

# Distinct Proteomic Profiling of Plasma Extracellular Vesicles from Moderate-to-Severe Atopic Dermatitis Patients

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**Background:** Atopic dermatitis (AD) is a chronic, inflammatory cutaneous disorder characterized by a T helper 2 (Th2) immune response phenotype. Extracellular vesicles (EVs) are a heterogeneous family of cell-derived membranous structures, which transport cellular components such as DNA and proteins, and are involved in multiple physiological and pathological processes. Increasing evidence has shown that EVs secretion took part in the pathogenesis of AD. However, the proteomic studies of plasma-derived EVs in AD patients have not been reported.

**Objective:** In this study, we investigated the diversity of plasma EVs collected from AD patients and healthy individuals and suggested that the candidates for uniquely or differentially expressed proteins in plasma EVs could be a diagnostic marker in AD.

**Methods:** The plasma EVs were collected from 12 patients with moderate-to-severe AD and 13 healthy subjects. Proteomic analysis was performed by using a comprehensive nanoLC-MS/MS method.

**Results:** Proteomic analysis revealed that a total of 1478 proteins in plasma EVs were found to be common proteins in AD, whereas a total of 1597 proteins in plasma EVs were found to be common proteins in HC. Eighty-six proteins in plasma EVs showed more than 2.5-fold up-regulation, while a total of 225 proteins in plasma EVs showed less than 1/2.5-fold down-regulation with a significant difference ( $p < 0.05$ ) among AD compared with HC. The candidates for differentially expressed proteins in plasma EVs have been described as a connectivity PPI network related to several KEGG pathways, including pathways in platelet activation, complement, and so on.

**Conclusion:** SLP-76 tyrosine phosphoprotein (SLP76) involved in platelet activation may significantly contribute to the pathogenesis of AD. We will further verify the role of SLP67 in AD via animal and cell experiments to provide a promising therapeutic or diagnostic target.

**Keywords:** atopic dermatitis, extracellular vesicle, plasma, proteomic profiling

## Introduction

Atopic dermatitis (AD) is a common, chronic, inflammatory cutaneous disorder presenting recurrent eczematous lesions and intense pruritus that affects children and adults. It is also characterized by a T helper 2 (Th2) immune response phenotype.<sup>1</sup> AD has a substantial physical and psychosocial impact on patients and relatives.<sup>2,3</sup> The estimated prevalence of AD ranges from 15% to 20% in children and 7% to 10% in adults.<sup>4,5</sup> It is the leading cause of the global burden

from skin disease.<sup>1</sup> The pathophysiology of AD is multifactorial and complex, involving genetic component, dysfunction of epidermal barrier, skin microbiome and immune dysregulation.<sup>2</sup>

Extracellular vesicles (EVs) are a heterogeneous family of cell-derived membranous structures, including exosomes and microvesicles.<sup>6</sup> They transport cellular components such as microRNAs, mRNAs, DNA and proteins, and are involved in multiple physiological and pathological processes. It has been reported that EVs play essential roles in immune response, tumor pathogenesis, neurodegenerative pathogenesis and pathogenic infections.<sup>7–10</sup> EVs also have the ability to serve as biomarkers, vaccine and drug carriers engineered.<sup>11</sup> Increasing evidence has shown that EVs secretion takes part in the pathogenesis of skin diseases, such as systemic lupus erythematosus and AD.<sup>12</sup>

Proteomics is the characterization of proteome, involving expression, structure, functions, interactions and modifications of proteins in a cell, tissue or an organism. Proteomics plays a vital role in early disease diagnosis, prognosis and the development of disease. Furthermore, it is also crucial for drug development as target molecules.<sup>13,14</sup> Although multiple sample resources are available, peripheral blood has been regarded as an ideal source due to its reproducibility of measurement and feasibility of access.<sup>15</sup> However, 99% of serum proteins, including albumin, immunoglobulins, and complement factors, will mask some biomarker candidates when measured by conventional proteomics. To break down these barriers, we focused on proteomic profiling of EVs.

In a recent study, protein analysis of exosomes derived from human adipose tissue-derived mesenchymal stem cells (ASC-exosomes) has shown that ASC-exosomes effectively restore epidermal barrier functions in AD by facilitating the synthesis of ceramides.<sup>16</sup> Cho et al<sup>17</sup> also prompted that ASC-exosomes could be engaged in immune regulation. Hong et al<sup>18</sup> have revealed that EVs derived from *Staphylococcus aureus* could induce AD-like inflammation in the skin. Yang et al<sup>19</sup> supported that microbial EVs-based metagenomic markers might detect the risk of AD development. These series of studies have shown an inextricable link between EVs and the pathophysiology of AD. However, the proteomic studies of plasma-derived EVs in AD patients have not been reported.

This study showed the diversity and abundance of plasma EVs collected from moderate-to-severe AD

patients and healthy individuals via proteomic analysis and suggested that the candidates for uniquely or differentially expressed proteins in plasma EVs could be a diagnostic marker in AD.

## Materials and Methods

### Sample Collection

Patients who met the following inclusion criteria were eligible: (1) patients age at least 18 years old or above with physician-diagnosed AD, (2) according to the criteria of Williams,<sup>20</sup> (3) having a SCORing Atopic Dermatitis (SCORAD) index<sup>21</sup> of at least 25 or higher (moderate-to-severe). Exclusion criteria included patients with suspected or established primary immunodeficiency, patients receiving systemic corticosteroids, immunotherapeutic agents, and/or anti-infective drugs. Patients whose SCORAD score dropped below 25 (mild AD) were excluded.

Plasma samples were obtained from 12 patients with moderate-to-severe atopic dermatitis (AD) (8 males and 4 females, SCORAD range 30.6–72.9, mean 59.89±12.25) and 13 healthy subjects (7 males and 6 females). Each of the clinical subjects showed very severe AD symptoms causing them to visit the hospital for treatment. Healthy control (HC) subjects were screened through a general health examination. The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Ethics Committee of Xiamen Chang Gung Hospital (Approval number XMCGRB2020039). All patients and donors agreed to participate in this study and signed the informed consent.

### Isolation of EVs

EVs were isolated from plasma samples by a series of ultracentrifugation (UC). Phosphate buffered saline (PBS) diluted the blood samples to decrease the viscosity. The diluted samples were centrifuged at 2000 × g for 30 min (4°C) to eliminate the debris in the blood. The supernatant was removed from the new tubes, which were further centrifuged at 10,000 × g for 45 min (4°C). The supernatant was filtered with 0.45 μm syringe filter (Millipore, USA) and the filtered supernatant was ultra-centrifuged at 100,000 × g for 70 min (4°C) (Optima L-100XP, Beckman Coulter). The exosome pellet was resuspended with 10 mL cold PBS and repeated the ultracentrifugation step. The final exosome pellet was resuspended with 100 μL of 0.22 μm-filtered PBS for subsequent analysis.

## Sample Preparation

The exosomes were lysed in 100 $\mu$ L 1% of Rapigest (m/v) in 50 mM of ammonium bicarbonate. The lysate was initially reduced to 20 mM of dithiothreitol (DTT) (Sigma) at 95°C for 5 min, and subsequently alkylated in 50 mM of iodoacetamide (IAA) (Sigma) for 30 min in the dark at room temperature (RT). After alkylation, the samples were centrifugated at 20000 g for 10 min, the supernatant was transferred to a 10 kDa centrifugal spin filter (Millipore) and sequentially washed with 200  $\mu$ L of 8M of urea for three times and 200 $\mu$ L of 50mM of ammonium bicarbonate for two times by centrifugation at 13500 g. Next, tryptic digestion was performed by adding trypsin (Promega) and Lys-C (Wako) at 1:50 (enzyme/substrate, m/m) in 150  $\mu$ L of 50 mM of ammonium bicarbonate at 37°C for 16 h. Peptides were recovered by transferring the filter to a new collection tube and spinning at 13500 g. To increase the yield of peptides, the filter was washed twice with 100 $\mu$ L of 50mM of ammonium bicarbonate. Peptides were desalted by StageTips and dried by speed vacuum at 45°C.

## Transmission Electron Microscope (TEM)

TEM with negative staining was used to observe the morphology of isolated exosomes. The purified exosome was diluted by two folds to 10 $\mu$ L and loaded onto a copper grid for 1 min and remove the excess exosome solution by filter paper carefully. The absorbed exosomes were stained with 10 $\mu$ L 2% uranyl acetate for 1 min, and the excess fluid was removed with filter paper. Grids were dried for few minutes at room temperature and exosomal images were captured by TEM at 80 kV (HT-7700, Hitachi High-Technologies Corporation, Japan).

## Nano-Flow Cytometry Measurement (NFCM)

The concentration and size of serum exosomes were analyzed by NFCM flow nano-Analyzer that following the manufacturer's instructions (N30E, nano-Analyzer, NanoFCM Inc., Xiamen, China). 200 nm Silica Nanosphere Cocktail was used to calibrate the concentration and size. 20  $\mu$ L from each sample was diluted by cold PBS (1:4 dilution). The 30 $\mu$ L diluted sample was single stained with 20  $\mu$ L Fluorescein Isothiocyanate (FITC) Mouse Anti-Human CD9 and FITC Mouse Anti-Human CD81 antibodies (BD Bioscience, USA) at 37°C for 30 minutes respectively. After incubation, the mixture was washed twice with PBS and

centrifuged at 110,000  $\times$  g for 70 min at 4°C (CP100MX, Hitachi High-Technologies Corporation, Japan). The supernatant was removed, and the pellet was resuspended with 50 $\mu$ L cold PBS. Events were recorded for 1 minute. The concentration, size distribution and surface proteins were quantified.

## Western Blot Analysis

According to the manufacturer's instructions, protein concentrations of EVs were evaluated using a Bicinchoninic Acid (BCA) Protein Assay Kit (Beyotime, China). Bovine Serum Albumin (BSA) was used as the standard. Antibodies for EV markers of CD9 (Abcam, ab92726, 1:1000), TSG101 (Abcam, ab30870, 1:1000) and Calnexin (Abcam, ab22595, 1:1000) were tested using Western Blotting, using anti-mouse secondary antibody (BioLegend, 400108, 1:5000). 10 $\mu$ g protein lysate was loaded onto 10% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels dependent on the molecular weight of target proteins. The separated proteins were transferred to a methanol-activated polyvinylidene difluoride (PVDF) membrane (Merck Millipore, USA). The membrane in the blocking buffer containing 5% skim milk powder in 1x tris-buffered saline with tween 20 (TBST), for one hour at room temperature and then incubated overnight with the primary antibody at 4°C. They were removing the primary antibodies by TBST three times of washing, following by another incubation with the secondary antibody for one hour at room temperature. Afterward, the membrane was incubated with an enhanced chemiluminescence (ECL) substrate (Merck Millipore, USA) for five minutes at room temperature, allowing for chemiluminescent signal capturing using the Chemiscope 3300 mini (Clinx, China).

## Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) Analysis

All MS experiments were performed on a nanoscale EASY-nLC 1200UHPLC system (Thermo Fisher Scientific) connected to an Orbitrap Fusion Lumos equipped with a nanoelectrospray source (Thermo Fisher Scientific). Mobile phase A contained 0.1% of formic acid (v/v) in water; mobile phase B contained 0.1% of formic acid in 80% of acetonitrile (ACN). The peptides were dissolved in 0.1% of formic acid (FA) with 2% of acetonitrile and separated on a reversed-phase high-performance liquid chromatography (RP-HPLC) analytical column (75  $\mu$ m $\times$ 25 cm)

packed with 2  $\mu\text{m}$  C18 beads (Thermo Fisher Scientific) using a linear gradient ranging from 9% to 28% of ACN in 90 min and followed by a linear increase to 44% B in 20 min at a flow rate of 300nL/min. The Orbitrap Fusion Lumos acquired data in a data-dependent manner alternating between full-scan MS and MS2 scans. The spray voltage was set at 2.2 kV and the temperature of ion transfer capillary was 300°C. The MS spectra (350–1800m/z) were collected with 120,000 resolution, automatic gain control (AGC) of  $4 \times 10^5$ , and 50 ms maximal injection time. Selected ions were sequentially fragmented in a 3 s cycle by high collision dissociation (HCD) with 30% normalized collision energy, specified isolated windows 1.6 m/z, 15,000 resolution. AGC of  $5 \times 10^4$  and 40 ms maximal injection time were used. Dynamic exclusion was set to 30 s. Unassigned ions or those with a charge of 1+ and >7+ were rejected for MS/MS.

## Mass Spectrometry Data Analysis

Raw data were processed using Proteome Discoverer (PD, version 2.2), and MS/MS spectra were searched against the reviewed SwissProt human proteome database. All searches were carried out with precursor mass tolerance of 20ppm, fragment mass tolerance of 0.02Da, oxidation (Met) (+15.9949Da) and acetylation (protein N-terminus) (+42.0106Da) as variable modifications, carbamidomethylation (Cys) (+57.0215Da) as fixed modification and three trypsin missed cleavages allowed. Only peptides with at least six amino acids in length were considered. The peptide and protein identifications were filtered by PD to control the false discovery rate (FDR) <1%. At least one unique peptide was required for protein identification.

## Functional and STRING Interaction Network Analysis

Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway were analysed with the Database for Annotation, Visualization and Integrated Discovery (DAVID version 6.8) (<https://david.ncifcrf.gov/>).<sup>22</sup> The biological process, molecular function and cellular component were further analysed using Panther software, online version (<http://www.pantherdb.org/>).

## Statistical Analysis

Statistical analysis was performed using the Student's *t*-test to compare the plasma EVs proteins from patients and healthy controls. The p-value of 0.05 was considered statistically significant.

## Results

### Demographic Characteristics of the Study Subjects

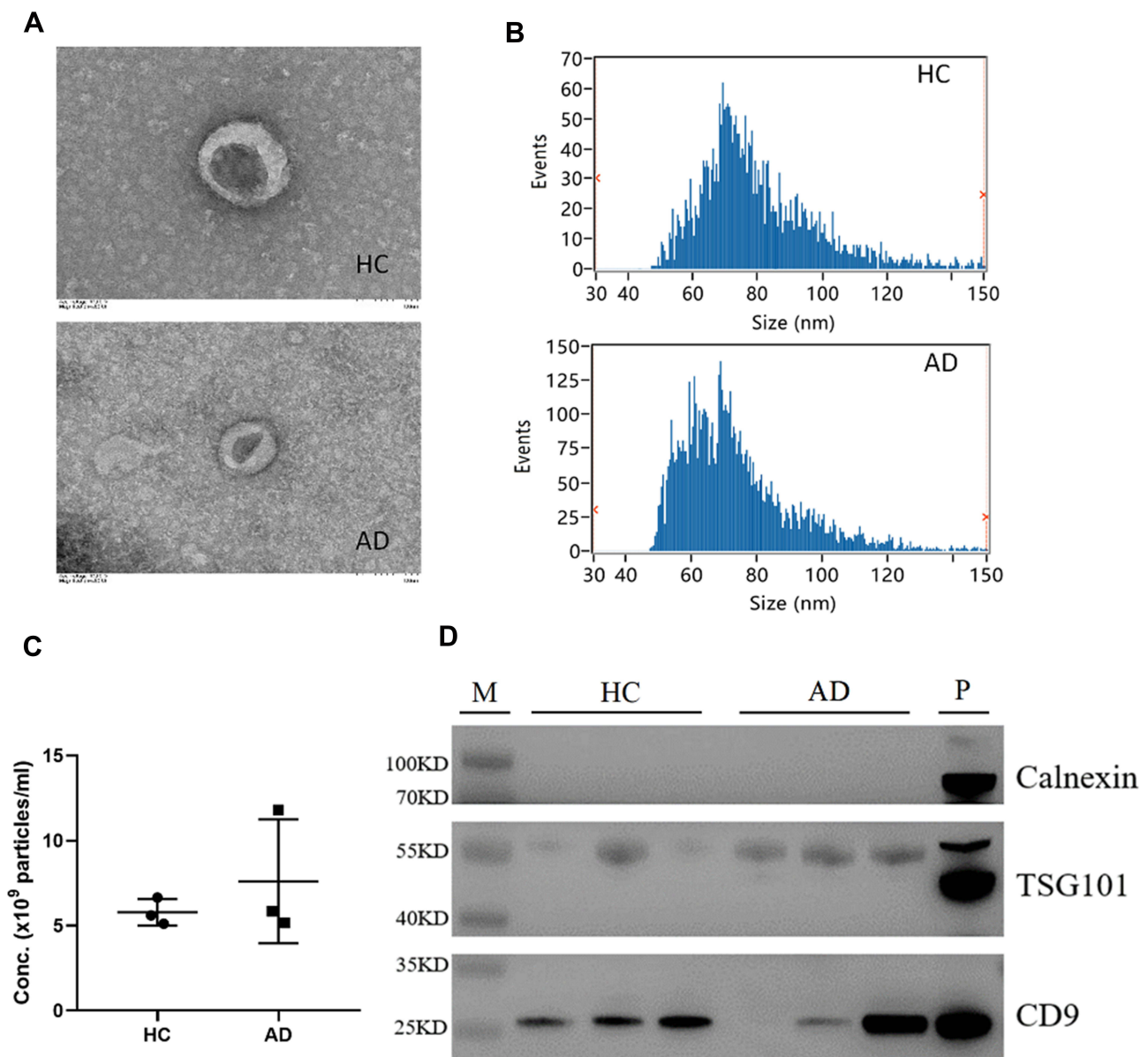
A total of 25 subjects were enrolled that included 12 AD patients and 13 healthy controls. The difference in sex and age between the healthy control and patient groups was not significant ( $P = 0.513$  and  $P = 0.252$ , respectively). Specific details are shown in [Table 1](#).

### Isolation and Characterization of EVs

Plasma EVs were isolated from HC and AD that were identified using TEM, nano-flow cytometer, and Western blot analysis. The characterization of typical cup-shaped vesicles was presented by TEM in EVs from the represented sample ([Figure 1A](#)). A nano-flow cytometer was used to measure the size and concentration of EVs. NFCM measurement revealed that the isolated EVs were 50–150 nm in size with an average size of  $80.66 \pm 1.45$  nm and  $74.12 \pm 0.63$  nm in HC and AD groups, respectively ([Figure 1B](#)). The EVs in the HC group were significantly larger in size compared to those in the AD group ([Figure 1B](#)). After normalization, particles concentration also was determined by  $5.79 \times 10^9 \pm 0.78 \times 10^9$  p/mL and  $7.61 \times 10^9 \pm 3.65 \times 10^9$  p/mL for HC and AD, respectively. Although particle concentration of AD is higher slightly than those of HC, the two groups are non-significantly different ([Figure 1C](#)). Western blotting was used to evaluate the purity of isolated EVs. Western blot analysis confirmed the presence of exosomal markers, such as CD9 and TSG101, whereas calnexin, as non-EV marker, was not detected ([Figure 1D](#)). Taken together, a series of centrifugation processes is successful to isolate EVs with purity and the amount of EVs is higher in AD patients.

**Table 1** The Characteristics of Atopic Dermatitis Patients (AD) and Healthy Controls (HC)

Variables	HC (n=13)	AD (n=12)	P value
Sex (Male/Female)	7/6 (53.8% vs 46.2%)	8/4 (66.7% vs 33.3%)	0.513
Age (years)			
Total	43.23±11.76	52.33±25.15	0.252
Male	37.14±6.67	59.75±22.66	0.175
Female	50.55±12.89	37.50±26.10	0.606
SCORAD index	–	59.89±12.25	–



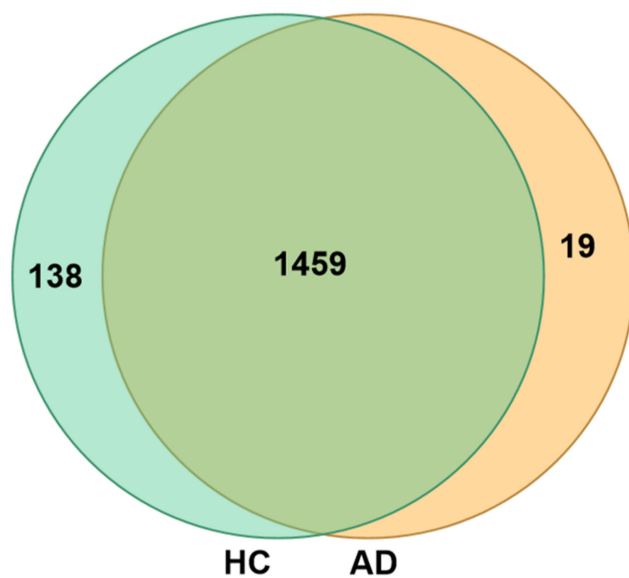
**Figure 1** Isolation and identification of EVs from plasma. Electron micrographs of EVs were observed by transmission electron microscopy (bar=100nm) (**A**); Nano-Flow Cytometry Measurement (NFCM) was used to detect the size (**B**) and concentration (**C**) of EVs; Western blot analysis to detect the expression of EV protein markers (CD9, TSG101) and one non-EV marker (Calnexin) (**D**).

**Abbreviations:** HC, healthy control group; AD, atopic dermatitis group.

## Proteomic Profiling of Plasma EVs

Isolated EVs were analysed using the nano LC-MS/MS method to compare the plasma EVs proteins of patients with moderate-to-severe AD and HC patients. The proteomic analysis revealed a large number of proteins in plasma EVs from both AD and HC groups. A total of 1478 proteins in plasma EVs were found to be common proteins in AD, whereas a total of 1597 proteins in plasma EVs were found to be common proteins in HC. A total of 1459 proteins in plasma EVs overlapped in comparing HC

with AD (**Figure 2**). The volcano plot indicated the differential encapsulation of plasma EVs proteins between AD and HC (**Figure 3A**). A total of 86 proteins in plasma EVs showed more than 2.5-fold up-regulation, while a total of 225 proteins in plasma EVs showed less than 1/2.5-fold down-regulation with a significant difference ( $p < 0.05$ ) among AD compared with HC. The heat map showed 20 up-regulation proteins and 20 down-regulation proteins with a significant difference ( $p < 0.05$ ) in AD plasma EVs (**Figure 3B** and **Table 2**).



**Figure 2** Comparative Venn diagram of plasma EVs proteins between HC group and AD group.

## Functional and Protein–Protein Interaction (PPI) Network Analysis

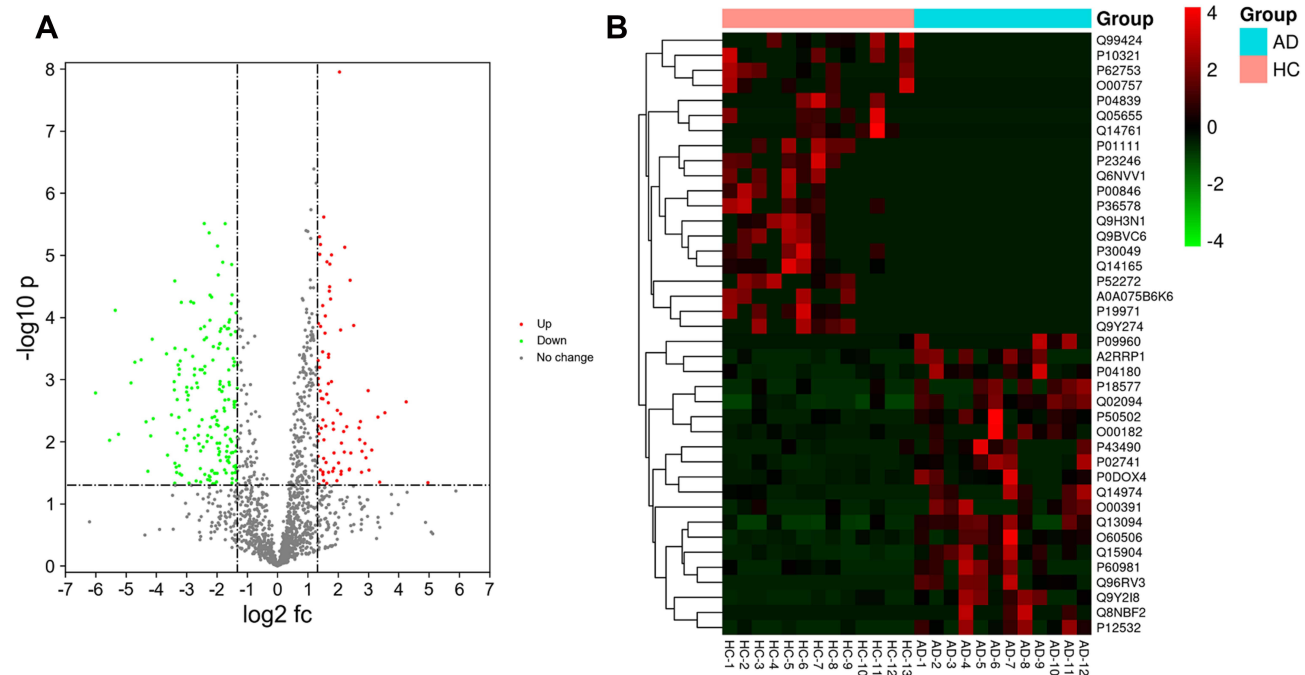
The GO analysis was performed to evaluate the candidates for differentially expressed proteins (Figure 4A–C) to obtain a comprehensive image of the proteins in plasma EVs from

moderate-to-severe AD patients. The candidates for differentially expressed proteins in plasma EVs were interrogated for a broad range of biological processes, including localization, regulation of biological quality, single-multicellular organism process, cell activation, response to stimulus, and so on. In the molecular function category, most of the proteins appeared to participate in protein binding and structural molecular activity. Cellular component proteins were mainly localized in the extracellular membrane-bounded organelle, cytoplasm, and extracellular region.

The candidates for differentially expressed proteins in plasma EVs were illustrated in the protein–protein interaction (PPI) network by STRING analysis. Notably, the candidates for differentially expressed proteins in plasma EVs have been described a connectivity PPI network (Figure 5A) relating to several KEGG pathways, including pathways in platelet activation, leukocyte transendothelial migration, complement and coagulation cascades, phagosome, pathogenic *Escherichia coli* infection, Rap1 signaling pathway, and so on (top 10 KEGG pathways are shown in Figure 5B).

## Discussion

It has been reported that EVs play an important role in cell-to-cell communication in normal physiology and



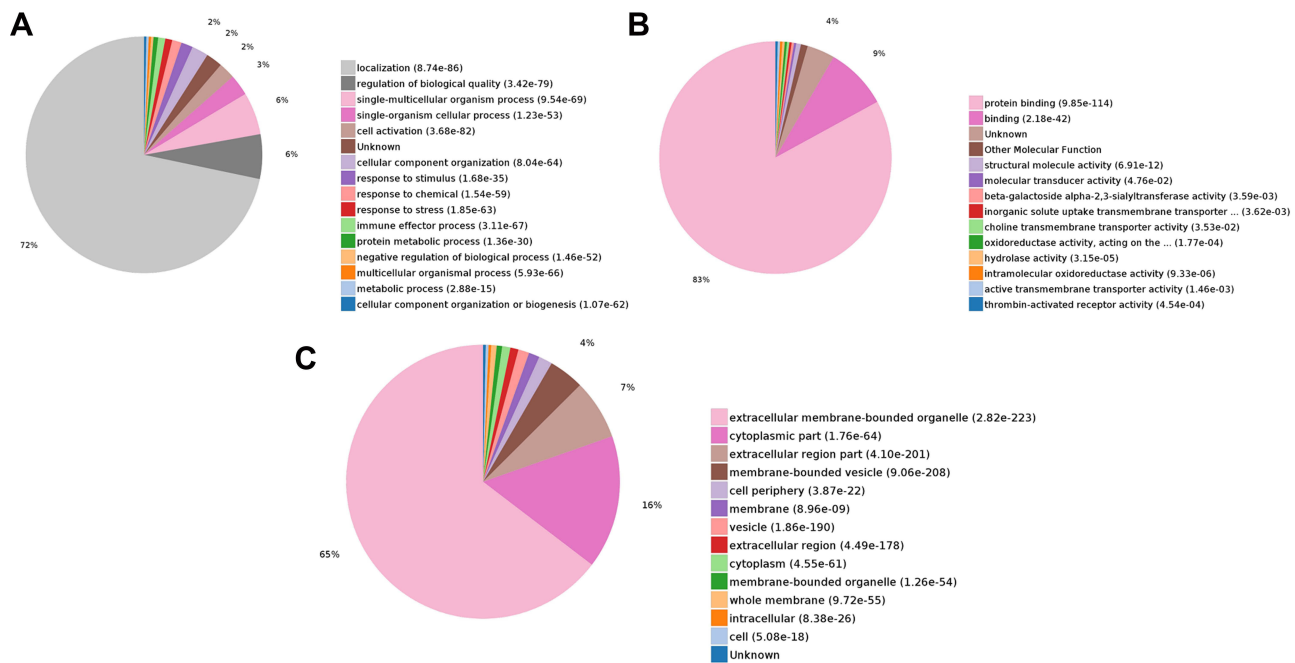
**Figure 3** Volcano plot demonstrated the differential encapsulation of plasma EVs proteins between AD and HC. Up/down-regulated EVs proteins were indicated in green and red colors, respectively. Proteins not classified as up/down-regulated were plotted in grey color. X-axis and Y-axis indicate a more than two-fold change (in log<sub>2</sub> scale) and  $-\log_{10}$  with significance ( $p < 0.05$ ) (A). Heat map of hierarchical clustering indicate the candidates for differentially expressed proteins in AD plasma EVs. Up/down-regulated proteins are indicated by red/green color, respectively (B). GraphPad Prism software, version 8 (<https://www.graphpad.com>) was used to generate the volcano plot and heat map.

**Table 2** The Candidates for Differentially Expressed Proteins in Plasma EVs from AD Patients

UniProt Accession	Protein Name	Gene Name	Regulation
Q99424	Peroxisomal acyl-coenzyme A oxidase 2	ACOX2	Down
P10321	HLA class I histocompatibility antigen, C alpha chain	HLA-C	Down
P62753	Ribosomal protein S6	RPS6	Down
O00757	Fructose-1,6-bisphosphatase isozyme 2	FBP2	Down
P04839	Cytochrome b-245 heavy chain	CYBB	Down
Q05655	Protein kinase C delta type	PRKCD	Down
Q14761	Protein tyrosine phosphatase receptor type C-associated protein	PTPRCAP	Down
P01111	GTPase NRas	NRAS	Down
P23246	Splicing factor, proline- and glutamine-rich	SFPQ	Down
Q6NVV1	Ribosomal protein L13a protein	RPL13AP3	Down
P00846	ATP synthase subunit alfa	MT-ATP6	Down
P36578	Ribosomal protein L4	RPL4	Down
Q9H3N1	Thioredoxin-related transmembrane protein I	TMX1	Down
Q9BVC6	Transmembrane protein 109	TMEM109	Down
P30049	ATP synthase subunit delta	ATP5F1D	Down
Q14165	Malectin	MLEC	Down
P52272	Heterogeneous nuclear ribonucleoprotein M	HNRNPM	Down
AOA075B6K6	Immunoglobulin lambda variable 4-3	IGLV4-3	Down
P19971	Thymidine phosphorylase	TYMP	Down
Q9Y274	Type 2 lactosamine alpha-2,3-sialyltransferase	ST3GAL6	Down
P09960	Leukotriene A-4 hydrolase	LTA4H	Up
A2RRP1	Neuroblastoma-amplified sequence	NBAS	Up
P04180	Phosphatidylcholine-sterol acyltransferase	LCAT	Up
P18577	Blood group Rh (CE) polypeptide	RHCE	Up
Q02094	Ammonium transporter Rh type A	RHAG	Up
P50502	Hsc70-interacting protein	ST13	Up
O00182	Galectin-9	LGALS9	Up
P43490	Nicotinamide phosphoribosyl transferase	NAMPT	Up
P02741	C-reactive protein	CRP	Up
P0DOX4	Immunoglobulin epsilon heavy chain	P0DOX4	Up
Q14974	Importin subunit beta-1	KPNB1	Up
O00391	Sulfhydryl oxidase I	QSOX1	Up
Q13094	Lymphocyte cytosolic protein 2	LCP2	Up
O60506	Heterogeneous nuclear ribonucleoprotein Q	SYNCRIP	Up
Q15904	V-type proton ATPase subunit S1	ATP6API	Up
P60981	Dextrin	DSTN	Up
Q96RV3	Pecanex-like protein I	PCNX1	Up
Q9Y218	WD repeat-containing protein 37	WDR37	Up
Q8NBF2	NHL repeat-containing protein 2	NHLRC2	Up
P12532	Creatine kinase U-type	CKMT1B	Up

pathological conditions.<sup>23</sup> Wang et al<sup>12</sup> reviewed the role of exosomes in inflammatory skin diseases and skin cancer, including systemic lupus erythematosus, psoriasis, atopic dermatitis, and melanoma. In conclusion, they indicated that EVs are involved in the onset or development of disease and may serve as diagnostic markers or predictive tools for prognosis and treatment responses. To date, increasing evidence has indicated that EVs are associated with the pathogenesis of AD. In this study, we compared

the proteomic profiles of plasma-derived EVs between AD patients and HC patients for the first time. Proteomic analysis revealed a total of 1478 proteins in plasma EVs found as common proteins in AD, whereas a total of 1597 proteins in plasma EVs were found to be common proteins in HC. From the result, a total of 19 proteins in plasma EVs from AD were the candidates for uniquely expressed proteins compared to those from HC. GO and KEGG analyses identified several functional terms that the



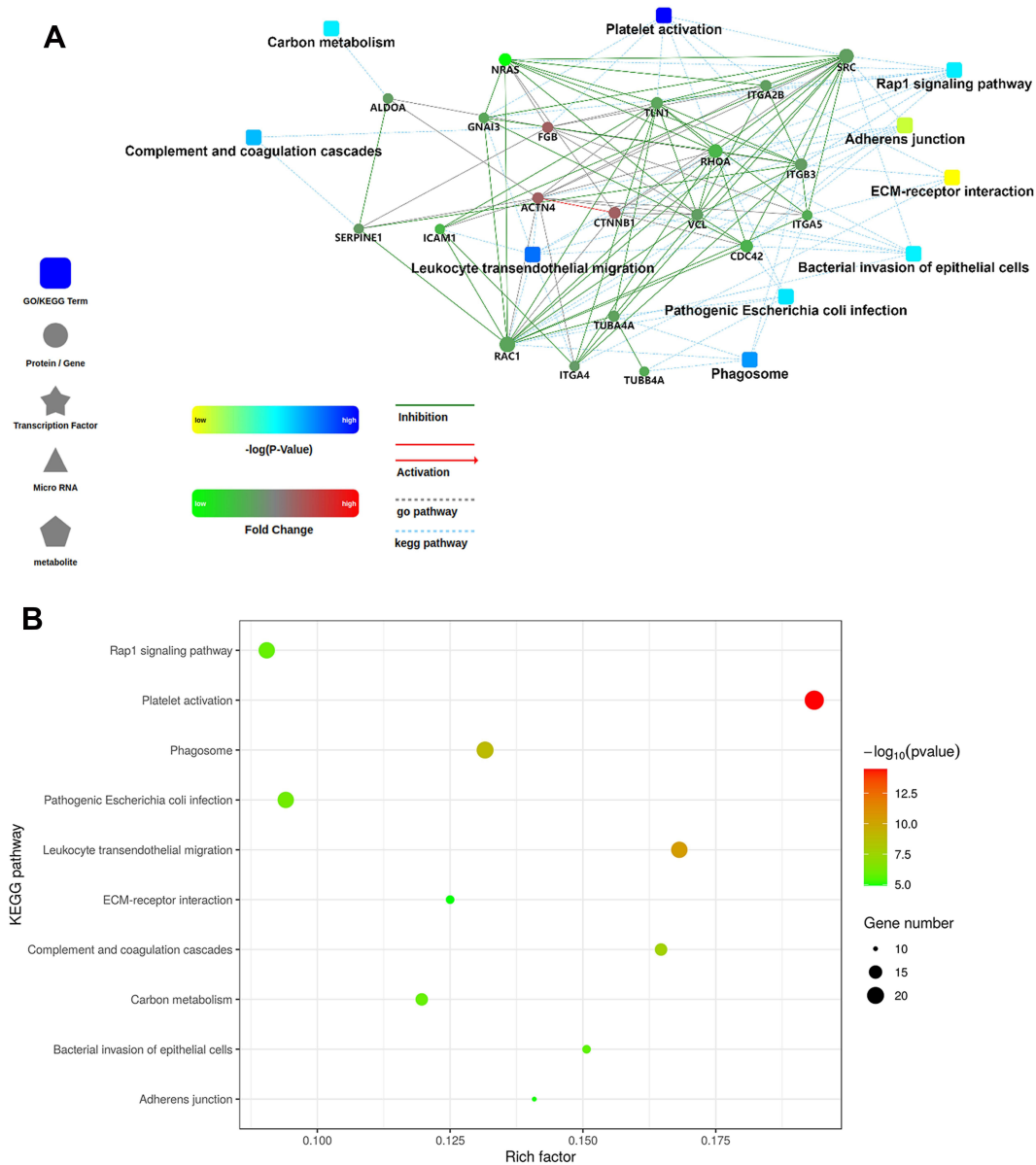
**Figure 4** GO analysis of the candidates for differentially expressed proteins in plasma EVs from AD. The candidates for differentially expressed proteins in plasma EVs from AD were analysed using Panther software and categorised according to biological process (A), molecular function (B), cellular component (C).

candidates enriched for differentially expressed proteins in plasma EVs. Attention was paid to the aforementioned KEGG pathways.

The KEGG analysis revealed that the candidates for differentially expressed proteins were involved in many pathways, including pathways in platelet activation, leukocyte transendothelial migration, complement and coagulation cascades, phagosome, and so on. Thereby, the critical role of platelet activation has become apparent in inflammatory diseases. This encapsulates and describes all the procedures that platelets undergo to provide the necessary cofactors for the clotting system.<sup>24</sup> As early as 2004, Kasperska-Zaj et al<sup>25</sup> showed that patients with atopic eczema/dermatitis syndrome (AEDS) might have enhanced blood platelet activity as expressed by  $\beta$ -thrombomodulin ( $\beta$ -TG) and platelet factor-4 (PF-4) level. A previous study also reported that elevated platelet activation markers, including  $\beta$ -TG, PF-4, vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) in AD patients, are correlated with concomitant allergic diseases.<sup>26</sup> Hilger et al<sup>27</sup> demonstrated that platelet activation could enhance stimulation and responsiveness to *Staphylococcus aureus*, which is involved in AD pathogenesis. Taken together, platelet activation may contribute to the pathogenesis of AD.

Our research showed that the proteins involved in platelet activation, including lymphocyte cytosolic protein 2, and fibrinogen  $\alpha/\beta/\gamma$  chain are upregulated. Among these proteins, lymphocyte cytosolic protein 2 (LCP2) has become a focus of our interest. LCP2, also called SLP-76 tyrosine phosphoprotein (SLP76), is expressed throughout the hematopoietic compartment.<sup>28</sup> It is a key protein involved in TCR signaling and other hematopoietic pathways.<sup>29</sup> Athari<sup>30</sup> confirmed that targeted therapy modulating cell signaling pathways, including high-affinity IgE receptor (FcRI)-LAT-SLP76, can be a powerful strategy to design new drugs to treat asthma. In addition, it has been reported that SLP76 is an essential signaling component to regulate Fc $\epsilon$ RI-induced IL-4 production in basophils,<sup>31</sup> while IL-4 provided by basophils plays a regulatory role in the initiation of Th2-type immune responses.<sup>32</sup> So SLP76 may be involved in the pathogenesis of Th2-associated disease. However, whether it is associated with the occurrence and development of AD has yet to be established.

In conclusion, proteomic analysis identified a total of 1478 common proteins in plasma EVs from AD patients from which 19 proteins were candidates for uniquely expressed proteins compared with those of HC. Additionally, GO and KEGG analyses showed a new protein-SLP67 that may significantly contribute to AD pathogenesis.



**Figure 5** PPI network and KEGG pathway analysis. The PPI network was analysed by STRING software to evaluate the candidates for differentially expressed proteins in plasma EVs from AD (**A**). The molecular relationships are indicated by solid lines with or without arrow (Inhibitory/activating relations are indicated by green/red color, respectively). The gray or blue dotted lines represent GO pathway or KEGG pathway, respectively. The shape of square or circle separately stands for GO/KEGG term or protein/gene. Color set point is described in the left panel. The candidates for differentially expressed proteins in plasma EVs from AD were analysed using KEGG software, and the top 10 KEGG pathways were demonstrated in the bubble map (**B**). The node size reflects the number of gene. Color set point is described in the right panel.

This is the first study to present proteomic analysis of plasma EVs from AD patients. In the future, we will further verify the role of SLP67 in AD via animal and cell experiments to provide a promising therapeutic or diagnostic target.

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## Disclosure

The authors report no conflicts of interest in this work.

## References

- Gooderham MJ, Hong HC, Eshtiaghi P, Papp KA. Dupilumab: a review of its use in the treatment of atopic dermatitis. *J Am Acad Dermatol*. 2018;78 (3 Suppl 1):S28–s36. doi:10.1016/j.jaad.2017.12.022
- Langan SM, Irvine AD, Weidinger S. Atopic dermatitis. *Lancet*. 2020;396(10247):345–360. doi:10.1016/S0140-6736(20)31286-1
- Silverberg JI. Comorbidities and the impact of atopic dermatitis. *Ann Allergy Asthma Immunol*. 2019;123(2):144–151. doi:10.1016/j.anai.2019.04.020
- Sacotte R, Silverberg JI. Epidemiology of adult atopic dermatitis. *Clin Dermatol*. 2018;36(5):595–605. doi:10.1016/j.clindermatol.2018.05.007
- Garg N, Silverberg JI. Epidemiology of childhood atopic dermatitis. *Clin Dermatol*. 2015;33(3):281–288. doi:10.1016/j.clindermatol.2014.12.004
- van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol*. 2018;19 (4):213–228. doi:10.1038/nrm.2017.125
- Vega VL, Rodríguez-Silva M, Frey T, et al. Hsp70 translocates into the plasma membrane after stress and is released into the extracellular environment in a membrane-associated form that activates macrophages. *J Immunol*. 2008;180(6):4299–4307. doi:10.4049/jimmunol.180.6.4299
- Zhao H, Yang L, Baddour J, et al. Tumor microenvironment derived exosomes pleiotropically modulate cancer cell metabolism. *eLife*. 2016;5:e10250. doi:10.7554/eLife.10250
- Asai H, Ikezu S, Tsunoda S, et al. Depletion of microglia and inhibition of exosome synthesis halt tau propagation. *Nat Neurosci*. 2015;18(11):1584–1593. doi:10.1038/nn.4132
- Dreux M, Garaigorta U, Boyd B, et al. Short-range exosomal transfer of viral RNA from infected cells to plasmacytoid dendritic cells triggers innate immunity. *Cell Host Microbe*. 2012;12(4):558–570. doi:10.1016/j.chom.2012.08.010
- He C, Zheng S, Luo Y, Wang B. Exosome theranostics: biology and translational medicine. *Theranostics*. 2018;8(1):237–255. doi:10.7150/thno.21945
- Wang WM, Wu C, Jin HZ. Exosomes in chronic inflammatory skin diseases and skin tumors. *Exp Dermatol*. 2019;28(3):213–218.
- Aslam B, Basit M, Nisar MA, Khurshid M, Rasool MH. Proteomics: technologies and their applications. *J Chromatogr Sci*. 2017;55 (2):182–196. doi:10.1093/chromsci/bmw167
- Domon B, Aebersold R. Mass spectrometry and protein analysis. *Science*. 2006;312(5771):212–217. doi:10.1126/science.1124619
- Koba T, Takeda Y, Narumi R, et al. Proteomics of serum extracellular vesicles identifies a novel COPD biomarker, fibulin-3 from elastic fibres. *ERJ Open Res*. 2021;7(1):00658–2020. doi:10.1183/2312-0541.00658-2020
- Shin KO, Ha DH, Kim JO, et al. Exosomes from human adipose tissue-derived mesenchymal stem cells promote epidermal barrier repair by inducing de novo synthesis of ceramides in atopic dermatitis. *Cells*. 2020;9(3):680. doi:10.3390/cells9030680
- Cho BS, Kim JO, Ha DH, Yi YW. Exosomes derived from human adipose tissue-derived mesenchymal stem cells alleviate atopic dermatitis. *Stem Cell Res Ther*. 2018;9(1):187. doi:10.1186/s13287-018-0939-5
- Hong SW, Kim MR, Lee EY, et al. Extracellular vesicles derived from *Staphylococcus aureus* induce atopic dermatitis-like skin inflammation. *Allergy*. 2011;66(3):351–359. doi:10.1111/j.1398-9995.2010.02483.x
- Yang J, McDowell A, Seo H, et al. Diagnostic models for atopic dermatitis based on serum microbial extracellular vesicle metagenomic analysis: a Pilot Study. *Allergy Asthma Immunol Res*. 2020;12(5):792–805. doi:10.4168/aaair.2020.12.5.792
- Williams HC, Burney PG, Hay RJ, et al. The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. I. Derivation of a minimum set of discriminators for atopic dermatitis. *Br J Dermatol*. 1994;131(3):383–396. doi:10.1111/j.1365-2133.1994.tb08530.x
- Kunz B, Oranje AP, Labreze L, Stalder JF, Ring J, Taieb A. Clinical validation and guidelines for the SCORAD index: consensus report of the European Task Force on Atopic Dermatitis. *Dermatology*. 1997;195(1):10–19. doi:10.1159/000245677
- Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4(1):44–57. doi:10.1038/nprot.2008.211
- Todorova D, Simoncini S, Lacroix R, Sabatier F, Dignat-George F. Extracellular vesicles in angiogenesis. *Circ Res*. 2017;120 (10):1658–1673. doi:10.1161/CIRCRESAHA.117.309681
- Rubenstein DA, Yin W. Platelet-activation mechanisms and vascular remodeling. *Compr Physiol*. 2018;8(3):1117–1156.
- Kasperska-Zaj A, Nowakowski M, Rogala B. Enhanced platelet activation in patients with atopic eczema/dermatitis syndrome. *Inflammation*. 2004;28(5):299–302. doi:10.1007/s10753-004-6054-z
- Nastalek M, Potaczek DP, Wojas-Pelc A, Undas A. Plasma platelet activation markers in patients with atopic dermatitis and concomitant allergic diseases. *J Dermatol Sci*. 2011;64(1):79–82. doi:10.1016/j.jdermsci.2011.07.001
- Hilger RA, Neuber K, König W. Conversion of leukotriene A4 by neutrophils and platelets from patients with atopic dermatitis. *Immunology*. 1991;74(4):689–695.
- Koretzky GA, Abtahian F, Silverman MA. SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond. *Nat Rev Immunol*. 2006;6(1):67–78. doi:10.1038/nri1750
- Lev A, Lee YN, Sun G, et al. Inherited SLP76 deficiency in humans causes severe combined immunodeficiency, neutrophil and platelet defects. *J Exp Med*. 2021;218(3). doi:10.1084/jem.20201062
- Athari SS. Targeting cell signaling in allergic asthma. *Signal Transduct Target Ther*. 2019;4:45.
- Hidano S, Kitamura D, Kumar L, Geha RS, Goitsuka R. SLP-76 is required for high-affinity IgE receptor- and IL-3 receptor-mediated activation of basophils. *Int Immunol*. 2012;24(11):719–727. doi:10.1093/intimm/dxs072
- Yoshimoto T, Yasuda K, Tanaka H, et al. Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. *Nat Immunol*. 2009;10(7):706–712. doi:10.1038/ni.1737

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