Journal of Inflammation Research

Dovepress
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ORIGINAL RESEARCH

Identification of Biomarker in Kidney Stone Disease by Integrating Transcriptomics and Olink Proteomics: A Case–Control Study

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Background: Kidney stone disease (KSD) is a prevalent disorder characterized by considerable morbidity and growing healthcare burden. However, the pathogenesis of kidney stone formation remains incompletely understood. This study aimed to explore the gene expression profiles and underlying mechanisms associated with calcium oxalate (CaOx) stone formation, as well as to identify potential urinary biomarkers for CaOx KSD.

Methods: Blood and urine samples were collected from patients diagnosed with KSD and matched healthy controls (HCs). A comprehensive transcriptomic profile of peripheral blood mononuclear cells (PBMCs) was developed utilizing RNA sequencing (RNA-seq). Additionally, the expression levels of 92 inflammation-related proteins in the urine were detected using Olink proteomics.

Results: A total of 1,063 differentially expressed genes (DEGs) were identified between the KSD and HC groups. Gene ontology (GO) and gene set enrichment analysis (GSEA) revealed that production of molecular mediator of immune response, inflammatory response, and oxidative stress were dysregulated in the KSD group. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis indicated that the most DEGs were enriched in extracellular matrix (ECM)-receptor interaction. Eight inflammation-related proteins exhibited significant alterations in the KSD group. Notably, the level of matrix metalloproteinase-1 (MMP-1) demonstrated the greatest fold change and highest mean decrease accuracy in the urine of the KSD group.

Conclusion: Our preliminary findings suggest the molecular mechanisms and functional pathways that might be involved in CaOx kidney stone formation and identify MMP-1 as a potential urinary biomarker associated with CaOx KSD.

Keywords: kidney stone disease, CaOx, transcriptomic, olink proteomics, MMP-1

Introduction

Kidney stone disease (KSD), also known as urolithiasis or nephrolithiasis, is one of the most prevalent urological conditions around the world.[1] The incidence of KSD has been increasing worldwide, with a prevalence of 7.54% in China.[2,3] Notably, KSD is characterized by a high recurrence rate, with a 50% chance of recurrence within 5 to 10 years and a 75% chance within 20 years.[4] Kidney stones can lead to ureteral obstruction, hematuria, severe pain, frequent urinary tract infections, acute kidney injury (AKI) and chronic kidney disease (CKD), culminating in significant impairment of renal function in affected individuals.[5]

Kidney stones can be divided into five types based on their mineralogical composition: calcium oxalate (CaOx; 65.9%), carbapatite (15.6%), urate (12.4%), struvite (magnesium ammonium phosphate; 2.7%), and brushite (1.7%).[6] Regardless of the type, the formation of kidney stones involves multiple steps including crystal nucleation, growth, and aggregation.[7] This complex process is associated with several intrinsic factors (such as genetic background) and extrinsic factors (for example, dietary habits, lifestyle and infections).[8,9] These complexities increase challenges in understanding the mechanisms of kidney stone formation. Experimental studies have suggested that inflammation, immune dysfunction, reactive oxygen species (ROS) accumulation, and oxidative stress play essential roles in kidney stone formation.[10–15]

https://doi.org/10.2147/JIR.S605025
Received: 3 March 2026
Accepted: 19 May 2026
Published: 11 June 2026

Additionally, kinds of programmed cell death, including ferroptosis, apoptosis, and necrosis have been found to participate in the kidney stone formation by molecular experiments.[16–18] Despite these findings, the global gene expression profile and functional alterations in patients with CaOx kidney stones are poorly understood.

Currently, the diagnosis of KSD is primarily based on a combination of clinical history, biochemical evaluation, and imaging techniques, with ultrasound (US) serving as the primary diagnostic tool and low-dose computed tomography (CT) regarded as the gold standard.[19] Patients frequently experience delays until the onset of severe pain or during routine physical examinations to establish a diagnosis. Furthermore, radiation exposure associated with CT makes this modality unsuitable for children, pregnant women, and patients who require repeated examinations. Protein biomarkers are useful for the diagnosis of various diseases.[20–23] Recent scientific advancements have made high-throughput techniques, such as mass spectrometry, a useful tool in protein identification. Nonetheless, these techniques still face challenges when analyzing proteins in complex situations or with limited sample volumes.[20] Olink technology, which employs the proximity extension assay (PEA) for DNA quantification via microfluidic quantitative real-time polymerase chain reaction or next-generation sequencing methods, is particularly advantageous because of its efficacy in small sample volumes.[23–26] Furthermore, Olink proteomics has demonstrated reliable reproducibility and stability for the detection of proteins in urine samples.[27,28] Therefore, Olink has emerged as a promising option for identifying the urinary biomarkers of KSD.

In this study, we used RNA sequencing (RNA-seq) to analyze peripheral blood mononuclear cells (PBMCs) from patients with CaOx kidney stones and matched healthy controls (HCs) to explore comprehensive gene expression profiles and underlying functional alterations associated with CaOx stone formation. Furthermore, we conducted a comparative analysis of inflammation-related proteins in the urine of KSD patients and HCs using Olink proteomics. This study offers preliminary insights into the molecular mechanisms underlying CaOx kidney stone formation and identifies potential urinary biomarkers of CaOx KSD.

Methods and Materials

Study Design and Study Participants

This is a case-control study. This study was approved by the Medical Ethics Committee of the Second Affiliated Hospital of Zhengzhou University (approval number KY2024087) and complied with the Declaration of Helsinki. Fourteen patients diagnosed with KSD were enrolled in this study. The diagnosis of KSD was established through imaging examinations and observations obtained via flexible ureteroscopy, with no evidence of urinary obstruction. Following surgical removal, the composition of kidney stones was determined by infrared spectroscopy analysis.[29,30] Twelve participants with no prior history of kidney stones were enrolled as HCs. Before inclusion, all the participants provided written informed consent.

Sample Collection

Peripheral venous blood was collected from all participants. The PBMCs were isolated according to the manufacturer's instructions (LTS1077-1, Tianjin Haoyang Biological Manufacture Co., Ltd, Tianjin, China). Total RNA was extracted from PBMCs using TRIzol reagent (15596026, Thermo Fisher Scientific, Waltham, MA, USA) and stored at −80 °C for further use. Morning urine samples from all participants were centrifuged at 1,000 × g for 5 min, and the supernatants were stored at −80 °C.

RNA-Seq and Data Processing

RNA-seq libraries were prepared and sequenced by Novogene Bioinformatics Technology Co. Ltd (Beijing, China). Raw reads were processed and mapped to the human (*Homo sapiens*) hg19 reference genome using HISAT2 (version 2.2.1). The mapped reads were then prepared for counting reads using featureCounts (version 2.0.6). Differential expression analysis was performed using the DESeq2 R package (version 1.42.0). The P value was adjusted using the Benjamini-Hochberg method. The threshold for differentially expressed genes (DEGs) was set at $|\log_2(\text{fold change})| > 1$ and adjusted $P < 0.05$. Gene set enrichment analysis (GSEA) was conducted by GSEA application (version 2.2.4). Gene

Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed using clusterProfiler R Package (version 4.8.1).

PEA Platform-Based Urine Proteomics

Urine samples were analyzed using Olink Inflammation Panels, with 92 inflammation-related proteins analyzed simultaneously. Briefly, each target protein binds to the oligonucleotide-labeled antibody, with a complementary DNA barcode. The resulting DNA sequence is subsequently detected and quantified using a microfluidic real-time PCR instrument (Biomark HD, Fluidigm, South San Francisco, CA, USA). The data were presented as Normalized Protein eXpression (NPX) units, which were arbitrary units on a log2-scale. Higher NPX values correspond to higher protein expression levels.[31] All assay validation data (detection limits, intra- and inter-assay precision data, etc). are available on the manufacturer's website (www.olink.com).

Proteomics Data Analysis

Differential expression analysis between KSD and HC groups was evaluated using Welch's *t*-test via "Olink Analyze" R Package (version 1.5.3.0). The results were corrected using the false discovery rate (FDR) method.[32] Differentially expressed proteins (DEPs) were identified with a *P* value cutoff of 0.05. The GO and KEGG enrichment analyses were visualized using the ggplot2 package (version 3.5.1). The correlation between the expression of two proteins was assessed using Pearson's Correlation Coefficient by OmicStudioClassic (version 1.60.0). The RandomForest package (version 4.7.1.1) was used to implement the RandomForest function. Receiver operating characteristic (ROC) curves were generated using the ROC R package (version 1.0.11) to evaluate the diagnostic performance of the biomarkers.

Statistical Analysis

Differences in age and body mass index (BMI) between the KSD and HC groups were assessed by Student's *t*-test using GraphPad Prism 8 software (GraphPad Software, SanDiego, CA, USA). Age and BMI are presented as the mean ± standard deviation (SD). A P value < 0.05 was considered statistically significant. For transcriptomic data, differential expression analysis of genes was performed using the DESeq2 R package (version 1.42.0). The P value was adjusted using Benjamini-Hochberg method. The thresholds for DEGs were set at $|\log_2(\text{fold change})| > 1$ and adjusted $P < 0.05$. For Olink proteomics data, differential expression analysis was evaluated using Welch's *t*-test via "Olink Analyze" R Package (version 1.5.3.0). The results were corrected using the FDR method. DEPs were identified with a *P* value cutoff of 0.05.

Results

Characteristics of the Participants

The clinical characteristics of the study participants are summarized in Table 1. Fourteen patients with KSD and 12 HCs were enrolled in this study. All participants were male, and there were no statistically significant differences in age and BMI between the two groups. Regarding 14 patients with KSD, all kidney stones were exclusively composed of CaOx with no other components identified, with 9 pure calcium oxalate monohydrate (COM) stones, and 5 mixed COM/calcium oxalate dihydrate (COD) stones. The distribution of stone location revealed that 36% of the patients had stones solely in the left kidney, 28% had stones exclusively in the right kidney, and 36% had stones present in both kidneys.

Overview of the Transcriptomic Analysis

To better understand the gene expression profiles and functional alterations in patients with KSD, transcriptomic analysis of PBMCs was performed. The samples segregated well between the KSD and HC groups, as shown by the principal component analysis (PCA) and heatmap of DEGs (Figure 1A and C). A total of 58,735 genes were identified in the samples. Among these, 1,063 genes were significantly altered, with 278 genes upregulated and 785 genes downregulated (Figure 1B and Supplementary Table S2).

Table 1 Clinical Characteristics of the Participants

Basic Information		KSD Group (n=14)	HC Group (n=12)	*P* Value
Age, years	Mean ± SD	30.64±12.71	29.08±9.69	0.73
Gender	Male	100%	100%	1.00
	Female	0%	0%	1.00
BMI	Mean ± SD	25.12±3.55	23.40±2.45	0.12
Station of stone	Left kidney alone	36.00%	–	–
	Right kidney alone	28.00%	–	–
	Both kidneys	36.00%	–	–
Stone components	CaOx	100.00%	–	–
	COM	64.29%	–	–
	COD	0.00%	–	–
	Mixed COM/COD	35.71%	–	–
	Carbapatite	0.00%	–	–
	Urate	0.00%	–	–
	Others	0.00%	–	–

Abbreviations: KSD, kidney stone disease; HC, healthy control; SD, standard deviation; BMI, body mass index; CaOx, calcium oxalate, COM, calcium oxalate monohydrate; COD, calcium oxalate dihydrate.

Dysregulated Functional Pathways in KSD Group

GO enrichment analysis was performed to explore the changes in biological processes (BPs) and molecular functions (MFs) in the KSD group. As shown in Figure 2A, the most significantly upregulated BP in the KSD group was the production of molecular

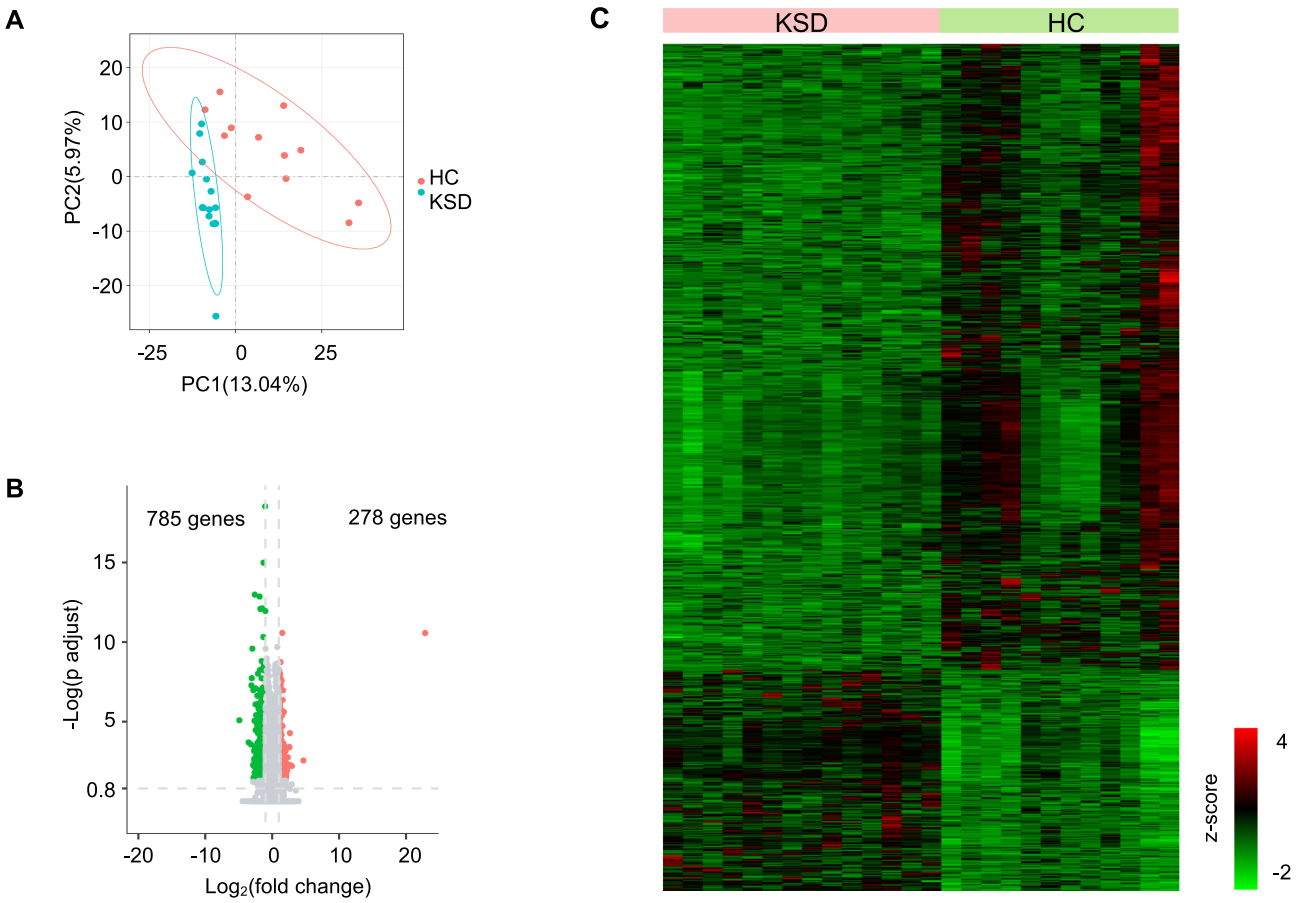

Figure 1 Overview of transcriptomic analysis. (**A**) PCA of gene expression between KSD and HC groups. (**B**) Volcano plot of all genes detected in the PBMCs of KSD and HC groups. (**C**) Heatmap of DEGs ($|\log_2(\text{fold change})| > 1$ and adjusted $P < 0.05$) detected in the PBMCs of KSD and HC groups.
Abbreviations: PCA, principal component analysis; KSD, kidney stone disease; HC, healthy control; PBMC, peripheral blood mononuclear cells; DEG, differentially expressed gene.

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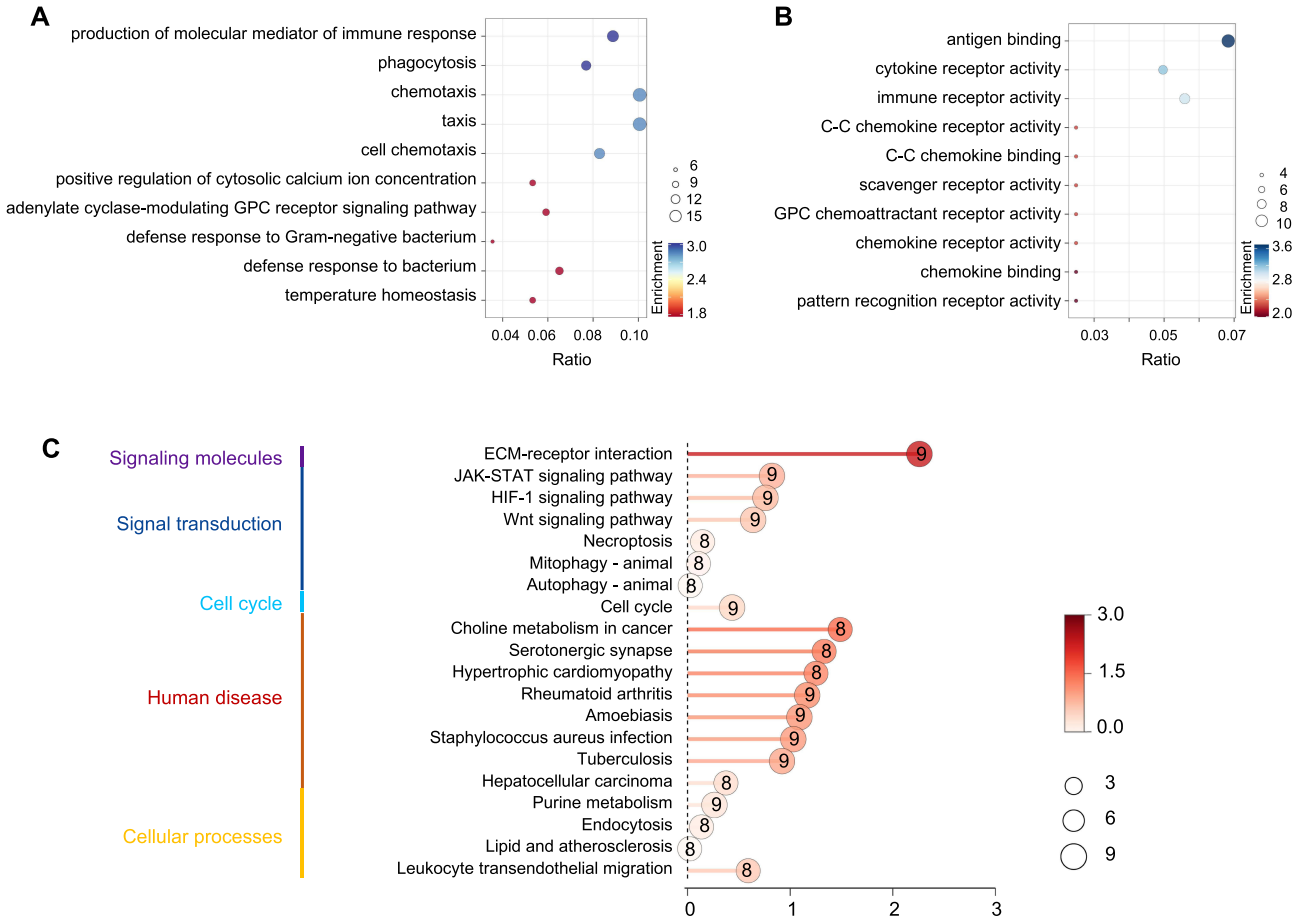

Figure 2 Functional analysis of DEGs between KSD and HC groups. (**A**) GO database BP term enrichment analysis of DEGs (|log_2(fold change)| > 1 and adjusted $P < 0.05$) between KSD and HC groups. Top 10 upregulated BPs are shown. (**B**) GO database MF term enrichment analysis of DEGs (|log_2(fold change)| > 1 and adjusted $P < 0.05$) between KSD and HC groups. Top 10 upregulated MFs are shown. (**C**) KEGG enrichment analysis of DEGs (|log_2(fold change)| > 1 and adjusted $P < 0.05$) between KSD and HC groups. Top 20 KEGG pathways are shown.

Abbreviations: DEG, differentially expressed gene; KSD, kidney stone disease; HC, healthy control; GO, gene ontology; BP, biological process; MF, molecular function; GPC, G protein-coupled; KEGG, kyoto encyclopedia of genes and genomes.

mediator of immune response. GO-MF analysis also revealed the activation of immune receptor activity (Figure 2B). Notably, positive regulation of cytosolic calcium ion concentration was significantly upregulated, as shown by GO-BP (Figure 2A). Consistent with previous research, defense response to Gram-negative bacterium and defense response to bacterium were also upregulated in patients with KSD (Figure 2A).[33] Furthermore, compared to the HC group, cytokine receptor activity chemotaxis, C-C chemokine receptor (CCR) activity, C-C chemokine binding, chemokine receptor activity, and chemokine binding were all significantly upregulated (Figure 2B).

KEGG enrichment analysis was conducted to further investigate the functions of DEGs. The most DEGs were enriched in extracellular matrix (ECM)-receptor interaction, as illustrated in Figure 2C. Similarly, dysregulation of ECM-receptor interaction in Randall's plaques was also identified in patients with kidney stones.[34] Additionally, the JAK-STAT, HIF-1, and Wnt signaling pathways, as well as necroptosis, mitophagy, and autophagy, were all dysregulated in the KSD group (Figure 2C).

Dysregulated Inflammation-Related Pathways in KSD Group

Considering the important role of inflammation in stone formation,[35–39] the top 30 inflammation-related GO-BP enrichment analyses for DEGs were presented in a bubble plot (Figure 3A). The inflammatory response, cytokine production, and leukocyte activation were the main modules of GO-BP. To comprehensively present the functional alterations in the KSD group, GSEA, which incorporates the established biological knowledge and expression levels

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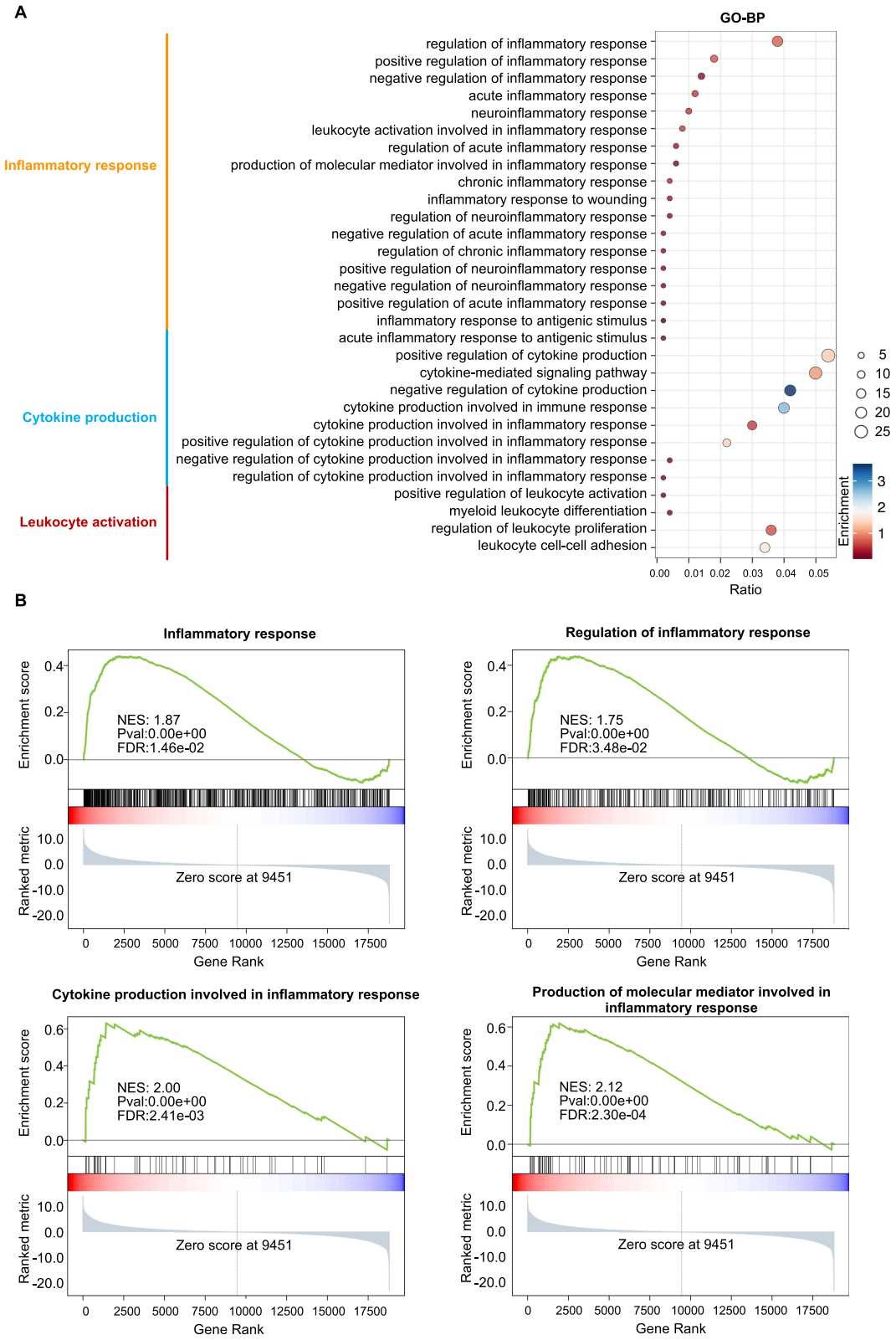

Figure 3 Enrichment analysis of inflammation-related terms. (**A**) GO database inflammation-related BP term enrichment analysis of DEGs ($|\log_2$(fold change)$| > 1$ and adjusted $P < 0.05$) between KSD and HC groups. Top 30 inflammation-related BPs are shown. (**B**) Visualization of GSEA results.

Abbreviations: GO, gene ontology; BP, biological process; DEG, differentially expressed gene; KSD, kidney stone disease; HC, healthy control; GSEA, gene set enrichment analysis.

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of all genes, was also performed.[40] The enrichment analysis results from GSEA, focusing on inflammation-related GO-BP, were partially presented in Figure 3B. Notably, the four most prominent gene abundance sets were inflammatory response, regulation of inflammatory response, cytokine production involved in inflammatory response, and production of molecular mediator involved in inflammatory response. Cytokines such as *toll like receptor 4* (*TLR4), interleukin 4* (*IL-4), CCR2, IL-18, myeloid differentiation primary response 88* (*MyD88*) were the core upregulated genes enriched in cytokine production involved in the inflammatory response (Supplementary Table S5).

Dysregulated Oxidative Stress-Related Pathways in KSD Group

Oxidative stress is seen as an important contributor to kidney stone formation.[39,41] Consistently, GO-BP enrichment analysis revealed alterations in the response to oxidative stress and the cellular response to oxidative stress in the KSD group (Figure 4A). GSEA analysis further confirmed the functional changes in the oxidative stress response and the regulation of cellular response to oxidative stress (Figure 4B and C). The lead genes involved in oxidative stress response were *CYBB, CAT, SP1, NFIX, NFKB1, MGST1, TXNRD1* and *MAPK14*, all of which were upregulated in the KSD group (Figure 4C). Similarly, the primary genes associated with the regulation of cellular responses to oxidative stress, including *PINK1, ALOX5, MAPKAP1, ABCD1, CD36, OXR1* and *NCOA7*, were also upregulated (Figure 4C). Moreover, the upregulation of oxidative stress may contribute to tubular epithelial cell damage and induce apoptosis.[41–45] Functional analysis consistently observed differences in GO-BP terms, including the intrinsic apoptotic signaling pathway in response to oxidative stress and negative regulation of oxidative stress-induced neuron intrinsic apoptotic signaling pathway, in the KSD group (Figure 4A).

Overview of Olink Proteomic Analysis

Given the crucial role of inflammation in kidney stone formation, we simultaneously detected the expression levels of 92 inflammation-related proteins in the urine supernatants of the KSD and HC groups using the Olink panel. PCA analysis revealed a good segregation between the two groups (Figure 5A). Eight inflammation-related proteins were significantly different ($P < 0.05$) between the KSD and HC groups, with 5 upregulated and 3 downregulated in the KSD group (Figure 5B). Correlation analysis was performed to explore the interactions between these DEPs. As shown in Figure 5C, matrix metalloproteinase-1 (MMP-1) and interleukin 24 (IL-24) showed the strongest negative correlation. KEGG enrichment analysis indicated that the MAPK, NF-kappa B, and PI3K-Akt signaling pathways were dysregulated in the KSD group (Figure 5D).

Identification of Urinary MMP-1 as the Potential Biomarker of KSD

The expression levels of the DEPs in the KSD and HC groups were represented in a boxplot (Figure 6A). Among the dysregulated proteins, MMP-1 was the most significantly upregulated protein in the urine of the KSD group compared with that in the HC group (Figure 6B). Random forest analysis is frequently employed for feature selection, providing a method for measuring the importance of each variable in a decision tree.[46] As shown in Figure 6C, the random forest feature selection identified MMP-1 as the top-ranked protein for distinguishing KSD, with the highest mean decrease accuracy of 8. Notably, MMP-1 showed a good performance in diagnosing KSD, with the area under the ROC curve (AUC) of 0.6786 (Figure 6D). Collectively, these results suggested that urinary MMP-1 might serve as a potential biomarker for CaOx KSD.

Discussion

KSD is the third most common urological disease following urinary tract infections and prostate diseases, with an incidence rate of approximately 15%, which continues to rise. As previously reported, KSD results from the underlying genetic and/or metabolic alterations.[47] Therefore, elucidating the genetic and functional changes is of great importance in determining the mechanisms underlying CaOx stone formation and development. In this study, we reported transcriptomic changes in PBMCs of patients with CaOx kidney stones and identified dysregulated signaling pathways. The level of MMP-1 in the urine was significantly higher in KSD group, suggesting it a potential marker associated with CaOx KSD.

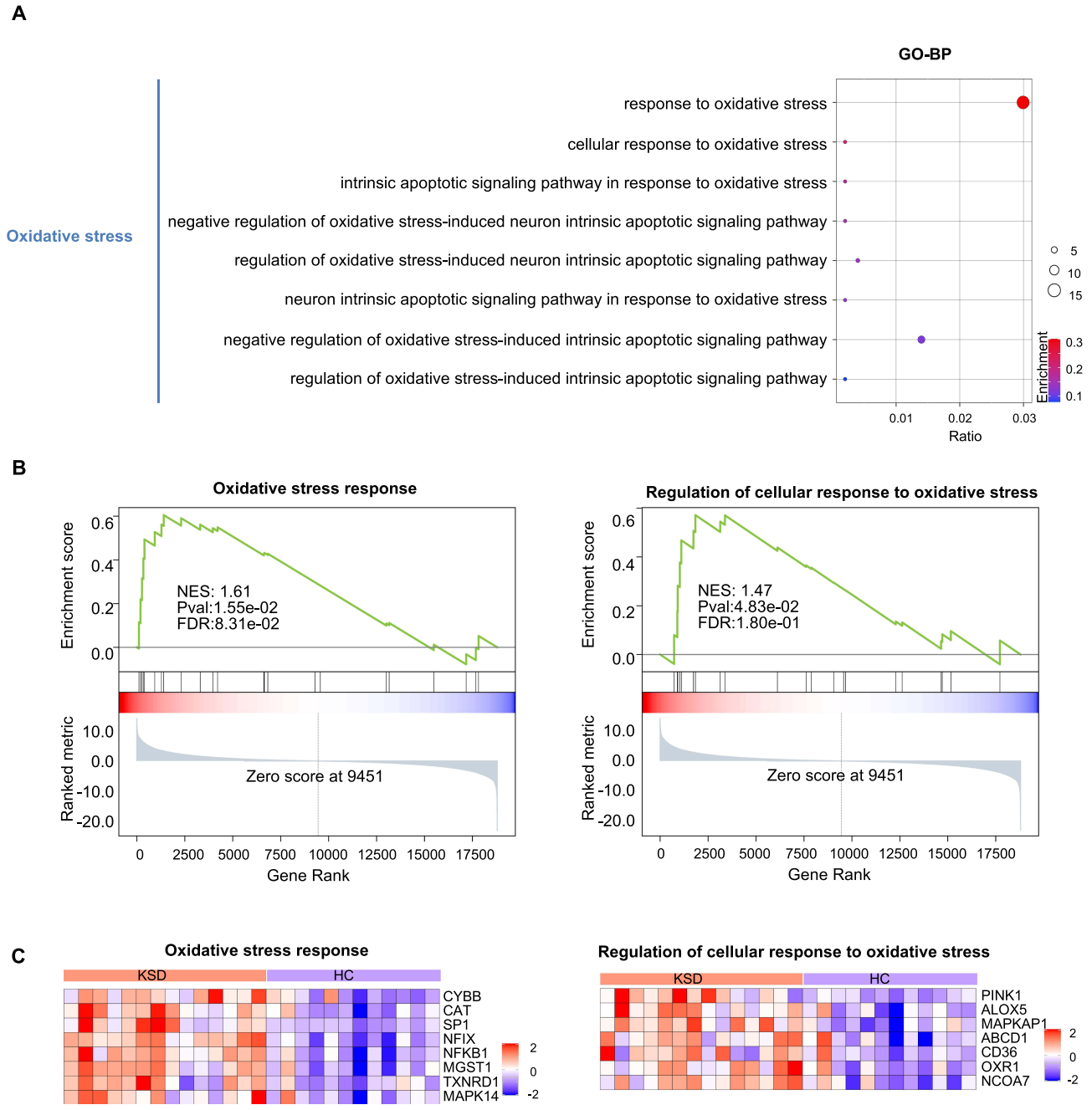

Figure 4 Enrichment analysis of oxidative stress-related terms. (**A**) GO database oxidative stress-related BP term enrichment analysis of DEGs ($|\log_2$(fold change)$| > 1$ and adjusted $P < 0.05$) between KSD and HC groups. (**B**) Visualization of GSEA results. (**C**) Heatmap of lead genes enriched in oxidative stress response and regulation of cellular response to oxidative stress.

Abbreviations: GO, gene ontology; BP, biological process; DEG, differentially expressed gene; KSD, kidney stone disease; HC, healthy control; GSEA, gene set enrichment analysis.

The kidney, ranking second only to the heart in terms of its mitochondrial content and oxygen consumption, is one of the most energy-demanding organs. Maintaining mitochondrial homeostasis, which allows mitochondria to detect and adapt to varying energy demands and nutrient availability, is key to proper functioning of renal cells.[48,49] Mitochondrial dysfunction, specifically oxidative stress, has been implicated in various kidney diseases, including KSD.[41,50,51] Several sets of proteomic data have demonstrated that kidney stones altered the expression levels of proteins associated with oxidative stress.[52–54] Moreover, experimental studies have verified that oxidative stress accelerated the CaOx crystals deposition.[55] In this study, as indicated by the GO-BP and GSEA enrichment analyses, response to oxidative stress,

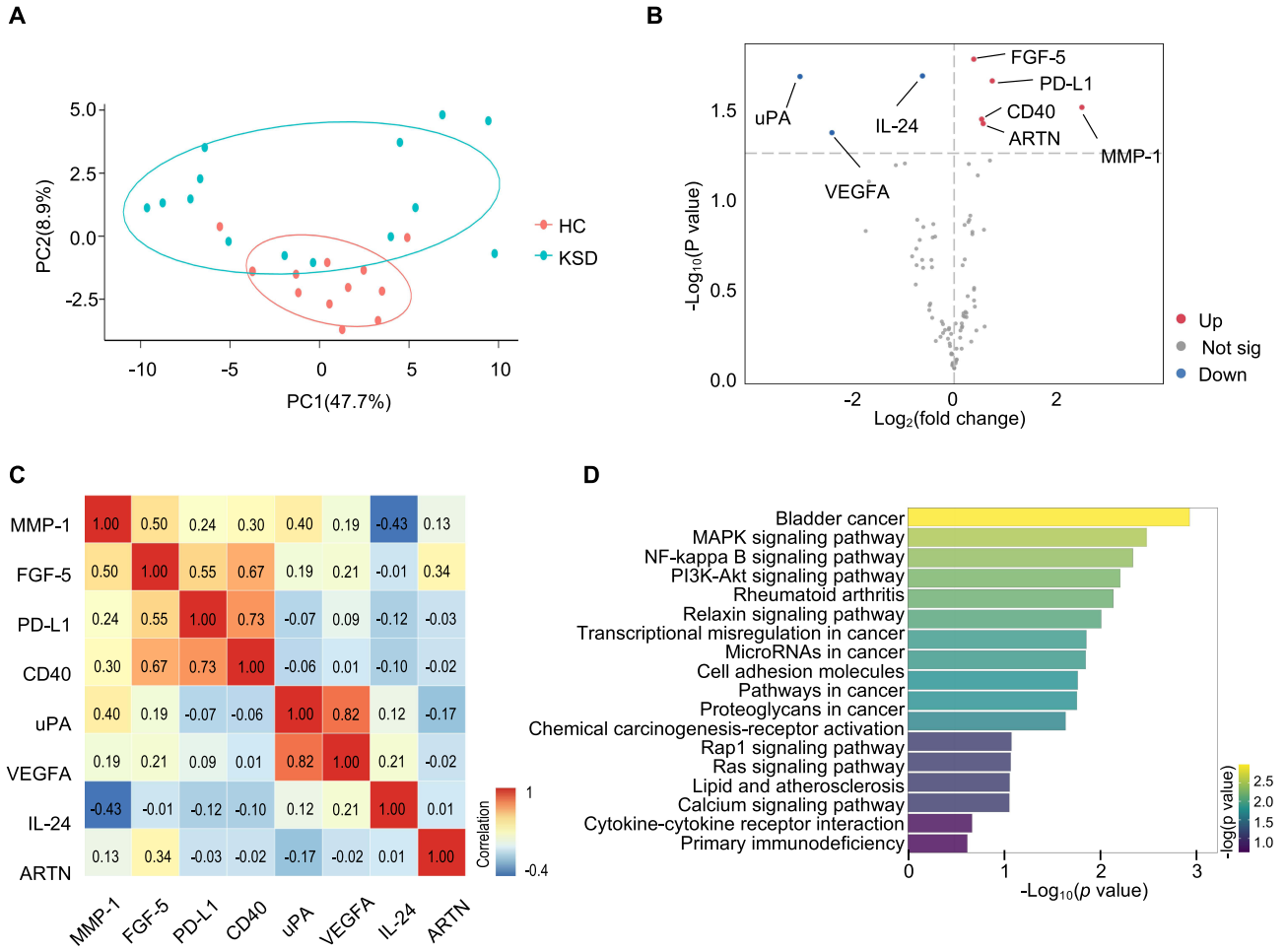

Figure 5 Overview of Olink proteomic analysis. (**A**) PCA of 92 inflammation-related proteins detected in the urine of KSD and HC groups. (**B**) Volcano plot of all 92 inflammation-related proteins in the urine of KSD and HC groups. (**C**) The correlation among MMP-1, FGF-5, PD-L1, CD40, uPA, VEGFA, IL-24, and ARTN. (**D**) KEGG enrichment analysis of differentially expressed inflammation-related proteins between KSD and HC groups.

Abbreviations: PCA, principal component analysis; KSD, kidney stone disease; HC, healthy control; KEGG, kyoto encyclopedia of genes and genomes; MMP-1, matrix metalloproteinase-1; FGF-5, fibroblast growth factor 5; PD-L1, programmed death-ligand 1; uPA, urokinase-type plasminogen activator; VEGFA, vascular endothelial growth factor A; IL-24, interleukin 24; ARTN, artemin.

cellular response to oxidative stress, and oxidative stress response were dysregulated in the KSD group (Figure 4A and B). As a product of oxidative stress, ROS could directly stimulate the transient receptor potential (TRP) calcium channels. The expression levels of genes related to TRP calcium channels, such as *TRPC2, TRPC6*, and *TRPM6* significantly increased in the KSD group by comparing to HC group (Supplementary Table S1). Dysregulation of these genes could lead to increased calcium influx, exacerbating mitochondrial calcium overload.[56] In the KSD group, there was a consistent upregulation in the positive regulation of cytosolic calcium ion concentration, as indicated by GO-BP terms (Figure 2A and Supplementary Table S3). CLDN5 and CLDN23 are members of the Claudin family, which are tight junction membrane proteins that comprise the paracellular pore and therefore regulate the permeability of the paracellular pathway to calcium ions.[57] Transcriptomic data showed that the expression levels of *CLDN5* and *CLDN23* were dysregulated, which could perturb paracellular calcium ion transport and the subsequent calcium reabsorption. Dysregulation of calcium reabsorption might lead to higher supersaturation of calcium stones, which accelerated the formation of kidney stone.[58,59] Based on these findings, we propose that oxidative stress might be a key factor in the pathogenesis of CaOx kidney stone formation.

Numerous pathways have been reported to be involved in kidney stone formation.[60] GO and KEGG enrichment analyses revealed that inflammation-related signaling pathways, including NF-κB, MAPK, Rap1, Wnt and JAK-STAT signaling pathways, were dysregulated in the KSD group (Figure 2C, Supplementary Tables S3 and S4). Activation of

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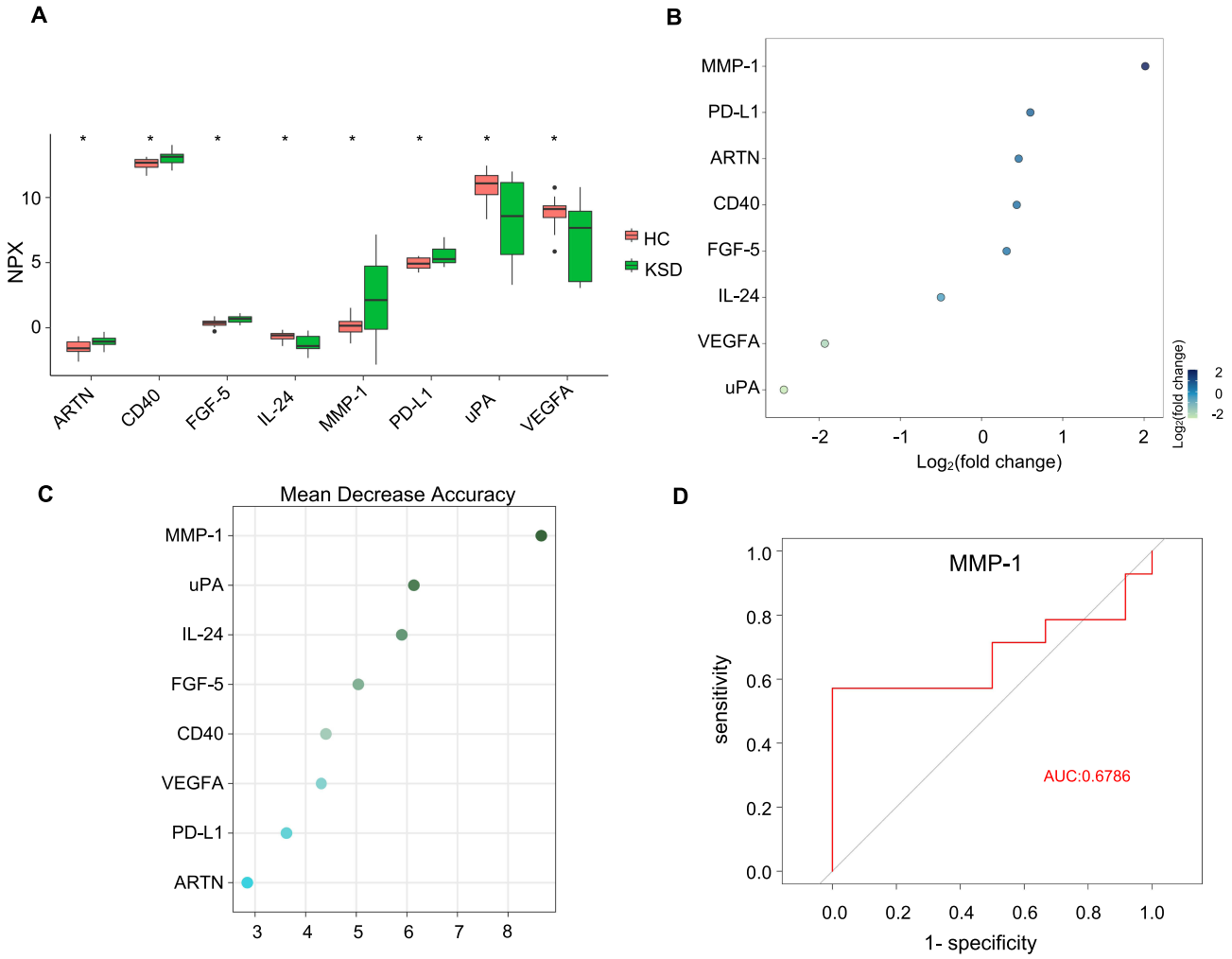

Figure 6 Identification of urine MMP-1 as the potential marker for KSD by Olink proteomics. (**A**) Expression levels of DEPs in the urine of KSD and HC groups. (**B**) The bubble plot of DEPs in the urine of KSD and HC groups. (**C**) The variable importance of DEPs by Random forest model. (**D**) ROC curve of MMP-1. The results were mean ± SD. Compared to HC group, *$P < 0.05$.

Abbreviations: MMP-1, matrix metalloproteinase-1; DEP, differentially expressed protein; KSD, kidney stone disease; HC, healthy control; ROC, receiver operating characteristic.

the NF-κB and MAPK signaling pathways would cause the release of various pro-inflammatory factors, including adhesion molecules (such as *CD44, ICAM-1*, Supplementary Table S1) to promote crystal adhesion, chemokines (such as *CCR2*, Supplementary Table S1) to stimulate macrophage M1 polarization and accelerate crystal deposition, cytokines (such as *CCL2, TNF*, Supplementary Table S1) to induce renal tissue injury, and the activation of the nod-like receptor protein 3 (NLRP3) inflammasome (Supplementary Table S1).[11,61–64] The assembly and activation of the NLRP3 inflammasome complex triggered the upregulation of *IL-1β, IL-18*, and *NLRP3* (Supplementary Table S1) and the release of the corresponding mature proteins, causing direct renal cellular damage and inflammation, thereby exacerbating systemic renal damage and dysfunction, and subsequently facilitating crystal adherence and retention.[37–39,65] The JAK-STAT, Wnt and Rap1 signaling pathways have been implicated in kidney stone formation.[66–68] The activation of these pathways induced the release of inflammatory cytokines, causing an inflammatory cascade and ultimately renal tissue damage. Several cell death pathways have also been reported to participate in renal damage and kidney stone formation.[60,69,70] Transcriptomic data and KEGG enrichment analysis suggested that renal cell injury and death may be associated with necroptosis, apoptosis, ferroptosis, and autophagy pathways (Figure 2C and Supplementary Table S4) and higher expression levels of *CAS7, CAS9, MLKL, RIPK3, GSDMD, GSDME*, and *BACH1* (Supplementary Table S1). These mechanisms of injury and cell death generated considerable cellular debris and degradation products that were

involved in the development of Randall's Plugs and Randall's plaques, acting as a base for crystal deposition and eventually forming CaOx kidney stones.[18]

Fibroblast growth factor (FGF-5), programmed death-ligand 1(PD-L1), CD40, and artemin (ARTN) were significantly upregulated in the urine of the KSD group, as revealed by Olink proteomics (Figure 5B and 6A). The upregulation of FGF-5 was consistent with existing evidence that elevated level of FGF-5 was associated with an increased risk of kidney stone formation.[71] FGF-5 would bind to various isoforms of fibroblast growth factor receptors (FGFRs) and interact with downstream mediators including MAPK1/3 during inflammatory response.[72,73] In our results, the expression levels of *FGFR1/2/3* and *MAPK1* were increased (Supplementary Table S1), and the positive regulation of MAPK cascade and MAPK signaling pathway were dysregulated (Supplementary Tables S3 and S4) in the KSD group. Previous studies have consistently found that the activation of MAPK pathway might promote CaOx crystal deposition and facilitate stone formation.[61] PD-L1, which is expressed by various immune cells including macrophages, was upregulated in the urine of the KSD group.[74] Several inflammatory mediators, including TNF-α, and various pathways, including the JAK/STAT and RAS/MAPK pathways, are involved in the regulation of PD-L1 expression via different downstream transcription factors, such as STAT1/3 and NF-κB.[75–78] Therefore, the activation of NF-κB, JAK-STAT, and MAPK signaling pathways in the KSD group could account for the increased levels of PD-L1 (Figure 2C, Supplementary Tables S3 and S4).[79] CD40, a membrane glycoprotein belonging to the tumor necrosis factor receptor (TNFR) superfamily,[80] was found to be increased in the urine of the KSD group (Figure 5B and 6A), corroborating previous reports of higher CD40 level in the urine of patients with KSD.[81,82] Elevated levels of CD40 interacted with the CD40 ligand (CD40L), resulting in an increased release of monocyte chemoattractant protein-1 (MCP-1, also known as CCL2), stimulation of NF-κB, and ultimately an inflammatory response.[83] Our findings consistently showed that *CD40LG* (CD40 ligand) and *CCL2* were up-regulated to varying degrees in the KSD group (Supplementary Table S1). We also observed elevated ARTN expression in the urine of the KSD group (Figure 5B and 6A), which might reflect an inflammatory response in stone formation.[84,85] Moreover, elevated level of ARTN, a neurotrophic factor, might contribute to pain symptoms in patients with kidney stones.[85–87]

We also found that the expression levels of urokinase-type plasminogen activator (uPA), IL-24, and vascular endothelial growth factor A (VEGFA) were significantly reduced in the urine of the KSD group (Figure 5B and 6A). Previous studies have demonstrated that uPA might exert a protective effect by inhibiting inflammatory signaling pathways such as the NF-κB signaling pathway.[88,89] The decreased expression of uPA reflected the ongoing inflammatory process (Figure 5B and 6A), further contributing to kidney stone formation.[90] IL-24, a member of the IL-10 family, played a crucial role in inflammation.[91] In addition to its pro-inflammatory role, IL-24 activated suppressors of cytokine signaling proteins (SOCS), repressed the Th17 cytokine program, and subsequently inhibited inflammatory responses.[92,93] Therefore, the reduced IL-24 level in urine (Figure 5B and 6A), combined with the downregulation *SOCS1* and *SOCS2* (Supplementary Table S1), provided further evidence for the ongoing inflammatory response. Unlike *SOCS1* and *SOCS2, SOCS3* was overexpressed in the KSD group, as shown by transcriptomic data (Supplementary Table S1). Overexpression of SOCS3 showed the ability to suppress VEGFA secretion.[94–96] This may explain the reduced level of VEGFA in the urine of KSD group. Moreover, existing evidence indicated that downregulation of VEGFA did not inhibit ongoing inflammation.[97] So, the markedly reduced levels of uPA, IL-24, and VEGFA corroborated the increased inflammation associated with KSD.

Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, are secreted by various cell types including leukocytes.[98] The first member, MMP-1, is expressed at relatively low level under normal physiological conditions.[99,100] However, MMP-1 expression could be induced by oxidative stress, ROS accumulation, and upregulation of MCP-1 binding to CCR2 during the inflammatory response.[99,101] Therefore, oxidative stress, along with elevated levels of *MCP-1* and *CCR2*, may account for the increased level of MMP-1 in the KSD group (Figure 4 and Supplementary Table S1). The upregulation of MMP-1 could directly break down the epithelial barrier by hydrolyzing epithelial cell junction proteins, permitting leukocyte influx into the sites of inflammation.[102] This process might contribute to the dysregulation of the positive regulation of leukocyte migration, regulation of leukocyte migration, and leukocyte activation involved in the inflammatory response in the KSD group, as suggested by the GO enrichment

 https://doi.org/10.2147/JIR.S605025

analysis (Supplementary Table S3). Overall, the MMP-1 might be a marker of inflammation and leukocyte activation associated with CaOx kidney stones.

Our study has several limitations. First, the number of blood and urine specimens used to generate transcriptomics and Olink proteomics was relatively small. Future studies with larger sample size would enhance the reliability and generalizability of the results. Second, the lack of experimental validation represents another limitation that will be addressed in future studies.

Conclusion

In conclusion, this study elucidated a global transcriptomic profile of PBMCs in patients with CaOx kidney stones and identified 1,063 DEGs compared with HC group. Functional enrichment analysis revealed that these DEGs were primarily enriched in production of molecular mediator of immune response, inflammatory response, oxidative stress, and extracellular matrix (ECM)-receptor interaction. Furthermore, we identified 8 DEPs in the urine of KSD and HC groups. Notably, MMP-1 demonstrated the greatest fold change and the highest mean decrease accuracy, serving as a potential biomarker for CaOx KSD. These findings provide a preliminary insight for future investigations into CaOx kidney stone formation and the development of targeted therapeutic interventions for patients with CaOx kidney stones.

Data Sharing Statement

The RNA-seq data generated for this study (raw sequencing and processed) are available at NCBI GEO with the accession number GSE 318167. The data used to support the findings of this study are available within this article and its supplementary tables or from the corresponding authors on request.

Author Contributions

Ning Wang: Conceptualization, Data curation, Funding acquisition, Investigation, Resources, Visualization, Writing – original draft, Writing – review & editing. Bo Zhou: Data curation, Investigation, Writing – review & editing. Changbao Xu: Conceptualization, Supervision, Writing – review & editing. Bin Hao: Conceptualization, Supervision, Writing – review & editing. All authors gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This research was funded by the Science Research Start-up Fund for Doctor of The Second Affiliated Hospital of Zhengzhou University (202104091).

Disclosure

The authors declare that they have no conflict of interest.

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