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Maternal pre-pregnant obesity is associated with cord blood metabolomics in a multi-ethnic cohort

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Data described in the manuscript, code book, and analytic code will be made available upon request pending.

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Background

Maternal obesity has become a growing global health concern that impacts fetal health and subsequently predisposes the offspring to medical conditions later in life. However, the metabolic link between maternal pre-pregnant obesity and offspring has not yet been fully elucidated.

Objective

This study aims to investigate metabolomics changes in fetal cord blood associated with obese (BMI>30) and normal pre-pregnant weight (18.5<BMI<25) mothers.

Design

In this study, we conducted a case-control study using coupled untargeted and targeted metabolomics approach, from the newborn cord blood metabolomes associated with a matched discovery cohort of 28 cases and 29 controls for maternal pre-pregnant obesity. The subjects are recruited from multi-ethnic populations in Hawaii, including rarely reported Native Hawaiian and other Pacific Islanders (NHPI). The results are subsequently validated in by an indepdent cohort of 12 cases and 18 controls.

Results

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Maternal obesity is the most important factor contributing to differences in cord blood metabolomics. Using elastic net regularization based logistic regression model, we identify 29 metabolites as early-life biomarkers manifesting intrauterine effect of maternal obesity, with accuracy as high as 0.947 even after adjusting for clinical confounding (maternal ethnicity etc). This obese model is validated in a separate cohort (N=30) with accuracy of 0.822. Among the metabolites, six metabolites of them (galactonic acid, butenylcarnitine, 2-hydroxy-3-methylbutyric acid, phosphatidylcholine diacyl C40:3, 1,5-anhydrosorbitol, and phosphatidylcholine acyl-alkyl 40:3) are individually significantly different between the maternal obese vs. norm-weight groups. Interstingly, hydroxy-3-methylbutyric acid shows significnatly higher levels in cord blood from the NHPI group in the dicovery cohort, compared to asian and caucasian groups. This trend is also observed in the validation cohort.

Conclusions

The work here demonstrates the significant associations between maternal pre-pregnant obesity and offspring metabolomics alternation at birth, revealing the inter-generational impact of maternal obesity.

Keywords

Metabolite; metabolomics; obesity; native hawaiian; polynesian; bioinformatics; analysis; mother

Introduction

Obesity is a global health concern. While some countries have a relative paucity of obesity, in the United States, obesity affects 38% of adults (1, 2). As such, maternal obesity has risen to epidemic proportions in recent years and can impose significant risk to both the mother and unborn fetus. Recently, research has

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focused on the association of maternal health during pregnancy and the subsequent effects on the future health of offspring (3). Since the inception of Barker's hypothesis in the 1990's, efforts to connect intrauterine exposures with the development of disease later in life has been the subject of many studies (4). Both obesity and its accompanying morbidities, such as diabetes, cardiovascular diseases and cancers, are of particular interest as considerable evidence has shown that maternal metabolic irregularities may have a role in genotypic programming in offspring (5, 6). Identifying markers of predisposition to health concerns or diseases would present an opportunity for early identification and potential intervention, thus providing life-long benefits (7-9).

Previous studies have found that infants born to obese mothers consistently demonstrate elevation of adiposity and are at more substantial risk for the development of metabolic disease (10). While animal models have been used to demonstrate early molecular programming under the effect of obesity, human research to elucidate the underlying mechanisms in origins of childhood disease is lacking (11). In *Drosophila melanogaster*, offspring of females given a high-sucrose diet exhibited metabolic aberrations both at the larvae and adult developmental stages (12, 13). Though an invertebrate model, mammalian lipid and carbohydrate systems show high level of conservation in *Drosophila melanogaster* (14, 15). In a mouse model of maternal obesity, progeny demonstrated significant elevations of both leptin and triglycerides when compared with offspring of control mothers of normal weight (5). The authors proposed that epigenetic modifications of obesogenic genes during intrauterine fetal growth play a role in adaption to an expected future environment. Recently, Tillery *et al.* used a primate model to examine the origins of metabolic disturbances and altered gene expression in offspring subjected to maternal obesity (16). The offspring consistently displayed significant increases in triglyceride level and also fatty liver disease on histologic preparations. However, human studies that explore the fetal metabolic consequences of maternal obesity are still in need of investigation.

Metabolomics is the study of small molecules using high throughput platforms, such as mass spectroscopy (17). It is a desirable technology that can detect distinct chemical imprints in tissues and body fluids (18).

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The field of metabolomics has shown great promise in various applications including early diagnostic marker identification (19), where a set of metabolomics biomarkers can differentiate samples of two different states (eg. disease and normal states). Cord blood metabolites provide information on fetal nutritional and metabolic health (20), and could provide an early window of detection to potential health issues among newborns. Previously, some studies have reported differential metabolite profiles associated with pregnancy outcomes such as intrauterine growth restriction (21) and low birth weight (22). For example, abnormal lipid metabolism and significant differences in relative amounts of amino acids were found in metabolomic signatures in cord blood from infants with intrauterine growth restriction in comparison to normal weight infants (21). In another study higher phenylalanine and citrulline levels but lower glutamine, choline, alanine, proline and glucose levels were observed in cord blood of infants of low-birth weight (22). However, thus far no metabolomics studies have been reported to specifically investigate the impact of maternal obesity on metabolomics profiles in fetal cord blood (21-24).

This study aims to investigate metabolomics changes in fetal cord blood associated with obese (BMI>30) and normal pre-pregnant weight (18.5<BMI<25) mothers. Uniquely, we recruited mothers from the multi-ethnic population in Hawaii, including Native Hawaiian and other Pacific Islanders (NHPI). NHPI is a particularly under-represented minority population across most scientific studies. To ensure the quality of the study, we enrolled the mothers undergoing elected C-sections without any clinically known gestational diseases. In addition to the cord blood samples of their babies at birth, we collected comprehensive EMR records from the subjects, other maternal and paternal parameters such as ethnicities. To confirm the scientific rigor, we validated the model and observations from another case-control cohort of 30 subjects. This study has discovered the metabolomic links between cord blood and maternal pre pregnant obesity and identified potential early-life metabolite biomarkers associated with maternal obesity.

Methods

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125

126 **Study population**

127 We performed a multi-ethnic case-control study at Kapiolani Medical Center for Women and Children,
128 Honolulu, HI from June 2015 through June 2017. The study was approved by the Western IRB board
129 (WIRB Protocol 20151223). To avoid confounding of inflammation accompanying labor and natural births
130 (25) we recruited women scheduled for full-term cesarean section at ≥ 37 weeks gestation. All subjects
131 fasted for at least 8 hours before the scheduled cesarean delivery. Patients meeting inclusion criteria were
132 identified from pre-admission medical records with pre-pregnancy BMI ≥30.0 (cases) or 18.5-25.0
133 (controls). The pre-pregnancy BMIs were also confirmed during the enrollment. Women with preterm
134 rupture of membranes (PROM), labor, multiple gestations, pre-gestational diabetes, hypertensive disorders,
135 cigarette smokers, HIV, HBV, and chronic drug users were excluded. Clinical characteristics were
136 recorded, including maternal and paternal age, maternal and paternal ethinicities, mother's pre-pregnancy
137 BMI, net weight gain, gestational age, parity, gravidity and ethnicity. For the discovery cohort, a total of
138 57 subjects (28 cases and 29 controls) were recruited. Additionally, to confirm the results, we recruited 30
139 subjects (12 cases and 18 controls) from the same site but different time interval (July 2017 to June 2018).

140

141 **Sample collection, preparation and quality control**

142 Cord blood was collected under sterile conditions at the time of cesarean section using Pall Medical cord
143 blood collection kit with 25 mL citrate phosphate dextrose (CPD) in the operating room. The umbilical cord
144 was cleansed with chlorhexidine swab before collection to ensure sterility. The volume of collected blood
145 was measured and recorded before aliquoting to conicals for centrifugation. Conicals were centrifuged at
146 200g for 10 minutes, with break off, and plasma was collected. The plasma was centrifuged at 350g for 10
147 minutes, with break on, aliquoted into polypropylene cryotubes, and stored at -80C.

148

149 **Metabolome profiling**

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The plasma samples were thawed and extracted with 3-vol cold organic mixture of ethanol: chloroform and centrifuged at 4 °C at 13500 rpm for 20 min. The supernatant was split for lipid and amino acid profiling with an Acquity ultra performance liquid chromatography coupled to a Xevo TQ-S mass spectrometry (UPLC-MS/MS, Waters Corp., Milford, MA). Metabolic profiling of other metabolites including organic acids, carbohydrates, amino acids, and nucleotides were measured with an Agilent 7890A gas chromatography coupled to a Leco Pegasus time of flight mass spectrometry (Leco Corp., St Joseph, MI). The raw data files generated from LC-MS (targeted) and GC-MS (untargeted) were processed with TargetLynx Application Manager (Waters Corp., Milford, MA) and ChromaTOF software (Leco Corp., St Joseph, MI) respectively. Peak signal, mass spectral data, and retention times were obtained for each metabolite. The detected metabolites from GC-MS were annotated and combined using an automated mass spectral data processing (AMSDP) software package (26). The levels of lipids and amino acids detected from LC-MS were calculated with calibration curves established with reference standards.

Metabolomics data processing

We conducted data pre-processing similar to the previous report (27). Briefly, we used K-Nearest Neighbors (KNN) method to impute missing metabolomics data (28). To adjust for the offset between high and low-intensity features, and to reduce the heteroscedasticity, the logged value of each metabolite was centred by its mean and autoscaled by its standard deviation (29). We used quantile normalization to reduce sample-to-sample variation (30). We applied partial least squares discriminant analysis (PLS-DA) to visualize how well metabolites could differentiate the obese from normal samples. To explore the contribution of different clinical/physiological factors to metabolomics data, we conducted source of variation analysis. We used comBat Bioconductor R package (31) to adjust for the batch effects in the metabolomics data.

Classification modeling and evaluation

To reduce the dimensionality of our data (230 metabolites vs 57 samples), we selected the unique metabolites associated with separating obese and normal status. To achieve this, we used a penalized

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logistic regression method called elastic net that was implemented in the glment R package (32). Elastic net method selects metabolites that have non-zero coefficients as features, guided by two penalty parameters alpha and lambda (32). Alpha sets the degree of mixing between lasso (when alpha=1) and the ridge regression (when alpha=0). Lambda controls the shrunk rate of cofficients regardless of the value of alpha. When lambda equals zero, no shrinkage is performed and the algorithm selects all the features. As lambda increases, the coefficients are shrunk more strongly and the algorithm retrives all features with non-zero coefficients. To find optimal parameters, we performed 10-fold cross-validation that yield the smallest prediction minimum square error (MSE). We then used the metabolites selected by the elastic net to fit the regularized logistic regression model. Three parameters were tuned: cost, which controls the trade-off between regularization and correct classification, logistic loss and epsilon, which sets the tolerance of termination criterion for optimization.

To construct and evaluate the model, we divided samples into 5 folds. We trained the model on four folds (80% of data) using leave one out cross validation (LOOCV) and measured model performance on the remaining fold (20% of data). We carried out the above training and testing five times on all folds combination. We plotted the receiver-operating characteristic (ROC) curve for all folds prediction using pROC R package. To adjust confounding other clinical covariants such as ethnicity, gravidity and parity, we reconstructed the metabolomics model above by including these factors.

Analysis on metabolite features

We used Classification And REgression Training (CARET) R package to rank metabolites based on the model-based approach (33). In this approach, each metabolite was assigned a score that estimates its contribution to the model performance (34). These scores were scaled to have a maximum of 100. We performed metabolomic pathway analysis on metabolites chosen by the elastic net method using Consensus Pathway DataBase (CPDB). We used rcorr function implemented in Hmisc R package to compute the correlations among clinical and metabolomics data.

Data availability

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The metabolomics data generated by this study is deposited to Metabolomics workbench (Study ID ST001114).

Results

Cohort subjects characteristics

Our disovery cohort consisted of three ethnic groups: Caucasian, Asian and Native Hawaiian and other Pacific Islander (NHPI). Women undergoing scheduled cesarean delivery were included based on the previously described inclusion and exclusion criteria (Methods). Demographical and clinical characteristics in obese and control groups are summarized in Table 1. In the Caucasian group (10 mothers), 6 were categorized as non-obese and 4 as obese. In the Asian group (23 mothers), 16 were categorized as non-obese and 7 as obese. In the NHPI group (24 mothers), 7 (24%) were categorized as non-obese and 17 (61%) as obese. The variation in recruitment of cases versus controls in each ethnic background reflects the demographics in Hawaii. Compared to mothers of normal pre-pregnant BMI, obese mothers have significantly higher pre-pregnancy BMI (33.51+/- 4.49 vs 21.89 +/- 1.86 kg/m2, p=9.18e-11). Mothers have no statistical difference regarding their ages (32.10 +/- 4.88 vs 32.48 +/- 5.66, p=0.7) or gestational age (39.04 weeks+/- 0.22 vs 38.93 +/- 0.45 p=0.38), excluding the possibility of confouding from these factors. Babies of obese mothers have significantly (P=0.03) higher birth weight compared to the normal pre pregnant weight group, consistent with earlier observations (35, 36).

Preliminary assessment of metabolomics results

We detected a total of 230 metabolites, including 79 untargeted and 151 targeted metabolites (11 amino acids, and 140 lipids). To explore which clinical/physiological covariates are associated with the variations in the metabolomics, we conducted source of variation analysis. Indeed, maternal obesity is the predominatly most important factor contributing to metabolomic difference, rather than other factors

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(Figure 1A). To test if these metabolites allow clear separation between the obese and normal-weight subjects, we used elastic net regularization based logistic regression, rather than the partial least squares (PLS) model, a routine supervised multivariate method which only yielded modest accuracy AUC=0.62 (Figure 1S). Elastic net regularization overcomes the limitation of either ridge and lasso regularization alone, and combines their strengths to identify an optimized set metabolites [25]. Using the optimized regularization parameters (Figure. 2S), we identified a total of 29 metabolite features, which together yields the highest predictive performance with AUC=0.97, 95 % CI=[0.904-0.986] in 20% hold-out test dataset (Figure 1B). Among them, six metabolites have large contributions to the separations between case/control, with an importance score of at least 70% individually (Figure 1C). These are galactonic acid, butenylcarnitine (C4:1), 2-hydroxy-3-methylbutyric acid, phosphatidylcholine diacyl C40:3 (PC aa C40:3), 1,5-anhydrosorbitol, and phosphatidylcholine acyl-alkyl 40:3 (PC ae C40:3). Thus, metabolites selected by the elastic net method indeed improved the prediction power of the model.

Calibrated maternal-obese predictive model with consideration of confounding

For statiscal rigor, it is important to consider possible confounders, such as maternal/paternal ethnicity and parity (Table 1) during the analysis. Towards this, we conducted two investigations. First, we explored the correlations among the demographic factors and metabolomics data. It is evident that several metaboloties are correlated with maternal and paternal ethnicity, gravidity, and/or parity (Figure 2-A). For example, maternal ethnicity is positively correlated with 2-hydroxy-3-methylbutyric acid. Secondly, we built a logistic regression model using the above-mentioned four covariates alone (parity, gravidity, maternal and paternal ethnicity). This model yields a modest AUC of 0.701 95% CI=[0.55-0.82] (Figure 3S-A), again suggesting existence of confounding. These observations prompted us to recalibrate the 29-metabolite elastic net model, by adjusting the metabolomics model using all collected clinical covariants (Figure 2B). The resulting modified model remains to have very high accuracy, with AUC= 0.947, 95% CI= [0.87-0.97].

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In the new model, besides the original 6 metabolite features, maternal ethnicity and paternal ethnicity also have importance scores greater than 70% (Figure 2C).

Metabolomite features and their pathway and enrichment analysis

The 29 metabolite features selected by the model belong to acylcarnitine, glycerophospholipid, amino acids and organic acids classes. Their log fold changes ranged from -0.45 (Hydroxyhexadecenoylcarnitine, or C16:1-OH) to 0.66 (2-hydroxy-3-methylbutyric acid) (Figure 3A). Among them, 15 metabolites are higher in obese associated cord blood samples, including 2-hydroxy-3-methylbutyric acid, galactonic acid, PC ae C40:3, Propionylcarnitine (C3), PC aa C40:3, O-butanoyl-carnitine (C4:1), Hexanoylcarnitine (C6 (C4:1 - DC)) , Phosphatidylcholine diacyl C40:2 (PC aa C40:2), benzoic acid, 1,5-anhydrosorbitol, Isovalerylcarnitine (C5), PC ae C40:2, L-arabitol, Octadecenoylcarnitine (C18:1) (Figure 3A, Table 2) . The remaining 14 metabolites are lower in obese associated cord blood samples: malic acid, L-aspartic acid, citric acid, PC ae C34:0, isoleucine, PC ae C36:2, oleic acid, PC aa C36:5, PC ae C34:3, PC ae C40:6, C5:1-DC, 2-hydroxybutyric acid, myoinositol, and C16:1 -OH (Figure 3A, Table 2). The individual metabolite levels of Hexanoylcarnitine (C6(C4:1-DC)), O-butanoyl-carnitine (C4:1), PC aa C40:3, Propionylcarnitine (C3), PC ae C40:3, galactonic acid, and 2-hydroxy-3-methylbutyric acid increased significantly in obese cases ($p<0.05$, t-test).

To elucidate the biological processes in newborns that may be effected by maternal obesity, we performed pathway enrichment analysis on the 29 metabolite features, using Consensus pathway database (CPDB) tool (37). We combined multiple pathway databases including KEGG, Wikipathways, Reactome, EHNM and SMPDB. A list of 10 pathways are enriched with adjusted p-value $q<0.05$ (Figure 3B). Among them, alanine and aspartate metabolism is the most significantly enriched pathway ($q=0.004$). Transmembrane transport of small molecules and SLC-mediated transmembrane transport are also significantly enriched ($q=0.004$ and $q=0.01$ respectively).

The influence of ethinicity on metabolite levels

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Our earlier correlational analysis suggested that maternal ethnicity may be correlated with 2-hydroxy-3-methylbutyric acid level (Figure 2A). To confirm this, we conducted 2-way ANOVA statistical tests and indeed obtained significant p-value (P=0.023, chi-square test). We thus stratified the levels of 2-hydroxy-3-methylbutyric acid by ethnicity (Figure 4). There is no significant difference in normal pre pregnant-weight subjects across the three ethnic groups (Figure 4A). However, in cord blood samples associated with obese mothers, the concentration of 2-hydroxy-3-methylbutyric acid is much higher in NHPI, as compared to Caucasians (p=0.05) or Asians (p=0.04) (Figure 4B). 2-hydroxy-3-methylbutyric acid originates mainly from ketogenesis through the metabolism of valine, leucine and isoleucine (38). Since all subjects have fasted 8 hours before the C-section, we expect the confounding from diets is minimized among the three ethnical groups. Thus the higher 2-hydroxy-3-methylbutyric acid level may indicate the higher efficiency of ketogenesis in babies born from obese NHPI mothers.

Validation on an independent cohort

To test the robustness of our results, we applied our model on a new cohort of 30 patients (18 normal-weight and 12 obese). We then performed new metabolomics measurements and processed the data as earlier. Using the model built on 57 samples, we tested its performance on the new 30 samples, and obtained an AUC of 0.822 (95% CI= [0.74-0.89]), confirming the reproducibility of our findings. Moreover, we observed a similar trend of higher concentration of 2-hydroxy-3-methylbutyric acid in NHPI compared to Asians and Caucasians (p=0.001) in the obese group, whereas no statistical difference between ethnicities exists in the control group. Moreover, within this cohort, four of the six metabolites that had large contributions to the separations between case/control (importance score > 70%) in the discovery cohort, has consistent trend of changes in the validation cohort.

Discussion

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This study aims to distinguish key cord blood metabolites associated with maternal pre-pregnancy obesity. The novelty of the study is manifested in several folds. First, we have collected a unique multi-ethnic population in Hawaii over a period of 3 years (2015-2018), which includes Asian, NHPI and Caucasians, following very strict inclusion/exclusion criteria (esp. on matching gestational weight gain). Secondly, we utilize state of the art metabolomics technology platform coupling GC-MS and LC-MS platforms, which allows us to detect hundreds of metabolites simultaneously. Lastly, we use the state of art method called elastic net based logistic regression that drastically improves the classification accuracy on cord blood metabolomics data.

To ensure the quality of metabolomics data, our study set most stringent inclusion and exclusion crtieria to exclude as many confouding factors as possible. To avoid the confounding from labor and vaginal delivery, we only targeted mothers having elective C-sections. We also excluded obese mothers who had known complications during pregnancy, such as pre-gestational diabetes, smoking, and hypertension. These criteria helped to improve the quality of the metabolomics data. To minimize confounding due to maternal diet, all subjects fasted 8 hours before the Cesarean section.

Such careful experimental design did yield good data quality, as the source of variation analysis did show that maternal obesity is the only dominate factor contributing to metabolomics diffeence in the cord blood. Additionally, we conducted rigorous statistical modeling and found that metabolites can distinguish the two maternal groups with accuracy as high as AUC=0.97 under cross-validation (or 0.947 after adjusting for confounding effects). Metabolomics pathway analysis on the metabolite features in the model identified 10 significant pathways. Among them, alanine and aspartate metabolism was previously reported to be associated with obesity (39). Transmembrane transport was identified as another significant pathway. The transmembrane transport pathway corresponds to the acylcarnitine metabolites in the features. Acylcarnitines are known transmembrane transporters of fatty acids across the mitochondrial membrane (40). Among all metabolites and physiological/demographic features selected by the combined model, galactonic acid has the largest impact on the model performance (importance score =86%). Galactonic acid,

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was previously shown to be associated with diabetes in a mouse model, due to a proposed mechanism of oxidative stress (41). On the other hand, maternal ethnicity has the largest impact among physiological factors (importance score =84%).

A very few cord blood metabolomics studies have been carried out to associate with maternal obesity directly, or birth weight (22, 42, 43). In a recent Hyperglycemia and Adverse Pregnancy Outcome (HAPO) Study, Lowe et al. reported that branched-chain amino acids such as valine, phenylalanie, leucine/isoleucine and AC C4, AC C3, AC C5 are associated with maternal BMI in a meta-analysis over 4 large cohorts (400 subjects in each) (43). In another study to associate cord blood metabolomics with low birth weight (LBW), Ivorra et al. found that newborns of LBW (birth weight < 10th percentile, n = 20) had higher levels of phenylalanine and citrulline, compared to the control newborns (birth weight between the 75th-90th percentiles, n = 30) (22). They also found lower levels of choline, proline, glutamine, alanine and glucose in new borns of LBW, however, there was no significant differences between the mothers of the two groups. In our study, isoleucine is also identified as one of the 29 metablite features related to maternal obesity; although alanine iteself is not selected by the model to be a maternal obesity biomarker in cord blood, we did find that alanine and aspartate metabolism are enriched in the cord blood samples associated with maternal obesity group.

Notably, our study has identified 5 metabolites which are previously not reported in the literature with association to obesity or maternal obesity: galactonic acid, L-arabitol, indoxyl sulfate, 2-hydroxy-3-methylbutyric acid and citric acid. Except citric acid, all the other four metabolites are increased in obese associated cord blood samples. 2-hydroxy-3-methylbutyric acid concentrations varied by ethnicity, but only in babies born from obese pre-pregnant mothers. 2-hydroxy-3-methylbutyric acid is known to accumulate in high levels during ketoacidosis and fatty acid breakdown. Therefore, the higher elevation of 2-hydroxy-3-methylbutyric acid is likely due to increased cellular ketoacidosis and fatty acid breakdown in new borns from obese pre-pregnant mothers. To the best of our knowledge, this is the first study that shows differences in the 2-hydroxy-3-methylbutyric acid concentration levels among different ethnicities. Additionally,

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Indoxyl sulfate is a metabolite of the amino acid tryptophan. As tryptophan is commonly found in fatty food, red meat and cheese, it is possible that high levels of indoxyl sulfate detected in the cord blood associated with obese pre-pregnant mothers could be due to the maternal high fat diet. Oppositely, citric acid, a compound associated with the citric acid cycle (44), is decreased in the cord blood associated with obese pre-pregnant mothers. This could be related to the lower vegitable and fruit consumptions among obese pre-pregnant mothers. In all, the data suggest that maternal obesity may impact offspring cord blood metabolites. Further research into the specific mode of action of these metabolites would be beneficial in understanding its association with maternal obesity.

This study may benefit from some improvmenet in the future follow-up s. We determined the subjects' ethnicity by self-reporting rather than genotyping, due to the restriction of the currently approved IRB protocol. Additionally, there has been debates on the use of BMI as an indicator of obesity (45), more direct measures of body fat could be considered such as skin-fold thickness measurements, bioelectrical impedance and energy x-ray absorptiometry (46, 47). Nevertheless, this study has established relationships between cord blood metabolomics with maternal pre-pregnant obesity, which in turn is associated with social economical disparities.

Conclusion

In this study, we identified 29 metabolites that are associated with maternal obesity, 5 of which are previously unreported in the literature. These metabolites have the potential to be maternal obesity-related bio-markers in newborns that warranty dietary interventions in early-life.

Author Contributions

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LXG envisioned the project, obtained funding, designed and supervised the project and data analysis. RJS, IC, PAB and SJC collected the samples. AG prepared the plasma samples. FMA analyzed the data. GX performed the metabolomics experiments. RJS, FMA, PAB, AG, GX, SJC and LXG wrote the manuscript. All authors have read, revised, and approved the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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Tables

Table 1: Demographical and clinical characteristics in obese and control groups

	Control(n=29)	**Case(n=28)**	**P-value***
	Mean (SD)		
Maternal age, years	32.48 (5.66)	32.10 (4.88)	0.78
Paternal age, years	34.68(7.14)	35.21(6.43)	0.79
Pre-pregnancy BMI, kg/m2	21.89(1.86)	33.51(4.49)	1.12 e-14
Gestational Age, Weeks	39.04(0.218)	38.93(0.45)	0.3812
Net weight gain	30.85(10.92)	29.4(13.55)	0.7335
Baby weight (kg)	3.29(0.32)	3.54(0.5)	0.03
Head Circle (cm)	34.89(1.10)	35.55(1.36)	0.05
Baby length (cm)	51.3(1.9)	51.4(2.36)	0.8
Parity			0.03
0	5	2	
1	16	7	
2	7	10	
3 and above	1	9	

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Gravidity			0.12
1	5	1	
2	12	5	
3	7	8	
4 and above	5	14	
Maternal Ethnicity			0.01
Caucasian	6	4	
Asian	16	7	
Pacific island	7	17	
Paternal Ethnicity			0.03
Caucasian	8	3	
Asian	14	9	
Pacific island	7	16	

*Categorical variables were compared using chi-square test, whereas continuous variables were compared using *t* test.

Table 2: A list of metabolites associated with obese-control maternal status and selected by elastic net regularization based logistic regression. The metabolites are sorted by the average log fold change of cases over controls.

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Metabolites	Chemical name	Fold change [a] (case-control)		Univariate Analysis [b]	
		logFC	P_value	Cofficient	P_value
2-hydroxy-3-methylbutyric acid	2-hydroxy-3-methylbutyric acid	0.6609	0.0119	0.65592	0.062950865
Galactonic acid	Galactonic acid	0.6337	0.0158	0.640515	0.06565148
PC ae C40:3	Phosphatidylcholine acyl-alkyl C40:3	0.6249	0.0173	0.762691	0.035189439
C3	Propionylcarnitine	0.5598	0.033	-0.1467	0.648143485
PC aa C40:3	Phosphatidylcholine diacyl C40:3	0.5561	0.0342	-0.33489	0.318665241
C4:1	O-butanoyl-carnitine, butenylcarnitine	0.556	0.0342	-0.44274	0.168989046
C6 (C4:1 -DC)	Hexanoylcarnitine, Fumarylcarnitine	0.5355	0.0414	-0.28551	0.337718
PC aa C40:2	Phosphatidylcholine diacyl C40:2	0.4793	0.0679	0.532796	0.113517583
Benzoic acid	Benzoic acid	0.4549	0.0831	0.279734	0.350259256
1,5-Anhydrosorbitol	1,5-Anhydrosorbitol	0.3664	0.1628	0.636374	0.24536415
C5	Isovalerylcarnitine, Valerylcarnitine, Methylbutyrylcarnitine	0.3654	0.1638	-0.38664	0.196793118
PC ae C40:2	Phosphatidylcholine acyl-alkyl C40:2	0.3242	0.2168	-0.71475	0.042908449
L-Arabitol	L-Arabitol	0.2685	0.3062	0.360549	0.266082992

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C18:1	Octadecenoylcarnitine	0.228	0.385	0.253734	0.427416515
Indoxyl sulfate	Indoxyl sulfate	0.1792	0.4948	-0.06239	0.827985019
Malic acid	Malic acid	-0.006	0.9811	0.010217	0.977502972
L-Aspartic acid	L-Aspartic acid	-0.036	0.8899	-0.18507	0.549849292
Citric acid	Citric acid	-0.058	0.8242	-0.08235	0.790831897
PC ae C34:0	Phosphatidylcholine acyl-alkyl C34:0	-0.091	0.7295	0.712	0.058228623
Isoleucine	Isoleucine	-0.158	0.5473	-0.56607	0.089720981
PC ae C36:2	Phosphatidylcholine acyl-alkyl C36:2	-0.193	0.4629	-0.1802	0.553764206
Oleic acid	Oleic acid	-0.2	0.4465	0.183252	0.536574067
PC aa C36:5	Phosphatidylcholine diacyl C36:5	-0.218	0.4059	-0.4694	0.174139565
PC ae C34:3	Phosphatidylcholine acyl-alkyl C34:3	-0.22	0.4008	0.319963	0.31966488
PC ae C40:6	Phosphatidylcholine acyl-alkyl C40:6	-0.261	0.3193	0.741937	0.01875932
C5:1-DC	Glutaconylcarnitine, Mesaconylcarnitine	-0.271	0.3021	-0.26351	0.409158971
2-Hydroxybutyric acid	2-Hydroxybutyric acid	-0.323	0.219	0.250888	0.404894782
Myoinositol	Myoinositol	-0.386	0.1416	0.47233	0.144462991
C16:1 -OH	Hydroxyhexadecenoylcarnitine	-0.447	0.0884	0.809254	0.093896414

[1]Fold change was calculated as mean (log2 (obese)) – mean (log2 (control))

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[b]Univariate logistic regression of each Elanet-selected metabolite adjusted for maternal age, ethnicity, parity, and gravidity.

Legends for figures

Figure 1: Source of variation and accuracies of logistic regression models and important features selected by the metabolomics model. (A) ANOVA plot of clinical factors using the metabolites levels in cord blood samples. Averaged ANOVA F-statistics are calculated for potential confounding factors, including obesity, gravida, parity, paternal and maternal age and ethnicity. (B) Model accuracy represented by classification Receiver Operator Curves (ROCs). (C) The ranking of contributions (percentage) of selected metabolomics features in the model.

Figure 2: (A) Correlation coefficients among demographical/physiological factors and the metabolomics data. Blue colors indicates positive correlations and red indicated negative correlations. (B) Receiver Operator Curves (ROCs) of the combined model with metabolomics and physiological/demographic data. (C). The ranking of contributions (percentage) of selected features in the model (B).

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Figure 3: Analysis of the 29 selected metabolites. (A) Heatmap of selected metabolites separated by maternal group. * indicates metabolites that shows significant p-values (P<0.05, t-test) individually. (B) Pathway analysis of the 29 metabolites. X-axis shows size of metabolomic pathway. Y-axis shows the adjusted p-value calculated from CPDB tool. The size of the nodes represents the size of metabolomic pathway (number of metabolites involved in each pathway). The color of the nodes represents the source of these pathways.

Figure 4: Violin plot of 2-hydroxy-3-methylbutyric acid among 3 ethnic groups in the discovery cohort. Association between 2-hydroxy-3-methylbutyric acid and the ethnicity in (**A)** normal (n=29) and (**B)** obese (n=28) subjects.

Figure 5: Validation with another cohort. (**A)** Accuracy on classifying cases vs controls in the validation cohort, using the model built on the discovery cohort as shown in Fig 2(B). **(B-C)** violin plots of 2-hydroxy-3-methylbutyric acid in NHPI vs (Asians/Caucasians). Asians (n=2) and Caucasians (n=3) were combined, as the number of patients of these ethnicities in the obese group is small. (**A)** normal (n=18) and (**B)** obese (n=12) subjects are displayed.

Supplementary Figures

Supplementary Figure 1: Discrimination of obese and normal groups by Partial Least Squares (PLS) method. **(A)** Discriminant analysis score plot for obese cases (Green) and normal (Red). (**B)** The accuracy of the 10 fold cross-validation of the PLS-DA model. R2 is the sum of squares captured by the model; Q2 is the cross-validation of R2.

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Supplementary Figure 2: Selection of metabolites using elastic net regularization. **(A)** Tuning alpha parameter, the parameter representing the degree of mixing between lasso and the ridge regularization. Y-axis is the root mean square error of the 10-fold cross-validation. X-axis is the range of alpha values, with the optimal alpha =0.22. **(B)** Tuning lambda, the parameter controlling the shrunk rate of coefficients in the linear model. Y-axis is the misclassification error of the 10 fold cross validation. X-axis is the range of lambda, with the optimal lambda=0.008. **(C)** The shrinkage coefficients of the metabolites using tuned alpha and lambda.

Supplementary Figure 3: Accuracies of logistic regression models and important features selected by the clinical model. (A) Model accuracy represented by classification Receiver Operator Curves (ROCs). (B) The ranking of contributions (percentage) of selected clinical features in the model.

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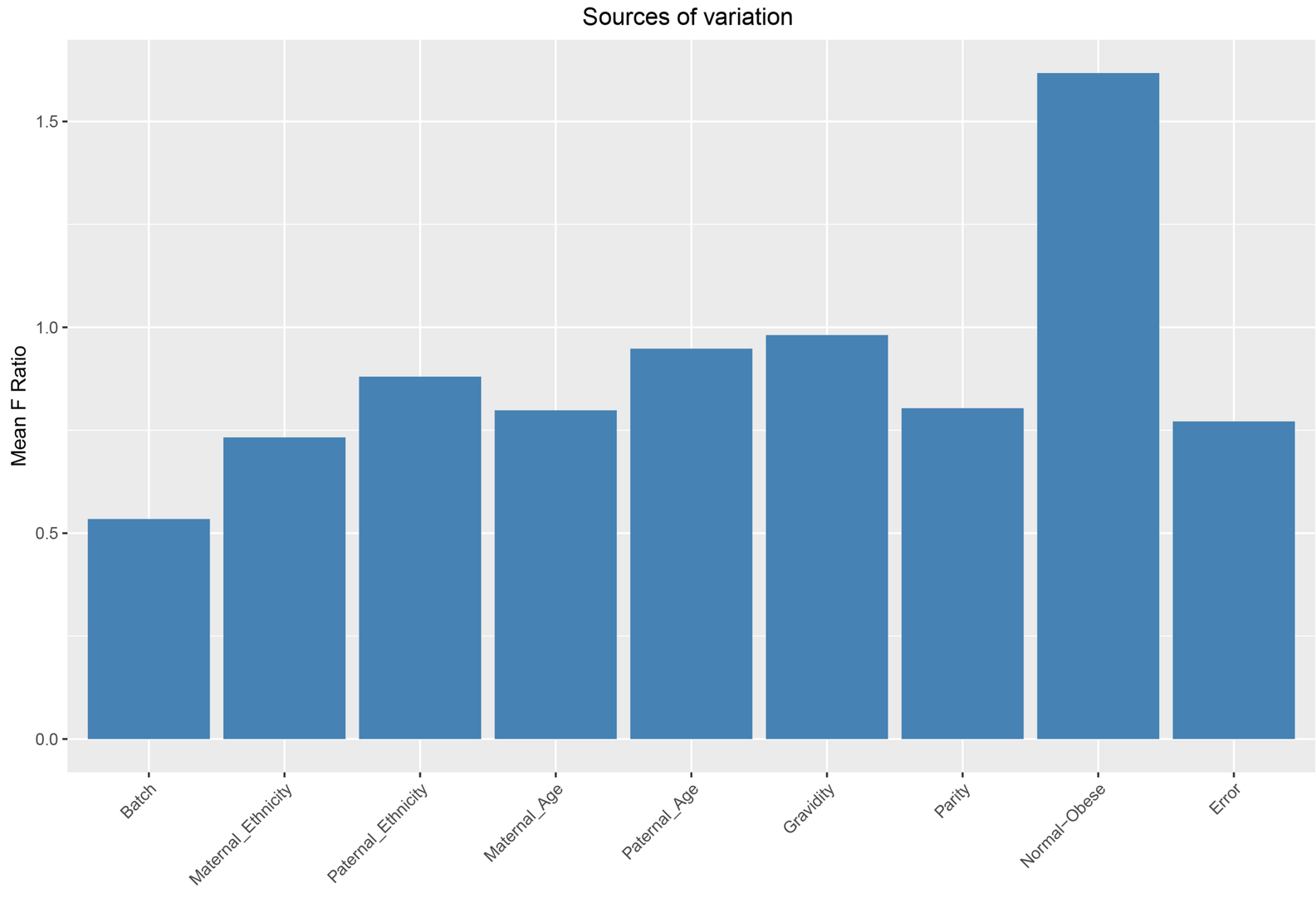

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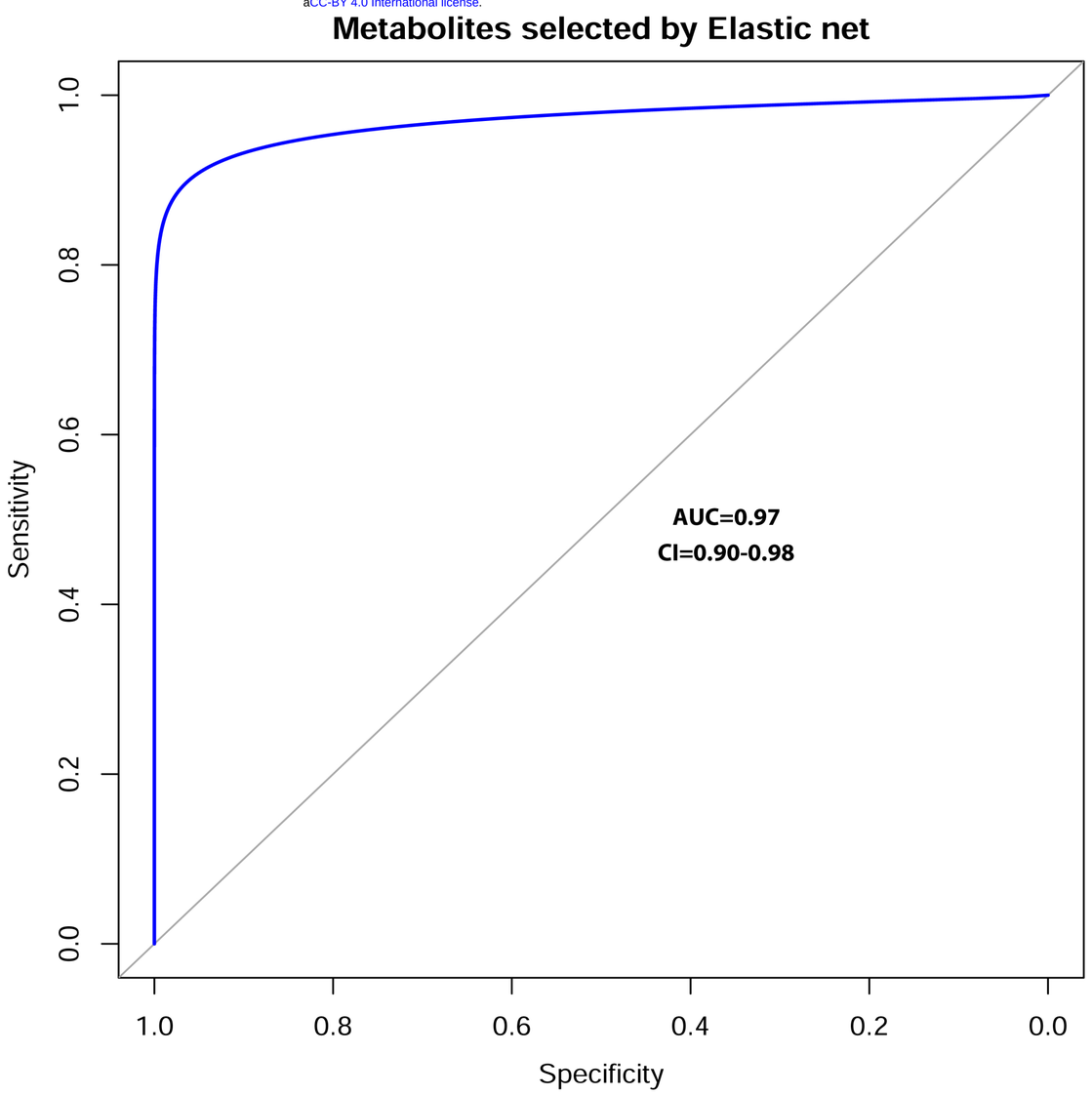

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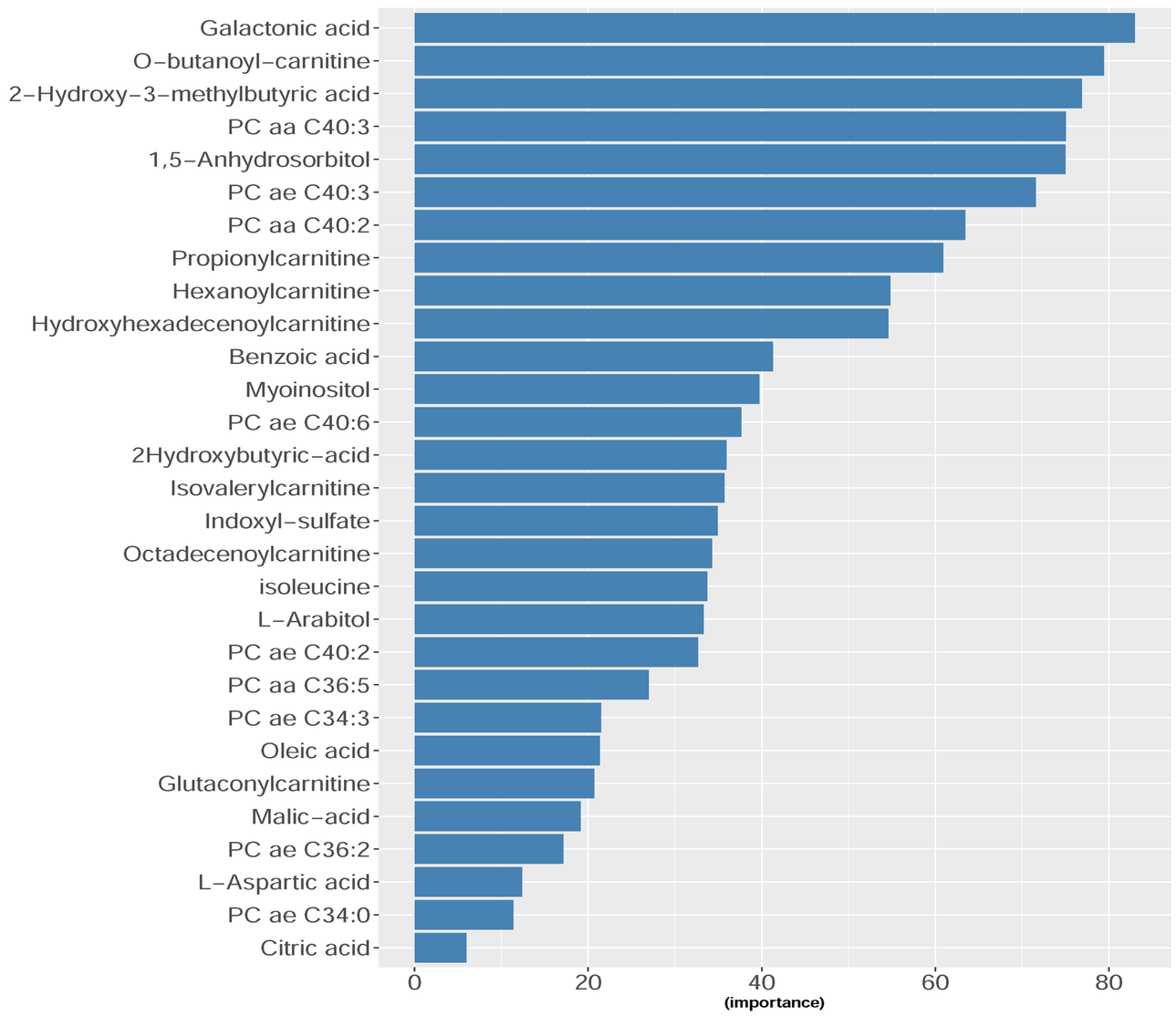

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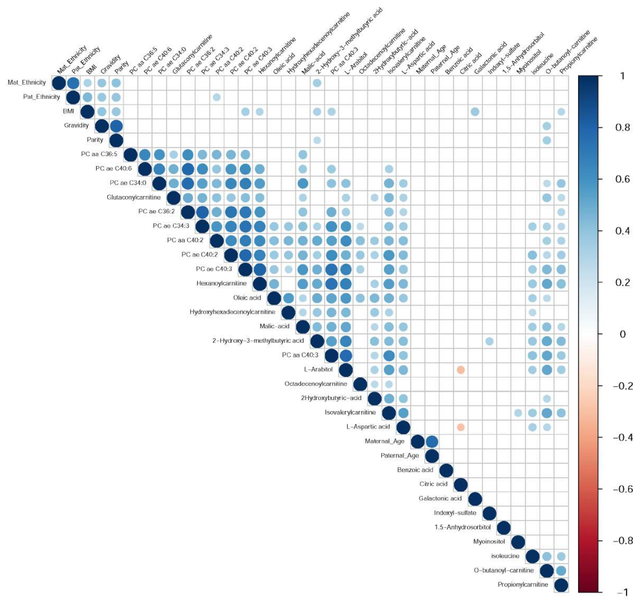
A



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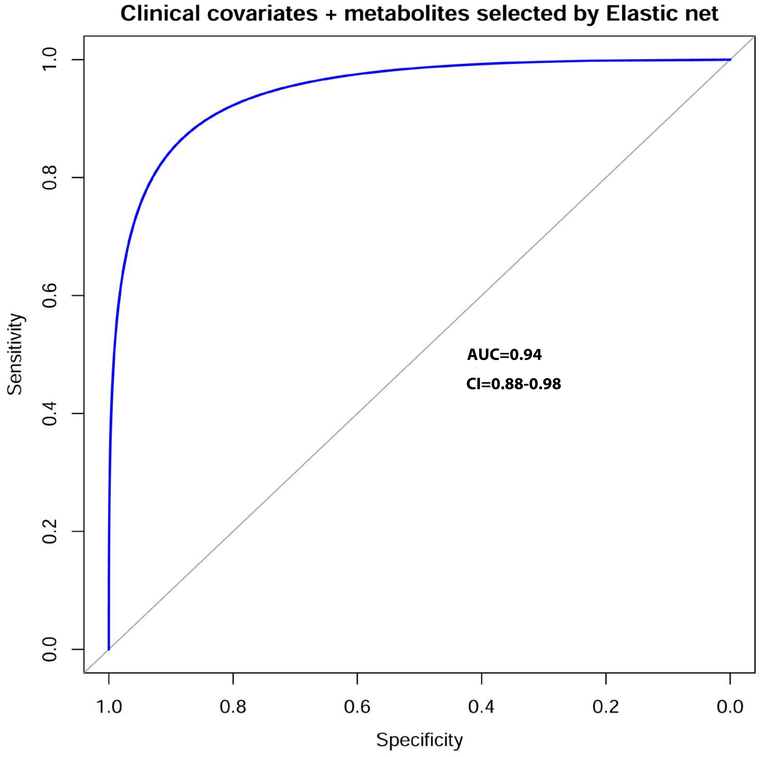

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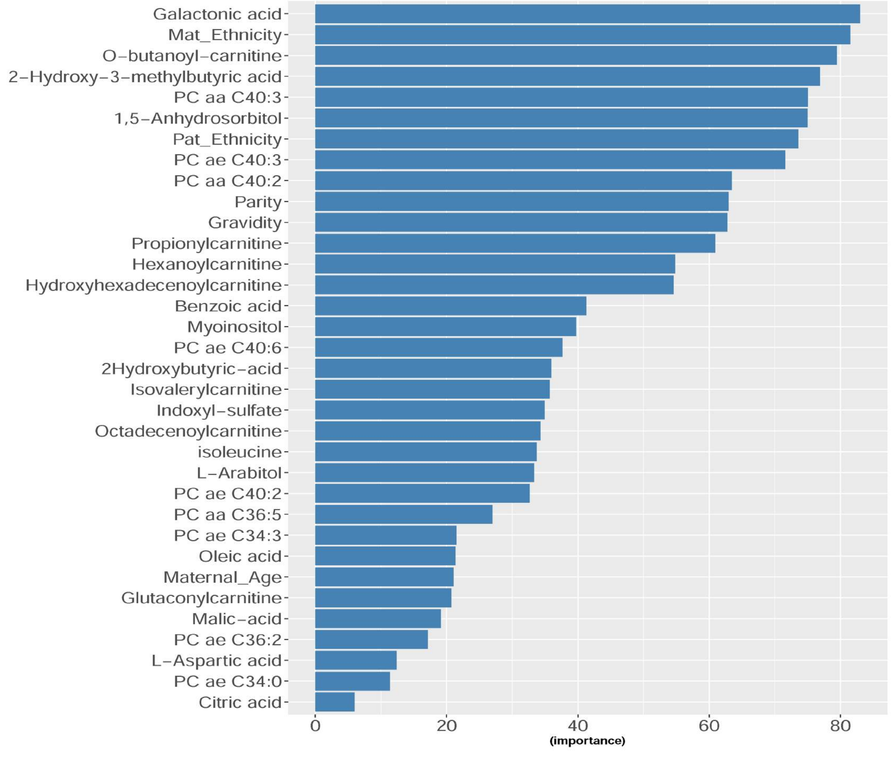

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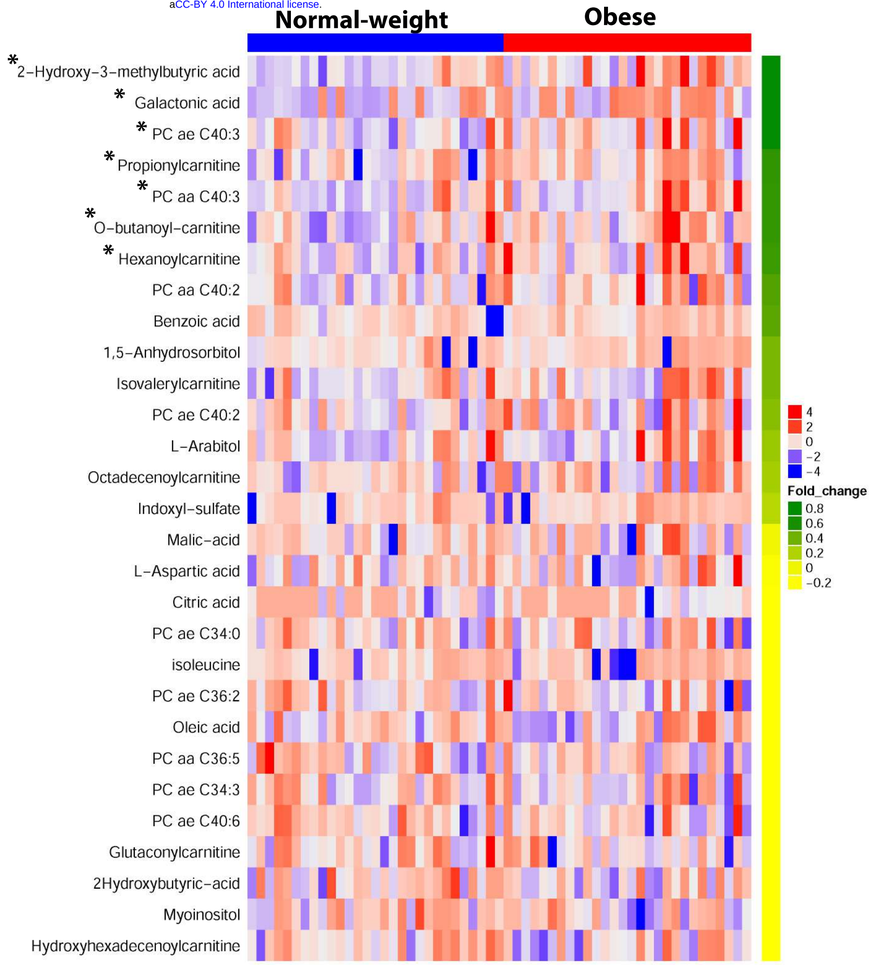

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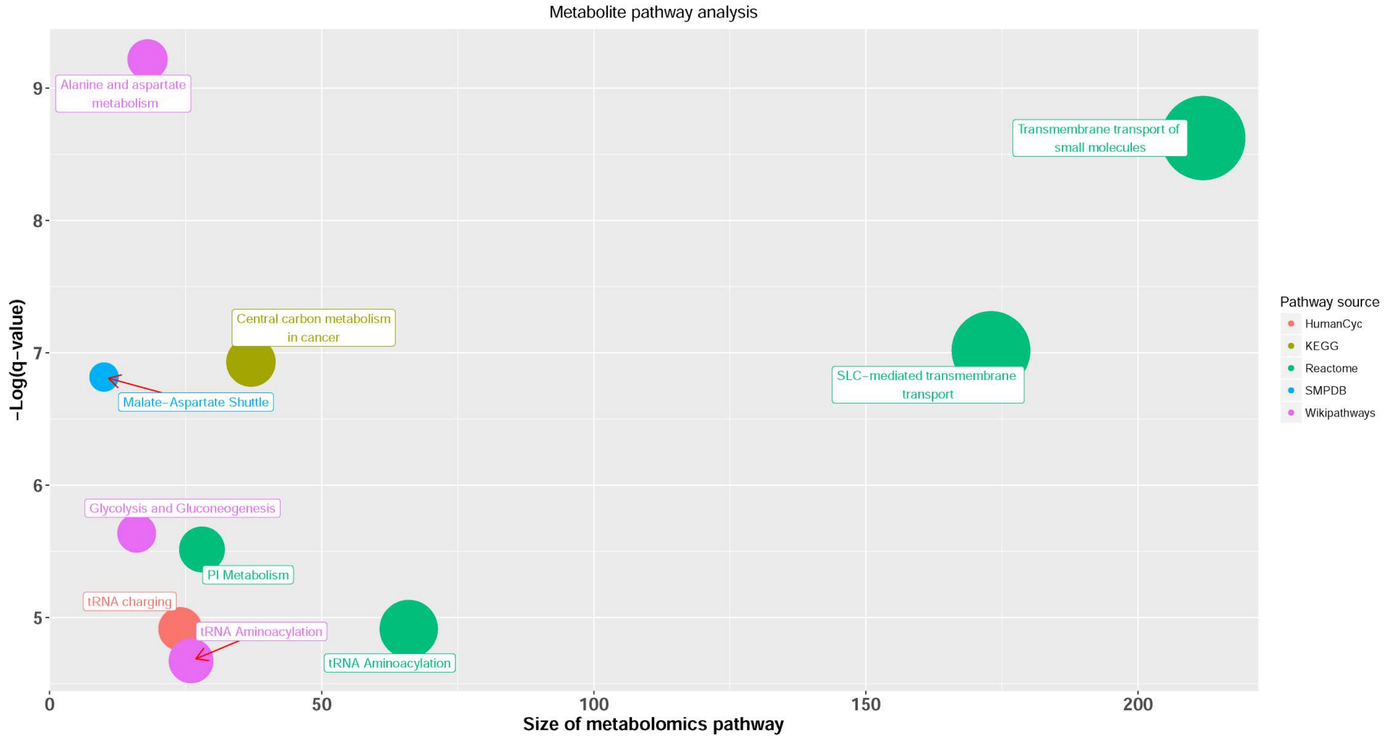

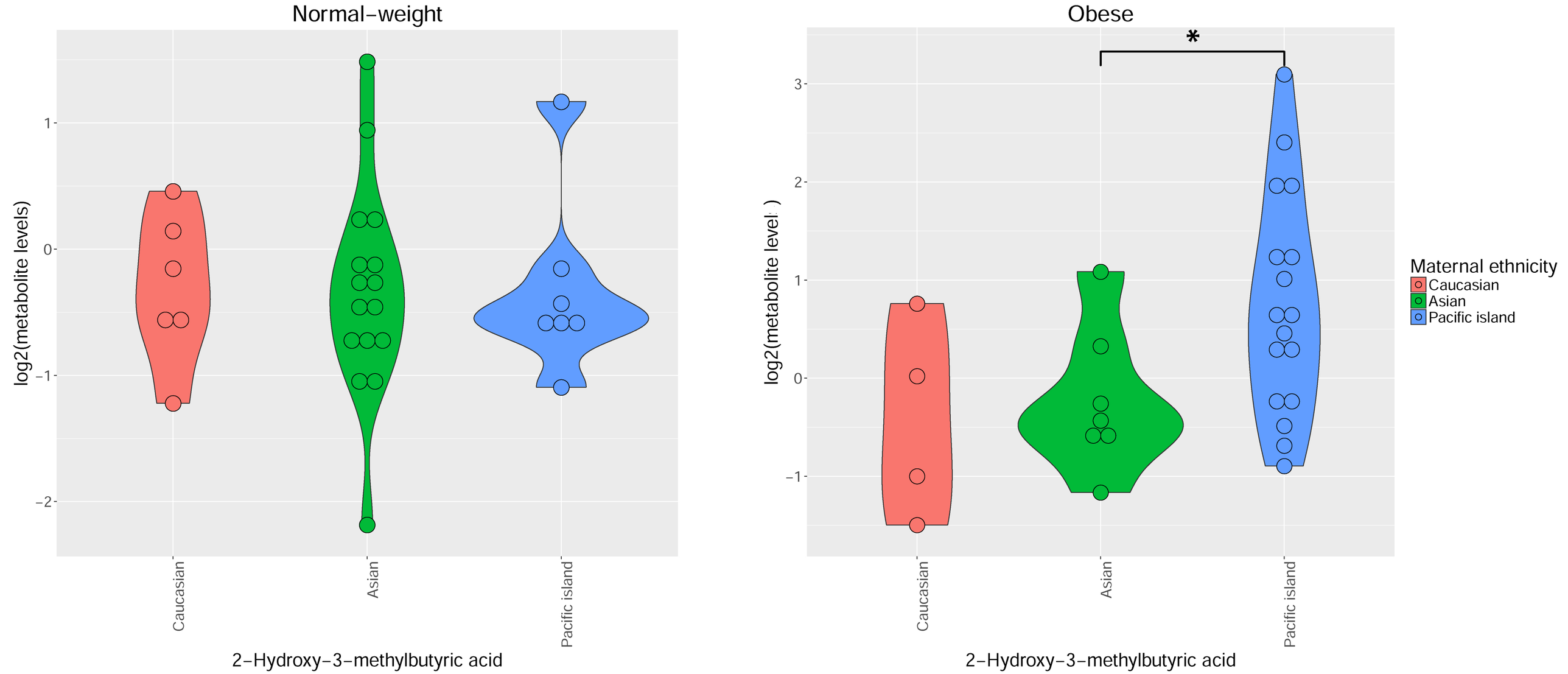
Normal−weight
Obese
log2(metabolite levels)
log2(metabolite level)
1
0
−1
−2
3
2
*
Caucasian
Asian
Pacific island
2−Hydroxy−3−methylbutyric acid
Maternal ethnicity
Caucasian
Asian
Pacific island

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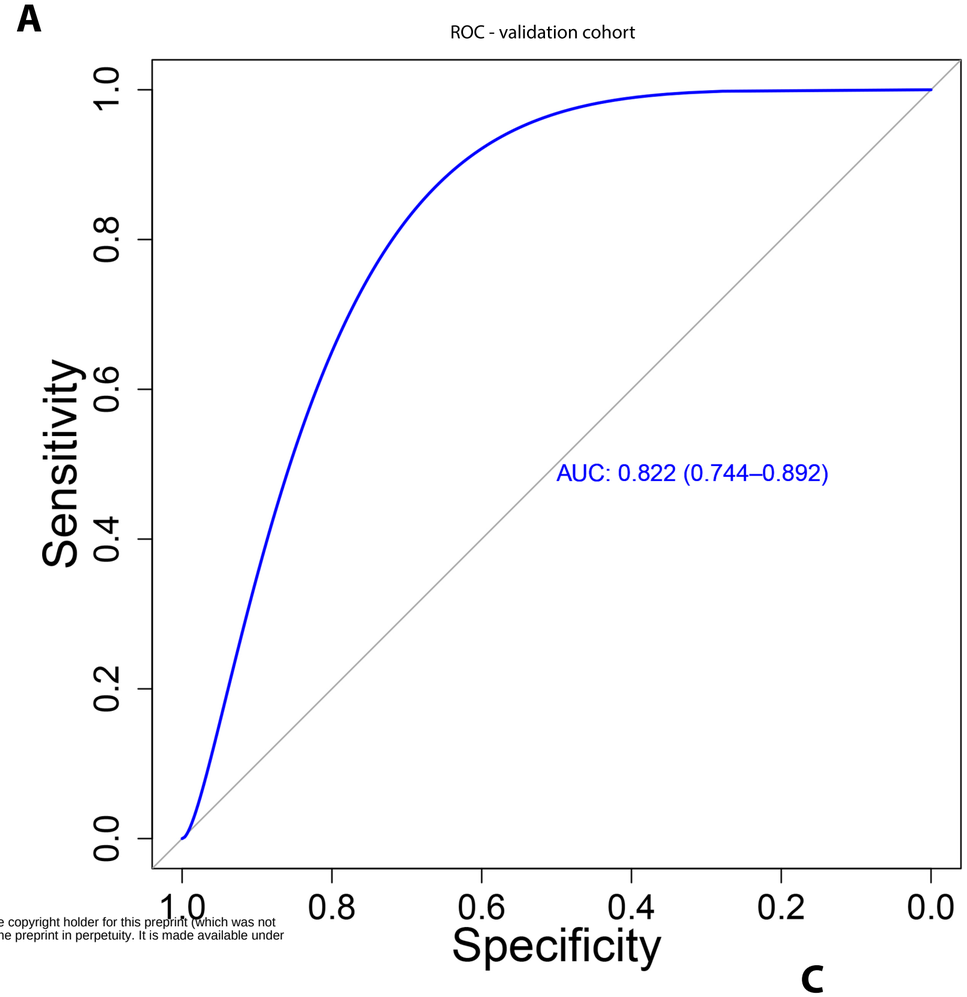

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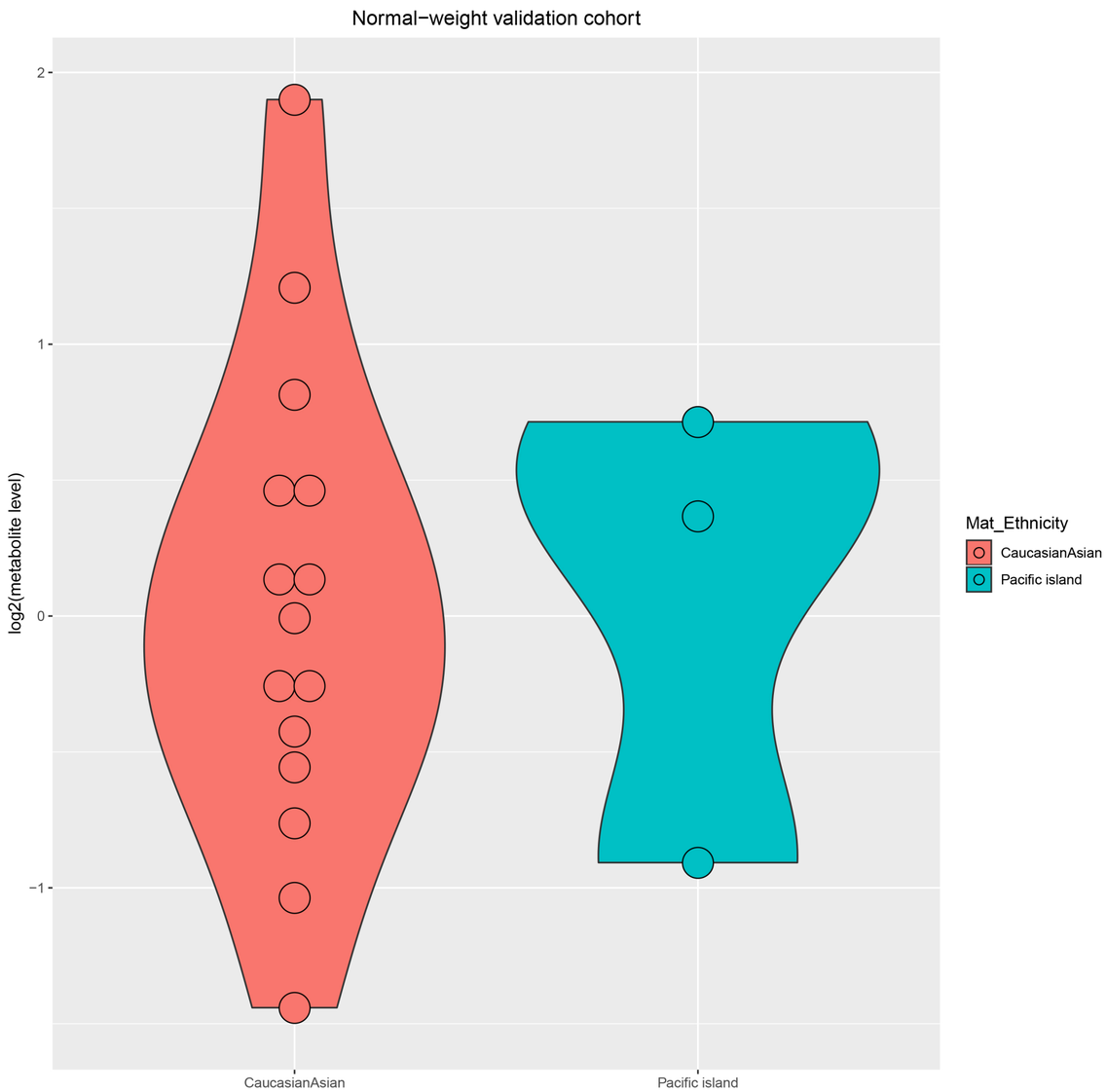

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