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Delineating the Role of the Urinary Metabolome in the Lithogenesis of Calcium-Based Kidney Stones

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Abstract

OBJECTIVE—To delineate the role of the urinary metabolome in the genesis of urinary stone disease (USD).

METHODS—Untargeted metabolomics was utilized in comparative analyses of calcium-based stones (CBS) and spot urine samples from patients with a history of USD with or without urinary stone activity based on radiologic imaging. Stone and urine metabolomes were stratified by composition and radiographic stone-activity, respectively. Additionally, we quantified highly abundant metabolites that were present in either calcium oxalate (CaOx) or calcium phosphate (CaPhos) stones and also significantly enriched in the urine of active stone formers (SF) compared to non-active SF. These data were used to delineate either a direct involvement of urinary metabolites in lithogenesis or the passive uptake of biomolecules within the stone matrix.

RESULTS—Urinary metabolomes were distinct based on radiographic stone-activity and the 2 types of CBS. Stratification by radiologic stone activity was driven by the enrichment of 14 metabolites in the urine of active SF that were also highly abundant in both CaOx and CaPhos stones, indicative of a potential involvement of these metabolites in lithogenesis. Using the combination of these 14 metabolites in total, we generated a model that correctly classified patients as either active vs non-active SF in a prospectively recruited cohort with 73% success.

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CONCLUSION—Collectively, our data suggest specific urinary metabolites directly contribute to the formation of urinary stones and that active SF may excrete higher levels of lithogenic metabolites than non-active patients. Future studies are needed to confirm these findings and establish the causative mechanisms associated with these metabolites.

Urinary stone disease (USD) afflicts 1 in 11 Americans and incurs an annual expenditure of approximately $10 billion in healthcare costs.[1] USD is a chronic disease with long asymptomatic periods and high recurrence rate.[2] More than 90% of cases involve calcium-based stones (CBS),[3] most of which are labeled as idiopathic.[4] While a few studies have examined the urinary metabolome in the context of USD,[5,6] many questions remain about the interactions between urinary metabolites and lithogenesis.

Urine is a source of nearly 5000 metabolites, collectively called the metabolome, many of which have been associated with disease.[7] Urinary metabolites can come from the host, microbiome,[7] as well as from the diet or biochemical interactions, and can strongly influence biomineralization.[8] Similarities in urinary chemistry abnormalities exist among patients with different urinary stone compositions. For instance, low urinary citrate is associated with the development of uric acid or CBS.[9] Moreover, a prospective study of stone formers with and without radiographic stones demonstrated that USD patients with different stone compositions exhibited a lower urinary concentration of osteopontin.[10]

We have previously demonstrated significant differences in the urinary metabolome of patients with no history of USD compared to symptomatic USD of diverse composition.[5] However, it is unclear whether the metabolites enriched in the urine of patients with symptomatic USD directly promote lithogenesis, or are just passively absorbed into the stone matrix. Delineation of these hypotheses is important for understanding mechanisms of lithogenesis and can help stratify patient risk levels for recurrence.

To address questions about the lithogenic potential of the urinary metabolome, we sought to: (1) Characterize the non-crystalline metabolome of CBS and urine of SF with or without radiographic stone appearance; and (2) Delineate hypotheses about the influence of urinary metabolites on lithogenesis. We hypothesize that specific metabolites within the urinary tract may facilitate stone formation through the direct interaction with mineralized components present in the stones.

MATERIALS AND METHODS

Project workflow is presented in Fig. S1.

Recruitment of Participants

Population to Delineate Metabolome of CBS.—To identify stone metabolites, surgically-extracted stones were collected, washed and sent for composition analysis with infrared spectroscopy. Only CBS were considered for metabolomics analysis, which represent about 90% of all USD cases, to focus hypotheses while covering the most commonly manifested stone types.[3] Pure CaOx or pure CaPhos stones were used to ensure a clear demarcation in stone composition. No other clinical data was collected.

Population to Delineate Urinary Metabolome of Active vs Non-Active Stone Formers.—Two independent cohorts were recruited for urine specimens to delineate hypotheses surrounding direct and passive metabolite-stone interactions by focusing on patients with a history of USD that visited the Kidney Stone Clinic at Cleveland Clinic to evaluate stone burden by radiographic imaging. These patients either did or did not exhibit radiographic stone activity with imaging. Recruitment of independent populations for the stone and urine analyses allowed for robust hypothesis testing while removing biases associated with individual variabilities in each cohort. The inclusion criteria were patients of both sexes, >18 years old, any ethnicity, history of 1 episodes of USD, any composition of previous calculi, stone free after their latest stone episode (no visible stones on imaging) and patients were to be radiologically evaluated to determine stone activity. Patients without imaging on the same day of urine collection and those with an active urinary tract infection were excluded. For imaging, either ultrasonography or non-contrast enhanced computer tomography (NCCT) was used as requested by the treating physician as part of standard operating procedures. Patients were classified as radiographically active, if stones 4 mm were observed on imaging, or non-active if no stone was observed. This stone size threshold was used to overcome the sensitivity limitation of ultrasonography.[11] Clinical data collected from USD patients were number of previous episodes, method of last episode stone clearance, and time since last USD episode. Given limitations in identifying stone composition based on radiographic imaging, our study is limited to identifying urinary metabolites associated with different types of stones, rather than metabolites specifically linked to CBS. Urine samples were collected and stored based on an established protocol for urine metabolomics research.[12] To minimize diurnal variation and effect of diet on the urinary metabolome, fasting patients were asked to give the first morning, midstream urine samples the same day of clinical follow-up imaging. Urine samples were stored at 4°C, less than 3hours before centrifuging at 14000 RPM for 5 minutes and saving 1-mL of supernatant at −80°C until processing. All procedures were approved by the Institutional Review Board of Cleveland Clinic (IRB# 18–586).

Untargeted Metabolomics.—Urine supernatant and 125 mg of powder-stone samples were diluted 1:4 in a 50% acetonitrile solution containing 2 internal standards, using previously validated protocols.[13] Samples were vortexed to solubilize adherent metabolites and centrifuged at 14,000 g for 5 minutes to precipitate proteins and the supernatant recovered. Supernatant of the stone samples was filtered (0.2 micron) to exclude crystalline components. External standards were added to all samples to ensure run consistency. Negative controls including extraction solutions with added standards were run at the beginning, middle, and end of the run. Untargeted metabolomics was performed on an ultra-high performance liquid chromatography tandem mass spectrometry system coupled to a Q Exactive HF hybrid quadrupole-orbitrap mass spectrometer.

Untargeted metabolomics data is semi-quantitative and requires normalization to a common factor.[13] As such, data from urine samples were normalized to total creatinine quantified through mass spectrometry and data from stones were normalized to total mass used for extraction. Normalized data were analyzed in Metabolyzer software.[14] Spectral features were defined by molecular mass and retention time and given putative identification

by comparison to metabolites in the curated databases KEGG, HMDB, BioCyc, and LIPIDMAPS[14] with validation of identification given by correctly identifying added internal and external standards from mass and retention time. Prior to comparative analyses, metabolites present in negative controls or in fewer than 25% of samples for any one population were removed. Additionally, samples that had concentrations of internal or external standards that deviated more than 1.5 fold from the inter-quartile range for all samples, were removed.

Statistical Analysis

Power analysis to calculate the sample size for the cohort of active or non-active patients for urinary metabolome analysis was conducted in the PWR package (https://cran.r-project.org/web/packages/pwr/index.html) in R statistical software (version 4.1.0; https://www.r-project.org/), using results from a previous urinary metabolome study in patients with or without USD.[5]

The CBS samples were stratified into CaOx and CaPhos groups based on composition analysis. Urine samples were stratified into radiographically active vs non-active. Urine specimens collected for metabolomic analysis were collected, processed and assayed in 2 batches by the same individuals, 1 year apart. The urinary metabolomic data was corrected for batch effects with the BER package (https://cran.r-project.org/contrib/main/00Archive/ber/) in R,[15] which uses a linear regression model that identifies the location and scale of batch effects in the metabolomics data.

To determine if the metabolomes differed by sample type, stone composition, or radiographic stone activity, the dissimilarity of whole metabolome was quantified through a binomial dissimilarity matrix analysis.[16] For stone-urine comparisons, an unweighted analysis, which considers the presence or absence of metabolites, was used given the differences in normalization procedures. For stone-stone or urine-urine comparisons, a weighted analysis, which considers presence or absence of metabolites combined with creatinine-normalized concentration. Dissimilarity between groups was compared by PERMANOVA with 999 permutations, using the Vegan package (version 2.5–7; https://cran.r-project.org/web/packages/vegan/index.html)[17] in R.

To determine the specific metabolites that differentiated each group, a permuted Welch's *t* test was conducted. All *P*–values were corrected via the Benjamini–Hochberg[17] step–up procedure for false discovery rate (FDR) correction. To delineate either the direct interaction of metabolites with the stone matrix vs passive uptake, the metabolites significantly enriched in the urine of active or non-active SF were compared to metabolites that were highly prevalent in CaOx or CaPhos stones, defined as present in >80% of samples for each stone composition.

To further validate the relevance of metabolites, we defined threshold concentration values for the metabolites that were both enriched in either the radiographically active or non-active groups and were highly abundant in CaOx or CaPhos stones. Thresholds were quantified by taking the average concentration of the selected metabolites in each group ± standard error, then finding the middle value between metabolite concentration in the active population

minus standard error and the metabolite concentration in the non-active population plus standard error. Patients with a metabolite concentration above or below the threshold were classified as active or non-active for that metabolite, respectively (Fig. S2). Final classification was based on the majority classifications of the metabolites. These threshold-based predictions were compared to the actual imaging result to determine the error rate between the predicted phenotype and the phenotype based on imaging.

RESULTS

Demographics

A total of 10 CaOx stones and 13 CaPhos stones were assessed. Additionally, 60 patients were recruited for urinary metabolome analyses with 40 patients exhibiting an active stone burden and 20 non-active patients. Power analysis for the comparison of the urinary metabolome between active and non-active patients revealed an 86% probability of detecting a significant difference in these populations if one exists. Clinical data of the patients recruited for metabolomics of urine specimens are presented in Table 1. Groups did not differ by age, biological sex, method of last stone removal, and time since last active episode, minimizing any biases associated with life history or the natural history of the stones. However, there was a significant difference between groups in the diagnostic imaging modality between groups (Table 1).

Urine and Stone Metabolome Composition

Normalized values of raw metabolomic data are provided in Table S1. The processed and filtered spectral features with number of metabolites in each population and putative identification are presented in Table S2. The sample metabolomes differed significantly from negative controls and exhibited significantly greater levels of variance compared to duplicate control samples (Fig. S3). In total, we detected between 2800–4500 urinary metabolites and over 5500 metabolites in the stone matrix (Table S2). The urinary metabolomes, based on specimens collected and processed 1 year apart, exhibited a significant batch effect (Fig. S4a). However, after batch correction in the BER package, batch effects were effectively removed (Fig. S4b). For both the stone and urinary metabolomes, a core metabolome was apparent, whereby most of the metabolites were present in all or nearly all of the samples assessed (Fig. 1A–B).

Metabolomic profiles stratified by stone type and radiographic stone activity status are presented in Figs. 1C and S5. Metabolites commonly present in the urinary metabolome,[18] such as oxalate, acetate, uric acid, and creatinine, were almost universally detected in both the urine and stone samples (Fig. S6). Metabolomes differed significantly by sample type (Fig. 2A; Permanova P-value = .001); stone composition (Fig. 2B; Permanova P-value = .004); and radiographic stone activity (Fig. 2C; Permanova P-value = .002).

The differences in the metabolomic composition of the stone types were driven by 722 metabolites enriched in either the CaOx or CaPhos stones (Fig. 2D). Differences in the urinary metabolome between active vs non-active SF were driven by 187 metabolites

enriched in either group (Fig. 2E). The complete list of significantly different metabolites between groups is given in Table S3.

Interactions Between Urinary and Stone Metabolomes

To help delineate potential interactions between the urinary metabolome and stone burden, we cross-referenced metabolites significantly enriched in the urine of active and non-active SF to those that were highly prevalent in CaOx or CaPhos stones, defined as being present in >80% of stone samples. The 80% threshold was selected to focus only on metabolites strongly associated with the stone matrix. This approach revealed that of the 73 metabolites enriched in active SF, 27 were highly prevalent in CaOx stones and 14 in CaPhos stones. All 14 highly prevalent metabolites in CaPhos stones were also highly prevalent in CaOx stones (Table 2). In contrast, none of the metabolites enriched in the non-active SF were found to be highly prevalent in either CaOx or CaPhos stones (Table 2). Ten of the 14 metabolites that were significantly enriched in the urine of active SF and highly prevalent in CBS were putatively identified (Table 2). To further validate that these specific metabolites could be drivers of stone activity, we developed a predictive radiographic activity assay after establishing threshold values for metabolite concentrations using data from the initial cohort (Fig. S2). With this assay, we predicted radiographic stone activity with 73.3% success rate (Table 2). All of the error in the prediction was generated by falsely classifying active SF as non-active SF.

DISCUSSION

Case-control studies have revealed significant differences in the whole metabolome between individuals with an active episode of USD and those with no history of the disease.[5,6] The current study sought to delineate hypotheses about urinary metabolites present in the stone matrix, as potential promoters of lithogenesis or as passive components absorbed into organic matrix of the the stone. Several metabolomic characteristics distinguish the alternative hypotheses. Under the direct promoter hypothesis, we would expect unique metabolomes between CaOx and CaPhos stones. In contrast, under the passive absorption hypothesis, we would expect no such metabolomic differences. Here we show clear differences in the metabolome of CaOx vs CaPhos stones, which supports the direct promoter hypothesis (Fig. 2B). Interestingly, there were clear differences in the metabolites present in stones of both types and the urine (Fig. 2A). These data may reflect the fact that the urinary metabolome is transient, influenced by day-to-day activities[7] whereas kidney stones are a longer-term record of bioactivity.[19] Importantly, our urine specimens come from fasting patients, eliminating much of the variability.[12]

The second characteristic that distinguishes the direct promoter and passive absorption hypotheses refers to differences in the urinary metabolome of individuals with a history of USD, with or without radiographic stone activity. We observed a significant difference in the urinary metabolome profile based on the radiographic presence of a stone (Fig. 2C). Taken alone, these data suggest that having a stone may impede metabolite excretion through passive absorption into the stone matrix, as a result of differential binding affinities between metabolites and components of the stone matrix. However, these data cannot rule out the

hypothesis that individuals with radiographically active stones have higher concentrations of metabolites that do actively promote lithogenesis, necessitating further analysis.

To validate the passive uptake hypothesis, we compared the metabolites significantly enriched in the urine of active vs non-active SF to those that were highly prevalent in the CBS. With this analysis, under the stone promoter hypothesis, we expect to see an overlap of metabolites enriched in the urine of patients with radiographic activity and those that are highly prevelant in the stones, with the assumption that higher levels of stone-promoting urinary metabolites would facilitate urinary stone development. In contrast, with passive absorption, we would expect that stones will become part of the stone matrix without promoting lithogenesis. This would produce the counter-intuitive result that there would be an overlap in metabolites enriched in non-active SF and those that are highly prevalent in stones. Comparison of metabolites differing between active and non-active SF clearly favored the stone-promoter hypothesis (Table 2), such that there was an overlap of several of the metabolites present in the group with stone activity and the metabolites highly prevalent in stones, but there was no overlap between the metabolites in the non-active group and the metabolites prevalent in stones. These results are in contrast to the interpretation achieved using the urine metabolome data alone, but consistent with the comparative stone metabolome data. Collectively, results suggest that the urine of active SF has a higher concentration of potentially lithogenic metabolites than non-active SF.

Among the potentially lithogenic metabolites identified, 10 were given putative identification. Of these, butanal,[20] N-butanoyl-lhomoserine lactone,[21] and methylpropenyl-ketone[22] have been correlated with pathogenic bacteria activity, supporting a potential role of the urinary microbiome in stone formation.[7] Six metabolites were of apparent host origins. These include the metabolite 6-methylmercaptopurine, which has previously been associated with lithogenic activity.[23] Hexanolglycine has been closely linked with metabolic syndrome.[24] Two of the metabolites are involved in estrogen metabolism pathways, 2-hydroxyestradiol-3-methyl ether, and methylene-dioxycoumestan.[25] Previous work has demonstrated that renal cells treated with estrogen exhibit reduced CaOx crystal binding which may contribute to stone prevention,[26] so weaker estrogenic activity represented by these metabolites could be associated with a diminished protective effect of estrogen. Two additional metabolites were derivatives of arginine, dimethyl-L-arginine and 2-oxoarginine. Dimethyl-L-arginine is a biomarker of proteolysis[27] and high levels are associated with chronic kidney disease, end-stage renal disease, and coronary calcifications.[28] The metabolite 2-oxoarginine is formed during the catabolism of arginine and has been linked to sepsis in rodent models.[29] Ferulate is ubiquitously produced in plants and likely represents some residuals from the diet.[30] Future studies will need to address the causality of the metabolites identified here for stone formation.

Some limitations affect the present study. Some clinical aspects not considered like degree of renal impairment, use of antimicrobials or other medications, could affect the urine chemistry. There was a difference in imaging modality between the active and non-active stone formers. However, biases between these modalities were minimized by adjusting our selection criteria based on stone size. Additionally, active and non-active stone formers were recruited independent of stone composition, since chemical stone composition based

just on radiology is unreliable. Given this limitation, our study could only identify urinary metabolites that generally associate with stones/lithogenesis. A targeted analysis, differentiating patients based on types of stones would identify metabolites associated with stone composition. While the results indicate that the 14 metabolites identified through untargeted metabolomics represent candidate lithogenic agents, the causal relationship of these metabolites for stone formation has not been established and warrants additional studies.

CONCLUSION

The metabolome of CaOx vs CaPhos stones differs significantly, as does the urinary metabolome of radiologically active and non-active SF. Collectively, our data suggest that stone activity may be driven by high levels of lithogenic metabolites in the urinary tract that can potentially promote lithogenesis. Additional studies are needed to validate the lithogenic activities of the 14 metabolites identified here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment.

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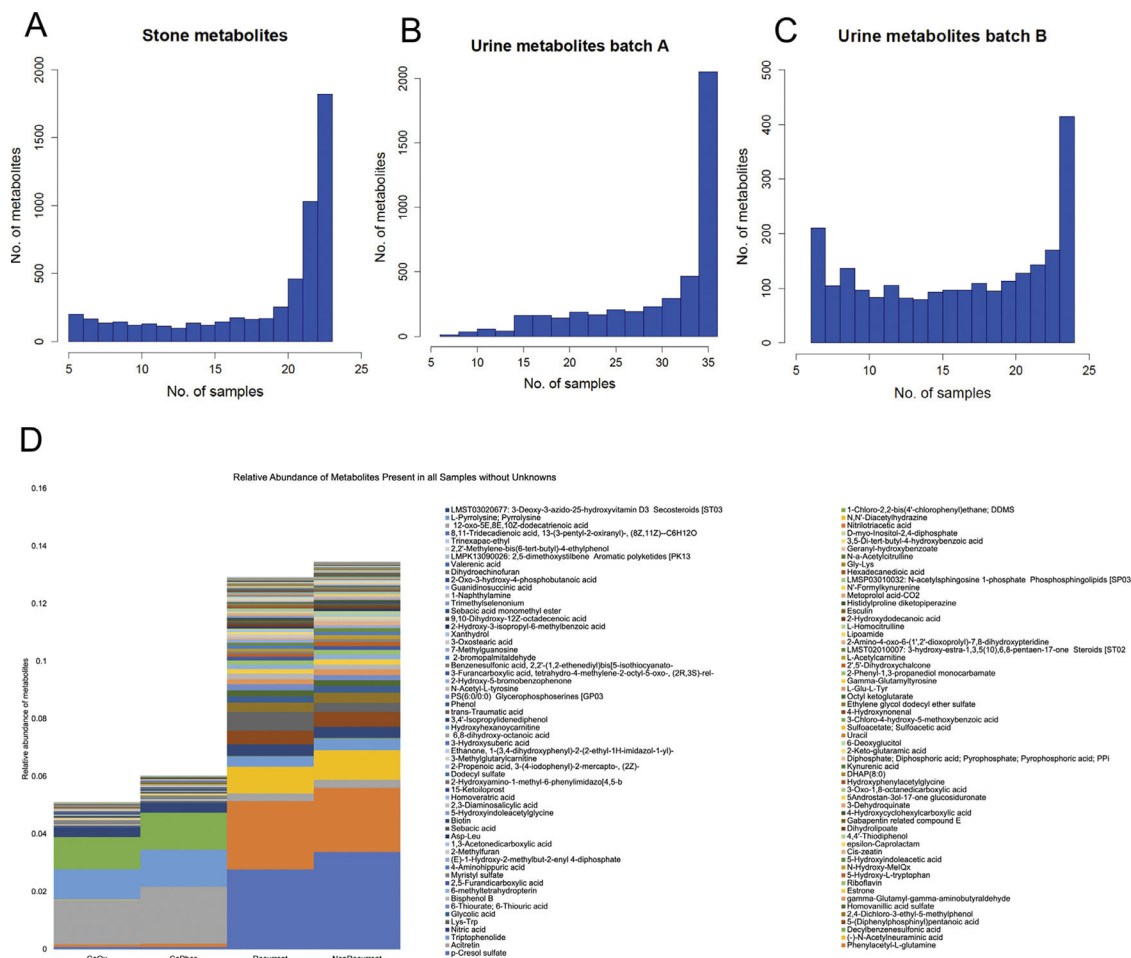
Figure 1.
Distribution of metabolite presence from untargeted metabolomics runs. (A) Metabolites
extracted from CaOx & CaPhos stones; (B) Metabolites extracted from the urine of patients;
Data indicate that most metabolites in stones and urine are broadly distributed among
the population. (C) Metabolomic profile of the kidney stones and urine, stratified by
stone composition and radiographic recurrence status. Metabolites are only listed if they
comprised >0.1% of the metabolome and were given putative ID.
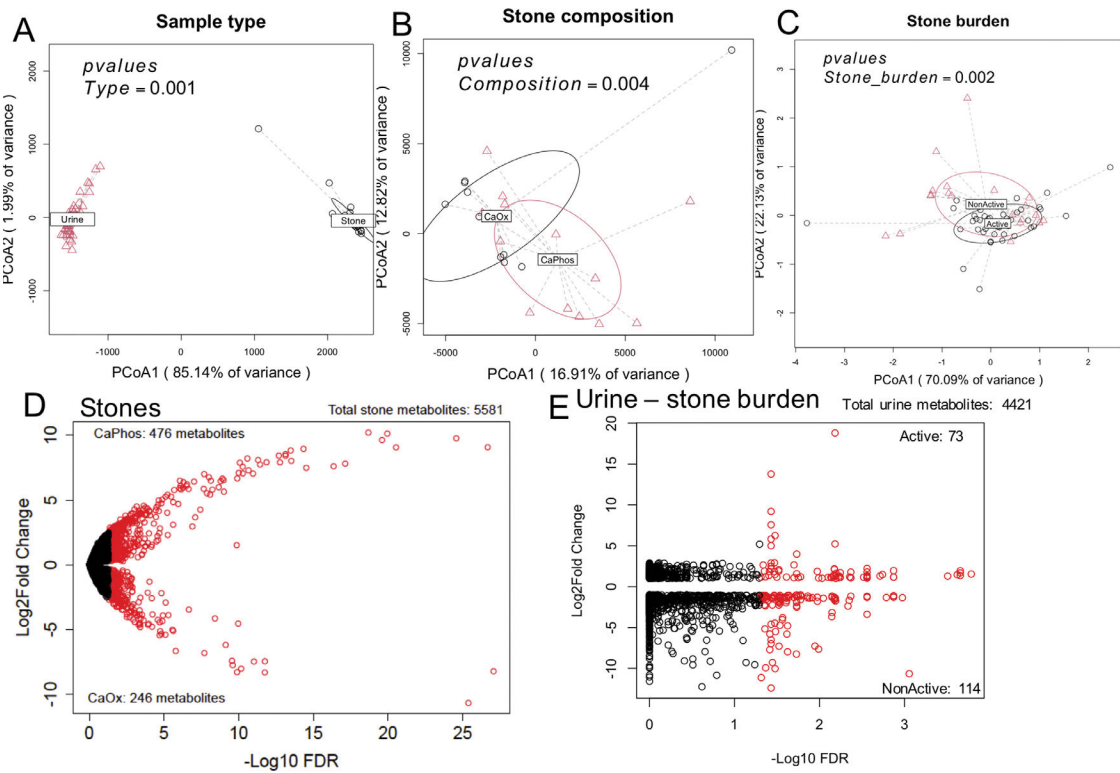
Figure 2.
Whole metabolome comparisons. PCoA plot based on a Binomial distance matrix of urine and kidney stone samples (A), CaOx and CaPhos samples (B), and the urine of active and non-active SF (C). PERMANOVA was used to statistically compare metabolome composition between groups. Differential abundance analysis to identify specific metabolites enriched in CaOx or CaPhos stones (D), or active or non-active SF (E). For differential abundance analysis, an FDR-corrected permuted Welch's *t* test was conducted on mass (stone) or creatinine (urine) normalized metabolite levels. Red dots indicate metabolites that were significantly different between groups, whereas black dots are insignificant metabolites. Listed are the total number of metabolites in the dataset, along with the number of metabolites enriched in either group. Statistical analyses were conducted in Vegan (version 2.5–7; https://cran.r-project.org/web/packages/vegan/index.html) and figures were drawn with R statistical software (version 4.1.0; https://www.r-project.org/).

Table 1.

Demographic and clinical characteristics of patients providing urine speciemens

Patient Characteristics	Active SF	Non-Active SF	*P*-Value	Statistic
No. of patients	40	20	NA	NA
Age +/− SD	58 +/− 12	58 +/− 10	.22	Students *t* test
% Male	50%	60%	.67	Relative risk, Fisher exact text
# of previous stone episodes, median, (IQR)	1.5 (1–2)	1.75 (1–2)	.513	Students *t* test
Mean of last episode stone removal				
Surgically (URS, PNL)	50%	60%	.217	Relative risk, Fisher exact text
Pass stone	50%	40%		
Time since last episode in months, median (IQR)	22(5–59)	24(6–142)	.89	Students *t* test
Imaging modality for actual diagnosis				
% Unenhanced CT scan	65%	20%	<.001	Relative risk, Fisher exact text
% Ultra-sonography	35%	80%		
Stone characteristics				
Stone size, median, (IQR) (mm)	7 (4–17)	NA	NA	NA
Mean number, (IQR)	1.4 (1–3)	NA	NA	NA
Location				
% Renal	87%	NA	NA	NA
% Ureteral	13%	NA	NA	NA
% Unilateral	71%	NA	NA	NA
% Bilateral	29%	NA	NA	NA

IQR = Interquartile Range.

Table 2.

Interaction between stone burden and stone metabolome

Group	Total No. of Significant Metabolites	CaOx (>80%)	CaPhos (>80%)	Both CaOx & CaPhos
Active	73	27	14	14
Non-Active	114	0	0	0

Mass: charge ratio	Retention time	Putative ID
73.07	14.33	Butanal
99.53	17.37	Unknown
100.11	17.28	Unknown
−355.05	1.86	2-Hydroxy-1,3-dimethoxy-8,9-methylenedioxycoumestan
−201.02	10.67	6-Methylmercaptopurine
203.15	12.8	Dimethyl-L-arginine
−170.08	10.64	N-butanoyl-lhomoserine lactone
−238.07	10.64	Unknown
−172.1	11.08	Hexanoylglycine
−210.08	6.91	Unknown
−83.05	2.31	Methyl propenyl ketone
195.07	11.65	Ferulate
−172.07	10.48	2-Oxoarginine
−301.18	8.55	2-Hydroxyestradiol-3-methyl ether

	Active (Predicted)	Non-Active (Predicted)	Group Error
Active (Actual)	24	16	40.00%
Non-Active (Actual)	0	20	0
		Total Error	26.67%

(Top) Number of metabolites significantly enriched in the Active or Non-Active groups that were also present in >80% of either CaOx or CaPhos stones. Numbers for the initial and validation cohorts are separated by the semicolon. (Middle) Mass: charge ratio, retention time, and putative IDs of the metabolites that were both significantly enriched in the urine of Active SF and highly prevalent in both CaOx & CaPhos stones. (Bottom) Results of stone burden classification based on the concentration of 14 metabolites significantly enriched in urine specimens from Active SF and highly prevalent in both CaOx and CaPhos stones. Numbers of patients in rows reflect the numbers in the active or non-active groups.