Peripheral markers of blood–brain barrier damage

Nicola Marchia, Marco Cavaglia, Vincent Fazio, Sunil Bhudia, Kerri Hallene, Damir Janigro

Department of Neurological Surgery, Cerebrovascular Research Center, NB20, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, USA
Department of Thoracic and Cardiac Surgery, The Cleveland Clinic, Cleveland, OH 44199, USA

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Abstract

Neurological diseases are often associated with cerebrovascular dysfunction and changes in blood–brain barrier (BBB) function. This is important for two seemingly conflicting reasons. On the one hand, a leaky BBB may lead to brain disease by allowing extravasation of cells and molecules normally segregated in the periphery, while on the other hand an intact BBB may hamper drug delivery to the ailing brain. Under both circumstances, it would be desirable to follow closely over time BBB “tightness”. Several lines of evidence have suggested that the astrocytic protein S100β is a potentially useful peripheral marker of BBB permeability. Other markers of brain-to-blood barriers have been recently discovered by a proteomic approach. These proteins are virtually absent in normal blood, appear in serum from patients with cerebral lesions, and can be easily detected. We will present clinical and laboratory evidence supporting the use of these markers as modern neurodiagnostic tools.

Keywords: S100β; BBB (blood–brain barrier); Blood–CSF barrier; Endothelium; Cerebral ischemia; MRI (magnetic resonance imaging); Neurological disorders; NSE (neuron-specific enolase); TTR (transthyretin); Neurodiagnostic tools

The discovery that the entry of many substances into the brain is restricted has been generally attributed to Paul Ehrlich, who observed in the early 1880s that aniline dyes injected into animals intravenously colored all the organs except for the brain and spinal cord [1]. The singular failure of the brain to be stained at autopsy during jaundice must have been observed before Ehrlich’s work, but apparently did not steer inquiry into its mechanisms.

Not all the blood vessels in the brain constitute a blood–brain barrier (BBB): only capillary vessels are endowed with a full-blown BBB phenotype. Vessels of increasing diameter have comparably increasing levels of leakiness and thus superficial vessels of large diameter are the leakiest while penetrating pial vessels and descending penetrating vessels tend to have an intermediate barrier function. Since most animals, including vertebrates, have some form of barrier separating their blood circulation from the brain or the central nervous system, it has been speculated that profound evolutionary pressure existed to create such a complex organ [2,13]. At present, however, it is not clear why such sophistication exists, and we can only...
speculate on the teleological pressure that manifests in the specialization observed in endothelial cells (EC) of the brain.

The ontogeny of the mammalian blood–brain barrier may be related to the absolute need for controlled homeostasis of the central nervous system. It has been known for many years that the ionic and molecular composition of the cerebrospinal fluid (CSF) differs considerably from the composition of the plasma and remains remarkably constant in spite of intravascular changes. For example, proteins are few in the CSF, and ionic gradients for potassium and calcium ions exist across the blood–brain barrier. Glucose constitutes the main energy supply to the brain and is avidly consumed by neuronal cells. To ensure a constant supply of glucose to the brain, even under conditions where plasma glucose is low, the blood–brain barrier maintains nearly a 10-fold gradient for glucose entry into the brain [3]. Other substances are actively transported into and out of the brain, including amino acids, metallic ions, etc.

Homeostatic control of the brain parenchyma by vascular endothelial cells is also achieved by a function that is seemingly opposed to their “barrier” function. For example, the abluminal membrane of the endothelium facing the glia end feet is endowed with a high concentration of sodium/potassium transporter molecules that permit absorption of excess potassium from the brain into the blood [4–8]. Failure of such a mechanism could lead to an abnormal accumulation of potassium into the brain leading to neurological disorders such as seizures. Control of parenchymal pH may also depend on the integrity of brain endothelial cells.

1. Anatomy and physiology of the normal blood–brain barrier

The BBB is primarily composed of microvascular EC linked by tight junctions that largely prevent molecular communication between the plasma and the brain or central nervous system [9–12]. Only capillary vessels have complete BBB properties. As vessel diameter increases, leakiness also increases: superficial vessels of large diameter are the leakiest, and penetrating pial vessels and descending penetrating vessels have intermediate degrees of barrier function. Surprisingly, in spite of the large body of evidence on the neurophysiological properties of the central nervous system, comparatively little is known about the physiology and pathophysiology of microvascular EC.

The long axis of these cells is perpendicular to blood flow, and they are exquisitely sensitive to shear stress. Recent evidence has shown that shear stress promotes the expression of numerous genes involved in various aspects of endothelial cell function [12,14–16]. It is unknown whether flow-induced changes in EC affect neighboring glia, and whether these changes affect expression of astrocytic proteins that may be detected in plasma. Some of the unique properties of the BBB are induced by the perivascular glia. This is known from two lines of evidence. First, it is evident that shear stress alone cannot be responsible for BBB properties in EC, because systemic microvascular EC are exposed to comparable levels of shear stress. Second, glia cells have been shown to induce blood–brain barrier properties in vessels of systemic origin [17,18]. Thus, the blood–brain barrier is commonly understood to be constituted of both endothelial cells and glial end feet. Perivascular pericytes and microglia may also be considered active components of the blood–brain barrier.

The microvascular endothelium shares a common basement membrane with astrocytes and pericytes. Beyond the basement membrane in the parenchymal vessels of the brain lies a close investment of end feet from neuroglial cells, predominantly astrocytes. Astrocytes and their processes invest more than 90% of endothelial capillaries, and their end feet are projected tightly around the endothelial cells. Therefore, the glial end feet are a natural candidate for mediating communication between neurons and capillaries. Astrocytic proteins are synthesized and released next to capillaries, but it is thought that they extravasate into the plasma only when the BBB is breached.

2. Protein permeability at the BBB

Brain-derived proteins may be useful markers of BBB integrity because they have several possible mechanisms of passage across the BBB. Under physiologic conditions, the production of CSF from
plasma involves efficient removal of plasma proteins by filtering mechanisms in the choroid plexus. The resulting CSF contains extremely small quantities of proteins [19]. However, many neurological disorders are associated with elevated CSF protein levels. Furthermore, CSF-specific proteins exist [20].

Proteins in CSF can be detected by directly sampling CSF, which requires invasive techniques such as lumbar puncture or intraoperative sampling from ventricles or subarachnoid space. BBB integrity can also be assessed by contrast-enhanced computed tomography or MRI (see above). An obvious limitation of intrathecal detection methods is that they are

Fig. 1. Gradients and topography of peripheral markers of BBB/brain damage. See text for details. The micrographs show GFAP and S100β immunopositive astrocytes and NSE immunoreactivity in human neocortex (temporal lobe). Note that GFAP and S100β immunoreactivity is highly concentrated in proximity to blood vessels (indicated by small arrows). A large NSE+ neuron neighboring a vessel is also shown. Bars indicate 20 μm. n = neuron, a = astrocyte.
invasive and that the sample itself may be contaminated by the procedure. Accurate non-invasive techniques would clearly be preferable, particularly to analyze multiple longitudinal samples. A small group of proteins are found exclusively or almost exclusively in the cerebrospinal fluid. Any disruption in BBB integrity may allow protein leakage in both directions (see Fig. 1). Thus, testing serum levels of CSF proteins represents a non-invasive means for evaluating BBB integrity that may be of diagnostic value [21]. Fig. 2 shows the predicted behavior of proteins in conditions of intact vs. impaired BBB function. Scenarios A and B demonstrate the observed elevation in S100β serum levels as the result of BBB opening following and prior to neuronal damage (respectively). Scenario C exemplifies the use of a chemical means (mannitol infusion) that has been used to produce a transient opening of the BBB without producing accompanying neuronal damage.


Substantial progress has been made in the understanding of the pathophysiology and mechanisms involved in the attenuation of BBB permeability. In many diseases that affect the brain, the cerebral endothelium plays an active part in the disease process with the BBB becoming disrupted, or modified, in such a way that there is a dramatic increase in vascular

Fig. 2. Possible mechanisms of release of putative markers of neuronal function or blood–brain barrier damage across the BBB. (A) Marker (red dots) may be released after neuronal injury; (B) before injury across a leaky blood–brain barrier, with additional release after injury; or (C) in the absence of neuronal damage. Hypothesis A predicts that a marker that is released or synthesized in response to injury will appear in the plasma compartment only after brain injury. In contrast, if BBB dysfunction precedes injury and if the marker is present in CSF but not plasma under normal conditions, appearance in plasma will be temporally related to opening of the blood–brain barrier, with a possible additional release due to increased synthesis or release in the CSF. However, if the BBB opening is not followed by significant increase in marker’s availability in the CNS from neuronal damage, the appearance in plasma will be brief in duration.
permeability. Theoretically, several ways exist in which various molecules can pass the endothelium. These include intercellular routes, vesicular transport or direct transcellular penetration through damaged endothelium. BBB dysfunction may be a cause or consequence of a particular disease process. Cerebral vessels from pathological brain tissue often resemble peripheral endothelium, with significant increases in vesicular transport and abnormal tight junctions. Diseases in which increased BBB permeability have been reported include neoplasia, ischemia, hypertension, dementia, epilepsy, infection, multiple sclerosis and trauma. The effect of a disease on BBB function will secondarily affect the cerebral blood flow and vascular tone in the brain, which further influences transport across the BBB. Besides the effects of increased vascular permeability on the brain parenchyma, a question of great significance is whether, in certain neuropathological conditions, the BBB disturbance constitutes the main pathogenic factor itself, which then triggers a sequence of events molding the final pathological state.

While loss of blood brain barrier (BBB) function is an etiologic component of many neurological diseases an intact BBB may restrict the delivery of certain therapeutic substances to the brain. Thus, measuring BBB function may be important to diagnose disease progression and monitor time-dependent changes in BBB integrity when chemotherapeutic penetration may be enhanced. At present, only invasive and expensive techniques such as contrast-enhanced magnetic resonance imaging, CT-scan and lumbar puncture are available to clinically assess BBB integrity. An alternative approach has been proposed, consisting of detection of changes in blood composition that indicate BBB disruption [21].

Current BBB assessment by imaging or cerebrospinal fluid sampling is based on direct or indirect determination of protein permeability across the BBB. CNS proteins are normally asymmetrically distributed, with generally much higher concentration in plasma than in CSF. Thus, the appearance of plasma proteins in CSF is a hallmark of numerous CNS disorders with presumed or overt BBB disruption. Only a few proteins are synthesized exclusively by, or are present in higher concentrations in CSF or interstitial compartment compared to the blood. These CSF markers may appear or increase their plasma concentration after passage across a failed BBB. Therefore, measuring levels of CSF proteins in plasma may be a reliable way to monitor blood to CNS barrier integrity without the use of invasive methods.

4. Neuronal damage vs. glial/BBB damage

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 damage or death. This research has shown that neuronal sensitivity to insult is region-and disease-specific. As example, ischemic insult selectively affects the CA1 region of the hippocampus, leaving the neighboring dentate gyrus and CA3 practically intact [22]. In contrast, damage resulting from epileptic seizures is more prominent in CA3 hippocampal subfield [23]. In addition to these patterns of specificity, it has also been observed that neuronal cell death does not occur concomitantly with the insult, but rather after a delay.

In acute insults such as ischemia [24,25], 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. Ideal markers of BBB permeability and of neuronal damage share several characteristics (Table 1): both should be virtually undetectable in normal subjects and should show distinct alterations in response to insults that are correlated with the severity of the damage.

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 BBB openings develop early after the initial arterial occlusion [26–32], clinicians would have a unique opportunity to administer drugs that are normally BBB-impermeant (e.g., nerve growth factors) before neurons were 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. When a patient experiences both blood–brain barrier opening and neuronal damage, plasma levels of both markers would be expected to exceed normal levels [54]. Also, as we will discuss below, S100 protein levels appear to be directly correlated with BBB integrity rather than with neuronal damage, while another neuronal protein, monomeric-transthyretin (TTR) represents a potential marker of opening to the blood–CSF barrier [48].

Because of this focus on neuronal damage, much of the previous research on biochemical markers has focused on markers that measure neuronal damage. However, most neurologic diseases are accompanied by increased BBB permeability, and thus the markers thought to indicate neuronal damage may in fact indicate BBB defects. Marker proteins under investigation have included neuron-specific enolase (NSE), glial fibrillary acidic protein (GFAP), NSE and S100β; (see Fig. 1). S100β seems particularly promising. In normal subjects, NSE is more concentrated in plasma and S100β is primarily present in central nervous system fluids [33]. Thus, opening the blood–brain barrier in the absence of neuronal damage would be expected to markedly increase plasma S100β levels while leaving NSE levels unchanged.

5. Markers of blood–brain barrier integrity: S100β

S100β is primarily synthesized in the brain by the end feet process of the astrocytes and is quickly released from the brain in the blood when the BBB is disrupted [21,34–37]. S100β has also been found in other tissues but at lower concentrations [38–40]. While S100β appearance in plasma correlated well with BBB openings, S100β has been shown to increase in plasma, CSF or both as a consequence of other pathologies not limited to the CNS. S100β may also detect brain damage, or indicate advanced metastasis in melanoma patients [41–45].

The fact that S100β can increase in serum independent of brain (or neuronal) damage was demonstrated indirectly by a team of scientists who studied the affects of boxing as well as other high cardiovascular output activities on the levels of S100β in serum. Interestingly, this study found that a significant increase in S100β was observed in serum of subjects undertaking activities that involved repetitive, jarring movement or contact to the head (such as boxing, sparring, running and jogging), but essentially no increase was observed in persons exerting themselves through exercise on a stationary bicycle. Clearly, these activities did neither cause nor promoted brain damage but the rise in S100β protein in running activities may be due to astroglial activation, astroglial destruction or blood–brain barrier disruption or a combination of the three. This finding also indicated that the source of S-100β was not influenced greatly from excretion of the protein from extra-cranial tissue.

Another study demonstrated dissociation between serum S100β and brain damage. S100β was measured in sera from patients diagnosed with major depression [46]. This study discovered a positive correlation of the severity of depression with S100β levels; patients given antidepressive therapy (prescription anti-depressants) experienced significantly decreased levels of serum S100β.

A direct demonstration proving that S100β may increase in serum in spite of an obvious lack of

<table>
<thead>
<tr>
<th>BBB integrity</th>
<th>S100β</th>
<th>NSE</th>
<th>GFAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Low or undetectable levels in plasma in normal subjects</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>(2) Normally present in CSF</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>(3) [Plasma] &lt; [CSF]</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>(4) Increase [CSF] in response to insult</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>(5) Normally excluded by BBB</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>(6) Expected flux after BBBD</td>
<td>blood-to-brain</td>
<td>brain-to-blood</td>
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Table 1: Properties of selected proteins and their use as potential markers

additional brain damage was provided by studies where S100β was measured at short intervals (minutes) in patients undergoing osmotic opening of the blood–brain barrier [21,34,47,48]. These authors demonstrated that S100β elevation correlated temporally with BBB disruption; these findings were also confirmed radiologically by Kanner et al. [47]. To test the connection between S100β and blood–brain barrier integrity, NSE and S100β were measured in serum of patients with primary central nervous lymphoma who underwent blood–brain barrier disruption by intra-arterial mannitol infusion before receiving methotrexate infusion. Mean serum levels of S100β increased significantly after mannitol infusion and again after methotrexate infusion, and they remained elevated through recovery. However, NSE serum levels remained constant throughout the procedure and during recovery. Previous investigators have shown that blood–brain barrier disruption with intra-arterial methotrexate does not lead to brain damage [49–51]. To rule out the possibility that the increased plasma S100β levels were caused by the methotrexate, the authors measured S100β and NSE in the blood of three patients who were given methotrexate without blood–brain barrier disruption. Under these conditions (no BBB disruption) the levels of both markers remained within normal ranges. It was concluded that the increase in S100β immediately after the blood–brain barrier disruption was almost certainly too soon to be the result of synthesis and release from “reactive” glia and that S100β may be an early marker of blood–brain barrier disruption that is not necessarily related to either neuronal or glial brain damage. These findings of course do not challenge the traditional understanding that NSE is related to neuronal damage.

In addition to S100β, another putative marker of blood–brain barrier function was unveiled in these studies. The CSF protein, monomeric-TTR was also temporally linked to S100β movement from brain to serum [48]. However, since the authors observed a slight, but significant delay (within minutes) between the peak serum elevation of S100β (immediately following chemical opening of the BBB) and TTR (occurring shortly thereafter following the administration of chemotherapy), it was concluded that TTR was a marker of blood-to-CSF rather than a BBB marker (see Fig. 3).

![Figure 3](image-url)  
**Fig. 3.** Different distribution between S-100β and TTR in the brain. In the brain, S-100β is primarily synthesized by the astrocytes surrounded the BBB, whereas TTR is synthesized by the choroid plexuses and is found in the ventricular CSF. This topographic segregation may explain the different roles of these markers.
6. Mathematical modeling of serum S100\(\beta\) levels after brain damage or BBB leakage

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 \([43,44,52,53]\). How could these seemingly contrasting two findings be explained? If one utilizes values from the literature related to S100\(\beta\) levels in tissue as well as the measured dimensions of various body cavities, a kinetic model for S100\(\beta\) release in serum can be deduced \([34,38]\). The predictive value for these parameters were independently confirmed by mathematical modeling of the range of steady-state concentrations that S100\(\beta\) serum would reach when the BBB is greatest (schematically represented in Fig. 4A) or when brain damage occurs concomitantly. This model was used to assess the

![Image](https://example.com/image.png)

**Fig. 4.** S100\(\beta\) levels after BBB disruption: (A, B) schematic representation and mathematical modeling of S100\(\beta\) kinetics (see Ref. [10] for details). Sigmoidal (Boltzmann) fit of S100\(\beta\) serum levels measured after hemispheric BBBD. The average of 36 openings is shown reflecting leakage produced by opening of the BBB of one hemisphere. The asymptotic value determined was 0.176 ng/ml. (C) Tri-dimensional representation of Eq. (2). The initial values of S100\(\beta\) serum and S100\(\beta\) CSF were 0.05 and 2 ng/ml, respectively. Note that S100\(\beta\) obtained after hemispheric BBB disruption depend on both CSF and blood volumes. Similar plots were constructed at different S100\(\beta\)CSF levels to estimate the contribution of neuronal damage to plasma levels (box 3, C) under conditions of breached BBB. (D) shows the results of these calculations. Figure was reproduced and modified with permission from Ref. [34].
dependency of S100β steady state on serum and CSF volumes as well as CSF levels of the protein. The initial values used were derived from the literature (e.g., S100β CSF = 2 ng/ml and S100β serum = 0.05 ng/ml). Data were fitted according to the following equation:

\[
S100β_{s-s} = \frac{(S100β_{CSF} * 1/2CSF_{vol}) + (S100β_{ser} * Serum_{vol})}{1/2CSF_{vol} + Serum_{vol}}
\]

(1)

where S100β_{s-s} is the steady-state serum concentration after hemispheric opening of the barrier; S100β_{ser/CSF} are the reference concentrations of S100β in serum and CSF expressed in ng/ml; CSF_{vol} and serum_{vol} are volumes of these compartments expressed in liters. The resulting three-dimensional plot is shown in Fig. 4C to demonstrate the dependence of S100β_{s-s} on CSF and blood volume. As expected, the peak levels of S100β_{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 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 bi-hemispheric BBB damage, we used the following equation (Fig. 4D):

\[
S100β_{s-s} = \frac{(CSF_{vol} * S100β_{CSF}) + (serum_{vol} * 0.05)}{CSF_{vol} + serum_{vol}}
\]

(2)

Values for CSF and serum volume were arbitrarily set at 0.15 and 1.5 l, respectively, to reflect the average volumes for serum and cerebrospinal fluid. The red region in Fig. 4D represents S100β_{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 Buttner et al. [55], 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 in patients affected by a variety of neurological diseases [38,43,44,52,54–60]. These levels were compared in the same study with S100_{serum}. Again, a perfect correlation of CSF/serum ratios with data obtained from our model was found.

Taken together, these experimental results and mathematical modeling demonstrate that the maximal levels of S100β_{s-s} achievable after BBB failure are around 0.34 ng/ml (Fig. 4C). Thus, levels of S100β_{s-s} exceeding this value may be due to other factors, such as non-CNS release [57], synthesis ex novo due to damage, or other mechanisms.

7. Conclusions

S100β, neuron-specific enolase and other putative markers of brain damage have been shown to correlate with outcome in a variety of neurological disorders [57–59,61,62]. The interpretation of the clinical significance of the appearance of S100β in serum of neurological patients is complicated by the fact that 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. The presence of this endothelial barrier minimizes the extravasation of a variety of molecules including CSF (or serum) S100β. 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.

The fact that S100β_{serum} may be used as marker of BBB integrity is 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.

A possible explanation of the dual message that levels of S100β_{serum} may convey was derived mathematically [34]. According to these authors, low levels of S100β are normally present at the blood-to-brain interface and in the CSF while disruption of the BBB interface and in the CSF while disruption of the BBB
will result in sudden appearance of cerebral $S_{100\beta}$ in serum. It was possible to estimate the steady-state levels of $S_{100\beta}$ that are when (1) The BBB is completely leaky; (2) levels of $S_{100\beta_{CSF}}$ do not increase over time due to neuronal damage; and (3) $S_{100\beta}$ and serum concentrations are constant. Furthermore, similar analysis was performed for $S_{100\beta_{CSF}}$ levels typical of a broad range of cerebral dysfunction. Serum levels of $S_{100\beta}$ exceeding the “BBB ceiling” may implicate brain damage or release from non-CNS sources.

In conclusion, interpretation of recent results and existing literature compelled us to reinterpret the significance of $S_{100\beta}$ as marker of brain damage. Experimental, clinical and theoretical data show that: (1) $S_{100\beta}$ is a marker of both BBB and neuronal damage; (2) threshold serum values indicating brain damage can be estimated; (3) conditions exist when $S_{100\beta_{serum}}$ is low in spite of massive brain damage; and (4) detection of slightly elevated levels of $S_{100\beta_{serum}}$ may be an early sign of future neuronal damage, triggered or accompanied by blood–brain barrier failure.

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