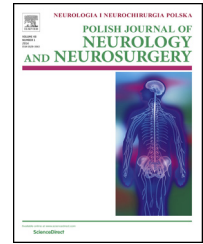


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## Review article

# Looking for novel, brain-derived, peripheral biomarkers of neurological disorders

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## ABSTRACT

The role of blood brain barrier (BBB) is to preserve a precisely regulated environment for proper neuronal signaling. In many of the central nervous system (CNS) pathologies, the function of BBB is altered. Thus, there is a necessity to evaluate a fast, noninvasive and reliable method for monitoring of BBB condition. It seems that revealing the peripheral diagnostic biomarker whose release pattern (concentration, dynamics) will be correlated with clinical symptoms of neurological disorders offers significant hope. It could help with faster diagnosis and efficient treatment monitoring. In this review we summarize the recent data concerning exploration of potential new serum biomarkers appearing in the peripheral circulation following BBB disintegration, with an emphasis on epilepsy, traumatic brain injury (TBI) and stroke. We consider the application of well-known proteins (S100 $\beta$  and GFAP) as serum indicators in the light of recently obtained results. Furthermore, the utility of molecules like MMP-9, UCHL-1, neurofilaments, BDNF, and miRNA, which are newly recognized as a potential serum biomarkers, will also be discussed.

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## 1. Introduction

The brain is one of the most vulnerable organs in the body. Direct contact between the blood and cells of the central nervous system could be dangerous for this organ. To avoid such contact, brain endothelial cells form specific barriers that can separate brain tissue from the blood. It is estimated that

blood–brain barrier is a result of 4–500 million years of vertebrate evolution, associated with concomitant neural tissue centralization [1]. However, invertebrates like *Drosophila melanogaster* also developed vertebrate-like chemoprotective mechanism of central nervous system (CNS) [2]. There are three barriers that protect the neural tissue against uncontrolled exchange of various substances with the blood or cerebrospinal fluid. First is the blood–brain-barrier (BBB),

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formed by the cerebro-vascular endothelial cells and endfeet of astrocytes that isolate blood and brain interstitial fluid (ISF). Second is the choroid plexus epithelium that comprises barrier between blood and ventricular cerebro-spinal fluid (CSF). Finally, the third one is the arachnoid epithelium that separates blood and subarachnoid CSF [3]. The most important function of the brain barriers is to protect the micro-environment of the neural cells from unfavorable agents that circulate in the blood. In fact, BBB forces the transcellular route of traffic because the tight junctions between endothelial cells are impermeable for most substances circulating in the blood [4]. Nevertheless, the BBB is easily permeable for the small molecules such as oxygen or other lipophilic agents like ethanol, valproic acid or many antipsychotic drugs. It is important to note that in the circumventricular organs the endothelium is leaky [3]. That attribute of the BBB is forced by the function which neurons play in circumventricular organs as they are specialized for neurosecretion (posterior pituitary, pineal gland) or chemosensitivity (area postrema, subfornical organ). The access to the brain is not only physically limited by BBB. The enzymes and specific transporters including peptidases, nucleotidases, monoamine oxidase, p450 cytochromes, P-glycoprotein (p-GP), multidrug resistance proteins (MRPs), and many others provide the “metabolic barrier” [5]. The active protection is achieved by inactivation of toxic compounds or by active efflux from endothelium back to circulation. The hydrophilic molecules, especially when they are large, such as peptides and proteins, can be transferred by specific receptor-mediated or adsorptive-mediated transcytosis. Many agents circulating in the plasma or secreted by the cells are able to change the BBB permeability. For example, among agents that can disturb the tightness of BBB (increase its permeability) may be ranked: bradykinin, ATP, ADP, adenosine, TNF $\alpha$  or interleukins (IL-1, IL-6) [6].

Changes in the functioning of BBB, especially an increase in permeability, are also seen in many pathological conditions. The most typical ones are a massive increase in the permeability that occurs in meningitis or brain inflammation. Similar changes, although not so evident, are observed in many other pathological conditions such starvation (e.g. increase in the glutamate transporters localized on abluminal membrane), traumatic brain injuries, stroke, epilepsy, neurodegenerative disorders (e.g. Alzheimer and Parkinson disease), multiple sclerosis, brain neoplasms or hypoxia [7]. Thus, there is an unmet need to look for therapeutic methods or drugs that are able to reverse the BBB dysfunction.

On the other hand, increased expression of transporters localized on the BBB is a significant problem in the treatment of some diseases. It is postulated that hyperexpression of these transporters (e.g. p-GP) may reduce availability of the therapeutic drug in the brain tissue, thus limiting effectiveness of treatment of epilepsy or brain neoplasms [8,9]. Some authors postulate, for example, that the problem of drug resistant epilepsy originates from the changes in BBB function and limited availability of the antiepileptic drugs in brain tissue (in epileptic focus) [8]. Therefore, in some clinical conditions it might be more advisable not to seal up the damaged BBB, but rather make it more permeable. It is crucial in the treatment of brain tumors, where appropriate penetration of cytotoxic drugs is one of the critical conditions of

effective treatment. One example of such an approach may be intracarotid infusion of a hypertonic arabinose or mannitol solution in patients with metastatic or primary brain tumors to increase drug penetration [10].

BBB is a structure that not only limits access to brain tissue but also inhibits the transfer of brain-specific proteins, peptides and neurotransmitters to the blood. Some of those compounds are specific only for the brain and their appearance in the circulation may be a proof of a damage in the brain tissue and of an increased permeability of BBB. For many years, a lot of effort has been put to identify the peripheral markers of neurological disorders, whose concentrations correlate with the extent of neuronal damage and could help to differentiate particular neurological disorders and also to predict clinical outcomes in a particular patient. Among the candidates for that purpose were for example S100 $\beta$  protein and GFAP (Glial fibrillary acidic protein) [11,12]. In this short review we concentrate on biomarkers that appear in the peripheral circulation as a result of disturbances in the BBB integrity during specific pathological conditions, with special attention paid to epilepsy, traumatic brain injury (TBI) and stroke. Due to rules regarding the length of a manuscript we have decided to focus our consideration on new biomarkers, and those with a long history of research which introduction to the clinics may significantly improve diagnosis and treatment of the most common neurological disorders. However, we bear in mind the existence of many interesting indicators which could play a role in dealing with other neurological disorders and psychiatric diseases as well e.g. c-Tau in Alzheimer and Parkinson's disease, NSE in stroke, MAP2 in Creutzfeldt-Jacob disease and stroke or BDNF in depression.

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## 2. Review of potential peripheral biomarkers for BBB disintegration

### 2.1. S100 $\beta$

S100 $\beta$  is a member of S100 protein family responsible for cytoskeleton structure, Ca<sup>2+</sup> homeostasis, cell proliferation, protein phosphorylation and degradation. S100 $\beta$  is present mostly in the cytoplasm and the nucleus of astrocytes and gets extravasated into the bloodstream only when the BBB is disrupted [13]. Changed concentration of S100 $\beta$  in peripheral blood has been linked with many diseases in the CNS with an emphasis on conditions with BBB leakage.

S100 $\beta$  is the most extensively studied protein in the context of its potential utility in diagnosis and treatment of various conditions of the CNS, TBI especially. There are excellent reviews summarizing current knowledge regarding revealed advantages and limitations of S100 $\beta$  use [14,15]. For this reason a detailed review of results discussed before in many scientific papers will not be a subject of our consideration. However, it has to be mentioned that after many years of research and establishing a cut off concentration of 0.1  $\mu$ g/ml in the first 6 h after accident, the Scandinavian Neurotrauma Committee (SNC) introduced assessment of plasma S100 $\beta$  in guidelines for adult head injury. It has been shown that proper analysis of S100 $\beta$  results in patients with mild TBI without extracranial

trauma and other risk factors, reduce the number of computed tomography scan (CT scan) by up to 30% [11].

S100 $\beta$  may be perceived as a reliable candidate to become a peripheral marker of BBB disintegration because of its low mass, easy detection and stability in the bloodstream. However, S100 $\beta$  may be produced by extracerebral tissues and cells like adipose tissue, chondrocytes, lymphocytes, bone marrow cells, or melanocytes and obtained results have to be interpreted with great caution [16]. Additionally, many studies do not show association between serum S100 $\beta$  level and clinical symptoms. In children, for example, higher concentration has not correlated with worse outcome and complete neurological recovery was observed [17]. For this reasons S100 $\beta$  itself has a rather limited clinical application today. However, recent data highlight the inclusion of S100 $\beta$  in the set of markers specific for each neurological condition, which in combination could contribute to better diagnosis, monitoring and treatment of CNS conditions [18,19].

## 2.2. GFAP

The next protein that could be considered as biomarker of BBB disruption is GFAP. GFAP is a filament protein abundantly expressed in cytoskeleton of mature astrocytes, where it maintains shape and structure, coordinates cells mobility and contributes to the transduction of molecular signals [20]. GFAP is a well-established astrocyte marker. Its expression increases in tissues with neurodegenerative disorders [21], neurological diseases [22] and after brain injury [23,24]. Astrocytes disintegration leads to BBB disruption and facilitates GFAP release from tissue to the bloodstream. Potential usefulness of the GFAP in the diagnosis and treatment of neurological disorders has been widely investigated for years and revealed some limitations. Recent data, however, shed new light on the possibility of applying GFAP as a diagnostic tool.

GFAP has been proposed as a marker for ischemic stroke and intracerebral hemorrhage (ICH) distinction. Twenty four hours after symptoms onset, GFAP concentration was below the limit of detection in patients with ischemic stroke [25,26]. Furthermore, the level of GFAP was higher in ICH compared to ischemic stroke patients [12,25,27–29]. In ischemic stroke patients serum GFAP level has been increasing over time, mirroring progress of cell necrosis and was correlated with the size of brain lesions [30]. Measurement of serum GFAP concentration may rule out ICH from heterogeneous stroke population very early on after symptoms onset. Proper and fast diagnosis of patients with stroke-like symptoms has a beneficial impact on treatment efficacy.

GFAP was also considered as a marker for TBI. Concentration of serum GFAP was found to increase after TBI [31–35]. GFAP levels were higher in patients with focal mass lesions than in those with diffuse injury [34]. Its higher peripheral values corresponded to worse health condition manifested by low points number in Glasgow Coma Scale (GCS) after admission [34] and with poor outcome by Glasgow Outcome Scale (GOS) assessed 6 months after brain damage [33,34,36]. GFAP level was increased in patients with CT positive scans for intracranial lesions compared to CT negative scans after mild TBI. Additionally, in patients after TBI treated with hypother-

mia, a high level of GFAP at admission decreased gradually each day. Observed changes were induced by reduced cerebral metabolism, diminished oxygen consumption, and stabilization of BBB following decreased body temperature [31]. It indicates that assessment of GFAP concentration may have application in the controlling of different methods of treatment efficiency after TBI. Furthermore, GFAP may be a favorable marker to distinguish patients with intracranial lesions from lesions free patients [37].

Summarizing, GFAP is highly specific for brain protein and biochemically stable in the blood. Recent studies emphasize its potential role as a marker in conditions other than those highlighted in this review, like diagnosis and prognosis of patients with glioblastoma multiforme [38,39]. More research is needed to evaluate the role of GFAP use in medicine. For now, especially promising are reports about the role of GFAP in distinguishing ICH from strokes of different etiology and positive correlation between serum concentration and injury volume in patients after TBI.

## 2.3. MMP-9

MMP-9 is zinc-calcium dependent endopeptidase included in the family of matrix metalloproteinases [40]. The main source of MMP-9 are white blood cells and neutrophils. Oligodendrocyte precursor cells, astrocytes, neurons, microglia and endothelium also have the ability to synthesize MMP-9 [41]. MMP-9 is responsible for modification of extracellular matrix (ECM) as a response to many physiological and pathological stimuli [42]. MMP-9 degrades ECM proteins: collagen IV, V, laminin, fibronectin and proteins creating tight junction in endothelium of BBB: ZO-1 and occludins. Thus, MMP-9 contributes to BBB remodeling and disruption [41]. Elevated MMP-9 serum levels were demonstrated in some pathological conditions of CNS connected with BBB leakage like multiple sclerosis (MS) [43], ischemic stroke [44] and TBI [45]. Therefore it is considered as a potential serum biomarker of BBB disruption. Additionally, some effort has been made to correlate MMP-9 serum release pattern with symptoms of neurological disorders.

In the hypoxic brain, MMP-9 is a relevant proteinase involved in the degradation of microvascular structures of the BBB and was considered as a marker for stroke [46]. In patients after acute ischemic stroke, an increased serum MMP-9 level was observed. Furthermore, MMP-9 correlated with clinical stroke severity as assessed by The National Institutes of Health Stroke Scale (NIHSS) 24 h after symptoms onset [47]. It has been shown that higher MMP-9 were associated with an increased death risk and major disability after stroke [48,49]. Furthermore, during the first 24 h plasma MMP-9 concentration correlated with S100 $\beta$ , suggesting an association between MMP-9 and the rate of brain injury [48]. Thus MMP-9 may be perceived as a reliable marker of stroke severity and outcome.

Elevated concentrations of MMP-9 have been noted in serum of epileptic patients. It was found that peak levels of serum MMP-9 occur after the first few hours and then it decreases slowly to the control level 3 days after seizures. However, so far no relationship has been found between increased MMP-9 levels and pathogenesis or etiology of seizures [50]. Further studies are needed to associate periph-

eral MMP-9 concentration with clinical symptoms in epilepsy in order to improve diagnosis and settlement of treatment.

In patients after TBI, serum MMP-9 concentrations were increased [45,51] thus MMP-9 was taken into account as a serum marker of BBB disruption in that condition. Plasma MMP-9 were elevated after TBI before induction of hypothermia as a treatment. After hypothermia onset levels of MMP-9 in the blood returned to the initial level [45]. These data suggest increased synthesis and release of MMP-9 as an effect of brain trauma. Furthermore, MMP-9 may be perceived as a reliable marker of treatment efficacy after TBI.

The role of MMP-9 in remodeling of BBB in stroke, epilepsy, TBI and other neurological diseases is well established. Reviewed research points to a new role of MMP-9 in the diagnosis and monitoring of CNS condition. Nevertheless, further studies are needed to evaluate utility of MMP-9 as serum marker of neurological diseases.

#### 2.4. Neurofilaments

Neurofilaments are specific for neuron intermediate filaments forming cytoskeleton. Neurofilaments are particularly abundant in axons, where they play an important role in the growth of axons during development, the maintaining of shape and size of axons, and the transmission of electrical signals. There are four subunits of neurofilaments in the CNS: heavy neurofilaments (NF-H; 200 kDa), medium neurofilaments (NF-M; 150 kDa), light neurofilaments (NF-L; 70 kDa) and  $\alpha$ -internexin (66 kDa) [52]. Each of them contains from 6 to 8 tandemly repeated sequences of three amino acids: lysine-serine-proline. Specific phosphorylation of serine residues which is carried out in the axons protects the protein from proteases [53]. It has been shown that neurofilaments in native and phosphorylated form could cross the BBB and serve as a marker of axonal loss.

The potential utility of neurofilaments in native and phosphorylated form (pNF) has been researched in patients after TBI. Serum NF-L level was increased in TBI patients compared to healthy controls [54,55]. Initially elevated NF-L level predicted poor clinical outcome. Furthermore, serum NF-L correlated with pupil reactivity and Marshall CT classification scores [54]. A positive correlation has been found between elevated NF-L and the size of axonal injury [55]. Serum pNF-H level was also elevated in patients after TBI and was negatively correlated with GCS after admission and 7 days after injury – higher pNF-H level with lower GCS scores. Furthermore, an increased blood pNF-H concentration correlated with greater brain lesions and mortality after 3 months [56]. Peripheral release pattern of neurofilaments is associated with multiple clinical symptoms after TBI.

Neurofilaments were considered as markers for other neurological conditions. In children with febrile seizure, longer seizure duration was associated with higher serum pNF-H values interpreted as a clinically irrelevant axonal loss [57]. NF-L has been found to be useful in monitoring the treatment efficacy and axonal damage in MS patients [58,59]. Further studies are needed to reveal clinical utility of neurofilaments as markers. However, neurofilaments are able to cross the BBB and their peripheral concentrations are associated with neurological symptoms, which prompts us to further consider

their potential utility in the diagnosis and treatment in the future.

#### 2.5. UCH-L1

Ubiquitin carboxy-terminal hydrolase L1 (UCHL-1) is a cytoplasmic enzyme from ubiquitin carboxy-terminal hydrolases family, expressed mainly in neurons and to a lesser degree in the gonads. UCHL-1 is a small molecule, weighting 24 kDa and representing 5% of soluble protein in the nervous tissue [60]. It is an important element of axonal transport and, by a strong interaction with cytoskeleton proteins, plays an important role in the axons integrity [61]. A faulty UCHL-1 function has been shown to contribute to the pathology of many CNS diseases. Furthermore, UCHL-1 can cross the disrupted BBB. In the recent years, a growing interest in the use of UCHL-1 as a serum biomarker for BBB disintegration is observed.

After TBI, increased serum UCHL-1 concentrations have been shown [35,36,62,63]. UCHL-1 concentration was elevated in moderate to severe TBI compared to mild TBI patients [35,36,62]. Furthermore, UCHL-1 level was higher in patients with CT assessed abnormalities than in patients with normal CT scan results [35,37]. It has been revealed that UCHL-1 level above 40 pg/mL detects patients with acute intracranial lesions confirmed by CT scans [37]. It is believed that serum UCHL-1 assessment could diminish the number of negative CT scans. High prognostic value of UCHL-1 for poor outcome assessed 3 months [35] and 6 months after TBI was observed [62]. However, some limitations associated with the use of UCHL-1 as a marker were also noted. UCHL-1 does not add predictive value to commonly used prognostic tools like GCS [36].

The utility of UCHL-1 measurement in diagnosis and treatment of epilepsy has also been considered in the research up to date. In epileptic patients, an elevated concentration of plasma UCHL-1 after 12 h from seizure was noted. It is believed that an increased plasma UCHL-1 concentration in early time frame mirrors BBB disruption during acute phase after seizure. UCHL-1 level correlated with seizure number and severity [64]. Results suggest that UCHL-1 may reflect neurons injury after seizures and indicate BBB leakage. It may help clinicians to monitor treatment efficacy and make better therapeutic decisions.

UCHL-1 is a relatively small protein highly expressed in neurons. It has been shown that an increased release to the peripheral compartment indicates neuron damage and BBB disruption. More research is needed to combine UCHL-1 release to the peripheral compartment with clinical symptoms and disease progression monitoring.

#### 2.6. BDNF

Brain-derived neurotrophic factor (BDNF) is a member of neurotrophic factors family. BDNF regulates neuronal cell morphology, synaptogenesis and has a neuroprotective role as well. The role of BDNF, besides its physiological impact, has been described in many pathological conditions, such as epilepsy or neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS) [65,66]. BDNF is widely researched for its potential role as a therapeutic target. BDNF

**Table 1 – Serum biomarkers with a role in the diagnosis and treatment of CNS diseases.**

Protein	Source	Disease	Potential utility	Reference
S100 $\beta$	Astrocytes	TBI	Reduction of the CT scans in the diagnosis of TBI	[11]
GFAP	Astrocytes	Stroke	ICH differential diagnosis	[12]
		TBI	Correlation with injury volume	[34]
pNF-H	Axons	TBI	Correlation: negative with GCS and positive with brain lesions	[56]
UCHL-1	Neurons	Epilepsy	Reflection of neuron injury	[64]
		TBI	High prognostic value for poor outcome	[35,62]
BDNF	Neurons	Epilepsy	Disease severity indicator, seizures differential diagnosis	[67,69]
miRNA	Astrocytes, neurons, cerebro-vascular endothelial cells	Epilepsy	Indicator of drug resistant epilepsy	[75]

crosses the BBB and in the recent years a correlation between its release to the serum and clinical symptoms has been searched.

Blood level of BDNF was decreased in epilepsy patients compared to healthy control and in patients with psychogenic nonepileptic seizure [67]. It has been shown that serum BDNF correlated with disease severity [68]. Serum level of BDNF mirrored epilepsy duration, white matter integrity, and poor cognitive function in temporal lobe epilepsy (TLE) patients [69]. BDNF may be a good marker for differentiating epileptic seizures from nonepileptic ones. Furthermore, it may be a valuable marker of epilepsy severity.

Potential clinical relevance of BDNF is still being evaluated. Interestingly, serum BDNF was found to be a promising marker in psychiatric disorders. In patients with major depressive disorders, initially low serum BDNF became normalized after antidepressant treatment [70]. Furthermore, in meta-analysis of 52 studies, serum BDNF was an indicator of disease activity in bipolar disorder [71]. The research exploring potential usefulness of BDNF as a diagnostic tool may also contribute to revealing new data on its previously unexplored role in neurological and psychiatric diseases.

## 2.7. miRNAs

miRNAs are small noncoding RNA involved in posttranscriptional gene expression [72]. They have been recognized as a therapeutic target and a diagnostic tool in multiple neurological disorders. miRNA can be detected in the blood after crossing BBB in a form of miRNA containing exosomes [73]. Serum miRNA concentration has been analyzed in humans with epilepsy [74]. It revealed that miRNA has been differentially expressed in epilepsy patients compared to healthy control [75]. Furthermore, one of the miRNAs, named hsa-miR-106b-5p, was established as a high sensitivity and specificity marker for epilepsy diagnosis. In drug resistant epilepsy, five miRNAs serum have changed their concentration in epilepsy patients compared to healthy participants, showing hsa-miR-301a-3p as having the biggest diagnostic value for drug resistant epilepsy [75]. miRNA was also assessed as a potential marker for epileptogenesis after different initial triggers like TBI. Rno-miR-9a-3p has been proposed as a potential marker for intensified differentiation of normal neurons into epileptic ones. However, further studies, especially the human one, are needed to evaluate the role of miRNA in epileptogenesis detection [76].

There is a growing body of evidence pointing to potential usefulness of serum miRNA as a diagnostic and therapeutic target in neurological conditions. Besides epilepsy, serum miRNAs were evaluated in MS, where diminished expression of miR-572 was observed in MS patients [77], in migraine patients, where miR-382-5p were increased in pain-free period [78], and in ischemic stroke, where miRNA-221-3p and miRNA-382-5p serum concentrations were elevated compared to healthy control [79]. Furthermore, miRNAs are thought to be a useful marker for diagnosis and monitoring of progression of neurodegenerative disorders like Alzheimer and Parkinson's diseases [80,81]. Studies considering introduction of miRNA to clinical diagnosis are a novel and unrevealed area. However, existing data seems to be very promising and extra effort has to be made to evaluate the role of miRNA in the diagnosis and treatment of CNS diseases.

## 2.8. Biomarker panel assay

Serum indicators as a single have a limited application today. Therefore, in order to increase diagnostic value of peripheral biomarkers, the application of a set of molecules – panel assay, has been proposed. In ischemic patients, applying panel assay (MMP-9, BNF – brain natriuretic factor, D-dimer, S100 $\beta$ ) resulted in 86% sensitivity in detecting all strokes and 94% sensitivity in differentiation diagnosis of ICH [82]. For ischemic stroke, five markers study (S100 $\beta$ , B-type neurotrophic growth factor, von Willebrand factor, MMP-9, and monocyte chemoattractant protein-1) provided 92% sensitivity detection in samples collected within 6 h of symptom onset [83]. It has been shown that multiple protein assay in patients with suspected stroke increased the percentage of correct diagnosis from 77%, according to CPSS (Cincinnati Prehospital Stroke Scale), to 86% after application of both tests results [18]. Advantages of applying multiple biomarker assay has been revealed in patients after TBI. The improved intracranial injury diagnosis was observed, which in turn facilitated better definition of TBI severity [19]. Given the heterogeneity of CNS disorders, a single biomarker application may not be able to handle a given task. Thus, more attention has to be paid to research associated with application of multiple molecule assays.

## Summary

Disrupted BBB is a hallmark of many diseases in the CNS. Therefore, there is a need to evaluate a non-invasive, fast and

specific assay methods for control of brain neurovascular unit condition. Recent studies have identified several potential biomarkers whose appearance in the bloodstream mirrors the state of BBB. There are promising data showing multiple correlations between release of the brain-specific biomarkers and clinical symptoms (Table 1). However, the complexity and heterogeneity of CNS system diseases urge us to think that clinically useful information may be obtained only from a panel of biomarkers specific for each neurological condition.

### Conflict of interest

None declared.

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