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Review article

FLUID-BASED BIOMARKERS IN NEURODEGENERATION: CURRENT FINDINGS AND FUTURE DIRECTIONS

SOLUBILNI BIOMARKERI NEURODEGENERATIVNIH PROCESA

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Abstract

With the increase in life expectancy, the prevalence of neurodegenerative disorders is expected to rise in many countries of the world. The need for reliable biomarkers in neurodegenerative diseases is crucial to improving timely and accurate clinical diagnostics, facilitating the development of disease-modifying therapies, and monitoring patient progress. In many cases, complex pathology of neurodegenerative diseases can be reflected in the extracellular fluid, allowing for the development of soluble biomarkers that can mirror neuropathology in the cerebrospinal fluid or blood. This review is an overview of the current findings, the latest developments in the field, as well as applications of fluid-based biomarkers in neurodegenerative diseases.

Keywords:
fluid biomarkers, neurodegeneration

Sažetak

Sa produženjem životnog veka u mnogim zemljama se očekuje porast prevalencije neurodegenerativnih bolesti. Utvrđivanje pouzdanih biomarkera neurodegenerativnih bolesti je ključno za unapređenje blagovremene i adekvatne kliničke dijagnoze, razvoj terapija koje utiču na tok bolesti i praćenje pacijenata. Složena patologija neurodegenerativnih bolesti prikazuje se u vanćelijskoj tečnosti, omogućavajući razvoj solubilnih biomarkera koji će oslikavati neuropatološke promene u cerebrospinalnoj tečnosti i krvi. Cilj ovog preglednog rada je da ukaže na trenutna saznanja i pravce razvoja primene biomarkera neurodegenerativnih bolesti u telesnim tečnostima.

Ključne reči:
biomarkeri u tečnostima, neurodegeneracija

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According to the World Health Organisation (WHO) the prevalence of neurological diseases is increasing, making them a major contributor to the disease burden worldwide, estimating that, by the year 2050, they will become the second leading cause of death. Amongst the most prevalent of these diseases, with the numbers expected to increase, are some of the neurodegenerative diseases (NDDs) such as Alzheimer's disease (AD), one of the most common neurological diseases, and the most common NDD, Parkinson's disease (PD), motor neuron disease etc. (1). They are characterized by synaptic and neuronal dysfunction and loss, as well as the deposition of altered protein variants throughout the nervous system inside and outside of cells (2). Most of the NDDs are chronic in nature, with changes in protein expression and deposition in the brain, starting years, sometimes decades, before the onset of symptoms (3). As previously mentioned AD and PD, along with amyotrophic lateral sclerosis (ALS), motor neuron disease, Huntington's disease (HD), frontotemporal dementia (FTD) and spinal muscular atrophy (SMA) are all counted amongst NDDs (2).

The main risk factor for NDD development is considered to be aging. Genomic and epigenetic alterations, impairment of proteostasis, mitochondrial dysfunction, cellular senescence, impaired signaling between and inside the cells and altered metabolism found in aging are all thought to be behind the susceptibility of neurons to degeneration (4, 5). With the prolonging of the average life span and the aging of the global population, it is expected that these diseases will only increase in significance and prevalence over the coming years (6).

Considering the prevalence and varying clinical manifestations of NDDs, as well as developing therapeutic strategies, various methods needed to be developed for monitoring and correctly diagnosing them in the most efficient and effective manner, one of them being appropriate biomarkers (3).

By definition 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" (7). A biomarker should have certain attributes, in the case of NDDs: it should detect a fundamental feature of neuropathology and be validated in neuropathologically-confirmed cases; have a certain predetermined sensitivity (% of subjects with the disease with the positive test) and specificity (% of subjects without the disease with the negative test); be reliable, reproducible, non-invasive, simple to perform, and inexpensive (8). Beyond assisting in the preliminary and definite diagnosis, screening asymptomatic patients, and monitoring disease progression, biomarkers are utilized in treatment strategy optimization as well as exclusion/inclusion factors for clinical trials (3). In broader terms, some authors even count the clinical presentation of the disease as a biomarker, however, generally speaking, this term applies to imaging, biochemical, histological, -omics and genetic characteristics of a disease (9). This review will focus on biochemical biomarkers measured in the cerebrospinal fluid (CSF) and in blood/plasma.

Cerebrospinal fluid, being the extracellular fluid of the neuronal tissue, reflects the changes inside the cells caused by neurodegeneration, making it a suitable sample for measurement of biomarkers (10). Although the CSF biomarkers and available positron-emission tomography (PET) imaging have so far proven to be good markers of amyloid and tau pathology, they have their limitations: the invasiveness, cost and availability. In the last couple of years, with the development of more sensitive technologies, peripheral blood-based biomarkers are taking the spotlight, offering a less invasive and more affordable route compared to the CSF and PET biomarkers, while also being able to reflect the situation in the central nervous system (CNS) (11, 12). However, determining these proteins in blood has its flaws that warrant consideration, there is production of these proteins in tissues other than the brain, resulting in levels different than those in the CNS, furthermore there are also different substances such as enzymes or metabolites present in blood that could interact and interfere with these measurements (10).

The focus of this review will be the most frequently determined biomarkers for various NDDs. As mentioned previously, pathological changes in neurodegeneration include a multitude of processes. Bearing that in mind, biomarkers can be broadly classified into several categories, as described in **table 1**.

Table 1. Classification of biomarkers.

Type of pathology	Biomarkers
Markers of pathological process	Amyloid pathology - Aβ40, Aβ42, Aβ40/42
	Alpha synuclein pathology - t-aSyn, p-aSyn, o-aSyn
	Tau pathology - p-tau 181, 217, 231
Axonal loss and neurodegeneration	t-tau, NfL
Synaptic disfunction	Neurogranin, SNAP-25
Inflammation and glial activation	GFAP, TREM2, YKL-40

Aβ - amyloid β; aSyn - alpha synuclein; p-tau – phosphorylated form of tau protein; TDP 43 - TAR DNA binding protein 43; t-tau – total tau protein; NfL - neurofilament light chain; Ng - neurogranin; GFAP - glial fibrillary acidic protein, TREM2 - Triggering receptor expressed on myeloid cells 2; YKL-40 - Chitinase-3-like protein 1.

Biomarkers of pathological processes

Amyloid β (Aβ) is a small protein, derived from the transmembrane amyloid precursor protein (APP), that can be found in many tissues. The APP has important physiological functions during brain development, as well as in neuronal plasticity, memory and neuroprotection in the mature and aging brain (13). The proteolytic processing of the APP along the amyloidogenic pathway, by β and γ secretases, generates Aβ (14). Depending on the cleavage site of the γ secretase, the length of Aβ reaction product can vary, creating peptide isoforms 37 - 42 amino acids long. Longer isoforms, Aβ42 and Aβ43, are less soluble, leading to aggregation and the creation of extracellular plaques characteristic of AD (12, 15). According to the Amyloid hypothesis, the formation of the neuritic or senile Aβ plaques is the starting occurrence in AD, driving further neurodegeneration (16). The changes in Aβ42 levels in the CSF appear before the positive Aβ PET scans in the earliest stages of neurodegeneration, making them the first measurable marker of amyloid pathology (17, 18). However, CSF contains many Aβ isoforms, of which Aβ40 is the most abundant, in concentration about ten times higher than Aβ42 (19). In AD, the produced Aβ42 is insoluble and is deposited into amyloid plaques. Therefore, the availability of soluble Aβ42 in the extracellular fluid is decreased, resulting in lower concentrations of Aβ42 in CSF or blood, with Aβ40 levels mostly unaltered in both fluids (20–22). Furthermore, the CSF Aβ42/Aβ40 ratio reduction is more pronounced than in CSF Aβ42 alone (23). Recent literature suggests that the ratio between Aβ42 and Aβ40, rather than the proteins on their own, both in CSF and blood is what corresponds better with amyloid pathology and should be used to distinguish AD from other types of cognitive impairment, as well as to predict the cognitive decline of patients (20–22, 24). When comparing the CSF and plasma Aβ ratios in AD patients a difference in results is evident. The decrease of the CSF Aβ42/Aβ40 ratio is about 50%, compared to the Aβ42/Aβ40 ratio determined in blood which decreases by around 10 - 15%, possibly as a result of peripheral production of the Aβ (11). One of the suggested ways of increasing the accuracy of Aβ measurements in blood is by combining the Aβ42/Aβ40 with p-tau or GFAP values (25, 26).

Lower CSF Aβ42 levels were also found in patients with PD and are believed to have predictive value of cognitive decline, and worsening of motor and autonomic symptoms (27). An increase in plasma Aβ was recorded in patients with vascular diseases, both in the brain and on the periphery, with an inverse relationship of Aβ42/Aβ40 in plasma with blood pressure in the elderly (28, 29).

Alpha-synuclein (aSyn) is a protein primarily expressed in the presynaptic terminals of neurons and in some non-neuronal cells, such as erythrocytes. It plays a key role in vesicle release and recycling in nerve terminals. The misfolding and accumulation of aSyn in neurons, leading to their degeneration and loss of function, is a hallmark of synucleinopathies - a subgroup of neurodegenerative diseases (NDDs) that includes Parkinson's disease (PD), Lewy body dementia (LBD), and multiple system atrophy (MSA). The aSyn aggregates in these diseases differ, involving various conformational strains of aSyn, suggesting that detecting the specific type of aSyn present could help differentiate between these diseases (30–32).

In cerebrospinal fluid (CSF), total, oligomeric, and phosphorylated aSyn have been measured in the search for reliable biomarkers for synucleinopathies and other NDDs. In PD, a trend similar to that seen with Aβ in AD has been observed: a decrease in aSyn levels compared to controls. However, the sensitivity and specificity of these tests alone have not been adequate due to significant overlap between patient and control groups. As a result, the ratio between different aSyn forms has emerged as a more reliable measure (3, 33). Longitudinal studies on CSF total aSyn showed no significant change in concentration over time in either PD or control patients (34).

When it comes to measuring aSyn in blood, the results are more ambiguous. A 2023 meta-analysis revealed that various studies report conflicting outcomes: some show elevated aSyn levels in PD patients, others show lower levels, and some show no change compared to controls (35). Another meta-analysis from 2022 found an overall increase in total aSyn in the early stages of PD, though no clear differences were observed in oligomeric or phosphorylated aSyn forms (36). Evidence for aSyn levels in the blood of patients with other diseases is also inconsistent (35). These findings suggest that plasma or serum aSyn is not yet as reliable a biomarker as Aβ or tau, and further research is needed.

The development of aSyn seeding aggregation assays in CSF has provided a new tool for detecting proteins with prion-like misfolding and aggregation properties. These assays have shown good accuracy in distinguishing PD or LBD patients from healthy controls or those with other NDDs, as well as identifying individuals in prodromal stages before diagnosis. Given the inconsistencies and inadequate sensitivity of other assays, these methods are currently the most conclusive tests available (32).

Although the literature search found limited studies on aSyn concentrations in fluids of non-PD patients, a 2015 study was identified that compared blood aSyn levels across different age groups in males. This study demonstrated a decrease in aSyn concentrations with age (37, 38). One of the key age-related changes is the decline in cellular responses to protein accumulation, leading to a reduced ability to degrade proteins, which may contribute to an increased risk of protein aggregation, as seen in PD (39). Since age is a known factor in aSyn accumulation and related neurodegeneration, the decrease in soluble aSyn in the blood could be explained by its sequestration in the nervous system in less soluble forms over time, even in individuals without PD pathology.

Markers of neurodegeneration

Tau is one of the first characterized microtubule-associated proteins (MAPs), found primarily in neuronal axons, whose physiological roles include stabilizing microtubules (MTs), facilitating axonal transport, and modulating synaptic plasticity. Under physiological conditions tau supports neuronal plasticity by regulating the structural dynamics of MTs necessary for neuronal growth and function (40). Dysregulation and loss of the microtubule-stabilizing function has been intricately linked to several NDDs, including FTD, progressive supranuclear palsy (PSP), Pick's disease (PiD), and corticobasal degeneration (CBD), therefore termed tauopathies (41).

Tau protein is encoded by the *MAPT* (Microtubule-associated protein tau) gene, due to alternative splicing many isoforms are produced in the human central and peripheral nervous system (six brain-specific tau isoforms) (40). There are numerous posttranslational modifications of this protein, including over 40 phosphorylation sites. Different tau isoforms can aggregate, leading to the formation of neurofibrillary tangles (NFTs) in the neurons, glia and extracellularly, characteristic of tauopathies. Apart from tauopathies, NFTs and higher levels of tau are one of the hallmarks of AD (41, 42). Different soluble tau species (both total tau and in phosphorylated forms – p-tau181, p-tau217, p-tau231) can be reliably measured in the CSF. Increased levels of total-tau protein can be found in AD, but also in other NDDs, whereas phosphorylated tau forms are shown to be selectively increased in AD, but not in other NDDs (3).

The most widely utilised soluble biomarker of them is p-tau181. It has been shown that p-tau181 correlates and predicts amyloid and tau pathologies, that it's a specific AD marker, differentiating AD from other tauopathies e. g. PD, FTD, etc. and shows a gradual increase across the AD continuum (42, 43). Recent literature shows that p-tau217 has a higher degree of discrimination between AD and other NDDs when compared to p-tau181, both in the CSF and in the plasma. It has also been shown to have a better correlation to the amyloid and tau PET results (3, 44). Palmquist et al. found that p-tau217 in plasma was increased in AD but not in other tauopathies, such as PSP and CBD, suggesting that this form of tau is associated with Aβ pathology. The study also showed that p-tau217 levels rise before the onset of symptoms before the changes on the tau-PET are visible, and continue rising with disease progression both in sporadic and autosomal-dominant AD (45). However, recent findings pointed out that an increase in p-tau231 precedes other p-tau isoforms in AD, with similar predictive properties as p-tau181, positioning it as possibly the earliest of the biomarkers to be detected in AD preclinical stages. Phoshpo-tau231 has also been shown to more accurately differentiate cognitively unimpaired Aβ+ from Aβ–individuals than CSF p-tau181 and CSF t-tau (11, 46).

The latest research introduces a new parameter, with better accuracy - %p-tau. This number represents the ratio of p-tau on a particular phosphorylation site (e.g. 217) to all the other p-tau isoforms detected but not phosphorylated on that site (47–49).

Results of a cross-sectional study showed that blood p-tau, in all isoforms, was significantly more closely associated with Aβ PET changes than tau-PET changes, further fortifying the stance that p-tau is a marker of amyloid pathology (50). Another isoform of tau: microtubule-binding region of tau, containing residue 243 (MTBR-tau243), has been proposed as the form that most closely correlates to tau PET while also having the lowest amyloid PET correlation (51). Furthermore, the tau/Aβ42 ratios combine measures of two pathological processes into a single diagnostic biomarker, ensure better reliability than Aβ42 alone, have stronger concordance with amyloid-PET, and are less prone to methodological errors compared to Aβ42 measurements alone (3).

One of the newer promising biomarkers is the neurofilament light chain (NfL). Neurofilaments are intracellular intermediate filaments, found in both the central and peripheral nervous system, consisting of neurofilament light chain (NfL) of ~68 kDa, Nf medium chain of ~150 kDa, and Nf heavy chain of ~190 – 210 kDa. Of the three subunits, NfL has been the focus of most clinical biomarker studies, probably due to its relative abundance and solubility in CSF and blood, compared to other neurofilament subunits. Upon axonal injury, NfL gets released into brain interstitial fluid, which freely communicates with the CSF, as well as with blood, through arachnoid villi and perivascular drainage systems. It has been shown that CSF and blood NfL concentration is increased in a range of disorders accompanied by axonal injury, including most neurodegenerative and some neuroinflammatory disorders (3, 11, 52).

Increased NfL concentrations in the CSF have been found in various NDDs: AD (53), ALS (54), FTD, PD, and NfL appear to be a marker of neurodegeneration irrespective of the underlying cause (55). However, several factors, including age, influence NfL levels, with an annual age-related increase in NfL concentration of ~3% (56–58). Idland et al. found higher CSF NfL correlated with a higher chance of hippocampal atrophy in old people with a low risk of AD, suggesting that NfL was not a predictor of AD but rather age-related neurodegeneration (59). Systematic review and meta-analysis showed that NfL concentration in CSF displayed strong performance in distinguishing AD, FTD, and ALS from cognitively unimpaired controls, with NfL providing the greatest separation for ALS, followed by FTD, then AD (60). Furthermore, studies have shown that in NDDs blood NfL levels associate well with the NfL concentrations measured in CSF, and imaging changes (59, 61).

Studies in older populations show that in normal cognition (NC) groups higher plasma NfL levels do not correlate with worse cognition, however in the Mild cognitive impairment (MCI) groups there is a correlation between the elevation of plasma NfL and worse composite

cognitive score or neuroimaging results (62–64). In the elderly, combined lower plasma Aβ42/Aβ40 ratio and higher NfL were associated with greater decline of gait speed and cognitive functions over time (65).

Comparable performance of NfL in blood and CSF demonstrates its promise as a non-invasive biomarker of neurodegeneration. Due to connections and predictive values of NfL in cognitive impairment in NDDs, the recommendations are to use this biomarker as an indicator of neurodegeneration intensity and disease progression, rather than a specific marker for a particular disease (3, 11).

As NfL is primarily a marker of degeneration of myelinated axons, NfL is gaining increasing attention as a biomarker of disease progression and therapeutic responses in multiple sclerosis. It has been shown that in MS relapse of poor therapeutic response, increased levels of NfL in CSF and plasma appear before the MRI changes (55, 66).

Markers of neuroinflammation

Glial fibrillary acidic protein (GFAP) is an intermediate filament used as an astrocyte marker. It should be noted that some cells that would be characterized as astrocytes do not express detectable levels of this protein, while there are cells other than astrocytes that do express it (67). In general increase in GFAP levels in CSF and blood occurs after astrocyte damage or increased activation so it can be used as a biomarker of brain injury or reactive astrocytosis (3, 11). The GFAP can also be used to distinguish astrocytomas from other types of glial tumors (67).

Reactive astrocytosis has been found in AD, and its distribution matches the distribution of amyloid plaques found postmortem. More recent studies identify astrocytosis as an early phenomenon in AD, which decreases as plaque pathology increases (68). The increase of GFAP in blood is more pronounced than CSF GFAP in AD patients, as well as being a better discriminator between Aβ positive and Aβ negative patients (69).

Recent research confirms that even though blood NfL and GFAP do not have good specificity for AD, they have a good correlation to cognitive decline and can be good predictors of AD progression in MCI individuals (70, 71). Plasma GFAP levels are elevated in NC older adults who have an increased risk of AD development (72) and could be used as predictors of dementia development (73). Elevated GFAP levels have also been recorded in cases of FTD as well as Lewy body dementia, suggesting that this protein could also be a marker for other types of dementia other than AD (42).

Triggering receptor expressed on myeloid cells 2 (TREM2) is expressed on the surface of microglia in the CNS. Cleavage of the extracellular domain of this receptor creates the soluble TREM2 (sTREM2) which can be detected in both the CSF and blood. Increased levels of this protein were found in AD and MCI patients, although the predictive value for disease progression remains insufficiently elucidated (74). Increased levels of sTREM2 were detected in the CSF and blood of ALS patients (75). Even though there was no difference of sTREM2 in PD patients and healthy controls, an increase of the protein levels has been detected through the duration of PD, suggesting that sTREM2 could possibly be a disease progression rather than a diagnostic marker in PD (76).

Chitinase-3-like protein 1 (CHI3LI) also known as YKL-40 is a glycoprotein involved with innate immune system activation, and with that is considered to be a marker of inflammation (77). Elevated levels of this protein have been found in the CSF of early AD patients, allowing discrimination against HC (78). However, the results regarding blood samples, as well as different stages of AD and other NDDs are less consistent, mostly implying there is no significant change between these groups (77–80).

Markers of synaptic dysfunction

Neurogranin (Ng) is a calmodulin binding protein expressed in postsynaptic dendritic terminals of the cerebral cortex, hippocampus and striatum neurons, used as a marker of synaptic dysfunction (81, 82). Increasing levels of Ng have been detected in the CSF of AD and MCI patients, but not most other NDDs (82, 83). It has been proposed as a diagnostic and predictive biomarker for AD. The results in the blood are less clear, a decrease in Ng levels has been noted in AD, but some work also suggests that plasma Ng is not a suitable marker for AD (84, 85). The role of Ng as a biomarker in other NDDs remains to be defined (84). Other CSF synaptic biomarkers, such as SNAP-25, synaptotagmin, GAP-43, and β-synuclein may support AD diagnoses and monitoring of disease progression/treatment response.

The complexity of predicting, diagnosing and/or monitoring NDDs, as well as the importance of biomarkers in these processes, can be seen in the newest Criteria for diagnosis and staging of Alzheimer's disease established by the the Alzheimer's Association Working Group. The new criteria for biological staging focuses on biomarker profiles, relying on the updated ATX classification scheme and is independent of the clinical staging of AD. The A and T categories reflect core biomarkers amyloid and phospho-tau, and X marks one of the following categories: Biomarkers of non-specific processes involved in AD pathology: N - injury, disfunction or degeneration of neuropil (NfL); I - inflammation (GFAP); Biomarkers of non-AD co pathology: V - vascular injury; S - α synuclein (24).

Determination of biomarkers has long been, and for some of them still is, a methodological challenge. Some of the protein biomarkers are present in body fluids as different species or isoforms, which makes their quantification difficult, they can also be present in small concentrations, making detection challenging and the samples more susceptible to mishandling, with high inter-individual levels of these proteins. Throughout the years, several methods have been established in order to measure soluble biomarkers, with the last two decades making significant technological advancements in the field.

Immunoassays are widely applied for biomarker measuring, amongst the first to be used for this was the standard Enzyme-linked immunosorbent assay (ELISA). This method captures the targeted protein, binds it to a solid surface and forms a complex with an antibody linked to an enzyme, forming a highly specific antibody-antigen complex. The concentration of the targeted protein is then determined by detecting the product of the reaction catalysed by the bound enzyme, most often by photometry. The main limitation of this method is the inability of the test to detect low concentrations of proteins as well as the complexity of the protocols leaving plenty of room for error and the lack of multiplexing abilities. In the hopes of overcoming this, automated systems were developed by several companies, as well as highly sensitive technologies such as the Single molecule array (SIMOA) (12, 45, 86). This immunosorbent assay has the ability to detect single molecules of protein, binding them in an immunocomplex on a single bead, and measure fluorescence from a single enzyme labelled immunocomplex. Although the precision of this method is undeniable, its complexity for everyday use and high-cost limit its availability (87–89).

Mass spectrometry (MS) is considered the gold standard for protein quantification. It is based on separating charged particles moving through electric fields based on their mass-to-charge ratio. The advantages of this platform are sensitivity and specificity, the ability to multiplex and easily scale up the number of samples analyzed, as well as the ability to detect substances in low concentrations. It allows for targeted protein quantification, through selected reaction monitoring or parallel reaction monitoring, for known biomarkers, but also leaves room for exploratory monitoring needed for new biomarker detection and proteomics research (88, 90, 91).

The methodology for aSyn measuring is proving to be particularly difficult. Lately the alpha-synuclein seeding assays (SAA) have emerged as the leading way for measuring aSyn. They are based on the properties of the misfolded peptide to induce propagation and further formation of aSyn deposits. Both real-time quaking-induced conversion assay (RT-QuIC) and protein misfolding cyclic amplification (PMCA) fall under this category of assays (92, 93).

The complexity and the range of clinical presentations with the ever-evolving therapeutic strategies and clinical trials make the proper diagnosis of NDDs of imperative importance. Soluble biomarkers are one of the tools in this endeavor, having the potential to enable diagnosis of the disease in its preclinical stages, before the onset of symptoms, and allowing clinicians to start early treatment or trial enrollment in the hopes of slowing or stopping disease progression. Along with the advancements in the field of blood biomarkers, and the improvements and innovations in technologies used to measure them, they are making their way into clinical guidelines for diagnostic criteria and are an ever-relevant topic and focus of much research to come.

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