100Peripheral markers of brain damage and blood-brain barrier dysfunction

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Abstract: Occurrence of brain damage is frequently associated with abnormal blood-brain barrier (BBB) function. Two brain-specific proteins, S100β• and neuron-specific enolase (NSE) are released systemically in a variety of neurological disease, but S100β levels sometimes rise in the absence of neuronal damage, suggesting that S100β is a marker of BBB rather than neuronal damage. We measured both proteins in the serum of patients undergoing iatrogenic BBB disruption with intrarterial mannitol, followed by chemotherapy. Serum S100β increased significantly after mannitol infusion ($p < 0.05$) while NSE did not. Furthermore, in a model of intracerebral hemorrhage, S100β increases in CSF did not lead to serum changes at a time when the BBB was intact. Modeling of S100β release from the CNS suggested that low (< 0.34 ng/ml) serum levels of S100β are consistent with BBB opening without CNS damage, while larger increases imply synthesis and release from presumable damaged glia. Thus, S100β in serum is an early marker of BBB openings that may precede neuronal damage and may influence therapeutic strategies. Secondary, massive elevations in S100β are indicators of prior brain damage and bear clinical significance as predictors of poor outcome or diagnostic means to differentiate extensive damage from minor, transient impairment.

Keywords: S100, cerebrovascular disease, diagnostics, endothelium, magnetic resonance imaging, neurological disorders, cerebral ischemia

1. Introduction

Loss of blood brain barrier (BBB) function is hallmark of many neurological diseases. Perhaps paradoxically, BBB integrity is frequently associated with reduced delivery of pharmacologic substances into the brain. Thus, measuring BBB function may be important to diagnose disease progression and monitor time-dependent loss of BBB integrity when chemotherapic penetration may be more effective. At present time, only invasive and expensive techniques such as contrast-enhanced magnetic resonance imaging, CT-scan and lumbar puncture are available to test clinically BBB integrity. An alternative approach has been proposed, i.e., detection of changes in blood composition that indicates BBB disruption [46]. We will here present in a mini review format, evidence suggesting that peripheral detection of brain specific proteins may be used to monitor changes in BBB integrity.

2. Blood-brain barrier, brain-specific proteins and neurological disease

The blood-brain barrier is primarily composed of microvascular endothelial cells (EC) linked by tight junctions that largely prevent molecular communication be-

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Table 1
Permeability of various substances across the blood-brain barrier.
See references [15,38,41,52,66–68,77] for details

Substance	Permeability (cm/sec)
Potassiumions	$< 10^{-7}$
Glucose	$> 10^{-6}$
Sucrose	$\sim 10^{-7}$
Proteins	$< 10^{-8}$, negligible
Diazepam	10^{-4}

tween blood and the brain. Some of the unique properties of the BBB are induced by perivascular glia. Thus, the blood-brain barrier is constituted of both endothelial cells and glial end feet [10,61]. Perivascular pericytes and microglia may also be considered active components of the blood-brain barrier [53,55]. Astrocytes and their processes invest more than 90% of endothelial capillaries, and their end feet are projected tightly around the endothelial cells [22]. Astrocytic proteins are synthesized and released next to capillaries, but owing to the negligible trans-endothelial permeability to proteins, they extravasate into the serum only when the BBB is breached (see Table 1).

Candidates for passage from glia to plasma are two distinct proteins more or less specifically expressed by CNS astrocytes: glial fibrillary acid protein (GFAP) and S100β. Upon immunocytochemical detection, these proteins outline the shape of intraparenchymal blood vessels (Fig. 1). Many neurological disorders and lesions are associated with increased BBB permeability: they include primary and metastatic brain tumors, ischemia, hypertension, dementia, epilepsy, infection, multiple sclerosis, and trauma [5,7,12,13,23,26,27,30, 38,48]. Under these conditions, both GFAP and S100β are upregulated further supporting the hypothesis that astrocytic proteins may be used to peripherally detect changes that occur in the brain parenchyma [14,20,33, 40,46,50].

Although the estimated association between disease and BBB disruption is clear, the nature of this association is not always evident. An important question is whether impaired BBB function is a result of the condition, or whether in some conditions the BBB disturbance is itself the primary pathogenic factor [25,39]. In the latter case, rapid identification of BBB impairment might allow preventive therapy to be given before neurological damage develops. Predictable and reliable ways to assess damage would also be useful for monitoring neurological status, predicting outcome, and adjusting therapy. Brain-derived proteins may be useful markers of BBB integrity because they have several possible mechanisms of passage across the BBB.

Proteins in CSF can be detected by directly sampling CSF, which requires invasive techniques such as lumbar puncture or intrasurgical sampling from the ventricles or the subarachnoid space. Obvious limitations of intrathecal detection methods are that they are invasive, and that the sample itself may be contaminated by the procedure. Blood-brain barrier integrity can also be assessed by contrast-enhanced computed tomography or MRI [9,19,37]. Accurate non-invasive techniques would clearly be preferable, particularly in chronic diseases that are tracked with multiple longitudinal samples. Protein levels in normal CSF are very low, but the traditional understanding that it is a protein-free fluid, like the aqueous humor of the eye or normal urine, is mistaken [68]. A small group of proteins are found exclusively or almost exclusively in the cerebrospinal fluid [66–68]. Any disruption in blood-brain barrier integrity may allow protein leakage in both directions. Thus, testing seru levels of CSF proteins may be of diagnostic value [46].

3. Putative markers of brain damage may actually indicate blood-brain barrier leakage

Most research into brain damage has focused on neuronal damage, because this is the cause of most deficits from neurological disease. In fact, "brain damage" has often been used as a synonym for neuronal death. Neuronal sensitivity to insult is region- and disease-specific. For example, ischemic insults will selectively affect the CA1 region of the hippocampal formation, leaving the neighboring dentate gyrus and CA3 practically intact [65]. Interestingly, CA1 sensitivity to neuronal damage also extends to vascular cells [11]. Thus, BBB failure may be a local phenomenon perhaps paralleling other topographic variations within the brain, e.g., differences between gray and white matter, cortical vs. basal ganglia, etc. In addition to these patterns of specificity, it has also been shown that neuronal cell death does not occur concomitantly with the insult but rather after a delay. In acute insults such as ischemia [47], the delay provides a potential therapeutic window for neuroprotective intervention. In chronic and progressive neurological diseases such as multiple sclerosis, the delay may be even longer.

Because of this focus on cellular damage, much of the previous research on biochemical markers has focused on markers that measure neuronal damage [29, 42,60]. However, most neurologic diseases are accompanied by increased BBB permeability, and thus the
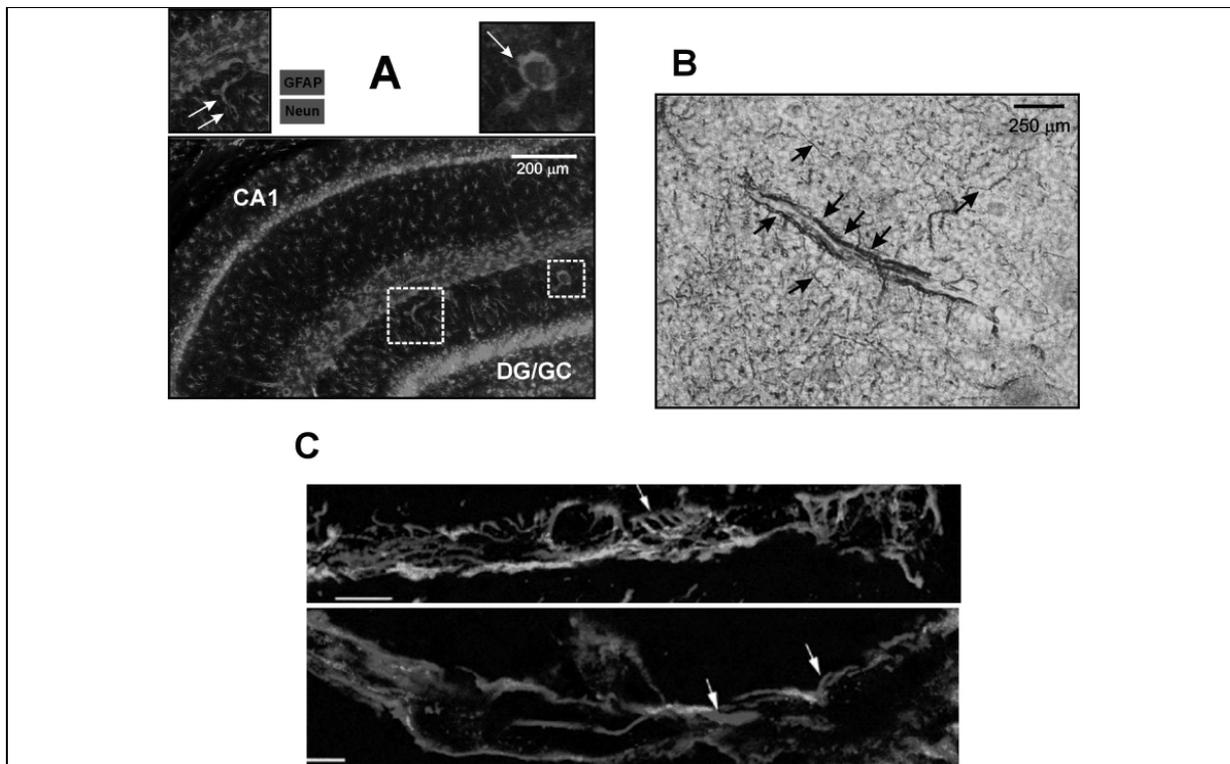
Fig. 1. Relationship between perivascular glia, neurons and cerebral blood vessels: distribution of immunoreactivity for astrocyte-specific markers S100β and GFAP. A) GFAP immunoreactivity (red), neuronal cell bodies (green) and capillaries in the rat hippocampus. The enlargements in the insets show capillaries and arterioles clearly outlined by GFAP immunoreactivity, emphasizing the proximity of perivascular glia to the lumen. B) GFAP immunoreactivity outlines large penetrating pial vessels in human cortex. C) S100β immunoreactivity (in red) co-localizes with intracellular GFAP (in green).

markers thought to indicate neuronal damage might in fact indicate BBB defects. In fact, ideal markers of BBB permeability and of neuronal damage share several characteristics: both should be virtually undetectable in normal subjects and should show distinct alterations in response to insult that correlate with the severity of the damage (See Table 2).

Distinguishing between BBB defects and neuronal damage has enormous clinical relevance. For example, in acute CNS disturbances such as ischemic stroke, the delay between insult and irreversible neuronal cell death offers a window of therapeutic opportunity. If, as suggested by numerous studies [1,18,56,57,64,72], BBB openings develop early after the initial arterial occlusion, clinicians would have a unique opportunity to administer drugs that are normally BBB-impermeant (e.g., nerve growth factors) before neurons are damaged. The duration of these openings may be unpredictable, so a peripheral, non-invasive, easily repeatable test would be extremely useful. In chronic neurological diseases, such as multiple sclerosis, BBB openings

may have both therapeutic and etiologic significance. Severity of symptoms has been suggested to correlate with BBB function in these conditions, and promising therapies using brain-derived proteins have failed largely because the compounds are poorly transported across the BBB (see Table 1 and [3,16,34,62]).

Marker proteins under investigation have included neuron-specific enolase (NSE), GFAP, and S100β see Table 3). In normal subjects, NSE is more concentrated in plasma while S100β is primarily present in central nervous system fluids [29,68]. Thus, opening the blood-brain barrier in the absence of neuronal damage is expected to markedly increase serum S100 levels while leaving NSE levels unchanged. When a patient experiences both blood-brain barrier opening and neuronal damage, plasma levels of both markers are expected to exceed normal levels [42]. S100β levels were investigated in a variety of pathologies as well as after delivery of seemingly healthy babies [21,54,82]. Interestingly, it was assumed that infants' brain contributed significantly to cord blood values of this marker. In

Table 2
Desirable properties of markers of BBB leakage vs. brain damage. See text for details and discussion

Brain damage	Vascular (BBB) Damage
Expressed or released only when neuroglial damage occurs	Normally present in CSF or interstitial fluid
Normally absent in serum	Normally absent in serum
Expressed in neurons or glia	Expressed at the blood-brain interface
Detectable at low levels	Detectable at low levels
Appears in CSF and blood only when damage occurs	Appears in blood only when the BBB is breached

sharp contrast with this assumption are the facts that venous (fetal) and arterial (mostly maternal) blood levels were identical and the discovery of a higher $S100\beta$ level in vaginal deliveries vs. elective cesareans. This may reflect changes in mother's BBB function (e.g., due to increased intracranial pressure during vaginal delivery) rather than "newborn brain damage". Age dependent changes have also been described [59,75].

4. Serum S100 as a marker of BBB leakage: direct evidence of a link between serum $S100\beta$ and BBB integrity

$S100\beta$ and NSE are not extravasated into the peripheral circulation of healthy individuals, but they may be released following a variety of cerebral lesions and injuries, including brain tumors, stroke, severe head injury, or multiple sclerosis. Thus, they have been for many years considered markers of CNS damage [29]. However, the time course of $S100\beta$ appearance in serum is not entirely consistent with this hypothesis, because blood $S100\beta$ levels have been reported to increase in the absence of or before neuronal damage [70]. Recent evidence has also shown that brain $S100\beta$ increases poorly correlate with serum levels, further suggesting that appearance of $S100\beta$ is related to BBB integrity (see below). Current knowledge about BBB permeability to proteins predicts that these CSF proteins will occur only when the BBB is breached. Thus, the early appearance of CSF proteins in serum may be caused by changes in BBB permeability rather than directly by neuronal damage.

To test the connection between $S100\beta$ and blood-brain barrier integrity, we measured both NSE and $S100\beta$ in the serum of patients with primary central nervous system lymphoma who underwent iatrogenic blood-brain barrier disruption by intra-arterial mannitol infusion before receiving methotrexate infusion [49]. Mean serum levels of $S100\beta$ increased significantly after mannitol infusion and again after methotrexate infusion, and they remained elevated through recovery (Fig. 2A). Blood-brain barrier with intra-arterial

methotrexate does not lead to brain damage [69]. In agreement with this finding, NSE serum levels remained constant throughout the procedure (Fig. 2A). To rule out the possibility that the increased serum $S100\beta$ levels were caused by the methotrexate and not BBBD, we measured $S100\beta$ and NSE in the blood of three patients who were given intrarterial methotrexate without blood-brain barrier disruption. We found that in these patients, levels of both $S100\beta$ and NSE remained within normal ranges [46].

We concluded that the increase in $S100\beta$ level immediately after the blood-brain barrier disruption was almost certainly too soon to be the result of synthesis and release from "reactive" glia. We also concluded that $S100\beta$ protein may be an early marker of blood-brain barrier disruption that is not necessarily related to either neuronal or glial brain damage. This finding does not change the traditional understanding that NSE is related to neuronal damage (Fig. 2B).

5. Modeling serum $S100\beta$ levels after brain damage: Experimental results

While these results clearly demonstrated a relationship of serum $S100\beta$ with BBB function, previous findings by others demonstrated a positive correlation with brain damage [28,35,36,71]. How could these seemingly contrasting two findings be explained? We hypothesized that high levels of serum $S100\beta$ correlate with brain damage while lesser increases above normal values are associated with BBB leakage in the absence of parenchymal damage. This was tested by a dual approach, one based on analysis of experimental data (Fig. 2A) and the other on mathematical modeling built on data from this and other labs (see below).

Based on previous work, we made the following assumptions: 1) $S100\beta$ extravasation from CNS to blood follows a distribution kinetic similar to the pharmacodistribution of a drug administered intravenously by slow infusion (see Fig. 3A and 3B see also reference [6]); 2) The half-life of $S100\beta$ was assumed to be 30 minutes but similar conclusions were drawn with

Table 3
Putative peripheral markers of neuronal and blood-brain barrier damage.
Their characteristics and comparison of useful properties as markers of BBB
vs. brain damage

Characteristics	S100β	GFAP	NSE
Desirable for marker of brain damage			
Present in normal CSF	Yes	Yes	Yes
Normally blocked by BBB	Yes	Yes	Yes
Normal plasma level $<<$ CSF level	Yes	No	Yes
Plasma level increases after insult to the BBB	Yes	No	No
Desirable for BBB marker			
Levels very low in normal CSF	Yes	Yes	Yes
CSF Level increases after insult	Yes	Yes	Yes
Plasma levels correlate with damage	Yes	No	Yes

BBB indicates blood-brain barrier. CSF indicates cerebrospinal fluid. NSE
indicates neuron-specific enolase. GFAP indicates glial fibrillary acidic
protein. See [2,4,63,79–81] for details.

half life values of 2 hrs [24]; 3) Serum volume was determined to be in our samples 25–30% of blood volume; CSF volume was between 0.14 L and 0.19 L, and 4) S100β serum and S100βCSF were initially set at 0.05 ng/mL (Fig. 2A) and 2 ng/mL respectively [68]. We first wished to determine at which time point S100β serum will reach steady state after blood-brain barrier disruption. Figure 3A shows a schematic representation of the pharmacokinetic model used to derive S100β values from β (rate of clearance of the protein), V_d (the distribution volume), and K_o (transfer constant from brain to blood). K_o is obviously negligible when the BBB is intact, and reaches its maximal value when the BBB is fully breached. This equation was used to determine the time point at which $S100\beta_{\mathrm{serum}} = S100\beta_{\mathrm{steady-state}}$.

The initial three time points of the data shown in Fig. 2A are related to S100β values after blood-brain barrier disruption. These values were fitted with a Boltzmann equation to extrapolate steady state values of serum S100β after BBB disruption:

$$S100\bullet_{\mathrm{serum}} = A2 + (A1 - A2)/$$
$$[1 + \exp^{(x-x0/dx)}] \qquad (1)$$

$A1$ and $A2$ represent fitting constants, x_0 is the center of the sigmoidal fit and dx represents the time constant (in minutes). The results of these computations are shown in Fig. 3B. The data point extrapolated by this equation at 120 minutes represents steady state values for $S100\bullet_{\mathrm{serum}}$ [6] corresponding to an S-100$\bullet$ serum concentration at steady state of 0.176 ng/ml. This steady-state value thus represents the maximum level of $S100\beta_{\mathrm{serum}}$ reachable after opening of the blood-brain barrier. Note the data points used for the fit were obtained after *hemispheric* BBB disruption.

Thus, the asymptotic value obtained is the maximum S100β obtainable after approximately 1/2 of the BBB was breached.

6. Mathematical modeling of serum S100β levels after brain damage

These values were independently confirmed by mathematical modeling of the range of steady-state concentrations that S100β serum would reach when the BBB is maximally leaky (schematically represented in Fig. 3A). This model was used to assess the dependency of $S100\beta_{\mathrm{steadystate}}$ on serum and CSF volumes as well as CSF levels of the protein. The initial values used were those described above (e.g., $S100\bullet_{\mathrm{CSF}} = 2$ ng/mL and $S100\bullet_{\mathrm{Serum}} = 0.05$ ng/mL). Data were fitted according to the following equation:

$$S100\beta_{\mathrm{s-s}} = [S100\beta_{\mathrm{CSF}} * 1/2CSF_{\mathrm{vol}}$$
$$+ S100\beta_{\mathrm{ser}} * Serum_{\mathrm{vol}}]/ \qquad (2)$$
$$1/2CSF_{\mathrm{vol}} + Serum_{\mathrm{vol}}$$

where $S100\beta_{\mathrm{s-s}}$ is the steady state serum concentration after hemispheric opening of the barrier, $S100\beta_{\mathrm{ser/CSF}}$ are the reference concentrations of S100β in serum and CSF expressed in ng/ml, CSFvol and serumvol are volumes of these compartments expressed in liters. The resulting three-dimensional plot is shown in Fig. 3C to demonstrate the dependence of $S100\beta_{\mathrm{s-s}}$ on CSF and blood volume.

As expected, the peak levels of $S100\beta_{\mathrm{s-s}}$ are achieved when CSF volume is greatest and serum lowest. As predicted by our direct experimental observation and fitting, these values were again close to 0.18 ng/ml (green arrow) which closely parallels the
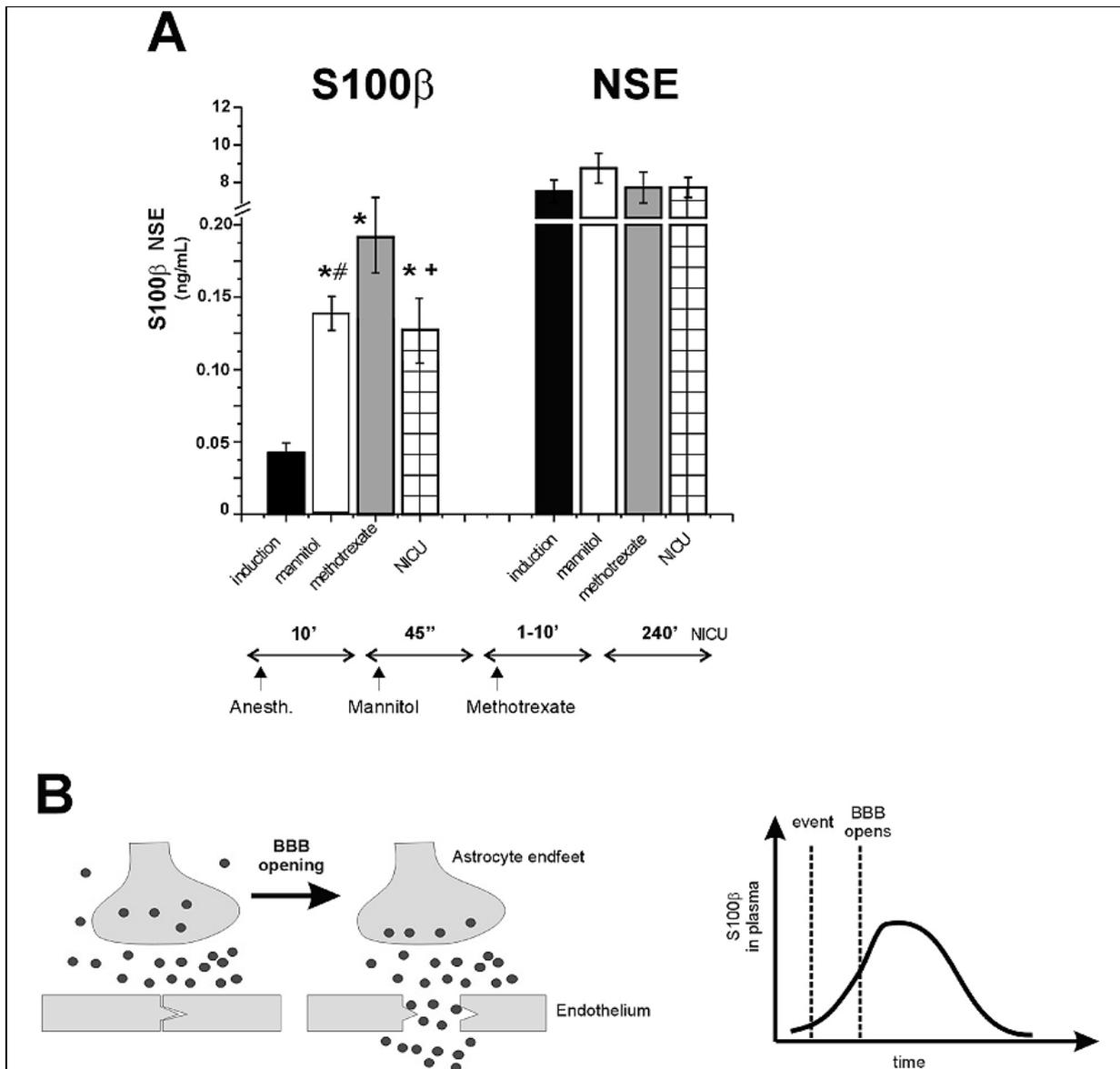
Fig. 2. S100β levels in serum correlate with BBB opening in the absence of neuronal damage. A) Serum levels of S100β rise as a result of osmotic opening of the blood-brain barrier, not of ongoing neuronal damage. The bar graph shows mean serum levels of S100β assessed after 32 blood-brain barrier disruptions in five patients. Also shown are mean serum levels of NSE measured in 18 openings ($n = 3$ patients). Error bars show standard error of the mean. Results show that S100β levels increased after the administration of intra-arterial mannitol and remained elevated, whereas levels of NSE did not change significantly. * indicates level was significantly different from level at induction, and # indicates level was significantly different from level at methotrexate administration ($p < 0.05$; paired t-test).. B) Interpretation of results; see text for details.

amount leaked from the CNS after hemispheric BBB disruption.

These data and fits were based on CSF S100β levels typical of uninjured brain. To estimate the steady-state values of S100β_{Serum} at different S100β_{CSF} and under condition of bilateral BBB damage, we used the following equation (Fig. 3D):

$$S100\beta_{\text{s-s}} = [CSF_{\text{Vol}} * S100\beta_{\text{csf}}$$
$$+ Serum_{\text{Vol}} * 0.05]/[CSF_{\text{Vol}} \quad (3)$$
$$+ Serum_{\text{Vol}}]$$

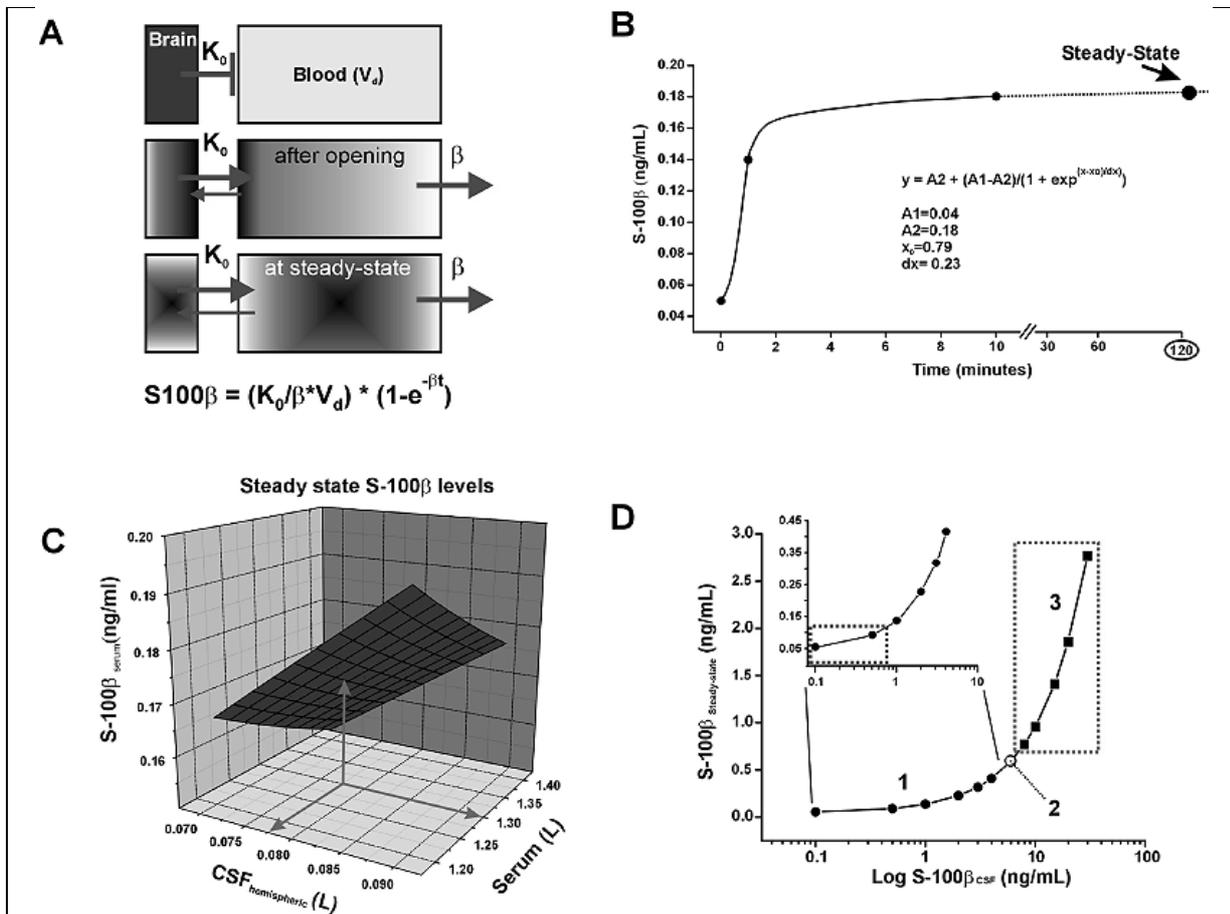Values for CSF and serum volume were arbitrarily set

Fig. 3. S100β levels after BBB disruption. A) Sigmoidal (Boltzmann) fit of S100β_{serum} levels measured after hemispheric BBBD. The average of 36 openings is shown reflecting leakage produced bu opening of the BBB of one hemisphere. The asymptotic value determined was 0.176 ng/ml. B) Tri-dimensional representation of Eq. (2). The initial values of S100β_{serum} and S100β_{CSF} were 0.05 and 2 ng/ml respectively. Note that S100$\beta_{\text{s-s}}$ obtained after hemispheric BBB disruption depend on both CSF and blood volumes. Similar plots were constructed at different S100β_{CSF} levels to estimate the contribution of neuronal damage to plasma levels (box 3 Fig. 3C) under conditions of breached BBB. C) shows the results of these calculations (see Eq. (3).

at 0.15 L and 1.5 L respectively to reflect the average volumes for serum and cerebrospinal fluid. The red region in Fig. 3D represents S100$\beta_{\text{s-s}}$ within a range that includes normal values and levels that may be achieved by breaching the BBB in absence of damage (our findings). The data point 2 refers to data from Martens et al. [51] where experimentally measured CSF values of 6 μg/L corresponded to serum levels of around 0.6 μg/L. Note that these values were identical to those predicted by our model. The boxed blue region (3) represents S100β_{csf} levels measured by de Vries et al., in patients affected by a variety of neurological diseases [17]. These levels were compared in the same study with S100β_{serum}. Again, a perfect correlation of CSF/serum ratios with data obtained with our model was found.

Taken together, these experimental results and mathematical modeling demonstrate that the maximal levels of S100$\beta_{\text{s-s}}$ achievable after BBB failure are around 0.34 ng/ml (Fig. 3C). Thus, levels of S100$\beta_{\text{s-s}}$ exceeding this value may be due to other factors, such as non-CNS release [43], synthesis ex novo due to damage, or other mechanisms.

7. Brain damage in the absence of BBB damage: Interpretation of false negative values

Our working hypothesis was that useful peripheral markers of ongoing or past CNS damage will appear in serum in virtue of a leaky blood-brain barrier. This indirectly implies that, if the BBB is intact, serum lev-

els of S100β will remain low even under conditions of ongoing brain damage (i.e., elevated CSF S100β Others have shown that intracerebral hemorrhage causes massive elevations of S100β_{CSF} without appreciable changes in serum levels early after injury [17], suggesting that the BBB may be intact acutely after intracerebral hemorrhage (ICH). This was tested by us in an animal model of ICH (Fig. 4).

Adult pigs were bilaterally injected with autologous blood in the white matter underlying the cortex. CSF and blood were sampled prior to, immediately after experimental ICH, and following surgical evacuation of the clot. BBB permeability to proteins was determined by a method developed by Cavaglia and Janigro [11]. This method consists of intrarterial injection of FITC-labeled albumin and subsequent evaluation of capillary leakage by confocal microscopy. Two hours following intracerebral injection of blood, BBB integrity was not significantly affected as determined by evaluation of vessel permeability in the perilesional region (Fig. 4A). This was further confirmed by measurements of the permeability to potassium. At this time point, brain potassium was significantly higher than blood K^+, as predicted by damage to brain and intact BBB (data not shown). Thus, early after damage the cerebrovascular endothelium maintained barrier properties to both protein and small ions.

S100β_{CSF} levels increased rapidly after experimental ICH (Fig. 4B; $n = 6$). These levels declined after surgical evacuation of the bilateral clots. Serum levels, however, remained largely unchanged. Taken together, these results show that when BBB function is preserved appearance of peripheral markers of BBB damage is either delayed or prevented. This is schematically outlined in Fig. 4C.

8. Discussion and conclusions

Diagnostic tools have been successfully used for many years to detect changes in cardiovascular function. It is thus not surprising that a quest for peripheral markers of brain function has ensued. S100β, neuron-specific enolase, and other putative markers of brain damage have been shown to correlate with outcome in a variety of neurological disorders [29,42,76,78,84].

The cerebral circulation, unlike the coronary vascular network, is characterized by tight junctions between endothelial cells. The presence of tight junctions is the molecular basis of the so-called blood-brain barrier, a specialized endothelial structure effectively shielding

the brain from systemic influences [39,58,73]. The presence of this endothelial barrier minimizes the extravasation of a variety of molecules including CSF (or serum) S100β (see Table 1). Thus, detection of passage of albumin from serum to brain is the preferred clinical method to evaluate BBB intactness by either direct measurements (lumbar puncture) or contrast-enhanced CT-MRI where albumin is chemically linked to radio-opaque ions (e.g., gadolinium). The opposite approach, detection of S100β protein in serum, is also possible in virtue of the fact that this protein is almost exclusively present in brain astrocytes [53,67,68]. Kapural et al. [46] have demonstrated that S100β_{serum} may be used as mark of BBB integrity. This finding was not necessarily in disagreement with the notion that S100β is a marker of brain damage, since both phenomena (BBB failure and brain damage) are temporally and topographically associated. Kanner et. Al have recently shown a clear correlation between MRI enhancement and peripheral levels of S100β [45].

9. Significance of quantitative evaluation of serum S100β

A possible explanation of the dual message that levels of S100β_{serum} may convey is shown in Figs 2, 3 and 4. According to this hypothesis, low levels of S100β are normally present at the blood-to-brain interface and in the CSF. This is supported by ample evidence (e.g., Fig. 1; see also [53,66,67]). Thus, disruption of the BBB will result in sudden appearance of cerebral S100β in serum. This was confirmed in BBB disruption experiments (Fig. 2; [40,46,50]). The extravasation of S100β depends on the existence of a gradient from CSF to serum [66,67], and the levels in those compartment in normal individuals are known. Thus, it was possible to estimate the steady-state levels of S100β that are when 1) The BBB is completely leaky; 2) levels of S100β_{CSF} do not increase over time due to neuroglial damage; and 3) CSF and serum concentrations are constant. Furthermore, similar analysis was performed for S100β_{CSF} levels typical of a broad range of cerebral dysfunction (Fig. 3C).

The mathematical analysis performed according to equations 1, 2 and 3 shown in Fig. 3A & 3B, demonstrate that increases in S100β up to $\sim$0.34 ng/ml are the plateau levels reachable by opening of BBB in absence of neuronal damage. Thus, serum levels of S100β exceeding this ceiling may implicate brain damage or release from non-CNS sources. This model approach
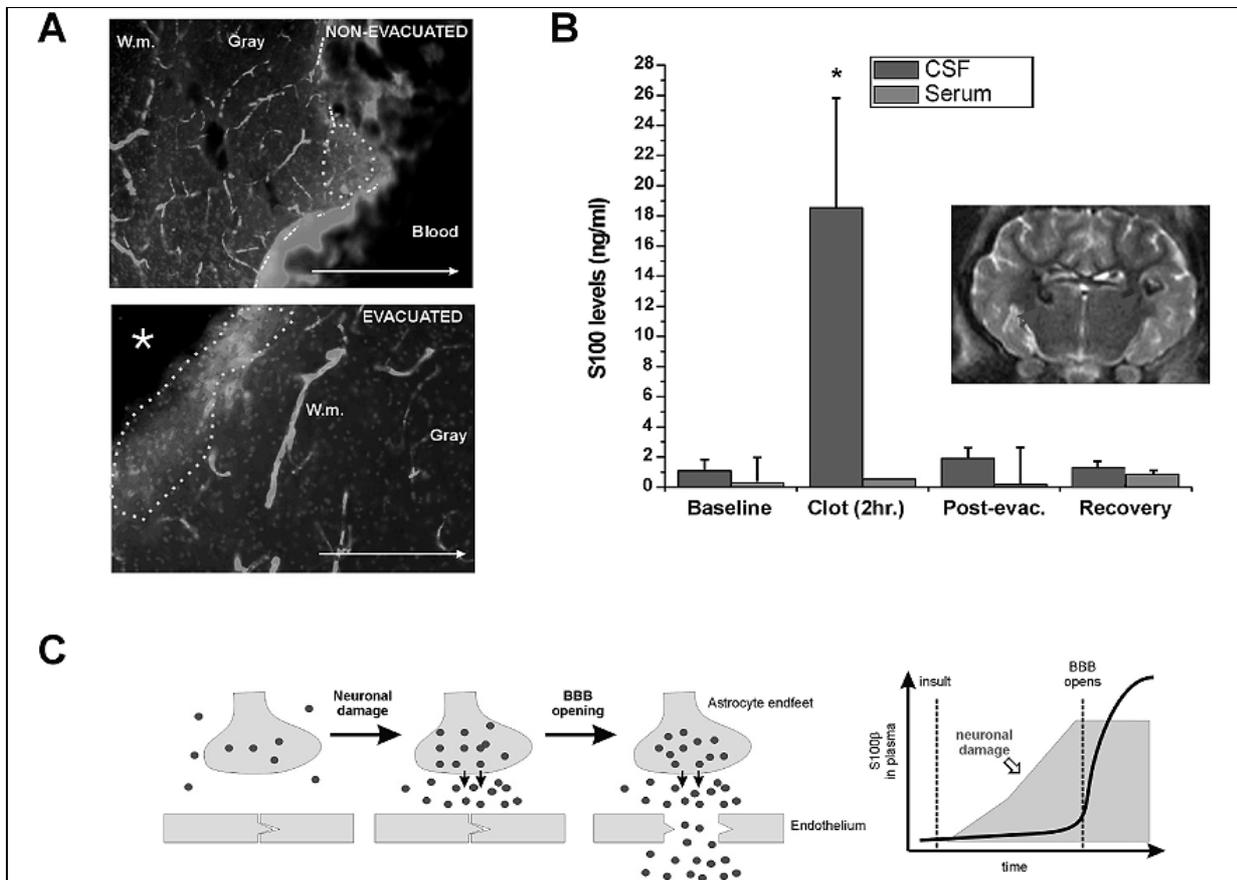
Fig. 4. S100β levels after intracerebral hemorrhage. A) FITC-labeled albumin is sequestered intraluminally 2 hrs. after bilateral injection of autologous blood (as indicated in B). Note that even in close proximity of the clot (outlined by a dashed line) the BBB remained intact. BBB integrity was observed in both evacuated and non-evacuated hemispheres. B) Lack of correlation of S100$\beta_{\rm serum}$ with S100$\beta_{\rm CSF}$ after experimental intracerebral hemorrhage. Note that significant increases of CSF S100β were not accompanied by comparable changes in serum levels. The mean of six experiments is shown; * indicates $p < 0.02$. C) Interpretation of results. Under conditions of intact BBB, extravasation of biochemical markers of brain damage is limited by the low transendothelial permeability to macromolecules. Therefore, neuronal damage precedes appearance in peripheral blood of any proteic marker of brain damage. The latter will extravasate into plasma at later times if the BBB is breached.

led to results that match experimental and clinical data. For example, negative outcome in acute cerebral infarction was associated with serum S100β levels of $\sim$0.7 ng/mL. These values, according to our model, correspond to S100$\beta_{\rm CSF}$ of $\sim$7–8 ng/mL in accordance to S100β levels measured by Martens et al. [51]. Accordance between experimental data and our model were also found for benign mass lesion and malignant neoplasms [17] (Fig. 3D).

Quantitative evaluation of S100$\beta_{\rm serum}$ is not, however, infallible. In fact, when the barrier is intact, S100β fails to appear in serum even when S100$\beta_{\rm CSF}$ is greatly increased (Fig. 4)[17]. Thus, caution must be taken when interpreting negative S100$\beta_{\rm serum}$ values when a brain lesion is suspected. Additional stud-

ies will allow understanding under which pathological conditions the BBB remains intact thus hampering detection of peripheral markers of brain damage (see Table 4).

10. Unresolved issues and future directions

One of the main findings presented here is the fact that levels of S100β below a certain threshold are likely to correlate with BBB damage, whereas larger increases can only be attributable to concomitant damage to the brain and blood-brain barrier. The levels of S100β consistent with brain damage may also be further subdivided to indicate different pathologies, as suggested

Table 4
Current limitations to the use of peripheral markers of damage and research priorities to develop screening tools of widespread clinical usefulness

	Present limitation	Strategy	Significance
1)	Undetermined threshold level(s)	Large scale studies with standardized tests	Differentiate brain damage from BBB leakage, malignant melanoma
2)	Overlapping properties of $S100\beta$ (BBB and damage)	See 3, 4	Establish temporal and causal relationship between BBB failure and neuronal damage
3)	Discovery of new markers of BBB leakage	Proteomics[1], cMRI-CT	BBB-specific markers unrelated to neuroglial damage as early predictors of disease
4)	Discovery of new markers of brain damage	Proteomics, genomics (cDNA arrays; DNA SNPs	Discriminate BBB damage from BBB leakage and brain damage
5)	Discovery of non-protein, non-DNA markers of brain damage	HPLC, Mass spectroscopy, "metabolomics"	BBB-permeant markers will avoid false negatives
6)	Decrease time required to perform tests	Modern diagnostics	Emergency situations (ER, OR)
7)	Development of "stand-alone[2]" tests	Modern diagnostics	Emergency situations, rural settings, home tests

[1]See (18).
[2]Tests based on immobilized antibodies on supports that can be readily used and discarded, as are, for example, modern pregnancy tests based on detection of proteins in urine. cMRI-CT: Contrast-based MRI-CT. DNA SNPs, single nucleotide polymorphisms.

for head injury [35,36,83]. Finally, levels exceeding those associated with neurological disorders have been measured in serum of patients affected by malignant melanoma [8,32,44,74]; these exorbitant levels are indicators of terminal stages of the disease where CNS infiltration is common [31]. It is therefore important to determine disease-specific cutoff values with a great level of accuracy and reproducibility. Patient-specific estimates may become necessary since both volemia and CSF volume are important factors in the interpretation of serum $S100\beta$ at least when BBB failure is involved Fig. 3B A consequence of this is the necessity to use standardized and consistent tests in various hospitals and emergency settings. The devices used to perform these tests must use homogenous, based on automated techniques to allow large-scale data collection and comparison across centers. There are currently several tests for detection of $S100\beta$ all based on immunological detection by ELISA or similar approaches. The sensitivity and specificity of these tests are likely to be different and sometimes, as in the case of manually performed ELISA, operator-dependent. A more focused and equivalent procedure needs to be developed and used.

Finally, if the goal of "BBB markers" is the early diagnosis of a variety of neurological diseases (including recurrence and onset of primary and metastatic brain tumors), we need the development of a rapid, easy to use test that does not require extensive laboratory equipment. The ideal test can be repetitively performed perhaps by an unattended patient, as for example is the case for tests to determine glucose levels in diabetics or for detection of pregnancy. A diagnostic future for BBB markers also depends on the discovery of more specific markers that lack properties of indicators of brain damage. Recently, it has been shown that the monomeric form of transthyretin, a CSF protein, fulfills some of these properties [50].

In conclusion, interpretation of recent results and existing literature compelled us to reinterpret the significance of $S100\beta$ as marker of brain damage. Experimental, clinical and theoretical data show that: 1) $S100\beta$ is a marker of both BBB and neuronal damage; 2) threshold serum values indicating brain damage can be estimated; 3) conditions exist when $S100\beta_{\text{serum}}$ is low in spite of massive brain damage; and 4) detection of slightly elevated levels of $S100\beta_{\text{serum}}$ may be an early sign of future neuronal damage, triggered or accompanied by blood-brain barrier failure.

Acknolwedgements

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