Thrombosis Research (2008) **122**, 167–173

ELSEVIER

THROMBOSIS RESEARCH

intl.elsevierhealth.com/journals/thre

REGULAR ARTICLE

Early signaling of inflammation in acute ischemic stroke: Clinical and rheological implications

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Received 16 July 2007; received in revised form 16 September 2007; accepted 17 October 2007
Available online 3 December 2007

KEYWORDS
Stroke;
Inflammation;
High-sensitivity interleukin-6;
Erythrocyte aggregation

Abstract

Introduction: Several studies have highlighted the role of interleukin-6 (IL-6) as an early signal of the inflammatory response following acute ischemic stroke. This study examines the potential advantage of employing high-sensitivity (hs)-IL-6 as a possible biomarker at the early stages of acute stroke for identifying an acute phase response and its potential rheological and clinical implications.
Methods: Venous blood was obtained from 186 stroke patients within 24 h of hospital admission and 3–5 days thereafter in order to characterize an inflammatory and hemorheological profile (including erythrocyte aggregation). Neurological state was assessed by the National Institutes of Health Stroke Scale (NIHSS) and the modified Rankin scale (mRs).
Results: While most biomarkers displayed elevated concentrations with time, serum concentrations of hs-IL-6 declined 3–5 days following acute stroke. Initially elevated levels of hs-IL-6 at presentation further correlated with unfavorable clinical outcomes (by NIHSS and mRs) at both time points. Analysis of variance in the different quartiles identified an hs-IL-6 gradient-dependent correlation at both time

Abbreviations: IL-6, interleukin-6; hs-Il-6, high-sensitivity IL-6; CRP, C-reactive protein; EAT, erythrocyte aggregation test; ESR, erythrocyte sedimentation rate; hs-CRP, high-sensitivity C-reactive protein; hs-IL-6, high-sensitivity interleukin-6; EP, erythrocyte percentage; VR, vacuum radius; BMI, body mass index; NIHSS, National Institutes of Health Stroke Scale; mRs, modified Rankin scale.

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0049-3848/$ - see front matter
doi:10.1016/j.thromres.2007.10.019

points, such that the higher the initial hs-IL-6 concentration, the higher the elevation in inflammatory biomarkers and the poorer the neurological state at both time points ($p<0.001$ for NIHSS and $p=0.001$ for mRs, for trend across quartiles).
Conclusions: This study demonstrates the potential of employing hs-IL-6 as an early stage biomarker for the prognosis of acute ischemic stroke. Such an advance would provide the means to identify at an early stage the patients who would require closer clinical surveillance and/or administration of therapeutic interventions.

There are numerous lines of evidence to suggest detrimental effects of inflammation on the risk of stroke [1,2] or the clinical outcome of this acute event [3–6]. Several of these studies concentrated on the role of high-sensitivity interleukin-6 (hs-IL-6) as an early signal of the inflammatory response known to follow the occurrence of acute ischemic stroke [7,8]. An association was subsequently established between circulating IL-6 and brain infarct volume, stroke severity, or clinical outcome up to 6 months post-stroke [8,9]. Furthermore, hs-IL-6 has been associated with endothelial cell activation, stimulation of C-reactive protein (CRP) and fibrinogen synthesis [10,11]. These later-acting biomarkers have likewise been related to both disease severity and poorer clinical outcome in acute ischemic stroke patients [12,13]. It has been suggested that elevated CRP and fibrinogen concentrations might have deleterious effects on both inflammation promotion [13] and blood rheology [14]. CRP might be involved in the perpetuation and augmentation of the inflammatory response, while hyperfibrinogenemia might play a key role in plasma hyperviscosity and erythrocyte aggregation, and both are associated with an abnormal hemorheological profile [15].

We reasoned that the detection and quantification of these inflammatory factors could have practical implications in light of the beneficial effects attributed to interventions which reduce inflammation [16,17] and improve rheology [18–21]. The present study examines the clinical implications in taking advantage of the hs-IL-6 biomarker at the early stages of acute ischemic stroke in order to identify the intensity of the acute phase response as well as the hemorheological state and the severity of the disease. Our intent was to better characterize the inflammation factors involved in acute ischemic stroke.

Materials and methods

Study group

A total of 186 patients with documented acute ischemic stroke were enrolled into the study. They had all been admitted to the Department of Emergency Medicine at the Tel-Aviv Sourasky Medical Center, Tel Aviv, Israel between January 2002 and April 2005. Exclusion criteria included stroke resulting from trauma or an invasive procedure, cerebral hemorrhage, history of malignant tumor, chronic inflammatory disease, autoimmune disease, coagulation disorders, signs and symptoms of acute chronic infection, or treatment with anti-inflammatory agents, excluding acetylsalicylic acid (up to 325 mg/day). None of the included patients received thrombolytic therapy. Signed informed consent was obtained from each participant or from an immediate family member of a patient who was suffering from aphasia, in compliance with the local Institutional Ethics Committee.

Experimental procedure

To test for inflammatory biomarkers and assess erythrocyte aggregation, venous blood was obtained from all stroke patients

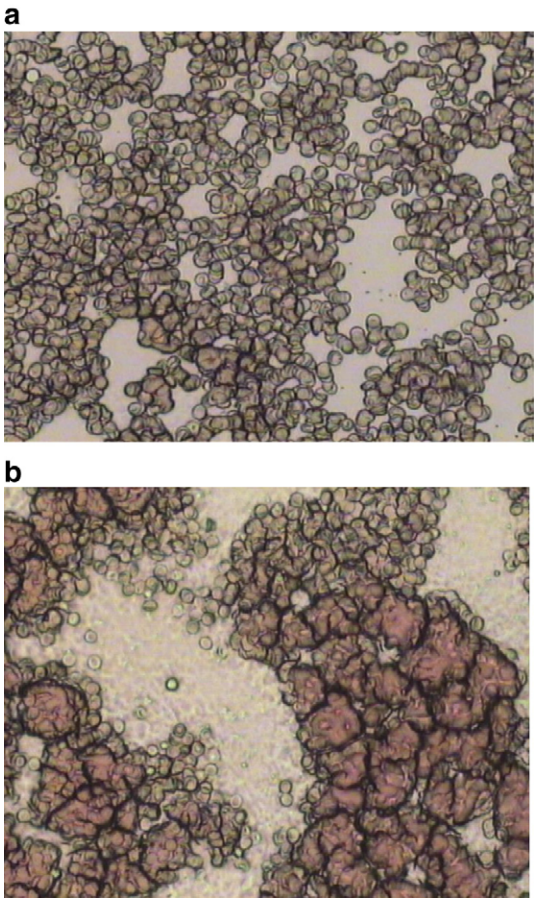

Figure 1 A typical picture (×200, resolution of 0.4 μm/pixel) of a peripheral blood slide from a stroke patient at admission (Time 1, a) and between 3–5 days after stroke onset (Time 2, b).

Table 1 The mean (±SD) of the biomarkers including the degree of erythrocyte aggregation, expressed as vacuum radius (VR) at admission (Time 1) and 3–5 days later (Time 2)

	Time 1	Time 2	*p*-value
ESR, mm/h	26.6 (18.4)	27.5 (21.8)	0.807
Fibrinogen, mg/dl	347.8 (79)	369.7 (91.9)	0.0002
hs-CRP, mg/L	7.0 (7.8)	12.1 (23.2)	0.057
hs-IL-6, pg/ml	4.4 (3.6)	3.6 (2.5)	0.0002
VR, mm	7.3 (5.5)	9.2 (7.3)	0.003

ESR indicates erythrocyte sedimentation rate; hs-CRP, high-sensitivity C-reactive protein; hs-IL-6, high-sensitivity interleukin-6; VR, vacuum radius.

within 24 h of hospital admission (Time 1, mean 27.4 h from stroke symptom onset) as well as 3–5 days afterwards (Time 2). The stroke subtypes were classified according to the TOAST classification as follows: large-artery atherosclerosis, cardioembolism, small-vessel occlusion, stroke of other determined etiology, and stroke of undetermined etiology [22]. Finally, the patients neurological state was assessed at both time points using the National Institutes of Health Stroke Scale (NIHSS) [23] and the modified Rankin Scale (mRs) [24] by a certified senior vascular neurologist (IB or LS).

Laboratory methods

Inflammatory biomarkers

The intensity of the acute phase response was determined by assessing the levels of inflammatory sensitive biomarkers at Time 1 and at Time 2. The biomarkers tested included the erythrocyte sedimentation rate (ESR) by Westergen's [25] method, concentrations of high sensitive C-reactive protein (hs-CRP) by the Behring BN II Nephelometer (DADE Behring, Marburg, Germany) [26], Plasma IL-6 levels were determined in triplicates, and mean values calculated, by the enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA (detection limit 0.156 pg/ml)) and quantitative fibrinogen by Clauss's [27] method and a Sysmex 6000 (Sysmex Corporation, Hyaga, Japan) analyzer.

Erythrocyte aggregation test (EAT)

The erythrocyte aggregation (EA) was determined by using a simple slide test and image analysis as described elsewhere [28–30]. In brief, venous blood was obtained into a syringe containing sodium citrate (one volume of 3.8% sodium citrate and three volumes of whole blood). One drop of blood was trickled onto a slide inclined at an angle of 30° and allowed to seep down with gravity, leaving behind a fine film of blood. The slides were left to dry in that slanted position at room temperature. A technician employed an image analysis system (INFLAMET™ Inflamet Ltd., Tel Aviv, Israel) to scan the slides which remained unmarked to prevent bias. The variable assessed was the "vacuum radius" (VR), which describes the area (in microns) formed between the aggregated cells such that the higher the aggregation the larger the area measured and vice versa [29,30] (Fig. 1).

Statistical analysis

All continuous data were summarized and displayed as mean ± SD. Results of the different biomarkers at Time 1 and Time 2 were compared by Student's *t* test for normal distributed variables and the Mann–Whitney *U* test for non-normal distributed variables. Analysis of variance (ANOVA) was conducted with the Dunnett's T3 or Scheffe post hoc comparison tests and used to compare between the levels of inflammatory biomarkers and the neurological scales. In addition, we further divided the patient cohort into four groups (quartiles) according to the levels of hs-IL-6 they exhibited at Time 1, and these variance tests were also used to determine the statistical differences between the quartiles. The χ^2 test was used to assess associations among categorical variables. Correlations between the different inflammatory biomarkers were determined using the two-tailed Spearman rank correlation. Significance was

Table 2 Population characteristics according to quartiles of Day 1 plasma concentration of interleukin-6

Variable	hs-IL-6 Quartiles (pg/ml)				*p*-value	*p* for trend
	<1.95	1.96–3.33	3.4–6.11	>6.15		
n	46	47	47	46		
Age, years (SD)	65.2 (12.9)	67.4 (12.7)	67.2 (12)	71.3 (12)	0.128	**0.030**
BMI, kg/m^2	25.9 (3.6)	26.5 (4.8)	29.1 (5.4)	26.6 (4.7)	**0.01**	0.157
Gender, males (%)	69.6	66	72.3	65.2	0.874	0.830
Current smokers (%)	31.1	21.3	40.4	20.5	0.11	0.669
Diabetes (%)	34.8	36.2	40.4	28.3	0.675	0.629
Hyperlipidemia (%)	41.3	36.2	42.6	34.8	0.841	0.684
Hypertension (%)	54.3	67.4	74.5	67.4	0.225	0.140
hs-IL-6, pg/ml (SD)*	1.37 (0.6)	3.10 (2.5)	3.89 (1.8)	5.5 (2.4)	**<0.001**	**<0.001**
CRP, mg/L (SD) [a]	3.7 (8.3)	8.1 (9.3)	14.2 (34.4)	28.3 (30.8)	**<0.001**	**<0.001**
ESR, mm/h (SD) [a]	20.9 (29.2)	25.7 (18.3)	26.6 (16.6)	41.5 (22.5)	**0.002**	**0.001**
Fibrinogen, mg/dl (SD) [a]	315.3 (77.9)	359.3 (91.4)	376.8 (79.6)	430.5 (87.7)	**<0.001**	**<0.001**
VR, mm (SD) [a]	5.8 (3.8)	8.5 (6.5)	10.6 (9)	14.4 (9.5)	**<0.001**	**<0.001**
NIHSS	3.89 (2.8)	3.83 (2.3)	5.63 (4.4)	7.07 (5.4)	**<0.001**	**<0.001**
mRs	1.96 (1.7)	1.89 (1.3)	2.58 (1.4)	2.8 (1.5)	**0.007**	**0.001**
NIHSS [a]	3.11 (3.0)	3.06 (2.7)	4.46 (3.9)	7.18 (8.1)	**0.001**	**<0.001**
mRs [a]	1.52 (1.4)	1.69 (1.5)	2.48 (1.5)	2.64 (1.7)	**0.008**	**0.001**

IL-6 indicates high-sensitivity interleukin-6; BMI, body mass index; hs-CRP, high-sensitivity C-reactive protein; ESR, erythrocyte sedimentation rate; VR, vacuum radius; NIHSS, National Institutes of Health Stroke Scale; mRs, modified Rankin scale.

[a] Variables measured 3–5 days from stroke onset (Time 2).

set at $p<0.05$. SPSS/WIN (version 13.0, SPSS INC, Chicago, IL, USA) software was used to carry out all statistical analyses.

Results

Of the 186 acute stroke patients enrolled in this study (mean ± SD age 67.7 ± 12.5 years, 127 males), 120 had lacunar stroke, 48 had large-artery atherosclerotic stroke, 13 had cardioembolic stroke, 4 had stroke of undetermined etiology and 1 with a stroke of other determined etiology. In addition to stroke, 34.9% of the patients had diabetes mellitus, 38.7% had hyperlipidemia, 65.9% had hypertension and 28.4% were current smokers.

Patients had elevated inflammatory biomarkers and increased erythrocyte aggregation (expressed as the VR) at Time 2 compared to Time 1, with the exception of plasma hs-IL-6 concentration, which decreased significantly with time ($p=0.0002$, Table 1). The high concentration of serum hs-IL-6 at Time 1 correlated with a more severe stroke presentation as expressed by NIHSS and mRs both at Time 1 ($r=0.238$, $p=0.0009$ and $r=0.254$, $p=0.001$, respectively) and Time 2 ($r=0.268$, $p=0.001$ and $r=0.292$, $p=0.001$, respectively). This correlation in neurological scores remained significant after adjustment for the vascular risk factors of age, diabetes mellitus, hypertension, hyperlipidemia, present smoking, and treatment of aspirin, Angiotensin Converting Enzyme (ACE) inhibitor and HMG-CoA reductase inhibitors at both Time 1 ($r=0.211$, $p=0.028$ and $r=0.215$, $p=0.026$, respectively) and Time 2 ($r=0.377$, $p<0.001$ and $r=0.242$, $p=0.012$, respectively).

In order to explore the significance of the decrease observed only for the hs-IL-6 biomarker, we divided the patients into four subgroups (quartiles) according to their hs-IL-6 baseline plasma levels at Time 1. Analysis of these quartiles revealed that inflammatory and rheological (erythrocyte aggregation and ESR) biomarkers at Time 2 all differed from hs-IL-6 quartiles at Time 1. Interestingly, all Time 2 inflammatory biomarkers displayed a significant elevation in parallel with the initial high levels of hs-IL-6 at Time 1, from the lowest quartile to the highest (Table 2, Fig. 2). This trend was also evident neurologically: the higher baseline IL-6 quartile groups displayed higher NIHSSs compared with the lowest quartile ($p<0.001$ for the trend across quartiles at both time points, Table 2). The same held true for the functional impairment score, measured as mRs ($p=0.001$ for the trend across quartiles at both time points, Table 2).

We have further performed a subgroup analysis of the inflammatory biomarkers by stroke etiology: small and large arteries (Table 3). IL-6 measurement was the only inflammatory biomarker who discriminate between large and small stroke etiology ($p=0.018$).

Finally, comparison of neurological scores between the two different time points revealed that while individuals categorized

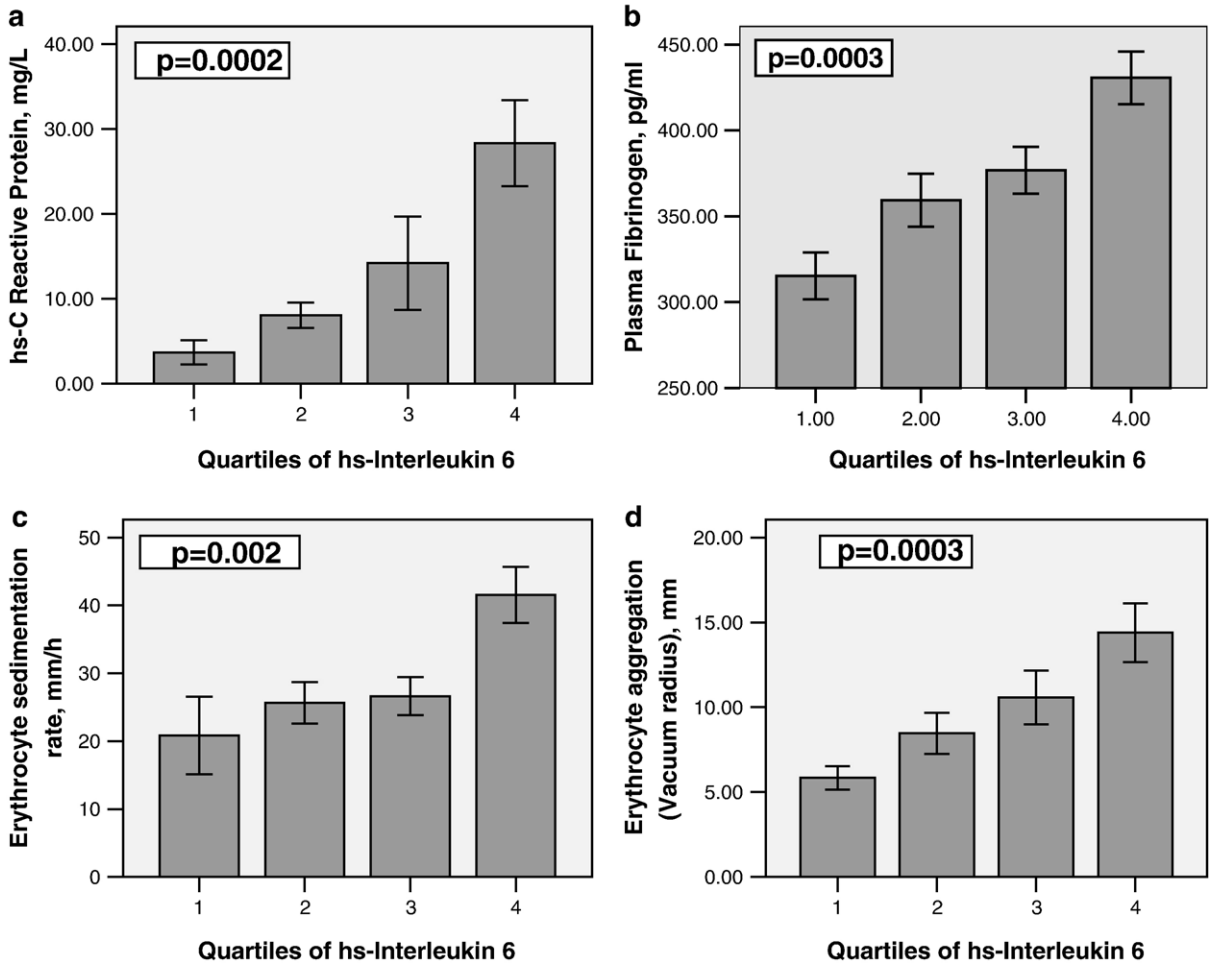

Figure 2 Time 2 (between 3–5 days after stroke onset) inflammatory biomarkers according to quartiles of Time 1 (at admission) plasma concentration of interleukin-6. (a) High-sensitivity C-reactive protein (hs-CRP). (b) Plasma fibrinogen. (c) Erythrocyte sedimentation rate (ESR). (d) Erythrocyte aggregation.

Table 3 Inflammatory biomarkers according to stroke etiology

	Large arteries (large-artery atherosclerotic or cardioembolic stroke)	Small arteries (lacunar stroke)	*p*-value
n	61	120	
ESR, mm/h	26.56 ± 18.13	28.0 ± 18.8	0.584
Fibrinogen, mg/dl	350.16 ± 87.20	346.67 ± 74.32	0.770
hs-CRP, mg/L	10.02 ± 15.58	10.0 ± 27.79	0.534
VR, mm	7.9 ± 7.24	7.19 ± 5.24	0.84
hs-IL-6, mg/L	5.33 ± 4.09	4.14 ± 3.23	**0.018**

as having the lower hs-IL-6 quartile (at Time 1) displayed an improvement of 20% (in both NIHSS and mRs) 3–5 days later, individuals in the highest hs-IL-6 quartile (at Time 1) displayed minimal or no improvement (5.6% for the mRs and −1.5% for the NIHSS) at Time 2.

Discussion

The findings of this study demonstrate the potential value of acquiring hs-IL-6 measurements at the early stages of acute ischemic stroke in order to identify patients at risk for an inflammatory response and who would therefore require closer clinical surveillance and/or administration of therapeutic interventions. Patients with relatively high hs-IL-6 concentrations upon admission had higher concentrations of inflammatory biomarkers and potential hemorheological determinants at a later stage of the disease (Table 1). Moreover, there was less short-term clinical improvement following acute insult in those individuals who displayed increased hs-IL-6 concentrations upon admission.

The successive increases in concentrations of both hs-CRP and fibrinogen have also been implicated in a less favorable clinical outcome in patients with ischemic stroke [12,13]. Given that these proteins are effectors in the inflammation process (often stimulating the activity of other proteins [12,13,31]), identifying a potential for increased concentrations of these proteins at an early stage of the disease could be meaningful in terms of improving prognosis at a later stage. Given the close correlations between the severity of the disease and its course, the hs-IL-6 level of concentration at an early stage and the subsequent elevation in concentrations of CRP and fibrinogen might argue against the potential advantage in conducting early hs-IL-6 measurements in the usual clinical setting. This is particularly true when taking into account that while CRP and fibrinogen assays are available at real-time and at a low cost in any laboratory, hs-IL-6 concentration assays are costly and the required facilities are relatively sparse. It is, however, highly conceivable that introducing therapeutic interventions at the earliest stages of the inflammatory cascade could go a long way towards preventing the potential detrimental effects these effector proteins might induce [12,13,31]. We therefore believe that it would be worthwhile to take advantage of the increasing levels of hs-IL-6 as an early biomarker for the prognosis of acute ischemic stroke in order to implement timely and appropriate patient management.

One of the main findings is that early IL-6 detection was the only inflammatory biomarker that discriminates between large and small stroke etiology at the very early stage following hospital admission. Interestingly, we find no significant difference between these groups regarding the ESR, Fibrinogen, hs-CRP and erythrocyte aggregation. This similarity of acute phase proteins may be due to the early stage of evaluation, when there was not enough time to develop acute phase response following larger brain infarction. Yet, another explanation is that both groups of patients arrive with similar burden of inflammation, a point that should await further studies.

Substantiation of our claim of the importance of early detection of the acute phase response could be presented in the hemorheological context. Increased synthesis of fibrinogen is associated with enhanced erythrocyte aggregation, an event that has detrimental consequences in terms of microcirculatory flow [32], tissue deoxygenation [33], endothelial dysfunction [34] as well as functional capillary density [35]. In addition to being a determinant of blood viscosity [36], fibrinogen is also a major determinant of erythrocyte aggregation [37]. Indeed, interventions intended to reduce the concentrations of this protein in order to obtain a better hemorheologic profile have been suggested in the past [18,21,38,39]. By taking advantage of the early stage hs-IL-6 biomarker, one might have the opportunity to intervene at a relatively early stage, before the deleterious effects of hyperfibrinogenemia have taken hold. The potential importance of this finding is exemplified by a recent study documenting the negative consequence of higher fibrinogen concentrations in terms of anti platelet aggregation therapy [40].

In our current study, we employed two methods in order to determine the intensity of erythrocyte aggregation, namely, Westergren's indirect method [41] and our direct one [30,42]. These methods were chosen due to their simplicity and low cost. The potential clinical application of the data that these rheological determinants could provide has been supported by recent studies that emphasized the

importance of microcirculatory flow in the presence of large vessel obstructions [43,44].

Our study was limited in its inability to measure hs-IL-6 immediately upon the occurrence of stroke but rather within the first 24 h of hospital admission. A recent study reported that IL-6 levels increased in fewer than 6 h following acute stroke, reaching maximum levels within 24 h and subsequently plateauing by days 3–4, after which they began to slowly decrease [7,45]. Therefore, measuring hs-IL-6 within the first 24 h most probably provides the peak levels of hs-IL-6 for a given patient. In effect, it is possible that measurements taken within the first 12 h would not identify the patients at higher risk for unfavorable outcomes and might not contribute any additive value, a consideration that remains to be determined.

We conclude that the employment of hs-IL-6 as an early stage biomarker of acute ischemic stroke could indicate the presence of an acute phase response and its associated detrimental hemorheological environment in association with a subsequent poorer clinical outcome. If further confirmed, these observations may pave the way towards introducing a preclinical approach to assist clinicians in identifying patients at risk for an inflammatory response at the earliest stages of the disease.

Acknowledgment

Esther Eshkol is thanked for editorial assistance.

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