# Association of Serum Biomarkers with Post-Thrombolytic Early Neurological Improvement in Stroke: A Comprehensive Protein Microarray Analysis From INTRECIS Study

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**Research Article**

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## Abstract

# Objective

Early neurological improvement (ENI) after intravenous thrombolysis is associated with favorable outcome, but associated serum biomarkers were not fully determined. We aimed to investigate the issue in a prospective cohort.

# Methods

In INTRECIS study, five centers were designed to consecutively collect the blood sample from enrolled patients. Enrolled patients with ENI and without ENI were matched by propensity score matching with the ratio of 1:1. Preset 49 biomarkers were measured by protein microarray analysis. Enrichment of Gene Ontology and pathway, and protein-protein interaction network were analyzed in the identified biomarkers.

# Results

Of 358 patients, 19 occurred ENI, who were assigned as ENI group, while 19 matched patients without ENI were assigned as Non ENI group. A total of 9 biomarkers were found different, among which levels of chemokine (C-C motif) ligand (CCL)-23, chemokine (C-X-C motif) ligand (CXCL)-12, insulin-like growth factor binding protein (IGFBP)-6, interleukin (IL)-5, lymphatic vessel endothelial hyaluronan receptor (LYVE)-1, plasminogen activator inhibitor (PAI)-1, platelet-derived growth factor (PDGF)-AA, suppression of tumorigenicity (ST)-2, and tumor necrosis factor (TNF)-α were higher in ENI group, compared with those in Non ENI group.

# Interpretation:

Our finding indicated that pretreatment serum CCL-23, CXCL-12, IGFBP-6, IL-5, LYVE-1, PAI-1, PDGF-AA, ST-2, and TNF-α levels were associated with post-thrombolytic ENI in ischemic stroke. The role of these biomarkers warrant further investigation.

# Registration-URL

: https://www.clinicaltrials.gov; Unique identifier: NCT02854592.

## Introduction

Intravenous thrombolysis with alteplase is an effective treatment for acute ischemic stroke within 4.5 hours of symptom onset.[1–2] Early neurological improvement (ENI) in 24 hours after intravenous

thrombolysis is associated with vessel recanalization and predicts functional outcome at 90 days.[3–4] Exploring predictors of ENI is critical for identifying potential patients who can benefit from intravenous thrombolysis.

In the previous studies, female, blood glucose < 8 mmol/L, absence of cortical involvement on brain computerized tomography at 24 hours after thrombolysis, development of leptomeningeal collaterals, hyperdense artery sign and fall in systolic blood pressure at early 24 hours after thrombolysis were found to be associated with ENI in ischemic stroke.[5–9] Given less serum biomarkers associated with post-thrombolytic ENI,[10–13] the issue need to be comprehensively investigated.

In INTRECIS (INtravenous Thrombolysis REgistry for Chinese Ischemic Stroke within 4.5 hours of onset),[14] five centers were pre-designed to consecutively collect blood samples prior to intravenous thrombolysis for additional exploratory research. In the present study, we measured baseline serum levels of 49 well-known biomarkers in thrombolytic patients with ENI vs Non ENI, and tried to identify associated biomarkers with ENI and their interactions through protein microarray analysis.

# Results

Overall, 358 thrombolytic patients were consecutively screened in the present study and 234 patients were excluded for different reasons: 160 with urokinase or non-standard dose of alteplase, 10 with endovascular intervention, 3 with incomplete clinical data, and 61 without blood samples collection. Finally, 124 patients were recruited into the current study, including 19 with ENI, and 105 without ENI in 24 hours after intravenous thrombolysis. With the ratio of 1:1, 19 patients without ENI were matched to Non ENI group for comparative analysis (Figure 1). There was no significant difference in the baseline characteristics between two groups (Table 1).

Table 1
Demographic and Baseline Characteristics

| Variables | ENI (n=19) | Non ENI (n=19) | P Value |
|---|---|---|---|
| Demographics | | | |
| Age, years, mean ± SD | 65.4±9.21 | 68.4±14.86 | 0.452 |
| Gender, male, n (%) | 7(36.8) | 8(42.1) | 0.740 |
| Current smoking, n (%) | 8(42.1) | 11(57.9) | 0.330 |
| Alcohol consumption, n (%) | 5(26.3) | 6(31.6) | 0.721 |
| Medical history, n (%) | | | |
| Stroke | 3(15.8) | 4(21.1) | 0.676 |
| Hypertension | 10(52.6) | 10(52.6) | 1.000 |
| Diabetes mellitus | 5(26.3) | 5(26.3) | 1.000 |
| Atrial fibrillation | 4(21.1) | 4(21.1) | 1.000 |
| Congestive heart failure | 3(15.8) | 4(21.1) | 0.676 |
| Previous use of antiplatelet | 2(10.5) | 3(15.8) | 0.631 |
| Baseline scales, mean ± SD | | | |
| Systolic blood pressure, mmHg | 158.8±24.63 | 153.0±25.09 | 0.478 |
| Diastolic blood pressure, mmHg | 87.3±9.68 | 90.0±15.95 | 0.527 |
| Blood glucose, mmol/L | 9.19±4.68 | 7.68±2.92 | 0.276 |
| Symptom onset to thrombolysis time, min | 160.1±49.11 | 157.0±53.87 | 0.854 |
| NIHSS score at admission | 9.4±5.69 | 8.1±5.65 | 0.462 |
| TOAST classification, n (%) | | | 0.185 |
| LAA | 11(57.9) | 10(52.6) | |
| SAO | 5(26.3) | 4(21.1) | |
| CE | 1(5.3) | 5(26.3) | |
| UND | 2(10.5) | 0(0.0) | |

CE, cardiogenic embolism; ENI, early neurological improvement; LAA, large artery atherosclerosis; NIHSS, National Institute of Health Stroke Scale; SAO, small artery occlusion; SD, standard deviation; TOAST, the Trial of Org 10172 in Acute Stroke Treatment; UND, undetermined cause.

# Baseline Biomarkers Identification

Compared with the Non ENI group, 9 significantly different biomarkers in ENI group were observed ($P < 0.05$, Table 2). Higher baseline serum levels of chemokine (C-C motif) ligand (CCL)-23, chemokine (C-X-C motif) ligand (CXCL)-12, insulin-like growth factor binding protein (IGFBP)-6, interleukin (IL)-5, lymphatic vessel endothelial hyaluronan receptor (LYVE)-1, plasminogen activator inhibitor (PAI)-1, platelet-derived growth factor (PDGF)-AA suppression of tumorigenicity (ST)-2, and tumor necrosis factor (TNF)-α were identified in END group than Non ENI group. The scatter plot and volcano plot showed results of all measured biomarkers (Figures 2A and 2B). The heatmap and column plot showed results of the identified biomarkers (Figures 2C and 2D).

Table 2
Detected Pretreatment Serum Levels of Identified Biomarkers

| Biomarkers | Pretreatment serum levels | | P Value | Fold change |
|---|---|---|---|---|
| | ENI (n=19) | Non ENI (n=19) | | |
| Mean ± SD, pg/ml | | | | |
| CCL-23 | 505.32±416.14 | 224.57±141.14 | 0.006 | 1.99 |
| CXCL-12 | 1244.39±5178.59 | 20.33±20.97 | 0.004 | 5.00 |
| IGFBP-6 | 53450.38±8664.92 | 42904.08±8448.38 | 0.002 | 1.25 |
| IL-5 | 212.50±203.96 | 96.42±92.19 | 0.009 | 2.46 |
| LYVE-1 | 1389.22±144.92 | 1089.21±242.02 | <0.001 | 1.30 |
| PAI-1 | 17523.08±4691.52 | 11918.16±4343.32 | <0.001 | 1.52 |
| PDGF-AA | 2434.21±479.85 | 1810.86±649.86 | <0.001 | 1.41 |
| ST-2 | 146.39±198.39 | 48.56±62.75 | 0.009 | 3.73 |
| TNF-α | 153.72±221.95 | 58.29±89.10 | 0.006 | 5.77 |

CCL-23, chemokine (C-C motif) ligand 23; CXCL-12, chemokine (C-X-C motif) ligand 12; ENI, early neurological improvement; IGFBP-6, insulin-like growth factor binding protein 6; IL-5, interleukin 5; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; PAI-1, plasminogen activator inhibitor 1; PDGF-AA, platelet-derived growth factor AA; SD, standard deviation; ST-2, suppression of tumorigenicity 2; TNF-α, tumor necrosis factor α.

# Protein Function Analysis Of Association In Identified Biomarkers

The comprehensive gene ontology (GO) enrichment analysis was used to gain a deeper insight into the main functions of the identified biomarkers. The GO analysis consisted of biological process, molecular

function, and cellular component analysis. The biological process analysis showed that CCL-23, CXCL-12, TNF-α, and PAI-1 were mostly related to the mononuclear cell migration (Figure 3A). The molecular function analysis showed that CCL-23, CXCL-12, TNF-α, IL-5, and PDGF-AA, were mostly associated with the receptor ligand activity (Figure 3B). The cellular component analysis showed that PAI-1 and PDGF-AA, were mostly included in the platelet alpha granule lumen (Figure 3C). The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed that CCL-23, CXCL-12, TNF-α, IL-5, and ST-2 were included in cytokine-cytokine receptor interaction (Figure 3D).

Based on the information of Search Tool for the Retrieval of Interacting Genes (STRING) database, the PPI network constructed by the above 9 identified biomarkers was obtained (Figure 4). The results showed that TNF-α (degree = 6) could interact with the most biomarkers, followed by CXCL-12 (degree = 5), IL-5 (degree = 3), CCL-23 (degree = 2), PAI-1 (degree = 2), ST-2 (degree = 2), LYVE-1 (degree = 1) and PDGF-AA (degree = 1).

# Discussion

To our best knowledge, this is the first study to comprehensively investigate serum biomarkers associated with post-thrombolytic ENI through microarray protein analysis. A total of 9 biomarkers were found higher in ENI group than Non ENI group, including CCL-23, CXCL-12, IGFBP-6, IL-5, LYVE-1, PAI-1, PDGF-AA, ST-2, and TNF-α.

Up to date, only a few biomarkers, such as ADAMTS13 activity, Aquaporin-4, leukocyte count, and neutrophil to lymphocyte ratio, were found to be associated with post-thrombolytic ENI in stroke.[10–13] In the present study, the 9 identified biomarkers have never been previously reported. Of those, IGFBP-6 and IL-5 were reported to be associated with inhibiting development of atherosclerotic plaques,[16–19] LYVE-1 and PDGF-AA were beneficial to vascular remodeling and neural regeneration, respectively,[20–22] while CCL-23, CXCL-12, ST-2, and TNF-α were reported to promote and worsen the inflammatory response: CCL-23, CXCL-12, and TNF-α played pro-inflammatory role;[23–25] and ST-2 prevented anti-inflammatory effect of IL-33.[26–27] In addition, PAI has an effect on inhibiting fibrinolysis in patients treated with tissue plasminogen activator.[28–29]

Based on functional enrichment analysis, mononuclear cell migration, receptor ligand activity, platelet alpha granule lumen, and cytokine-cytokine receptor interaction were significantly enriched items. Contributing to the inflammation after brain injury, CCL-23 and CXCL-12 modulate immune response through promoting migration of monocytes to the local sites of injury.[23–24] As the result of monocytes migration, releasing inflammatory cytokines from microglia, such as TNF-α, modulate infarct evaluation.[25] It is worthy to note that these cytokines seemed contradictory with ENI given their proinflammatory effects. Taken together, the role of these newly identified biomarkers in ENI is complex and need be further determined given that the elucidation of their complex relationships will provide an insight into our understanding of the mechanisms underlying ENI.

The strength of this study was to explore key biomarkers associated with post-thrombolytic ENI through comprehensively screening preset biomarkers in a prospective cohort and find several new biomarkers which have never been reported previously. However, we acknowledge that our study has several limitations. First, limited sample of the present study weaken the power of results. Second, these identified biomarkers were not confirmed by other methods, such as western blot analysis, enzyme-linked immunosorbent assay, or other in vivo experiments. Thirdly, although the effect of identified biomarkers was independent of other preset biomarkers, the effect of an acute phase reaction or comorbidity on these biomarkers can't be ruled out. Finally, complex roles of identified biomarkers need further be investigated.

# Conclusion

Our finding indicated that higher baseline serum levels of CCL-23, CXCL-12, IGFBP-6, IL-5, LYVE-1, PAI-1, PDGF-AA, ST-2, and TNF-α were associated with post-thrombolytic ENI in acute ischemic stroke. The value of these newly identified biomarkers warrant further investigation.

# Methods

### Study Population and Procedure

From August 2018 to July 2019, patients receiving intravenous thrombolysis within 4.5 hours after symptoms onset were consecutively screened to collect blood samples prior to thrombolysis from five pre-set stroke centers in the INTRECIS study. INTRECIS is a nationwide, multicenter, prospective, and registry study of consecutive adult patients who were eligible for treatment with intravenous thrombolysis within 4.5 hours of onset of symptoms. Details of the study design and results of the primary outcomes have been reported recently.[14] Briefly, the consecutive patients who received standard dose of alteplase (0.9 mg/kg, maximum 90 mg; manufacturer: Boehringer Ingelheim) within 4.5 hours after symptoms onset were enrolled. The exclusion criteria were as follows: (1) patients received urokinase or non-standard dose of alteplase, (2) patients received endovascular intervention, (3) patients lacked complete clinical data, (4) blood samples were not collected prior to intravenous thrombolysis. All the patients and/or their legally gave written informed consent for data collection. According to the occurrence of ENI, enrolled patients were divided into two groups: (1) ENI group: patients occurred ENI; (2) Non ENI group: patients did not occur ENI. Furthermore, propensity score matching was performed between groups with the ratio 1:1, caliper of 0.1, and a nearest-neighbor matching strategy, and operated with control factors including age, gender, current smoking, alcohol consumption, systolic blood pressure, diastolic blood pressure, blood glucose, symptom onset-to-treatment time, National Institutes of Health Stroke Scale (NIHSS) score at admission, the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification,[15] previous use of antiplatelet, and medical history.

The baseline characteristics and clinical data of recruited patients were obtained from electronic database: age, gender, current smoking, alcohol consumption, systolic blood pressure, diastolic blood

pressure, blood glucose, symptom onset-to-thrombolytic time, NIHSS scores, TOAST classification, previous use of antiplatelet, history of stroke, hypertension, diabetes mellitus, atrial fibrillation, and congestive heart failure. ENI was defined as a decrease of ≥4 on the NIHSS scores within 24 hours after thrombolysis from baseline in the present study.[2]

### Ethics Approval

The study was centrally approved by the Institution Human Research Ethics Committees of General Hospital of Northern Theater Command and performed in accordance with the Declaration of Helsinki.

### Blood Sampling and Biomarkers Measurements

About four milliliters of peripheral venous blood samples were collected from each patient just prior to intravenous thrombolysis. The blood samples were centrifuged at 1000 × g for 10 minutes at 4℃, and then transferred into an 1.8 milliliter cryotube and stored at -80℃ until measurement.

According to the manufacturer's instructions, pre-customized protein microarray analysis (Raybiotech Inc) was used to simultaneously detect and quantify 49 biomarkers in the collected blood samples, which were preset based on published data. Identified biomarkers were defined as those variations with $P < 0.05$, and fold change > 1.20 or < 0.83. Functional enrichment analysis and protein-protein interactions network were performed to explore the possible mechanism between identified biomarkers

### Statistical Analysis

Descriptive statistics was performed to compare variables between two groups. Continuous variables with normal distribution were described as means and standard deviation. Continuous variables included age, systolic blood pressure, diastolic blood pressure, blood glucose, symptom onset to thrombolysis time, NIHSS score, and detected serum concentration of biomarkers. The t tests were used to analyze the normally distributed continuous variables. Categorical variables were described as number and proportions. Categorical variables included gender, current smoking, alcohol consumption, medical history, previous use of antiplatelet, and TOAST classification. The Pearson $\chi^2$ tests were used to analyze the categorical variables.

The *p* value is obtained from the moderated t-statistic with false discovery rate of adjustment for multiple testing. In all analysis, differences were considered statistically significant with a $p < 0.05$. The free statistical language R (version 3.10.3) was used for the outcomes and graph in the propensity score matching and microarray analysis.

# Declarations

### Acknowledgment

We thank all participating hospitals, relevant clinicians, and statistician. We also thank all patients who participated in the INTRECIS study.

### Author Contributions

Hui-Sheng Chen designed the study and critically revised the manuscript. Wei-Hong Meng designed the study. Yu Cui conducted the analyses and drafted the manuscript. Xin-Hong Wang, Yong Zhao, Shao-Yuan Chen, Bao-Ying Sheng, and Li-Hua Wang contributed to the implementation of blood sample collection.

### Conflict of Interest

None declared.

### Sources of Funding

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### Data Access Statement

Data are available on reasonable request.

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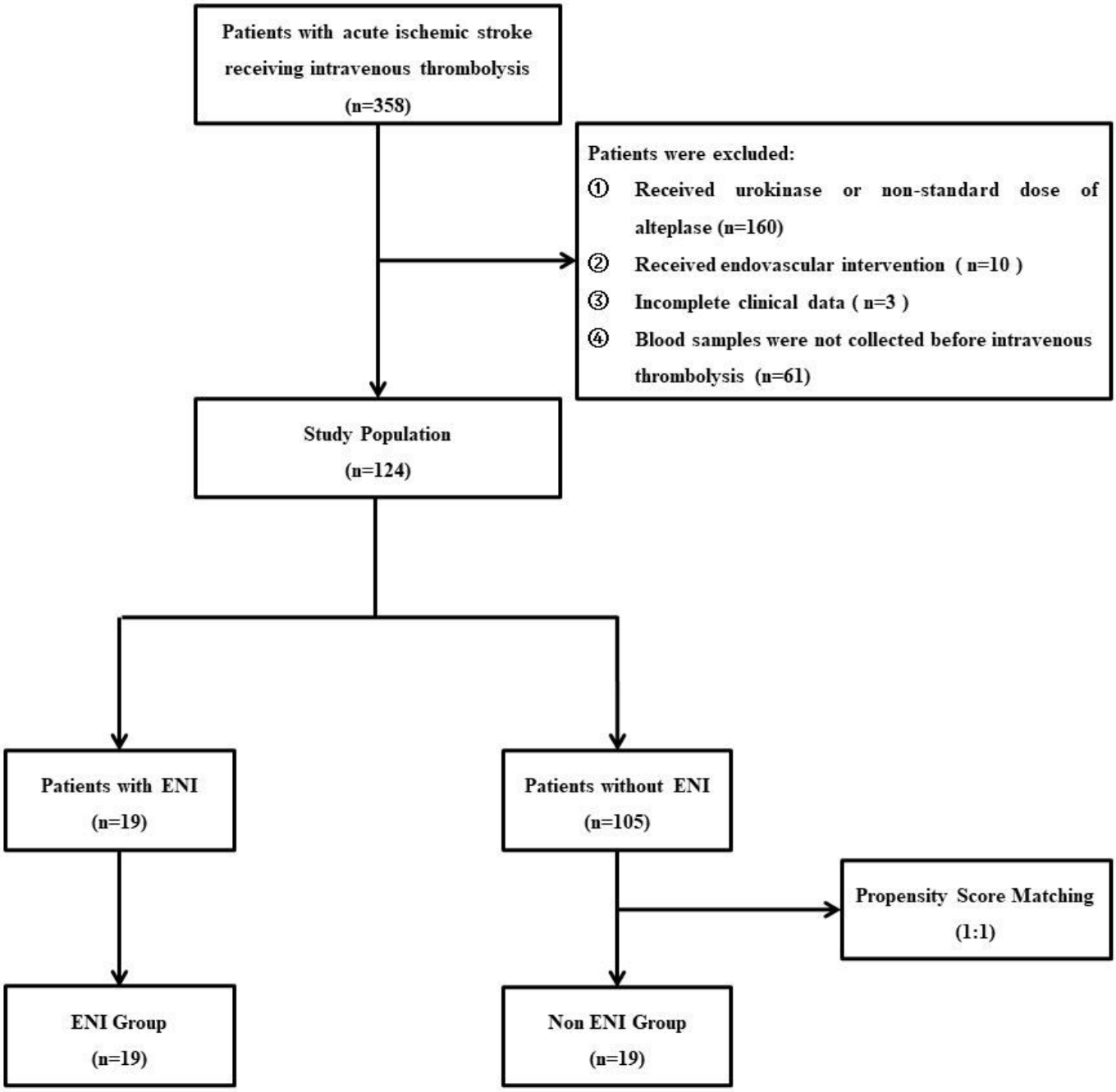
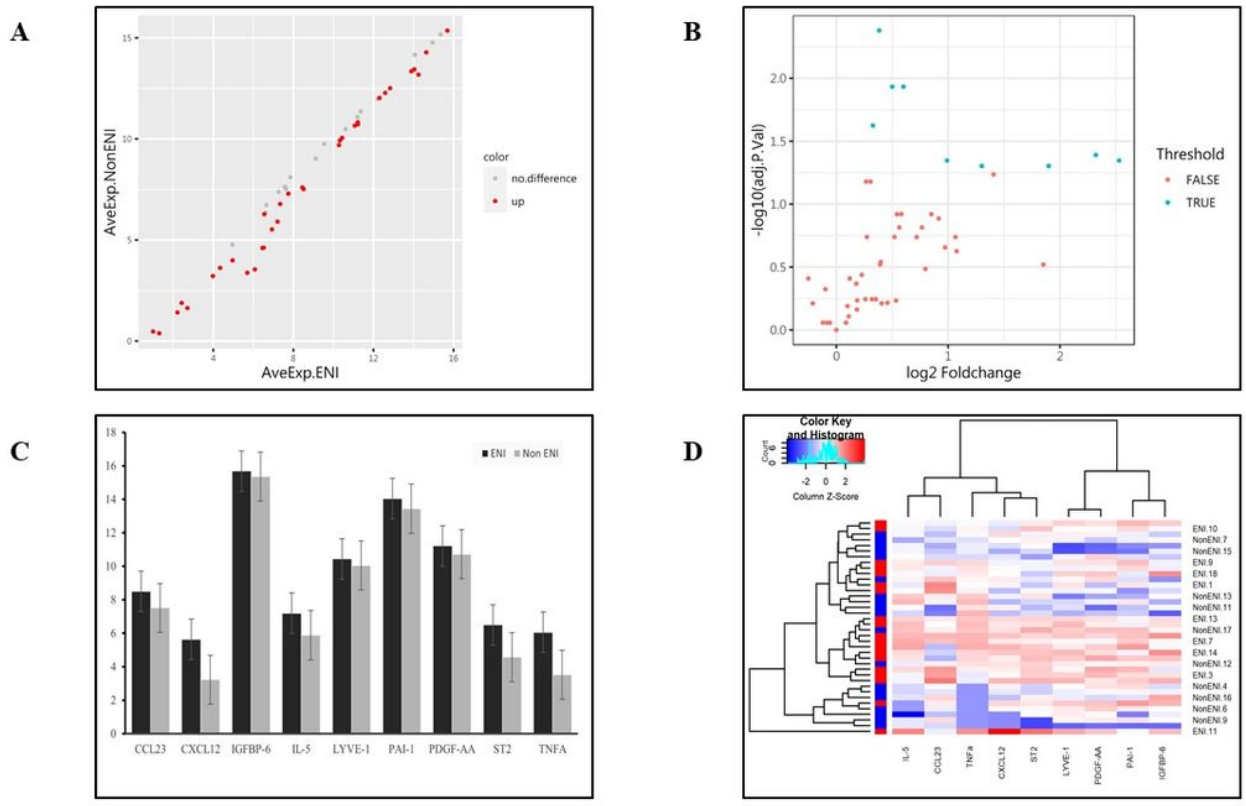
# Figures




## Figure 1

**Flow diagram.** ENI, early neurological improvement.




## Figure 2

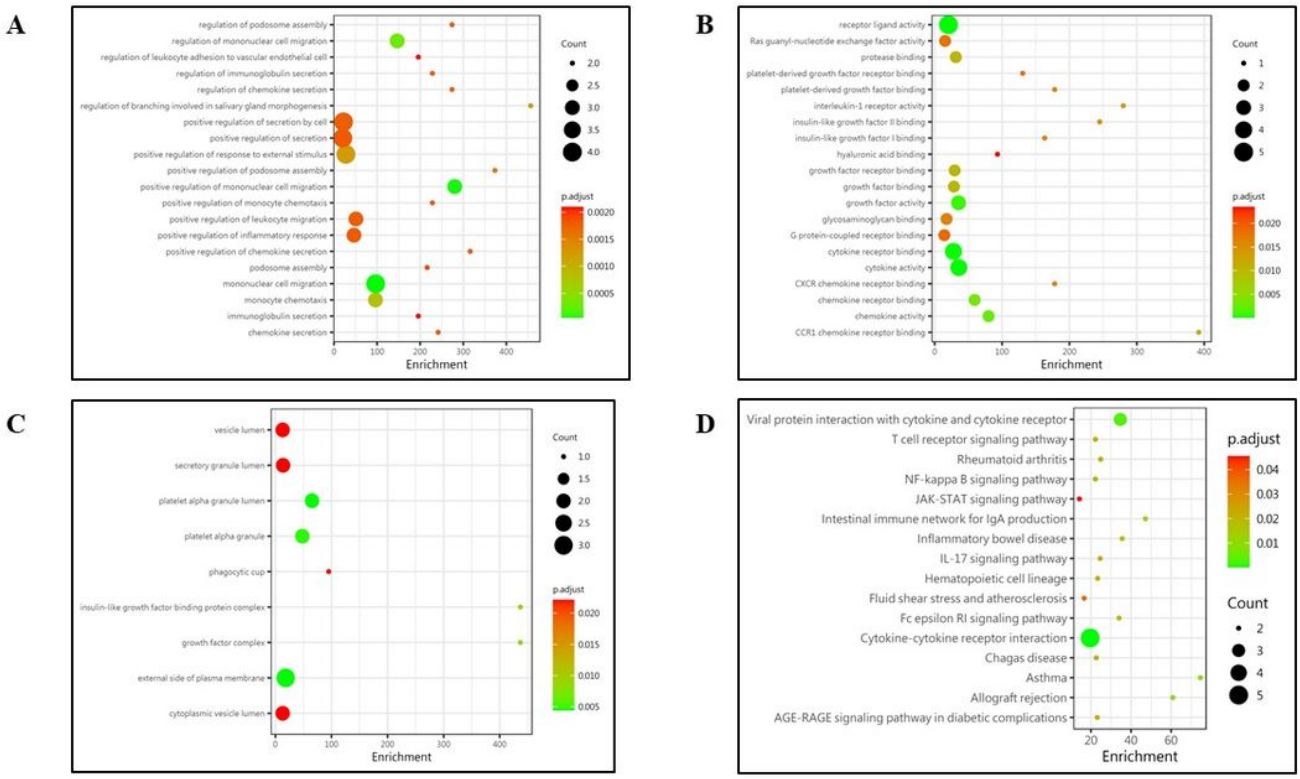
**Results of detected biomarkers in the microarray analysis.**

(A) The scatter plot for detected biomarkers; the X-axis represent the average of log2 serum levels in ENI group, while the Y-axis represent the average of log2 serum levels in Non ENI group; compared with Non ENI group, the gray point represents biomarkers with similar serum levels in ENI group, while the red point represents biomarkers with higher serum levels. ENI, early neurological improvement.

(B) The volcano plot for detected biomarkers; the X-axis represents the log2 fold-change value, while the Y-axis represents the –log10 P value; the cyan point represents the biomarkers with significant difference, while the red point represents the biomarkers without significant difference. ENI, early neurological improvement.

(C) The column plot for identified biomarkers; the X-axis represents identified biomarkers, the Y-axis represents average of log2 serum levels in two groups; the deep color represents ENI group, while the light color represents Non ENI group. CCL-23, chemokine (C-C motif) ligand 23; CXCL-12, chemokine (C-X-C motif) ligand 12; ENI, early neurological improvement; IGFBP-6, insulin-like growth factor binding protein 6; IL-5, interleukin 5; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; PAI-1, plasminogen activator inhibitor 1; PDGF-AA, platelet-derived growth factor AA; ST-2, suppression of tumorigenicity 2; TNF-α, tumor necrosis factor α.

(D) The heatmap for identified biomarkers; the red color represents biomarkers with higher serum levels, while the blue color represents the biomarkers with lower serum levels; the darker the color, the more significant the difference of biomarkers. CCL-23, chemokine (C-C motif) ligand 23; CXCL-12, chemokine (C-X-C motif) ligand 12; ENI, early neurological improvement; IGFBP-6, insulin-like growth factor binding protein 6; IL-5, interleukin 5; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; PAI-1, plasminogen activator inhibitor 1; PDGF-AA, platelet-derived growth factor AA; ST-2, suppression of tumorigenicity 2; TNF-α, tumor necrosis factor α.




## Figure 3

**Protein function analysis of identified biomarkers.**

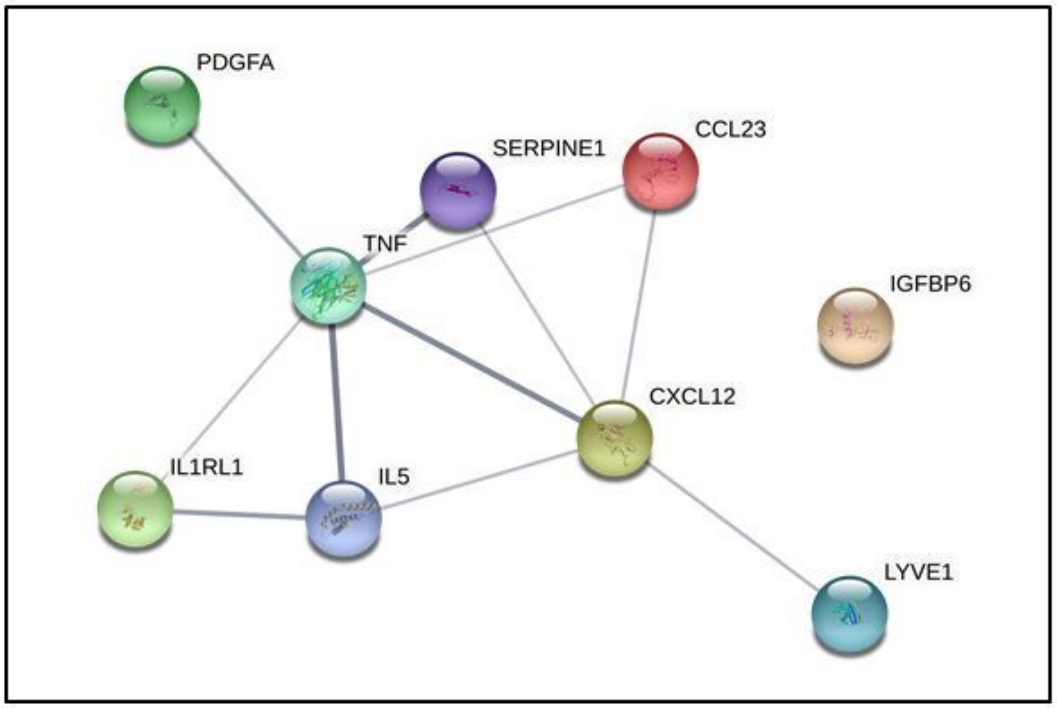
(A) top 20 significantly enriched biological process of identified biomarkers; the X-axis represents the enrichment, while the Y-axis represents the biological process.

(B) the molecular function enriched by identified biomarkers; the X-axis represents the enrichment, while the Y-axis represents the molecular function.

(C) the cellular component enriched by identified biomarkers; the X-axis represents the enrichment, while the Y-axis represents the cellular component.

(D) the pathway enriched by identified biomarkers; the X-axis represents the enrichment, while the Y-axis represents the pathway.

The deeper the color, the larger the P value; the larger the circle, the bigger the counts.




## Figure 4

### Protein-protein interaction network of identified biomarkers.

CCL-23, chemokine (C-C motif) ligand 23; CXCL-12, chemokine (C-X-C motif) ligand 12; IGFBP-6, insulin-like growth factor binding protein 6; IL-5, interleukin 5; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; SERPINE1, plasminogen activator inhibitor 1 (PAI-1); PDGF-AA, platelet-derived growth factor AA; IL1RL1, suppression of tumorigenicity 2 (ST-2); TNF-α, tumor necrosis factor α.