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Biomarkers in Medicine

Tears – more to them than meets the eye: why tears are a good source of biomarkers in Parkinson's disease

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Tears are a known source of biomarkers for both ocular and systemic diseases with particular advantages; specifically, the noninvasiveness of sample collection and a unique and increasingly better-defined protein composition. Here, we discuss our rationale for use of tears for discovery of biomarkers for Parkinson's disease (PD). These reasons include literature supporting changes in tear flow and composition in PD, and the interconnections between the ocular surface system and neurons affected in PD. We highlight recent data on the identification of tear biomarkers including oligomeric α-synuclein, associated with neuronal degeneration in PD, in tears of PD patients and discuss possible sources for its release into tears. Challenges and next steps for advancing such biomarkers to clinical usage are highlighted.

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Tears are a biofluid that is secreted by the lacrimal functional unit which includes the main and accessory lacrimal glands, meibomian glands, goblet cells, cornea, conjunctiva and which is under control of the interconnected nervous system [1]. The components of the lacrimal functional unit and their relationships are shown in Figure 1 . Tears have the extremely important role of maintaining and protecting the corneal surface and health of the eye. The tear film has traditionally been considered to consist of three layers [2]. The outermost layer is a lipid layer consisting of polar and non-polar lipids and phospholipids that is primarily secreted by the meibomian glands, with the function of reducing tear film evaporation and preventing contamination [3]. The middle aqueous layer of the tear film consists of water, electrolytes and proteins that are secreted by the main and accessory lacrimal glands, and by corneal and conjunctival epithelial cells [4]. This layer has multiple functions including protection of the cornea from pathogens through activity of antibacterial and antiviral proteins, clearance of dead cells, toxins and foreign bodies, provision of oxygen and nutrients to underlying tissues, maintenance of tear pH and osmolarity and keeping the ocular surface moist [2]. The inner layer of the tear film is a mucous layer, consisting of mucins, electrolytes and water secreted by goblet cells, corneal and conjunctival epithelial cells and to a lesser extent, the lacrimal gland [4,5]. This layer plays a major role in lubricating the ocular surface as well as protecting the eye from pathogens, external environmental factors and mechanical stress [4,6]. Recently the interpretation of a three-layered structure has been revised in favor of a structure where the aqueous and mucous layers are considered as overlapping, referred to as the muco-aqueous layer [7]. Typically, the tear film thickness in a healthy human eye is around 2–5.5 μm measured using ocular coherence tomography (OCT), encompassing 8 ± 3 μl in volume measured using fluorophotometric assessment [2]. Tear drainage occurs naturally through the nasolacrimal ducts which drain into the nasal cavity, and typically from there into the gastrointestinal tract.

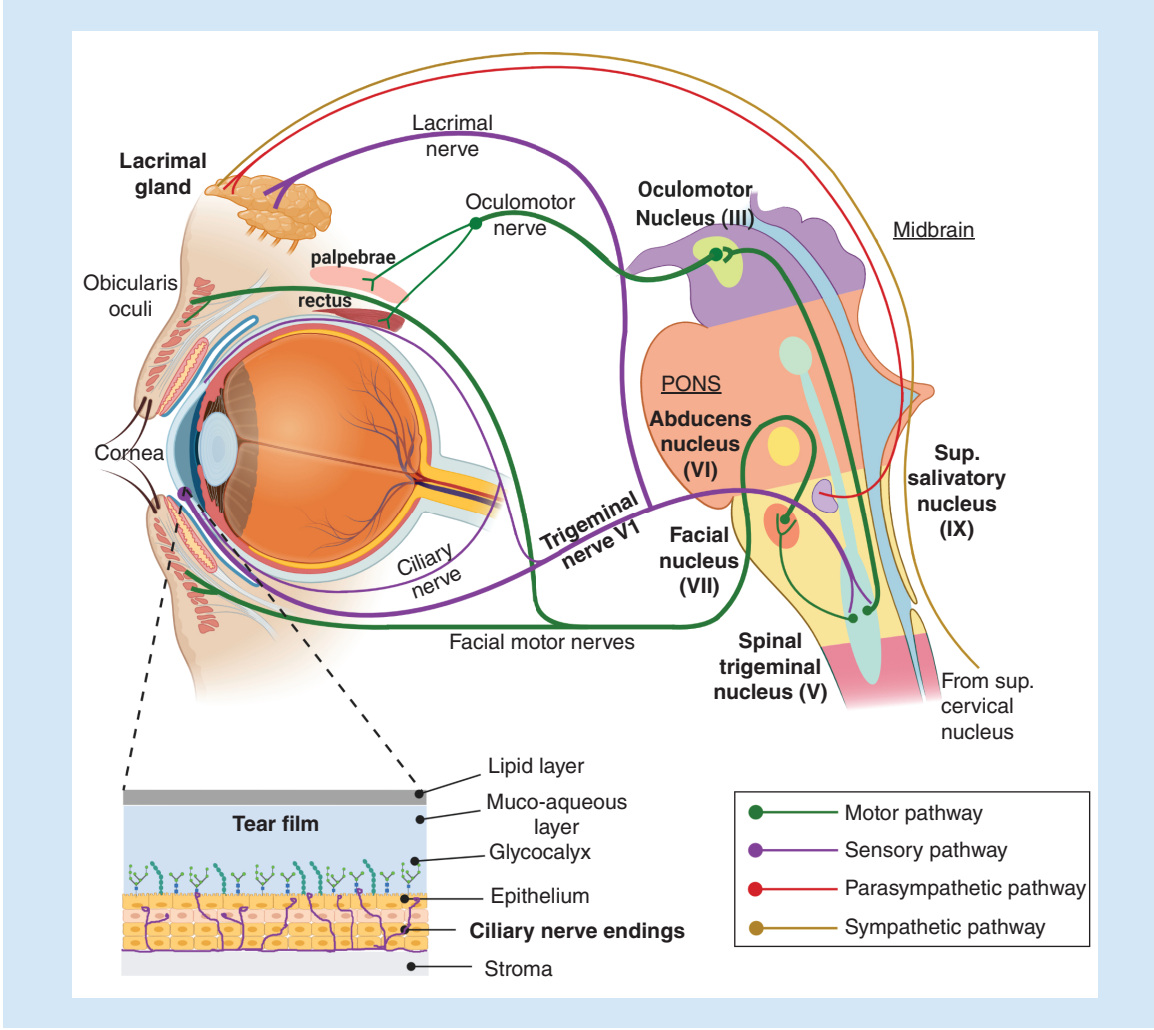

Figure 1. Interaction between ocular surface, ocular muscles and the brain. The ocular apparatus receives neural input from the brainstem, pons and the midbrain in addition to the visual cortex. The trigeminal nuclei are a group of nuclei that run along the brainstem and have centers in the pons and midbrain. The lacrimal nerve which originates from the trigeminal nucleus in the brain stem is directly connected to the gland, relaying sensory input. Ciliary nerves, extensions of the trigeminal nerves, innervate the cornea. These nerves have highly branched nerve endings that innervate the corneal epithelium and extend to the ocular surface (inset). Facial motor nerves that provide efferent fibers to the eyelids (orbicularis oculi), originate from the facial nucleus at the base of the pons, which in turn receives output from the trigeminal nucleus. Additionally, the muscles controlling the eye movements – levator palpebrae and recti, receive motor input from the oculomotor and abducens nuclei via the oculomotor nerve. Correctional feedback for voluntary eye movements is provided by the substantia nigra and the caudate nuclei in the midbrain via the abducens nuclei. The directionality of neural input is indicated by the • showing the nerve cell body and >, showing the nerve terminus. The illustration was in part adapted from [91] and created with biorender.com.

To date, tears have been classified into four types: basal, reflex, emotional and closed-eye tears. Basal tears coat the eye continuously without any direct stimulation and are critical for ocular surface health. Reflex tears are usually evoked by a reflex mechanism, triggered by stimulation of sensory nerves at the ocular surface, the somata of which are located in the trigeminal nerve ganglion and projected to brain centers that regulate lacrimation (superior salivatory nucleus) and blinking (facial motor nucleus VII) [8] (Figure 1). Emotional tears are, like reflex tears, evoked by stimulation of the lacrimal gland, however, the neural input originates in the emotional centers of the brain. Finally, closed-eye tears are the tears that accumulate during sleep, playing a particular role in clearance of cell debris and maintenance of homeostasis [2].

The composition of each type of tears, when collected, is known to be distinct due to differential involvement of the lacrimal gland functional unit. In the case of basal versus reflex tears, the difference in composition is due

to the involvement of different receptors in both the corneal sensory nerves and in the regulation of the lacrimal gland [8,9], while emotional tears differ from the other types in that they contain distinct chemo-signals, such as pheromones [10]. Tear turnover rate, which is related to drainage, also influences tear composition. This rate is decreased for closed-eye tears relative to other types of tears, while leakage of proteins from blood vessels in the conjunctiva is increased, causing an increase in total protein concentration associated largely with increased serum-derived proteins such as albumins [11].

According to analyses of the human tear proteome, lipidome and metabolome, tears express close to 1500 proteins, 100 small-molecule metabolites and 150 lipid species from six lipid classes [12–14]. In addition there is also a high quantity of nucleic acids including miRNAs [15], making tears an excellent sample for biomarker discovery. Many studies have explored tear biomarker association with diverse ocular diseases such as dry eye disease, vernal conjunctivitis, diabetic macular edema, diabetic retinopathy, meibomian gland dysfunction, Sjögren's syndrome, thyroid eye disease and infection, as changes in the tears can reflect disease pathophysiology and progression [16–21]. Relative to the plasma proteome, the tear proteome is markedly different; more than two-thirds of proteins are detected uniquely in the tears relative to plasma. Of the one-third of tear proteins that overlap with plasma proteins, the concentrations of some of these proteins, including lactoferrin, lysozyme, secretory immunoglobulin A (sIgA), immunoglobulin M (IgM), VEGF and TNF-α, is remarkably higher in tears than in plasma [13,22]. The presence of plasma proteins in tears provides a unique opportunity to study systemic diseases with and without ocular complications. To date, tears have also been used for biomarker discovery in several systemic diseases including multiple sclerosis, cancer, diabetes mellitus, rheumatoid arthritis, Alzheimer's disease and in our study in Parkinson's disease (PD) [16,17,20,23–27].

Many biofluids have been used as sources of potential biomarkers including, most notably plasma, cerebrospinal fluid (CSF) and saliva. Tears offer several advantages over these biofluids, first and foremost of which is the ease of sample collection. Tears can be collected noninvasively using Schirmer's strips (small calibrated strips of porous paper routinely used clinically to measure tear flow). These strips wick samples onto the paper matrix, allowing elution of proteins of interest from the strip [28]. The Schirmer's test may cause temporary and mild discomfort while the strip is inserted in the eye. Despite this minor drawback, it has been used clinically for over 100 years since it was first described by Schirmer in 1903 [29]. There are no published data on longer lasting side effects of the test. Alternatively, tears can be collected non-invasively using glass or plastic microcapillary tubes [30]. Both methods have advantages and disadvantages. Collection using capillary tubes enables a more precise determination of tear volume while limiting potential matrix interactions and protein retention within the paper strip. However, the collection takes a longer period of time than the Schirmer's strip, and only a limited volume can be obtained. As it is important to avoid contact of the capillary with the eye lids and the ocular surface, such collection also requires experienced personnel to perform the procedure, especially in patients with tremors. Patients may also feel uncomfortable having a sharp object held close to their eyes. Furthermore, collection of reflex tears using this method would require another source of stimulation, such as a cotton swab inserted in the nostril [31]. The Schirmer's test, while generating a foreign body sensation during collection, is generally well tolerated by patients. Furthermore, it can be readily managed within the everyday workflow of the clinic, and does not require significant training. According to studies conducted by Small *et al.* [32] and Dumortier and Chaumeil [33], data obtained from tear obtained using Schirmer's strips is more reliable and comparable relative to data collected with capillaries. However, when using Schirmer's strips for tear collection, contamination with epithelial cells may occur [34]. Furthermore, recovery from the strip may vary by the protein of interest, and may also be affected by the elution method used [32,35].

CSF collection, hospitalization and/or use of a specialized operator is not required for tear sample collection and there are typically no associated side effects. Tear collection is similarly far less invasive than blood collection. Despite low tear volume, the total protein concentration of human tears is reported to range between 6–11 mg/ml, higher than saliva (0.4–4.4 mg/ml) [36], although some variation in protein abundance has been reported [20,37,38]. Further, unlike plasma, the depletion of abundant proteins such as albumin is not necessary for analysis [19]. Although lactoferrin is abundant in tears, studies conducted in our laboratory detecting single proteins, as well as proteomics and lipidomics analysis of tears by other groups, suggests that it is feasible to obtain good quality data from tears without lactoferrin depletion. To detect α-synuclein in the tear lipidome and proteome studies conducted in other laboratories did not require tears to undergo any depletion process to achieve good quality data without lactoferrin depletion [20,38,39]. Tear samples are also not significantly contaminated with microorganisms or blood products as may be the case with saliva and CSF, respectively.

Evidence for alterations in the ocular surface system of PD patients

There is widespread recognition of the need for biomarkers to enable earlier and more accurate diagnosis of the devastating, chronic, progressive neurological disorder, PD. PD is the second most common neurodegenerative disease, and is characterized by the occurrence of motor symptoms including resting tremor, rigidity and slowness with postural instability later in the disorder [40]. The motor symptoms of PD arise from the degeneration of dopaminergic neurons in the substantia nigra, a brain center regulating motor function. There is currently no definitive diagnostic biomarker for PD; the diagnosis is based on clinical signs and symptoms. However, motor symptoms often do not occur before a loss of 30–70% of the dopaminergic neurons in the substantia nigra has occurred, which may be decades after the initial onset of cell and molecular neuropathology [41]. A definitive diagnosis of PD can only be obtained from a postmortem autopsy when the presence of pathological lesions called Lewy bodies (aggregates of an abnormal form of the protein α-synuclein) can be confirmed in the substantia nigra. In PD, Lewy bodies are found not only in the substantia nigra but also in parasympathetic and sympathetic ganglia, also causing disturbances in autonomic function [42]. These non-motor symptoms of PD often occur many years prior to motor symptoms.

To aid in the assessment of longitudinal disease progression, several scales have been developed, including the I–V graded Hoehn and Yahr Scale (H&Y) in which the severity of disease is graded into stages with a value of 0 indicative of no signs of disease and values from I to V reflective of the severity of disease presentation (I, least severe and V, most severe) [43,44]. This scale is commonly used in combination with the unified Parkinson's disease rating scale (UPDRS) which evaluates key areas of disability related to mental activity, impact of disease on daily activities, motor function and complications of treatment. Points are assigned based on the individual's response, as well as observation and physical examination, resulting in a total cumulative score ranging from zero (no disability) to 199 (total disability) [45]. These scales are useful for monitoring progression of symptoms and medication treatment responses; however, there is limited data regarding their performance in early stages of the disease, which is when most disease modifying therapy trials require them [46].

Physicians have noted, anecdotally, that PD patients often complain of ocular dryness and discomfort. Secretion by the lacrimal gland, the major contributor to the muco-aqueous tear film, is regulated by autonomic sympathetic and parasympathetic nerves, whose activity is in turn regulated by reflex input from sensory nerves in the ocular surface [8]. This system may, therefore, be highly sensitive to autonomic dysfunction. A number of tests are commonly used to evaluate ocular surface health such as: measures of tear flow (including the Schirmer's test described above); tear break up time (TBUT) which measures tear film stability; and fluorescein or Rose Bengal corneal staining, the extent of which is inversely correlated with corneal health.

The results of the few studies that have evaluated ocular surface health including tear parameters in PD populations are summarized here. Although some of these studies include a more extensive clinical work up of ocular complaints, we have focused on the results related to Schirmer's test, TBUT, blink rate and the correlation of these parameters to disease severity using the H&Y scale. Collection of tears using an unanesthetized Schirmer's test yields primarily reflex tears, whereas collection after topical anesthesia (anesthetized Schirmer's test) yields basal tears. Two early studies investigated only the differences in tear secretion measured using an unanesthetized Schirmer's test in PD patients and healthy control subjects [47,48]. In a study of 51 PD patients and 75 healthy control subjects, Kwon *et al.* showed that tear secretion in PD patients was decreased relative to healthy controls; however they did not evaluate the correlation to disease severity [48]. Bagheri *et al.* observed that 25 PD patients with a H&Y stage III–IV had reduced tear secretion compared with 36 healthy control subjects, but did not find a significant difference between a group of 14 patients with a H&Y stage I–II exhibiting less severe motor symptoms [47]. In a study of 30 PD patients and 31 healthy control subjects, Biousse *et al.* were the first to evaluate a large number of ocular surface parameters with early, untreated disease (mean duration of diagnosed PD, 22.0 ± 8 months) [49]. Despite not detecting a significant difference in anesthetized Schirmer's values in PD patients relative to healthy control subjects, they found that the most common complaints in patients were ocular surface irritation including dry eyes and blepharitis, a complaint corroborated by findings of decreased TBUT and a reduced blink rate.

In contrast, Söğütlü Sarı *et al.* showed that unanesthetized Schirmer's test values but neither blink rate nor TBUT were reduced in a study of 37 patients and 37 healthy controls [50]. However, in this study, no correlation between Schirmer's test values and the H&Y scores was observed [50]. Another study by Demirci *et al.* in 40 patients and 40 healthy controls found that anesthetized Schirmer's test values, TBUT and blink rate were all lower in PD

patients [51]. In a study of 56 patients, 45 of whom had H&Y scores of I–II and 11 with H&Y scores of 2.5–3, versus 34 healthy control subjects, Tamer *et al.* measured tear flow with the unanesthetized Schirmer's test as well as TBUT, corneal fluorescein staining, Rose Bengal staining and other indications of ocular surface abnormality [52]. As the results of each test were grouped as normal or abnormal for each subject, the overall numbers of tests with abnormal results were significantly increased in PD patients. Abnormalities in each separate parameter measured were found to occur at a higher rate in PD patients relative to healthy controls, while Schirmer's values, TBUT and blink rate reductions were all inversely correlated with the H&Y score [52].

In a study comparing tear function in patients with different neurological pathologies including Alzheimer's disease (n = 20), multiple sclerosis (n = 20), PD (n = 30), Friedreich's ataxia (n = 10) and epilepsy (n = 21) with age-matched healthy controls for each patient group, Örnek *et al.* [53] found that the mean, TBUT levels were significantly shorter in patients with Alzheimer's disease and multiple sclerosis, while unanesthetized Schirmer's test values were only lower in epilepsy patients when each group was compared with their controls. However, when compared across the different neuro-pathologies, mean TBUT levels in the Alzheimer's, multiple sclerosis and PD groups were significantly shorter than levels in the Fredrich's Ataxia and epilepsy groups, while the mean unanesthetized Schirmer's test values were lower in the Alzheimer's and PD groups than in the multiple sclerosis, Fredrich's Ataxia and epilepsy groups.

Although these reports on tear secretion and other indications of dry eye disease are not completely consistent, the majority show that tear flow measured by Schirmer's test, tear quality as measured by TBUT, and blink rate are decreased in PD relative to controls. Basal tear secretion (anesthetized Schirmer's values) was reduced in one out of two studies, and reflex tears were reduced in four out of six studies while TBUT and blink rate were decreased in three out of four studies. The correlation of these changes with disease severity remains unclear, as some of the apparent discrepancies between the studies may be due to heterogeneity in disease severity. When considering tear flow, it is important to note that several systemic medications used to treat PD including anticholinergic (antimuscarinic agents), anticholinergic-like (amantadine) and antidepressants may negatively impact tear secretion [54]. Most of the PD patients in the studies discussed above, with the exception of the study by Biousse *et al.* [49], were being treated with these medications, and as such, the observed reductions in tear secretion could have been due to the PD pathology itself, drug side effects, or both.

Reduced blink rate and evidence of meibomian gland dysfunction or disease in PD patients has also been confirmed in multiple studies. Blinking is a motor function and a reduced blink rate may be associated with the decrease in dopamine levels [55]. Thus, complaints about dry eye in PD patients are most likely due to a combination of reduction of reflexive tearing in combination with decreased TBUT and reduced blink rate which promote increased tear evaporation.

The tears of PD patients are enriched with aggregated α-synuclein, the principal component of the Lewy body

Our interest in the tear film as a source of potential PD biomarkers was sparked by the studies and results outlined above detailing compromised tear production and tear quality in PD. We further reasoned that changes in potential innervation of the lacrimal gland, in particular, through cholinergic dysregulation associated with PD development, could directly influence the composition and volume of tears. We tested this hypothesis in a study recently published in *Biomarkers in Medicine* exploring the identity of putative biomarkers in basal tears of PD patients when compared with healthy control subjects [20]. We focused on α-synuclein in its monomeric form and in oligomers, a precursor to the aggregates found in the Lewy bodies. These two constituents have been explored in diverse biofluids as biomarkers of PD [56–58]. We found that levels of oligomeric α-synuclein were significantly elevated in tears of PD patients when compared with healthy controls. Surprisingly, this elevation was more pronounced in males in comparison with females. Monomeric α-synuclein, levels were also modestly but significantly decreased in basal tears of PD patients when compared with healthy control subjects, leading to a significantly increased ratio of oligomeric α-synuclein to monomeric α-synuclein in tears with an area under the receiver operating characteristic (AROC) curve of 0.72 in all PD patients (0.73 in male PD patients). No notable changes in four other proteins evaluated were identified in basal tears beyond a modest but significant increase in the PD-related protein, DJ-1, only in male PD patients relative to male healthy control subjects, which did not affect the AROC. Further, no changes in Schirmer's test values were seen in basal (anesthetized) tears in these two populations.

Intriguingly, in our follow-up study in the same PD patients (81 PD and 57 HC subjects from the original study), reflex tears also revealed marked and significant increases in oligomeric α-synuclein which occurred to an

equal extent in males and females, but was of a significantly greater magnitude than that in basal tears ($p = 0.006$) in PD patients. This change contributed to a higher AROC value between PD and HC in reflex tears [38]. Moreover, analysis of additional features of the reflex tears in 84 PD patients showed a significant increase in CCL2/RANTES ($p = 0.003$), a protein implicated in PD, as well as a significant reduction in Schirmer's test values ($p = 0.001$) relative to 84 healthy control subjects [38]. The combination of the changes in these potential biomarkers in reflex (unanesthetized) tears was a more powerful indicator of PD than any single individual change [38]. It is well known that the tear composition differs in response to the neural stimulation of the lacrimal gland in reflex versus healthy tears. However, whether the use of topical anesthesia also impacts tear composition by a direct effect on the ocular surface has not been extensively studied.

What is the source of oligomeric α-synuclein in PD patient tears?

α-synuclein is predominantly an intracellular neural protein [59]. Therefore, leakage or secretion from damaged neurons is a likely source of its aggregates in any biofluid including tears. Figure 1 outlines the multiple points of interaction and origin of the neural circuitry engaging components of the lacrimal functional unit including the lacrimal gland, the cornea and other tissues such as the ciliary body and ocular orbit muscles adjacent to the ocular surface system. The lacrimal nerve arises from the ophthalmic division (V1) of the trigeminal nerve and provides sensory innervation to the lacrimal gland while the autonomic supply comes from the maxillary division (V2). Parasympathetic input to the lacrimal gland originates at the superior salivatory nucleus in the pons, whereas the sympathetic input originates at the superior cervical nucleus. The cornea receives sensory afferent fibers from the trigeminal ganglion via two long ciliary nerves [60]. The eyelids receive motor efferent fibers from the facial nucleus (completing the corneal blink reflex circuit) and oculomotor nucleus and sensory innervation from the trigeminal nucleus. Voluntary eye movements are controlled by six muscles that receive motor input from the oculomotor and abducens nuclei, whereas gaze correction involves the cortex and regions of the midbrain, including the substantia nigra [61].

PD patients experience a range of eye-related issues well beyond the features of ocular surface and dry eye disease already discussed. Research suggests that there is degeneration of the optic nerve [62] remodeling of the foveal region of the retina [63], increased meibomian gland disease [64], deficits in voluntary eye movements [65] and decreased corneal nerve density, corneal sensitivity and blink reflexes [66–68]. Reduced corneal thickness and increased nerve branching in the cornea is also reported [66–68]. Finally, voluntary eye movements such as saccades are impaired in PD [69]. These findings collectively suggest that in PD there are significant global alterations in the integrity of the neural connections of the visual system. Thus, abnormal aggregates of α-synuclein shed passively or actively from damaged neurons could be transferred to non-neuronal tissues prior to release into tears. The tissues with the direct ability to contribute proteins to tear fluid are the lacrimal gland and cornea.

The acinar cells of the lacrimal gland are specialized secretory epithelial cells constituting ~80–85% of the mass of the lacrimal gland and are responsible for the secretion of most proteins into tears [2]. Protein secretion from acinar cells is effected across the apical membrane into ducts that drain to the surface of the eye. Secretion includes contributions from secretory and transcytotic pathways that are both sensitive to regulation by neurotransmitters [45–48]. The components secreted from the regulated secretory pathway are primarily synthesized in the acinar cells and packaged into mature secretory vesicles from the *trans*-Golgi network to await the stimuli triggering exocytosis. α-synuclein has very low gene expression in lacrimal gland, relative to its expression in brain. Our unpublished data reveal that mouse lacrimal glands show α-synuclein (*Snca*) gene expression of 0.6% relative to the expression seen in mouse brain (defined as 100%). These traces of α-synuclein expression most likely reflect the low abundance of innervating neurons present in lacrimal gland homogenates. Given these findings, it is unlikely that tear oligomeric α-synuclein is produced in acinar cells and secreted via the regulated secretory pathway.

We thus hypothesize that the increased oligomeric α-synuclein in tears of PD patients may reach the tears through the transcytotic pathway. The transcytotic pathway accommodates fluid phase- and receptor-mediated endocytosis of materials from the interstitium, followed by vesicular transport from the basolateral to the apical region of the cell where contents are exocytosed into tears. This is a robust pathway in acinar cells, responsible for the vigorous receptor-mediated transport of secretory IgA and IgM constituting the major mucosal immune defense mechanism in tears [70]. Increased oligomeric α-synuclein could be bound or captured from interstitial fluid, endocytosed, transcytosed and exported by acinar cells. Affected neurons innervating the lacrimal gland in PD patients may also release oligomeric α-synuclein locally, leading to a change in local accumulation at synaptic sites which may then be transported into the tear fluid (Figure 2A).

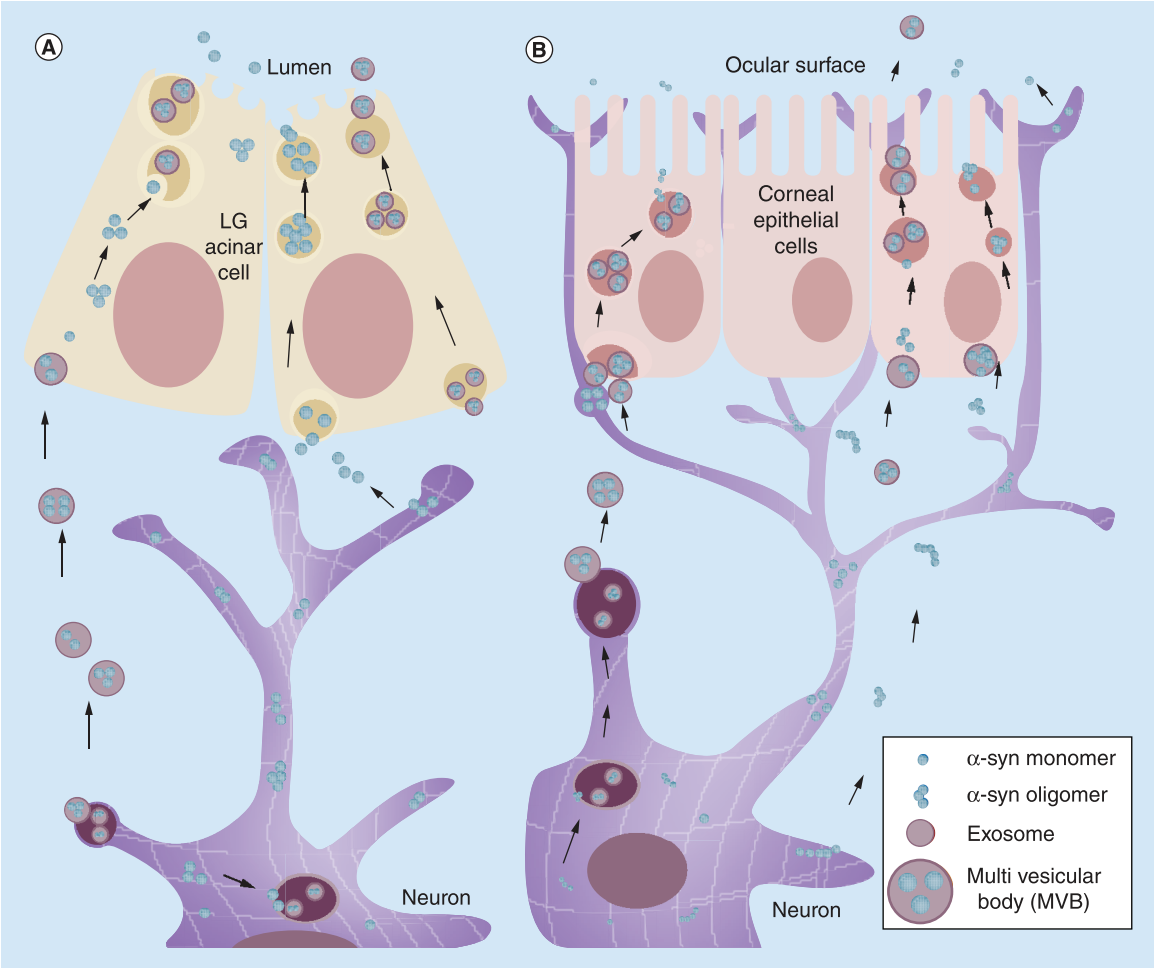

Figure 2. Model for trafficking of oligomeric α-synuclein from the interstitium to tears in lacrimal acinar cells and corneal epithelial cells. Oligomeric α-synuclein can be increased in the extracellular space or tissue interstitium either free or associated with or inside exosomes. **(A)** Acinar cells in the lacrimal gland may take up oligomeric α-synuclein via fluid-phase or receptor-mediated endocytosis and transcytose these proteins to the lumen where the oligomers are secreted into tears (cell on the right). Exosome-associated oligomeric α-synuclein may also be internalized by the fusion of the exosome membrane with the basolateral plasma membrane or by fluid-phase or receptor-mediated endocytosis (both cells). Free-floating α-synuclein oligomers in the cytoplasm may also be packaged into multi-vesicular bodies and be secreted as exosomes into the lumen. **(B)** When secreted in exosomes, oligomeric α-synuclein can be internalized by corneal epithelial cells either by fluid-phase or receptor-mediated endocytosis, or the exosomal membrane may fuse with the basolateral plasma membrane and deliver oligomeric α-synuclein into the cytoplasm. Exosome-free oligomeric α-synuclein may also be directly taken up by fluid-phase or receptor-mediated endocytosis at the basolateral plasma membrane and transcytosed to the ocular surface. Both the models depict damaged nerve endings in the lacrimal gland **(A)** and corneal epithelium **(B)** providing a local source of oligomeric α-synuclein, but this may also be increased in general in plasma, which is accessible to acinar cells. The illustration was created with biorender.com.

It is also possible that oligomeric α-synuclein reaches the tears via the cornea, one of the most densely-innervated tissues in the body [8]. The presence of α-synuclein and its aggregates in the cornea is relatively understudied in PD. One study failed to detect any α-synuclein in healthy human corneas using a western blotting approach, but it is possible that levels were below the threshold of detection with this technique [71]. Another study showed strong immunostaining for α-synuclein in the corneal epithelium and endothelium and punctate staining in the stroma in healthy human corneas [72]. As the cornea is highly enriched in peripheral sensory nerve endings, and the presence of Lewy bodies has been shown in other peripheral sensory nerve terminals in PD patients [73], corneal ciliary nerves are also a potential source of oligomeric α-synuclein present in PD patient tears. Like acinar cells, corneal epithelial cells have a transcytotic pathway that may provide a venue for exocytosis into tears (Figure 2B). Alternatively,

oligomeric α-synuclein could be shed directly into tears from damaged ciliary nerve endings intercalated into the corneal epithelial layer (Figure 2B).

Is oligomeric α-synuclein secreted into tears free in solution or within extracellular vesicles?

Damaged axons that release their contents including oligomeric α-synuclein may increase the local concentration of this form of the protein such that it would be transported in increasing amounts by acinar or corneal epithelial cells. Some studies have also suggested that oligomeric α-synuclein may be generally elevated in plasma of PD patients [74], which could also contribute to increased abundance in tears due to the significant inherent transcytotic activity in the acinar cells in particular. Under these scenarios, the oligomeric α-synuclein secreted into tears would be present-free in solution.

Tears also contain extracellular vesicles including exosomes [75–77], 40–150 nm vesicles derived from endocytic compartments that are released into almost all biofluids [78]. Our unpublished data on human tear exosomes isolated by size exclusion chromatography shows that tears have an exosome concentration of ~ 200 million particles per ml. Tear exosomes also show positive reactivity for the known exosome membrane markers, TSG101 and CD9, and have a median diameter of 135 nm. The exosomal pathway has recently become of interest for its role in the disposal of aggregated intracellular proteins, and for the transport of RNA- and potentially protein-based 'messages' to neighboring, as well as distant cells, as an intercellular signaling pathway, akin to the function of the endocrine system. There is a growing literature suggesting that cell-to-cell transmission of α-synuclein oligomers, and therefore the potential spread of synuclein pathology, may occur by packaging of these oligomers into exosomes and the subsequent secretion and intercellular spread of these exosomes [79]. A recent study in multiple sclerosis patients and healthy control subjects showed high similarities between the extracellular vesicles isolated from tears and CSF regarding the protein composition specific to the cells of origin, and suggesting a strong connection between the CSF and tears [80].

Both monomeric and oligomeric α-synuclein can be released by cells freely or packaged inside exosomes [81] However, exosome-associated α-synuclein is internalized more readily, and is more toxic to cells than the free-floating form [81]. CSF exosomes isolated from PD patients can trigger α-synuclein oligomerization in cell lines, leading to cell death [82]. A recent study has reported that serum exosomes isolated from PD patients induced protein aggregation and microglial activation, degeneration of dopaminergic neurons and movement deficits in BALB/c mice [83]. It is still unclear whether these exosomes are produced primarily by neurons or by other cell types. One study using a neuronal exosome marker, L1 CAM showed that neuronal exosome-associated α-synuclein was significantly higher in the plasma of PD patients, suggesting a neuronal source [84].

A second potential route of secretion of oligomeric α-synuclein into tears is therefore through transcytosis of internalized exosomes. These exosomes could be endocytosed intact at the basolateral membrane of acinar cells through fluid phase endocytosis, or even receptor-mediated mechanisms, transcytosed, and released intact at the apical membrane. In this scenario, oligomeric α-synuclein in tears would be present largely in exosomes. The ways in which exosomal oligomeric α-synuclein may be endocytosed and transported to tears by acinar and corneal cells are also shown schematically in Figure 2. These models are directly testable in future studies utilizing *in vitro* model systems and by investigating whether oligomeric α-synuclein in tears exists free in solution or within exosomes. If oligomeric α-synuclein is indeed identified in exosomes, the cellular source of the material may be further identified through analysis of additional exosomal membrane markers associated with the cell of origin. Finally, free oligomeric α-synuclein which has been reported to have some cell-penetrating properties [85,86] may be transported across the basolateral membrane of acinar cells, recognized as an 'abnormal' or aggregated protein, packaged into exosomes, and then secreted into tears (Figure 2A).

Challenges in the use of tears as a biomarker source

One challenge in the use of tears as a biomarker is that there are four different classifications of tears – basal, reflex, emotional and closed eye. The multiple points of proximity and engagement of the nervous system and components of the ocular surface system (Figure 1) are potentially beneficial from the perspective of capturing changes in nerve function and/or gathering proteins scavenged from damaged neurons. This engagement may have relevance moving forward in the investigation of biomarkers for other neurodegenerative diseases associated with protein aggregation such as Alzheimer's disease and Huntington's disease. However, this complex interplay necessitates the collection of the 'right' type of tears, as the protein composition and the magnitude of any observed changes may vary by classification of tears. Reflex tears may be more useful as a biomarker source relative to basal

tears in some diseases, and vice versa. We have observed this directly with findings that basal and reflex tears both show increased oligomeric α-synuclein, but that the increase in reflex tears is of greater magnitude and produce a higher area under the receiver operating characteristic curve (AROC), while manifesting additional changes that may aid in discrimination of PD patients [20,38]. Thus, adherence to regimented tear collection protocols and comparison of tear types will be important.

Although tear collection is non-invasive and atraumatic, an additional challenge of using tears as a biomarker source is the limited volume that is obtained in each collection, particularly in older individuals. Although the volume acquired is generally sufficient for many immunoassays, to extract RNA or DNA, and to isolate extracellular vesicles or exosomes, analysis of any individual protein will be dependent on the sensitivity of the particular method of analysis. The development of devices that integrate collection and analysis functions and eliminate the need for tear elution from a collection device into a secondary container may mitigate some of these challenges. The Inflammadry® test (Quidel, CA, USA), which measures MMP9 above a certain threshold in a disposable immunoassay, is such an example. This device is still limited by the threshold of detection (≥40 ng/ml), although many patients with mild to moderate dry eye have lower levels of MMP-9 which may still be clinically significant. The development of more sensitive methods for detecting specific proteins of interest and/or non-invasive methods which can secure multiple tear collections without irritating the ocular surface would thus be of great benefit.

Finally, the tear profile may be affected by sex due to hormonal influence on the lacrimal gland, and by age due to the death of many signaling receptors in the corneal and conjunctival epithelium that are responsible for tear secretion [87,88]. The literature also suggests diurnal and seasonal variations in the tear protein profile [89,90]. While these effects may not drive the large fold-changes in protein levels that would be expected for any useful biomarker, it is important that these variations are carefully analyzed to understand the inherent variability in the level of any protein of interest.

Our vision for the future of tear biomarkers in PD

In PD, substantial and irreversible loss of dopaminergic neurons may occur in the substantia nigra decades before the onset of motor symptoms that would prompt a formal disease diagnosis. Given the acute need to identify patients at the earliest stages of PD before irreversible neuronal damage has occurred, our vision is to develop a tear-based assay for early diagnosis of PD patients based on detecting oligomeric α-synuclein, and/or other tear protein biomarkers. To accomplish this goal, we have several immediate challenges. Our initial study analyzed the tear composition of a relatively homogeneous population of patients, largely at H&Y stage two (mild to moderate disease severity). To understand if the levels of oligomeric α-synuclein in tears are positively correlated with disease severity, we must be able to measure oligomeric α-synuclein in PD patients at different H&Y stages representing different degrees of PD progression/severity. Such an analysis would provide insights regarding the threshold of detection that might prompt an undiagnosed individual with abnormal levels to undergo a more extensive evaluation.

A biomarker may not only reflect the presence of disease, but also aid in monitoring disease progression and/or response to therapy, if that therapy was in fact disease modifying and could effect changes in the underlying neuropathological substrate of the disease. Diagnosis of PD at an earlier stage in patients is only the first step; demonstrating that the amount of oligomeric α-synuclein in tears is reflective of disease status must be followed by a test of whether a reduction in the amount reflects a response to therapy. Such a function would dramatically expand the utility of this and any other tear-based diagnostic test for PD.

Finally, we have just begun to mine the potential of tears in PD and in other systemic diseases. Two-thirds of the proteins found in the tearome are distinct from those in plasma. Many studies have focused beyond identification of a single biomarker and to consider combinations of several biomarker which can vastly improve sensitivity and specificity. A recent analysis of the tearome revealed that about 30 proteins that are involved in lipid metabolism, oxidative stress and immune responses were significantly changed in PD patients when compared with healthy controls. Many of these proteins are associated with PD and/or other neurological disorders and have been reported in serum or CSF of PD patients [37]. Continued efforts to identify changes in other protein components of the tear film in PD may enable development of a tear biomarker panel which could be further customized to discriminate more rigorously between PD associated with different synucleinopathies, atypical Parkinsonian syndromes and discern disease severity. We have already made progress in this area with the findings of additional biomarkers, beyond oligomeric α-synuclein, in reflex tears of PD patients that improve the sensitivity and specificity of our tear-based analysis [38]. Much work remains to be done in these critical areas as defined above.

Executive summary

- Tears are a complex mixture containing various proteins and lipids with a critical role in ocular surface health.
- There are different types of tears, generated by variations in the source of neural input to the contributing tissues.
- As a biofluid for biomarker discovery, tears have advantages as they are easily accessible, of defined composition and largely contaminant-free.
- Parkinson's disease (PD) patients have many associated visual problems, suggesting that tear fluid composition might differ in PD patients.
- Oligomeric α-synuclein is significantly increased in tears of patients with PD relative to healthy control subjects, while other changes in the tear fluid are noted.
- Increased oligomeric α-synuclein in tears may be derived from damaged interstitial neurons in the cornea or lacrimal gland through active transport.
- Future research may focus on an understanding of how changes in oligomeric α-synuclein and other tear parameters vary with disease severity, and identification of panels of tear biomarkers with higher selectivity and specificity.

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Cerebrospinal fluid biomarkers in neurodegenerative disorders

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Neurodegenerative diseases represent a daunting challenge in clinical diagnosis and management. Biomarkers that might aid in the diagnosis of these devastating and globally important diseases are urgently sought and required. Here we describe the application and state of development of a range of cerebrospinal fluid biomarkers in common neurodegenerative disorders including Alzheimer's disease, frontotemporal dementia and prion diseases.

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Alzheimer's disease

Cerebrospinal fluid (CSF) biomarkers and their use in neurodegenerative disorders represent an emerging area of research. The clinical use of CSF biomarkers for Alzheimer's disease (AD) is better supported than for other neurodegenerative diseases. CSF biomarkers are useful as screening tools and as supplementary information to diagnostic investigations but are lacking as diagnostic tools in isolation.

For AD, established markers are T-tau, P-tau and $A\beta_{42}$ [1]. CSF $A\beta_{42}$ is elevated, while CSF T-tau and P-tau are lowered [2]. Their significance results from their direct correspondence to the AD amyloid/tau hypothesis and evidence of effective clinical use. Of the markers discussed here, T-tau, P-tau and $A\beta_{42}$ are some of few to actually form part of diagnostic criteria; this triplet of biomarkers is part of both the International Working Group IWG-2 Criteria for AD and the National Institute on Aging-Alzheimer's Association (NIA-AA) Criteria for AD and mild cognitive impairment (MCI) resulting from AD [3]. In both criteria, CSF T-tau, P-tau and $A\beta_{42}$ are part of the same criterion as amyloid imaging, in allowance for dissociation between CSF biomarkers and imaging studies [3,4].

Recent studies have examined biomarker ratios and frequently found them to be superior to their individual constituents. The most prominent ratio is CSF $A\beta_{42}/A\beta_{40}$, which has demonstrated greater sensitivity and specificity for AD [2,5], greater accuracy in predicting abnormal amyloid deposition in patients with MCI [6], and greater correlation to amyloid imaging, than CSF $A\beta_{42}$ alone [7]. The CSF $A\beta_{42}/A\beta_{38}$ ratio has also demonstrated diagnostic promise in at least one study [6]. The same study also identified dissociations between $A\beta_{42}/A\beta_{40}$ and $A\beta_{42}/A\beta_{38}$ ratios, individual biomarkers ($A\beta_{42}$, $A\beta_{40}$ and $A\beta_{38}$) and findings on MRI (hippocampal atrophy, white matter lesions, etc.) [6]. As individual biomarkers, CSF $A\beta_{40}$ and CSF $A\beta_{38}$ have received little attention and there is no evidence to indicate that they are comparable to CSF $A\beta_{42}$. One study found correlation between levels of CSF $A\beta_{38}$ and CSF $A\beta_{40}$, indicating that they may be comparable to each other [8] – in light of dissociations between $A\beta_{42}/A\beta_{40}$ and $A\beta_{42}/A\beta_{38}$ ratios, this is curious. Correlations between elevated CSF $A\beta_{40}$ and CSF $A\beta_{38}$, and worsening gait variability have also been observed; this correlation did not extend to CSF $A\beta_{42}$ [9]. Perhaps CSF $A\beta_{42}$ is useful in screening and diagnosis while CSF $A\beta_{40}$ and CSF $A\beta_{38}$ are useful for screening and diagnosis when part of ratios, and useful for prognosis when used individually. Until there is further investigation of CSF $A\beta_{40}$, CSF $A\beta_{38}$, and their corresponding ratios with CSF $A\beta_{42}$, however, such statements remain conjecture.

T-tau has also been implemented into ratios; for instance, the CSF T-tau/$A\beta_{42}$ ratio is able to predict progression from preclinical to clinical AD [2]. Findings regarding CSF T-tau ratios are limited, however, with the bulk of research focusing on Aβ peptides. Recent developments have focused on the different forms of P-tau. CSF P-tau is measured in the form of either P-tau_{181} or P-tau_{231} (i.e., tau phosphorylated at the threonine 181 or 231 position,

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respectively) [2,10]. Studies thus far identify P-tau$_{231}$ as the more accurate of the two [11]; however, recent work suggests that both forms of P-tau could have their own unique roles in diagnosis. Elevated CSF P-tau$_{231}$ has been investigated as a biomarker to differentiate AD from vascular dementia [12,13], but further work is required. Elevated CSF P-tau$_{181}$ has been suggested as a biomarker for suspected non-AD pathology; Pouclet-Courtmanche *et al.* identified normal CSF Aβ_{42}, normal CSF Aβ_{42}/Aβ_{40} and abnormal CSF P-tau$_{181}$ as a characteristic profile for suspected non-AD pathology in patients presenting with memory loss [14]. Further examination of such associations and dissociations in AD, AD variants and other neurodegenerative disorders whose presentations overlap with AD would further inform the use of CSF biomarkers in clinical settings.

Dissociation between CSF biomarkers and imaging studies has been observed. In the context of tau imaging, Mattson *et al.* observed that although tau deposition increased in progression from preclinical to clinical AD, CSF T-tau and P-tau remained static [15]. This ties in with others' observations that while imaging studies demonstrate progression, CSF biomarkers do not – they are 'static' [3]. For instance, many CSF biomarkers in AD (including Aβ_{42}, T-tau and P-tau) have been able to predict progression from preclinical to clinical AD as they are already elevated in prodromal patients [16]. This success extends to the combination of CSF biomarkers with imaging [17] and the use of CSF biomarkers in the diagnosis of early-onset AD (AD in patients 65 years or younger) [18]. Further evaluation of how CSF biomarkers relate to imaging studies and time is required. Overall, the current evidence supports the use of CSF Aβ_{42}, CSF T-tau and CSF P-tau as screening or diagnostic tools; if they do not change with time, little is to be gained beyond use at initial presentation.

Emerging biomarkers

Recent work has investigated the behaviour of biomarkers that are nonspecific to AD pathology. Study of these biomarkers and their relationships to the classical AD triplet could represent further improvement and efficacy in the clinical application of CSF biomarkers. Prominent examples of such biomarkers are YKL-40 (also known as CHI3L, YKL-40 is a marker of glial inflammation [19]), VILIP-1 (also known as VLP-1, a marker of neuronal damage [20]) and NFL (nonspecific marker of neurodegeneration [10,21]).

Elevated CSF YKL-40 has been most successful as a predictor of progression from MCI to clinical AD [16,22]. Elevated CSF YKL-40 has also been associated with pathological processes associated, or hypothesized to be associated, with AD; elevated CSF YKL-40 has been associated with markers of humoral immunity [22] and abnormal increases in blood-brain barrier permeability [22]. This perhaps indicates a role for CSF YKL-40 in clinical trials or research, while the precise clinical role of CSF YKL-40 remains uncertain. The same can be said of CSF VILIP-1 - findings of VILIP-1 in AD fall into two groups: increased levels or no change [10,22,23]. Associations between VILIP-1 and YKL-40 have also been observed [22]. Specificity of these markers for AD requires assessment.

CSF NFL has received significant attention in the literature across many neurodegenerative diseases, including AD. NFL forms part of the cytoskeleton and is released into the CSF when axons are damaged [21]; increased CSF NFL is thus a marker for neuronal injury and neurodegeneration [10,21]. Its utility in AD diagnosis is debatable: some studies identify significant increases in CSF NFL [10], whilst others observed no such increase once age and statistical corrections are considered [21]. Furthermore, the specificity of increased CSF NFL for AD is questionable, with CSF NFL showing increases in other neurodegenerative diseases [24,25]. Additionally, one study observed no correlation between CSF NFL and CSF Aβ_{42}, indicating that NFL may indicate neurodegeneration independently of Aβ pathology [26]. CSF NFL has shown potential is distinguishing disease variants [27] but further work is required. The apparent nonspecificity of CSF NFL indicates that it may be more useful as a screening tool for neurodegenerative disease in general; however, more specific CSF biomarkers are already available as screening tools for several neurodegenerative diseases. Despite the attention it has received in the literature, the clinical utility of CSF NFL for AD (and other neurodegenerative diseases) remains questionable.

Beyond YKL-40, VILIP-1 and NFL, other emerging biomarkers include Ng (synaptic protein) and UCH-L1 (neuron-specific cell cycle enzyme). CSF Ng is significantly increased in AD [28]. Initial evidence indicates that this increase is AD-specific [29]. Studies found that CSF Ng could predict progression to clinical AD in MCI patients [30], and, in combination with T-tau, differentiate amnestic AD from AD variants [31]. CSF Ng has also been correlated with CSF T-tau and CSF Aβ_{42} [26], the deposition of tau neurofibrillary deposits [32], and the deposition of β-amyloid plaques [32]. CSF UCH-L1 has been found to increase in AD patients versus controls, patients with other dementias and patients with MCI [11]. UCH-L1 is also implicated in AD pathophysiology as a constituent of neurofibrillary tangles; however, the specificity of UCH-L1 to these tangles is uncertain [11,33]. CSF UCH-L1 also shows positive correlations with CSF P-tau and NSE, both markers of neurodegeneration [11].

Findings of CSF Ng and CSF UCH-L1 are sufficient to prompt further investigation, but insufficient to support clinical application at this time.

Many other potential CSF biomarkers have been investigated (e.g. NSE [34,35], CSF MCP-1, CSF/serum albumin ratios, vascular growth factors, and various forms of sAPP [1,10]) but findings have been insufficiently robust [10].

Frontotemporal dementia

Frontotemporal dementia (FTD) has three clinical subtypes: behavioural variant FTD, semantic dementia and progressive nonfluent aphasia. Overlapping clinical features create difficulties in clinical differentiation. These difficulties are further complicated by FTD's pathological heterogeneity (tau versus TDP-43). To this end, the biomarker with the most promise is CSF P-tau$_{181}$/T-tau; reduced CSF P-tau$_{181}$/T-tau is able to distinguish TDP-43-pathology from tau-pathology [36–38]. The proposed usage of CSF biomarkers in FTD is in distinguishing different FTD variants and distinguishing FTD from AD with frontal presentations. The classical AD triplet may be useful in distinguishing AD from FTD (particularly frontal AD), with the concentrations of Aβ_{42}, T-tau and P-tau in FTD being comparable with controls [24]. Ratios may be more useful, with CSF Aβ_{42}/P-tau$_{181}$ demonstrating the greatest ability to distinguish AD from FTD [39].

Nonspecific markers for FTD have had promising results. Findings of CSF YKL-40 and CSF NFL in FTD are comparable those in AD. CSF YKL-40 is elevated in FTD [40,41]. YKL-40 has also shown positive correlations with CSF T-tau [41], and one study has found CSF YKL-40 distinguished tau-positive patients from controls [42]. Few studies have compared CSF YKL-40 in different neurodegenerative diseases such as AD and FTD. One investigation found CSF YKL-40 levels in AD patients to be significantly higher than those with FTD and controls [42]. NFL has been the topic of considerable FTD research, with different applications suggesting a relationship to disease severity [43] and survival [44]. Similarly to AD, the specificity of CSF NFL to FTD remains debatable; however, one study found that increased CSF NFL levels in FTD patients could distinguish FTD from Lewy body disease, Parkinson's disease (PD) dementia, AD, FTD-associated motor neuron disease (MND); and behavioral variant FTD from progressive supranuclear palsy (PSP) [24]. CSF NFL cannot distinguish different FTD subtypes (clinical or pathological) [36]. It has also been suggested that CSF NFL can be used together with CSF YKL-40 and other markers to distinguish FTD from AD; at least one study has suggested the use of sβAPP ratios with YKL-40 and NFL in separating FTD from AD [40]. Results of CSF sβAPP as an individual FTD biomarker have been inconsistent and further study is required [24,40]. Further assessment of both markers is needed – inconsistencies and uncertainties of disease specificity limit clinical application of CSF YKL-40 and CSF NFL in FTD as well as AD.

Prion diseases

Prion diseases are fatal neurodegenerative diseases caused by the formation and propagation throughout the brain of misfolded proteins known as prions [45]. Typically, physiological PrP^{C} transforms into pathological PrP^{Sc}, producing disease [46]. Prion diseases include Creutzfeldt-Jakob Disease, kuru, fatal familial insomnia and others [45].

Recent studies have focused on measuring levels of prion protein, PrP^{Sc}, in CSF. To this end, a technique called real-time quaking-induced conversion (RT-QUIC) has been developed. The 'quaking' refers to intermittent agitation applied to a 96 well plate as part of the assay [47]. RT-QUIC offers improved sensitivity and specificity in comparison to nonspecific biomarkers [47]. RT-QUIC is able to detect the presence of PrP^{Sc} in biological samples such as CSF [47]. RT-QUIC can detect PrP^{Sc} in human CSF with sensitivities and specificities of 80–96% and 99–100%, respectively [48]; one study has successfully used RT-QUIC in the diagnosis of sporadic prion diseases [49]. RT-QUIC and CSF PrP^{Sc} demonstrate significant potential. Further assessment in clinical settings is still required prior to widespread application.

Prior to RT-QUIC, the focus was on nonspecific CSF biomarkers for prion disease. The most successful nonspecific marker is the 14-3-3 protein. 14-3-3 protein is a neuronal surface protein and CSF biomarker for neuronal cell death [50]. 14-3-3 protein has been useful as a surrogate marker of prion disease, but its specificity and sensitivity remain questionable [50]. The clinical utility of 14-3-3 protein may be improved by combination with other CSF biomarkers (Aβ_{42}, T-tau, P-tau, NFL, etc.); however, research is still preliminary. CSF ratios of Aβ_{42}, T-tau and P-tau have provided inconsistent results in the diagnosis of prion disease [51,52], and correlations between CSF T-tau, 14-3-3 and NFL have been found [53]. CSF T-tau and P-tau may be more useful than CSF Aβ_{42} in prion diseases. Associations with Aβ_{42} are tenuous – one study reported low CSF Aβ_{42} in the absence of Aβ plaques [51]. Again, further work is needed prior to confident clinical application.

Motor neuron disease

Though investigation into the clinical utility of CSF biomarkers in MND is still required, preliminary studies are promising. Of most prominent are neurofilament proteins – specifically NFL and pNFH [25,54]. Increased levels of NFL and pNFH have been consistently observed in CSF samples from patients with MND [25]. Similarly to AD, FTD and others, the specificity of elevated CSF NFL for MND remains uncertain. Furthermore, the absence of increased CSF NFL and/or pNFH is insufficient to rule out an MND diagnosis [25]. Both NFL and pNFH vary with rate and stage of disease progression [25], and are indistinguishable from controls in asymptomatic disease [55]. pNFH is less vulnerable to variations and artifacts in laboratory methods than NFL [56], and may be more useful. CSF TDP-43 might be more helpful [54]. Promising results have been found with lipids [57] and the Sonic Hedgehog protein (a morphogen involved in the development and maintenance of the CNS) [58].

Parkinson's disease

CSF biomarkers for PD have received little attention. Some nonspecific patterns have been identified: increased NFL, decreased α-synuclein and decreased levels of 3,4-dihydroxyphenylacetate (a dopamine metabolite) [59]. Other dopamine metabolites and the application of metabolomics have shown promise pending further investigations [59]. Amyloid, tau and oxidative stress markers may have prognostic use: amyloid and tau markers show relationships with cognitive decline; oxidative stress markers show correlations with duration and severity of disease [59].

Specific markers have been examined with minimal success. Studies have focused on gene markers such as protein deglycase DJ-1. *PARK7*, the gene encoding DJ-1, results in autosomal recessive early-onset PD [60]. Studies of CSF DJ-1 in PD have produced inconsistent results [60,61]. There has been suggestion of combining CSF DJ-1 with CSF T-tau and/or P-tau [62] but this has yet to be pursued. UCH-L1 (encoded by *PARK5*) and ubiquitin have also been investigated, also to limited success [63,64].

Other extrapyramidal syndromes

Extrapyramidal syndromes (EPS) include multiple system atrophy (MSA), corticobasal degeneration (CBD) and PSP. These diseases are of two pathological subtypes: α-synucleinopathies (MSA) and tauopathies (CBD, PSP) [65]. Their diagnosis is complicated by a wide spectrum of overlapping clinical features. CSF analysis may offer improvements to diagnosis. Magdalinou *et al.* found CSF NFL, sAPPα and α-synuclein to be helpful: NFL levels were higher in MSA, CBD and PSP than PD; sAPPα levels were higher in MSA, CBD and PSP than both PD and AD; α-synuclein was lower in MSA than PD [65]. Studies using NFL to characterize tauopathies from PD have been successful while those using P-tau and T-tau to differentiate tauopathies (PSP and CBD) from controls have produced conflicting results [24]. CSF DJ-1 and CSF T-tau/$A\beta_{42}$ have been found to be useful in distinguishing MSA from PD [62,66]. Additionally, T-tau and $A\beta_{42}$ were able to successfully discriminate CBD from the corticobasal syndrome variant of AD [66]. Other markers have shown promise (e.g., YKL-40), but further assessment is required [24]. Due to the limited literature on CSF biomarkers for EPS, clinical application is not yet practical.

Limitations

The primary limitation in this area is the unmet need to address procedural and methodological inconsistencies; inconsistent cut-off values, lack of assay standardization, overlapping case definition – further studies, with larger, representative samples, are required to overcome these obstacles.

Future perspective

Commonly cited future directions are the need for standardized cut-off values and improving methodological consistency across different laboratories. This report would like to expand on these and identify more specific gaps in knowledge that require attention. First, clearer understanding of which biomarkers are useful at screening, diagnosis and/or prognosis is required. Further research into the clinical utility of different combinations of markers and/or ratios for different diseases and clinical settings is also needed. By addressing these gaps in knowledge, it is possible that within the next decade characteristic CSF biomarker profiles may be developed for each neurodegenerative disease – this would be of great benefit to diagnosis and prognosis. Second, more comprehensive understanding of how CSF biomarkers and ratios associate or dissociate with the findings of imaging studies and other investigations is required. Third, there should be closer examination of how the relationships between CSF biomarkers/ratios and the findings of other investigations change with time. CSF biomarkers do not change with time [3] while imaging findings do – further evaluation of different temporal changes, as well as other investigations (blood biomarkers,

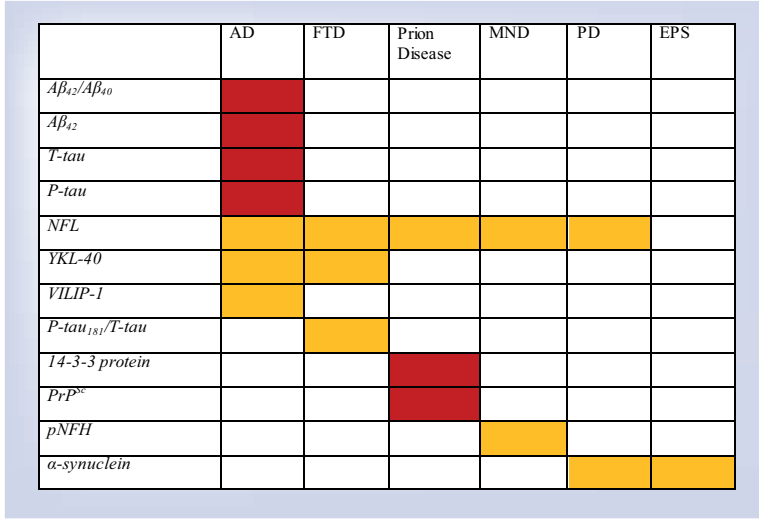

Figure 1. Cerebrospinal fluid biomarkers in neurodegenerative disorders. Red = useful. Gold = promising, requires further study. AD: Alzheimer's disease; EPS: Extrapyramidal syndrome; FTD: Frontotemporal dementia; MND: Motor neuron disease; NFL: Neurofilament light protein; pNFH: Phosphorylated neurofilament heavy chain; PD: Parkinson's disease.

functional and cognitive assessments, etc.) would be beneficial to our understanding of disease staging, progression and prognosis. Promising initial results have been observed with plasma T-tau [10], miRNA [67] and NFL [28], but further investigations are required. Finally, dissociation between ratios and individual markers must also be addressed; it would be beneficial to understand the relationships between individual markers, ratios and other investigations for different neurodegenerative diseases.

We think that more CSF biomarkers will be discovered, and their utility investigated such that a proteomic profile will be developed in every patient; this profile will be useful in diagnosis and prognosis. That is, personalized neurology, such as artificial intelligence using computer algorithms, will streamline diagnosis and treatment of neurodegenerative processes.

Conclusion

CSF biomarkers have been investigated to varying degrees for many neurodegenerative disorders, as summarized in Figure 1. Nonspecific markers such as YKL-40 and NFL have demonstrated potential in AD, FTD, prion diseases, MND, PD and EPS, but further work is needed prior to clinical application. Specific markers have been more successful for AD ($A\beta_{42}$, T-tau and P-tau) and prion diseases (PrP^{Sc}). Ratios such as CSF $A\beta_{42}/A\beta_{40}$ may be superior to their individual constituents, pending further investigation.

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Executive summary

- There are many obstacles to overcome in the diagnosis of neurodegenerative diseases. Identifying characteristic cerebrospinal fluid (CSF) biomarker profiles would be highly beneficial to overcoming these obstacles; however, many gaps and inconsistencies in the literature remain.

Alzheimer's disease

- Currently, CSF biomarkers have the most relevance to Alzheimer's disease (AD). AD is characterized by elevated CSF $A\beta_{42}$, decreased CSF T-tau and decreased CSF P-tau. Ratios such as CSF $A\beta_{42}/A\beta_{40}$ are increasingly prevalent and likely to supersede their individual constituents.
- CSF biomarkers do not show progression, while imaging studies do. CSF biomarkers become abnormal in early disease stages and remain static from then on, promoting their use as screening or diagnostic tools but not as prognostic tools. Further work is required.
- Markers that are not specific to AD disease processes, such as YKL-40, VILIP-1, NFL, Ng, UCH-L1 and others, are becoming more popular but the specificity of their changes to AD, and how these changes relate to classical AD markers, is unknown.

Frontotemporal dementia

- The struggle to identify specific markers for frontotemporal dementia is worsened by the multiple clinical and pathological subtypes of frontotemporal dementia. Some success has been found with tau ratios, for example, CSF P-tau_{181}/T-tau, but further work is required.
- Results have been more promising with combinations of markers such as YKL-40 and NFL. The applicability of these results is limited by unclear case definitions and lack of reproduced findings in the literature.

Prion disease

- Success has been found with the development of a new assay technique called real-time quaking-induced conversion, which is able to detect pathological prion proteins (PrP^{Sc}) in biological samples such as CSF.
- 14-3-3 protein has been a popular biomarker for prion disease; however, its specificity and sensitivity are questionable. Preliminary findings indicate that the utility of 14-3-3 protein may be improved by combining 14-3-3 with other biomarkers (e.g. $A\beta_{42}$, T-tau, NFL, etc.).

Motor neuron disease

- Little attention has been given to CSF biomarkers for motor neuron disease. The most promising are NFL and pNFH. The specificity of elevated NFL and elevated pNFH for motor neuron disease is uncertain.
- Other biomarkers such as lipids and Sonic Hedgehog protein have had promising initial results.

Parkinson's disease

- It has been difficult to identify CSF biomarkers for Parkinson's disease. Some patterns with nonspecific markers such as NFL and α-synuclein have been observed, but further work is required. It has also been suggested that oxidative stress markers, $A\beta_{42}$, T-tau or P-tau could be used.
- There have been attempts to use specific CSF biomarkers for Parkinson's disease-associated genes (e.g. *PARK5*, *PARK7*), with limited success.

Other extrapyramidal syndromes

- This group of neurodegenerative diseases is characterized by differing pathologies (α-synucleinopathies, tauopathies) and overlapping clinical features. Some attempts have been made to identify patterns of both specific and nonspecific biomarkers but findings are yet to be reproduced.

Limitations

- Unclear definitions and inconsistencies surrounding clinical features, cut-off values and assay techniques limit the generalization and clinical applicability of CSF biomarkers in neurodegenerative diseases.

Future perspective

- Clearer understanding of at which stage of diagnosis each biomarker(s) is useful is required.
- Minimal research has been done comparing different combinations of biomarkers and ratios for each neurodegenerative disease. It is important to engage in such comparisons if characteristic biomarker profiles for each disorder are to be developed.
- Understanding of how CSF biomarkers relate to other investigations, and how these relationships change with time, are needed. This would clarify the use of CSF biomarkers at various stages of diagnosis and/or prognosis.

Conclusion

- CSF biomarkers have been investigated to varying degrees of thoroughness for a range of neurodegenerative disorders.
- Nonspecific markers such as YKL-40 and NFL show potential across many neurodegenerative disorders. Profiles of these nonspecific markers in each neurodegenerative disorder have yet to be developed.
- Specific markers have been identified for AD ($A\beta_{42}$, T-tau and P-tau) and prion diseases (PrP^{Sc}).

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Short Communication

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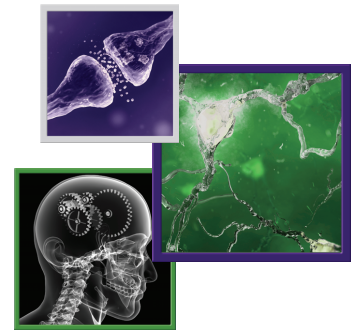
Neurodegenerative Disease Management



Definition and quantification of six immune- and neuroregulatory serum proteins in healthy and demented elderly

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Aim: Blood-based biomarkers related to immune- and neuroregulatory processes may be indicative of dementia but lack standardization and proof-of-principle studies. **Materials & methods:** The blood serum collection protocol as well as the analytic procedure to quantify the markers BDNF, IGF-1, VEGF, TGF-β 1, MCP-1 and IL-18 in blood serum were standardized and their concentrations were compared between groups of 81 Alzheimer's disease patients and 79 healthy controls. **Results:** Applying standardized methods, results for the quantification of the six markers in blood serum are stable and their concentrations significantly differ for all analytes except VEGF between patients diagnosed with Alzheimer's disease and healthy controls. **Conclusion:** Analyzing a panel of six markers in blood serum under standardized conditions may serve as a diagnostic tool in primary dementia care in the future.

Lay abstract: Neurodegenerative processes underlie and account for a large portion of dementia cases in the elderly, with Alzheimer's disease being the most common neurodegenerative disorder. Yet, it is challenging for nonspecialized physicians to assess whether a patients' cognitive impairment originates in underlying neurodegenerative processes. We show that the combination of six biomarkers quantified in blood can indicate the presence of Alzheimer's disease, since these markers are not altered in healthy elderly people. Blood biomarkers indicative of neurodegenerative processes may be useful in the future to compile a personalized diagnostic and treatment plan to assess the basis of, and properly treat, cognitive impairment.

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Keywords: Alzheimer's disease • biomarker • blood serum • clinical diagnosis • cytokines • dementia • inflammation • neurodegeneration

There is considerable evidence to suggest that inflammatory responses may be involved in neurodegenerative cascades leading to dementia [1]. The most common type of dementia, Alzheimer's disease (AD), is a progressive neurodegenerative disorder characterized by cognitive dysfunction and selective neuronal death in the brain.

Also reports show that systemic infection and inflammation affect chronic neurodegeneration [2]. However, these associations are often inconsistent between studies [2–4], thus it is clear that to achieve a reliable description of the role of cytokines and its levels in neurodegeneration, a high degree of methodical standardization is needed [5]. For this reason, there is an increased need for studies that allow for the comparison of findings regarding the concentrations of cytokines in patients' blood samples which is technically demanding. It is well known that

improper design or use of blood collection devices can adversely affect the accuracy of laboratory test results in general [6]. Components from blood collection tubes, such as stoppers, lubricants, surfactants and separator gels [7] can leach into specimens and/or absorb analytes from a specimen. Special tube additives may also alter analyte stability. Because of these interactions with blood specimens, blood collection devices are a potential source of pre-analytical error in laboratory testing. Thus, the optimization and standardization of collecting samples is a first crucial step for the definition of inflammatory markers in neurodegenerative diseases, such as AD. Moreover, the standardization of the quantification method is as important as the blood collection, since the absolute values differ between supplier and technology used for the quantification of the blood born markers [8,9]. Already the selection of the blood fraction for the analysis of inflammatory markers is a nontrivial choice. There are a number of studies proposing blood-based biomarkers for AD, and studies that use different blood fractions yielding noncomparable results [8]. A blood-based algorithm for detecting biomarkers in serum will likely not be the same as one in plasma, and accurate laboratory testing requires an understanding of the complex interactions between collection devices and blood specimens.

For AD, the lack of a sensitive, readily obtainable biomarker is a major impediment to therapeutic development and clinical trial design [10]. Also, as worldwide societies are aging with increasing numbers of affected individuals, a number of disease modifying therapies such as vaccination strategies are under investigation [11], and better diagnostic tools in the biomarker field will be of great benefit. Collecting venous blood for a subsequent biomarker analysis would be a minimally invasive routine practice during medical examination in primary care or clinical settings. Accordingly, this would likely reduce patient burden, time and costs.

At present, the neurobiologically validated diagnosis of AD is based on a combination of multiple modalities, including MRI, cerebrospinal fluid (CSF) biomarkers, clinical memory tests and possibly Amyloid-PET [12,13]. Typically, these diagnostic methods are exclusively available at specialized centers; however, the general practitioner is mostly the first point of contact for a patient presenting with memory problems. All diagnostic procedures mentioned above are time-consuming, expensive and can be stressful for the patient. In particular, the collection of CSF via a lumbar puncture is often perceived as an invasive practice. This limits the utility of CSF biomarkers in clinical practice, despite their substantial sensitivity and specificity [14]. Diagnostic tools that allow for a timely, neurobiologically validated diagnosis of AD may lead to an earlier and more effective treatment, as well as a closer monitoring of the disease.

It has been reported that proteins associated with the adaptive and innate immune system are altered in neurological diseases [15–17], and specifically in AD [18,19]. Taking into account these general contextual considerations and results from our own practical and technical evaluations, we have identified the following six immune- and neuroregulatory proteins to be analyzed: BDNF, IGF-1, VEGF, TGF-ß1, MCP-1 and IL-18. We describe these markers as being robustly and reproducibly measurable at reasonable levels in serum samples. We have chosen serum as the target blood fraction as it has been shown to be most suitable for immunodetection of neurotrophins and inflammatory cytokines in general [20].

Materials & methods

Study participants

Patients and controls were enrolled in a study that started in 2014. The 79 healthy elderly (controls) between 60 and 70 years of age included in the study were selected from a cohort recruited from a healthy blood donor population of a Blood Transfusion Center.

A total of 81 AD patients (AD) between 57 and 95 years of age (ten patients older than 90 years) were selected from a cohort recruited from the geriatric department of a hospital. Patients had previously been diagnosed with probable AD by the referring general practitioner.

After admission to the hospital, the AD diagnosis was based on the NINCDS- ADRDA Alzheimer's Criteria [21]. Exclusion criteria included having a history malignant diseases, severe renal insufficiency, HIV infection, acute infections, autoimmune diseases, neurodegenerative diseases other than AD, acute stroke and history of stroke with residual symptoms, delirium, substance abuse and psychotic disorders. A cerebral computed tomography was performed on all AD patients, MRI and/or PET for aid to the clinical diagnosis have not been performed. The diagnostic regimen included a detailed anamnesis, including disease history and family history for all patients. A standard neuropsychological examination was performed with all patients. This included the Mini Mental State Examination (MMSE) and the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) for a subset

Table 1. Commercial ELISA Kits, sample dilutions and assay specifications.

Name	Catalog No.	Manufacturer	Sample dilution	Sensitivity (LLOQ)	Interassay variability (CV)	Intra-assay variability (CV)
Human free BDNF immunoassay	DBD00/SBD00	Bio-Techne GmbH (R&D)	1:20	20.0 pg/ml	9.0%	5.0%
Human IGF-1 immunoassay	DG100/SG100	Bio-Techne GmbH (R&D)	1:100	26.0 pg/ml	7.9%	4.0%
Human VEGF immunoassay	DVE00/SVE00	Bio-Techne GmbH (R&D)	Undiluted	9.0 pg/ml	7.3%	5.4%
Human TGF-β1	DB100B/SB100B	Bio-Techne GmbH (R&D)	1:40	4.61 pg/ml	8.3%	2.4%
LEGEND MAX™ human MCP-1/CCL2	438807/438808	Biolegend	Undiluted	1.6 pg/ml	3.9%	6.2%
Human IL-18 ELISA Kit	7620	Bio-Techne GmbH (R&D)/MBL	1:5	12.5 pg/ml	7.5%	8.0%

CV: Coefficient of variation; LLOQ: Lower limit of quantification.

of patients. The average MMSE score of AD patients was 18.0 out of 30 points, the MMSE has not been applied in the control group.

The present study was approved by a local ethics committee. Written informed consent was obtained from all study participants.

To estimate the error in sensitivity and specificity for our algorithm-based classification based on our data as a function of sample size, we have used a bootstrapping resampling technique. Calculations yielded that we need about 40 subjects in both the patient and healthy control groups in order to achieve the targeted selectivity and sensitivity with confidence, and that up to 80 patients would give an improvement, but little is gained beyond this sample size (Supplementary Data). We thus conclude that with 81 patients and 79 controls the study is sufficiently powered.

Blood sampling

Blood samples were taken from the median cubital vein and collected in neutral 7.5 ml S-Monovette® without additives (Sarstedt, Nürnbrecht, Germany). The samples were allowed to clot for 60 min and protected from light and heat before being centrifuged at room temperature for 10 min at 2000 × *g* to segregate the serum. The obtained serum was stored in aliquots at -80°C until analysis. Hemolytic, lipemic or icteric samples were excluded, as such quality deficits can potentially influence assay results. For the substudy on different blood collection tubes, blood from 89 patients was sampled into neutral 7.5 ml S-Monovette tubes without additives and at the same time into serum gel-tubes (Sarstedt).

Measurement of BDNF, IGF-1, VEGF, TGF-β1, MCP-1 & IL-18

The protein levels of the six biomarkers were assessed using ELISAs. After a thorough literature research and testing of commercially available ELISA kits approved for investigation use only (IUO), we chose six assays that provided stable and reproducible results (Table 1). The quantifications were performed according to the manufacturer's instructions with the application of the specific sample dilutions shown in Table 1. These sample dilutions were optimized and established for each biomarker by our work group. All assays are linear within the dynamic range of the respective assay, specifications on assay variability and sensitivity are given in Table 1. In addition to the assay-specific protein standards, an internal control was measured within each assay run. Assay results were quantified using a microplate reader (ELX808, BioTek Instruments Inc., Winooski, VT, USA). The protein concentrations for BDNF, IGF-1 and TGF-β1 are expressed in ng/ml, and in pg/ml for VEGF, MCP-1 and IL-18. For the substudy on different blood collection tubes, samples from the two collection tubes were analyzed in parallel.

Statistical analysis

Data of each single biomarker in the two gropus were analyzed using unpaired t-tests with Welch's correction. p-values of ≤ 0.05 were considered significant.

For the computation of the algorithm to predict the group of a given patient, we used a logistic regression model on input data (data from all six variables) that has been discretized by a K-Means discretizer into three intervals.

Table 2. Comparison of serum protein levels of the six analytes using different blood collection tubes.

Analyte	Type of blood collection tube	Average value (SD)	Coefficient of variation	Correlation coefficient
BDNF (ng/ml)	Neutral tubes	13.21 (9.06)	49.52%	0.688
	Tubes with gel	18.72 (8.03)**		
IGF (ng/ml)	Neutral tubes	76.92 (29.74)	10.31%	0.977
	Tubes with gel	75.15 (30.68)*		
VEGF (pg/ml)	Neutral tubes	443.35 (408.40)	39.54%	0.862
	Tubes with gel	598.06 (470.17)**		
TGF (ng/ml)	Neutral tubes	29.45 (33.27)	215.03%	0.665
	Tubes with gel	27.39 (38.31)*		
MCP (pg/ml)	Neutral tubes	89.41 (78.93)	31.04%	0.928
	Tubes with gel	117.55 (79.29)**		
IL-18 (pg/ml)	Neutral tubes	287.22 (165.36)	9.64%	0.988
	Tubes with gel	293.04 (171.58) n.s.		

*$p \leq 0.05$; **$p \leq 0.001$.
n.s.: Not significant; SD: Standard deviation.

The result is a 1D value for each dataset and a general classifier that best separates group. The model is trained and then validated with half of the datasets in each group, respectively. Receiver operating characteristic (ROC) plots were obtained from the data used for validation. Data analysis and plots were computed using Python (version 2.7.9; Python Software Foundation, OR, USA), the Python sciPy package and the Python matplotlib library for generating plots and images. For the sub-study on different blood collection tubes, average values and standard deviations (SD) were calculated for each value for the different tubes (Table 2). To compare the values obtained with the different tubes, we calculated coefficients of variation as well as Pearson's correlation coefficients (n = 89, except for TGF-β1, for TGF-β1 n = 20 samples were below the limit of detection when using tubes with gel). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ for all analyses.

Results

Comparison of BDNF, IGF-1, VEGF, TGF-β1, MCP-1 & IL-18 concentrations in serum using different blood collection tubes & stability of the results

When analyzing the levels for the six analytes in serum collected in neutral tubes versus serum collected in tubes containing gel (n = 89), we found that the average values largely differ for most analytes and that the coefficients of variation are high (Table 2), except for IL-18. For BDNF, VEGF, TGF-β1 and MCP the coefficients of variation are above 30%, thus the usage of blood collection tubes largely influences the values obtained, also for TGF-β1 using tubes containing gel was extremely low, often close to or even below the limit of quantification of the respective assay. We thus conclude that the gel used in tubes interacts and/or binds to the analytes and thus continued the study, using solely neutral tubes without separating gel.

To analyze the stability and reproducibility of the results for each analyte from serum collected in tubes without gel, we analyzed aliquots of the same serum pools (n = 2 serum pools) and aliquots of individual sera (n = 13) in a large number of analyses over the time span of 7–15 months (4–13 independent analyses, depending on the amount of the serum available). The absolute values for each analyte were highly stable in a given sample over all measurements (Supplementary Data 2).

Characteristics of AD patients & controls

In this study, 81 AD patients (27 males and 54 females) aged 57–95 years (mean age of 81.9 years) and 79 (51 males and 28 females) aged 60–70 years (mean age of 64.5 years) were included. The mean age was significantly different between groups ($p < 0.001$; Table 3). The mean MMSE score of AD patients was 18.0 out of a possible 30 points, and the majority of patients could thus be classified as having moderate dementia. Study participants in the control group have not been further characterized regarding cognitive measures.

To control for bias regarding the unbalanced distribution of females in males between groups and to adjust for the age difference between group, we calculated an algorithm with 30 patients of each group that have been

Table 3. Characteristics of Alzheimer's disease patients and controls.

Characteristics	Control	Dementia/AD
n	79	81
Age (years; SD)	64.5 (2.7)	81.9 (7.8)*
Gender ratio (m/f)	51/28	27/54
MMSE (SD)	n.d.	18.0 (4.3)

*$p \leq 0.001$.
AD: Alzheimer's disease; f: Female; m: Male; MMSE: Mini Mental State Examination; SD: Standard deviation.

Table 4. Serum protein levels of six biomarkers in control subjects and dementia/Alzheimer's disease patients.

Group	N	BDNF (ng/ml)	IGF-1 (ng/ml)	VEGF (pg/ml)	TGF-β1 (ng/ml)	MCP-1 (pg/ml)	IL-18 (pg/ml)
Control	79	25.0 (± 7.2)	95.4 (± 24.1)	409.6 (± 225.1)	31.4 (± 8.4)	126.4 (± 61.5)	238.6 (± 101.9)
AD	81	11.1 (± 7.9)***	66.6 (± 32.3)***	465.7 (± 581.1)	20.9 (± 18.2)***	79.3 (± 130.4)**	324.8 (± 282.9)*

Data are expressed as mean (± standard deviation).
*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.
AD: Alzheimer's disease.

sex-matched (15 males and 15 females in each group) and adjusted for age (mean age [±SD] controls: 67.2 [1.4] years, mean age AD-group: 74.3 [5.7] years; $p < 0.001$).

Levels of BDNF, IGF-1, VEGF, TGF-β1, MCP-1 & IL-18 in serum of controls compared with AD patients

First, each biomarker was analyzed separately to assess its individual change in AD patients compared with controls. Unpaired t-tests with Welch's correction revealed significant mean differences in BDNF ($t[157] = 11.61$; $p \leq 0.001$), IGF-1 ($t[148] = 6.392$; $p \leq 0.001$), TGF-β1 ($t[113] = 4.678$; $p \leq 0.001$), MCP-1 ($t[115] = 2.937$; $p \leq 0.005$) and IL-18 ($t[101] = 2.575$; $p \leq 0.05$) serum levels between patients and controls, VEGF ($t[104] = 0.809$ not significant [n.s.]) serum levels were not significantly different between groups. In patients, the mean protein levels of BDNF (Figure 1A), IGF-1 (Figure 1B), TGF-β1 (Figure 1D) and MCP-1 (Figure 1E) were significantly reduced compared with controls. In contrast, mean IL-18 (Figure 1F) serum levels were significantly increased in patients (Table 4).

Next, we tested whether the combination of these proteins would allow to accurately classify each study subject as belonging to either the patient or control group, as each protein alone has limited classification value due to its within-group variability. This algorithm-based classification analysis revealed that the combination of the six serum proteins could identify AD patients with 76% sensitivity and controls with 95% specificity. The analysis of the accuracy of the biomarkers combined to identify subjects who have the disease (sensitivity) and subjects who do not have the disease (specificity) was evaluated using the algorithm-based values for group separation (Figure 2A). The receiver operating characteristic (ROC) analysis (Figure 2B) based on our algorithm-based classification confirms these values.

As the subjects have not been sex-matched between groups and the mean age was significantly different between groups, reduced sex-matched datasets were selected, adjusted for age. Still, patients and controls could be identified with 80% sensitivity and 80% specificity (Figure 3).

Discussion

In the presented study, we describe the identification of six blood proteins (BDNF, IGF-1, VEGF, TGF-β1, MCP-1 and IL-18) as being robustly and reproducibly measured in serum samples using commercially available ELISA kits; and, except for VEGF, as significantly differing in their serum concentrations between AD patients and controls. Our results do not contradict but rather are in line with the hypothesis that a dysfunctional immune system plays a central role in the pathogenesis of neurodegenerative diseases, such as AD [22]. While we do not show a direct correlation with microglial activity, the data presented here support the notion that immunoregulatory proteins might play a role in AD to control to certain microglia [23], especially since TGF-β1 has been shown to be a major factor in the control of inflammatory processes, involving microglia [24]. It has been proposed that ongoing formation of Aβ and positive feedback loops between microglia, cytokines and amyloid precursor protein processing compromise the regulation of the inflammatory process. In the course of a neurodegenerative disease, neurons die

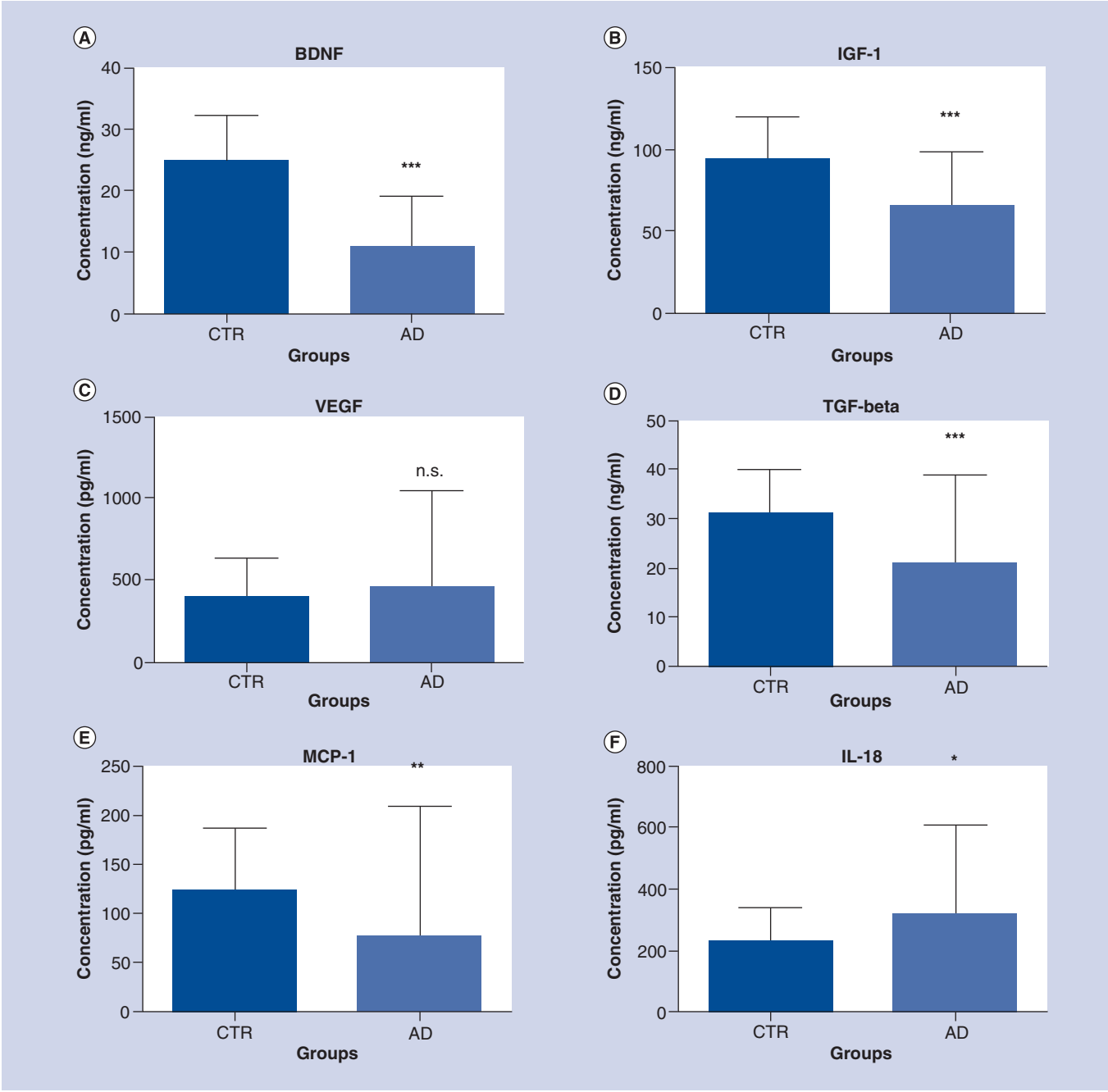

Figure 1. Serum protein levels of the six analytes. Depicted are the group levels for the concentrations of the single analytes in blood serum for the groups of controls and Alzheimer's disease patients. **(A)** BDNF, **(B)** IGF-1, **(C)** VEGF, **(D)** TGF-β1, **(E)** MCP-1 and **(F)** IL-18 data are depicted as mean ± standard deviation.
*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ compared with CTR.
AD: Alzheimer's disease; CTR: Control group.

to a large extent because of a local chronic inflammation. This inflammation might indeed be caused by amyloid or tau deposits but could also have alternative origins [22,25].

The six biomarkers identified and quantified in this assay relate to the peripheral and innate immune system and directly correlate to the activity of the immune system and brain function [26]: BDNF regulates age-associated pathways as well as neuronal plasticity [27], TGF-β1 regulates neurogenesis [28] and IGF-1 is a neuroprotective factor whose role in AD is still controversial [29]. Also VEGF has neuroprotective effects and lowered levels in AD have

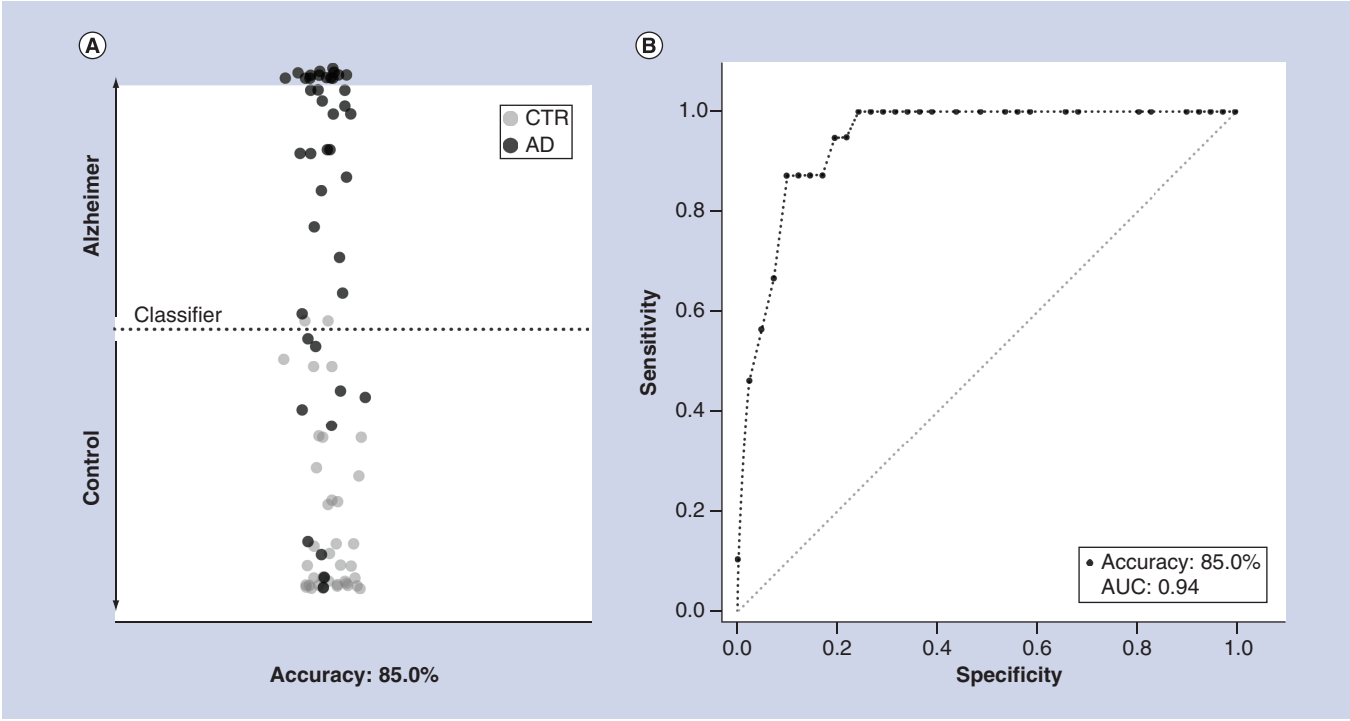

Figure 2. A visualization of the results from the logistic regression model to separate Alzheimer's disease patients from control (control group). (A) The plot shows the value (y-axis) obtained after applying the logistic regression model on datasets from AD and CTR, each dot represents the data from a single patient/control. The classifier indicates the best separation between groups. The arrows on the left indicate the group according to the algorithm. Data are 1D, the values in the second dimension (x-axis) are only varied for visualization purposes. **(B)** A receiver operating characteristic analysis based on the score calculated applying the algorithm to combine all six markers into a single score.
AD: Alzheimer's disease; AUC: Area under the curve; CTR: Control group.

previously been described [30]. Although we do not see a statistical significance between groups for VEGF, we do find rather low VEGF levels in the majority of AD patients, and about 25% of AD patients with largely increased VEGF levels (data not shown; see the high SD in Figure 1C), potentially indicating cerebral amyloid angiopathy in some AD patients [31]. Furthermore, the levels of MCP-1, a factor regulating the inflammatory response, depend on the stage of the disease [32]: increased MCP-1 levels were previously found in MCI and mild AD, but not in severe AD patients as compared with controls. IL-18 directly links to pathophysiological pathways in AD since it can alter the processing of Alzheimer precursor protein [33], thus directly influencing Aβ levels. Also, the IL-1-β/IL-18 regulates the inflammasome pathway and thus could contribute to neuroinflammatory processes in AD [34].

In general, with age, the immune system gradually deteriorates, a process called immunosenescence [35]. The neuroprotective environment is due to factors secreted by the immune cells (B and T cells), including chemokines, cytokines and growth factors. It is known that the development of Alzheimer's disease neurobiologically starts more than 20 years before the onset of clinical symptoms [36], and this may be related to the emergence of immunosenescence. Since with age, the immunological system becomes less effective, protein deposits are not removed anymore, inflammation increases and neurons die [37]. Whether these effects play a role in the disease course of AD needs to be clarified in studies involving patients at different disease stages, which is currently ongoing.

Results presented here suggest that in AD, the adaptive and innate immune systems are compromised. Together with the age-related senescence of microglia, characterized by exaggerated pro-inflammatory response and impaired phagocytosis and chemotaxis, this may further promotes neurodegeneration and impaired neurorepair [38]. Nevertheless, the markers described here might be used as biomarkers not only for detecting overt AD when symptoms are clear, but also in early-stage AD, as such detrimental inflammatory processes may precede dementia symptoms and be detected earlier [1]. This is of particular interest when considering that an effective AD treatment might have been impossible until now, as the disease may have already progressed to a stage of irreversible neuronal damage between the appearance of the first clinical symptoms and the time of AD diagnosis. An ideal biomarker test reliably

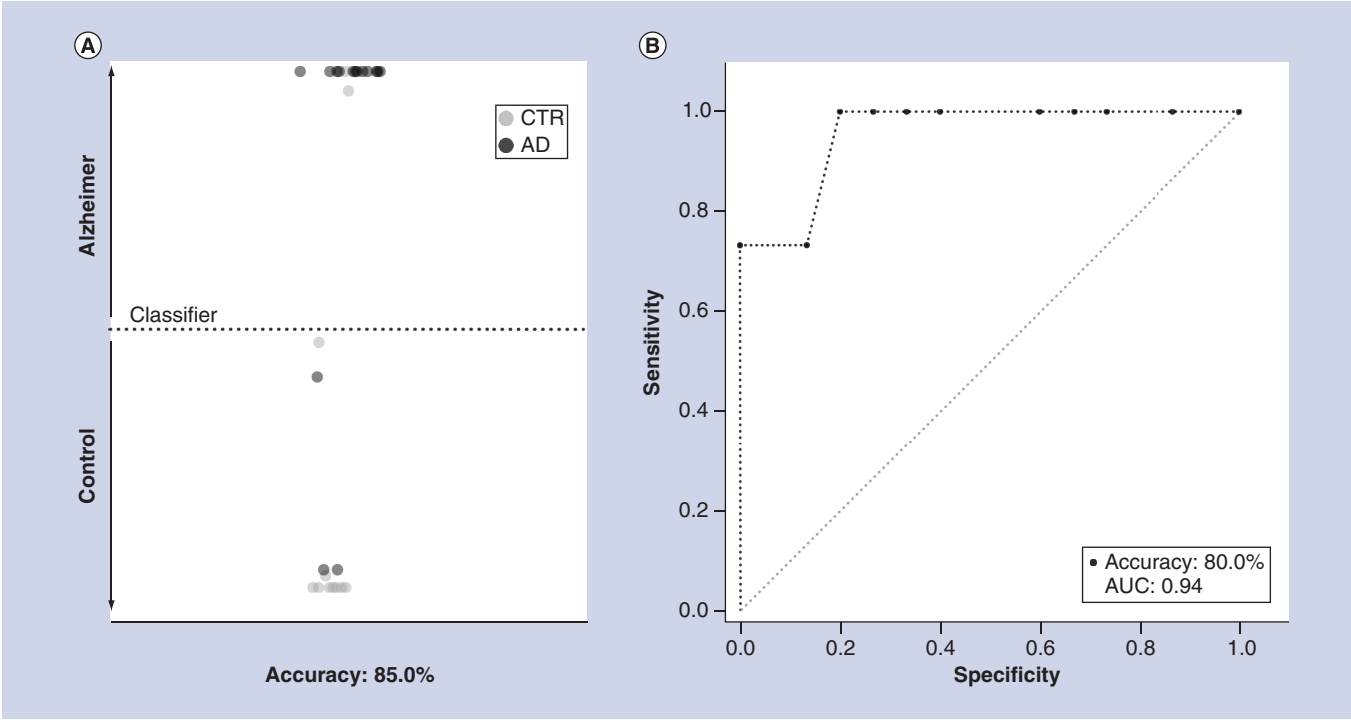

Figure 3. A visualization of the results from the logistic regression model to separate Alzheimer's disease patients from control (control group) in a sex-matched and age-adjusted dataset. (A) The plot shows the value (y-axis) obtained after applying the logistic regression model on datasets from AD (n = 30) and CTR (n = 30), each dot represents the data from a single patient/control. The classifier indicates the best separation between groups. The arrows on the left indicate the group according to the algorithm. Data are 1D, the values in the second dimension (x-axis) are only varied for visualization purposes. **(B)** A receiver operating characteristic analysis based on the score calculated applying the algorithm to combine all six markers into a single score.
AD: Alzheimer's disease; AUC: Area under the curve; CTR: Control group.

indicates important processes of the underlying pathology in any stage of AD and at the same time substantiates the clinical diagnosis. According to the proposed model of AD [39], various biomarkers become abnormal in a temporally ordered manner. First, changes in β-amyloid-related biomarkers appear early in the disease, even before clinical symptoms manifest, reaching a plateau by the time clinical symptoms emerge. Therefore, early diagnosis of AD patients at the presymptomatic stage improves the chances of an effective intervention, which may prevent or delay the onset of the disease by years [40].

All study participants in the study presented here were Caucasians, in other words, they were white-skinned and of European origin. In a recently published study with more than 1000 multiethnic participants [10] it has been shown that ethnicity does not influence the results of blood tests targeting cytokines, we thus rather exclude a bias toward Caucasian study participants in our study that impacts the results.

Although the study presented here supports the use of the six biomarker-panels merely for a distinction between asymptomatic subjects and symptomatic dementia patients, further clinical studies should include study cohorts from different clinical settings and involve AD patients at different disease stages, especially at early disease stages. Also, further studies should include a control group as well as disease controls age-matched to the groups of patients, since groups in the study presented here were not age-matched. In addition, since the controls did not have a neuropsychological examination, we cannot exclude that some individuals in the control group could have early stage dementia. Currently, studies controlling for all these factors are ongoing. As in this study, we aim to present a proof-of-principle for the applicability of the test to distinguish clearly demented patients from controls, we assume that a possibly limited number of patients with early-stage dementia in the control group do not have a considerable effect in the data analysis.

Conclusion

We conclude that following standardized protocols, the reproducible quantification of immune- and neuroregulatory blood serum proteins is possible in healthy controls and in AD patients. This brings up the possibility to implement these proteins as biomarkers for AD. The protein set defined here might be a valuable candidate for further analytical and clinical validation. Since neurobiologically any neurodegenerative disorder develops slowly even long before onset of cognitive symptoms, the markers described here should be further compared with the well-defined classical AD biomarkers tau, p-tau and the amyloid peptides 40 and 42, which are implemented in the definition of AD in a research context [41].

Future perspective

Large efforts are currently dedicated to the development of blood biomarkers for AD. At present, the markers reflecting disturbed amyloid metabolism and neuronal death received most of the attention, not only in blood, but also in the CSF.

Since the quantification of these markers in CSF has technically recently reached a high standard, most probably the first markers to be reliably quantified in blood will also be amyloid peptides or markers indicating neuronal death such as Tau proteins or other proteins related to the neuronal cytoskeleton such as neurofilament light chain.

However, these markers only reflect a restricted aspect of the disease. As the course of the neurodegenerative diseases is regulated by numerous additional cellular pathways such as immunological processes, autophagy, apoptosis, neurogenesis and neuroprotective processes, markers reflecting these aspects will evolve, especially for disease staging and biomarker-driven identification of patients at risk to develop a neurodegenerative disorder. The latter will be especially important, since the recent failures of clinical trials to treat AD in symptomatic stages indicate that new treatment options need to be implemented as early as possible in the disease course, ideally before onset of clinical symptoms.

Yet, the standardization of quantification and the definition of cut-off values for blood biomarkers to achieve a reliable diagnosis is one of the most important aspects in blood biomarker research and major efforts will go into the development of technical guidelines and operating procedures.

Summary points

- The standardization of a blood collection protocol permits a reliable quantification of neuroinflammation-related and neuroregulatory molecules in blood serum.
- The gel commonly used in blood serum tubes interacts and/or binds to the analytes and thus largely influences absolute values of the quantified analytes.
- Results for the quantification of neuroinflammation-related and neuroregulatory molecules in blood serum are stable and reproducible when adhering to strict standardized pre-analytical and analytical procedures.
- Average concentrations of the molecules BDNF, IGF-1, TGF-β 1, MCP-1 and IL-18 in blood differ between a group of healthy elderly and a group of AD patients AD, while VEGF levels do not differ significantly between groups.
- The level of every single analyte alone does not sufficiently identify patients as AD patients or controls.
- We developed an algorithm-based classification including values from all six markers to separate groups of AD patients and controls.
- We applied an algorithm-based classification yielding the result that the combination of the six serum proteins could identify AD patients with 76% sensitivity and controls with 95% specificity in our cohorts.
- Further validation studies may indicate whether the six proteins may prove to form a characteristic signature in the serum of AD patients indicating neurodegenerative processes.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: https://www.futuremedicine.com/doi/suppl/10.2217/nmt-2019-0003

Author contributions

O Gunter and JP Sigle recruited and examined patients; C Weinert performed experiments; J Kallarackal performed statistical analyses; CG Schipke, P Scotton, A Finzen, AF Heidl and D Kabelitz planned the study and analyzed data; CG Schipke, P Scotton

and AF Heidl analyzed data and wrote the manuscript. All authors worked together on data interpretation, critically revised the manuscript, agree to be accountable for all aspects of the work and finally approved the published version of the manuscript.

Disclosure

High-Tech Gründerfonds, Germany had no role in study design, in the collection, analysis and interpretation of data, in the writing of the report, in the decision to submit the article for publication.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The present study was approved by a local ethics committee. Written informed consent was obtained from all study participants.

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Translating blood biomarkers into the clinic for neurodegenerative diseases with Henrik Zetterberg

Written by: Sharon Salt, Editor

Tremendous efforts have been made in the last few years with regards to blood biomarkers for neurodegenerative diseases. From the discovery of serum neurofilament light as a sensitive biomarker for neuronal loss to advancements in imaging techniques, the field is moving forward at an extremely quick pace.

In this video interview, we spoke with Henrik Zetterberg at the Wolfson Closing Symposium on Intervening in Neurodegeneration (19 June 2019, University College London, UK) to find out more about the current status of the field. Henrik spoke to us about how the challenges surrounding replication and reproducibility might be addressed, in addition to discussing the issues around specificity with serum neurofilament light. Take a look at our questions below and watch the video to hear his responses..

Summary

00:05 – Please could you introduce yourself and tell us about your current research focuses?

00:22 – Can you tell us about some of the most promising new areas of research in the search for blood biomarkers for the pre-clinical phase of Alzheimer's disease?

01:36 – One of the biggest challenges surrounding blood-based biomarkers for neurodegenerative disease is the lack of replication and reproducibility. What could be done to overcome these obstacles?

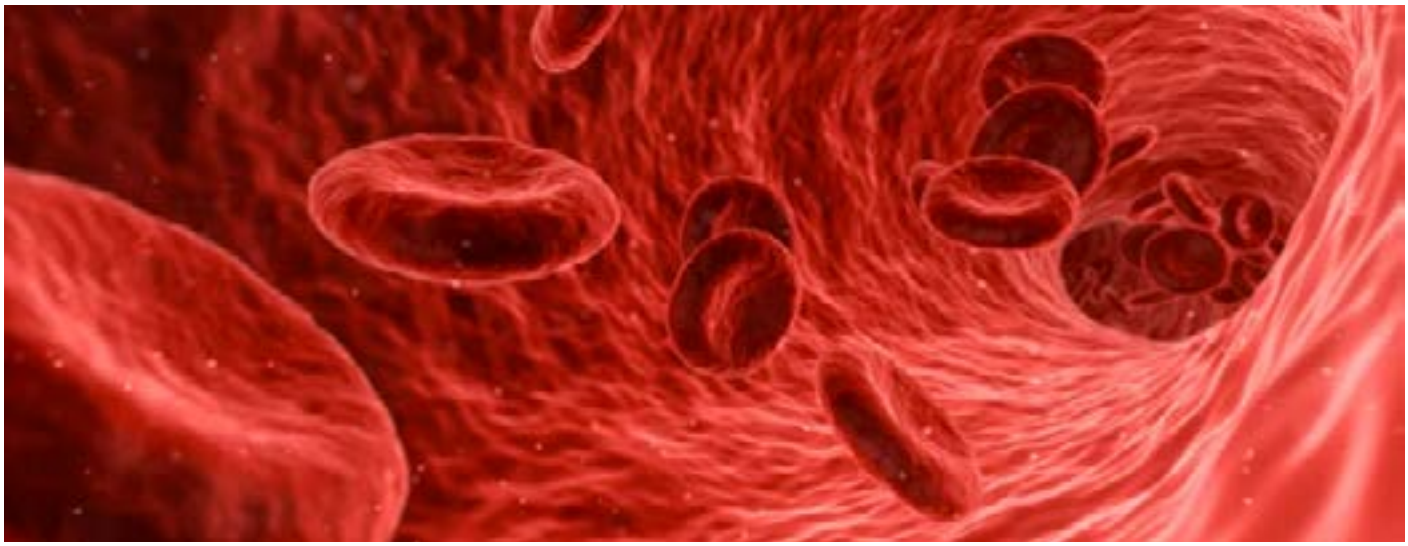
03:19 – In relation to serum neurofilament light, how might the challenge of specificity be addressed?

04:51 – What further steps are required for the clinical translation of blood-based biomarkers?

Neuro Central

Blood biomarkers for neurological disease research: current status, challenge and future outlook

Written by Liu Shi (Department of Psychiatry, University of Oxford, UK)



Biomarker discovery for neurological diseases is burgeoning due to the advances in technologies that permit molecular measures taken from the brain, cerebrospinal fluid (CSF), plasma, saliva, urine and so on. Biomarkers could be biochemical changes (proteins, metabolomics and lipids), genetic alteration or changes in structural or functional features. They could help the diagnosis and detect the progression of these diseases, referred to as diagnostic and prognostic markers, respectively. Furthermore, biomarkers could help to measure the efficacy of the treatments, known as predictive markers.

The importance of biomarkers in neurological diseases should not be underestimated, particularly considering the large social and economic burden presently attributed to these diseases. This article describes the current status of blood-based biomarker research in neurological disorders, particularly in Alzheimer's disease (AD), as well as addresses the main challenges and future direction of this field.

Current status of blood biomarker development in neurological disease.

CSF is one of the main resources for biomarker development for neurological diseases given that CSF surrounds the brain and spinal cord. For example, the levels of amyloid and tau in CSF have been used in diagnosing AD [1]. However, these measures are challenging because of invasiveness, cost and limited availability [2,3]. In part due to these limitations, increasing numbers of studies have attempted to find biomarkers in blood; a tissue that is easily accessible and suitable for repeated measures throughout the disease course or over the time-frame of an interventional study. Previous reviews have summarized much of this growing research effort to find biomarkers for AD diagnosis [4–11], as well as for other neurological disease [12–16]. Though it looks promising, it is important to note that there is no blood-based biomarker used in clinic for neurological disease diagnosis yet, mainly due to the lack of replication.

Challenge: why do most blood biomarkers fail replication?

Although a number of plasma biomarkers of diagnosis, disease severity and progression have been identified, a key concern for the field has been the lack of reproducibility of these results. The reason for such non-reproducibility might be caused by the heterogeneity of the disease itself as well as the complexity of blood. Furthermore, a number of other major factors could also lead to the failure of replication. They include pre-analytical sample handling, analysis of different blood fractions, use of different analytical platforms and inappropriate statistical analysis [17]. For example, Huebinger et al. compared the concentration of

100 proteins in matched samples of serum and plasma from 39 AD patients. They found that only 40 proteins were highly correlated between blood fractions while the remaining proteins were only moderately or weakly correlated, including some of considerable interest in AD [18].

Solutions: better study design, standardization and multimodal biomarkers

To address the challenges of blood-based biomarker development, several aspects should be taken into consideration. First is the study design. Most biomarker studies use a case-control study design, namely the cases included in this design had established disease. Diagnosis of established neurological disease is not difficult, whereas diagnosing the disease in the very early stages is challenging. In contrast to case-control approaches, phenotype-based approaches are more reliable. The latter approach aims to find biomarkers related to early pathology of neurological disease. Let's take AD for an example: one of the key early pathologies of AD is amyloid deposition in brain. Therefore, finding blood biomarkers relating to brain amyloid deposition could help the early diagnosis of AD.

Second is the standardization of measurement of these biomarkers. Since all these factors could influence the results, at the very least such parameters should be recorded, and standardization of methodologies would be desirable. Currently, an international working group led by O'Bryant has provided the initial starting point for such guidelines and standardized operating procedures [19]. It is only through cooperation and collaboration of all academic societies that we will make progress.

Last but not the least is the usage of multimodel biomarkers. The complexity and the long prodromal phase of neurological disease demands the combination of different kinds of biomarkers together including metabolite or lipidomic markers, genomics including miRNAs and epigenetic changes, transcriptomics and other markers. The challenges of data management and analysis of any one of these approaches will be considerable but the real value might emerge when combinatorial analysis becomes possible.

In conclusion, integrated, collaborative efforts are needed to standardize a multimodal set of biomarkers with dynamic range, optimize the methods, and conduct sufficiently powered, multi-site studies so that these tools progress rapidly to clinical qualification by regulatory agencies. It is now critical to set standards for validation in the field so that promising biomarkers can be applied in clinical trials and clinical practice.

Disclaimer

The opinions expressed in this feature are those of the author and do not necessarily reflect the views of Neuro Central or Future Science Group.

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Emerging concepts in dementia research: using blood spectroscopy as a diagnostic tool

Written by Mary Paraskevaidi (University of Central Lancashire, UK; Imperial College London, UK)

Mary Paraskevaidi is a Postdoctoral Research Associate at the University of Central Lancashire (UK) and Imperial College London (UK) and is working in the field of disease investigation and diagnosis with the application of analytical chemistry techniques. She completed her PhD in Biomedical Sciences at Lancaster University (UK) and the University of Central Lancashire, during which she developed an interest in translational research and neurodegenerative disorders.

In this interview, Mary speaks to us about her research using spectroscopic methods to detect Alzheimer's disease (AD) in blood plasma – a technique that she mentions has demonstrated accurate diagnosis of not only AD patients in advanced disease states, but also those with very early symptoms

Could you tell us more about the talk you were supposed to be giving at the Alzheimer's Research UK Conference (17–18 March 2020, Newport, UK) on emerging concepts in dementia research?

It was with great sadness to find out that ARUK 2020 had to be cancelled, as it would have been a great opportunity for experts in the Alzheimer's field to come together and share their research. However, growing concerns over COVID-19 rendered its cancellation necessary and people should really act responsibly and take the right precautions against this unprecedent situation.

With regards to the talk I would give at the conference, we have performed a substantial amount of work in the field of AD in an effort to develop a new blood test that could be utilized for an accurate and timely diagnosis.

AD is a complex, multifactorial disorder with a number of genetic, epigenetic and environmental factors playing an important role in its development and progress, ultimately leading to premature neuronal death.

With a rapidly aging population across the world, AD is expected to affect approximately 75 million people in 2030, which necessitates, now more than ever, an early and accurate diagnosis. An early diagnosis is expected to expedite the recruitment of patients into clinical trials as well as the prescription of promising drug candidates, before severe brain damage occurs.

Currently, an accurate diagnosis remains challenging and is based on clinical presentation as well as imaging and biofluid biomarkers, such as those derived from cerebrospinal fluid (CSF) and blood. Specific CSF markers have been shown to have optimal diagnostic accuracy, however, the nature of their collection is invasive, which limits their wider use in routine clinical practice.

Over the last decade, and with the advent of sensitive analytical technologies, there is emerging evidence that blood could serve as an information-rich sample for AD diagnosis. Different blood biomarkers, such as amyloid-β, tau and neurofilament light, have shown promise.

What we propose as an alternative approach is the use of a spectroscopic technique, which can be used to analyze a biological sample and provide information for a range of different biomolecules (e.g., proteins, lipids and carbohydrates), rather than focusing on individual biomarkers. This approach is anticipated to be beneficial for more accurately detecting multifactorial diseases, such as AD or cancer.

In summary, in my talk I would discuss the use and advantages of such technologies in the field of AD and also present some of our results on this.

Part of your work focuses on the detection and early diagnosis of neurodegenerative diseases using biological fluids – can you tell us more about this? What advancements have been made in the field?

There is an intensive effort from numerous research groups to develop effective AD tests using biological fluids. As already mentioned, the use of CSF – even though clinically used in many parts of the world – is not ideal, as it requires an invasive and painful sampling procedure that is rarely performed by general practitioners. Therefore, a lot of focus has been placed instead on the study of blood as a minimally invasive and easily collected alternative sample.

As part of my research, I have looked into spectroscopic methods with regards to detecting AD in blood plasma. Spectroscopy exposes a sample to electromagnetic radiation, which causes characteristic chemical motions of the biomolecules within the sample, therefore allowing generation of specific patterns, known as 'biological fingerprints'. Altered 'fingerprints' emerging from an existing disease can thus be used as a diagnostic tool when compared to those from healthy individuals.

Blood spectroscopy has been used in our studies to compare individuals with AD, as well as other dementias, to healthy volunteers serving as the study's controls. The diagnostic accuracy (sensitivity and specificity) achieved by this test was comparable to, if not better than, other currently used tests, while at the same time it has the advantage of being inexpensive, fast and simple as a method, without the need for laborious assays or costly reagents.

It is also worth noting that we managed to accurately diagnose not only AD patients in an advanced disease state, but also those who had very early symptoms, a fact which is critical as it would allow an early intervention. In addition, this blood test differentiated AD patients from patients with another common subtype of dementia, known as dementia with Lewy bodies, a crucial distinction for providing a more accurate prognosis and administering a suitable treatment.

This work has great potential to be developed into a minimally invasive, cost-effective blood test for the differential diagnosis of AD. Further future research should aim to establish whether the proposed approach could also detect individuals at a pre-symptomatic phase and whether this method could serve as a screening test in high-risk cohorts. As a future direction we aim to study buccal cells as a potential non-invasive source of AD biomarkers.

How close are we to seeing this translated into the clinic? What obstacles are yet to be overcome? In your opinion, how might these challenges be addressed?

Translation into the clinic would require further follow-up clinical trials in larger number of patients in order to replicate and confirm our initial results. Assuming results of these trials show non-inferior or even superior accuracy to that of existing tests, that would permit further introduction of these methods into clinical practice. I believe that clinical translation would require a number of years. Also, prospective studies should focus on the recruitment and investigation of pre-symptomatic individuals to demonstrate the efficacy of the method as a screening tool.

Some of the obstacles that have hindered clinical implementation so far include lack of standardization of techniques and sample preparation, as well as lack of large-cohort multicenter studies, all of which are necessary to validate results before novel methods are used as part of life-changing decisions within a clinical environment. As with every analytical technique for diagnostics and/or biomarker discovery, many requirements and phases (pre-analytical, analytical and post-analytical) need validation in independent datasets and by independent researchers before clinical approval.

Blood spectroscopy has the potential to be translated from a laboratory-based tool to a necessary and informative clinical tool. Continued development towards automation of technological processes, in combination with the advent of portable, clinic-based instruments, will undoubtedly facilitate large-scale studies in the years to come.

Lastly, what developments are you most excited to see in the field of dementia in the next 5–10 years' time?

I am personally hopeful for the future of dementia research. The advent of innovative, sensitive diagnostic approaches is what most excites me in the field. Currently, AD is incurable, a fact which has partly been attributed to its late diagnosis, when brain damage has already become widespread. Consequently, it is of crucial importance that an accurate diagnosis is given at an early stage, even before the appearance of symptoms. It is anticipated that any potential drug candidate will work more effectively in such cases. The use of artificial intelligence towards a rapid detection of subtle changes that might identify individuals who are destined to develop the disease in the future, is also a very promising area of research.

Finally, the development of efficient treatments is eagerly expected within the next decade. Preliminary results from ongoing clinical trials have shown some promise, with conclusive results from Phase II trials against amyloid-β currently being anticipated.