Journal of Inflammation Research

Dovepress
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ORIGINAL RESEARCH

Serum Olink Targeted Proteomics Identifies IL-17A as a Prospective Inflammatory Marker for the Prediction and Diagnosis of Kawasaki Disease

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Purpose: Kawasaki disease (KD) is an acute febrile vasculitis and the leading cause of acquired heart disease in children. However, early diagnosis of KD remains challenging, and its pathogenic mechanisms are yet to be fully elucidated. This study utilized Olink Targeted Proteomics to analyze serum protein profiles and identify potential early diagnostic biomarkers for patients with KD.

Methods: Based on febrile children final diagnosis, they were categorized into either the KD group or the febrile control (FC) group. Serum samples from each group were randomly selected and analyzed using the Olink Target 96 Inflammation panel. A retrospective analysis of clinical data was also conducted. By integrating the results of the Olink analysis with clinical data, receiver operating characteristic (ROC) curve analysis was performed to determine the diagnostic potential and critical thresholds of the identified biomarkers.

Results: This study identified 25 differentially expressed proteins, with 18 upregulated and 7 downregulated proteins in the KD group. Using LASSO regression analysis, we identified 5 protein biomarkers, IL-17A, CCL23, SCF, TWEAK, and NT-3, that could be used to distinguish KD from FC. Among these, IL-17A exhibited the greatest fold change. Additionally, a subset of the participants underwent serum cytokine testing within the first 5 days of fever onset during hospitalization. Our retrospective analysis of this clinical data found that IL-17 levels were significantly elevated in children subsequently diagnosed with KD.

Conclusion: Our results suggest that inflammation-associated serum proteins are strongly linked to KD. Among the identified biomarkers, IL-17 family, especially IL-17A, showed the best correlation, providing clinicians with a new potential biomarker for early diagnosis of KD.

Keywords: Kawasaki disease, inflammation, Olink Targeted Proteomics, IL-17A

Introduction

Kawasaki disease (KD), which is primarily affects in children under 5 years of age, is a pediatric vasculitis disease.[1] Although the etiology of KD remains unclear, it is generally accepted that KD is an unknown stimulus that triggers an immune-mediated inflammatory cascade in genetically susceptible children. In clinical practice, KD is diagnosed entirely based on established criteria (fever lasting ≥ 5 days and meeting clinical criteria for skin and mucosal inflammation).[2] Sometimes, it is difficult to diagnose because of the absence of certain symptoms. Untreated KD patients may experience coronary aneurysms or dilatation, ischemic heart disease, or even sudden mortality in up to 30% of cases.[3] Intravenous immunoglobulin (IVIG) treatment, if administered early, has reduced the incidence of coronary artery aneurysms (CAAs) from 25% to approximately 4%.[4] Therefore, identifying biomarkers with high specificity and sensitivity to aid in the early diagnosis of KD will be beneficial for averting major KD sequelae.

Received: 22 November 2024
Accepted: 22 February 2025
Published: 4 March 2025

In recent years, various proteomics-based studies have been conducted to identify possible biomarkers for the early diagnosis and treatment of KD. Kimura et al identified leucine-rich α-2-glycoprotein as a potential indicator for promoting KD using mass spectrometry-based proteomics analysis.[5] Furthermore, Qian et al combined white blood cell proteomics and serum metabolomics to investigate the molecular profiles of acute KD patients with coronary artery lesions (CALs). Their study identified 35 differentially abundant metabolites, including increased levels of ALPL, NAMPT, and S100P proteins, as well as decreased levels of C1QB proteins and apolipoprotein family members, which are promising candidate marker proteins and metabolites for revealing KD progression.[6] However, none of these biomarkers have been adopted for routine clinical practice. There may be unidentified serum proteins that serve as highly sensitive and specific biomarkers for distinguishing KD from other febrile conditions. Identifying such biomarkers is crucial for improving the clinical diagnosis of KD in febrile individuals.

Olink-targeted proteomics is an advanced technique based on Proximity Extension Assay. It not only has good reproducibility and stability but can also provide diverse detection panels for different diseases, assisting in the identification of clinical biomarkers.[7,8] Through the Olink Targeted Proteomics, researchers have discovered a set of promising blood biomarker diseases.[9,10] These findings underscore the potential of this technology in advancing precision medicine. Uncontrolled chronic inflammation, characterized by immune cell infiltration into the arterial wall, along with the gradual remodeling and degeneration of vascular tissue, which plays a central role in the development of KD.[11] This inflammatory response plays a critical role in the pathogenesis of KD, emphasizing the importance of identifying biomarkers that reflect these pathological changes.

In our study, the Olink Target-96 Inflammation Panel was used to screen serum samples from KD or febrile controls. By integrating the proteomic results with retrospective clinical data, we aimed to identify one or more protein biomarkers that could be used for early diagnosis of KD (Figure 1).

Materials and Methods

Ethics Approval

This study was approved by the Ethics Committee of Xuzhou Central Hospital (XZXY-LK-20230227-021) and followed the Helsinki Declaration. All the guardians signed an informed consent form.

Subjects

We obtained serum samples and clinical information from 150 pediatric patients with fever for ≥ 5 days between March 2023 and October 2024 at Xuzhou Central Hospital. Patients were classified into either the KD group (n=54) or the febrile control (FC) group (n=96) based on their final diagnosis. From each group, 15 serum samples were randomly selected for Olink Target 96 Inflammation panel.

Inclusion criteria: KD Group: (1) Age range: 6 months to 6 years; (2) Diagnosis of KD based on the American Heart Association's diagnostic criteria;[4] (3) Course of illness within 11 days of symptom onset. FC Group: Children with infectious fever who were hospitalized during the same period (their diseases mainly include: acute tonsillitis, acute pharyngitis, bronchopneumonia, infantile rash, infectious mononucleosis, sepsis, pharyngitis, etc). Exclusion Criteria: (1) Patients in the recovery stage of KD; (2) Recent treatment with immunosuppressants and/or IVIG; (3) Presence of serious underlying diseases; (4) Incomplete laboratory data.

Serum Sample Collection

Blood samples (2 mL) were collected from patients on the first day of admission. Serum was extracted by centrifugation at 3000 rpm for 15 min and stored at −80°C for further analysis.

Inflammation-Related Biomarkers Screening

In accordance with the manufacturer's instructions, the Olink Target 96 Inflammation panel was utilized to quantify serum protein levels. The operating principle of Olink technology is based on the Proximity Extension Assay method. Two labeled oligonucleotide probes are used; they approach and bind to the target inflammatory protein and are then

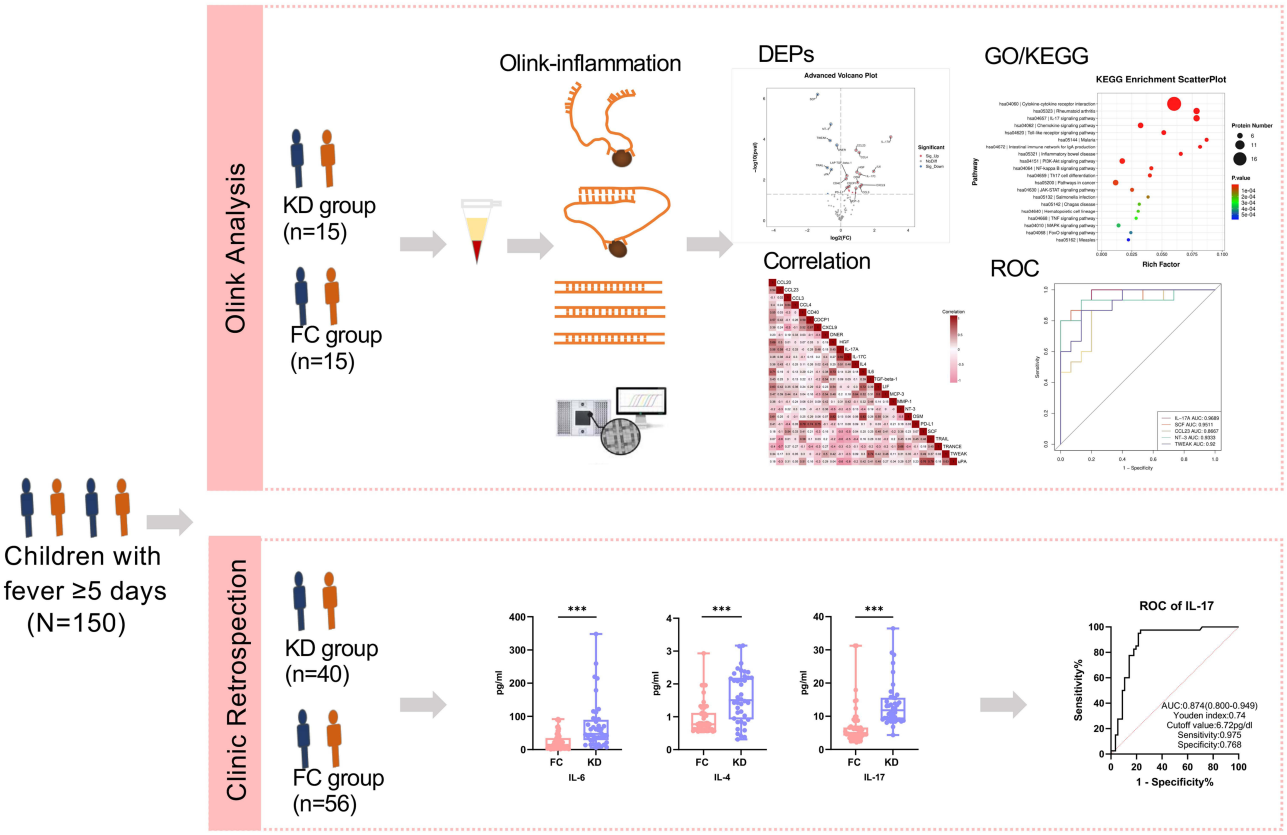

Figure 1 Study strategy and schematic illustration of Serum Olink proteomics. Serum samples and clinical data were collected from 150 pediatric patients presenting with fever lasting ≥ 5 days. Based on the final diagnosis, the patients were categorized into the KD group (n=54) and the FC group (n=96). 15 serum samples were randomly selected from each of the KD and FC groups for Olink Targeted Proteomics analysis. Among the enrolled children, 40 KD patients and 56 FC patients underwent cytokine tests (covering 12 items) prior to enrollment. By integrating the results of the Olink analysis with the clinical data from the cytokine tests, IL-17A was identified as a potent predictor of KD. (***$p<0.001$).

detected through PCR amplification. After quality control and log2 standardization, the data is presented as standardized protein expression (NPX), with the median representing protein expression.

The R package "Olink Analyze" was employed to identify differentially expressed proteins (DEPs) between the FC and KD group. To visualize the distribution and clustering of these DEPs, we employed the ggplot2 package to construct heat maps and volcano plots. Furthermore, the Glmnet package was used to perform Least Absolute Shrinkage and Selection Operator (LASSO) regression analysis with 10-fold cross-validations to select the model with the best predictive performance or the lowest cross-validation error, which can be considered the most optimal diagnostic model. The diagnostic efficacy of the DEPs was evaluated using a Receiver Operating Characteristic (ROC) curve. The area under the curve (AUC) served as a metric for diagnostic accuracy, where a larger area indicated superior diagnostic performance.

Function Enrichment Analysis and Correlation Analysis

We used the ggplot2 package to conduct in-depth enrichment analyses, specifically targeting Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. In this process, differentially expressed proteins (DEPs) were carefully associated with their corresponding entries in the GO and KEGG databases. A hypergeometric testing method was applied to identify significant enrichments. Subsequently, Pearson correlation analysis was carried out to clarify the relationship between the expression levels of two proteins. After that, Cytoscape software (version 3.9.1) was used to construct and visualize a Protein-Protein interaction (PPI) network for the DEPs.

https://doi.org/10.2147/JIR.S506154

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 27 and the R programming language. Depending on the nature of the data being compared, an unpaired Student's *t*-test, rank-sum test (such as Mann–Whitney *U*-test), or chi-square test was employed to assess the statistical significance of the differences between the KD and FC groups. Receiver Operating Characteristic (ROC) curve analysis was employed to evaluate the diagnostic effectiveness of the biomarkers and determine the optimal cut-off value. Statistical significance was determined when the *p*-value was less than 0.05.

Results

Clinical Characteristics of All Participants

There were 54 and 96 patients in the KD and FC groups. Table 1 shows the general clinical features and conventional laboratory results for all subjects. No significant differences were noted between the two groups in terms of sex distribution, age, fever duration, platelet count, or white blood cell count ($p>0.05$). However, KD patients exhibited notably decreased levels of hemoglobin ($p<0.001$), albumin ($p<0.001$), and Na^+ ($p<0.001$), accompanied by elevated levels of aminotransferase ($p<0.001$) and C-reactive protein ($p<0.001$).

Identification of the Inflammation-Related Biomarker

The Olink inflammation panel was utilized to assess the expression levels of 92 inflammation-related proteins in both the KD and FC groups. As shown in Table 2 and Figure 2A, 25 DEPs were identified in the KD group, including 18 upregulated and 7 downregulated proteins. Figure 2B presents a heatmap of the expression levels of the aforementioned DEPs across all samples. Significant differences in the expression of DEPs were observed between the KD and FC groups (Figure 2C). Among these, interleukin-17A (IL-17A) exhibited the most substantial fold change (fold change = 2.15, $p<0.001$) (Table 2).

Function Enrichment Analysis and Correlation Analysis

To delve deeper into the potential functions of the 25 DEPs, GO and KEGG enrichment analyses were performed. GO enrichment analysis revealed that these distinct proteins were primarily involved in inflammatory response, immune response, signal transduction, and various cellular processes (Figure 3A). KEGG enrichment analysis suggested that the differentially expressed proteins identified in this study were mainly implicated in multiple signaling cascades, including the IL-17 signaling pathway, cytokine-cytokine receptor interaction, and chemokine signaling pathway (Figure 3B). Additionally, we performed correlation analysis and constructed a PPI network to explore the interactions among the aforementioned proteins. As shown in Figure 3C, a significant correlation was observed between IL-17A and IL-17C

Table 1 Baseline Characteristics of Enrolled Patients

Variable	KD (n=54) [a]	FC (n=96) [a]	*p* Value [b]
Age (months)	19 (12, 47)	32 (20, 46)	0.069
Female/male	19/35	39/57	0.511
Duration of fever (days)	6 (5, 7)	6 (5, 7)	0.114
Number of clinical features	4 (3, 5)	2 (1, 3)	<0.001
Hemoglobin (g/L)	108.7±8.8	122.2±8.8	<0.001
Platelet ($\times10^9$/L)	363±108	338±75	0.105
White blood cell ($\times10^3$/μL)	13.0±4.4	11.9±5.2	0.173
C-reactive protein (mg/L)	47.9 (23.7, 76.6)	9.6 (3.3, 20.1)	<0.001
Alanine aminotransferase (U/L)	24 (15, 57)	14 (11, 19)	<0.001
Albumin (g/L)	41 (38, 44)	45 (42, 47)	<0.001
Na^+ (mmol/L)	136 (133, 138)	138 (137, 141)	<0.001

Notes: [a] Mean (SD); Median (P25, P75). [b] Unpaired Student's *t*-test; Wilcoxon rank sum test; Pearson's Chi-squared test.

Table 2 Differentially Expressed Serum Inflammatory Proteins Between the KD and FC Groups

Olink ID	Protein Symbol	Name	*P* Value	Fold Change
OID00500	SCF	Kit ligand	<0.001	0.86
OID00554	NT-3	Neurotrophin-3	<0.001	0.77
OID00485	IL-17A	Interleukin-17A	<0.001	2.15
OID00555	TWEAK	Tumor necrosis factor ligand superfamily member 12	<0.001	0.94
OID01213	DNER	Delta and Notch-like epidermal growth factor-related receptor	<0.001	0.97
OID00530	CCL23	C-C motif chemokine 23	<0.001	1.09
OID00498	CCL4	C-C motif chemokine 4	<0.001	1.13
OID00488	TRAIL	Tumor necrosis factor ligand superfamily member 10	0.003	0.91
OID00481	u PA	Urokinase-type plasminogen activator	0.003	0.95
OID00482	IL-6	Interleukin-6	0.004	1.37
OID00522	HGF	Hepatocyte growth factor	0.004	1.09
OID00483	IL-17C	Interleukin-17C	0.005	1.34
OID00480	TGF-beta-1	Transforming growth factor beta-1	0.006	1.05
OID00542	CD40	Tumor necrosis factor receptor superfamily member 5	0.009	1.03
OID00494	OSM	Oncostatin-M	0.013	1.12
OID00490	CXCL9	C-X-C motif chemokine 9	0.018	1.14
OID00476	CDCP1	CUB domain-containing protein 1	0.021	1.12
OID00532	CCL3	C-C motif chemokine 3	0.021	1.15
OID00518	PD-L1	Programmed cell death 1 ligand 1	0.024	1.06
OID00474	MCP-3	C-C motif chemokine 7	0.025	1.23
OID00546	IL-4	Interleukin-4	0.032	1.75
OID00547	LIF	Leukemia inhibitory factor	0.035	1.63
OID00510	MMP-1	Interstitial collagenase	0.041	1.06
OID00556	CCL20	C-C motif chemokine 20	0.043	1.11
OID00521	TRANCE	Tumor necrosis factor ligand superfamily member 11	0.047	0.86

levels (R=0.82, $p<0.001$). Furthermore, IL-6 and IL-17A received higher scores in the PPI network, suggesting that these proteins may play pivotal roles in the pathogenesis of KD (Figure 3D).

Significant Diagnostic Values of DEPs

LASSO regression analysis was used to further investigate the potential protein biomarkers that could differentiate KD from FC. We identified five protein biomarkers: IL17-A, CC-chemokine ligand 23 (CCL23), stem cell factor (SCF), TNF-like weak inducer of apoptosis (TWEAK) and Neurotrophin-3 (NT-3) (Figure 4A and B). Figure 4C shows that compared to the FC group, IL-17A and CCL23 serum expression levels in the KD group were significantly increased, while those of SCF, NT-3, and TWEAK were significantly decreased. Subsequently, we evaluated the diagnostic effectiveness of the five protein biomarkers in predicting KD using ROC analysis. The AUC values of IL-17A, CCL23, SCF, NT-3, and TWEAK were greater than 0.8, with actual values of 0.969 (0.918–1.0), 0.867 (0.737–0.996), 0.951 (0.874–1.0), 0.933 (0.833–1.0), 0.920 (0.827–1.0), respectively (Figure 4D). Compared with the other four DEPs, IL-17A has a larger AUC, demonstrating superior diagnostic performance.

Differences of Cytokines Between KD and FC in Clinical Practice

After reviewing the clinical data of the 150 participants in this study, it was discovered that 40 children in KD group and 56 children in FC group had undergone Cytokine Tests (12 items, TNF-α, IFN-α, IFN-γ, IL-6, IL-4, IL-17, IL-1β, IL-2, IL-5, IL-8, IL-10, and IL-12p70) prior to enrollment, despite having a fever duration of less than 5 days. By combining the results of Olink analysis with clinical data from the Cytokine Tests, we found the serum concentration of IL-17, IL-6 and IL-4 in the KD group [11.81 (9.04, 15.57), 47.21 (28.60, 89.67), 1.51 (0.92, 2.20)] were significantly higher compared with children in the FC group [4.58 (4.18, 6.60), 13.69 (4.55, 34.68), 0.61 (0.77, 1.12)] ($p<0.001$) (Figure 5A). Notably, IL-17 demonstrated excellent diagnostic performance, with an area under the curve (AUC) of

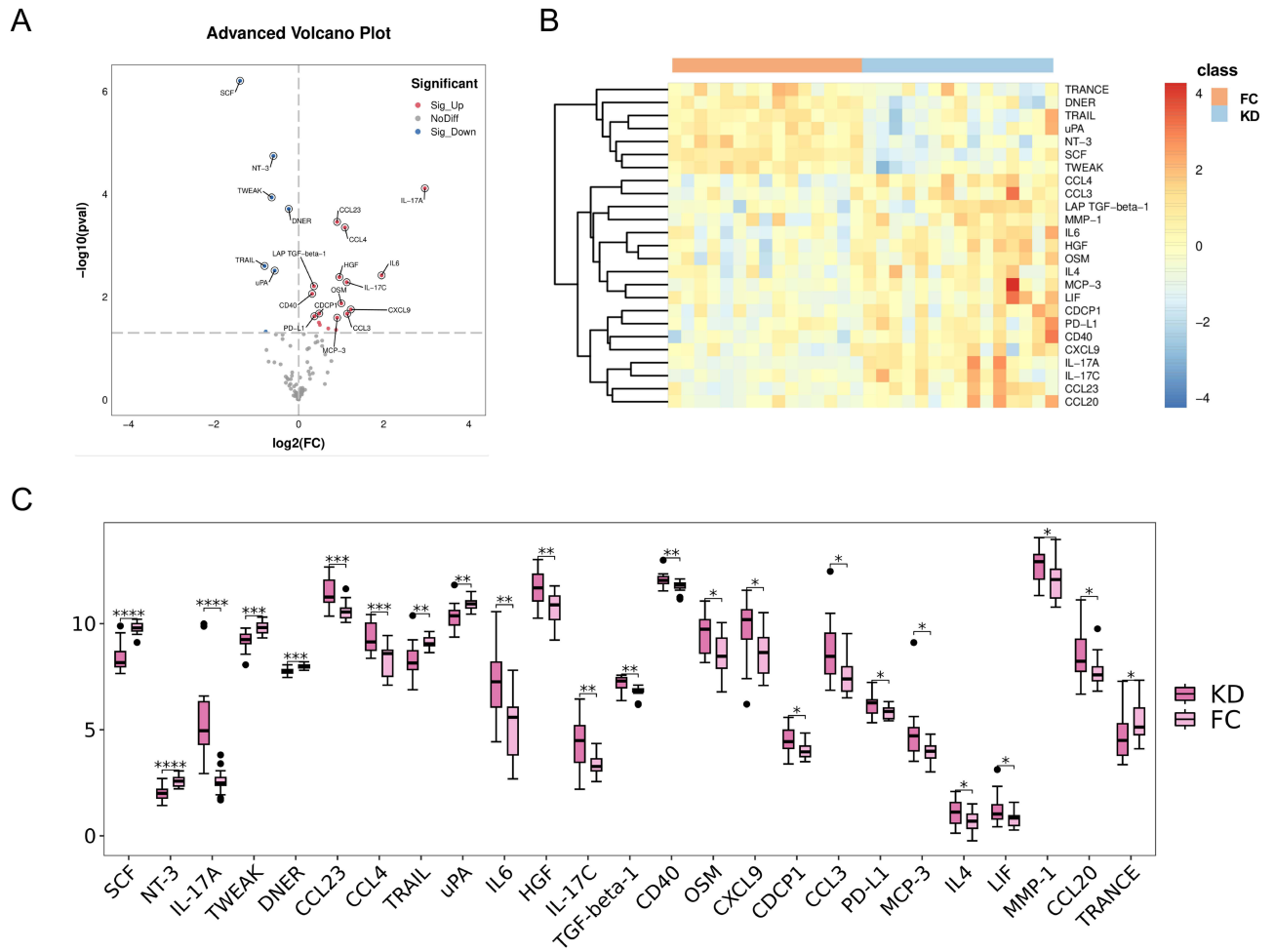

Figure 2 Inflammation-related DEPs between the KD and FC Groups. (**A**) Volcano Plot: A volcano plot was used to visualize 92 inflammation-related biomarkers. (**B**) Heatmap: A heatmap was constructed to display the expression patterns of the 25 DEPs. (**C**) Box Plot: A box plot was generated for the 25 DEPs. Solid dots outside the box represent values that are 1.5 times the interquartile range above or below the quartiles. (*p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001).

0.874 [95% CI, 0.800–0.949], sensitivity of 97.5%, and specificity of 76.8%. The optimal cut-off value for IL-17 was 6.72 pg/mL, as determined by the Youden index (Figure 5B and C).

Discussion

In 1967, Japanese pediatrician Tomisaku Kawasaki first described mucocutaneous lymph node syndrome, which he presented as a new disease.[12,13] Since then, KD cases have been reported across various racial and ethnic groups in over 60 countries worldwide. Japan has the highest incidence of KD. Recently published data from a nationwide survey in Japan reported an increasing rate over time, from 218.6 cases per 100,000 people in 2008 to 371 cases per 100,000 people in 2019.[14,15] The incidence of KD in children under 5 years of age varies internationally. In Shanghai, the largest city in China, the incidence of KD has increased approximately threefold over the past 20 years in longitudinal surveys.[16,17]

KD is an inflammatory vasculitis that affects small- and medium-sized arteries; however, its pathogenesis remains poorly understood. Most researchers assume that uncontrolled chronic inflammation, including immune cell infiltration into the arterial wall as well as gradual remodeling and death of vascular tissue, plays a central role in the development of KD.[11] Elevated levels of proinflammatory cytokines, such as monocyte chemotactic protein (MCP)-1, IL-6, IL-8, and IL-1β, have been implicated in KD pathogenesis and CALs.[18,19] Wang et al[20] found that IL-6 and IFN-γ were independent

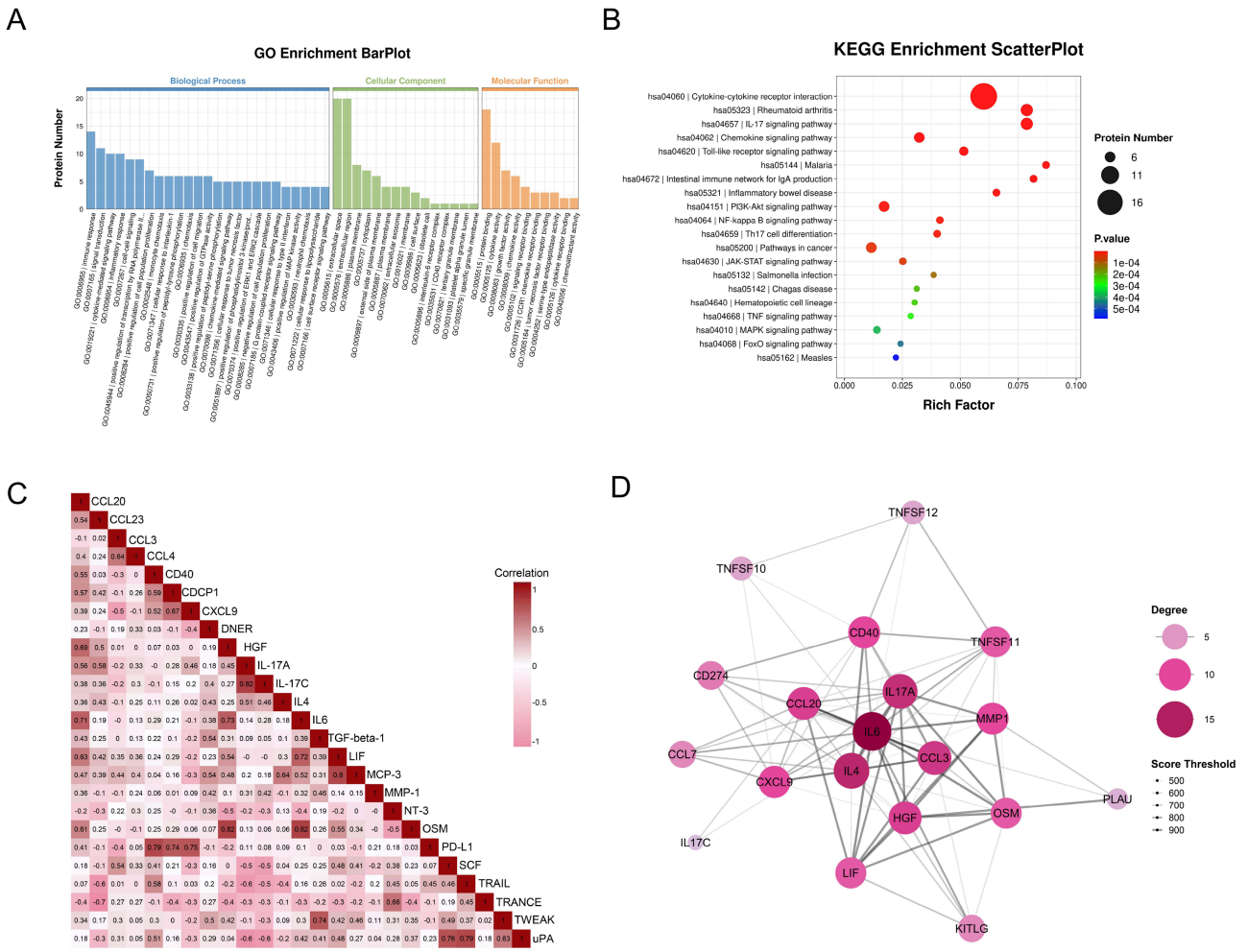

Figure 3 Functional analysis of inflammation-related DEPs between the KD and FC groups. (**A**) GO enrichment of the DEPs. (**B**) KEGG enrichment of the DEPs. (**C**) Correlation coefficient diagram of the DEPs. (**D**) PPI network diagram of the DEPs.

risk factors for IVIG resistance in KD, further linking KD to abnormal inflammatory responses and highlighting the potential of inflammatory protein factors as biomarkers.

In this study, we compared serum inflammation-related protein profiles between the KD and FC groups using Olink Targeted Proteomics and identified 25 DEPs. These DEPs were enriched in several inflammation-related pathways such as the IL-17 signaling pathway, cytokine-cytokine receptor interaction, and chemokine signaling pathway. Among the DEPs, IL-17A, SCF, CCL23, NT-3, and TWEAK emerged as potential biomarkers for KD. IL-17A was the most significantly upregulated inflammation-related protein in the KD group (fold-change=2.15, $p<0.001$). LASSO regression analysis revealed the exceptional predictive efficacy of IL-17A, with an AUC value exceeding 0.9. Retrospective analysis of clinical data revealed that IL-17 levels were significantly elevated in KD patients during the early febrile phase, with an optimal diagnostic cut-off value of 6.72 pg/mL.

IL-17A, a proinflammatory cytokine and member of the IL-17 family (IL-17A to F), plays a critical role in autoimmune diseases, inflammatory responses, and host defense.[21] It can recruit immune cells to increase the proinflammatory effect and cause endothelial cells, epithelial cells, monocytes/macrophages, and fibroblasts to produce a range of proinflammatory cytokines and chemokines.[22] Substantial evidence suggests that IL-17 involved in many diseases, for example psoriasis,[23] asthma[24] and rheumatoid arthritis.[18] Additionally, some researchers have discovered links between KD and IL-17. By detecting plasma levels of Th17-related cytokines, such as IL-6, IL-17A, TGF-β, and IL-10, KUO.et al found that plasma levels of IL-17A were significantly higher in KD patients.[19] KD patients exhibit

https://doi.org/10.2147/JIR.S506154

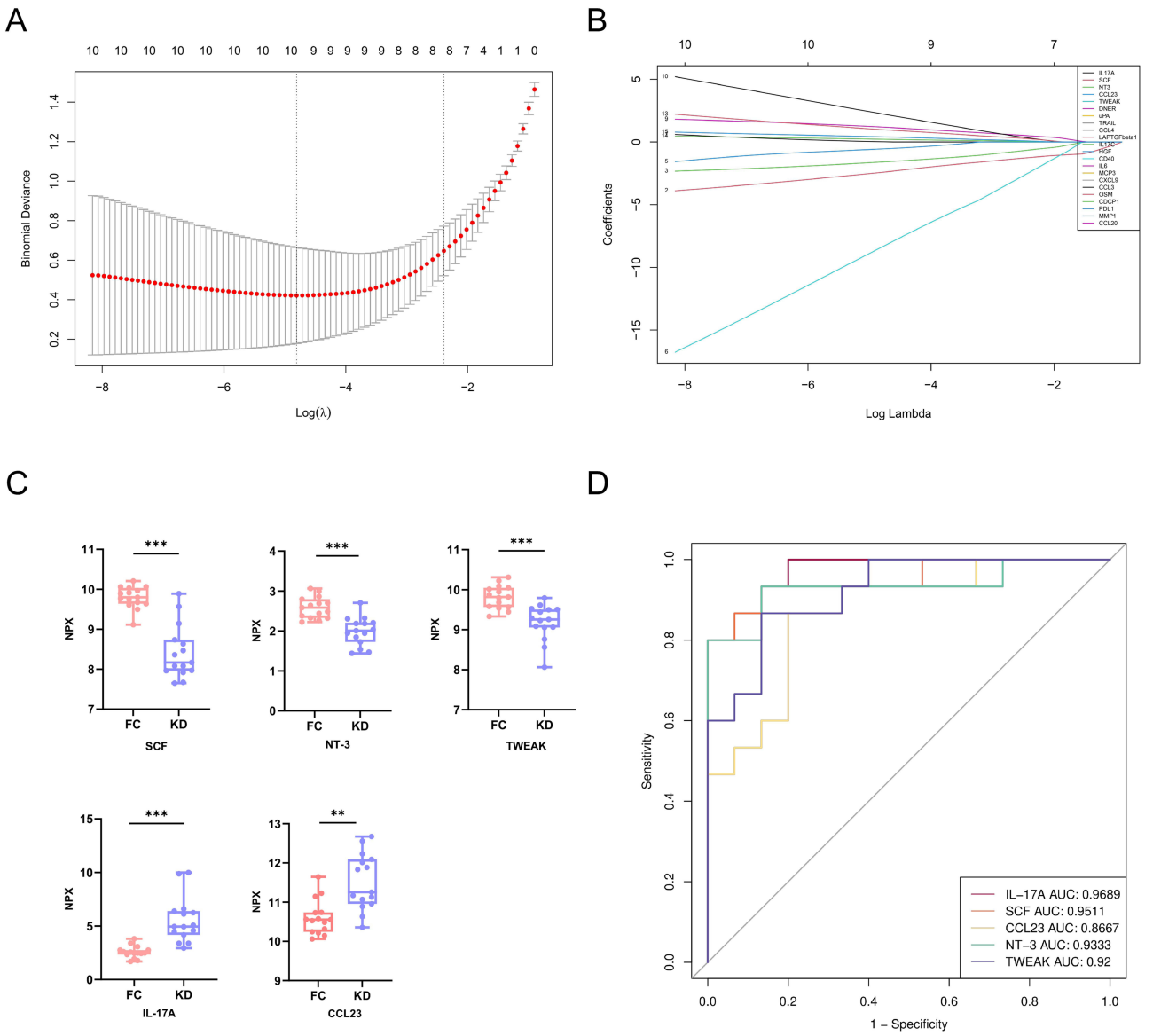

Figure 4 Screening of candidate protein biomarkers for the early diagnosis of KD. (**A** and **B**) Candidate proteins were selected through LASSO regression analysis. (**C**) Boxplot of the five candidate protein biomarkers (IL-17A, SCF, CCL23, NT-3 and TWEAK) in the discovery set. (***p*<0.01, ****p*<0.001) (**D**) The diagnostic performance of the five proteins was evaluated.

higher levels of IL-17 family cytokines, including IL-17A and IL-17C, compared to those with other pediatric inflammatory disorders, such as multisystem inflammatory syndrome in children, juvenile dermatomyositis, and macrophage activation syndrome.[25] In addition, IL-17A blockers have made significant progress in the treatment of certain immune-related diseases, such as psoriasis, psoriatic arthritis, and ankylosing spondylitis.[26] This study suggests that targeting the IL-17 signaling pathway may provide insights for the treatment of KD and even refractory KD.

The roles of SCF, CCL23, NT-3, and TWEAK in Kawasaki disease (KD) warrant further exploration. As a dimeric molecule, SCF exerts its biological functions by binding to and activating the c-Kit receptor, playing a crucial role in angiogenesis and cardiovascular health.[27] Matsui et al reported that endothelial cells treated with SCF may trigger proangiogenic response, increase motility and promote capillary formation.[28] Wang et al found that SCF promotes the survival of vascular smooth muscle cells and inhibits cell apoptosis by activating c-Kit.[29] A prospective population-based study has found that individuals with higher levels of SCF in their bodies have a reduced risk of cardiovascular disease and mortality.[30] However, the research on the relationship between SCF and KD remains limited, and existing findings

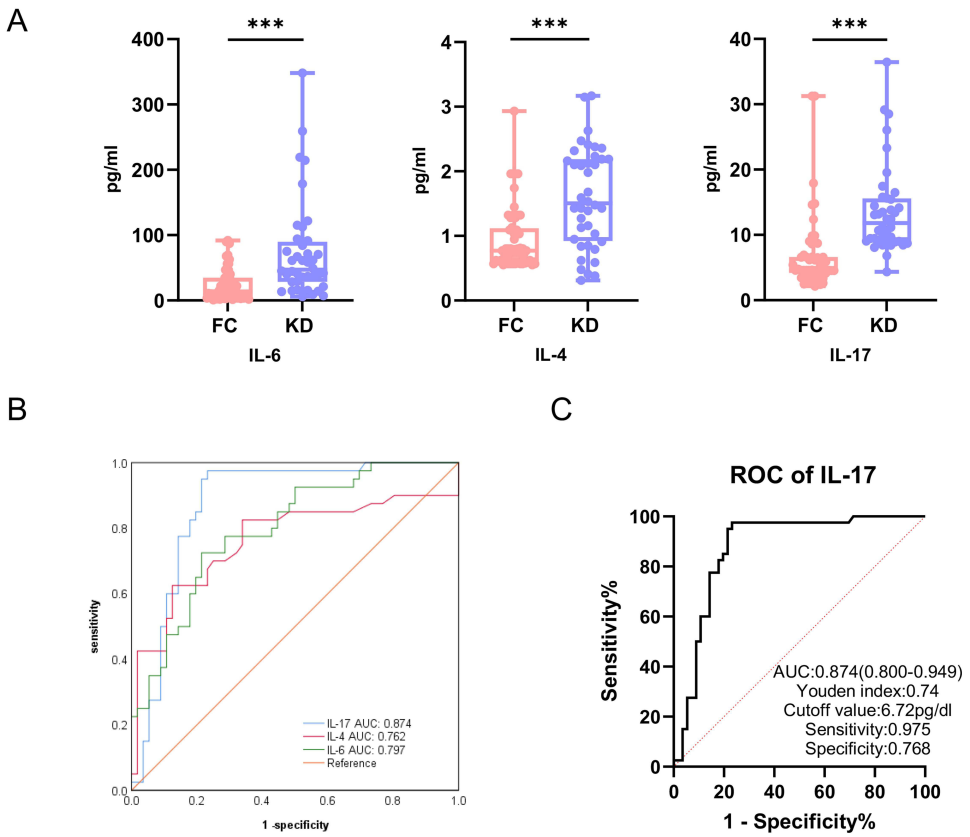

Figure 5 Differences in cytokines between the KD and FC Groups in clinical practice. (**A**) The IL-17, IL-6 and IL-4 serum expression level in Cytokine Tests. (***p<0.001) (**B**) Diagnostic performance of the three proteins. (**C**) ROC curve of IL-17.

are somewhat controversial. In our study, we observed a significant downregulation of SCF in the serum of children with KD, suggesting that SCF may play a protective role in the disease.

CCL23, secreted by a variety of immune cells, including neutrophils, monocytes, eosinophils, dendritic cells, and macrophages, exerts its biological effects on endothelial and immune cells through the CCR1 receptor.[31–33] Elevated levels of CCL23 in the serum may stimulate the recruitment of peripheral immune cells and the release of proinflammatory cytokines, thereby amplifying inflammatory responses.[34–36] Recent studies have highlighted the pivotal role of CCL23 in the inflammatory processes of several diseases, including chronic rhinosinusitis, eosinophilic airway inflammation, and chronic kidney disease.[32,34,37] Our findings revealed a significant increase in CCL23 levels in the serum of children with KD. The clinical significance of this elevation, particularly in relation to CALs and KD prognosis, warrants further investigation.

NT-3, a member of the NT family, is thought to be an essential trophic element in the control of nervous system development, survival, and function.[38] Cristofaro et al provided an initial insight into the proangiogenic potential of NT-3, positioning it as a promising candidate for the treatment of ischemic diseases.[39] However, there is currently limited research on the role of NT-3 in KD. TWEAK, which binds to Fn14 (a type I transmembrane receptor), plays a pivotal role in the activation and regulation of various cellular responses including the production of inflammatory cytokines, angiogenesis, and proliferation. Previous studies have demonstrated an imbalance in the expression of TWEAK/Fn14 in immune diseases such as osteoporosis, systemic lupus erythematosus, and inflammatory bowel disease.[40–42] Although TWEAK's role has been studied in several immune disorders, its specific involvement in KD remains unclear. Our study provides new insights into the pathogenesis of KD, suggesting that TWEAK may play a significant role in this.

This study has several limitations. First, the sample size used for Olink detection is relatively small, which may limit the generalizability of the findings. Second, we were not to conduct a detailed classification of KD, such as distinguishing between complete and incomplete KD, assessing the presence of CAAs, or performing a prognostic follow-up. To

address these limitations, we planned to participate in a multicenter study aimed at validating these biomarkers in a larger and more diverse patient population.

Conclusion

In conclusion, our study identified 25 DEPs in the serum of children with KD. The enrichment of these DEPs in inflammatory signaling pathways underscores the central role of inflammation in KD progression. IL-17A, SCF, NT-3, CCL23, and TWEAK emerge as promising diagnostic biomarkers for KD, with IL-17A demonstrating the highest diagnostic efficacy. Notably, IL-17 levels significantly increase during the initial febrile phase of KD, further supporting the potential of IL-17A as an early diagnostic target for KD.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Acknowledgments

This study was supported by the Xuzhou Medical Leading Talent Training Project (No. XWRCHT20210039).

Disclosure

The authors declare that they have no competing interests.

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