

# Identification of Serum BST1 as a Biomarker to Predict Coronary Artery Lesions in Children with Kawasaki Disease Based on 4D-DIA Quantitative Proteomics

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**Background:** Kawasaki disease (KD) is a leading cause of acquired heart disease in children, with coronary artery lesions (CAL) as its most severe complication. This observational study aimed to investigate the expression of bone marrow stromal cell antigen-1 (BST1) in KD patients and its value for predicting CAL.

**Methods:** Serum samples were collected from KD patients before intravenous immunoglobulin (IVIG) treatment and healthy controls (HC) in Xi'an Children's Hospital from July 2023 to August 2024. KD Patients were stratified into CAL and non-CAL (nCAL) groups based on echocardiography. In the discovery cohort (8 KD-CAL patients, 7 KD-nCAL patients, and 4 HC), Sera were analyzed via four-dimensional data-independent acquisition (4D-DIA) quantitative proteomics. Differentially expressed proteins (DEPs) were identified and analyzed for bioinformatics. In the validation cohort (35 KD-CAL patients, 61 KD-nCAL patients, and 30 HC), serum BST1 was validated by enzyme-linked immunosorbent assay (ELISA). Receiver operating characteristic (ROC) curve was constructed to assess the performance of BST1 in predicting CAL.

**Results:** A total of 2575 proteins were identified by 4D-DIA proteomics, with 807 DEPs in nCAL vs HC and 883 DEPs in CAL vs HC. Compared to nCAL, 213 DEPs were identified in CAL patients, among which BST1 was significantly upregulated only in the CAL group. Functional analyses revealed enrichment in innate immune response, cell adhesion, RNA processing pathways, complement and coagulation cascades, extracellular exosomes, and cellular metabolism. ELISA confirmation showed elevated BST1 levels in CAL patients, which positively correlated with Z-scores. ROC analysis demonstrated high prognostic accuracy for CAL (AUC=0.9052).

**Conclusion:** Serum BST1 has the potential to be a predictive biomarker for CAL in KD patients.

**Keywords:** Kawasaki disease, CAL, KD-CAL, BST1, proteomics, 4D-DIA, ROC curve

## Introduction

Kawasaki disease (KD) is an acute febrile illness predominantly affecting children under the age of five, characterized by systemic vasculitis of unknown etiology.<sup>1</sup> The incidence of KD has gradually increased over the past few decades, making it the leading cause of acquired heart disease in developed nations.<sup>2</sup> Without prompt treatment, a significant proportion, approximately 25%-30%, of KD patients are at high risk for developing coronary artery lesions (CAL).<sup>3</sup> These KD-associated CAL (KD-CAL) account for nearly 5% of acute coronary syndrome cases in young adults under 40 years of age and can lead to severe adverse events such as myocardial infarction and sudden death.<sup>4</sup> The diagnosis of KD, which lacks definitive pathological evidence, currently depends on the identification of key clinical signs and the exclusion of other diseases with similar symptoms and known etiologies.<sup>5</sup> Consequently, the identification of effective

biomarkers during the acute phase of KD is imperative for the early prediction of CAL, which is critical for guiding treatment decisions and prognostic assessments.<sup>6</sup>

Serum contains a vast array of circulating proteins that play crucial roles in regulating physiological functions, and changes in the expression levels of serum proteins mirror the dynamic nature of disease pathology, such as KD.<sup>7</sup> Therefore, a thorough analysis of the serum proteome is of significant value in unraveling KD mechanisms and discovering potential predictors. Four-dimensional data-independent acquisition (4D-DIA) quantitative proteomics is a state-of-the-art method that extends traditional proteomics, which not only expedites scanning speed but also enhances detection sensitivity, significantly amplifying the performance metrics of proteomics analysis. In recent years, as its comprehensive and in-depth analytical capabilities, 4D-DIA technology has been used to explore disease pathophysiological processes and identify potential diagnostic or prognostic biomarkers.<sup>8</sup> Despite its potential, to the best of our knowledge, 4D-DIA technology has not been applied in the context of KD, particularly for the prediction of CAL. Consequently, the development of serum-based 4D-DIA proteomics in KD could offer new insights into the disease pathogenesis and biomarker discovery, which is a significant area of investigation, especially considering the current lack of effective biomarkers for assessing the risk of KD-CAL.

In this study, we leveraged the advanced 4D-DIA quantitative proteomics to identify potential biomarkers associated with KD-CAL, aiming to enhance our understanding of the pathophysiology underlying this condition. The differentially expressed proteins (DEPs) identified through 4D-DIA revealed distinct alterations in biological processes pertinent to KD through the pathway analyses, such as Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO). Among these, we selected bone marrow stromal cell antigen-1 (BST1) for further validation using ELISA in an independent cohort, including 96 KD patients and 30 healthy controls (HC), and evaluated for its prognostic performance.

## Materials and Methods

### Study Cohort and Sample Collection

Patients with KD were enrolled based on the 2017 American Heart Association (AHA) criteria.<sup>9</sup> The exclusion criteria were stringent, excluding children who were not in the initial phase of the disease or had a disease duration greater than 10 days, those with other congenital heart malformations, and those who had already received treatment prior to admission. Echocardiography was employed to measure coronary artery diameters before treatment, using the protocol described in our previous work.<sup>10</sup> Subsequently, KD patients were divided into two groups based on the presence or absence of CAL using Z-score calculator established by Kobayashi et al (<https://raise.umin.jp/zsp/calculator/>):<sup>11</sup> those with coronary artery lesions (the CAL group, Z-score  $\geq 2.0$ ) and those without coronary artery lesions (the nCAL group, Z-score  $< 2.0$ ). The discovery cohort was composed of serum samples from 15 pediatric KD patients before IVIG infusion (7 nCAL and 8 CAL) and 4 HC, which were subjected to 4D-DIA proteomic analysis. The validation cohort were composed of samples from 96 KD patients prior to IVIG treatment (61 nCAL and 35 CAL) and 30 HC, which were subjected to ELISA analysis. All the KD patients with CAL completed echocardiographic re-evaluation at 1 month post-treatment and showed resolution of coronary damage (Z-score  $< 2$ ). This study adheres to the RECORD guidelines.<sup>12</sup>

Participants were serially and consecutively enrolled from our hospital between July 2023 and August 2024. Blood samples were collected within 5 days of fever onset before IVIG administration. We collected 2 mL of non-anticoagulated blood in plain tube. Within 30 min of venepuncture the sample was centrifuged at  $2000\times g$  for 10 min at  $4^{\circ}\text{C}$  to obtain serum, which was immediately transferred to Eppendorf tube, snap-frozen, and stored at  $-80^{\circ}\text{C}$  until batch-wise analysis.

### Sample Preparation and 4D-DIA Analysis

The proteomics analysis was performed by Shanghai Luming Biological Technology Co., Ltd. Briefly, EasyPept DeepP™ kit (Omicron, China) was used to enrich serum proteins in the samples. The total peptide concentration was measured using the nanodroplet method. TimsTOF Pro mass spectrometer and nanoElute system (Bruker, Germany) was used for both shotgun proteomics and DIA experiments. To ensure reliability and reproducibility, iRT standards from Biognosys

(ThermoFisher) were added to the sample peptides at a 1:20 ratio for retention time calibration, with a coefficient of variation (CV) of less than 4%.

## Database Search and Bioinformatics Analysis

We used the default settings of Spectronaut Pulsar 18.4 (Biognosys, Switzerland) for database search and spectral library generation. The DIA data were analyzed using Spectronaut to search the constructed spectral library. Differentially expressed proteins (DEPs) were categorized based on their biological functions using GO and KEGG. Additionally, we employed InterPro, Reactome, and WikiPathways<sup>13</sup> to identify the active biological pathways of DEPs.

## Validation of Biomarker BST1 by ELISA

The quantitative proteomic finding was validated using ELISA. Serum concentrations of BST1 were determined in accordance with the manufacturer's instructions, employing a commercial human BST1 ELISA kit (Cloud-Clone, USA). Optical density readings were taken at a wavelength of 450 nm using a microplate reader (Royto, China). The ELISA-derived data were subsequently normalized against the total protein content to account for variations in sample concentration.

## Statistical Analysis

Data analysis was conducted using R software (version 4.2.0) and Prism 7.0 software (GraphPad, USA). Continuous variables are presented as the mean  $\pm$  standard deviation (M $\pm$ SD). For comparing three groups, the differences were determined by one-way ANOVA with Tukey's post hoc test. Paired *t*-tests were utilized to evaluate changes within subjects before and after IVIG treatment. Categorical data between groups were analyzed using the  $\chi^2$  test or Fisher's exact test. Receiver operating characteristic (ROC) curves were constructed to assess the prognostic accuracy of BST1. The Pearson correlation analysis was performed to explore the relationship between serum BST1 and the maximum Z-score. A two-tailed *p*-value <0.05 was statistically significant.

## Results

### Demographic and Clinical Characteristics of Subjects

Two participant cohorts were enrolled in this study. Each cohort consists of three groups, namely HC (Healthy controls), nCAL (KD patients without CAL), and CAL (KD patients with CAL). The demographic and clinical profiles for the discovery cohort and validation cohort are detailed in Table 1.

### Proteomic Profiling of Sera from KD Patients Using 4D-DIA

The schematic flowchart delineates the study's design and methodology, encompassing participant categorization, experimental procedures, and the discovery of potential protein biomarker for KD with CAL (Figure 1), which illustrates the two-stage strategy: (i) 4D-DIA screening in a small discovery set to identify candidate biomarkers, and (ii) targeted ELISA confirmation in a larger, independent validation cohort to establish the clinical utility of BST1 for predicting CAL before IVIG therapy. Quality assessment of the samples indicated that the relative molecular weights of all detected proteins (Figure 2A), the lengths of all peptides (Figure 2B) and the degree of within-group data dispersion and between-group differences (Figure 2C) were within normal ranges, suggesting that the samples were generally stable. Then, the 3D distribution diagram of principal component analysis (PCA) (Figure 2D), the heatmap of sample correlation (Figure 2E) and the hierarchicalclustering dendrogram of sample Euclidean distance were plotted (Figure 2F). The results demonstrated clear inter-group differences and strong intra-group correlations, which are indicative of the samples meeting the criteria for 4D-DIA quantitative analysis.

DEPs among various groups was performed in the discovery cohort. A threshold for statistical significance was set at a fold change >2 or < -2, coupled with a *p*-value <0.05. Utilizing 4D-DIA, an extensive spectral library was established, encompassing 24033 peptides and 2575 proteins. Among the proteins quantified, a total of 807 proteins showed significantly differential expression between the nCAL group and the HC group, with 405 proteins being upregulated

**Table I** Demographic and Clinical Characteristics of HC and KD Patients with or Without CAL in the Discovery and Validation Cohort

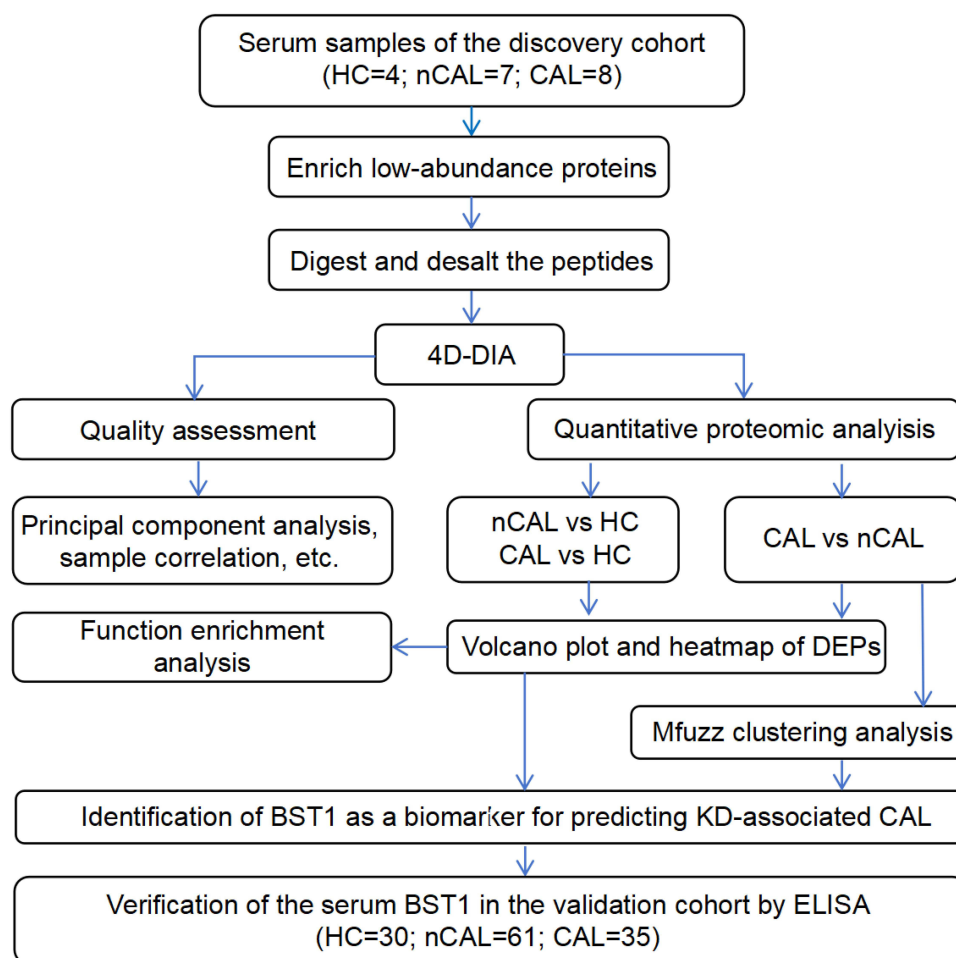
| Characteristics                      | Discovery Cohort |               |               |         | Validation Cohort |                |                            |         |
|--------------------------------------|------------------|---------------|---------------|---------|-------------------|----------------|----------------------------|---------|
|                                      | HC<br>(n=4)      | nCAL<br>(n=7) | CAL<br>(n=8)  | p value | HC<br>(n=30)      | nCAL<br>(n=61) | CAL<br>(n=35)              | p value |
| Gender<br>(male/female)              | (3/1)            | (4/3)         | (4/4)         | 0.686   | (19/11)           | (35/26)        | (25/10)                    | 0.390   |
| Age<br>(months)                      | 38.00 ± 19.66    | 34.71 ± 22.40 | 21.00 ± 9.071 | 0.200   | 39.31 ± 24.13     | 40.67 ± 25.31  | 34.04 ± 33.27              | 0.517   |
| CRP<br>(mg/L)                        | 0.375 ± 0.150    | 34.61 ± 39.52 | 32.63 ± 39.32 | 0.282   | 0.552 ± 0.897     | 52.23 ± 34.66  | 75.83 ± 61.08 <sup>a</sup> | <0.0001 |
| WBC<br>(×10 <sup>9</sup> /L)         | 7.745 ± 2.277    | 7.856 ± 2.365 | 11.73 ± 9.181 | 0.430   | 7.716 ± 2.163     | 12.35 ± 4.521  | 14.03 ± 5.51               | <0.0001 |
| Neutrophils<br>(×10 <sup>9</sup> /L) | 2.788 ± 0.841    | 3.249 ± 2.002 | 5.424 ± 5.268 | 0.410   | 3.300 ± 1.460     | 8.004 ± 4.020  | 9.380 ± 4.942              | <0.0001 |
| Monocytes<br>(×10 <sup>9</sup> /L)   | 0.560 ± 0.400    | 0.597 ± 0.258 | 0.845 ± 0.426 | 0.330   | 0.519 ± 0.165     | 0.786 ± 0.424  | 0.879 ± 0.500              | 0.0014  |
| Lymphocytes<br>(×10 <sup>9</sup> /L) | 4.103 ± 1.206    | 3.570 ± 1.557 | 4.029 ± 1.458 | 0.783   | 3.631 ± 1.464     | 3.240 ± 1.876  | 3.392 ± 1.876              | 0.483   |
| Platelets<br>(×10 <sup>9</sup> /L)   | 357.3 ± 128.4    | 405.1 ± 134.5 | 531.5 ± 177.0 | 0.149   | 302.6 ± 71.59     | 326.6 ± 105.5  | 384.5 ± 134.7 <sup>a</sup> | 0.007   |
| AST (U/L)                            | 33.50 ± 2.646    | 57.57 ± 68.76 | 196.8 ± 293.7 | 0.300   | 34.67 ± 7.783     | 59.52 ± 117.2  | 32.91 ± 21.06              | 0.219   |
| ALT (U/L)                            | 13.00 ± 2.160    | 101.9 ± 173.4 | 207.6 ± 242.7 | 0.261   | 15.50 ± 4.637     | 44.97 ± 64.21  | 43.14 ± 45.13              | 0.028   |

**Notes:** Data are expressed as means ± standard deviation or number for categorical variables. <sup>a</sup> CAL vs nCAL, *p* <0.05.

and 402 being downregulated (Figure 3A and B). Furthermore, 470 upregulated and 413 downregulated DEPs were observed when comparing the CAL group with the HC group (Figure 3C and D). Additionally, between CAL and nCAL, 213 DEPs were identified, consisting of 122 upregulated and 91 downregulated proteins (Figure 3E and F). Notably, CXCL10, EPS8, FBN2, SKIC2 and EPB41L1 are the top 5 proteins with the most pronounced upregulation in nCAL vs HC, and interestingly, these 5 proteins also exhibited marked upregulation in CAL vs HC, which indicated that regardless of the occurrence of CAL, these proteins may be common molecules involved in the pathogenesis of KD. Strikingly, through these three volcano plots, we noticed BST1 as the sole molecule that is elevated in the CAL group but not in the nCAL group, and it exhibits the highest expression in CAL vs nCAL, suggesting that BST1 may be a specific biomarker for the risk of CAL.

## GO Function and KEGG Pathway Analysis of DEPs in KD Patients

We further conducted GO analysis to identify the predominant biological pathways associated with DEPs across the different groups. When comparing nCAL with HC, GO analysis indicated that the DEPs were notably enriched in a range of biological processes (BP), such as innate immune response, cell adhesion, mRNA splicing via spliceosome, and defence response to bacterium (Figure 4A). Cellular Component (CC) analysis of the DEPs in the nCAL group showed an enrichment in locations like the cytosol, extracellular exosome, extracellular region, and extracellular space (Figure 4B). Molecular Function (MF) analysis suggested that the DEPs in the nCAL group were significantly associated with functions such as RNA binding and identical protein binding (Figure 4C). Moreover, when comparing CAL to HC, the analysis of BP showed that DEPs were predominantly involved in processes such as innate immune response, cell adhesion, and RNA splicing, which was similar to the nCAL vs HC (Figure 4D). CC analysis revealed that DEPs were



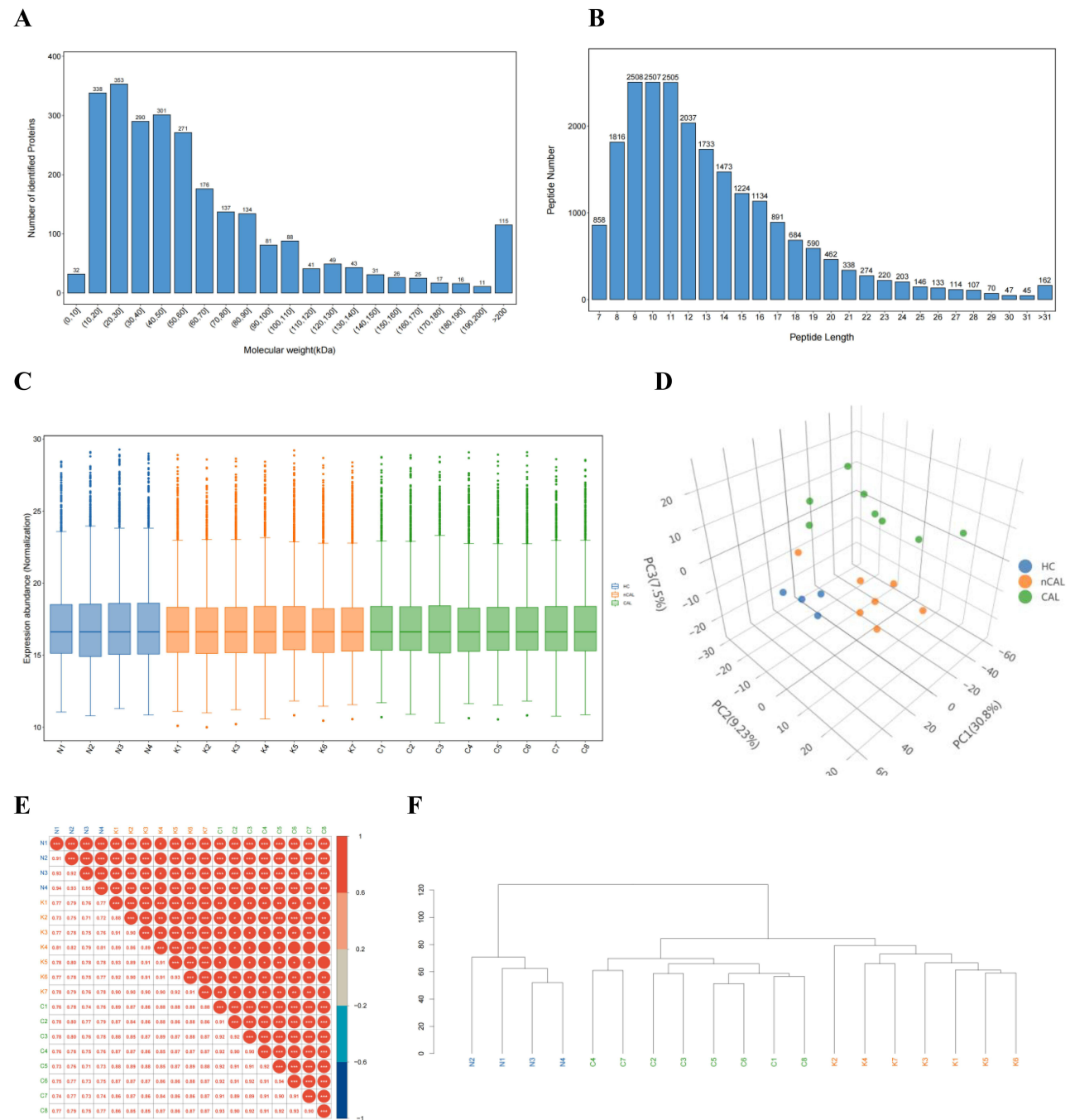
**Figure 1** The flowchart of this study.

also mainly enriched in components like the cytosol, extracellular exosome, extracellular region, and extracellular space (Figure 4E). In terms of MF, DEPs were significantly enriched in functions including RNA binding and identical protein binding (Figure 4F).

Subsequently, we conducted KEGG pathway analyses to investigate the DEPs between KD patients with or without CAL and HC. The top 10 pathways for each comparison group were identified based on the *p*-value. On the one hand, the KEGG pathway analysis revealed that, compared to HC, the upregulated proteins in both nCAL and CAL were notably enriched in infectious and RNA processing pathways, such as spliceosome, endocytosis, ribosome, salmonella infection and COVID-19-associated processes (Figure 5A). On the other hand, the KEGG analysis indicated that the down-regulated proteins in both nCAL and CAL were significantly enriched in pathways related to PI3K-AKT signaling, phagosome, hematopoietic cell lineage and complement and coagulation cascades when compared to HC (Figure 5B). Taken together, GO and KEGG analyses demonstrated a significant difference of serum proteome profile between KD patients and HC, while there were common GO function and KEGG pathway in KD no matter the presence or absence of CAL, essentially offering valuable insights into the immune landscape of KD-related systemic vasculitis.

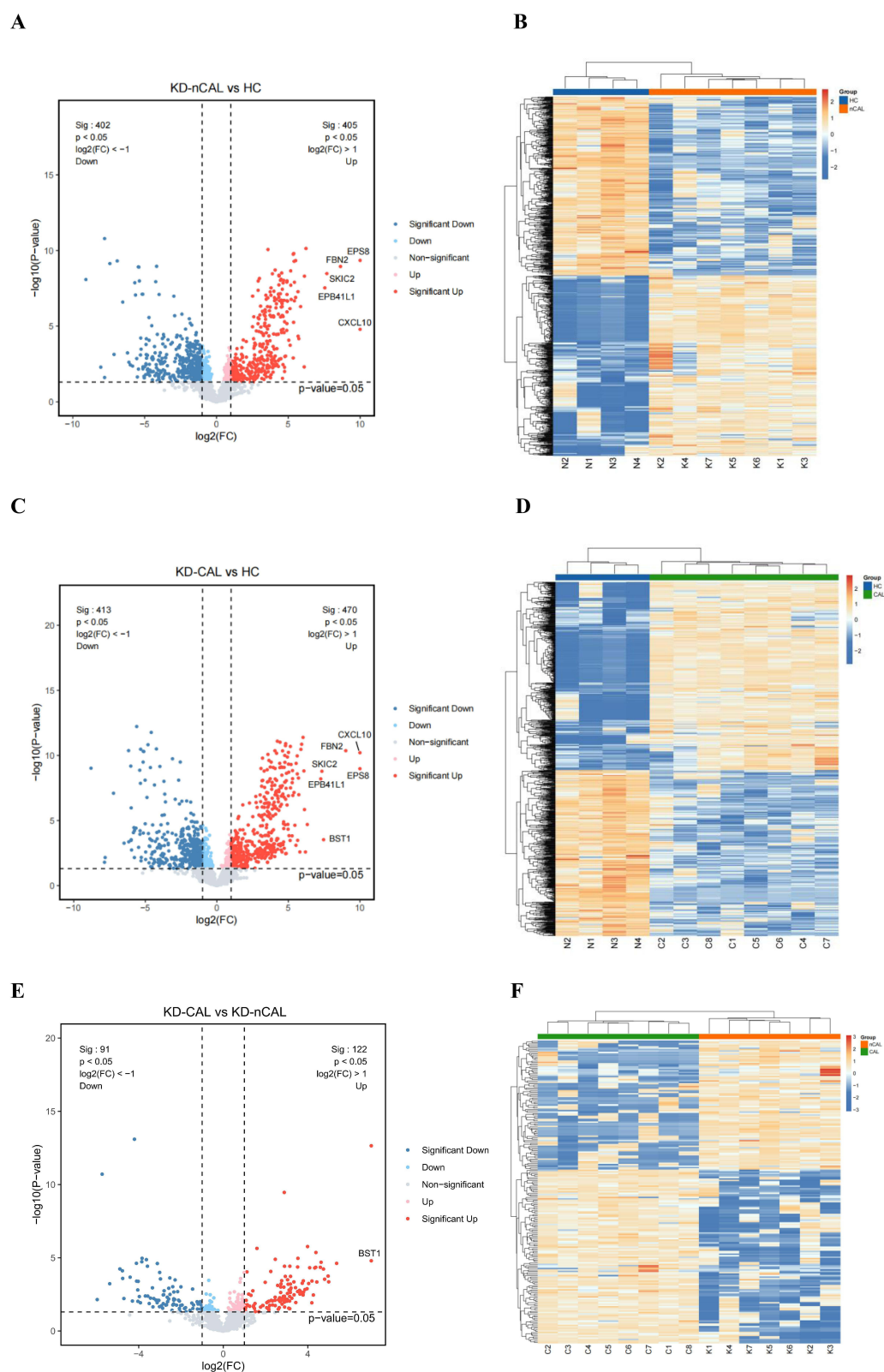
## Functional Enrichment Analysis of DEPs in KD-CAL Patients

As CAL is the most severe complication associated with KD, the protein profile of KD patients may vary significantly among individuals. Our primary objective was to scrutinize the functional alterations in serum proteins among KD patients with CAL, and importantly, to uncover potential biomarkers linked to CAL. To delve deeper into the heterogeneity within the KD patient population, a subgroup analysis focusing on CAL was performed. The PCA distribution plot demonstrated a clear divergence

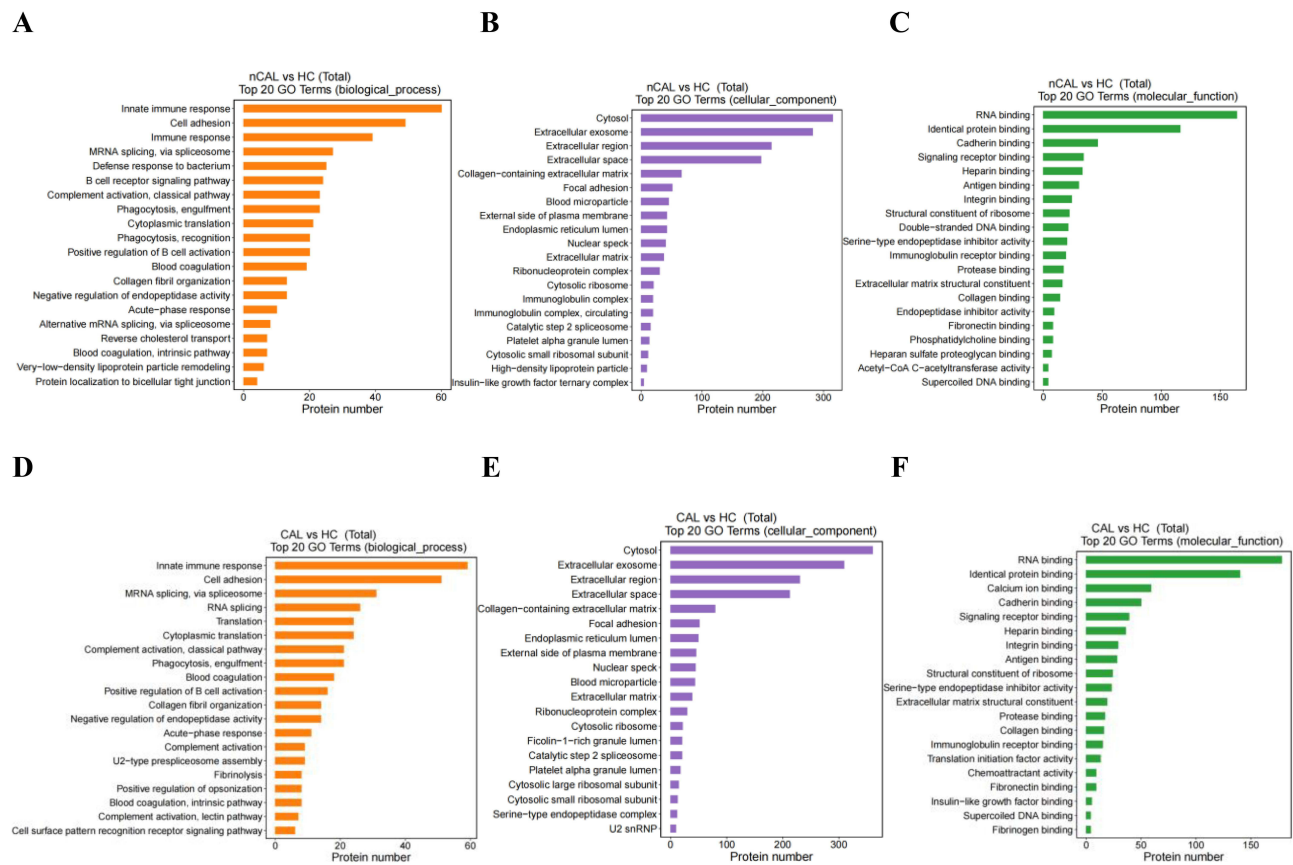


**Figure 2** Sample information and quality control. **(A)** The relative molecular weight distribution of identified proteins. **(B)** The peptide length distribution of identified proteins. **(C)** The boxplot of the degree of within-group data dispersion and between-group differences. **(D)** The 3D PCA diagram of the three sets of samples. **(E)** The heatmap of sample correlation. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . **(F)** The hierarchical clustering dendrogram of sample Euclidean distance.

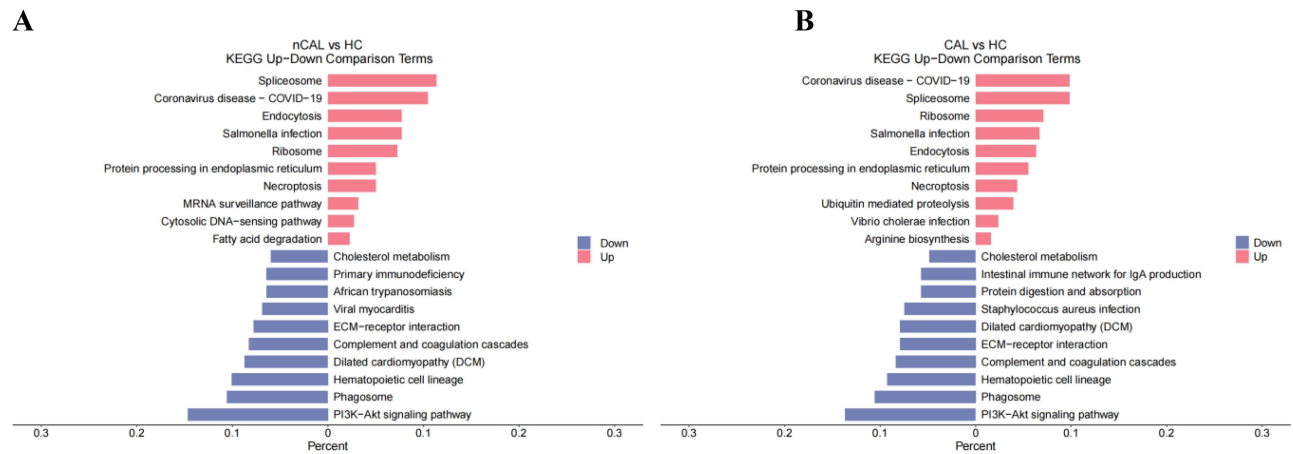
between the two groups (Figure 6A). Furthermore, we performed GO and KEGG analysis on the significantly DEPs between the CAL group and the nCAL group. GO analysis indicated that DEPs were mainly associated with extracellular exosome, cadherin binding, RNA binding and ATP (adenosine triphosphate) binding (Figure 6B). Furthermore, the upregulated DEPs were mainly enriched in “exit from mitosis, regulation of translation in response to stress, and RNA splicing” (BP), “extracellular exosome, cytosol, and focal adhesion” (CC), and “cadherin binding, RNA binding and ATP binding” (MF) (Figure 6C), while the downregulated DEPs in the CAL group were significantly associated with “fatty acid  $\beta$  oxidation, protein homotetramerization” (BP), “extracellular exosome, cytosol, and proteasome complex” (CC), and “acetyl-CoA



**Figure 3** Quantitative proteomic study in HC and KD patients. **(A)** Volcanic visualization of DEPs between the nCAL group and the HC group. **(B)** Heatmap of all 807 DEPs clustered into the nCAL group and the HC group. **(C)** Volcanic visualization of DEPs between the CAL group and the HC group. **(D)** Heatmap of all 883 DEPs clustered into the CAL group and the HC group. **(E)** Volcanic visualization of DEPs between the CAL group and the nCAL group. **(F)** Heatmap of all 213 DEPs clustered into the nCAL group and the CAL group.

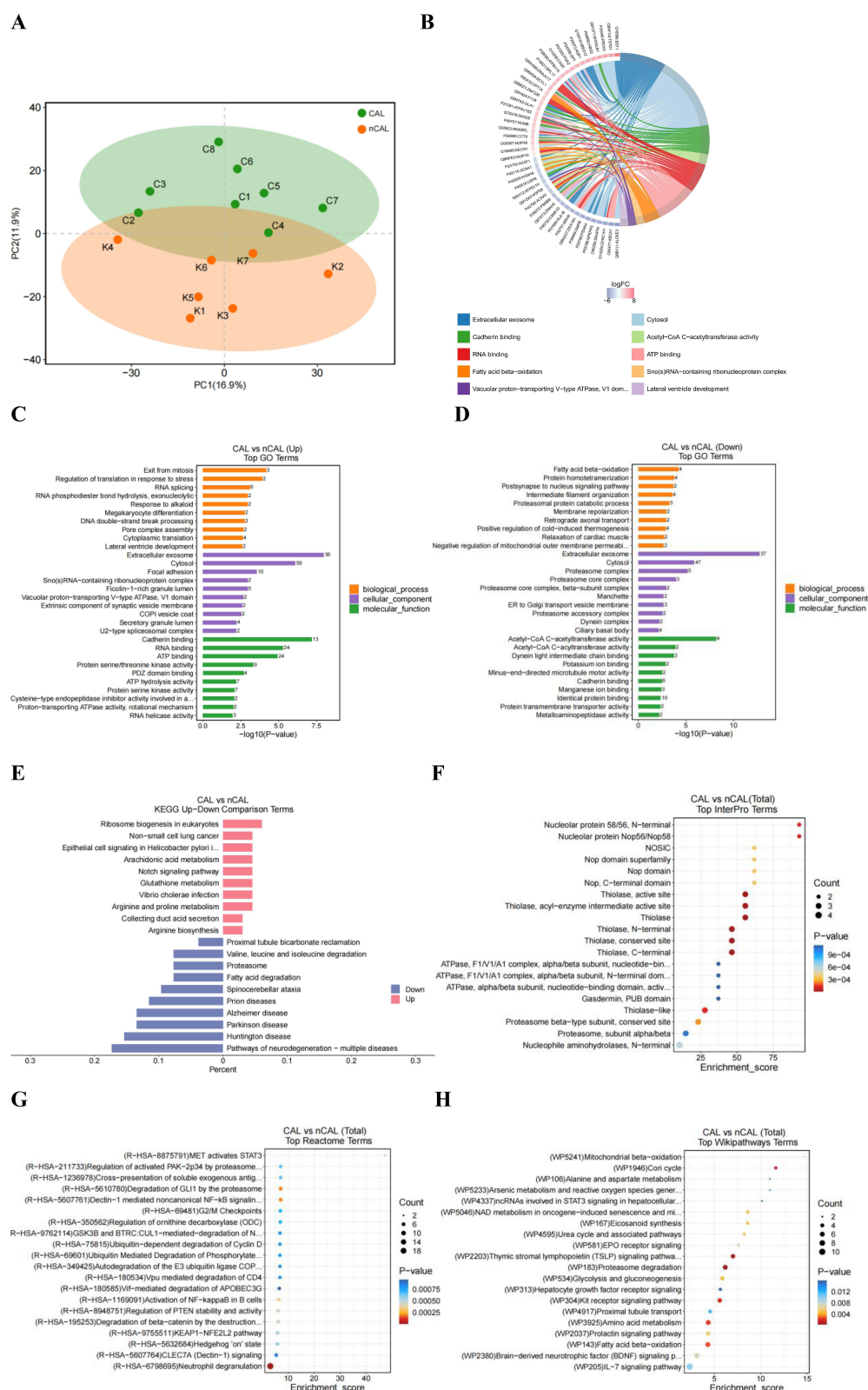


**Figure 4** GO functional enrichment analysis of DEPs. **(A)** Top 20 term of biological processes between nCAL and HC. **(B)** Top 20 term of cellular components between nCAL and HC. **(C)** Top 20 term of molecular function between nCAL and HC. **(D)** Top 20 term of biological processes between CAL and HC. **(E)** Top 20 term of cellular components between CAL and HC. **(F)** Top 20 term of molecular function between CAL and HC.



**Figure 5** KEGG pathway enrichment analysis of DEPs. **(A)** KEGG enrichment analysis of the up-and downregulated DEPs between nCAL and HC. **(B)** KEGG enrichment analysis of the up-and downregulated DEPs between CAL and HC.

acetyltransferase activity, acetyl-CoA acyltransferase activity” (MF) (Figure 6D). Moreover, KEGG analysis indicated that the upregulated proteins in KD-CAL patients were significantly enriched in “ribosome biogenesis, infectious disease, metabolism, and Notch signaling pathways”, and the downregulated proteins were related to “neurodegenerative diseases, fatty acid degradation and proteasome” when compared to KD-nCAL patients (Figure 6E). Additionally, InterPro, Reactome, and Wikipathways were performed to further explore the functions of DEPs between CAL and nCAL, respectively. InterPro



**Figure 6** Enrichment analysis of DEPs in KD patients with or without CAL. **(A)** PCA analysis of protein expression data of the CAL group and the nCAL group. **(B)** Chord plot of GO enrichment analysis between CAL and nCAL. **(C)** GO enrichment analysis of the upregulated DEPs between CAL and nCAL. **(D)** GO enrichment analysis of the downregulated DEPs between CAL and nCAL. **(E)** KEGG enrichment analysis of the up- and downregulated DEPs between CAL and nCAL. **(F)** InterPro enrichment analysis of DEPs between CAL and nCAL. **(G)** Reactome enrichment analysis of DEPs between CAL and nCAL. **(H)** Wikipathways enrichment analysis of DEPs between CAL and nCAL.

enrichment analysis showed that the DEPs were enriched in proteasome, ATP metabolism, and thiolase (acetyl-CoA acetyltransferase) (Figure 6F). Reactome enrichment analysis demonstrated that the DEPs were mainly associated with neutrophil degranulation (Figure 6G). Wikipathways enrichment analysis found that the DEPs were related with proteasome degradation, fatty acid  $\beta$  oxidation, and TLSP signaling pathways (Figure 6H). Collectively, these findings suggest that future research in KD-CAL should focus on the interplay between these processes, particularly the role of DEPs in immune response, cellular metabolism, and protein degradation, which may offer new therapeutic targets and biomarkers for early diagnosis and treatment.

To further elucidate the relationship between serum proteins and KD progression, we employed the fuzzy c-means algorithm for unsupervised clustering, resulting in eight distinct clusters (Figure 7A). Among these clusters, we primarily focused on “Cluster 2”, as the proteins in this cluster were elevated only in the CAL group, with no changes observed between the nCAL and HC groups. This cluster comprised a total of 54 molecules (Figure 7B), and GO analysis revealed significant enrichment in extracellular exosomes, cadherin and ATP binding (Figure 7C). KEGG analysis indicated major enrichment in pathways related to metabolism, COVID-19, and endocytosis (Figure 7D). Taken together, these alterations in biological processes and molecular functions suggest that KD-CAL may involve the activation of inflammation and immune responses, changes in cell-cell communication, metabolic reprogramming, and dysregulation of cellular endocytic pathways. These changes may collectively contribute to the development of vascular inflammation and arterial lesions, providing potential pathophysiological mechanisms and therapeutic targets for future research.

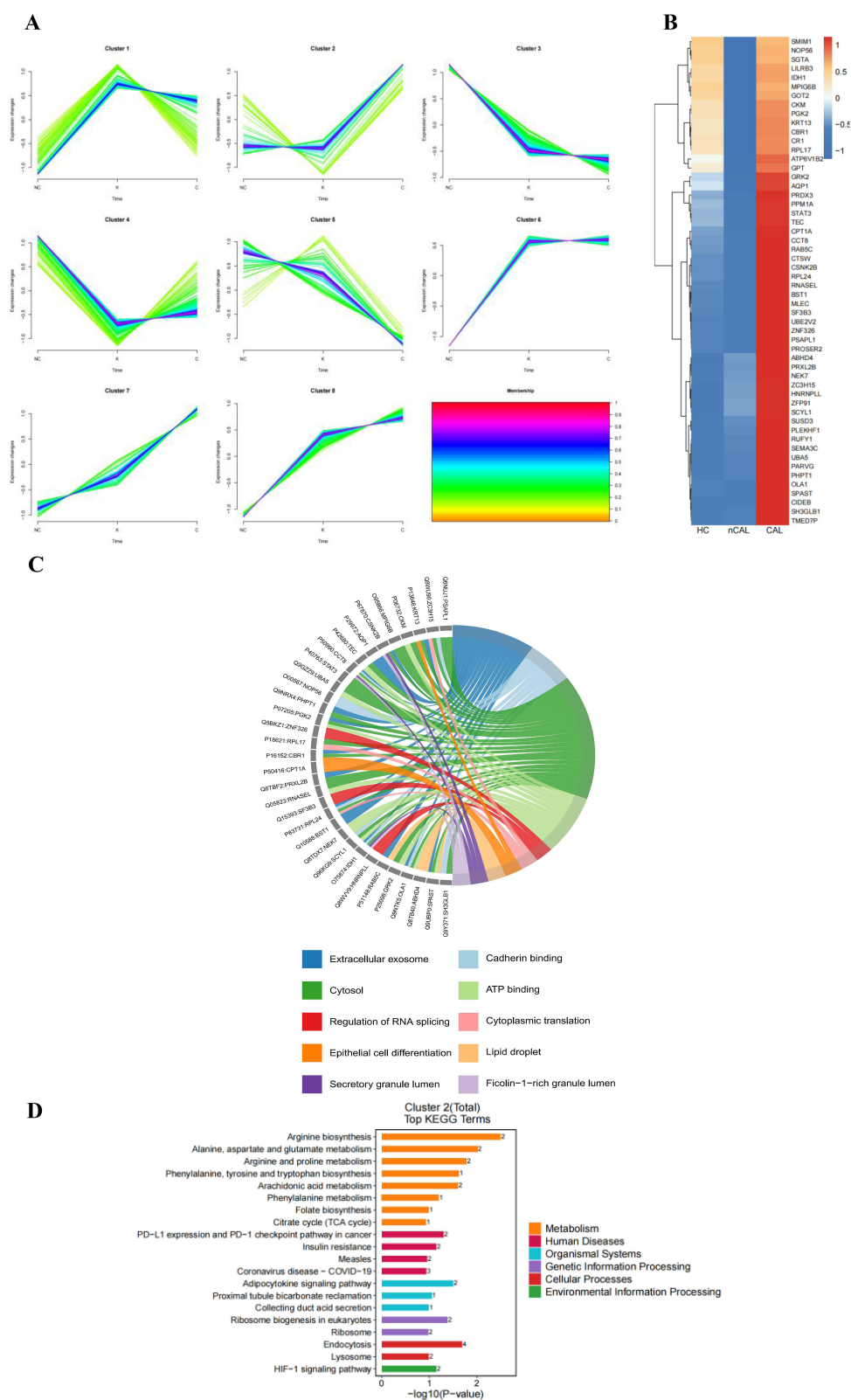
## Validation of Serum BST1 as a Predictor for KD-CAL in an Independent Validation Cohort

As previously mentioned, BST1 is the molecule of our interest, which is elevated only in the CAL group. Therefore, to further elucidate the clinical relevance of BST1, we conducted an ELISA assay in the validation cohort. The ELISA data confirmed a marked elevation in the serum concentrations of BST1 among KD patients. The levels of BST1 were significantly elevated in the nCAL group compared to the HC group. Furthermore, BST1 levels in the CAL group were also significantly higher than those in both the nCAL and HC groups (Figure 8A). Moreover, a ROC curve analysis was utilized to evaluate the predictive power of BST1 for CAL. The analysis showed that the area under the curve (AUC) for serum BST1 in predicting CAL was 0.9052 (95% confidence interval: 0.8439 to 0.9664,  $p < 0.0001$ ), with a sensitivity of 85.71% and a specificity of 80.33% (Figure 8B), indicating the substantial prognostic potential of BST1 for identifying CAL. The optimal cut-off value for BST1 was 3.19 ng/mL, as determined by the Youden index (0.6604). Additionally, we evaluated changes in BST1 levels after IVIG treatment in KD-CAL patients and found that serum concentrations of BST1 were significantly reduced after effective IVIG therapy (Figure 8C). To further investigate the clinical implications of BST1 in KD, we examined the relationship between BST1 and Z-score, and found that the serum BST1 levels were positively correlated with the maximum Z-score (Figure 8D), indicating BST1's potential in reflecting the severity of KD-CAL.

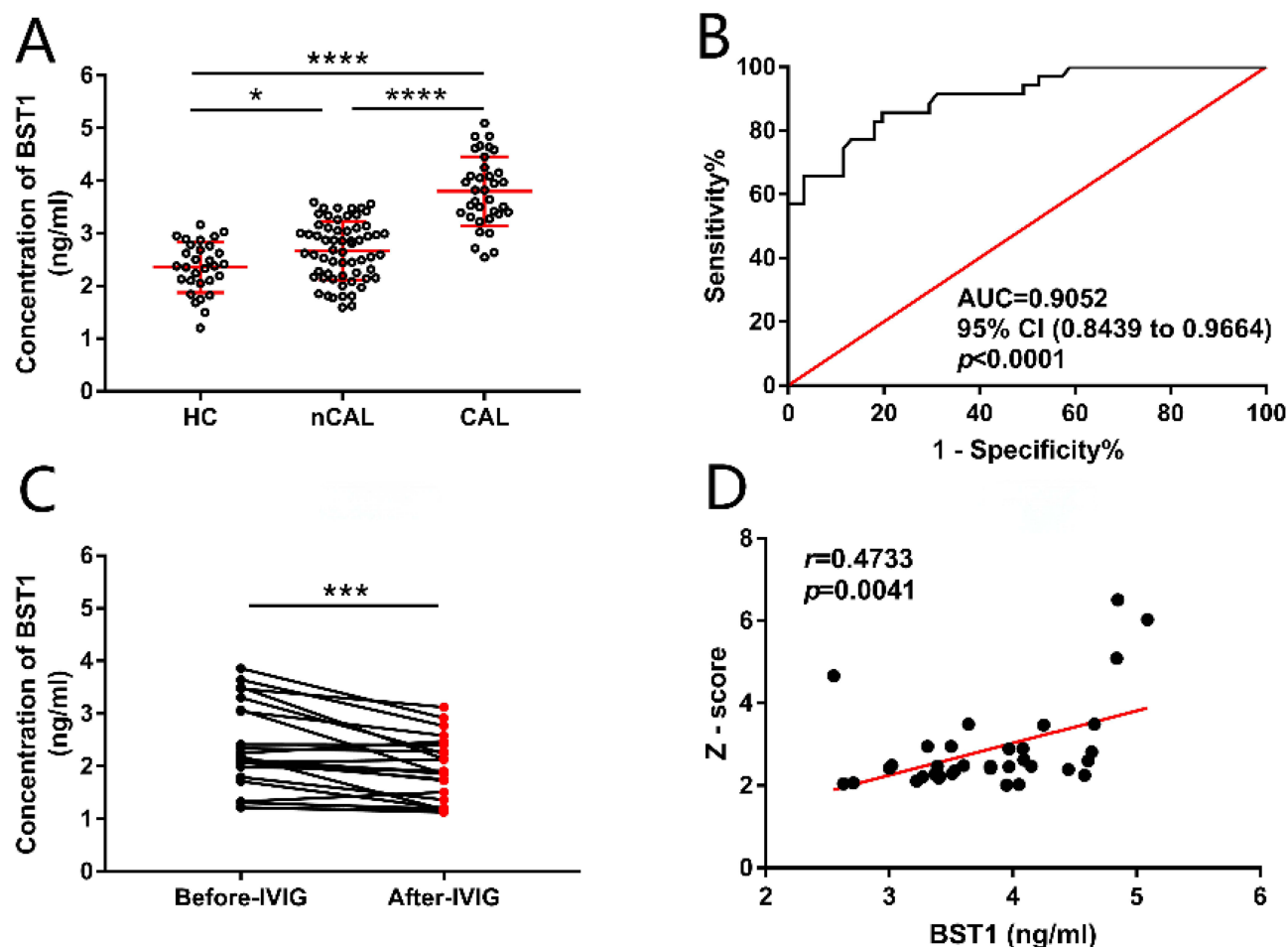
## Discussion

The screening and identification of specific markers in peripheral circulation to predict KD-CAL is currently an important field of research.<sup>7</sup> Here, we utilized 4D-DIA to evaluate the serum proteome in KD patients. To our best knowledge, this is the first study employing of 4D-DIA proteomics to dissect the proteomic profiles in KD patients with or without CAL. Our study uncovered aberrant protein expression patterns in KD and highlighted notably increased serum BST1 levels in KD patients who developed CAL. Moreover, to ascertain the clinical applicability of BST1 as a predictive marker, we leveraged an independent validation cohort to assess its prognostic significance. The serum BST1 levels demonstrated robust predictive accuracy for CAL development, reinforcing its potential as a reliable biomarker for assessing the risk of CAL in KD patients.

In our study, the significant differential expression of 807 proteins between the nCAL group and the HC group, with 405 upregulated and 402 downregulated proteins, suggests a profound perturbation in the serum proteome of KD patients. Notably, the top five upregulated proteins—CXCL10, EPS8, FBN2, SKIC2, and EPB41L1—are consistently overexpressed in both nCAL and CAL groups, indicating their potential involvement in KD pathogenesis, irrespective of



**Figure 7** Quantitative proteomic study in HC and KD patients with or without CAL. **(A)** The eight clusters of proteins with different expression dynamics among the different groups were computed via Mfuzz analysis. **(B)** Heatmap of the 54 DEPs in "cluster 2" among the different groups. **(C)** Chord plot of GO enrichment analysis of the 54 DEPs in "cluster 2". **(D)** KEGG enrichment analysis of the 54 DEPs in "cluster 2".



**Figure 8** Validation of BST1 in sera from KD patients. **(A)** Validation of the BST1 concentration in HC, nCAL and CAL group. **(B)** ROC curve of serum BST1 in the nCAL and CAL groups from the validation cohort. **(C)** BST1 levels in KD patients before- and after-IVIG treatment in the KD patients. **(D)** Correlation between serum BST1 levels and Z-score. Data are mean  $\pm$  SD. \* $p$  < 0.05; \*\*\* $p$  < 0.001; \*\*\*\* $p$  < 0.0001. One-way ANOVA with Tukey's multiple comparisons test **(A)**. Paired t-test **(C)**. Pearson correlation **(D)**.

CAL development. The exclusive elevation of BST1 in the CAL group, to the highest extent when compared directly between CAL and nCAL, positions BST1 as a promising biomarker for CAL risk.

Our GO analysis highlights the enrichment of DEPs in innate immune responses, cell adhesion, mRNA splicing, and defense against bacterial infections, underscoring the inflammatory and immune-mediated nature of KD.<sup>14</sup> The consistent functional enrichment observed across nCAL and CAL groups points to a shared pathophysiological mechanism in KD, despite the heterogeneity in CAL manifestation. The KEGG pathway analysis further supports the notion of an altered immune and inflammatory response in KD, with upregulated proteins in both nCAL and CAL groups being significantly associated with infectious disease pathways and RNA processing, such as the spliceosome. This aligns with the known hyper-inflammatory state in KD<sup>15</sup> and suggests a potential role for these pathways in disease progression. Conversely, downregulated proteins in KD patients implicate pathways related to PI3K-AKT signaling and phagosome function, which may reflect the resolution of inflammation and clearance of pathogens, processes that are likely disrupted in KD. Of note, the PI3K-AKT (phosphoinositide 3-kinase/protein kinase B) signaling pathway has emerged as a critical player in the pathophysiology of KD, particularly in the context of vascular homeostasis imbalance.<sup>16</sup>

Notably, KEGG analysis showed that both nCAL and CAL groups in our study exhibit a significant enrichment of DEPs associated with COVID-19 (coronavirus disease 2019) pathways. This connection is further supported by the emergence of pediatric inflammatory multisystem syndrome temporarily associated with SARS-CoV-2 (PIMS-TS), also known as multi-system inflammatory syndrome in children (MIS-C).<sup>17</sup> The clinical presentation of MIS-C shares several features with KD,

including persistent fever, shock, and multi-organ dysfunction, with a notable involvement of the cardiovascular system.<sup>18</sup> This indicates a potential overlap in the immunopathological mechanisms between KD and MIS-C. Furthermore, the enrichment of DEPs in pathways such as spliceosome, endocytosis, and ribosome, which are also implicated in COVID-19-associated processes, further supports the notion of a shared inflammatory and immune response in KD and COVID-19.<sup>19</sup> Moreover, the downregulation of DEPs in pathways related to PI3K-AKT signaling, phagosome function, and complement and coagulation cascades in KD patients, as compared to healthy controls, may reflect an impaired immune response and clearance of pathogens, which is also a feature of severe COVID-19 cases.<sup>20</sup> This suggests a potential commonality in the dysregulation of immune and inflammatory responses between KD and COVID-19. Future research should focus on elucidating the precise mechanisms underlying this association, which could provide insights into the development of novel therapeutic strategies targeting the shared pathways in KD and COVID-19-related inflammatory syndromes in children.

In this study, we revealed that the upregulation of DEPs associated with extracellular exosomes, cadherin binding, RNA binding, and ATP binding, as well as processes such as “exit from mitosis” and “RNA splicing” suggest a profound disturbance in cellular regulation and energy metabolism in KD patients with CAL. These findings align with the known hyper-inflammatory state in KD and point to potential dysregulation in cellular processes that govern cell cycle progression and response to stress.<sup>21</sup> Furthermore, the downregulation of DEPs involved in “fatty acid  $\beta$  oxidation” and “protein homotetramerization” along with the enrichment of proteasome complex, indicates a possible impairment in energy metabolism and protein homeostasis in KD patients with CAL. This is further supported by the KEGG analysis, which highlights the enrichment of upregulated proteins in pathways related to metabolism and infectious disease, and downregulated proteins in neurodegenerative diseases and fatty acid degradation.

The InterPro and Wikipathways enrichment analyses have also shed light on the complex biological processes involved in KD, particularly highlighting the importance of protein degradation and metabolic dysregulation. Additionally, as demonstrated by Reactome enrichment analysis, the association of DEPs with neutrophil degranulation is particularly intriguing. Neutrophils are the first line of defense in innate immunity, and their degranulation releases a plethora of antimicrobial proteins and inflammatory mediators.<sup>22</sup> This finding may reflect the intense neutrophil activation in KD, which is consistent with the prominent vasculitis and inflammation observed in the disease.<sup>23</sup> The degranulation process is crucial for the neutrophil's role in host defense, and its dysregulation is a hallmark of KD and may contribute to the pathogenesis of KD-CAL.<sup>24</sup> Furthermore, the unsupervised clustering analysis, particularly the focus on “Cluster 2”, reveals a group of proteins that are specifically elevated in the CAL group. The significant enrichment in extracellular exosomes, cadherin and ATP binding, metabolism, COVID-19, and endocytosis pathways suggests that these biological processes may be critical in the development of CAL.

BST1, also known as CD157, is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein that belongs to the adenosine diphosphate (ADP)-ribosyl cyclase family, which is involved in the ectoenzymatic activities of ADP-ribosyl cyclase and nicotinamide adenine dinucleotide (NAD<sup>+</sup>) glycohydrolase.<sup>25</sup> It plays a multifaceted role in the immune system and has been implicated in various diseases, making it a potential biomarker for diagnosis and prognosis.<sup>26</sup> Soluble BST1 (sBST1) have been detected in sera and other biological fluids under some disease conditions, such as rheumatoid arthritis,<sup>27</sup> malignant pleural mesothelioma,<sup>28</sup> tuberculous pleurisy,<sup>29</sup> and multiple sclerosis.<sup>30</sup> Furthermore, sBST1 has been identified as a key player in orchestrating leukocyte and specific tumor cell type trafficking, modulating essential neutrophil and monocyte functions<sup>31</sup> such as adhesion to the extracellular matrix, cell motility, and transmigration.<sup>32</sup> Recently, studies have demonstrated that BST1 plays a critical role in promoting inflammation by facilitating neutrophil migration and mediating inflammatory responses associated with kidney diseases,<sup>33</sup> neuropsychiatric disorders,<sup>34</sup> acute liver failure,<sup>35</sup> and pediatric sepsis.<sup>36</sup>

Here, the results of our study provide significant insights into the role of BST1 in KD, particularly in the context of CAL, and its potential as a biomarker. The elevated serum concentrations of sBST1 in KD patients with CAL, as confirmed by ELISA, underscore its potential clinical relevance. The high AUC value from the ROC curve analysis, indicating a strong predictive power of BST1 for CAL, suggests that BST1 could be a valuable prognostic tool in the early identification of CAL in KD patients. Moreover, the reduction in serum sBST1 concentrations following IVIG treatment further highlights the treatment's impact on this specific protein, implying that BST1 may also serve as a marker of treatment response.

There are some limitations in our study that need to be considered. Firstly, due to the heterogeneity in serum composition in KD patients, the small sample size from a single center may introduce bias and weaken our findings. To bolster the validity of the identified candidate biomarker, future studies should aim to validate it within larger, multicenter cohorts. Secondly, to gain a comprehensive understanding of the molecular mechanisms underlying BST1's role in KD pathogenesis, more extensive and detailed investigations are necessary. Thirdly, our study did not include animal models to validate the human data obtained. Incorporating animal experiments in future research could provide valuable insights into the BST1-mediated pathophysiological mechanisms in KD-CAL.

## Conclusions

In summary, this study is the first to apply 4D-DIA quantitative proteomics to identify serum protein alterations associated with CAL in children with KD. Our findings highlight that DEPs are significantly enriched in pathways related to immune dysregulation, cellular metabolism, and protein degradation. Moreover, we have discovered that BST1 functions not only as a baseline physiological regulator of the immune system, but also as a pathophysiological amplifier that drives coronary vascular inflammation and consequently serves as a powerful predictor for CAL.

## Abbreviations

4D-DIA, Four-dimensional data-independent acquisition; BST1, Bone marrow stromal cell antigen-1; CAL, Coronary artery lesions; CRP, C-reactive protein; DEP, Differentially expressed proteins; ELISA, Enzyme-linked immunosorbent assay; HC, Healthy controls; IVIG, Intravenous immunoglobulin; KD, Kawasaki disease; KEGG, Kyoto encyclopedia of genes and genomes; GO, Gene ontology; ROC, Receiver operating characteristic.

## Data Sharing Statement

The datasets analyzed during the current study are available in the China National Center for Bioinformation repository, [<https://ngdc.cncb.ac.cn/omix/release/OMIX008635>].

## Ethics Statement

This study was approved by the Ethics Committee of Xi'an Children's Hospital (Approval number: 20230613-16). Informed consent was obtained from the guardians of each participant. All methods were performed in accordance with the relevant guidelines and regulations, and all procedures adhered to institutional and national ethical standards and the 2013 Declaration of Helsinki.

## Author Contributions

Shuwan Zhang: Conceptualization, methodology, writing draft, project administration, funding acquisition; Junhua Huang: Investigation, validation; Mengfei Zhang and Chuanmei Zhao: Data curation, resources; Wenjing Wu: Formal analysis, supervision; Wei Chen: Conceptualization, supervision. All authors made a significant contribution to the work reported; took part in revising or critically reviewing the article; 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.

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## Disclosure

The authors report no conflicts of interest in this work.

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