Supplemental Material

Title: Identification of Novel Biomarkers and Pathways for Coronary Artery Calcification in Non-diabetic Patients on Hemodialysis Using Metabolomic Profiling

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SUPPLEMENTAL METHODS

Selection of study population

In the PACE cohort (n=568), diabetes status and blood samples were not available in 158 participants because these participants did not complete the study visit. Every participant in the PACE cohort had hypertension. In current study, we compared the serum metabolites of non-diabetics with a CAC score>100 (case) with non-diabetics with a CAC score of 0 (control). Compared to the study population, participants with diabetes were older (56 ± 11 vs. 53 ± 16 years) and more likely to have CAD (45% vs. 27%; Table S1). Participants without available CAC scores (76% had diabetes) were more likely to have CAD (40%) compared to the study population. Non-diabetic participants with a CAC score between 0-100 had similar demographic characteristics with the study population.

Details on CAC measurement

CAC was measured at the baseline visit by computed tomography (Toshiba Aquilon One, Japan), acquired prospectively in synchrony with the electrocardiogram at 70-80% RR interval, near the end of diastole and before atrial contraction.[1] Coronary calcium was quantified by Agatston score. CAC scores were assessed and then validated by a second reviewer.

Details on the measurement of covariates

Potential confounders included demographics, education level, smoking history, comorbidities, medication use, dialysis access, and dialysis clearance. Participants' demographic factors (age, sex, and race), education level, smoking history, and medication use were self-reported. Comorbidities including diabetes mellitus, coronary artery disease, and hypertension were adjudicated by a committee of physicians. Dialysis clearance was assessed by single-pool Kt/V (spKt/V).

We also measured serum markers of mineral metabolism, circulating inhibitors of cardiovascular calcification, calciprotein particle size and transformation time, and C-reactive protein. All measurements were performed in the same serum samples as the metabolomics, except for serum

calcium, phosphorous, and intact parathyroid hormone (PTH) levels, which were averaged from 3-months of lab values collected before a dialysis session. C-terminal fibroblast growth factor (FGF23, CV=13%; Immutopics, San Clemente, CA, USA) and soluble klotho (CV=2.3%; Immuno-biological Laboratories, Takasaki, Japan) were measured using enzyme-linked immunosorbent assays (ELISA). For circulating inhibitors of cardiovascular calcification, osteoprotegerin[2] (CV=9%; Alpaco Diagnostics, Salem, NH, USA), dephosphorylated and uncarboxylated matrix glutamate (Gla) protein (dp-ucMGP)[3,4] (CV=11%; VitaK BV, Maastricht, Netherlands), and fetuin-A[5] (CV=18%; Epitope Diagnostics, San Diego, CA, USA) were measured using ELISA. The size of secondary calciprotein particle aggregates (CPP2; CV=13%) and the time of transformation from primary to secondary calciprotein particle (T_{50}; CV=10%) were measured using dynamic light scattering as previously described.[6] High sensitivity C-reactive protein (CV=7%; BNII Nephelometer; Siemens Healthcare, Germany) was measured using ELISA.

SUPPLEMENTAL TABLES

Table S1. Comparing baseline characteristics of study population with unselected participants

	Study population (n=99)	Diabetes (n=239)	No CAC score (n=291)	CAC score between 0-100 & non-diabetics (n=31)
Age, year (missing=200)	53 ± 16	56 ± 11*	56 ± 12	52 ± 11
Women, n (%) (missing=158)	38 (38)	98 (41)	132 (42)	16 (52)
African American, n (%) (missing=158)	71 (72)	169 (71)	223 (71)	23 (74)
Diabetes mellitus, n (%) (missing=158)	0 (0)	239 (100)*	239 (76)*	0 (0)
Hypertension, n (%) (missing=158)	99 (100)	239 (100)	316 (100%)	31 (100)
Coronary artery disease, n (%) (missing=158)	27 (27)	107 (45)*	127 (40)*	6 (19)

*$p<0.05$ compared to the study population. Age is provided as mean ± standard deviation and were tested using two-sample t-test. The rest are categorical variables, which are presented as absolute number with percentage and were tested with chi-square test.

Table S2. Summary of metabolites that differed between cases and controls

	Fold difference	Unadjusted p	Volcano plot	PLS-DA	Demographics-adjusted p
Arginine and proline metabolism					
Arginine	1.14	0.03		x	0.68
Ornithine	1.17	0.008		x	0.57
Succinic acid	1.44	0.005		x	0.21
Glycine and serine metabolism					
Betaine	1.30	0.0047	x	x	0.17
Tyrosine metabolism					
L-tyrosine	1.19	0.007		x	0.49
Methionine metabolism					
N-formyl-L-methionine	2.82	0.004	x	x	0.18
Amino acid degradation					
Methylguanidine	0.62	0.005		x	0.76
Fatty acid metabolism					
Suberic acid	1.25	0.004	x	x	0.16
Nonadecanoic acid	1.10	0.02		x	0.40
Bile acid synthesis					
Chenodeoxycholic acid	2.33	0.002	x	x	0.049
Deoxycholic acid	2.32	0.001	x	x	0.04
Glycolithocholic acid	2.44	0.004	x	x	0.04
Creatine phosphate pathway: muscle breakdown					
Creatinine	0.77	0.003	x	x	0.68
Glycolysis, glyoxylate and tricarboxylic acid cycle metabolism					
Citric acid	1.25	0.002	x	x	0.13
Food component					
Lumazine	1.22	0.002	x	x	0.49

Abbreviation: PLS-DA, partial least squares discriminant analysis

Using Mann-Whitney U test and a set threshold of fold difference (1.0) and raw p-value (0.005), we identified 9 metabolites that differed between cases and controls and showed them in the volcano plot. Using PLS-DA, we identified top 15 metabolites based on VIP scores. All 9 metabolites revealed by the volcano plot were also identified by the PLS-DA. After adjusting for demographics (age, sex, and race), only 3 bile acids remained associated with CAC status ($p<0.05$).

Table S3. Tobit regression of log-transformed CAC score*

Metabolites[#]						
	Unadjusted		Demographics-adjusted		Fully adjusted	
	Coefficient (95% CI)	p	Coefficient (95% CI)	p	Coefficient (95% CI)	p
Chenodeoxycholic acid	0.27 (0.07 – 0.46)	0.01	0.15 (0.03 – 0.27)	0.02	0.13 (0.01 – 0.25)	0.03
Deoxycholic acid	0.28 (0.06 – 0.50)	0.01	0.16 (0.04 – 0.29)	0.01	0.14 (-0.001 – 0.28)	0.05
Glycolithocholic acid (per 0.01 unit)	0.10 (0.04 – 0.16)	0.01	0.05 (0.01 – 0.09)	0.01	0.01 (-0.05 – 0.06)	0.74
Pathways[#]						
	Unadjusted		Demographics-adjusted		Fully adjusted	
	Coefficient (95% CI)	p	Coefficient (95% CI)	p	Coefficient (95% CI)	p
Arginine/proline metabolism	0.91 (0.19 – 1.62)	0.01	0.61 (0.12 – 1.11)	0.02	0.58 (0.11 – 1.05)	0.02
Urea cycle	1.61 (0.89 – 2.34)	<0.001	0.65 (0.003 – 1.29)	0.049	0.65 (0.02 – 1.29)	0.04
Bile acid synthesis	-0.02 (-0.75 – 0.71)	0.96	0.11 (-0.38 – 0.61)	0.65	0.22 (-0.29 – 0.73)	0.40

*CAC score was log-transformed after adding 1 (i.e. Ln(CAC score +1)). [#]per 1 unit higher in relative intensity unless otherwise specified for analyses of metabolites; per 1 unit higher in the first principal component score of each pathway for analyses of pathways.

Demographics included age, sex, and race. Full models were adjusted for demographics, coronary artery disease, use of calcium-based phosphate binder, renin-angiotensin-aldosterone system blockage and statin for all 3 bile acids and bile acid synthesis pathway, and were adjusted for demographics and statin use for arginine/proline metabolism and urea cycle pathways.

Table S4. Metabolized analyzed in arginine/proline metabolism, urea cycle and bile acid synthesis

Arginine and proline metabolism (m=15)		Urea Cycle (m=10)		Bile acid synthesis (m=12)	
Metabolites	HMDB ID	Metabolites	HMDB ID	Metabolites	HMDB ID
L-arginine*	0000517	L-arginine*	0000517	Glycine	0000123
Ornithine*	0000214	Ornithine*	0000214	Glycocholic acid	0000138
Citrulline*	0000904	L-glutamine*	0000641	Palmitic acid	0000220
L-aspartic acid	0000191	Citrulline*	0000904	Taurine	0000251
Fumaric acid	0000134	L-alanine	0000161	Chenodeoxycholic acid*	0000518
Oxalacetic acid	0000223	L-aspartic acid	0000191	Glycochenodeoxycholic acid	0000637
L-glutamic acid	0000148	Fumaric acid	0000134	Taurochenodeoxycholic acid	0000951
Succinic acid*	0000254	Oxalacetic acid	0000223	Deoxycholic acid*	0000626
4-hydroxy-L-proline*	0006055	Pyruvic acid	0000243	Glycodeoxycholic acid	0000631
S-adenosylmethionine	0001185	L-glutamic acid	0000148	Taurodeoxycholic acid	0000896
Flavin adenine dinucleotide	0001248			Glycolithocholic acid*	0000698
Glycine	0000123			Flavin adenine dinucleotide	0001248
L-proline	0000162				
Creatine	0000064				
S-adenosylhomocysteine	0000939				

*$p<0.05$, comparing cases with controls for each metabolite. Abbreviation: HMDB, human metabolome database identification

Table S5. Correlations of bile acid synthesis and arginine/proline metabolism pathways and their key metabolites with serum markers of mineral metabolism (Ca, Phos, Mg, PTH, FGF23, klotho), circulating inhibitors of calcification (osteoprotegerin, dp-ucMGP, fetuin-A, CPP2, T_{50}) and inflammation (CRP).

	Ca	Phos	Mg	PTH	FGF23	Klotho	Osteoprotegerin	Dp-ucMGP	Fetuin-A	CPP2	T50	CRP
Bile acid synthesis pathway	-0.14	0.13	0.03	0.17	0.24*	-0.09	0.33**	0.03	-0.08	0.17	-0.23*	-0.15
Glycocholic acid	-0.06	0.14	-0.03	0.06	0.25*	-0.03	0.2*	0.04	-0.01	0.1	-0.12	-0.17
Chenodeoxycholic acid	0.03	0.05	0.08	0.07	-0.12	-0.17	0.09	-0.11	0.07	-0.02	-0.05	-0.24*
Glycochenodeoxycholic acid	-0.25*	0.08	-0.001	0.12	0.16	-0.06	0.29**	0.1	-0.02	0.11	-0.15	-0.16
Taurochenodeoxycholic acid	-0.14	0.01	0.02	0.13	0.24*	0.08	0.23*	0.08	-0.03	0.11	-0.16	-0.14
Deoxycholic acid	0.02	0.06	0.08	0.09	-0.11	-0.17	0.08	-0.09	0.07	-0.02	-0.03	-0.22*
Glycodeoxycholic acid	-0.25*	0.09	0.01	0.11	0.16	-0.06	0.28**	0.1	-0.02	0.11	-0.14	-0.16
Taurodeoxycholic acid	-0.19	0.002	0.09	0.15	0.2	0.06	0.22*	0.08	-0.08	0.14	-0.15	-0.17
Glycolithocholic acid	0.11	0.12	0.16	0.23*	-0.03	-0.08	0.03	-0.14	0.15	-0.05	-0.02	-0.23*
Argine/proline metabolism pathway	-0.17	0.22*	0.17	0.03	0.27*	-0.0002	0.33**	0.22*	0.1	-0.04	-0.03	-0.15
L-arginine	0.08	0.1	0.05	0.01	-0.06	-0.15	0.05	0.07	-0.04	-0.01	0.001	0.23*
Ornithine	-0.19	0.06	0.01	-0.07	0.02	-0.26*	0.44**	0.07	-0.1	0.09	-0.03	-0.18
Citrulline	0.09	0.24*	0.31**	0.01	0.21*	-0.13	0.25*	0.05	0.08	-0.001	0.06	-0.13
Succinic acid	0.03	-0.07	-0.1	-0.1	0.06	0.04	0.37**	0.23*	0.09	-0.12	0.22*	-0.15
4-hydroxy-L-proline	0.01	-0.04	0.05	-0.05	0.05	-0.04	0.09	-0.06	-0.07	0.14	0.1	-0.03
L-glutamic acid	-0.11	-0.03	0.06	0.08	-0.05	-0.02	-0.08	0.03	0.25*	-0.34**	0.12	-0.09
L-proline	-0.14	0.28*	0.22*	0.13	0.24*	0.002	-0.03	0.12	0.2*	-0.02	-0.05	-0.12

Spearman's rank correlations were used and presented as Rho in the table. The first principal component scores were used to represent the pathways. *p<0.05; **p<0.005. Abbreviations: Ca, calcium; Phos, phosphorous; Mg, magnesium; PTH, intact parathyroid hormone; FGF-23, fibroblast growth factor-23; klotho, soluble klotho; dp-ucMGP, Dephosphorylated and uncarboxylated matrix Gla protein; CPP2, secondary calciprotein particle; T_{50}, half maximal transformation of primary to secondary calciprotein particle; CRP, C-reactive protein.

SUPPLEMENTAL FIGURES

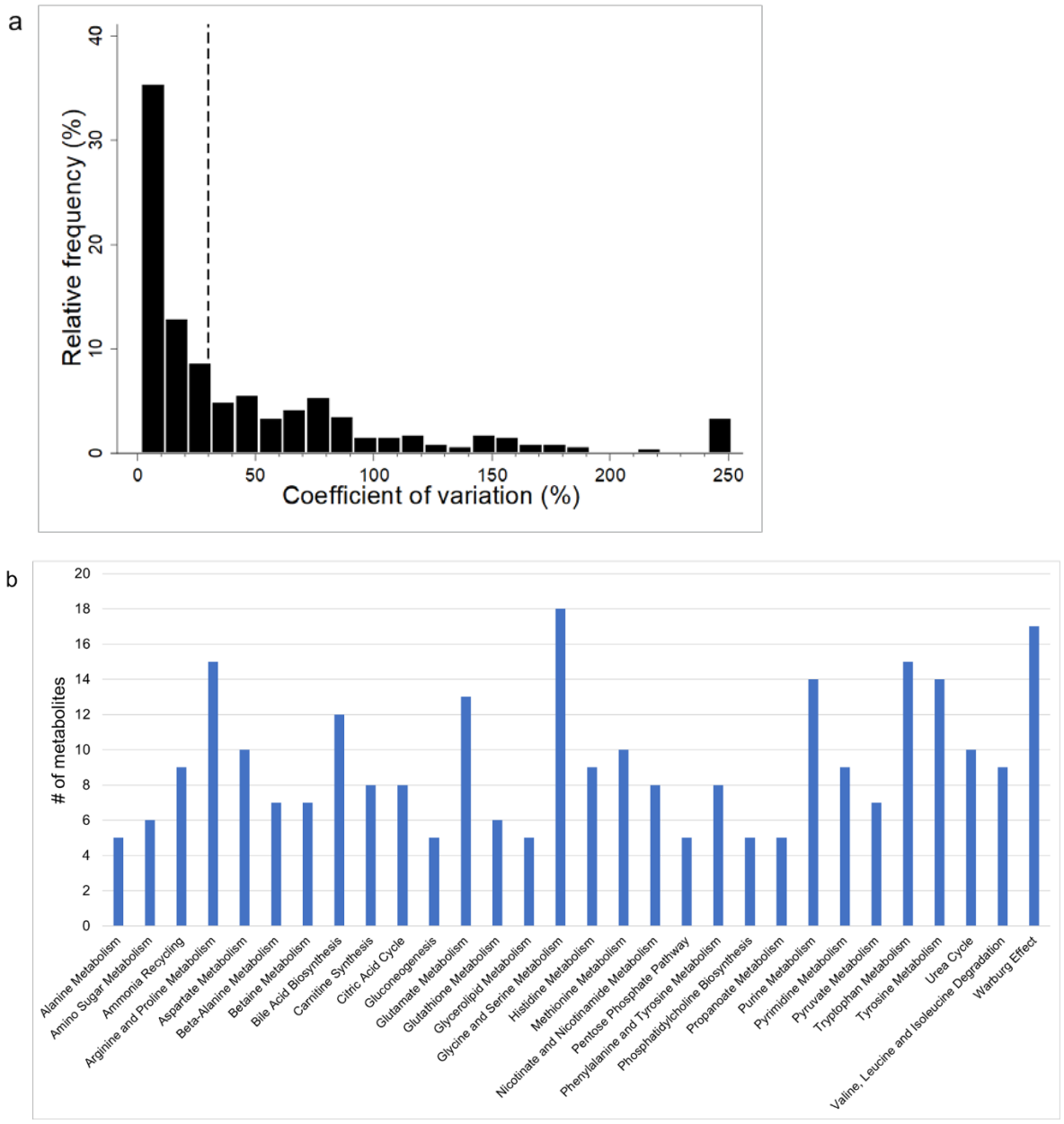


Figure S1. a) Distribution of coefficients of variation (CVs) for all metabolites measured (m=452). Reference line (dash line) is at CV of 30%. Approximately 55% of metabolites (m=247) had CV <30%, and were analyzed. b) Pathways and number of metabolites (m=247) analyzed. Pathways were identified using the Small Molecule Pathway Database. Only pathways that contains at least 5 metabolites analyzed are shown. Some metabolites appear in multiple pathways. The pathways that have the most metabolites measured and analyzed included glycine/serine metabolism, Warburg effect, arginine/proline metabolism, and tryptophan metabolism.

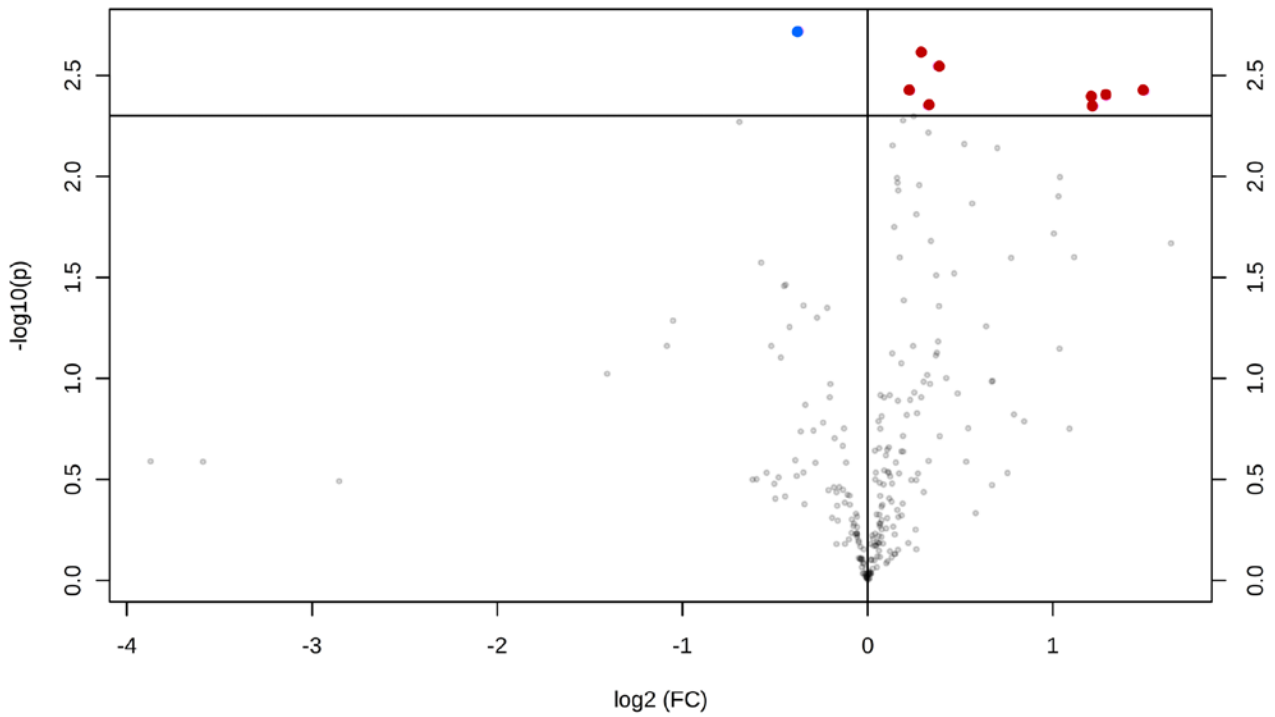


Figure S2. Volcano plot identified metabolites that differed between cases and controls. Compared to controls, cases had 8 metabolites that were higher in relative intensity (red dots) and 1 metabolite that was lower in relative intensity (blue dot). These metabolites and their fold differences are listed in Table S1. Mann-Whitney U tests were used with p-value threshold of <0.005 and a fold difference threshold of 1.0 to obtain approximately 10 metabolites.

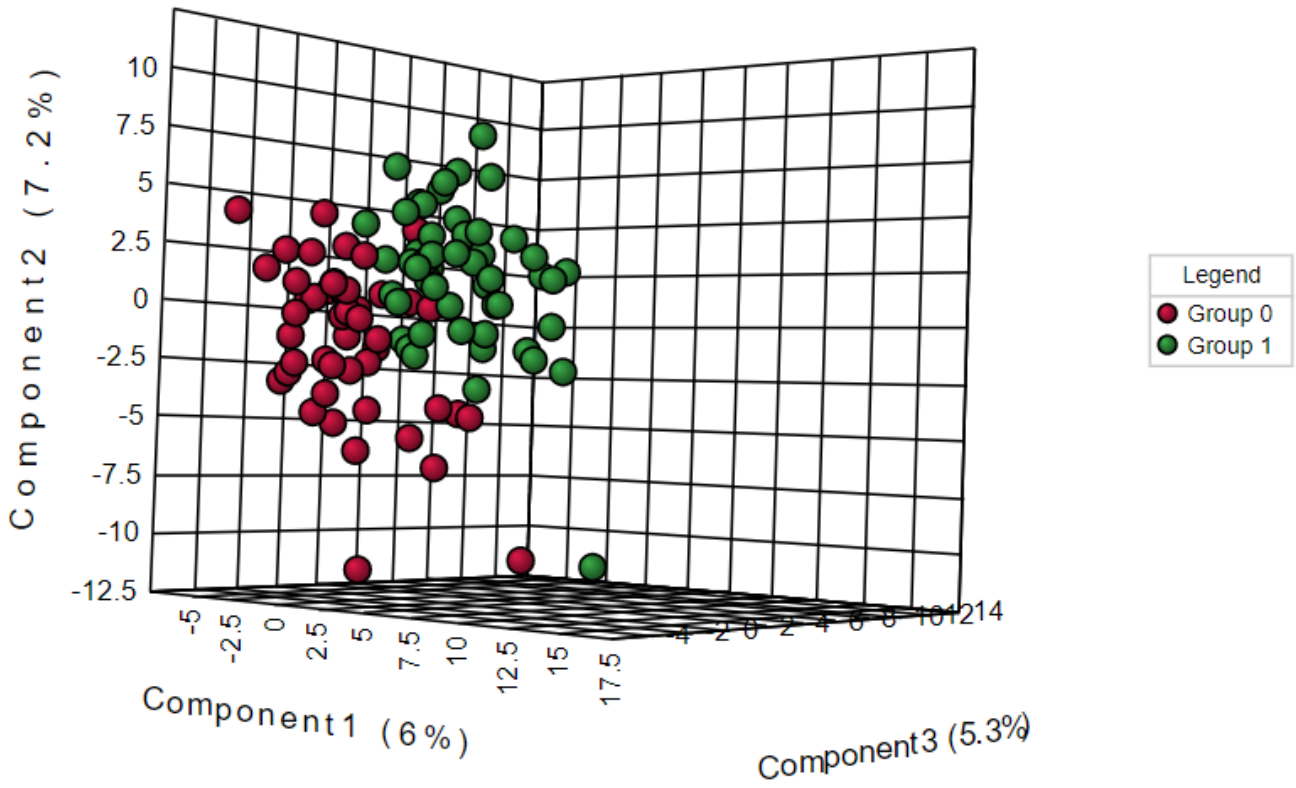


Figure S3. Synchronized 3-dimensional plot of partial least squares-discriminant analyses. Cases are in green and controls are in red.

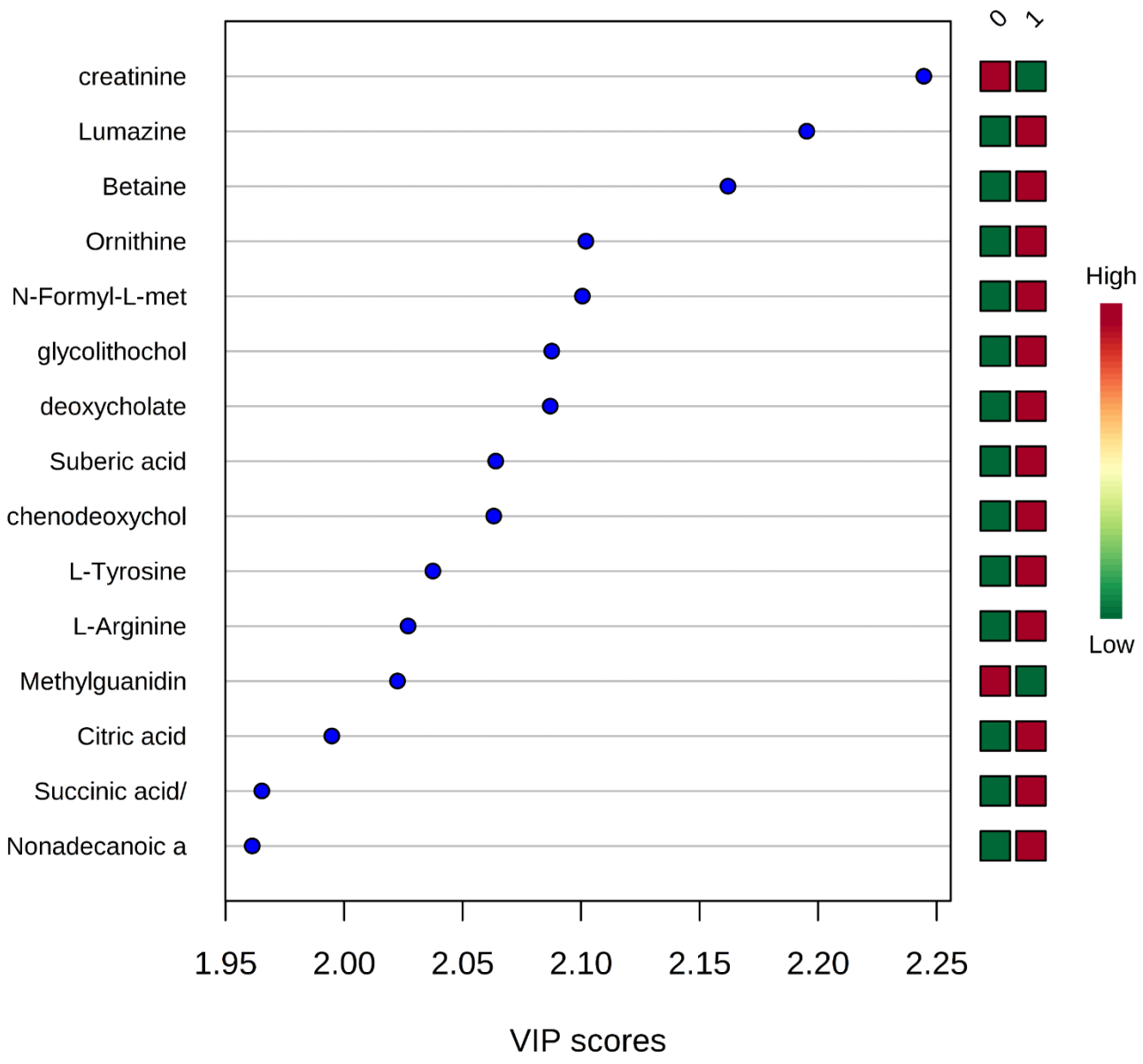


Figure S4. Top 15 serum metabolites listed by their Variable Importance in Projection scores (using the first principal component) using partial least squares discriminant analyses. The colored boxes on the right indicate the relative concentrations of the corresponding metabolites in cases (labeled as 1) and controls (labeled as 0). Red indicates high relative concentration and green indicates low relative concentration. Abbreviation: VIP, variable importance in project.

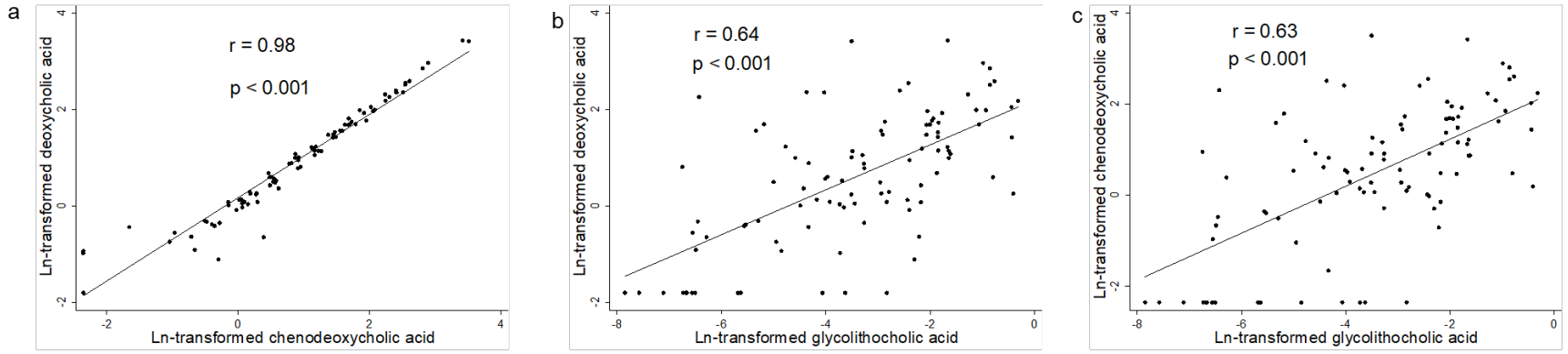


Figure S5. Correlation among 3 bile acids. Bile acids positively correlated with each other. Pearson Correlation was used after log-transformation of bile acid levels.

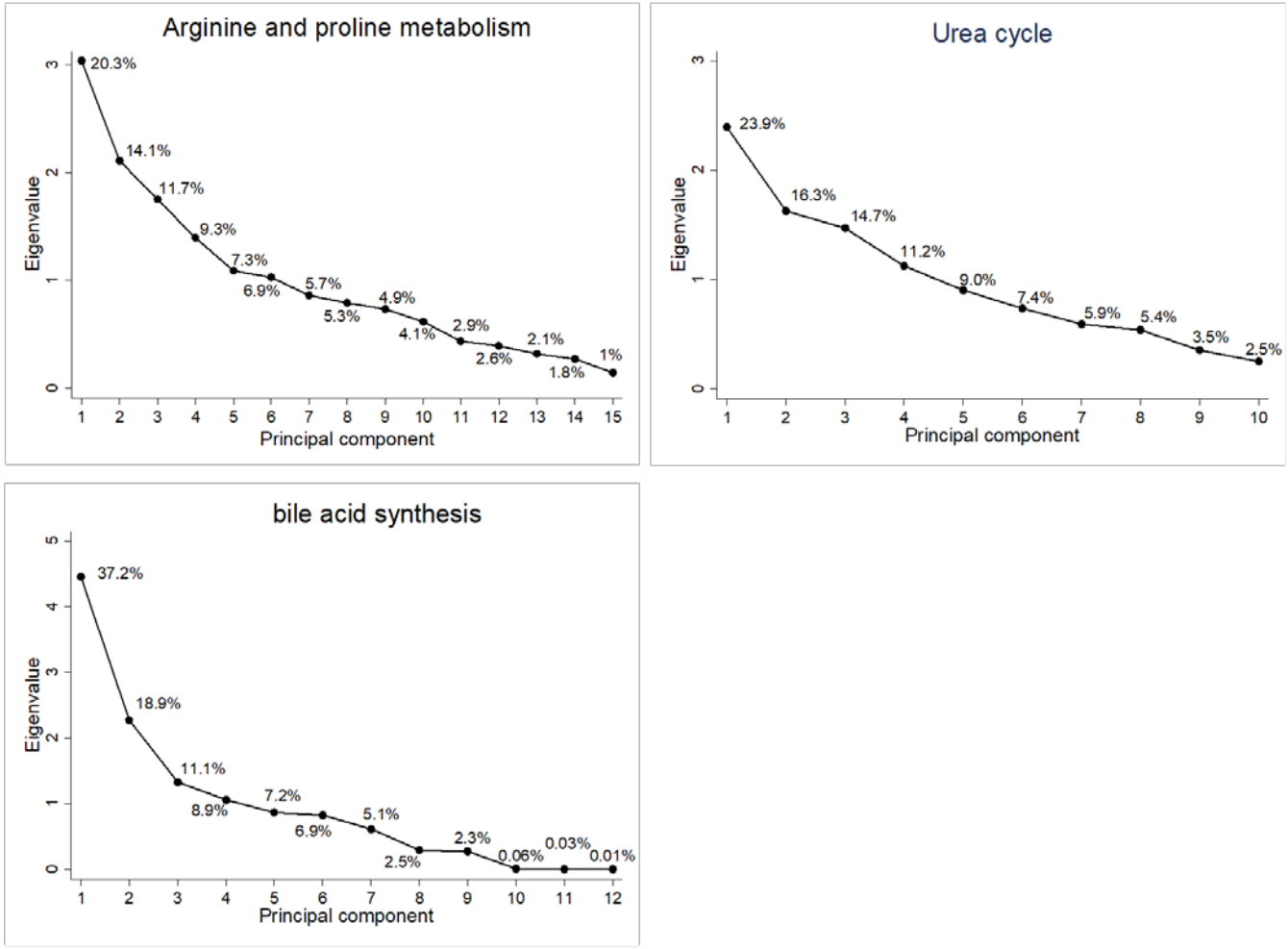
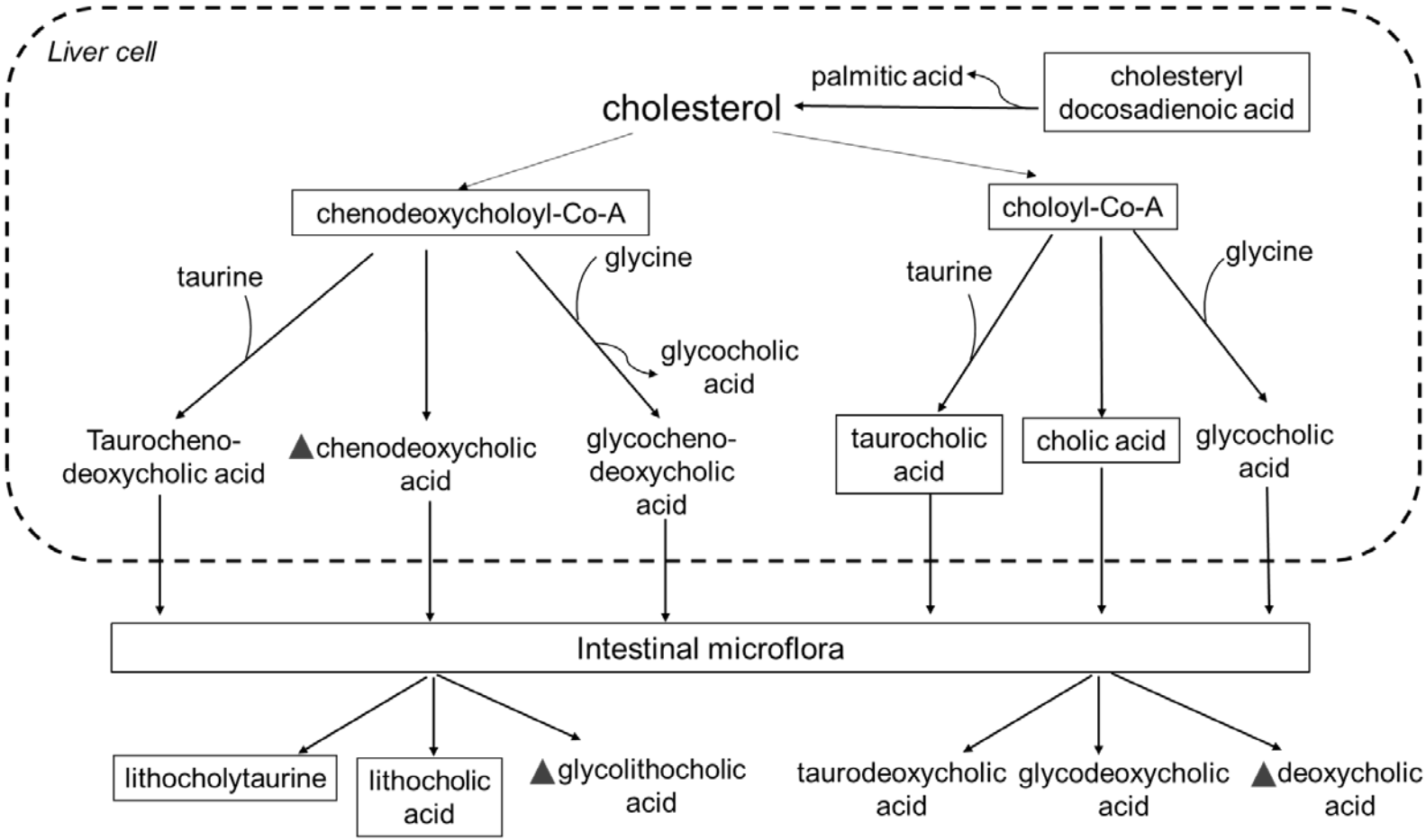


Figure S6. Scree plots of arginine/proline metabolism (m=15), urea cycle (m=10) and bile acid synthesis (m=12). Principal component analysis was used to generate principal component scores of arginine/proline metabolism, urea cycle and bile acid synthesis. X-axis is the principal component number; y-axis is the eigenvalue. Variance explained by individual principal component is annotated on each curve.

a.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taurine
glycine
Taurocheno-deoxycholic acid
chenodeoxycholic acid
glycocheno-deoxycholic acid
taurocholic acid
cholic acid
glycocholic acid
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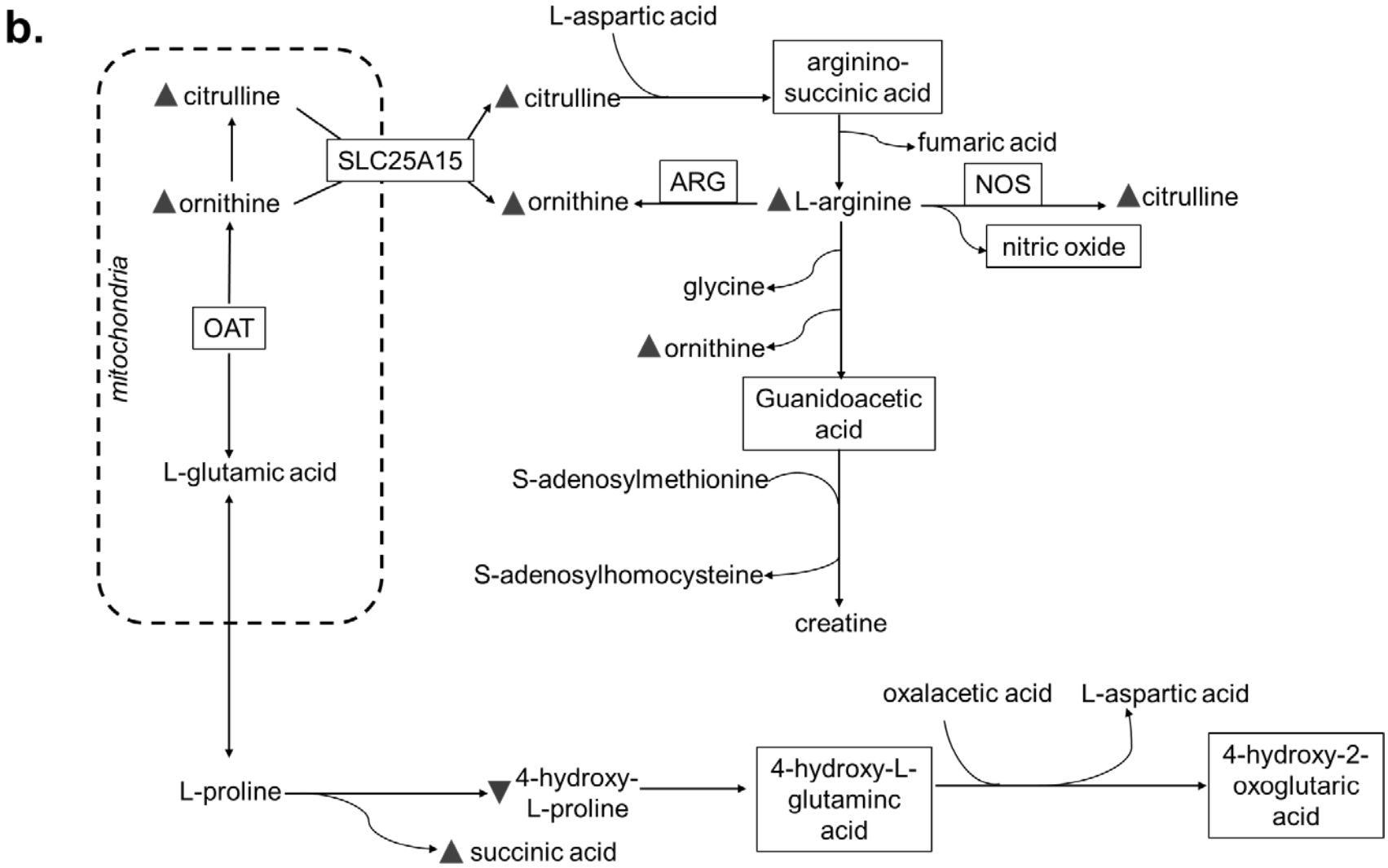

Figure S7. Bile acid synthesis and intracellular arginine and proline metabolism, simplified version (based on the Small Molecule Pathway Database). (a) Bile acid synthesis. Chenodeoxycholic acid is a primary bile acid as it is directly synthesized from cholesterol in liver. After being synthesized, chenodeoxycholic acid is then secreted into bile and intestinal tract, where it is converted by intestinal bacteria to lithocholic acid, which is a secondary bile acid. To increase its solubility and decrease toxicity, lithocholic acid is conjugated with glycine to form glycolithocholic acid. Cholic acid is another primary bile acid. In the intestinal tract, cholic acid is

converted to deoxycholic acid, a secondary bile acid. We found that higher levels of chenodeoxycholic, deoxycholic, and glycolithocholic acids were associated with the presence and severity of CAC. (b) Intracellular arginine and proline metabolism. Arginine/proline metabolism is one of the central pathways for the biosynthesis of arginine and proline from glutamine. We found that arginine/proline metabolism was independently associated with CAC status. Compared to controls, cases had higher levels of L-arginine, ornithine, citrulline, and succinic acid and lower levels of 4-hydroxy-L-proline ($p<0.05$). Assuming intracellular metabolism of these amino acids mirrors their extracellular metabolism and serum levels, their directionality seems to suggest that there was an increased synthesis of L-arginine and decreased metabolism of proline in participants with CAC.

Note: Metabolites that either were not measured or had a CV≥30% are in boxes. The rest are metabolites analyzed. Total cholesterol level was measured separately in fasting blood samples on a non-HD day. Arrowheads (▲▼) indicate the directionality when comparing the cases with controls ($p<0.05$). Abbreviations: OAT, ornithine aminotransferase; SLC25A15, mitochondrial ornithine transporter 1; ARG, arginase; NOS, nitric oxide synthase.

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