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

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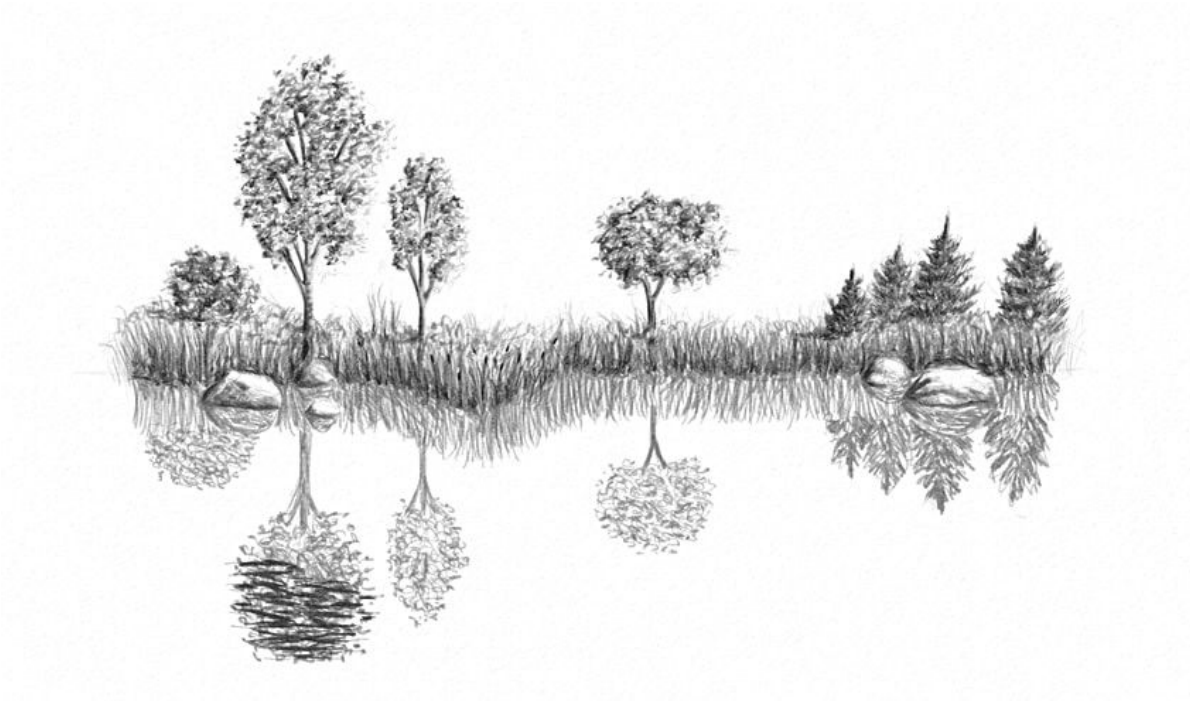
2020

Part 2. Chapter 5.

Comparison of multiplex platforms for cytokine assessments and their potential use for biomarker profiling in multiple sclerosis



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Cytokine 91 (2017) 145–152

Abstract

Background

The levels of pro and anti-inflammatory cytokines can be altered in different autoimmune pathologies, such as multiple sclerosis (MS). It is likely that cytokines in bodily fluids can provide a good reflection of ongoing disease patho-physiology. In this study we aimed to validate multiplex cytokine platforms and evaluate whether these cytokines are differentially expressed in MS.

Methods

Assay validation for simultaneous quantification of IL-1β, IL-6, IL-8 and TNF-α in serum and CSF were performed using both the Luminex-xMAP (Luminex) and Meso Scale Discovery (MSD) platforms. Next, the relation of the pro-inflammatory cytokine 4-plex with disease progression, symptoms and subtypes was studied in paired serum and CSF of MS patients (n=56), and compared with healthy controls (n=203), with the use of the MSD-platform.

Results

The MSD-platform showed overall better assay characteristics such as, sensitivity, recovery and linearity compared to the Luminex for the 4-plex cytokines in CSF and serum. IL-6, IL-8 and TNF-α ($p<0.001$) levels were significantly increased in MS serum compared to healthy controls. Moreover, serum IL-1β levels correlated with expanded disability status scale (EDSS) scores ($r=-0.34$ $p<0.05$). Additionally, IL-6 and IL-8 CSF levels were both significantly decreased in MS patients compared to non-inflammatory neurological disease controls. Noteworthy, higher IL-8 CSF levels than IL-8 serum levels were observed for MS patients, indicating intrathecal activation of macrophages in MS.

Conclusion

We have demonstrated that the pro-inflammatory 4-plex kit of the MSD-platform shows better assay characteristics in comparison with Luminex kit for quantification of these cytokines in serum and CSF. Overall, the increased levels of IL-6, IL-8 and TNF-α in serum of MS patients compared to healthy controls, support the use of multiple cytokines for future MS biomarker and disease progression research

1. Introduction

Despite the prolonged efforts to identify unique biomarker(s) for multiple sclerosis (MS), no specific subtyping and prognostic biomarkers have been discovered [1]. Ideally, a quantifiable laboratory marker(s) assists clinicians with rapid MS diagnosis, allows subtype discrimination from onset, reflects disease progression and assists the clinician with choice of therapy.

MS is a heterogeneous chronic demyelinating disease of the CNS primarily affecting young adults [2],[3]. Possibly a loss of self-tolerance to an unknown particle of the myelin sheath initiates an inflammatory cascade by attracting auto-reactive CD4+/CD8+ T-lymphocytes to sites with a high density of small venules and veins [3],[4]. T-lymphocytes, myelin phagocytizing macrophages, B-cells and complement factors are present within the lesions of 58% of MS patients [4]. Other types of lesions contain a different subset of immune cells and are probably associated with different mechanisms of demyelination and neuro-degeneration [3],[4]. However, within individuals the immune-patterns of the lesions are identical [4].

The involvement of both innate and or adaptive immune systems in MS etiology and disease progression offer the opportunity to assess MS specific immune profiles that could be used as biomarker profiles that potentially reflect disease onset and or progression.

Cytokines are secreted proteins and fundamental mediators between the immune cells, and can affect neuronal and glial cells [5]. Cytokines together with chemokines coordinate the immune response to infection, tissue damage, repair and self-tolerance [6],[7]. These short lived inflammatory components have pleiotropic functions and a low basal presence in body fluids [6],[7]. Because of the pleotropic and in concert functioning characteristics of cytokines, there is limited value of evaluating a single cytokine. Therefore, it is more likely that quantification of multiple cytokines can serve as a profile that reflects disease progression and potentially allows MS subtype discrimination and controls.

Novel multiplex assays can quantify multiple analyses in the same sample at the same time in small volumes. Two of the well-known multiplex platforms currently used are Luminex-xMAP (Luminex) and Meso Scale Discovery (MSD). In the Luminex format primary antibodies are bound to uniquely dyed polystyrene beads, which are distinguished by flow cytometry. In theory

the Luminex platform allows a quantification of 100 different analytes at the same time. In MSD, primary antibodies are bound to specified carbon spots. Upon electrochemical stimulation of bound ruthenium-conjugated secondary antibodies light is emitted and measured with a CCD camera.
Limited studies have directly compared cytokine quantification between these multiplex platforms for different body fluids and especially in cerebrospinal fluid (CSF) [2],[8]. In this study we aimed to compare the assay characteristics of Luminex and MSD in serum and CSF, and evaluated whether these cytokines are differentially expressed in MS subtypes and controls.

2. Material & Methods

2.1. Quality control assessments

Quality control experiments were performed using left-over serum and CSF, obtained freshly from the routine diagnostics laboratory of the VUMC medical hospital, and pooled in polypropylene tubes (Sarstedt, Germany) (within 3 hours' post-collection), aliquoted and stored at -80 C° prior use. Blood was drawn using BD vacutainer serum tubes (BD, New Jersey, USA) and CSF was collected in polypropylene tubes (Sarstedt) post lumbar puncture. All samples and standard curves were measured in duplicates.

2.1.1. Sensitivity

The lower limit of detection was calculated by adding 2 times the standard deviation (SD) to the mean of blank. The lower limit of quantification was calculated by adding 10 times the SD to the mean of blank samples. The lower limit of detection and quantification for MSD were assessed by quantification of 10 blank samples and for Luminex 3 different blank samples

2.1.2. Intra- and inter assay accuracy and precision

The intra-assay coefficient of variation (CV%) for the MSD kit was determined by assessment of 55 serum and CSF samples. For the Luminex kit 15 CSF samples were used for intra-assay assessment. We defined a CV% lower than 20% as acceptable.

The mean inter-assay variability was determined by assessment of CV% of repeated sample measurements for serum and CSF. The inter-assay reproducibility for the MSD platform was assessed with three different serum samples using 12 different MSD plates and for CSF, one sample was measured in three different MSD plates. The inter-assay variability of the Luminex platform was not determined because of the low number of plates assessed for this platform.

2.1.3. Recovery experiments

To determine the recovery of both platforms three different spike concentrations of the provided calibration blends were added to assay diluents, cerebrospinal fluid (CSF) and serum. For the MSD platform the different three spikes were 1000 pg/ml, 100 pg/ml and 10 pg/ml in all the different media. For Luminex the spikes were 100 pg/ml, 62.5 pg/ml and 10 pg/ml. The readout from CSF and serum spikes (observed values) minus the background, were divided by the theoretical spiked values to obtain the spike recovery. Recovery rates between 80%-120% were defined as acceptable.

2.1.4. Cross calibration Recovery

We spiked 200 pg/ml, 75 pg/ml and 10 pg/ml of the MSD and Luminex kit calibration blends into either Luminex assay diluent or MSD assay diluents, respectively. The readout was done on the MSD platform. Recovery was determined by dividing the observed value minus the background, by the theoretical expected spike values minus the background. These experiments were performed in duplicate and were repeated in an independent experiment.

2.1.5. Cross calibration linearity experiments

For the linearity assessments, cross-spiking MSD and Luminex assay buffers were done by spiking MSD calibration blend into the Luminex assay buffer, and the Luminex calibration blend into MSD assay buffer. These solutions were serially diluted by a factor of 4, and recovery was calculated by the factor of dilution times the observed concentration divided by the actual measured concentration of the undiluted samples.

2.1.6. Inter-platform comparisons

CSF of 15 MS patients was measured on both platforms to compare the results for the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α. For the MSD CSF assessment, 1% ultra-pure BSA/PBS as diluent buffer was used. For Luminex the provided protocol and assay buffers were used as described.

2.2. Meso Scale Discovery assay

The human pro-inflammatory II 4-plex kit (ultrasensitive) kit, IL-1β, IL-6, IL-8 and TNF-α (Meso Scale Discovery, Maryland, USA) was used for platform comparisons and sample quantification. The provided manufacturers guidelines were followed. The MSD kits were run as follows; the calibration curve with a range of 0.61 pg/ml to 2500 pg/ml was prepared in supplied assay diluent (diluent 2). Prior to the addition of 25-µl sample or calibration blend, arrays were incubated with 25 µl assay diluent for 30 min with shaking at room temperature (RT). The samples and the calibration blends were added in duplicates and incubated for 2 hours (shaking at RT). The arrays were washed with phosphate-buffered saline (PBS) plus Tween-20 [0.05%] (PBST) and subsequently incubated with 25 µl of detection antibody for 2 hours (shaking at RT). Next, the arrays were washed with PBST and immediately after adding 150 µl of the reading buffer, the read out was performed with a Sector Imager 2400A (MSD). The cytokine concentrations were determined with Discovery Workbench 3.0.18 using a log-log curve fit model. All samples were measured in duplicate.

2.3. Luminex assay

The human pro-inflammatory 4-plex high sensitive custom kit (IL-1β, IL-6, IL-8 and TNF-α) (Merck Millipore, Darmstadt, Germany) was applied using a Bioplex 200 platform (Bio-Rad laboratories, California, USA). All reagents were applied and prepared according to the manufacturers guidelines. The 4-plex high sensitive kit was run as follows;

The plate was incubated with wash buffer for 10 minutes (shaking at RT). Subsequently the washing buffer was removed by vacuum filtration and 25 µl of pre-coated mixed beads was added to each well and subsequently rinsed twice. Next, 50 µl of the prepared standard with a range 0.13 pg/ml to 2000 pg/ml together with the two provided kit controls were added. Next, 50 µl of assay buffer was added to background control well and sample wells and 50 µl of sample were added to the wells. Subsequently the plate was sealed and incubated at 4 C° (shaking) overnight. The next day, the unbound well content were removed and beads were twice washed with washing buffer. Next, 50 µl of detection antibodies solution was added and incubated for 1 hour (RT). Next, the beads were incubated with 50 µl of Streptavidin-

Phycoerythin solution for 30 min (RT). After two wash steps, 150 µl of sheath fluid (Bio-Rad) was added and the signal was read with Bioplex manager software. All samples were measured in duplicate.

2.4. Study participants

Approval for the study was obtained from the Ethics Committee of the VU Medical Center and written informed consent was obtained from all patients [9]. These samples were collected over a decade ago [9]. Blood was drawn using BD vacutainer serum tubes (BD) and aliquoted in polypropylene tubes (Sarstedt) and stored at -80 C°. CSF was collected following a lumbar puncture, aliquoted in polypropylene tubes (Sarstedt) and stored at 80 C°. Samples were collected during remission and patients with clinical relapse were excluded from our study [9]. A total of 57 MS patients were selected from this historical cohort (average age 45.2 years (SD ± 8.5), 23 males and 33 females) [9]. Of these patients 11 were diagnosed with primary progressive, 22 with secondary progressive and 23 with relapse remitting MS. Of the 57 patients, 7 patients were on disease modifying therapies 6 months prior or at the time of the blood-draw. Paired CSF and serum of these patients were analyzed, of one of the patients no serum, and of two other patients no CSF samples were available. The average expanded disability status scale (EDSS) for the RRMS patients was 2.0 (SD± 1.4); SPMS 5.6 (SD± 1.9) and PPMS 5.0 (SD± 2.2). The RRMS group average for the fatigue severity scale (FSS) was 41 (SD± 9.9), SPMS 47.9 (SD± 9.9) and PPMS 48.5 (SD± 9.9). T1 and T2 total lesion load were collected as previously described [9].

Serum of 203 healthy controls were selected from a different cohort, however similar blood collection protocol and storage were followed [10].

2.5. Statistics

Cytokine concentrations were not normally distributed, and therefore Log10 transformed data were used. Group comparisons with regard to different MS subtypes and disease-related clinical scales were analyzed using ANCOVA with age corrections. Spearman's Rho correlation analysis was performed to assess to association between the cytokine levels and different clinical and MRI scales.

For inter-method comparisons, Bland-Altman plots were formed and subsequently linear-regression analysis on Log10 transformed data was performed to examine levels of agreement between the two platforms. A significance criteria of < 0.05 was set. All analyses were conducted using SPSS 20.0 and Graphpad prism version 6.03.

3. Results

3.1. Intra-assay precision

The intra-assay coefficient of variation (CV) on the MSD platform for IL-1β, IL-6, IL-8 and TNF-α in serum were respectively 19%, 6%, 7% and 7%, and thus within the acceptance. The CSF samples used for intra-assay assessments had either undetectable IL-1β levels or levels lower than the LLOD with CVs% higher than the accepted criteria, therefore no further analyses of IL-1β CSF levels were made.

The MSD platform for CSF showed only acceptable CV rates for IL-6 (12%) and IL-8 (11%), whereas unacceptable reproducibility rates were observed for TNF- α (31%) (figure 1).

The intra-assay CV for IL-6, IL-8 and TNF-α in CSF for the Luminex platform were respectively 19%, 9% and 18% and thus within the acceptance criteria.

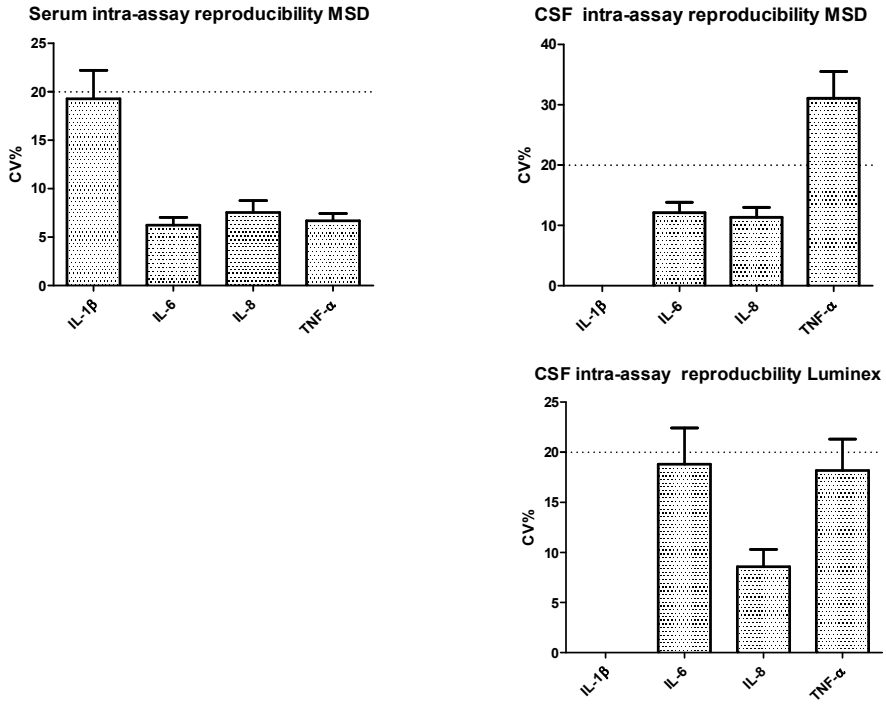

Figure 1. Intra-assay reproducibility of cytokines in serum (MSD) and CSF (MSD and Luminex). The boxes are mean CV values and the whisker depict SEM. The dashed horizontal lines represent the 20% CV acceptance cutoff

3.2. Inter-assay precision

The inter-assay CV of the MSD platform in serum for IL-6, IL-8 and TNF-α were lower than the acceptance criteria of 20%. The CV for IL-1β showed rates of 54% and 114% in serum and CSF respectively. Moreover, the inter-assay in-precision of TNF-α in CSF was 45.6% (figure 2).

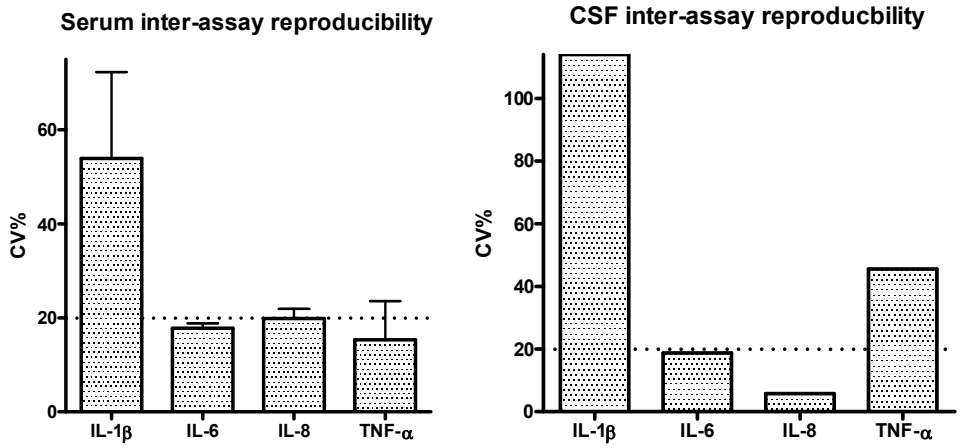

Figure 2. Graphical representation of mean inter-assay reproducibility of the MSD platform in serum and CSF. The boxes are mean CV values and the whiskers are standard error of the mean (SEM). The dashed horizontal lines represent the 20% CV acceptance cutoff

3.3. Assessment of sensitivity

Sensitivity for both platforms was assessed with the use of the provided blank assay buffers and subsequently calculations of the limit of blank (LoB), limit of detection (LoD) and limit of quantification (LoQ). It was not possible to assess the LoD and LoQ for the Luminex platform because of high variations in CV of the blank samples (n=3) for all the four cytokines, therefore the lowest standard point in the applied standard curve is shown and is used for the calculations of LoD (table 1). It should be noted that the extrapolation of the MSD standard curve allowed the assessment of the depicted LoB, and all quantified blank samples showed CV lower than the acceptance criteria of 20%. Overall the MSD platform showed lower CVs close to the LoD border, which we did not observe for the Luminex platform, indicating higher sensitivity for the MSD platform.

Table 1. Overview of sensitivity assessment of the pro-inflammatory cytokines quantified with MSD and Luminex platforms

	IL-1β (pg/ml)		IL-6 (pg/ml)		IL-8 (pg/ml)		TNF-α (pg/ml)	
	MSD	**Luminex**	**MSD**	**Luminex**	**MSD**	**Luminex**	**MSD**	**Luminex**
Limit of Blank (LoB)	0.27	0.13	0.12	0.13	0.15	0.13	0.14	0.13
Limit of Detection (LoD)	0.43	0.19	0.27	0.22	0.21	0.20	0.24	0.17
Limit of qunatification (LOQ)	1.2		0.62		0.73		0.64	

3.4. Recovery experiments

To ensure accurate measurements in serum and CSF matrixes for both multiplex platforms, spiking in both serum and CSF was performed. The MSD platform showed for all of the medium (100 pg/ml) and high (1000 pg/ml) spikes, acceptable recovery percentages, whereas the lowest (10 pg/ml) concentration spike results were overall not within the criteria of 80-120% (Table 2). Quantifications on Luminex platform of both serum and CSF spikes were not within the acceptance criteria of 80-120% (Table 2). All spikes in serum measured with the Luminex kit showed lower concentrations than the expected values, whereas CSF spikes showed higher concentrations than the actual expected values, indicating a possible matrix effect in this assay for CSF and serum cytokine quantifications.

Table 2. Spike recovery (%) of MSD and Luminex platform for the pro-inflammatory cytokines in CSF and serum

		Spike CSF				Spike Serum			
		IL-1β	**IL-6**	**IL-8**	**TNF-α**	**IL-1β**	**IL-6**	**IL-8**	**TNF-α**
MSD	**High**	110.1	113.3	111.5	118.0	88.0	86.0	93.0	94.0
	Mid	103.4	116.5	128.0	113.0	81.1	89.5	83.0	92.0
	Low	101.1	*139.5*	*175.5*	91.5	*76.4*	*288.0*	88.0	*73.0*
Luminex	**High**	*201.8*	*498.6*	*185.4*	*121.2*	*54.9*	*17.3*	*70.8*	*58.1*
	Mid	*217.4*	*187.0*	*328.4*	*160.5*	*64.9*	*20.9*	108.6	*77.9*
	Low	*164.1*	*561.9*	*923.6*	*129.7*	*49.6*	*11.7*	*76.0*	*50.4*

3.5. Cross calibration blends recovery experiments

To get insight into whether the calibration blend applied on the Luminex platform was accurate, we performed spike and linearity recovery for the Luminex kit by spiking Luminex's kit calibration blend into the assay buffer of the MSD kit. Moreover, in order to rule out possible assay buffer related issues with the Luminex kit, we spiked different concentrations of the MSD calibration blend into the Luminex kit assay buffer and subsequently quantified both experiments on the MSD platform.

The spikes prepared with the Luminex calibration blend in MSD assay diluent were overall not within the acceptable recovery rates for the IL-1β, IL-8 and TNF-α spikes, only IL-6 showed acceptable recovery rates for the high and medium spikes (Table 3). The spikes prepared with the MSD calibration blend into Luminex kit assay buffer showed overall acceptable rates except for the low spikes of IL-8 and TNF-α (Table 3). Noteworthy is that IL-1β analyte from the Luminex calibration blend showed five times higher concentration than the theoretical calculated concentrations when using the MSD assay buffer. Whereas the quantification of the remaining cytokines from the same calibration blend were closer to the expected theoretical concentrations.

Next, we assessed the linearity recovery by spiking and performing serial dilution for the Luminex and MSD cross calibration blends. The recovery rates for the 4 times diluted Luminex calibration blend showed recovery rates within the 80%-120% cut-offs, however 16 times dilution showed poor recovery rates (Table 4). For the MSD the recovery percentages were within the accepted ranges for both the different dilutions.

Both the earlier recovery spike experiments performed in CSF and serum, and cross calibration spikes and linearity assessments indicate that the MSD platform shows overall acceptable recovery rates for the different analytes within the chosen cut-offs, which in most of the spike recovery cases were not observed for the Luminex kit.

Table 3. Recovery (%) of cross calibration spikes of both the calibration blends measured on the MSD platform

	IL-1β	IL-6	IL-8	TNF-α
Luminex Calibrator (200pg/ml)	*510.5*	107.6	*134.8*	*70.2*
Luminex Calibrator (75pg/ml)	*500.9*	108.4	*133.2*	*67.5*
Luminex Calibrator (10pg/ml)	*562.5*	*137.*0	*151.4*	*74.5*
MSD Calibrator (200pg/ml)	95.5	106.2	111.4	98.6
MSD Calibrator (75pg/ml)	97.7	95.0	100.9	94.0
MSD Calibrator (10pg/ml)	119.6	119.9	*120.7*	*121.2*

Table 4. Recovery percentages of linearity assessment experiments performed with MSD and Luminex calibrator blends in Luminex and MSD assay buffers respectively

	IL-1β	IL-6	IL-8	TNF-α
Luminex calibrator blend 4x dilution (50pg/ml)	85.3	80.8	83.7	*75.6*
Luminex calibrator blend 16x dilution (12.5pg/ml)	*78.5*	*79.6*	*72.5*	*60.7*
MSD calibrator blend 4x dilution (50pg/ml)	116.8	113.8	104.7	105.6
MSD calibrator blend 16x dilution (12.5pg/ml)	105.2	91.9	84.6	87.4

3.6. Inter-platform comparisons of CSF

In order to examine whether both platforms were in agreement regarding cytokine concentrations in CSF we examined a set of samples on both platforms. IL-1β concentrations were too low to measure in CSF on both platforms. We observed significant association on log transformed data for IL-6 and TNF-α ($p<0.05$) (figure 3). This means that the Luminex platform has the tendency to show higher concentrations of IL-6 and TNF-α with increasing IL- 6 and TNF-α concentrations in CSF samples compared to the concentrations as measured by the MSD platform.

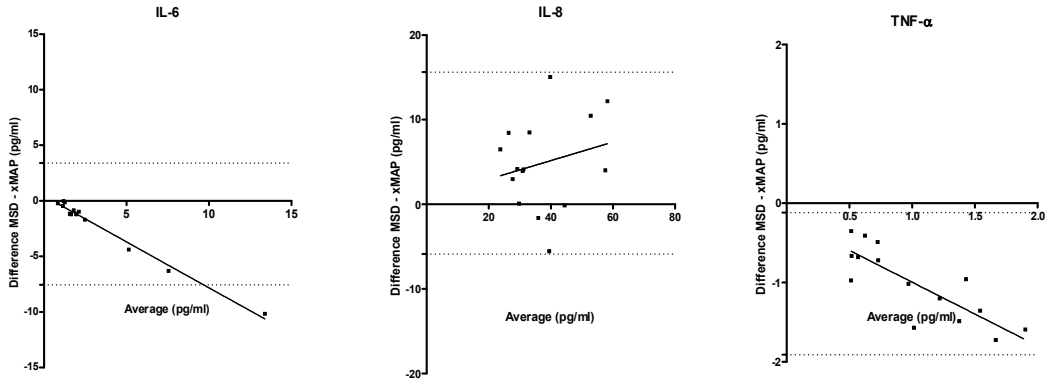

Figure 3. Bland-Altman plot of mean differences between IL-6, IL-8 and TNF-α quantified with MSD and Luminex platforms. On the y-axis the mean differences between MSD and Luminex are depicted, whereas on the x-axis the average between these platforms is shown. The dashed horizontal lines represent 95% confidence interval. Moreover, linear regression lines are depicted for assessment of significant biases between these platforms.

3.7. Cytokine profiles in patients with multiple sclerosis

Based on the overall good validation results obtained with the MSD platform we continued using the MSD platform for cytokine assessment in serum and CSF of MS patients. To determine whether the pro-inflammatory cytokines were differentially expressed in MS we quantified IL-1β, IL-6, IL-8 and TNF-α in paired serum and CSF of MS patients, serum of healthy controls and CSF of non-inflammatory neurological disease controls (NINDC) (Table 5). Next, we correlated the outcomes with clinical data such as expanded disability status scale (EDSS) and fatigue severity scale (FSS) (Table 5). IL-1β concentrations in CSF were overall too low to quantify, therefore no subsequent analysis on IL-1β in CSF was performed.

A significant increase in serum for all MS subtypes compared to healthy controls for IL-6 (ANCOVA with age correction, RRMS $p<0.001$, SPMS $p<0.001$ and PPMS $p<0.001$) and IL-8 (ANCOVA with age correction, RRMS $p<0.001$, SPMS $p<0.001$ and PPMS $p<0.05$) was observed (figure 4). For TNF-α in serum only a significant increase in PPMS compared to healthy control was observed (ANCOVA, with age correction, $p<0.05$).

A significant negative correlation of IL-1β values in serum with EDSS was observed (Spearman's rho, $p<0.05$, $r=-0.34$). No other significant associations were observed between serum or CSF cytokine with the clinical scales or MRI parameters, such as T1 and T2 total lesion load.

As control group for MS CSF samples, we used CSF of NINDC. For both IL-6 (Mann Whitney U, $p<0.01$) and TNF-α (Mann Whitney U, $p<0.05$) a significant decrease was observed for RRMS compared to NINDC. Interestingly, significant higher levels of IL-8 in CSF were observed compared to the paired MS serum samples.

Table 5. Sample demographics assessed for IL-1β, IL-6, IL-8 and TNF-α using the MSD platform

	RRMS	SPMS	PPMS	Healthy Controls	Non-inflammatory controls
Sex (F/M)	14/9	11/12	8/3	85/118	13/13
Age mean (SD)	42 (7.8)	46 (9.6)	50 (3.3)	54 (6.4)	44 (12.8)
EDSS mean (SD)	2 (1.4)	5.5 (2)	5 (2.2)	-	
FSS mean (SD)	41 (10)	47 (10)	48 (10)	-	

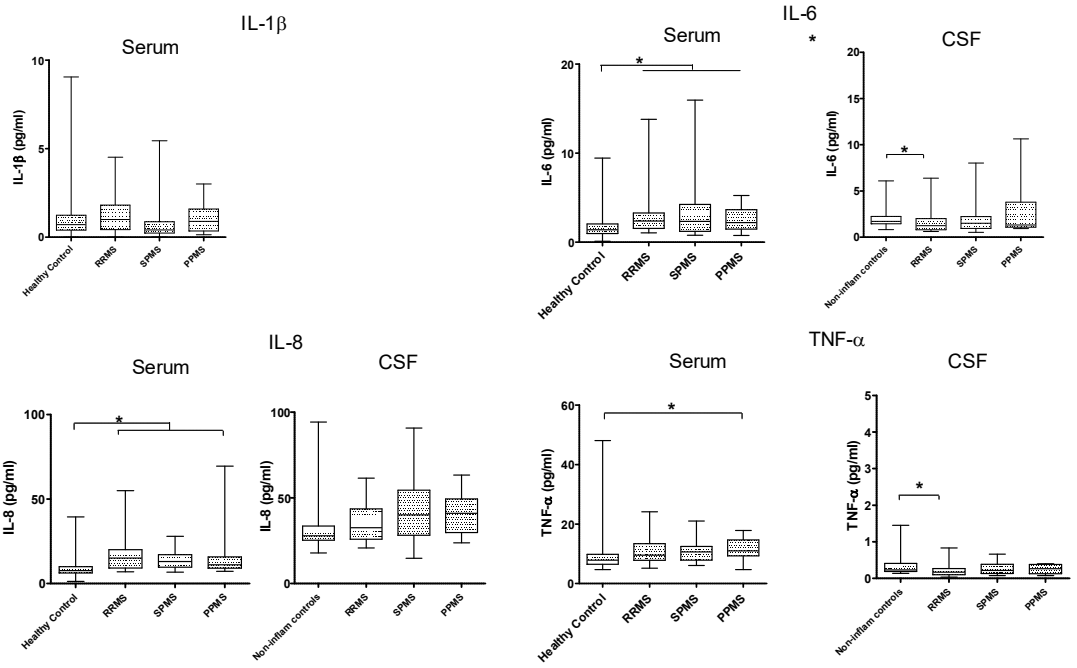

Figure 4. Paired CSF and **s**erum cytokine levels of different MS subtypes compared to healthy controls and NINDC controls. Data were corrected for age. Higher IL-6, IL-8 and TNF-α serum levels were observed for MS patients compared to healthy individuals in serum. No differences are observed for any of the cytokines between the different MS subtypes. The length of the boxes represent 25% and 75% percentiles, and the whiskers are the minimum and the maximum values.

4.Discussion

The objective of this study was to validate whether MSD and Luminex platforms could reliably quantify IL-1β, IL-6, IL-8 and TNF-α in serum and CSF and to assess whether these were differentially expressed in different MS subtype discrimination and in comparison to healthy controls. In general, assay characteristics, such as precision, sensitivity, and recovery in serum and CSF for the MSD platform were within the accepted research criteria. Additionally, we observed higher IL-6 and IL_8 levels for all MS subtypes compared to healthy controls in serum.

In overall terms of assay performance, our results are in line with previous findings, in which MSD was found to be more sensitive for similar and other cytokines compared to the Luminex platform, with higher accuracy and reproducibility near the LoD cut-off than Luminex [8],[11]. It is possible that the custom kits are less extensively validated and this could explain the observed poor assay characteristics for the Luminex kit in serum. It should be noted that we also observed inaccuracy for the calibrator blend of the Luminex kit, based on the poor recovery results observed for the cross-recovery experiments. In which five times higher IL-1β levels than the theoretical value was observed for the Luminex blend when analysed with the MSD kit. Alternatively, it is possible that the antibody mixture used in the MSD platform was able to bind IL-1β Luminex calibrator more efficiently compared to the Luminex kits antibody combination. In order to assess whether it is the inaccuracy of the calibration blend or the binding efficiency of the antibody pair of the MSD kit is the cause, another cross-calibration blend experiment could be performed in which the MSD calibration blend is assessed on the Luminex platform using the Luminex kit. These results demonstrate that validation of analytical performance is important, and custom-made kits need to be validated for the matrix of interest.

Interestingly, we observed that the Luminex kit has the tendency to yield higher IL-6 and TNF-α concentrations with increasing concentrations in CSF compared to the MSD kit. Variation in absolute values for cytokine analysis has been reported between different ELISA kits, in which only a single analyte is analysed, as well as for the Luminex platform with different kit suppliers [12]. The most likely explanation is that the observed variance in absolute concentration lies in differences between the antibody pairs used by

different suppliers, each being manufactured, tested and optimized differently, with no regulatory guidelines for cross comparison of methods and no well-established reference standards to confirm accuracy for these cytokines.
Limitations of this study are that we used fewer Luminex kits than MSD kits during assay validation, and therefore less data, e.g. on inter-assay variation could be obtained for these kits. However, we could reliably estimate the recovery and linearity, the results of which were sufficient to decide for continuation with MSD in clinical samples. In addition, both of the kits were not produced for analysis of CSF, and the conditions for CSF were therefore possibly not optimized. MSD advised to apply a BSA/PBS buffer instead of their provided assay buffers, which we did not use for the Luminex kit. Therefore, it is possible that the discrepancies observed in CSF for both the platforms could partially be explained by the difference in applied assay buffer matrix for the MSD kit which we did not use for the Luminex kit.
Overall, the assay validation characteristics for the MSD kit were better compared to the Luminex kit for serum and CSF. Based on these results we conclude that the MSD kit is more accurate and reproducible for quantification of IL-6, IL-8 and TNF-α in serum and CSF.

Analysis of the pro-inflammatory cytokines associated with MS, in line with previous studies, indicated for both IL-6 and IL-8 in serum significant different levels in MS subtypes compared to healthy controls or non-inflammatory controls [13]–[16]. Additionally, IL-6 and TNF-α showed significant decrease in CSF of RRMS patients compared to non-inflammatory controls. Noteworthy, IL-8 levels were approximately two times higher in CSF of MS samples compared to their matched MS serum levels. This is in agreement with an earlier study, which showed decreased IL-8 CSF levels after using disease-modifying therapy [17]. However, this decrease was not observed for serum IL-8 levels in MS patients [17]. This could indicate that local macrophage activity could be restored by using disease modifying therapies [18],[19]. These results support the potential use of cytokine profiles as diagnostic biomarkers and or as therapeutic response marker. However, other cytokines potentially associated with MS should be assessed in order to have a specific and more complete MS cytokine profile overview. Moreover, assessment of effects of immune modifying therapies

currently used for MS therapy on cytokine and chemokines will aid in obtaining insight into biological response mechanisms.

The increase in IL-6, IL-8 and TNF-α in serum of MS patients supports the idea of altered inflammation status of different cytokines in MS patients compared to healthy controls. The significant decrease of IL-6 and TNF-α in CSF RRMS patients compared to controls supports the general hypothesis that inflammatory pathways are more involved in the RRMS subtype compared to SPMS and PPMS [20]. Whether these cytokines can be seen as a specific profile for MS needs to be determined by comparing with other (auto)immune CNS pathologies.

4.1. Conclusion

In summary, the assay validation characteristics for the MSD kit were better compared to the Luminex kit for serum and CSF. Based on these results we conclude that the MSD kit is a more accurate and reproducible kit for quantification of IL-6, IL-8 and TNF-α in serum and CSF. The analysis of cytokine and chemokine profiles by multiplex platforms to capture the imbalance in the immune system is promising for future studies for MS and other auto-immune pathologies.

Acknowledgments

This work was supported by Fonds NutsOhra (Grant no. ZonMw 89000005). This study has been performed on behalf of the Treating Fatigue in Multiple Sclerosis: Aerobic Training, Cognitive Behavioural Therapy, Energy Conservation Management (TREFAMS-ACE) Study Group

Abbreviations

MSD: Meso Scale Discovery
MS: multiple sclerosis
CNS: central nervous system
CSF: cerebrospinal fluid
EDSS: Expanded Disability Status Scale

Authors' contributions

AM, HT and CT and MAB participated in the study design, and the experiments were performed by AM and HT. JK and NW, respectively provided the MS cohort and healthy controls. All authors have discussed the results and conclusions and reviewed the manuscript. All authors have read and approved of the final manuscript.

Conflict of interests

The authors declare no conflict of interests in regards with the publication of this study

Disclosure

A. Malekzadeh has nothing to disclose
H. Twaalfhoven has nothing to disclose
N. Wijnstok has nothing to disclose
J. Killestein has accepted speaker and consulting fees from Merck-Serono, Biogen Idec, Teva, Genzyme and Novartis.
C. Teunissen serves on the advisory board of Fujirebio and Roche, received research consumables from Euroimmun, IBL, Fujirebio, Invitrogen and Mesoscale Discovery, and performed contract research for IBL, Shire, Boehringer, Roche and Probiodrug; and received grants from the European Commission, the Dutch Research Council (ZonMW), Association of

Frontotemporal Dementia/Alzheimer's Drug Discovery Foundation, ISAO and the Alzheimer's Drug Discovery Foundation.

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