Original Article

Proteomic investigation and biomarker identification of lung and spleen deficiency syndrome in HIV/AIDS immunological nonresponders

Yi-Ting Wang[1], Shao-Xiu Ji[1], Xin-Yi Xia[2], Ting-Jun Wan[1], Jia-Xi Zou[1], Bai-Xue Li[1], Qian-Nan Hu[1], Xin Chen[1], Jie Mu[1], Quan-Sheng Feng[1], Li Wen[1]

[1]College of Basic Medicine, Chengdu University of Traditional Chinese Medicine, Chengdu, China; [2]College of Basic Medicine, Shandong University of Traditional Chinese Medicine, Jinan, China

Contributions: (I) Conception and design: YT Wang, QS Feng; (II) Administrative support: QS Feng; (III) Provision of study materials or patients: YT Wang, SX Ji, XY Xia, TJ Wan; (IV) Collection and assembly of data: YT Wang, JX Zou, BX Li, QN Hu, X Chen; (V) Data analysis and interpretation: YT Wang, J Mu, L Wen; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Li Wen; Quan-Sheng Feng. Chengdu University of Traditional Chinese Medicine, Chengdu 611137, China.
Email: wenliccn@163.com; fengqs118@163.com.

Background: Human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS) immunological nonresponders (HIV/AIDS-INRs) whose $CD4^+$ cell counts do not rebound after highly active antiretroviral therapy (HAART) treatment usually experience severely impaired immune function and high mortality. Traditional Chinese medicine (TCM) has many advantages in the field of AIDS, especially its promotion of patients' immune reconstitution. Accurate differentiation of TCM syndromes is a prerequisite for guiding an effective TCM prescription. However, the objective and biological evidence for identification of the TCM syndromes in HIV/AIDS-INRs remains lacking. Lung and spleen deficiency (LSD) syndrome, a typical HIV/AIDS-INR syndrome, was examined on in this study.

Methods: We first performed a proteomic study of LSD syndrome in INRs (INRs-LSD) using tandem mass tag combined with liquid chromatography-tandem mass spectrometry (TMT-LC-MS/MS) and screened them against the healthy and undocumented identifiable groups. The TCM syndrome-specific proteins were subsequently validated based on bioinformatics analysis and enzyme-linked immunosorbent assay (ELISA).

Results: A total of 22 differentially expressed proteins (DEPs) were screened in INRs-LSD compared to the healthy group. Based on bioinformatic analysis, these DEPs were found to be mainly associated with the immunoglobin A (IgA)-generated intestinal immune network. In addition, we examined the TCM syndrome-specific proteins alpha-2-macroglobulin (A2M) and human selectin L (SELL) with ELISA and found that they were both upregulated, which was consistent with the proteomic screening results.

Conclusions: A2M and SELL were finally identified as potential biomarkers for INRs-LSD, providing a scientific and biological basis for identifying typical TCM syndromes in HIV/AIDS-INRs and an opportunity to build a more effective TCM treatment system for HIV/AIDS-INRs.

Keywords: Human immunodeficiency virus and acquired immune deficiency syndrome (HIV/AIDS); immunological nonresponders (INRs); biomarker; proteomics; lung and spleen deficiency syndrome

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Introduction

Human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS) together constitute a major global public health issue. Highly active antiretroviral therapy (HAART) is currently the most effective treatment for suppressing HIV replication and can reduce AIDS-related morbidity and mortality (1). However, some HIV/AIDS patients never experience a rebound in their $CD4^+$ T-cell counts despite the virus not being detected after HAART treatment. These patients are known as HIV/AIDS immune reconstitution-incompetent patients or HIV/AIDS-immunological nonresponders (HIV/AIDS-INRs) (2). Compared to individuals with complete immune reconstitutions, INRs are at a severely impaired stage of immune function and are more likely to develop cardiovascular disease, liver- and kidney-related diseases, and metabolic syndrome or malignancies, leading to a higher mortality rate (3-6). Moreover, a weakened immune system is susceptible to other viruses, and the superimposed infection of multiple viruses can further affect the immune system (7). The mechanisms underlying the development of HIV/AIDS-INRs are complex and influenced by many factors, such as age, gender, genetics, metabolic characteristics, drug effects, timing of HAART, baseline $CD4^+$ T-cell levels, viral load, and cytokines (8). Therefore, the clinical symptoms may differ among INRs, and specific therapeutic plans are needed for individual INRs.

Therefore, HAART currently is faced with several insoluble issues, including immune reconstitution and syndrome-specific treatment, but the unique advantages of traditional Chinese medicine (TCM) may compensate for these deficits in modern care. It has been proven that TCM can alleviate clinical symptoms, improve quality of life, and reduce HAART resistance and toxicity in the field of AIDS (9-11); more importantly, it has been shown capable of promoting the immune reconstruction of HIV/AIDS-INRs (12,13). Moreover, TCM can be tailored to treat the specific syndrome of HIV/AIDS-INRs. TCM syndromes, known as *Zheng* in Chinese, refers to a process of summarizing and distinguishing comprehensive signals and symptoms of individuals from a particular stage of disease (14). Thus, the acute differentiation of TCM syndromes is the key to guiding the effective prescription of TCM. However, the identification of TCM syndromes in HIV/AIDS-INRs is currently based on traditional inspection, listening and smelling, inquiring, and pulse taking (15). Due to the experience and subjectivity of herbalists, the criteria for diagnosis are often heterogenous. Hence, it is important to objectively define TCM syndrome in HIV/AIDS-INRs. Lung and spleen deficiency (LSD) syndrome is a typical TCM syndrome of HIV/AIDS-INRs, as identified by epidemiological questionnaires applied in our previous work (16), and we sought to turn our research focus to INRs-LSD in the present study. INRs-LSD usually present with symptoms of diarrhea, fever, herpes, mouth sores, skin ulcers, cough, fatigue, and loss of appetite, among others (see supplementary information: Appendix 1).

Proteomics has developed rapidly in recent years and is of great value in medical and therapeutic drug target research (17). Proteomics has been widely used in early diagnosis, prognosis, and monitoring of disease progression, and has made considerable progress in the study of molecular markers for various diseases (18-21), including cancer (22-24). By analyzing the expression of all proteins in an organism, proteomics holistically observes the dynamic changes of proteins and thus has parallels with the holistic concept of TCM. Proteomics has been applied to the investigation of a variety of TCM symptoms, revealing differences in protein expression, structure, function, and interaction between healthy individuals and patients (25). Some scholars have identified urinary

Highlight box

Key findings

- We identified the potential biological markers of typical traditional Chinese medicine (TCM) syndromes in HIV/AIDS immunological nonresponders (INRs).

What is known and what is new?

- Chinese medicine has the ability to promote immune reconstitution in patients in the field of AIDS. However, objective and biological evidence for identifying TCM evidence in HIV/AIDS INRs is still lacking.
- We identified 22 differentially expressed proteins (DEPs) between INRs with lung and spleen deficiency (LSD) syndrome (INRs-LSD) and a healthy control group Through bioinformatics analysis, it was found that these DEPs were mainly associated with the intestinal immune network for immunoglobin A production. Furthermore, we validated the TCM syndrome-specific proteins using enzyme-linked immunosorbent assay.

What is the implication, and what should change now?

- Modern histological techniques should be combined to explore and study the immunological basis of each evidence type and that between the evidence types.

protein biomarkers of abdominal allergic purpura using proteomics (26). In this study, tandem mass tag combined with liquid chromatography-tandem mass spectrometry (TMT-LC-MS/MS) was used to screen and identify the differentially expressed proteins (DEPs) in INRs-LSD. These DEPs were then analyzed with bioinformatics assays. Enzyme-linked immunosorbent assay (ELISA) was further conducted to validate the potential biomarkers in order to provide an objective basis for the clinical diagnosis of the syndrome. We present the following article in accordance with the MDAR reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-322/rc).

Methods

Study population

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the Regional Ethics Review Committee of Sichuan Traditional Chinese Medicine (ethical approval No. 2018KL-062; registration No. ChiCTR1800015290). Informed consent was obtained from all participants.

From September 2018 to April 2020, 29 HIV/AIDS-INRs, including those in the AIDS stage and asymptomatic stage, were enrolled from the Center for Disease Control of Butuo County and Zhaojue County People's Hospital in Liangshan Prefecture, Sichuan Province, China. These included 19 INRs-LSD (9 participants for TMT-LC-MS/MS proteomics and 10 participants for ELISA analyses), 10 nonsyndromic INRs (NS-INRs), and 20 healthy volunteers.

Diagnostic, inclusion, and exclusion criteria

Diagnostic, inclusion, and exclusion criteria for HIV/AIDS and HIV/AIDS-INR are detailed in the supplemental materials. The diagnostic criteria for the LSD syndrome group were as follows. The primary symptoms included (a) a lack of complexion and the presence of fatigue, low voice, and lethargy in speaking; (b) shortness of breath and asthma, cough with thin sputum, and prolonged cough; and (c) anorexia, bloating, and diarrhea. The secondary symptoms included (a) a pale tongue with white smooth coating and (b) a weak pulse. Patients were diagnosed with LSD syndrome if they met any of the following criteria: (I) with all the primary symptoms; (II) with the primary symptoms of b and c; or (III) with the primary symptoms of the b, c, and any of the secondary symptoms.

Sample collection

Whole blood specimens of peripheral blood (5 mL) were collected from the groups, left at room temperature for 1 h, and centrifuged at 3,500 rpm for 10 min. The supernatant was then aspirated in a lyophilized storage tube and then quickly transferred to a −80 ℃ refrigerator for storage for subsequent analysis.

Tandem mass tag combined with liquid chromatography-tandem mass spectrometry

TMT-labeled reagent was added to each sample peptide, and the reaction was incubated for 1 h at room temperature and then burst with hydroxylamine for 15 min. Subsequently, the TMT-labeled peptides were graded using high-pH reversed-phase high performance liquid chromatography (HPLC). The peptides were eluted in a gradient at a flow rate of 300 nL/min under appropriate conditions, and the effluent was collected. Finally, the samples were fully dissolved and uploaded onto a Q Exactive mass spectrometer (Thermo Fisher Scientific, Massachusetts, USA) to analyze the fractured samples using LC-MS/MS and the full scan range of mass spectra, with the mass to charge ratio (m/z) ranging from 350–1,600. The parent ions were fragmented using a high-energy collision-induced dissociation (HCD) method for secondary mass spectrometry sequence determination and were simultaneously quantified with the ratio of reporter ions. The raw files of the mass spectrometric assays were generated.

Bioinformatics analysis

The differential proteins were screened in the Gene Ontology (GO) database (http://geneontology.org/) and Universal Protein database (https://www.uniprot.org/). The following criteria were used to screen the significant DEPs: a 1% false discovery rate (FDR), fold proteins with fold change greater than or equal to 1.5 or fold change less than or equal to two-thirds, and a P value less than or equal to 0.05. GO annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed on the significant DEPs, the interaction relationships between DEPs were identified using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (http://string-db.org/), and the obtained data were imported into Cytoscape software to visualize the interaction network.

ELISA

To further validate the DEPs screened in the LSD syndrome group, at 3 replicates per sample, the serum protein expression levels were measured in INRs-LSD and 10 healthy controls using the human alpha-2-macroglobulin (A2M) ELISA kit (CSB-E08959h) and human selectin L (SELL) ELISA kit (CSB-E04653h) from Wuhan Huamei Bioengineering Co. (Wuhan, China).

Statistical analysis

SPSS 25.0 software (IBM Corp., New York, USA) was used for the statistical analysis of data, and the baseline information of enrolled patients were analyzed using 1-way analysis of variance (ANOVA) and the Mann-Whitney test, with the results of data analysis being expressed as the mean ± standard deviation (SD). $P<0.05$ was considered a statistically significant difference.

Results

Clinical data statistics

In this study, 19 patients in the LSD syndrome group, 10 patients in the nonsyndromic (NS) group, and 20 people in the healthy control group were included. There was no significant difference in age or sex between the 3 groups (Table S1). There was also no significant difference in HAART hours, viral load, immunological indexes, blood routine, or liver and kidney function indexes in the LSD syndrome group compared with the NS group, although the aspartate aminotransferase level differed (Table S1).

Proteomics analysis

We performed serum proteomics analysis with TMT-LC-MS/MS for both the LSD syndrome group and the NS group. The DEPs were screened at a 1% FDR. A total of 22 DEPs were screened in the LSD syndrome samples, 18 of which were upregulated and 4 downregulated (*Table 1*, Figure S1). A total of 25 DEPs were screened in the NS samples, 23 of which were upregulated and 2 downregulated (*Table 2*, Figure S1).

Annotation analysis of the DEPs

To further clarify the functions of DEPs, we performed bioinformatics analysis of 22 DEPs in the patients with LSD syndrome and 25 DEPs in the NS patients. GO analysis revealed that the DEPs in the LSD syndrome group were mainly localized in early endosome and chylomicron, representing biological processes mainly related to the negative regulation of lipase activity, and the identified molecular functions indicated that the DEPs were mainly involved in lipase inhibitor activity (*Figure 1A*). The DEPs in the NS patient group were mainly localized in the mitochondrial envelope, representing biological processes mainly related to cytokine production involved in immune response, and the identified molecular functions indicated that the DEPs were mainly involved in phospholipid transporter activity (*Figure 1B*). KEGG pathway analysis showed that the DEPs in the LSD syndrome groups were mainly enriched in the intestinal immune network for immunoglobin A (IgA) production (hsa04672) (*Figure 2A*). In contrast, the DEPs in the NS group were mainly enriched in African trypanosomiasis (hsa05143) (*Figure 2B*).

Analysis of protein-protein interaction networks

The interaction relationships between DEPs were identified in the STRING database, and the obtained data were imported into Cytoscape software to visualize the interaction network and construct a protein-protein interaction (PPI) network for these DEPs. We found that most of the DEPs had interactions with each other, and some of the DEPs could show some specific functions in the LSD syndrome group and the NS group (*Figure 3*). In the LSD syndrome group, apolipoprotein C3 (APOC3), apolipoprotein A2 (APOA2), A2M, SELL, and immunoglobulin lambda-like polypeptide 1 (IGLL1) were at key positions in the PPI. In the NS group, those in key PPI positions were von Willebrand factor (VWF), hyaluronan-binding protein 2 (HABP2), serpin family a member 7 (SERPINA7), and complement factor properdin (CFP). These DEPs were considered to be potential biomarkers for the LSD syndrome group and the NS group.

Validation of potential biomarkers with ELISA

The baseline indicators for the LSD syndrome group and the healthy control group are shown in Table S2. By analyzing the enrichment results of DEPs between the LSD syndrome group and the healthy control (HC) group, we found that these DEPs were mainly enriched in the immune system. To validate the TMT-LC-MS/MS results and further identify the biomarkers for INRs-LSD, we selected

Table 1 The DEPs between the LSD and HC group

Proteins	Description	Regulation	Score	Coverage	Peptides	FC	P
SHBG	Sex hormone-binding globulin isoform 1 precursor [*Homo sapiens*]	Up	39.635	18%	7	2.5	0.00028
PRG2	Bone marrow proteoglycan isoform 1 preproprotein [*Homo sapiens*]	Up	6.699	9%	2	1.917	0.00234
B2M	Beta-2-microglobulin isoform X1 [*Homo sapiens*]	Up	30.83	19%	3	1.897	0.00970
LOC102723407	Immunoglobulin heavy variable 4-38-2-like [*Homo sapiens*]	Up	29.334	14%	3	1.844	0.02229
LGALS3BP	Galectin-3-binding protein precursor [*Homo sapiens*]	Up	148.961	34%	17	1.732	0.01897
CETP	Cholesteryl ester transfer protein isoform 1 precursor [*Homo sapiens*]	Up	32.095	14%	8	1.723	0.00000
CHGA	Chromogranin-A isoform 1 preproprotein [*Homo sapiens*]	Up	6.282	4%	2	1.718	0.01067
PIGR	Polymeric immunoglobulin receptor precursor [*Homo sapiens*]	Up	41.029	15%	11	1.705	0.00486
LRG1	Leucine-rich alpha-2-glycoprotein precursor [*Homo sapiens*]	Up	90.859	41%	11	1.664	0.00123
CD163	Scavenger receptor cysteine-rich type 1 protein M130 isoform A precursor [*Homo sapiens*]	Up	27.698	8%	10	1.636	0.01080
IGLL5	Immunoglobulin lambda-like polypeptide 5 isoform 1 [*Homo sapiens*]	Up	146.862	42%	12	1.635	0.02923
FETUB	Fetuin-B isoform 1 precursor [*Homo sapiens*]	Up	53.836	32%	12	1.631	0.00046
SOD2	Superoxide dismutase [Mn], mitochondrial isoform A precursor [*Homo sapiens*]	Up	8.344	10%	2	1.609	0.01317
VCAM1	Vascular cell adhesion protein 1 isoform A precursor [*Homo sapiens*]	Up	50.798	17%	12	1.54	0.04096
PI16	Peptidase inhibitor 16 precursor [*Homo sapiens*]	Up	57.646	25%	10	1.529	0.00482
IGLL1	Immunoglobulin lambda-like polypeptide 1 isoform C [*Homo sapiens*]	Up	27.654	27%	4	1.522	0.00383
SELL	L-selectin precursor [*Homo sapiens*]	Up	48.782	17%	6	1.511	0.01578
A2M	Alpha-2-macroglobulin isoform X1 [*Homo sapiens*]	Up	1208.161	64%	82	1.504	0.02831
APOA2	Apolipoprotein A-II preproprotein [*Homo sapiens*]	Down	77.946	47%	7	0.607	0.00225
APOC3	Apolipoprotein C-III precursor [*Homo sapiens*]	Down	62.599	59%	7	0.547	0.00492
APOA1	Apolipoprotein A-I isoform 1 preproprotein [*Homo sapiens*]	Down	517.655	87%	45	0.509	0.00351
APOC1	Apolipoprotein C-I precursor [*Homo sapiens*]	Down	61.047	40%	7	0.415	0.00456

DEP, differentially expressed protein; LSD, lung and spleen deficiency; HC, health control; FC, fold change.

2 proteins (A2M and SELL) for validation, as they were the specific DEPs of LSD syndrome and also associated with the immune system. The results showed that both A2M and SELL concentrations were significantly higher in the LSD syndrome group than in the HC group (*Figure 4*), which was consistent with the results of TMT-LC-MS/MS.

Table 2 The DEPs between the NS and HC group

Proteins	Description	Regulation	Score	Coverage	Peptides	FC	P
PRDX2	Peroxiredoxin-2 [*Homo sapiens*]	Up	29.041	28%	6	2.197	0.00696
SHBG	Sex hormone-binding globulin isoform 1 precursor [*Homo sapiens*]	Up	39.635	18%	6	2.11	0.00541
CD163	Scavenger receptor cysteine-rich type 1 protein M130 isoform A precursor [*Homo sapiens*]	Up	27.698	8%	10	1.961	0.00096
PRG2	Bone marrow proteoglycan isoform 1 preproprotein [*Homo sapiens*]	Up	6.699	9%	2	1.845	0.01603
IGFBP4	Insulin-like growth factor-binding protein 4 precursor [*Homo sapiens*]	Up	9.296	14%	3	1.833	0.02083
CHGA	Chromogranin-A isoform 1 preproprotein [*Homo sapiens*]	Up	6.282	4%	2	1.825	0.00584
B2M	Beta-2-microglobulin isoform X1 [*Homo sapiens*]	Up	30.83	19%	3	1.825	0.00509
VCAM1	Vascular cell adhesion protein 1 isoform a precursor [*Homo sapiens*]	Up	50.798	17%	12	1.76	0.01566
VWF	von Willebrand factor preproprotein [*Homo sapiens*]	Up	406.585	26%	62	1.749	0.00982
IGLL5	Immunoglobulin lambda-like polypeptide 5 isoform 1 [*Homo sapiens*]	Up	146.862	42%	12	1.711	0.04282
LCP1	Plastin-2 isoform X1 [*Homo sapiens*]	Up	8.112	4%	3	1.696	0.00001
FETUB	Fetuin-B isoform 1 precursor [*Homo sapiens*]	Up	53.836	32%	12	1.667	0.00033
HEG1	Protein HEG homolog 1 isoform X1 [*Homo sapiens*]	Up	13.82	2%	3	1.659	0.00075
SOD2	Superoxide dismutase [Mn], mitochondrial isoform A precursor [*Homo sapiens*]	Up	8.344	10%	2	1.655	0.02576
PIGR	Polymeric immunoglobulin receptor precursor [*Homo sapiens*]	Up	41.029	15%	11	1.645	0.01376
HABP2	Hyaluronan-binding protein 2 isoform 1 preproprotein [*Homo sapiens*]	Up	85.367	26%	12	1.629	0.00013
LRG1	Leucine-rich alpha-2-glycoprotein precursor [*Homo sapiens*]	Up	90.859	41%	11	1.608	0.00037
SERPINA7	Thyroxine-binding globulin precursor [*Homo sapiens*]	Up	140.116	38%	16	1.582	0.00094
CP	Ceruloplasmin precursor [*Homo sapiens*]	Up	760.474	62%	60	1.577	0.00211
ALCAM	CD166 antigen isoform 1 precursor [*Homo sapiens*]	Up	11.659	8%	4	1.571	0.01176
CETP	Cholesteryl ester transfer protein isoform 1 precursor [*Homo sapiens*]	Up	32.095	14%	8	1.565	0.00000
LGALS3BP	Galectin-3-binding protein precursor [*Homo sapiens*]	Up	148.961	34%	17	1.546	0.03781
CFP	Properdin precursor [*Homo sapiens*]	Up	38.045	17%	7	1.546	0.02750
APOA1	Apolipoprotein A-I isoform 1 preproprotein [*Homo sapiens*]	Down	517.655	87%	45	0.595	0.02628
APOC1	Apolipoprotein C-I precursor [*Homo sapiens*]	Down	61.047	40%	7	0.415	0.00456

DEP, differentially expressed protein; NS, nonsyndromic; HC, health control; FC, fold change.

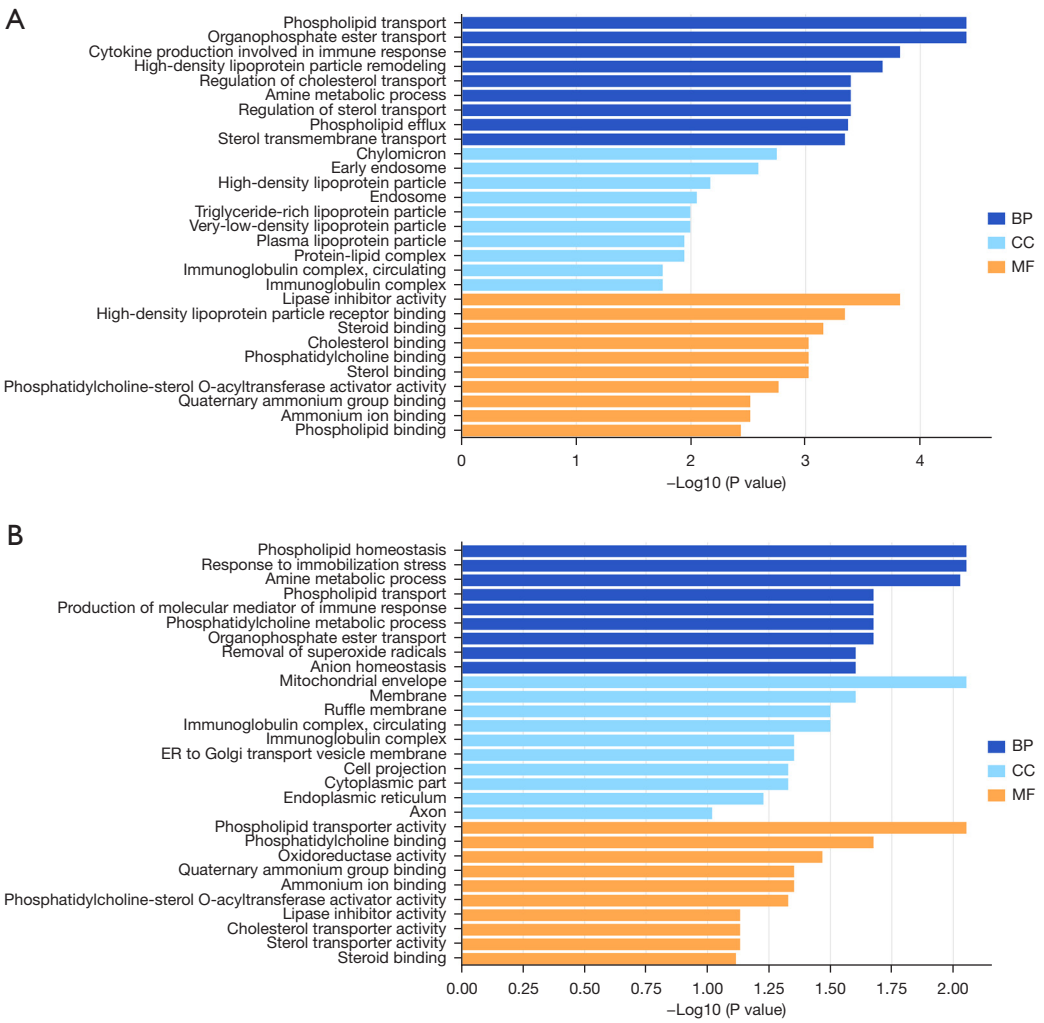

Figure 1 GO annotation of the LSD (A) and NS (B) groups. BP, biological process; CC, cell composition; MF, molecular function; GO, Gene Ontology; LSD, lung and spleen deficiency; NS, nonsyndromic.

Discussion

For those with AIDS, researchers have tried a variety of treatments, such as human umbilical cord mesenchymal stem cell (MSC) therapy (27), enteral nutritional supplementation (28), and intestinal gut microbial agents (29). As an alternative therapy, TCM can improve the efficacy of HAART for HIV/AIDS and has a high safety profile (30). By analyzing the combined expression of all proteins in an organism, proteomics observes the dynamic changes of proteins through a holistic dimension, which is in line with the holistic concept of TCM (31). Proteomics is now widely used in the determination of TCM syndrome and symptoms to detect differences in protein expression, structure, function, and interactions between healthy individuals and specific syndrome and symptoms, which can provide a scientific and biological basis for TCM syndromes and ultimately reveal the material basis and pathogenesis of diseases (25). The concept of proteomics has also been applied in the field of Chinese medicine research to observe the changes of protein levels in the body through the intervention of Chinese medicine in specific syndromes to reveal the targets of Chinese medicine and to explore the molecular level regulation of the development of syndromes.

In this study, we first compared the differences of routine blood and urine tests, clinical biochemical values, and immunological indexes between the LSD syndrome group and NS group. Subsequently, both the LSD syndrome and

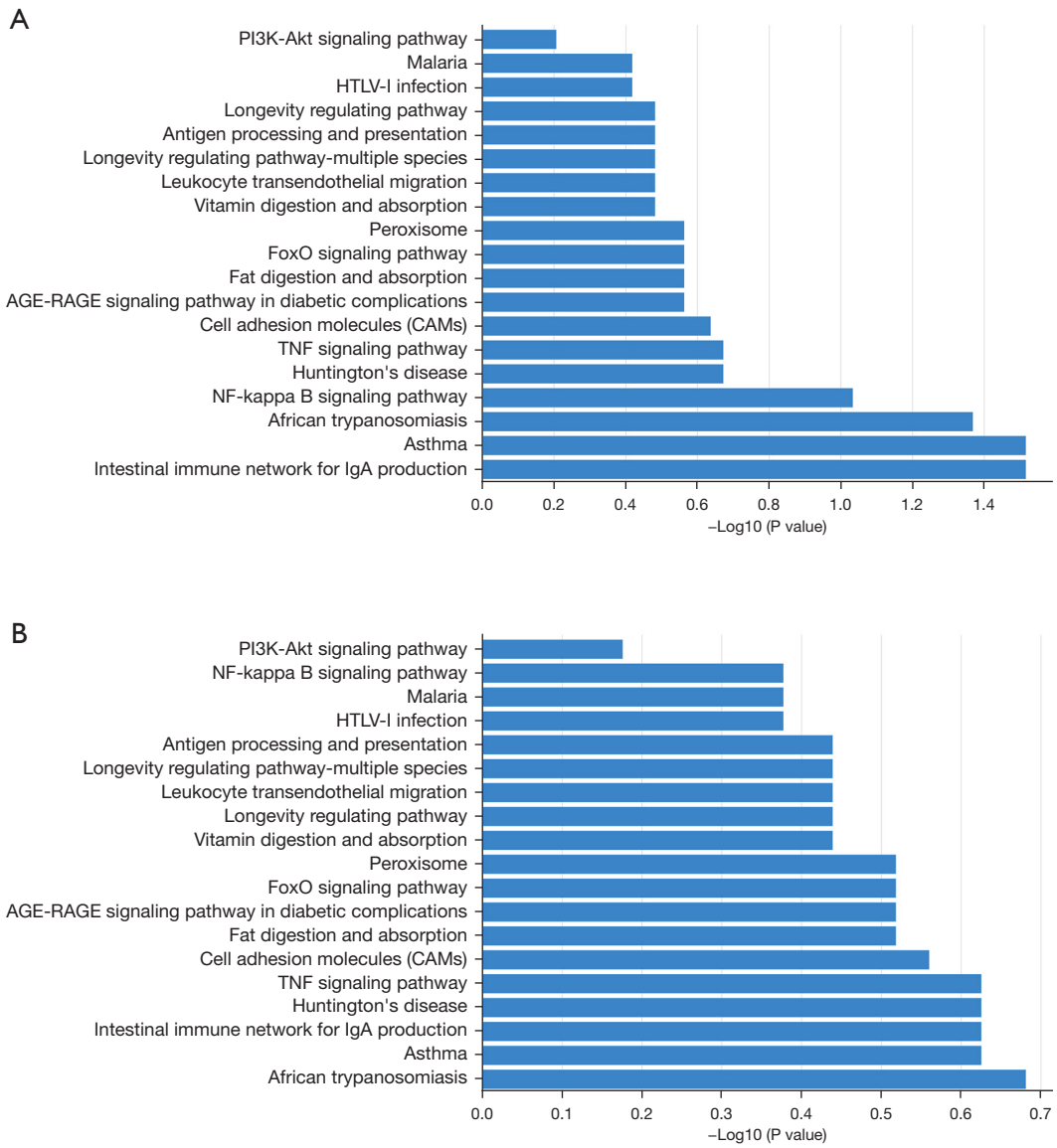

Figure 2 KEGG pathway analyses of the DEPs in the LSD (A) and NS (B) groups. IgA, immunoglobin A; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEP, differentially expressed protein; LSD, lung and spleen deficiency; NS, nonsyndromic.

NS group in AIDS-INRs were examined with TMT-LC-MS/MS proteomic analysis and comparatively analyzed with bioinformatics. Finally, the specific DEPs of the LSD syndrome group were validated with ELISA, and thus potential biomarkers were identified, with the aim of these potentially providing a biological basis for recognizing TCM syndromes and symptoms in AIDS-INRs. TMT is a widely used technique for quantitative proteomic labeling, which enables the labeling of peptides via binding to the N-terminal group of the peptide and the amino group of the lysine side chain as well as the comparison of proteomes between different samples via labeling different samples with different molecular weight reagents (32). The TMT-LC-MS/MS technique can achieve accurate qualitative and quantitative analysis of proteins in 2 to 10 different samples simultaneously and has the advantages of high detection throughput and high quantitative accuracy (33). Therefore, in this study, we used the TMT-LC-MS/MS technique for proteomic studies. In comparative KEGG pathway analysis, an intestinal immune network for IgA production

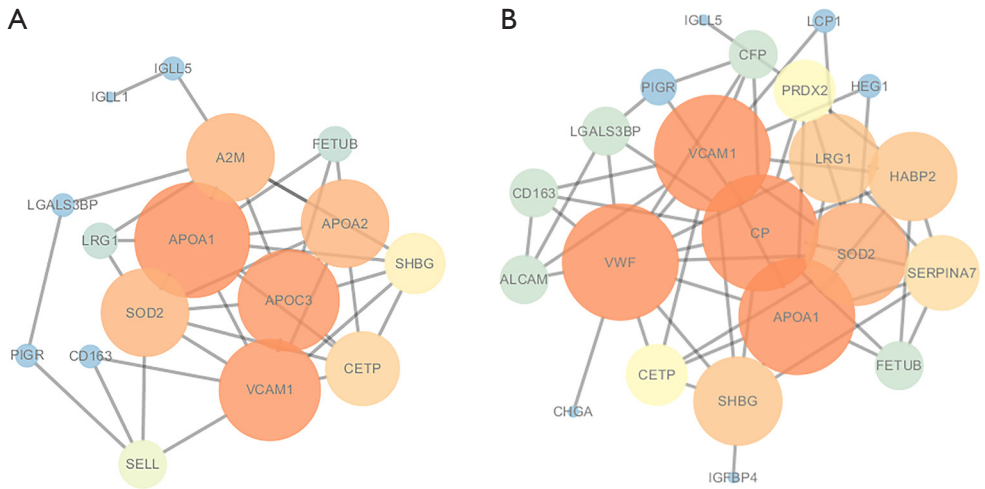

Figure 3 PPI network of the DEPs in LSD (A) and NS (B) groups. A larger circle size and a greater number of nodes indicate a higher density of protein interactions. PPI, protein-protein interaction; DEP, differentially expressed protein; LSD, lung and spleen deficiency; NS, nonsyndromic.

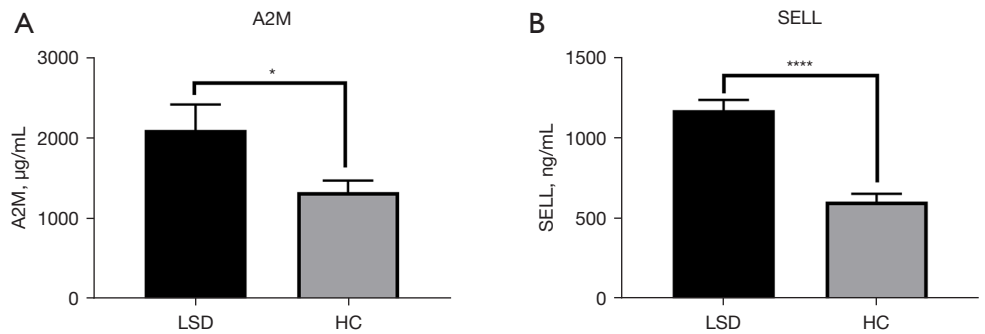

Figure 4 Validation of the potential biomarkers for LSD syndrome. Statistical analyses of the relative levels of A2M and SELL between the LSD and HC groups. *, P<0.05, ****, P<0.0001. A2M, alpha-2-macroglobulin; SELL, selectin L; LSD, lung and spleen deficiency; HC, healthy control.

was more closely associated with LSD than it was with NS. In TCM, there is a mutually effectual interior-exterior relationship between the lung and the large intestine (34). The spleen is the root of acquired nature and regulates the digestion and absorption of nutrients, which is inseparable from the gastrointestinal tract. Within the framework of modern medicine, the gastrointestinal tract is part of the mucosal immune system, and most of the lymphocytes in the gastrointestinal tract can synthesize and secrete immunoglobulin plasma cells, which are the main source of IgA synthesis in the body. Moreover, IgA exerts a protective effect on the integrity of the intestinal barrier, which in turn has an important impact on the elimination of antigens (35). Patients with LSD syndrome often exhibit gastrointestinal symptoms such as diarrhea, abdominal distension, and loss of appetite, which are closely related to the intestinal immune network, and this is consistent with the results of the KEGG pathway analysis in this study.

The ELISA results showed that A2M levels were significantly elevated in patients with LSD syndrome compared to the healthy control group. A2M inhibits broad-spectrum serine and threonine metalloproteinases and inflammatory cytokines and is a major component of the eukaryotic innate immune system (36). It is currently used mainly in neurodegenerative pathologies (37), cerebrovascular diseases (38), inflammation-related diseases (39),

and cancer-related immune modulation (40). A2M is a cytokine transporter protein that expresses transforming growth factor-β1 (TGF-β1), which exerts anti-inflammatory activity. Therefore, A2M concentrations increase with increasing protease levels at the site of inflammation (41). Interleukin 10 (IL-10) is an immunomodulatory cytokine with anti-inflammatory activity, and A2M can trigger an anti-inflammatory response by binding to IL-10 (42). INRs-LSD are often accompanied by a series of chronic inflammatory responses, such as prolonged diarrhea, cough, and low fever. Upregulated A2M often suggests an inflammatory response *in vivo*, which is consistent with the upregulation of A2M expression observed in this study. Among the participants examined, LSD-INRs had lower platelet levels compared to NS-INRs, which may also suggest the presence of inflammation *in vivo*. Thymosin can enhance cellular immune function and regulate immune homeostasis, and under normal conditions, thymosin exerts its immune effects through binding to zinc (43). While A2M has a higher binding affinity for zinc than does thymosin, it has been shown that in patients with cervical cancer, there is reduced peripheral zinc bioavailability, reduced plasma active thymosin level, reduced natural killer cell activity, reduced IL-2 level, and increased A2M level, suggesting that increased A2M competing with thymosin for zinc binding may lead deficiency and impaired immune function *in vivo* (44). Since A2M has a high binding capacity for thymosin zinc, it may play a key role in immune efficiency. In this study, A2M was upregulated in patients in the LSD syndrome group, suggesting impaired immune efficiency *in vivo*, which is consistent with the characteristics of AIDS-INRs.

According to ELISA, SELL levels were also significantly higher in the LSD syndrome group compared to the HCs. SELL, or L-selectin, is a type I transmembrane glycoprotein and cell adhesion molecule that is a member of the selectin family, can be expressed on most leukocytes, and is a major regulator of leukocyte adhesion, migration, and signaling (45). Currently, SELL is most commonly used for inducing T-lymphocyte homing and has many antitumor applications (46). Chronic inflammation may promote tumor growth through myeloid-derived suppressor cells (MDSCs) to induce SELL expression and promote metastasis of the tumors to the lymph nodes (47). MDSCs can use multiple mechanisms to block innate and adaptive antitumor immunity and suppress $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes (48). One study showed that MDSC levels in peripheral blood of patients with HIV/AIDS with lung spleen qi deficiency syndrome were negatively correlated with $CD4^+$ T-cell levels, suggesting that MDSC may affect immune function in AIDS by regulating SELL expression (49). The population targeted in this study was HIV/AIDS-INRs, who are at a higher risk of opportunistic infections and are more likely to be associated with the incidence of tumors and other diseases than are those with HIV/AIDS. Furthermore, LSD syndrome is inherently a qi deficiency, which is associated with clinical symptoms of qi and blood deficiency, the inability to produce blood, and even worse immune function. In this study, we found that SELL was upregulated in AIDS-INRs with LSD syndrome compared with the HC group, suggesting that SELL is upregulated when immune deficiency is present. Thus, SELL may serve as a target for the treatment of HIV/AIDS and the reconstitution of immune status, possibly through the downregulation of its expression.

Conclusions

Based on the DEPs discovered with proteomics and on bioinformatics analyses, it was found that there were biological differences between HCs and HIV/AIDS-INRs with the typical TCM syndrome of LSD. Furthermore, TCM syndrome-specific DEPs including A2M and SELL were identified as the serum biomarkers for LSD syndrome in INRs. These findings may help contribute to the scientific and objective evidence for the differentiation and clinical diagnosis of LSD syndrome in INRs, providing a biological basis for a deeper understanding of TCM syndromes and more opportunities for the stable application TCM's strengths in the treatment of HIV/AIDS-INRs. Due to the small number of cases included, which is a limitation this study, we will further expand the number of cases for analysis in follow-up research.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-322/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-322/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and approved by the Regional Ethics Review Committee of Sichuan Traditional Chinese Medicine, China (ethical approval No. 2018KL-062). Informed consent was obtained from all participants.

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Appendix 1

1. Diagnostic and inclusion criteria

a. Inclusion criteria for HIV/AIDS and HIV/AIDS-INR

The inclusion criteria for HIV/AIDS and the Western medical diagnostic criteria for HIV/AIDS-INR were derived from the "HIV/AIDS Treatment Guidelines (2018 Edition) published by the Chinese Medical Association AIDS Specialty Group and Infectious Diseases Branch.

Diagnosis is made when one of the following conditions is met:

i. positive HIV antibody screening test and positive HIV supplementation test (positive antibody supplementation test or positive qualitative nucleic acid test or quantitative nucleic acid >5000 copies/mL);
ii. positive HIV isolation test.

The following HIV/AIDS-INR diagnostic criteria were derived from the Expert Consensus on Collaborative Treatment of HIV/AIDS, Immune Non-Responders issued by the AIDS Prevention and Control Branch of the Chinese Academy of Traditional Chinese Medicine:

i. meeting the diagnostic criteria of HIV/AIDS;
ii. CD4+<300/μL or CD4+ growth rate less than 20% compared to baseline;
iii. HIV-RNA <50 copies/ml for more than 18 months.

All patients enrolled were required to meet the above HIV/AIDS-INR diagnostic criteria as well the following criteria:

i: between the ages of 18 and 65 years;
ii: HAART treatment duration of more than 2 years and plasma HIV load <50 copies/mL for more than 18 months;
iii: signed informed consent for participation.

b. Diagnosis criteria for LSD syndromes of HIV/AIDS-INR

Diagnostic criteria for HIVAIDS-LSD syndrome refer to the Chinese Medicine Treatment Protocol for Adults with AIDS (2016 version) issued by the State Administration of Traditional Chinese Medicine

The primary symptoms are as follows:

i: poor complexion, fatigue, low voice, and lazy speech;
ii: shortness of breath and asthma, cough with thin sputum, and prolonged cough;
iii: loss of appetite, poor appetite, bloating, and loose stools.

The secondary symptoms are as follows:

i: pale tongue with white smooth coating;
ii: weak pulse.

Any patient meeting 1 of the following criteria could be diagnosed as a HIV/AIDS-INR LSD.

i: all the primary symptoms present;
ii: primary symptoms ii and iii present;
iii: primary symptoms ii and iii and at least 1 of the secondary symptoms present.

c. Diagnosis criteria for NS of HIV/AIDS-INR

None syndrome (NS) refers meeting the HIV/AIDS diagnostic criteria of Western medicine but with a syndrome score of zero as and no obvious symptoms related to the tongue or pulse.

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2. Exclusion criteria

Participants who met the inclusion criteria but had any 1 of the following were excluded from the study:

i: uncontrolled, severe opportunistic infections;
ii: administration of immunomodulatory agents within 1 month prior to enrollment;
iii: in a pregnant or lactating state;
iiii: coinfection with hepatitis C virus or hepatitis D virus;
iiiii: combined with malignancy or cirrhosis;
iiiiii: with severe allergies or allergies to certain herbal ingredients.

3. Clinical evaluation of YDSK and NS patients

Table S1 Clinical evaluation of LSD and NS patients

Projects	LSD (n=9)	NS (n=10)	Control (n=10)	P value
Gender (female/male)	4/5	6/4	4/6	
Age (years)	(40.22±5.19)	(38.0±6.32)	(36.3±9.89)	0.517
HAART hours (months)	(48.22±28.40)	(59.10±24.19)		0.380
Viral load (cp/mL)	(17.78±21.08)	(3.20±10.12)		0.063
CD4 (cells/μL)	(176.11±79.23)	(166.10±40.37)		0.729
Erythrocyte (10^{12} /L)	(4.14±0.39)	(4.21±1.12)		0.288
Hemoglobin (g/L)	(142.44±14.36)	(147.60±17.61)		0.497
Leukocyte (10^9 /L)	(6.04±2.46)	(7.38±3.54)		0.540
Absolute neutrophil count (10^9/L)	(3.79±2.13)	(4.92±2.98)		0.462
Absolute lymphocyte count (10^9/L)	(1.71±0.39)	(1.78±0.74)		0.825
Blood platelet (10^9 /L)	(189.78±41.04)	(261.90±110.63)		0.083
Alanine aminotransferase (U/L)	(33.37±13.27)	(23.36±8.80)		0.067
Aspartate aminotransferase (U/L)	(33.96±7.67)	(26.91±11.03)		0.045
Gamma-glutamyl transpeptidase (U/L)	(53.70±32.53)	(53.42±29.61)		0.985
Blood urea nitrogen (mmol/L)	(4.88±1.77)	(4.75±2.09)		0.744
Serum creatinine (μmol/L)	(74.49±14.60)	(75.82±12.32)		0.832

Data are presented as the mean ± standard deviation. YDSK, Yang deficiency of spleen and kidney; LSD, lung and spleen deficiency; NS, nonsyndromic; HAART, highly active antiretroviral therapy.

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4. Clinical information of participants for ELISA validation

Table S2 Clinical information of participants for ELISA validation

Projects	LSD (n=10)	Control (n=10)
Gender (female /male)	4/6	3/7
Age (years)	(40.60±6.70)	(25.90±2.13)
HAART hours (months)	(48.60±24.28)	
Viral load (cp/mL)	(4.00±12.65)	
CD4 (cells/μL)	(233.40±52.10)	
Erythrocyte (10^{12}/L)	(4.56±0.95)	
Hemoglobin (g/L)	(150.10±26.08)	
Leukocyte (10^9/L)	(5.63±1.77)	
Absolute neutrophil count (10^9/L)	(3.08±1.30)	
Absolute lymphocyte count (10^9/L)	(2.02±0.58)	
Blood platelet (10^9/L)	(186.30±63.69)	
Alanine aminotransferase (U/L)	(45.31±19.26)	
Aspartate aminotransferase (U/L)	(45.60±14.24)	
Gamma-glutamyl transpeptidase (U/L)	(43.21±22.34)	
Blood urea nitrogen (mmol/L)	(4.67±1.88)	
Serum creatinine (μmol/L)	(74.49±13.29)	

Data are presented as mean ± standard deviation. LSD, lung and spleen deficiency HAART, highly active antiretroviral therapy.

5. Volcano map of DEPs

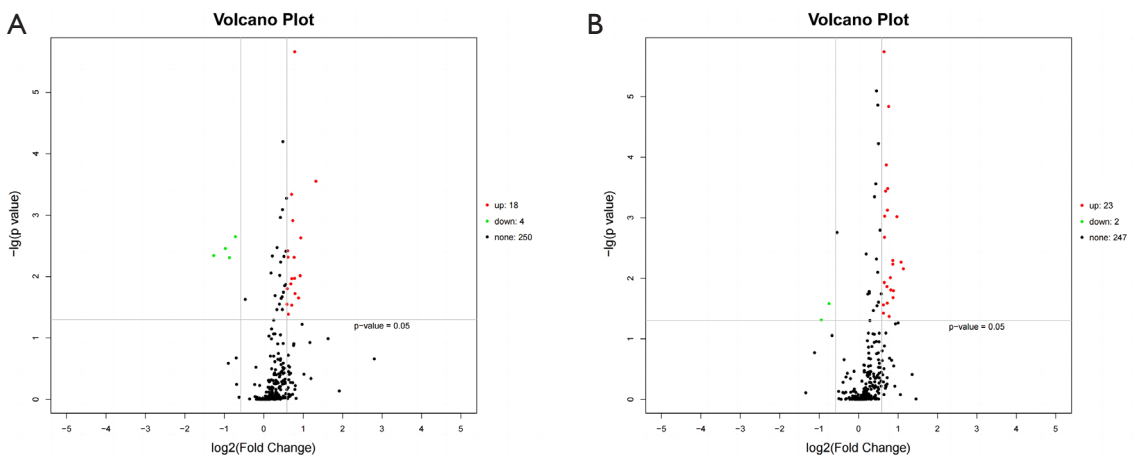

Figure S1 (A) Volcano plot of DEPs between the LSD-INR and HC groups. (B) Volcano plot of the DEPs between the NS-INR and HC groups. DEP, differentially expressed protein; LSD, lung and spleen deficiency; INR, immunological nonresponder; HC, healthy control; NS, nonsyndromic.

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