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ORIGINAL COMMUNICATION

Nucleosomes as a new prognostic marker in early cerebral stroke

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Abstract *Background* The prognostic relevance of blood markers in cerebral stroke is still a matter of controversial debate. *Patients and Methods* In sera of 63 patients, nucleosomes, neuron-specific enolase (NSE), S100 protein, and C-reactive protein (CRP) were determined daily during the first week after cerebral stroke. Infarction volume was quantified by CT or MRI and the clinical status by Barthel Index (BI) at admission, discharge, and after 12 months (prognosis). All markers were evaluated by univariate and multivariate analysis on their prognostic relevance. *Results* During observation time (12 months), three patients died and 33 reached complete recovery. Infarction volume, nucleosomes, NSE, S100, and CRP correlated significantly with clinical status at admission. The same markers except CRP and initial BI correlated with recovery after 12 months. Almost all patients with initial BI ≥ 50 reached complete recovery. In patients with initially severe defects (BI < 50), nucleosomes and S100, both at day 3, were found to be prognostically relevant. At 100%-specificity for non-recovery, only nucleosomes maintained their prognostic power (sensitivity 52.6%; p = 0.014), whereas S100 did not (sensitivity 16.7%; p = 0.25). In multivariate analysis, nucleosomes and BI at admission showed independent prognostic relevance (p = 0.039). *Conclusion* Circulating nucleosomes and clinical scores provide independent prognostic information concerning the later outcome in patients with initially severe defects after stroke.

Key words prognosis · stroke · nucleosomes · DNA · S100

Introduction

Stroke represents the third leading cause of death worldwide and the first leading cause of disability in adults. Prognostic markers available during the initial phase after acute stroke would be helpful for the further effective management of the patients. Clinical scores and stroke volume showed considerable prognostic relevance [1, 2]. Blood levels of parameters such as S100 protein and neuron-specific enolase (NSE) might provide additional prognostic information [3–6]. Recently, circulating DNA measured during the first hours after acute stroke was reported to be highly predictive for the 28–days and 6-months mortality [7]. These results were surprising because circulating DNA is a non-specific cell death indicator and ischemic cell damage is considered to be a

dynamic process affected with considerable interindividual variations. Therefore in the present study, we analyzed the kinetics of circulating nucleosomes during the whole first week after stroke on their prognostic impact and compared them with clinical scores, infarction volume, NSE, S100 and C-reactive protein. DNA in blood is mainly present as histone-bound nucleosomal complexes which are better conserved from further digestion by endonucleases [8, 9]. Though DNA here was quantified by an immunological nucleosome assay, a good correlation with the real-time PCR method as gold standard for DNA measurements was shown recently [10]. Unlike the previous DNA study [7] we focused on the recovery of the patients 12 months after stroke. This might be particularly relevant to identify those patients who need further or more intensive rehabilitation to improve the later outcome.

Patients and Methods

Patients

63 consecutive patients under the care of the Stroke Unit of the University Hospital Munich-Grosshadern were enrolled in the study from 2002 to 2004. All patients had had a cerebral ischemic stroke within 24 h before admission to the hospital and received intravenous heparin treatment; 13 among them additionally s.c. with recombinant tissue plasminogen activator (rtPA). The disability status of the patients was scored by the Barthel Index (BI, 1) at time of admission, at discharge, and after 12 months (range 6–18 months) to objectify the individual outcome. Details of the patient's characteristics are listed in Table 1.

Inclusion criteria of our study were 1) acute stroke that had occurred within 24 h before admission, 2) BI available at admission and 3) at least three blood samplings during the first week. Exclusion criteria were neoplasms, chronic inflammatory diseases and cytostatic therapy at time of stroke, hemoglobin <9 mg/dL and stroke-specific symptoms >24 h before admission. The study was approved by the local ethic committee. Informed written consent was obtained either from the patient or a relative in all cases.

Table 1 Clinical characteristics of the patients

Age (years):	Median 67.9	Range: 32–88
Infarction volume (ccm):	Median 11.0	Range: 0–294.5
Time of hospitalisation (days):	Mean 6.9	Range: 3–16
		Number
Gender:	Male	36
	Female	27
Stroke localisation:	Middle cerebral artery	50
	Posterior cerebral artery	3
	Thalamus	2
	Posterior cerebellar artery	2
	Brain stem	6
Etiology:	Cardio-embolic	27
	Arterio-embolic	23
	Microangiopathic	6
	Traumatic dissection	4
	Unknown	3
Risc factors:	Arterial hypertension	45
	Hypercholesteremia	22
	Diabetes mellitus	14
	Nicotine abuse	19
	Coronary heart disease	11
Therapy:	Heparin s.c.	63
	Additional lysis with rtPA	13
Barthel Index at admission (points):	0–<25	26
	25–<50	6
	50–<75	14
	75–<100	8
	100	9
Barthel Index at discharge (points):	0–<25	16
	25–<50	9
	50–<75	6
	75–<100	4
	100	15
Barthel Index after 12 months (points; prognosis):	0–<25	7
	25–<50	2
	50–<75	4
	75–<100	7
	100	33

Methods

Nucleosomes were measured in serum at time of hospitalisation and daily during the first week. A strict preanalytical protocol was followed including early centrifugation of the samples, subsequent addition of 10 mM EDTA and storage at −70 °C [11]. Nucleosomes were quantified in batches containing all samples from a patient using the Cell-Death-Detection ELISA^{Plus} (Roche Diagnostics, Germany) as described earlier [10, 11]. Measurements of NSE and S100 in serum were performed on Elecsys 2010 (Roche Diagnostics, Germany); CRP was quantified on Olympus AU 2700 (Olympus, Germany).

The extent of the morphological damage was determined in the first hours after admission to the hospital by computed tomography (CT) and/or during the first days by diffusion-weighted magnetic resonance imaging (MRI). The analysis of volume measurements was performed by an experienced radiologist for the MRI imaging on a Linux working station using MedX-3.4.1-software (by Sensor Systems, www.sensor.com); for patients who were imaged by CT, the quantification was performed on the PACS station (Magic View B1, Siemens Medical Systems, Forchheim, Germany). In every instance, the ischemic region was manually outlined on each image slice: The area of the outlined region was multiplied by the slice thickness for either CT- or MR images, and the interslice gap (in MR images of 0.25), and then summed to give the total lesion volume.

Statistics

For statistical analysis, concentrations of nucleosomes, NSE, S100 and CRP determined at admission and daily during the first week after stroke were considered. For all parameters and time points, median concentrations, range, and 75th-percentiles are given.

For correlation with BI at admission, at discharge, and after 12 months, as well as with infarction volume, the measurements were focused on day 1, 3 and 6. In case of non-available blood samples at these time points, the concentrations of day 4 (instead of day 3), and days 7 or 5 (instead of day 6) were considered. Correlations of clinical variables and biochemical parameters were assessed by Spearman's rank-correlation coefficient.

Furthermore, the prognostic impact of biochemical markers was calculated under consideration of the BI at admission (partial correlation) by Spearman's rank-correlation coefficient. Influence

of treatment modality (additional therapy with rtPA) on prognosis was tested by Wilcoxon-Test. For prognostic evaluation, BI after 12 months was defined as an indicator of long-time recovery.

Patients with an initial BI above the median of all patients investigated (BI ≥ 50) were not considered for further prognostic evaluation because all but one achieved complete recovery (BI = 100). The most promising prognostic parameters in patients with BI < 50 at admission were investigated on their power to predict the non-recovery (BI < 100) after 12 months. The probability of not reaching BI = 100 (sensitivity) was calculated using as cutoffs the median of all patients with BI < 50 and the 100%-specificity marker concentration for reaching BI = 100, respectively. Relative risks and 95% confidence intervals are given and significance was assessed by the chi square test. Finally, the independence of the two relevant prognostic parameters (nucleosomes and S100) from BI at admission was tested by the Cochran-Mantel-Haenszel statistic.

A p-value of $p < 0.05$ was considered statistically significant. All p-values are two-sided. Statistical analyses were performed with software of SAS (version 8.2, SAS-Institute Inc. Cary, NC, USA).

Serum measurement of laboratory markers and outcome assessment by Barthel Score and stroke volume as well as statistical calculation were all done independently by different persons. Results were collected in different data bases and only joined for the statistical evaluation to ensure that there was no interference between clinical and laboratory results.

Results

During the first week after stroke, all biochemical markers showed a considerable increase with a maximum at days 3 to 5 and followed by a continuous decrease. (Figure 1). Levels of nucleosomes, NSE, and S100 of days 3 and 6 as well as CRP of day 3 correlated significantly with initial BI and with infarction volume. (Table 2). Strong correlations were also observed between BI at admission and BI at discharge ($R = 0.823$; $p < 0.0001$) and between BI at admission and infarction volume ($R = -0.562$; $p < 0.0001$). Concerning the follow-up evaluation, 3 patients died during the observation time and 7 patients were lost to follow-up. Of the remaining 53 patients, complete recovery corresponding with BI = 100 was found in 33 patients; in 11 patients BI was between 50 and 90 and in 6 patients BI was still ≤10 points corresponding with persistent severe functional defects.

In univariate analysis, the following measures correlated with the later outcome (BI after 12 months): BI at admission, infarction volume, nucleosomes (days 3, 6), NSE (days 3, 6), and S100 (days 3, 6): however, CRP did not correlate. Partial correlation of blood variables with prognosis under continuous consideration of BI at admission revealed only nucleosomes and S100 (both day-3) as prognostically relevant markers independently from the initial clinical status (Table 2). Lysis therapy with recombinant tissue plasminogen activator showed no impact on the concentration of the markers or on the outcome after 12 months.

Almost all patients with initial BI ≥ 50 (N = 27) reached complete recovery (N = 26), only one patient had BI = 90. However, in patients with initial BI < 50, the later outcome was heterogeneous (Figure 2). In the patient group with an initial BI < 50 prognostic relevance was found for BI at admission, S100 protein at days 3 and 6 ($p = 0.015$ and $p = 0.011$, respectively), and a borderline significant correlation for nucleosomes at day 3 ($p = 0.057$). In clinical routine, it would be necessary to identify those patients who will not reach complete recovery (BI < 100) to intensify individual treatment programs. When cutoff-values of the relevant markers were defined at the median of all patients with BI < 50, nucleosomes ($p = 0.048$) and BI at admission ($p = 0.027$) were found to be prognostic measures, but not S100 ($p = 0.143$). When cutoff-values were defined at the 100%-specificity for not reaching BI = 100, nucleosomes ($p = 0.014$) were maintained as a prognostic marker (Figure 3).

Finally in analysis using Mantel-Haenszel statistics including initial BI and nucleosomes, nucleosomes revealed prognostic power independent of the initial clinical status ($p = 0.039$). Particularly in patients with 10 < BI < 50, nucleosomes showed the best additional prognostic information (relative risk = 2.5; Table 3). For S100 no prognostic relevance in addition to the clinical information was found ($p = 0.564$).

All results concerning the DNA and S100 evaluation were confirmed when those 10 patients who died or were lost to follow-up were additionally included in uni- and multivariate analyses. As most patients lost to follow-up had BI < 20, and as all but one in the first evaluation with a similar preconditions had not reached complete recovery, these 10 patients were considered as "non-recovery patients" in this second evaluation.

Discussion

As stroke is one of the most frequent causes of deaths worldwide and the leading cause of permanent disability in adults, early diagnosis, estimation of the severity and later prognosis remain a challenge [12]. Prognostic information is particularly relevant to estimate the risk of severe complications and how intensive the rehabilitation program should be for individual patients. Several clinical scores are known to be predictive of the later outcome, but also blood markers might contain additional prognostic information.

Cell death is one essential factor which contributes to the functional lesions after cerebral stroke. Whereas necrosis predominates in the ischemic core, mainly apoptosis is observed in the penumbra [13].

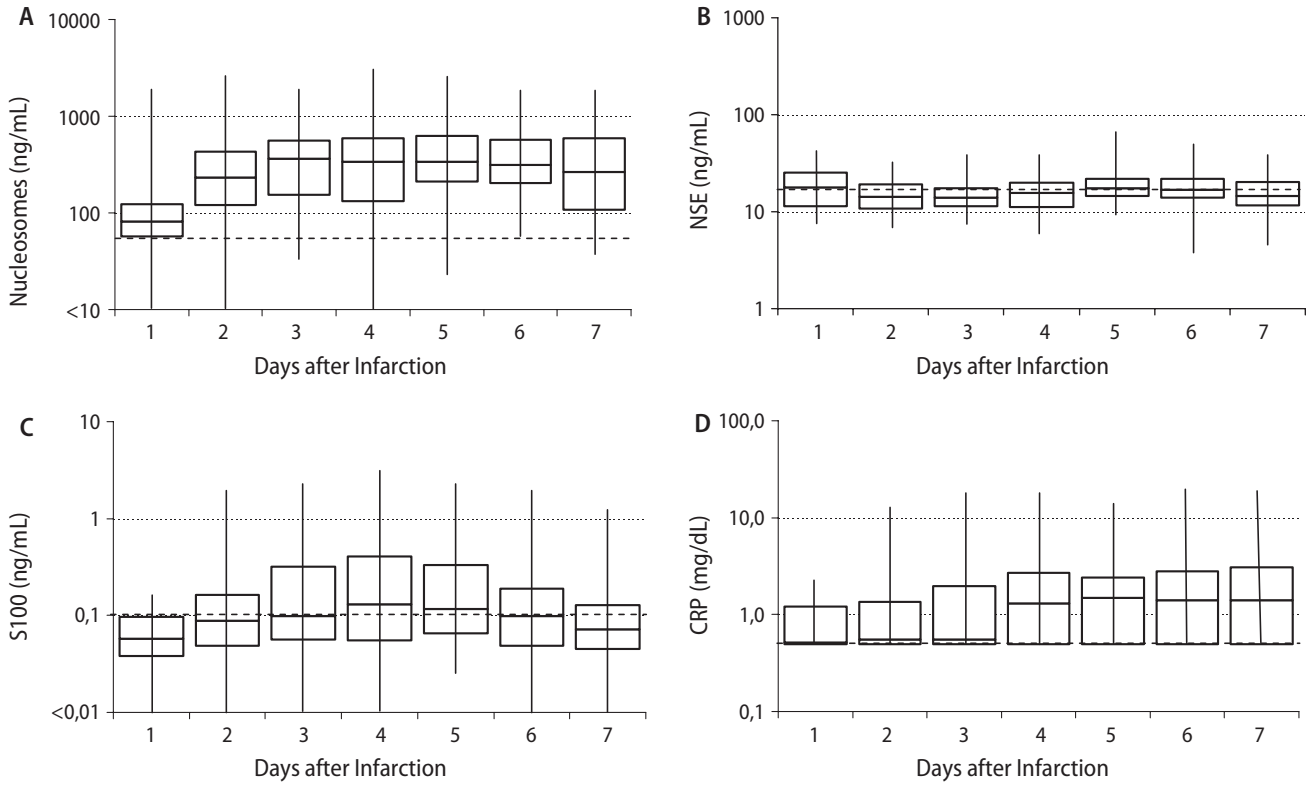

Fig. 1 Concentration of A) nucleosomes, B) NSE, C) S100 and D) C-reactive protein during the first week after stroke (median, 25 and 75-percentiles, ranges). For comparison, 95th percentiles of normal controls are given (.....)

Table 2 Correlations of biochemical variables with Barthel Index at admission and infarction volume as well as with prognosis (Barthel Index after 12 months) in univariate analysis and under continuous consideration of BI at admission (partial correlation) (N = number; R = correlation coefficient; P = P-value; ns = not significant)

		Correlation with BI at Admission			Correlation with Infarction Volume			Correlation with Prognosis			Partial correlation with Prognosis	
		N	R	P	N	R	P	N	R	P	R	P
Nucleosomes	Day 1	35	−0.157	ns	33	0.141	ns	33	−0.039	ns		
	Day 3	63	**−0.378**	**0.0023**	59	**0.474**	**0.0001**	53	**−0.452**	**0.0007**	**−0.287**	**0.0391**
	Day 6	55	**−0.296**	**0.0284**	51	0.264	0.0612	47	**−0.430**	**0.0025**	−0.280	0.0591
NSE	Day 1	24	−0.099	ns	22	0.360	ns	23	−0.204	ns		
	Day 3	58	**−0.423**	**0.0009**	54	0.264	0.0541	49	**−0.416**	**0.0030**	−0.106	ns
	Day 6	54	**−0.448**	**0.0007**	50	**0.305**	**0.0313**	46	**−0.398**	**0.0061**	−0.129	ns
S100	Day 1	25	−0.159	ns	23	−0.091	ns	24	−0.164	ns		
	Day 3	61	**−0.631**	**<0.0001**	57	**0.550**	**<0.0001**	51	**−0.625**	**<0.0001**	**−0.296**	**0.0367**
	Day 6	55	**−0.506**	**<0.0001**	51	**0.527**	**<0.0001**	47	**−0.525**	**0.0001**	−0.271	0.0687
CRP	Day 1	7	−0.010	ns	6	−0.213	ns	7	0.196	ns		
	Day 3	47	**−0.498**	**0.0004**	43	0.273	0.0764	40	−0.286	0.0742	0.120	ns
	Day 6	41	−0.287	0.0694	38	0.274	0.0956	36	−0.272	ns		

Sometimes both cell death forms are detected in the same cell which led to the creation of a new term "aponecrosis" [14]. NSE as marker for neuronal cell destruction and S100 for glial activation and/or cell death are known to reflect these processes after stroke in blood circulation [3–6]. Recently, circulating DNA levels during acute stroke were reported to be highly predictive of the 28-days and 6-months mortality after stroke [7]. As circulating DNA is considered a non-specific cell death measurer and, in blood, it can not be differentiated whether it originates from dying neurons and crossed the blood-brain-barrier, or

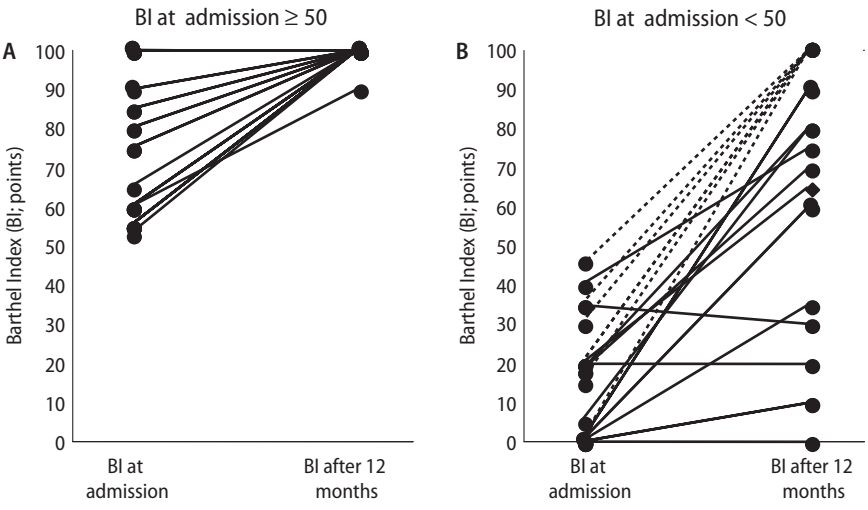

Fig. 2 Outcome after 12 months of A) patients with initial BI ≥ 50 and B) those with initial BI < 50. Among the latter ones, only a part achieved complete recovery (.....)

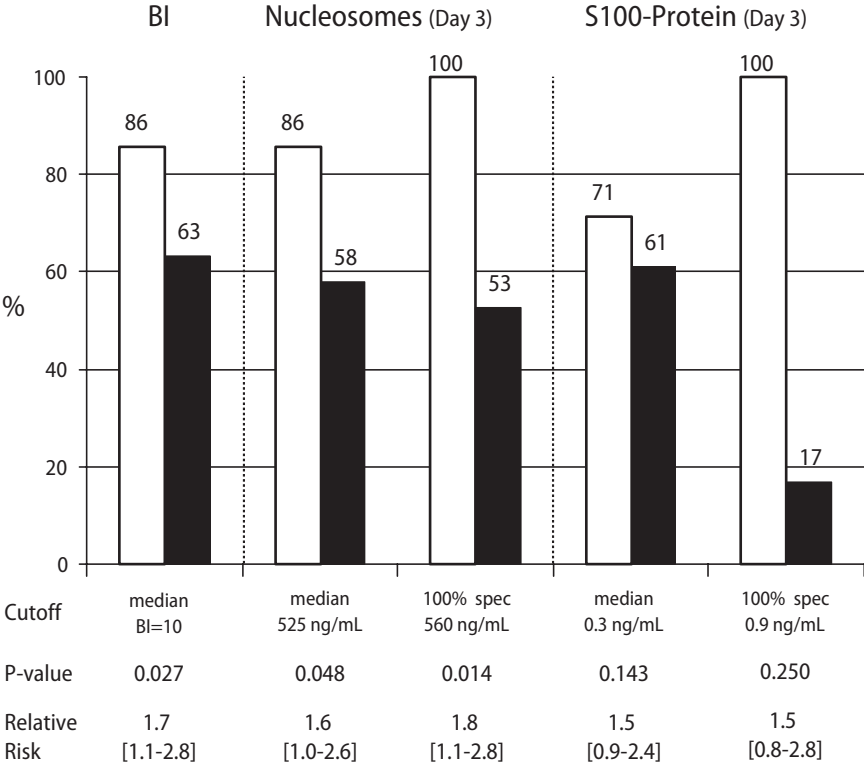

Fig. 3 Specificities (□) and sensitivities (■) of nucleosomes and S100 for the prediction of non-recovery (BI < 100) in the subgroup of patients with initial BI < 50 using as cutoffs A) the median of all patients in this subset, and B) the 100%-specificity for not reaching BI = 100 after 12 months. Relative risks are given and significance was assessed by X^2-test. For BI at admission, values <cutoff (10 points) are defined as ''positives'', because they are prognostically unfavourable

whether it simply reflects local or generalized inflammatory responses, these results were surprising at first glance. Because development of ischemia is dynamic, changes in DNA concentrations are expected to be relevant for the later outcome. Recently, we reported that the kinetics of circulating nucleosomes correlate significantly with clinical status and infarction volume [15]. However, the prognostic relevance of these changes and their relation with other markers have not been shown so far.

NSE and S100 in cerebrospinal fluid and blood were correlated with the infarction volume and with prognosis after cerebral hypoperfusion [4, 16]. While Missler et al. reported the correlation of S100 plasma peak with the resulting infarction volume [4], Büttner et al. found a correlation between S100 and severity of stroke but not with functional prognosis [17]. Foerch et al. finally found S100 to predict the malignant course of middle cerebral artery-infarction [18]. By contrast, NSE did not correlate with the clinical out-

Table 3 Analysis of independency of nucleosomes from BI at admission for the prediction of non-recovery using Mantel-Haenszel statistics

	Nucleosomes (Day 3)	Complete Recovery (BI = 100 after 12 months) N = 7	Non-Recovery (BI < 100 after 12 months) N = 19	Relative Risk [95% Confidence Interval]	Overall Relative Risk [95% Confidence Interval]
BI > 10 at admission (N = 13)	<560 ng/mL	6 (60.0%)	4 (40.0%)	2.5 [1.2–5.3]	1.5 [1.1–2.2]
	≥560 ng/mL	0 (0%)	3 (100%)		
BI ≤ 10 at admission (N = 13)	<560 ng/mL	1 (16.7%)	5 (83.3%)	1.2 [0.8–1.7]	
	≥560 ng/mL	0 (0%)	7 (100%)		

come, even if tendencies were observed [4, 5]. However, kinetics of both markers correlated with the infarction volume and the severity of stroke [5, 18, 19].

Concerning non-specific markers, Winbeck et al. found an increase of CRP concentrations 12–24 hours after onset of stroke symptoms predicting a poor outcome [20]. Similarly, Di Napoli et al. reported a worse outcome after one year in those patients with initially elevated CRP levels [21]. By contrast, Canova et al. could not identify differences in CRP concentrations among patients with various grades of cerebral ischemia [22].

In the present study, we found considerable correlations between the kinetics of circulating nucleosomes during the first week after cerebral infarction with the disability status, infarction volume and with the later outcome after 12 months. Similar results were obtained for NSE and S100. By contrast, CRP only correlated with clinical status at admission, however, not with prognosis. While at the first day after onset of symptoms, biochemical markers were not found to be elevated, the best correlations either with the clinical status (BI at admission) and with prognosis (BI after 12 months) were observed for the concentrations at day 3 for all parameters. Cellular degradation processes, including those starting with delay, and inflammatory responses seem to be most active at this time point or at least result in the highest release of markers into circulation. It is noteworthy that thrombolysis treatment with recombinant tissue plasminogen activator had neither impact on the markers' concentrations nor on the further outcome of the patients.

Because almost all patients with initially slight clinical deficits (BI ≥ 50) reached complete recovery, any other marker would have been superfluous in this setting. By contrast, the outcome in patients with severe clinical deficits (BI < 50) was very heterogeneous implying that additional markers would be helpful for estimating prognosis. Most notably, nucleosomes and S100 (both at days 3) were identified as prognostic factors in this setting. As clinical factors are known to be strong prognostic factors, additive biochemical variables would be only acceptable in clinical practice if they provide very high specificity for defined indications.

To identify patients who would achieve complete recovery would not be affected with any therapeutic consequence. However, selection of those patients who currently have not been cured yet would possibly lead to intensified therapeutic and rehabilitation efforts. After showing the general prognostic power of nucleosomes and S100 at day 3 using the median as cutoff, we additionally chose the concentration at the 100% specificity for the non-recovery as cutoff to minimize the false positive results: Then, S100 only showed a minor sensitivity of 16.7% whereas nucleosomes maintained a notable sensitivity of 52.6%. Thus in univariate analysis of this patient's subset, nucleosomes were the only relevant biochemical prognostic marker along with BI at admission as prognostic clinical factor. The impact of S100 for prognosis was limited due to some high values which were observed in patients with favorable outcome, too. Similar results were presented by a very recent report on the prognostic relevance of circulating DNA and S100 in patients with negative neuroimaging findings within the first 24 hours of stroke symptom onset [23]: Although in that study blood samples were taken during the very initial stroke phase, only DNA showed prognostic value for the spontaneous neurological improvement or good outcome after 6 months but not S100 due to a lower specificity.

When analyzing the independent prognostic relevance of nucleosomes from BI at admission, the relative risk for not reaching BI = 100 was only 1.2 in patients with initial BI < 10 and elevated nucleosome levels. Because only few patients with these severe deficits gained complete recovery at all, the prognostic value of any additional biochemical marker would be limited. However, in patients with initial BI between 10 and 50 points, increased levels of nucleosomes implicated a relative risk of 2.5 for not reaching complete recovery as compared with those with low nucleosomes concentrations. This means that the additional determination of nucleosomes

would be particularly useful in patients with moderate to severe deficits to identify those who would need more intensified therapy.

For the evaluation of the clinical status, several scores besides the Barthel Index (BI) such as the National Institute of Health Stroke Scale (NIHSS), the Scandinavian Stroke Scale (SSS) or the modified Rankin Scale (mRS) are used. At starting time of the study, the usefulness and limitations of the various scores were still in debate. In recent years, NIHSS and mRS have shown to reflect more appropriately the clinical deficits and might be used in further prospective trials.

Summarizing our results, many clinical and biochemical parameters are well suited as general prognostic factors. When a high specificity for predicting non-recovery of stroke patients is required, BI at admission and nucleosomes at day 3 provide independent prognostic information particularly for patients with initially $10 < BI < 50$.

We are aware that the limitations of this study are the small sample size and the explorative character of the statistical evaluation. However, if these results are confirmed by larger studies, nucleosomes might be included as valuable prognostic parameter in prospective therapeutic trials.

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