Biological reflections in body fluids of multiple sclerosis progression and multiple sclerosis-related fatigue

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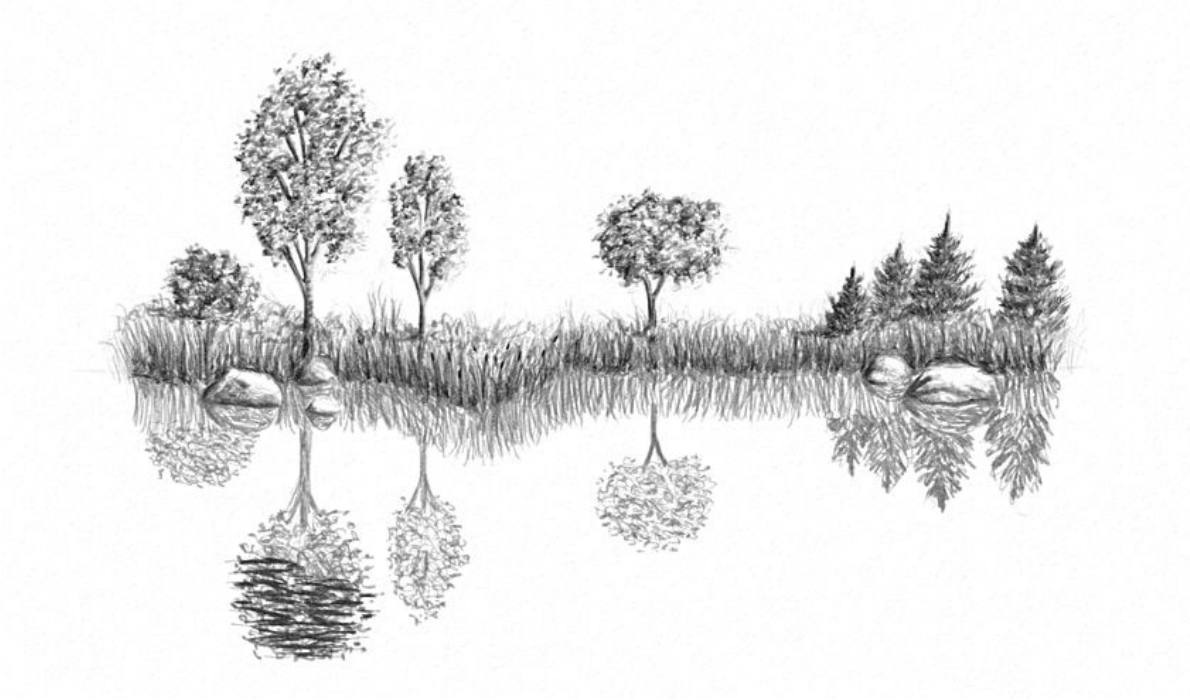
Part 2. Chapter 4.

Challenges in multi-plex and mono-plex platforms for the discovery of inflammatory profiles in neurodegenerative diseases



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Abstract

Pro and anti-inflammatory cytokines are involved in disease onset and pathophysiology of multiple sclerosis, Alzheimer's disease and Parkinson's disease. It is likely that panels of multiple cytokines provide a good reflection of disease status and can be used as biological markers in body fluids. Different multi-plex platforms, Luminex-xMAP and Meso Scale Discovery, are able to detect multiple analytes in the same sample at the same time.
In this literature based review, we offer an overview of the multi-plex platforms and compare them with the golden standard ELISA in their ability to accurately and sensitively detect cytokines in cerebrospinal fluid (CSF) and blood (serum/plasma). The detectability and levels of cytokines in multiple sclerosis, Alzheimer's disease and Parkinson's disease are promising but also show discrepancies between studies. The current immuno-assays lack sensitivity for detection of various cytokines that have low concentrations of cytokines in CSF and blood, and therefore technical improvements are needed. With such improvements the use of large panels of cytokines as inflammatory profiles may offer additional value in diagnosis, prognosis and therapeutic response in neurodegenerative diseases.

1. Introduction

During the last two decades a vast body of research has focused on inflammation markers associated with disease onset and treatment responses for different neurological diseases [1-5]. This has offered new insights into the inflammatory pathways and their association with disease onset and pathophysiology [4]. In general, it has been accepted that the inflammatory response of the host in reaction to invasion, infection or injury caused by foreign substances, or the hosts own immune system can be both beneficial and harmful for disease onset or its clinical course [4,6,7]. This duality of the inflammatory system plays an important role in neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD) and multiple sclerosis (MS) [8-11]. Whether inflammation is the cause or the consequence of the pathogenic process, quantification of inflammatory cytokines could lead to the identification of new disease specific biomarkers.
MS is considered to be an auto-immune disease, which often occurs in young adults [12]. For MS an up-regulation of the T-helper-1 (Th1) cytokines, for example Interferon gamma (IFN-γ) and Tumour necrosis factor alpha (TNF-α), is observed in cerebrospinal fluid (CSF) and blood [8,9]. Whereas the Th2 cytokines, such as interleukin-10 (IL-10) and IL-4 are considered to be down-regulated in CSF and blood [8,13]. A common symptom associated with MS is fatigue, and the pro-inflammatory cytokines TNF-α and IFN-γ have been shown to be associated with fatigue in MS [14,15]. In MS auto-reactive T-cells, especially CD4+-T cells recruit, for as yet unknown reasons, CD8+ and B-cells to the inflammatory sites in the CNS [8,9]. This eventually leads to demyelination and possibly neuronal death which leads to gliosis [16,17].
In AD and PD the presence, of microglia and astrocytes around aggregates are shown in post-mortem tissue [10,18]. In AD the microglial cells surround the amyloid plaques and synthesize pro-inflammatory cytokines, attracting monocytes to the inflammatory site [10,19,20]. In PD the microglia enhance the loss of the dopaminergic neurons at substantia nigra, causing muscle rigidity and muscle tremor [19,21]. Many different studies have shown the association of inflammatory cytokines with the onset and course of neurodegenerative disease, but we have not been able to use the cytokine profiles as biomarkers yet [8-10,19-22].

So far cytokine profiles have not been clinically used as biomarkers for any of the earlier mentioned neurodegenerative diseases. One of the reasons could be that most of the studies have used so called mono-plex Enzyme-Linked Immuno-Sorbent Assay (ELISA) for individual cytokines as quantification tool, which limits the number of cytokines studied. The technological developments in the last decades have resulted in the development of multi-plex platform assays which allow for the simultaneous quantification of multiple cytokines in a single aliquot of the specimen. Several companies have produced multi-plex platforms based on flow cytometry and electrochemiluminescence technologies. The popularity of these multi-plex platforms has increased in the last decade, but their application to neurodegenerative diseases and particularly cytokine profiling is still limited.
The aim of this review is to give insight into: 1) the technical challenges that are associated with the multi-plex platforms in comparison with the mono-plex ELISA. With this we aim to offer researchers the possibility to make informed choices about the available platforms; 2) the cytokine profiles in body fluids that are considered to be associated with the neurodegenerative diseases MS, AD and PD. To this end, we selected studies in international journals cited in Pubmed according to the following criteria: a) cytokine detection with mono-plex ELISA platform; b) cytokine detection with multi-plex platforms; c) comparisons in accuracy and sensitivity for detecting cytokines in CSF and blood between multi-plex platforms and ELISA; d) cytokines associated with MS, AD or PD in blood or CSF. By reviewing the papers retrieved, we aim to raise awareness for the multi-parametric approach to detect cytokine profiles that could be used as neurodegenerative disease biomarkers

2. General characteristics of multi and mono-plex immuno-assays

2.1 Enzyme-linked Immuno-Sorbent Assay (ELISA)

Early in the 1970's a PhD graduate student by the name Eva Engvall proposed to combine the Radio-Immuno Assay (RIA) format with the enzyme labeling of antibodies and the coating of antibodies to plastic. This resulted in the development of the Enzyme-linked Immuno-Sorbent Assay (ELISA) [23-26]. The ELISA has been applied in a broad range of fields within and outside human medicine, such as experimental research, veterinary medicine, home pregnancy tests and in art restoration. The broad use of ELISA resulted in the interest of manufacturers, which resulted in the production of the commercial ELISA kits and automated pipetting, multichannel, washing and reader devices [27].

In a typical double antibody ELISA (sandwich immunoassay), a primary antibody is attached to the bottom of a well, enabling both capturing and antigen specificity. A secondary antibody linked to an enzyme, often horseradish peroxidase (HRP), binds to a different region of the antigen and thus provides a second step of specificity and detection of the antigen of interest. This method enables accurate and reproducible quantification of the antigens of interest. ELISA has become the standard tool for diagnostics for example HIV and autoimmune diseases, because of the reproducible and accurate characteristics [28,29]. Other technical possibilities of ELISA are reviewed in [30].

Apart from the advantages there are several weaknesses associated with ELISA. The performance is largely dependent on antibody quality, i.e. affinity, specificity and avidity, the selection of appropriate blocking and dilution buffers and the incubation periods. Other weaknesses are that traditional ELISAs lack sensitivity to detect low cytokine concentrations, are relatively time consuming, require relatively large quantities of sample and can only detect one cytokine in a single experiment [27,31,32]. Another limiting factor of ELISA is that colorimetric reagents are used for read outs, which may reduce the dynamic range for these assays. Dynamic range is the linear relationship between the cytokine concentration and the absorbance

reading. Some companies provide ultrasensitive ELISA kits for cytokine measurements, which are optimized for detection of low concentrations of cytokines. These kits contain specially selected buffers and use different incubation times. However these kits are rather expensive and the dynamic range is narrow. As with all immune-based assays, ELISAs are also prone to cross-reactivity and matrix-effect. Cross-reactivity is the possibility of the antibodies binding to more than one antigen, thereby causing an erroneous result and often a false-positive effect [33,34]. Matrix effect is described as potential interference of the sample matrix, for example plasma, to interfere with the antigen binding. In addition to all the above mentioned issues, samples containing high concentrations of cytokines have to be diluted. This not only dilutes the cytokines, but also the soluble cytokine receptors and inhibitors potentially present in the sample and thus may interfere in the chemical equilibrium, resulting in enhanced binding of the cytokine to the antibody. This phenomenon hypothetically could thus cause false positive results by overestimation of the cytokine concentrations in diluted in comparison with non-diluted samples [32].

Properties of ELISA cytokine kits of two major commercial providers, Invitrogen (Carlsbad, CA) and eBioscience (San Diego, CA), were compared for their dynamic range and sensitivity. In general, and as judged from the information provided by the manufacturers, the properties of different cytokine ELISA kits show a high degree of similarity. Nevertheless, when applied in different laboratories, results may differ to a large extent [6]. For example, a comparison of TNF-α and IL-1β kits of three different commercial companies showed different results in plasma [6]. This indicates that the properties of antibodies and standards used in the different kits are not necessarily comparable. Multiple reasons for this could prevail, such as differences in epitope recognition, the use of different colorimetric substrates, experience of operator and different read out instruments [35]. Furthermore the lack of internationally accepted standard preparations of cytokines in body fluids, could explain why different inter-laboratory results are observed.

2.2 Multi-plex platforms

The idea that assays of panels of multiple cytokines provide a better reflection of biological processes has led to the development of multi-plex arrays with the purpose of quantifying multiple cytokines in the same sample at the same time. These formats make use of flow cytometry or electrochemiluminescence technologies. The most commonly used flow cytometric platforms, also known as multi-plex bead array assays (MBAA) is the Luminex-xMAP (Luminex Corporation, TX).

The MBAAs follow the principles of sandwich immunoassays. A primary antibody is linked to a polystyrene bead. Subsequently, a secondary antibody labeled with biotin or directly labeled with phycoerythrin (PE) binds the antigen. Each bead is internally dyed with a red fluorophore. The beads can be identified by the fluorescence ratio of the internal red dye and the amount of protein bound can be identified by the PE emitted fluorescence. The xMAP technology, in theory at least allows unique recognition of 100 different dyed beads and in theory thus 100 different analytes.

The Meso Scale Discovery (MSD) platform makes use of electrochemiluminescence technology. In this platform up to 10 antibody coated carbon electrodes integrated in 96 well plates provide the solid phase of the sandwich immunoassays. Following binding of the antigen, a ruthenium-conjugated secondary antibody, emitting light upon electrochemical stimulation, provides the means for quantification. The signal is then amplified by microscopy and the images are captured and analyzed by MSD-specific software [33,36].

Multi-plex platforms have several advantages compared to the mono-plex ELISA; a) Multiple parameters can be measured at the same time, b) less sample volume is needed, which is especially relevant to limited fluids such as cerebrospinal fluid (CSF), c) they are relatively efficient in time (Table 1). As with all technologies the reproducibility, precision and accuracy must be evaluated. The companies provide this information for their multi-plex cytokine kits; however as with the mono-plex ELISA the inter-assay variability between the different laboratory and kits are high [32,37,38]. Furthermore, the inter-method comparison between the MBAA and MSD with the current standard ELISA needs to be addressed before these multi-plex platforms can be used for diagnostics and experimental research fields. Which will be discussed in the next paragraph. A major practical issue for the

multi-plex platforms is that these assays are more prone to matrix and cross-reactivity effects [33,34]. In multi-plex platforms the aim is to quantify multiple targets, the use of multiple antibodies for quantification of the antigen of interest increases the chance that one of the primary antibodies is affected by the matrix and thus the antigen-antibody binding might be interrupted for a certain binding complex. Although both the matrix-effect and the cross-reactivity may also occur in mono-plex ELISA, these effects are expected to be stronger in multi-plex platforms due to multiple antigen-antibody binding [33,37]. For example detection of multiple closely related members of protein families is unlikely due to the higher cross-reactivity events that could occur [33].

Table 1. Overview of the characteristics the multi/mono-plex platforms discussed

	MBAA	**MSD**	**ELISA**
Sample input per well per assay	25 µl	<25 µl	>100 µl
Dynamic range	<10000 pg/ml	<10000 pg/ml	< 2000 pg/ml
Time efficiency	1 day	1 day	2 days
Number of analytes detected simultaneously	100*	10	1

The above mentioned data is a visualization based on information provided by the manufacturers. The lower detection limit is not shown here, which we show in Table.2 as the sensitivity. The dynamic range shown for ELISA was observed for certain kits and is not an average. The * star sign indicates theoretical detection of 100 analytes, however this has not been reached in the experimental settings yet.

3. Multi and mono-plex comparisons

The challenge for multi-plex assays is to prove their specificity, sensitivity, linearity, accuracy, stability and robustness compared to the established technology, in this case the ELISA [39]. Specificity is the ability of detecting the antigen of interest in a complex mixture of closely related or similar analytes and sensitivity is the ability for detecting accurately the smallest amount of an analyte in a sample. High sensitivity is especially important for quantification of cytokines in biological fluids, this due to the relatively low baseline levels and short half-life of cytokines [40].

Overall, the multi-plex platform kit providers claim to have higher sensitivity for cytokines compared to the mono-plex ELISA kits (Table 2). Especially the lower limit of detection (LLOD) of the MSD platform has been shown to be lower, thus having higher sensitivity [31,41]. The accuracy is the closeness of the detected value to the theoretical value and linearity is obtaining the same outcome throughout different experimental dilutions. Several groups have reported comparable results in accuracy and linearity between the Luminex-xMAP assays and ELISA for cytokine quantification in cell culture supernatants [37,38,42]. While others claim poor correlation for the same subset of cytokines in bodily fluids [43,43,44]. This suggests that the protein-rich environment of the biological fluids potentially could cause cross-reactivity and matrix-effects in the multi-plex platforms, and thus affecting reliable quantification of cytokines in biological fluids [45]. One of the reasons could be that the beads in MBAA technology are able to circulate in the suspension, which in theory could inhibit antibody-antigen bindings by self-binding or aggregation [33,46]. Additional comparisons of the multi-plex platforms and ELISA showed poor correlations in serum, especially for lower concentration of cytokines. Whereas for MSD and MBAA multi-plex platforms comparable results in serum were obtained [47-49]. Yet others show that the different multi-plex platforms vary in their ability to measure cytokines in serum and question the detectability of certain cytokines in serum [48-50]. These inconsistent findings indicate that the different multi and mono-plex platforms vary in their results for cytokine quantification in body fluids. This inconsistency is even greater when MBAA and MSD are

compared with ELISA for the lower expressed cytokines. Although, this could be explained by the limited dynamic range and lower sensitivity and dynamic range of the mono-plex ELISA [14,38,50,51]. Finally, prevention of effects of possible pre-analytical factors such as extended processing delay or repeated freezing thawing, that may induce for example cytokine degradation should be taken into consideration by implementing described guidelines with respect to good laboratory practice and sample handling and storage [52-54].

Table 2. Detection limits of methods for quantification of inflammatory components in bodily fluids according to the manufacturers

	xMAP (pg/ml)	MSD (pg/ml)	ELISA (1) (pg/ml)	ELISA (2) (pg/ml)
IL-1β	1	0.18	3.9	4
IL-2	1	0.67	5.1	4
IL-4	1.3	0.31	2	2
IL-6	1	NA	2	2
IL-10	1	0.36	1	2
IL-12	NA	NA	2	NA
IL-12p40	NA	NA	2	NA
TNF-α	1	0.48	1.7	4
TGF-β	NA	NA	15.6	NA
MCP-1	NA	NA	20	7
MIP-1	NA	NA	2	3
IFN-γ	1	0.39	4	4

In case of not available (NA), the sensitivity was not given in the provided manual or not part of the multi-plex kits as shown in the table. For xMAP the Human cytokine Luminex custom 10-plex kit is shown. For MSD the Th1/Th2 10-plex ultrasensitive kit. The column with ELISA (1) the sensitivity of Invitrogen Human kits is described, whereas for ELISA (2) sensitivity of eBioscience human kits is depicted.

4. Cytokines associated with neurodegenerative diseases

Accurate early diagnosis of neurodegenerative diseases is often difficult for clinicians due to the heterogeneous and indistinguishable disease characteristics, such as mild cognitive impairment, dementia and movement impairments [55-57]. The search for biomarkers for Parkinson disease (PD) and multiple sclerosis (MS) has not yet resulted in clinical applicable biomarkers, except for oligoclonal bands in MS [58]. A combination of β-amyloid (1–42), phospho-tau-181 and total Tau is used for diagnosis of Alzheimer disease (AD) [55]. Currently the diagnosis of MS, PD and AD are mainly focused on a combination of physical examination, neuro-imaging, laboratory testing and neuropsychological evaluation [55-57,59]. The ideal biomarker needs to be disease specific, easy to quantify, reproducible in genetically diverse populations, correlate with disease progression and therapeutic response [60]. It is not likely to find one biomarker that fulfills all these characteristics; therefore it seems more likely that multiple biomarkers are needed for diagnosis and prognosis of neurodegenerative diseases. The immune system responds to tissue injury and could over-react and even cause autoimmunity, which for example could lead to MS [12,16]. The involvement of the inflammatory pathway in neurodegenerative diseases, gives an opportunity to potentially use cytokine profiles as disease biomarkers for diagnosis and disease monitoring.

Earlier research on cytokines associated with multiple sclerosis (MS) mainly focused on single cytokine detection, animal model (experimental autoimmune encephalomyelitis, EAE) or mRNA levels of cytokines in blood derived peripheral blood mononuclear cell (PBMC) [12,16,61-64]. Overall, in these studies the pro-inflammatory cytokines IL-1β, IL-6, IL-12, IFN-γ and TNF-α were identified in CSF of MS patients [8,9,61,63]. In serum of MS patients, TNF-α, IL-4 and IL-12 were also shown to be up-regulated [8,65]. It should be noted that for IL-4 and IL-6, different results are observed between the different studies. For example, some studies reported a higher expression of IL-4 has in serum while others show no difference in CSF and blood [8,63,64,66]. A reason for this might be that the detectability of IL-4

was different between the studies, supporting the idea that different ELISA kits could result in different cytokine quantities. Additionally, some groups reported an increase in IL-6 whereas other reported no difference [8,63,67]. Moreover, MS is a heterogonous disease with different subtypes such as relapses remission (RRMS), progressive MS (PPMS) and secondary progressive (SPMS). Even within the same subtype of MS, differences in cytokine expression could be observed between patients. It is considered that pro-inflammatory components are associated with the relapses, while anti-inflammatory cytokines are considered to be associated with remission phase in RRMS [68]. A recent study on association of multiple cytokines and chemokines in response to IFN-β therapy showed that IL-17F was significantly present in serum of non-responsive RRMS patients. This supports the idea that cytokines potentially can be used as biomarkers for therapeutic response [69]. Two studies used the Luminex-xMAP technology for detection of different cytokines and chemokines, which previously were shown to be associated with MS by ELISA, failed to confirm any association of these cytokine profiles in serum and CSF of MS patients [8,58,63,65,68,70,71]. However, these two studies have used a small sample size which affects the strength of the studies. In general the presence of inflammatory components in MS remains inconclusive. This could be solved by: a) cooperation of MS centers worldwide to increase the number of patients per study and thus study power; b) better characterization of studied groups, by performing MS subtype specific studies. This to understand the underlying mechanisms of the inflammatory components per subtype; c) furthermore it is required to characterize the control groups; d) perform population stratifications, this to avoid possible genetic background differences associated with immune system [72,73]; e) avoiding possible life-style and circadian rhythm associations on cytokine levels in body fluids to obtain reliable data throughout different studies [72,74,75].

The etiology of Alzheimer's disease (AD) is not well understood. The presence of amyloid beta-peptide (Aβ) and intracellular hyper-phosphorylated tau fibrillary aggregates are the pathological hallmarks of AD [10,18,76]. These hallmarks are mainly detected in the cortex, basal forebrain,

amygdala and the hippocampus [10,76]. Microglial activation and neuro-inflammation is observed in AD plaques. Continued activation of inflammation is considered to damage surrounding tissue and exacerbate AD pathology [10,18,76]. The involvement of the inflammatory components synthesized by astrocytes and microglia gives the opportunity to use cytokine profiles that may be associated with AD as biomarkers for disease progression. The earlier studies of inflammatory cytokines in AD have shown some contradictory and unexpected results. Some groups claim that TGF-β is upregulated in serum of AD patients, while no up-regulation of any of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1 is observed [77,78]. Others claim a higher concentration of IL-1, IL-6 and TNF-α in AD sera (Table 3) [18,79]. These differences could be explained by methodological differences and lack of case-control participants, often having 20 or less cases, which limits the study power. However, several relatively new studies have been able to detect AD with an accuracy of 90% or higher, by implementing a multi-parameter approach using xMAP technology for measuring different cytokines and analytes in CSF [18,79-82]. It should be noted that different subsets of analytes were used, with the highest accuracy for detection being a 5-plex consisting of IL-1, IL-6, epidermal growth factor (EGF), TNF- α and granulocyte colony-stimulating factor (G-CSF) [80]. In conclusion, it is tempting to speculate that combination of cytokine profiles with the traditional AD biomarkers can improve detection accuracy.

The hallmark of Parkinson's disease (PD) is the aggregation of misfolded α-synuclein proteins. Aggregation of these proteins in neurons is called Lewy bodies, and often these bodies occur in the substantia nigra which results in loss of dopaminergic neurons [51,76]. The infiltration of microglial and astrocytes suggests the involvement of the inflammatory system in PD [11,51,76,83]. Earlier studies have shown the presence of cytokines in PD lesions and CSF of PD patients [11,51,76,83]. Overall, an increase of IL-1β, IL-2, IL-4, IL-6 and TNF-α is observed in CSF of PD patients (Table 3) [11,51,83-85]. It should be noted that contradictory results were reported for the aforementioned pro-inflammatory cytokines in blood [86,87]. This could be due to the paracrine nature, short half-life and the presence of soluble

cytokine receptors in blood, suggesting that CSF might be more suitable for detection of cytokine profiles for the all above mentioned neurodegenerative diseases.

Whether cytokine profiles can be used as potential diagnostic biomarkers remains unclear, especially due to the different observed results between studies. The question remains, if it is possible to link specific cytokine profiles to a neurodegenerative disease. For example, cytokines IL-1 and IL-6 are associated with traumatic brain injury (TBI) and an up-regulation is observed in both CSF and serum upon brain injury [88,89]. It is known that IL-6 is only expressed during development and in case of pathophysiology or injury in the adult CNS [90]. So detecting IL-6 in CSF could potentially say something about a state of irregularity or acute damage in the CNS. However to be able to pinpoint a certain disease or injury to cytokines profiles, more knowledge of the underlying involvement of the immune cells and their synthesis of inflammatory components in neurodegenerative diseases is required. The focus of these studies should mainly be multi-parametric based approach. For now it seems that due to inconclusive data derived from both ELISA and multi-plex platforms, cytokine profiles are not likely to be used as diagnostic markers. However these cytokine profiles can add more accuracy to other biomarkers, and potentially can be used as markers for monitoring disease progression and therapeutic response.

Table 3. Cytokines considered to be associated with the neurodegeneration diseases MS, AD, and PD.

	Multiple sclerosis [8,61-64,67]	Alzheimer disease [18,77-80]	Parkinson disease [11,21,83-85]
IL-1β	↑	↑*	↑
IL-2	_	_	↑
IL-4	↑↓*	_	↑
IL-6	↑-	↑*	↑
IL-10	↓	_	_
IL-12	↑*	_	_
TNF-α	↑*	↑*	↑
TGF-β	↓	↑	_
MCP-1	↑	_	_
MIP-1	_	↑	_
IFN-γ	↑	_	_

* cytokines were detected in both CSF and serum for MS and AD. All cytokines for PD were detected in CSF. - indicates that no association for these cytokines in CSF-serum with the respective disease has been reported.

5. Summary

The aim of this review is to shed light on the multi-plex bead array assays and Meso Scale Discovery multi-plex technologies for the quantification of cytokines. We especially wanted to raise awareness for the technical challenges that are accompanied with the quantification of cytokines with these relatively new technologies. Issues to be considered in this context are sensitivity, reproducibility, and accuracy, especially when compared to the golden standard ELISA.

Overall, the use of the MSD and MBAA platforms for establishing neurodegenerative disease cytokine profiles could offer biomarkers for disease onset, progression and/or response to treatment. The multi-plex platforms discussed in this review can be promising technical tools for quantification of cytokine profiles that are associated with neurodegenerative diseases. This is due to their characteristics such as higher sensitivity, time efficiency, less sample input and multiple analyte detection. Currently, comparable results for the different platforms have been obtained with cell culture derived supernatants. For blood, only the abundantly expressed cytokines showed similar data between the different multi-plex platforms. Furthermore, the use of large panels of cytokines that are considered to be associated with MS, AD and PD in patients remain debatable due to the observed contradictory results observed between studies.

Before these new platforms can be broadly used for detection of cytokine profiles, several recommendations should be taken into account; a) avoidance of possible pre-analytical errors by handling according to guidelines with respect to good laboratory practice and sample handling and storage ; b) in case of using multiple cytokines for diagnostic reportings, assessment of possible study biases and technical accuracy should be performed. A methodological overview of the necessities for diagnostic reportings is shown in the Studies of Diagnostic Accuracy initiative (STARD) [91]; c) although beyond the scope of this review, statistical methodology between different multi-plex platform based studies differ and assessment of the used statistical models in a validation cohort should be considered; d) the correct controls

should be performed for the multi-plex kits, to control for possible cross-reactivity and matrix-effect. Additionally, with increase in usage of multi-plex platforms, an increase in the experience of the operators could enhance further discussion which can lead to standardized guideline and technological and methodological improvements for multi-plex platforms. The establishment of internationally accepted standard preparations and reference methods should also be helpful in this respect.
In conclusion, the multi-plex platforms offer an opportunity to detect cytokine profiles that can potentially be used for diagnosis of neurodegenerative disease. However, because of the observed differences and for some cytokines the lack of sensitivity, it seems unlikely that with the current immune assay platforms cytokine profiles on their own can be used as diagnostic tools. Nevertheless, these cytokines profiles could potentially be used for monitoring disease progression and therapeutic response. Further developments in this exciting area are awaited.

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