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Label-Free Quantification based Proteomic Analysis of Serum Obtained from Henoch–Schönlein Purpura Patients before and after Zhenbao Pill Treatment

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WN gave the idea and provided the facilities and guidance and also helped in writing the manuscript. BB collected the samples and provided the data. NQ and JG performed the experiments and wrote the manuscript. ED analyzed the data. SW helped in writing the manuscript. CG helped in experimentation. HG helped in bioinformatics analysis. HJ helped in sample collection. BR helped in figure preparation. TH helped in data analysis. All authors reviewed the article.

Key words
Henoch–Schönlein purpura, Zhenbao pill, Immunity, LFQ proteomics, Therapeutic strategies

ABSTRACT

Henoch–Schönlein Purpura (HSP), also known as immunoglobulin A vasculitis, is an immune disorder that is predominantly seen in children (8–20/100000 each year) worldwide. It is characterized by inflammation and bleeding of tiny blood vessels in the skin, joints, intestines, and kidneys. The most distinctive characteristic of this type of vasculitis is a purple rash that often affects the thighs and buttocks. The Zhenbao pill, containing several medicinally important compounds, has been found to be effective against a variety of neurological and immunological illnesses. We used a label-free quantification (LFQ) proteomics approach to investigate the effect of the Zhenbao pill on the differential serum protein expression of HSP patients. Out of 14 significantly differentially expressed proteins, four proteins immunoglobulin lambda variable 3-27, immunoglobulin kappa variable 3-11, neural cell adhesion molecule L1-like protein, and immunoglobulin heavy variable 3-33 were upregulated, whereas 10 proteins i.e., protein Z-dependent protease inhibitor, C4b-binding protein alpha chain, heparin cofactor 2, complement C4-A, complement C4 beta chain, vitamin K-dependent protein Z, coagulation factor VII, proteoglycan 4, lipopolysaccharide-binding protein (LBP), thrombospondin-4, and plasminogen activator inhibitor 1 were downregulated. Bioinformatics analysis of these differentially expressed proteins revealed that they play a critical role in immune-related responses. Parallel reaction monitoring validation also substantiated the results of the LFQ proteomics approach by analyzing the expression of LBP and SERPINA10. This work identifies the proteins and protein-related molecular pathways that are responsible for the efficacy of Zhenbao pill in the management of HSP.

INTRODUCTION

Henoch–Schönlein purpura (HSP) is commonly found in children, with a prevalence of 8–20 children per 100,000 each year. Its incidence is higher in males than in females (1.5:1) and children between the ages of 3 and 8 years are particularly vulnerable (Di Pietro *et al*., 2019; Leung *et al*., 2020; Rostoker, 2001). HSP is a systemic small-vessel vasculitis caused by immunoglobulin A (IgA) accumulation in the vessel walls. This results in damage to the skin, joints, intestines, and kidneys (Bluman and Goldman, 2014; de Almeida *et al*., 2007; Leung *et al*., 2020; Namgoong, 2020). However, its exact etiology remains unknown; usually, a severe infectious illness is followed by HSP with a seasonal pattern (non-summer months), which might indicate a contagious spread (Rostoker, 2001; Trnka, 2013).

Additionally, HSP can be diagnosed with one or more of the following four symptoms; the appearance of petechiae or purpura that are frequently palpable, with lower limb predominance (Leung *et al*., 2020; Rostoker, 2001; Trnka, 2013). Various signs and symptoms associated with HSP include renal involvement, abdominal pain, arthralgia, increased 24-h albumin excretion, morning serum creatinine levels greater than 30 mol/L, and positive dipstick or positive histopathologic results for hematuria (Trnka, 2013). Proliferative glomerulonephritis with evident IgA deposits and leukocystoclastic vasculitis seen on kidney biopsy and predominant IgA deposits seen on skin biopsy are two examples of autoimmune responses

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that may manifest during the course of the disease (Bluman and Goldman, 2014; Oni and Sampath, 2019).

Male patients express scrotal soreness, fever, edema and cardiac, pulmonary, or neurological signs (Rostoker, 2001). HSP is a temporary disorder which is usually limited to 6–8 weeks but can cause complications (Bluman and Goldman, 2014). Renal disorder occurs in 37% of patients, although 1% of patients progress to end-stage renal failure (Ozen *et al*., 2010; Rostoker, 2001). Gastrointestinal symptoms affect 66% of students and include abdominal pain (44%), intestinal bleeding (22%) and intussusceptions (≤3%) (Gardner-Medwin *et al*., 2002; Rostoker, 2001). Nonsteroidal anti-inflammatory drugs and acetaminophen may be used to relieve moderate joint pain and fever, respectively (Bluman and Goldman, 2014; Duvuru and Stone, 2013; Rostoker, 2001). However, when consumed by patients with gastrointestinal or renal complications, symptoms may get aggravated (Bluman and Goldman, 2014; Rostoker, 2001). Therefore, the use of nonsteroidal anti-inflammatory drugs is avoided in managing HSP patients (Bluman and Goldman, 2014; Rostoker, 2001).

Various therapeutic approaches have been used to treat HSP; however, none have yet proven effective. The Zhenbao pill consists of 29 Chinese herbal medicines, including pearl, bezoar, amomum, saffron, cassia seed, and licorice, which promotes blood circulation, calms nerves, and activates meridians (Jia *et al*., 2021). It has been reported to be anti-inflammatory drug effective against various disorders (Luo *et al*., 2020). The Zhenbao pill has been used against certain neurological disorders, including hemiplegia sequelae and stroke. Studies have revealed that the Zhenbao pill leads to the eradication of oxygen free radicals, promotes microcirculation and repairs damaged neurons (He *et al*., 2018a; Liu *et al*., 2015; Wu *et al*., 2007). One study found that the Zhenbao pill had a protective effect on the nerves of rats; the Basso–Beattie–Bresnahan score of rats with spinal cord injury (SCI) was improved after Zhenbao pill administration, and a reduction in the proportion of Treg cells was also observed (Bluman and Goldman, 2014; Rostoker, 2001). It has recently been established that SCI repair can be promoted directly by reducing Treg cell numbers and indirectly by alleviating immunosuppression (Raposo *et al*., 2014). The mechanism behind this is related to the inhibition of Treg cell numbers, which leads to the reduction of transforming growth factor-β levels by inhibiting the miR-214-induced heat shock protein 27 expression during SCI repair (He, Yongxiong *et al*., 2018a). The above literature illustrates that Zhenbao pill has been found effective against various immune related disorders. However, further research is required to elucidate the underlying molecular mechanism of action of the Zhenbao pill in these immune disorders.

A label-free quantification (LFQ)-based proteomics (Al-Shweiki *et al*., 2017) investigation was conducted to understand the effect of the Zhenbao pill on protein-based molecular pathways in the serum of HSP patients before and after its administration. This investigation will aid in understanding the molecular processes that are responsible for its effectiveness against immunological dysfunctions and evaluating its potential as an effective therapeutic strategy against HSP.

MATERIALS AND METHODS

Patients and serum samples

The research protocol was approved by the Ethics Committee of the Affiliated Hospital of Inner Mongolia University for the Nationalities (NM-LL-2019-01-05-01). HSP patients were diagnosed in the Affiliated Hospital of Inner Mongolia University for the Nationalities. Diagnostic criteria of HSP were in accordance with the guidelines for HSP diagnosis and treatment, which include: skin purpura, joint swelling and pain, abdominal pain and hematochezia. The main manifestations of renal involvement are hematuria and proteinuria.

Inclusion other complications including infection, cancer, hypertension, diabetes and other systemic diseases to meet the criteria. Written informed consent was obtained from all the participants. The detailed information pertaining to the HSP patients, including their demographics, and main manifestation are provided in Table I.

Table I. Clinical serum samples for the identification of HSP-associated patients.

Characteristics	Before Zhenbao pill treatment (n=30)	After Zhenbao pill treatment (n=30)
Age	27.27±18.37	27.27±18.37
Male	19(63.3%)	19(63.3%)
Female	11(36.7%)	11(36.7%)
Skin purpura	100%	0%
Hematuria	53.3%	0%
Proteinuria	50%	0%

HSP patients admitted to this study were provided daily with 3-5 g of Zhenbao pill (based on the body weight). The effect of HSP treatment with Zhenbao pill was followed every two weeks up to four weeks by examining the urine albumen and blood.

In this study, 30 blood samples from HSP patients were collected. Among them, 12 blood samples were randomly selected for proteomics. Before centrifugation

at 4°C (1000×*g*), the samples were allowed to coagulate at room temperature to obtain the serum. All serum samples were tested at the same time, and they were stored at -80 °C in the freezer until use. Whole of the experimental scheme is shown in Figure 1.

Table include characteristics, Before Zhenbao pill treatment, after Zhenbao pill treatment.

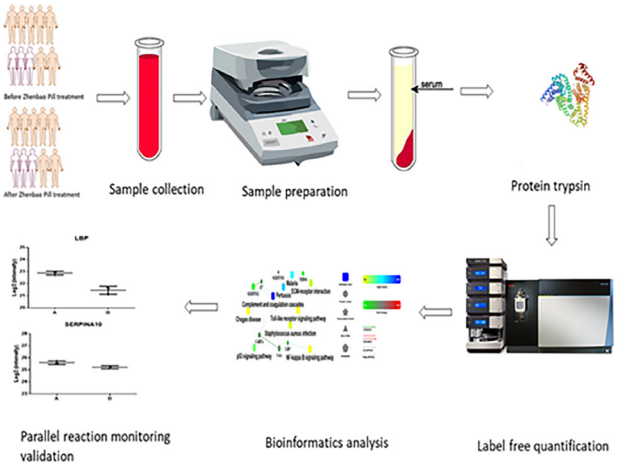

Fig. 1. Flow chart of the experimental scheme. HSP patients were treated with Zhenbao pill, samples were collected and processed. A label-free quantification proteomics was performed and data was analyzed with bioinformatics tools. Proteins were validated by PRM.

Serum treatment and protein digestion

The serum samples were thawed and depleted of albumin, IgG, and haptoglobin by using the albumin/IgG SpinTrap columns (GE Healthcare) according to the manufacturer's protocol. Total protein concentration was determined using the Bradford protein assay (Quick Start Bradford protein assay kit; Bio-Rad, USA), and 15 µg of protein from each sample was loaded and separated using 12% SDS-PAGE gels, which were subsequently stained with the Coomassie blue reagent (Bio-Rad). Gel bands were then cut into 1–2-mm^3 cubes, washed twice with destain buffer [500 µL, 50 mM NH_4HCO_3 in acetonitrile (ACN,1:1), v/v] for 20 min, dehydrated with ACN (500 µL), and dried in a SpeedVac (ThermoFisher). The proteins were reduced in 10 mM dithiothreitol (50 µL) at 56 °C for 30 min. Alkylation was completed using 55 mM iodoacetamide (50 µL) for 30 min in the dark at room temperature. Gel bands were shrunk with ACN (500 µL) for 15 min. The gel bands were completely dried before trypsin digestion and rehydrated using 0.01 µg/µL trypsin (15–20 µL). The proteins were then incubated at 37 °C overnight. The gel fragments were heated in a 40 °C water bath for 60 min and sonicated for 3 min every 30 min. Subsequently, the supernatant was transferred to clean tubes, and the gel fragments were extracted with 200 µL 50% ACN/2.5% trifluoroacetic acid (v/v) at 30 °C for 60 min and sonicated for 3 min every 30 min. The peptides were dried in a SpeedVac and re-solubilized in 0.1% formic acid (FA).

LC-MS/MS analysis

The peptides were separated using a nano-liquid chromatography system (EASY-nLC 1200, Thermo Fisher Scientific) in conjunction with an Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific). Trapped samples were separated on an Acclaim PepMap C18 trap column (100 mm×2 cm, 5 mm) after separation on an Acclaim PepMap C18 separation column (100 mm×50 cm, 2 mm). The samples were pumped through the columns at a flow rate of 600 nL/min. Mobile phase A included 1.9 % ACN and 0.1 % FA in H_2O(v/v), whereas mobile phase B contained 1.9 % H_2O and 0.1 % FA in ACN (v/v). The gradient began at 6% B, increased to 9% B in 8 min, and then to 9–14 % buffer B for 8–24 min, 14–30 % buffer B for 24–60 min, 30–40 % buffer B for 60–75 min, 40–95 % buffer B for 75–78 min, and 95 % buffer B for 78–85 min, before returning to 6% B within 1 min and equilibrating for 4 min.

MS spectra were collected using the following parameters: automated gain control (AGC) target, 1e6; injection duration, 50 ms; mass range, 400–1500 mass to charge ratio (m/z); and resolution, 35,000. The MS/MS scans were performed on the top ten precursor ions using a normalized collision energy of 30 % and a dynamic exclusion time of 30 s. MS spectra were obtained in the positive mode with a resolution of 17,500, an AGC target of 2e5, and an injection period of 50 ms.

Protein identification and LFQ

MaxQuant version 1.5.2.8 was applied to evaluate the raw data, which were compared to the Uniprot Human reference database (20,377 protein entries, version of June 2020). The search parameters included a 20-ppm mass tolerance for precursors, a 20-ppm mass tolerance for fragments, trypsin cleavage, and up to two miscleavages. The minimum peptide length was set to seven amino acid residues, and carbamidomethylation of cysteine residues was treated as a fixed modification, whereas oxidation (M) and acetylation (protein N-terminal) were treated as variable modifications. At both the peptide and protein levels, the incidence of false discovery was less than 1%. LFQ and intensity-based absolute protein quantification (iBAQ) were utilized. The two groups were compared using a log2 transformation of the iBAQ results and a two-sample *t*-test. Differentially expressed proteins (DEPs)

must fulfill the following criteria: fold change more than 1.5 or equal to 0.67 and a *p* value less than 0.05.

Bioinformatics analysis of DEPs

Analysis of DEPs was carried out by using OmicsBean (http://www.omicsbean.cn/) (Iqbal *et al*., 2018, 2019), a multi-omics data analysis tool that includes Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, Gene Ontology (GO) enrichment, and protein–protein interaction (PPI) network analyses.

Parallel reaction monitoring (PRM) validation

Only DEPs identified with a high confidence peptide sequence were selected for parallel reaction monitoring (PRM) validation based on proteomics data. Three biological replicates were included, each of which was performed with 1-µg samples. First, trypsin-digested peptides were dissolved in buffer A (1.9 % ACN and 0.1 % FA in H_2O,v/v) and eluted using an Acclaim PepMap C18 (75 µm × 25 cm) with gradient buffer B (1.9 % H_2O and 0.1 % FA in CAN, v/v; 6% to 9% B in 8 min, 9% to 14% B over 16 min, 14% to 30% B for 36 min, 30% to 40% B for 15 min, 40% to 95% B for 5 min, holding at 95% B for 7 min, decreased to 6% B in 1 min, and kept at 5% for the last 4 min) at a flow rate of 300 nl/min. The eluted peptides were analyzed with a nano spray ionization source and Q-Exactive HFx (Thermo Fisher Scientific). MS1 spectra were measured in the orbitrap at 35,000 resolutions, with a mass range from 400 to 1500 m/z, maximum injection time of 30 ms, and dynamic exclusion of 45 s. The data-dependent mode cycle was set to trigger MS/MS on up to the top 20 most abundant precursors per cycle at an MS^2 resolution of 17,500, AGC target of 2e5, maximum injection time of 50 ms, isolation window of 1.6 m/z, and HCD collision energy of 27. Precursor ions with charge states z = 2–6 was selected for MS/MS collision-induced dissociation fragmentation. The PRM data were processed using the Skyline software (version 20.2.0.286, MacCoss Lab, University of Washington, United States) (Adams *et al*., 2020). The results for each peptide were quantified according to the MS/MS ion peak area from its corresponding transitions, and statistical significance was set at $p < 0.05$ using the Student's *t*-test.

RESULTS

LFQ-based proteomics approach produced interesting results. A total of 389 DEPs were identified, of which 14 proteins were significantly differentially expressed (*p* value < 0.05; Fig. 2B). Four proteins immunoglobulin lambda variable 3-27, immunoglobulin kappa variable 3-11, neural cell adhesion molecule L1-like protein, immunoglobulin heavy variable 3-33 were upregulated, whereas 10 proteins protein Z-dependent protease inhibitor, C4b-binding protein alpha chain (C4BP), heparin cofactor 2, complement C4-A, vitamin K-dependent protein Z, coagulation factor VII, proteoglycan 4, lipopolysaccharide-binding protein (LBP), thrombospondin-4, and plasminogen activator inhibitor 1 were downregulated (Table II).

Table II. List of significantly differentially proteins.

No	UniprotID	Gene name	Protein name	B/A_pvalue	B/A_ratio	Status
1	P01718	*IGLV3-27*	Immunoglobulin lambda variable 3-27	0.026815693	70.91378837	Up-regulated
2	P04433	*IGKV3-11*	Immunoglobulin kappa variable 3-11	0.046764105	1.792455007	Up-regulated
3	O00533	*CHL1*	Neural cell adhesion molecule L1-like protein	0.04464601	1.626221087	Up-regulated
4	P01772	*IGHV3-33*	Immunoglobulin heavy variable 3-33	0.021642784	1.611622973	Up-regulated
5	Q9UK55	*SERPINA10*	Protein Z-dependent protease inhibitor	0.024283293	0.662118878	Down-regulated
6	P04003	*C4BPA*	C4b-binding protein alpha chain	0.037989633	0.622913933	Down-regulated
7	P05546	*SERPIND1*	Heparin cofactor 2	0.020392997	0.585974119	Down-regulated
8	P0C0L4	*C4A*	Complement C4-A	0.023744338	0.583900664	Down-regulated
9	P22891	*PROZ*	Vitamin K-dependent protein Z	0.004611638	0.504156321	Down-regulated
10	P08709	*F7*	Coagulation factor VII	0.003094434	0.496020472	Down-regulated
11	Q92954	*PRG4*	Proteoglycan 4	0.022630567	0.412207053	Down-regulated
12	P18428	*LBP*	Lipopolysaccharide-binding protein (LBP)	0.035372975	0.286501172	Down-regulated
13	P35443	*THBS4*	Thrombospondin-4	0.008671686	0.075547246	Down-regulated
14	P05121	*SERPINE1*	Plasminogen activator inhibitor 1	0.017846436	0.059730775	Down-regulated

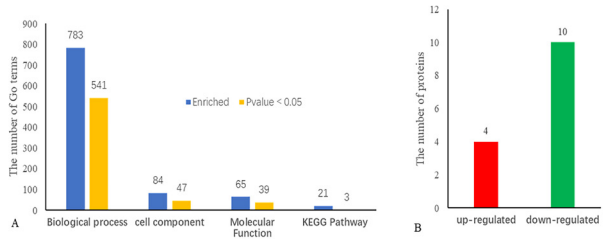

Fig. 2. (A) Summary of the total number of GO hits. (B) Total number of upregulated and downregulated proteins using LFQ analysis.

Bioinformatics analysis was performed by OmicsBean bioinformatics tool to retrieve complete biological information of these DEPs. A summary of the total GO hits is shown in Figure 2A, which illustrates the total quantitative biological processes (BP), cellular components (CC), molecular functions (MF), and KEGG pathways. Fifteen enriched BP are shown in Figure 3, illustrating the involvement of these DEPs in immune-related dysfunctions after Zhenbao pill treatment. These processes include response to external stimulus, response to stress, positive regulation of the immune system process, leukocyte migration, response to wounding, regulation of response to external stimulus, regulation of response to wounding, protein maturation, vesicle-mediated transport, regulation of protein metabolic process, import into cell, protein processing, regulation of protein maturation, regulation of inflammatory response and regulation of protein processing. The involvement of these DEPs in the CC of the Zhenbao pill-treated cells is shown in Figure 4A. Most of the enriched proteins were found to be involved in extracellular components, whereas few were involved in immunologic responses. KEGG analysis (Fig. 4B) revealed involvement of the complement and coagulation cascade, which directly involves immunity-related functions. Figure 5 shows the PPI network of DEPs. Several biological pathways related to these DEPs interact with each other. These include the complement and coagulation cascades, NF-kappa B signaling pathway, p53 signaling pathway, toll-like receptor signaling pathway, and ECM signaling pathway.

PRM validation

The expression of 14 DEPs was underwent PRM analysis to further validate the mass spectrometric results. Owing to the requirements of protein characteristics and abundance, we obtained abundant values of six proteins using quantitative data of target peptide fragments, including C4BPA, SERPIND1, F7, C4A, LBP, and SERPINA10. Among them, SERPINA10 and LBP successfully validated the proteomics results. As shown in Figure 6, these proteins showed exactly the same trend as when quantified by using the LFQ proteomics method, although the fold change values varied between the two techniques. Significant differences were observed between the groups (Supplementary Tables S1-S3).

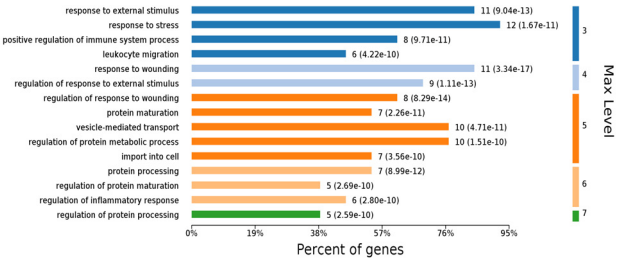

Fig. 3. Biological processes involving the differentially expressed proteins. Each bar includes the number of genes and p-value calculated with Fish exact test with Hypergeometric algorithm. Max level means maximal annotated level of this term in the GO graph.

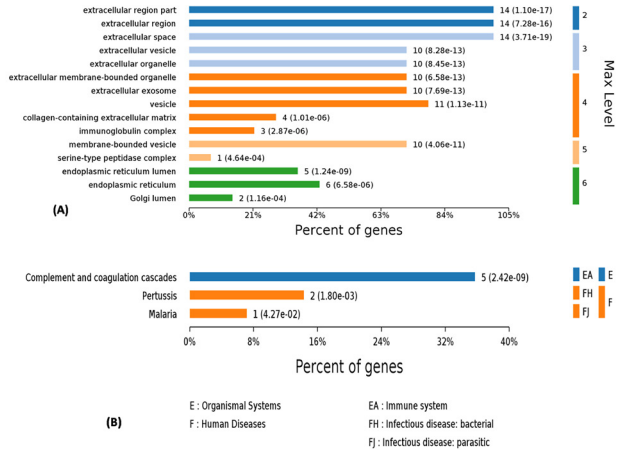

Fig. 4. (A) Cellular components, (B) KEGG pathways involving the differentially expressed proteins.

DISCUSSION

This study investigated the impact of the Zhenbao pill on protein expression in the serum of patients with HSP. Although the mechanism of HSP remains unclear, various hypotheses have been proposed to highlight the pathophysiology of HSP. There are many different types of immunoglobulin A nephritis (IgAN), immunoglobulin A vasculitis (IgAV), and immunoglobulin. Vasculitis nephropathy (IgAVN) may develop in people. It is possible that all forms contain the same entities. IgAV, IgAVN, and IgAN may have different origins, depending on the type of tissue in which they are detected. Immune complexes in IgAN may be formed due to increased galactose-deficient

IgA1 levels (Gd-IgA1), which can be explained by a combination of hereditary and environmental variables. IgA1 is responsible for the IgAN recognition of the endothelial cell antigen (Heineke *et al*., 2017; Knoppova *et al*., 2016; Novak *et al*., 2015).

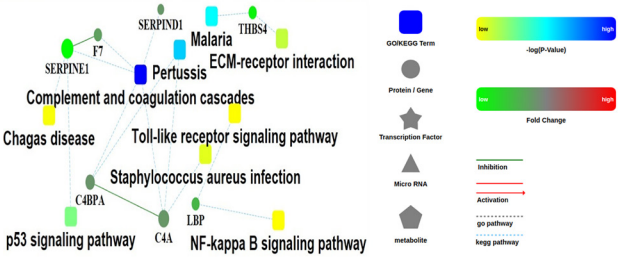

Fig. 5. Protein protein interactions network of the differentially expressed proteins.

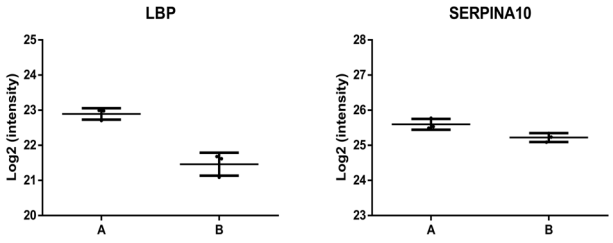

Fig. 6. Validation of differentially expressed proteins (DEPs) using parallel reaction monitoring analysis. Two candidate DEPs were used to verify the LFQ proteomics results.

Gd-IgA1 can bind to immune complexes, such as CD89 and CD71 and to complement components that could be receptors of IgA. Human mesangium cells are activated through the deposition of Gd-IgA1-containing immune complexes, resulting in abnormal renal function (Heineke *et al*., 2017; Knoppova *et al*., 2016; Novak *et al*., 2015). IgAV and IgAVN seem to have overlapping pathological mechanisms. The interplay between IgA and FcRI receptors may activate and attract neutrophils to areas of inflammation and is subject to changes due to environmental factors or through specific molecular pathways (Yeo *et al*., 2018). This leads to an increase in the levels of IgA1 AECA in blood; IgA1 AECA then bind to tiny vessels. The interaction of AECA with endothelial cells results in the production of interleukin-8, a neutrophil chemoattractant (Lau *et al*., 2010; Yeo *et al*., 2018).

The interaction between IgA1 and FcRI stimulates neutrophil recruitment. Processes, such as CDC, ADCC, NETosis, and ROS generation, harm vascular endothelial cells. LTB4 is generated by IgA-activated neutrophils, which activates and attracts additional neutrophils. Furthermore, neutrophils release TNF, which stimulates endothelial cells and reveals antigens that are normally buried. After identification by AECA antibodies, these antigens may eventually bind to the endothelium antigens. Neutrophil activation eventually results in inflammation and vascular hemorrhage, as seen in IgAV and IgAVN (Heineke *et al*., 2017; Yang *et al*., 2002, 2004, 2006, 2012).

HSP is an autoimmune condition triggered by pathogens, environmental factors, or hereditary factors (Yeo *et al*., 2018). Specific immune system abnormalities are responsible for HSP pathogenesis. As seen in Figure 2A, the Zhenbao pill dysregulated proteins that further influenced immune-related biological processes, demonstrating that the Zhenbao pill may have a therapeutic effect on HSP. Zhenbao pill is made up of almost 29 Chinese herbal medicines, including cassia seed, pearl, amomum, saffron, licorice, and bezoar, which help in activating meridians, promoting blood circulation, and calming nerves. The Zhenbao pill can promote microcirculation, repair damaged neurons, and remove oxygen free radicals (Kubo *et al*., 2007; Liu *et al*., 2015; Wu *et al*., 2007).

LFQ proteomics analysis revealed the upregulation of 3 immunoglobulin proteins: Immunoglobulin kappa variable 3-11, immunoglobulin lambda variable 3-27 and immunoglobulin heavy variable 3-3, after treatment with the Zhenbao pill. The immune system releases immunoglobulins or Y-shaped proteins to identify and phagocytose foreign bodies, such as viruses, bacteria, and other pathogens. Such infection is responsible for the production of unique antigen molecules that are identified by antibodies (Aleyd *et al*., 2015; He, Yongxiong *et al*., 2018a, b; He *et al*., 2018; Liu *et al*., 2015; Nicholson, 2016; Wu *et al*., 2007). Paratopes are present in antibodies; each antigen has a specific epitope that mimics a lock and key mechanism, enabling the two structures to be linked with the accuracy of the pinpoint. Paratoperase is found at the tip of the antinodes (Y) (Sela-Culang *et al*., 2013). An antibody is either an infected cell or a microbe that is assaulted by other components in the immune system, or directly neutralized by the binding mechanism by blocking the virus part that is critical for invasion (Aleyd *et al*., 2015).

Antibodies are a component of the adaptive immune system, along with B and T cells. A B cell-attached form and a soluble form, both of which may be detected in extracellular fluids, such as blood plasma. Initially, all antibodies are in the form of B cell receptors (BCR), which are connected to the surface of a BCR. To produce soluble antibodies with a similar paratope to those secreted by the plasma cells, the antigens bind with the BCR to remain in the body for a long time and produce immunity against the

antigen (Janeway, 2001). Additionally, soluble antibodies can be released into the tissue fluid, blood stream, and other secretions. These fluids are historically referred to as humor and are responsible for exhibiting humoral immunity or antibody-mediated immunity (Litman *et al*., 1993). Upregulation of these immunoglobulins may have a protective role during HSP infections when treated with Zhenbao pill.

Neural cell adhesion molecule L1-like protein (CHL1) is a multidomain type 1 membrane glycoprotein, belonging to the immunoglobulin superfamily, whose expression is perturbed when HSP patients are treated with the Zhenbao pill. It performs similar physiological activities in neural system development as CHL1 (Borghesi and Milcarek, 2006). CHL1 is produced as two isoforms, and isoform two is distinguished by the presence of an extra mini-exon 8 (Karstens *et al*., 2020). These isoforms have no physiological significance. CHL1 is involved in nerve cell regeneration and the growth, proliferation, and migration of neurons and serves to preserve motoneurons (Holm *et al*., 1996; Pier, 2004) (Guseva *et al*., 2018; Holm *et al*., 1996; Karstens *et al*., 2020). The increased CHL1 levels in patients with HSP after treatment with the Zhenbao pill illustrates the recovery processes of vessels and skin tissues.

Hepatocyte-derived C4BP is another protein with differential expression that serves as an essential component of the classic complement pathway. Here, it serves as a cofactor for serine protease factor I (F) in the degradation of C4b (Guseva *et al*., 2018; Jakovcevski *et al*., 2009). It inhibits the formation of the C4b2a complex (C3 convertase) that plays an important role in the inflammatory process following the activation of the signaling pathway. In human plasma, there are many distinct C4BP owing to the combinations of its C4BPα chain (75 kDa) and C4BPβ chain (Jakovcevski *et al*., 2009). Six or seven chains (C4BP) and either one or no chains (C4BP) are present in C4BP, depending on the model (Jakovcevski *et al*., 2009). In C4b, the anticoagulant protein S (PS) is bound by C4BP, whereas C4BP binds to the same protein. In addition to be a cofactor of activated protein C (APC), PS is a vitamin K-dependent plasma glycoprotein (Mr 75 000) (APC). Blood coagulation factors, FVa and FVIIIa, are inactivated by APC (Dahlbäck, 1991; Rezende *et al*., 2004; Vehar and Davie, 1980). Therefore, PS may also directly block the prothrombinase complex by binding to FVa and FXa (Vehar and Davie, 1980; Walker, 1981).

Heparin cofactor II is another dysregulated protein found in patients with HSP treated with the Zhenbao pill that is involved in various immune functions. It is associated with leukocyte-mediated protein breakdown, which releases cytokines as the inflammatory response (Heeb *et al*., 1994). Although it is a thrombin inhibitor, the absence of HCII does not result in significantly higher levels of thrombosis (Heeb *et al*., 1993, 1994).

Levels of PAI-1 were downregulated in this study. This protein inhibits the activity of urokinase plasminogen activator (uPA) that plays a role in plasmin formation by cleaving palminogen (Mahmood *et al*., 2018). Plasmin accelerates the breakdown of the extracellular matrix directly or indirectly with the help of matrix metalloproteinases. In this scenario, PAI-1 inhibits uPA via active site binding, preventing plasmin formation. Additional inhibition is mediated by PAI-1 binding to the uPA/uPA receptor complex, resulting in the degradation of the latter (Heeb *et al*., 1994; McMahon and Kwaan, 2015). Thus, PAI inhibit the serine proteases tPA and uPA/urokinase and consequently fibrinolysis, the physiological process that degrades blood clots. PAI-1 inhibits the activity of matrix metalloproteinases, which play a crucial role in the invasion of malignant cells through the basal lamina. PAI-1 is mainly produced by the endothelium (cells lining blood vessels) but is also secreted by other tissues, such as adipose tissue (Qureshi *et al*., 2016).

CONCLUSION

The present study is an attempt to explore some sound therapeutic strategy against HSP. Therefore, Zhenbao pill has been used in HSP patients and their proteomic profile is checked using LFQ proteomics approach. Expression of various proteins of vital importance is found to be perturbed in HSP patients when treated with Zhenbao pill. For example, proteins involved in positive regulation of the immune system, leukocyte migration, response to wounding, vesicle mediated transport, regulation of protein metabolic process, protein activation cascade, protein processing, regulation of protein maturation, regulation of inflammatory response, acute inflammatory response, regulation of protein processing, and regulation of complement activation are immune related cascades affected when treated with Zhenbao pill. In summary, Zhenbao pill may prove to be effective in certain immune-related functions and may be beneficial as a new therapeutic agent against HSP in future.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20220623100642

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Adams, K.J., Pratt, B., Bose, N., Dubois, L.G., St John-Williams, L., Perrott, K.M., Ky, K., Kapahi, P., Sharma, V., MacCoss, M.J., Moseley, M.A., Colton, C.A., MacLean, B.X., Schilling, B., Thompson, J.W., and Alzheimer's Disease Metabolomics, C., 2020. Skyline for small molecules: A unifying software package for quantitative metabolomics. *J. Proteome Res*., **19**: 1447-1458. https://doi.org/10.1021/acs.jproteome.9b00640

Al-Shweiki, M.H.D.R., Mönchgesang, S., Majovsky, P., Thieme, D., Trutschel, D., and Hoehenwarter, W., 2017. Assessment of label-free quantification in discovery proteomics and impact of technological factors and natural variability of protein abundance. *J. Proteome Res*., **16**: 1410-1424. https://doi.org/10.1021/acs.jproteome.6b00645

Aleyd, E., Heineke, M.H., and van Egmond, M., 2015. The era of the immunoglobulin A Fc receptor FcαRI; its function and potential as target in disease. *Immunol. Rev*., **268**: 123-138. https://doi.org/10.1111/imr.12337

Bluman, J., and Goldman, R.D., 2014. Henoch-Schönlein purpura in children: Limited benefit of corticosteroids. *Can. Fam. Phys*., **60**: 1007-1010.

Borghesi, L., and Milcarek, C., 2006. From B cell to plasma cell: Regulation of V(D)J recombination and antibody secretion. *Immunol. Res*., **36**: 27-32. https://doi.org/10.1385/IR:36:1:27

Dahlbäck, B., 1991. Protein S and C4b-binding protein: Components involved in the regulation of the protein C anticoagulant system. *Thromb. Haemost*., **66**: 49-61. https://doi.org/10.1055/s-0038-1646373

De Almeida, J.L., Campos, L.M., Paim, L.B., Leone, C., Koch, V.H., and Silva, C.A., 2007. Renal involvement in Henoch-Schönlein purpura: A multivariate analysis of initial prognostic factors. *J. Pediatr*., **83**: 259-266. https://doi.org/10.2223/JPED.1638

Di Pietro, G.M., Castellazzi, M.L., Mastrangelo, A., Montini, G., Marchisio, P., and Tagliabue, C., 2019. Henoch-Schönlein purpura in children: Not only kidney but also lung. *Pediatr. Rheumatol. Online J*., **17**: 75. https://doi.org/10.1186/s12969-019-0381-y

Duvuru, G., and Stone, J.H., 2013. Chapter 39. Henoch-schönlein purpura. In: *Current diagnosis and amp; treatment: rheumatology, 3e* (eds. J.B. Imboden, D.B. Hellmann and J.H. Stone). The McGraw-Hill Companies, New York, NY.

Gardner-Medwin, J.M., Dolezalova, P., Cummins, C., and Southwood, T.R., 2002. Incidence of Henoch-Schönlein purpura, Kawasaki disease, and rare vasculitides in children of different ethnic origins. *Lancet*, **360**: 1197-1202. https://doi.org/10.1016/S0140-6736(02)11279-7

Guseva, D., Jakovcevski, I., Irintchev, A., Leshchyns'ka, I., Sytnyk, V., Ponimaskin, E., and Schachner, M., 2018. Cell adhesion molecule close homolog of L1 (CHL1) guides the regrowth of regenerating motor axons and regulates synaptic coverage of motor neurons. *Front. Mol. Neurosci*., **11**: 174. https://doi.org/10.3389/fnmol.2018.00174

He, Y., Li, M., Wujisiguleng, Lv, B., Huan, Y., Liu, B., Wang, D., Yu, H., Zhang, L., and Shi, Z., 2018b. Zhenbao Pill reduces Treg cell proportion in acute spinal cord injury rats by regulating TUG1/miR-214/HSP27 axis. *Biosci. Rep*., **38**: BSR20180895. https://doi.org/10.1042/BSR20180895

He, Y., Lv, B., Huan, Y., Liu, B., Li, Y., Jia, L., Qu, C., Wang, D., Yu, H., and Yuan, H., 2018. Zhenbao pill protects against acute spinal cord injury via miR-146a-5p regulating the expression of GPR17. *Biosci. Rep*., **38**: BSR20171132. https://doi.org/10.1042/BSR20171132

Heeb, M.J., Mesters, R.M., Tans, G., Rosing, J., and Griffin, J.H., 1993. Binding of protein S to factor Va associated with inhibition of prothrombinase that is independent of activated protein C. *J. biol. Chem*., **268**: 2872-2877. https://doi.org/10.1016/S0021-9258(18)53854-0

Heeb, M.J., Rosing, J., Bakker, H.M., Fernandez, J.A., Tans, G., and Griffin, J.H., 1994. Protein S binds to and inhibits factor Xa. *Proc. natl. Acad. Sci. U. S. A*., **91**: 2728-2732. https://doi.org/10.1073/pnas.91.7.2728

Heineke, M.H., Ballering, A.V., Jamin, A., Ben Mkaddem, S., Monteiro, R.C., and Van Egmond, M., 2017. New insights in the pathogenesis of immunoglobulin A vasculitis (*Henoch-Schönlein purpura*). *Autoimmun. Rev*., **16**: 1246-1253. https://doi.org/10.1016/j.autrev.2017.10.009

Holm, J., Hillenbrand, R., Steuber, V., Bartsch, U., Moos, M., Lübbert, H., Montag, D., and Schachner, M., 1996. Structural features of a close homologue of L1 (CHL1) in the mouse: A new member of

the L1 family of neural recognition molecules. *Eur. J. Neurosci*., **8**: 1613-1629. https://doi.org/10.1111/j.1460-9568.1996.tb01306.x

Iqbal, J., Zhang, K., Jin, N., Zhao, Y., Liu, Q., Ni, J., and Shen, L., 2018. Effect of sodium selenate on hippocampal proteome of 3×Tg-AD mice exploring the antioxidant dogma of selenium against alzheimer's disease. *ACS Chem. Neurosci*., **9**: 1637-1651. https://doi.org/10.1021/acschemneuro.8b00034

Iqbal, J., Zhang, K., Jin, N., Zhao, Y., Liu, X., Liu, Q., Ni, J., and Shen, L., 2019. Alzheimer's disease is responsible for progressive age-dependent differential expression of various protein cascades in retina of mice. *ACS Chem. Neurosci*., **10**: 2418-2433. https://doi.org/10.1021/acschemneuro.8b00710

Jakovcevski, I., Siering, J., Hargus, G., Karl, N., Hoelters, L., Djogo, N., Yin, S., Zecevic, N., Schachner, M., and Irintchev, A., 2009. Close homologue of adhesion molecule L1 promotes survival of Purkinje and granule cells and granule cell migration during murine cerebellar development. *J. comp. Neurol*., **513**: 496-510. https://doi.org/10.1002/cne.21981

Janeway, C., 2001. *Immunobiology 5: The immune system in health and disease*. Garland Publication, New York.

Jia, Y., Chen, X., Chen, Y., Li, H., Ma, X., Xing, W., and Zhao, K., 2021. Zhenbao pill attenuates hydrogen peroxide-induced apoptosis by inhibiting autophagy in human umbilical vein endothelial cells. *J. Ethnopharmacol*., **274**: 114020. https://doi.org/10.1016/j.jep.2021.114020

Karstens, K.F., Bellon, E., Polonski, A., Wolters-Eisfeld, G., Melling, N., Reeh, M., Izbicki, J.R., and Tachezy, M., 2020. Expression and serum levels of the neural cell adhesion molecule L1-like protein (CHL1) in gastrointestinal stroma tumors (GIST) and its prognostic power. *Oncotarget*, **11**: 1131-1140. https://doi.org/10.18632/oncotarget.27525

Knoppova, B., Reily, C., Maillard, N., Rizk, D.V., Moldoveanu, Z., Mestecky, J., Raska, M., Renfrow, M.B., Julian, B.A., and Novak, J., 2016. The origin and activities of IgA1-containing immune complexes in IgA nephropathy. *Front. Immunol*., **7**: 117. https://doi.org/10.3389/fimmu.2016.00117

Kubo, T., Hata, K., Yamaguchi, A., and Yamashita, T., 2007. Rho-ROCK inhibitors as emerging strategies to promote nerve regeneration. *Curr. Pharm. Des*., **13**: 2493-2499. https://doi.org/10.2174/138161207781368657

Lau, K.K., Suzuki, H., Novak, J., and Wyatt, R.J., 2010. Pathogenesis of Henoch-Schönlein purpura nephritis. *Pediatr. Nephrol*., **25**: 19-26. https://doi.org/10.1007/s00467-009-1230-x

Leung, A., Barankin, B., and Leong, K., 2020. Henoch-schönlein purpura in children: An updated review. *Curr. Pediatr. Rev*., **16**: 265-276. https://doi.org/10.2174/18756336MTA2lNDYc2

Litman, G.W., Rast, J.P., Shamblott, M.J., Haire, R.N., Hulst, M., Roess, W., Litman, R.T., Hinds-Frey, K.R., Zilch, A., and Amemiya, C.T., 1993. Phylogenetic diversification of immunoglobulin genes and the antibody repertoire. *Mol. Biol. Evol*., **10**: 60-72.

Liu, R.Y., Wu, W.J., Tan, R., Xie, B., Zhong, Z.D., He, J.P., Chen, Y., and Kang, X.L., 2015. Intervention effect of Tibetan patent medicine Ruyi Zhenbao pills in acute ischemic stroke in rats. *China J. Chinese Materia Med*., **40**: 556-559.

Luo, Y.M., Ren, X.Q., Yang, X.Q., Song, H.R., Li, R., Gao, M.H., Li, Y.R., Zhou, R.R., Ma, L., Zhang, S.J., Dong, R.J., Ge, D.Y., Wang, C.G., Ren, Q.J., and Tao, X.H., 2020. Tibetan medicine Ru-yi-Zhen-bao Pills exhibits anti-migraine effect through mediating PAG anti-nociceptive channel. *J. Ethnopharmacol*., **249**: 112437. https://doi.org/10.1016/j.jep.2019.112437

Mahmood, N., Mihalcioiu, C., and Rabbani, S.A., 2018. Multifaceted role of the urokinase-type plasminogen activator (uPA) and its receptor (uPAR): Diagnostic, prognostic, and therapeutic applications. *Front. Oncol*., **8**: 24. https://doi.org/10.3389/fonc.2018.00024

McMahon, B.J., and Kwaan, H.C., 2015. Components of the plasminogen-plasmin system as biologic markers for cancer. *Adv. exp. Med. Biol*., **867**: 145-156. https://doi.org/10.1007/978-94-017-7215-0_10

Namgoong, M., 2020. Management of IgA vasculitis nephritis (Henoch-Schonlein purpura nephritis) in Children. *Child. Kidney Dis*., **24**: 1-13. https://doi.org/10.3339/jkspn.2020.24.1.1

Nicholson, L.B., 2016. The immune system. *Essays Biochem*., **60**: 275-301. https://doi.org/10.1042/EBC20160017

Novak, J., Rizk, D., Takahashi, K., Zhang, X., Bian, Q., Ueda, H., Ueda, Y., Reily, C., Lai, L.Y., Hao, C., Novak, L., Huang, Z.Q., Renfrow, M.B., Suzuki, H., and Julian, B.A., 2015. New insights into the pathogenesis of IgA nephropathy. *Kidney Dis.* (Basel), **1**: 8-18. https://doi.org/10.1159/000382134

Oni, L., and Sampath, S., 2019. Childhood IgA

vasculitis (Henoch Schonlein Purpura)-advances and knowledge gaps. *Front Pediatr*., **7**: 257. https://doi.org/10.3389/fped.2019.00257

Ozen, S., Pistorio, A., Iusan, S.M., Bakkaloglu, A., Herlin, T., Brik, R., Buoncompagni, A., Lazar, C., Bilge, I., Uziel, Y., Rigante, D., Cantarini, L., Hilario, M.O., Silva, C.A., Alegria, M., Norambuena, X., Belot, A., Berkun, Y., Estrella, A.I., Olivieri, A.N., Alpigiani, M.G., Rumba, I., Sztajnbok, F., Tambic-Bukovac, L., Breda, L., Al-Mayouf, S., Mihaylova, D., Chasnyk, V., Sengler, C., Klein-Gitelman, M., Djeddi, D., Nuno, L., Pruunsild, C., Brunner, J., Kondi, A., Pagava, K., Pederzoli, S., Martini, A., and Ruperto, N., 2010. EULAR/PRINTO/PRES criteria for Henoch-Schönlein purpura, childhood polyarteritis nodosa, childhood Wegener granulomatosis and childhood Takayasu arteritis: Ankara 2008. Part II: Final classification criteria. *Annls Rheum. Dis*., **69**: 798-806. https://doi.org/10.1136/ard.2009.116657

Pier, G., 2004. *Immunology, infection, and immunity*. ASM Press, Washington, D.C. https://doi.org/10.1128/9781555816148

Qureshi, T., Goswami, S., McClintock, C.S., Ramsey, M.T., and Peterson, C.B., 2016. Distinct encounter complexes of PAI-1 with plasminogen activators and vitronectin revealed by changes in the conformation and dynamics of the reactive center loop. *Protein Sci*., **25**: 499-510. https://doi.org/10.1002/pro.2841

Raposo, C., Graubardt, N., Cohen, M., Eitan, C., London, A., Berkutzki, T., and Schwartz, M., 2014. CNS repair requires both effector and regulatory T cells with distinct temporal and spatial profiles. *J. Neurosci*., **34**: 10141-10155. https://doi.org/10.1523/JNEUROSCI.0076-14.2014

Rezende, S.M., Simmonds, R.E., and Lane, D.A., 2004. Coagulation, inflammation, and apoptosis: Different roles for protein S and the protein S-C4b binding protein complex. *Blood*, **103**: 1192-1201. https://doi.org/10.1182/blood-2003-05-1551

Rostoker, G., 2001. Schönlein-henoch purpura in children and adults: Diagnosis, pathophysiology and management. *BioDrugs*, **15**: 99-138. https://doi.org/10.2165/00063030-200115020-00004

Sela-Culang, I., Kunik, V., and Ofran, Y., 2013. The structural basis of antibody-antigen recognition. *Front. Immunol*., **4**: 302. https://doi.org/10.3389/fimmu.2013.00302

Trnka, P., 2013. Henoch-Schönlein purpura in children. *J. Paediatr. Child Hlth*., **49**: 995-1003. https://doi.org/10.1111/jpc.12403

Vehar, G.A., and Davie, E.W., 1980. Preparation and properties of bovine factor VIII (antihemophilic factor). *Biochemistry*, **19**: 401-410. https://doi.org/10.1021/bi00544a001

Walker, F.J., 1981. Regulation of activated protein C by protein S. The role of phospholipid in factor Va inactivation. *J. biol. Chem*., **256**: 11128-11131. https://doi.org/10.1016/S0021-9258(19)68566-2

Wu, B., Liu, M., Liu, H., Li, W., Tan, S., Zhang, S., and Fang, Y., 2007. Meta-analysis of traditional chinese patent medicine for ischemic stroke. *Stroke*, **38**: 1973-1979. https://doi.org/10.1161/STROKEAHA.106.473165

Yang, Y.H., Chang, C.J., Chuang, Y.H., Hsu, H.Y., Yu, H.H., Lee, J.H., Wang, L.C., Lin, Y.T., and Chiang, B.L., 2012. Identification and characterization of IgA antibodies against β2-glycoprotein I in childhood Henoch-Schönlein purpura. *Br. J. Dermatol*., **167**: 874-881. https://doi.org/10.1111/j.1365-2133.2012.11068.x

Yang, Y.H., Huang, Y.H., Lin, Y.L., Wang, L.C., Chuang, Y.H., Yu, H.H., Lin, Y.T., and Chiang, B.L., 2006. Circulating IgA from acute stage of childhood Henoch-Schönlein purpura can enhance endothelial interleukin (IL)-8 production through MEK/ERK signalling pathway. *Clin. exp. Immunol*., **144**: 247-253. https://doi.org/10.1111/j.1365-2249.2006.03076.x

Yang, Y.H., Lai, H.J., Huang, C.M., Wang, L.C., Lin, Y.T., and Chiang, B.L., 2004. Sera from children with active Henoch-Schönlein purpura can enhance the production of interleukin 8 by human umbilical venous endothelial cells. *Annls Rheum. Dis*., **63**: 1511-1513. https://doi.org/10.1136/ard.2003.016196

Yang, Y.H., Wang, S.J., Chuang, Y.H., Lin, Y.T., and Chiang, B.L., 2002. The level of IgA antibodies to human umbilical vein endothelial cells can be enhanced by TNF-alpha treatment in children with Henoch-Schönlein purpura. *Clin. exp. Immunol*., **130**: 352-357. https://doi.org/10.1046/j.1365-2249.2002.01964.x

Yeo, S.C., Cheung, C.K., and Barratt, J., 2018. New insights into the pathogenesis of IgA nephropathy. *Pediatr. Nephrol*., **33**: 763-777. https://doi.org/10.1007/s00467-017-3699-z