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Neuronal biomarkers: An emerging path towards neuronal disease diagnosis in veterinary practice

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Abstract
Central nervous system (CNS) disorders till date are the major burden worldwide and associated with high rates of mortality and morbidity in animal community. Rapid diagnosis and initiation of appropriate treatment are vital to reduce the risk of poor outcome; however, diagnostic techniques are lacking behind for the accurate diagnosis of disease/ disorders caused by certain infection, assess severity of injury, and predict prognosis. Various Central nervous system and peripheral nervous system related biomarkers are in regular use in human medicine. However, the application of these neuronal biomarkers in veterinary practice is in primitive stage. The present review article discusses the clinical implications of various biomarkers more specific to diseases and disorders related to nervous system in animals *viz*., Glial Fibrillary Acidic Protein (GFAP), neurofilament subunit NF-H, myelin basic protein (MBP), S100β, neuron specific enolase (NSE), tau protein, alpha II spectrin, ubiquitin carboxy-terminal hydrolase L1 (UCHL1), creatine kinase BB, Matrix metallopeptidases (MMP) etc., present in the body fluids of animals related to the nervous disorders in animal practice. However, a single biomarker does not have sufficient sensitivity and specificity to diagnose and determine prognosis, a combination of biomarkers may prove to be more valuable than a single biomarker. Interpretation of mechanisms by which combinations of these biomarkers are affected by disease and intervention may assist the development of novel therapeutic approaches for CNS diseases in veterinary clinical management of neurological complications.

Keywords: Nervous system, biomarkers, veterinary practice

Introduction
A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of a physiological as well as a pathological process or pharmacological response to a therapeutic intervention. The classical biomarkers could measure the alterations in the blood pressure, blood lactate levels following exercise and blood glucose in diabetes mellitus [1]. According to the Biomarkers Definitions Working Group paper, "A biomarker is a characteristic, that is, objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention" [2].
Understanding the source, diagnosis, severity, prediction, or result of animal therapies can be aided by biomarkers, which can be linked to each stage of the gene-to-protein processing or metabolites generated during subsequent intracellular processes [3].
Central nervous system (CNS) infections are the major burden of disease worldwide and are associated with high rates of mortality and morbidity. Rapid diagnosis and initiation of appropriate treatment are vital to reduce the risk of poor outcome; however, diagnostic techniques are lacking behind for the accurate diagnosis of disease/ disorders caused by certain infection, assess severity of injury, and predict prognosis. The identification of biomarkers for structural neurological damage may be a useful tool in tackling these obstacles.
Neurological sequelae in survivors include physical, sensory, and cognitive disability, could be permanent disabilities [4]. Biomarkers in disease diagnosis, to quantify the injury and to monitor progress are used frequently for other organ systems, but their use in CNS pathology is in its infancy. Several studies have examined biomarkers of neurological tissue injury in different CNS pathologies which throw light on the underlying pathophysiological processes while they are occurring; however, data are sparse [5].
Blood, urine, cerebrospinal fluid (CSF), and tissue samples from the pathologic site have been the main source of biomarkers for CNS disease. Blood and urine are easily collected but CNS tissues and CSF sampling are more difficult and could be end with the risk of significant

morbidity or mortality. However, the blood-brain barrier (BBB), a highly selective barrier, which means that pathologic processes in the CNS are not truly reflected in the blood, unless its permeability got altered [3].

Studies in traumatic brain injury (TBI), subarachnoid haemorrhage, dementia, Alzheimer disease, stroke, cardiac arrest, and various other pathologies have found that biomarkers of CNS injury hold promise as diagnostic and prognostic markers in human practice. Their role in infections of the CNS has generated less research [5].

History of biomarkers in CNS injury

Since the 1950s, interests on biomarkers of neuronal injuries have increased significantly. Most of the earliest work involves studies in traumatic brain injury (TBI) and cerebral ischemia. Rudman *et al.* [6] published a classical study on cerebrospinal fluid (CSF) cyclic adenosine monophosphate (AMP) levels in TBI as a putative marker of the depth of coma after injury. Tsai *et al.* [7] reported that CSF-IFN (Interferon) appears to be a valid marker for Canine Distemper Virus (CDV) persistence in the canine CNS. Vaagenes *et al.* [8] studied the levels of brain Creatine Kinase (CK) in CSF in dogs after cardiac arrest and shows that peak activity at 48–72 hrs post-arrest correlated with poor outcome. This was translated to human 6 years later and found that the increase in CK activity in CSF reflects permanent brain damage. Visser *et al.* [9] studied the relationship between Scrapie in Sheep and serum total CK brain isoenzyme (CK-BB) activity which did not aid the clinical diagnosis of Scrapie. Płonek *et al.* [2] summarized the application of the described biomarkers in human and veterinary medicine.

Classification of CSF Biomarkers

A biomarker present in the CSF is derived either from the central nervous system (CNS) parenchyma or systemic circulation. Petzold [11] classified the biomarkers present in the CSF into six classes: I) Pigments, II) Metabolic biomarkers, III) Cell-type specific biomarkers (including neurofilaments, glial fibrillary acidic protein, myelin proteins, myelin basic protein (MBP), and circulating nucleic acids, IV) CNS specific biomarkers, V) Free radicals, and VI) Inflammatory and immunological biomarkers.

I. Pigments

The change in CSF colour due to increased level of certain pigments (haemoglobin or Bilirubin) during subarachnoid haemorrhages (SAH) using Spectrophotometer. Micro-rebleeds may be too small to be visible on CT, so the quantification of CSF pigments may help in prognostic implications of micro-rebleeds [11].

II. Metabolic biomarkers

They are the intermediate and end products of the metabolic pathways of cells. In most of the cell types the basic metabolic pathways are shared and can be used as good markers. However, these intermediate and end products are not specific for any particular cell type or pathologic process. For example, CSF glucose and pyruvate are routinely measured. CSF lactate is also relevant in certain conditions. During ischemia, there is an elevation in lactate and reduction in pyruvate indicating a lowering of oxygen supply to the cells [11].

III. Cell specific biomarkers

The biomarkers include proteins, lipids or nucleic acids which are expressed exclusively by specific type of cell. They may indicate the amount of cell damage of a given cell population during nervous tissue damage hence of great clinical significance.

1. Glial fibrillary acidic protein (GFAP)

The glial fibrillary acidic proteins are monomeric intermediate filament proteins found in astrocytes and are the component of astrocytic cytoskeleton which are thought to play a role in modulating astrocytic motility and shape, white matter architecture, myelination, and the integrity of the BBB. They are specific to the CNS [12] and can be used to monitor the glial pathology [13]. It is thought that a damage of astroglial cells due to mechanical injury or necrosis causes GFAP to leak through the BBB into the bloodstream, where it can be detected. A study of GFAP in human patients with various neurological disorders found the GFAP mean blood values were highest in patients with bacterial meningitis, subarachnoid haemorrhage and status epilepticus [14]. GFAP has been shown to have excellent specificity and moderate sensitivity for TBI (Traumatic brain injury), while also having good specificity for computed tomography (CT) confirmed brain injury [15] and shown to have higher levels in patients with mass lesions compared with diffuse injury [16]. These raise the redundancy of this biomarker, as CT scans are readily available in most hospitals. In a recent prospective cohort study of 67 patients, serum GFAP levels on admission and during the first 5 days of injury were increased in patients with severe TBI and were predictive of neurological outcome at 6 months. However, they do not add predictive power to commonly used prognostic variables in a TBI population of varying severities. Overall, GFAP has the potential to be a useful biomarker, but more studies need to be done.

Sato *et al.* [17] demonstrated increased serum GFAP levels in dogs with progressive myelomalacia and intervertebral disc disease. Ide *et al.* [18] assessed GFAP in a number of canine neuroepithelial tumours like low grade astrocytomas, medulloblastoma, and primitive neuroectodermal tumour [2]. The production of anti-GFAP autoantibodies was also detected in dogs with autoimmune disease [19]. Toda *et al.* [20] found dogs with necrotising meningoencephalitis to have an increased level of CSF anti-GFAP autoantibodies.

2. Phosphorylated neurofilament heavy chain (pNF-H)

Neurofilaments form a major cytoskeletal component in axons; they consist of light chain (NF-L), medium chain (NF-M), and heavy chain (NF-H). NF-H is one of the most abundant protein components of neurons is found only in neurons and contains many repeated lysine-proline-serine sequences in which essentially all of the serine residues are phosphorylated. This feature makes it resistant to proteases, so that it remains as an undegraded form in the CSF and serum after release from damaged axons. In humans, the phosphorylated NF-H (pNF-H) level in blood has been reported to be increased in amyotrophic lateral sclerosis, SCI, and TBI [21].

Because blood is quicker, safer, and more convenient to obtain than cerebrospinal fluid (CSF) sample collection, the assay of serum NF-H has the potential to be a novel, specific, and convenient tool for assessing axonal damage in patients with SCI [22], as well as in diffuse axonal TBI.

Nishida *et al.* [3] examined this neurofilament in dogs with spinal cold injury (SCI) following intervertebral disc disease and found that mean pNF-H serum levels were significantly

higher in dogs with no pain perception than in those with intact pain perception. They were also higher in dogs that did not regain ambulation following surgery compared to those regain. Toedebusch *et al.* [23] conducted a study on CSF and serum pNF-H as a diagnostic biomarker in Canine Degerative Myelopathy (CDM).

Blood pNF-H values in dogs that were paraplegic with absent deep pain perception (DPP) were significantly increased compared with those in dogs with paraplegia and intact DPP and control dogs [3]. Since pNF-H is not an immediate indicator of CNS damage, it may be potentially used in dogs in combination with other biomarkers such as S100β or UCHL-1 that are detectable in the CSF and blood within the first hours after injury [24].

3. Myelin basic protein (MBP)

Myelin basic protein accounts for 30 % of the total protein found in myelin and are considered specific to the nervous system. MBP is produced by oligodendrocytes/Schwann cells and is a major constituent of the axonal myelin sheath, and is therefore predicted to be a marker of white matter injury. It has been detected in the CSF of humans with multiple sclerosis, SCI, and TBI. It is the only structural protein that is crucial for the formation of CNS myelin, signalling, interactions with the cytoskeleton, and the regulation of the expression of myelin [25]. In rats, intact MBP diminished significantly in the contused cortex as early as 2 hours after TBI, reached its lowest level (a 75-fold reduction) at 48 hours [26], and then returned to basal levels at 3 to7 days. It is also of interest due to the immune response it is thought to induce in the course of certain demyelinating disorders, such as multiple sclerosis in humans. Its presence in the CSF is linked to diseases involving myelin breakdown [27].

MBP CSF levels were found to be increased in dogs with low and high-speed trauma and correlated with the extent of damage in the hypothalamus and hippocampus suggested its significance as an early stage biomarker of traumatic stress disorder [28]. Oji *et al.* [29] observed higher concentration of CSF-MBP concentration in German shepherd dogs with degenerative myelopathy. The authors recommended the analysis of MBP concentrations in the CSF as a supplementary tool in the diagnosis of demyelinating lesions. Summers *et al.* [30] studied the elevated levels of MBP in the CSF of Beagle dogs infected with canine distemper with demyelination. Levine *et al.* [31] studied the correlation between MBP and the functional outcome following intervertebral disc herniation (IVDH) in dogs and found that dogs with a CSF concentration of MBP more than3 ng/mL had worse outcomes compared to animals with a lower MBP concentration. It is suggested that the biomarker may be used as a prognostic indicator following IVDH In dogs.

In an effort to improve the value of this test, an investigation was made into combining these results with those available from CSF Creatine Kinase (CK) measurements. Successful long-term functional recovery occurred in 98% of cases when CSF CK activity was less than or equal to 38 U/L and MBP concentration was less than or equal to 3 ng/mL [32].

4. Central nervous system-specific biomarkers

The following biomarkers are produced by more than one cell type. They are considered to be used as biomarkers of neuroaxonal damage or gliosis.

1. S100 β

S100 β is a calcium binding protein mainly found in CNS. The protein was found in ependymal cells, oligodendrocytes, microglial and Schwann cells, as well as non-glial cells such as vascular endothelial cells, lymphocytes, and neurons. Under normal conditions, S100 β is concentrated in CSF, but due to the leak that occurs upon BBB disruption, serum S100 β levels increase [33]. Serum levels of S100β were reported to peak within 6 h following TBI in humans [34]. Therefore, the serum concentrations of this protein may normalise after this time. In an analysis of the levels of S100 β in dogs following circulatory arrest and found the CSF concentration to increase up to 18 h after reperfusion [35]. More research is needed to determine the application of this biomarker in veterinary medicine. Hayashi *et al.* [36] found that S100β levels in CSF of non-ambulatory dogs with IVDD treated with Electroacupuncture and found the elevated levels of S100β in late motor recovered (76±17.0 days) dogs compared with early motor recovered (6.7±7.8 days). Unfortunately, lack of specificity, and the presence of extracerebral sources of S100 β in peripheral blood, limits the diagnostic value. Moreover, elevated serum S100 β has also been detected in patients with extracranial pathology, such as traumas and burns [33].

2. Neuron Specific Enolase (NSE)

NSE is a protein reported to be found only in neurons of the CNS, PNS, and neuroendocrine cells. It acts as a glycolytic isoenzyme converting 2-phosphoglycerate into phosphoenolpyruvate. Neuron structural damage leading to cell death causes NSE leakage into the extracellular space allowing its detection in serum and CSF [37]. Serum and CSF levels of NSE were found to be correlated with the severity of the neurological state on admission and with the outcome in patients with TBI. Cao *et al.* [38] demonstrated that NSE levels increased after 2 h in rats following an induced SCI compared to control rats. Within 6 h, the serum NSE levels were significantly higher in rats with moderate and severe SCI compared to those with mild SCI. CSF- NSE concentrations are increased in dogs with GM1 gangliosidosis [39] and in dogs with meningoencephalitis [40]. Elias *et al.* [41] compared the use of NSE in dogs with and without encephalitis, to detect neuronal damage and found that NSE can be measured in serum samples of dogs to monitor neuronal lesions in encephalitis. However, at present it does not aid in differentiating different causes of neuronal injury.

3. Tau protein: (Cleaved tau (c-tau) protein)

Cleaved tau (c-tau) protein has a microtubule structure, is localized in axons where it binds to axonal microtubules and forms axonal microtubule bundles, which are important structural elements in the axonal cytoskeleton. Following injury, c-Tau protein is cleaved by calpain-1 and 3, becomes insoluble and forms aggregates, known as neurofibrillary tangles. Such tau inclusions were identified in numerous degenerative diseases, which were collectively named tauopathies [42]. In animals, tau phosphorylation has been studied in the brains of growing and aged cats and dogs [43]. It has also been studied in the course of IVDH. In the case of IVDH, CSF tau protein levels were found to directly related to the severity of spinal cord injury [44].

Based on the preliminary study carried out by Roerig *et al.* [45], Blomme and Waring [46] speculate that CSF tau levels may have a potential application as a prognostic biomarker not only of IVDH, but also of acute brain and spinal cord injury. Roerig *et al.* [45] found that CSF tau concentrations were significantly higher in dogs showing plegia compared to

healthy dogs and dogs with paresis. The CSF protein tau levels are associated with the severity of spinal cord injury and serves as an prognostic indicator in dogs with IVDH.

4. Alpha II-spectrin

Alpha II-spectrin is a protein present in neurons and is commonly found in axons and presynaptic terminals. Although Alpha II-spectrin is not CNS specific, it is regarded as a potential CNS injury biomarker due to its abundance in neurons. Studies associating breakdown products of this protein with neurological diseases were carried out in patients with TBI. Weiss *et al.* [47] analysed calpain (involved in necrotic cell death) and caspase-3 (involved in apoptotic cell death) cleaved alpha II-spectrin breakdown products in dogs during hypothermic circulatory arrest (HCA) and cardiopulmonary bypass (CPB). They found that the levels of alpha II-spectrin breakdown product correlated with mild histological and neurological changes.

5. Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1)

UCH-L1 is a multi-functional, proteolytically stable protein with high abundance in the brain comprising 1% -5% of its total soluble protein [48]. It presents in human brain at concentrations at least 50-fold greater than in other organs [49]. However, its exclusivity was questioned when it was also found in non-neuroendocrine carcinomas, such as those of the breast, kidney, prostate, pancreas, lung and colon [50]. Remarkably, this neuronal protein can be readily detected in CSF and blood very early after brain injury, status epilepticus and carbon monoxide poisoning which allow it to be used as valuable time-window biomarkers for potential neuroprotective strategies.

A single study of the levels of UCH-L1 in dogs was performed by Arnaoutakis *et al.* [51] who found that dogs with hypothermic circulatory arrest and those undergoing cardiopulmonary bypass displayed elevated CSF UCH-L1 levels within 8 h. However, further research into the usefulness of this biomarker in veterinary neurological disorders is warranted. Zhang *et al.* [52] studied in pigs with a hypothesis that UCH-L1 may be a potential biomarker for Deep Hypothermic Circulatory Arrest (DHCA)-induced ischemic neuronal apoptosis and observed that the elevation in UCH-L1 concentration is consistent with the severity of neural apoptosis following DHCA.

6. Creatine kinase BB

Creatine kinase isoenzymes are generally found in areas of high energy production or its demand. Creatine kinase BB (CKBB) is found in astrocytes and neurons [53] and has been used as a biomarker of CNS damage [54]. Sawashima *et al.* [55] found a correlation between high-voltage slow activity in electroencephalography and CKBB in the serum of dogs with progressive brain disease. CKBB serum levels showed a statistically significant difference between healthy dogs and those with central neurological disease. Serum CKBB levels were not increased in cats with feline infectious peritonitis (FIP). Similarly, CKBB in sheep with neurological disorders associated with coenurosis and scrapie was not increased [56]. Consequently, the usefulness of this biomarker in veterinary neurology remains uncertain.

7. Matrix metallopeptidases (MMPs)

MMPs belong to a large family of proteolytic enzymes that degrades basement membrane components such as collagen IV, laminin and fibronectin, which are the major constituents of BBB. Increased CSF MMP-9 activity was detected in 6 of 35 dogs with thoracolumbar IVDH, whereas it was detected in 1 of 8 control dogs [57]. However, the correlation with functional recovery has not been investigated and MMP-9 was also detected in dogs with different diseases, such as leishmaniasis [58]. MMP-9 has been detected in dogs with intracranial tumors such as meningiomas, gliomas, pituitary tumours, choroid plexus tumours, and lymphoma [59]. Dogs with intracranial tumours were significantly more likely than those without tumours to have detectable MMP-9 in the CSF. The data collected from a small number of canine and feline meningiomas suggested that MMP is not correlated with morphologic malignancy patterns [3].

Biomarkers of BBB dysfunction

Unfortunately, there is not a reliable technique for directly and noninvasively measuring BBB integrity and function; therefore, peripheral markers of BBB dysfunction, which are indicative of cerebrospinal (CSF) molecules in serum and/or serum components in the CNS.

1. CSF/serum albumin ratio

The CSF/serum albumin ratio has been used to examine BBB disruption in patients with severe TBI and remains the standard biomarker for BBB integrity. An increased CSF/serum albumin ratio has been reported in patients with severe TBI and indicates that albumin from the blood has passed into the CSF, indicative of BBB damage [60]. The use of this index is potentially limited, however, because the large variability of CSF albumin in normal animals (at least in dogs and horses) results in a large variability in the values for this index [61].

2. Tight junction proteins

Tight junction proteins, such as the integral membrane protein occludin, have been suggested as another possible biomarker of mTBI-associated BBB dysfunction. It is likely that the levels of such proteins would be disrupted in BBB injury. Unfortunately, as occludin is not specific to the brain, its use as biomarker for diagnosis or confirmation of mTBI has limitations. Other families of brain-specific tight junction proteins may prove to be more selective biomarkers of BBB integrity, but research in this area is lacking [33].

3. S100β

S100 β is an astrocyte-specific CNS protein that is known to be upregulated in several neurological conditions, including ischemia. Under normal conditions, S100 β is concentrated in CSF, but due to the leak that occurs upon BBB disruption, serum S100 β levels increase. Though the exact functions of S100 β remain elusive, sensitivity of S100β as a marker of BBB leakage is comparable to that of the CSF/serum albumin ratio [62].

4. Plasma-soluble prion protein

Cellular prion protein (PrPc) is a ubiquitous glycoprotein with the highest concentration in the CNS [63]. Because PrPc is located entirely within the extracellular domain of the plasma membrane, it is reasonable to suspect that it may be released during a concussive event through BBB disruption. Indeed, transcription of the prion protein gene (PRNP) is upregulated in a rat model of concussion following blast exposure, resulting in increased serum levels of PrPc [64].

Biomarkers of neuroinflammation

1. Interleukins and other acute-phase inflammatory response proteins

Pilot study of 16 paediatric mTBI (mild traumatic brain injury) patients showed that increased concentrations of both IL-6 and MMP9 had a sensitivity of 81% and specificity of 94% for mTBI diagnosis [65]. Further studies on these substances may yield potentiality as potential biomarkers.

2. High Mobility Group Box 1 (HMGB1) Protein

HMGB1 are known CSF indicators of the neuronal inflammation associated with encephalitis in dogs and they will be a new indicator of encephalitis [66].

CSF and Blood Biomarkers for Intracranial tumours

A limited number of biomarkers (beyond tumor cells specifically) have been evaluated in CSF from dogs with intracranial tumors, including MMP 2 and 9, uric acid, and fibrinolytic activity (Ddimers), as well as vascular endothelial growth factor (VEGF) in plasma, but findings to date are similar to those in humans with regard to limitations of sensitivity and specificity [67].

Limitations of Neuronal Biomarkers

One of the limitations of biomarkers is that they may detect the physiologic and pathologic changes in the body resulting from a disease process but they do not detect the disease itself. Variability is a major concern. Biomarker studies are normally conducted on body fluid or tissue samples collected from patients and healthy subjects of a range of ages, sexes, and breeds [68]. It is also important that difference in types and locations of the lesions can affect the results. The timing for sampling and measuring biomarkers is crucial to accurate interpretation. Some of the recent reviews concluded that no single biomarker alone has been proven to have substantial clinical use and more research needs to be undertaken [68, 69].

Conclusions

Biomarkers play a crucial role in human and veterinary medicine. They assist in understanding physiological and pathological biological processes and their clinical effects. They can also aid in assessing treatment protocols or predicting their outcomes. The main advantage of assessing biomarkers is the ability to detect a disease process at its early stage, or even at a subclinical level, as well as monitor the treatment success. At the same time, biomarkers may vary inter-individually, within a group or among various species. The biomarkers mentioned above have only been studied in individual neurological disorders of animals, the majority of which were experimentally induced. Further research into their use in developmental, auto-immunological, and neoplastic CNS and PNS disorders is needed. A single biomarker does not have sufficient sensitivity and specificity to diagnose and determine prognosis, a combination may prove valuable. Interpretation of mechanisms by which combinations of biomarkers are affected by disease and intervention may assist the development of novel therapeutic approaches for CNS diseases both in animal and human medical management of neurological complications.

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