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Brain plasticity and neuroinflammatory protein biomarkers with circulating MicroRNAs as predictors of acute brain injury outcome – A prospective cohort study

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ABSTRACT

Background: Brain recovery mechanisms after injuries like aneurysmal subarachnoid hemorrhage (aSAH), ischemic stroke (IS), and traumatic brain injury (TBI) involve brain plasticity, synaptic regeneration, and neuroinflammation. We hypothesized that serum levels of the p75 neurotrophic receptor (p75NTR) and associated signaling proteins, as well as differentially expressed (DE) microRNAs, could predict recovery outcomes irrespective of injury type.

Methods: A prospective patient cohort with ischemic stroke (IS, $n = 30$), aneurysmal subarachnoid hemorrhage (aSAH, $n = 31$), and traumatic brain injury (TBI, $n = 13$) were evaluated (total $n = 74$). Serum samples were collected at two post-injury intervals (early: 1–3 days, late: 4–8 days), and outcomes were assessed after three months using the modified Rankin Scale (mRS), categorizing outcomes as favorable (mRS 0–3) or unfavorable (mRS 4–6). Six proteins were measured using ELISAs: p75NTR, NGF, sortilin, IL1β, TNFα, and cyclophilin. DE microRNAs were identified using DESeq2, and their target genes were predicted. Serum molecules between patients with differing outcomes were compared using a Kolmogorov-Smirnov test, 2-tailed *t*-test and multivariate linear discriminant analysis (LDA).

Abbreviations: aSAH, Subarachnoid hemorrhage; IS, ischemic stroke; TBI, traumatic brain injury; p75NTR, p75 neurotrophin receptor; NGF, nerve growth factor; miRNA, microRNA; IL1β, interleukin-1β; TNFα, tumor necrosis factor α; LPS, lipopolysaccharide; mRS, modified Rankin Scale; LDA, linear canonical discriminant analysis; DE, differentially expressed; ROC, receiver operating characteristic curves; AUC, are under the curve; GCS, Glasgow Coma Scale; SORT1, sortilin; PPIA, Cyclophilin A..

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Results: Favorable ($n = 46$) and unfavorable ($n = 28$) outcome cohorts were balanced with age and sex ($p = 0.25$ and 0.63). None of the studied proteins correlated with age. Combinatory LDA of the six protein biomarkers indicated strong prognostic value for favorable outcomes (OR 2.09; AUC = 70.3%, $p = 0.0058$). MicroRNA expression changes over time were identified in the aSAH, TBI, and IS groups ($p < 0.05$, FDR corrected). Twenty-three microRNAs were commonly DE across all brain injury groups when comparing favorable and unfavorable outcomes ($p < 0.05$). LDA of four microRNAs targeting the studied proteins showed high prognostic accuracy (OR 11.7; AUC = 94.1%, $p = 0.016$).
Conclusions: The combined prognostic microRNA and protein biomarker models demonstrated accurate outcome prognostication across diverse injury types, implying the presence of a common recovery mechanism. DE microRNAs were found to target the studied molecules, suggesting a potential mechanistic role in recovery. Further investigation is warranted to study these molecules in prognostication, as well as therapeutic targets for enhancing recovery.

1. Introduction

Subarachnoid hemorrhage (aSAH), ischemic stroke (IS), and traumatic brain injury (TBI) are among the most common and devastating forms of acute brain injuries [1,2]. These conditions often result in severe neurological deficits and can have a profound impact on affected individuals' quality of life. Despite significant advances in medical care and management, the clinical outcome of these conditions remains highly variable and difficult to predict [3].

p75NTR is a transmembrane protein highly expressed in the nervous system that plays a critical role in various cellular processes including neuronal survival, apoptosis, plasticity, and neuroinflammation [4–7]. Interestingly, p75NTR belongs to a tumor necrosis factor superfamily [4]. p75NTR is known to interact with a range of ligands, including neurotrophins such as nerve growth factor (NGF), and to activate multiple signaling pathways [4,8]. In recent years, there has been growing interest in the role of p75NTR in neuroinflammation, a process involved in the pathogenesis of various neurological disorders [9–12]. Several studies have shown that p75NTR can modulate the production and release of pro-inflammatory cytokines such as interleukin-1β (IL1β), tumor necrosis factor α (TNFα), and cyclophilin A, and thereby contribute to the inflammatory response in the brain [13–17]. Furthermore, EVT901, a novel piperazine-derived compound, was discovered to interfere with p75NTR oligomerization and block p75NTR signaling by various ligands, such as prion peptide and amyloid-β [18]. In vivo studies on rats showed that EVT901 reduced lesion size, protected cortical neurons and oligodendrocytes, and improved neurological function after TBI [18]. Another recent study investigated the effects of EVT901 in a mouse model of TBI and found that EVT901 reduces the expansion of peripheral pro-inflammatory monocytes and their response to lipopolysaccharide (LPS) in vitro, suggesting a peripheral EVT901 effect that blunts inflammation [19]. Furthermore, blocking p75NTR with EVT901 was neuroprotective and reduced the number of multiple subsets of pro-inflammatory monocytes that enter the injury site at one and six weeks post-injury [19]. Sortilin, a transmembrane protein, has been shown to interact with p75NTR to regulate its trafficking and signaling. Recent studies have highlighted the importance of the sortilin-p75NTR complex in various cellular processes, including apoptosis [20].

MicroRNAs (miRNAs) are recognized as small non-coding RNA molecules that regulate gene expression and are involved in a wide range of physiological processes, including neuronal function and plasticity [21]. Several studies have suggested that changes in the expression of specific miRNAs may be associated with the severity and clinical outcome of acute brain injuries [22,23]. Understanding the role of miRNA signaling and its associations with p75NTR in acute brain injuries could potentially lead to the development of new prognostic or therapeutic strategies to improve outcomes in these devastating conditions with limited therapeutic options. By combining findings of differentially expressed (DE) miRNAs and their targets, it is feasible to uncover important regulatory mechanism candidates that may influence disease progression and recovery, offering deeper insights into potential intervention points and targeted research in future.

In this study, we hypothesized that alterations in serum p75NTR levels and its mechanistically linked signaling proteins (NGF, IL1β, TNFα, cyclophilin A, sortilin) might act as biomarkers for outcomes from diverse acute brain injuries regardless of the type of injury. The selection was supported by extensive data mining with in silico analyses of p75NTR and its associated functional interaction molecules previously published [8]. We further propose that using a discovery next-generation sequencing approach, temporal changes detected in circulating miRNAs post-injury could target the mRNAs of these studied proteins and potentially predict injury outcomes or enhance the prediction capabilities of protein biomarkers.

2. Material and methods

2.1. Study design and participants

The prospective cohort ($n = 74$) consisted of consecutively collected patients of IS ($n = 30$), aSAH ($n = 31$), and TBI ($n = 13$) patients (Fig. 1).

Inclusion criteria were aneurysmal subarachnoid hemorrhage (diagnosed with CT-angiography or DSA), ischemic stroke (either embolic, thrombotic, or cryptogenic that was diagnosed clinically by a neurologist and in CT/MRI) or traumatic brain injury causing subdural hematoma requiring surgical evacuation (CT and neurosurgeon's decision to operate). Age > 18 years old and informed consent was required. The consecutively recruited patients were admitted and treated in a tertiary University Hospital of Turku, Finland, between 2016 and 2019.

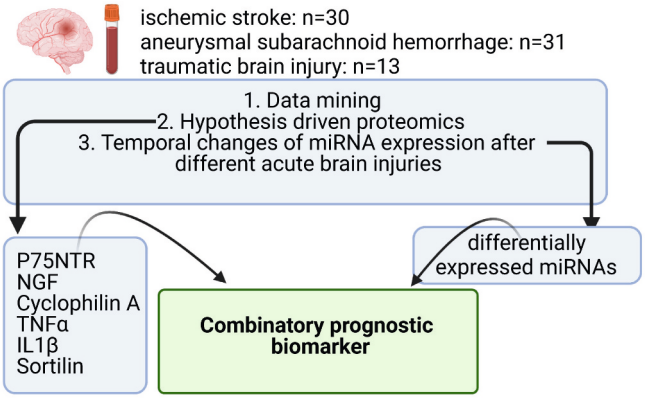

Fig. 1. Flow chart of study design. The prospective cohort (n = 74) consisted of consecutively collected patients of ischemic stroke (IS) (n = 30), aneurysmal subarachnoid hemorrhage (aSAH) (n = 31), and traumatic brain injury (TBI) (n = 13) patients. At first, datamining and extensive gene network analyses were performed to generate hypotheses for protein biomarker selection. Protein biomarker concentrations and differentially expressed (DE) miRNAs were measured for biomarker development. Serum samples were used in all analyses. P75NTR = p75 neurotrophin receptor, NGF = nerve growth factor, TNFα = tumor necrosis factor alpha, IL1β = interleukin-1β.

Standard clinical treatment was given according to in-house protocols that are in line with current recommendations for treating patients with aSAH, IS and TBI [24–26]. Peripheral venous samples were collected early at 1–3 days and late at 4–8 days after the insult. Three-month structured outcome evaluation was performed in an out-patient clinic with aSAH patients. Outcomes of IS and TBI patients were evaluated with structured telephone interviews. Modified Rankin Scale (mRS) was used to determine the outcome (favorable mRS 0–3 and unfavorable mRS 4–6). Patients who deceased during the hospitalization were noted at the time with mRS 6. Eleven patients declined to give consent for the study. One patient gave consent but decided later to withdraw from the study, thus samples and other data were not used in the study. Lastly, six enrolled patients were excluded from the protein and miRNA biomarker detection measurements due to that only very early samples being available (1–2 days after the insult). No study patients were lost to follow-up.

2.2. Serum isolation

Standard 10 ml venous blood serum collection tubes (BD Vacutainer No Additive, REF 364915) were used for blood collection. After the blood draw, each samples allowed to rest at room temperature for 30 to 60 min to allow for the clot to form. The serum was then isolated by centrifuging the blood sample at the end of the clotting time (30–60 min) in a horizontal rotor (swing-out head) for 15 min at 2200*g* at room temperature. Subsequently the serum was aliquoted in three 10 ml clean tubes (BD Vacutainer No Additive, REF 364915) for storage at −80 °C.

2.3. Timing of samples collected and analyzed

We analyzed protein biomarkers only at the late time point (post-insult days 4–8). Protein model development, including receiver operating characteristics (ROC) analytics and linear discriminant analysis (LDA), was performed using samples from the late time point. DE miRNAs were analyzed by comparing early (post-insult days 1–3) to late time points, thereby identifying miRNAs that significantly changed over time. For the miRNA prognostic model development, we used normalized expression values (early compared to late) to capture temporal changes. This approach enhances the biological relevance of our findings, as it reflects dynamic changes in miRNA expression that may be more indicative of underlying pathophysiological processes and recovery mechanisms.

2.4. Protein biomarker selection and assessment

Based on a systematically generated hypothesis from the past 20 years of published p75 literature [8], we selected to measure concentrations of p75NTR and five mechanistically and network-linked molecules (Fig. 2, **Supplemental Table S1**) using commercially available enzyme-linked immunosorbent assays (Invitrogen®, Catalog numbers: EH138RB, EHNGFR, EH433RB, EHNGF, BMS224–2, KHC3011). P75NTR was chosen as the central molecule due to its established importance in previous literature, its crucial role in animal models, and its relevance as a drug treatment target [7,13,18–20,27,28]. In addition to p75NTR, the other five proteins (NGF, IL1β, TNFα, cyclophilin A, and sortilin) were selected based on extensive literature evidence and their strong connections to p75NTR signaling in previous studies further supported by our group's extensive published in silico studies of the p75NTR functional interaction network [5,8,11,14,16,17,29]. This comprehensive approach ensures that our candidate selection is robust and relevant to the pathophysiology of acute brain injuries.

For protein biomarkers, serum samples were collected in one time-point at 4–8 days after the insult and were analyzed for concentrations of p75NTR, nerve growth factor (NGF), sortilin, interleukin-1β (IL1β), tumor necrosis factor α (TNFα) and cyclophilin A. An experienced researcher performed loading of the plates and was unaware of the subjects' clinical outcomes. Measurements were performed with a Varioskan® Flash analyzer running SkanIt Software version 2.4.3 RE. The samples were loaded in duplicate wells, and the results were averaged. Four-parameter logistic regression analysis was performed to estimate the sample concentration. Batch/plate effect was not detected (CV < 10%). One plate per disease group was used, resulting in a total of 18 ELISAs performed (six per disease group). Seventy-four patients underwent each ELISA test (IS $n = 30$, aSAH $n = 31$ and TBI $n = 13$ patients).

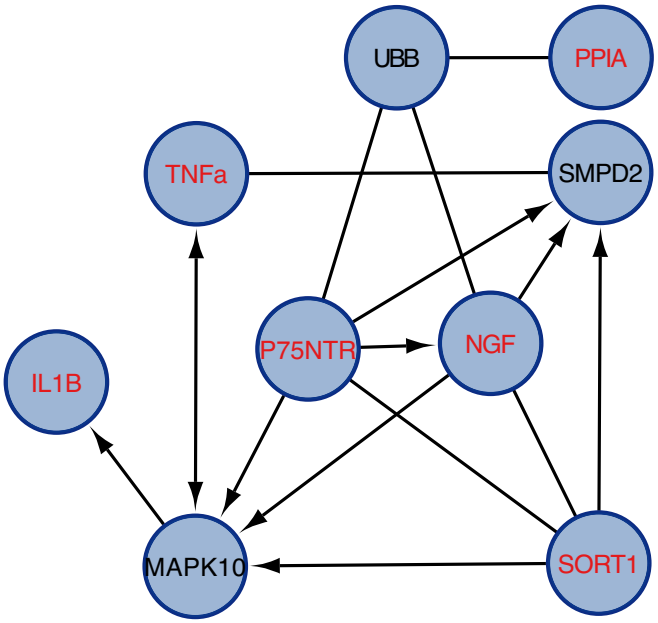

Fig. 2. Focused functional interaction network of p75NTR and mechanistically linked proteins analyzed with ReactomeFIViz in Cytoscape. Red label indicates imputed proteins. Black label indicates linker molecules. P75NTR = p75 neurotrophin receptor, NGF = nerve growth factor, TNFa = tumor necrosis factor alpha, IL1B = interleukin-1β, PPIA = cyclophilin A, SORT1 = sortilin, MAPK10 = mitogen-activated protein kinase 10, UBB=Ubiquitin B, SMPD2 = Sphingomyelin Phosphodiesterase 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.5. MiRNA extraction from serum and sequencing

MiRNAs were extracted from the serum samples using the QIAseq miRNA Library Kit (QIAGEN, Hilden, Germany) from the same patient cohort. A sub-cohort of eight patients per disease group weas randomly selected from each disease group (total $n = 24$). Extraction of miRNAs was performed from early (1–3 days) and late (4–8 days) serum samples ($n = 48$).

The quality of the samples was ensured using Bioanalyzer 2100, Agilent. Sample concentration was measured with Qubit Fluorometric Quantitation, Life Technologies. Library preparation was performed according to the library preparation protocol (QIAseq miRNA Library Kit (QIAGEN). Human Brain Total RNA AM7962 (ThermoFisher Scientific) was used as a positive control. The sequencing run was performed using Illumina NovaSeq 6000 SP platform using single-end 75-bp reads. Raw sequencing quality was assessed using FastQC.

2.6. Bioinformatics and statistics

2.6.1. Network and pathway analyses

Network and pathway analyses were performed with ReactomeFIViz, a plugin for Cytoscape (v.3.9.1), to analyze the Reactome functional interaction network and pathways (Fig. 2, **Supplemental Table S1**), which combines curated human pathways with predicted interactions from ReactomeFIViz database (v. 8.0.5) to provide a

comprehensive understanding of molecular interactions [30]. FDR-corrected $p < 0.05$ was considered to be statistically significant.

2.6.2. Protein biomarkers

For the cohort ($n = 74$), six circulating serum molecules were measured and compared between favorable outcome (mRS 0–3) and unfavorable outcome (mRS 4–6) patients. Upon testing our data for normality, we found that most biomarkers were not normally distributed, with the exception of TNFα. Consequently, we adapted our statistical analysis approach to address these distributional properties. Outliers were detected using the ROUT (Q = 1%) method [31]. For individual biomarkers that did not meet the normality assumption, we performed the Kolmogorov-Smirnov test, a non-parametric test that does not assume normality and is sensitive to differences in both the location and shape of the distributions. For TNF alpha, which met the normality assumption, we performed the standard *t*-test to compare the groups. To ensure the robustness of our combined biomarker analysis, we normalized the data using the Yeo-Johnson transformation, suitable for handling zero values [32]. After applying the Yeo-Johnson transformation, we performed Linear Discriminant Analysis (LDA). Univariate (all the circulating biomarkers, age, sex, and brain injury type) and multivariate (all the circulating biomarkers) LDA was performed, and canonical scores were used to build combinatory biomarkers with logistic modeling predicting outcomes [33]. Receiver operating characteristic (ROC) curve was generated and area under the curve (AUC) computed for the combination of model. Sensitivity and specificity was calculated using Youden method [34]. There were no missing data points. The population size was determined based on the size of previous biomarker discovery studies to ensure sufficient statistical power for detecting statistical differences [33,35,36]. Statistical analyses were performed using SAS 9.4 (SAS Institute Inc., 2016, Cary, NC, US) and Prism 9.4.0 (GraphPad Software, LLC).

2.6.3. Combination of miRNAs and protein biomarkers

MiRNAs of randomly selected prospective sub-cohort ($n = 24$) consisting of patients with IS ($n = 8$), aSAH (n = 8), and TBI (n = 8) were analyzed at two time points after the injury (early at 1–3 days and late at 4–8 days), and the outcome was assessed after 90 days using the modified Rankin Scale (favorable outcome: mRS 0–3, unfavorable outcome: mRS 4–6).

To identify potential miRNAs, we extracted miRNAs from the serum samples ($n = 48$, early and late), and used DESeq2 to identify differentially expressed (DE) miRNAs [37]. The differentially expressed miRNAs were identified between early and late samples and also further analyzed according to the patient outcome using R Bioconductor (https://www.bioconductor.org) package DESeq2 with a statistical significance level of FDR corrected *p*-value <0.05 [37]. We then predicted putative target genes for these miRNAs using the miRWalk database platform (with bonding prediction probability higher than 95%) via an online interface (http://mirwalk.umm.uni-heidelberg.de) [38]. Normalized expression values of identified DE miRNAs were used to perform linear canonical discriminant analysis (LDA). We used the resulting canonical scores to build a combinatory biomarker with logistic modeling to predict the outcome. Protein biomarkers from the same sub-cohort patients were used to combine protein and miRNA biomarkers.

3. Results

3.1. Demographics and disease characteristics of enrolled patients

The brain injury types comprised three distinct disease groups ($n = 74$), including aneurysmal subarachnoid hemorrhage (31/74, 41.9%), traumatic brain injury (13/74, 17.6%), and ischemic stroke (30/74, 40.5%) (**Supplemental Table S2 and S3**). Analysis of demographic and disease characteristics revealed a slightly higher proportion of male patients in the cohort (40/74, 54.1%). The mean age of patients in the cohort was 58.4 ± 12.7 years. At the scene of the initial ictus, the Glasgow Coma Scale (GCS) was 11.8 ± 4.4. Of the patients, 62.2% (46/74) achieved a favorable outcome (mRS 0–3), while 37.8% (28/74) suffered from an unfavorable outcome [4–6], as evaluated by the three-month mRS. The overall three-month mortality rate was 21.6% (16/74).

The cohort further studied was dichotomized into favorable (mRS 0–3, $n = 46$) and unfavorable (mRS 4–6, $n = 28$) outcome groups. The groups compared were balanced for age ($p = 0.25$) and sex ($p = 0.63$) (Table 1). The age of patients in the unfavorable group was 60.6 ± 13.1 years and in the favorable group 57.1 ± 12.4 years. Males comprised 58.7% of the favorable group and 46.4% of the unfavorable group (p = 0.63). Regarding the type of brain injury, 39.1% of the favorable group and 46.4% of the unfavorable group had aSAH (p = 0.63), 13.1% of the favorable group and 25.0% of the unfavorable group had TBI ($p = 0.22$), and 47.8% of the favorable group and 28.6% of the unfavorable group had IS ($p = 0.14$).

3.2. Protein biomarkers

The concentrations of circulating protein biomarkers including p75 neurotrophin receptor (p75NTR), nerve growth factor (NGF), interleukin-1β (IL1β), tumor necrosis factor α (TNFα), sortilin, and cyclophilin A, were quantitatively measured in serum samples at the late timepoint (4–8 days). A trend of negative correlation between IL1β and

Table 1
Patient characteristics and concentrations of p75 neurotrophin receptor (p75NTR), interleukin-1β (IL-1β), cyclophilin A and tumor necrosis factor α (TNFα), sortilin and neural growth factor (NGF) from the acute brain injury cohort ($n = 74$). Modified Rankin Scale (mRS): favorable 0–3, unfavorable mRS 4–6. Two-sample *t*-test (continuous) or Chi square test or Fisher's exact test (categorical) for *p*-values.

Variables	Favorable ($n = 46$)	Unfavorable ($n = 28$)	p-value
Age in years			0.25‡
Mean ± SD	57.1 ± 12.4	60.6 ± 13.1	
Min–Max	23.0–75.0	30.0–74.0	
Median (IQR)	59.5 (47.0–67.3)	65.0 (50.0–70.8)	
Sex			0.63
Male	27 (58.7)	13 (46.4)	
Female	19 (41.3)	15 (53.6)	
Type of brain injury			
aSAH	18 (39.1)	13 (46.4)	0.63
TBI	6 (13.1)	7 (25.0)	0.22
IS	22 (47.8)	8 (28.6)	0.14
p75NTR (ng/ml)			0.024
Mean ± SD	1.23 ± 3.09	7.29 ± 14.0	
Min–Max	0.00–18.4	0.00–40.0	
Median (IQR)	0.18 (0.00–1.00)	0.43 (0.00–4.38)	
IL-1β (pg/ml)			0.149
Mean ± SD	20.55 ± 15.80	31.25 ± 29.13	
Min–Max	4.18–63.01	3.93–137.70	
Median (IQR)	15.45 (7.02–33.53)	25.80 (12.32–36.96)	
Cyclophilin A (ng/ml)			0.279
Mean ± SD	200.50 ± 339.70	305.00 ± 387.3	
Min–Max	0–1000	0–1000	
Median (IQR)	13.92 (0–278.40)	147.40 (5.91–448.80)	
TNFα (pg/ml)			0.415
Mean ± SD	20.16 ± 4.82	19.29 ± 4.29	
Min–Max	10.04–28.10	10.86–27.44	
Median (IQR)	20.23 (16.65–23.96)	19.23 (16.90–21.39)	
Sortilin (pg/ml)			0.026
Mean ± SD	113.90 ± 173.10	147.20 ± 176.40	
Min–Max	0.00–597.50	0.00–400.0	
Median (IQR)	20.28 (2.56–165.70)	39.25 (1.84–400.0)	
NGF (pg/ml)			0.001
Mean ± SD	2134 ± 3628	3250 ± 3684	
Min–Max	0.00–12,258	0.00–10,000	
Median (IQR)	351.90 (22.71–2081)	1870 (12.63–7021)	

‡ No correlation between age and any studied protein molecule.

age ($p = 0.069$) was identified. No correlation between any other studied protein biomarker and age was detected (**Supplementary Fig. S1**). Elevated levels of p75NTR were observed in patients who experienced unfavorable outcomes with a statistically significant difference ($p = 0.024$) (Table 1, Fig. 3A). Increasing trend in concentration of circulating IL1β was detected in patients with unfavorable outcomes ($p = 0.149$) (Fig. 3B). Interestingly, the levels of NGF ($p = 0.001$) and sortilin ($p = 0.026$) were higher in the patients with unfavorable outcome (Fig. 3C and E). Cyclophilin A and TNFα did not reach statistical significance (Fig. 3D and F).

Linear discriminant analysis (LDA) of these six protein targets showed a significant model predicting favorable outcome (OR (95% CI) 2.09 (1.24–3.53); AUC = 70.3%, 95% CI = (0.579–0.827), $p = 0.0058$) (Fig. 4). Our analysis using the Youden method showed that the sensitivity of our model was 74% and the specificity was 71% (J = 0.453). LDA of these six proteins resulted in an equation with canonical scores:

$$0.303[\text{cyclophilin a}] + 0.833[\text{IL1}\beta] - 0.079[\text{sortilin}] + 0.630[\text{p75NTR}] - 0.160[\text{NGF}] + 0.068[\text{TNF}\alpha]$$

In the univariate analyses age ($p = 0.31$, OR (95% CI) 1.0 (0.98–1.06)) and sex ($p = 0.29$, OR (95% CI) 0.61 (0.24–1.54)) were not statistically significant.

In addition, a consistent pattern across biomarkers with no significant differences in concentrations between the disease groups was identified, as observed for p75NTR, NGF, sortilin, and cyclophilin across both favorable and unfavorable outcome groups (Supplementary Fig. S2). IL1β showed a difference due to relatively lower concentrations in the IS group compared to TBI and aSAH groups (Supplementary Fig. S2).

3.3. Differentially expressed miRNAs

3.3.1. Temporal changes in miRNA expression

From the same patient cohort, we randomly selected eight patients per disease group for analyzing the circulating miRNAs in serum ($n = 24$). Samples were collected and analyzed in two time points enabling the detection of miRNA expression level changes across time (total number of data points = 48, DE between early (1–3 days) versus late (4–8 days) samples).

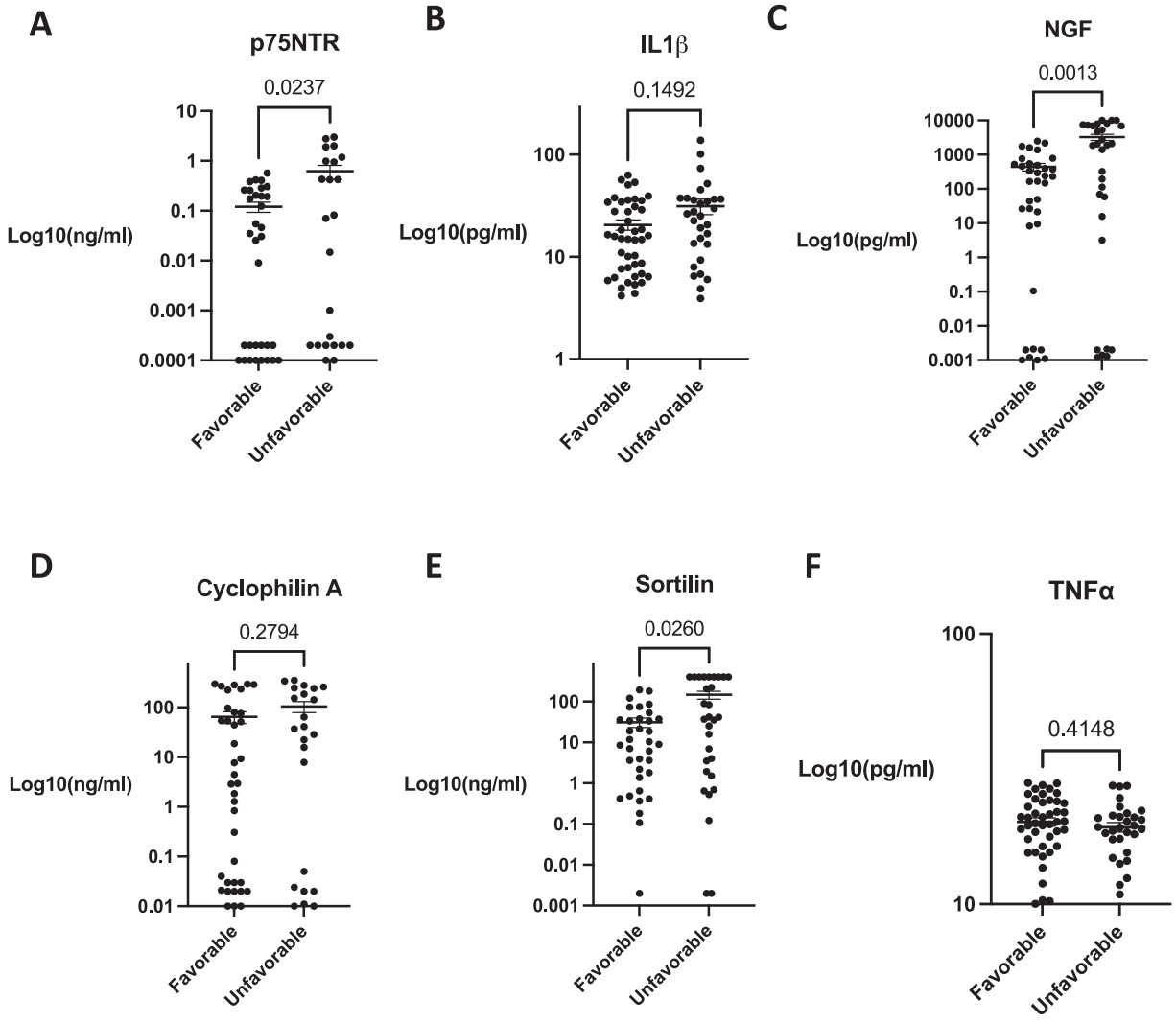

Fig. 3. Protein biomarkers after acute brain insult in the combined cohort ($n = 74$) of aneurysmal subarachnoid hemorrhage ($n = 31$), traumatic brain injury ($n = 13$), and ischemic stroke ($n = 30$) patients. Among six protein molecules identified, the p75 neurotrophin receptor (p75NTR) showed higher serum levels of p75NTR in patients suffering from the unfavorable outcome ($p = 0.024$) (A). Circulating interleukin-1β (IL1β) showed an increasing trend in patients with unfavorable outcomes ($p = 0.149$) (B). Nerve growth factor (NGF) and sortilin concentrations were higher in unfavorable group ($p = 0.001$ and $p = 0.026$ respectively) (C and E). Cyclophilin A and tumor necrosis factor α (TNFα) did not reach statistical significance (D and F). The serum molecule concentrations were compared between favorable (modified Rankin Scale 0–3) and unfavorable outcomes (modified Rankin Scale 4–6) using a 2-tailed 2-sample *t*-test for TNFα. For other proteins a non-parametric Kolmogorov-Smirnov test was used. Data represent mean ± SEM.

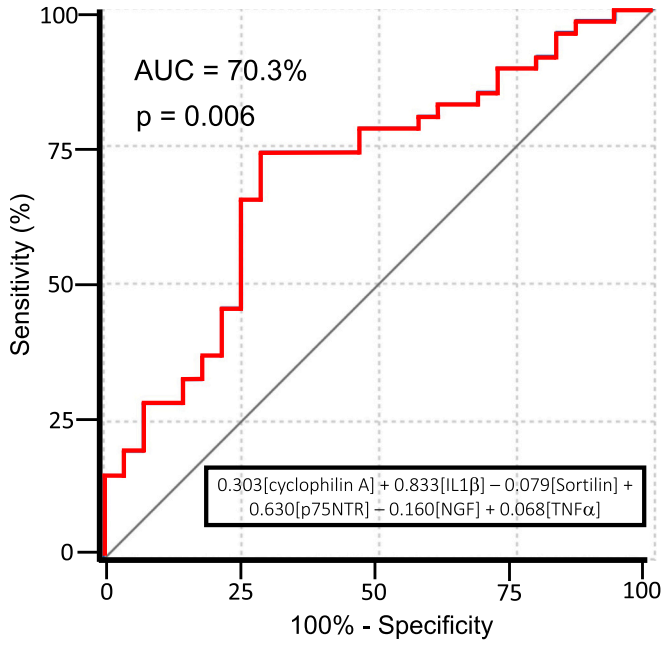

Fig. 4. Linear discriminant analysis (LDA) of these six protein targets showed significant model predicting favorable outcome (OR (95% CI) 2.09 (1.24–3.53); Area under the curve (AUC) = 70.3%, 95% CI = (0.579–0.827), p = 0.0058). LDA of these six brain plasticity and neuroinflammatory linked proteins resulted an equation with canonical scores: 0.303[cyclophilin A] + 0.833[IL1β] – 0.079[sortilin] + 0.630[p75NTR] – 0.160[NGF] + 0.068[TNFα]. Sensitivity of our model was 74% and the specificity 71%. Sensitivity of our model was 74% and specificity 71%, with a Youden Index (J) of 0.453.

Eleven differentially expressed (DE) miRNAs were identified as being temporally altered ($p < 0.05$, FDR corrected). Three DE miRNAs were detected to be altered temporally in the aSAH group, four in the TBI group, and two in the IS group ($p < 0.05$, FDR corrected) (**Supplemental Table S4**). All the miRNAs identified were unique in the disease groups, none of them were shared as being common between the diseases (**Supplemental Table S4**). Hsa-miR-1275 had a markedly altered fold change in the aSAH group, whereas hsa-miR-148b-5p fold change was identified as markedly altered in the TBI group. More modest alterations in fold changes across time were identified in the IS group.

3.3.2. *Common miRNAs associated with clinical outcome*

We combined all miRNA expression data from all different disease groups, divided the patients into two categories according to their outcome (favorable outcome mRS 0–3, $n = 15$), unfavorable outcome mRS 4–6, $n = 9$) and performed DE analysis detecting temporal expression changes of miRNAs. The analysis identified 23 miRNAs that were common to all disease groups and differentiated the favorable and unfavorable groups ($p < 0.05$) (Table 2). Thirteen miRNAs were downregulated and eight miRNAs were upregulated. Putative target gene analysis showed that four miRNAs were highly targeting to our protein biomarkers studied with significant support from the literature (Table 2, **Supplemental material**). Hsa-miR-146b-3p, hsa-miR-485-3p, hsa-miR-5010-5p and hsa-miR-485-5p were identified to target *p75NTR*, *NGF*, *SORT1* (sortilin), *PPIA* (cyclophilin A), *IL1β*, and *TNFα* (Table 2, Fig. 5A). Thus, these four miRNAs were selected for further candidates to analyze as prognostic biomarkers.

We then proceeded to investigate the association between selected circulating miRNAs and patients' outcomes using miRNAs normalized expression values (temporal change) that were compared between favorable and unfavorable outcomes. Circulating miRNAs hsa-mirR-146-3p, hsa-miR-5010-5p and hsa-miR-485-5p were identified to be upregulated in patients with favorable outcome ($p < 0.05$) (Fig. 5B-D). However, Hsa-miR-485-3p did not reach statistical significance

Table 2
Analyzed 23 commonly differentially expressed microRNAs (miRNAs) across all studied brain injury types when compared between dichotomized favorable (mRS 0–3) and unfavorable (mRS 4–6) outcome groups ($p < 0.05$). MirWalk was used analyzing the target genes with predicted >95% binding probability. The four first miRNAs were used in biomarker development.

miRNA	miRNA targets predicted >95% binding probability	miRNA targets validated*	Binding site	Log2 FC	p-value
hsa-miR-146b-3p	*SORT1, p75NTR, TNFα*	No	CDS, 3'UTR	−5.44	0,003
hsa-miR-485-3p	*p75NTR, IL1β, SORT1, NGF, TNFα*	No	3'UTR, 5'UTR	−2.88	0.008
hsa-miR-5010-5p	*p75NTR, IL1β, PPIA, NGF, TNFα*	Yes	3'UTR, CDS	−6.60	0.009
hsa-miR-485-5p	*SORT1, IL1β, PPIA*	No	3'UTR	−4.18	0.030
hsa-miR-6803-3p	*PPIA*	No	3'UTR	−6.66	0.006
hsa-miR-885-5p	*NGF*	No	CDS	−3.89	0.010
hsa-miR-323a-3p	*SORT1*	No	3'UTR	−5.23	0.011
hsa-let-7i-3p	*NGF*	No	5'UTR	5.02	0.012
hsa-miR-1270	*NGFR, PPIA*	No	3'UTR	−4.95	0.015
hsa-miR-4516	*SORT1, TNFα*, NGF	No	3'UTR, 5'UTR, CDS	1.32	0.016
hsa-miR-1247-5p	*NGFR*	No	3'UTR	3.55	0.021
hsa-miR-3124-5p	*NGFR, NGF*	No	3'UTR, CDS	−5.95	0.029
hsa-miR-1-3p				−2.52	0.033
hsa-miR-598-3p				−4.87	0.034
hsa-miR-551a	*PPIA, SORT1*	No	3'UTR, CDS	3.83	0.035
hsa-miR-3178	*SORT1, PPIA*	No	3'UTR, 5'UTR	3.33	0.038
hsa-miR-374a-5p				−3.88	0.041
hsa-miR-18a-3p	*TNFα, SORT1*	No	3'UTR	3.46	0.043
hsa-miR-197-5p	*SORT, IL1β*	No	3'UTR, CDS	4.55	0.044
hsa-miR-365a-3p				−4.41	0.045
hsa-miR-365b-3p				−4.40	0.046
hsa-miR-192-5p				−0.84	0.046
hsa-miR-27a-5p	*PPIA, SORT1, NGFR*	No	3'UTR, 5'UTR, CDS	4.76	0.047

* miRTarBase: experimentally confirmed miRNA-target interactions. P75NTR = p75 neurotrophin receptor, NGF = nerve growth factor, TNFα = tumor necrosis factor α, IL1β = interleukin 1β, PPIA = cyclophilin A, SORT1 = sortilin, mRS = modified Ranking scale, CDS = coding DNA sequence.

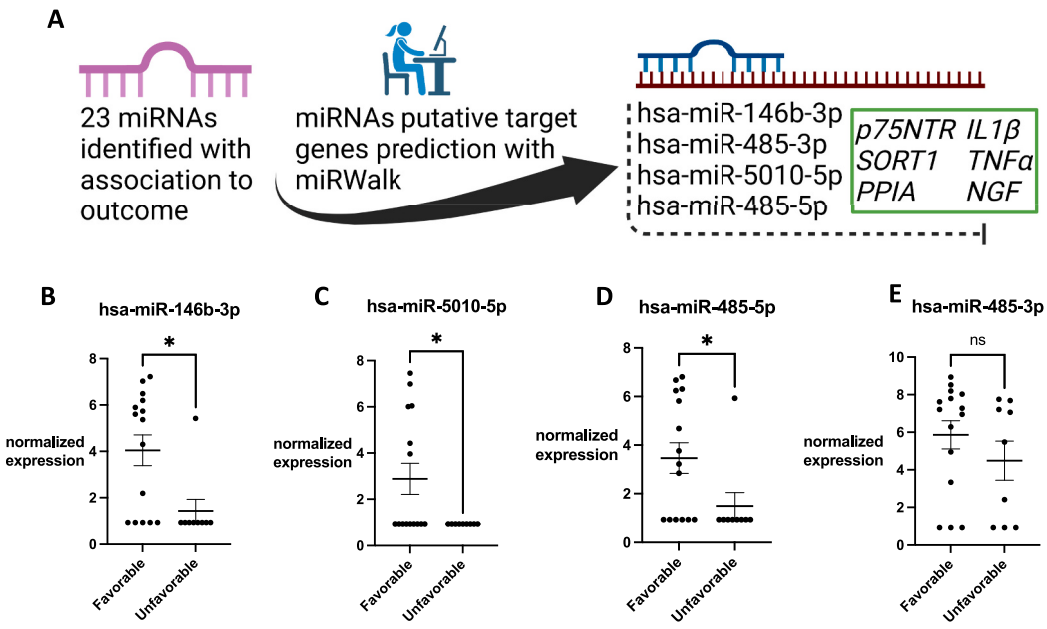

Fig. 5. Identified microRNAs (miRNAs) after acute brain insult in the combined cohort ($n = 24$) of aneurysmal subarachnoid hemorrhage ($n = 8$), traumatic brain injury (n = 8), and ischemic stroke (n = 8) patients. MiRNAs identified mechanistically linked to studied protein targets (A). Circulating miRNAs hsa-miR-146b-3p, hsa-miR-5010-5p, and hsa-miR-485-5p were identified to be up regulated in patients with favorable outcomes (B—D). Hsa-miR-485-3p did not reach statistical significance (E). The serum miRNA normalized expression values were compared between favorable (modified Rankin Scale 0–3) and unfavorable outcomes (modified Rankin Scale 4–6) using a 2-tailed 2-sample *t*-test. $^*P < 0.05$. Data represent mean ± SEM.

(Fig. 5E).

3.4. Combinatory biomarker

The performance of four miRNAs (hsa-miR-146b-3p, hsa-miR-485-3p, hsa-miR-5010-5p, and hsa-miR-485-5p) in linear discriminant analysis was evaluated using the receiver operating characteristic (ROC) curve. The area under the curve (AUC) was calculated to be 94.1%, with a 95% confidence interval (CI) of (0.849–1.00) and a *p*-value of 0.016, indicating good discriminatory ability of the miRNAs (OR (95% CI) 11.7 (2.39–226)) (Fig. 6). The linear discriminant analysis of these four miRNAs resulted in an equation with canonical coefficient scores:

$$0.636[\text{hsa} - \text{miR} - 146\text{b} - 3\text{p}] + 0.576[\text{hsa} - \text{miR} - 485 - 3\text{p}] + 0.652[\text{hsa} - \text{miR} - 5010 - 5\text{p}] + 0.372[\text{hsa} - \text{miR} - 485 - 5\text{p}]$$

This equation can be used to prognosticate the presence of the favorable outcome, based on the expression levels of these four miRNAs.

Furthermore, the prognostic model was developed using the canonical scores of a combined miRNA biomarker and one of six potential protein biomarkers, (p75NTR, NGF, sortilin, IL1β, TNFα, and cyclophilin A). However, analysis of the data revealed that none of the protein plus miRNA biomarker models were superior to the miRNA-only model in predicting outcome.

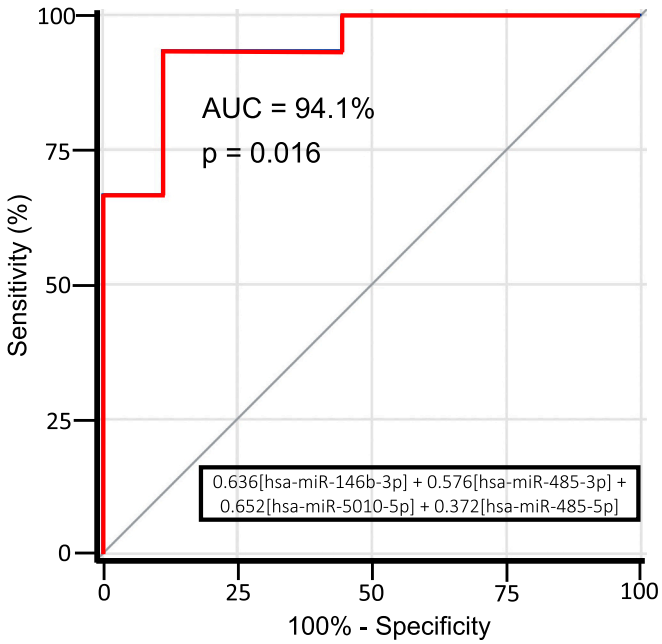

Fig. 6. The receiver operating characteristic curve of identified four miRNAs (hsa-miR-146b-3p, hsa-miR-485-3p, hsa-miR-5010-5p, hsa-miR-485-5p) in linear discriminant analysis (LDA) predicting favorable outcome: OR (95% CI) 11.7 (2.39–226); Area under the curve (AUC) = 94.1%, 95% CI = (0.849–1.00), $p = 0.016$. LDA of these four miRNAs resulted in an equation with canonical scores: 0.636[hsa-miR-146b-3p] + 0.576[hsa-miR-485-3p] + 0.652[hsa-miR-5010-5p] + 0.372[hsa-miR-485-5p].

4. Discussion

This study analyzed circulating protein biomarkers and DE miRNAs in patients with IS, TBI, and aSAH to identify potential biomarkers associated with favorable or unfavorable outcomes. The study identified combinatory protein biomarker of p75NTR, NGF, IL1β, TNFα, sortilin and cyclophilin A as good prognostic protein biomarker associated with outcome, while four miRNAs (hsa-miR-146b-3p, hsa-miR-485-3p, hsa-miR-5010-5p, and hsa-miR-485-5p) were found to target these protein biomarkers and were identified as a powerful prognostic biomarker for outcome. We identified that all these circulating miRNAs were upregulated in the favorable outcome group, whereas targeted protein biomarkers were downregulated in the favorable outcome group. This may indicate biologically relevant links between the studied proteins and miRNAs. By focusing on temporal changes in miRNA expression, we identified candidate miRNAs whose dynamic alterations imply

important mechanistic links to the underlying pathophysiological processes. This approach enhances the biological relevance of our findings, as it reflects the evolving nature of disease mechanisms and recovery from brain injuries. Furthermore, the performance of these four miRNAs in linear discriminant analysis showed a highly accurate discriminatory ability. However, in this study, the analysis did not reveal any significant prognostic model combining both protein and miRNA biomarkers.

4.1. Identified circulating biomarkers

The miRNAs hsa-miR-146b-3p, hsa-miR-485-3p, hsa-miR-5010-5p, and hsa-miR-485-5p were identified to target specific protein biomarkers and were found to be differentially expressed in patients with favorable versus unfavorable outcome across different brain injury types. miR-146b-3p has been shown to have a role in various biological processes, including inflammation and cell differentiation [39,40]. Downregulation of miR-485-3p has been shown to promote the progression and development of cerebral infarction by mediating the RAF/P38MAPK/COX-2 signal transduction pathway, which is involved in inflammation [40]. Interestingly, in our study, we identified increased expression of hsa-miR-146b-3p and downregulation of its putative target proteins in a favorable outcome group. This may indicate a biologically relevant link in the recovery of different acute brain injuries.

MiR-485-5p has been identified to promote neuron survival after cerebral ischemia/reperfusion injury [41]. MiR-485-5p expression was significantly decreased after injury, and interestingly, over-expression of miR-485-5p protected neuronal cells from cell death. In our study we identified over-expression of hsa-miR-485-5p in the favorable outcome group. The identified miR-485-5p-mediated neuronal survival was achieved by activating the Rac1/Notch2 signaling pathway, and this pathway is also linked in synaptic plasticity [41–43]. Similarly, hsa-miR-485-3p has been identified to modulate neuronal survival and neuroinflammatory responses [44,45].

Hsa-miR-5010-5p was over-expressed as well in the favorable outcome group in our study. Hsa-miR-5010-5p is involved in the regulation of lipid metabolism in the early stages of Alzheimer's disease [46]. Specifically, it was found to be positively correlated with lipids arachidonic acid (FA (20:4)) and dihomo-gamma-linolenic acid (FA (20:3)) but negatively correlated with the triglyceride TG (17:0/17:0/17:0). These findings suggest that miRNAs, such as hsa-miR-5010-5p, could play a crucial role in the dysregulation of lipid metabolism within the brain. Although the exact mechanisms and implications of this relationship are not yet fully elucidated, it is possible that alterations in lipid metabolism might contribute to the pathogenesis of acute brain injuries [47].

Neuroinflammatory and brain plasticity processes play a crucial role in the pathogenesis and recovery of acute brain injuries such as aSAH, TBI, and IS (15, 48). The neurotrophin receptor p75NTR is involved in neuronal cell death and neuroinflammation in acute brain injuries [4,5,13]. Blocking p75NTR signaling has been shown to improve outcomes in animal models of TBI and IS [18,28]. Our study found that circulating p75NTR was down-regulated in the group with favorable outcomes, consistent with animal studies showing that increased p75NTR signaling leads to unfavorable outcomes [18,19,28]. We also observed the upregulation of miRNAs targeting p75NTR, suggesting a potential interaction between miRNAs and p75NTR.

The primary hypothesis of this study centered around the idea that specific serum protein biomarkers would manifest alterations irrespective of the type of brain injury sustained. This hypothesis was rooted in the broader understanding of shared neurobiological mechanisms, such as neuroinflammation, synaptic regeneration, and brain plasticity, that are activated in response to diverse brain insults. The uniformity in the concentrations of these biomarkers across the injury groups suggests that they could be part of a common neurobiological response to brain injuries, regardless of the etiology of the injury.

4.2. Prognostic models

In this study, the performance of four miRNAs (hsa-miR-146b-3p, hsa-miR-485-3p, hsa-miR-5010-5p, and hsa-miR-485-5p) was evaluated using LDA. The results indicated that these miRNAs possess good discriminatory ability with an AUC of 94.1% and a 95% CI of (0.849–1.00), along with a p-value of 0.016 (OR 11.7 (2.39–226)). The canonical coefficient scores derived from the LDA led to the development of an equation that could potentially predict the presence of a favorable outcome based on the levels of these miRNAs. Although the combinatory protein biomarker did not yield the performance of the miRNA-model (OR 2.09 (1.24–3.53); AUC = 70.3%, 95% CI = (0.579–0.827), $p = 0.0058$), it is sufficient to emphasize the potential pathobiological role of studied molecule network in acute brain injuries. Furthermore, a prognostic model incorporating both miRNA and protein biomarkers was developed, including p75NTR, NGF, sortilin, IL1β, TNFα, and cyclophilin A.

4.3. Implications for the biomarker equation and group combination

The robustness of the biomarker equation is notably strengthened by the general consistency observed in biomarker concentrations across disease groups. Since the concentrations of most biomarkers do not vary significantly between aSAH, TBI, and IS, it implies that the derived equation – based on the combinatorial linear discriminant analysis (LDA) of these six proteins – can be applied universally across different brain injury types. This may have substantial implications; a unified model can be used for prognostication without the need to develop disease-specific models. Furthermore, the fact that most biomarker concentrations didn't differ significantly among the injury cohorts further validates the approach of combining data from these different diseases. Combining data from these disease groups not only increases the sample size, enhancing the statistical power and robustness of findings but also highlights the potential universal pathways of brain recovery post-injury. This is a significant stride towards understanding the shared neurobiological underpinnings following brain injury and opens avenues for developing interventions that target these common pathways.

These results underscore the potential utility of the identified proteins and miRNAs as prognostic biomarkers offering new insights into patient stratification and personalized treatment approaches. Further validation of these findings in larger patient cohorts is warranted to confirm the validity of the protein and miRNA-based model and to explore the possible integration of these into clinical practice.

4.4. Limitations

While the study provides valuable insights into the potential prognostic value of circulating protein biomarkers and miRNAs in predicting outcomes for brain injury patients, several limitations need to be acknowledged and considered while interpreting the results. First, the sample size of the study is relatively small ($n = 74$), which may limit the generalizability of the results and introduce unexpected bias, reducing the power of the prediction model. A larger, and more diverse patient cohort would be necessary to validate these findings thoroughly.

Second, the study population comprises patients with three distinct types of brain injuries, potentially introducing heterogeneity in the disease pathophysiology and molecular profiles. However, testing three different types of brain injuries allowed us to identify common reflective recovery mechanisms candidates and prognostic biomarkers that are robust across various pathophysiological conditions. This heterogeneity enhances the strength and generalizability of our findings, suggesting that the identified biomarkers may have broader applicability in predicting outcomes for a wide range of brain injuries.

Third, the analysis of miRNA expression changes across time was performed in a small subgroup of patients ($n = 24$), potentially limiting

the generalizability of the findings. Future studies should consider investigating temporal changes in miRNA expression in a larger cohort and at additional time points to provide a more comprehensive understanding of the miRNA dynamics following brain injury.

Finally, the study has identified a set of miRNAs that demonstrate significant associations with patient outcomes, but the mechanistic links between these miRNAs and the observed clinical outcome remain unclear. Further studies are required to elucidate the biological functions of these miRNAs as well as their potential roles in the pathophysiology of brain injuries.

Despite these limitations, the study provides a promising foundation for the development of novel prognostic biomarkers in brain injury patients and provides targets for mechanistic validation in animal models. Further research is warranted to address these limitations and to validate and expand upon the findings in larger patient populations.

5. Conclusions

The results of this study suggest that measuring serum levels of p75NTR and its mechanistically linked proteins can predict outcomes in patients with different types of acute brain injuries. This finding indicates that there may be a shared mechanism for recovery across various types of brain insults. The DE miRNAs identified in this study were found to target the studied molecules, further supporting the notion that miRNAs may play a role in outcome. The combinatory biomarker developed using these miRNAs performed well in predicting outcomes across different types of brain injuries, which suggests that the identified temporally altered miRNAs may be involved in the recovery process regardless of the type of injury. Additionally, the common miRNAs identified in this study may provide new targets for mechanistic validation in disease models of stroke and TBI, which could lead to the development of novel therapeutic targets for acute brain injuries. However, validation in larger cohorts and studies in animal models are necessary to confirm these findings.

Study approval and ethics

The Turku University Hospital institutional review board and ethics committee approved this study (T291/2016), which was conducted in accordance with the Declaration of Helsinki and its subsequent amendments. Written informed consent was obtained from all participants or their legal representatives if the subject was unable to provide consent due to severe acute illness. The study adhered to the laws and regulations of Finland.

Consent for publication

Not applicable.

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CRediT authorship contribution statement

Antti Sajanti: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Yan Li:** Writing – original draft, Methodology, Formal analysis. **Santtu Hellström:** Writing – original draft, Visualization, Formal analysis, Data curation. **Ying Cao:** Writing – original draft, Methodology, Formal analysis. **Romuald Girard:** Writing – original draft. **Juzoh Umemori:** Writing – review & editing, Data curation. **Janek Frantzén:** Writing – review & editing. **Fredrika Koskimäki:** Writing – review & editing, Data curation. **Seán B. Lyne:** Writing – review & editing. **Johannes Falter:** Writing – review & editing. **Tomi Rantamäki:** Writing – review & editing. **Riikka Takala:** Writing – review & editing. **Jussi P. Posti:** Writing – review & editing. **Susanna Roine:** Writing – review & editing, Data curation. **Sulo Kolehmainen:** Software, Resources. **Abhinav Srinath:** Writing – review & editing. **Miro Jänkälä:** Writing – review & editing. **Jukka Puolitaival:** Writing – review & editing. **Melissa Rahi:** Writing – review & editing, Data curation. **Jaakko Rinne:** Writing – review & editing. **Eero Castrén:** Writing – review & editing, Resources. **Janne Koskimäki:** Writing – original draft, Visualization, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

The anonymized data from this study can be made available upon request to qualified researchers who have obtained appropriate institutional review board (IRB) approval. Requests should be directed to the corresponding author. The raw genomic sequencing data used in this study have been deposited in the NCBI Gene Expression Omnibus (GEO) database under accession number GSE233775 and are publicly available for download.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jns.2024.123169.

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