Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Serum proteomics reveals the proteomic characteristics involved in the progressive feature of acute ischemic stroke (AIS)

Minchao Lai
The First Affiliated Hospital of Shantou University Medical College
Xiaojun Zhang
Shantou University
Danya Zhou
Shantou University Medical College
Xiaojuan Zhang
Shantou University Medical College
Mengting Zhu
Shantou University Medical College
Qingxian Liu
Shantou University Medical College
Dian Wang (g_dwang@stu.edu.cn)
STU

Research

Keywords: acute progressive ischemic stroke (APIS), acute non-aggressive ischemic stroke (ANPIS), serum proteomics, biomarkers, serum amyloid A1 (SAA1), S100 calcium binding protein A9 (S100-A9)

Posted Date: November 8th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1040149/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Abstract

Background

To distinguish acute progressive ischemic stroke (APIS) from acute non-progressive ischemic stroke (ANPIS) is clinically critical for the precise treatment and prevention of acute ischemic stroke (AIS) deterioration. Serum proteins could reflect the unique pathophysiological processes of APIS. It is expected to find serum protein biomarkers to identify APIS at the early state, especially when typical symptoms have not been present in conventional clinical indices.

Methods

We recruited 178 subjects, including 133 AIS patients and 45 healthy controls. The discovery set, which included 10 age- and sex- matched cases from each of the APIS, ANPIS and control groups, was used for serum proteomic analysis with a mass spectrometry-based proteomics. The disrupted proteins of APIS were screened, of which, those common to ANPIS and specific to APIS were particularly concerned. Potential biomarkers of APIS were screened based on the theoretical knowledge to explain the mechanism and diagnostic value, and subsequently tested in the validated set (25 APIS patients, 88 ANPIS patients, and 35 controls).

Results

We found that 46 serum proteins were disturbed in the APIS patients; 23 differentially expressed proteins (DEPs) were common to these two groups, and 23 DEPs were associated with APIS alone. Enrichment analysis suggested that complement activation, cytolysis, proteolysis, positive regulation of fibrinolysis, blood coagulation were specifically associated with APIS; while inflammatory response, neutrophil chemotaxis, oxygen transport, and positive regulation of cell death process were common to AIPS and ANPIS. Serum amyloid A1 (SAA1) and S100 calcium binding protein A9 (S100-A9) jointly conferred a moderate value (AUC = 0.799) to diagnose APIS and to distinguish APIS from ANPIS (AUC = 0.699).

Conclusions

This study provides human serum proteomic evidence that inflammation, oxidative stress, and necrosis in APIS are common to ANPIS; the progressive feature of APIS may be mainly associated with complement activation, more serious inflammation, and lipid metabolic disorder; SAA1 and S100-A9 may be considered as potential biomarker panel of APIS.

Background

Worldwide, stroke is the second leading cause of death, responsible for approximately 10% total mortality; and it is also the major cause of disability, accounting for almost 5% of all disability-adjusted life-year [1, 2]. Ischemic stroke is the most common subtype of stroke, and it often occurs urgently and appears as a sudden loss of brain function due to the reduction or even total blockage in blood flow to a specific area of the brain [1, 3]. Acute progressive ischemic stroke (APIS) is a common and serious clinical subtype of acute ischemic stroke (AIS), characterized as gradual progression and worsening in stroke symptoms [4]. Nowadays, substantial improvement has been achieved in AIS treatment since the application of intravenous thrombolysis and endovascular clot retrieval, which can remove the block and restore blood flow to the insulted brain tissues [5, 6]. However, neurologists are still facing with huge challenges from high morbidity and mortality of AIS, partly owing to poor outcome in the treatment of APIS [7]. Nevertheless, the underlying mechanism of APIS remains unclear, which deserves further studies.

Proteomics is an effective strategy to comprehensively explore the pathophysiological mechanism of disease, and it has been widely applied in clinical and basic medicine researches [8]. In recent years, proteomics has been used as an effective way to identify proteins involved in the pathogenesis of stroke [9]. However, few studies have explored the mechanism of AIS at serum proteome level [10]. Serum is the predominant specimen for clinical studies as it can be easily accessed; it contains measurable potential protein biomarkers, uncovering pathological changes that are associated with the mechanism of disease. Therefore, serum proteomic analysis is helpful in clarifying the mechanism of APIS and finding its novel potential biomarkers.

Here, we carried out a serum proteome investigation on patients with acute progressive ischemic stroke (APIS) and acute non-progressive ischemic stroke (ANPIS), and age- and sex- matched controls (10 cases per group). Differentially expressed proteins (DEPs) among these three groups were screened; where the disturbed proteins of APIS common to ANPIS and those associated with APIS alone were particularly

concerned. Based on the related DEPS, enrichments were analysed, attempting to explore the underlying pathophysiological processes of APIS. Potential biomarkers of APIS were identified based on the theoretical knowledge to explain the mechanism and diagnostic value; the latter was subsequently tested in the validated set.

Materials & Methods

The subjects

This study was approved by the ethics committee of Shantou University Medical College, and written informed consent was obtained from all the subjects. Totally, 178 subjects were enrolled in this study, which included 45 controls and 133 patients with AIS. The AIS patients were further divided into acute progressive ischemic stroke (APIS) group and acute non-progressive ischemic stroke (ANPIS) group according to their clinical conditions. The enrolled criterion of the patients was the acute phase of ischemic stroke (recruited within seven days of the onset of ischemic stroke symptoms); those with progression of ≥ two points on the National Institute of Health Stroke Scale (NIHSS) in the initial five days after stroke onset were considered as APIS [4]. The exclusion criteria included: (i) the presence of severe renal and liver diseases, malignancy, infection, trauma, autoimmune diseases, and hypothyroidism; and (ii) inability to comply with neuroimaging examination. The controls were relatively healthy subjects, which were enrolled with the same exclusion criteria as those of the patients. The NIHSS and mRS scores were calculated at admission and/or discharge to assess the severity of AIS and therapeutic outcome, as well as to assist us in distinguishing the APIS patients from ANPIS ones. The subjects' basic clinical information, including gender, age, complications and data of relative clinical examinations, was collected or evaluated during the hospitalization.

Thirty subjects, namely, ten sex- and age-matched cases from each group, were selected as the discovery set and used for serum proteomic analysis. Other subjects, which included 25 APIS patients, 88 ANPIS patients, and 35 controls were used as the validation set to assess the diagnostic value of the potential biomarkers. The detailed clinical characteristics of all subjects are described in Table 1 and Table 2.

Sample collections

The fasting blood samples of all subjects were obtained by venipuncture and collected in glass tubes containing coagulant. The serum was separated by centrifugation at 3000 rpm for five min at four °C and stored at -80 °C until use.

Serum protein extraction and digestion

Serum samples in the discovery set were used for the proteomic analysis. Briefly, total protein concentration in the serum was measured by the bicinchoninic acid (BCA) method (Beyotime, China). Then, the High-Select^{TM} HAS/Immunoglobulin Depletion Resin kit (Thermo Scientific, USA) was used to remove human serum albumin (HSA) and immunoglobulin in the serum according to the protocol provided by the manufacturer. Thereafter, 50 μg of protein from each sample was treated with filter-aided sample preparation (FASP) methods, followed by reduction, alkylation with dithiothreitol (DTT, 10 mM) and iodoacetamide (IAA 50 mM), respectively. Then, the Sequencing Grade Modified Trypsin (Promega, USA), with a protease: protein ratio of 1:50 (w/w), was used to digest the protein at 37 °C overnight. The resultant peptides were subsequently desalted on Pierce™ C18 Tips (Thermo Scientific, USA) and dried under vacuum centrifugation in succession and stored at -80 °C until detection.

LC-MS/MS analysis

An LTQ-Orbitrap mass spectrometry platform, coupled with EASY-nLC 1000 liquid chromatography (Thermo Scientific™), was used for serum proteomic analysis. An analytical column (75 μm ID with 15 μm tip, 10.5 cm bed length, Reprosil-PUR C18 3 μm 120 Å, New Objective) was utilized for analytical separation with eluent A (0.1% formic acid in water) and eluent B (0.1% formic acid in acetonitrile). For each sample, we loaded 2 μg of desalted peptides onto the column. The flow rate was 300 nL min $^{-1}$. The gradient was 5-9% eluent B for 2 min, 9%-27% eluent B for 90 min, 27%-40% eluent B for 13 min, 40%-90% eluent B for 5 min, and 90%-100% eluent B for 10 min. First stage mass spectrum data (scan range 350-1800 m/z) were acquired from the Orbitrap at resolution of 60,000. The AGC target was set as 3e6 with a maximum injection time of 200 ms. Top 20 multiply charged precursor ions were selected for collision induced dissociation (CID) analysis in the linear ion trap with centroid data type. Activation times were 30 ms for CID fragmentation with normalized collision energy of 35.0 [11].

Label-free quantification

Proteomic data were analyzed by the MaxQuant software (Version 1.6.6.0). Proteins were identified by searching MS and MS/MS data of peptides against a decoy version of the UniprotKB (June 2020). The search parameters were specified as follows: the mass tolerance was set to 20 ppm for precursor ions and 20 mmu for fragment ions. Trypsin was specified as the digesting enzyme, and two missed cleavages were allowed. A false positive detection rate (FDR) was calculated using a decoy database search. The results were filtered using the

following settings: only highly confident peptides, with a global FDR < 1% based on the target decoy approach, were included in the results. The most confident centroid method was used with an integration window of 20 ppm. For protein quantitation, only unique peptides were used to quantify proteins.

Bioinformatic analysis

Differentially expressed proteins (DEPs) were selected in R (Version 3.6) with the criteria of *p*-value < 0.05 and fold change > 1.2 or < 0.83 [12], which would be used for Gene Ontology (GO) and pathway enrichment analysis in the DIVID (https://david.ncifcrf.gov/), Metascape (https://metascape.org), and String (https://string-db.org/) database. The significant biological processes (BPs), molecular functions (MFs), cellular components (CCs), and pathways in enrichments were illustrated by GraphPad Prism8.0 and aligned with Microsoft Visio 2013.

Validation of potential biomarkers of APIS

After screening, serum amyloid A1 (SAA1) and S100 Calcium-Binding Protein (S100-A9) were selected as potential biomarkers for APIS. Human Serum Amyloid A1 DuoSet ELISA kit (R&D: DY3019-05) and Human S100-A9 Heterodimer DuoSet ELISA kit (R&D: DY8226-05) were used to measure the absolute concentration of SAA1 and S100-A9 in the serum of the validation set, respectively, following the protocol provided by the manufacturer.

Data processing

Continuous variables are presented as mean ± SEM and categorical variables are presented as number or percentage. Differences between two groups were assessed by using the Student's *t*-test or $\chi 2$ test. One-way ANONA was performed for comparisons among three groups. Statistical analysis was carried out using SPSS version 17.0 (IBM Corp). A *p*-value of < 0.05 was considered statistically significant.

Binary logistic regression was performed to analyze the synergetic effect of several potential biomarkers. The receiver operating characteristic curve (ROC) analysis was performed by using the MedCalc statistical software for selecting the optimal cut-off point for each potential biomarker, where area under the curve (AUC), specificity, and sensitivity were used to evaluate their diagnostic value.

Results

Clinical Features of the Subjects

A total of 178 subjects, including 133 AIS patients and 45 healthy controls, were enrolled in this study. The discovery set contained 10 age- and sex- matched cases from each of the APIS, ANPIS and control groups. Basic clinical information showed that the APIS patients had higher incidence of atrial fibrillation, history of arrhythmias, and intracranial artery stenosis than the ANPIS patients (30% *vs.* 0%, 70% *vs.* 20%, and 70% *vs.* 30%, respectively). In addition, compared with the ANPIS patients, the APIS patients also had higher NIHSS scores at admission and discharge (7.5 *vs.* 2.2, 11.9 *vs.* 1.6, respectively). Noticeably, even receiving conventional treatments, APIS patients' NHISS and mRS (modified Rankin Scale) scores have still remarkably increased (7.5→11.9), particularly during the worsening of stroke symptoms (NHISS=13.7). Consequently, the APIS patients required longer hospitalization than the ANPIS patients (19.2 *vs.* 9.3).

The APIS patients also exhibited elevated serum levels of (i) white blood cell, neutrophils ratio (%), erythrocyte sediment rate (ESR), C-reactive protein (CRP) and hs-CRP, indicative of more serious inflammation and stress condition during APIS; (ii) homocysteine, suggesting dysfunction or disturbance in coagulation, platelet aggregation, and blood lipid metabolism; and (iii) BUN and Cr, implying kidney dysfunction (Table 2). Therefore, clinically, the APIS patients had more critical conditions and worse prognosis than the ANPIS patients.

The validation set, which included 25 APIS patients, 88 ANPIS patients, and 35 controls, had similar clinical characters as their counterparts in the discovery set (Table1 and Table 2).

The basic results of proteomic analysis

Totally, 796 proteins were successfully identified in the serum samples of the subjects after removing the high-abundant proteins (Fig. 1). Of these, 161 proteins that had detailed quantitative information and annotation terms were used to screen DEPs (Table S1). As a result, 46 proteins were disturbed in AIPS, of these, 23 were common to ANPIS, and the other 23 were specific to APIS (Table 3).

APIS proteomic profile common to that of ANPIS

To obtain comprehensive insight into the proteomic feature of APIS that common to ANPIS, we made GO and pathway enrichment analyses of the common DEPs (13 were upregulated and 10 were downregulated) between these two groups. The significant terms in the enrichments are given in Fig. 2 and Table S2.

The significant BPs mainly focused on platelet degranulation, innate immune response, acute-phase response, oxygen transport, hydrogen peroxide catabolic process, neutrophil chemotaxis, cellular oxidant detoxification, phosphatidylcholine metabolic process, leukocyte migration involved in inflammatory response, and positive regulation of cell death (Fig. 2 and Table S2). Accordingly, the significant MFs mostly focused on oxygen transporter activity, oxygen binding, protease binding, aryl esterase activity, antioxidant activity, peroxidase activity, immunoglobulin receptor binding, and serine-type endopeptidase activity. The significant CCs were largely related to blood microparticle, exosome, platelet alpha granule lumen, extracellular matrix, and external side of plasma membrane. The network chart of enriched ontology cluster illustrates that hydrogen peroxide catabolic process, acute-phase response and humoral immune response are the most significant pathways (Fig. 3).

The proteomic feature specifically associated with APIS

The current study has found that 23 DEPs were specific to APIS; of these, 13 were upregulated and 10 were downregulated (Table 3). The crucial mechanistic factors leading to progressive quality of APIS were analyzed using the aforementioned strategy.

The significant terms included complement activation, proteolysis, positive regulation of fibrinolysis, negative regulation of very-low-density lipoprotein particle clearance, cholesterol transport, receptor-mediated endocytosis, plasminogen activation, phospholipid efflux, intrinsic pathway blood coagulation, serine-type endopeptidase activity, lipase inhibitor activity, complement binding, insulin-like growth factor binding, protein complex binding, and fibronectin binding, extracellular region, extracellular space, extracellular exosome, blood microparticle, membrane attack complex, chylomicron, and lipoprotein particle (Fig. 2 and Table S3). In addition, the network chart of enriched ontology clusters indicates complement and coagulation cascades, neutrophil degranulation, and blood coagulation response are the most significant functions involved in the worsening progress of AIS symptoms (Fig. 3).

Diagnostic potential of SAA1 and S100-A9

In this study, potential serum protein biomarkers were selected by the following strategies. Firstly, DEPs with high fold change between the APIS patients and controls were chosen. Then, those proteins that could reasonably explain the pathophysiological mechanism of APIS were given a priority consideration. As a result, SAA1 and S100-A9, both met the two requirements, were selected as potential biomarkers, and then their absolute concentrations were measured to analyze the diagnostic value. The mean concentrations of SAA1 in the APIS, ANPIS, and control groups were 373.59 ng/mL, 284.58 ng/mL, and 199.45 ng/mL, respectively, which were significantly different among these three groups (Fig. 4). The SAA1 contents in the AIS patients correlated with several clinical indicators (Table 4). The concentrations of S100-A9 in these three groups were 734.44 pg/mL, 658.74 pg/mL, and 607.05 pg/mL, respectively. SAA1 demonstrated good potential to identify APIS (AUC = 0.729, p=0.002, cut-off value > 386.72 ng/mL) and to distinguish APIS from ANPIS (AUC =0.635, p = 0.036, cut-off value > 233.25 ng/mL). Although the contents of S100-A9 were not significantly different among these three groups; however, its content in the APIS group was the highest among them, and it illustrated moderate potential to diagnose APIS (AUC = 0.675, p = 0.013). Lastly, to determine whether these biomarkers had synergetic effect to diagnose APIS or AIS, we performed a binary logistic regression analysis and found that they jointly yielded AUC of 0.799, sensitivity of 63.6%, and specificity of 92.6% to diagnose APIS; moreover, they jointly yielded AUC of 0.699, sensitivity of 72.7%, and specificity of 69.0% to distinguish APIS from ANPIS (Fig. 4).

Discussion

The prognosis of AIS is still not so desirable, which could largely be attributed to critical conditions and worse consequence in the APIS patients [13]. A better understanding of the pathogenesis is a prerequisite for improving clinical outcome in APIS therapy. Proteomics enables comprehensive explanation of the pathophysiological mechanism of disease. Serum is more feasible in clinical practices; their combination, namely, serum proteomics introduces a promising strategy for mechanistic understanding of molecular events in APIS. However, serum proteome analysis faces the interference from high-abundant proteins [14]. Removing of high-abundant proteins is likely to destroy other small molecular proteins. Our study exemplifies a good try using serum proteomics to explore the mechanism of APIS. After removing high-abundant proteins, we have identified 796 proteins. Of these, 59 proteins were differentially expressed between the AIS (APIS and ANPIS) patients and controls (Table 3), which concealed a rich information associated with the pathophysiological processes of AIS, especially with those of APIS.

Potential Biomarkers Of Apis

Biomarkers of disease or pathophysiological process should meet two requirements. First, they are able to explain the mechanism of disease reasonably. Second, they have a good diagnostic potential to identify the onset of disease and (or) to evaluate the prognosis of disease. From the perspective of mechanism, we found that APIS has more serious inflammation than ANPIS. Consistently, SAA1 and S100-A9, both represented inflammation and stress [24], and had higher fold changes in APIS, were selected as potential biomarkers of APIS.

SAA1 is the major component of amyloid. Inducible expression of SAA1 is a hallmark of the acute-phase response of vertebrates to environmental challenges such as tissue injury and surgery [25]. Following inflammatory response, the liver secretes a large amount of SAA1, leading to a marked increase in SAA1 level [24]. Accumulating evidence has revealed that SAA1 plays an important role in the regulation of inflammation, lipid metabolism, and propagation of the primordial acute phase response [26]. The current data showed that the APIS patients had higher SAA1 content than the ANPIS and controls, and thereby indicating more serious inflammation and ischemia-related injury in the APIS setting. Consistently, SAA1 content demonstrated a moderate potential to diagnose APIS and to distinguish APIS from ANPIS (Fig. 3).

S100-A9 is a calcium- and zinc-binding protein, which is commonly combined with S100A8 as a complex. The complex has been found to be highly expressed in the neutrophil, monocyte, extracellular milieu under inflammatory conditions, and to be involved in multiple I/R-induced stress responses and suppression of mitochondrial function [27]. Here, we found that S100-A9 concentration in the APIS was the highest among the three groups. In support, the clinical data demonstrated that the APIS patients had higher levels of inflammatory indicators (ESR, WBC and neutrophils percentage) than the ANPIS patients, suggestive of much more serious inflammation in the setting of APIS. In terms of diagnostic value, S100-A9 showed moderate potential to diagnose APIS (Fig. 3).

In addition, we found that the combination of SAA1 and S100-A9 illustrated a higher diagnostic value than either of them (Fig. 3). Although SAA1 and S100-A9 had been found to play critical roles in other clinical conditions (e. g. myocardial infarction, infection, etc.) [27]; however, the current study provides the possibility to increase the diagnostic specificity when used these two proteins simultaneously.

Conclusions

In this study, we have described a common landscape of serum proteome between APIS and ANPIS, which are mainly associated with inflammation, oxidative stress, and necrosis. More importantly, we have further identified a serum proteomic feature may be involved in the progressive feature of APIS, which are largely relevant to complement activation, more serious inflammation, and lipid metabolism disturbance. SAA1 and S100-A9 may serve as potential serum biomarker panel to diagnose APIS and to distinguish APIS from ANPIS. These findings confer potential novel therapeutic targets and diagnostic indicators for APIS.

Abbreviations

APIS, acute progressive ischemic stroke; ANPIS, acute non-progressive ischemic stroke; AIS, acute ischemic stroke; DEP, differentially expressed protein; GO, gene ontology; SAA1, Serum amyloid A1; S100-A9, S100 calcium binding protein A9; ROC, the receiver operating characteristic curve; AUC, area under the curve.

Declarations

Ethics approval and consent to participate: The study was carried out in conformity with the ethical standards established in the 1964 Declaration of Helsinki and its later amendments and was approved by the Ethics Committee of Shantou University Medical College (No. SUMC-2021-76-K). Informed consent was obtained from all study subjects.

Consent for publication: All authors consent to the publication of this manuscript.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no conflict of interest.

Funding: This study was supported by the Shantou science and technology plan project (Shan Science Government No: [2017]166-03).

Authors' contributions: Dian Wang, XiaoJun Z, Danya Zhou, Xiaojuan Zhang performed the experiments. Dian Wang and Minchao Lai wrote the manuscript. Minchao Lai and Dian Wang analyzed the data. Qingxian Liu, Mengting Zhu, Xiaojuan Zhang, Dian Wang and Minchao Lai collected the serum and clinical information. Dian Wang designed the study and takes responsibility for the work in its entirety.

Acknowledgements: The authors acknowledge the study subjects for their participation in the study.

References

1. Khoshnam SE, Winlow W, Farzaneh M, Farbood Y, Moghaddam HF. Pathogenic mechanisms following ischemic stroke. Neurological sciences: official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology. 2017;38(7):1167–86.
2. Collaborators GBDLRoS, Feigin VL, Nguyen G, Cercy K, Johnson CO, Alam T, et al. Global, Regional, and Country-Specific Lifetime Risks of Stroke, 1990 and 2016. The New England journal of medicine. 2018;379(25):2429–37.
3. Phipps MS, Cronin CA. Management of acute ischemic stroke. Bmj. 2020;368:l6983.
4. Philipps J, Thomalla G, Glahn J, Schwarze M, Rother J. Treatment of progressive stroke with tirofiban--experience in 35 patients. Cerebrovascular diseases. 2009;28(5):435–8.
5. Powers WJ. Acute Ischemic Stroke. The New England journal of medicine. 2020;383(3):252–60.
6. Campbell BCV, De Silva DA, Macleod MR, Coutts SB, Schwamm LH, Davis SM, et al. Ischaemic stroke. Nature reviews Disease primers. 2019;5(1):70.
7. Kwan J, Hand P. Early neurological deterioration in acute stroke: clinical characteristics and impact on outcome. QJM: monthly journal of the Association of Physicians. 2006;99(9):625–33.
8. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature. 2003;422(6928):198–207.
9. Zhou A. Proteomics in stroke research: potentials of the nascent proteomics. Journal of investigative medicine: the official publication of the American Federation for Clinical Research. 2016;64(8):1236–40.
10. Penn AM, Saly V, Trivedi A, Lesperance ML, Votova K, Jackson AM, et al. Differential Proteomics for Distinguishing Ischemic Stroke from Controls: a Pilot Study of the SpecTRA Project. Translational stroke research. 2018;9(6):590–9.
11. Xie D, Wu J, Wu Q, Zhang X, Zhou D, Dai W, et al. Integrating proteomic, lipidomic and metabolomic data to construct a global metabolic network of lethal ventricular tachyarrhythmias (LVTA) induced by aconitine. Journal of proteomics. 2021;232:104043.
12. Huang T CM, Vitek O. MSstatsTMT: Protein Significance Analysis in shotgun mass spectrometry-based proteomic experiments with tandem mass tag (TMT) labeling. R package version 1.4.3, http://msstats.org/msstatstmt/. 2019.
13. Seners P, Baron JC. Revisiting 'progressive stroke': incidence, predictors, pathophysiology, and management of unexplained early neurological deterioration following acute ischemic stroke. Journal of neurology. 2018;265(1):216–25.
14. Lee PY, Osman J, Low TY, Jamal R. Plasma/serum proteomics: depletion strategies for reducing high-abundance proteins for biomarker discovery. Bioanalysis. 2019;11(19):1799–812.
15. Zheng Y, Zhou Z, Han F, Chen Z. Special issue: Neuroinflammatory pathways as treatment targets in brain disorders autophagic regulation of neuroinflammation in ischemic stroke. Neurochemistry international. 2021;148:105114.
16. Yang Y, Han Y, Sun W, Zhang Y. Increased systemic immune-inflammation index predicts hemorrhagic transformation in anterior circulation acute ischemic stroke due to large-artery atherosclerotic. The International journal of neuroscience. 2021:1–7.
17. Nash KM, Schiefer IT, Shah ZA. Development of a reactive oxygen species-sensitive nitric oxide synthase inhibitor for the treatment of ischemic stroke. Free radical biology & medicine. 2018;115:395–404.
18. Allen CL, Bayraktutan U. Oxidative stress and its role in the pathogenesis of ischaemic stroke. International journal of stroke: official journal of the International Stroke Society. 2009;4(6):461–70.
19. Yang Y, Zhu L, Zhang B, Gao J, Zhao T, Fang S. Higher Levels of C-Reactive Protein in the acute phase of stroke Indicate an Increased Risk for Post-stroke Depression: A Systematic Review and Meta-Analysis. Neuroscience and biobehavioral reviews. 2021; S0149-7634(21)00361-4.
20. Zhang X, Yin J, Shao K, Yang L, Liu W, Wang Y, et al. High serum complement component C4 as a unique predictor of unfavorable outcomes in diabetic stroke. Metabolic brain disease. 2021; Sep 4. Online ahead of print.
21. Pekna M, Stokowska A, Pekny M. Targeting Complement C3a Receptor to Improve Outcome After Ischemic Brain Injury. Neurochemical research. 2021; 46(10):2626–2637..
22. Alawneh JA, Moustafa RR, Baron JC. Hemodynamic factors and perfusion abnormalities in early neurological deterioration. Stroke. 2009;40(6):e443-50.
23. Nakamura A, Otani K, Shichita T. Lipid mediators and sterile inflammation in ischemic stroke. International immunology. 2020;32(11):719–25.
24. Yu J, Zhu H, Taheri S, Mondy W, Bonilha L, Magwood GS, et al. Serum Amyloid A-Mediated Inflammasome Activation of Microglial Cells in Cerebral Ischemia. The Journal of neuroscience: the official journal of the Society for Neuroscience. 2019;39(47):9465–76.

25. Azurmendi L, Lapierre-Fetaud V, Schneider J, Montaner J, Katan M, Sanchez JC. Proteomic discovery and verification of serum amyloid A as a predictor marker of patients at risk of post-stroke infection: a pilot study. Clinical proteomics. 2017;14:27.
26. Chang C, Pan Y, Du H, Wang X, Li X. Serum amyloid A1 can be a novel biomarker for evaluating the presence and severity of acute coronary syndrome. Clinical biochemistry. 2020;85:27–32.
27. Li Y, Chen B, Yang X, Zhang C, Jiao Y, Li P, et al. S100a8/a9 Signaling Causes Mitochondrial Dysfunction and Cardiomyocyte Death in Response to Ischemic/Reperfusion Injury. Circulation. 2019;140(9):751–64.

Tables

Table 1. Basic information of the subjects

Indices	The discovery set (n = 30)				The validation set (n = 148)			
	APIS	ANPIS	Controls	Comp*	APIS	ANPIS	Controls	Comp*
	n = 10	n = 10	n = 10		n = 25	n = 88	n = 35	
Age	67.0 ± 9.3	65.7 ± 11.0	65.8±6.0	N	66.7 ± 11.2	65.5 ± 11.3	53.5 ± 14.4	N
Sex	5/5(M/F)	5/5(M/F)	5/5(M/F)	N	17/5	57/31	21/14	N
Hypertension (Y/N)	7/3	8/2	3/7	N	13/12	32/56	N	N
Diabetes mellitus (Y/N)	4/6	4/6	3/7	N	2/23	10/78	N	N
Atrial fibrillation (Y/N)	3/7	0/10	0/10	N	1/24	1/87	N	N
History of arrhythmias (Y/N)	7/3	2/8	0/10	p = 0.025	0/25	0/88	N	N
Intracranial artery stenosis (Y/N)	7/3	3/7	NA	N	18/7	44/44	NA	p = 0.041
NIHSS-Admission	7.5 ± 2.2	2.2 ± 0.9	NA	p = 0.006	6.3 ± 3.7	3.7 ± 3.3	NA	p = 0.002
NIHSS-Discharge	11.9 ± 11.7	1.6±1.6	NA	p < 0.001	6.1 ± 4.2	2.7 ± 2.1	NA	p = 0.007
NIHSS-progressive	13.7 ± 11.3	NA	NA	NA	9.7 ± 5.2	3.9 ± 0.9	NA	p < 0.001
mRS-Discharge	3.3 ± 1.7	1.7±0.9	NA	p = 0.010	3.2 ± 1.5	1.7 ± 1.3	NA	p < 0.001
Recurrent (Y/N)	2/8	3/7	NA	N	1/24	1/87	NA	N
Days of hospitalization (d)	19.2 ± 4.6	9.3± 2.9	NA	p < 0.001	15.5 ± 8.9	10.3 ± 4.9	NA	p < 0.001
Time from stroke onset (h)	24.3 ± 9.7	20. 5± 7.7	NA	N	41.6 ± 33.1	54.4 ± 26.8	NA	N

APIS, acute progressive ischemic stroke group (n=10); ANPIS, acute non-progressive ischemic stroke group (n=10); controls, the control group (n=10); *comparisons within the same cohort, between the APIS group and ANPIS group except sex and age. NIHSS-Ad, NIHSS score at admission; NIHSS-Dis, NIHSS score at discharge; NIHSS-progressive, NIHSS score when stroke symptoms deteriorate or progressive; mRS, modified Rankin Scale; d, day; Y/N, Yes/no; h, hour; NA, not available or not applicable. N, not significantly different.

Table 2. Blood biochemical indices of the AIS patients

AAIS, acute progressive ischemic stroke; ANAIS, acute non-progressive ischemic stroke; Com*, t-test. #, the subjects' information was the same as table1.

Indices	The discovery set #			The validation set #		
	APIS	ANPIS	Comp*	APIS	ANPIS	Comp*
White blood cell ($\times 10^9$)	8.46 ± 2.21	7.41 ± 2.52	N	8.42 ± 2.34	7.80 ±2.30	N
Neutrophils (%)	72.63 ± 9.21	67.11 ± 12.10	N	71.89 ± 3.57	64.18 ± 3.88	P < 0.001
Complement C3 (g/L)	0.96 ± 0.05	0.73 ± 0.14	N	0.90 ± 0.13	0.87 ± 0.23	N
Complement C4 (g/L)	0.22 ± 0.02	0.14 ± 0.02	p = 0.048	0.24 ± 0.08	0.19 ± 0.07	N
Homocysteine (μmol/L)	35.77 ± 5.33	14.27 ± 3.79	N	23.36 ±34.65	18.50 ± 36.32	N
Fasting blood glucose (mmol/L)	8.25 ± 4.04	6.42 ± 2.24	N	7.60 ± 4.03	6.33 ± 2.19	N
Erythrocyte sediment rate (mm/h)	32.88 ± 23.27	27.57± 9.50	N	18.25 ±10.77	15.4 ± 9.71	N
C-reactive protein (mg/L)	18.33 ± 1.87	12.10 ± 1.78	N	18.29 ± 27.58	6.89 ± 9.94	p = 0.008
High sensitive CRP (mg/L)	10.42 ± 8.84	4.46 ± 4.09	N	4.04 ± 3.60	3.12 ± 2.11	N
Fibrinogen (g/L)	3.21 ± 0.96	3.09 ± 0.86	N	3.27 ± 1.00	2.99 ± 0.71	N
Thyroid stimulating hormone (mIU/L)	0.87 ± 0.44	1.99 ± 1.03	p = 0.025	1.21 ± 0.77	1.58 ± 1.34	N
Alanine aminotransferase (U/L)	27.10± 16.70	18.00 ± 6.10	N	20.56 ± 13.24	19.31 ± 10.06	N
Aspartate aminotransferase (U/L)	23.90 ± 7.46	20.91 ± 7.14	N	25.47 ± 11.66	21.99 ±6.70	N
Blood urea nitrogen (mmol/L)	6.81 ± 2.67	4.46 ± 0.87	p = 0.008	5.60 ± 2.21	5.53 ± 1.76	N
Creatinine (μmol/L)	111.70 ± 23.74	96.00 ± 14.62	p = 0.047	92.91 ±26.98	92.99 ±28.98	N
Total cholesterol (mmol/L)	5.15 ± 1.11	4.54 ± 1.14	N	4.93 ± 1.11	4.78 ± 1.16	N
Low density lipoprotein-c (mmol/L)	3.26 ± 0.75	2.83 ± 0.84	N	3.22 ± 0.88	3.10 ± 0.86	N

Table3. The disturbed serum proteins in the APIS patients and (or) ANPIS patients

Disturbed proteins			Disturbed in APIS		Disturbed in ANPIS		Common or specific
IDs	Protein names	Symbols	*p*-value	FC	*p*-value	FC	
P00915	Carbonic anhydrase 1	CA1	<0.001	2.56	<0.001	2.43	Common
P01011	Serpin family A member 3	SERPINA3	<0.001	1.35	0.014	1.23	Common
P01834	Immunoglobulin kappa constant	IGKC	0.048	0.72	0.022	0.69	Common
P01861	Immunoglobulin heavy constant gamma 4	IGHG4	0.002	1.85	0.001	1.90	Common
P02042	Hemoglobin subunit delta	HBD	<0.001	3.69	<0.001	3.25	Common
P02750	Leucine rich alpha-2-glycoprotein 1	LRG1	0.002	1.48	0.047	1.28	Common
P02751	fibronectin 1	FN1	0.027	1.56	0.043	1.71	Common
P02765	Alpha 2-HS glycoprotein	AHSG	0.004	0.77	0.001	0.73	Common
P02775	Pro-platelet basic protein	PPBP	0.040	0.81	0.008	0.77	Common
P04217	alpha-1-B glycoprotein	A1BG	0.007	0.65	0.037	0.73	Common
P04275	Von Willebrand factor	VWF	0.029	1.45	0.025	1.64	Common
P05543	Serpin family A member 7	SERPINA7	<0.001	0.71	0.001	0.76	Common
P06276	Butyrylcholinesterase	BCHE	<0.001	0.60	<0.001	0.64	Common
P06702	S100 calcium binding protein A9	S100A9	0.023	1.57	0.003	1.91	Common
P06727	Apolipoprotein A4	APOA4	0.021	0.73	0.001	0.64	Common
P0C0L4	Complement C4A	C4A	0.012	1.34	0.006	1.37	Common
P0DJI8	Serum amyloid A1	SAA1	<0.001	4.44	<0.001	4.34	Common
P14151	Selectin L	SELL	0.031	0.84	0.011	0.81	Common
P27169	Paraoxonase 1	PON1	0.001	0.68	0.023	0.77	Common
P59666	Defensin alpha 3	DEFA3	0.003	1.65	<0.001	2.27	Common
P68871	Hemoglobin subunit beta	HBB	0.001	2.06	0.001	2.00	Common
P69905	Hemoglobin subunit alpha 1	HBA1	<0.001	2.50	0.002	2.25	Common
Q96PD5	Peptidoglycan recognition protein 2	PGLYRP2	0.045	0.84	0.014	0.81	Common
P02766	Transthyretin	TTR	<0.001	0.70			only in APIS
P03952	Kallikrein B1	KLKB1	0.033	1.27			only in APIS
P06681	Complement C2	C2	0.006	1.26			only in APIS
P17936	Insulin like growth factor binding protein 3	IGFBP3	0.001	0.62			only in APIS
P22352	Glutathione peroxidase 3	GPX3	0.003	0.78			only in APIS
P35858	Insulin like growth factor binding protein	IGFALS	0.006	0.75			only in APIS
P43652	Afamin	AFM	<0.001	0.69			only in APIS
P61626	Lysozyme	LYZ	0.049	0.72			only in APIS
Q96KN	Carnosine dipeptidase 1	CNDP1	<0.001	0.65			only in APIS
P02655	Apolipoprotein C2	APOC2	0.008	1.55			only in APIS

Q06033	inter-alpha-trypsin inhibitor heavy chain 3	ITIH3	0.011	1.55			only in APIS
P00746	Complement factor D	CFD	0.002	1.59			only in APIS
P00751	Complement factor B	CFB	0.021	1.18			only in APIS
P01859	Immuneoglobulin heavy constant gamma 2	IGHG2	0.033	0.70			only in APIS
P02654	Apolipoprotein C1	APOC1	0.005	0.54			only in APIS
P02748	Complement C9	C9	0.003	1.56			only in APIS
P03951	Coagulation factor XI	F11	0.041	1.99			only in APIS
P07357	Complement C8 alpha chain	C8A	0.042	1.34			only in APIS
P07358	Complement C8 beta chain	C8B	0.006	1.44			only in APIS
P23142	Fibulin 1	FBLN1	0.029	1.26			only in APIS
P35542	Serum amyloid A4, constitutive	SAA4	0.016	1.39			only in APIS
P36955	serpin family F member 1	SERPINF1	0.028	1.23			only in APIS
P68133	Actin, alpha 1, skeletal muscle	ACTA1	0.002	0.62			only in APIS
Q66K66	Transmembrane protein 198	TMEM198			0.044	0.63	only in ANPIS
P04196	Histidine rich glycoprotein	HRG			0.003	0.74	only in ANPIS
P02679	fibrinogen gamma chain	FGG			<0.001	0.22	only in ANPIS
P01871	Immunoglobulin heavy constant mu	IGHM			<0.001	0.40	only in ANPIS
P01009	Serpin family A member 1	SERPINA1			0.017	0.86	only in ANPIS
P27918	Complement factor properdin	CFP			0.026	0.75	only in ANPIS
P02741	C-reactive protein	CRP			0.039	2.10	only in ANPIS
P02647	Apolipoprotein A1	APOA1			0.022	0.79	only in ANPIS
P01876	Immunoglobulin heavy constant alpha 1	IGHA1			0.004	0.63	only in ANPIS
P51884	Lumican	LUM			0.018	0.80	only in ANPIS
P18428	Lipopolysaccharide binding protein	LBP			0.005	1.60	only in ANPIS
P02675	Fibrinogen beta chain	FGB			<0.001	0.16	only in ANPIS
Q9UGM5	Fetuin B	FETUB			0.008	0.69	only in ANPIS

APIS, acute progressive ischemic stroke; ANPIS, acute non-progressive ischemic stroke.

Table 4. Correlations of SAA1 and S100-A9 with clinical indices in AIS patients

Indices	CRP		N%		ESR		Age		Fib		PLT		SP	
	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*
SAA1	0.342	0.002	0.184	0.005	0.343	0.008	0.269	0.003	0.314	0.001	-	-	-	-
S100-A9	0.239	0.034	0.252	0.007	-	-	-	-	-	-	0.206	0.02	0.244	0.009

SAA1, serum amyloid A1; S100-A9; S100 Calcium-Binding Protein; CRP, C-reactive proteins; ESR, erythrocyte sediment rats; Fib, fibrinogen; PLT, platelet; SP, patients' systolic pressure at admission. R, Spearman's correlation coefficient; *p*, significance in spearman correlation.

Figures

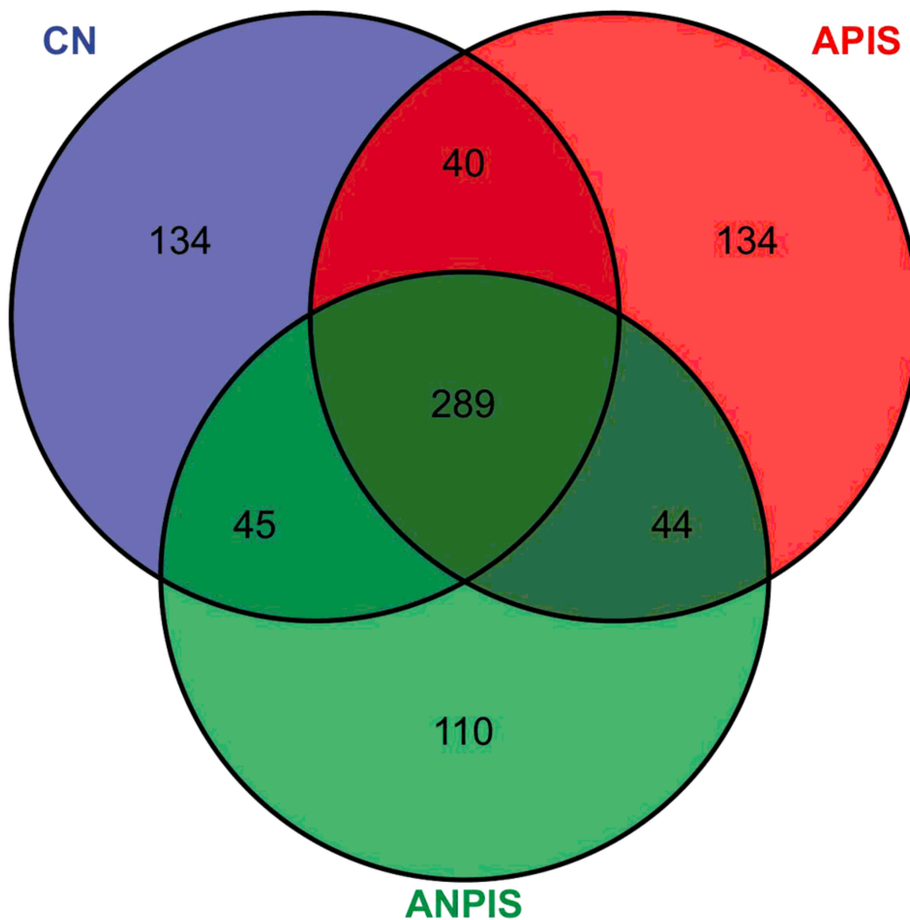

Figure 1

A Venn diagram illustrating all identified proteins by MS. The current study has successfully identified 507, 488, and 508 proteins in the serum samples of the APIS, ANPIS, and Control group, respectively; of these, 289 proteins were common to all groups. APIS, acute progressive ischemic stroke; ANPIS, acute non-progressive ischemic stroke, CN, controls.

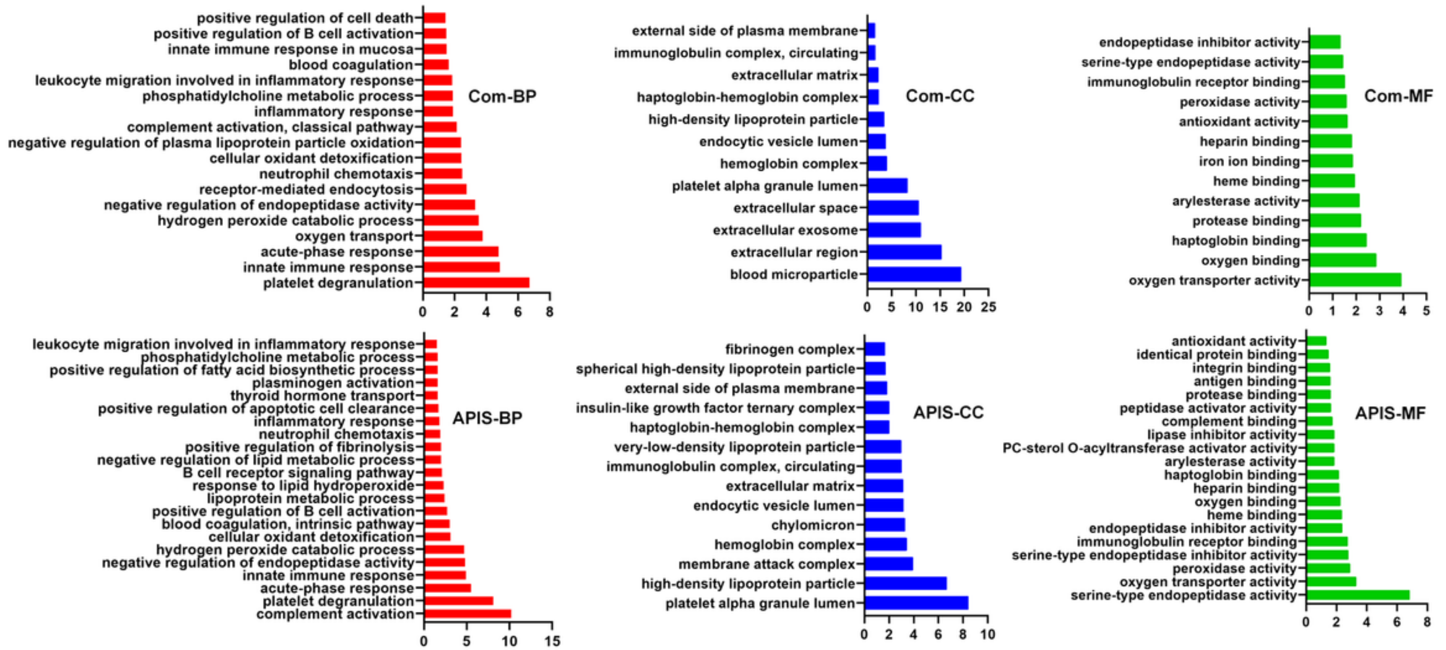

Figure 2

Gene Ontology (GO) enrichment of DEPs. The Bar Charts were produced by GraphPad prism (8.0.2) and were arrayed by Office Visio (2013). Com represents DEPs in APIS common to ANPIS; APIS represents DEPs associated acute progressive ischemic stroke; BP, biological process; MF, molecular function; CC, cellular component.

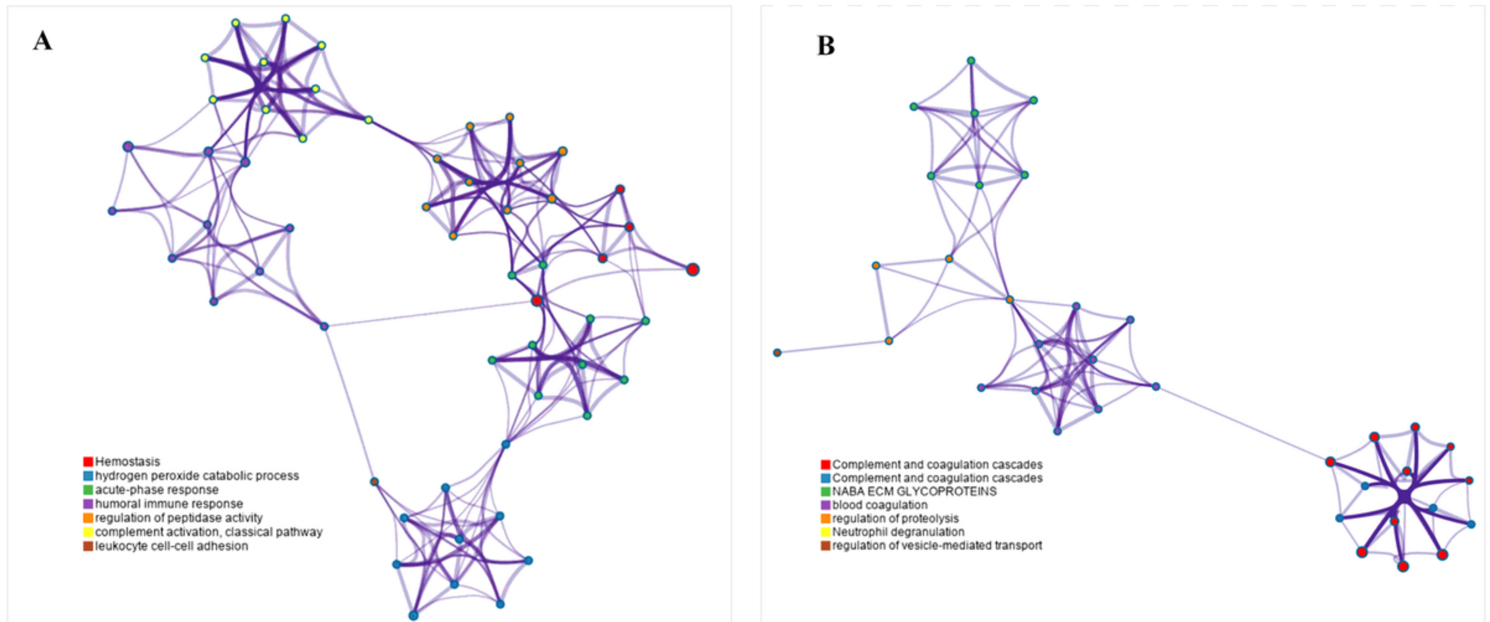

Figure 3

Network charts of enriched ontology clusters. The charts were produced by the Metascape database. The relationships between each enriched GO terms have been selected and rendered as a network plot, where terms with a similarity > 0.3 were connected by edges. The terms with the best p-values from each of the 20 clusters were selected, with the constraint that there are no more than 15 terms per cluster and no more than 250 terms in total. The network is visualized using Cytoscape5, where each node represents an enriched term and is colored first by its cluster ID. EMC, extracellular matrix.

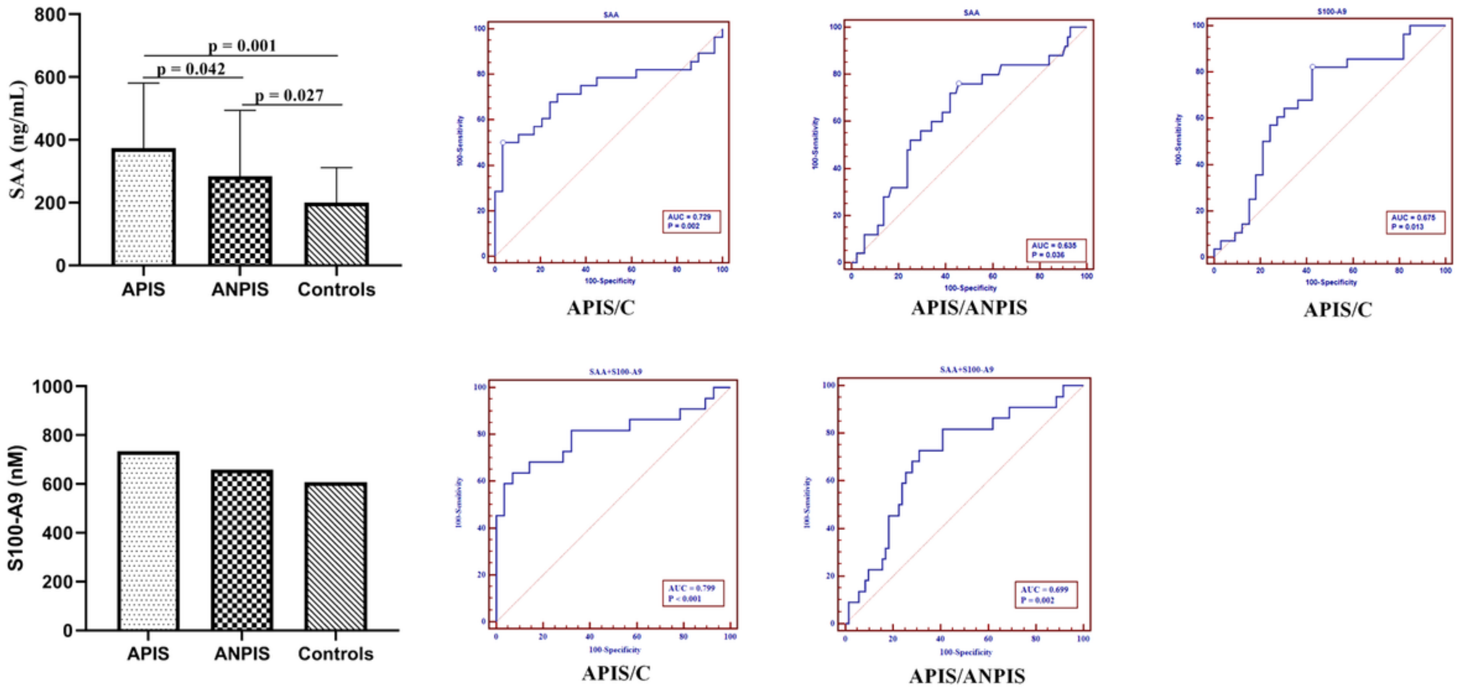

Figure 4

Absolute Concentration of SAA1 and S100-A9 in the subjects of the validation set and their diagnostic potential. The receiver operating characteristic curves (ROCs) were produced by the MedCalc statistical software. APIS/C represents the value of SAA1 or S100-A9 alone, or their combination to diagnose APIS. APIS/ANPIS represents the diagnostic value to distinguish APIS from ANPIS.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterials111.docx