

Plasma Olink Proteomics Reveals the Dysregulated Cytokines in Kidney Stone Disease

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Article

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Abstract

Background

Kidney stone disease (KSD) is a prevalent global health issue with a continuously rising incidence. Extensive clinical research suggests that the formation of kidney stones may be closely associated with immune-inflammatory responses. In this study, we aim to reveal the profile of cytokines in the serum which can reflect the immune-inflammatory activity during the progression of KSD.

Methods

We established a glyoxylate-induced nephrolithiasis mouse model validated through histopathology (H&E/Pizzolato's staining) and renal function markers (Scr/BUN). Inflammation-related cytokines were examined using Olink Target 48 Mouse Cytokine panel, with bioinformatics analysis (GO /KEGG /PPI) of differentially expressed proteins. Clinical validation was performed through ELISA measurement of hepatocyte growth factor (HGF) in the serum from human subjects (40 patients/40 controls).

Results

The mouse KSD model demonstrated successful calcium oxalate crystal deposition with significant tubular damage ($p < 0.001$). Olink proteomics identified 24 differentially upregulated cytokines, including interleukins such as IL-1 β and IL-6, chemokines such as CCL2, interferons such as IFN- γ , and growth factors such as HGF. GO/KEGG enrichment analysis showed that these molecules were significantly enriched in immune inflammation-related processes such as cytokine-mediated signaling pathways and leukocyte migration, as well as cytokine-cytokine receptor interaction and IL-17 signaling pathway. PPI network analysis identified CD274, CTLA4, IL-6, IL-2, and IL-1 β as core molecules, suggesting that these molecules play a key role in the pathogenesis of kidney stones. ELISA confirmed significant HGF elevation in patient sera ($p < 0.05$).

Conclusion

This first application of Olink technology in KSD research reveals an enhanced inflammatory cytokine signature.

1. Introduction

Kidney stone disease (KSD) is a prevalent urological disorder and a significant global health issue, affecting approximately 12% of the population [1]. In recent years, its prevalence and incidence have been increasing worldwide [2]. Urinary tract stones are estimated to affect 10%–15% of the general population over their lifetime, with a notably higher prevalence in men compared with women[3].

Accumulating evidence suggests that the risk of nephrolithiasis rises with advancing age, reaching its maximum incidence among individuals aged 40–60 years [4]. This pattern is closely linked to alterations in lifestyle and dietary behaviors. In addition to causing intense pain, kidney stones may result in severe clinical complications including urinary tract obstruction and chronic renal damage [5]. Collectively, these issues significantly compromise patients' quality of life and place a heavy economic burden on both patients and society at large. Globally, there were 106 million cases of urolithiasis in 2021, contributing significantly to disease burden and elevating risks of mortality and disability [6].

KSD are often associated with chronic low-grade inflammation. Renal stones induce alterations in serum cytokine profiles.[7] Traditional ELISA assays face limitations in simultaneously detecting multiple cytokines and are often inadequate for identifying low-abundance cytokines. Olink proteomics technology offers a robust approach for revealing global cytokine profiles. In recent years, Olink technology, utilizing Proximity Extension Assay (PEA), has enabled highly sensitive quantification of low-abundance proteins [8]. Owing to its high reproducibility, stability, and minimal sample requirement, serum inflammatory biomarker profiling based on Olink proteomics has been applied to the study of various diseases, such as abdominal aortic aneurysm, preeclampsia, and type 2 diabetes [9–11]. Our goal is to utilize Olink proteomics technology to comprehensively unveil the dysregulated cytokines in KSD, thereby providing an experimental basis for deep understanding the immune-inflammatory mechanism of KSD.

2. Material and methods

2.1. Chemicals

Glyoxylic acid (GA, Sigam, USA) and pure water were used to prepare glyoxylic acid solution with a concentration of 1 g/100 ml in proportion. Each mouse was given glyoxylic acid according to the standard of 100 mg/kg.

2.2. Animals

Animal experiments were approved by the Ethics Committee of Zhejiang University (approval number: ZJU20240947) and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Eight-week-old male C57BL/6J mice were provided by the Laboratory Animal Research Center of Zhejiang University (Zhejiang, China). All mice were kept in 12hour bright/dark cycles at 25°C with ad libitum access to food and water. All mice were weighed before the experiment started and then randomly assigned to two groups. Control Group (CTL) consisted of normal mice received intraperitoneal injection of pure water only (n=11). Glyoxylic acid treatment group (GA) mice received intraperitoneal injection of Glyoxylic acid (n=9, 100 mg/kg) for 7 consecutive days as previous reported [12]. Each mouse received about 200 µl of glyoxylic acid solution. At the end of the experiments, mice were euthanized via intraperitoneal injection with an overdose of sodium pentobarbital at a dose of 150

mg/kg. Subsequently, transcardial perfusion with phosphate-buffered saline (PBS) was performed for subsequent tissue isolation and collection. Kidneys were harvested. One portion of the kidneys were removed and fixed with paraformaldehyde and embedded in paraffin for hematoxylin and eosin (H&E) and Pizzolato's staining (n=6/group). Blood serum was collected from the orbits of the mice for serum creatinine (Scr) and blood urea nitrogen (BUN) content tests.

2.3 Tubular damage score

Kidney samples were fixed in 10% buffered formalin, embedded in paraffin, and cut into 4- μ m sections. The sections were stained with hematoxylin and eosin (H&E).

Tubular damage was scored semiquantitatively from 0 to 5 as previously described [13]:

0 = normal histology;

1 = degeneration without necrosis;

2–5 = necrosis, regeneration, tubular dilatation, degeneration, protein casts, and interstitial lymphocytic infiltration involving <25%, <50%, <75%, and >75% of epithelial cells, respectively.

2.4 Pizzolato's staining

Kidney tissues were rapidly fixed in 10% formalin. After dehydration in a graded ethanol series (70%, 80%, 96%, 100%), the samples were cleared with xylene and embedded in paraffin. Tissue sections of 4 μ m were stained with hematoxylin and eosin (H&E) for histopathological evaluation. Pizzolato's staining was performed to detect calcium oxalate crystals in renal tissue. In brief, sections were incubated with a mixture of 5% silver nitrate and 30% hydrogen peroxide at a 1:1 ratio. After exposure to ultraviolet light for 30 min, sections were rinsed thoroughly with distilled water. After that, the glass slides were washed with distilled water and stained with nuclear fast red for 5 minutes, and then the tissue sections were dehydrated in 95% alcohol and anhydrous alcohol. Environmental protection sealing tablets, using polarization microscope for microscopic examination and image acquisition and analysis [14].

2.5 Blood analysis

Blood samples were collected from the orbital venous plexus into plain tubes without anticoagulant supplementation, and left to clot naturally at room temperature. Serum specimens were then harvested by centrifugation at 3000 rpm for 15 minutes. Serum urea and creatinine concentrations were determined using commercially available assay kits, strictly in accordance with the manufacturer's protocols. All biochemical analyses were conducted on a Chemwell automatic biochemistry analyzer.

2.6 Olink Proteomic analysis

Protein expression levels were detected using the Olink Target 48 Mouse Cytokine panel (Olink Proteomics AB, Uppsala, Sweden), based on the Proximity Extension Assay (PEA) technology. In this

assay, paired oligonucleotide-labeled antibody probes bind specifically to their corresponding target proteins. Once the paired probes are brought into close proximity, their oligonucleotide sequences hybridize pairwise. DNA polymerase is subsequently added to trigger proximity-dependent DNA amplification, generating a unique PCR target fragment for each individual protein. Each sample plate was subjected to quality control assessment based on the standard deviation of NPX values derived from incubation and detection controls. Only data from experimental runs that fully met the established QC criteria were included for downstream analysis. Briefly, each target protein was recognized by two specific antibodies conjugated with unique complementary DNA barcodes. These barcodes were then quantified using a high-throughput microfluidic real-time PCR system (Biomark HD, Fluidigm, South San Francisco, CA, USA). Final protein quantification results were presented in standard units (pg/ml), with absolute quantification performed via a 4-parameter logistic (4-PL) curve-fitting model.

2.7 Bioinformatics Analysis

All GO and KEGG analyses and visualizations were performed in R (version 4.2.1). The R package cluster Profiler (v4.4.4) was used for enrichment analysis, and org. Mm. eg.db was employed for ID conversion. The analysis was conducted using the mouse species (*Mus musculus*). After converting the input molecular list IDs, enrichment analysis was performed using the cluster Profiler package. Statistical significance for enrichment was defined as an adjusted p-value (p_{adj}) < 0.05.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed to identify the significant pathways associated with differentially expressed proteins, using the clusterProfiler package [15]. For enrichment analyses, all significantly differentially expressed proteins were mapped to individual Gene Ontology (GO) terms and KEGG pathways in the corresponding databases. A hypergeometric test was applied to screen GO terms and KEGG pathways that were significantly enriched in the differential protein set, compared with the specified background reference [16]. Enrichment analysis results were compared under two distinct backgrounds: the total protein set, and the 24 cytokines included in the Olink Target 48 Mouse Cytokine panel. Spearman's correlation analysis was conducted to assess the correlation between the expression levels of paired proteins. Protein-protein interaction (PPI) networks of the 24 differentially expressed proteins were constructed and visualized using the STRING database.

2.8 Patients and Health people enrollment

This study protocol was approved by the Ethics Committee of the Second People's Hospital of Aksu Prefecture, Xinjiang Uygur Autonomous Region, China (Approval No.: akseyyllsc-ky202402). Written informed consent was obtained from all participants prior to sample collection and study involvement.

The GA group consisted of 40 patients with renal calculi who were admitted to the Department of Urology of the hospital. Inclusion criteria: Aged 18–65 years; renal calculi directly detected and diagnosed by examinations such as ultrasound, CT or MRI. Exclusion criteria: Complicated with severe

immune-related diseases; adults over 65 years old; special populations such as pregnant women and cancer patients.

The CTL group included 40 healthy people from the Physical Examination Center of the hospital. Inclusion criteria: Aged 18–65 years; healthy people who were excluded from renal calculi by examinations such as ultrasound, CT or MRI. There were no significant differences in baseline data such as age, gender and group between the two included populations.

2.9 Plasma Sample Collection

Approximately 2 mL of peripheral venous blood was collected from each patient into Eppendorf tubes; Then, the serum was extracted by centrifugation at 3000 rpm for 15 min and frozen in a -80°C refrigerator for ELISA examination.

2.10 ELISA Validation

After Olink Target 48 Mouse Cytokine Panel detection, there were 24 proteins with significant statistical differences, so we randomly selected one cytokine (HGF) for ELISA verification. 80 serum samples, including 40 patients with kidney calculi's disease and 40 healthy people were used. The Human HGF ELISA Kit (enzyme-linked immunosorbent assay) (A1H0150641, Hangzhou Lianke Biotechnology Co., Ltd., Hangzhou, Zhejiang) was used for ELISA detection.

2.11 Statistical analysis

GraphPad Prism (version 6.0; Sand Diego, USA) was used to analyze the revelations of the study by student T test analysis. The values are presented in terms of mean $\pm$ SEM. Findings demonstrating $p < 0.05$ were considered significant. Statistical analyses were performed at Olink using R V.4.1.2 (2021-11-01), apart from baseline characteristics that were analyzed in-house using RStudio V.2022.12.0+353. Values are given as means $\pm$ SD. $P < 0.05$ were considered statistically significant.

3 Results

3.1 Successful Establishment of the KSD Mouse Model

Pizzolato staining combined with polarized light microscopy was performed on mouse kidney sections to assess crystal deposition. Extensive calcium oxalate (CaOx) crystal accumulation was observed in the renal tissues of mice treated with glyoxylate for 7 consecutive days (Figures 1A–C). Mice in the control (CTL) group exhibited intact and normal renal histological structure. In contrast, the glyoxylate (GA) treatment group showed distinct pathological renal injury, including marked tubular dilatation, tubular epithelial cell necrosis and degeneration, as well as obvious inflammatory cell infiltration into the tubular epithelial lining (Figures 1A, D). Compared with CTL group, the kidney index of GA group increased significantly whereas the body weight of mice decreased significantly (Figures 1E, F). GA group showed the significant increases in Scr and BUN level ($P < 0.001$), (Figures 1G H).

3.2 Olink Target 48 Mouse Cytokine panel result

Olink proteomic analysis was conducted to quantify and compare the expression levels of 45 inflammation-associated factors between the GA group and the CTL group. Among all tested targets, two cytokines – Fractalkine and vascular endothelial growth factor A (VEGFA) – were undetectable in both study groups. Based on this finding, we hypothesize that these two cytokines may not be involved in the pathological progression of nephrolithiasis. In total, 24 inflammation-related proteins were identified as significantly differentially expressed between the GA group and CTL group. Detailed profiles of these differentially regulated cytokines are documented in Table S1. According to the Log2 Fold Change (Log2FC) value, the Top ten cytokines with great differences are shown in Table 1.

Table 1 Top 10 Most Differentially Expressed Cytokines Based on Log2FC

UniProt	Assay	log2(FC)	p.value	Adjusted_pval	updown
P08505	Il6	2.2566529	0.000194	0.000928927	UP
Q62401	Ccl12	1.6009038	0.000178	0.000928927	UP
P10749	Il1b	1.5611567	0.00061	0.002621787	UP
P01580	Ifng	1.5553168	0.023807	0.048747941	UP
P18893	Il10	1.4629446	4.85E-07	1.04E-05	UP
Q7TNI7	Il17f	1.4247627	6.55E-05	0.000469191	UP
Q9JJN1	Fgf21	1.3071379	0.038209	0.074682078	UP
P10148	Ccl2	1.2766944	1.74E-07	7.47E-06	UP
P06804	Tnf	1.2460653	0.000174	0.000928927	UP

These significant differentially expressed cytokines accounted for 55.81% of the total (Figure 2C). The scatter plot of principal component analysis (PCA) scores of all samples is shown in Figure 2A. The volcanic visualization of 43 inflammation-related cytokines and chemokines is shown in Figure 2B and the heatmap of them is shown in Figure 2D. Finally, Figure 2E shows that Pie chart of subcellular localization analysis of the significant differentially expressed cytokines and chemokines.

The box diagram of 24 up-regulated differential cytokines is shown in Figure 3. Up-regulated cytokines include Interleukin-IL-6 / IL-1 β / IL-10 / IL-17f / IL-16 / IL-27 / IL-33 / IL-26, C-C motif chemokine (Ccl12 / Ccl2 / Ccl4 / Ccl11 / Ccl17), Interferon gamma (IFN- γ / IFN- α 2) C-X-C motif chemokine (Cxcl1 / Cxcl2), Granulocyte-macrophage colony-stimulating factor (CSF1 / CSF2), Hepatocyte growth factor (HGF), Programmed cell death 1 ligand 1 (CD274), Cytotoxic T-lymphocyte protein 4 (CTLA4), Tumor necrosis factor (Tnf), Fibroblast growth factor 21 (Fgf21). Among them, Interleukin-6 (IL-6) and C-C motif chemokine 12 (Ccl12) had the largest Log2 FC, 2.26 and 1.60, respectively. The correlation between

these difference 24 up-regulated differential cytokines and chemokines is represented by a numerically label thermogram as shown in figure 4.

To further explore the potential biological functions of the screened differentially expressed cytokines, GO and KEGG pathway enrichment analyses were performed (Figure 5). The enrichment results revealed that these dysregulated cytokines were predominantly enriched in multiple GO terms closely linked to immune and inflammatory responses, including GO-BP: cytokine-mediated signaling pathway, leukocyte proliferation–leukocyte migration. GO-CC: perikaryon, recycling endosome, plasma membrane signaling receptor complex. GO-MF–cytokine activity–cytokine receptor binding–chemokine activity. KEGG pathway enrichment analysis further identified several significantly enriched signaling pathways, including Cytokine-cytokine receptor interaction, IL-17 signaling pathway, and Viral protein interaction with cytokine and cytokine receptor (Figure 5).

KEGG pathway enrichment analysis was performed using the online database (available at: www.kegg.jp/kegg/kegg1.html).

Additionally, we performed a correlation analysis and characterized a PPI network to explore the interactions among the above-mentioned cytokines. We found that Cd274 and CTLA4, IL-6 and IL-2, IL-1β and IL-2, had the highest degree scores in the PPI network, indicating they may have important roles in renal calculus (Figure 6).

3.3 The result of ELISA Validation

This study included 80 subjects, including 40 in the GA group and 40 in the CTL group. There was no statistical significance in the baseline data of gender/age/race between the two groups ($P \geq 0.05$, Table 2). The results of HGF ELISA showed that the content of HGF protein in GA group was significantly higher than that in CTL group ($P < 0.05$, Table 2).

Table 2: The result of ELISA Validation

	GA(n=40)	CTL(n=40)	<i>P</i> -value
Sex			
Male	28 (50%)	28(50%)	1
Female	12 (50%)	12(50%)	
Ethnicity			
Han	28(50.9%)	27(49.1%)	0.809
Uygur	12 (48.0%)	13 (52.0%)	
Age (years)	45(31.5,52.0)	39(30.0,50.8)	0.491
HGF (pg/ml)	597.44(429.90,1048.23)	441.61(369.86,707.59)	0.041

4 Discussion

As the first study applying the Olink Target 48 Mouse Cytokine panel in KSD, we established a valid glyoxylate-induced mouse nephrolithiasis model (significant calcium oxalate deposition and renal tubular damage, $p < 0.001$). Olink analysis identified 24 upregulated serum cytokines (e.g., HGF, IL-6) in KSD mice; bioinformatics analyses revealed their involvement in immune-inflammatory pathways, with CD274, CTLA4, IL-6, IL-2, and IL-1 β as core hub molecules. ELISA confirmed higher serum HGF in KSD patients ($p < 0.05$), suggesting its potential as a serum inflammatory marker.

The findings of this study are consistent with the existing consensus that immune-inflammatory responses are closely related to KSD pathogenesis. Our study found upregulated IL-1 β and IL-6 in KSD mice, which are involved in the IL-17 signaling pathway [17]. Our findings of elevated serum inflammatory cytokines are consistent with a clinical study demonstrating increased urinary inflammatory mediators in patients with urinary stones [18].

In terms of innovations, this is the first study to apply Olink proteomic technology in KSD research. Compared with traditional methods, Olink technology has high throughput and sensitivity, enabling comprehensive detection of the serum inflammatory cytokine profile. Previous KSD studies focused on single or few cytokines without systematic analysis of the cytokine network [19], while our study identified 24 upregulated cytokines and clarified their core molecules and signaling pathways via bioinformatics.

Notably, ELISA validation confirmed significantly elevated serum HGF in KSD patients ($p < 0.05$), which is crucial for exploring HGF's role in KSD pathogenesis. HGF is a multifunctional cytokine mainly secreted by Kupffer cells, endothelial cells and MSCs (with negligible production by renal parenchymal cells, exerting key roles in angiogenesis, tissue repair, anti-apoptosis and inflammatory regulation through paracrine/autocrine mechanisms [20]. Regarding KSD, existing studies are limited and controversial: one study reported lower serum HGF in KSD patients [21], inconsistent with our findings (possibly due to differences in study populations), while another showed HGF protects against calcium oxalate crystal-induced renal damage [22]. This suggests elevated HGF in our study may be a compensatory response to renal injury, though its specific regulatory mechanism in KSD requires further functional verification.

Additionally, this study identified CD274 and CTLA4 as core hub molecules through PPI network analysis, which have not been widely reported in previous KSD-related inflammatory studies, thus providing new research directions for exploring the immune-inflammatory mechanisms of KSD.

Limitations: First, only a single time point was detected in this study, failing to dynamically track the temporal correlation between cytokine changes and KSD progression. Second, the Olink Target 48 panel only detects 48 inflammatory cytokines, which may lead to the omission of other key regulatory molecules. Third, the clinical sample size was small, and patients were not stratified by disease severity

or duration. Fourth, only HGF was validated clinically, without verifying other differentially expressed cytokines identified in the animal model. Fifth, this study only identified differentially expressed cytokines and core hub molecules, without conducting functional validation experiments.

In summary, this study, the first to apply Olink proteomic technology in KSD research, identified a comprehensive signature of cytokines in KSD. These findings confirm the close association between immune-inflammatory responses and KSD, enrich the understanding of KSD's molecular mechanisms, and provide new targets for KSD's early diagnosis and treatment.

Declarations

Acknowledgement

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Compliance with Ethical Standards

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Competing Interests

The authors declare no competing interests.

Ethics Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Animal experiments were approved by the Ethics Committee of Zhejiang University (approval number: ZJU20240947) and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee. Clinical Study Protocol was approved by the ethics committee of the Second People's Hospital of Aksu Prefecture, XinJiang, China (approval number: akseyyllsc-ky202402).

Informed Consent

Written informed consent was obtained from all individual participants included in the study.

Author contributions

Ge Zhang: Animal experiments and testing, manuscript writing.

Chuanjiang Ling: Project initiation, funding acquisition, manuscript review.

Zhipeng Liu: Screening of clinical research subjects, data analysis.

Qiang Yao, Cui Shi: Informed consent implementation and specimen collection.

Juan He, Bingjuan Zhang: Specimen processing and storage.

Qingchun Zhang: Recruitment of healthy volunteers.

Chang Liu: Coordination of research implementation, ethical supervision, manuscript revision.

All authors have read and approved the final manuscript.

Data availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Figures

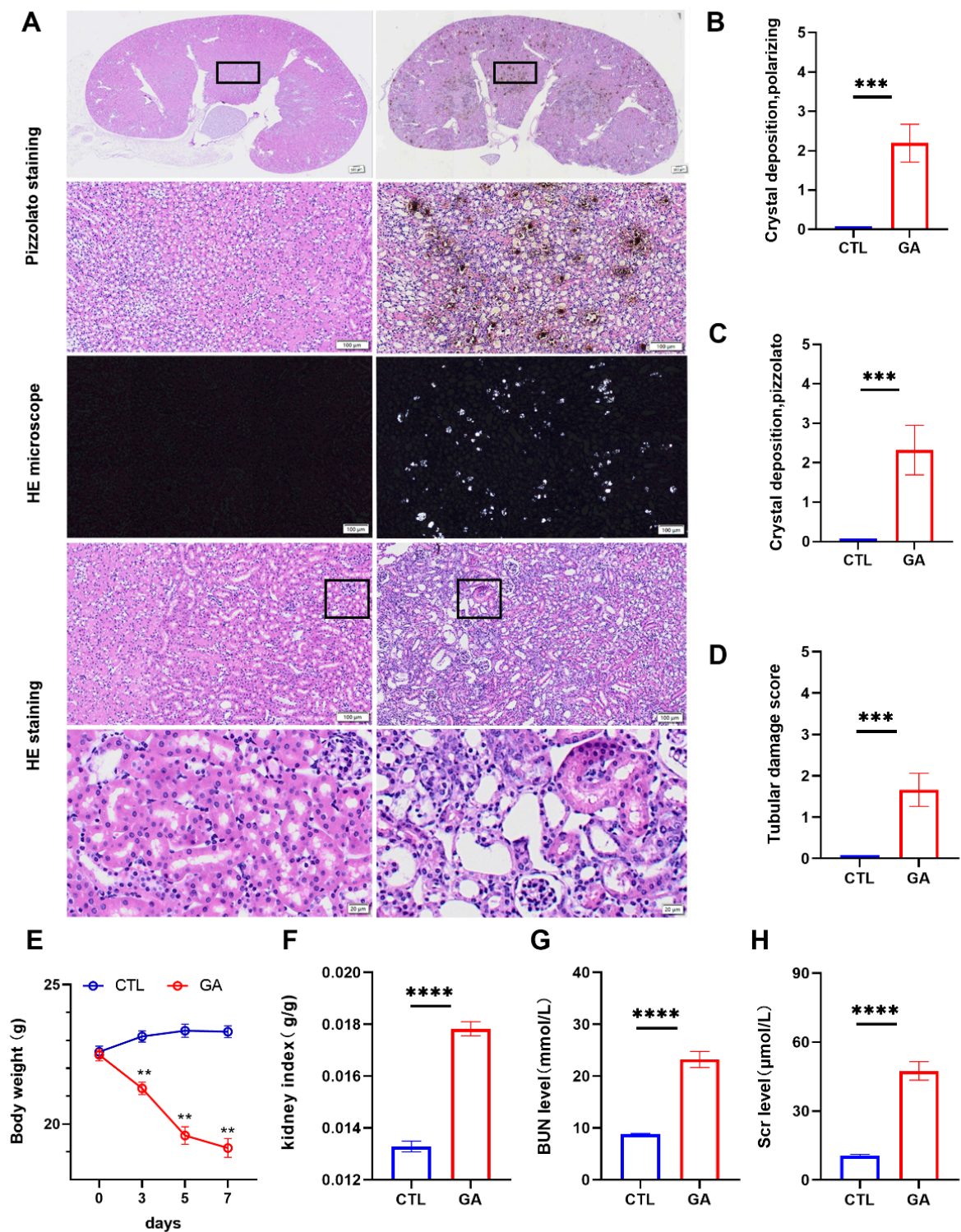


Figure 1

Kidney calculi mouse model was successfully constructed. (A) The CaOx crystal deposition in kidneys of mice was detected by pizzolato staining (Scale bar = 500μm and 100μm) and H&E staining (Scale bar = 100 μm); The renal histological changes in mice were observed by HE staining (Scale bar = 100 μm and 20 μm); (B–D) Corresponding analysis of polarization microscopy, pizzolato staining, HE staining(n=6). (E–F) Changes of the body weight and the kidney index of mice. Kidney index is the ratio of kidney weight

to mouse weight. Body weight was measured every 3 days beginning in the acclimation period (0 days) and ending at 7 days. “0” represents the acclimation period. (G–H) The levels of SCr, BUN in serum of mice were determined by corresponding kits. The results were expressed as mean ± SEM. Statistical comparisons were performed using Unpaired t test (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

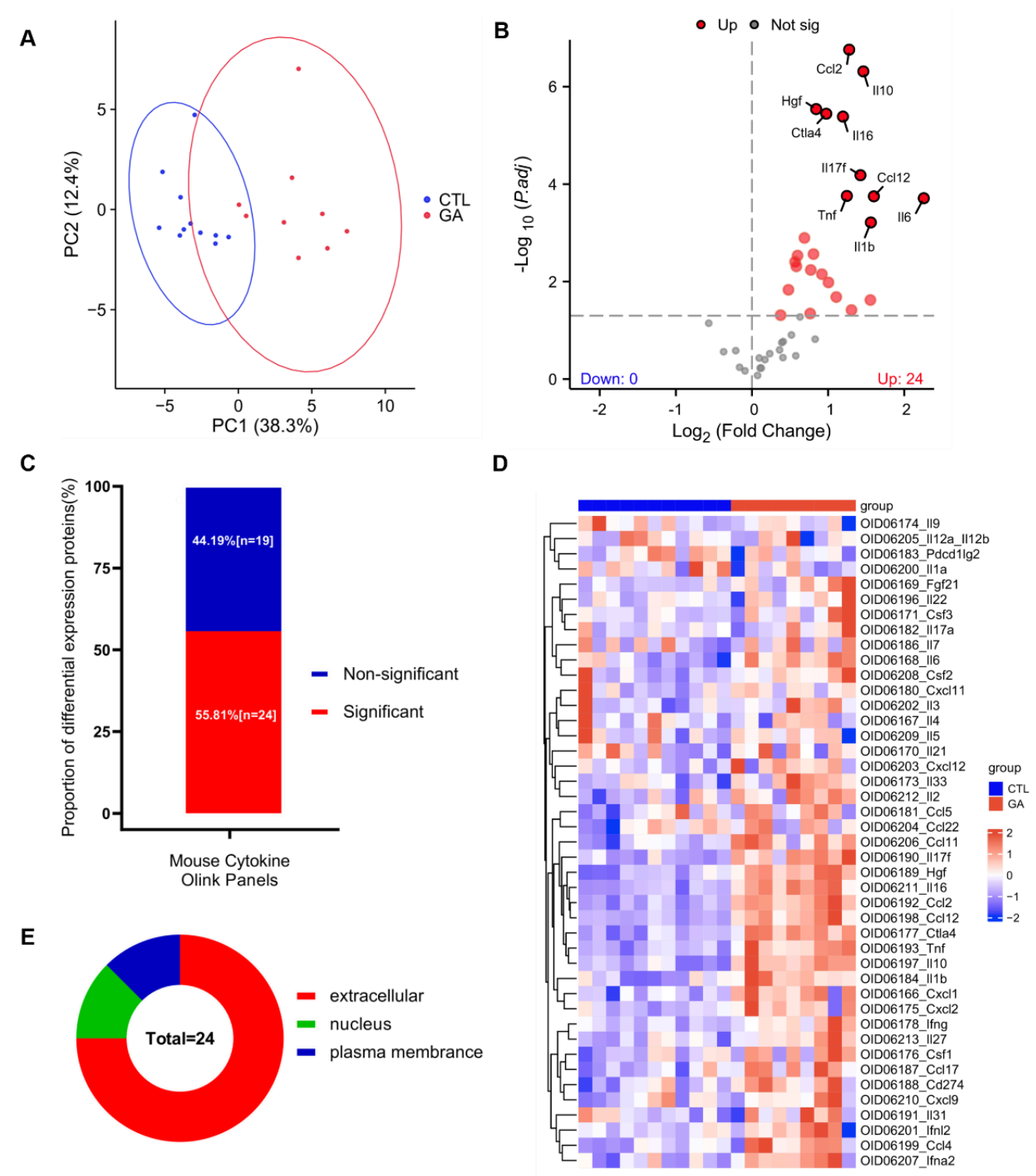


Figure 2

All differentially expressed cytokines between the CTL and GA groups(n=9~11). (A) the scatter plot of PCA scores of all samples. (B) Volcanic visualization of detected 43 cytokines. (C) Proportion of differential expression cytokines (D)Heatmap of detected 43 cytokines. (E) Pie chart of subcellular localization analysis of detected 43 cytokines

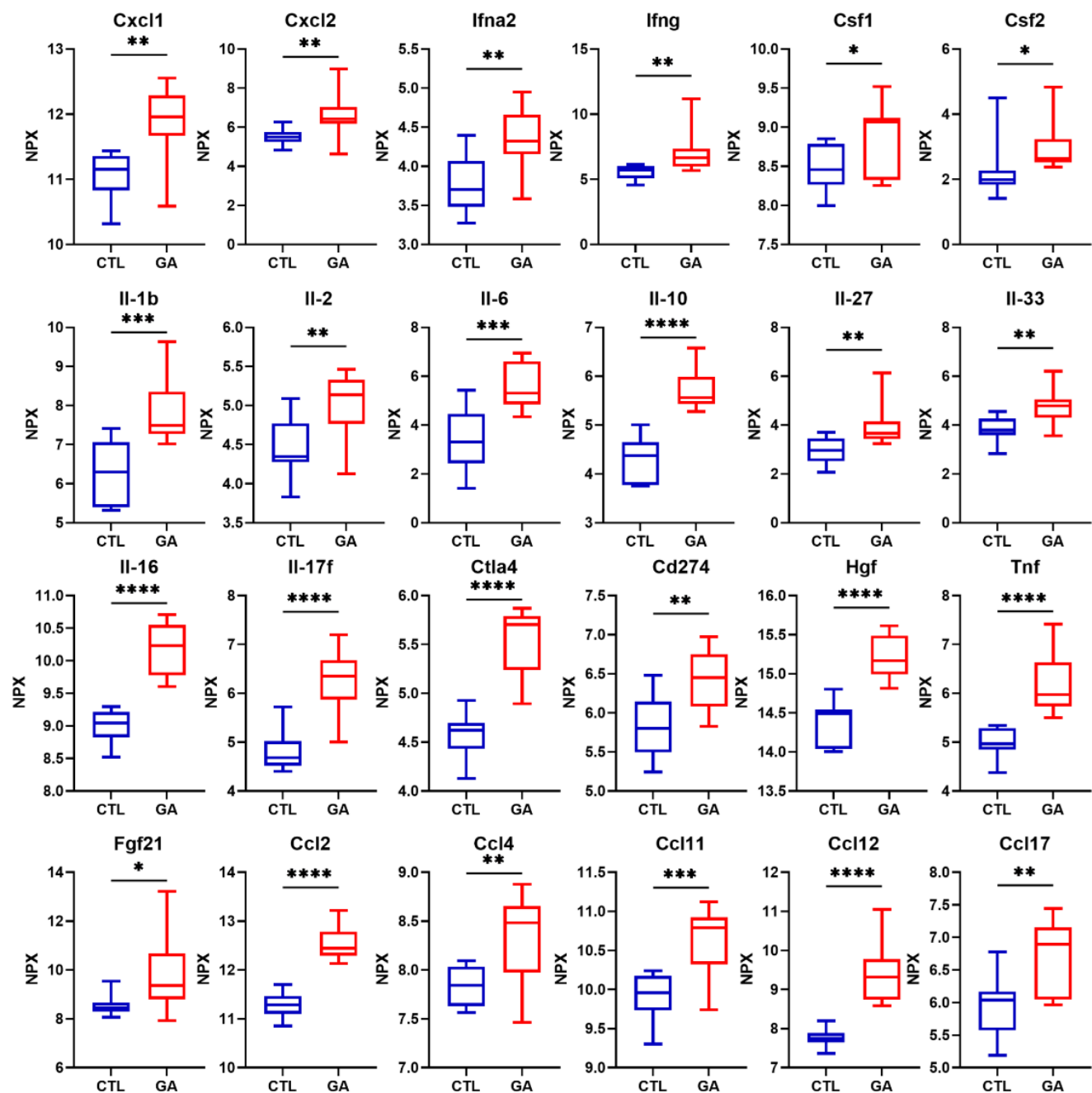


Figure 3

The comparison of the detected 24 cytokines with significant differences between the CTL and GA groups. Box plot of the detected 24 cytokines with significant differences. Data are represented as NPX,

which are normalized protein expression values on a Log2 scale. (*P < 0.05, **P < 0.01, ***P < 0.001).

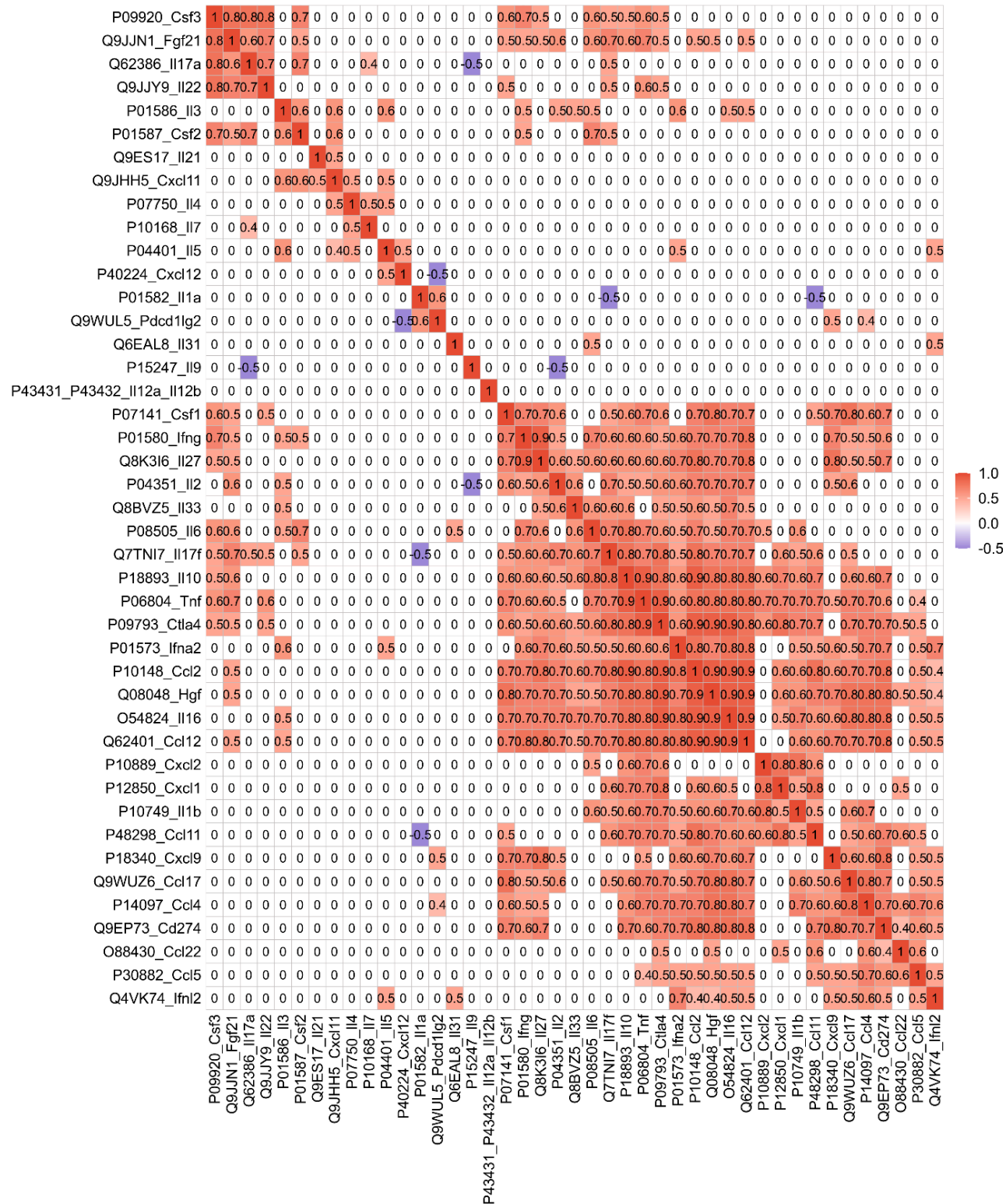


Figure 4

The thermogram of correlation between differential cytokines between the CTL and GA groups.

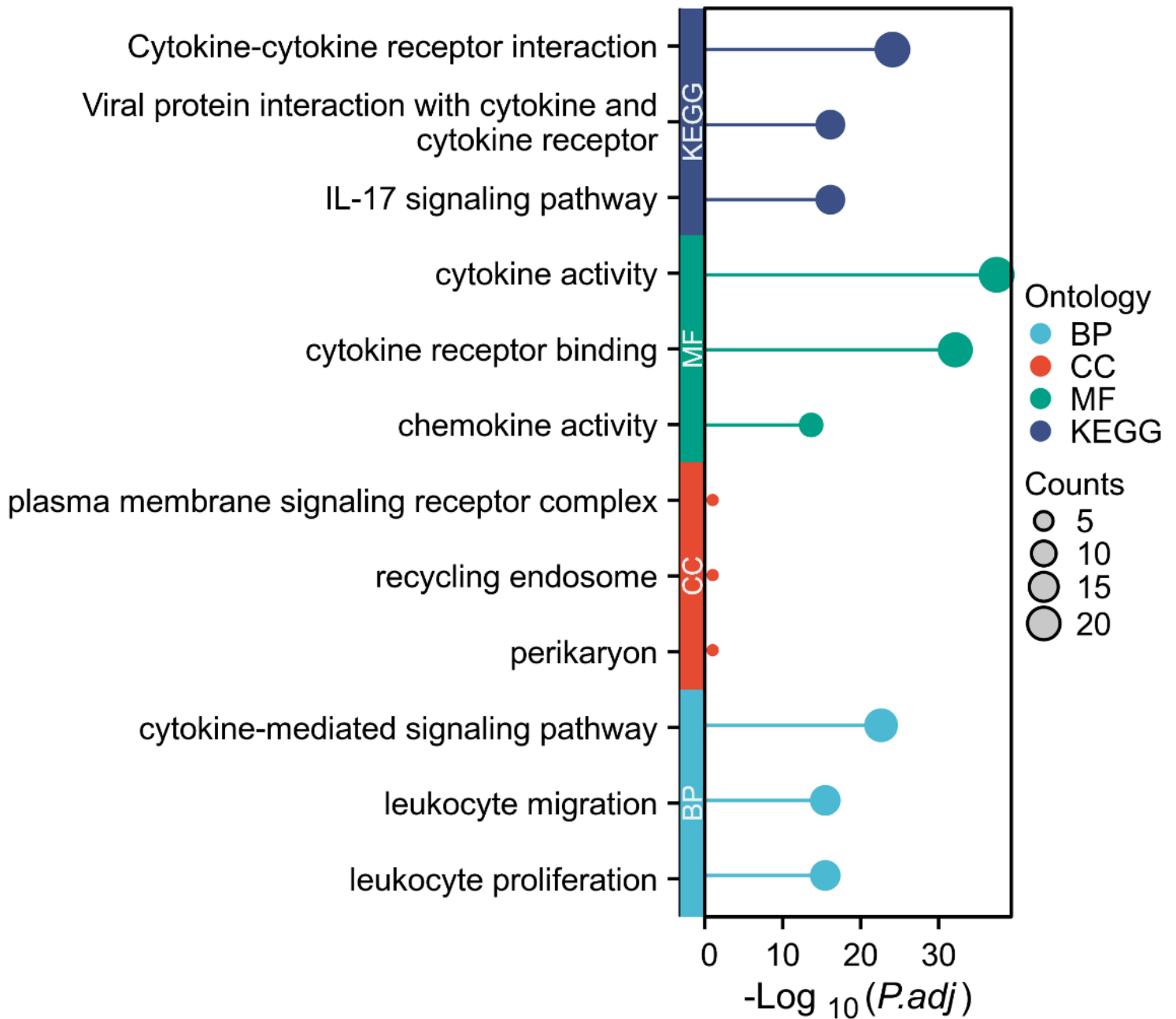


Figure 5

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the differentially detected cytokines between the CTL and GA groups.

KEGG pathway enrichment analysis was performed using the online database (available at: www.kegg.jp/kegg/kegg1.html).

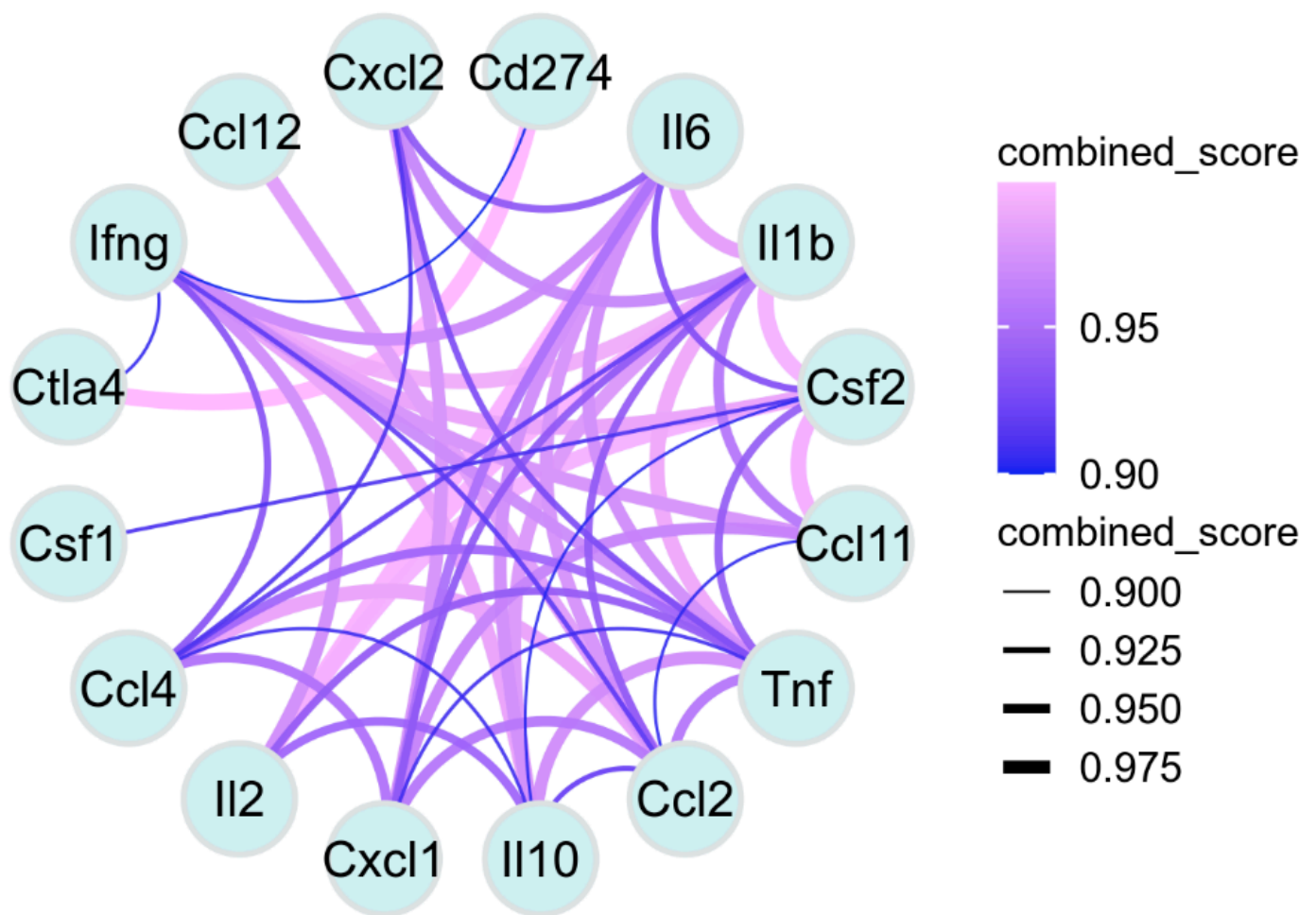


Figure 6

Protein-protein interaction (PPI) network for 24 cytokines: Protein-protein interaction analysis was carried out on the input molecular list based on the STRING database (<https://string-db.org>), and the threshold value was ultra-high reliability (0.90).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1.xls](#)
- [image1.png](#)