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(54) Title: SMALL MOLECULE METABOLITES FOR THE DIAGNOSIS OF BACTERIAL VAGINOSIS AND ASSESSMENT OF DISEASE RISK

(57) Abstract: The invention disclosed herein generally relates to methods and kits for diagnosing, assessing disease risk, treating, and preventing bacterial vaginosis (BV) and associated conditions. Additional embodiments include methods for developing metabolic profiles associated with increased disease risk, and developing new approaches to treat BV based on interrupting metabolic networks.



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SMALL MOLECULE METABOLITES FOR THE DIAGNOSIS OF BACTERIAL VAGINOSIS AND ASSESSMENT OF DISEASE RISK

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims priority to U.S. Provisional Application 62/146,867 filed on April 13, 2015, which is hereby incorporated by reference in its entirety.

GOVERNMENT SUPPORT

[0002] This invention was made with government support under A1061628 and HG005816 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] The invention disclosed herein generally relates to methods and kits for diagnosing, assessing disease risk, treating, and preventing bacterial vaginosis (BV) and associated conditions. Additional embodiments include methods for developing metabolic profiles associated with increased disease risk, and developing new approaches to treat BV based on interrupting metabolic networks.

BACKGROUND

[0004] Bacterial vaginosis (BV) is a common but highly enigmatic condition that is associated with adverse outcomes for women and their neonates. BV is characterized by shifts in the vaginal microbiota from *Lactobacillus* dominant to a microbiota with diverse anaerobic bacteria. Small molecule metabolites in the vagina influence host physiology, affect microbial community composition, and impact risk of adverse health outcomes.

[0005] The composition of small molecule metabolites in the human vagina is largely shaped by bacterial metabolism of human derived nutrients. These bacterial metabolites may impact human cell function, inflammation, and disease susceptibility. However, few studies have comprehensively examined the associations between vaginal bacteria and metabolites, and no study has used quantitative PCR to link concentrations of fastidious vaginal bacteria to metabolite concentrations. Individual metabolites associated

with BV status have been identified and shown to be useful markers of BV such as lactate (no BV) and succinate (BV) (1-7). However, there are hundreds of other compounds that have not been identified in vaginal fluid and linked to particular bacterial communities or clinical outcomes.

[0006] The composition of the human vaginal microbiota ranges from communities dominated by a limited of *Lactobacillus* species to complex communities of diverse anaerobes, most evident with the common condition BV (8-11). BV affects up to 29% of women in the United States (12), and has been associated with several adverse reproductive and health outcomes including elevated risks for acquisition of sexually transmitted infections (13-15), transmission of HIV to male sex partners (16), preterm birth (17), pelvic inflammatory disease (18), and cervicitis (19). The microbiota in BV is heterogeneous; different groups of women have vaginal microbiotas dominated with different bacteria such as BV-associated bacterium-1 (BVAB1), *Leptotrichia/Sneathia* species, *Prevotella* spp., *Gardnerella vaginalis*, or *Lactobacillus iners*, while others are colonized with diverse bacteria and no single dominant bacterium (11, 20). BV is diagnosed in clinical settings using Amsel criteria in which a set of four signs or symptoms are evaluated, with at least three required for a diagnosis of BV (21). Different bacteria are correlated with each of these four clinical criteria (11). Small molecule metabolites can affect at least three of these clinical criteria including vaginal discharge, pH, and amine odor. The diagnosis of BV can also be made using Gram stain interpretation of vaginal fluid smears with enumeration of bacterial morphotypes (22).

[0007] Polyamines such as putrescine and cadaverine are found in women with BV and contribute to the fishy amine odor in this condition (1, 23-25). These polyamines are likely produced from decarboxylation of amino acids mediated by bacteria (23). Chen et al. demonstrated that in vitro production of polyamines by mixed anaerobic vaginal bacteria was inhibited in the presence of metronidazole, suggesting a role for bacterial production of polyamines (26). Likewise, trimethylamine has a fishy odor, is found in women with BV, and is also thought to be a product of bacterial metabolism (27). These amines become volatile when pH is elevated, a property utilized in the clinical diagnosis of BV when employing the "whiff test" (4, 28, 29). The amine odor can also be noted *in vivo* with elevated pH during menses and after vaginal intercourse with men, as the pH of blood and semen are close to 7 leading to enhanced volatilization. It has been suggested that the amines

are associated with increased vaginal transudation of fluid and squamous cell exfoliation, resulting in the thin homogeneous grayish-white discharge that is typically associated with BV (4).

[0008] Mass spectrometry (when coupled with chromatography) offers the opportunity to measure small molecule metabolites in the vagina, and assign identity to hundreds of compounds simultaneously. One recent study described the metabolite profiles in eight women with BV using this approach, and classified these women in to two groups based on their vaginal metabolite compositions (30). Additional insights into the functions of these bacterial consortia can be gained by linking bacterial community composition and concentrations of bacteria to metabolic signatures.

[0009] Current methods of diagnosing BV have numerous disadvantages. Most common methods of diagnosing BV and their disadvantages currently include: (1) Clinical settings: Amsel clinical criteria which include a set of four signs or symptoms of which at least three are required for a positive diagnosis. These include pH, a positive “whiff test,” greater than 20% clue cells (vaginal epithelial cells coated with bacteria) and a thin homogeneous discharge. This diagnostic procedure requires a trained clinician and a microscope is needed to evaluate clue cells; (2) Research settings: Vaginal fluid Gram stains and evaluation of the morphotypes based on the Nugent method or variations of the Ison & Hay method. The Nugent method is the gold standard in research settings, however, a highly trained microscopist is needed for evaluating the slides; (3) Detection of bacterial nucleic acids: Gen-Probe has developed a test targeting bacterial nucleic acids from bacteria that cause BV. Testing nucleic acids by PCR is typically expensive. Becton Dickinson has a BD Affirm VPIII system that can detect high levels of *Gardnerella vaginalis* DNA. However, *G. vaginalis* is not specific for the diagnosis of BV, as it is present in 70% of women without BV; (4) Detection of bacterial sialidase: OSOM BVBlue test that measures bacterial sialidases that are elevated in BV. While one study showed that the test was sensitive and specific (Bradshaw et al., 2005 JCM 43:1304-1308), other studies have not been able to demonstrate high sensitivity (Madhivanan et al., 2014 Infect Dis Obstet Gynecol Article ID 908313; Hillier <https://clinicaltrials.gov/ct2/show/results/NCT00682851?sect=X01256>.)

[0010] Thus there is a need for improved methods and kits for diagnosing, assessing disease risk, treating, and preventing BV and associated conditions.

SUMMARY

[0011] The present invention describes methods and kits for diagnosing, assessing disease risk, treating and preventing BV and associated conditions.

[0012] The method for diagnosing BV in a subject can include collecting a sample from the subject, assessing a small molecule metabolomic profile of a metabolomic signature by measuring levels of at least two indicative metabolites, and comparing the metabolomic profile with a diagnostic metabolomic profile of the signature. A substantial match between the metabolomic profile and the diagnostic metabolomic profile can be indicative of BV.

[0013] The method for monitoring disease progression of BV can include collecting a first sample from the subject; assessing a small molecule metabolomic profile of a metabolomic signature by measuring levels of at least two indicative metabolites; comparing the metabolomic profile with a diagnostic metabolomic profile of the signature; collecting a second sample from the subject; assessing a small molecule metabolomic profile of a metabolomic signature in the second sample by measuring levels of at least two indicative metabolites; and comparing the metabolomic profile with a diagnostic metabolomic profile of the signature. The disease progression can be based on substantial matches between the metabolomic profiles and the diagnostic metabolomic profiles. The second sample can be collected after a period of time from the first sample. The period of time can be at least three days.

[0014] The methods can include Chromatographic separation and/or mass spectroscopy. The substantial match can be at least 80%.

[0015] A kit for diagnosing BV in a subject in which the diagnosis is made with a test apparatus and include reagents for collecting a test sample from a subject; and reagents for measuring the profile of a metabolomics profile by measuring levels of indicative metabolites.

[0016] The sample can be vaginal fluid or Cervicovaginal lavage fluid (CVL).

[0017] The indicative metabolites can include two or more metabolites selected from the group listed in Table 1.

[0018] The indicative metabolites can include lipids, amino acids, nucleotides, co-factors, vitamins, carbohydrates, energy molecules and redox molecules.

[0019] The indicative metabolites can include amino acids such as 5-Aminovaleric Acid, Cadaverine, D-Leucic Acid, Glutaric Acid, Histamine, N-

Acetylputrescine, Putrescine, Tryptamine, Tyramine, Anthranilate, Arginine, Asparagine, Aspartic Acid, Creatine, Cysteine, Cystine, Glycine, Histidine, iso-Leucine, Leucine, Lysine, Methionine, Methylsuccinate, Ornithine, Phenylalanine, Pimelic acid, Reduced glutathione, Serine, Threonine, Tryptophan, Tyrosine, Xanthurenate. The indicative metabolites can include fatty acids such as 13-HODE, 12-HETE, Glycerol-3-P, Arachidonate, Carnitine. The indicative metabolites can include nucleotides such as Oxypurinol, Uracil, Adenosine, AMP, Hypoxanthine, and Urate. The the indicative metabolites can include carbohydrates such as Glyceraldehyde, N-Acetylneuraminate, PEP, F16BP/F26BP/G16BP, Glucuronate, Glucose, Glutamic acid, Lactate, Oxalic Acid, and Sorbitol.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] Those of skill in the art will understand that the drawings, described below, are for illustrative purposes only. The drawings are not intended to limit the scope of the present teachings in any way.

[0021] Figure 1 is a dendrogram showing associations of 30% of most variable metabolites with BV status, determined using Amsel and Nugent criteria. Clustering of the metabolites resulted in four groups (Clusters I-IV). The clustering algorithm was not informed by BV status. Study participant IDs are provided on the x-axis and metabolites are listed on the y-axis. The heat map depicts log-transformed concentrations of the most variable metabolites between the clusters. Values ranged from -6.956 to 4.818.

[0022] Figure 2 depicts the association between metabolites and bacterial abundance. Hierarchically clustered Pearson correlation coefficients are displayed in a heat map to demonstrate associations of vaginal bacteria (relative abundance) detected using broad-range PCR and pyrosequencing (x-axis) with the abundance of 30% most variable metabolites (y-axis). Correlation values ranged from -0.7 to 0.84.

[0023] Figure 3 depicts the association of specific vaginal bacteria with metabolites. Hierarchically clustered Pearson correlation coefficients are displayed in a heat map to demonstrate associations of key vaginal bacterial concentrations (x-axis) measured using taxon directed qPCR with the 30% most variable metabolites (y axis). Correlation values range from -0.71 to 0.85. Two sub-groups of BV associated bacteria were observed, and clustering patterns were similar to that noted in Figure 2 using untargeted approach for bacterial community analysis. *L. crispatus* and *L. jensenii* exhibited correlation patterns that were similar and opposite to those by BV-associated bacteria.

[0024] Figure 4 is a model depicting metabolites associated with individual clinical criteria used in BV diagnosis (21). Stars denote metabolites that were positively or negatively associated with BV status. BV status is indicated on the x-axis of box plots. The y-axis of box plots represent scaled concentrations of metabolites. The lines in box plots represent the mean and whiskers denote 95% confidence intervals.

[0025] Figure 5 depicts shifts in concentrations of key vaginal bacteria and metabolites with treatment for BV. Longitudinal data is presented for four study participants (19, 21, 23 & 25) who were cured of BV post treatment with metronidazole at the one month follow-up visit. Bacterial concentrations are displayed as 16S rRNA copies per swab on the y-axis of the XY-plots. Scaled metabolite concentrations (y-axis) of 14 metabolites associated with individual clinical criteria (from Figure 4 & Table 3) have been classified as positively associated with BV and are higher in women BV and negatively associated with BV and are lower in women with BV. Y-axis scales are different for each subject reflecting differences in metabolite concentrations. All four participants had high concentrations of lactobacilli at their follow-up visits and increased concentrations of metabolites negatively associated with BV. Women with high concentrations of *L. crispatus* at follow-up had high concentrations of metabolites negatively associated with BV. Women with high concentrations of *L. iners* also showed increased concentrations of metabolites negatively associated with BV, but these shifts were not as dramatic as increases seen in women with *L. crispatus* dominant communities.

[0026] Figure 6 is a model representing putative putrescine metabolism pathways in bacterial vaginosis. Box plots show selected metabolites that were detected in studies. BV status is indicated on the x-axis of box plots. The y-axis of box plots represent scaled concentrations of metabolites. The lines in box plots depict the mean, and whiskers denote 95% confidence intervals. Arginine typically serves as a precursor for the generation of polyamines putrescine, spermidine and spermine. Levels of putrescine were higher in BV, while levels of spermine were lower. Higher levels of succinate are a hallmark in BV. Recently, a novel putrescine utilization pathway has been discovered in *Escherichia coli* via γ -aminobutyraldehyde (GABA), resulting in succinate production. Higher levels of N-acetylputrescine were observed in BV, which can also lead to GABA formation. Arrows next to metabolites indicate metabolites detected in the Examples, and show whether concentrations were higher or lower in women with BV.

[0027] Figure 7 is a group of graphs depicting bacteria and metabolite concentrations in women with no changes in BV status. Limited longitudinal data are presented for 4 women who returned for follow-up visits 4 weeks after baseline sample collection per study protocol. BV status was evaluated by Amsel criteria and confirmed by Gram stain. Participants diagnosed with BV were treated with metronidazole. Bacterial concentrations are displayed as 16S rRNA copies per swab on the y-axis of the XY-plots. Scaled metabolite concentrations (y-axis) of 14 metabolites associated with individual clinical criteria (from Figure 4 & Table 3) have been classified as positively associated with BV and negatively associated with BV. Y-axis scales are different for each subject reflecting differences in metabolite concentrations. (A) Study participants 4 and 7 did not have BV at either time point and had high concentrations of lactobacilli at both time points. High concentrations of metabolites negatively associated with BV were also noted at baseline and follow-up. (B) Study participants 12 and 28 had recurrent BV. In both participants, concentrations of metabolites associated with BV declined at the follow-up visit, but there was no associated increase in metabolites negatively associated with BV. This longitudinal analysis showed that stability of the microbiota was associated with few shifts in metabolites even after patients received antibiotic therapy.

[0028] Figure 8 depicts an association between metabolites and bacterial abundance in the validation study. Hierarchically clustered Pearson correlation coefficients demonstrate associations of vaginal bacteria detected by broad-range PCR and pyrosequencing (x-axis) with metabolites (y-axis, 50% of the most variable metabolites based on inter-quartile range, and 12-HETE, arginine, ornithine, N acetylneuraminate and succinate). Correlation values ranged from -0.86 to -0.86.

[0029] Figure 9 depicts metabolite profiles in women with intermediate Nugent scores. Non-metric multi dimensional scaling (MDS) plot summarizing metabolite profiles in women with and without BV classified by Nugent score. The MDS plot was created using non-metric multi-dimensional scaling of Euclidean distance between log-transformed metabolite concentrations, and represented in 2- dimensional space. Green dots denote women with Nugent scores 0-3, purple dots indicate women with intermediate scores 4-6 and red dots show women with scores 7-10. Women with intermediate Nugent scores have metabolite profiles ranging from those that are similar to women without BV (Nugent 0-3) to profiles which are more BV-like.

[0030] Figure 10 depicts a comparison of metabolite concentrations in swab samples and CVL. A limited number of swab samples (n=8) were included in the validation study to determine the extent of dilution of metabolites in CVL samples. Figure 10 summarizes the log2 fold change of CVL relative to swab as a function of average log2 abundance. Of the 101 metabolites detected using the NW-MRC platform, no metabolite was detected only in swab samples.

DETAILED DESCRIPTION OF THE INVENTION

[0031] Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art.

[0032] The present invention relates to methods and kits for diagnosing, assessing disease risk, treating and preventing BV and associated conditions.

[0033] In some embodiments, the invention relates to a method of diagnosing bacterial vaginosis (BV) in a subject including the steps of collecting a sample from the subject; assessing a small molecule metabolomic profile of a metabolomic signature by measuring levels of metabolites; comparing the metabolomic profile with a diagnostic metabolomic profile of the signature. In some embodiments, a substantial match between the metabolomic profile and the diagnostic metabolomic profile is indicative of bacterial vaginosis.

[0034] In some embodiments, the invention relates to a method of monitoring disease progression of BV in a subject including the steps of: collecting a first sample from the subject; assessing a small molecule metabolomic profile of a metabolomic signature by measuring levels of metabolites; comparing the metabolomic profile with a diagnostic metabolomic profile of the signature; wherein the disease progression is based at least in part upon a substantial match between the metabolomic profile and the diagnostic metabolomic profile; collecting a second sample from the subject; assessing a small molecule metabolomic profile of a metabolomic signature by measuring levels of metabolites; comparing the metabolomic profile with a diagnostic metabolomic profile of the signature; wherein the disease progression is based at least in part upon a substantial match between the metabolomic profile and the diagnostic metabolomic profile; wherein the disease progression is based at least in part upon a substantial match between the metabolomic profile and the diagnostic metabolomic profile; wherein the second sample is collected after a period of time

from the first sample. The period of time can be 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 2 weeks, 3 weeks, 1 month, 2 months 3 months, 4 months, 5 months or more.

[0035] In some embodiments, the invention relates to a kit for diagnosing BV in a subject in which the diagnosis is made with a test apparatus. The kit can include reagents for collecting a test sample from a subject; and reagents for measuring the profile of a metabolomics profile by measuring levels of metabolites.

[0036] In some embodiments, sample is vaginal fluid or Cervicovaginal lavage fluid (CVL).

[0037] In some embodiments, the metabolites include one or more metabolites selected from the group listed in Table 1. The method can include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more metabolites from the group listed in Table 1.

[0038] In some embodiments, the metabolites include one or more of metabolites of lipids, amino acids, nucleotides, co-factors, vitamins, carbohydrates, energy molecules or redox molecules. In some embodiments, the metabolites include 1, 2, 3, 4, 5, 6, 7, 8, or all of lipids, amino acids, nucleotides, co-factors, vitamins, carbohydrates, energy molecules or redox molecules.

[0039] In some embodiments, the metabolites include one or more amino acids selected from 5-Aminovaleric Acid, Cadaverine, D-Leucic Acid, Glutaric Acid, Histamine, N-Acetylputrescine, Putrescine, Tryptamine, Tyramine, Anthranilate, Arginine, Asparagine, Aspartic Acid, Creatine, Cysteine, Cystine, Glycine, Histidine, iso-Leucine, Leucine, Lysine, Methionine, Methylsuccinate, Ornithine, Phenylalanine, Pipecolate, Reduced glutathione, Serine, Threonine, Tryptophan, Tyrosine, Xanthurenate, and the like. The method can include 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or more of these amino acids.

[0040] In some embodiments, the metabolites can include one or more fatty acids selected from 13-HODE, 12-HETE, Glycerol-3-P, Arachidonate, Carnitine, and the like. The method can include 0, 1, 2, 3, 4, or all of these fatty acids.

[0041] In some embodiments, the metabolites can include nucleotides selected from Oxypurinol, Uracil, Adenosine, AMP, Hypoxanthine, Urate, and the like. The method can include 0, 1, 2, 3, 4, 5, or all of these nucleotides.

[0042] In some embodiments, the metabolites can include carbohydrates selected from Glyceraldehyde, N-Acetylneuraminate, PEP, F16BP/F26BP/G16BP, Glucuronate,

Glucose, Glutamic acid, Lactate, Oxalic Acid, Sorbitol, and the like. The method can include 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 or all of these carbohydrates.

[0043] In some embodiments, the a substantial match between the metabolomic profile and the diagnostic metabolomic profile is 50%, 60%, 70%, 80%, 90%, or more.

[0044] In some embodiments, the method comprises Chromatographic separation or mass spectroscopy, or both.

[0045] In some embodiments, the method includes pyrosequencing of the test sample. In some embodiments, the pyrosequencing targets the V3-V4 region of the 16S-rRNA gene.

Table 1 Heat map of statistically significant biochemicals profiled. For paired comparisons, shaded cells indicate $p \leq 0.05$ (darker grey filled boxes indicate that the mean values are significantly higher for that comparison; lighter grey boxes have values that are significantly lower). **Bold** text indicates $0.05 < p < 0.10$.

2										Statistical value		Mean Values	
Welch's Two-Sample t-Test										Welch's Two-Sample t-Test			
PATHWAY SORT	SUPER PATHWAY	SUB PATHWAY	BIOCHEMICAL NAME	PLATFORM	COMP ID	Pos Neg	Fold Change	Pos / Neg		Neg	Pos		
1	Amino acid	Glycine, serine and threonine metabolism	glycine	GC/MS	11777	0.34		< 0.001	0.0000	3.739741	1.263827		
2			sarcosine (N-Methylglycine)	GC/MS	1516	4.44		< 0.001	0.0000	0.427684	1.898044		
7			serine	GC/MS	1648	0.20		< 0.001	0.0002	6.580896	1.285634		
9			N-acetylserine	LC/MS pos	37076	0.41		< 0.001	0.0007	1.707820	0.697719		
16			threonine	GC/MS	1284	0.23		< 0.001	0.0002	5.900021	1.329807		
17			N-acetylthreonine	LC/MS neg	33939	0.19		< 0.001	0.0000	3.111569	0.593361		
21			betaine	LC/MS pos	3141	0.70		0.0243	0.0119	1.202041	0.844976		
23			alanine	GC/MS	1126	0.80		0.1348	0.0520	1.891140	1.513845		
24			beta-alanine	GC/MS	55	1.62		0.2366	0.0857	0.810754	1.310248		
26		Alanine and aspartate metabolism	N-acetylalanine	LC/MS neg	1585	2.18		0.0351	0.0165	0.915336	1.991908		
29	aspartate		GC/MS	15996	0.22		0.0013	0.0009	5.004044	1.116333			
31	N-acetylaspartate (NAA)		LC/MS pos	22185	6.21		< 0.001	0.0000	0.405221	2.517588			
38	glutamate		LC/MS pos	57	0.44		0.0072	0.0042	2.753214	1.204244			
42	Glutamate metabolism	glutamine	LC/MS pos	53	0.56		0.1241	0.0485	1.649106	0.931688			
43		pyroglutamine*	LC/MS pos	32672	0.75		0.0776	0.0329	1.288365	0.968558			
44		gamma-aminobutyrate (GABA)	GC/MS	1416	0.02		0.0148	0.0077	81.695175	1.687678			
45		N-acetylglutamate	LC/MS pos	15720	3.72		< 0.001	0.0000	0.556992	2.073273			
48	Histidine metabolism	histidine	LC/MS neg	59	0.20		< 0.001	0.0003	4.207027	0.836916			

50	urocanate	LC/MS pos	607	0.65	0.8376	0.2431	1.282119	0.833274
53	histamine	GC/MS	1574	0.72	0.7823	0.2313	2.076850	1.486302
60	cadaverine	GC/MS	15308	27.38	< 0.001	0.0000	0.146588	4.012971
66	lysine	GC/MS	1301	0.08	< 0.001	0.0000	11.290963	0.915742
67	2-aminoadipate	GC/MS	6146	1.04	0.2273	0.0827	1.358665	1.407328
69	pipecolate	LC/MS pos	1444	52.72	< 0.001	0.0000	0.070170	3.699688
74	N6-acetyllysine	LC/MS pos	36752	5.70	< 0.001	0.0000	0.352662	2.009462
77	phenyllactate (PLA)	LC/MS neg	22130	2.24	0.5252	0.1668	1.685189	3.772378
78	phenylalanine	LC/MS pos	64	0.15	< 0.001	0.0000	4.537531	0.683247
79.5	Isobar: 1-phenylethylamine, phenylethylamine	LC/MS pos	38763	5.78	< 0.001	0.0000	4.475218	25.796219
80	phenylacetate	LC/MS neg	15958	3.72	< 0.001	0.0001	0.175127	0.652184
82	p-cresol sulfate	LC/MS neg	36103	0.68	0.5443	0.1721	1.931141	1.309778
90	tyrosine	LC/MS pos	1299	0.11	< 0.001	0.0000	6.775518	0.747969
91	3-(4-hydroxyphenyl)lactate	LC/MS neg	32197	0.54	0.0103	0.0056	3.235635	1.732232
94	tyramine	LC/MS pos	1603	21.24	< 0.001	0.0000	0.333968	7.092416
106	4-hydroxyphenylacetate	GC/MS	541	51.37	< 0.001	0.0000	0.049353	2.535028
111	N-acetylphenylalanine	LC/MS neg	33950	2.24	0.0222	0.0110	0.633846	1.421726
115	phenylacetylglutamine	LC/MS pos	35126	0.54	0.1103	0.0441	2.078089	1.129685
118.5	3-(4-hydroxyphenyl)propionate	GC/MS	39587	71.71	< 0.001	0.0000	0.037424	2.683498
119	3-phenylpropionate (hydrocinnamate)	LC/MS neg	15749	14.82	< 0.001	0.0002	0.119842	1.752567
121	phenol sulfate	LC/MS neg	32553	0.80	0.7819	0.2313	0.985803	0.792313
124	kynurenate	LC/MS neg	1417	1.43	< 0.001	0.0003	0.779062	1.115300
125	kynurenine	LC/MS pos	15140	0.46	0.0058	0.0035	1.245910	0.571509
126	tryptophan	LC/MS pos	54	0.19	< 0.001	0.0000	4.789226	0.915774
127	indolelactate	GC/MS	18349	0.38	0.0198	0.0101	2.103397	0.792366
131	tryptophan betaine	LC/MS pos	37097	1.07	0.9049	0.2548	1.250748	1.336430
136	tryptamine	LC/MS pos	6104	24.84	< 0.001	0.0000	0.634729	15.642847

147	3-indoxyl sulfate	LC/MS neg	27672	0.89	0.8494	0.2453	0.997218	0.884641
148	indolepropionate	LC/MS neg	32405	1.54	0.0174	0.0089	0.425099	0.653345
149	3-methyl-2-oxobutylate	LC/MS neg	21047	2.41	< 0.001	0.0000	0.305080	0.734092
150	3-methyl-2-oxovalerate	LC/MS neg	15676	2.30	< 0.001	0.0000	0.440157	1.010772
156	alpha-hydroxyisocaproate	LC/MS neg	22132	5.83	< 0.001	0.0000	0.504073	2.939890
157	isoleucine	LC/MS pos	1125	0.52	< 0.001	0.0002	2.412487	1.256762
158	leucine	LC/MS pos	60	0.31	< 0.001	0.0000	3.289422	1.005091
162	N-acetyl-leucine	LC/MS pos	1587	3.24	< 0.001	0.0003	0.500713	1.624179
163	N-acetyl-valine	LC/MS pos	1591	2.23	< 0.001	0.0005	0.432821	0.963793
167	valine	LC/MS pos	1649	0.77	0.0282	0.0134	1.618634	1.239967
170	4-methyl-2-oxopentanoate	LC/MS neg	22116	3.11	< 0.001	0.0000	0.356438	1.109900
175	alpha-hydroxyisovalerate	GC/MS	33937	16.12	< 0.001	0.0000	0.087300	1.407149
177	isobutyryl carnitine	LC/MS pos	33441	0.21	< 0.001	0.0000	2.624396	0.553982
178	2-hydroxy-3-methylvalerate	LC/MS neg	36746	16.48	< 0.001	0.0000	0.088556	1.636135
179	2-methylbutyryl carnitine	LC/MS pos	35431	0.16	< 0.001	0.0000	2.672136	0.439636
188	cysteine	GC/MS	31453	0.03	< 0.001	0.0000	15.954344	0.509295
191	cystine	GC/MS	31454	0.11	< 0.001	0.0002	0.698290	0.079583
193	methionine sulfoxide	LC/MS pos	18374	0.31	< 0.001	0.0000	2.456314	0.750162
194	N-formylmethionine	LC/MS neg	2829	1.20	0.9900	0.2758	0.652447	0.784818
195	hypotaurine	GC/MS	590	0.59	0.1398	0.0533	1.801513	1.061490
196	taurine	GC/MS	2125	1.16	0.8229	0.2419	1.311479	1.517780
199	methionine	LC/MS pos	1302	0.25	< 0.001	0.0001	3.820236	0.945843
201	N-acetyl-methionine	LC/MS neg	1589	0.99	0.0803	0.0338	1.822001	1.799556
203	2-hydroxybutyrate (AHB)	GC/MS	21044	8.61	< 0.001	0.0000	0.291261	2.508011
212	dimethylarginine (SDMA + ADMA)	LC/MS pos	36808	1.33	0.1164	0.0461	0.946267	1.262746
213	arginine	LC/MS pos	1638	0.10	< 0.001	0.0001	4.932915	0.512710
216	ornithine	GC/MS	1493	0.05	< 0.001	0.0002	19.529267	0.971406

217	urea	GC/MS	1670	0.31	0.0066	0.0039	3.618807	1.108288
218	proline	LC/MS pos	1898	1.49	0.0915	0.0382	1.768739	2.631250
219	5-aminovaleate	GC/MS	18319	36.22	< 0.001	0.0000	0.248177	8.988925
220	citrulline	LC/MS pos	2132	3.28	< 0.001	0.0003	0.652030	2.135567
221	N-acetylornithine	LC/MS pos	15630	1.88	0.1094	0.0440	1.173158	2.202987
224	trans-4-hydroxyproline	GC/MS	1366	0.81	0.8755	0.2492	1.710068	1.382874
232	creatine	LC/MS pos	27718	0.38	< 0.001	0.0000	1.899790	0.714386
233	creatinine	LC/MS pos	513	0.66	0.0102	0.0056	1.415647	0.932472
235	2-aminobutyrate	GC/MS	1577	3.64	0.0032	0.0021	0.842538	3.065685
238	5-methylthioadenosine (MTA)	LC/MS pos	1419	1.49	0.0812	0.0341	0.856503	1.275299
239	putrescine	GC/MS	1408	22.90	< 0.001	0.0000	0.314180	6.910507
240	N-acetylputrescine	LC/MS pos	37496	19.83	< 0.001	0.0000	0.126190	2.502043
241	agmatine	GC/MS	15496	18.45	< 0.001	0.0000	0.226448	4.178625
242	spermidine	LC/MS pos	485	1.21	0.3210	0.1121	1.002315	1.217463
244	spermine	LC/MS pos	603	0.43	< 0.001	0.0003	2.466098	1.058128
251	glutathione, reduced (GSH)	LC/MS pos	2127	0.03	< 0.001	0.0000	7.943949	0.247406
253	5-oxoproline	LC/MS pos	1494	0.51	< 0.001	0.0001	1.812848	0.918718
254	glutathione, oxidized (GSSG)	LC/MS pos	38783	0.46	0.0494	0.0222	2.418668	1.109746
261	glycylvaline	LC/MS pos	18357	0.41	0.0035	0.0022	1.828693	0.748924
262	glycylglycine	GC/MS	21029	0.10	< 0.001	0.0000	1.241861	0.129154
264	glycylproline	LC/MS pos	22171	0.16	< 0.001	0.0000	4.315719	0.695861
265	glycylisoleucine	LC/MS pos	36659	0.43	0.0018	0.0012	2.270640	0.984348
266	glycylleucine	LC/MS pos	34398	0.23	< 0.001	0.0000	3.702514	0.869842
269	glycylphenylalanine	LC/MS neg	33954	0.18	< 0.001	0.0000	2.415152	0.429368
270	glycyltyrosine	LC/MS neg	33958	0.36	0.0023	0.0015	1.309452	0.474025
272	alanylalanine	LC/MS neg	15129	0.55	0.0212	0.0108	1.844075	1.023070
273	alanylthreonine	LC/MS neg	37085	0.26	0.0016	0.0010	1.516159	0.388090
274	alanylvaline	LC/MS pos	37084	0.27	0.0026	0.0017	3.456289	0.921019

278	alanyl-leucine	LC/MS pos	37093	0.12	< 0.001	0.0000	5.516745	0.645927
279	alanylgarginine	LC/MS neg	37096	0.34	0.0077	0.0044	1.095681	0.374877
281	alanylglutamate	LC/MS pos	37064	0.16	< 0.001	0.0000	3.480754	0.571405
283	alanylisoleucine	LC/MS pos	37118	0.74	0.1699	0.0636	1.171897	0.871067
283.5	alanylphenylalanine	LC/MS pos	38679	0.14	< 0.001	0.0000	4.253526	0.599824
285	alanyltirosine	LC/MS neg	37098	0.20	< 0.001	0.0001	1.565923	0.319435
286	aspartylphenylalanine	LC/MS pos	22175	0.36	< 0.001	0.0005	1.470863	0.526500
286.5	aspartate-glutamate	LC/MS neg	37461	0.30	< 0.001	0.0002	1.467490	0.439940
287	alpha-glutamylglutamate	LC/MS pos	22166	0.30	< 0.001	0.0003	2.474494	0.739946
289	prolyl-leucine	LC/MS neg	31914	0.77	0.0247	0.0121	0.599613	0.463184
291	isoleucylisoleucine	LC/MS pos	36761	0.64	0.0280	0.0134	1.522233	0.976674
292	isoleucylleucine	LC/MS pos	36760	0.67	0.1117	0.0444	1.572229	1.055765
295	leucylleucine	LC/MS neg	36756	0.32	0.1026	0.0419	1.752017	0.552354
299	threonylphenylalanine	LC/MS pos	31530	0.12	< 0.001	0.0000	6.378384	0.783527
300	phenylalanylphenylalanine	LC/MS pos	38150	0.60	0.1004	0.0412	1.023343	0.612418
303	pyroglutamylvaline	LC/MS neg	32394	0.42	0.0034	0.0021	1.198512	0.497466
304	valinylglutamate	LC/MS pos	32454	0.18	< 0.001	0.0000	4.490319	0.795339
305	Isobar: glycylglutamate; glutamylglycine	LC/MS neg	34466	0.69	0.0094	0.0053	1.373884	0.951120
366	glucosamine	GC/MS	18534	0.28	0.0059	0.0035	1.108914	0.307987
372	erythronate*	GC/MS	33477	0.72	0.3424	0.1180	1.013308	0.732922
374	N-acetylneuraminic acid	LC/MS pos	32377	3.14	< 0.001	0.0002	0.685260	2.150771
379	fucose	GC/MS	15821	1.62	0.0686	0.0295	1.442967	2.342404
389	fructose	GC/MS	31266	0.28	0.0012	0.0008	1.756132	0.493023
391	galactose	GC/MS	12055	5.63	< 0.001	0.0004	0.318466	1.791958
397	maltose	GC/MS	15806	0.46	0.7206	0.2164	6.579565	3.020671
398	mannitol	GC/MS	15335	0.02	0.0010	0.0007	5.584213	0.114066

410	sorbitol	GC/MS	15053	0.70	0.0552	0.0241	0.535369	0.372560
417	maltotriose	GC/MS	15877	0.23	0.0095	0.0053	8.598705	2.015523
423	maltotetraose	LC/MS neg	15910	0.28	0.0016	0.0011	11.307067	3.204442
425	maltopentaose	LC/MS neg	35163	0.08	< 0.001	0.0000	2.031463	0.164349
426	maltohexaose	LC/MS neg	35170	0.12	< 0.001	0.0001	1.779392	0.221209
430	1,5-anhydroglucitol (1,5-AG)	GC/MS	20675	0.15	< 0.001	0.0000	2.539897	0.376257
432	glycerate	GC/MS	1572	0.24	0.0129	0.0068	3.611720	0.867426
434	glucose-6-phosphate (G6P)	GC/MS	31260	2.74	0.8379	0.2431	0.699393	1.913208
436	glucose	GC/MS	20488	1.30	0.4973	0.1586	2.138502	2.781033
442	Isobar: fructose 1,6-diphosphate, glucose 1,6-diphosphate	LC/MS neg	36984	0.18	< 0.001	0.0000	2.519516	0.441328
450	pyruvate	GC/MS	599	0.53	0.0996	0.0411	2.309850	1.228426
451	lactate	GC/MS	527	0.13	< 0.001	0.0000	5.553953	0.749216
462	ribitol	GC/MS	15772	0.42	0.0220	0.0110	0.981060	0.414604
463	threitol	GC/MS	35854	5.38	< 0.001	0.0000	0.194599	1.047048
466	gluconate	GC/MS	587	0.11	< 0.001	0.0002	2.464444	0.270641
468	ribose	GC/MS	12080	1.02	0.1081	0.0437	2.115984	2.168290
473	ribulose	GC/MS	35855	0.12	0.0011	0.0007	1.047534	0.122005
477	UDP-glucuronate	LC/MS neg	2763	0.34	< 0.001	0.0002	1.681324	0.577494
479	xylitol	GC/MS	4966	1.44	0.7366	0.2194	0.478526	0.687893
491	citrate	GC/MS	1564	0.18	0.0689	0.0295	7.980905	1.404370
499	tricarballoylate	LC/MS neg	15729	2.90	0.0089	0.0050	0.964367	2.795634
501	succinate	GC/MS	1437	11.68	< 0.001	0.0000	0.356008	4.156583
504	fumarate	GC/MS	1643	0.87	0.4414	0.1431	1.404028	1.226511
507	malate	GC/MS	1303	0.50	0.9955	0.2761	2.411262	1.216911
511	phosphate	GC/MS	11438	0.79	0.4293	0.1410	1.974197	1.568821

512.5		linoleate (18:2n6)	LC/MS neg	1105	0.64	0.2888	0.1027	2.503180	1.612596
516		linolenate [alpha or gamma; (18:3n3 or 6)]	LC/MS neg	34035	0.34	0.0777	0.0329	3.130635	1.065817
517		diomo-linolenate (20:3n3 or n6)	LC/MS neg	35718	0.44	0.0012	0.0008	2.434987	1.078852
519		docosapentaenoate (n3 DPA; 22:5n3)	LC/MS neg	32504	0.55	< 0.001	0.0005	1.692179	0.931787
521		docosahexaenoate (DHA; 22:6n3)	LC/MS neg	19323	0.54	0.0231	0.0114	1.407097	0.758958
528		pelargonate (9:0)	LC/MS neg	12035	1.28	0.0437	0.0200	0.967598	1.240067
535		myristate (14:0)	LC/MS neg	1365	1.65	< 0.001	0.0001	0.796121	1.324662
536		myristoleate (14:1n5)	LC/MS neg	32418	1.25	0.0422	0.0196	1.045900	1.305066
537		pentadecanoate (15:0)	LC/MS neg	1361	1.57	0.0082	0.0047	0.838910	1.315387
538		palmitate (16:0)	LC/MS neg	1336	0.78	0.6337	0.1956	1.357113	1.061208
539		palmitoleate (16:1n7)	LC/MS neg	33447	1.43	0.1048	0.0426	1.205405	1.723318
541		margarate (17:0)	LC/MS neg	1121	0.62	0.0049	0.0030	1.807274	1.114219
542		10-heptadecenoate (17:1n7)	LC/MS neg	33971	1.03	0.6744	0.2057	1.522486	1.567355
543		stearate (18:0)	GC/MS	1358	0.38	0.0051	0.0030	2.870164	1.082576
545		oleate (18:1n9)	GC/MS	1359	0.36	0.0029	0.0019	3.131461	1.122614
547		cis-vaccenate (18:1n7)	GC/MS	33970	0.53	0.0989	0.0410	1.308343	0.692943
556		arachidate (20:0)	GC/MS	1118	0.54	0.1506	0.0570	1.215563	0.651867
559		eicosenoate (20:1n9 or 11)	LC/MS neg	33587	1.42	0.6726	0.2057	1.455978	2.066571
561		diomo-linoleate (20:2n6)	LC/MS neg	17805	1.38	0.9759	0.2728	1.476627	2.035057
565		arachidonate (20:4n6)	LC/MS neg	1110	0.60	0.0075	0.0043	2.562764	1.549759
569		docosadienoate (22:2n6)	LC/MS neg	32415	1.37	0.8702	0.2487	1.276007	1.749032
573		lignocerate (24:0)	GC/MS	1364	0.98	0.9003	0.2545	0.958237	0.940437
589		n-Butyl Oleate	GC/MS	36802	0.64	0.0506	0.0223	0.854271	0.544793
591		4-hydroxybutyrate (GHB)	GC/MS	34585	26.89	< 0.001	0.0000	0.084776	1.695504
602		2-hydroxystearate	LC/MS neg	17945	1.57	0.3032	0.1068	1.381444	2.167484
603		13-HODE	LC/MS neg	37374	3.90	0.0042	0.0026	1.448723	5.656509

Lipid

604		2-hydroxypalmitate	LC/MS neg	35675	1.24	0.8312	0.2430	2.094107	2.590470
610	Fatty acid, dicarboxylate	2-hydroxyglutarate	GC/MS	37253	12.82	< 0.001	0.0000	0.573594	6.896975
616		azelate (nonanedioate)	LC/MS neg	18362	0.67	0.4340	0.1413	1.578876	1.061955
621		undecanedioate	LC/MS neg	35671	0.81	0.0497	0.0222	1.094301	0.885314
622		3-carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPF)	LC/MS neg	31787	0.86	0.3299	0.1147	0.440869	0.378743
632.5		13-methylmyristic acid	LC/MS neg	38293	1.95	< 0.001	0.0000	0.710272	1.385212
632.65	Fatty acid, branched	methyl palmitate (15 or 2)	LC/MS neg	38768	1.23	0.1820	0.0678	0.966898	1.188506
632.7		17-methylstearate	LC/MS neg	38296	1.22	0.5782	0.1806	1.227807	1.500760
672	Eicosanoid	12-HETE	LC/MS neg	37536	7.65	< 0.001	0.0002	0.796090	5.614170
678	Endocannabinoid	oleic ethanolamide	LC/MS neg	38102	4.72	< 0.001	0.0000	0.384114	1.811836
679		palmitoyl ethanolamide	LC/MS neg	38165	4.97	< 0.001	0.0000	0.470637	2.337596
683	Fatty acid metabolism (also BCAA metabolism)	propionylcarnitine	LC/MS pos	32452	0.24	< 0.001	0.0000	2.834756	0.690499
685		butyrylcarnitine	LC/MS pos	32412	0.34	< 0.001	0.0000	2.199301	0.738763
691		deoxycarnitine	LC/MS pos	36747	22.97	< 0.001	0.0000	0.124527	2.860155
692	Carnitine metabolism	carnitine	LC/MS pos	15500	0.32	< 0.001	0.0000	2.285080	0.720942
693		3-dehydrocarnitine*	LC/MS pos	32654	1.07	0.9085	0.2549	1.056017	1.133051
694		acetylcarnitine	LC/MS pos	32198	0.20	< 0.001	0.0000	3.840583	0.784791
746	Glycerolipid metabolism	ethanolamine	GC/MS	34285	1.59	0.0504	0.0223	1.337565	2.130038
747		phosphoethanolamine	GC/MS	12102	0.70	0.2959	0.1047	1.231679	0.856039
750		glycerol	GC/MS	15122	0.44	0.0462	0.0209	2.884838	1.279821
752		glycerol 3-phosphate (G3P)	GC/MS	15365	0.25	< 0.001	0.0001	3.593932	0.881919
753		glycerophosphorylcholine (GPC)	LC/MS pos	15990	0.18	< 0.001	0.0000	7.439241	1.342709
760	Inositol metabolism	myo-inositol	GC/MS	19934	0.31	< 0.001	0.0001	3.091537	0.969853
767		scyllo-inositol	GC/MS	32379	0.49	0.0108	0.0058	1.564517	0.762610
771	Ketone bodies	1,2-propanediol	GC/MS	38002	0.01	0.1276	0.0495	81.309337	1.067614
778	Lysolipid	2-palmitoleoylglycerophosphoethanolamine*	LC/MS neg	36619	0.53	0.0037	0.0023	1.407027	0.744857

780	1-stearoylglycerophosphoethanolamine	LC/MS neg	34416	0.20	< 0.001	0.0000	2.538105	0.507276
781	1-oleoylglycerophosphoethanolamine	LC/MS neg	35628	0.59	0.0444	0.0201	1.906482	1.123846
782	2-oleoylglycerophosphoethanolamine*	LC/MS neg	35687	0.59	0.0515	0.0226	1.788931	1.054957
783	1-linoleoylglycerophosphoethanolamine*	LC/MS neg	32635	0.57	0.0276	0.0134	1.609657	0.920543
784	2-linoleoylglycerophosphoethanolamine*	LC/MS neg	36593	0.47	< 0.001	0.0001	1.606901	0.759890
785	1-arachidonoylglycerophosphoethanolamine*	LC/MS neg	35186	0.56	0.0114	0.0061	1.424438	0.795836
786	2-arachidonoylglycerophosphoethanolamine*	LC/MS neg	32815	0.47	0.0020	0.0013	1.349214	0.628647
794	1-palmitoylglycerophosphocholine	LC/MS neg	33955	0.25	< 0.001	0.0000	1.663882	0.411784
799	1-stearoylglycerophosphocholine	LC/MS pos	33961	1.26	0.1396	0.0533	1.067878	1.345730
802	2-oleoylglycerophosphocholine*	LC/MS pos	35254	0.80	0.4231	0.1401	0.836825	0.673232
824	1-oleoylglycerophosphoserine	LC/MS neg	19260	0.93	0.3897	0.1319	1.893220	1.758314
825.5	1-palmitoylplasmalethanolamine*	LC/MS neg	39270	0.36	< 0.001	0.0003	2.917835	1.051735
833	1-stearoylglycerol (1-monostearin)	GC/MS	21188	0.30	0.0116	0.0061	1.079715	0.323913
853	sphinganine	LC/MS pos	17769	0.98	0.6974	0.2110	2.095458	2.044173
855	sphingosine	LC/MS pos	17747	1.02	0.1177	0.0464	2.767111	2.823340
864	palmitoyl sphingomyelin	GC/MS	37506	0.85	0.3346	0.1158	1.625145	1.387937
879	lathosterol	GC/MS	33488	0.73	0.2682	0.0958	0.889059	0.645631

881			cholesterol	GC/MS	63	0.76	0.2047	0.0752	1.697102	1.283848
883			dihydrocholesterol	GC/MS	21131	2.78	0.3959	0.1331	0.822348	2.286362
891			dehydroisoandrosterone sulfate (DHEA-S)	LC/MS neg	32425	0.98	0.6553	0.2015	0.620480	0.607584
895			androsterone sulfate	LC/MS neg	31591	1.09	0.5726	0.1796	0.539983	0.587072
935			4-androsten-3beta,17beta-diol disulfate 1*	LC/MS neg	37202	1.04	0.5474	0.1724	0.690855	0.718376
947		Purine metabolism, (hypo)xanthine/inosine containing	xanthine	GC/MS	3147	1.91	0.4941	0.1582	1.005427	1.916714
950			hypoxanthine	LC/MS neg	3127	0.37	< 0.001	0.0003	3.114551	1.147087
951			inosine	LC/MS neg	1123	0.07	< 0.001	0.0000	1.748493	0.119613
955		Purine metabolism, adenine containing	adenine	LC/MS pos	554	0.70	0.0572	0.0248	1.459897	1.021916
956			adenosine	LC/MS pos	555	0.33	0.0132	0.0069	2.679957	0.894570
964			adenosine 5'-monophosphate (AMP)	LC/MS pos	32342	0.36	< 0.001	0.0000	1.631900	0.588981
973		Purine metabolism, guanine containing	guanine	LC/MS pos	32352	0.40	0.2234	0.0817	3.619263	1.459953
976			N2,N2-dimethylguanine	LC/MS pos	37081	1.66	0.0428	0.0198	0.790915	1.311851
977			guanosine	LC/MS pos	1573	0.31	0.0044	0.0027	2.315595	0.723458
988		Nucleotide	2'-O-methylguanosine	LC/MS pos	36811	1.61	0.0362	0.0169	0.429850	0.691926
994			urate	LC/MS neg	1604	0.53	0.0097	0.0053	1.241189	0.657312
1016			thymine	GC/MS	604	18.13	< 0.001	0.0000	0.177007	3.209986
1021		Pyrimidine metabolism, thymine containing	3-aminoisobutyrate	GC/MS	1566	1.24	0.4286	0.1410	1.024298	1.269410
1022			uracil	GC/MS	605	0.62	0.1577	0.0594	2.927632	1.811774
1024			uridine	LC/MS neg	606	0.36	< 0.001	0.0000	2.435443	0.880983
1025		Pyrimidine metabolism, uracil containing	pseudouridine	LC/MS neg	33442	0.26	< 0.001	0.0000	2.247591	0.580166
1030.2			uridine monophosphate (5' or 3')	LC/MS pos	39879	0.81	0.1877	0.0696	0.955846	0.778221
1040		Cofactors	ascorbate (Vitamin C)	GC/MS	1640	0.01	< 0.001	0.0002	11.476273	0.106009

1041	aldarate metabolism	dehydroascorbate	GC/MS	1659	0.53	0.0431	0.0198	0.402364	0.215056
1055	Hemoglobin and porphyrin	heme*	LC/MS pos	32593	0.32	0.4722	0.1518	4.510335	1.437990
1063		nicotinamide	LC/MS pos	594	0.30	< 0.001	0.0002	2.563745	0.756635
1066	Nicotinate and nicotinamide metabolism	nicotinamide adenine dinucleotide (NAD+)	LC/MS pos	5278	0.20	< 0.001	0.0000	1.328450	0.268022
1074		adenosine 5'diphosphoribose	LC/MS neg	558	0.40	0.0220	0.0110	1.648350	0.667043
1077		nicotinate	LC/MS pos	1504	1.87	< 0.001	0.0000	0.439218	2.139249
1085	Pantothenate and CoA metabolism	pantothenate	LC/MS pos	1508	1.04	0.3467	0.1189	1.248344	1.302237
1088		3'-dephosphocoenzyme A	LC/MS neg	18289	1.65	0.0048	0.0029	0.305462	0.505276
1098		flavin adenine dinucleotide (FAD)	LC/MS neg	2134	2.05	< 0.001	0.0001	0.565367	1.159047
1099	Riboflavin metabolism	riboflavin (Vitamin B2)	LC/MS pos	1827	1.79	0.1402	0.0533	0.628271	1.124005
1105	Tocopherol metabolism	alpha-tocopherol	GC/MS	1561	0.76	0.0302	0.0143	0.560465	0.428338
1117	Vitamin B6 metabolism	pyridoxate	LC/MS neg	31555	1.07	0.7353	0.2194	0.989351	1.058418
1123		hippurate	LC/MS neg	15753	0.08	0.0105	0.0057	5.395996	0.453304
1126.5	Benzoate metabolism	3-hydroxyhippurate	LC/MS neg	39600	0.33	0.0282	0.0134	1.316128	0.435932
1127		4-hydroxyhippurate	LC/MS neg	35527	1.08	0.8552	0.2453	0.790986	0.855582
1134		catechol sulfate	LC/MS neg	35320	0.46	0.3141	0.1102	1.497728	0.687864
1157		glycerol 2-phosphate	GC/MS	27728	0.18	< 0.001	0.0002	1.215266	0.221673
1161		heptaethylene glycol	LC/MS pos	38154	0.05	0.6818	0.2071	92.519657	4.360554
1162	Chemical	hexaethylene glycol	LC/MS pos	38133	0.07	0.3874	0.1317	20.503810	1.421641
1163		octaethylene glycol	LC/MS pos	38155	0.05	0.3993	0.1331	107.214971	5.896527
1164		pentaethylene glycol	LC/MS pos	38158	0.56	0.1946	0.0719	2.046873	1.150927
1180		2-pyrrolidinone	GC/MS	31675	0.34	0.1244	0.0485	1.378630	0.466938
1216		4-acetaminophen sulfate	LC/MS neg	37475	0.26	0.4002	0.1331	0.699167	0.182977
1217		4-acetamidophenol	LC/MS pos	12032	0.86	0.8519	0.2453	0.426936	0.366874
1218	Drug	p-acetamidophenylglucuronide	LC/MS neg	33423	0.11	0.3983	0.1331	2.333262	0.260924
1219		2-hydroxyacetaminophen sulfate*	LC/MS neg	33173	0.50	0.2670	0.0958	0.553826	0.279180

1220	2-methoxyacetaminophen sulfate*	LC/MS neg	33178	0.91	0.5959	0.1847	0.455487	0.413317
1221								
1268.6								
1281								
1341	Food component/Plant	LC/MS neg	21151	0.84	0.4548	0.1468	0.387694	0.327339
1343								
1344	Xanthine metabolism	LC/MS pos	18254	1.44	0.2391	0.0862	0.665644	0.960747
1345								
1349								
1360	Sugar, sugar substitute, starch	GC/MS	20699	0.94	0.7059	0.2128	0.963717	0.901261

Table 1 (continued)

BIOCHEMICAL NAME	CAS	PUBCHEM	KEGG	HMDB	RI	MASS
glycine	56-40-6;	5,257,127,750	C00037	HMDB00123	1166	101.9
sarcosine (N-Methylglycine)	107-97-1;	10,887,311,726	C00213	HMDB00271	1182.9	116
serine	56-45-1;	59,516,857,581	C00065	HMDB03406	1389.1	204
N-acetylserine	97-14-3;	65249		HMDB02931	1012	148
threonine	72-19-5;	69,710,196,288	C00188	HMDB00167	1412.3	218.1
N-acetylthreonine		4651717	C01118		846	160.1
betaine	107-43-7;	247		HMDB00043	721	118.2
alanine	56-41-7;	59,507,311,724	C00041	HMDB00161	1147.6	115.9
beta-alanine	56-41-7; 107-95-9;	2,394,755,801	C00099	HMDB00056	1451.8	174
N-acetylalanine	97-69-8;	88064	C02847	HMDB00766	882	130.1
aspartate	56-84-8;	5960	C00049	HMDB00191	1529.7	232

N-acetylaspargate (NAA)	997-55-7; 997-55-7 ;	65065	C01042		HMDB00812	1222	176.1
glutamate	56-86-0;	611	C00025		HMDB003339	700	148.1
glutamine	56-85-9;	69,920,865,961	C00064		HMDB00641	684	147.2
pyroglutamine*		134508				764	129.2
gamma-aminobutyrate (GABA)	56-12-2;	6,992,099,119	C00334		HMDB00112	1539.7	304.1
N-acetylglutamate	5817-08-3;	1549099	C00624		HMDB01138	1492	190.1
histidine	5934-29-2;	7,733,651,426	C00135		HMDB00177	757	154.1
urocanate	104-98-3;	736715	C00785		HMDB00301	1164	139.1
histamine	51-45-6;	774	C00388		HMDB00870	1809.7	174.1
cadaverine	462-94-2; 1476-39-7;		C01672		HMDB02322	1780.4	174
lysine	56-87-1;	5962	C00047		HMDB00182	1836.7	317.2
2-aminoadipate	542-32-5; 1118-90-7;	469	C00956		HMDB00510	1686.5	260.1
pipecolate	4043-87-2;	849	C00408		HMDB00070	1120	130.1
N6-acetyllysine	692-04-6;	699,197,892,832	C02727		HMDB00206	1134	189.1
phenyllactate (PLA)	828-01-3;	3848	C05607		HMDB00779	2237	165.1
phenylalanine	63-91-2;	69,256,656,140	C00079		HMDB00159	2056	166.1
Isobar: 1-phenylethylamine, phenylethylamine			C02455, C05332		HMDB02017, HMDB12275	2114.7	122.1
phenylacetate	103-82-2;		C07086		HMDB00209	2127	135.1
p-cresol sulfate	3233-57-7;	4615422	C01468			2896	187.1
tyrosine	60-18-4;	60,576,942,100	C00082		HMDB00158	1516	182.1
3-(4-hydroxyphenyl)lactate	6482-98-0;	9378	C03672		HMDB00755	1395	181.1
tyramine	60-19-5;	5610	C00483		HMDB00306	1503	138.1
4-hydroxyphenylacetate	156-38-7;	4693933	C00642		HMDB00020	1630.6	179
N-acetylphenylalanine	2018-61-3;	74839	C03519		HMDB00512	2590	206.2
phenylacetylglutamine	28047-15-6;	306137	C05597		HMDB06344	2868	265.2
3-(4-hydroxyphenyl)propionate	501-97-3;	10394	C01744		HMDB02199	1727.9	179.1

3-phenylpropionate (hydrocinnamate)	501-52-0;		C05629		HMDB00764	2830	149.1
phenol sulfate	937-34-8;	74426	C02180			2199	173.1
kynurenate	492-27-3;	3845	C01717		HMDB00715	2243	188.1
kynurenine	2922-83-0;	1,611,666,971,029	C00328		HMDB00684	1902	209.1
tryptophan	73-22-3;	69,235,166,305	C00078		HMDB00929	2445	205.1
indolelactate	832-97-3;	92904	C02043		HMDB00671	1964.9	202
tryptophan betaine	20671-76-5;	442106	C09213			2464	247.1
tryptamine	61-54-1;	1150	C00398		HMDB00303	2323	161.1
3-indoxyl sulfate	2642-37-7;	10258			HMDB00682	2258	212
indolepropionate	830-96-6 ;	3744			HMDB02302	2795	188.2
3-methyl-2-oxobutyrat	3715-29-5;	49	C00141		HMDB00019	1489	115.1
3-methyl-2-oxovalerate	51829-07-3;	47	C00671		HMDB03736	2106	129.2
alpha-hydroxyisocaproate	10303-64-7;	83697	C03264		HMDB00746	1854	131.2
isoleucine	73-32-5;	791	C00407		HMDB00172	1614	132.1
leucine	61-90-5;	70,457,986,106	C00123		HMDB00687	1674	132.2
N-acetyl-leucine	1188-21-2;	70912	C02710		HMDB11756	3444	174.1
N-acetylvaline	96-81-1;	66789			HMDB11757	2654	160.1
valine	72-18-4;	69,710,186,287	C00183		HMDB00883	1040	118.1
4-methyl-2-oxopentanoate	816-66-0;	70	C00233		HMDB00695	2200	129.2
alpha-hydroxyisovalerate	600-37-3;	99823			HMDB00407	1208	145.1
isobutyrylcamitine	25518-49-4;					1941	232.2
2-hydroxy-3-methylvalerate	488-15-3;	164623			HMDB00317	1787	131.1
2-methylbutyrylcamitine	31023-25-3;	6426901			HMDB00378	2439	246.1
cysteine	52-90-4;56-89-3;	58,626,419,722	C00097		HMDB00574	1560.1	218
cystine	56-89-3;	595	C00491		HMDB00192	2015.3	218
methionine sulfoxide	3226-65-1;	1,589,801,548,907	C02989		HMDB02005	729	166.1
N-formylmethionine	4289-98-9;	439750	C03145		HMDB01015	1541	176.1

hypotaurine	300-84-5;	107812	C00519	HMDB00965	1598.5	188
taurine	107-35-7;	11,234,068,592	C00245	HMDB00251	1616.4	254.1
methionine	63-68-3;	69,920,876,137	C00073	HMDB00696	1252	150.1
N-acetylmethionine	65-82-7;	448580	C02712	HMDB11745	1805	190.1
2-hydroxybutyrate (AHB)	3347-90-8;	440864	C05984	HMDB00008	1169.4	130.9
dimethylarginine (SDMA + ADMA)		123831	C03626	HMDB01539, HMDB03334	812	203.2
arginine	1119-34-2;	5,246,487,232	C00062	HMDB00517	650	175.2
ornithine	3184-13-2;	6262	C00077	HMDB03374	1763.8	141.9
urea	57-13-6;	117,616,150,869	C00086	HMDB00294	1223.9	171
proline	147-85-3;	1,457,426,971,047	C00148	HMDB00162	796	116.1
5-aminovaleate	660-88-8;	6,992,101,138	C00431	HMDB03355	1620.8	174
citrulline	372-75-8;	833	C00327	HMDB00904	715	176.1
N-acetylornithine	6205-08-9;	6,992,102,439,232	C00437	HMDB03357	875	175.2
trans-4-hydroxyproline	51-35-4;	58,106,971,053	C01157	HMDB00725	1537	140
creatine	57-00-1;	586	C00300	HMDB00064	758	132.1
creatinine	60-27-5;	588	C00791	HMDB00562	730	114.1
2-aminobutyrate	1492-24-6;	4,396,916,971,251	C02261	HMDB00650	1215.7	130
5-methylthioadenosine (MTA)	2457-80-9;	439176	C00170	HMDB01173	2427	298.1
putrescine	110-60-1;		C00134	HMDB01414	1705.8	174
N-acetylputrescine	18233-70-0;	122356	C02714	HMDB02064	895	131.1
agmatine	2482-00-0;	199	C00179	HMDB01432	1528	174
spermidine	124-20-9;	1102	C00315	HMDB01257	533	146.2
spermine	71-44-3;	1103	C00750	HMDB01256	506	203.2
glutathione, reduced (GSH)	70-18-8;	124886	C00051	HMDB00125	1274	308.1
5-oxoproline	98-79-3;	7405	C01879	HMDB00267	1446	130.1
glutathione, oxidized (GSSG)	103239-24-3;	6,535,911,215,652	C00127	HMDB03337	1535	307.3
glycylvaline	1963-21-9;	27,248,076,994,979			1572	175.1

glycylglycine	556-50-3;	154,889,711,163	C02037	HMDB11733	1757.9	174
glycylproline	704-15-4;	30,136,256,993,386		HMDB00721	1115	173.1
glycylisoleucine	19461-38-2;				2080	189.1
glycylleucine	869-19-2;	928,431,548,899	C02155	HMDB00759	2236	189.1
glycylphenylalanine	3321-03-7;	154,934,492,953			2193	221.2
glycyltyrosine	658-79-7;	928,296,994,980			1446	237.2
alanylalanine	1948-31-8;	54,603,626,992,112	C00993	HMDB03459	815	159.2
alanylthreonine		426318			827	189
alanylvaline		137276			1513	189
alanylleucine		259583			2150	203
alanylarginine		9964828			1079	244.1
alanylglytamate		656476			766	219
alanylisoleucine		4,173,585,246,008			2011	203.1
alanylphenylalanine	3061-90-3;	2080			2422	237.2
alanyltyrosine		5,723,194,099,800			1642	251
aspartylphenylalanine	13433-09-5;	93078		HMDB00706	2538	281.1
aspartate-glutamate	6157-06-8;	4130574			603	261.2
alpha-glutamylglutamate	3929-61-1;	439500	C01425		857	277.1
prolylleucine	52899-07-7;	4,441,097,082,009			2985	227.2
isoleucylisoleucine	42537-99-5;				2678	245.1
isoleucylleucine	26462-22-6;	11644431			2856	245.1
leucylleucine	3303-31-9;	768,076,992,072	C11332		3895	243.2
threonylphenylalanine		40,997,994,099,798			2500	267.2
phenylalanylphenylalanine	2577-40-4;	69,930,906,993,089			3317	313.2
pyroglutamylvaline		152416			1755	227.2
valinylglutamate	3062-07-5;	7009623			1346	247.1
Isobar: glycylglutamate; glutamylglycine					634	203.1

glucosamine	66-84-2;	441477	C00329	HMDB01514	1833	203
erythronate*	13752-84-6;	2781043		HMDB00613	1546.9	292.1
N-acetylneuraminate	131-48-6;		C00270	HMDB00230	787	310
fucose	2438-80-4;	3034656	C00382	HMDB00174	1682.2	204
fructose	57-48-7;	5984	C00095	HMDB00660	1762.7	204
galactose	59-23-4;	3037556	C01582	HMDB00143	1793.8	203.9
maltose	6363-53-7;	439341	C00208	HMDB00163	2142.1	204.1
mannitol	69-65-8;	6251	C00392	HMDB00765	1839	319.1
sorbitol	6706-59-8;	107428	C00794	HMDB00247	1843	319.1
maltotriose	1109-28-0;	439586	C01835	HMDB01262	2419	204
maltotetraose	34612-38-9;	446495	C02052	HMDB01296	965	665.1
maltopentaose	34620-76-3;	3710145	C06218	HMDB12254	1100	827
maltohexaose	34620-77-4;	5288409	C01936	HMDB12253	1224	989
1,5-anhydroglucitol (1,5-AG)	154-58-5;		C07326	HMDB02712	1788.7	217
glycerate	600-19-1;	752	C00258	HMDB00139	1360.7	189
glucose-6-phosphate (G6P)	103192-55-8;		C00668	HMDB01401	2042.7	387.2
glucose	50-99-7;	79025	C00293	HMDB00122	1866.8	217.1
Isobar: fructose 1,6-diphosphate, glucose 1,6-diphosphate					572	339
pyruvate	127-17-3;	107735	C00022	HMDB00243	1130.6	217
lactate	79-33-4;	612	C00186	HMDB00190	1102.8	116.9
ribitol	488-81-3;		C00474	HMDB00508	1692.4	217
threitol	2418-52-2;	169019	C16884	HMDB04136	1513	217.1
gluconate	527-07-1;	10690	C00257	HMDB00625	1879.4	333

ribose	50-69-1;	5311110	C00121	HMDB00283	1639.2	204
ribulose	488-84-6;	79021	C00309	HMDB00621, HMDB03371	1662	306.1
UDP-glucuronate	28053-08-9; 63700-19-6;	17473	C00167	HMDB00935	594	579.1
xylitol	87-99-0;	6912	C00379	HMDB00568	1677.6	307.2
citrate	77-92-9;	311	C00158	HMDB00094	1763.4	273.1
tricarballoylate	99-14-9;	14925			592	175
succinate	110-15-6;	1110	C00042	HMDB00254	1348	247
fumarate	100-17-8;		C00122	HMDB00134	1382.1	245
malate	6915-15-7;	525	C00149	HMDB00156	1502	233
phosphate	7664-38-2 ;	1061	C00009	HMDB01429	1307.7	298.9
linoleate (18:2n6)	60-33-3;	5280450	C01595	HMDB00673	5533	279.3
linolenate [alpha or gamma; (18:3n3 or 6)]			C06427	HMDB01388	5450	277.3
dihomo-linolenate (20:3n3 or n6)		5312529	C03242	HMDB02925	5600	305.4
docosapentaenoate (n3 DPA; 22:5n3)	2234-74-4 ;		C16513	HMDB01976	5574	329.4
docosahexaenoate (DHA; 22:6n3)	6217-54-5;	445580	C06429	HMDB02183	5518	327.3
pelargonate (9:0)	112-05-0;	5461016	C01601	HMDB00847	4847	157.2
myristate (14:0)	544-63-8;	11005	C06424	HMDB00806	5439	227.3
myristoleate (14:1n5)	544-64-9 ;	5281119	C08322	HMDB02000	5338	225.3
pentadecanoate (15:0)	1002-84-2;	13849	C16537	HMDB00826	5522	241.3
palmitate (16:0)	57-10-3;	985	C00249	HMDB00220	5619	255.3
palmitoleate (16:1n7)	373-49-9;	445638	C08362	HMDB03229	5477	253.3
margarate (17:0)	506-12-7;	10465		HMDB02259	5733	269.3
10-heptadecenoate (17:1n7)	29743-97-3;	5312435			5558	267.3
stearate (18:0)	57-11-4;	5281	C01530	HMDB00827	1994.6	341.3
oleate (18:1n9)	112-80-1;	445639	C00712	HMDB00207	1984.4	339.2

cis-vaccenate (18:1n7)	693-72-1;	5282761	C08367			1987	339.3
arachidate (20:0)	506-30-9;	10467	C06425		HMDB02212	2071.2	369.3
eicosenoate (20:1n9 or 11)					HMDB02231	5955	309.4
dihomo-linoleate (20:2n6)	2091-39-6;	6439848	C16525			5722	307.3
arachidonate (20:4n6)	506-32-1;	444899	C00219		HMDB01043	5525	303.4
docosadienoate (22:2n6)	7370-49-2 ;	5282807	C16533			6017	335.4
lignocerate (24:0)	557-59-5;	11197	C08320		HMDB02003	2211	425.4
n-Butyl Oleate	142-77-8;	5354342				2045	265.3
4-hydroxybutyrate (GHB)	502-85-2;	10413	C00989		HMDB00710	1277	233.1
2-hydroxystearate	629-22-1;	69417	C03045			5705	299.4
13-HODE	29623-28-7;	6443013	C14762		HMDB04667	5251	295.3
2-hydroxypalmitate	764-67-0;	92836				5508	271.3
2-hydroxyglutarate	40951-21-1;	43	C02630		HMDB00606	1576	247
azelate (nonanedioate)	123-99-9;	2266	C08261		HMDB00784	1322	187.2
undecanedioate	1852-04-6;	15816			HMDB00888	2376	215.1
3-carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPF)	86879-39-2;	123979				2815	239.1
13-methylmyristic acid	2485-71-4;	151014				5498	241.3
methyl palmitate (15 or 2)						5698	269.4
17-methylstearate	2724-59-6;	3083779				5987	297.4
12-HETE	73837-14-6;				HMDB06111	5295	319.3
oleic ethanolamide	11-58-0;111-58-0;	5283454			HMDB02088	6553	324.5
palmitoyl ethanolamide		4671				6416	298.4
propionylcamitine	17298-37-2 ;	107738	C03017		HMDB00824	1589	218.2
butyrylcamitine	25576-40-3;	213144				2007	232.2
deoxycamitine	6249-56-5;	725	C01181		HMDB01161	759	146.1
camitine	461-05-2;	10917				702	162.2
3-dehydrocamitine*	10457-99-5;	6991982	C02636		HMDB12154	1020	160.2

acetylcamitine	5080-50-2;	7045767	C02571		HMDB00201	1203	204.2
ethanolamine	141-43-5;		C00189		HMDB00149	1074	102
phosphoethanolamine	1071-23-4;	52,323,241,015	C00346		HMDB00224	1577.3	299.1
glycerol	56-81-5;	753	C00116		HMDB00131	1311	205
glycerol 3-phosphate (G3P)	29849-82-9;	754	C00093		HMDB00126	1719.7	357.1
glycerophosphorylcholine (GPC)	28319-77-9;	657272	C00670		HMDB00086	694	258.1
myo-inositol	87-89-8;		C00137		HMDB00211	1924.9	217
scyllo-inositol	488-59-5;		C06153		HMDB06088	1893.8	318.2
1,2-propanediol	57-55-6;		C00717,C02912,C00583,C01506,C02917		HMDB01881	1041	117
2-palmitoleoylglycerophosphoethanolamine*						5611	450.3
1-stearoylglycerophosphoethanolamine	69747-55-3;	9547068			HMDB11130	6200	480.4
1-oleoylglycerophosphoethanolamine		9547071			HMDB11506	5928	478.3
2-oleoylglycerophosphoethanolamine*						5848	478.3
1-linoleoylglycerophosphoethanolamine*					HMDB11507	5725	476.3
2-linoleoylglycerophosphoethanolamine*						5650	476.4
1-arachidonoylglycerophosphoethanolamine*					HMDB11517	5731	500.3
2-arachidonoylglycerophosphoethanolamine*						5674	500.3
1-palmitoylglycerophosphocholine	17364-16-8;	86554				6046	570

1-stearoylglycerophosphocholine	19420-57-6;	497299				5844	524.4
2-oleoylglycerophosphocholine*						5640	522.4
1-oleoylglycerophosphoserine		9547099				5690	522.3
1-palmitoylplasmenylethanolamine*						6153	436.4
1-stearoylglycerol (1-monostearin)	123-94-4;	24699	D01947			2186.6	399.4
sphinganine	3102-56-5;	3126	C00836	HMDB00269		5175	302.3
sphingosine	123-78-4;	5353955	C00319	HMDB00252		5197	300.2
palmitoyl sphingomyelin		9939941				2524	311.3
lathosterol	80-99-9 ;	65728	C01189	HMDB01170		2337	458.5
cholesterol	57-88-5;	6432564	C00187	HMDB00067		2316.9	329.3
dihydrocholesterol	80-97-7;	6665		HMDB00908		2320.8	215.2
dehydroisoandrosterone sulfate (DHEA-S)		12594	C04555	HMDB01032		4771	367.2
androsterone sulfate	2479-86-9 ;		C00523	HMDB02759		5011	369.2
4-androsten-3beta,17beta-diol disulfate 1*				HMDB03818		3735	224.3
xanthine	69-89-6;	1188	C00385	HMDB00292		1889.9	353
hypoxanthine	68-94-0;	790	C00262	HMDB00157		1313	135.1
inosine	58-63-9;					1630	267.2
adenine	73-24-5;	190	C00147	HMDB00034		1003	136.1
adenosine	58-61-7;	60961	C00212	HMDB00050		1650	268.1
adenosine 5'-monophosphate (AMP)	149022-20-8;	15938965	C00020	HMDB00045		1210	348.1
guanine	73-40-5;	764	C00242	HMDB00132		1022	152.1
N2,N2-dimethylguanine	1445-15-4;	74047				1764	180
guanosine	118-00-3;	6802	C00387	HMDB00133		1676	284
2'-O-methylguanosine	2140-71-8;		C04545			1926	298

urate	69-93-2;120K5305;	C00366	HMDB00289	769	167.1
thymine	65-71-4;	C00178	HMDB00262	1429.1	255
3-aminoisobutyrate	10569-72-9;	C05145	HMDB03911	1252.2	101.9
uracil	66-22-8;	C00106	HMDB00300	1370.4	241
uridine	58-96-8;	C00299	HMDB00296	1467	243.1
pseudouridine	1445-07-4;	C02067	HMDB00767	1104	243.1
uridine monophosphate (5' or 3')				1079	325
ascorbate (Vitamin C)	134-03-2;	C00072	HMDB00044	1850.1	332.1
dehydroascorbate	490-83-5;	C05422	HMDB01264	1800	245.1
heme*	14875-96-8;			4985	616.2
nicotinamide	98-92-0;	C00153	HMDB01406	1267	123.1
nicotinamide adenine dinucleotide (NAD+)	53-84-9;	C00003	HMDB00902	1370	664
adenosine 5'diphosphoribose	68414-18-6;	C00301	HMDB01178	964	558.1
nicotinate	59-67-6;	C00253	HMDB01488	1241	124.1
pantothenate	137-08-6;	C00864	HMDB00210	2218	220.1
3'-dephosphocoenzyme A	3633-59-8;	C00882	HMDB01373	2010	686.2
flavin adenine dinucleotide (FAD)	146-14-5;84366-81-4;	C00016	HMDB01248	2413	784.1
riboflavin (Vitamin B2)	83-88-5;	C00255	HMDB00244	3111	377.2
alpha-tocopherol	59-02-9;10191-41-0;	C02477	HMDB01893	2305.4	502.5
pyridoxate	82-82-6;	C00847	HMDB00017	2210	182.1
hippurate	495-69-2;	C01586	HMDB00714	2136	178.1
3-hydroxyhippurate			HMDB06116	1670	194.1
4-hydroxyhippurate	2482-25-9;			1439	194

catechol sulfate		3083879	C00090			1928	188.9
glycerol 2-phosphate	819-83-0;	2526	C02979,D01488		HMDB02520	1691.8	243
heptaethylene glycol	5617-32-3;	79718				2836	327.2
hexaethylene glycol	2615-15-8;	17472				2651	283.2
octaethylene glycol	5117-19-1;	78798				3054	371.3
pentaethylene glycol	4792-15-8;	62551				2471	239.2
2-pyrrolidinone	616-45-5 ;	12025			HMDB02039	1190.9	142
4-acetaminophen sulfate	10066-90-7;32113-41-0;	83939	C06804		HMDB01859	1779	230.1
4-acetamidophenol	103-90-2;	1983	C06804		HMDB01859	2238	152.1
p-acetamidophenyglucuronide	120595-80-4;	4022661			HMDB10316	1377	326.1
2-hydroxyacetaminophen sulfate*						1703	246.1
2-methoxyacetaminophen sulfate*						1977	260.1
3-(cystein-S-yl)acetaminophen*		5233914				1960	271.2
benzoylcegonine	519-09-5;	442997	C10847			3100.7	290.2
saccharin	81-07-2;	5143	D01085			2013	182.1
stachydrine	4136-37-2;	115244	C10172		HMDB04827	860	144.1
caffeine	58-08-2;	2519	C07481		HMDB01847	2820	195.1
paraxanthine	611-59-6;	4687	C13747		HMDB01860	2444	181.2
theobromine	83-67-0;	5429	C07480		HMDB02825	2136	181.1
1,7-dimethylurate	33868-03-0;	91611	C16356		HMDB11103	1607	195.1
erythritol	149-32-6;		C00503		HMDB02994	1517.5	217

[0046] Having described the invention in detail, it will be apparent that modifications, variations, and equivalent embodiments are possible without departing from the scope of the invention defined in the appended claims. Furthermore, it should be appreciated that all examples in the present disclosure are provided as non-limiting examples.

EXAMPLES

[0047] The following non-limiting examples are provided to further illustrate embodiments of the invention disclosed herein. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent approaches that have been found to function well in the practice of the invention, and thus can be considered to constitute examples of modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

EXPERIMENTS (“COHORT A”)

[0048] Study population and sample collection: The initial study population was derived from two clinic sites in Seattle with enrollment between November 2003 and June 2010: the Public Health, Seattle and KingCounty Sexually Transmitted Diseases Clinic (STD clinic; n=30) and the University of Washington's Women's Research Clinic (WRC; n=30). A case-control study comprising 40 women with BV and 20 women without BV was conducted. BV was defined by both Amsel criteria (21) and Gram stain interpretation using the Nugent method (22); women were classified BV-negative if negative by both diagnostic measures. Cases and controls were randomly selected from both clinics in a 2:1 ratio, with the goal of having a better representation of women with BV given the increased bacterial heterogeneity associated with this condition. All women provided written informed consent, and the studies were approved by Review Boards at the Fred Hutchinson Cancer Research Center and the University of Washington.

[0049] Studies were aimed at using mass spectrometry to link specific metabolites with particular bacteria detected in the human vagina by PCR. BV was associated with strong metabolic signatures across multiple pathways affecting amino acid, carbohydrate and lipid metabolism, highlighting the profound metabolic changes in BV. These signatures were associated with the presence and concentrations of particular vaginal bacteria including some bacteria yet to be cultivated, thereby providing clues as to the microbial origin of many metabolites.

[0050] A pelvic examination with speculum was performed for collection of samples. Polyurethane foam swabs (Epicenter Biotechnologies, Madison, WI) were brushed against the lateral vaginal wall and stored at -80°C until DNA extraction for bacterial PCRs. Vaginal fluid was also collected for Gram staining, pH, saline microscopy and potassium hydroxide preparation. Cervicovaginal lavage fluid (CVL) was collected by instilling 10 mL sterile saline into the vagina, aspirating, and storing at -80°C prior to processing for metabolomics studies.

[0051] DNA Extraction and quantitative PCR: DNA was extracted using the Ultra Clean Soil DNA Kit or the Bacteremia Kit (Mobio, Carlsbad, CA), which gave similar results. DNA was eluted in 150 µL buffer and diluted 1:1 in 1 mM Tris, 0.1 mM EDTA (TE) buffer. Sham swabs without human contact were processed as controls to assess contamination from reaction buffers or sample collection swabs. Samples were evaluated for presence of PCR inhibitors using a qPCR assay targeting a segment of exogenously added jellyfish DNA; inhibition was defined as a delay in the threshold cycle of >2 cycles compared with no template controls (68). Control assays targeting the human 18S rRNA gene were performed to ensure that swabs contained human tissue (68). DNA from each sample was subjected to a panel of 14 qPCR assays to measure concentrations of bacteria. The bacteria targeted included three members of the *Clostridia/es* order which are highly specific for BV, designated BV-associated bacterium-1 (BVAB-1), BVAB-2, and BVAB-3 (8). Other vaginal bacteria targeted included *Gardnerella vaginalis*, *Leptotrichia/Sneathia* spp., *Megasphaera*-like bacterium (Type 1 & Type 2), *Atopobium vaginae*, *Lactobacillus crispatus*, *Lactobacillus jensenii*, and *Lactobacillus iners* (69, 70). Four additional qPCR assays targeting an *Eggerthella*-like bacterium, *Prevotella amnii*, *Prevotella timonensis* and *Prevotella buccalis* using 16S rRNA gene-specific primers and taxon-directed hydrolysis probes were developed. Assay conditions, primer and probe sequences for

qPCR assays developed in this study are presented in Table 2. Core PCR reagents were obtained from Applied Biosystems (Carlsbad, CA), and master mixes contained buffer A (1 mM), deoxynucleotide triphosphates (1 mM), magnesium (3 mM), AmpErase uracil-N-glycosylase (0.05 U), and AmpliTaq Gold polymerase (1-1.5 U) per reaction. Primers were added at 0.8 μ M per reaction and final probe concentration was 150 μ M. Assays underwent 45 cycles of amplification on the StepOnePlus™ Real-time PCR System (Life Technologies, Grand Island, NY). Plasmid standards were run in duplicate from 106 to 2.5 copies. Specificity and sensitivity testing was conducted as previously described (69). Two microliters of DNA (diluted 1:1 with TE buffer) were added to each qPCR reaction and values are reported as 16S rRNA gene copies per swab.

[0052] Table 2. Primer and probe sequences

PCR Assay	PCR Conditions	Amplicon Size	Primer/Probe	Primer/Probe Sequence
<i>Eggerthella</i> - like	56°C annealing, 39 s	84 bp	Egger-like_126F	5'-GACCAACCTGCCTCTTACATT-3'
	72°C extension, 30 s		Egger-like_210R	5'-G CATA CATCATGTG ATATGTG C-3'
			Egger-like_155-178_pb	5'-FAMAAAAGAAATTCTGGCTAATACCAA - MG BN FQ-3'
<i>Prevotella buccalis</i>	57°C annealing, 39 s	171 bp	Pbuccalis_455F	5'-G CG CGACGTGTCGTG CA-3'
	72°C extension, 30 s		Pbuccalis_626R	5'-CCGGTTGAGCCGGTACA-3'
			P. buccalis_587-604_pb	5'-FAM-CG CCAG RTAAG CGTGTGGMG BN FQ-3'
<i>Prevotella timonensis</i>	64°C anneal/extend, 60	90 bp	Ptimonensis_578F	5'-GAG CGTAG G CTGTCTATTAAG C-3'
			Ptimonensis_668R	5'-CTTCCTG CATACTCAAGTCG A C
			Pti m onensis_609-629_p b	5'-FAM-ATTACCGGCTCAACCGGTGGMG BN FQ-3'
<i>Prevotella amnii</i>	59°C annealing, 39 s	69 bp	Pamnii_989F	5'-GGCTTGAATTG CAGATGTTTATAT-3'
	72°C extension, 30 s		Pamnii_1058R	5'-CCATGCAGCACCTTCACAAAT-3'
			Pa mnii_1014-1033_pb	5'-FA M-AG ATGATATATTCCTTCG G - MG BN FQ-3'

MG - Minor groove binder, FAM - fluorescein label, BN FQ - Black Hole Non-fluorescent quencher

[0053] Broad-range PCR and pyrosequencing of 16S rRNA gene amplicons: Broad-range 16S rRNA gene PCR with pyrosequencing was performed using 454 Life Sciences FLX technology (Roche, Branford, CT) targeting the V3-V4 region of the 16S-rRNA gene for a subset of samples from 20 women with BV and 10 women without BV, a subset of the cohort described previously (11). Sequence reads were classified using a phylogenetic placement tool pplacer (71) and a curated reference set of vaginal bacteria (11). All sequence reads were deposited in the NCBI Short Read Archive (SRA051298) (11). Measurement of levels of biochemicals: Chromatographic separation and full-scan mass spectroscopy (MS) were performed using the Metabolon (Durham, NC) platform (72-74). Briefly, CVL samples were extracted to remove the protein fraction while allowing maximal recovery of small molecules using the MicroLab STAR® system (Hamilton, Reno, NV). The resulting extract was split into equal parts for analysis by gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS). Samples were dried by placing them on a Zymark TurboVap® (American Laboratory Trading, East Lyme CT) and stored at -80°C. Aliquots of water and solvents used in the extraction were included as controls to assess the contribution of the separation process and extraction methods to the compound signals. Additional samples with known compositions were also included with every run to ensure that there were no process deviations in each run. A reference library of 1000 purified standards with known chemical structures was used to identify the small molecules from the samples by matching chromatographic properties and mass spectral signatures.

[0054] Data analysis: Metabolite data were median-centered, and missing values imputed as the minimum observed for that metabolite; median values were then log-transformed. Pyrosequencing taxonomic counts and qPCR measures were log-transformed. Welch's two sample t-tests were used to compare metabolites between BV-positive and BV-negative groups. An estimate of false discovery rate (q-value) was calculated to account for multiple comparisons. A low q-value (<0.1) indicates high confidence in a result. Eighty-three metabolites (30%) with largest variability (inter-quartile range) were used to hierarchically cluster samples using Euclidean distance (Figure 1); identification of four sample groups maximizes silhouette width. To explore the relationship between relative abundances of metabolite and pyrosequencing reads (Figure 2) or qPCR measures (Figure 3), Pearson correlations were calculated between

metabolite and pyrosequencing reads or qPCR levels; these were used in hierarchical clustering using Euclidean distance. Data transformation was performed in R (<http://crans-project.org>) (75). Lasso regression with binomial response was used to identify metabolites strongly associated with Amsel clinical criteria (Table 3). The number of metabolites retained was set to the largest value within 1 standard deviation of the cross-validation error. Analysis was performed with the R package glmnet (76).

[0055] Table 3. Association of metabolites with individual clinical criteria

Bacterial Taxa	Regression co-efficient'
BV Status	
deoxycarnitine	0.5
phenylalanine	-0.299
N-acetylputrescine	0.254
pipecolate	0.148
cadaverine	0.025
Elevated pH	
cadaverine	0.0657
N-acetylputrescine	0.0577
lactate	-0.0505
glutamate	-0.0249
sphingosine	-0.0137
tyrosine	-0.0083
tyramine	0.0033
Presence of Clue Cells	
deoxycarnitine	0.2519
glycylproline	-0.203
glutathione, reduced (GSH)	-0.0311
pipecolate	0.0024
Presence of Amine Odor	
N-acetylputrescine	0.302
lactate	-0.072
phenylalanine	-0.049
Vaginal Discharge	

agmatine	0.14
cadaverine	0.11

METABOLITE SUMMARY OF COHORT A

[0056] Characteristics of the 60 women from cohort A are presented in Table 4. There was low prevalence of other infections including *Candida* (8%), *Trichomonas vaginalis* (3%), *Chlamydia trachomatis* (3%), and *Neisseria gonorrhoeae* (0%). Cases included women with BV by both Amsel criteria and Nugent score; controls did not have BV by both criteria. Elevated pH > 4.5 was the only Amsel criterion noted in all cases (n=40); vaginal fluid from 20% of control women had pH > 4.5 (n=4). Presence of >20% clue cells and a positive whiff test (amine odor) were both observed in 83% of cases (n=33); clue cells and a positive whiff test were not detected in the control group. A thin homogeneous discharge that is present in some women with symptomatic BV was noted in 85% of cases (n=34), and 5% (n=1) of women in the control group.

[0057] Table 4. Characteristics of study participants according to case or control status

Characteristic	Cohort A			Cohort B		
	Total (n=60)	Cases (n=40)	Controls (n=20)	Total (n=60)	Cases (n=40)	Controls (n=20)
Age , Median (range)	26 (17-56)	26 (19-42)	26 (17-56)	29 (19-49)	29 (19-45)	30 (21-49)
Race (self-defined) ¹						
Black or African American	13 (22)	10 (25)	3 (15)	24 (40)	22 (55)	2 (10)
White	41 (68)	27 (68)	14 (70)	28 (47)	14 (35)	14 (70)
Other	4 (7)	2 (5)	2 (10)	7 (12)	4 (10)	3 (15)
Don't Know/Declined to provide data	2 (3)	1 (3)	1 (5)	1 (1)	0 (0)	1 (5)
Menses , at visit	4 (7)	3 (8)	1 (5)	5 (8)	4 (10)	1 (5)
Vaginal douching , past week ²	4 (7)	3 (8)	1 (5)	3(5)	2 (5)	1 (5)
Antibiotic use , past month ³	5 (8)	5 (13)	0 (0)	5 (8)	3 (7.5)	2 (10)
Hormonal contraceptive , past month ⁴	7 (60)	3 (8)	4 (20)	18 (30)	9 (23)	9 (45)
Sexual activity						
Sex, past 3 months ⁵	55 (92)	37 (93)	18 (90)	50 (83)	33 (83)	17 (85)
Sex with female partner ⁶	13 (22)	9 (23)	4 (20)	9 (15)	7 (18)	2 (10)
Sex with male partner ¹	50 (83)	33 (83)	17 (85)	49 (82)	34 (85)	15 (75)

Amsel criterion - Vaginal Discharge⁸						
Normal	18 (30)	5 (13)	13 (65)	18 (30)	0 (0)	18 (90)
Abnormal	35 (58)	34 (85)	1 (5)	35 (58)	34 (85)	1 (5)
Other	7 (11)	1 (3)	6 (30)	7 (12)	6 (15)	1 (5)
Amsel criterion - pH						
4.5 and less	16 (27)	0 (0)	16 (80)	16 (27)	1 (2)	15 (75)
Greater than 4.5	44 (73)	40 (100)	4 (20)	44 (73)	39 (98)	5 (25)
Amsel criterion - Clue Cells						
Absent	21 (35)	2 (5)	19 (95)	18 (30)	0 (0)	18 (90)
Less than 20% (few)	5 (8)	4 (10)	1 (5)	4 (7)	2 (5)	2 (10)
Greater than 20% (many)	33 (55)	33 (83)	0 (0)	38 (63)	38 (95)	0 (0)
Amsel criterion - Whiff Test						
Negative	27 (45)	7 (18)	20 (100)	28 (47)	8 (20)	20 (100)
Positive	33 (55)	33 (83)	0 (0)	32 (53)	32 (80)	0 (0)
Other infections						
<i>Trichomonas vaginalis</i>	2 (3)	2 (5)	0 (0)	1 (2)	1 (2)	0 (0)
<i>Chlamydia trachomatis</i> ⁹	2 (3)	1 (3)	1 (5)	0 (0)	0 (0)	0 (0)
<i>Neisseria gonorrhoeae</i> ⁹	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
<i>Candida</i>	5 (8)	1 (3)	4 (20)	4 (7)	3 (8)	1 (5)

Data represent number (%) of participants. Cases included women with BV by both Amsel criteria (21) and by interpretation of Gram stain using Nugent score (22). Controls included women who did not have BV by both criteria. ¹Study participants were able to select more than one category for race. Black/African American and white indicate women who selected only these categories. ²Douching data not available for 1 study participant who did not provide data. ³Antibiotic use data not available for 4 study participants. ⁴Hormonal contraceptive data not available for 4 study participants who did not provide data. ⁵Sex data not available for 2 study participants who did not provide data. ⁶Female sex partner data not available for 9 study participants who did not provide data. ⁷Male sex partner data not available for 2 study participants who did not provide data. ⁸Abnormal vaginal discharge indicates those with a thin homogenous discharge that is consistent with BV. Women with vaginal discharge that was not normal, or did not have a thin homogeneous discharge were placed in the other category. ⁹*Chlamydia trachomatis* and *Neisseria gonorrhoeae* data not available for 5 study participants.

[0058] 279 known metabolites was detected in vaginal fluid from women with and without BV. There were significant differences in levels of 173 metabolites (62% of the 279 detected) on comparison of women with and without BV (q-value = 0.02). Women with BV had higher levels of 55 biochemicals (20%), and lower levels of 118 biochemicals (42%). A summary of metabolites altered in BV based on pathway is provided in Table 5.

[0059] Table 5. Summary of biochemicals altered based on super pathways

Super Pathway¹	Total Altered	Biochemicals higher in BV Number (%)	Biochemicals lower in BV Number (%)
Amino Acid	90	32 (35.6)	35 (38.9)
Peptide	28	0 (0.0)	24 (85.7)
Carbohydrate	27	3 (11.1)	15 (55.6)
Energy	6	2 (33.3)	0 (0.0)
Lipid	74	12 (16.2)	27 (26.5)
Nucleotide	17	3 (17.6)	8 (47.1)
Cofactors & Vitamins	13	3 (23.1)	6 (46.2)
Xenobiotics	24	0 (0.0)	3 (12.5)

¹Biochemicals categorized according to KEGG pathways.

[0060] Hierarchical clustering depicting this set of the most variable metabolites measured in CVL samples from all women resulted in the creation of four groups, of which 2 clusters were populated by women with BV. Cluster-I had 12 women without BV, and Cluster-II comprised 15 women with BV. Cluster-III included women without BV. Cluster-IV included 25 women with BV and 1 woman without BV (Subject 58) (Figure 1). Vaginal fluid from Subject 58, the outlier in Cluster-IV, had an elevated pH of 5.6 and a Nugent score of 6, defined as intermediate on this scale.

EXAMPLE 3

ALTERATIONS IN METABOLISM (“COHORT A”)

[0061] Amino acids: 19 of 20 important amino acids (95%) that are incorporated into proteins were detected using the Metabolon platform (Table 1). Levels of 18 of 19 amino acids detected (94.7%) were lower among cases; this was statistically significant for 16 of 18 amino acids (84.9%). There were higher concentrations of amino acid catabolites in women with BV (Table 1). Examples of such catabolites include: cadaverine ($p < 0.001$) and pipecolate ($p < 0.001$) in the lysine degradation pathway; tyramine ($p < 0.001$), 4-hydroxyphenylacetate ($p < 0.001$) and 3-

(4-hydroxyphenyl)propionate ($p<0.001$) in the tyrosine pathway; tryptamine ($p<0.001$) in the tryptophan pathway; and citrulline ($p<0.001$) in the arginine pathway. There was marked elevation of the polyamine putrescine ($p<0.001$), along with lower levels of putrescine precursors arginine ($p<0.001$) and ornithine ($p<0.001$) in women with BV. The polyamine spermine ($p<0.001$), a typical product of putrescine degradation, was lower in BV. Enhanced protein/amino acid catabolism in BV was further supported by detection of lower levels of dipeptides ($n=28$); this was statistically significant for 24 dipeptides (85.7%). Levels of oxidized ($p<0.001$) and reduced ($p=0.049$) glutathione were lower in BV.

[0062] Carbohydrates: Of the four aminosugars detected, N-acetylneuraminate, commonly known as sialic acid, was higher in BV ($p<0.001$), while glucosamine levels were lower ($p=0.0059$). Glucose oligosaccharides of varying length including maltotriose ($p=0.0095$), maltotetraose ($p=0.0016$), maltopentaose ($p<0.001$), and maltohexaose ($p<0.001$) were all lower in BV. Likewise, lower levels of simple sugars and sugar alcohols such as lactate ($p<0.001$), fructose ($p=0.0012$), and mannitol ($p=0.001$) were noted; galactose ($p<0.001$) and threitol ($p<0.001$) were significantly higher in BV. Succinate levels were also higher in BV ($p<0.001$).

[0063] Nicotinamide adenine dinucleotide (NAD): NAD, an essential co-factor for energy metabolism, was below the limit of detection in 82.5% of BV cases and in only 10% of controls ($p<0.001$). Nicotinamide levels, a precursor to NAD biosynthesis, were also lower in BV ($p=0.06$), while nicotinate ($p=0.002$) levels were higher.

[0064] Lipids: Multiple components of lipid metabolism were affected in BV. The arachidonic acid catabolite 12-hydroxyeicosatetraenoic acid (12-HETE) was higher in BV ($p<0.001$) while arachidonate was lower ($p=0.0075$). Deoxycarnitine, a precursor to carnitine was higher in BV ($p<0.001$), while carnitine was lower ($p<0.001$). Ascorbic acid, which is essential to the synthesis of carnitine, was lower in BV ($p<0.001$). Acyl-carnitines such as acetylcarnitine ($p<0.001$), propionylcarnitine ($p<0.001$), and butyrylcarnitine ($p<0.001$) were also lower in BV. Of the four monohydroxy fatty acids detected, levels of 4-hydroxybutyrate ($p<0.001$) and 13-hydroxyoctadecadienoic acid (13-HODE) ($p=0.0042$) were higher in BV. Biochemicals involved in glycerol metabolism including glycerol ($p=0.046$) and glycerol-3-phosphate ($p<0.001$) were lower in BV.

EXAMPLE 4

ASSOCIATION BETWEEN METABOLITES AND BACTERIAL ABUNDANCE AND CONCENTRATIONS IN COHORT A

[0065] Association between metabolites and bacterial abundance: Pearson correlation coefficients were used to investigate associations between bacterial abundance and metabolite abundance in vaginal samples. Specifically, the abundance of 30% of the most variable metabolites and relative abundance of bacteria detected by broad-range PCR with pyrosequencing was analyzed in a subset of 20 women with BV and 10 women without BV. A group of ten BV-associated bacteria were highly correlated with metabolites typically associated with BV including succinate, cadaverine, putrescine, tyramine and deoxycarnitine (Figure 2). *Megasphaera* sp. type 1 had the highest correlation coefficient with the fatty acid, 12-HETE (0.48). A second cluster of BV-associated bacteria including BVAB1, BVAB3, *Megasphaera* sp. type 2 and several *Prevotella* species such *P. amnii*, *P. disiens*, *P. buccalis* and *P. bivia* showed similar positive associations with the above-mentioned metabolites, but the correlations were less pronounced. The three lactobacilli, *L. crispatus*, *L. jensenii* and *L. iners* clustered together and exhibited metabolite correlation patterns that overall were in contrast to the patterns found with BV-associated bacteria.

[0066] Association between metabolites and bacterial concentrations: Bacteria were selected for qPCR based on relative abundance measured by broad-range PCR and pyrosequencing in a subset of samples (Figure 2), and on associations made in previous studies that have demonstrated sensitivity, specificity (31) and significance of these bacteria in BV (11, 20, 32). Associations of concentrations of key vaginal bacteria with the 30% most variable metabolites were investigated using Pearson correlation coefficients (Figure 3). *L. crispatus*, *L. jensenii* and *L. iners* clustered together, while the BV-associated bacteria clustered as two groups. Group-1 comprised *Leptotrichia/Sneathia* spp., BVAB2, *Megasphaera* spp., *Prevotella timonensis*, *Atopobium vaginae*, *Gardnerella vaginalis*, *Eggerthella*-like bacterium and *Prevotella buccalis*. Group-2 comprised BVAB1, BVAB3 and *Prevotella amnii*. Clustering patterns noted here also reflected patterns observed in the association analysis of metabolites with bacteria detected by broad range PCR and pyrosequencing. *L. crispatus* and *L. jensenii*, lactobacilli typically associated with vaginal health, exhibited metabolite correlation patterns that

were similar; these were in striking contrast to those exhibited by BV-associated bacteria (Figure 3). *L. iners* exhibited correlation patterns that were intermediate between those of BV-associated bacteria and those of *L. crispatus* /*L. jensenii*. Concentrations of *L. crispatus* and *L. jensenii* had strong positive correlations with several amino acids and dipeptides, but were negatively correlated with amino acid catabolites (tyramine, pipecolate, cadaverine) and polyamines (putrescine, agmatine). *L. iners* was negatively correlated with some amino acids and dipeptides (glutamate, glycylleucine), but positively correlated with others (proline, threonine, aspartate, serine, and valinylglutamate). *L. crispatus* and *L. jensenii* were positively correlated with sugars such as maltose, maltotriose, and maltohexose, lipid metabolism biochemicals such as arachidonate and carnitine, as well as lactate, urea, and reduced glutathione. These two lactobacilli were negatively correlated with N-acetylneuraminate, succinate, the carnitine precursor deoxycarnitine, the eicosanoid 12-HETE, the fatty acid 13-HODE, and the nucleobase uracil. BV-associated bacteria typically exhibited correlation patterns with metabolites that were opposite to that exhibited by *L. crispatus* and *L. jensenii*. Among BV-associated bacteria, BVAB1 displayed specific differences in metabolite correlation patterns when compared with BV-associated bacteria from Group-1. BVAB1 was negatively correlated with N-acetylaspartate, 12-HETE, fatty acids such as eicosenoate and dihomolinate; these metabolites had positive associations with Group-1 BV-associated bacteria. BVAB3 and *P. amnii*, members of Group-2 BV-associated bacteria, exhibited metabolite correlation patterns that were similar to each other.

EXAMPLE 5

ALTERATIONS IN KEY VAGINAL BACTERIA AND METABOLITE CONCENTRATION WITH CHANGES IN BV STATUS IN COHORT 1

[0067] Limited longitudinal data are presented for 8 women who returned for follow-up visits 4 weeks after baseline sample collection per study protocol (Figure 5, Figure 7). Participants diagnosed with BV were treated with metronidazole. Study participants 19, 21, 23 and 25 were cured of BV at the follow-up visit (Figure 5). All four participants had high concentrations of lactobacilli at their follow-up visits and increased concentrations of metabolites negatively associated with BV. Women with high concentrations of *L. crispatus* at follow-up had

high concentrations of metabolites negatively associated with BV. Women with high concentrations of *L. iners* also showed increased concentrations of metabolites negatively associated with BV, but these shifts were not as dramatic as increases seen in women with *L. crispatus* dominant communities (Figure 5). Study participants 4 and 7 did not have BV at either time point and had high concentrations of lactobacilli at both time points (Figure 7). High concentrations of metabolites negatively associated with BV were also noted at baseline and follow-up. Study participants 12 and 28 had recurrent BV after antibiotic treatment. In both participants, concentrations of metabolites associated with BV declined at the follow-up visit, but there was no associated increase in metabolites negatively associated with BV (Figure 7). In this longitudinal analysis, changes in the microbiota were associated with shifts in metabolites in the same subject, whereas stability of the microbiota was associated with fewer changes even after patients received antibiotic therapy.

EXAMPLE 6

VALIDATION STUDY (COHORT B)

[0068] Study population and sample collection: Cohort B in the independent validation study also comprised 40 women with BV and 20 women without BV enrolled in the STD clinic between April 2011 and August 2013. BV definitions and sample collection procedures were the same as Cohort A. An additional 10 women with intermediate (4-6) Nugent scores were included to determine if their metabolite profiles differed from those who had BV (Nugent 7-10) or those who had scores of Nugent 0-3. Of these 10 women, 6 were BV negative and 4 were BV positive by Amsel clinical criteria. All women provided written informed consent, and the study was approved by Institutional Review Board at the Fred Hutchinson Cancer Research Center.

[0069] DNA Extraction and molecular methods: DNA was extracted using the Bacteremia Kit (Mobio, Carlsbad, CA) and eluted in 150 μ L buffer and diluted 1:1 in 1 mM Tris, 0.1 mM EDTA (TE) buffer. The V3-V4 region of the 16S-rRNA gene was targeted for broad-range 16S-rRNA gene PCR with pyrosequencing using 454 Life Sciences Titanium technology (Roche, Branford, CT). Reads were processed using the same methods as Cohort A, and reads were deposited in the NCBI Short Read Archive (SRP056030).

[0070] Measurement of levels of biochemicals: Sample preparation: Commercially available pooled serum (Innovative Research, Novi, MI) was used as quality control (QC) sample and aqueous metabolites were extracted using methanol (250 μ L). The supernatant (200 μ L) obtained post-centrifugation (20,800 $\times$ g for 10 min) was dried for 90 min using a Vacufuge Plus evaporator (Eppendorf, Hauppauge, NY) and reconstituted in 600 μ L 40% Solvent A (40 mM ammonium acetate in H₂O + 0.3% acetic acid) and 60% Solvent B (acetonitrile + 0.3% acetic acid) containing 5.13 μ M L-tyrosine-¹³C₂ and 22.54 μ M sodium-L-lactate-¹³C₃ (Cambridge Isotope Laboratory). The isotope-labeled internal standards helped monitor LC-MS assay performance. Samples were filtered through 0.45 μ m PVDF filters (Phenomenex, Torrance, CA) prior to LC-MS analysis. Acetonitrile, ammonium acetate, methanol, and acetic acid (LC-MS grade) were obtained from Fisher Scientific (Pittsburgh, PA) and stable isotope-labeled tyrosine and lactate internal standards (>99% pure) from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). The QC sample was injected every 10 vaginal samples. Vaginal swab samples and CVL (100 μ L) were processed using the same procedure as the QC sample, but extracted using 850 μ L of methanol. Sham samples (saline solution and swabs without human contact) were included to assess background signals (chemical impurities present in materials used to collect vaginal fluid). Blank saline solution and swab samples were injected once each for every 10 vaginal fluid samples.

[0071] Chromatography Conditions: The LC system was composed of two Agilent 1260 binary pumps, an Agilent 1260 auto-sampler and Agilent 1290 column compartment containing a column-switching valve (Agilent Technologies, Santa Clara, CA). Each sample was injected twice, 10 μ L for analysis using negative ionization mode and 2 μ L for analysis using positive ionization mode. Both chromatographic separations were performed in HILIC mode on two parallel SeQuant ZIC-cHILIC columns (150 $\times$ 2.1 mm, 3.0 μ m particle size, Merck KGaA, Darmstadt, Germany). While one column was performing the separation, the other column was reconditioning in preparation for the next injection. The flow rate was 0.300 mL/min, auto-sampler temperature was kept at 4°C, the column compartment was set at 40°C, and total separation time for each ionization mode was 18 min. The mobile phase was composed of Solvents A and B; the gradient conditions for both separations were identical and are as follows:

0-2 min, 25% Solvent A, 75% Solvent B; 2-5 min, 25 to 70% Solvent A, linear gradient; 5-9 min, 70% Solvent A; 9-11 min 70 to 25% Solvent A, linear gradient; 11-18 min, 25% Solvent A.

[0072] Mass Spectrometry (MS) and data processing: After the chromatographic separation, MS ionization and data acquisition were performed using an AB Sciex QTrap 5500 mass spectrometer (AB Sciex, Toronto, ON, Canada) equipped with electrospray ionization (ESI) source. The instrument was controlled by Analyst 1.5 software (AB Sciex, Toronto, ON, Canada). Targeted data acquisition was performed in multiple-reaction-monitoring (MRM) mode. Additional assays for 19 metabolites of interest based on results from Cohort A were developed wherein biochemicals using the Metabolon platform (Table 6) were measured. 104 and 76 MRM transitions in negative and positive mode, respectively, were monitored (180 transitions total) (Table 6). The source and collision gas was N₂ (99.999% purity). The ion source conditions in negative mode were: Curtain Gas (CUR) = 25 psi, Collision Gas (CAD) = high, Ion Spray Voltage (IS) = - 3.8KV, Temperature (TEM) = 500 °C, Ion Source Gas 1 (GS1) = 50 psi and Ion Source Gas 2 (GS2) = 40 psi. The ion source conditions in positive mode were: Curtain Gas (CUR) = 25 psi, Collision Gas (CAD) = high, Ion Spray Voltage (IS) = 3.8KV, Temperature (TEM) = 500 °C, Ion Source Gas 1 (GS1) = 50 psi and Ion Source Gas 2 (GS2) = 40 psi. The extracted MRM peaks were integrated using MultiQuant 2.1 software (AB Sciex, Toronto, ON, Canada). Standard compounds corresponding to measured metabolites were purchased from Sigma-Aldrich (Saint Louis, MO, USA) and Fisher Scientific (95-99% pure).

[0073] Data analysis: Metabolite data and pyrosequencing reads were processed and analyzed as described for Cohort A.

Table 6. **Cohort B - University of Washington NW-MRC Platform** Cells highlighted in the darker grey indicate metabolites that are higher in BV and statistically significant; lighter grey indicates lower in BV and statistically significant; bold indicates metabolites that are trending to significance.

Biochemical Name	Platform	Comp ID	KEGG	HMDB	Mean Values		Welch's Two Sample t-Test			Pos/Neg	Super Pathway	Measured using Metabolon Platform
					Neg	Pos	p-value	q-value	Fold Change			
12-HETE	LC/MS neg	5312983	C14777	HMDB06111	0.549761	2.153909	0.0058	0.0225	3.918		Lipid	Yes
13-HODE	LC/MS neg	5282948	C14767	HMDB61708	0.296921	4.872555	0.0004	0.0023	16.419		Lipid	Yes
1-Methyladenosine	LC/MS pos	27476	C02494	HMDB03331	13.89231	1.019602	0.2533	0.3109	0.073		Nucleotide	No
2-Amino adipate	LC/MS neg	469	C00956	HMDB00510	1.083608	1.236584	0.5614	0.6032	1.141		Amino Acid	Yes
2-Hydroxyglutarate	LC/MS neg	43	C02630	HMDB59655	3.932806	1.095001	0.0389	0.0655	0.278		Lipid	Yes
4-Hydroxybutyrate	LC/MS pos	10413	C00989	HMDB00710	0.949666	1.009497	0.1147	0.1655	1.063		Lipid	Yes
5-Aminovaleric Acid	LC/MS pos	138	C00431	HMDB03355	0.099272	3.554135	<0.001	<0.001	35.803		Amino Acid	Yes
Adenine	LC/MS neg	190	C00147		1.42114	0.997052	0.3296	0.3871	0.702		Nucleotide	Yes
Adenosine	LC/MS pos	60961	C00212	HMDB00050	2.789559	0.829478	0.0190	0.0392	0.297		Nucleotide	Yes
Adenylosuccinate	LC/MS neg	195	C03794	HMDB00536	2.850905	1.945559	0.4879	0.5415	0.682		Nucleotide	No
Adipic Acid	LC/MS neg	196	C06104	HMDB00448	0.969576	1.041395	0.3615	0.4197	1.074		Caprolactam Degradation	No
Alanine	LC/MS pos	5950	C00041	HMDB00161	1.837288	0.955452	0.1784	0.2435	0.520		Amino Acid	Yes
Allantoin	LC/MS neg	204	C01551	HMDB00462	1.512457	0.908111	0.1484	0.2082	0.600		Nucleotide	No
Alpha-Ketoglutaric Acid	LC/MS neg	51	C00026	HMDB00208	0.973055	1.024005	0.5515	0.5989	1.052		Energy	No
AMP	LC/MS neg	15938965	C00020	HMDB00045	2.399105	0.886976	0.0053	0.0225	0.370		Nucleotide	Yes
Anthranilate	LC/MS neg	227	C00108	HMDB01123	2.854806	0.943821	0.0137	0.0337	0.331		Amino Acid	No
Arachidonate	LC/MS neg	444899	C00219	HMDB60102	1.034647	0.975074	0.0160	0.0377	0.942		Lipid	Yes
Arginine	LC/MS pos	232	C00062	HMDB00517	5.047576	0.863601	0.0065	0.0233	0.171		Amino Acid	Yes
Ascorbic Acid (Vit. C)	LC/MS neg	54670067	C00072	HMDB00044	11933.54	9069.362	0.7750	0.7987	0.760		Cofactors and Vitamins	Yes
Asparagine	LC/MS pos	6267	C00152	HMDB00168	19.02775	1.777006	0.0257	0.0509	0.093		Amino Acid	No
Aspartic Acid	LC/MS pos	5960	C00049	HMDB00191	4.385856	0.750243	0.0165	0.0379	0.171		Amino Acid	Yes

Biotin	LC/MS neg	171548	C00120	HMDB000030	4.346047	0.965599	0.0681	0.1090	0.222	Cofactors and Vitamins	No
Cadaverine	LC/MS pos	273	C01672	HMDB02322	0.050142	3.857881	<0.001	<0.001	0.340	Amino Acid	Yes
Carnitine	LC/MS pos	2724480	C00487	HMDB00062	3.279169	0.764733	0.0274	0.0521	0.233	Lipid	Yes
Choline	LC/MS pos	305	C00114	HMDB00097	2.077764	1.520472	0.5026	0.5517	0.732	Cofactors and Vitamins	No
Citraconic Acid	LC/MS neg	643798	C02226	HMDB00634	1.018945	1.092198	0.3098	0.3681	1.072	Amino Acid	No
Citrulline	LC/MS neg	833	C00327	HMDB00904	1.667773	0.977009	0.2058	0.2598	0.586	Amino Acid	Yes
Creatine	LC/MS pos	586	C00300	HMDB00064	2.444336	0.842549	0.0104	0.0309	0.345	Amino Acid	Yes
Creatinine	LC/MS pos	588	C00791	HMDB00562	2.874441	0.953764	0.0347	0.0603	0.332	Amino Acid	Yes
Cystamine	LC/MS pos	2915	N/A	N/A	2.252528	0.949694	0.0690	0.1090	0.422	Amino Acid	No
Cysteine	LC/MS pos	5862	C00097	HMDB00574	12.56652	1.389119	0.0279	0.0522	0.111	Amino Acid	Yes
Cystine	LC/MS pos	67678	C00491	HMDB00192	11.15403	0.579027	0.0145	0.0349	0.052	Amino Acid	No
Cytosine	LC/MS pos	597	C00380	HMDB00630	1.312866	1.543991	0.7135	0.7586	1.176	Nucleotide	No
Dimethylglycine	LC/MS pos	673	C01026	HMDB00092	0.918006	2.008823	0.0294	0.0529	2.188	Cofactors and Vitamins	No
D-Leucic Acid	LC/MS neg	439960	C03284	HMDB00624	0.748823	4.282549	0.0131	0.0336	5.734	Amino Acid	No
Epinephrine	LC/MS pos	5816	C00788	HMDB00068	1.038287	0.952361	0.0498	0.0824	0.917	Amino Acid	No
Erythrose	LC/MS neg	439574	C01796	HMDB02649	1.208405	1.314378	0.4795	0.5381	1.088	Carbohydrate	No
F16BP/F26BP/G16BP	LC/MS neg	172313/ 105021/ 82400	C00354/C00 665/C01231	HMDB01058/ HMDB01047/ HMDB03514	2.395754	0.864547	0.0009	0.0046	0.361	Carbohydrate	Yes
Fumaric Acid/Maleic Acid	LC/MS neg	444972/ 444266	C00122/ C01384	HMDB00134/ HMDB00176	1.030087	1.09067	0.4138	0.4696	1.059	Energy	Yes
G1P/G6P/F6P/F1P	LC/MS neg	439165/ 5958/ 69507/ 10400369	C00103/ C00092/ C00085/ C01094	HMDB01586/ HMDB01401/ HMBD00124/ HMDB01076	1.014726	1.302086	0.2004	0.2595	1.283	Carbohydrate	Yes
Glucoronate	LC/MS neg	444791	C00191	HMDB00127	3.565885	0.862001	0.0039	0.0177	0.242	Carbohydrate	No
Glucose	LC/MS neg	79025	C00031	HMDB00122	3.2199	0.582469	<0.001	<0.001	0.181	Carbohydrate	Yes
Glutamic acid	LC/MS pos	611	C00025	HMDB03339	3.943463	0.950986	0.0081	0.0264	0.241	Amino Acid	Yes
Glutamine	LC/MS pos	5961	C00303	HMDB00641	2.096949	0.920155	0.0851	0.1283	0.439	Amino Acid	Yes
Glutaric Acid	LC/MS neg	743	C00489	HMDB00661	0.483471	1.974837	<0.001	0.0001	4.085	Amino Acid	No
Glyceraldehyde	LC/MS neg	751	C02426	HMDB01051	0.597584	1.553495	<0.001	0.0001	2.680	Carbohydrate	No

Glycerate	LC/MS neg	752	C00258	HMDB00139	1.841234	1.003125	0.0750	0.1148	0.545	Carbohydrate	Yes
Glycerol-3-P	LC/MS neg	754	C00093	HMDB00126	0.420476	1.980146	<0.001	<0.001	4.709	Lipid	Yes
Glycine	LC/MS pos	750	C00037	HMDB00123	3.851766	0.854576	0.0289	0.0529	0.222	Amino Acid	Yes
Guanidinoacetate	LC/MS neg	763	C00581	HMDB00128	2.062227	1.084617	0.1909	0.2537	0.526	Amino Acid	No
Guanosine	LC/MS pos	6802	C00387	HMDB00133	5.489016	0.849731	0.0389	0.0655	0.155	Nucleotide	Yes
Histamine	LC/MS pos	774	C00388	HMDB00870	0.360377	5.06166	<0.001	<0.001	4.945	Amino Acid	Yes
Histidine	LC/MS pos	6274	C00768	HMDB00177	3.183509	0.991506	0.0127	0.0336	0.311	Amino Acid	Yes
Homovanillate	LC/MS neg	1738	C05582	HMDB00118	1.061823	1.063368	0.9792	0.9863	1.001	Amino Acid	No
Hypoxanthine	LC/MS neg	790	C00262	HMDB00157	2.5559	0.792758	0.0133	0.0336	0.310	Nucleotide	Yes
iso-Leucine	LC/MS pos	791	C00407	HMDB00172	2.822668	0.886257	0.0112	0.0319	0.314	Amino Acid	Yes
Kynurenate	LC/MS neg	3845	C01717	HMDB00715	0.861798	1.163729	0.1817	0.2446	1.350	Amino Acid	Yes
Lactate	LC/MS neg	612	C001861	HMDB00190	1.529119	0.521579	<0.001	<0.001	0.341	Carbohydrate	Yes
Leucine	LC/MS pos	6106	C00123	HMDB00687	3.152967	0.687161	0.0009	0.0046	0.218	Amino Acid	Yes
Linolenic Acid	LC/MS neg	5280450	C01595	HMDB00673	1.514934	1.302946	0.7589	0.7901	0.860	Lipid	Yes
Lysine	LC/MS pos	5962	C00047	HMDB00182	7.517767	0.753601	0.0071	0.0245	0.100	Amino Acid	Yes
Malonic Acid/3-HBA	LC/MS neg	867/441	C00383/ C01089	HMDB00691/H MDB00357	0.894359	1.181646	0.0116	0.0319	1.321	Energy	No
Methionine	LC/MS pos	6137	C01733	HMDB00696	10.96199	1.212818	0.0188	0.0392	0.111	Amino Acid	Yes
Methylsuccinate	LC/MS neg	10349	C08645	HMDB01844	14.1235	1.039721	0.0060	0.0226	0.074	Amino Acid	No
N-Acetylneuraminic acid	LC/MS neg	445063	C00270	HMDB00230	0.359184	1.490695	<0.001	0.0001	4.150	Carbohydrate	Yes
N-Acetylputrescine	LC/MS pos	122356	C02714	HMDB02064	0.332351	5.445013	0.0008	0.0046	46.383	Amino Acid	Yes
Niacinamide	LC/MS pos	936	C00153	HMDB01406	1.771045	1.271236	0.4096	0.4696	0.718	Cofactors and Vitamins	No
Nicotinate (Niacin)	LC/MS neg	938	C00253	HMDB01488	0.916006	1.026653	0.0656	0.1069	1.121	Cofactors and Vitamins	Yes
Ornithine	LC/MS pos	6262	C01602	HMDB03374	14.05848	0.768175	0.0087	0.0275	0.055	Amino Acid	Yes
Oxalic Acid	LC/MS neg	971	C00209	HMDB02329	3.292335	0.619838	0.0003	0.0018	0.188	Carbohydrate	No
Oxaloacetate	LC/MS neg	970	C00036	HMDB00223	0.87623	1.162188	0.1093	0.1600	1.326	Energy	No
Oxidized glutathione	LC/MS neg	975	C00127	HMDB03337	2.985064	1.499132	0.1624	0.2247	0.502	Amino Acid	Yes
Oxypurinol	LC/MS neg	4644	C07599	HMDB00786	0.255491	1.923879	0.0000	0.0000	7.530	Nucleotide	No
Pantothenate	LC/MS neg	6613	C00864	HMDB00210	2.612067	1.311789	0.2381	0.2969	0.502	Amino Acid	Yes

PEP	LC/MS neg	1005	C00074	HMDB00263	0.94683	2.131465	0.0250	0.0505		Carbohydrate	No
Phenylalanine	LC/MS pos	6140	C02057	HMDB00159	4.385514	0.568173	0.0001	0.0004	2.251	Amino Acid	Yes
Pipecolate	LC/MS pos	6931663	C00408	HMDB00070	7.316458	0.69474	0.0180	0.0392	0.095	Amino Acid	Yes
Proline	LC/MS pos	145742	C00148	HMDB00162	2.313779	1.453898	0.2626	0.3157	0.628	Amino Acid	Yes
Propionate	LC/MS neg	1032	C00163	HMDB00237	0.898881	1.848487	0.0999	0.1485	2.056	Carbohydrate	No
Putrescine	LC/MS pos	1045	C00134	HMDB01414	0.087085	2.737776	<0.001	<0.001	31.438	Amino Acid	Yes
Pyruvate	LC/MS neg	1060	C00022	HMDB00243	1.601785	1.049359	0.1999	0.2595	0.655	Carbohydrate	Yes
Reduced glutathione	LC/MS neg	124886	C00051	HMDB00125	5.968484	1.030784	0.0267	0.0519	0.173	Amino Acid	Yes
Ribose-5-P	LC/MS neg	439167	C00117	HMDB01548	1.034082	1.011506	0.7387	0.7772	0.978	Carbohydrate	No
Serine	LC/MS pos	5951	C00065	HMDB00187	5.933157	0.782622	0.0185	0.0392	0.132	Amino Acid	Yes
Shikimic Acid	LC/MS neg	8742	C00493	HMDB03070	3.145483	1.477166	0.1259	0.1791	0.470	Amino Acid	No
Sorbitol	LC/MS pos	107428	C00794	HMDB00247	9.470836	0.684095	0.0073	0.0245	0.072	Carbohydrate	Yes
Spermidine	LC/MS pos	1102	C00315	HMDB01257	1.065615	1.11261	0.8640	0.8815	1.044	Amino Acid	Yes
Succinate/Methylmalonate	LC/MS neg	1110/ 487	C00042/ C02170	HMDB00254/ HMDB00202	0.493851	0.968	<0.001	0.0001	1.960	Energy	Yes
Taurine	LC/MS pos	1123	C00245	HMDB00251	1.653867	0.9972	0.2032	0.2598	0.603	Amino Acid	Yes
Threonine	LC/MS pos	6288	C00188	HMDB00167	5.380847	0.780656	0.0178	0.0392	0.145	Amino Acid	Yes
Trimethylamine-N-oxide (TMAO)	LC/MS pos	1146	C00565	HMDB00906	3.160464	1.040264	0.0117	0.0319	0.329	Redox	No
Tryptamine	LC/MS pos	1150	C00398	HMDB00303	1.583814	53.24717	0.0028	0.0133	33.623	Amino Acid	Yes
Tryptophan	LC/MS pos	6305	C00078	HMDB00929	7.565738	0.73977	0.0104	0.0309	0.098	Amino Acid	Yes
Tyramine	LC/MS pos	5610	C00483	HMDB00306	0.148074	3.726405	<0.001	0.0001	25.166	Amino Acid	Yes
Tyrosine	LC/MS pos	6057	C00082	HMDB00158	9.126862	0.632987	0.0056	0.0225	0.069	Amino Acid	Yes
Uracil	LC/MS neg	1174	C00106	HMDB00300	1.044215	2.825119	0.0299	0.0529	2.795	Nucleotide	Yes
Urate	LC/MS neg	1175	C00366	HMDB00289	2.842325	0.801884	0.0057	0.0225	0.282	Nucleotide	Yes
Uridine	LC/MS pos	6029	C00299	HMDB00296	3.811939	0.963101	0.0717	0.1114	0.253	Nucleotide	Yes
Valine	LC/MS pos	6287	C00183	HMDB00883	2.192665	1.277254	0.2555	0.3109	0.583	Amino Acid	Yes
Xanthine	LC/MS neg	1188	C00385	HMDB00292	0.987576	0.988861	0.9863	0.9863	1.001	Nucleotide	No
Xanthurenate	LC/MS neg	5699	C02470	HMDB00881	3.145443	0.755646	0.0026	0.0130	0.240	Amino Acid	No

EXAMPLE 6

VALIDATION STUDY (COHORT B)

[0074] An important question is whether metabolites identified by the global metabolic screen in the primary study (Cohort A) are reproducible in other samples using a different platform to measure the metabolites. Hence, in the validation study (Cohort B) a targeted set of metabolites that were identified as significant in the primary study including amino acids, amino acid catabolites, sialic acid, and eicosanoids 12-HETE and 13-HODE (Table 6) were evaluated. The validation cohort included 40 women with BV and 20 women without BV; BV was diagnosed by both Amsel criteria and Nugent score (Table 1). Of the 101 metabolites that were detected by the Northwest Metabolomics Research Center (NW-MRC) platform, 57 metabolites (56.4%) were significantly different between women with and without BV ($q < 0.05$). Of the 57 metabolites, 37 were lower in BV (64.9%) and 20 were higher (35.1%). Most observations made in the primary study were confirmed in the validation study. Examples include lower levels of amino acids in BV, and this was significant for 16/20. Similarly, amino acid catabolites cadaverine ($p < 0.001$), tyramine ($p < 0.001$) and tryptamine ($p = 0.003$) were higher in women with BV. The polyamine putrescine was higher in BV ($p < 0.001$), while precursors arginine ($p = 0.007$) and ornithine ($p = 0.009$) were lower. N-acetylneuraminate (sialic acid) ($p < 0.001$) and succinate ($p < 0.001$) were higher, and lactate was lower ($p < 0.001$). Components of lipid metabolism, including higher levels of 12-HETE ($p = 0.006$) and 13-HODE ($p = 0.0004$), and lower levels of the 12-HETE precursor, arachidonate ($p = 0.016$) were also corroborated. In some cases such as ascorbate ($p = 0.775$) and oxidized glutathione ($p = 0.162$), similar trends were observed in the primary and validation studies (both lower in BV), but fold change was not statistically significant in the validation study. Likewise, glucose was higher in women without BV ($p < 0.001$), but not statistically significant in the primary study. Pipecolate was lower in BV ($p = 0.018$) in the validation study, but found to be higher in BV in the primary study ($p < 0.001$), and was associated with the presence of clue cells.

[0075] As in the primary study, Pearson correlation coefficients were used to link bacteria detected by broad-range PCR and pyrosequencing with the metabolites detected in the validation study (Figure 8). BV-associated bacteria were highly correlated with metabolites

associated with BV such as amino acid catabolites tyramine and histamine, polyamines putrescine and cadaverine, and fatty acids 13-HODE and 12-HETE. In contrast, the lactobacilli were correlated with intact amino acids and glucose among other metabolites. Clustering patterns were similar to that observed in the primary study with the BV associated bacteria clustering as two groups and the lactobacilli clustering together. *L. iners* exhibited correlation patterns that were intermediate between the BV-associated bacteria and the lactobacilli (Figure 8).

EXAMPLE 6

SUMMARY

[0076] Research on the human microbiome has been largely focused on describing the composition of microbial communities under conditions of health and disease. Current knowledge of the functional properties of vaginal microbes is just emerging (30, 33). Small molecule metabolites (the metabolome) reflect the enzymatic pathways and complex metabolic networks that drive microbial transformation of host-derived products. The physiological state of the vagina was examined using approaches that integrate microbial community composition with metabolite profiles present in human vaginal fluid. First, metabolites present in vaginal fluid from women with and without BV were determined. Second, vaginal bacterial species was associated with metabolite concentrations using broad-range 16S rRNA gene PCR (to assess representation) as well as taxon directed qPCR (to measure bacterial concentrations). Third, metabolites were correlated with individual clinical criteria used to diagnose BV. And fourth, a set of select metabolites of interest that were identified in the primary study were validated by measuring them in a second set of samples using a different metabolomics platform.

[0077] There is a notable delineation of metabolite profiles in women with and without BV across multiple pathways (Figure 1, Tables 1, 5, 6). The range and magnitude of these differences is striking. Within women with BV, there are at least two sub-types based on the metabolic profiles reflecting different concentrations rather than presence/absence of particular metabolites (Figure 1). In a recent metabolomics study of eight women with symptomatic BV, clustering analysis resulted in two groups, differentiated by 48 metabolites (30). In the studies described in the Examples, 12/48 metabolites that were reported by Yeoman et al. were detected. Of the 12 metabolites, tryptophan, urea and valine contributed to the

differences in the four clusters; the other 9 were not among the top 30% of most variable metabolites presented in Figure 1. These data support previous studies that have demonstrated that BV is associated with high concentrations of putrescine and cadaverine, and low concentrations of lactate (2, 3, 5, 6, 23-27, 30, 34, 35). However, these data provide additional insight into the global changes in metabolite profiles in BV reflecting numerous pathways, particularly amino acid metabolism.

[0078] Lower levels of amino acids and higher levels of amino acid catabolites suggest increased utilization of amino acids in BV. Alternatively, lower amino acid levels in BV could be explained by decreased production of amino acids, but this would not explain the increase in amino acid decarboxylation products. Concomitant with lower levels of amino acids, there were lower levels of dipeptides as well, supporting increased catabolism (Tables 1 & 5). Specific BV-associated bacteria were correlated with presence of amino acid catabolites, whereas *L. crispatus* and *L. jensenii* were associated with the presence of intact amino acids and dipeptides. These data support the notion that BV-associated bacteria may use amino acids as a carbon and nitrogen source, in contrast to lactobacilli which are known to metabolize sugars such as glycogen. In line with these findings, another study that performed comparative analysis of *Lactobacillus* genomes from the human vagina suggested that *Lactobacillus* genomes were underrepresented in amino acid transport and metabolism, while being overrepresented for carbohydrate transport and metabolism (36). Furthermore, genome analysis of *L. iners* AB-1 showed that it lacks genes necessary for the *de novo* synthesis of amino acids with the possible exception of serine, and that approximately 15% of its genome is dedicated to various transport mechanisms, suggesting that it acquires metabolites such as amino acids crucial for survival from the environment (37). This is consistent with our observations that the metabolite profile of *L. iners* is intermediate to those of *L. crispatus* /*L. jensenii* and the BV-associated bacteria.

[0079] The amino acid arginine typically serves as a precursor for the generation of polyamines putrescine, spermidine and spermine. Lower arginine levels and higher putrescine levels in BV support conversion of arginine to putrescine. However, lower spermine and higher succinate levels suggest the presence of alternate pathways in BV (Figure 6, Tables Si, S2). One possible explanation is that putrescine is converted to succinate via γ -aminobutyrate (GABA), a novel alternate putrescine utilization pathway that was recently discovered in *E. coli* (38, 39),

wherein putrescine is not converted to spermine. This is also supported by observations of higher levels of N-acetylputrescine in the study, which also leads to the formation of GABA (Figure 6). Additional pathways may account for the higher levels of succinate in BV.

[0080] Carnitine, a product of lysine or methionine degradation, was lower in BV while levels of the precursor, deoxycarnitine, were high. One possible explanation for lower levels of carnitine is conversion to TMA by BV-associated bacteria, a process shown to be mediated by bacteria in the gut (40). TMA is produced from L-carnitine found in meat, and absorbed and converted to trimethylamine oxide (TMAO) in the liver. Production of TMAO can be inhibited by suppressing the intestinal microbiota using antibiotics, demonstrating another role for bacteria in TMA-TMAO metabolism. An alternate explanation for low carnitine in BV is that production is inhibited by lack of ascorbate, which is a necessary cofactor for synthesis.

[0081] There were significant alterations in lipid metabolism in BV. Higher levels of 12-HETE and lower levels of its precursor arachidonate in BV observed in both the primary and validation studies (Tables 1, 6) suggests conversion of arachidonate to 12-HETE by BV-associated bacteria. 12-HETE is a signaling eicosanoid that can mediate inflammatory response pathways, is a biomarker for inflammation (41-43) and is a fatty acid that serves membrane structural roles as a component of phospholipids (44). BVAB1 was negatively associated with 12-HETE (-0.028), while other BV-associated bacteria (measured by qPCR), were positively associated with 12-HETE (0.143 to 0.55). Interestingly, 12-HETE has been shown to be correlated with human parturition wherein 12-HETE levels are significantly elevated in laboring women (45). Furthermore, BV is associated with preterm birth (46, 47). Clearly, not all BV-associated bacteria are positively associated with 12-HETE, which suggests that the different sub-types of BV may be correlated with distinct risk factors.

[0082] The vaginal epithelium is in a reduced state in BV (48). Nonetheless, there are suggestions of perturbed redox homeostasis in BV, implying oxidative stress in this reduced environment. Reduced glutathione (GSH) to oxidized glutathione (GSSG) ratios are a good estimate of the redox environment; a decrease in GSH to GSSG ratio is suggestive of oxidative stress (49). The GSH/GSSG ratio in women without BV was found to be +3.29, and in BV was 0.23 in the primary study, and similar ratios were noted in the validation study (Table 6). GSH/GSSG ratios are critical in maintaining a reduced environment in the gut (50). All BV-

associated bacteria (detected by qPCR) and *L. iners* were negatively correlated with presence of GSH. *L. crispatus* and *L. jensenii* were positively correlated with presence of GSH. In addition, GSH was negatively correlated with the presence of clue cells. Another metabolite indicative of oxidative stress is ascorbate (Vitamin C), a major antioxidant whose levels were significantly lower in BV. A third metabolite that may affect the redox status is spermine, whose concentrations are lower in BV. Spermine is present in semen and has antioxidant properties. Spermine is thought to protect spermatozoa, which contain high levels of polyunsaturated fatty acids that can be susceptible to reactive oxygen species (51). It is unclear why BV is associated with multiple metabolic indicators of oxidative stress when the environment is otherwise considered anaerobic and reduced. There is typically a lack of leukocytes in vaginal fluid from women with BV, so leukocytes are unlikely to contribute to the production of reactive oxygen species.

[0083] There were substantial changes in carbohydrate and energy metabolism in BV. One example includes higher levels of N-acetylneuraminate in vaginal fluid from women with BV (Table 1, 6). N-acetylneuraminate is the most ubiquitous sialic acid, and is a component of glycoproteins, glycolipids and oligosaccharides (52). A recent study also observed increased quantities of N-acetylneuraminate in vaginal fluid from women with BV (53). There have been many reports of increased activity of sialidases in BV (54-57); sialidases hydrolyze N-acetylneuraminic acid residues from oligosaccharides, glycoproteins and glycolipids. A point-of-care diagnostic test for BV that is currently available relies on the measurement of sialidase activity in vaginal fluids (58, 59). Importantly, high sialidase activity in BV has been associated with increased risks for preterm birth (55, 56, 60, 61). Glycoproteins such as mucins are present in mucus, and are reported to create a physical barrier between the host epithelium and bacteria (62, 63); this may be the source of the free N-acetylneuraminate present in vaginal fluid in BV. While little is known about the utilization of this metabolite by vaginal bacteria, a recent study showed that *G. vaginalis* has the metabolic machinery required for the transport and subsequent breakdown of N-acetylneuraminate as a carbon and energy source (53). Interestingly, N-acetylneuraminate has been shown to play a role in bacterial biofilms (64). Indeed, biofilms may play a role in BV, and *G. vaginalis* has been shown to be a key member of these biofilms (65-

67). Interfering with production or liberation of N-acetylneuraminate in the vagina may be one approach to prevent BV if this metabolite plays a critical role in biofilm formation.

[0084] The studies confirm that women with BV had high levels of succinate and low levels of lactate. Consistent with this, BV-associated bacterial concentrations were positively correlated with succinate, while the lactobacilli were negatively correlated. Lactate was also negatively correlated with the presence of an amine odor and elevated pH, two criteria used in the clinical diagnosis of BV (Figure 4, Table 3).

[0085] Additional experiments as part of the validation studies were performed to address whether dilution in CVL impacts detection of particular metabolites and found that no metabolite was detected only in swab samples. As expected due to dilution, lower concentrations of metabolites were detected in CVL when compared with swabs (Figure 10).

[0086] Metabolic signatures of BV are distinct and broad, consistent with dramatic changes in vaginal bacterial compositions. There are at least two metabolic sub-types in BV.

[0087] The various methods and techniques described above provide a number of ways to carry out the application. Of course, it is to be understood that not necessarily all objectives or advantages described can be achieved in accordance with any particular embodiment described herein. Thus, for example, those skilled in the art will recognize that the methods can be performed in a manner that achieves or optimizes one advantage or group of advantages as taught herein without necessarily achieving other objectives or advantages as taught or suggested herein. A variety of alternatives are mentioned herein. It is to be understood that some preferred embodiments specifically include one, another, or several features, while others specifically exclude one, another, or several features, while still others mitigate a particular feature by inclusion of one, another, or several advantageous features.

[0088] Furthermore, the skilled artisan will recognize the applicability of various features from different embodiments. Similarly, the various elements, features and steps discussed above, as well as other known equivalents for each such element, feature or step, can be employed in various combinations by one of ordinary skill in this art to perform methods in accordance with the principles described herein. Among the various elements, features, and

steps some will be specifically included and others specifically excluded in diverse embodiments.

[0089] Although the application has been disclosed in the context of certain embodiments and examples, it will be understood by those skilled in the art that the embodiments of the application extend beyond the specifically disclosed embodiments to other alternative embodiments and/or uses and modifications and equivalents thereof.

[0090] In some embodiments, the numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth, used to describe and claim certain embodiments of the application are to be understood as being modified in some instances by the term “about.” Accordingly, in some embodiments, the numerical parameters set forth in the written description and attached claims are approximations that can vary depending upon the desired properties sought to be obtained by a particular embodiment. In some embodiments, the numerical parameters should be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of some embodiments of the application are approximations, the numerical values set forth in the specific examples are reported as precisely as practicable.

[0091] In some embodiments, the terms “a” and “an” and “the” and similar references used in the context of describing a particular embodiment of the application (especially in the context of certain of the following claims) can be construed to cover both the singular and the plural. The recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (for example, “such as”) provided with respect to certain embodiments herein is intended merely to better illuminate the application and does not pose a limitation on the scope of the application otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the application.

[0092] Preferred embodiments of this application are described herein. Variations on those preferred embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. It is contemplated that skilled artisans can employ such variations as appropriate, and the application can be practiced otherwise than specifically described herein. Accordingly, many embodiments of this application include all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the application unless otherwise indicated herein or otherwise clearly contradicted by context.

[0093] All patents, patent applications, publications of patent applications, and other material, such as articles, books, specifications, publications, documents, things, and/or the like, referenced herein are hereby incorporated herein by this reference in their entirety for all purposes, excepting any prosecution file history associated with same, any of same that is inconsistent with or in conflict with the present document, or any of same that may have a limiting affect as to the broadest scope of the claims now or later associated with the present document. By way of example, should there be any inconsistency or conflict between the description, definition, and/or the use of a term associated with any of the incorporated material and that associated with the present document, the description, definition, and/or the use of the term in the present document shall prevail.

[0094] In closing, it is to be understood that the embodiments of the application disclosed herein are illustrative of the principles of the embodiments of the invention. Other modifications that can be employed can be within the scope of the application. Thus, by way of example, but not of limitation, alternative configurations of the embodiments of the application can be utilized in accordance with the teachings herein. Accordingly, embodiments of the present application are not limited to that precisely as shown and described.

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CLAIMS

1. A method of diagnosing bacterial vaginosis (BV) in a subject comprising the steps of
collecting a sample from the subject;
assessing a small molecule metabolomic profile of a metabolomic signature by measuring levels of at least two indicative metabolites;
comparing said metabolomic profile with a diagnostic metabolomic profile of said signature;
wherein a substantial match between said metabolomic profile and said diagnostic metabolomic profile is indicative of bacterial vaginosis.
2. The method of Claim 1 wherein the sample is vaginal fluid or Cervicovaginal lavage fluid (CVL).
3. The method of Claim 1 wherein the indicative metabolites comprise two or more metabolites selected from the group listed in Table 1.
4. The method of Claim 1 wherein the method comprises Chromatographic separation and mass spectroscopy.
5. The method of Claim 1 wherein the indicative metabolites comprise one or more metabolites selected from the group consisting of lipids, amino acids, nucleotides, co-factors, vitamins, carbohydrates, energy molecules and redox molecules.
6. The method of Claim 1 wherein the indicative metabolites comprise one or more amino acids selected from the group consisting of 5-Aminovaleric Acid, Cadaverine, D-Leucic Acid, Glutaric Acid, Histamine, N-Acetylputrescine, Putrescine, Tryptamine, Tyramine, Anthranilate, Arginine, Asparagine, Aspartic Acid, Creatine, Cysteine, Cystine, Glycine, Histidine, iso-Leucine, Leucine, Lysine, Methionine, MethylSuccinate, Ornithine, Phenylalanine, Pipecolate, Reduced glutathione, Serine, Threonine, Tryptophan, Tyrosine, Xanthurenate
7. The method of Claim 1 wherein the indicative metabolites comprise one or more fatty acids selected from the group consisting of 13-HODE, 12-HETE, Glycerol-3-P, Arachidonate, Carnitine.
8. The method of Claim 1 wherein the indicative metabolites comprise one or more nucleotides selected from the group consisting of Oxypurinol, Uracil, Adenosine, AMP, Hypoxanthine, and Urate.

9. The method of Claim 1 wherein the indicative metabolites comprise one or more carbohydrates selected from the group consisting of Glyceraldehyde, N-Acetylneuraminate, PEP, F16BP/F26BP/G16BP, Glucuronate, Glucose, Glutamic acid, Lactate, Oxalic Acid, and Sorbitol.

10. The method of Claim 1 wherein a substantial match is at least 80%.

11. A method of monitoring disease progression of bacterial vaginosis (BV) comprising the steps of

collecting a first sample from the subject;

assessing a small molecule metabolomic profile of a metabolomic signature by measuring levels of at least two indicative metabolites;

comparing said metabolomic profile with a diagnostic metabolomic profile of said signature;

collecting a second sample from the subject;

assessing a small molecule metabolomic profile of a metabolomic signature by measuring levels of at least two indicative metabolites;

comparing said metabolomic profile with a diagnostic metabolomic profile of said signature;

wherein the disease progression is based at least in part upon substantial matches between said metabolomic profiles and said diagnostic metabolomic profiles;

wherein the second sample is collected after a period of time from the first sample.

12. The method of Claim 11 wherein the period of time is at least three days.

13. The method of Claim 11 wherein the sample is vaginal fluid or Cervicovaginal lavage fluid (CVL).

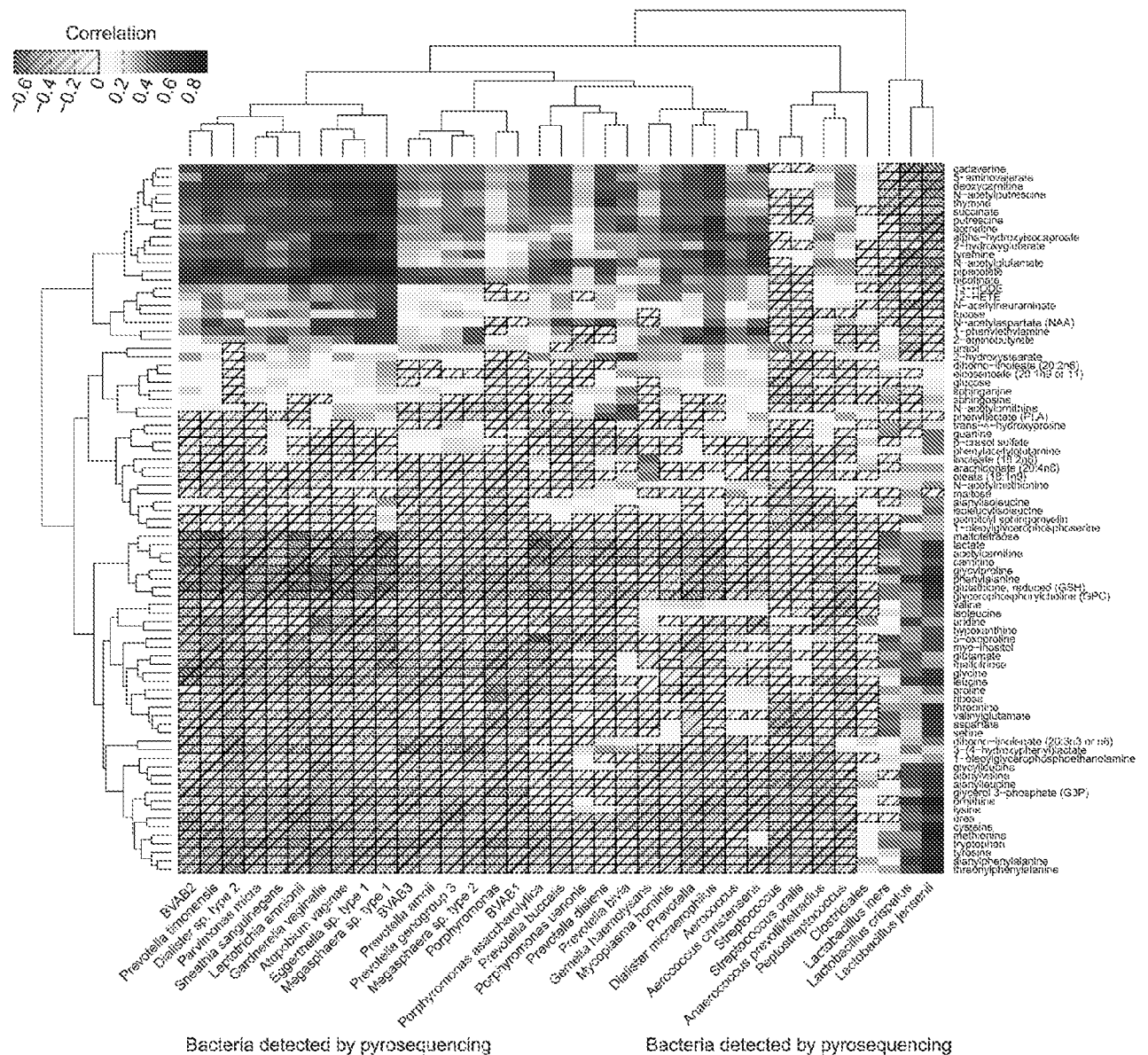
14. The method of Claim 11 wherein the indicative metabolites comprise one or more metabolites selected from the group listed in Table 1.

15. A kit for diagnosing BV in a subject in which the diagnosis is made with a test apparatus, the kit comprising: reagents for collecting a test sample from a subject; and reagents for measuring the profile of a metabolomics profile by measuring levels of indicative metabolites.

16. The kit of Claim 15 wherein the test sample is vaginal fluid or CVL.

17. The kit of Claim 15 wherein the indicative metabolites are selected from those listed in Table 1.

FIG. 1



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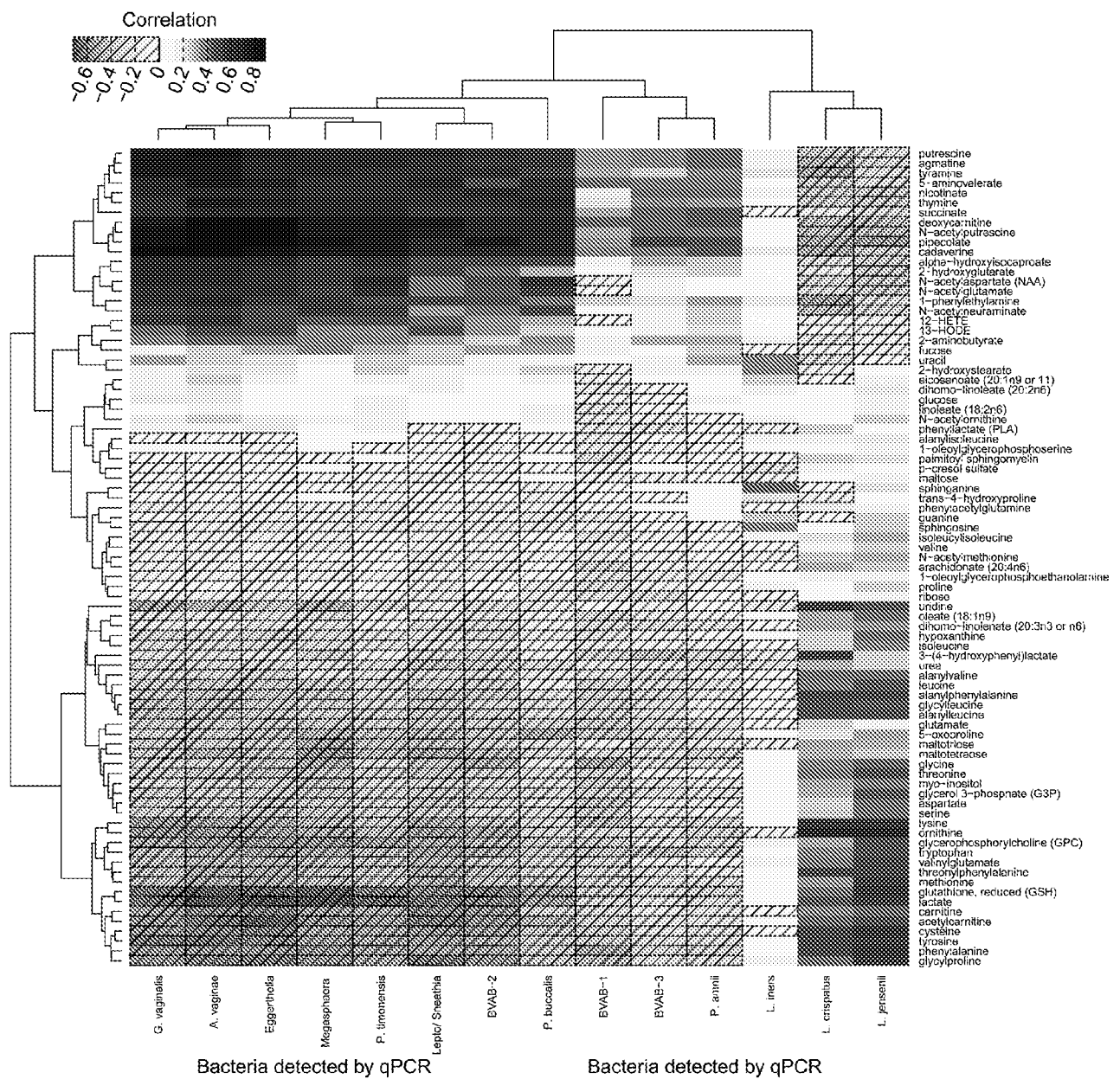
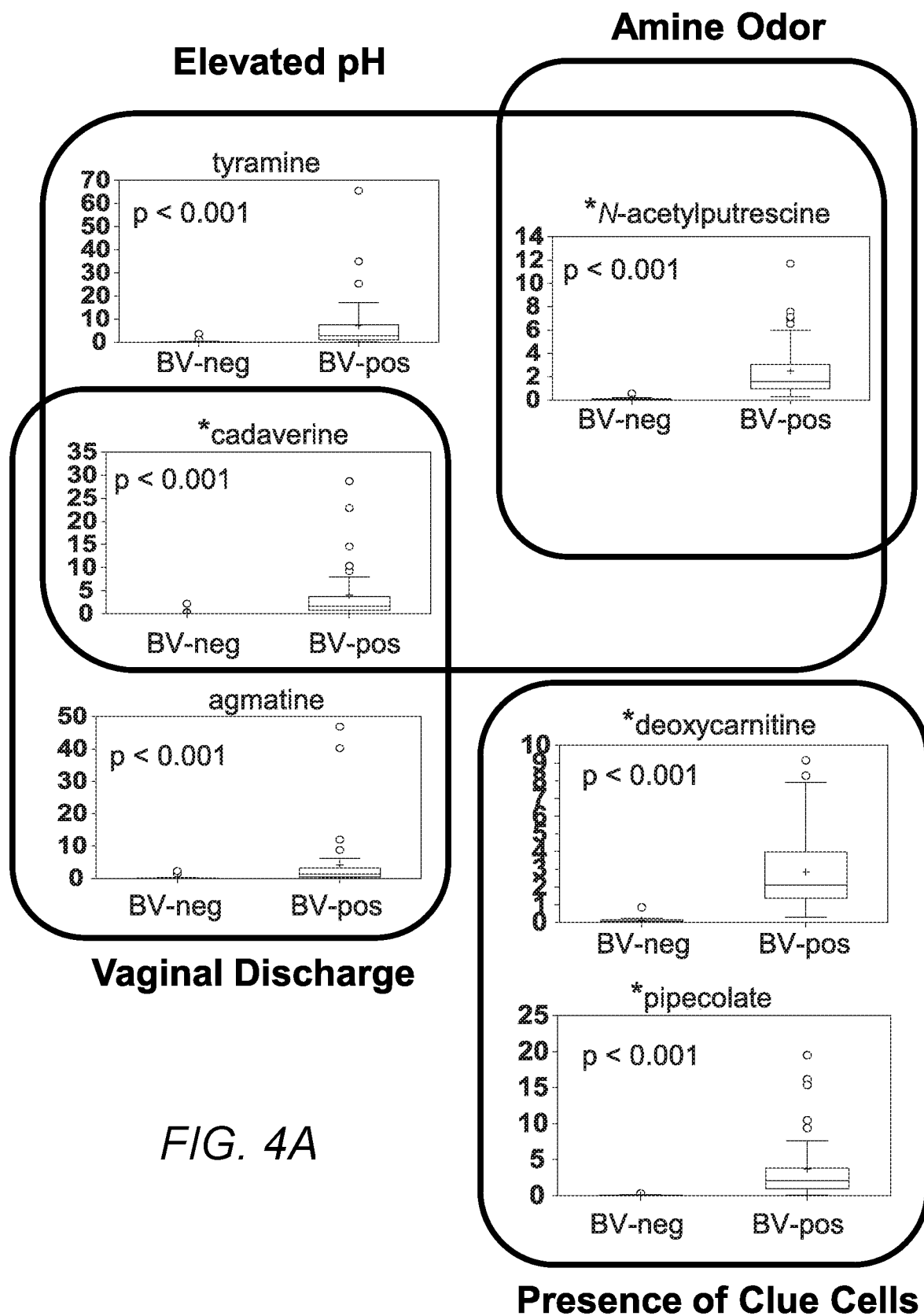
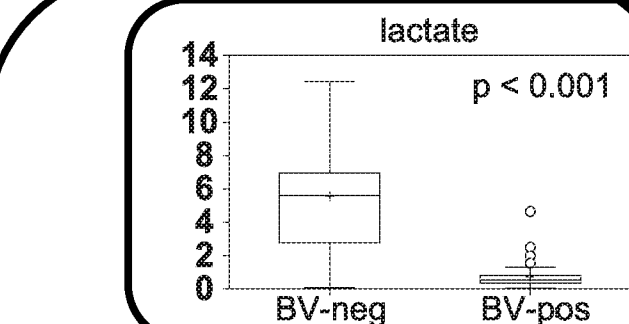
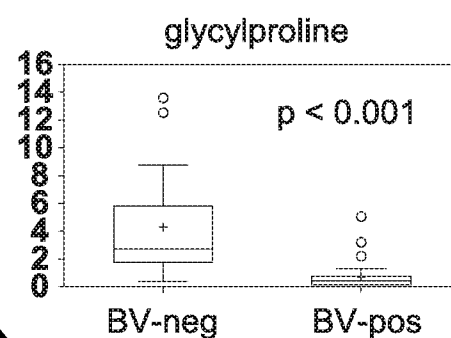
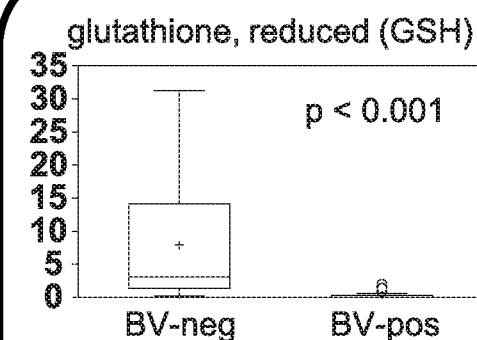
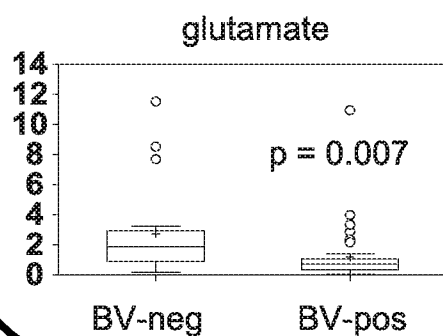
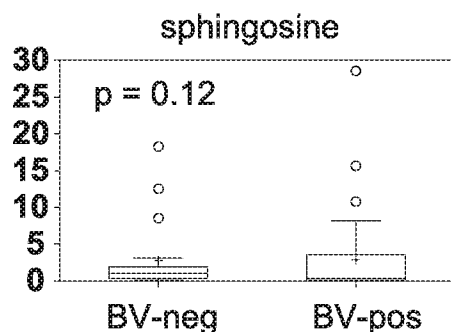
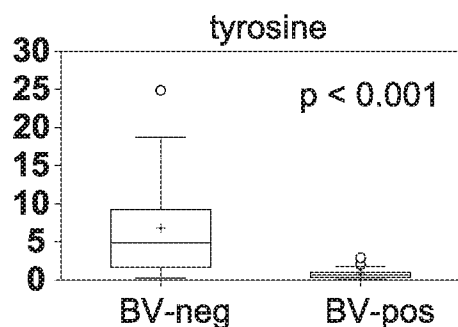
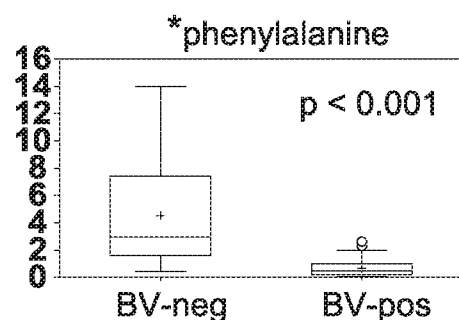


FIG. 3

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Metabolites positively associated with BV clinical criteria

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Metabolites negatively associated with BV clinical criteria**Elevated pH****Amine Odor****Presence of Clue Cells****FIG. 4B**

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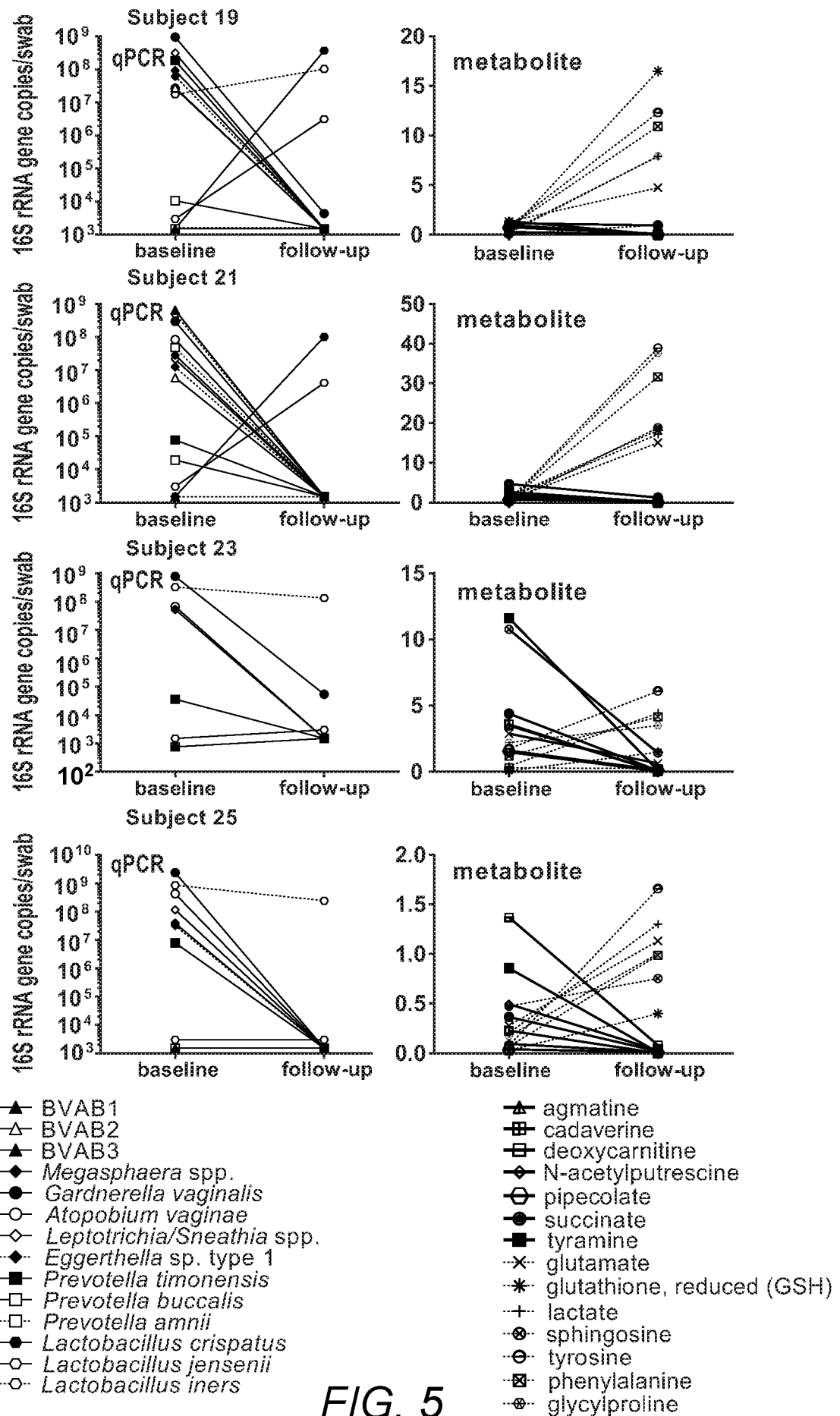


FIG. 5

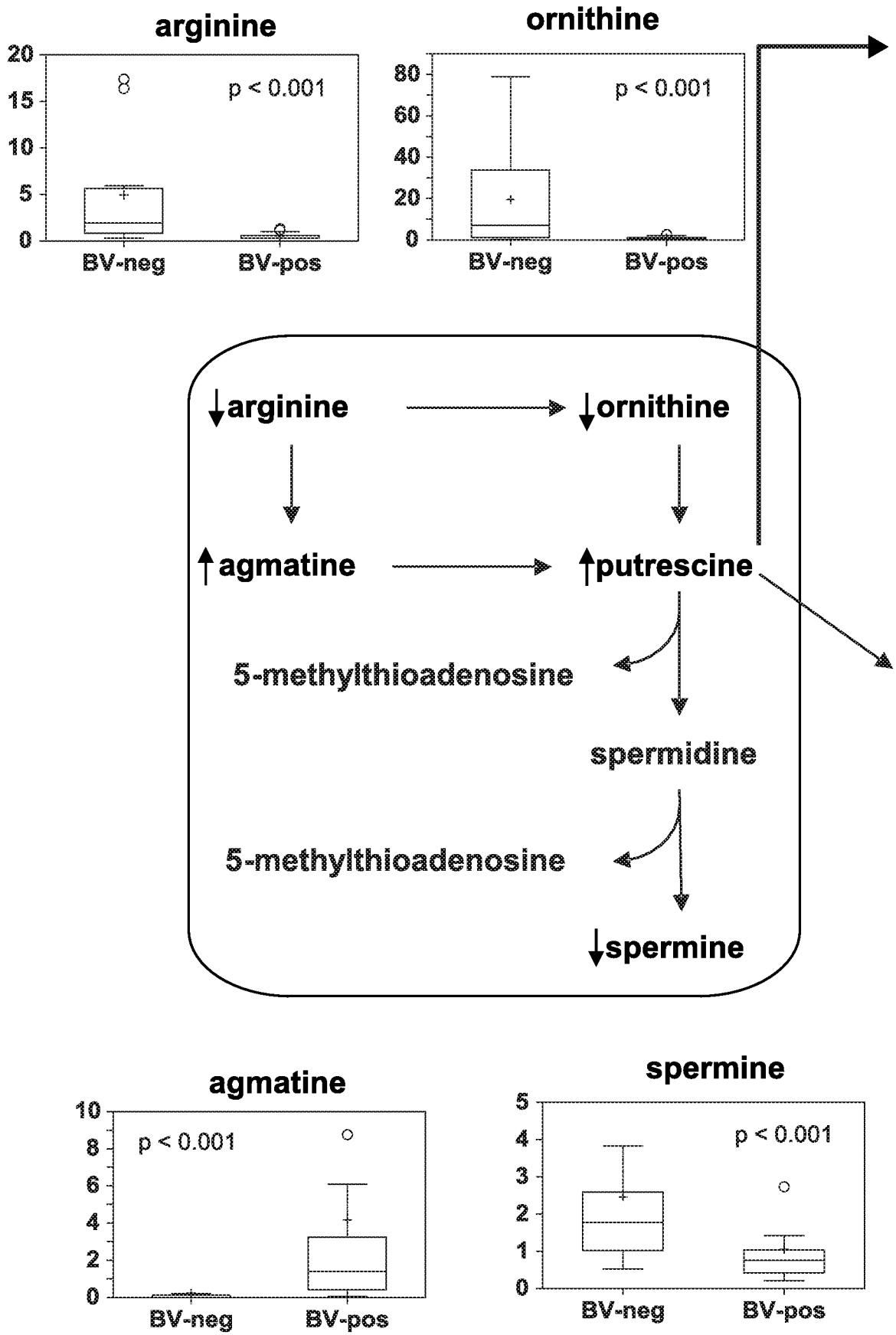


FIG. 6A

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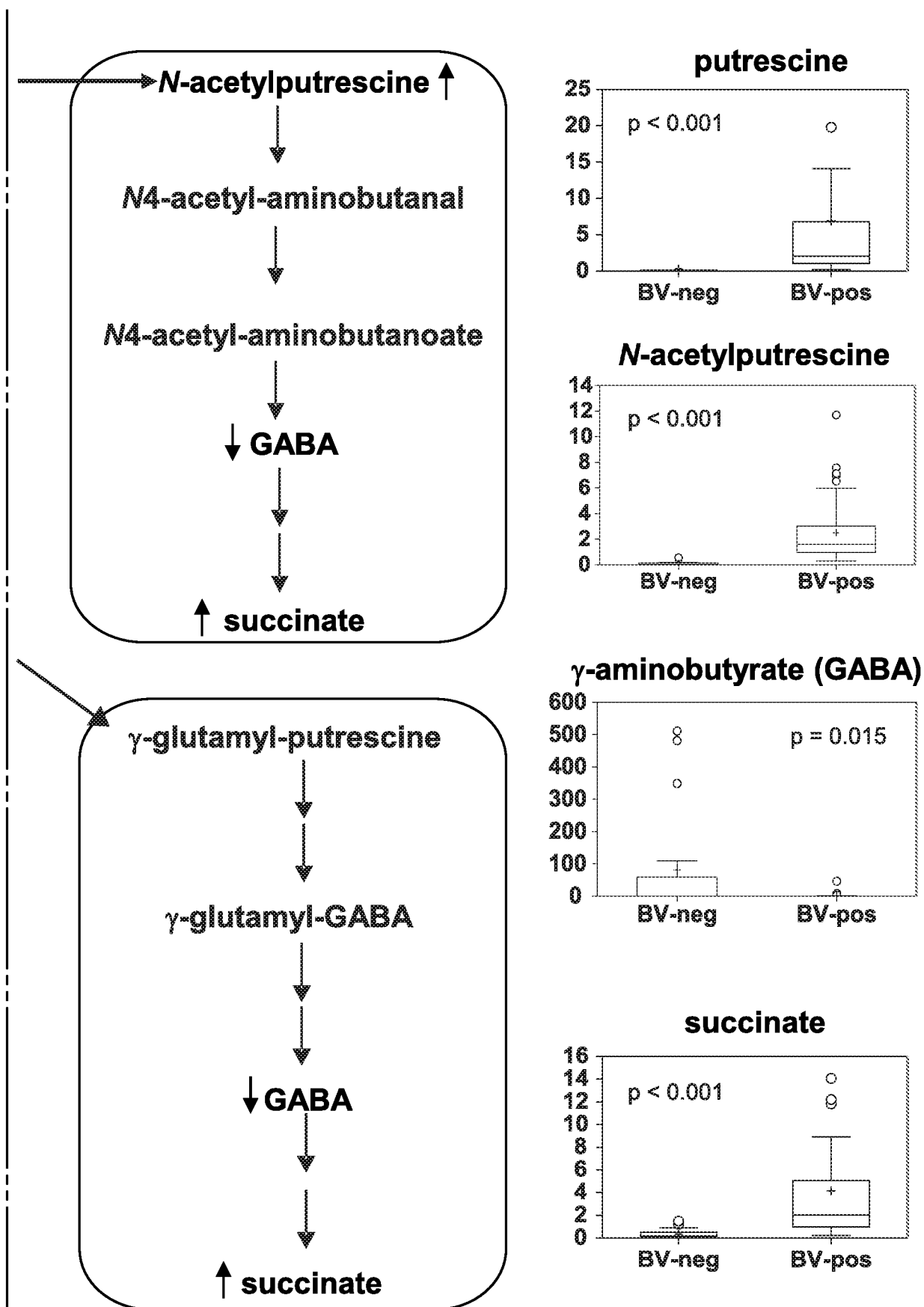


FIG. 6B

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BV negative at baseline and follow-up

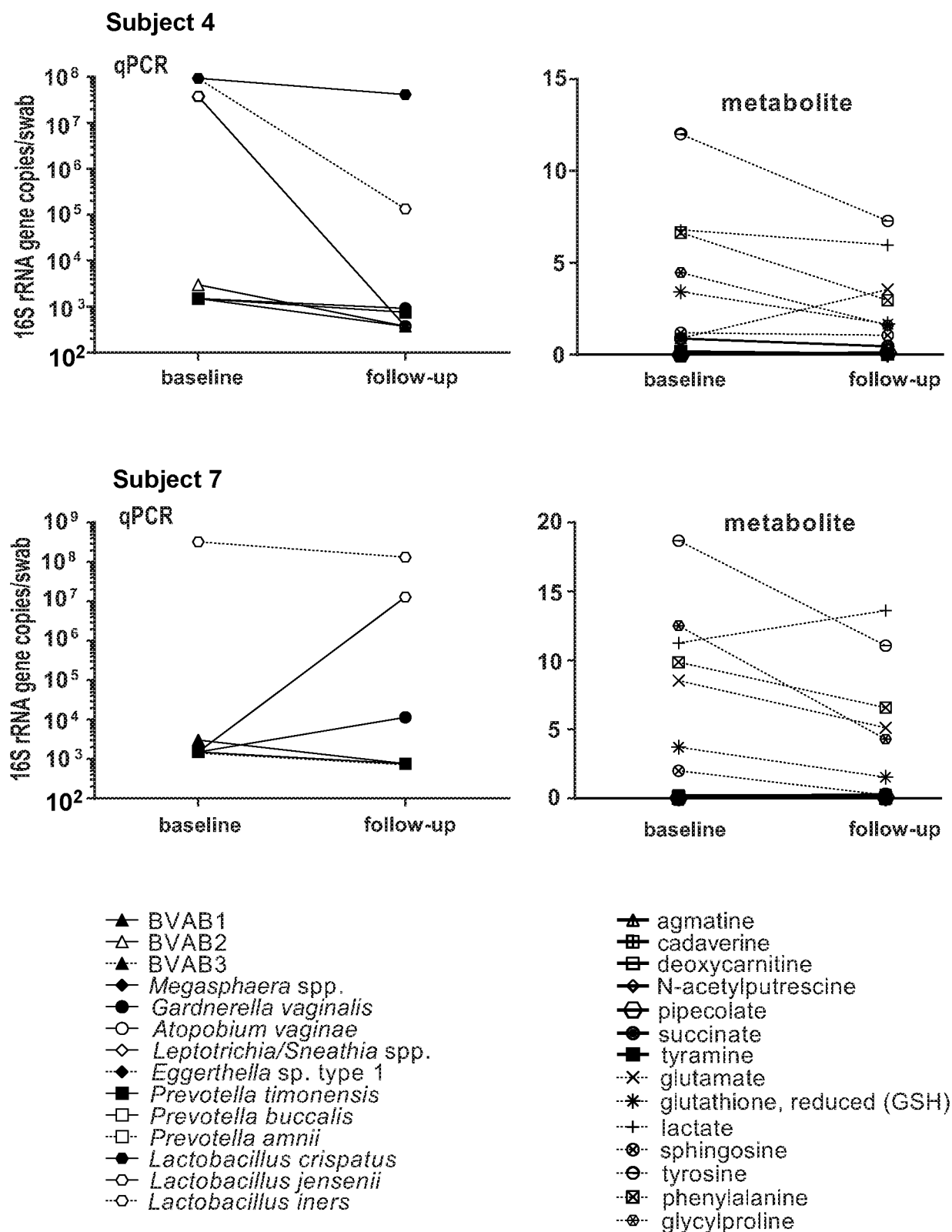


FIG. 7A

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Recurrent BV

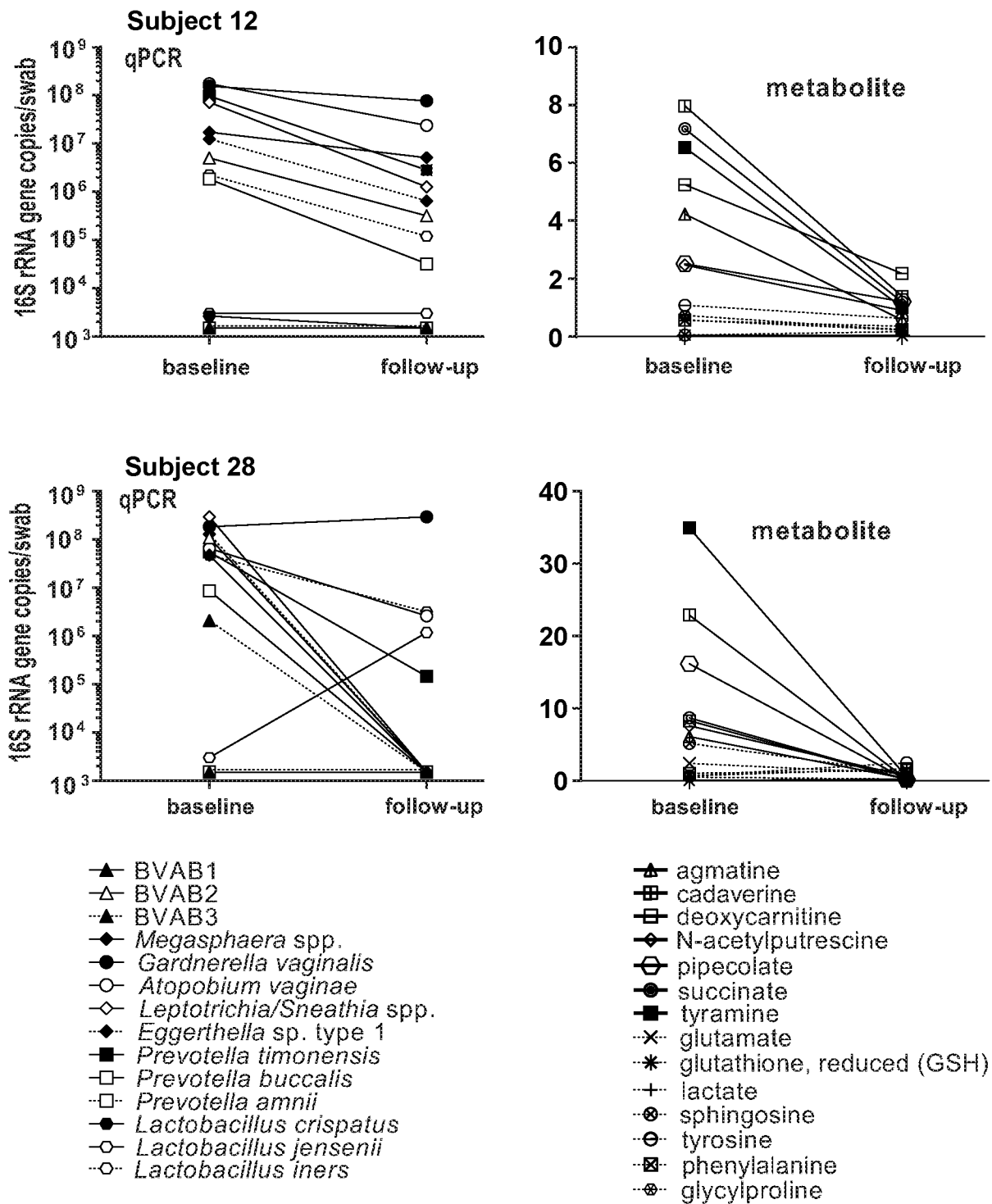


FIG. 7B

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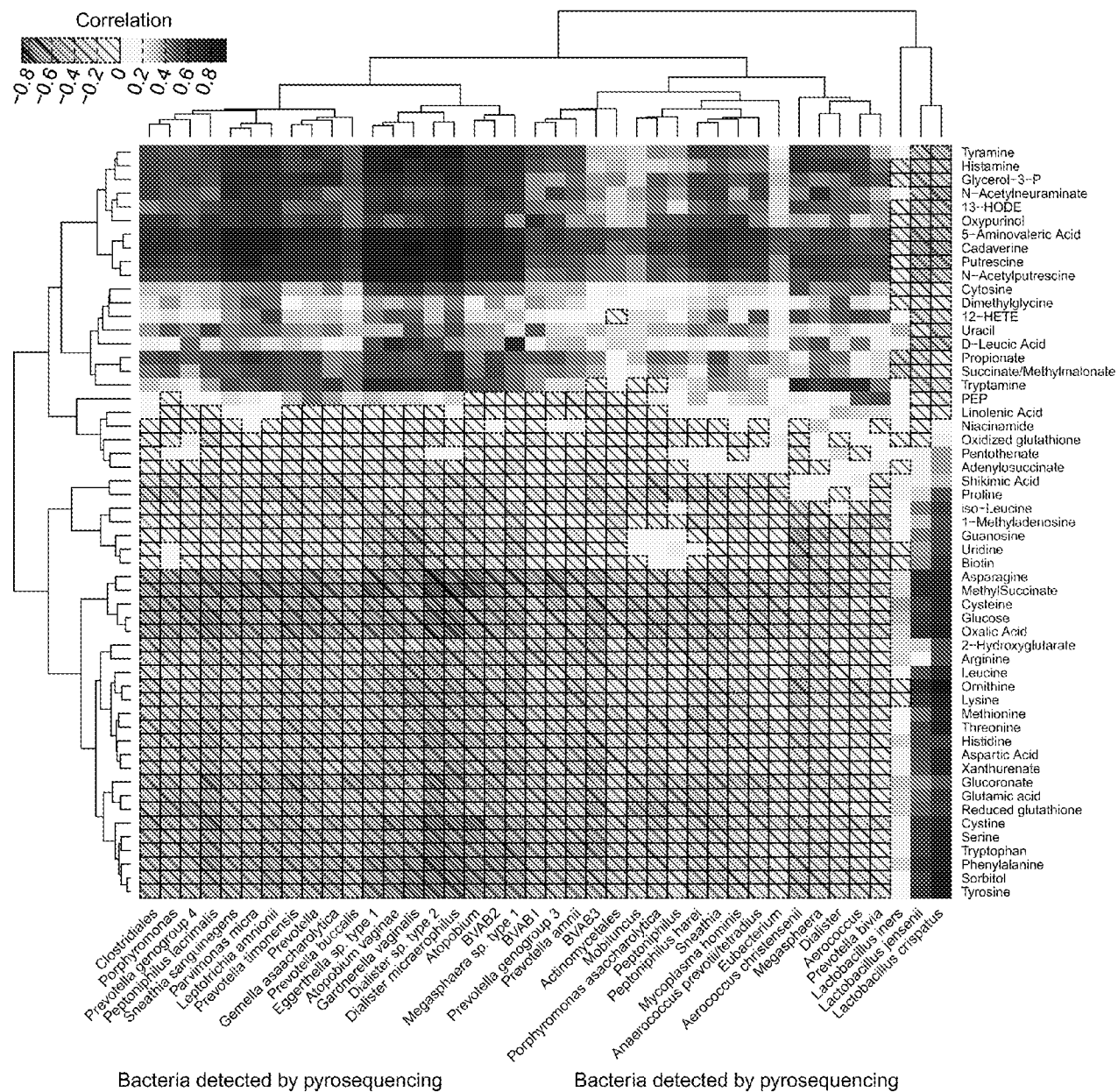
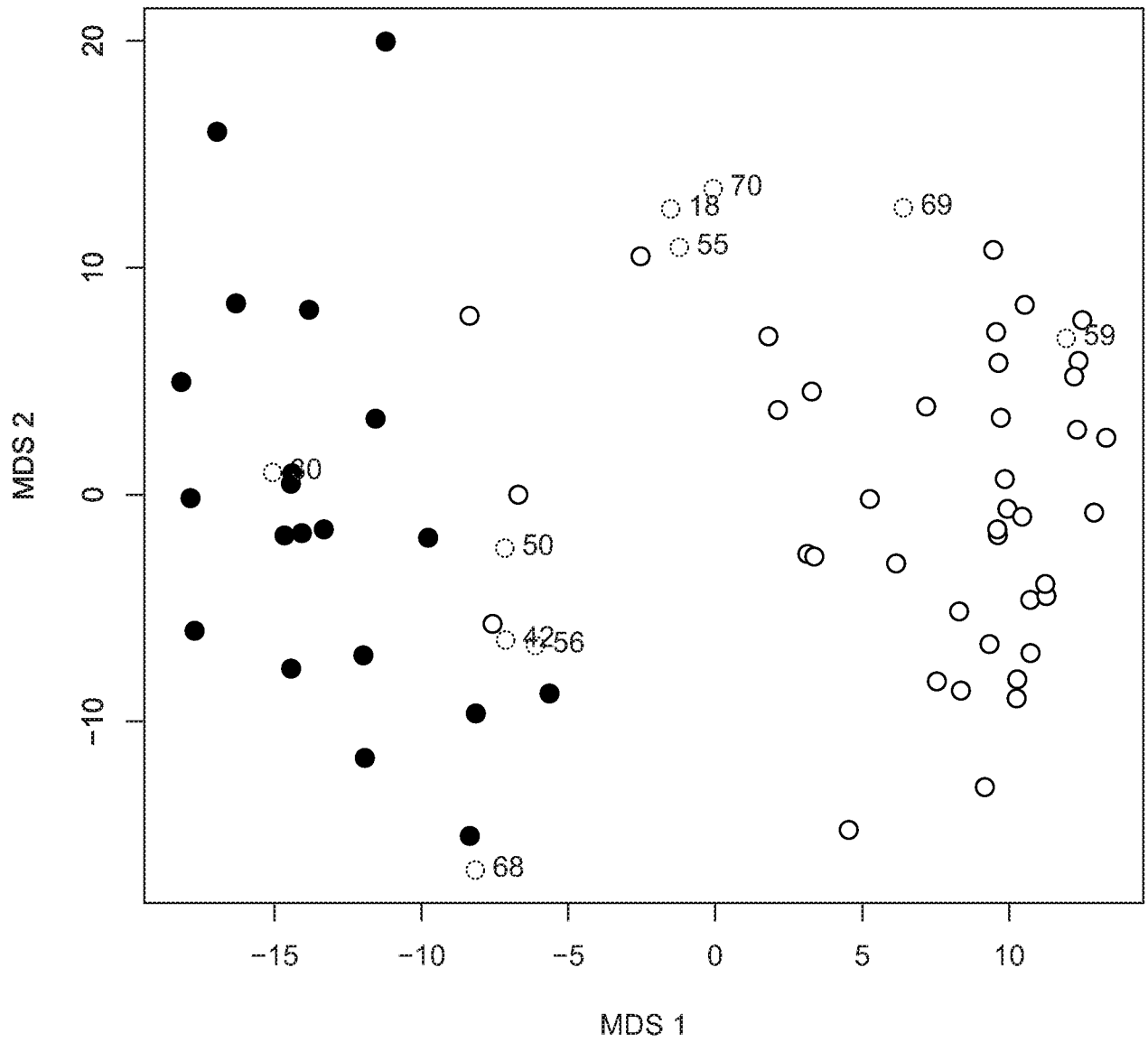
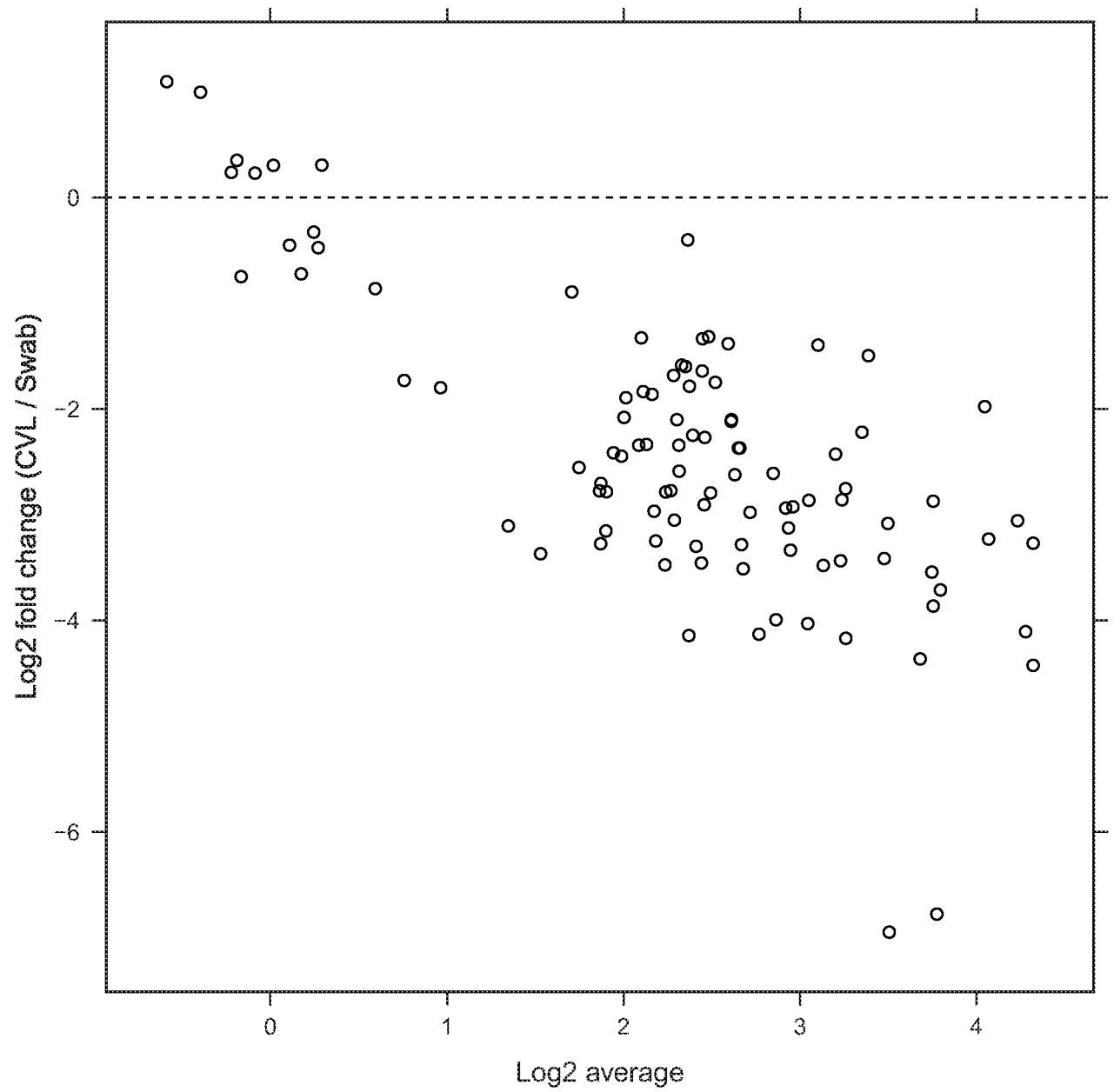


FIG. 8

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*FIG. 9*

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*FIG. 10*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/27266

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/68, C12P 19/34 (2016.01)

CPC - C12Q 1/689

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): C12Q 1/68, C12P 19/34 (2016.01)

CPC: C12Q 1/689

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/6.15; 435/91.2Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, PubWest, Google Scholar, Google Patents: Bacterial vaginosis, metabol*, diagnos*, fluid, vagin*, cervivobaginal lavage, mass spectroscopy, chromatograph*, 5-Aminovaleric Acid, Cadaverine, Dleucic Acid, Glutaric Acid, Histamine, N-Acetylputrescine, Putrescine, Tryptamine, Tyramine, Anthranilate, Arginine, Asparagine, Aspartic Acid, Creatin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---	YEOMAN et al., A Multi-Omic System-Based Approach Reveals Metabolic Markers of Bacterial Vaginosis and Insight into the Disease, PLOS One, February 2013, Vol. 8, No. 2; Author's Manuscript used. pages 1-18; page 3, col 2, para 4; Page 6, col 1, para 2; Page 10, col 2, para 1-4; Table 4; page 12, col 1, para 3; page 13, col 2, para 1; Page 15, col 2, para 3	1-6, 9-10 -----
Y	WOOD et al., Methylophilic Bacteria in Trimethylaminuria and Bacterial Vaginosis, Handbook of Hydrocarbon and Lipid Microbiology, 2010, page 3227-3240; page 3236, para 4	7-8, 11-17
Y	LAGHI et al., Rifaximin Modulates the Vaginal Microbiome and Metabolome in Women Affected by Bacterial Vaginosis, Antimicrobial Agents and Chemotherapy, June 2014, Vol. 58, No. 6, pages 3411-3420; page 3416, col 1, para 2	7
Y	US 2014/0220572 A1 (BANNER HEALTH) 07 August 2014 (07.08.2014); [0064], [0082], [0083]	8
Y		11-17

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

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

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

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

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Date of the actual completion of the international search

07 June 2016 (07.06.2016)

Date of mailing of the international search report

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