the test sample, wherein the set of biomarkers are one or more metabolites selected from the metabolites in Table 7, wherein the presence of the set of biomarkers in the test sample is an indication that the subject has cognitive impairment and treatment is initiated. In one embodiment, the set of biomarkers comprises PE (36:2), LPC (16:0), LPC (16:1), LPC (18:2), LPC (20:3), LPC (20:5), capric acid, and 17-
15 methyltestosterone. In another embodiment, the test sample is a blood sample. In yet another embodiment, the cognitive impairment is assessed by cognitive variables comprising PVT lapses and errors, PVT speed (1/RT), DSST total number correct, and DSTC (total number correct).

20 BRIEF DESCRIPTION OF THE DRAWINGS

For the purpose of illustrating the invention, there are depicted in the drawings of certain embodiments of the invention. However, the invention is not limited to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

25 FIG. 1A schematically illustrates the protocols of animal Study A (left panel) and animal Study B (right panel). Animals in Study A were subjected to sleep restriction (SR) by forced activity (FA) for 5 days (SR1-SR5) while a forced activity control group was subjected to FA for half the time at double speed (2 x FA) for 5 days (FA1-FA5). White bars indicate periods of forced activity. Blood samples for metabolomics were taken
30 just before the end of the light phase on day 0 (baseline), day 1 (acute SR), and day 5 (chronic SR). Animals in Study B (right panel) were similarly sleep-restricted, followed by 3 days of unrestricted recovery sleep (Rec1-Rec3). Blood samples were taken as on day 0

(baseline), day 1 (acute SR), and day 5 (chronic SR) and at Rec1 (acute recovery), and Rec3 (extended recovery).

FIG. 1B illustrates the overlap in metabolites noted as significantly different on SR day 1 and SR day 5. FA denotes a forced activity control from Study A. Suffix a and b denotes Study A and Study B, respectively.

FIG. 1C illustrates the Z-score plots of metabolites (ordered by mean change) that were significantly different between SR and baseline time points in Study A. Numeric identifiers indicate unidentified metabolites, and details of their retention index and quantified m/z values are available in Table 5.

FIG. 2A illustrates metabolites altered only under acute sleep restriction. Each data point represents the mean of 10 measurements across each group for each feature. FA1- forced activity control day 1, SR1 – sleep restriction day 1. Suffix a and b denotes Study A and Study B, respectively. The darker colors in the heatmaps indicate a reduction compared to baseline whereas lighter colors indicate an elevation compared to baseline.

FIG. 2B illustrates metabolites altered only under chronic sleep restriction. The metabolites are divided into those that recovered (upper panel) and those that remained altered (lower panel) following 3 days recovery sleep.

FIG. 2C illustrates metabolites altered under both acute and chronic sleep restriction. The metabolites are divided into those that recovered (upper panel) and those that remained perturbed (lower panel).

FIG. 3A schematically illustrates the protocol for human sleep restriction study consisting of two baseline (BL) nights followed by five nights of sleep restriction and one night recovery sleep.

FIG. 3B is a heatmap illustrating metabolites that are significantly different between baseline and SR among those that recovered to pre-SR levels. Each data point represents the mean of measurements from 10 individuals across each group for each feature.

FIG. 3C is a heatmap illustrating metabolites that are significantly different between baseline and SR among those that remained perturbed and did not recover to pre-SR levels. Each data point represents the mean of measurements from 10 individuals across each group for each feature.

FIG. 4A is a Venn diagram indicating two identified metabolites common to both rat and human studies.

FIG. 4B is a table showing statistical values of oxalic acid (i.e. oxalate) and Diacylglycerol (DG) 36:3 for rat and human datasets.

5 FIG. 4C is a bootstrapped hierarchical clustering tree indicating metabolites most correlated to oxalate. Values indicate the approximately unbiased probability percent computed by multiscale bootstrap resampling. Those found to be significant are labeled with an asterisk.

10 FIG. 5 is a chart illustrating the fraction of each lipid class found to be significant as a function of all lipids measured in that class.

FIG. 6A illustrates absolute mass spectral counts for each measured metabolite across the three conditions measured for human plasma (BL-baseline; SR5-sleep restriction day 5; Rec-recovery). Each animal's measurements are connected by a solid line.

15 FIG. 6B illustrates absolute mass spectral counts for each measured metabolite across the three conditions measured for rat plasma (BL-baseline; SR5-sleep restriction day 5; Rec3-recovery day 3). Each animal's measurements are connected by a solid line.

20 FIG. 7 is a principal component analysis plot from the rat study. Each point represents a single plasma sample from an individual animal, and is colored by the timepoint as indicated. The position of each point is determined by the multivariate combination of all measured metabolites for each sample.

25 FIGS. 8A-8D illustrate OPLS regression analysis of the plasma metabolome and individual cognitive variables: Four different OPLS regression models were generated, one for each of the four cognitive variables. Each cognitive variable was plotted against the predicted values of the same variable computed from a seven-fold cross validation. FIG. 8A is a Venn diagram illustrating the overlap of significantly associated metabolites (OPLS VIP>1.0) across the four models. Eight metabolites were commonly associated with all four variables. FIG. 8B is a scatter plot showing the Psychomotor Vigilance Test lapses (≥ 500 ms reaction times) and errors [false starts (errors of commission)] (PVT lapses + errors) plotted against the predicted values of the same variable. Results from significant OPLS models are shown with R^2 values indicated. FIG. 8C is a scatter plot illustrating

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Psychomotor Vigilance Test mean response speed or reciprocal response time (PVT 1/RT) plotted against the predicted values of the same variable. Results from significant OPLS models are shown with R^2 values indicated. FIG. 8D is a scatter plot illustrating Digit Symbol Substitution Task (DSST) variable plotted against the predicted values of the same variable. Results from significant OPLS models are shown with R^2 values indicated.

FIGs. 9A-9B illustrate the relative number of lipid species and their direct association with individual cognitive variables. FIG. 9A is a radial plot illustrating the relative number of lipids associated with each cognitive variable. The lipids were extracted from respective OPLS models; the number of individual species was normalized to the total number detected in each class followed by normalization of the total number of lipids associated with each variable. FIG. 9B illustrates the average loading values of each lipid class plotted against each cognitive variable.

FIG. 10 illustrates the fraction of small molecular weight metabolites and their association with individual cognitive variables: Radial plot showing the fraction of each small molecule class associated with each cognitive variable. The small molecules were extracted from respective OPLS models; the number of individual species was normalized to the total number of small molecules associated with each variable.

FIGs. 11A-11E illustrate models of the differences in lipid levels from baseline to sleep restriction day 5: The absolute difference of lipid levels from baseline to SR5 was modeled with the absolute difference of each cognitive variable from baseline to SR5 using OPLS regression. Only PVT lapses and errors and DSTC yielded significant models. FIG. 11A is a scatter plot illustrating PVT lapses and errors plotted against the predicted values of the same variable computed from a seven-fold cross validation. Results from significant OPLS models are shown with R^2 values indicated. FIG. 11B is a scatter plot illustrating DSTC plotted against the predicted values of the same variable computed from a seven-fold cross validation. Results from significant OPLS models are shown with R^2 values indicated. FIGs. 11C-11E are segments of a cluster analysis illustrating those lipids and other metabolites closely clustering with lipids which significantly correlated with the cognitive variables.

FIG. 12 is a schematic illustrating the experimental protocol. Subjects participated in either a sleep restriction or control protocol. In the sleep restriction condition, subjects received two baseline nights of 10h or 12h time-in-bed (TIB) per night

(BL1-2; 2200h–0800h/1000h) followed by five nights of sleep restriction of 4h TIB per night (SR1-5; 0400h–0800h) and one night of 12h TIB recovery sleep (R1; 2200h–1000h). In the control condition, subjects underwent the same procedures as in the sleep restriction condition, except they were allowed 10h TIB every night (BL1-CD6; 2200h–0800h). A
5 cognitive test was administered every 2h while awake. Blood samples for metabolomics (M) were collected in the morning following fasting as indicated. Black bars = sleep periods; white bars = wake periods; and gray bar = 0800h–1000h additional time-in-bed sleep at BL2 in sleep restriction protocol.

FIG. 13 illustrates the total number of small molecules and lipids detected in
10 the study (left panel) and the number of various small molecule and lipid species detected (right panel).

DETAILED DESCRIPTION OF THE INVENTION

The present invention includes methods and uses of a novel set of
15 biomarkers for identifying a subject suspected of having sleep deprivation. Further, the present invention includes a method of detecting the biomarkers in a biological sample, and a kit useful in the practice of invention.

Definitions

20 As used herein, each of the following terms has the meaning associated with it in this section. Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, and organic chemistry are those
25 well-known and commonly employed in the art.

As used herein, the articles “a” and “an” refer to one or to more than one (*i.e.* to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

As used herein, the term “about” will be understood by persons of ordinary
30 skill in the art and will vary to some extent on the context in which it is used. As used herein when referring to a measurable value such as an amount, a concentration, a temporal duration, and the like, the term “about” is meant to encompass variations of $\pm 20\%$ or $\pm 10\%$,

more preferably $\pm 5\%$, even more preferably $\pm 1\%$, and still more preferably $\pm 0.1\%$ from the specified value, as such variations are appropriate to perform the disclosed methods.

As used herein, the term “baseline level” refers to the concentration of a metabolite in a biological sample prior to sleep deprivation, in other words, under well-
5 rested conditions.

As used herein, the term “biomarker” refers to a metabolite that can be used to determine sleep deprivation of a subject.

As used herein, the term “significantly different” indicates that the difference between baseline level and test level of a biomarker is no less than 20%.

10 As used herein, the term “cognitive impairment” refers to a subject having trouble remembering, learning new things, concentrating, attending and responding.

As used herein, the terms “comprising,” “including,” “containing” and “characterized by” are exchangeable, inclusive, open-ended and does not exclude additional, unrecited elements or method steps. Any recitation herein of the term
15 “comprising,” particularly in a description of components of a composition or in a description of elements of a device, is understood to encompass those compositions and methods consisting essentially of and consisting of the recited components or elements.

As used herein, the term “consisting of” excludes any element, step, or ingredient not specified in the claim element.

20 As used herein, the term “consisting essentially of” does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim.

“Instructional material,” as that term is used herein, includes a publication, a recording, a diagram, or any other medium of expression that can be used to communicate
25 the usefulness of the composition and/or compound of the invention in a kit. The instructional material of the kit may, for example, be affixed to a container that contains the compound and/or composition of the invention or be shipped together with a container that contains the compound and/or composition. Alternatively, the instructional material may be shipped separately from the container with the intention that the recipient uses the
30 instructional material and the compound cooperatively. Delivery of the instructional material may be, for example, by physical delivery of the publication or other medium of expression communicating the usefulness of the kit, or may alternatively be achieved by

electronic transmission, for example by means of a computer, such as by electronic mail, or download from a website.

As used herein, the terms “sleep deprivation” and “sleep debt” are used interchangeably. Both refer to a condition of not having enough sleep.

5 As used herein, a “subject” may be a human or non-human mammal or a bird. Non-human mammals include, for example, livestock and pets, such as ovine, bovine, porcine, canine, feline and murine mammals. Preferably, the subject is human.

As used herein, the term “test level” refers to the concentration of a biomarker in a biological sample from a subject who will be evaluated as to whether the
10 subject may have sleep deprivation.

As used herein, an “instructional material” includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of the compositions and methods of the invention. The instructional material of the kit of the invention may, for example, be affixed to a container
15 which contains the reagents, and/or composition of the invention or be shipped together with a container which contains the reagents, and/or composition. Alternatively, the instructional material may be shipped separately from the container with the intention that the instructional material and the compound be used cooperatively by the recipient.

Throughout this disclosure, various aspects of the invention may be
20 presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range and, when appropriate, partial integers of the numerical values
25 within ranges. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

30 **Abbreviations:**

AA – Amino acids and related molecules; Carb – carbohydrates and related molecules; Carnitines – Fatty acid conjugated carnitines/free carnitines; CE – Cholesterol

and related molecules and esters; DG – Diglycerides and Diacylglycerol species; FA/Lipids – Fatty acids and lipid related metabolites; LPC and lysoPC – Lysophosphatidylcholine and Lysophosphatidylcholine species; LPE –Lysophosphatidylethanolamine; OA – Short chain organic acids and related metabolites; PC – Phosphatidylcholine and Phosphatidylcholine species; PE – Phosphatidylethanolamine; Plasmalogen – Plasmalogen PE and PCs; SM – Sphingomyelin; TG – Triglycerides and Triacylglycerol species.

Description

In one aspect, the present invention includes a method of identifying and treating a subject suspected of having sleep deprivation. The method comprises the following steps: determining the test level of a set of biomarkers in a test sample obtained from a subject, where the set of biomarkers are one or more metabolites selected from the metabolites in Table 1; and comparing the test level of the set of biomarkers in the test sample with a baseline level of the set of biomarkers. If the test level of the set of biomarkers in the test sample is significantly different from the baseline level of the set of biomarkers in a way that indicates sleep deprivation, then treatment is initiated. Such treatment may comprise sleeping, where the sleeping occurs with or without pharmaceutical intervention. The level of the tested biomarkers may be elevated or may be reduced when compared with the baseline levels. The data presented in Tables 1, 3A-3B, 4, 5, 7, 8, and 9 or Figures 1C, 2A-2C, 3B-3C, 4B, 6A-6B, 8A-8D, 9A-9B, 10, and 11A-11D, document how the levels of the tested biomarkers vary compared with baseline levels for subjects with sleep deprivation.

Table 1: Important Metabolites

	Metabolites	p	FDR
1	Tryptophan	<0.001	0
2	TG (54:3)	<0.001	0
3	Phenylalanine	<0.001	0
4	PC (38:3)	<0.001	0
5	PC (36:6)	<0.001	0
6	LPC (20:3)	<0.001	0
7	LPC (14:0)	<0.001	0
8	dodecanol	<0.001	0
9	Carnitine C5:0	<0.001	0
10	Acylcarnitine C12:0	<0.001	0
11	Acylcarnitine C10:0	<0.001	0
12	PE (36:1)	0.002	0.08635898
13	PC (38:2)	0.002	0.08635898
14	octadecanol	0.002	0.08635898
15	myristic acid	0.003	0.11226667
16	TG (53:1)	0.005	0.16192307
17	TG (52:2)	0.005	0.16192307
18	pipecolic acid	0.005	0.16192307
19	oxalic acid	0.005	0.16192307
20	LPC (16:1)	0.005	0.16192307
21	Acylcarnitine C18:1	0.005	0.16192307
22	lysoPC 16:0	0.006	0.1742069
23	TG (46:0)	0.007	0.18135385
24	PC (32:1)	0.007	0.18135385
25	lysoPC 18:2	0.007	0.18135385
26	DG (36:3)	0.007	0.18135385
27	methionine	0.008	0.18974648
28	TG (54:4)	0.009	0.19430768
29	TG (52:3)	0.009	0.19430768
30	TG (48:1)	0.009	0.19430768
31	DG (36:2)	0.009	0.19430768

Notes:

DG- Diacylglycerol species

TG- Triacylglycerol species

PC – Phosphatidylcholine species

lysoPC/LPC – lysophosphatidylcholine species

The baseline level of a biomarker can be obtained by analyzing a biomarker in a biological sample from a subject, when the subject is not sleep-deprived (i.e. when the

subject has slept for a certain number of hours prior to the baseline level determination). Normal sleep time can vary from one subject to another. In one embodiment, the sleep time is in the range of about 5 hours to about 10 hours. In one instance, the sleep time is about 6 hours. In another instance, the sleep time is about 7 hours. In yet another instance, the sleep time is about 8 hours. In yet another instance, the sleep time is about 9 hours.

The baseline level of a biomarker may vary from one subject to another. In some instances, the baseline level of a biomarker from a first subject can be used to determine sleep deprivation for a second subject when the first subject and the second subject have same or very similar physiological features. However, for more accurate identification, it is preferably to use the baseline level from the same subject under test.

The difference between the test level and baseline level needs to be more than about 20% to make an accurate determination of sleep deprivation. In one instance, the difference is about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 100%, about 110%, about 120%, about 130%, about 140%, about 150%, about 160%, about 170%, about 180%, about 190%, about 200%, about 250%, about 300%, about 350%, about 400%, about 450%, about 500%, or about 550%.

A set of biomarkers, as used for identifying sleep deprivation, refer to one or more metabolites selected from Table 1. In certain embodiments, a set of biomarkers comprises 1 to 20 metabolites selected from Table 1. In one embodiment, a set of biomarkers comprise one metabolite selected from Table 1. In another embodiment, a set of biomarkers comprise two metabolites selected from Table 1. In yet another embodiment, a set of biomarkers comprise three metabolites selected from Table 1. In yet another embodiment, a set of biomarkers comprise four metabolites selected from Table 1. In yet another embodiment, a set of biomarkers comprise five metabolites selected from Table 1. In yet another embodiment, a set of biomarkers comprise six metabolites selected from Table 1. In yet another embodiment, a set of biomarkers comprise seven metabolites selected from Table 1. In yet another embodiment, a set of biomarkers comprise eight metabolites selected from Table 1.

In one embodiment, a set of biomarkers comprise oxalic acid and DG (36:3). In one instance, when the test level of oxalic acid is about 45% reduced compared to the baseline level and the test level of DG (36:3) is reduced compared to the baseline level, the subject is determined to have sleep deprivation.

In yet another embodiment, a set of biomarkers comprises lysophosphatidylcholine (LPC) 14:0, lysophosphatidylcholine (LPC) 20:3 and PC 38:3. In one non-limiting example, if LPC 14:0 and LPC 20:3 and PC 38:3 levels are increased about 50%, relative to baseline, the subject is determined to have sleep deprivation.

5 In one embodiment, the subject is a human.

The biological sample described herein may be urine or blood. Blood includes whole blood, blood plasma, and blood serum. In one embodiment, the biological sample is blood plasma.

The test level of a set of biomarkers can be elevated or reduced or both
10 compared to the baseline level of the same subject. In one embodiment, the set of biomarkers comprises one or more metabolites having a higher test level. In another embodiment, the set of biomarkers comprise one or more metabolites having a reduced test level. In yet another embodiment, the set of biomarkers comprises one or more metabolites having a higher test level and one or more metabolites having a reduced test level. In some
15 instances, one or more metabolites recover after recovery sleep.

In another aspect, the present invention includes a method of identifying a subject suspected of having cognitive impairment as a result of sleep deprivation. The method comprises: a) obtaining a test sample from the subject; and b) determining the presence of a set of biomarkers in the test sample, wherein the set of biomarkers are one or
20 more metabolites selected from the metabolites in Table 7, wherein the presence of the set of biomarkers in the test sample is an indication that the subject has cognitive impairment and treatment is initiated. Such treatment may include sleeping, with or without pharmaceutical intervention. In one embodiment, the set of biomarkers for predicting a subject suspected of having cognitive impairment comprises phosphatidylethanolamine
25 (PE) (36:2), LPC (16:0), LPC (16:1), LPC (18:2), LPC (20:3), LPC (20:5), capric acid and 17-methyltestosterone. In another embodiment, the subject is a human.

Cognitive impairment is assessed using cognitive tests comprising the following objective evaluations: the Digit Symbol Substitution Task (DSST), a computerized version of the cognitive performance task bearing the same name in the
30 Wechsler Adult Intelligence Scale (Wechsler, 1997); the Digit Span task, a test of working memory storage capacity, given in both the forward and backward versions (Wechsler, 1997) and summed to produce a total number correct measure for analysis; and the 10-

minute Psychomotor Vigilance Test (PVT), a cognitive test of sustained attention utilizing reaction times as an assay of behavioral alertness (Dinges and Powell, 1985; Lim, 2008; Basner and Dinges, 2011). Subjects remained seated throughout the cognitive test periods. Outcome measures were as follows: DSST total number correct; DS total number correct (sum of forward and backward versions) (DSTC), PVT lapses (≥ 500 ms reaction times) + errors [false starts (errors of commission)] (PVT lapses + errors), and PVT mean response speed or reciprocal response time (PVT 1/RT). DSST, DSTC, PVT lapses + errors, and PVT 1/RT are also called cognitive variables.

The methods described herein can be readily implemented in software that can be stored in computer-readable media for execution by a computer processor. For example, the computer-readable media can be volatile memory (e.g., random access memory and the like) and/or non-volatile memory (e.g., read-only memory, hard disks, floppy disks, USB flash drives, portable hard drives, compact discs, and any other forms of electronic memory available to the skilled artisan).

Additionally or alternatively, the methods described herein can be implemented in computer hardware such as an application-specific integrated circuit (ASIC).

Additionally or alternatively, the methods described herein can be also readily implemented in a system comprising an assay determining the test level of a set of biomarkers described herein; a computer hardware; and a software program stored in computer-readable media extracting the test level from the assay; and outputting the result whether the subject has sleep deprivation.

Methods for Detecting

Detection of a metabolite described in Table 1 and Table 7 is well known in the art. The test level and the baseline level of a metabolite described in Table 1 and Table 7 can be determined by one of ordinary skill in the art without undue experimentation. Nonlimiting examples of methods that may be used to determine metabolite concentration may include gas chromatography–mass spectrometry (GC-MS); hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS); and charged surface hybrid column-quadrupole time of flight-mass spectrometry (CSH-qTOF-MS).

Kit

The present invention also includes a kit for identifying sleep deprivation or cognitive impairment as a result of sleep deprivation in a subject. A variety of kits having different components are contemplated by the current invention. In one embodiment, the
5 kit for identifying sleep deprivation comprises reagents to detect and quantify a set of biomarker comprising one or more metabolites selected from Table 1, and instruction material for using the kit. In another embodiment, the kit for identifying sleep deprivation comprises reagents to detect and quantify test level and baseline level of oxalic acid and DG (36:3) in a blood sample of a human, and instruction material for using the kit. In yet
10 another embodiment, the kit for identifying cognitive impairment as a result of sleep deprivation comprises reagents to detect and quantify a set of biomarker comprising one or more metabolites selected from Table 7, and instruction material for using the kit. In another embodiment, the kit for identifying cognitive impairment as a result of sleep deprivation comprises reagents to detect and quantify test level of PE (36:2), LPC (16:0),
15 LPC (16:1), LPC (18:2), LPC (20:3), LPC (20:5), capric acid and 17-methyltestosterone in a blood sample of a human, and instruction material for using the kit.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures, embodiments, claims, and examples described herein. Such equivalents were considered to
20 be within the scope of this invention and covered by the claims appended hereto. For example, it should be understood, that modifications in reaction conditions, including but not limited to reaction times, reaction size/volume, and experimental reagents, such as solvents, catalysts, pressures, atmospheric conditions, e.g., nitrogen atmosphere, and reducing/oxidizing agents, with art-recognized alternatives and using no more than routine
25 experimentation, are within the scope of the present application.

It is to be understood that wherever values and ranges are provided herein, all values and ranges encompassed by these values and ranges, are meant to be encompassed within the scope of the present invention. Moreover, all values that fall within these ranges, as well as the upper or lower limits of a range of values, are also contemplated
30 by the present application.

The following examples further illustrate aspects of the present invention. However, they are in no way a limitation of the teachings or disclosure of the present invention as set forth herein.

EXAMPLES

5 The invention is now described with reference to the following Examples. These Examples are provided for the purpose of illustration only, and the invention is not limited to these Examples, but rather encompasses all variations that are evident as a result of the teachings provided herein.

Materials and Methods

10 **Detailed Protocol for Rat Studies**

Animals and housing

 The rat studies were performed with adult male Sprague Dawley rats (Harlan, Horst, The Netherlands). Animals were individually housed under a 12h:12h light-dark cycle with lights on from 5 am-5 pm. Ambient temperature was maintained at 21 ± 1 15 °C. Standard laboratory chow food and water were provided ad libitum except where noted. The experiment was approved by the Ethical Committee of Animal Experiments of the University of Groningen.

Experimental design

 In two separate studies, adult male rats were subjected to sleep restriction for 20 a 5-day period. The animals were sleep deprived 20h per day and were allowed to rest during the last 4h of the light phase. In both study A and B, blood samples were taken for metabolic profiling on the baseline day prior to sleep restriction (day 0) and after 1 and 5 days of sleep restriction (SR 1 and 5). In study B, additional samples were taken after 1 and 3 days of unrestricted recovery sleep (R1 and 3). All blood samples were taken at the end 25 of the light phase, i.e. the main resting phase in rats.

Sleep restriction and forced activity control

 Sleep restriction and control procedures were performed using established procedures adapted from previous studies (Novati A *et al.*, 2008, *Sleep* 31:1579–1585; Roman Vet *et al.*, 2005, *Sleep* 28:1505–1510). The rats were subjected to a protocol of sleep 30 restriction for 5 days allowing them to sleep 4h per day at the end of the light phase (1-5 pm) in their home cage. The remainder of the time, the animals were kept awake by placing them in a slowly rotating drum (40 cm in diameter) driven by an engine at constant speed

(0.4 m/min). To examine whether effects of the treatment were caused by forced locomotion rather than sleep restriction, the second study included a forced activity control group. Animals of this group were housed in the same type of drums but rotating at double speed for half the time (0.8 m/min for 10h). These animals therefore walked the same distance as sleep-restricted animals, but had sufficient time to sleep. The 10h forced activity was done in the dark phase, the main activity phase of the rats.

All rats in the two experiments had continuous access to food and water, including in the rotating drums, except for the 6h period of fasting preceding the blood collection.

Sleep-restricted rats in this model generally show a temporary suppression of growth relative to baseline or home cage controls but differences with forced activity controls are small (Barf RP *et al.*, 2010, *Int J Endocrinol* 2010:819414; Barf RP *et al.*, 2012 *Physiology & Behavior* 107:322–328). In rat experiment A of the present study, sleep-restricted rats and forced activity controls had on average lost 5.1 and 0.3 g of weight after the first day of the protocol (-1.6 and -0.1% of total body weight). After 5 days, the sleep-restricted rats had lost 5.7 g relative to baseline whereas forced activity controls had gained 7.9 g (-1.7% and +2.6% respectively). Repeated measures ANOVA did not indicate an overall weight difference between the 2 groups ($P > 0.10$) but did reveal a significant treatment x time interaction effect ($F(2,36) = 14.9$; $p < 0.001$). On day 1, there was no significant difference between sleep-restricted rats and forced activity controls in body weight or change in body weight relative to baseline. On day 5, there was a trend for lower body weight in the sleep-restricted animals as compared to the forced activity controls ($t(18) = 2.1$; $p = 0.054$).

In rat experiment A of the present study, food intake was measured in the sleep restricted animals and forced activity controls on day 0, 1 and 5 (SR: 19.3 ± 1.0 g, 21.0 ± 1.6 g and 18.7 ± 1.5 g, respectively; FA: 19.5 ± 0.6 g, 21.9 ± 0.9 g, and 15.5 ± 1.0 g, respectively). Repeated measures ANOVA did not reveal significant differences between the SR and FA rats ($P > 0.10$). Hence, overall changes in food intake cannot account for the differences in metabolic profile between these groups.

Previous studies with this model have shown that plasma levels of the stress hormone corticosterone are only mildly increased during sleep deprivation and rapidly return to baseline after sleep deprivation (Meerlo P *et al.*, 2002, *J Neuroendocrinol* 14:397–

402). The elevations are in the range of increases seen when animals are feeding or are exposed to a novel cage. Moreover, corticosterone levels in sleep restricted animals were found to be lower than those in forced activity controls. Therefore, it seems unlikely that effects of sleep restriction in the present study are explained by elevated levels of stress hormones. Blood sampling: animals were fasted for 6h prior to blood sampling by removing all food from the cages. Blood samples (0.5 ml) were taken by incision of the tail and collected within 1-2 min in cold Eppendorf tubes containing EDTA. Samples were centrifuged (4°C, 2600g, 15 min) and the supernatant was stored at -80°C for later analysis.

Methods and Experimental Design for Human Metabolomics Study

Subjects

Ten healthy subjects, aged 22–50yrs (27.5 ± 5.6 yrs; 5 females), participated in one of two sleep restriction experimental protocols and four healthy subjects, aged 22–50y (37.5 ± 3.1 y; one female), participated in a control protocol. In order to be eligible for study participation, subjects met the following inclusionary criteria: age range from 22–50 yrs; physically and psychologically healthy, as assessed by physical examination and history; no clinically significant abnormalities in blood chemistry; drug-free urine samples; good habitual sleep, between 6.5-8.5 h daily duration with habitual bedtimes between 2200h- 0000h, and habitual awakenings between 0600h-0900h (verified by sleep logs and wrist actigraphy for at least one week before study entry); absence of extreme morningness or extreme eveningness, as assessed by questionnaire (Smith CS *et al.*, 1989, *J Appl Psychol* 74:728–738); absence of sleep or circadian disorders, as assessed by questionnaire (Douglass AB *et al.*, 1994, *Sleep* 17:160–167) and polysomnography; no history of psychiatric illness and no previous adverse neuropsychiatric reaction to sleep deprivation; no history of alcohol or drug abuse; and no current use of medical or drug treatments (excluding oral contraceptives). Table 2 contains subject demographic data and clinical parameters. The protocols were approved by the Institutional Review Board of the University of Pennsylvania. For all subjects, written informed consent was obtained according to the principles expressed in the Declaration of Helsinki prior to entry; all subjects received compensation for participation.

Experimental Design

Subjects participated in one of two protocols in the Sleep and Chronobiology Laboratory at the Hospital of the University of Pennsylvania and were studied for 14 or 18

consecutive days continuously, in a laboratory protocol with daily clinical checks of vital signs and symptoms by nurses (with an independent physician on call). Only data from the first seven nights of the protocols—which were procedurally identical between studies—were analyzed. In both protocols, subjects received two baseline nights of 10h or 12h time-in-bed (TIB) per night (BL1-2; 2200h–0800h/1000h) followed by five nights of sleep restriction of 4h TIB per night (SR1-5; 0400h–0800h) and one night of 12h TIB recovery sleep (R1; 2200h–1000h; FIGs. 3A and 12). In the control protocol, subjects underwent the same procedures as in the sleep restriction condition, except they were allowed 10h TIB every night (2200h–0800h). After 10–12 h of fasting, blood samples for metabolomics (see Metabolomics section for details) in the sleep restriction condition were collected in the morning after the first night of baseline (BL) sleep, after the fifth night of sleep restriction (SR), and following the night of recovery sleep (Rec). To control for morning-evening effects, sampling occurred during the two hours following waketime (between 0800h–1000h on BL1 and SR5 and between 1000h–1200h on R1). In the control condition, blood sampling occurred at 0800h for all three time points (BL1, CD5, and CD6; FIG. 13). A cognitive test was administered every 2h while awake.

Throughout the study, laboratory conditions were highly controlled in terms of environmental conditions and scheduled activities. Ambient light was fixed at <50 lux during scheduled wakefulness, and <1 lux (darkness) during scheduled sleep periods.

Ambient temperature was maintained between 22°–24°C. Subjects were restricted from exercising or engaging in strenuous activities, although they were allowed to read, play video or board games, watch television, and interact with laboratory staff to help remain awake (no visitors were permitted). Subjects were continuously monitored by trained staff to ensure adherence. The light levels were held constant at <50 lux during scheduled wakefulness and <1 lux during scheduled sleep periods. Ambient temperature was maintained between 22°–24°C. Subjects had ad libitum access to food/drink throughout the protocol. Subjects were allowed to consume food and drink at any time during the protocol other than when they were completing neurobehavioral tests or sleeping or when they were undergoing a 10–12h of fasting prior to each metabolomic blood sample. Full descriptions of ad lib access can be found in Spaeth *et al.* (Spaeth AM *et al.*, 2013, *Sleep* 36:981–990). Because the fasting was strictly enforced, it is highly unlikely that dietary alterations affected the metabolomic measurements.

Cognitive Assessment

Subjects underwent computerized cognitive tests every 2h during scheduled wakefulness. The cognitive tests included the following objective evaluations: the Digit Symbol Substitution Task (DSST), a computerized version of the cognitive performance task bearing the same name in the Wechsler Adult Intelligence Scale (Wechsler Adult Intelligence Scale 3 - Technical Manual (1997) San Antonio: Harcourt Brace and Company); the Digit Span task, a test of working memory storage capacity, given in both the forward and backward versions (Wechsler, 1997) and summed to produce a total number correct measure for analysis; and the 10-minute Psychomotor Vigilance Test (PVT), a cognitive test of sustained attention utilizing reaction times as an assay of behavioral alertness (Dinges and Powell, 1985, *Behav Res Methods Instrum Comput* 17: 652–655; Lim, 2008, *Ann NY Acad Sci* 1129:305–322.; Basner and Dinges, 2011, *Sleep* 34:581–591). Subjects remained seated throughout the cognitive test periods and were behaviorally monitored. Subjects were instructed to perform to the best of their ability and to use compensatory effort to maintain performance. Daily values for each performance task were calculated by averaging scores from all the test bouts that day [1000h-2000h (for BL1 and SR5/CD5), 1200h-2000h (for R1), and 1000h-2000h (for CD6)]. Outcome measures were as follows: DSST total number correct; DS total number correct (sum of forward and backward versions) (DSTC), PVT lapses (≥ 500 ms reaction times) + errors [false starts (errors of commission)] (PVT lapses + errors), and PVT mean response speed or reciprocal response time (PVT 1/RT).

Metabolomics

Blood samples for metabolomics were collected after a 10-12 h overnight fast in the morning following the first night of baseline sleep, after the fifth night of sleep restriction and after the night of recovery sleep (FIG. 3A). To control for morning evening effects, sampling occurred during the two hours following waketime (between 0800h-1000h on BL1 and SR5 and between 1000h-1200h on R1). Samples were immediately centrifuged and plasma was stored in a -80°C freezer until analysis. Table 2 contains subject demographic data and clinical parameters.

Table 2: Demographic and clinical details from human subjects

Subject ID	Age (y)	Sex	Race	BMI (kg/m ²)	Glucose (mg/dL)	Cholesterol (mg/dL)	Triglycerides (mg/dL)	Morningness-Eveningness Composite Scale	Actigraphic Sleep Onset (h)*	Actigraphic Sleep Offset (h)*	Actigraphic Sleep Midpoint (h)*	PSG Total Sleep Time SR1 (h)	PSG Total Sleep Time SR5 (h)
A	25	M	AA	27.69	73.0	188.0	113.0	45	23.40	7.17	3.05	3.80	3.93
B	32	M	AA	25.40	76.0	158.0	47.0	55	23.53	6.98	3.03	2.73	3.99
C	32	F	AA	28.16	78.0	165.0	32.0	41	23.33	6.77	3.25	3.83	3.76
D	27	F	AA	25.36	74.0	145.0	42.0	45	23.28	7.05	2.97	3.83	3.97
E	24	F	AA	26.23	83.0	135.0	37.0	37	24.61	8.85	4.73	3.76	3.72
F	22	M	H	25.89	80.0	156.0	36.0	47	24.30	7.83	2.36	3.90	3.92
G	23	F	AA	24.98	81.0	215.0	69.0	45	23.37	6.83	3.10	3.80	3.88
H	23	F	AA	17.25	74.0	209.0	45.0	48	22.38	6.75	2.82	3.73	3.87
I	40	M	AA	27.14	81.0	160.0	295.0	45	23.43	6.70	3.07	3.74	3.78
J	27	M	AA	21.67	92.0	140.0	43.0	43	24.13	8.48	4.30	3.81	3.93
K	33	M	AA	25.06	71.0	138.0	52.0	44	24.43	7.23	4.07	NA	NA
L	40	M	AA	24.37	83.0	159.0	51.0	48	23.90	7.43	3.83	NA	NA
M	38	M	C	25.10	74.0	229.0	239.0	49	23.22	6.85	3.28	NA	NA
N	49	F	AA	28.66	80.0	172.0	78.0	49	22.40	6.37	3.67	NA	NA

M: Male; F: Female; AA: African American; H: Hispanic; C: Caucasian; BMI: Body Mass

5 Index; PSG: Polysomnography; SR1: Sleep Restriction Night 1; SR5: Sleep Restriction Night 5; NA: Not Applicable; *One week before study entry

*Detailed Metabolomics Methods***Sample preparation and mass spectrometry for metabolomics analysis:**

Following collection, samples were immediately centrifuged and plasma was stored in a -80°C freezer until analysis. The plasma samples from the studies described above were used in three aliquots for mass spectrometry. For each sample, GC-MS, HILIC-MS and CSH-lipidomics were performed in the metabolomics core of University of California, Davis, CA. Sample preparation protocols for three different modes of mass spectrometry are detailed below.

GC-MS:

30 µl of plasma sample was extracted using 1 ml 3:3:2 acetonitrile/isopropanol/ water solution. 450 µl of the supernatant was evaporated to dryness, resuspended in 450 µl of 50% acetonitrile, centrifuged and the supernatant was again evaporated to dryness. The residue was used for derivatization by adding 10 µl of 40 mg/ml methoxyamine hydrochloride followed by 1.5 hours of shaking and addition of 91 µl of MSTFA (N-methyl-N-(trimethylsilyl)-trifluoroacetamide)-FAME marker mixture (prepared by adding 1 ml MSTFA and 10 ml FAME). This sample was subjected to shaking at 37°C and transferred to a glass vial and submitted to GC-TOFMS. The mass spectrometry was performed using a Leco Pegasus II with Gerstel MPS II injector system. The column dimension was 30m×0.25mm×0.25mm (Restek Rtx-5sil MS with Integra-Guard).

HILIC-MS:

Plasma samples were extracted using a 3:3:2 acetonitrile/isopropanol/water (vol/vol) mixture using the same protocol as described above and submitted to mass spectrometry. Mass spectrometry was performed in an Agilent 6530 accurate mass qTOF LC-MS with an Agilent 1290 infinity UHPLC fitted with Water Acquity UPLC BEH HILIC column of dimension of 2.1×150mm×1.7µm.

Lipidomics using CSH-qTOF-MS:

20 µl of blood plasma was dissolved in 250 µl ice-cold methanol and 750 µl ice-cold methyl tertiary butyl ether (MTBE) followed by vortexing and addition of 188 ml of ice-cold distilled water. The sample was vortexed and centrifuged for 2 minutes at 14,000 × g. 350 µl of the upper organic fraction was dried and reconstituted in 65 ml 9:1 (vol/vol) methanol-toluene with CUDA (12- [[[(cyclohexylamino)carbonyl]amino]-dodecanoic acid) as

an internal standard. The samples were subjected to UPLC-qTOF-MS using Agilent 6530 Accurate Mass Q-TOF LC/MS with an Agilent 1290 Infinity UHPLC. An Acquity UPLC CSH C18 Column was used for this purpose; the column dimension was 1.7 μ m, 2.1mm x 100mm. The following solvent system was used: – A: 60/40 ACN:H₂O 0.1% formic acid and 10mM ammonium formate and B: 90/10 IPA/ACN 0.1% formic acid and 10mM ammonium formate. The solvent gradient started with 15% B that reached to a maximum of 99% at 11.5 minutes and decreased to 15% at 12 minutes and was kept constant at this value until the 15 minute mark.

Statistical analysis of the data:

The data were normalized by the sum of all identified peak heights from the total ion chromatogram (TIC) of individual samples, followed by 6 normalization by the average of all TICs. The data were further used for univariate statistical analysis using MeV 4.9 (Saeed AI *et al.*, 2003, *BioTechniques* 34:374–378). For each set of experimental groups (for study 1 – forced activity and sleep-restricted animals, for study 2 – sleep-restricted animals and for the human study – sleep-restricted individuals), the metabolites from baseline samples were compared using the sleep-restricted (or forced activity) samples using non-parametric unpaired t-tests using permutation. 1000 permutations were used in each case; $p < 0.01$ was considered significant for samples across studies with a threshold of $p < 0.1$ and false discovery rate (FDR) < 0.2 required within each study to eliminate possible bias from an individual study. Relationships were calculated from the human data using the pvcust package in R which provides approximately unbiased and bootstrap probability p-values to ascertain stability of interactions (Shimodaira H *et al.*, 2002, *Syst Biol* 51:492–508).

Metabolite Set Enrichment Analysis was performed on metabolites from rats and humans (Table 3A and Table 3B).

Orthogonal Partial Least Square (OPLS) regression models correlated multivariate metabolomic profiles with cognitive variables. The BL, SR5 and R1 samples (or equivalent time points for control samples) were all used together (N=42 for each OPLS model) in order to construct the regression models. The models were judged significant based on the CV-ANOVA ($p < 0.05$) and the cross-validation parameter Q^2 (by removing 1/7th of the sample in each round of cross validation). In order to construct OPLS models explaining association differences between cognitive variables and metabolites across sleep

restriction, the difference of the variables (Y = cognitive variables and X = metabolites) in 10 sleep restricted individuals was calculated and the resulting difference matrix was used to construct the models. Those metabolites significantly associated with each specific cognitive variable were selected based on the variable importance on projection (VIP>1.0).

5

Table 3A: Metabolite Set Enrichment Analysis of Rat Sleep Restriction Metabolites

	total	expected	hits	Raw p	Holm p	FDR
AMMONIA RECYCLING	18	0.305	2	0.035	1	1
PROTEIN BIOSYNTHESIS	19	0.322	2	0.0388	1	1
VALINE, LEUCINE AND ISOLEUCINE DEGRADATION	36	0.611	2	0.121	1	1
MALATE-ASPARTATE SHUTTLE	8	0.136	1	0.128	1	1
GLUTATHIONE METABOLISM	10	0.17	1	0.158	1	1
HISTIDINE METABOLISM	11	0.187	1	0.173	1	1
BETA OXIDATION OF VERY LONG CHAIN FATTY ACIDS	14	0.238	1	0.215	1	1
STARCH AND SUCROSE METABOLISM	14	0.238	1	0.215	1	1
SPHINGOLIPID METABOLISM	15	0.255	1	0.228	1	1
PROPANOATE METABOLISM	18	0.305	1	0.268	1	1
PHOSPHOLIPID BIOSYNTHESIS	19	0.322	1	0.28	1	1
PORPHYRIN METABOLISM	22	0.373	1	0.317	1	1
CITRIC ACID CYCLE	23	0.39	1	0.329	1	1
METHIONINE METABOLISM	24	0.407	1	0.341	1	1
GALACTOSE METABOLISM	25	0.424	1	0.352	1	1
GLYCINE, SERINE AND THREONINE METABOLISM	26	0.441	1	0.364	1	1
GLUCONEOGENESIS	27	0.458	1	0.375	1	1
BILE ACID BIOSYNTHESIS	49	0.832	1	0.579	1	1

Table 3B: Metabolite Set Enrichment Analysis of Human Sleep Restriction Metabolites

	total	expected	hits	Raw p	Holm p	FDR
PHENYLALANINE AND TYROSINE METABOLISM	13	0.173	1	0.161	1	1
NICOTINATE AND NICOTINAMIDE METABOLISM	13	0.173	1	0.161	1	1
PROTEIN BIOSYNTHESIS	19	0.253	1	0.227	1	1
PHOSPHOLIPID BIOSYNTHESIS	19	0.253	1	0.227	1	1

10

Results and Discussion

Metabolomic analysis of sleep-restricted rats

To determine the extent to which systemic metabolism is impacted by sleep restriction, the comprehensive plasma metabolic profiles of rats undergoing a sleep restriction protocol were measured (FIG. 1A). Blood samples were taken at baseline and after 1 and 5 days of sleep restriction (SR1 and SR5, respectively) in two separate studies. Study A included a concurrent forced activity control group subjected to forced activity during the waking period (with blood samples at baseline, FA1a and FA5a). These rats had total activity levels equivalent to those of sleep-restricted rats, but were allowed to sleep, providing a control for the effects of activity induced by the SR protocol. Study B included samples drawn from two recovery time points at day 1 and 3 post-SR (Rec1b and Rec3b). Metabolic profiles consisted of both polar and non-polar metabolite measurements using a combination of GC-qTOF-MS, as well as HILIC and reverse-phase LC-qTOF-MS measurements. A representative principal components analysis scores plot from the multivariate analysis of all metabolite measurements is provided as FIG. 7.

Reproducible metabolite markers of sleep restriction in rats

A total of 3380 unique metabolite species were detected across the two independent studies of rat plasma. Of these, 407 were identified. To compare the change in metabolic profile as a function of sleep restriction, univariate comparisons with multiple testing correction were performed between baseline (BL) and one-day sleep restriction (SR1) time points to assess acute effects, and between BL and five-day SR (SR5) time points to assess chronic effects. All features were significant at $p < 0.01$, $FDR \leq 0.05$ when both studies were considered together. In addition, to ensure that the response was robust and not highly influenced by one of the two studies (Wood J *et al.*, 2014, *BMJ* 348:g2215), each measurement from an individual study was required to meet thresholds of $p < 0.1$ and $FDR < 0.2$. Results from the combined data showing $p < 0.01$ (Table 4) were compared with the equivalent analysis of the control forced activity (FA) group, and the extent of overlap between the groups is detailed in FIG. 1B. Under acute SR, a total of 26 features were reproducibly observed to be attributable to forced activity, and so were removed from consideration. Of the 38 that were unique to SR (FIG. 1C, left), 28 were identified. The majority of these metabolites (18 out of 28) were lipids. Reduced non-lipid metabolites

included: isocitrate from the Krebs cycle / lipogenesis; urocanic acid, a product of histidine catabolism; oxalic acid from glycolate metabolism; and 2-deoxyerythritol and trihydroxypyrazine, two non-mammalian metabolites which may be of dietary or gut microbial origin. Non-lipid metabolites that were increased included: leucine, valine, N-methylalanine and cellobiotol.

Following five days of chronic SR, 44 features were significantly altered across studies A and B: 23 were unique to the SR condition (FIG. 1C, right), and 18 were named, of which 15 were lipids. Glycine was reduced while the Krebs cycle intermediate malate and sucrose were elevated.

Table 4: Significant metabolites from rat plasma analysis for both acute and chronic sleep restriction pooled from Studies A and B

Metabolite	p{acute}	FDR{acute}	p {chronic}	FDR {chronic}	Up/Down in individual studies (Significant on day)
2-deoxyerythritol	0.001	0.00156	0.393	0.443486	↓(1)
DG (36:3)	0.003	0.003656	0.003	0.00468	↓(1)
isocitric acid	0.023	0.025629	0.414	0.4485	↓(1)
lignoceric acid	0.006	0.007091	0.905	0.905	↓(1)
LPC (18:1)	0	0	0.001	0.00195	↓(1)
LPC (22:6)	0.002	0.0026	0	0	↓(1)
oxalic acid	0.001	0.00156	0.199	0.235182	↓(1)
trihydroxypyrazine	0.001	0.00156	0.017	0.022862	↓(1)
urocanic acid	0.023	0.025629	0.352	0.403765	↓(1)
leucine	0.003	0.003656	0.446	0.470108	↑(1)
N-methylalanine	0	0	0.002	0.003545	↑(1)
Pls-PC (34:1)A	0.001	0.00156	0	0	↑(1)
Pls-PC (38:5)	0	0	0	0	↑(1)
PC (40:4)	0	0	0.027	0.033968	↑(1)
PC (40:6) A	0.001	0.00156	0	0	↑(1)
Pls-PC (40:5)	0	0	0.025	0.0325	↑(1)
Pls-PE (36:4)	0.002	0.0026	0.009	0.012536	↑(1)
SM (33:1)	0	0	0.003	0.00468	↑(1)
SM (d18:2/23:0)	0	0	0	0	↑(1)
SM (d41:1)	0	0	0	0	↑(1)
TG (58:10)	0	0	0.003	0.00468	↑(1)
Valine	0.001	0.00156	0.823	0.844658	↑(1)
glycine	0	0	0.004	0.006	↓(5)
LPC (16:1)	0	0	0	0	↓(5)
PC (33:1)	0	0	0	0	↓(5)
PC (37:6)	0	0	0	0	↓(5)
Ceramide (d42:1)	0	0	0	0	↑(5)
LPC (18:0)	0.057	0.060081	0.042	0.051188	↑(5)
malic acid	0.757	0.757	0	0	↑(5)
Pls-PC (38:3)	0	0	0	0	↑(5)
SM (43:1)	0.009	0.010324	0.008	0.011556	↑(5)
SM (d18:1/16:0)	0.001	0.00156	0	0	↑(5)
SM (d18:1/21:0)	0	0	0.002	0.003545	↑(5)
SM (d42:1)	0	0	0	0	↑(5)
sucrose	0.429	0.434132	0	0	↑(5)
Pls-PC (38:4) A	0	0	0	0	↑(1,5)
Pls-PC (40:3)	0	0	0	0	↑(1,5)
SM (d16:1/20:0)	0	0	0	0	↑(1,5)
SM (d18:1/16:1)	0	0	0	0	↑(1,5)

Notes: These data in Table 4 were generated by pooling the results from Study A and Study B and then applying a paired t-test between baseline and chronic or acute samples

A strength of these studies is the total replication of both experimental and analytical procedures for the first six days in Studies A and B. With this replication, the common markers observed are resistant to experimental variations. There was significant variation between the two studies when examined in aggregate. For example, 64 metabolites were found in both studies to be similarly changed under acute SR, and 44 under chronic SR (FIG. 1B), while the number of metabolites significantly different from controls across both studies was ~400-740 in the acute case and 550-725 in the chronic case. This may result from variation in the environmental and/or sampling procedures or in the analytical analysis. While a limitation of this study, this caveat generally applies to “Omics” type experiments and reinforces the need for complete experimental replication as done here.

Temporal Dynamics of SR-specific metabolic alterations

To better understand the potential roles of these metabolites as markers of sleep debt, the temporal relationship between metabolites significantly altered under sleep-restricted conditions was examined. Measurements were z-scored and normalized to the baseline samples. FIG. 2A depicts the metabolites altered only under acute sleep restriction conditions from both studies A and B, as well as the day 1 FA group. Metabolite features were ordered using hierarchical cluster analysis. FIG. 2B highlights metabolites altered under chronic SR at day 5, and is divided into those metabolites which normalized to baseline levels by recovery day 3 (upper panel) and those that did not recover (lower panel). The metabolic results from recovery day 1 may represent an intermediate transitional measurement between more stable physiological states. Sucrose and malate are two notable metabolites that did not recover, both possibly related to energy metabolism, although sucrose levels are likely controlled by intestinal absorption. FIG. 5 shows the fraction of each lipid class found to be significant as a function of all lipids measured in that class and demonstrates that PCs, LPCs, PlsCho and SM species are significantly altered equivalently between acute and chronic situations. In contrast, polar metabolites were primarily altered following acute SR, with only glycine specifically altered following chronic SR. Those compounds altered across both acute and chronic conditions were identified, all of which

were phospholipids (FIG. 2C). Numeric identifiers indicate unidentified metabolites, and details of their retention index and quantified m/z values are available as Table 5.

Intriguingly, 3 SM species recovered, and 3 PlsCho species did not recover based on the statistical thresholds ($p < 0.05$, FDR < 0.2 compared to baseline). While the thresholds are somewhat arbitrary, as demonstrated by visual similarity between the recovered and non-recovered groups, the clustering of SM and PlsCho species suggests a regulated process in which specific lipid classes/species are preferentially returned to non-SR levels.

Table 5: Retention Index and Quantitated m/z for GC-MS Data

Unknown ID	Ret Index	quant m/z
201887	1200427	316
211972	473115	98
226844	641635	301
239966	806614	290
267756	795288	156
281172	589910	140
289052	515010	186
294986	884907	390
299159	588963	241
367991	525157	188
438045	735508	174
470909	710744	331
619709	619088	290
624867	357202	241

Altered metabolic phenotype in humans under SR

Recent work indicates that total sleep deprivation induces a measurable perturbation to the blood metabolome (Davies SK *et al.*, 2014, *Proc Natl Acad Sci USA* 111:10761–10766); however, little is known about the metabolic effects of sleep restriction. The metabolome in blood samples drawn from 10 subjects was analyzed and sampled according to the sleep restriction protocol in FIG. 3A, including a sample at baseline (BL), following 5 days of SR which included 4 hours of sleep opportunity (SR) each night, and

following a night of recovery sleep (Rec). A similar filter was applied ($p < 0.1$, $FDR < 0.2$) as with the rat data as the goal was to validate the rat results in a human population (DATA SET 1). Comparison of the human BL and SR samples revealed 92 features that were altered, of which 37 were identified (FIG. 3B; unidentified metabolites not shown for clarity). Of the 5 identified species, 32 were lipid or fatty-acid related compounds. With regard to polar species, the amino acids tryptophan (Trp), phenylalanine (Phe), as well as pipecolic acid and 1-Methyl-4-pyridone-5-carboxamide (4-Pyr) were elevated, while oxalic acid was reduced.

As with the rat study, the majority of metabolites (30 identified and 46 unknowns) recovered to pre-SR levels, while 7 identified and 11 unknowns did not recover 10 over the period measured. Aside from 4-Pyr, the non-recovered metabolites were either PCs (32:1, 38:2, 38:3), or acylcarnitines (C10:0, C12:0, and C18:1). The differences between baseline (pre-sleep loss) and recovery (post-sleep loss) could indicate incomplete recovery. *Polar metabolites reflective of neurotransmitter, vitamin B3 and gut microflora metabolism are altered by SR in humans*

15 Both Trp and Phe were elevated in human subjects during SR. Trp is a major precursor for serotonin and melatonin and is elevated under acute total sleep deprivation (Davies SK *et al.*, 2014, *Proc Natl Acad Sci USA* 111:10761–10766). Intriguingly, Trp is also the precursor to liver synthesis of nicotinic acid (vitamin B3), which interconverts with the amide form, nicotinamide. In support of an effect on this pathway, elevated levels of 4- 20 Pyr, a primary end metabolite of nicotinamide, was observed. Phe is also a precursor to several neurotransmitters, including dopamine, norepinephrine and epinephrine via tyrosine. An increase in Phe might partly reflect increases in catecholamines in response to sleep loss (Meerlo P *et al.*, 2008, *Sleep Medicine Reviews* 12:197–210). In humans, acute total sleep deprivation in the laboratory increases levels of stress hormones such as catecholamines and 25 cortisol (Minkel J *et al.*, 2014, *Health Psychol* 33:1430–1434). However, many laboratory studies have reported that sleep restriction, the type of sleep loss used in the study, does not produce significant effects on cortisol levels (Pejovic S *et al.*, 2013, *Am J Physiol Endocrinol Metab* 305:E890–6). Altogether, it is unlikely that effects of sleep restriction in the present study are explained by globally elevated stress hormones, although this possibility cannot be 30 entirely excluded.

Finally, gut metabolism also likely plays a role in the observed metabolic profile (Wikoff WR *et al.*, 2009, *Proc Natl Acad Sci USA* 106:3698–3703). For example, pipecolic acid is a metabolite of lysine unique to gut microflora and was elevated after SR.

Relevance of circadian control to the SR metabolome

Gene expression profiling has established that there is a complex, but defined, interaction between the circadian system and sleep homeostasis (Möller-Levet CS *et al.*, 2013, *Proc Natl Acad Sci USA* 110:E1132–41) in humans. A comparison of the metabolites perturbed in the study with studies of metabolites demonstrating circadian oscillations supports this relationship. Lipidomics analysis has elucidated a set of lipids that oscillate across biological sub-types defined by stratification by lipid composition (Chua EC-P *et al.*, 2013, *Proc Natl Acad Sci USA* 110:14468–14473). From this group of rhythmic metabolites, 8 overlapping species of significance were found including LPC 16:0; TGs (52:2, 52:3, TG 54:3, 54:4, 54:5), and DGs (36:2, 36:3). Other groups have reported a mixture of polar and non-polar metabolites that cycle in a circadian manner, and consistently found that the proportion of lipids is elevated. For example, Dallman *et al.* (Dallmann R *et al.*, 2012, *Proc Natl Acad Sci USA* 109:2625–2629), found 33/40 lipid species to be regulated in a circadian manner, of which carnitine C12:0 overlaps with the study. Similarly, Ang *et al.* (Ang JE *et al.*, 2012, *Chronobiol Int* 29:868–881), found 25/34 to be lipid species, with LysoPCs (18:2, 20:3) acylcarnitine 12:0, 18:0, and Phe overlapping with the results.

While the expression of clock genes in the rat protocol was not examined, it was previously found that a 20h forced desynchrony protocol does not alter the period of the body temperature rhythm (Strijkstra AM *et al.*, 1999, *Chronobiol Int* 16:431–440). This is consistent with work indicating that forced activity does not affect the clock in the SCN (Al-Safadi S *et al.*, 2014, *PLoS ONE* 9:e111166). Effects on peripheral clocks cannot be excluded, and some of the metabolic phenotypes could arise from loss of synchrony between central and peripheral clocks. Indeed, circadian asynchrony could be one of the consequences of sleep restriction and merits future investigation. In support of this idea, 1 week of 5.70 h per night sleep restriction protocol was shown to affect rhythms of the human blood transcriptome (Möller-Levet CS *et al.*, 2013, *Proc Natl Acad Sci USA* 110:E1132–41).

Comparison to metabolic profile of acute total sleep deprivation in humans

Similar to a recent study using acute total sleep deprivation by Davies *et al* (Davies SK *et al.*, 2014, *Proc Natl Acad Sci USA* 111:10761–10766), the majority of metabolites altered in the chronic sleep restriction study were lipid or fatty acid related species (38/41 vs 32/37 in the current study). Twelve of these were found to be common
5 between the two studies including PCs (32:1, 36:6, 38:4, 38:2, 38:3), lysoPCs (14:0, 16:1, 17:0) and acylcarnitines (C5:0, C10:0, C12:0). All the above-mentioned metabolites were elevated in the acute sleep deprivation study. In the study, SR led to increases in all except acylcarnitines C10:0 and C12:0, which were reduced. As these might reflect the energetic process of fatty acid oxidation, the discrepancy may be a function of the difference in
10 protocol (one day vs five days and total SD vs chronic SR) leading to a difference in relative energy requirements. In both studies, Trp was also found to be elevated. Investigation of the human plasma metabolome suggests that amino acid metabolism is particularly perturbed as a function of sleep restriction (Bell LN *et al.*, 2013, *Physiology & Behavior* 122:25–31). The study of Davies *et al* (Davies SK *et al.*, 2014, *Proc Natl Acad Sci USA* 111:10761–10766)
15 identified metabolites that increased but none that decreased under total sleep deprivation. Here decreases in a number metabolites were identified as a function of SR, which were not part of the analytical platform used in the Davies *et al* study (Davies SK *et al.*, 2014, *Proc Natl Acad Sci USA* 111:10761–10766). These included TGs, DGs, fatty alcohols, myristic acid, a PE species and oxalic acid.

20 *Oxalic acid and DG 36:3 are putative translational markers of sleep debt*

To examine the feasibility of using metabolite markers to assess sleep debt across species, the list of significantly altered metabolites was compared in rats and humans. Two identified species were found to be common across rat and humans: oxalic acid and DG 36:3 (FIGs. 4A and 4B). These metabolites are from highly disparate metabolic pathways
25 yet behave in the same manner, and thus may provide a robust measure of sleep debt (FIG. 4C, FIG. 6). Bootstrapped hierarchical cluster analysis of the entire set of measured metabolites indicates a significant branch that includes oxalate as well hypoxanthine, medium chain acylcarnitines (C6, C10, C12, C12:1), and arachidonic acid (FIG. 4C)). While these metabolites do not share obvious metabolic pathways, they may be linked by cellular
30 function such as peroxisomal processing as described below.

The primary sources of blood oxalate are diet-derived plant sources, vitamin C (ascorbate) degradation, and endogenous synthesis pathways in erythrocytes and liver (Marengo SR *et al.*, 2008, *Nat Clin Pract Nephrol* 4:368–377). Endogenous oxalate has conventionally been considered an end product of mammalian metabolism and primarily studied in the context of kidney stone formation. Recent evidence suggests that blood levels are also influenced by gut microbiota with over a dozen oxalate degrading gut bacterial species identified (Miller A *et al.*, 2013, *Pathogens* 2:636–652). While dietary oxalate with high bioavailability has been shown to increase plasma and urine levels (Holmes RP *et al.*, 2005, *J Urol* 174:943–7), excreted oxalate is minimally dependent on diet in an epidemiological cohort (Taylor EN *et al.*, 2008, *Clin J Am Soc Nephrol* 3:1453–1460). Based on these data, as well as the highly regulated nature of pre-sample fasting and diet for the rat and human studies, it was postulated that the observed reduction in blood oxalate is a result of either reduced synthesis or increased gut microbiota processing and not of dietary origin. Endogenous substrates for oxalate synthesis include glycolate in peroxisomes and hydroxyproline. Several days of total sleep deprivation does not have an impact on hydroxyproline levels in rats, nor differences thereof in this study, and thus any alterations in endogenous synthesis would be the result of peroxisomal glycolate perturbations. This idea is consistent with the other evidence for oxidative stress and peroxisomal activation described below. Another possible mechanism for reduced oxalate levels in blood is via increased urinary clearance. Acute total sleep deprivation increases diuresis (Kamperis K *et al.*, 2010, *Am J Physiol Renal Physiol* 299:F404–F411), and oxalate is readily filtered from plasma.

With regard to DG 36:3, it is not clear why this particular species (along with DG 36:2 in humans) but no other DGs are altered in response to SR. This metabolite is also regulated in a circadian fashion in humans, and as such may be an indicator of both circadian time and sleep debt.

Energetic and lipid metabolism is impacted differentially by SR across species

Systemic metabolic disruption as a function of sleep restriction has been reflected in model systems, such as in rats experimentally manipulated to sleep less, either through mechanical stimulation (Barf RP *et al.*, 2012, *Physiology & Behavior* 107:322–328) or through lesions in the ventrolateral preoptic area. These studies consistently find that

energy expenditure increases, without a concomitant change in food intake. Furthermore, plasma levels of glucose, insulin and leptin are reduced. By contrast, humans undergoing sleep restriction increase caloric intake and show alterations in energy expenditure (St-Onge M-P *et al.*, 2011, *Am J Clin Nutr* 94:410–416; Nedeltcheva AV *et al.*, 2009, *Am J Clin Nutr* 89:126–133; Brondel L *et al.*, 2010, *Am J Clin Nutr* 91:1550–1559; Shechter A *et al.*, 2013, *Am J Clin Nutr* 98:1433–1439; Buxton OM *et al.*, 2012, *Sci Transl Med* 4:129-43; Benedict C *et al.*, 2011, *Am J Clin Nutr* 93:1229–1236). Despite the differences in caloric intake, it is clear that sleep loss affects metabolic function across species.

Specific metabolic changes due to sleep deprivation are difficult if not impossible to decouple from metabolic demands of the organism under increased wakefulness. In the rat study, the effects of *activity*— which may account for much of the effect of wake *per se*— were controlled by including a forced activity group. While a number of metabolites were common to sleep-restricted and forced activity groups, those specific to SR were the focus of the study. For the human study, physical activity levels in the subjects were limited. Studies in humans show that energy expenditure (resting metabolic rate, etc.) contributes very little to metabolic changes during sleep restriction, and that energy intake is the largest contributor to metabolic homeostasis (Spaeth AM *et al.*, 2014, *Am J Clin Nutr* 100:559–566). Thus, separating the metabolic effects from sleep effects was achieved by enforcing a 10-12 h fasting period prior to metabolomic sampling, such that observed changes could be attributed to sleep/sleep restriction effects.

The metabolites altered in the study are reflective of energetic changes, particularly the overrepresentation of lipid species. Across both species, phospholipids (PC, LPC, PE, SM) were most elevated as a function of SR (20/25 in rats, and 14/15 in humans, FIG. 5). This raises the possibility that there is a common source for the elevated phospholipids, such as membrane breakdown and/or release from circulating lipoprotein particles. Elevated LPCs in blood are possibly derived from secreted phospholipase A2 acting on lipoprotein PC; lecithin–cholesterol–acyl-transferase in liver acting on LDL or HDL; or endothelial phospholipase A₂ acting on HDL. In turn, LPCs may act in a signaling capacity to stimulate PKC, NF-κB, or COX-2 (Sevastou I *et al.*, 2013, *Molecular and Cell Biology of Lipids* 1831:42–60). Consistent with this observation, sleep deprivation increases murine cerebral cortex NF-κB (Chen Z *et al.*, 1999, *Am J Physiol* 276:R1812–8). Also,

COX-2 products such as prostaglandin D2 have been noted as sleep-promoting in rat CSF (Ram A *et al.*, 1997, *Brain Res* 751:81–89), which may support the idea that sleep-promoting molecules are elevated during sleep loss. The data suggest a possible mechanism for sleep deprivation-induced increases in NF- κ B and prostaglandins. The results further indicate a significant clustering relationship between oxalate and arachidonic acid (FIG. 4C) which is the substrate for COX-2, although arachidonic acid was not significantly changed by the criteria.

Seven plasmalogen species were elevated in the rat under acute and/or chronic sleep restriction conditions indicative of oxidative stress. This finding is consistent with several studies suggesting that the purpose of sleep is restorative and involves metabolite clearance from the brain, and that sleep restores anti-oxidant balance in peripheral tissues (Everson CA *et al.*, 2005, *Am J Physiol Regul Integr Comp Physiol* 288:R374–83). While plasmalogens were not significantly changed in the human study, transcript studies have shown that pathways involved in oxidative stress and ROS are significantly induced in blood upon sleep loss. The lack of detectable lipid oxidation signal in humans may be due to increased capacity of human metabolism to regulate oxidative stress, or greater heterogeneity inherent in human datasets.

In contrast, the human data showed a marked reduction in 6 TG species (> 52 carbon chains) and 2 DG species (36 carbon chains) while the rat data demonstrated a reduction in a single DG species and elevation of TG 58:10. Reduction in total blood TGs of humans has been observed previously using a similar protocol (Reynolds AC *et al.*, 2012, *PLoS ONE* 7:e41218). Of note, the two TG species elevated in humans were relatively short (≤ 48 carbons, average chain length ≤ 16 carbons). This implies a possible carbon shift from longer to shorter chain TGs. The combination of an oxidative environment, κ -oxidation of long-chain fatty acids as implied by TAG shortening, as well as the observed association of acylcarnitines with oxalate (FIG. 4C) suggests a role distinct role for peroxisomes, possibly via peroxisome proliferator-activated receptors (PPARs).

Oxidative stress and the potential role of PPARs

Mounting evidence points to sleep loss inducing an oxidative metabolic state (Vollert C *et al.*, 2011, *Behavioural Brain Research* 224:233–240). One mechanism may be via the induction of PPARs, which play a critical role in lipid and glucose

metabolism. Bezafibrate, an anti-hyperlipidemic PPAR- α agonist, has been shown in a mouse model to affect both baseline sleep, with increased EEG delta power, and recovery sleep following sleep restriction, with a reduced rebound in EEG delta power. As a result, the authors suggested that activation of PPAR- α may provide a protective effect against sleep restriction. In the same model SR was shown to elevate brain mRNA levels of PPAR- α (Chikahisa S *et al.*, 2014, *Neuropharmacology* 79:399–404). Oleoylethanolamide (OEA), a potent activator of PPAR- α is also elevated in CSF, and to a lesser extent in plasma of sleep-deprived humans (Koethe D *et al.*, 2009, *J Neural Transm* 116:301–305). While OEA was not directly measured in the study, a number of metabolite changes in the human data, including the reduced oxalate levels, can be explained by a mechanism that invokes changes in PPAR α and more generally in peroxisomes.

The peroxisome is the central site of fatty acid alcohol processing (Lodhi IJ *et al.*, 2014, *Cell Metab* 19:380–392) which generates either diacylphospholipids or ether-linked phospholipids. Induction of peroxisome biogenesis is consistent with the observed decrease in both fatty alcohols (humans) and an increase in phospholipid species (humans and rats), particularly plasmalogens. Furthermore, the reduced levels of long-chain TGs and elevation in shorter chain TGs observed in humans may reflect peroxisomal β -oxidation processes that are preferential for longer chain fatty acids. Reduction in both oxalate (humans and rats) and glycine (rats, SR5) might be explained by depletion of glyoxylate, which is a common precursor to both substances in peroxisomes. Finally, the direct relationship between oxalate, hypoxanthine, and medium chain acylcarnitines (C6, C10, C12, C12:1) in cluster analysis (FIG. 4C) may also be explained by peroxisomal processes. Direct conversion of oxalate to hypoxanthine has been observed in extreme oxidative environments (Holian J *et al.*, 1967, *Chemical Communications (London)*:676–677), although it is not clear if this occurs *in vivo*.

Correlation of Multivariate Metabolomic and Lipidomic Profiles with Cognitive Variables.

To determine the set of metabolites and lipids that correlated with cognitive variables using multivariate analyses, OPLS regression models were constructed for each cognitive variable. Significant models were generated after initial screening of metabolites based on the variable importance on projection (VIP>1.0). CV-ANOVA p and Q² (cum) values of each model are listed in Table 6. PVT lapses and errors and PVT 1/RT variables

correlated with 75 and 116 metabolites, respectively; similarly, DSST and DSTC correlated with 101 and 113 metabolites, respectively (in each case, duplicates were corrected due to detection by both RPLC and HILIC-MS). These metabolites are listed in Table 7. FIG. 8A shows the overlap of significantly correlated metabolites across the four cognitive variables.

Importantly, eight metabolites were commonly associated with all four variables: PE (36:2), LPCs (16:0, 16:1, 18:2, 20:3 and 20:5), capric acid and 17-Methyltestosterone. OPLS models were constructed with these eight metabolites, which produced significant models for all cognitive variables except DSTC. The correlations between the X and Y values for PVT lapses and errors, PVT 1/RT and DSST are shown in FIGs. 8B-8D.

Table 6. Detailed statistics of the multivariate models. Orthogonal Partial Least Square (OPLS) regression models were generated using 42 samples (10 sleep restricted subjects at three time points and 4 non-sleep restricted (control) subjects at three time points) and individual cognitive variables (PVT lapses + errors, PVT 1/RT, DSST and DSTC). All models were generated after an initial screening of metabolites based on VIP>1.0.

Cognitive Variable	Q ² (cum)	CV-ANOVA p
PVT Lapses + Errors	0.58	1.51×10 ⁻⁵
PVT 1/RT	0.74	1.93×10 ⁻⁵
DSST	0.53	8.53×10 ⁻⁶
DSTC	0.56	3.65×10 ⁻⁶

Lipid Levels Correlate Significantly with Different Cognitive Variables.

Lipids constituted the majority of all metabolites that significantly correlated with PVT lapses and errors (67%), PVT 1/RT (82%), DSST (58%) and DSTC (69%). The total number of small molecular metabolites detected was comparable to the number of lipids detected (150 small molecules out of 330 total species; FIG. 13). FIG. 9A shows the relative contribution of lipid classes significantly associated with the four cognitive variables.

Carnitines, fatty acids and LPCs are associated with PVT lapses and errors. Ceramides and TGs are significantly associated with PVT 1/RT. Cholesteryl esters (CEs), cholesterol and PCs are significantly associated with DSST, while the major associations with DSTC were with LPCs, fatty acids and cholesterol and CEs. Specific lipid species significantly associated with each cognitive variable are listed in Table 8. The directionality of these associations was analyzed using the loading values from the specific OPLS models. The average loading of each lipid species corresponding to each variable is shown in FIG. 9B.

PVT lapses and errors positively correlated with almost all lipid species except for TGs. PVT 1/RT positively correlated with TGs, carnitines and DGs; DSST positively correlated with CEs and cholesterol, PCs, LPCs and TGs; and DSTC showed positive associations with TGs and DGs. By contrast, negative associations were observed between PVT lapses and errors and TGs; between PVT 1/RT and fatty acids, LPCs, PCs, plasmenyl PCs, SM, ceramide, and CEs and cholesterol; between DSST and carnitines, fatty acids, PE, SM, ceramide; and between DSTC and carnitines, fatty acids, LPCs, PCs, plasmenyl PCs, LPEs, SMs, ceramides, and CEs and cholesterol.

Small Molecules Significantly Associated with Different Cognitive Variables.

A number of small molecular weight metabolites also significantly associated with cognitive variables. FIG. 10 represents the fraction of each small molecule class that significantly associated with each cognitive variable. Table 9 shows the direct association of each molecule and each cognitive variable.

Association of the Plasma Metabolome with Sleep Loss.

The PVT, DSST and DS tests show remarkable sensitivity to sleep loss. However, large and substantial individual differences were observed in performance responses to sleep loss. The range of absolute value differences (SR5- baseline) was large for all performance variables: PVT lapses and errors: -1.13 to 8.23; PVT 1/RT: 0.09 to -0.97; DSST: 9.70 to -3.63; and DSTC: 2.67 to -7.57. Therefore, the change in metabolomic values at SR5 from baseline was correlated with the change in cognitive variable values at SR5 from baseline. For this purpose, OPLS models were constructed using changes in individual cognitive variables as the response and change in the metabolites as predictor variables. Only the lipid classes that represented a majority (>50%) of the correlated metabolites were considered (FIG. 9A). Therefore, PVT lapses and errors were modeled with fatty acids and LPCs, and PVT 1/RT values were modeled with fatty acids, TGs, ceramides and PCs. DSST values were modeled with fatty acids, CEs and PCs, and DSTC values were modeled with fatty acids, LPC and CEs. After 5 days of sleep restriction, changes in PVT lapses and errors and changes in DSTC from baseline produced significant models with changes in corresponding lipid levels. The observed versus predicted plots are shown in FIGs. 11A-B. Overall, positive changes in PVT lapses and errors (poorer performance with sleep loss) associated with increases in most of the lipid species modeled.

By contrast, poorer performance with sleep loss on the DSTC was associated with decreases in most of the lipid species modeled. Several LPCs showed significant correlations (Pearson's $r > 0.7 / < -0.7$) with PVT lapses and errors, including LPC (16:0), LPC (18:1), LPC (20:2), LPC (20:4) and LPC (22:6). Similarly, a number of lipid species were significantly correlated (Pearson's $r > 0.7 / < -0.7$) with the DSTC variable including arachidonic acid, LPC (16:0), LPC (18:0), LPC (18:1), LPC (18:2), LPC (20:1), LPC (20:2), LPC (20:3), LPC (20:4) and LPC (22:6). Although the multivariate models were not significant, PC (37:3) showed a strong univariate correlation with PVT 1/RT, and DSST was strongly correlated with PC (28:0), PC (36:3) and PC (37:3). Support tree analysis using the differences of the metabolites at SR5 compared to baseline revealed further interesting features. Most of the LPCs mentioned above [except LPC (20:1)] clustered together along with PC (37:3), 1-Methyl-4-pyridone-5-carboxamide and PE (38:2), mannitol, arachidonic acid, niacinamide and 3 acetylcarnitines (6:0, 10:1 and 12:1) (FIG. 11C). LPC (20:1) was closely clustered with PC (37:2), maleimide, capric acid and isocitric acid (FIG. 11D). Similarly, PC (28:0) clustered closely with 2-aminoadipic acid, PC (36:3) and glycolic acid (FIG. 11E).

Table 7: List of metabolites and lipids significantly correlated with each cognitive variable.

PVT Lapses and Errors		PVT 1/RT		DSST		DSTC	
Metabolite	Class	Metabolite	Class	Metabolite	Class	Metabolite	Class
beta-alanine	AA	asparagine	AA	asparagine	AA	5-hydroxynorvaline	AA
glycine	AA	cysteine-glycine	AA	citrulline	AA	5-methoxytryptamine	AA
leucine	AA	glutamic acid	AA	cysteine	AA	aspartic acid	AA
N-acetylglutamate	AA	glutamylthreonine	AA	cystine	AA	glutamylthreonine	AA
phenylalanine	AA	indole-3-lactate	AA	indole-3-acetate	AA	glycine	AA
trans-4-hydroxyproline	AA	trans-4-hydroxyproline	AA	L-arginine	AA	leucine	AA
arabinose	Carb	1,5-anhydroglucitol	Carb	Leucine Isomer	AA	Leucine Isomer	AA
fucose + rhamnose	Carb	2-deoxyerythritol	Carb	methionine	AA	pipecolic acid	AA

ribitol	Carb	glucose	Carb	N-acetyl glycine	AA	Tryptophan	AA
sucrose	Carb	glycerol-3-galactoside	Carb	N-methylalanine	AA	1,5-anhydroglucitol	Carb
Acetylcarnitine	Carnitines	Acylcarnitine C5:0	Carnitines	ornithine	AA	2-deoxyerythritol	Carb
Acylcarnitine C10:0	Carnitines	Acylcarnitine C6:0	Carnitines	threonine	AA	arabitol	Carb
Acylcarnitine C10:1	Carnitines	CE (18:1)	CE	tryptophan	AA	erythritol	Carb
Acylcarnitine C12:0	Carnitines	CE (18:2)	CE	1,5-anhydroglucitol	Carb	fucose	Carb
Acylcarnitine C12:1	Carnitines	CE (18:3)	CE	fucose	Carb	glucuronic acid	Carb
Acylcarnitine C16:0	Carnitines	Ceramide (d18:1/23:0)	Ceramide	glucuronic acid	Carb	N-acetylmannosamine	Carb
Acylcarnitine C18:0	Carnitines	17-Methyltestosterone	Cholesterol	levanbiose	Carb	sorbitol	Carb
Acylcarnitine C6:0	Carnitines	Pantothenic acid	Cofactor	sucrose	Carb	sucrose	Carb
17-Methyltestosterone	Cholesterol	DG (34:1)	DG	Acetylcarnitine	Carnitines	threitol	Carb
1-Methyl-4-pyridone-5-carboxamide	Cofactor	DG (36:2)	DG	Acylcarnitine C12:1	Carnitines	xylose	Carb
Niacinamide	Cofactor	DG (36:3)	DG	Acylcarnitine C3:0	Carnitines	Acetylcarnitine	Carnitines
glycerol	FA/Lipid	DG (38:5)	DG	Acylcarnitine C12:0	Carnitines	Acylcarnitine C12:0	Carnitines
arachidonic acid	FA/Lipid	DG (38:6)	DG	Acylcarnitine C4:0	Carnitines	Acylcarnitine C12:1	Carnitines
capric acid	FA/Lipid	capric acid	FA/Lipid	Acylcarnitine C6:0	Carnitines	Acylcarnitine C16:0	Carnitines
heptadecanoic acid	FA/Lipid	caprylic acid	FA/Lipid	carnitine	Carnitines	Acylcarnitine C18:0	Carnitines
isoheptadecanoic acid	FA/Lipid	glycerol-alpha-phosphate	FA/Lipid	CE (18:3)	CE	carnitine	Carnitines
linoleic acid	FA/Lipid	LPC (14:0)	LPC	CE (22:6)	CE	CE (18:2)	CE
linolenic acid	FA/Lipid	LPC (16:0)	LPC	cholesterol	CE	CE (18:3)	CE
myristic acid	FA/Lipid	LPC (16:1)	LPC	Ceramide (d18:1/23:0)	Ceramide	Cholesterol	CE
oleic acid	FA/Lipid	LPC (18:0)	LPC	Ceramide (d40:1)	Ceramide	Corticosterone	CE
palmitic acid	FA/Lipid	LPC (18:1)	LPC	Ceramide (d42:1)	Ceramide	Ceramide (d18:1/23:0)	Ceramide
LPC (14:0)	LPC	LPC (18:2)	LPC	Ceramide (d42:2)	Ceramide	Ceramide (d40:1)	Ceramide
LPC (16:0)	LPC	LPC (20:0)	LPC	17-Methyltestosterone	Cholesterol	Ceramide (d42:1)	Ceramide
LPC (16:1)	LPC	LPC (20:1)	LPC	Niacinamide	Cofactor	17-Methyltestosterone	Cholesterol
LPC (17:0)	LPC	LPC (20:2)	LPC	Pantothenic acid	Cofactor	1-Methyl-4-pyridone-5-carboxamide	Cofactor

LPC (17:1)	LPC	LPC (20:3)	LPC	tocopherol alpha	Cofactor	DG (36:2)	DG
LPC (18:0)	LPC	LPC (20:5)	LPC	glycerol	FA/Lipid	DG (38:5)	DG
LPC (18:1)	LPC	LPC (22:1)	LPC	glycerol-alpha-phosphate	FA/Lipid	glycerol-3-galactoside	FA/Lipid
LPC (18:2)	LPC	LPC (22:4)	LPC	capric acid	FA/Lipid	capric acid	FA/Lipid
LPC (18:3)	LPC	LPC (22:5)	LPC	caprylic acid	FA/Lipid	LPC (14:0)	LPC
LPC (19:0)	LPC	fumaric acid	OA	isoheptadecanoic acid	FA/Lipid	LPC (16:0)	LPC
LPC (20:0)	LPC	lactamide	OA	myristic acid	FA/Lipid	LPC (16:1)	LPC
LPC (20:1)	LPC	lactic acid	OA	palmitoleic acid	FA/Lipid	LPC (17:0)	LPC
LPC (20:2)	LPC	creatinine	Others	LPC (16:0)	LPC	LPC (17:1)	LPC
LPC (20:3)	LPC	salicylaldehyde	Others	LPC (16:1)	LPC	LPC (18:0)	LPC
LPC (20:4)	LPC	TMAO	Others	LPC (17:0)	LPC	LPC (18:1)	LPC
LPC (20:5)	LPC	PC (16:0/16:0)	PC	LPC (18:2)	LPC	LPC (18:2)	LPC
LPC (22:1)	LPC	PC (18:1/16:0)	PC	LPC (19:0)	LPC	LPC (18:3)	LPC
LPC (22:4)	LPC	PC (32:1)	PC	LPC (20:3)	LPC	LPC (19:0)	LPC
LPC (22:5)	LPC	PC (32:2)	PC	LPC (20:4)	LPC	LPC (20:0)	LPC
LPC (22:6)	LPC	PC (34:0)	PC	LPC (20:5)	LPC	LPC (20:1)	LPC
LPE (18:2)	LPC	PC (34:2)	PC	LPC (22:1)	LPC	LPC (20:2)	LPC
2-deoxyisotetronic acid	OA	PC (36:1)	PC	LPC (22:4)	LPC	LPC (20:3)	LPC
2-hydroxybutanoic acid	OA	PC (36:2)	PC	LPC (22:5)	LPC	LPC (20:4)	LPC
3-aminoisobutyric acid	OA	PC (36:3)	PC	LPC (22:6)	LPC	LPC (20:5)	LPC
3-hydroxybutanoic acid	OA	PC (36:4)	PC	2-deoxyisotetronic acid	OA	LPC (22:6)	LPC
aconitic acid	OA	PC (36:4)1	PC	3-aminoisobutyric acid	OA	LPE (18:2)	LPE
dehydroascorbic acid	OA	PC (36:5)	PC	aminomalonic acid	OA	3-aminoisobutyric acid	OA
maleimide	OA	PC (36:6)	PC	azelaic acid	OA	alpha ketoglutaric acid	OA
benzylalcohol	Others	PC (37:2)	PC	citric acid	OA	aminomalonic acid	OA
creatinine	Others	PC (37:3)	PC	isocitric acid	OA	glutaric acid	OA
taurine	Others	PC (38:2)	PC	isothreonic acid	OA	isothreonic acid	OA
uric acid	Others	PC (38:4)	PC	oxalic acid	OA	lactic acid	OA
PC (34:2)	PC	PC (38:5)	PC	alpha-terpineol	Others	phenylacetic acid	OA
PC (36:2)	PC	PC (40:7)	PC	Creatine	Others	benzoic acid	Others
PC (36:5)	PC	PE (34:2)	PE	creatinine	Others	benzylalcohol	Others
PC (37:2)	PC	PE (36:1)1	PE	hippuric acid	Others	creatinine	Others
PE (36:2)	PE	PE (36:2)	PE	phosphoethanol	Others	phenylethylamine	Others

				mine		ne	
PE (38:2)	PE	PE (38:2)	PE	phosphoric acid	Others	phthalic acid	Others
Plasmenyl PC (40:2)	Plasmalogen	Plasmenyl PC (40:2)	Plasmalogen	pseudo uridine	Others	p-cresol-	Others
Plasmenyl PC (42:2)	Plasmalogen	Plasmenyl PC (42:2)	Plasmalogen	salicylaldehyde	Others	PC (18:1/16:0)	PC
TG (52:4)	TG	Plasmenyl-PC (34:1)	Plasmalogen	uridine	Others	PC (28:0)	PC
TG (52:5)	TG	Plasmenyl-PC (34:2)	Plasmalogen	PC (32:2)	PC	PC (32:1)	PC
TG (56:4)	TG	Plasmenyl-PE (36:2)	Plasmalogen	PC (36:4)I	PC	PC (32:2)	PC
		SM (33:1)	SM	PC (37:4)	PC	PC (34:2)	PC
		SM (34:0)	SM	PC (38:4)I	PC	PC (36:2)	PC
		SM (d18:1/14:0)	SM	PC (38:5)	PC	PC (36:3)	PC
		SM (d18:1/16:0)	SM	PC (38:6)	PC	PC (36:4)	PC
		SM (d18:1/16:1)	SM	PC (40:5)	PC	PC (36:5)	PC
		SM (d18:1/20:0)	SM	PC (40:6)	PC	PC (36:6)	PC
		SM (d18:1/21:0)	SM	PC (42:6)	PC	PC (37:2)	PC
		SM (d18:1/22:0)	SM	PE (34:2)	PE	PC (37:3)	PC
		SM (d18:1/23:0)	SM	PE (36:2)	PE	PE (36:1)	PE
		SM (d18:1/24:0)	SM	PE (38:2)	PE	PE (36:2)	PE
		SM (d18:2/23:0)	SM	PE (38:4)	PE	PE (38:4)	PE
		TG (51:2)	TG	PE (38:6)	PE	Plasmenyl PC (40:2)	Plasmalogen
		TG (51:3)	TG	Plasmenyl-PE (36:2)	Plasmalogen	Plasmenyl PC (42:1)	Plasmalogen
		TG (52:2)	TG	SM (d18:1/21:0)	SM	Plasmenyl PC (42:2)	Plasmalogen
		TG (52:3)	TG	SM (d18:1/22:0)	SM	SM (33:1)	SM
		TG (52:4)	TG	TG (46:0)	TG	SM (d18:1/14:0)	SM
		TG (52:4)I	TG	TG (49:0)	TG	SM (d18:1/21:0)	SM
		TG (52:5)	TG	TG (49:2)	TG	SM (d18:1/23:0)	SM
		TG (53:1)	TG	TG (56:6)	TG	TG (52:2)	TG
		TG (53:2)	TG	TG (56:7)	TG	TG (52:3)	TG
		TG (53:3)	TG	TG (56:8)	TG	TG (52:4)	TG
		TG (53:4)	TG	TG (58:10)	TG	TG (52:4)I	TG
		TG (54:1)	TG	TG (58:6)	TG	TG (52:5)	TG
		TG (54:2)	TG	TG (58:8)	TG	TG (53:1)	TG
		TG (54:3)	TG	TG (58:9)	TG	TG (53:2)	TG
		TG (54:4)	TG	TG (60:11)	TG	TG (53:3)	TG
		TG (54:5)	TG			TG (54:2)	TG
		TG (54:6)I	TG			TG (54:4)	TG

TG (54:8)	TG	TG (54:6)I	TG
TG (56:2)	TG	TG (56:4)	TG
TG (56:3)	TG	TG (56:5)	TG
TG (56:4)	TG	TG (56:6)	TG
TG (56:5)	TG	TG (56:7)	TG
TG (56:6)	TG	TG (56:8)	TG
TG (56:7)	TG	TG (58:6)	TG
TG (56:8)	TG	TG (58:8)	TG
TG (58:3)	TG	TG (58:9)	TG
TG (58:4)	TG	TG (60:11)	TG
TG (58:6)	TG		
TG (58:8)	TG		
TG (58:9)	TG		
TG (60:11)	TG		

Table 8: Lipid species significantly (VIP>1.0) associated with individual cognitive variables from OPLS regression modeling.

PVT Lapses and Errors	PVT 1/RT	DSST	DSTC
Acetylcarnitine	Acylcarnitine C5:0	Acetylcarnitine	Acetylcarnitine
Acylcarnitine C10:0	Acylcarnitine C6:0	Acylarnitine C12:1	Acylcarnitine C12:0
Acylcarnitine C10:1	CE (18:1)	Acylarnitine C3:0	Acylcarnitine C12:1
Acylcarnitine C12:0	CE (18:2)	Acylcarnitine C12:0	Acylcarnitine C16:0
Acylcarnitine C12:1	CE (18:3)	Acylcarnitine C4:0	Acylcarnitine C18:0
Acylcarnitine C16:0	Ceramide (d18:1/23:0)	Acylcarnitine C6:0	capric acid
Acylcarnitine C18:0	DG (34:1)	capric acid	carnitine
Acylcarnitine C6:0	DG (36:2)	caprylic acid	CE (18:2)
arachidonic acid	DG (36:3)	carnitine	CE (18:3)
capric acid	DG (38:5)	CE (18:3)	Ceramide (d18:1/23:0)
heptadecanoic acid	DG (38:6)	CE (22:6)	Ceramide (d40:1)
isoheptadecanoic acid	LPC (14:0)	Ceramide (d18:1/23:0)	Ceramide (d42:1)
linoleic acid	LPC (16:0)	Ceramide (d40:1)	Cholesterol
linolenic acid	LPC (16:1)	Ceramide (d42:1)	Corticosterone
LPC (14:0)	LPC (18:0)	Ceramide (d42:2)	DG (36:2)
LPC (16:0)	LPC (18:1)	cholesterol	DG (38:5)
LPC (16:1)	LPC (18:2)	isoheptadecanoic acid	LPC (14:0)
LPC (17:0)	LPC (20:0)	LPC (16:0)	LPC (16:0)
LPC (17:1)	LPC (20:1)	LPC (16:1)	LPC (16:1)
LPC (18:0)	LPC (20:2)	LPC (17:0)	LPC (17:0)
LPC (18:1)	LPC (20:3)	LPC (18:2)	LPC (17:1)
LPC (18:2)	LPC (20:5)	LPC (19:0)	LPC (18:0)

LPC (18:3)	LPC (22:1)	LPC (20:3)	LPC (18:1)
LPC (19:0)	LPC (22:4)	LPC (20:4)	LPC (18:2)
LPC (20:0)	LPC (22:5)	LPC (20:5)	LPC (18:3)
LPC (20:1)	PC (16:0/16:0)	LPC (22:1)	LPC (19:0)
LPC (20:2)	PC (18:1/16:0)	LPC (22:4)	LPC (20:0)
LPC (20:3)	PC (32:1)	LPC (22:5)	LPC (20:1)
LPC (20:4)	PC (32:2)	LPC (22:6)	LPC (20:2)
LPC (20:5)	PC (34:0)	myristic acid	LPC (20:3)
LPC (22:1)	PC (34:2)	palmitoleic acid	LPC (20:4)
LPC (22:4)	PC (36:1)	PC (32:2)	LPC (20:5)
LPC (22:5)	PC (36:2)	PC (36:4)1	LPC (22:6)
LPC (22:6)	PC (36:3)	PC (37:4)	LPE (18:2)
LPE (18:2)	PC (36:4)	PC (38:4)1	PC (18:1/16:0)
myristic acid	PC (36:4)1	PC (38:5)	PC (28:0)
oleic acid	PC (36:5)	PC (38:6)	PC (32:1)
palmitic acid	PC (36:6)	PC (40:5)	PC (32:2)
PC (34:2)	PC (37:2)	PC (40:6)	PC (34:2)
PC (36:2)	PC (37:3)	PC (42:6)	PC (36:2)
PC (36:5)	PC (38:2)	PE (34:2)	PC (36:3)
PC (37:2)	PC (38:4)	PE (36:2)	PC (36:4)
PE (36:2)	PC (38:5)	PE (38:2)	PC (36:5)
PE (38:2)	PC (40:7)	PE (38:4)	PC (36:6)
Plasmenyl PC (40:2)	PE (34:2)	PE (38:6)	PC (37:2)
Plasmenyl PC (42:2)	PE (36:1)1	Plasmenyl-PE (36:2)	PC (37:3)
TG (52:4)	PE (36:2)	SM (d18:1/21:0)	PE (36:1)
TG (52:5)	PE (38:2)	SM (d18:1/22:0)	PE (36:2)
TG (56:4)	Plasmenyl PC (40:2)	TG (46:0)	PE (38:4)
	Plasmenyl PC (42:2)	TG (49:0)	Plasmenyl PC (40:2)
	Plasmenyl-PC (34:1)	TG (49:2)	Plasmenyl PC (42:1)
	Plasmenyl-PC (34:2)	TG (56:6)	Plasmenyl PC (42:2)
	Plasmenyl-PE (36:2)	TG (56:7)	SM (33:1)
	SM (33:1)	TG (56:8)	SM (d18:1/14:0)
	SM (34:0)	TG (58:10)	SM (d18:1/21:0)
	SM (d18:1/14:0)	TG (58:6)	SM (d18:1/23:0)
	SM (d18:1/16:0)	TG (58:8)	TG (52:2)
	SM (d18:1/16:1)	TG (58:9)	TG (52:3)
	SM (d18:1/20:0)	TG (60:11)	TG (52:4)
	SM (d18:1/21:0)		TG (52:4)1
	SM (d18:1/22:0)		TG (52:5)
	SM (d18:1/23:0)		TG (53:1)
	SM (d18:1/24:0)		TG (53:2)
	SM (d18:2/23:0)		TG (53:3)
	TG (51:2)		TG (54:2)

TG (51:3)	TG (54:4)
TG (52:2)	TG (54:6)1
TG (52:3)	TG (56:4)
TG (52:4)	TG (56:5)
TG (52:4)1	TG (56:6)
TG (52:5)	TG (56:7)
TG (53:1)	TG (56:8)
TG (53:2)	TG (58:6)
TG (53:3)	TG (58:8)
TG (53:4)	TG (58:9)
TG (54:1)	TG (60:11)
TG (54:2)	
TG (54:3)	
TG (54:4)	
TG (54:5)	
TG (54:6)1	
TG (54:8)	
TG (56:2)	
TG (56:3)	
TG (56:4)	
TG (56:5)	
TG (56:6)	
TG (56:7)	
TG (56:8)	
TG (58:3)	
TG (58:4)	
TG (58:6)	
TG (58:8)	
TG (58:9)	
TG (60:11)	

Table 9: Small molecular metabolites associated with individual cognitive variables and direction (+ or -) of association.

PVT Lapses and Errors			PVT 1/RT			DSST			DSTC		
Metabolite	Class	+/-	Metabolite	Class	+/-	Metabolite	Class	+/-	Metabolite	Class	+/-
beta-alanine	Amino acids	+	asparagine	Amino acids	-	asparagine	Amino acids	+	5-hydroxynorvaline	Amino acids	+
glycine	Amino acids	-	cysteine-glycine	Amino acids	+	citrulline	Amino acids	-	5-methoxytryptamine	Amino acids	+
leucine	Amino acids	+	glutamic acid	Amino acids	+	cysteine	Amino acids	+	aspartic acid	Amino acids	+
N-acetylglutamate	Amino acids	+	glutamylthreonine	Amino acids	+	cystine	Amino acids	-	glutamylthreonine	Amino acids	+
phenylalanine	Amino acids	+	indole-3-lactate	Amino acids	+	indole-3-acetate	Amino acids	-	glycine	Amino acids	+
trans-4-hydroxyproline	Amino acids	-	trans-4-hydroxyproline	Amino acids	+	Arginine	Amino acids	-	leucine	Amino acids	-
arabinose	Carbohydrate	+	1,5-anhydroglucitol	Carbohydrate	-	Leucine Isomer	Amino acids	-	Leucine Isomer	Amino acids	-
fucose + rhamnose	Carbohydrate	+	2-deoxyerythritol	Carbohydrate	+	methionine	Amino acids	+	pipecolic acid	Amino acids	+
ribitol	Carbohydrate	-	glucose	Carbohydrate	-	N-acetyl glycine	Amino acids	-	Tryptophan	Amino acids	-
sucrose	Carbohydrate	+	glycerol-3-galactoside	Carbohydrate	+	N-methylalanine	Amino acids	-	1,5-anhydroglucitol	Carbohydrate	-
1-Methyl-4-pyridone-5-carboxamide	Cofactor	+	Pantothenic acid	Cofactor	-	ornithine	Amino acids	-	2-deoxyerythritol	Carbohydrate	+
Niacinamide	Cofactor	+	fumaric acid	Organic acids	-	threonine	Amino acids	+	arabitol	Carbohydrate	+
2-deoxyisotetroic acid	Organic acids	+	lactamide	Organic acids	+	tryptophan	Amino acids	+	erythritol	Carbohydrate	+
2-hydroxybutanoic acid	Organic acids	-	lactic acid	Organic acids	+	1,5-anhydroglucitol	Carbohydrate	-	fucose	Carbohydrate	+
3-aminoisobutyric acid	Organic acids	-	creatinine	Others	+	fucose	Carbohydrate	-	glucuronic acid	Carbohydrate	+
3-hydroxybutanoic acid	Organic acids	+	salicylaldehyde	Others	-	glucuronic acid	Carbohydrate	-	N-acetylmannosamine	Carbohydrate	+
aconitic acid	Organic acids	+	TMAO	Others	+	levanbiose	Carbohydrate	-	sorbitol	Carbohydrate	+
dehydroascorbic acid	Organic acids	-				sucrose	Carbohydrate	-	sucrose	Carbohydrate	-
maleimide	Organic acids	+				Niacinamide	Cofactor	-	threitol	Carbohydrate	+
benzylalcohol	Others	-				Pantothenic acid	Cofactor	-	xylose	Carbohydrate	+

creatinine	Others	+	tocopherol alpha	Cofactor	-	1-Methyl-4- pyridone-5- carboxamide	Cofactor	-
taurine	Others	-	2- deoxyisotetro nic acid	Organic acids	-	benzoic acid	Others	-
uric acid	Others	+	3- aminoisobuty ric acid	Organic acids	+	benzylalcohol	Others	+
			aminomaloni c acid	Organic acids	+	creatinine	Others	+
			azelaic acid	Organic acids	-	phenylethylam ine	Others	-
			citric acid	Organic acids	-	phthalic acid	Others	+
			isocitric acid	Organic acids	-	p-cresol-	Others	+
			isothreonic acid	Organic acids	+			
			oxalic acid	Organic acids	-			
			alpha- terpineol	Others	+			
			Creatine	Others	-			
			creatinine	Others	-			
			hippuric acid	Others	-			
			phosphoethan olamine	Others	-			
			phosphoric acid	Others	-			
			pseudo uridine	Others	-			
			salicylaldehy de	Others	-			
			uridine	Others	+			

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While the invention has been disclosed with reference to specific embodiments, it is
5 apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

Claims

What is claimed is:

1. A method of identifying and treating a subject suspected of having sleep deprivation, the method comprising:
 - a. determining the test level of a set of biomarkers in a test sample obtained from the subject, wherein the set of biomarkers are one or more metabolites selected from the group consisting of: tryptophan, triacylglycerol (TG) (54:3), phenylalanine, phosphatidylcholine (PC) (38:3), PC (36:6), lysophosphatidylcholine (LPC) (20:3), LPC (14:0), dodecanol, carnitine C5:0, acylcarnitine C12:0, acylcarnitine C10:0, phosphatidylethanolamine (PE) (36:1), PC (38:2), octadecanol, myristic acid, TG (53:1), TG (52:2), pipecolic acid, oxalic acid, LPC (16:1), acylcarnitine C18:1, lysophosphatidylcholine (lysoPC) 16:0, TG (46:0), PC (32:1), lysoPC 18:2, diacylglycerol (DG) (36:3), methionine, TG (54:4), TG (52:3), TG (48:1) and DG (36:2).
 - b. comparing the test level of the biomarkers in the test sample with a baseline level of the biomarkers;

wherein when the test level is significantly different from the baseline level of the set of biomarkers in the direction predicted for sleep deprivation, the subject is suspected of having sleep deprivation and treatment is initiated.
2. The method of claim 1, wherein the subject is a human.
3. The method of claim 1, wherein the test sample is a blood sample.
4. The method of claim 1, wherein the set of biomarkers comprises two metabolites selected from the group consisting of: tryptophan, TG (54:3), phenylalanine, PC (38:3), PC (36:6), LPC (20:3), LPC (14:0), dodecanol, carnitine C5:0, acylcarnitine C12:0, acylcarnitine C10:0, PE (36:1), PC (38:2), octadecanol, myristic acid, TG (53:1), TG (52:2), pipecolic acid, oxalic acid, LPC (16:1), acylcarnitine C18:1, lysoPC 16:0, TG

(46:0), PC (32:1), lysoPC 18:2, DG (36:3), methionine, TG (54:4), TG (52:3), TG (48:1) and DG (36:2).

5. The method of claim 1, wherein the set of biomarkers comprises three metabolites selected from the group consisting of: tryptophan, TG (54:3), phenylalanine, PC (38:3), PC (36:6), LPC (20:3), LPC (14:0), dodecanol, carnitine C5:0, acylcarnitine C12:0, acylcarnitine C10:0, PE (36:1), PC (38:2), octadecanol, myristic acid, TG (53:1), TG (52:2), pipecolic acid, oxalic acid, LPC (16:1), acylcarnitine C18:1, lysoPC 16:0, TG (46:0), PC (32:1), lysoPC 18:2, DG (36:3), methionine, TG (54:4), TG (52:3), TG (48:1) and DG (36:2).
6. The method of claim 1, wherein the set of biomarkers comprises four metabolites selected from the group consisting of: tryptophan, TG (54:3), phenylalanine, PC (38:3), PC (36:6), LPC (20:3), LPC (14:0), dodecanol, carnitine C5:0, acylcarnitine C12:0, acylcarnitine C10:0, PE (36:1), PC (38:2), octadecanol, myristic acid, TG (53:1), TG (52:2), pipecolic acid, oxalic acid, LPC (16:1), acylcarnitine C18:1, lysoPC 16:0, TG (46:0), PC (32:1), lysoPC 18:2, DG (36:3), methionine, TG (54:4), TG (52:3), TG (48:1) and DG (36:2).
7. The method of claim 4, wherein the two metabolites are oxalic acid and DG (36:3).
8. The method of claim 1, wherein the baseline level of the biomarkers is determined in a sample obtained from the subject when the subject is not sleep deprived.
9. The method of claim 8, wherein the subject is a human.
10. The method of claim 9, wherein the subject has slept for between about 6 hours to about 10 hours prior to determining the baseline level.
11. The method of claim 10, wherein the subject has slept for about 7 hours prior to determining the baseline level.

12. The method of claim 10, wherein the subject has slept for about 8 hours prior to determining the baseline level.
- 5 13. The method of claim 10, wherein the subject has slept for about 9 hours prior to determining the baseline level.
14. The method of claim 1, wherein when the test level of the set of biomarkers is elevated when compared to the baseline level, the subject is suspected of having sleep deprivation.
- 10 15. The method of claim 1, wherein when the test level of the set of biomarkers is reduced when compared to the baseline level, the subject is suspected of having sleep deprivation.
16. A method of identifying and treating a subject suspected of having cognitive impairment as a result of sleep deprivation, the method comprising: a) obtaining a test sample from
 15 the subject; and b) determining the presence of a set of biomarkers in the test sample, wherein the set of biomarkers are one or more metabolites selected from the group consisting of: beta-alanine, glycine, leucine, N-acetylglutamate, phenylalanine, trans-4-hydroxyproline, arabinose, fucose + rhamnose, ribitol, sucrose, acetylcarnitine, acylcarnitine C10:0, acylcarnitine C10:1, acylcarnitine C12:0, acylcarnitine C12:1,
 20 acylcarnitine C16:0, acylcarnitine C18:0, acylcarnitine C6:0, 17-methyltestosterone, 1-methyl-4-pyridone-5-carboxamide, niacinamide, glycerol, arachidonic acid, capric acid, heptadecanoic acid, isoheptadecanoic acid, linoleic acid, linolenic acid, myristic acid, oleic acid, palmitic acid, LPC (14:0), LPC (16:0), LPC (16:1), LPC (17:0), LPC (17:1), LPC (18:0), LPC (18:1), LPC (18:2), LPC (18:3), LPC (19:0), LPC (20:0), LPC (20:1),
 25 LPC (20:2), LPC (20:3), LPC (20:4), LPC (20:5), LPC (22:1), LPC (22:4), LPC (22:5), LPC (22:6), lysophosphatidylethanolamine (LPE) (18:2), 2-deoxyisotetroneic acid, 2-hydroxybutanoic acid, 3-aminoisobutyric acid, 3-hydroxybutanoic acid, aconitic acid, dehydroascorbic acid, maleimide, benzylalcohol, creatinine, taurine, uric acid, PC (34:2), PC (36:2), PC (36:5), PC (37:2), PE (36:2), PE (38:2), plasmenyl PC (40:2), plasmenyl
 30 PC (42:2), TG (52:4), TG (52:5), TG (56:4), asparagine, cysteine-glycine, glutamic acid, glutamylthreonine, indole-3-lactate, 1,5-anhydroglucitol, 2-deoxyerythritol, glucose,

glycerol-3-galactoside, acylcarnitine C5:0, cholesterol and related molecules and esters (CE) (18:1), CE (18:2), CE (18:3), ceramide (d18:1/23:0), pantothenic acid, DG (34:1), DG (36:2), DG (36:3), DG (38:5), DG (38:6), caprylic acid, glycerol-alpha-phosphate, fumaric acid, lactamide, lactic acid, salicylaldehyde, TMAO, PC (16:0/16:0), PC (18:1/16:0), PC (32:1), PC (32:2), PC (34:0), PC (36:1), PC (36:3), PC (36:4), PC (36:4)1, PC (36:6), PC (37:3), PC (38:2), PC (38:4), PC (38:5), PC (40:7), PE (34:2), PE (36:1)1, plasmenyl-PC (34:1), plasmenyl-PC (34:2), plasmenyl-PE (36:2), SM (33:1), SM (34:0), SM (d18:1/14:0), SM (d18:1/16:0), SM (d18:1/16:1), SM (d18:1/20:0), SM (d18:1/21:0), SM (d18:1/22:0), SM (d18:1/23:0), SM (d18:1/24:0), SM (d18:2/23:0), TG (51:2), TG (51:3), TG (52:2), TG (52:3), TG (52:4)1, TG (53:1), TG (53:2), TG (53:3), TG (53:4), TG (54:1), TG (54:2), TG (54:3), TG (54:4), TG (54:5), TG (54:6)1, TG (54:8), TG (56:2), TG (56:3), TG (56:5), TG (56:6), TG (56:7), TG (56:8), TG (58:3), TG (58:4), TG (58:6), TG (58:8), TG (58:9), TG (60:11), citrulline, cysteine, cystine, indole-3-acetate, L-arginine, leucine isomer, methionine, N-acetylglycine, N-methylalanine, ornithine, threonine, tryptophan, fucose, glucuronic acid, levanbiose, acylarnitine C12:1, acylarnitine C3:0, acylcarnitine C4:0, carnitine, CE (22:6), cholesterol, ceramide (d40:1), ceramide (d42:1), ceramide (d42:2), tocopherol alpha, palmitoleic acid, aminomalonic acid, azelaic acid, citric acid, isocitric acid, isothreonic acid, oxalic acid, alpha-terpineol, creatine, hippuric acid, phosphoethanolamine, phosphoric acid, pseudo uridine, uridine, PC (37:4), PC (38:4)1, PC (38:6), PC (40:5), PC (40:6), PC (42:6), PE (38:4), PE (38:6), TG (46:0), TG (49:0), TG (49:2), TG (58:10), 5-hydroxynorvaline, 5-methoxytryptamine, aspartic acid, pipecolic acid, arabitol, erythritol, N-acetylmannosamine, sorbitol, threitol, xylose, corticosterone, alpha ketoglutaric acid, glutaric acid, phenylacetic acid, benzoic acid, phenylethylamine, phthalic acid, p-cresol-PC (28:0), PE (36:1), and plasmenyl PC (42:1), wherein the presence of the set of biomarkers in the test sample is an indication that the subject has cognitive impairment and treatment is initiated.

17. The method of claim 16, wherein the set of biomarkers comprises PE (36:2), LPC (16:0), LPC (16:1), LPC (18:2), LPC (20:3), LPC (20:5), capric acid and 17-Methyltestosterone.

18. The method of claim 16, wherein the subject is a human.

19. The method of claim 16, wherein the cognitive impairment is assessed by cognitive variables comprising PVT lapses and errors, PVT 1/RT, DSST, and DSTC.
20. The method of claim 16, wherein the test sample is a blood sample.

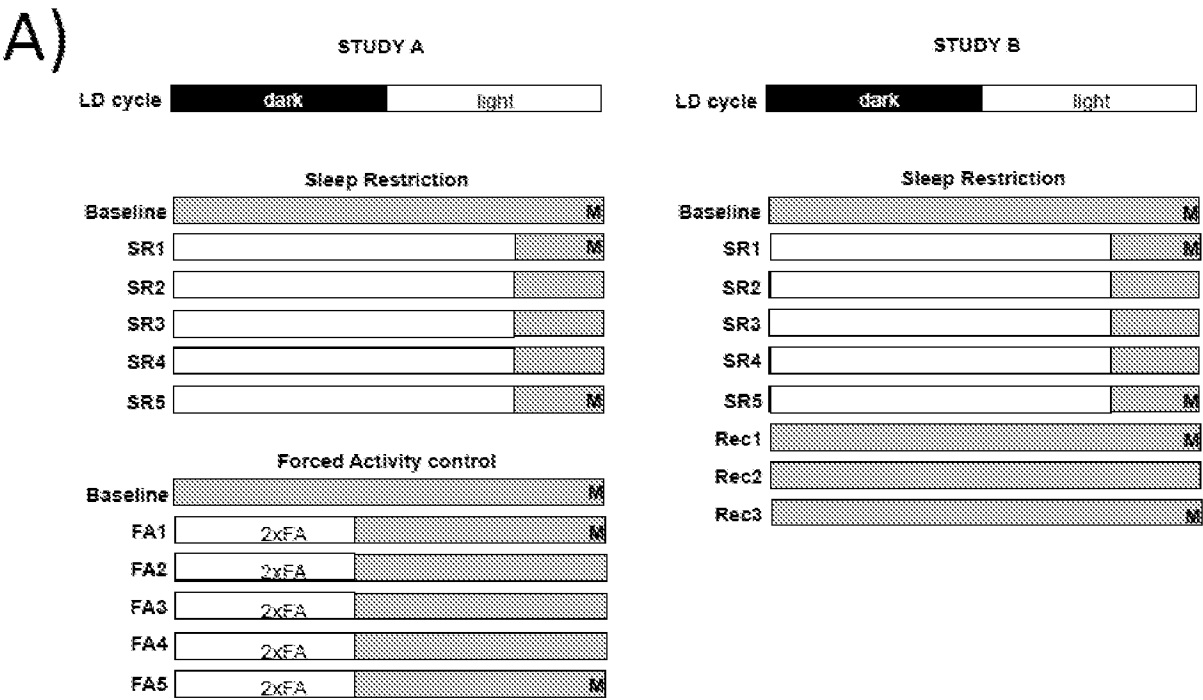


FIG. 1A

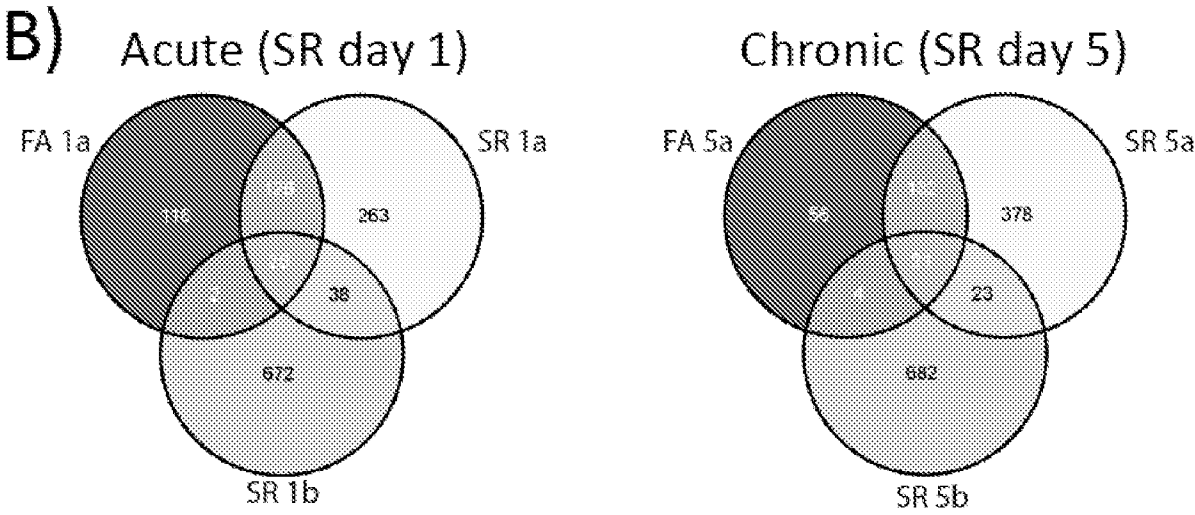


FIG. 1B

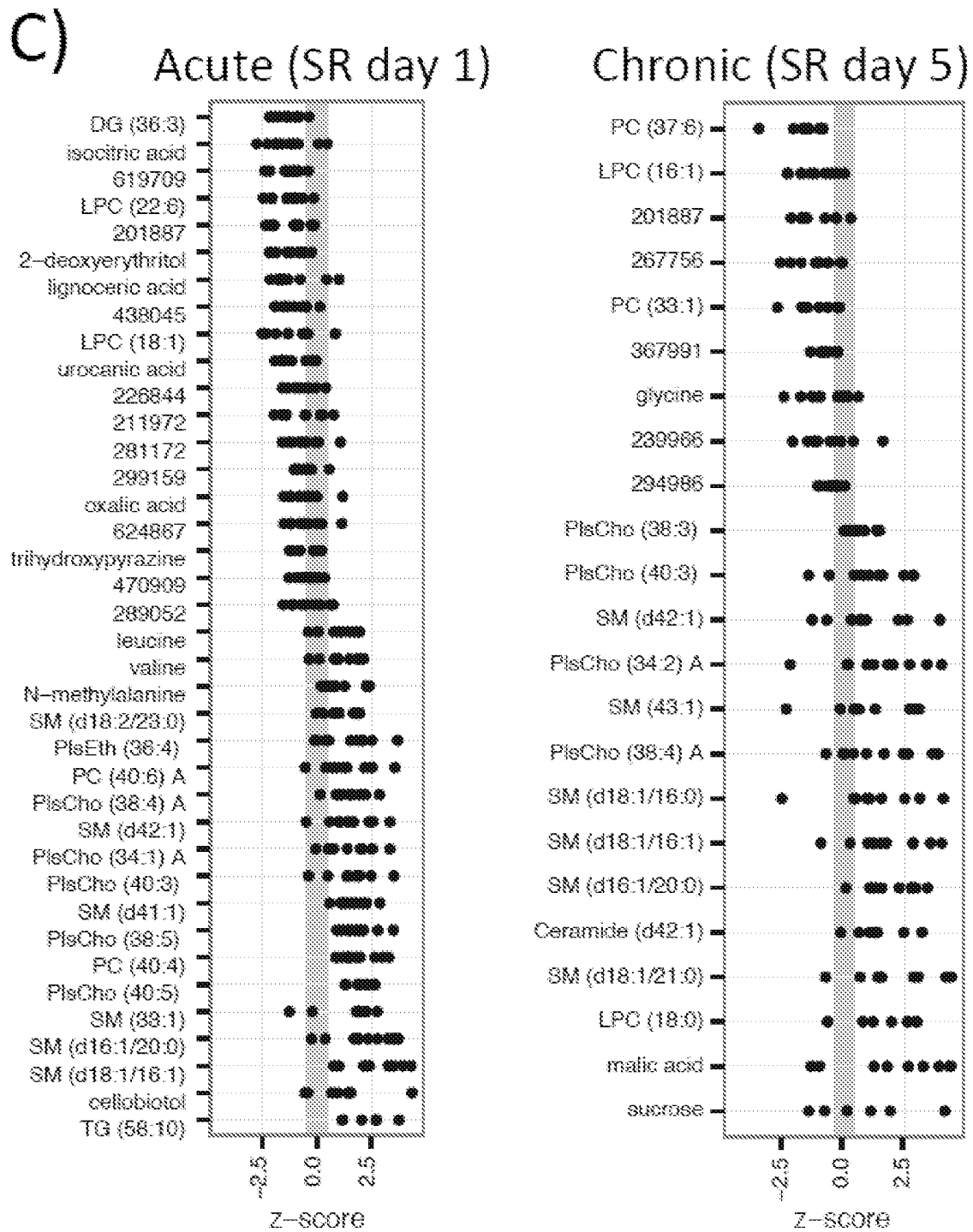


FIG. 1C

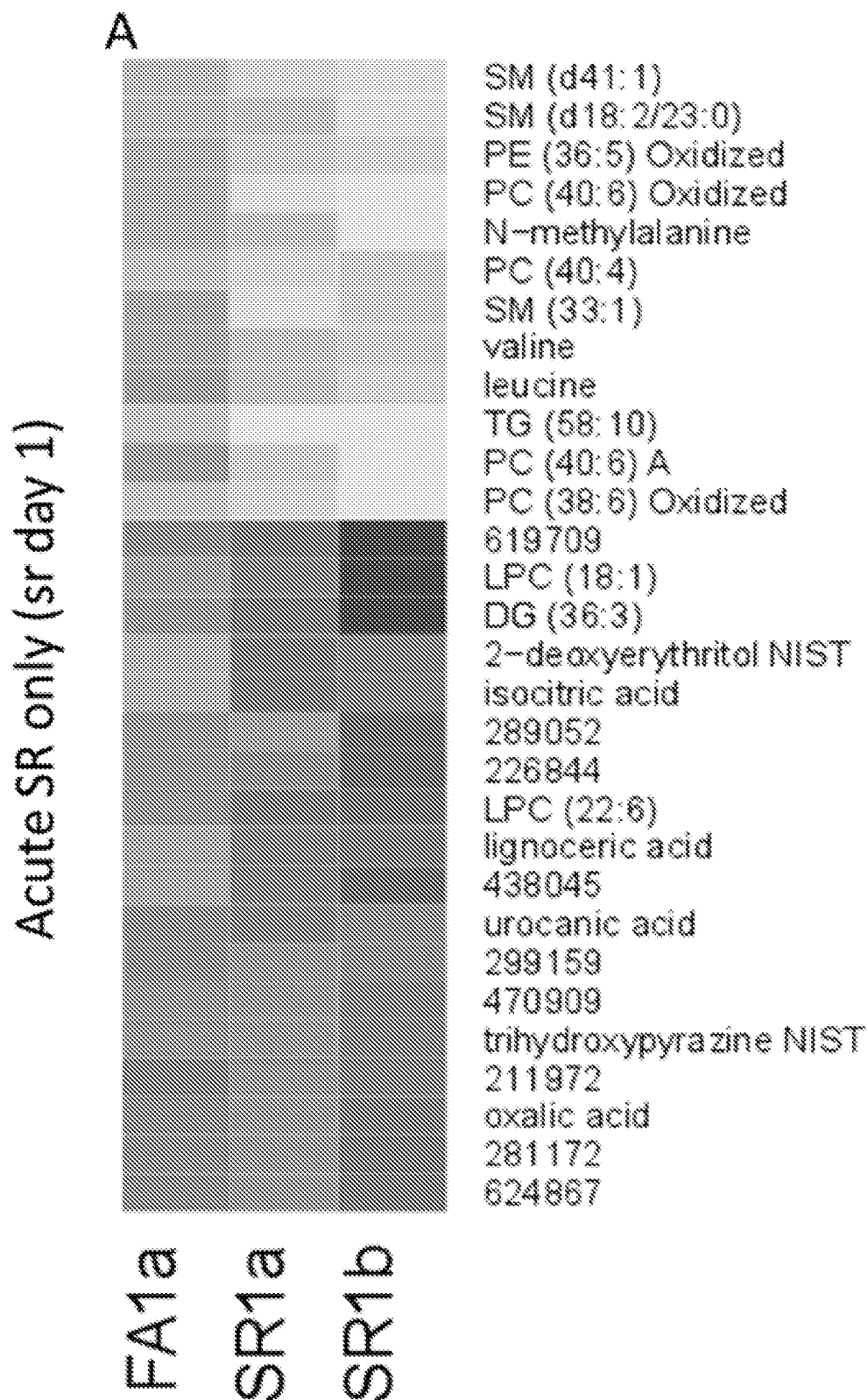
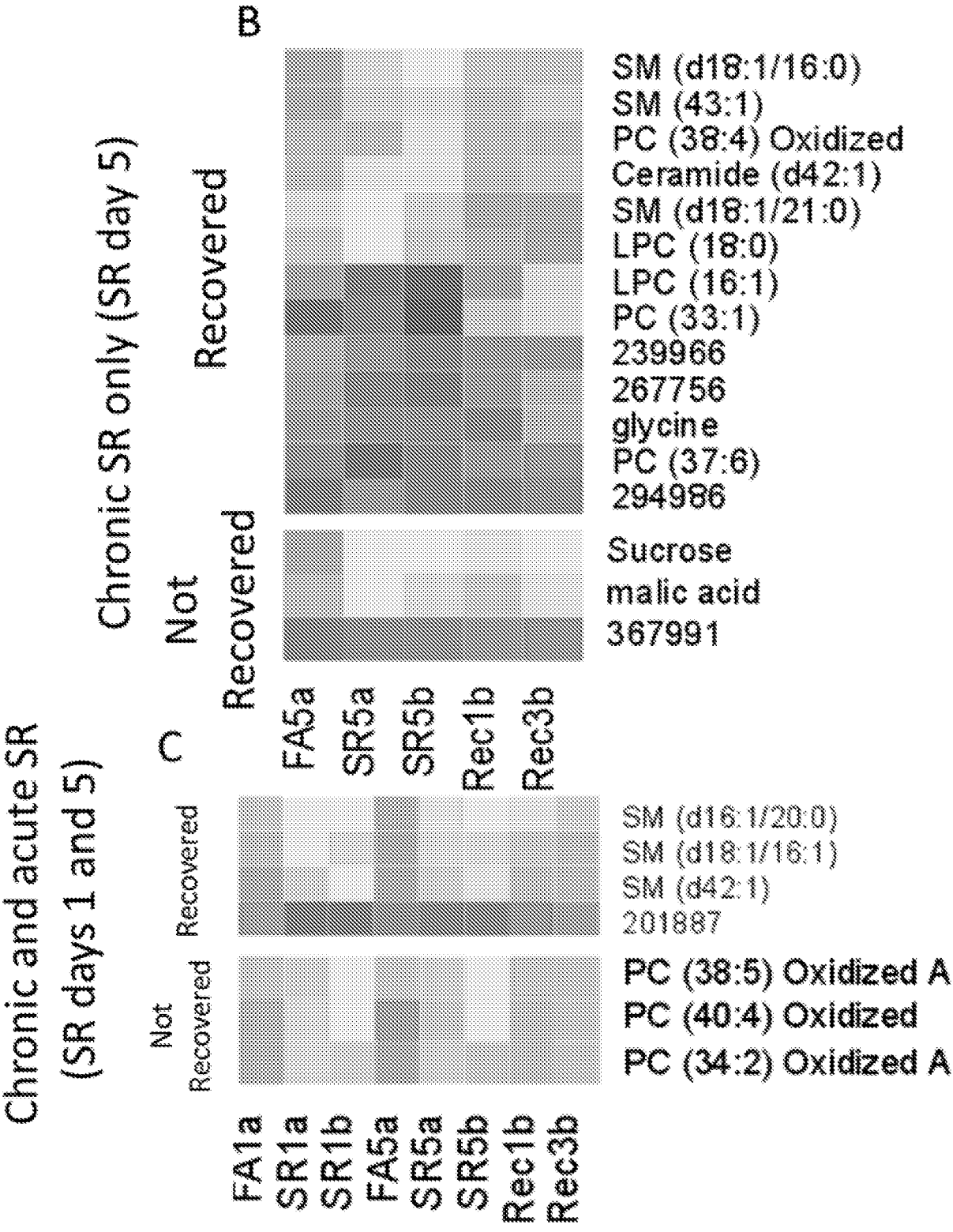


FIG. 2A



FIGs. 2B-2C

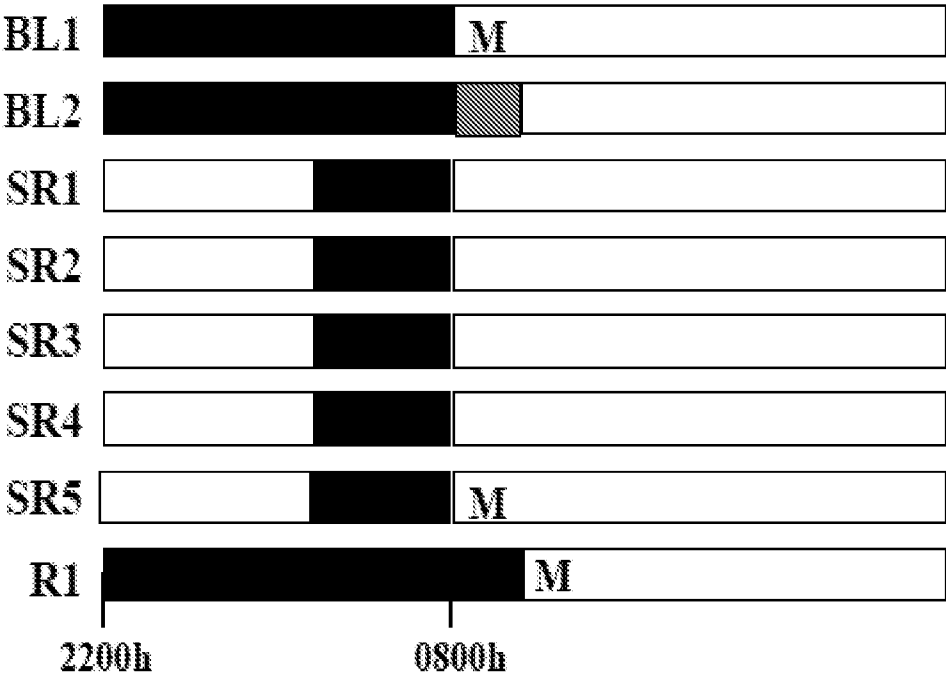


FIG. 3A

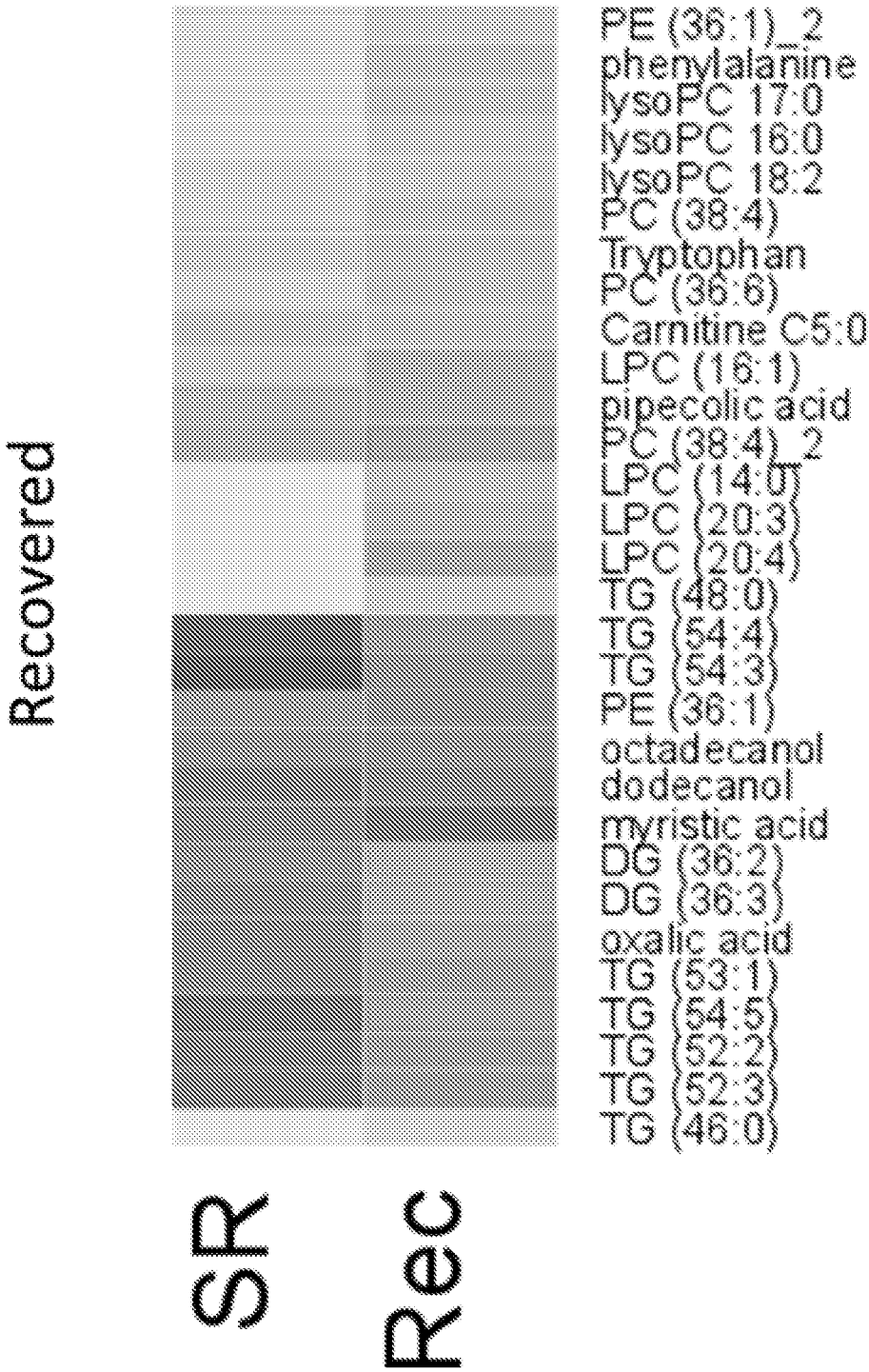


FIG. 3B

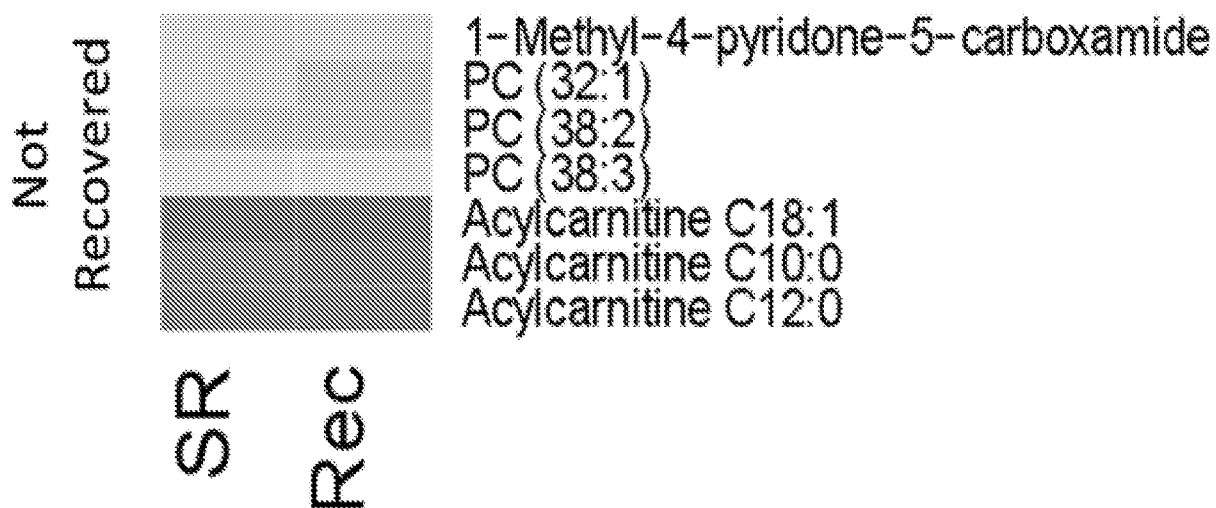


FIG. 3C

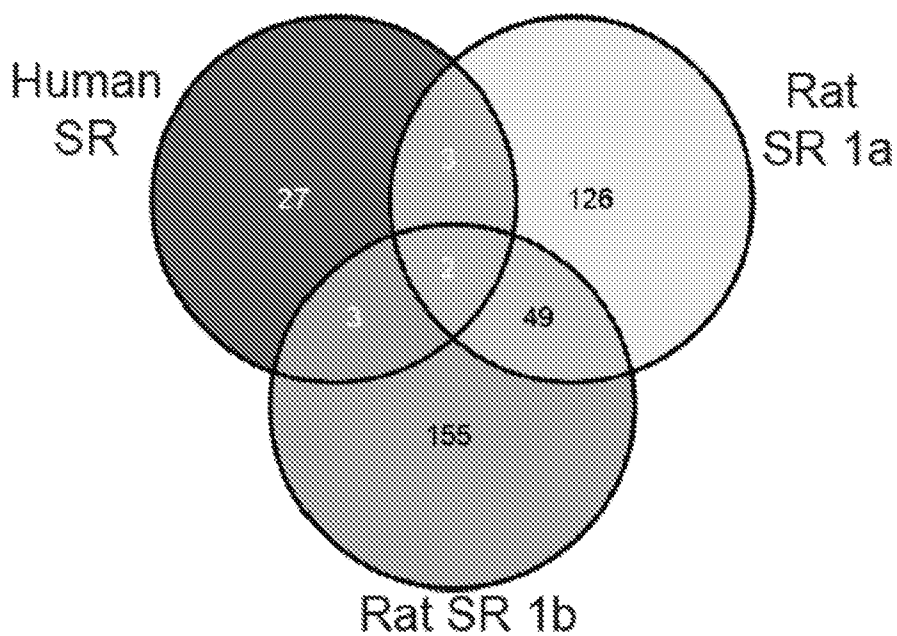


FIG. 4A

Metabolite	Rat			Human		
	P-value	FDR	Day	P-value	FDR	Day
Oxalic acid	<0.01	0.02	1↓	<0.01	0.19	5↓
DG 36:3	<0.01	0.008	1↓	<0.01	0.12	5↓

FIG. 4B

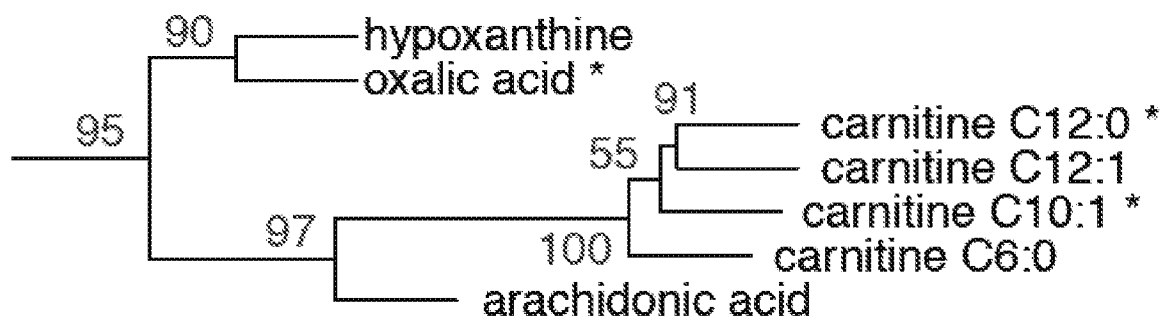


FIG. 4C

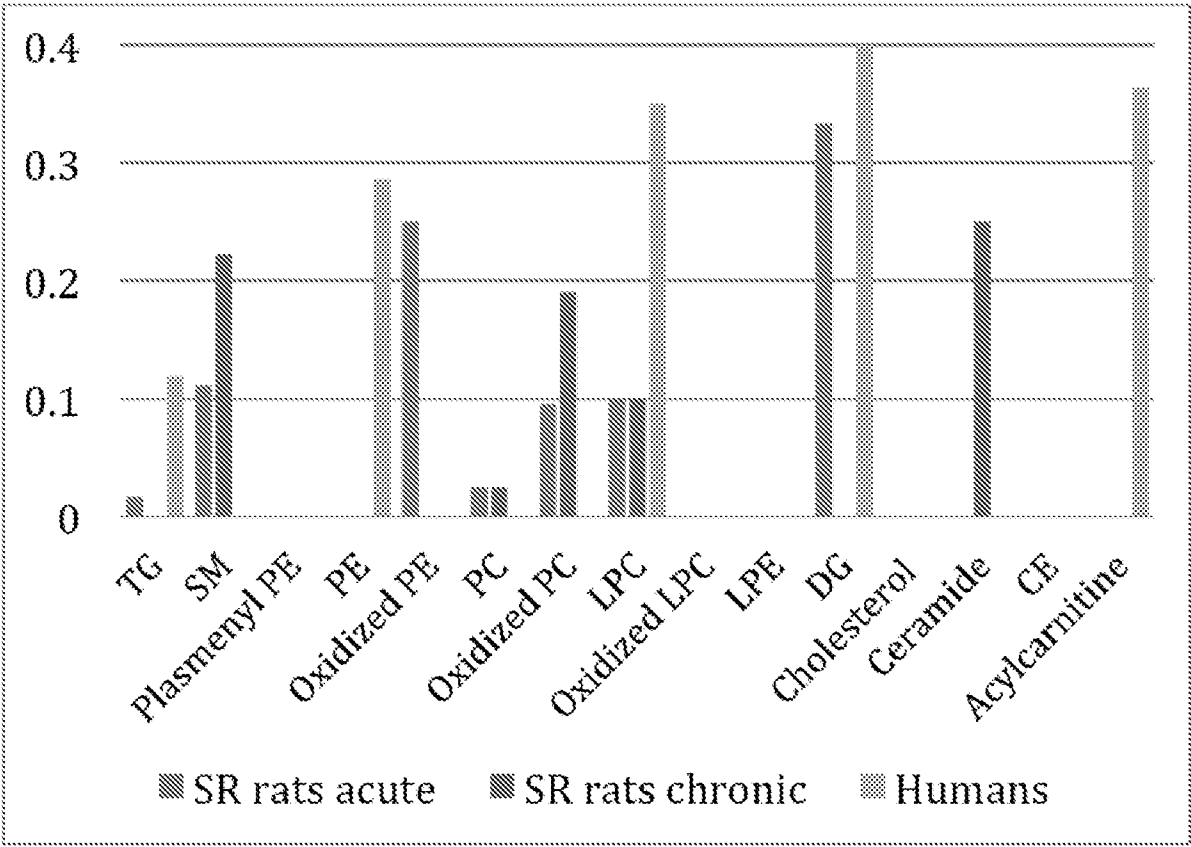


FIG. 5

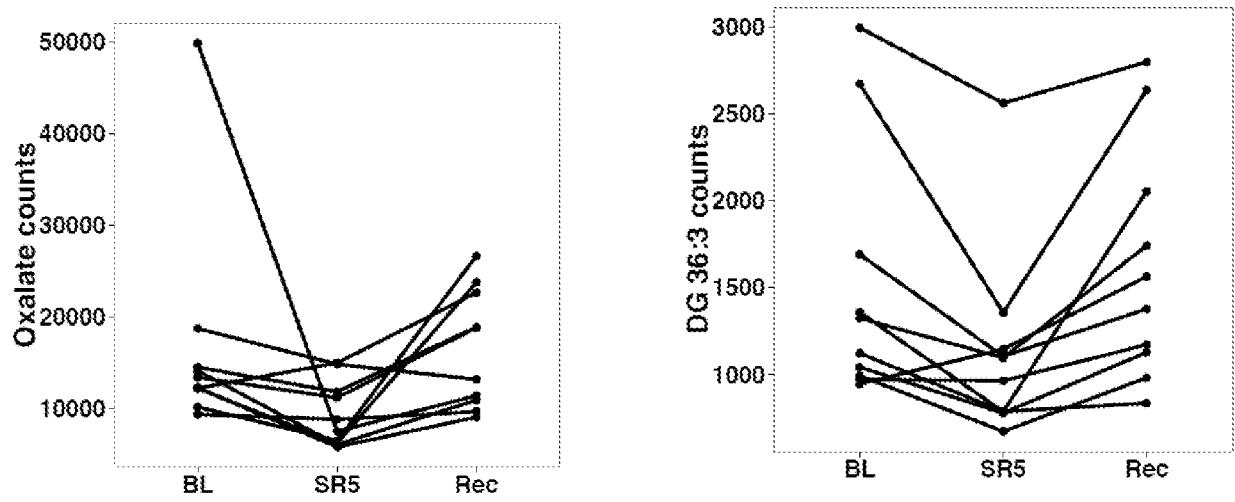


FIG. 6A

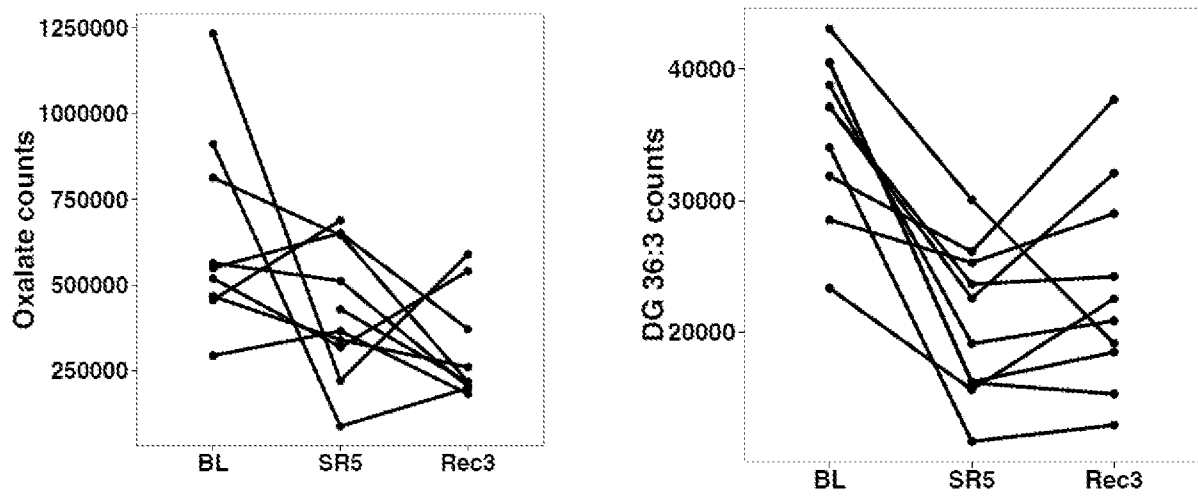


FIG. 6B

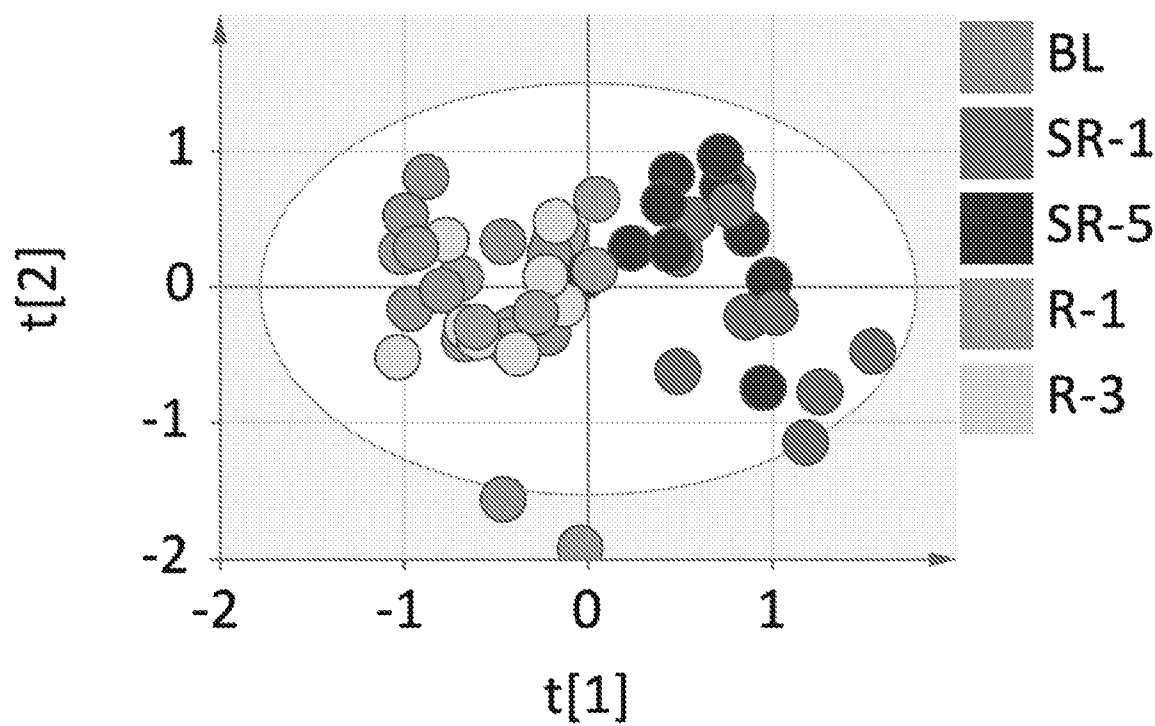


FIG. 7

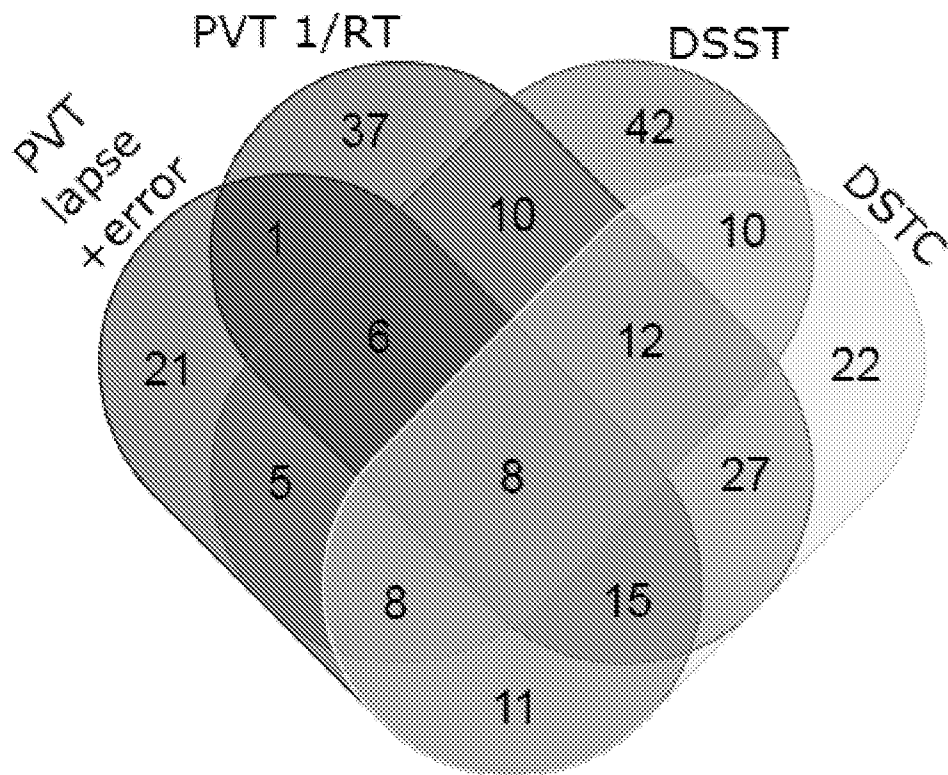


FIG. 8A

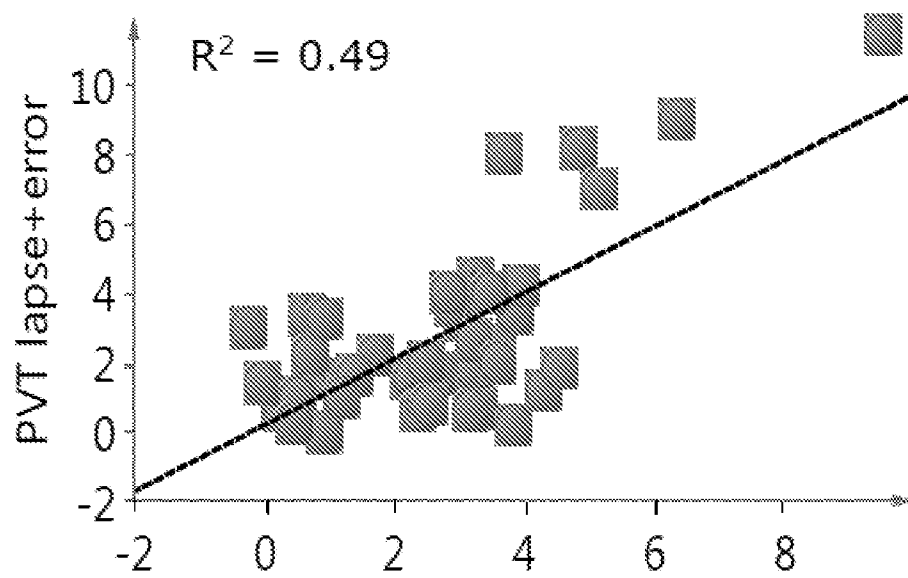


FIG. 8B

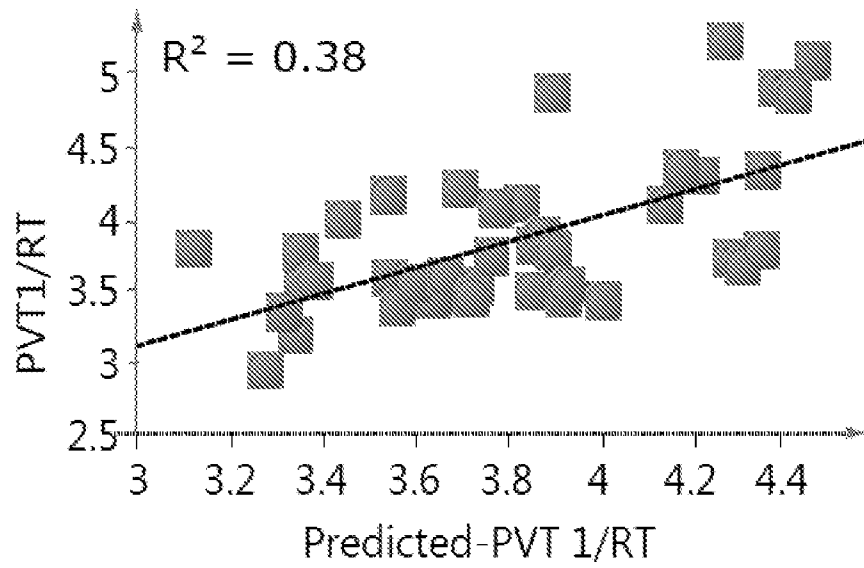


FIG. 8C

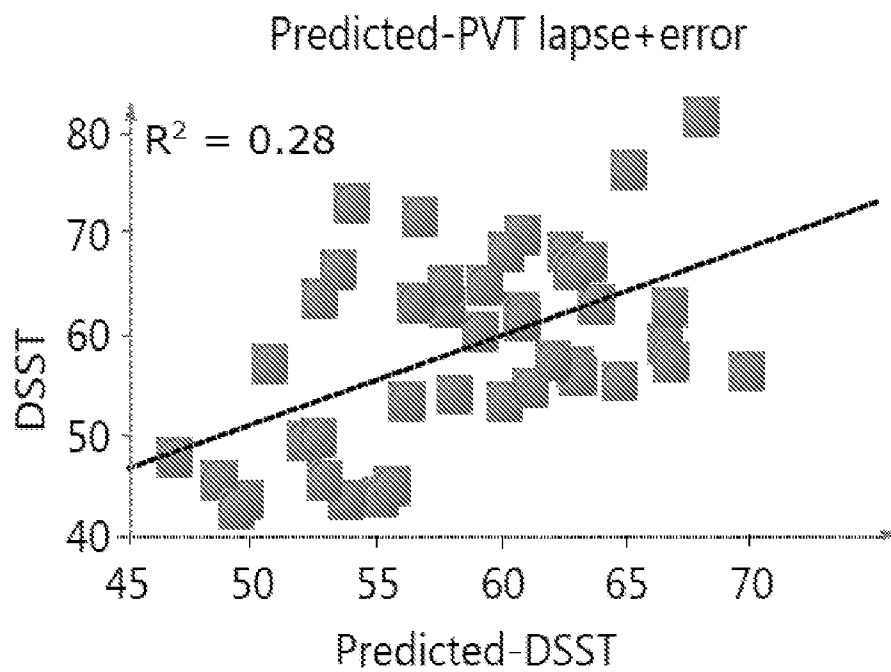


FIG. 8D

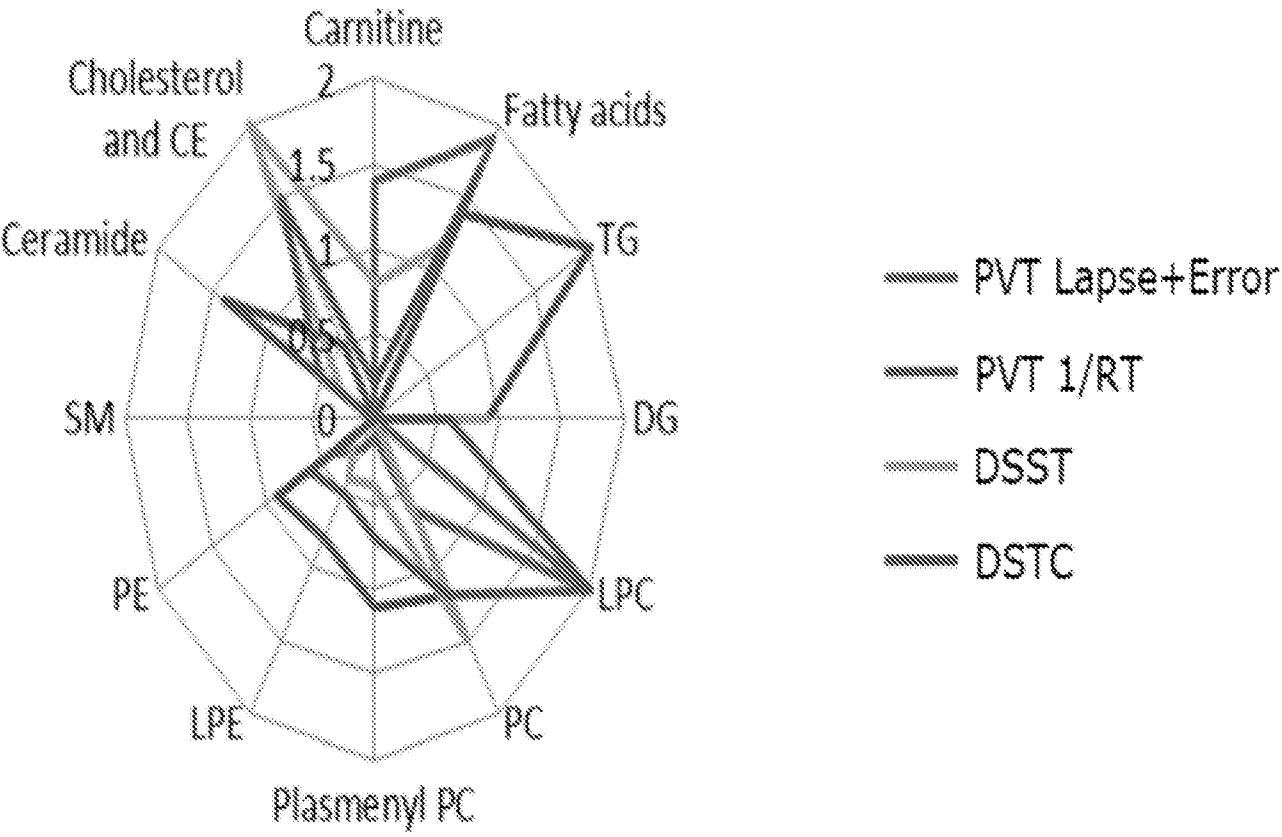


FIG. 9A

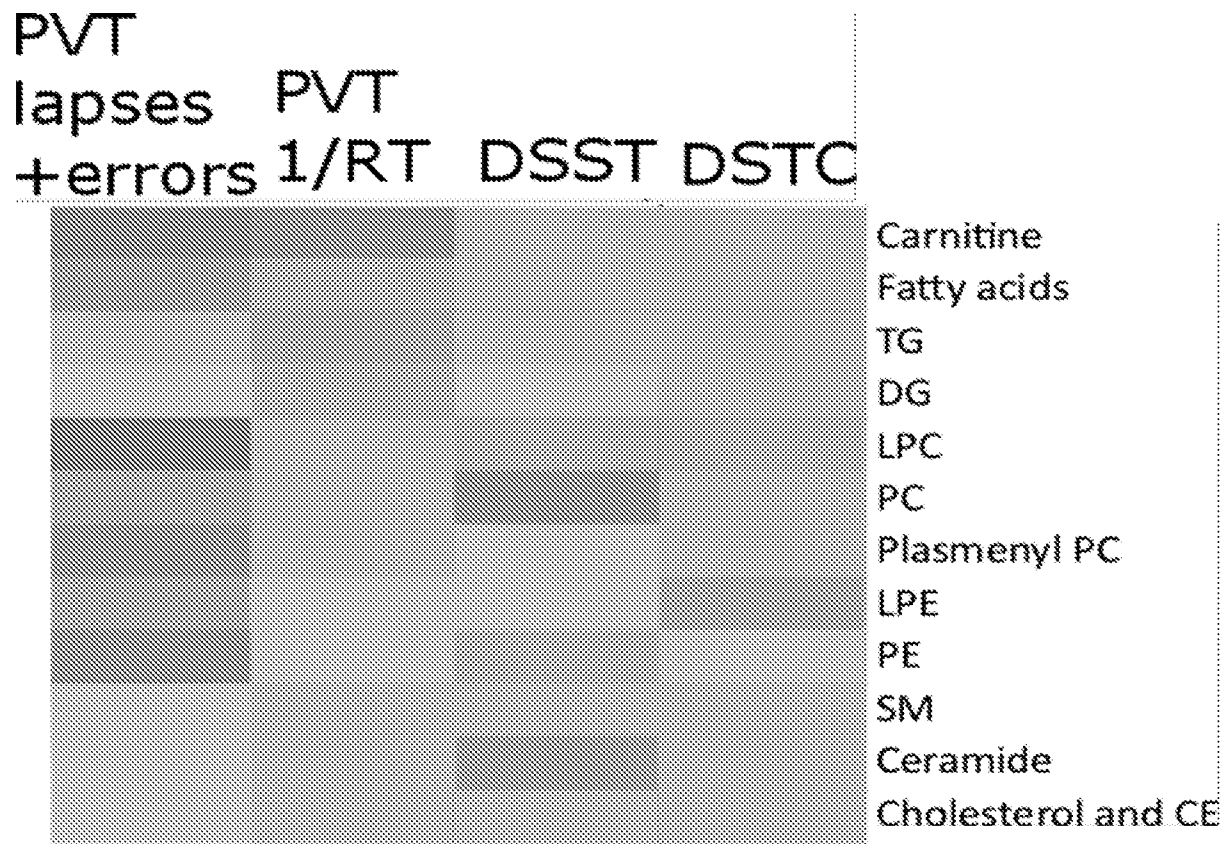


FIG. 9B

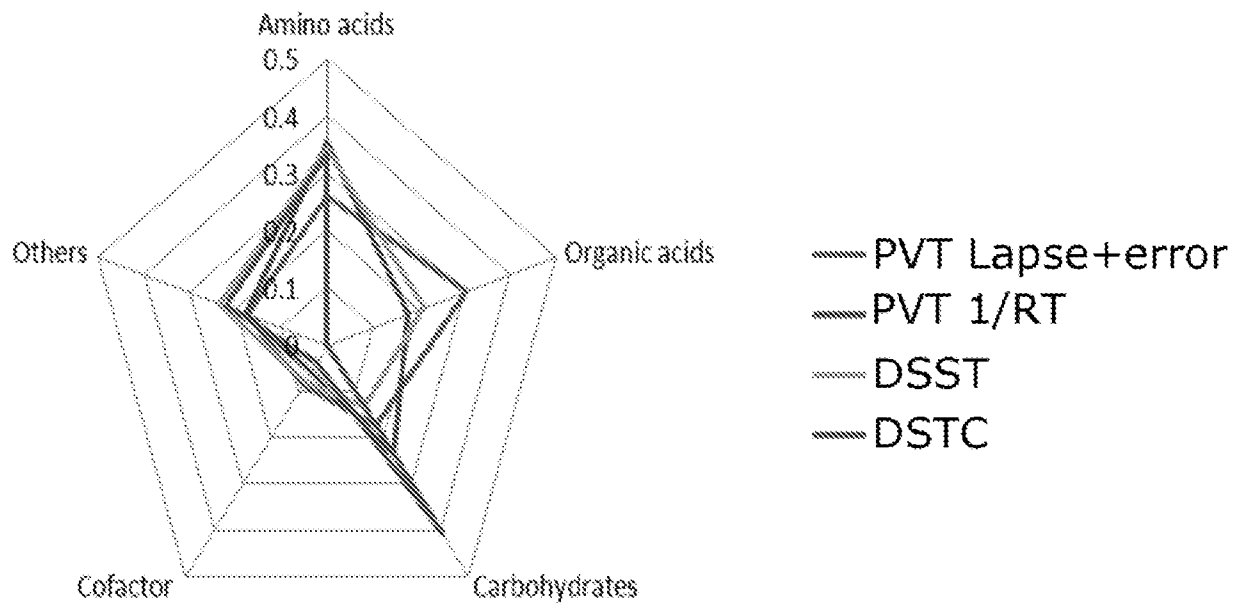
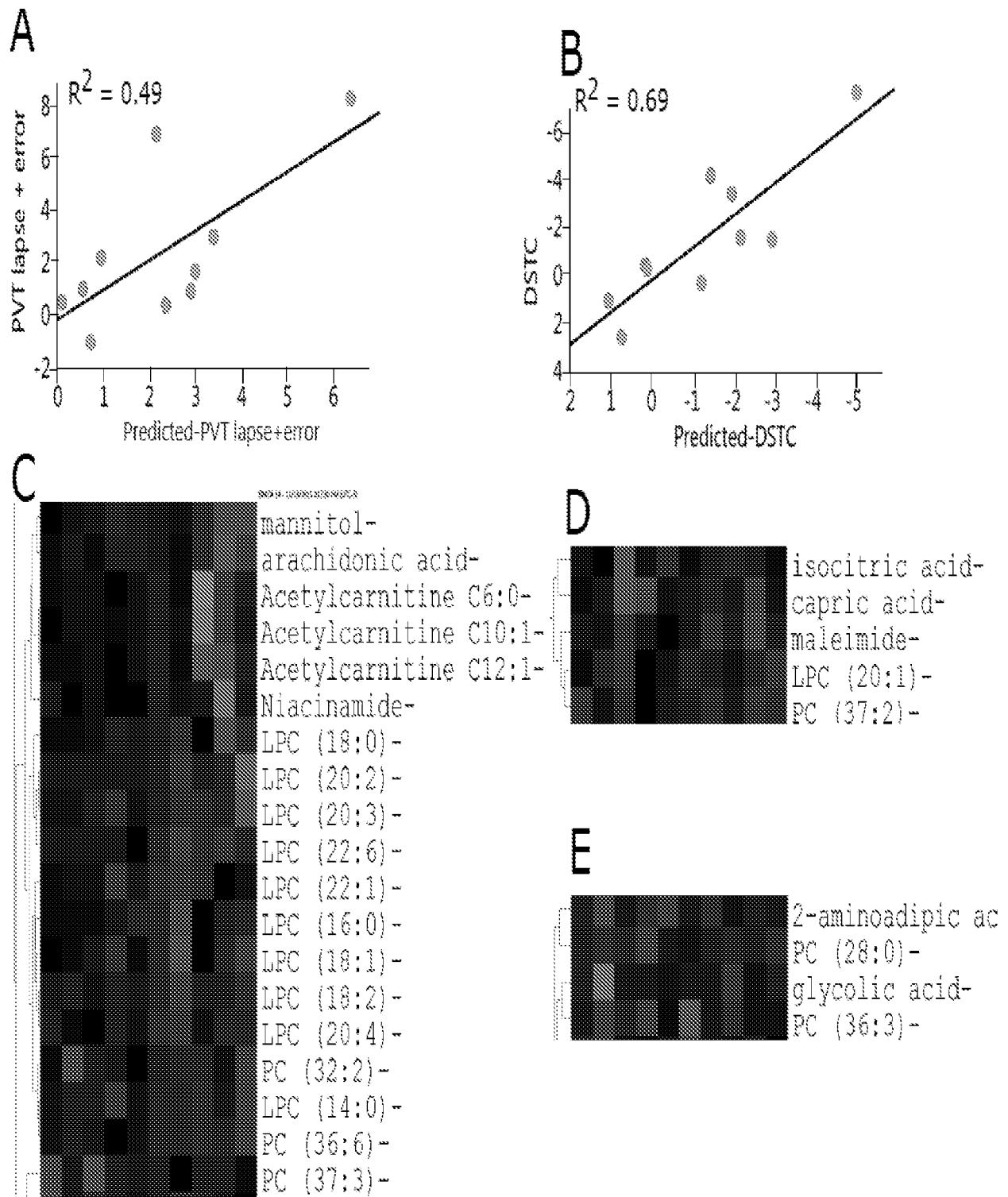


FIG. 10



FIGs. 11A-11E

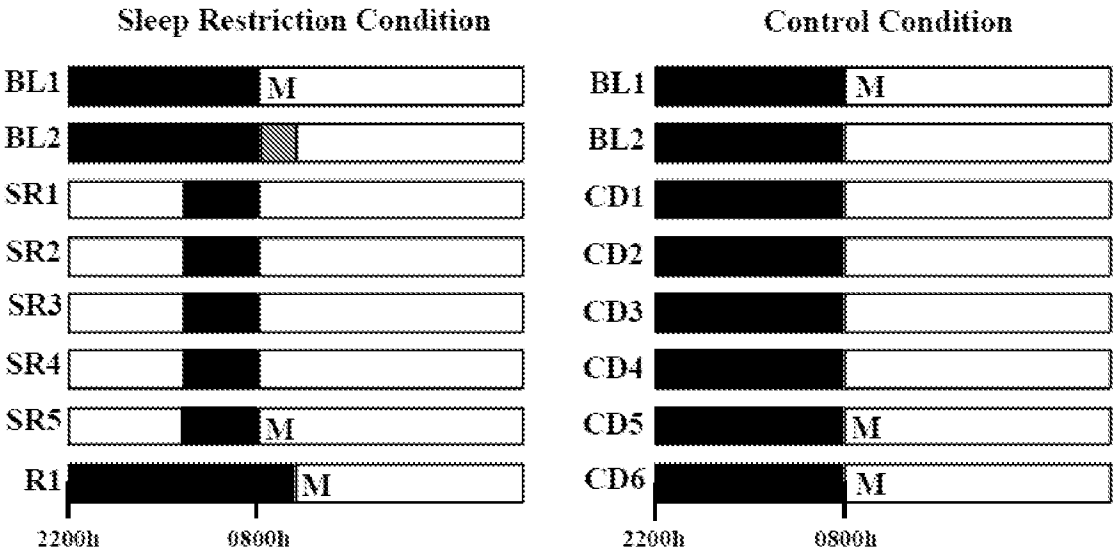


FIG. 12

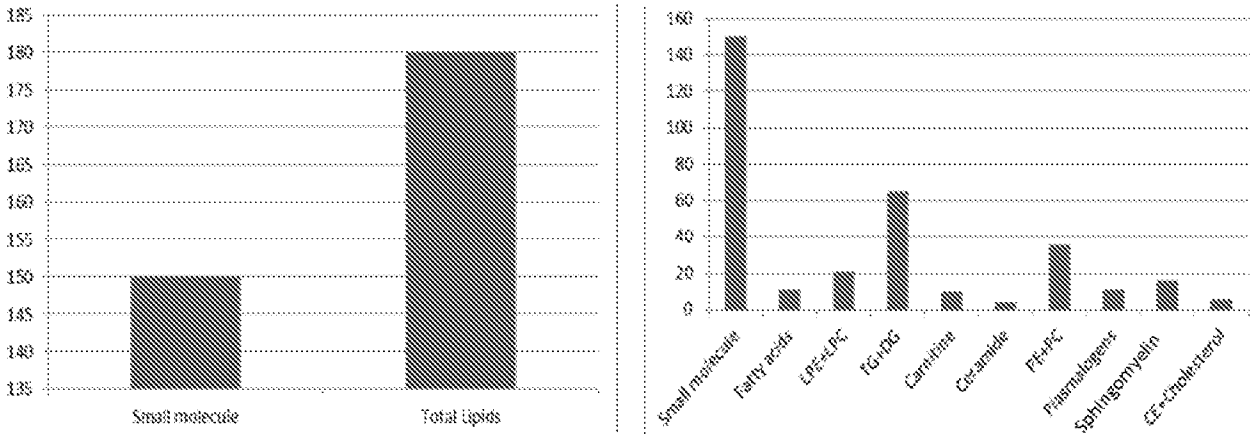


FIG. 13