Association of Microglia/macrophage Polarization With Perihematomal Edema and Clinical Outcome in Patients With Acute Intracerebral Hemorrhage

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Research Article

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Abstract

Background: Perihematomal edema (PHE) is a marker of secondary injury in intracerebral hemorrhage (ICH) and is associated with poor clinical outcomes. Microglial and macrophage activation, or their polarization could lead to a pro-inflammatory or anti-inflammatory response in stroke. However, little is known about the association between inflammatory cells and ICH. Thus, we characterized the inflammatory cell response, and assessed its association with parameters such as hematoma, PHE volume, and clinical outcome in patients with acute ICH.

Methods: Fifty-two patients with acute ICH were retrospectively enrolled in our study. All patients underwent surgery, and brain tissue from the PHE was acquired. Immunofluorescence staining was performed to evaluate microglia (CD11b+/TMEM119+), macrophages (CD11b+/TMEM119-), and the M1 (MHC+/CD11b+) and M2 (CD206+/CD11b+) phenotypes. The relative PHE (r-PHE) was the main marker for assessing PHE volume. The Wilcoxon test and Spearman correlation analysis were the main statistical analysis methods used.

Results: Microglia/macrophages, and their phenotypes were detected within 6 hours after stroke. Microglia and the M2 phenotype were negatively correlated with r-PHE, while macrophages and the M1 phenotype were positively correlated with r-PHE; however, these parameters were not associated with age, sex, or location. There was a positive correlation between the microglia and M2-phenotype levels (r = 0.443, p = 0.001) and between the macrophage and M1-phenotype levels (r = 0.458, p <0.001). Microglia (r = -0.295, p = 0.033) and M2-phenotype (r = -0.384, p = 0.005) levels were negatively correlated with the National Institutes of Health stroke scale (NIHSS) after treatment.

Finally, using lasso Poisson regression models, we developed a score for predicting the NIHSS score after treatment. Decision curve analysis showed notable net benefits of this score.

Conclusion: Microglial and macrophage activation, and their polarization were significantly associated with r-PHE and clinical outcomes in ICH, and could provide therapeutic insights for PHE management after hemorrhagic stroke.

1 Introduction

Intracerebral hemorrhage (ICH) is the second most common subtype of stroke, exhibiting high mortality and morbidity [1]. Trauma may lead to ICH and hematoma with subsequent brain injury. Secondary injury following ICH could result from inflammatory responses mediated by clotting components [2]. Perihematomal edema (PHE) is considered a radiological marker of secondary injury following ICH [3], and is associated with mortality and functional outcomes in ICH [3, 4].

Inflammation plays a critical role in the formation of PHE, with the activation of neutrophils and microglia/macrophages [3, 5]. Among these inflammatory cells, microglia, which are brain-resident innate phagocytes, are the first to respond to brain injuries after ICH [6]. They are activated to clear the

hematoma, but also secrete cytokines, chemokines, and other immunomodulatory molecules [3]. Moreover, the microglia can be polarized into two phenotypes: pro-inflammatory M1 and anti-inflammatory M2 [7]. Microglia polarization is closely related to microglial phagocytic function and may affect absorption of the hematoma and edema [8]. Proliferation and polarization of microglia/macrophages were detected in ischemic stroke experimental animal models [9], and exhibited a dynamic response [10]. These results indicate that balancing the polarization of microglia/macrophage could improve the clinical outcome of stroke patients. However, few studies have investigated the polarization of microglia/macrophage after hemorrhagic stroke.

In this study, immunofluorescence microscopy was used to evaluate the presence of microglia/macrophages and its polarization to the M1 and M2 phenotypes in the PHE after an intracerebral hemorrhage. The main aim of this study was to investigate the association between the M1/M2 phenotypes and clinical parameters, such as PHE. As polarization might be influenced by age [11] and sex [12], we also attempted to identify potential contributing factors of polarization as this could help in the development of novel therapeutic targets for PHE.

2 Materials And Methods

2.1 Study population

This was a retrospective study of ICH patients and patients with acute spontaneous ICH who required surgical treatment and were admitted to the Department of Neurosurgery at the People's Hospital of Anyang City between December 2018 and December 2019. All the enrolled ICH patients underwent an emergency surgery within 6 hours after the onset of stroke. All the patients required surgery and their operative indication satisfied one of the following criteria: presented with cerebral hernia, exacerbation of disturbance of consciousness, Glasgow coma scale (GCS) score less than 8 points, the computed tomography (CT) scan showing an intraventricular hemorrhage ≥ 50% in the lateral ventricle, obstructive hydrocephalus, cerebellar hemorrhage more than 10 ml, and compression or occlusion of the fourth ventricle. Patients were excluded if they had a history of traumatic brain injury or cerebrovascular disease within 1 month or if they had a coexisting disease including hematologic disorders, infection, autoimmune disease, and other malignancies. None of the patients received glucocorticoid treatment before the surgery. The Ethics Committee of The People's Hospital of Anyang City approved all the procedures in the study (KS-2018-07).The study was written informed consent was obtained from each patient, in accordance with the principles expressed in the Declaration of Helsinki.

The GCS and National Institutes of Health stroke scale (NIHSS) were used to evaluate the severity of stroke. CT was used to evaluate the baseline volume of hematoma and edema using the ABC/2 method [13, 14]. The PHE is indicated by the volume of hypodensity surrounding the hematoma on plain CT. We calculated the size of the PHE by subtracting the volume of the hyperdense area from the total volume of both the hypodense and hyperdense areas. Relative PHE (r- PHE) was employed, as it is an ideal parameter for assessing PHE, especially in larger ICH [15]. The r-PHE was defined as the ratio of the

volume of PHE to the hematoma size. Informed consent was obtained from the patients to acquire non-functional brain tissue within the PHE during open surgery. The ethics committees approved this study.

2.2 Immunofluorescence microscopy

Immunofluorescence microscopy was performed for the detection of microglia, macrophages, and M1/2 phenotypes. Cell surface markers were used to identify the following inflammatory cells: microglia, CD11b+/TMEM119+; macrophages, CD11b+/TMEM119-; M1-phenotype, MHC+/CD11b+; and M2-phenotype, CD206 +/CD11b+. Immunofluorescence microscopy procedures were conducted in accordance with the manufacturer's instructions. The brain tissues were quickly frozen in liquid nitrogen for 1 minute, and then stored at -80°C. Thereafter, the PHE samples were cut into 7-mm-thick sections. These sections were then thawed at room temperature before they were fixed in cold acetone for 10 minutes. The primary antibodies used were as follows: CD11b (ab1211, Abcam), TMEM119 (ab185337, Abcam), CD206 (ab64693, Abcam), and MHC (bs-4298R, Bioss). A fluorescence microscope (LSM-880, Zeiss) was employed for visualizing cells. The inflammatory cells were quantified as the total number based on the three most obvious regions. The number of microglia and/or M1/2-phenotype macrophages were evaluated based on positive immunostaining of their specific surface markers; the macrophage count was evaluated as the total number of CD11b+ cells minus the number of CD11b+/TMEM119+ cells. The ratio of M1/2-phenotype macrophages was calculated as the number of MHC+/CD206+ cells among the CD11b+ cells.

2.3 Statistics

R version 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria) and SPSS version 22.0 (IBM Corp., Armonk, NY) were the main tools used to analyze the data and to generate images. Differences in inflammatory cells based on sex and location were compared using the Wilcoxon test. Spearman analysis was used to analyze the correlation of continuous variables. The penalized Poisson regression model with lasso penalty was used to exclude the less valuable markers for predicting NIHSS after treatment with ten-fold cross-validations determining the optimal parameter λ. Decision curve analysis (DCA) was used for evaluating the net benefits of the score. A p-value of <0.05 was considered statistically significant.

3 Results

3.1 Characteristics of the study population

The characteristics of the study population are presented in Table 1. A total of 52 patients were enrolled in this study. The median age was 63 years, and 20 patients were female. All the hematomas were primarily located in the basal ganglia, except in eight cases, wherein the hematoma was very large and had spread to the temporal lobe. The volumes of the ICH and PHE were 49.08 ± 19.72 and 96.46 ± 32.67,

respectively. The r-PHE was 2.04 ± 0.46. The GCS score improved from 7 to 11 when compared from admission to discharge. The baseline NIHSS was 28.5 after treatment, improving from 14.5 before treatment.

3.2 Presence of inflammatory cells in the PHE

Immunofluorescence staining identified the presence of microglia and infiltration of macrophages in the PHE region (Fig. 1A). Moreover, we observed the polarization of microglia/macrophages at this very early stage of PHE (Fig. 1B, C). The number of microglia, macrophages, and M1/M2-phenotype cells were evaluated, based on their specific cell surface markers. We found that the number of microglia/macrophages and M1/2 phenotypes differed between individual samples (Table 2). However, our results did not show a correlation between the inflammatory cells and the patient's age, sex, or location (Table 2).

In the PHE regions, a positive correlation was found between microglia and M2 phenotype levels (r = 0.443, p = 0.001), and between macrophage and M1 phenotype levels (r = 0.458, p <0.001); contrarily, a negative correlation was noted between the microglia and macrophage levels (r = −0.551, p <0.001). There was a negative correlation between the ratio of the M1/2-phenotypes (r =−0.841, p <0.001; Fig. 2A). We found that r-PHE was closely positively associated with lower levels of microglia (r = 0.443, p = 0.001) and the M2 phenotype (r = 0.443, p = 0.001, Fig. 2B), and positively correlated with increased levels of macrophages (r = 0.507, p <0.001) and the M1 phenotype (r = 0.458, p = 0.001, Fig. 2C).

3.3 Association between inflammatory cells and severity of stroke

We divided patients into subgroups according to the severity of stroke, as follows: GCS (3–8, 9–12, 13–14) and NIHSS (<21, ≥21). Inflammatory cell levels did not vary between the subgroups (data not shown). Inflammatory cell levels did not show a correlation with the GCS score neither at the baseline nor after treatment (Fig. 3A). However, we observed that the M1 phenotype levels were negatively correlated with the baseline NIHSS (r = -0.301, p = 0.030, Fig. 3B). Moreover, microglia (r = -0.295, p = 0.033, Fig. 3C) and the M2 phenotype (r = -0.384, p = 0.005, Fig. 3D) levels were negatively correlated with NIHSS after treatment. As NIHSS was strongly correlated with the inflammatory cell levels, it was used as the main evaluating marker in our study.

To accurately predict the patient's NIHSS after treatment, lasso regression was performed to identify variables including age, sex, location, volume of hematoma, PHE and r-PHE, and inflammatory cells. Using the lasso Poisson regression models (Fig. 4A, B), we developed a prognostic score based on five features: (0.00577 × PHE) + (0.04132 × r-PHE) - (0.0072 × M2) - (0.0285 × GCS at baseline) + (0.0156 × NIHSS at baseline). The prognostic score was calculated for each patient, and was significantly higher in

patients with NIHSS ≥21 after treatment (Fig. 4C, p <0.001). Furthermore, DCA analysis showed that the prognostic score had a higher net benefit than other markers (Fig. 4D).

4 Discussion And Conclusions

In the present study, we first characterized the presence of microglia/macrophages and their polarization in the PHE region in patients with acute hemorrhagic stroke. There was a positive correlation between the microglia and M2-phenotype levels and between the macrophage and M1-phenotype levels, but a negative correlation between the microglia and macrophage levels. Although the presence of inflammatory cells was not correlated with clinical parameters including age, sex, and location, they were strongly correlated with the PHE size. Moreover, the microglia and M2 phenotype levels were significantly correlated with NIHSS after treatment, and may serve as prognostic markers for acute hemorrhagic stroke.

The positive staining for TMEM119 was used to distinguish resident microglia from macrophages [16]. However, this single marker could easily lead to a negative association between the microglia and macrophage levels in our study, and our findings could not fully clarify this association. Activated microglia secrete cytokines and chemokines for trafficking monocytes to brain in ischemic stroke [17] and further studies are needed to clarify a potential association in hemorrhagic stroke. Additionally, based on the positive correlation between the microglia and rzPHE and NIHSS score after treatment, we observed that microglia primarily had a potentially beneficial effect on stroke. However, studies based on animal models or ischemic stroke have associated microglial functions with both beneficial and detrimental outcomes in patients with stroke [17]. Additional studies are needed to validate the beneficial role of microglia in ICH. We also found a positive correlation between the macrophage levels and PHE size, which suggests that blood-derived macrophages might have a deleterious role in hemorrhagic stroke. In support of our results, previous studies in murine ICH models have associated monocytes/macrophages with worse disability, mediated by accelerated inflammation [18, 19].

We characterized M1/2 phenotypes by detecting MHC and CD206 from CD11b + cells. Previous studies have indicated that the M1-phenotype could potentially be polarized by aging [11] or sex hormones [20]. We did not observe an association of phenotypes with age or sex in the current study, which suggests their non-notable influence, at least in hemorrhagic stroke. M1 and M2 cells not only exert opposing effects, but the ratio was also negatively correlated according to our report. A temporal analysis of the M1/2 phenotypes showed that the M1/2 phenotype levels markedly varied in animal models of hemorrhagic stroke [18]. These results indicated that the process of polarization of microglia/macrophages could be completely different. The modulation of this process is important in improving patient survival and quality of life. Our results suggest that a unique polarization of microglia and macrophages occurs in acute hemorrhagic stroke.

There were several limitations of our study. First, a different pathophysiology is involved in deep ICH and lobar ICH. Our study only assessed the basal ganglia. Future studies should investigate the role of

microglia/macrophages in lobar ICH. Second, in vivo and in vitro studies are needed to validate the results and determine the mechanisms underlying microglia/macrophage polarization. Third, in most of our patients, the hematomas were very large and required urgent surgery. However, there is a large population of patients with ICH who do not need surgery, as they present with smaller hematomas. Microglia/macrophage polarization might differ in patients with smaller ICH. Last, our patient cohort lacked long-term follow-up data. The long-term prognostic value of inflammatory cells in ICH patients requires further study.

In conclusion, our results indicate that microglia, macrophages, and their phenotypes, measured by immunofluorescence, could provide insight on the mechanism of PHE formation in acute hemorrhagic stroke.

Declarations

Declarations of interest:

None.

Author Contributions

Chang-Xiang Yan: conception, design. Xue-Ming Shen: collection and assembly of data, data analysis and interpretation. All authors: manuscript writing, final approval of manuscript and accountable for all aspects of the work.

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Contribution to the Field Statement

Perihematomal edema is a marker of secondary injury in intracerebral hemorrhage, and is characterized by poor clinical outcomes. Microglia, macrophage, or their polarization could lead to pro-inflammatory or anti-inflammatory responses in stroke. However, the association between inflammatory cell levels and

intracerebral hemorrhage is unclear. Thus, we characterized inflammatory cells, hematoma, and perihematomal edema volume and clinical outcome in patients with acute intracerebral hemorrhage. Our retrospective study which included 52 patients revealed that microglia, macrophage, and their polarization were significantly associated with r-PHE and clinical outcome in intracerebral hemorrhage. In addition, we developed a score for predicting NIHSS after treatment. Decision curve analysis showed the notable net benefits of this score, which could provide therapeutic insights for perihematomal edema after hemorrhagic stroke.

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Figures

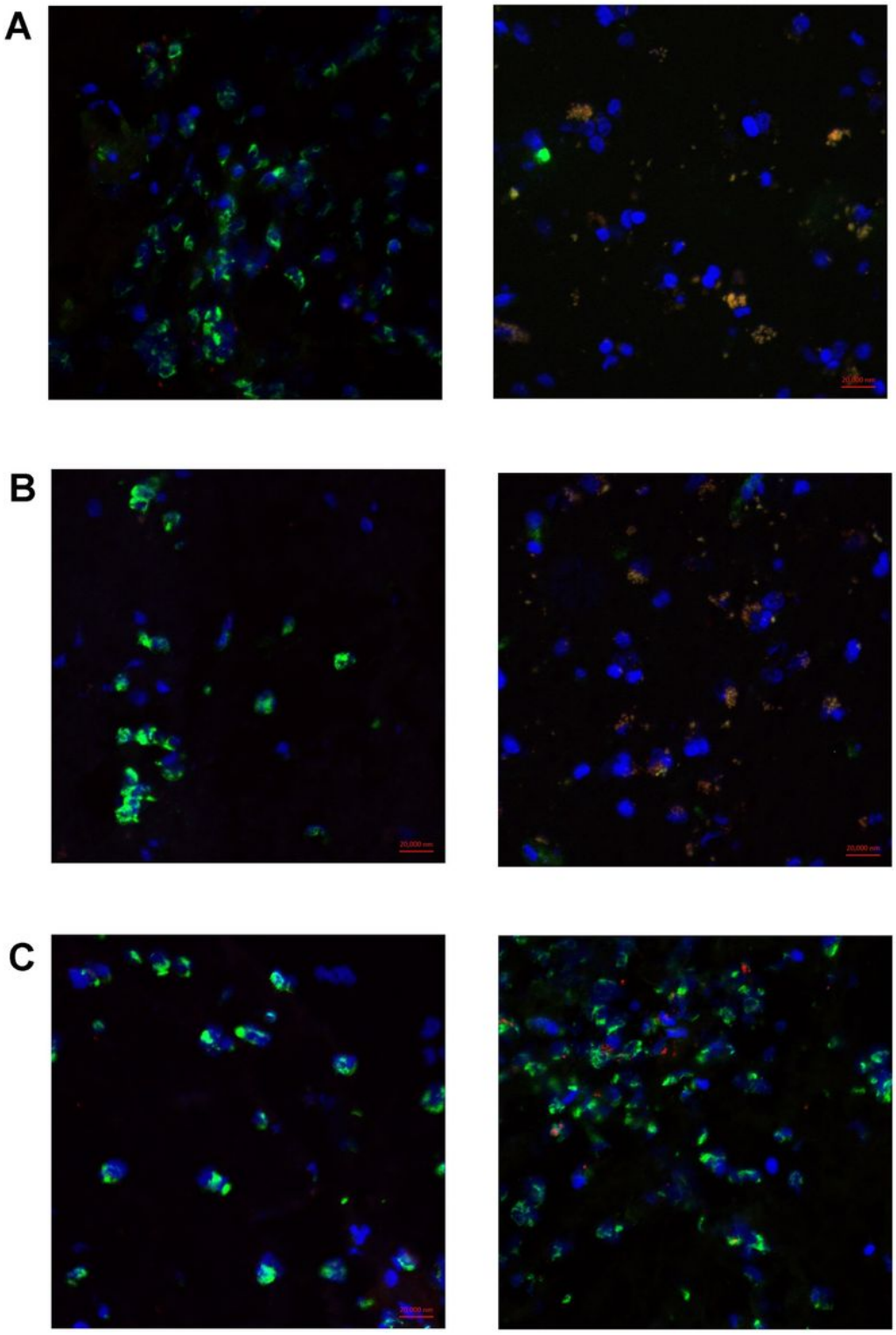

Figure 1

Representative confocal images of (A) CD11b+/TMEM119+ microglia, (B) MHC+/CD11b+ M1 and (C) CD206+/CD11b+ M2 phenotypes in the PHE regions from patients with acute ICH. The CD11b was stained with red, and TMEM119/MHC/CD206 was stained with green. The co- localization was presented as orange.

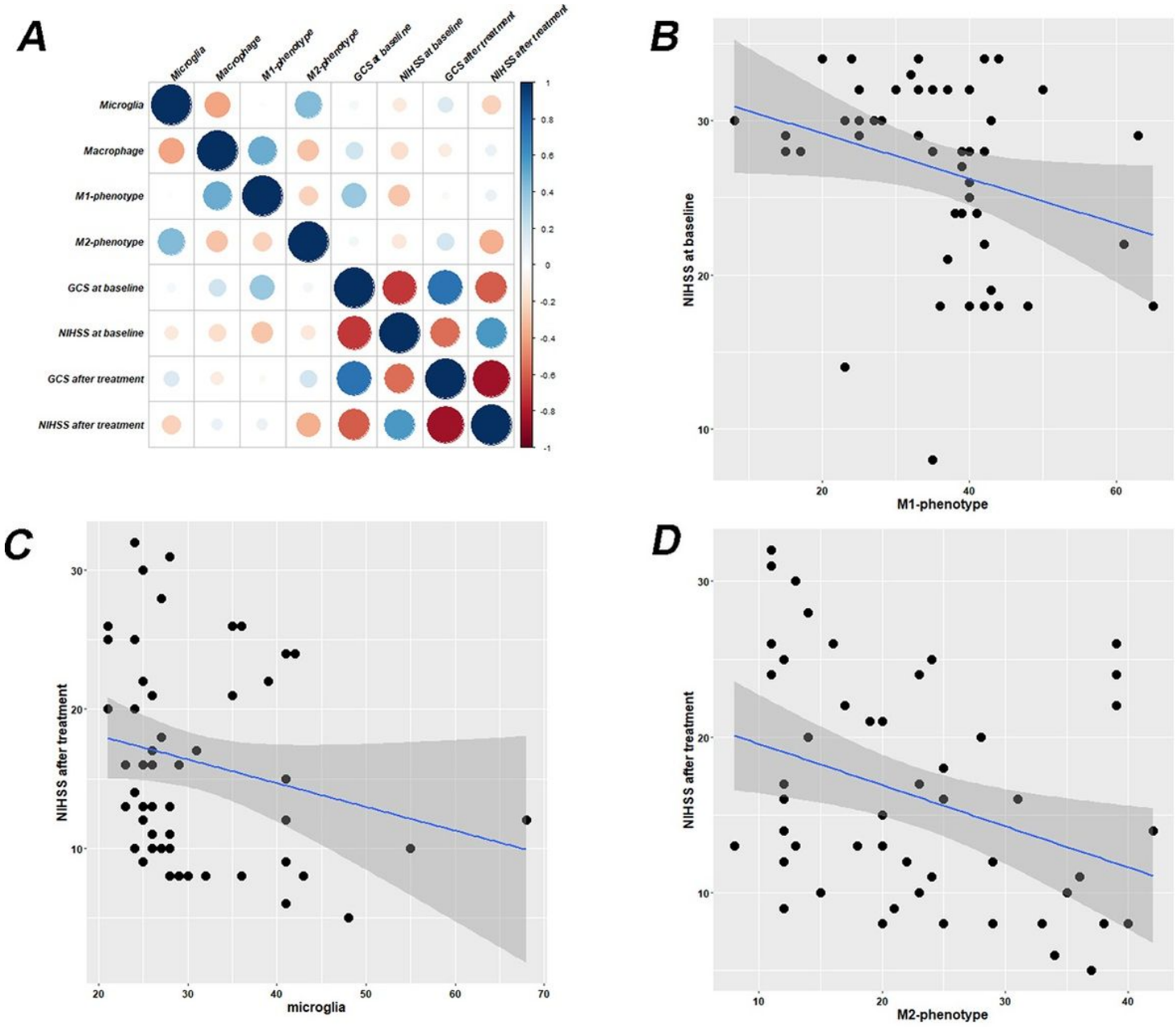

Figure 2

Correlation of microglia/macrophage, their polarization and ratios. Association between NIHSS at baseline and M1-phenotype (B), NIHSS after treatment and microglia (C), M2-phenotype (D).

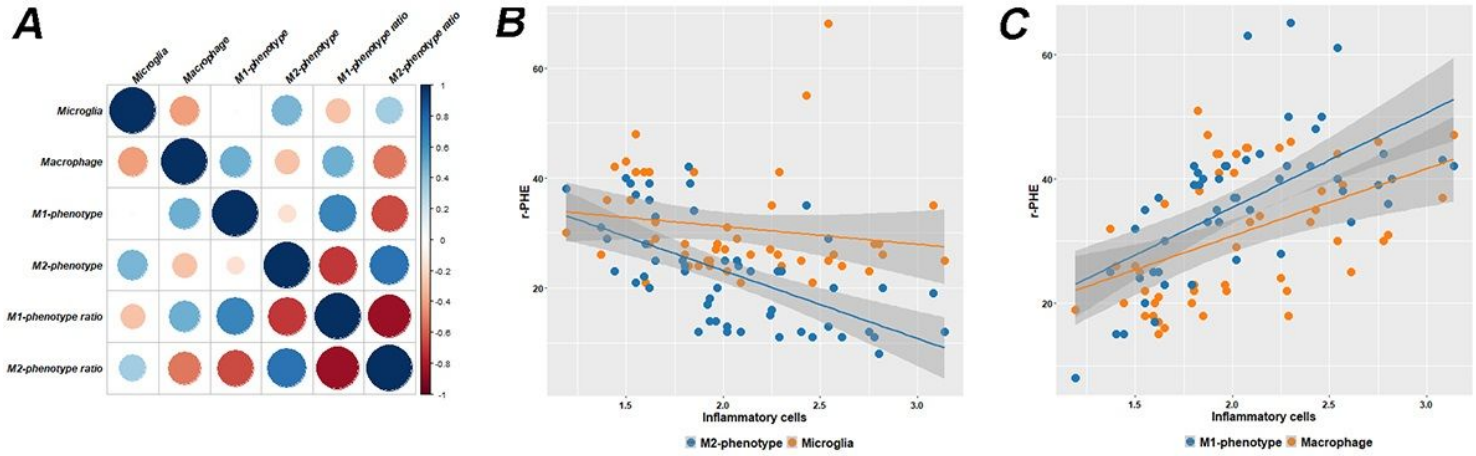

Figure 3

(A) The correlation of inflammatory cells in the PHE region. The association of r-PHE and microglia, M2-phenotype (B), and macrophage, M1-phenotype (C).

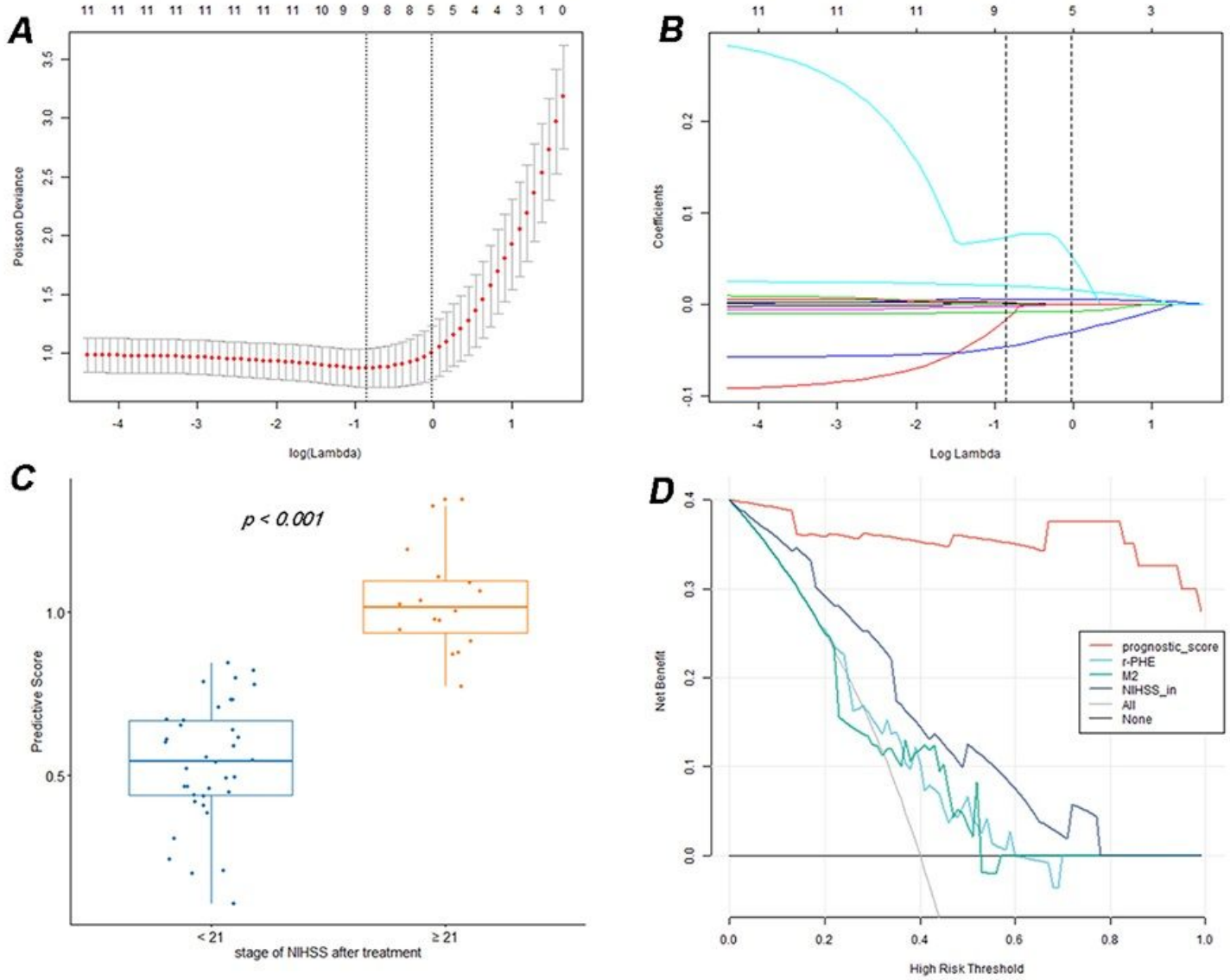

Figure 4

(A) Selection of lambda in the LASSO regression via 10-fold cross-validation; (B) LASSO coefficients were produced by Poisson regression analysis (in A). (C) The predictive scores were elevated in patients with NIHSS ≥21 after treatment. (D) DCA analysis of the predictive marker

Supplementary Files

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- Supplementtable.docx