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Short Communication

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Plasma phosphorylated neurofilament heavy chains as a potential marker for ischemic stroke patients

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Abstract

***Background**: The aim of the study was to determine the utility of plasma NfH in correlation with serum hsCRP for severity and short-term functional outcome prediction after ischemic stroke.*

***Methods**: 124 patients and 40 healthy controls were enrolled, serial plasma neurofilament heavy chains and hsCRP concentrations were measured and evaluated for TOAST subtype, stroke severity and functional outcome at discharge.*

***Results**: Serum level of hsCRP was significantly higher in patients versus controls ($p<0.05$) with no difference between TOAST subtypes. Plasma NfH concentration on day 5 was higher in CE stroke compared to LAA group and SVO group. A positive correlation between NfH levels on day 5 and mRS at discharge ($r=0.304$, $p=0.001$) and a gender stratification of hsCRP and mRS at discharge was found. Values of 6.04 mg/l for hsCRP and 46.4 ng/ml for NfH were found predictive for unfavorable short-term outcome, but after adjusting for age, sex and stroke severity, the prediction power was lost.*

***Conclusions**: Plasma concentration of NfH shows a significant increase over the first five days after ischemic stroke, in correlation with inflammatory status and short-term evolution.*

Keywords: phosphorylated neurofilament heavy chains, ischemic stroke

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Introduction

A large number of peripheral markers have been investigated in order to estimate the severity of the ischemic stroke and patient outcome (1), but none of these markers has been postulated as pathognomonic for this pathology, so far. Recent studies suggested the utility of cerebrospinal fluid (CSF) neurofilaments for cerebrovascular and spinal pathology (2,3), but this type of sampling is more invasive and prone to severe side effects. As an important structural protein in neurons, increased concentration of neuro-

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filaments in peripheral blood might mirror the neuronal destruction in various central nervous system (CNS) disorders and to be considered a potential marker for axonal damage, detectable now in peripheral blood by sensitive ELISA methods (4), being at the same time a causative agent for neurological diseases and a marker for disease progression (5). The aim of the study was to evaluate the utility of neurofilaments heavy chains (NfH) as a potential biomarker for evaluation of patients with ischemic stroke, in terms of stroke severity and functional outcome and to assess the relationship between plasma NfH and the concentration of high-sensitivity C Reactive Protein (hsCRP) as an inflammatory marker, as it is well known that inflammation triggers multiple pathogenic pathways after glucose-oxygen deprivation and hsCRP synthesis is induced by interleukin 6 (IL 6), a potent pro-inflammatory cytokine overexpressed in ischemic stroke (6).

Material and methods

124 ischemic stroke patients consecutively admitted during the first day after stroke onset and 40 healthy controls from the ambulatory were included in this study. The study protocol was approved by the Institutional Ethical Committee; only participants who have signed the informed consent were further enrolled in the study. The exclusion criteria for both patients and healthy controls were: age below 18, traumatic brain injury, hemorrhagic stroke, multiple sclerosis or stroke in the past three months, disease onset > 24 hours; known diagnosis of tumors, infections or chronic inflammatory diseases, pregnancy. Eight patients were excluded.

Clinical assessment

Stroke severity was assessed after admission using the National Institutes of Health Stroke Scale (NIHSS) on day 1, 5 and before discharge from the hospital and dichotomized in mild (NIHSS<8) and severe stroke (NIHSS≥8). Short-term functional outcome was assessed by modified Rankin Scale (mRS) at discharge from the hospital. Good outcome was defined as mRS ranging from 0-2, and poor outcome as mRS ranging from 3-6. Stroke subtype was classified according to TOAST (Trial of Org 10172 in Acute Stroke Treatment) criteria in large artery atherosclerosis (LAA), cardioembolic (CE) and small vessel occlusion (SVO) (7).

Laboratory testing

Blood was collected on the same days as NIHSS evaluation (1, 5 days) for dynamic evaluation of peripheral biomarkers of interest. Clot activator tubes for serum and Na-heparin tubes for plasma were used; after initial processing, the samples were aliquotted and stored at -80 °C until the parameters were analysed. Plasma NfH concentration was measured according to the manufacturer's instructions by ELISA Human Phosphorylated Neurofilament H (BioVendor – Laboratorní medicína a.s, Czech Republic), detection limit 23.5 pg/ml, within- and inter-run assay precision < 5%. Serum hsCRP concentrations were quantified by enhanced immunoturbidimetric assay hsCRP Vario on Architect 4000 (Abbott Laboratories Abbott Park, Illinois). For hsCRP, detection limit was 0.1 mg/L, within- and inter- run assay precision < 4%.

Statistical analysis

Categorical data were expressed as counts and percentage, continuous variables were expressed as mean ± standard deviation (SD) for parametric data and median and interquartile range (IQR) for non-parametric data. Mann-Whitney U test and Kruskal-Wallis test for non-parametric data or two-tailed t-test for parametric data were used for inter-groups comparisons. To assess the association between NIHSS, mRS and laboratory variables, Pearson's or Spearman's rank correlation analysis as appropriate, were applied. The

relationship between outcome and clinical variables was assessed with binomial logistic regression as appropriate statistical methods. The best threshold values of plasma NfH and hsCRP for poor outcome prediction was calculated using a receiver operating characteristic (ROC) curves. In development of the regression multivariate model we selected only variables with p-value less than 0.05 in univariate regression analysis along with clinical variable. A two-tail p-value <0.05 was considered statistically significant.

Results

Demographic and laboratory findings in control group and ischemic patients are represented in Table 1. Serum level of hsCRP was significantly higher in patients versus controls both for day 1 and 5 (*p=0.0002, p=0.0012* respectively), with no significant difference in ischemic group between these days. There was no difference in plasma NfH concentration between control group and ischemic group on admission (p=0.09), but the concentration increased significantly over the next five days in the ischemic group compared to the control group (*p=0.0004*).

According to TOAST classification, 36.2% (n=42) were CE, 60.3% (n=70) were LAA and only 3.4% (n=4) SVO. NIHSS on admission was higher in CE group, with a median of 5 (IQR 4-7) comparing to LAA group with a median of 4 (IQR 2-8) and SVO group with a median of 1.50 (IQR 0.5-3), without reaching the statistical threshold, p=0.08. There was no difference on day 1 for serum hsCRP between the three groups, with a median of 6.01 mg/L (IQR 3.13-13.5 mg/L) for LAA group, 7.63 mg/L (IQR 2.57-12.57 mg/L) for CE group and 1.50 mg/L (IQR 1.28-6.92 mg/L) for SVO group (p>0.05). No noticeable difference was found on day 5 in hsCRP levels between LAA and CE groups; in SVO group the values were significantly lower, but the number of subjects was very low. Plasma NfH concentration on day 5 was higher in CE stroke with a median of 182.6 ng/ml (IQR 23.5-310.57 ng/ml) compared to LAA stroke with a median of 49.2 ng/ml (IQR 23.5-285.4 ng/ml) and SVO stroke median of 23.5 ng/ml (IQR 23.5-54.5 ng/ml), p<0.05.

The laboratory parameters - hsCRP and NfH - in relations with stroke outcome at discharge are detailed in Table 2.

Table 1. Demographic and laboratory parameters. Values are expressed as median and inter quartile range (IQR); †- p for difference between day 1 and 5 hsCRP; ‡ p for difference between day 1 and 5 NfH in ischemic group.

	Control group (N=40)	Ischaemic group (N=116)	p
Age (years), Median, IQR	66 59.5-77.5	72 65-79	>0.05
Sex (female) (N; %)	23 (57.5%)	66 (56.9%)	>0.05
hsCRP (mg/L) Day 1	2.58 1.7-5.1	6.1 2.7-12.6	0.0002
Day 5	-	5.6 2.2-17.7	>0.05†
NfH (ng/ml) Day 1	23.5 23.5-102.8	23.5 23.5-159.0	>0.05
Day 5	-	70.6 23.5-302.1	0.01‡

Table 2. Clinical and laboratory characteristics , expressed as median and inter-quartile range (IQR), associated with short term evolution evaluated by mRS at discharge.

Parameters	mRs <3 (n-72)	mRS ≥3 (n-44)
Gender, female, n (%)	72 (48.6%)	44 (70.5%) *
Age (years)	69.4 (9.6)	75.52 (10.0) *
NIHSS on admission	3 (1-5)	8 (5-12) **
hsCRP day 1 (mg/l)	4.71 (2.25-11.57)	9.44 (4.66-20.21) *
hsCRP day 5 (mg/l)	3.88 (1.72-8.98)	8.41 (4.24-35.17) *
NfH day 1 (ng/ml)	23.5 (23.5-117.5)	23.5 (23.5-196.0) ♯
NfH day 5 (ng/ml)	26.5 (23.5-289.8)	159.3 (24.5-350.47)*

*p<0.05; **p<0.001; #NS - not significant

The concentrations of hsCRP were significantly higher both in day 1 and 5 in patients with mRS ≥3 comparing to patients with mRS<3. Fifth day plasma NfH concentration was significantly higher in poor outcome compared to good outcome patients at disgharge. Positive correlations between 5-th day NfH levels and NIHSS on admission (*r=0.194, p<0.05*) and mRS at discharge (*r=0.304, p=0.001*) were found. Clinical outcome and laboratory parameters were stratified by gender; although the age and NIHSS on admission and at discharge were similar in both male and female groups (p>0.05), women experienced more frequently poor outcome at discharge evaluated by mRS (median=2; IQR 1-4) compared to the male group (median =1; IQR 0-3) (p=0.03). A higher concentration of hsCRP on day 5 was revealed in the female group by comparison with the male group of ischemic patients (p=0.02).

On performing univariate regressions for unfavorable outcome at discharge (Table 3), all scrutinized variables, less the CRP for one day, manifested good statistical significance.

Considering threshold values for the unfavorable outcome and expanding to multivariate analysis, by applying logistic regression, the predictive capacity was preserved only selectively; OR 3.73 (95% CI 1.61 to 8.64) for hsCRP day 5 and OR 2.69; (95% CI 1.14 to 6.40) for NfH day 5. However, predictive capacity was lost after adjustment for sex, age and NIHSS on admission (Table 4).

Table 3. Univariate regression for considered independent variable for poor outcome evaluated by mRs at discharge.

Variables	Coefficient	S.E.	P value	OR crude	95% C.I.for OR crude	
					Lower	Upper
Age, years	0.066	0.021	0.002	1.068	1.024	1.115
Sex (female)	0,0924	0,406	0,002	2.521	1.137	5.586
NIHSS on admission	2.488	0.522	<0.0001	12.047	4.327	33.542
Stroke history	0.697	0.408	0.088	2.008	0.901	4.474
hsCRP day 1	0.013	0.012	0.272	1.013	0.989	1.037
hsCRP day 5	1.396	0.410	0.0007	4.042	1.809	9.034
NfH day 5	1.126	0.418	0.007	3.083	1.358	6.998

Table 4. Multivariate logistic regression for short term poor outcome prediction

Variable	Coefficient	S.E.	P value	O.R crude	95% CI for OR crude
Age (years)	0.043	0.0271	0.112	1.044	0.990 to 1.101
Sex (female)	1.102	0.528	0.037	3.012	1.068 to 8.491
NIHSS_on admission	2.623	0.664	0.0001	13.780	3.749 to 50.657
hsCRP_day 5_	0.538	0.522	0.302	1.713	0.616 to 4.767
NfH day 5	0.987	0.533	0.064	2.683	0.944 to 7.628

Discussions

Although there is an increasing evidence that NfH could predict severity and evolution in the pathology associated with neuroaxonal damage, there is still not sufficient data that this marker could be used in current practice for evaluation of patients with cerebral ischemia. Besides traumatic brain injury (8,9) and acute intracerebral hemorrhage (10), in the literature there are only a few studies regarding peripheral NfH in ischemic stroke. Traenka et al found that plasma NfL (light chains) concentration correlates with stroke severity mirrored by NIHSS in stroke occurred after cervical artery dissection, and NfL levels significantly associated with poor outcome evaluated after 3 months, but in this study the median time for serum sampling was 6 days, (IQR 3-9.5 days) (11). Even though the NfL are considered the core component of the neurofilaments and the most abundant in this structure, the absolute amounts of the three isoforms NfL, NfM (neurofilaments medium chains) and NfH are supposed to be comparable (4). In this regard, evaluation of either NfL or NfH could provide valuable information about neuronal damage after different kind of injuries, of which, NfH being more resistant to protease denaturation.

Our results regarding NfH concentration are in line with previous studies. Radwan et al. found a significant higher NfH level on day 7 comparing with day 1 (12), while Singh et al. revealed, after dynamic measurement of NfH at day 1, day 7 and after 3 weeks, that peripheral concentration of this marker is significantly lower on day 1 and raises continuously during the first week, up to 3 weeks (13) suggesting that there is ongoing axonal destruction after stroke onset. Another result that emerges from this study represents the positive correlation between serum hsCRP and plasma NfH concentration on day 5, this data supporting the idea that the increased degree of inflammation could be responsible for the magnitude of neuro-destruction.

In our study, cutoff values of 46.4 ng/ml for NfH and of 6.06 mg/L for hsCRP are found to be predictibile for poor short-term outcome. We also found that there was a significant difference in hsCRP concentration on day 5 between males and females with higher values in favor for the latter, despite the insignificant difference regarding NfH. This finding could be a resonable explanation for the fact that women are more susceptible to unfavorable evolution than the male group. Kim et al. also found that women are more prone to a poor outcome evolution comparing to male gender (14).

In univariate regressions for an unfavorable outcome at discharge, all scrutinized variables, except the hsCRP for day 1, manifested good statistical significance and hence their predictive capacity. The statistical significance remained valid for two variable regressions, respectively hsCRP day 5 and NfH day 5. However, adjusting for gender, age and NIHSS on admission, the correlation turned weak.

There are at least two implications of the above outcome: caution is needed when apply-

ing the model to predict and monitor the condition and on the other hand, the outcome may be improved by applying the model to a larger group. Further studies will be needed on a larger cohort, to validate NfH and hsCRP as prognostic markers for patient outcome.

Conclusions

The plasma concentration of NfH shows a significant increase over the first five days after ischemic stroke, in correlation with inflammatory status and short-term evolution.

Conflicts of interest

The authors declare that they have no conflict of interest.

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