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Validation of Serotransferrin in the Serum as Candidate Biomarkers for the Diagnosis of Pulmonary Tuberculosis by Label-Free LC/MS

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Cite This: *ACS Omega* 2022, 7, 24174−24183

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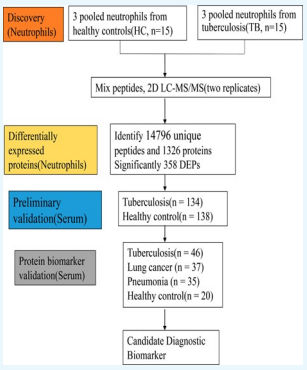
ABSTRACT: This study aimed to identify secreted protein biomarkers in serum from the label-free LC/MS proteomics of neutrophils in pulmonary tuberculosis (TB) patients for the diagnosis biomarkers of TB label-free LC/MS. The proteomic profiles of neutrophils from 15 active TB patients and 15 healthy controls (HCs) were analyzed using label-free LC/MS. We identified 358 differentially expressed proteins preliminarily, including 279 up-regulated proteins and 79 down-regulated proteins. Thirty-eight differentially expressed secreted proteins involved in the progress of platelet degranulation between TB patients and HCs were focused. Of these, serotransferrin (TRF), alpha-2-macroglobulin (AMG), alpha-1-antitrypsin (AAT), alpha-1-acid glycoprotein 1 (AAG), alpha-1-acid glycoprotein 2 (AGP2), and alpha-1B-glycoprotein (A1BG) were selected for further verification in the serum of additional 134 TB patients and 138 HCs by nephelometry and ELISA in the training set. Statistically significant differences of TRF ($P < 0.0001$), AAT ($P < 0.0001$), AAG ($P < 0.0001$), AGP2 ($P < 0.0001$), and A1BG ($P = 0.0003$) were observed. The serum concentration of TRF was down-regulated in TB patients compared with healthy controls, which was coincident with the proteomics results. An additional validation of TRF was performed in an independent cohort of patients with active TB ($n = 46$), patients with lung cancer ($n = 37$), 20 HCs, and patients with pneumonia ($n = 35$) in the test set by nephelometry. The serum expression levels of TRF in the TB patients showed lower levels compared with those in patients with pneumonia ($P = 0.0125$), lung cancer ($P = 0.0005$), HCs ($P < 0.0001$), and the non-TB controls ($P < 0.0001$). Furthermore, the AUC value of TRF was 0.647 with 90.22% sensitivity and 42.86% specificity in discriminating the TB group from the pneumonia group, 0.702 with 93.48% sensitivity and 47.16% specificity in discriminating the TB group from the lung cancer group, 0.894 with 91.30% sensitivity and 71.62% specificity in discriminating the TB group from all HCs, and 0.792 with 91.30% sensitivity and 58.90% specificity in discriminating the TB group from the non-TB controls. This study obtained the proteomic profiles of neutrophils in the TB patients and HCs, which contribute to a better understanding of the pathogenesis molecules existing in the neutrophils of pulmonary tuberculosis and provide candidate biomarkers for the diagnosis of pulmonary tuberculosis.



1. INTRODUCTION

Tuberculosis (TB) is a global infectious disease caused by *Mycobacterium tuberculosis* (Mtb), which is harmful to human health. According to the data of the World Health Organization (WHO), 5.8 million newly diagnosed TB cases were reported globally in 2020. About 1.3 million HIV-negative patients with TB died worldwide in 2020.[1] China is one of 30 countries in the world with a high burden of TB. The detection of TB is still heavily dependent on sputum smear, sputum culture, chest radiography (X-ray/computerized tomography (CT) scan), clinical symptomatology, Xpert MTB/RIF technology, tuberculin skin tests (TSTs), and interferon gamma release assays (IGRAs).[2] A sputum culture remains the gold standard for diagnosing TB, which can reach a higher positive rate than a sputum smear, while the culture requires 4 to 8 weeks for the growth of Mtb.[3] The radiological technologies and immunological diagnosis such as IGRAs and TSTs are easily influenced by some unknown, unspecific detection factors, and it is difficult to distinguish TB from other pulmonary diseases.[3,4] Therefore, there is an urgent need to develop a rapid and accurate diagnostic method for TB diagnostic and treatment.

In recent years, researchers have been screening potential diagnostic markers for TB by comparing and analyzing differentially expressed proteins in the plasma between serum of TB patients and controls by transcriptome sequencing or proteomic technology.[5] However, there are a large number of high-abundance proteins in plasma and serum in consideration of the complexity of plasma or serum samples. In order to avoid the interference of these high-abundance proteins on the subsequent mass spectrometry (MS) analysis results, these high-abundance proteins need to be removed before analysis and identification. This process will also lose a large number of

Received: February 10, 2022
Accepted: June 1, 2022
Published: July 6, 2022

low-abundance proteins, which makes it difficult to detect low-abundance proteins in plasma or serum.

In previous studies, we found oxidative burst in neutrophils (NEUs) significantly increased and chemotactic activity of neutrophils decreased in TB patients. The expression of costimulatory molecules in NEUs were also different from healthy individuals.[6] Moreover, NEUs could release soluble mediators and neutrophil extracellular traps (NETs) and cell-derived vesicles and thereby interact with lung-residing cells, notably alveolar macrophages, mucosal dendritic cells, pneumocytes, and lymphocytes to motivate and orchestrate tissue remodeling during Mtb infection.[7] From the perspective of transcriptomics, a whole blood transcriptional signature dominated by IFN-inducible genes was identified in active TB patients but not present in healthy controls (HCs).[8] However, the exact role of NEUs in TB remained unknown in proteomic assessment.

In this study, the proteomic profiles of NEUs in the TB patients and HCs was generated in NEUs from active TB and HCs using label-free LC/MS methods. Integrative analysis and bioinformatics analysis were performed. To identify some secreted proteins related to the progress of platelet degranulation in the TB patients, the candidate protein biomarkers were validated using nephelometry and ELISA in the serum of patients with TB, pneumonia, and lung cancer and HCs, which help us to better understand the pattern of neutrophils−platelet interactions and pathogenic mechanisms between TB and host. Further analysis of a receiver operating characteristic (ROC) curve revealed the sensitivity and specificity of the potential protein biomarkers, which may be used as potential diagnostic markers to identify active TB patients. These results also provide a new database of proteins in the NEUs of TB patients.

2. MATERIALS AND METHODS

2.1. Patients and Control Subjects. The study was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University and was carried out in compliance with the Helsinki Declaration. All participants recruited were at least 18 years old and gave informed consent. TB patients were recruited from the Jiangxi Chest Hospital and the First Affiliated Hospital of Nanchang University from September 2017 to November 2021. The NEUs from 15 active TB patients and NEUs from 15 HCs were enrolled in the discovery set for proteomics analysis. All TB patients fulfilled the clinical criteria from the WHO. HCs had not received anti-TB treatment and individuals with extrapulmonary TB, autoimmune disease, HBV, HCV, HIV, cancer, and other chronic disease were all excluded. The patients with lung cancer were included with different subtypes of lung cancer and at all stages. Pneumonia individuals satisfied the following criteria mentioned in the Guidelines for the Evaluation and Treatment of Pneumonia.[9] An independent cohort of 134 TB patients (94 males, 40 females) and 138 HCs (80 males, 58 females) were recruited from the Jiangxi Chest Hospital and the First Affiliated Hospital of Nanchang University (Nanchang, Jiangxi, China) during September 2017 to November 2021 in the training set. In addition, 138 independent cases of differential diseases (including 46 cases of TB, 37 cases of lung cancer, and 35 cases of pneumonia and 20 HCs) from the Jiangxi Chest Hospital and the First Affiliated Hospital of Nanchang University were enrolled in the test set during September 2017 to November 2021 for further validation.

2.2. Blood Sample Collection. We collected 5 mL of peripheral blood from each participant and centrifuged the blood specimens at 3500*g* for 10 min at room temperature within 4 h of collection. Then the serum was divided into 1.5 mL polypropylene tubes for immediate freezing at −80 °C. All samples avoided freeze−thawing cycles.

In addition, samples of peripheral blood (5 mL) were drawn by venipuncture and collected into EDTA tubes. The peripheral blood mononuclear cells (PBMCs) and NEUs fractions were isolated by density gradient centrifugation. Five milliliters of blood was diluted 1:1 with sterile saline and was centrifuged on Lymphocyte Separation Medium (MP Biomedicals, Solon, OH, U.S.A.) in a 15 mL polystyrene conical centrifuge tube for 30 min at 300*g* (RT). Cells were divided into four layers. The PBMCs were carefully collected by aspiration from the plasma−lymphocyte separation medium interface and washed once in phosphate buffered saline (PBS).

2.3. Purification of NEUs and Protein Extraction. After Ficoll-Paque gradient centrifugation of buffy coats or peripheral blood, followed by dextran sedimentation of granulocytes and hypotonic lysis of erythrocytes, NEUs were isolated to reach 99.7 ± 0.2% purity by positively removing all contaminating cells using the EasySep neutrophil enrichment kit (StemCell Technologies, Vancouver, BC, Canada) (Nicola Tamassia). The viability of the cells was monitored by trypan blue staining. All samples were sonicated three times on ice using a high-intensity ultrasonic processor (Scientz) in lysis buffer (8 M urea (Sigma), 1% Protease Inhibitor Cocktail (Calbiochem), 2 mM EDTA(Sigma)). The remaining debris was removed by centrifugation at 12 000*g* at 4 °C for 10 min. Finally, the supernatant was collected, and the protein concentration was determined with BCA kits (Beyotime Biotechnology) according to the manufacturer's instructions.

2.4. Trypsin Digestion and HPLC Fractionation. We pooled proteins from the NEUs from 15 individuals into one sample, namely, the H-NEU (healthy controls' neutrophils) and P-NEU (TB patients' neutrophils) groups, respectively (combined with different sexes and ages). Then equal proteins were mixed into a group for three biological replicates namely H-NEU1, H-NEU2, H-NEU3 and P-NEU1, P-NEU2, P-NEU3 groups, respectively. For digestion, the protein solution was reduced with 5 mM dithiothreitol (Sigma) for 30 min at 56 °C and alkylated with 11 mM iodoacetamide (Sigma) for 15 min at room temperature in darkness. The protein sample was then diluted by adding 100 mM NH_4HCO_3 to urea concentration less than 2M. Finally, trypsin (Promega) was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second 4 h-digestion. These tryptic peptides were fractionated into fractions by high-pH reverse-phase HPLC using Agilent 300 Extend C18 column (5 μm particles, 4.6 mm ID, 250 mm length). Briefly, peptides were first separated with a gradient of 8% to 32% acetonitrile (pH 9.0, Fisher Chemical) over 60 min into 60 fractions. Then, the peptides were combined into three fractions and dried by vacuum centrifuging.

2.5. LC-MS/MS Analysis. The tryptic peptides were dissolved in 0.1% formic acid (solvent A, Fluka), directly loaded onto a homemade reversed-phase analytical column (15 cm length, 75 μm i.d.). The gradient was composed of an increase from 6% to 23% solvent B (0.1% formic acid in 98%

https://doi.org/10.1021/acsomega.2c00837
ACS Omega 2022, 7, 24174−24183

Table 1. Detailed Information of 38 Secreted Differential Proteins Associated with the Progress of Platelet Degranulation and the Secreted Proteins of Mtb in P-NEU and H-NEU Groups[a]

protein description	gene name	mol. weight [kDa]	P-NEU/H-NEU ratio	regulation type
coagulation factor XIII A chain	F13A1	83.266	0.2645	down
galectin-3-binding protein	LGALS3BP	65.33	0.249	down
alpha-I-acid glycoprotein 1	ORM1	23.511	0.1345	down
fibrinogen beta chain	FGB	55.928	0.107	down
alpha-2-antiplasmin	SERPINF2	54.565	0.105	down
fibrinogen gamma chain	FGG	51.511	0.0985	down
alpha-1-antitrypsin	SERPINA1	46.736	0.0705	down
von Willebrand factor	VWF	309.26	0.0655	down
alpha-1-acid glycoprotein 2	ORM2	23.602	0.056	down
fibrinogen alpha chain	FGA	94.972	0.0435	down
plasminogen	PLG	90.568	0.037	down
interalpha-trypsin inhibitor heavy chain H4	ITIH4	103.36	0.03	down
alpha-1 -antichymotrypsin	SERPINA3	47.65	0.029	down
beta-2-glvcoprotein 1	APOH	38.298	0.028	down
plasma protease C1 inhibitor	SERPING1	55.154	0.0275	down
fibronectin	FNI	262.62	0.0265	down
apolipoprotein A-I	APOAI	30.777	0.0255	down
serotransferrin	TF	77.063	0.0245	down
alpha-1B-glycoprotein	A1BG	54.253	0.023	down
alpha-2-macroglobulin	A2M	163.29	0.02	down
vitamin K-dependent protein S	PROS1	75.122	0.0155	down
clusterin	CLU	52.494	0.01	down
alpha-2-HS-glycoprotein	AIISG	39.324	0.0085	down
kininogen-1	KNG1	71.957	0.008	down
histidine-rich glycoprotein	HRG	59.578	0.001	down
78 kDa glucose-regulated protein	HSPA5	72.332	5.345	up
prosaposin	PSAP	58.112	4.8725	up
annexin A5	ANXA5	35.936	4.768	up
peptidyl-prolyl cis−trans isomerase A	PPIA	18.012	3.671	up
vinculin	VCL	123.8	3.325	up
fructose-bisphosphate aldolase A	ALDOA	39.42	3.2505	up
WD repeat-containing protein 1	WDR1	66.193	2.8785	up
transgelin-2	TAGLN2	22.391	2.7095	up
filamin-A	FLNA	280.74	2.5725	up
alpha-actinin-4	ACTN4	104.85	2.563	up
pleckstrin	PLEK	40.124	2.552	up
profilin-1	PFN1	15.054	2.4195	up
alpha-actinin-1	ACTN1	103.06	2.263	up

[a]Filtered with threshold value of expression fold change >2 and P value <0.05. Mol. weight [kDa], protein molecular weight, unit [kDa]. H-NEU, healthy controls' neutrophils. P-NEU, TB patients' neutrophils.

acetonitrile) over 26 min, 23% to 35% in 8 min, and climbing to 80% in 3 min and then holding at 80% for the last 3 min, all at a constant flow rate of 400 nL/min on an EASY-nLC 1000 UPLC system.

The peptides were subjected to a NSI source followed by tandem mass spectrometry (MS/MS) in Q Exactive Plus (Thermo) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z scan range was 350 to 1800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70 000. Peptides were then selected for MS/MS using NCE setting as 28 and the fragments were detected in the Orbitrap at a resolution of 17 500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 15.0s dynamic exclusion. Automatic gain control (AGC) was set at 5×10^4. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository[10] with the data set identifier PXD033563.

2.6. Database Search and Bioinformatics Methods. The resulting MS/MS data were processed using the Maxquant search engine (v.1.5.2.8). Tandem mass spectra were searched against SwissProt Human database (V.56.9, 20402 sequences, Human) concatenated with reverse decoy database. Trypsin/P was specified as cleavage enzyme allowing up to two missing cleavages. The mass tolerance for precursor ions was set as 20 ppm in First search and 5 ppm in Main search, and the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on Cys was specified as fixed modification, oxidation on Met was specified as variable modifications. Label-free quantification method was label free quantification (LFQ), false discovery rate (FDR) was adjusted to <1% and minimum score for peptides was set >40. The entire genome serves as the reference set for their gene annotation enrichment analysis. A heatmap was visualized by

https://doi.org/10.1021/acsomega.2c00837
ACS Omega 2022, 7, 24174−24183

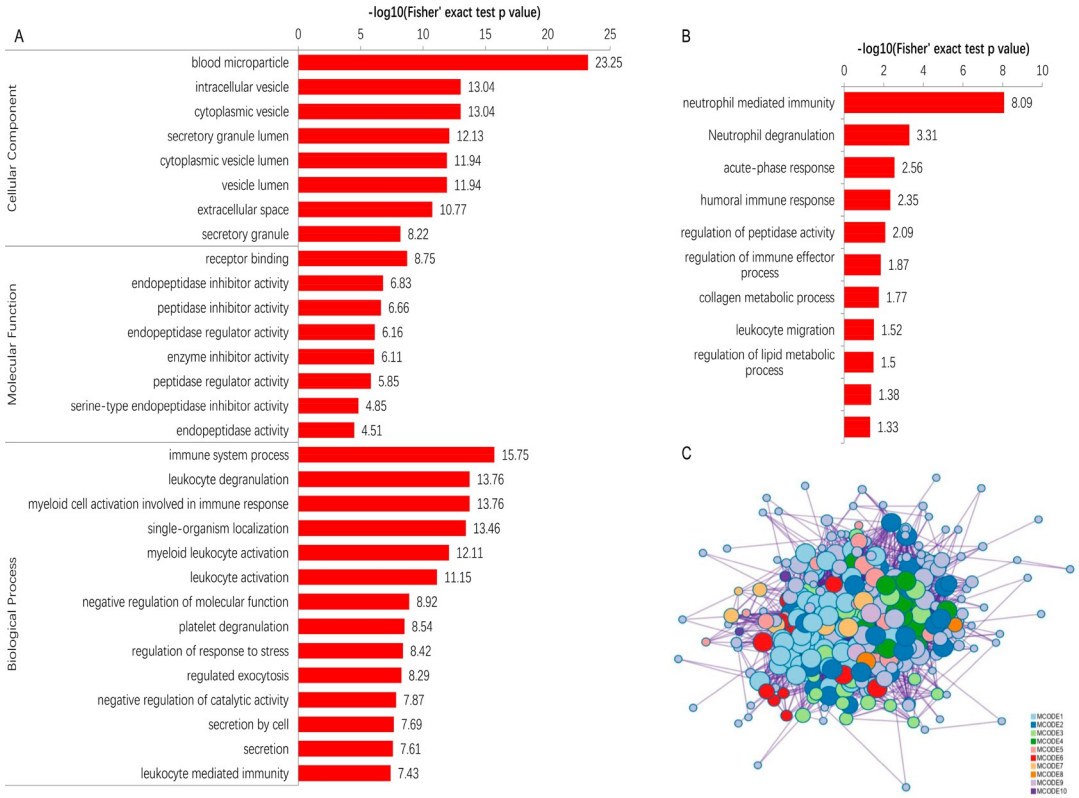


Figure 1. Bioinformatics analysis of 358 differentially expressed proteins. (A) Biological process (GO analysis), cellular component (GO analysis), molecular function (GO analysis). (B) KEGG pathway analysis. (C) Functional network analysis of differentially expressed proteins using the metascape search tool.

using the "heatmap.2" function from the "gplots" R-package. The functional distribution of proteins including their molecular function (MF), cellular component (CC), and biological process (BP) was determined by an online tool based on the Gene Ontology (GO) annotation project. Pathway analysis of differentially expressed proteins was elucidated using the Kyoto Encyclopedia of Genes and Genomes database (KEGG). The protein−protein functional network was analyzed using the metascape search tool through the Web site (http://metascape.org/).[11]

2.7. Nephelometry and ELISA Analysis. To validate the label-free LC-MS/MS Proteomic results, differentially expressed proteins were quantified in HCs, and patients with TB, lung cancer, and pneumonia using nephelometry and ELISA. Serotransferrin (TRF), alpha-2-macroglobulin (AMG), alpha-1-antitrypsin (AAT), and alpha-1-acid glycoprotein 1(AAG) were measured using nephelometry respectively (Immage 800, Beckman-Coulter). All kits are from Beckman Kurt Co. Ltd. The human alpha-1-acid glycoprotein 2 (AGP2) ELISA kit (Signalway Antibody, America. EK3957) with a detection limit of 0.31 ng/mL was applied to detect AGP2 concentration. The human alpha-1B-glycoprotein (A1BG) ELISA kit (Signalway Antibody, America. EK5697) with a minimum detectable dose of 10 pg/mL was employed to detect A1BG in serum at a 1:60 000 dilution factor. All assays were carried out according to the manufacturer's instructions.

2.8. Statistical Analysis. All the experimental data were analyzed using SPSS software (version 25.0, Chicago, IL, U.S.A.) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA U.S.A.). $P < 0.05$ was considered statistically significant. Parametric data were presented as the mean ± standard deviation (SD) and were investigated using the *t* tests for means. The nonparametric data were presented as the median ± interquartile range (IQR) and were analyzed using the Mann−Whitney *U* test for two groups and the Kruskal−Wallis *H* test for more than two groups. ROC curves were constructed to assess the diagnostic value of each biomarker.

3. RESULTS

3.1. Identification and Relative Quantification of Differential Proteins Expression by Label-Free LC-MS/MS. In the discovery set, proteins in NEUs from 15 HCs and 15 TB patients by label-free quantitative proteomics analysis were compared. On the basis of the label-free LC-MS/MS data, in total, 14796 unique peptides and 2313 proteins were identified, and 1326 proteins were quantified on the basis of the identification of one or more unique peptides across all three biological replicates in each group. The quality assessment is shown in Figure S1. The repeatability of the quantitation proteins between P-NEU and H-NEU groups is shown in Figure S2. The second biological replicate differed from the other two replicates and had a poor repeatability; therefore, we selected the other two biological replicates for further analysis. The fold change greater than 2.0 or less than 0.5 in relative abundance and *P*-value <0.05 were seen as the criteria to choose the differentially up-regulated and down-regulated proteins. We quantified 358 differentially expressed proteins including 279 up-regulated proteins and 79 down-regulated proteins between the P-NEU and H-NEU group as shown in Table S1. Between the two biological replicates, 376 up-regulated proteins and 166 down-regulated proteins were identified in the P-NEU1 and H-NEU1 group and 567 up-

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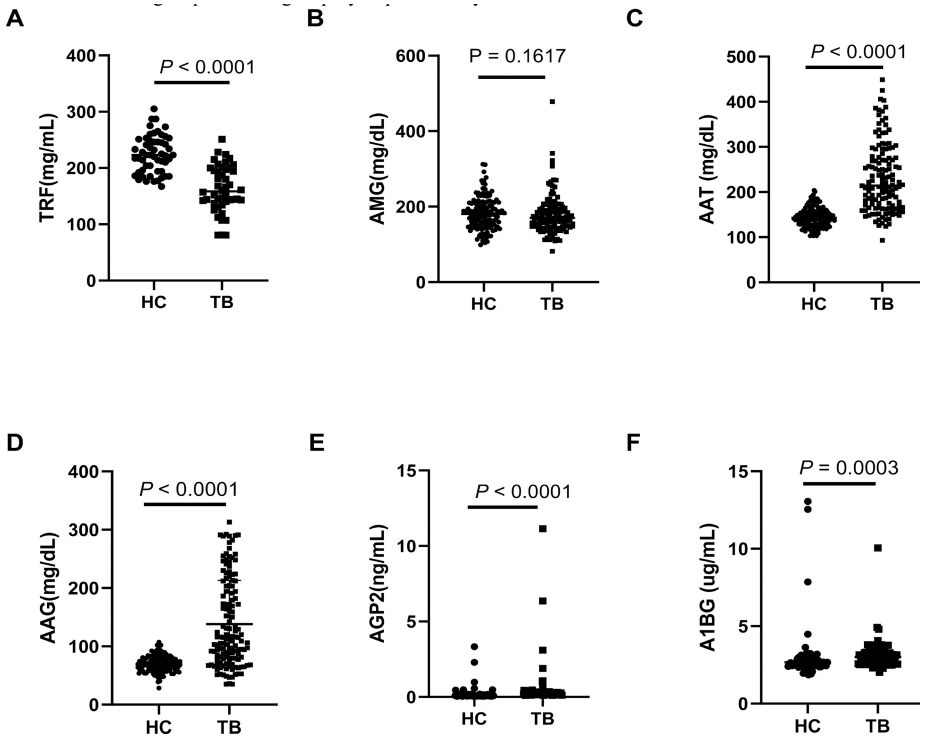

Figure 2. Serum levels of the serotransferrin (TRF), alpha-2-macroglobulin (AMG), alpha-1-antitrypsin (AAT), alpha-1-acid glycoprotein 1 (AAG), alpha-1-acid glycoprotein 2 (AGP2), and alpha-1B-glycoprotein (A1BG) by nephelometry and ELISA in the training set. Validation of the seven differentially expressed proteins TRF(A), AMG (B), AAT (C), AAG (D), AGP2 (E), and A1BG (F) are shown in the serum of the TB group and HC group by nephelometry and ELISA.

regulated proteins and 94 down-regulated proteins were identified in the P-NEU3 and H-NEU3 group. According to the differential expression of proteins, the secreted proteins of Mtb and the relation to the progress of platelet degranulation, we further screened 38 differentially expressed proteins in NEUs of TB patients and HCs, among which 13 were up-regulated (fold change >2.0) and 25 were down regulated (fold change <0.5) as shown in Table 1.

3.2. Bioinformatics Analysis of Differentially Expressed Proteins. We classified 358 differentially expressed proteins between patients with TB and HCs by GO analysis as MF, BP, and CC. Through the analysis of BP, we found that most of the differentially expressed proteins were involved in leukocyte degranulation 12.05%, platelet degranulation 12.05%, regulated exocytosis 10.84%, myeloid cell activation involved in immune response 8.43%, negative secretion by cell 8.43%, secretion 7.23%, myeloid leukocyte activation 7.23%, leukocyte activation 6.02%, regulation of response to stress 6.02%, regulation of catalytic activity 6.02%, negative regulation of molecular function 4.82%, leukocyte mediated immunity 4.82%, single-organism localization 3.61%, and immune system process 2.41%. The majority of the proteins have a different distribution, involving secretory granule lumen 18.33%, cytoplasmic vesicle lumen 16.67%, secretory granule 16.67%, cytoplasmic vesicle 13.33%, intracellular vesicle 11.67%, vesicle lumen 8.33%, blood microparticle 8.33%, extracellular space 6.67% and. According to the analysis of MF, the differentially expressed proteins were categorized into serine-type endopeptidase inhibitor activity 17.07%, endopeptidase activity 14.63%, endopeptidase inhibitor activity 14.63%, peptidase inhibitor activity 12.2%, endopeptidase regulator activity 12.2%, enzyme inhibitor activity 9.76%, receptor binding 9.76%, and peptidase regulator activity 9.76% (Figure 1A). In addition, KEGG analysis indicated that neutrophil mediated immunity, neutrophil degranulation, cute-phase response, humoral immune response, regulation of peptidase activity, regulation of immune effector process, collagen metabolic process, leukocyte migration, and regulation of lipid metabolic process were significantly associated with TB (Figure 1B). Furthermore, the protein−protein functional network diagram analysis demonstrated that differentially expressed proteins were closely interacted with each other (Figure 1C).

3.3. Validation of Differentially Expressed Proteins in Training Set by ELISA. On the basis of the experimental results, fold changes, bioinformatics data, the secreted proteins of Mtb, and availability of commercial kits, we next focused on six differentially expressed proteins related to the progress of platelet degranulation to confirm the proteomics data: six down-regulated proteins including TRF, AMG, AAT, AAG, AGP2, and A1BG. A total of 272 individuals (138 HCs and 134 TB) were recruited in the training set. As shown in Figure 2, the serum level of AAT was significantly increased in TB patients (231.38 ± 75.12 mg/dL, n = 134) compared with the HCs (144.94 ± 21.39 mg/dL, n = 138). Serum level of AAG was significantly increased in TB patients (113.50(125.6) mg/dL, n = 134) compared with the HCs (69.29 ± 13.78 mg/dL, n = 114). Also, the serum level of AGP2 was significantly increased in TB patients (0.5 ± 1.37 ng/mL, n = 89) compared with the HCs (0.19 ± 0.43 ng/mL, n = 89). The serum level of A1BG was significantly increased in TB patients (3.47 ± 2.96 ug/mL, n = 72) compared with the HCs (3.07 ± 1.91 ug/mL, n = 65). The serum concentration of AMG in HCs and TB patients were 182.33 ± 42.98 pg/mL (n = 134) and 179.11 ±

https://doi.org/10.1021/acsomega.2c00837
ACS Omega 2022, 7, 24174−24183

52.55 pg/mL (n = 125). respectively. However, the serum level of TRF was significantly decreased in TB patients (162.61 ± 40.95 mg/dL, n = 46) compared with the HCs (224.43 ± 33.13 mg/dL, n = 54). The results showed that serum levels of AAT (P < 0.0001), AAG (P < 0.0001), AGP2 (P < 0.0001), and A1BG (P = 0.0003) were significantly higher in the TB patients compared with the HCs while the serum concentration of TRF (P < 0.0001) was significantly lower in the TB group, compared with the HC group. No significant difference was observed in AMG (P = 0.1617) levels between TB patients and the HCs. Furthermore, only the serum concentration of TRF was coincident with the proteomics results.

3.4. The Diagnostic Value of TRF in Patients with TB, Pneumonia, and Lung Cancer and HCs. We further verified the diagnostic ability of TRF in an independent cohort of 46 patients with active TB, 20 HCs, 37 patients with lung cancer, and 35 patients with pneumonia. The results indicated that the serum concentration of TRF in the TB, HC, lung cancer, and pneumonia groups were 185.68 ± 44.31 mg/dL, 236.05 ± 34.97 mg/dL, 201.89 ± 72.73 mg/dL and 177(69) mg/dL, respectively. The serum level of TRF in the TB group was significantly lower than the HC, lung cancer, and pneumonia groups (P < 0.0001, P = 0.0005, P = 0.0125). When TB, HC, lung cancer, and pneumonia groups were combined as CON (non-TB controls), we found TRF was down-regulated in TB group (P < 0.0001) than the non-TB controls (Figure 3).

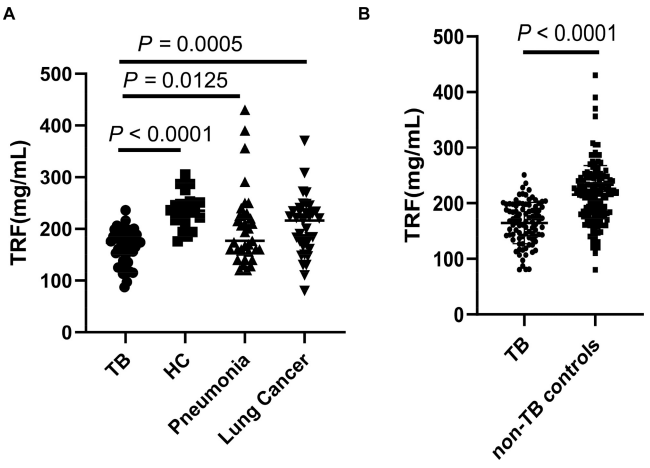

Figure 3. Serum levels of the TRF in the test set. (A) Serum levels of TRF were compared between the 46 TB patients, 20 healthy controls, 37 lung cancer patients and 35 pneumonia patients by ELISA and nephelometry. (B) Serum levels of TRF were compared between the 46 TB patients (TB) and 146 non-TB controls in the validation.

3.5. ROC Analysis. In order to evaluate the diagnostic value of the candidate biomarker TRF to distinguish TB from controls, we performed ROC curve analysis to obtain the area under the ROC curve (AUC) of TRF and the sensitivity and specificity based on the respective cut off value in patients with TB (n = 92), lung cancer (n = 37), pneumonia (35), non-TB controls (n = 146), and healthy controls (n = 74) in the validation. The AUC value of TRF was 0.647, 0.702, 0.894, and 0.792 to discriminate TB patients from pneumonia, lung cancer, HCs, and non-TB controls, respectively. In addition, TRF exhibited 90.22% sensitivity and 42.86% specificity in discriminating the TB group from the pneumonia group, 93.48% sensitivity and 47.16% specificity in discriminating the TB group from the lung cancer group, 91.30% sensitivity and 71.62% specificity in discriminating the TB group from all HCs, and 91.30% sensitivity and 58.90% specificity in discriminating the TB group from the non-TB controls, respectively (Figure 4, Table 2). These results revealed that TRF enabled higher diagnostic capacity in discriminating the TB group from all the other control groups.

4. DISCUSSION

It is still challenging to identify patients with active TB accurately and quickly, which is largely due to the limitations of the current diagnostic indicators used to distinguish TB from other diseases.[12] Several newly developed Mtb related molecules appeared to help us find biomarkers to distinguish active TB from chronic obstructive pulmonary disease (COPD), lung cancer, and latent tuberculosis infection (LTBI), such as microRNAs (miRNAs), long noncoding RNA (lncRNA), and circular RNA (circRNA).[13−18] As for nucleic acid amplification, XPert-MTB/RIF technology has high sensitivity and specificity in the diagnosis of TB, and it is convenient and rapid. However, there is the possibility of false positives, and it is impossible to distinguish dead bacteria from live bacteria.[19] However, low expression levels of these molecules existed in peripheral blood which may cause difficulty for detection. The technology for these small RNA requires complicated pretreatment methods and have limited clinical application value. Therefore, there is still a need for a diagnostic test for TB with high diagnostic efficiency, easy specimen acquisition, and less invasive procedures. It is known to all of us that it is a very effective method to diagnose TB by detecting peripheral blood biomarkers through sputum-free detection.

With the development and improvement of label-free quantitative proteomics technology, it is possible to study diseases from the whole protein expression level of body fluids, tissues or cells which can reflect the disease situation of the body so as to provide new specific molecular markers for early diagnosis of the disease and new clues for understanding the pathogenesis of the disease. Recently, serum protein biomarkers by proteomics technology for TB diagnosis have been widely studied. In particular. Xu et al.[20] identified three proteins including S100 calcium binding protein A9 (S100A9), extracellular superoxide dismutase [Cu−Zn], and matrix metalloproteinase 9 (MMP9) in serum acquired by iTRAQ-2DLC-MS/MS and ELISA and which may represent potential serological markers for TB to distinguish patients from HCs. Jiang et al.[21] found amyloid A (SAA), vitamin K-dependent protein Z (PROZ), and C4b-binding protein β (C4BPB) could discriminate the TB group from the healthy controls, pneumonia, COPD, and cured TB groups with high sensitivity and specificity. Liu et al.[22] evaluated proteins from severe TB, mild TB and by proteomic analyses and found α-1-acid glycoprotein 2 (ORM2), S100A9, interleukin-36 α (IL-36α) and superoxide dismutase (SOD1) were associated with the development of TB, and have the potential to distinguish between different stages of TB. Furthermore, Sun et al.[23] also detected distinct plasma protein biomarkers of TB, LTBI, and HCs by label-free quantitative proteomics technique. They established a new diagnostic model consisting of alpha-1-antichymotrypsin (ACT), alpha-1-acid glycoprotein (AGP1) and E-cadherin (CDH1) and it presented a relatively good capacity in discriminating TB patients from LTBI individuals. However, the available information on proteomics analysis

https://doi.org/10.1021/acsomega.2c00837
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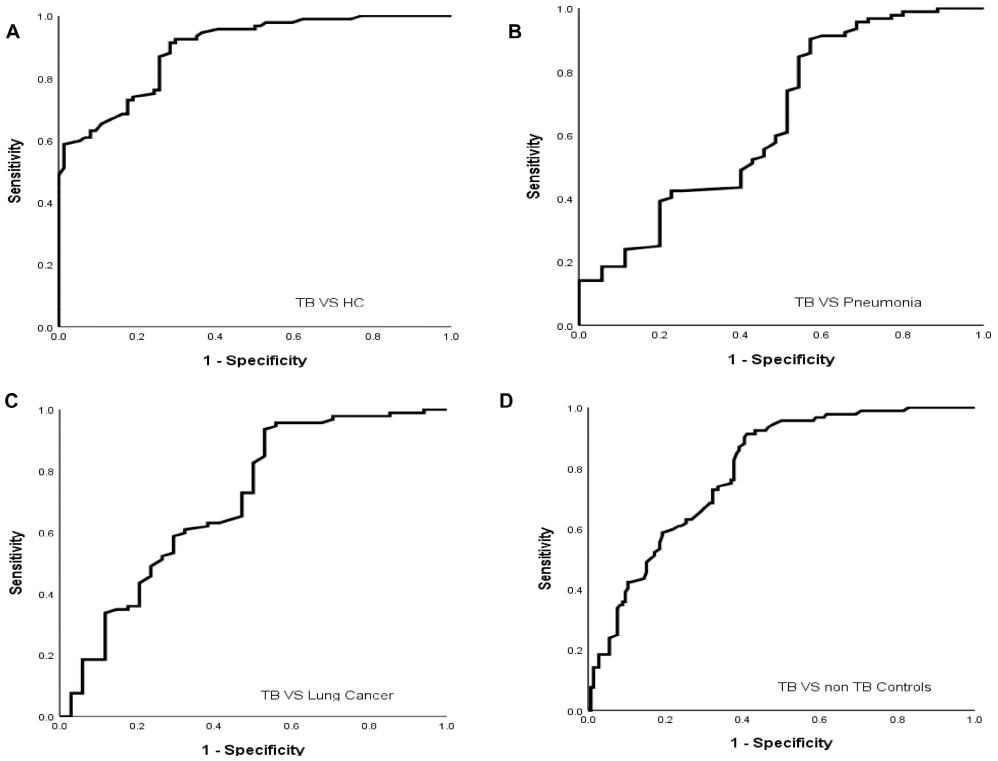

Figure 4. Efficacy of TRF in discriminating TB from healthy controls, lung cancer, pneumonia and non-TB controls. Receiver operating characteristic (ROC) curve analysis of TRF in discriminating TB (n = 92) from healthy controls (n = 74, panel A), lung cancer (n = 37, panel B), pneumonia (n = 35, panel C), non-TB controls (n = 146, panel D) in the validation.

Table 2. AUC Value, Sensitivity, and Specificity of TRF in Discriminating TB Patients from HCs, Lung Cancer, Pneumonia, and Non-TB Controls in the Training Set

TRF	sensitivity	specificity	AUC	95% CI	P value
TB vs pneumonia	90.22	42.86	0.647	0.557−0.730	0.0125
TB vs lung cancer	93.48	47.16	0.702	0.614−0.780	0.0005
TB vs HCs	91.30	71.62	0.894	0.836−0.936	<0.0001
TB vs non-TB controls	91.30	58.90	0.792	0.735−0.842	<0.0001

based on the cellular level between TB and HCs was limited until now. Most of these previous studies on TB biomarker screening focused on plasma or serum. Few studies had used this technique to characterize the proteins in lymphocytes or NEUs from TB patients. In TB, NEUs not only play a vital role as a first-line defense against pathogens after TB infection, but also participate in the TB-related tissue damage, resulting in the activation of LTBI.[8] However, neutrophils' protein biomarkers are rarely used, and their clinical value is rarely evaluated.

In the current study, we chose NEUs for the target to perform label-free quantitative proteomics technology. We finally identified 358 differentially expressed proteins including 279 up-regulated proteins and 79 down-regulated proteins in NEUs from TB patients compared with those from healthy controls (HCs) using label-free LC/MS methods. A total of 38 differentially expressed proteins involved in the progress of platelet degranulation were identified in TB patients and HCs. KEGG analysis revealed that proteins were involved in neutrophil mediated immunity, neutrophil degranulation, cute-phase response, humoral immune response and so on. Since we did not deplete any highly abundant proteins from the sample of neutrophils before LC-MS/MS analysis, some exosome proteins and highly abundant existing proteins maybe detected in our proteomics analysis.

Some researchers confirmed neutrophil-derived proteins maybe affect platelet function. Surface receptors P-selectin and PSGL-1, Glycoprotein Ibα (GPIbα) and αMβ2 integrin, αIIbβ3 integrin, Toll-like receptors (TLRs) could mediate platelet−neutrophil interactions. In addition, G-proteins, phospholipase C, Mitogen-activated protein kinases (MAPKs), Nuclear factor-κB (NF-κB) signaling and reactive oxygen species (ROS) could regulate the function of surface receptors required for platelet−neutrophil interactions.[24−26] Joshi et al.[27] elaborated that neutrophil-derived protein S100A8/A9 altered the platelet proteome in acute myocardial infarction and was associated with changes in platelet reactivity, potentially reflecting in vivo preactivation of platelets in thromboinflammatory states. To understand the secreted proteins from the neutrophils associated with the progress of platelet degranulation, further research was conducted to explore the focused six proteins. In the preliminary verification stage, the six secreted proteins TRF, AMG, AAT, AAG, AGP2 and A1BG were detected in serum of 134 TB patients compared with those in 138 HCs by ELISA and nephelometry. The serum expression of AAT, AAG, AGP2 and A1BG were higher expressed in the TB group than HCs while TRF were

https://doi.org/10.1021/acsomega.2c00837
ACS Omega 2022, 7, 24174−24183

lower expressed in the TB group than HCs. Actually, only the expression trend of TRF is consistent with the proteomic results, playing the roles of down-regulation in TB. The reason for the discrepancy of other proteins with proteomic results may be that the NEUs could secrete these proteins to the outside of the cell, resulting in the decrease of intracellular proteins and the increase of extracellular proteins after the activation. More studies are required to verify this supposition.

To investigate if TRF could distinguish TB from other respiratory system diseases, we further verified the diagnostic ability of TRF in an independent cohort of 46 patients with active TB, 20 HCs, 37 patients with lung cancer and 35 patients with pneumonia. Surprisingly, the serum expression levels of TRF in the TB group were significantly lower than the HCs, lung cancer, pneumonia group and non-TB groups ($P < 0.0001$, $P = 0.0005$, $P = 0.0125$, $P < 0.0001$). Transferrin is the major transferrin in plasma, providing iron required for cell differentiation and cell metabolism. Previous studies found that all cell growth was inseparable from transferrin.[28,29] Transferrin participated in many diseases such as iron deficiency anemia,[30] type 2 diabetes mellitus,[31] alzheimer's disease,[32] polycystic ovary syndrome (PCOS).[33] Specially, serum transferrin could reflect the iron status which was associated with poor treatment outcomes and mortality in TB.[34] Furthermore, serum transferrin could also be seen as markers to predict the mortality rate and discriminate TB from other diseases. Dai et al.[35] investigated biomarkers of iron homeostasis, including serum iron, ferritin and transferrin in HCs, and patients with TB, LTBI, non-TB pneumonia and cured TB (RxTB) and built a TB prediction model to best discriminate TB from HC, LTBI, RxTB and pneumonia in a large cohort of patients. Bapat et al.[36] explored the proteome changes of the host serum in response to Mtb infection by one-dimensional electrophoresis in combination with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). They showed alpha-2-macroglobulin (A-2-M), serotransferrin and haptoglobin were up-regulated in the malnourished patients with active TB and down-regulated in the malnourished patients compared with the healthy controls, which may be clinically relevant host biomarkers for TB diagnosis and disease progression in the malnourished population.

Moreover, a previous study also determined that transferrin levels were significantly lower in PTB and household contacts compared with HCs.[37] Sun et al[23] also confirmed plasma transferrin levels were significantly decreased in the PTB group samples than that in the and HC group, and the AUC value was 0.723. Consistent with these studies, our studies found serum transferrin levels were significantly lower in TB than healthy controls, pneumonia and lung cancer patients which indicated TRF may be promising markers to explore the pathogenesis of TB. In addition, ROC curve analysis showed that TRF could distinguish TB from other diseases well. The AUC value of TRF was 0.647 with 90.22% sensitivity and 42.86% specificity in discriminating the TB group from the pneumonia group, 0.702 with 93.48% sensitivity and 47.16% specificity in discriminating the TB group from the lung cancer group, 0.894 with 91.30% sensitivity and 71.62% specificity in discriminating the TB group from all HCs and 0.792 with 91.30% sensitivity and 58.90% specificity in discriminating the TB group from the non-TB controls.

To our knowledge, our study is the first to profile proteins fingerprinting from the perspective of NEUs in TB patients to identify candidate MTB secreted serum biomarkers using label-free LC/MS. However, there are still some limitations in our study. First, the sample sizes in the discovering set were not large enough, and the sample size in the test set was also small. Therefore, further studies for verifying the diagnostic performance of the regression model with larger sample size are required in the future. Second, there was a lack of controls with diverse pulmonary diseases in the discovery set, although pneumonia and lung cancer patients were included in the test set. Therefore, more inflammatory diseases (pneumonia, idiopathic pulmonary fibrosis (IPF), COPD. etc.) should be included as disease control group in the discovering set for further study. Finally, in the training set, we only selected a few down-regulated proteins. Therefore, it is necessary to research other up-regulated proteins in the future.

5. CONCLUSION

Our study uncovered proteomic profiles of neutrophils from TB patients and HC individuals, and identified 358 differentially expressed proteins between TB patients and HC individuals by label-free LC/MS. Five potential secreted diagnostic protein biomarkers associated with the process of platelet degranulation (TRF, AAT, AAG, AGP2 and A1BG) were significantly different in serum of TB patients from HCs. Furthermore, the serum expression of TRF was lower expressed in the TB patients than lung cancer, pneumonia, HCs and non-TB controls, and had high diagnostic capacity in discriminating the TB patients than others. The results of this proteomic analysis provide new information about the pathogenesis molecules existing in the neutrophils of patients with pulmonary tuberculosis and provide candidate biomarkers for the diagnose of pulmonary tuberculosis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00837.

Table S1: The detailed information on 358 differential proteins in P-NEU and H-NEU group (Filtered with threshold value of expression fold change >2 or <0.5 and P value <0.05); Figure S1: Quality assessment; Figure S2: The repeatability of the quantitation proteins between P-NEU and H-NEU group (PDF)

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https://doi.org/10.1021/acsomega.2c00837
ACS Omega 2022, 7, 24174−24183

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Project for Academic and Technical Leaders of Major Disciplines of Jiangxi Province (Grant No. 20172BCB22026), the project for high end talent of Science and Technology Innovation in Jiangxi "double thousand plan" (Grant No. jxsq2019201024, jxsq2019201094), the grants from the National Natural Science Foundation of China (Grant No. 82160308, 32060181), Science Fund for Distinguished Young Scientists of Jiangxi Province (Grant no. 20212ACB216006), the Science and Technology Plan of Jiangxi Provincial Health Commission (Grant no. 202130243, 202210217).

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ACS Omega 2022, 7, 24174−24183