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Getting Insights from Oral Metabolomics into Vascular Calcification in Patients with End-Stage Kidney Disease

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Abstract

This study investigated the association between oral metabolomics and vascular calcifi-cation (VC) in patients with end-stage kidney disease (ESKD). Thirty patients were stratified into a high VC group (Kauppila score ≥ 8) and a low VC group (Kauppila score < 8), matched for age, sex, and diabetes status. Differential metabolites were identified using t-tests and VIP scores, with group discrimination assessed by PLS-DA and heatmaps. Biomarker discovery involved uni- and multivariate analyses, and pathway enrichment was performed to explore underlying mechanisms. Correlations between the potential biomarkers and clinic-serologic features were examined. A total of 37 metabolites, in-cluding citrate, citraconic acid, and taurine, exhibited distinct distribution patterns be-tween the two groups. Key pathways included taurine and hypotaurine metabolism, purine metabolism, galactose metabolism, sphingolipid metabolism, phenylacetate metabolism, and β-oxidation of very long-chain fatty acids. Fifteen candidate biomarkers demonstrated strong predictive performance for high VC (maximum AUC = 0.994). Several metabolites were significantly correlated with Kauppila score, partly independent of calcium–phosphate metabolism. These findings suggest that oral metabolites and re-lated pathways are closely linked to VC and oral inflammation in ESKD, highlighting their potential as biomarkers for improved understanding and management of VC in ESKD.

INTRODUCTION

Vascular calcification (VC) is a major contributor to the markedly increased cardiovascular morbidity and mortality observed patients with end-stage kidney disease (ESKD) compared with the general population. Dysregulation of calcium and phosphate homeostasis has long been considered a key driver of VC in ESKD. However, effective strategies to prevent or reverse this process remain limited [1], as increased oxidative stress and chronic inflammation cannot be adequately addressed by managing mineral metabolism alone.

Periodontitis, a chronic inflammatory disease, is highly prevalent among ESKD patients, affecting over 90% of this population [2]. Growing epidemiological evidence suggests that periodontitis contributes to VC [3–5]. Recent animal studies have proposed several potential mechanisms underlying this association, including involvement of the osteoprotegerin (OPG)/RANK/RANKL signaling pathway, endoplasmic reticulum stress, apoptosis, and periodontal pathogens such as Porphyromonas gingivalis [6]. Moreover, emerging data indicate a bidirectional relationship in which periodontitis and VC may mutually exacerbate each other through systemic inflammation and metabolic dysregulation [7]. However, the mechanisms by which this localized oral pathology influences systemic disease processes remain unclear.

In this study, we aim to characterize oral metabolic alterations associated with VC in ESKD patients using metabolomics approaches and to evaluate their correlations with clinical manifestations. Through this investigation, we seek to explore the mechanisms by which periodontitis contributes to or exacerbates VC in ESKD, thereby providing new insights into potential therapeutic targets.

METHODS

Patient Selection and Assignment

Adult patients (age ≥ 18 years) undergoing maintenance hemodialysis or peritoneal dialysis at Beijing Tsinghua Changgung Hospital between March 2023 and August 2023 were screened and enrolled to receive free oral examinations and sample collection unless they met any of the following exclusion criteria: (1) inability to provide informed consent; (2) presence of severe coagulation disorders (INR > 2); (3) concurrent malignant tumors; (4) pregnancy or lactation; (5) active infections other than periodontitis; or (6) edentulous status. The study was reviewed and approved by the ethics committee of Beijing Tsinghua Changgung Hospital. Patients were assigned to either the moderate-to-severe VC group (Kauppila score ≥ 8, high VC group) or the mild-to-no VC group (Kauppila score < 8, low VC group) based on their abdominal aortic calcification scores. Priority was given to matching patients in the high VC group with those in the low VC group according to age, sex, and diabetes status. Of the 51 patients initially enrolled, 30 patients (15 high VC and 15 low VC) were successfully matched and included in the metabolomics analysis. Our study was approved by the Beijing Tsinghua Changgung Hospital Institutional Review Board (Protocol ID: 24117-0-01). The study was conducted in accordance with the Declaration of Helsinki ethical standards. All participants provided informed consent and signed the informed consent form.

Kauppila Score Measurement

Participants underwent abdominal aortic calcification scoring using the Kauppila method. X-ray slides were evaluated by a radiologist and subsequently reviewed by a senior radiologist to ensure accuracy and consistency.

Epidemiological and Clinical Data Collection

Epidemiological and clinical data were collected for all participants, including age, sex, comorbidities (e.g., hypertension, diabetes, heart disease, cerebrovascular disease, and hyperlipidemia), smoking history, alcohol consumption, and duration of dialysis. Laboratory parameters, including serum phosphorus, serum calcium, intact parathyroid hormone (iPTH), alkaline phosphatase (ALP), C-reactive protein (CRP), interleukin-6 (IL-6), procalcitonin (PCT), hemoglobin, glycated hemoglobin, blood lipids, and albumin, were recorded for the three months preceding gingival crevicular fluid collection.

Oral examinations were performed by calibrated periodontists using a standardized protocol. Periodontal pocket depth (PPD) and clinical attachment level (CAL) were measured to the nearest millimeter using a UNC-15 periodontal probe at six sites per tooth (mesiobuccal, mid-buccal, distobuccal, mesiolingual/palatal, mid-lingual/palatal, and distolingual/palatal). Bleeding on probing (BOP) was

assessed within 15 seconds after gentle probing (approximately 0.25 N) and recorded as present or absent. The gingival bleeding index (BI) was evaluated according to established scoring criteria.

For data aggregation, the mean PPD and CAL for each tooth were calculated from the six sites, and full-mouth mean values were subsequently derived for each participant. In addition, the maximum PPD and CAL values were recorded to reflect the most severe periodontal involvement. BOP was expressed as the percentage of bleeding sites relative to the total number of examined sites, and BI was calculated as the mean score across all sites. Missing teeth were excluded from the analysis. This approach allowed for comprehensive assessment of overall periodontal status while preserving sensitivity to localized disease severity.

Collection and Preparation of Gingival Crevicular Fluid (GCF) Samples

GCF samples were collected by professional dentists using sterile filter paper strips at the deepest PD site of every quarter. A total of 8 strips were collected from each subject. The strips were stored in labeled 1 mL EP tubes and immediately placed in liquid nitrogen. Samples were transferred to a -80°C freezer within 4 to 6 hours for long-term storage.

Samples were placed in 2 mL EP tubes and extracted with 300 μL of methanol. The mixture was vortexed for 5 minutes, sonicated for 10 minutes in ice water, and centrifuged at 12,000 rpm at 4°C for 15 minutes. A total of 280 μL of the supernatant was transferred to a fresh 1.5 mL EP tube. The process was repeated with an additional 300 μL of methanol, and the resulting supernatants were combined, vortexed for 30 seconds, and 500 μL was transferred to a fresh 1.5 mL EP tube. The remaining sample was completely dried using a vacuum concentrator. Non-targeted metabolomics analyses were performed using a liquid chromatography-high-resolution mass spectrometry (UPLC-Orbitrap) system. The first four samples (results provided in the Supplementary Material) and the subsequent 26 samples were processed and analyzed independently to minimize potential batch effects.

Data Integration and Analysis

Chromatographic separation was achieved using reverse-phase and amino columns. Data were collected in ddMS2 mode for both positive- and negative-ion analyses. An internal standard (IS) was added to each reconstituted sample to monitor sample preparation consistency and evaluate analytical stability at the individual sample level. Quality control (QC) samples, prepared by pooling aliquots from all study samples, were intermittently injected throughout the analytical sequence to assess the overall stability and reproducibility of the analytical platform.

Metabolomic data were processed and analyzed using the MetaboAnalyst platform and R (version 4.3.2). Data preprocessing included normalization, transformation, and scaling as appropriate. Differential metabolites were identified based on univariate analysis (Student's t-test, $p < 0.05$) combined

with multivariate analysis, with variable importance in projection (VIP) scores > 1 derived from partial least squares discriminant analysis (PLS-DA). The PLS-DA model was applied to evaluate group separation, and model robustness was validated using permutation tests. Both univariate and multivariate approaches were employed for biomarker identification. Pathway enrichment analysis was conducted to identify significantly altered metabolic pathways. Spearman's rank correlation analysis was used to assess associations between metabolite biomarkers and clinical characteristics. P values were adjusted for multiple testing using the false discovery rate (FDR) method (Benjamini–Hochberg). Metabolites with FDR-adjusted P values < 0.05 were considered statistically significant.

RESULTS

Patients' characteristics

A total of 28 hemodialysis (HD) patients and 2 peritoneal dialysis (PD) patients were enrolled. As summarized in Table 1, no statistically significant differences were observed between the high VC group and the low VC group in terms of age, gender, Kt/V, smoking status, dialysis vintage, comorbidities (e.g., diabetes, hypertension, coronary artery disease, cerebrovascular disease, etc.), or serum levels of calcium, magnesium, and phosphate. Notably, parameters such as pocket depth—a marker associated with periodontitis—and iPTH levels were significantly different between the two groups, apart from the Kauppila score ($P < 0.05$). Furthermore, although differences in valvular calcification rates and C-reactive protein (CRP) levels were observed between the groups, these differences did not reach statistical significance. Among the 30 patient samples, 4 were used in a pilot experiment (the results of which are provided in Supplementary Table 1 and Supplementary Fig. 1), and the remaining 26 were included in the final analysis. A detailed comparison of the clinical characteristics between the high VC and low VC groups in the final cohort is presented in Supplementary Table 2.

Table 1
Comparisons of Clinical Characteristics Between high and low VC groups

Factor	Low VC	High VC	p-value
N	15	15	
Age, mean (SD)	64.2 (8.4)	65.1 (6.6)	0.74
Male, n (%)	11 (73%)	12 (80%)	0.67
Kappila score, mean (SD)	3.0 (2.3)	14.9 (4.7)	< 0.001***
Valvular calcification, n (%)	7 (47%)	12 (80%)	0.058
Kt/V, mean (SD)	1.4 (0.2)	1.4 (0.3)	0.68
Alveolar bone resorption, mean (SD)	0.5 (0.1)	0.5 (0.2)	0.59
Bleeding index, mean (SD)	4.0 (0.0)	4.0 (0.0)	
Pocket depth (mm), mean (SD)	5.5 (0.6)	6.3 (1.3)	0.031*
Attachment loss, n (%)	15 (100%)	13 (100%)	
Tooth mobility, mean (SD)	2.5 (0.7)	2.3 (0.5)	0.63
Smoking, n (%)	8 (67%)	7 (58%)	0.67
Dialysis vintage (m), mean (SD)	57.3 (55.8)	56.9 (34.6)	0.98
Diabetes, n (%)	8 (53%)	11 (73%)	0.26
Hypertension, n (%)	15 (100%)	15 (100%)	
Coronary artery disease, n (%)	6 (40%)	5 (33%)	0.70
Cerebral vascular disease, n (%)	1 (7%)	4 (27%)	0.14
Magnesium, mean (SD)	1.1 (0.2)	1.1 (0.1)	0.89
Calcium, mean (SD)	2.2 (0.2)	2.1 (0.2)	0.36
Phosphate, mean (SD)	1.9 (0.6)	2.1 (0.6)	0.26
Intact parathyroid hormone, mean (SD)	189.2 (76.5)	380.3 (193.7)	0.001**
Hemoglobulin, mean (SD)	110.8 (8.1)	114.8 (10.5)	0.25
Albumin, mean (SD)	41.5 (2.8)	41.1 (2.1)	0.68
Ferritin, mean (SD)	284.3 (155.3)	269.1 (154.1)	0.79
C-reactive protein, mean (SD)	2.6 (1.8)	7.4 (8.9)	0.066

*P < 0.05, **P < 0.01, ***P < 0.001; Kt/V stands for dialysis dose.

Factor	Low VC	High VC	p-value
β2-microglobin, mean (SD)	28.1 (10.5)	29.4 (7.8)	0.72
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Kt/V stands for dialysis dose.			

Differential metabolites exploration

Using a non-targeted metabolomics approach, a total of 104 metabolites were identified. Following normalization by the sum of intensities, a t-test was performed to compare the levels of metabolites with a variable importance in projection (VIP) score greater than 1 between the two groups. As listed in Table 2, 37 metabolites were noted as differentially distributed between the two groups among which taurine was noted in both positive and negative ion analyses ($P < 0.05$).

Table 2
The list of 37 differential metabolites between high and low VC groups

Citrate	3-Sulfinoalanine
Citraconic Acid	3-Hydroxy-3-Methylglutarate
Maltotriose	N-Lauryldiethanolamine
N-Arachidonoyl Glutamic Acid	Caproylcholine
Inosine 5'-Phosphate	Sphinganine
Uridine 5'-Diphosphoglucose	Oleamide
4-Hydroxyphenylacetate	6-Hydroxyhexanoic Acid/Butoxyacetic Acid
Benzoic Acid	Inosine 5'-Monophosphate (IMP, Inosinic Acid)
PI(18:1_20:4)	2-Phenylglycine
PI(18:0_20:4)	Hexaethylene glycol
Lauric Acid	Melamine
PI(16:0_20:4)	1H-Indole-4-Carboxaldehyde
Phenylacetylglutamine	Trimethylamine N-Oxide(TMAO)
P-Cresol sulfate	Inosine
3α,7α,12α-Trihydroxy-5β-Cholestanoic Acid	Arachidonoylcholine
o-Cresol	Adenine
N-Palmitoyl Taurine	Mevalonic Acid
Taurine	Taurine
Tyr-Val	1-Methylnicotinamide

The heatmap demonstrates distinct patterns of metabolite distributions between the case and control groups (Fig. 1). In the sPLS-DA analysis conducted using the key metabolites, the cases were effectively separated from the controls, particularly along the top five components, with components 1 and 2 contributing the most to the differentiation (Fig. 2).

Pathway analysis of the differential metabolites

Pathway enrichment analysis was conducted using the KEGG database to identify metabolic pathways associated with the 37 differential metabolites. The top 25 enriched pathways are shown in Fig. 3a. Notably, taurine and hypotaurine metabolism, purine metabolism, galactose metabolism, sphingolipid metabolism, phenylacetate metabolism, and the beta-oxidation of very long-chain fatty acids emerged as the most significantly enriched pathways, characterized by either high enrichment ratios or the involvement of a substantial number of metabolites. Hierarchical clustering heatmaps (Fig. 3b) further highlight the distribution patterns of these metabolites across the identified pathways.

Biomarker Analysis

In the single-factorial biomarker analysis, 15 differential metabolites were identified, each exhibiting an area under the curve (AUC) greater than 0.8 for predicting high VC versus low VC. The predictive performance of these metabolites for moderate to high Kauppila scores is summarized in Supplementary Table 3. Pearson's correlation analysis demonstrated that the selected biomarkers were significantly correlated with CKD-MBD parameters, inflammatory mediators, and periodontal disease indicators (Fig. 4). Among the Kauppila scores, N-Lauryldiethanolamine, caproylcholine, sphinganine, citrate, oleamide, and citraconic acid showed positive associations ($P < 0.05$), while benzoic acid, butoxyacetic acid, and N-arachidonoyl glutamic acid exhibited negative correlations. More importantly, 8 metabolites remained correlated with the Kauppila score after adjusting for calcium, phosphate, and iPTH (see Supplementary Table 4). Similarly, caproylcholine, citrate, oleamide, Tyr-Val, and citraconic acid demonstrated consistent patterns of association with ABR levels. Furthermore, caproylcholine, sphinganine, oleamide, and hexaethylene glycol were significantly correlated with iPTH levels. Associations with CRP levels were observed for caproylcholine, citrate, N-arachidonoyl glutamic acid, and citraconic acid, with citraconic acid and N-arachidonoyl glutamic acid also showing correlations with serum albumin levels. Additional notable correlations were identified, including benzoic acid and butoxyacetic acid with hemoglobin levels, benzoic acid with dialysis vintage, and hexaethylene glycol with age. All reported correlations had P values < 0.05, underscoring their statistical significance. The metabolites involved in the top two pathways, each encompassing more than one detected metabolite, included taurine, 3-sulfinoalanine, inosinic acid, inosine, and adenine. These metabolites were subsequently subjected to correlation analyses (Supplementary Fig. 2). The results revealed that taurine, 3-sulfinoalanine, and inosine were all positively correlated with PPD ($P < 0.05$), while adenine exhibited a negative correlation with iPTH ($R^2 = -0.45$, $P = 0.020$).

As illustrated in Supplementary Fig. 3, combining multiple metabolites did not increase AUC, since a single metabolite, N-Lauryldiethanolamine, had already achieved a high AUC of 0.994.

DISCUSSION

This study represents the first investigation into the oral metabolomic profile associated with VC in patients with ESKD. The findings demonstrate that oral metabolites are associated with the severity of VC in ESKD patients and well correlated with calcium-phosphate metabolism, nutrition status, as well as periodontal inflammation. Moreover, these metabolites exhibit a strong discriminative ability to differentiate between varying degrees of VC, highlighting their potential as novel biomarkers for VC in chronic kidney disease (CKD). Multiple metabolites remained significantly associated with the calcification score after adjustment for calcium, phosphate, and iPTH, supporting a potential link between the oral cavity and VC that is independent of calcium–phosphate metabolism in patients with ESKD.

Several metabolites and their associated pathways identified in our study have been linked to both vascular and periodontal diseases, underscoring their potential role in mediating the interaction between these conditions. Bezamat et al. previously investigated the overlapping metabolic pathways between oral diseases, such as dental caries and periodontitis, and atherosclerotic cardiovascular disease (ASCVD) in 552 participants. Their findings highlighted sphingolipid metabolism as a common pathway between these diseases, which aligns with our study, where sphingolipid metabolism was notably observed in gingival crevicular fluid[8]. Sphingolipids are integral components of plasma membranes, influencing cell signaling, protein folding, and endoplasmic reticulum (ER) stress responses[9]. A key sphingolipid, sphingosine-1-phosphate (S1P), modulates ER stress through its synthesis, degradation, and receptor interactions, with intracellular levels differentially impacting stress responses. Given the critical role of sphingolipid metabolism in systemic diseases, its modulation has shown therapeutic potential for managing conditions such as nonalcoholic fatty liver disease, diabetes, Alzheimer's, Parkinson's, and cancer by targeting ER stress pathways.

Lipid metabolism disorders and oxidative stress are pivotal contributors to pathological calcification within vascular cells, significantly increasing the risk of cardiovascular diseases. In our study, several fatty acids and derivatives, including caproylcholine, 6-hydroxyhexanoic acid, mevalonic acid, and oleamide, were identified. These molecules have diverse roles in cell signaling and calcium regulation. For instance, in plants like cotton, caproylcholine levels increase under low vitamin K stress, which damages cell membranes and disrupts protein metabolism[10]. Similarly, oleamide modulates intracellular calcium homeostasis by inhibiting sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), thereby regulating SERCA activity[11]. Mevalonic acid interacts with osteogenesis and calcium transport through the metabolite/TRPM2/calcium signaling axis, suggesting potential implications for vascular health[12,13].

Of particular interest in our study, however, was taurine and hypotaurine metabolism, which exhibited the most significant effects among the pathways analyzed. Taurine and 3-sulfinoalanine, key metabolites

within this pathway, were found to be associated with periodontitis severity and Kauppila scores among our patients. Previous research has demonstrated taurine's pivotal role in calcium homeostasis and vascular health. By regulating calcium signaling through modulating channels, transporters, and intracellular flux, taurine prevents calcium overload—a crucial factor contributing to VC[14]. In addition to its regulatory effects, taurine provides antioxidant and anti-inflammatory benefits by scavenging reactive oxygen species (ROS) and mitigating oxidative stress, which are tightly linked to CKD-associated VC[15]. Furthermore, taurine inhibits inflammatory pathways such as NF-κB activation and cytokine release, thereby alleviating vascular damage and reducing calcium deposition[2,16]. Preclinical studies have revealed that taurine supplementation improves mitochondrial function, reduces oxidative damage, and restores vascular elasticity while decreasing calcium deposition[16]. Beyond its vascular benefits, taurine enhances endothelial function and lipid metabolism, which are vital for maintaining cardiovascular health[17]. Interestingly, recent evidence suggests that taurine, in combination with cannabidiol (CBD), synergistically reduces inflammatory markers and bone resorption in experimental models of periodontitis, highlighting its therapeutic potential in managing periodontal inflammation[18]. Additionally, N-chlorotaurine, a taurine derivative, has demonstrated antimicrobial activity against dental plaque bacteria, further supporting its use as an adjunctive treatment for periodontitis[19]. Collectively, taurine and related metabolites hold significant promise for treating VC by addressing underlying mechanisms of periodontitis, warranting further investigation.

Conversely, melamine, a known exogenous compound, was found to be positively associated with aortic calcification and alveolar bone resorption. Given its established role in inducing oxidative stress, renal injury, and disturbances in calcium metabolism, this association may reflect environmental or dietary exposure rather than endogenous metabolic alterations[20].

Dysregulated gut microbiota has emerged as a critical mechanism influencing VC, with microorganisms such as *Bacteroides* and *Prevotella* implicated in this process. Innovative strategies, such as bioengineered prebiotic-containing microspheres, have shown potential in reducing calcification in CKD rat models[21]. Furthermore, dietary inflammation driven by gut microbiota imbalance is a key driver of VC, with evidence supporting the benefits of probiotic therapies to improve gut health and mineral balance in animal studies[22,23]. Plant-based diets may help regulate gut microbiota composition and reduce VC risk in CKD patients[24]. Li et al. integrated Mendelian randomization, single-cell RNA sequencing, and bioinformatics to reveal causal relationships between the gut microbiome, immune regulation, and osteoporosis, identifying USP6NL, SELENOT, and TAF1A as novel therapeutic targets[25]. These findings underscore the intricate interplay between the gut microbiota, bone metabolism, and vascular health. The oral microbiota, a highly diverse microbial community, further contributes to VC via inflammatory responses, bacterial components, oxidative stress, and systemic effects. Although correlations between oral metabolites and VC were identified in our study, the relationship between these metabolites and the oral microbiota remains to be elucidated through integrated microbiome research.

This study has several limitations. First, it was conducted at a single center with a relatively small sample size, which may limit the generalizability of the findings. Second, the absence of validation using blood metabolite profiles restricts the ability to confirm the systemic relevance of the observed associations. Third, oral specimens such as gingival crevicular fluid are susceptible to multiple confounding factors. Although we attempted to minimize these influences through careful participant selection and standardized sampling procedures—including pre-sampling cleaning, consistent sampling sites, and controlled absorption times—residual variability cannot be completely excluded. In addition, the lack of a healthy control group limits the ability to define a normal oral metabolic profile. Future studies incorporating healthy controls, as well as integrating microbiomes and radiomics approaches, may provide a more comprehensive understanding of the relationship between oral metabolites and VC.

CONCLUSION

Oral metabolites and their associated pathways are strongly interconnected with both VC and oral inflammatory status in patients with ESKD. These findings lay a crucial groundwork for future investigations to elucidate the underlying mechanisms and advance the development of precise therapeutic strategies.

Declarations

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Author contributions: WW conceived the study and wrote the paper. WW and LW were involved in sample collection. XL was involved in metabolomics testing. WW, LW, XL, JL, and YL were involved in the study design, data analysis, and editing of the manuscript. All authors contributed to the article and approved the submitted version.

Data Availability Statement: The data used or analyzed in the study are available from the corresponding author upon reasonable request.

Conflict of interest: None.

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Compliance with Ethical Standards: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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Figures

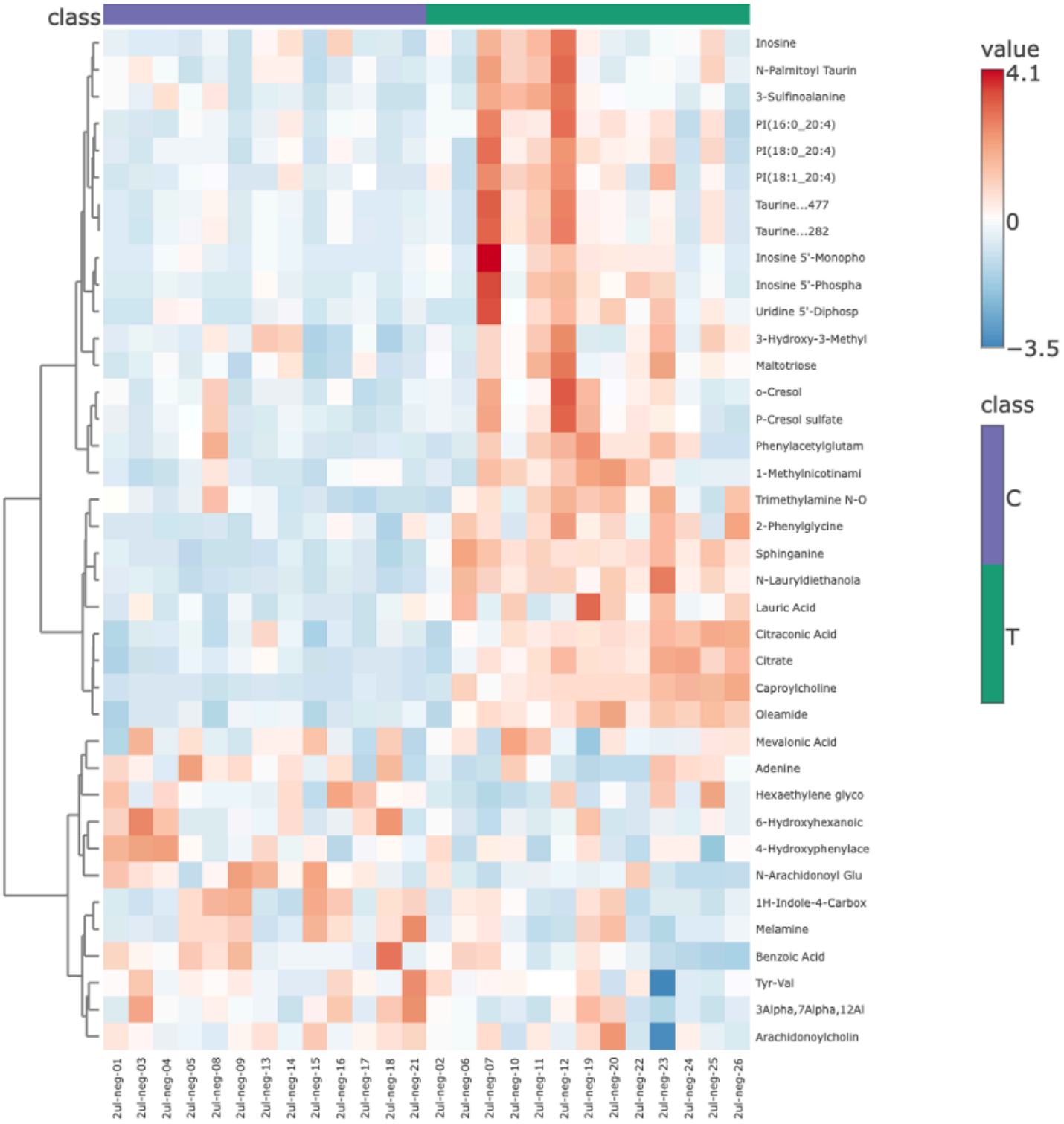


Figure 1

Hierarchical Clustering Heatmaps illustrating the distribution of differential metabolites in high VC (T) and low VC (C) groups

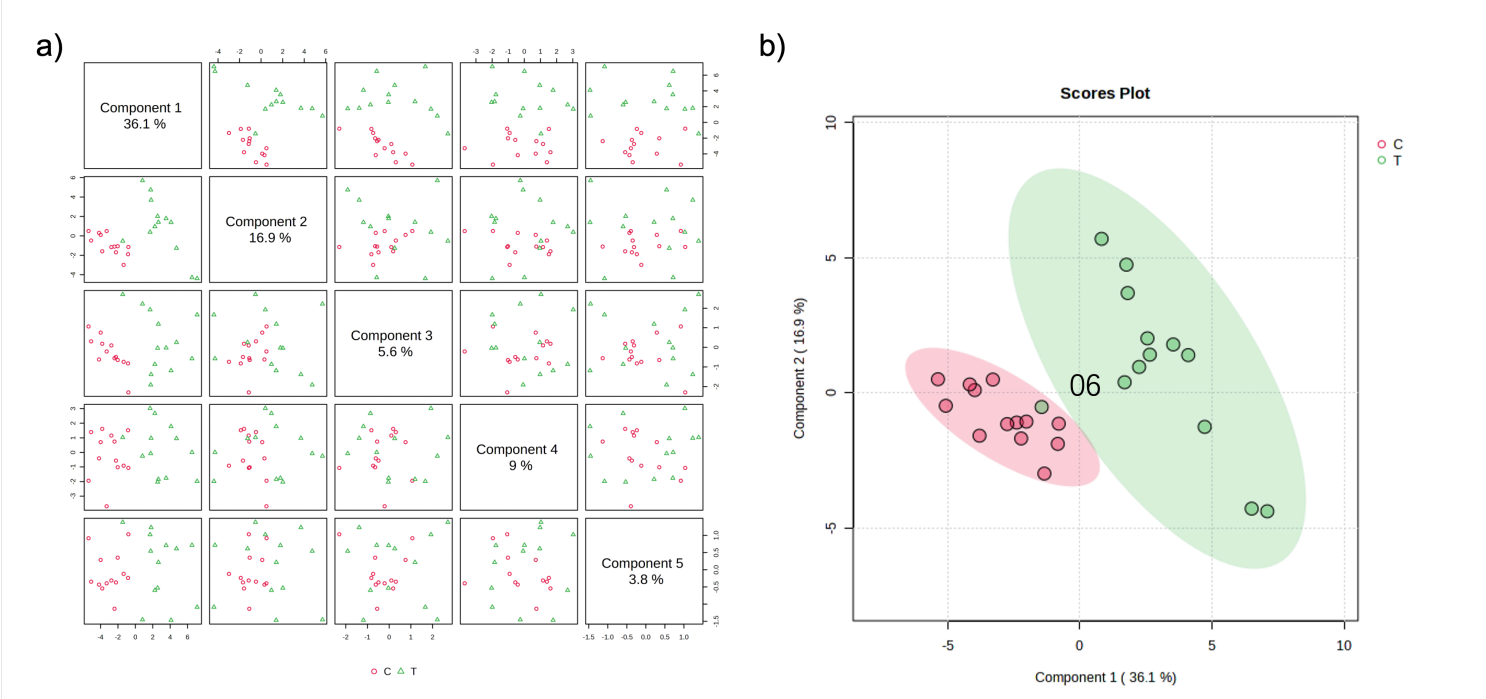


Figure 2

sPLS-DA analysis. a) The performance of the top five components in differentiation between high VC (T) and low VC (C) groups; b) The 2D score plot for Components 1 and 2.

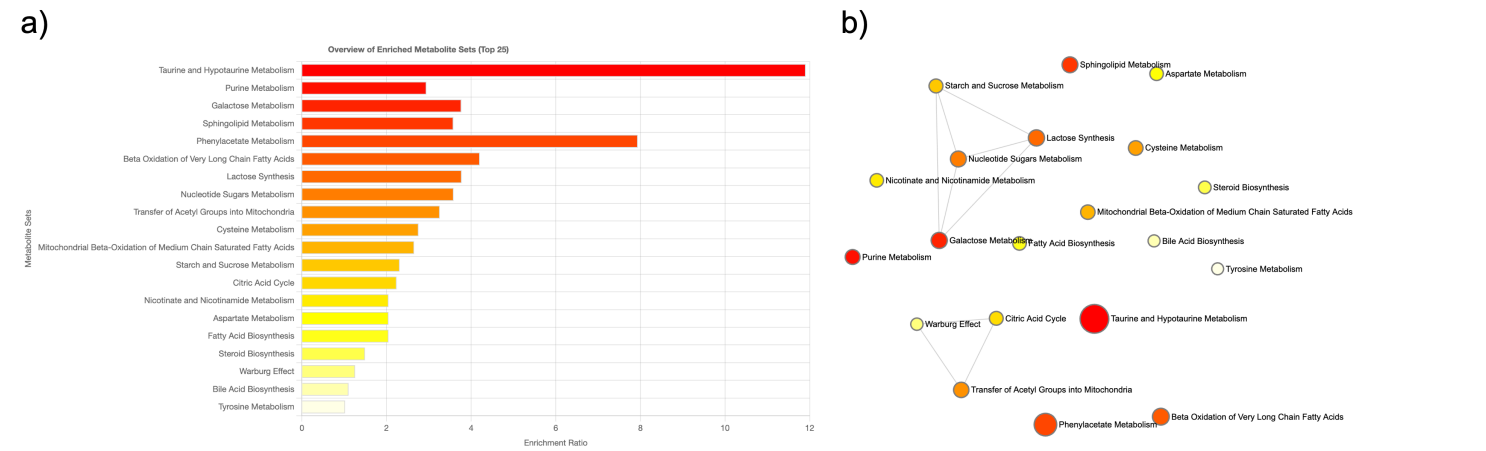


Figure 3

Enrichment Analysis of the 37 differential metabolites. a) The top 25 enriched pathways; b) Hierarchical clustering heatmaps for the pathways.

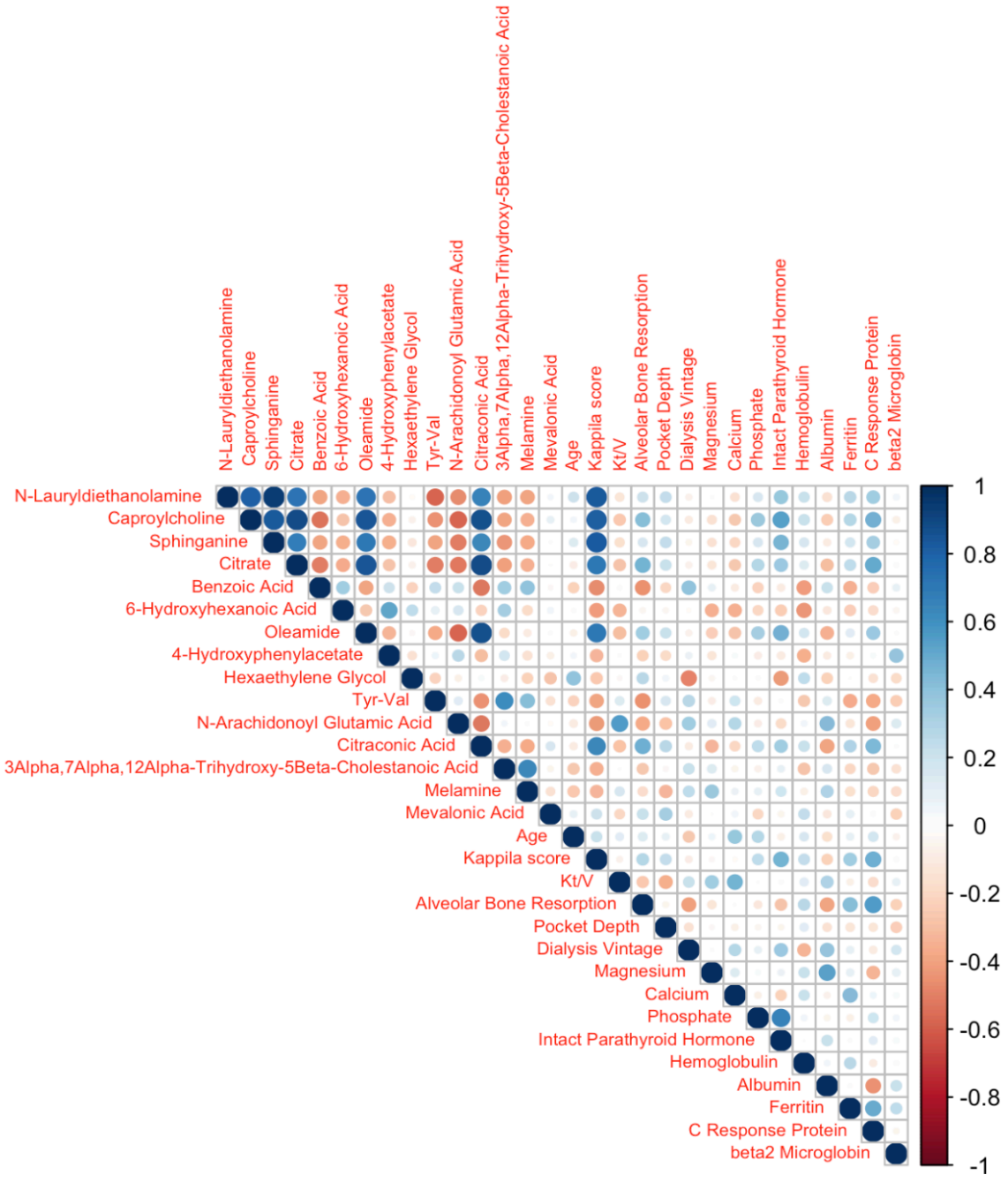


Figure 4

Heatmap for Correlation Analysis Between The 15 Top Biomarkers in Single-Factorial Analysis and Clinical Characteristics.

Supplementary Files

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- Supplementarydata.docx