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Inflammatory markers in Huntington's disease plasma—A robust nanoLC–MRM-MS assay development

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ABSTRACT

The development of an MRM assay for the measurements of six inflammatory markers is presented. We report a robust and sensitive quantitative assay with a relative standard deviation of <15% that accounts for the entire sample processing. The assay has a dynamic range with 4 orders of magnitude and the LOQs are in the attomolar range. We used plasma from Huntington's disease gene carriers and healthy controls to compare our MRM method with antibody based methods. Importantly, we found a good agreement between assays for the measurement of C-reactive protein, in contrast to complement component 3 and complement factor H.

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1. Introduction

The major mass spectrometry (MS) based protein analysis principles that have progressed over the years can be divided into: (1) global expression analysis, analyzing thousands of proteins and sequencing minute amounts of sample [1–3], and (2) targeted analysis targeting a specific and smaller set of proteins measured in dedicated assays such as multiple reaction monitoring (MRM) assays. In recent years, MRM has become very popular due to its generic concept and the ability to perform multiplex quantification [4,5].

MRM is the mass spectrometric scan type with the highest duty cycle that can monitor one or more specific ion transition(s) at high sensitivity. When it is combined with appropriate stable isotope-labeled internal standards, the MRM approach provides absolute quantification of the analyte concentration. Mass spectrometry based quantitative assays for small molecules have been used in both research and clinical laboratories and also in the pharmaceutical

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industry for years, and, more recently, the technique has been applied to protein quantification [6–9]. Although MS provides sequence information of the targets, i.e., molecular specificity, and has a potential to discriminate and measure different protein variants simultaneously, MS based protein quantification has not yet been adopted by clinical laboratories, mostly due to sensitivity issues, long analysis time and considerable costs of the analyses in comparison with immunoassays. Additionally, there is a lack of biomarkers measurable by MS-based assays that can improve the clinical outcome [10–12]. Several approaches have been presented to reduce sample complexity, thereby increasing sensitivity. The most promising techniques involve immune-enrichment at protein [13] or peptide level [9,14] prior the MS readout allowing detection and quantification of targets as low as pg/mL concentration in blood plasma. The robustness of the MRM technology has also been reported in inter-laboratory studies presenting high reproducibility that could fulfill the requirements for clinical applications [15,16]. The multiplexing ability of the technique has enormous advantage in contrast to immunoassays. Parallel measurement of different protein targets in one assay reduces both the net analysis time and cost, and decreases the measurement error. Recent technological advances make MRM protein assays a convincing alternative to the classical immune-based methods [17].

The complement system is part of the innate immune system, consisting of a number of circulating and membrane-associated proteins. The main function of the complement system is the protection of the host body against pathogens but a number of studies have suggested its involvement in the development of neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases and multiple sclerosis, as well as in other diseases, such as asthma and arthritis [18,19]. There is increasing evidence that complement activation has a role in the pathogenesis of neurodegenerative disorders, including Huntington's disease (HD) [20,21]. Complement components have been reported to be increased with disease progression in HD plasma [22] in accordance with increased complement activation in HD brains [23], however, no changes in plasma levels of complement components have also been reported [24].

In the present paper we report on quantitative mass spectrometry assay development for the measurement of complement components and C-reactive protein. We have investigated the analytical performance of a nanoLC–MRM-MS platform by using the stable isotope dilution strategy. Markers involved in innate immunity were measured in plasma in a cohort consisting of HD gene carriers and controls.

2. Experimental

2.1. Materials

Water (Chromasolv® Plus for HPLC), formic acid (reagent grade ≥ 95%), dithiothreitol and iodoacetamide were purchased from Sigma–Aldrich (Steinheim, Germany); acetonitrile (Hypergrade for LC–MS) was from Merck (Darmstadt, Germany). Sequence grade trypsin was purchased from Promega (Madison, WI). Light and heavy sequences of the target peptides with purity higher than 97% (AQUA Quant-Pro quality) were purchased from Thermo Fischer Scientific (Ulm, Germany). The C-terminal arginine or lysine was labeled with ^{13}C and ^{15}N in the heavy forms, providing an increased nominal mass of 10 and 8 Da, respectively.

2.2. Clinical materials

In the current study we used samples from 30 healthy controls, 30 premanifest HD and 30 early HD subjects. Non-fasting plasma samples were obtained from control subjects and genetically diagnosed HD patients in the morning and processed as previously described [25]. Participants were recruited through the HD Multidisciplinary Clinic of the National Hospital for Neurology and Neurosurgery, London, UK. Premanifest carriers of the gene were required to have a disease burden score greater than 250 at recruitment, approximating to less than 15 years to estimated onset, and a total motor score (TMS) of five or less in the Unified Huntington's Disease Rating Scale (UHDRS) motor assessment [26]. Participants in the control group were matched for age and sex to the combined premanifest and early HD groups. The study was conducted in accordance with the declaration of Helsinki and was approved by local ethics review boards; all subjects gave informed written consent.

2.3. Sample preparation for mass spectrometric analysis

The seven most abundant proteins were depleted in 10 μL of plasma in each case by using Multiple Affinity Removal Spin Cartridge, Human-7 (Agilent Technologies, Santa Clara, CA). Following speed vacuum centrifugation the two flow-through fractions from each depleted plasma sample were combined and dissolved in 200 μL of 50 mM ammonium bicarbonte buffer containing 8 M urea, pH 7.8. The proteins were reduced with 10 mM dithiothreitol (60 min, 37 °C) and alkylated with 50 mM iodoacetamide (30 min, at room temperature in dark). The excess of the reagents was removed by buffer exchange with 50 mM ammonium bicarbonate buffer, pH 7.8 using a 10 kDa cut-off spin filter (Millipore, Billerica, MA). The sample volume for the digestion was adjusted to 200 μL, the proteins were digested with sequence grade trypsin (ca. 1:100 enzyme:protein ratio) for 18 h at 37 °C and then the digestion was stopped by the addition of 10 μL of 10% formic acid. The digested plasma samples were dried by speed vacuum centrifugation and re-dissolved in 0.1% formic acid corresponding to 100 times dilution of the original plasma. Samples were then spiked with a mixture of heavy isotope-labeled peptide standards (12.5 fmol/μL final concentration for each peptide) and analyzed by nanoLC–MRM-MS.

2.4. Quantitative LC–MS/MS analysis

Isotopically labeled peptides for optimization of the assay were mixed and diluted with 5% ACN at a concentration of 25 fmol/μL for each synthetic peptide. The mixture was analyzed by nanoLC–MS/MS using a TSQ Vantage triple quadrupole mass spectrometer equipped with an Easy n-LC II pump (Thermo Scientific, Waltham, MA). 2 μL of samples

were injected onto an Easy C18-A1 pre-column (Thermo Scientific, Waltham, MA), and following on-line desalting and trapping at a pressure of 280 bar the peptides were separated on a 75 μm × 150 mm fused silica column packed with ReproSil C18 (3 μm, 120 Å from Dr. Maisch GmbH, Germany). Separations were performed in a 25-min linear gradient from 10 to 35% acetonitrile containing 0.1% formic acid at the flow rate of 300 nL/min. The MS analysis was conducted in positive ion mode at 1750 V applied spray voltage. The transfer capillary temperature was 270 °C and tuned S-lens value was used. SRM transitions were acquired in Q1 and Q3 operated at unit resolution (0.7 FWHM), the collision gas pressure in Q2 was set to 1.2 mTorr. Scheduled method was used for data acquisition with 4 min time windows and the cycle time was set to 1.5 s, while the maximum number of consecutive transitions was 50.

The SRM assay optimization was done with the aid of Skyline v1.2 software (MacCoss Lab). Primarily, high numbers of transitions, all possible b- and y-ion series, were chosen for each peptide at both 2+ and 3+ charge states. The best 3–6 transitions were selected for further analysis and three transitions, producing the most abundant signals without observed interferences in a real background matrix, were utilized for final quantification. Collision energies (CE) were optimized for each transition. The energy was ramped around the predicted value in 5 steps on both sides with 1 V increments [27].

For the sample analysis the same chromatographic conditions were used as described previously for the assay development and the optimized parameters were used during the MS/MS data acquisition. Identical SRM parameters were used for the heavy and natural forms of each peptide, while taking into account the Q1/Q3 mass differences due to the stable isotope labeling. Table 1 presents the protein and signal peptide sequences in our assay together with the monitored transitions.

To control the instrument performance a QC sample that consist of a mixture of heavy and light synthetic peptides at a concentration of 12.5 and 5 fmol/μL, respectively, was

Table 1 – Proteotypic peptide sequences and selected SRM transitions for the six analyzed plasma proteins.

Accession no.	Protein	Peptide sequence	Q1	Q3[a]	CE
P01024	Complement C3 (C3)	TGLQEVEVK	501.8 (2+)	731.4 ($y6^{1+}$)	18
				603.3 ($y5^{1+}$)	18
				474.3 ($y4^{1+}$)	20
		TGLQEVEVK	505.8 (2+)	739.4 ($y6^{1+}$)	18
				611.3 ($y5^{1+}$)	18
				482.3 ($y4^{1+}$)	20
P00751	Complement factor B (CFB)	EELLPAQDIK	578.3 (2+)	897.5 ($y8^{1+}$)	18
				784.5 ($y7^{1+}$)	18
				671.4 ($y6^{1+}$)	18
		EELLPAQDIK	582.3 (2+)	905.6 ($y8^{1+}$)	18
				792.5 ($y7^{1+}$)	18
				679.4 ($y6^{1+}$)	18
P08603	Complement factor H (CFH)	SPDVINGSPISQK	671.4 (2+)	943.5 ($y9^{1+}$)	26
				830.4 ($y8^{1+}$)	26
				716.4 ($y7^{1+}$)	26
		SPDVINGSPISQK	675.4 (2+)	951.5 ($y9^{1+}$)	26
				838.4 ($y8^{1+}$)	26
				724.4 ($y7^{1+}$)	26
P01031	Complement C5 (C5)	IDTQDIEASHYR	483.2 (3+)	990.5 ($y8^{1+}$)	20
				633.3 ($y5^{1+}$)	20
				562.3 ($y4^{1+}$)	20
		IDTQDIEASHYR	486.6 (3+)	1000.5 ($y8^{1+}$)	20
				643.3 ($y5^{1+}$)	20
				572.3 ($y4^{1+}$)	20
P02748	Complement C9 (C9)	TEHYEEQIEAFK	508.6 (3+)	607.3 ($y5^{1+}$)	19
				494.3 ($y4^{1+}$)	19
				365.2 ($y3^{1+}$)	21
		TEHYEEQIEAFK	511.2 (3+)	615.4 ($y5^{1+}$)	19
				502.3 ($y4^{1+}$)	19
				373.2 ($y3^{1+}$)	21
P02741	C-reactive protein (CRP)	ESDTSYVSLK	564.8 (2+)	696.4 ($y6^{1+}$)	20
				609.4 ($y5^{1+}$)	18
				446.3 ($y4^{1+}$)	20
		ESDTSYVSLK	568.8 (2+)	704.4 ($y6^{1+}$)	20
				617.4 ($y5^{1+}$)	18
				454.3 ($y4^{1+}$)	20

[a] The listed transitions were selected in the final assay as best transitions for quantification.

integrated into the running sequence. After the analysis of 8 patient samples one QC sample was injected, which was followed by three blank runs. These QC runs were evaluated and the variations of the absolute and relative peak areas were monitored over the entire study.

2.5. *Data analysis*

All raw data generated on the triple quadrupole mass spectrometer were imported to Skyline v1.2 software for data analysis. Quantification was based on the ratio of corresponding light and heavy peak areas. The peak integration was done automatically by the software, using Savitzky–Golay smoothing, and all the data were manually inspected to confirm the correct peak detection. Further statistical analysis was done using Microsoft Excel, R and Matlab v7.11 (Mathworks, Natick, MA).

3. Results

3.1. *Assay development*

For the build of the multiplex protein assay we selected one representative, unique proteotypic peptide for each target

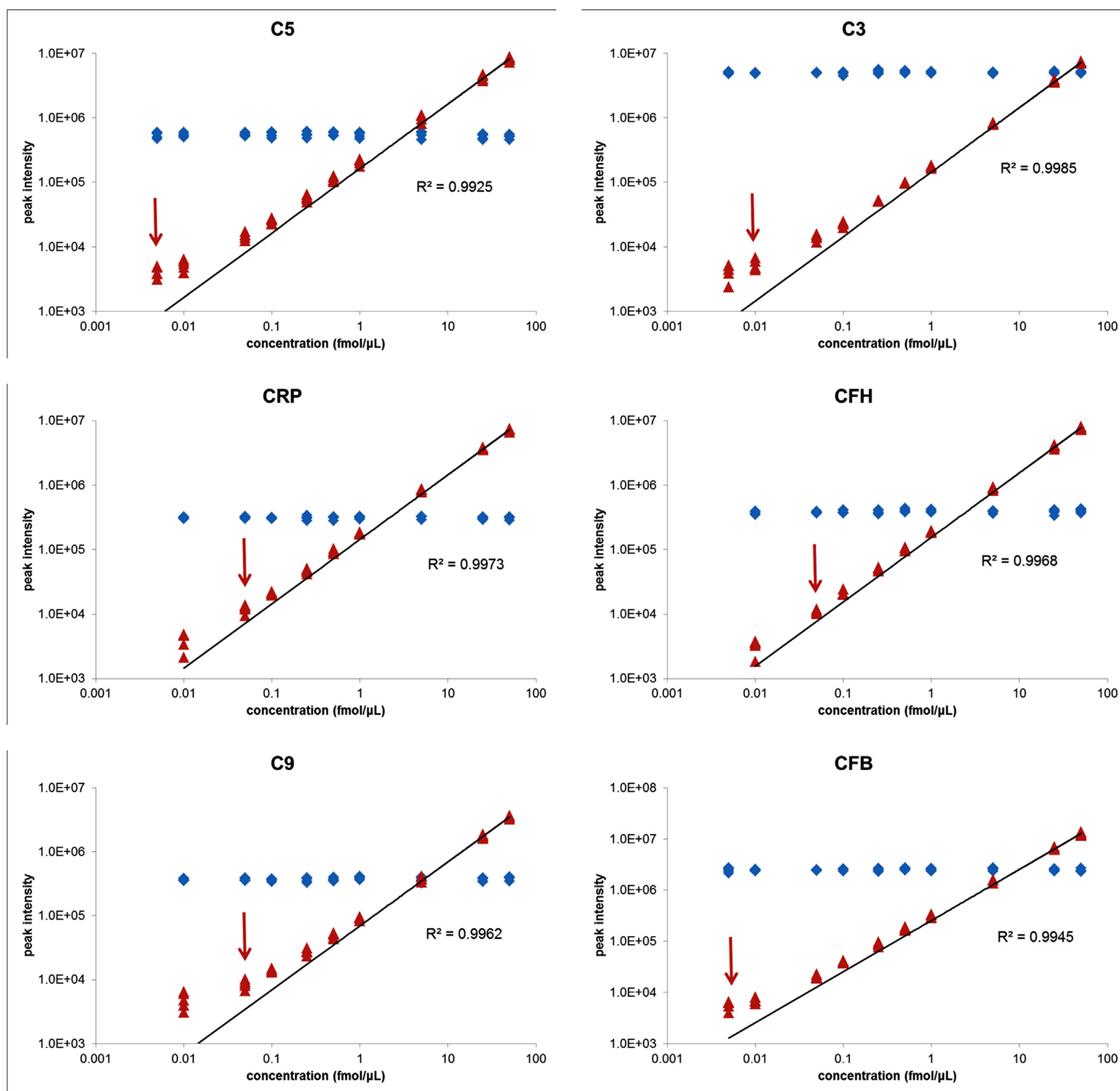

Fig. 1 – Linearity of the MRM assay determined by using heavy labeled IS peptides spiked into pooled plasma digest at various concentrations (0.005–50 fmol/μL). The measured levels of the endogenous peptides are shown as blue diamonds, whereas the LOQ (CV < 20%) is indicated with arrow.

protein and one precursor ion for each peptide. With the aid of Skyline software we selected the best transitions, preferably y-ions with higher mass to charge values free from matrix interferences and optimized the collision energies as well, and the three best transitions were used for quantification (Table 1). We also investigated the linearity within a concentration range of 0.2–200 fmol/μL in buffer solution and in real matrix. All signal responses were linear in the tested concentration range, represented by regression coefficients higher than 0.98. The limit of quantification (LOQ) was determined for each target peptide by creating and analyzing a dilution series of pooled plasma digest spiked with different amount of heavy internal standards ranging from 0.005 fmol/μL to 50 fmol/μL in five replicates. The LOQ of these peptides in blood plasma was estimated as the lowest concentration measured with CV <20% and was found to be in the attomolar range (10–100 attomoles on column), see Fig. 1.

3.2. *Reproducibility of the assay*

Prior to the clinical sample analysis we evaluated the reproducibility of the complete preparation workflow that includes depletion, digestion and spiking with heavy standards as well. Additionally, depletion spin cartridges from two different batches were compared and we could not find significant differences in their performance. Fig. 2 shows the comparison of the two cartridges based on the measurements of three biological replicates on each depletion cartridge. We obtained somewhat higher variations when using the first batch, but the coefficient of variation (CV) values were less than 20% for each target protein. The preparations of the clinical samples were performed on different days due to the high number of samples; therefore, we used a control plasma sample from a healthy volunteer, which was treated in the same way as clinical patient samples on each day. These processing controls were used for the evaluation of the reproducibility of the complete sample processing workflow. The variation of the spiking with heavy labeled internal standards was also evaluated. The CV of the total sample processing, which was calculated based on the triplicate measurements of four sample processing controls was equal or less than 15% for the 6 target peptides, whereas the relative standard deviation in testing the sample spiking with heavy standards was ≤10%.

Furthermore, ≈20% of the total samples was run in triplicates in order to evaluate the variation of the assay. The variation of the MRM measurement due to the isotopically labeled internal standards was very low, because the quantification is based on the endogenous signal relative to the internal standard signal. 40% of the triplicate measurements had relative standard deviation (RSD) equal or less than 2%, and in 75% of the measurements the RSD is equal or less than 5%, as it is displayed in Fig. 3.

The patient samples were analyzed randomly and quality control samples (mixture of light and heavy synthetic peptides) were run regularly using the same method to control the instrument performance. The variation of the calculated relative peak intensities in the control samples was below 5%, while the absolute signal intensities varied with 25% or less during the analysis of the complete patient sample set. This observation supports the benefit of using isotopically labeled internal standards. We observed similar values (≤25%) when the heavy labeled internal standard signal intensities in the entire set of analyzed samples were compared, while the retention time variation was less than 1%.

3.3. *Analysis of Huntington's disease patient samples*

We have analyzed EDTA plasma pooled from 16 individuals each and 90 individual patient samples from early and premanifest Huntington's disease patients and age matched control donors (Suppl. Table 1). The analysis of the individual samples confirmed the observations made by the investigation of the pooled samples, we recognized the same tendencies in protein levels in-between the 3 sample groups (Fig. 4).

Some of the markers were measured simultaneously with antibody-based assays and the results of the different methods were compared. Interestingly, we could find good agreement between the assays only in the measurement of C-reactive protein (CRP) but not in complement factor H (CFH) and complement component 3 (C3). The correlation coefficient

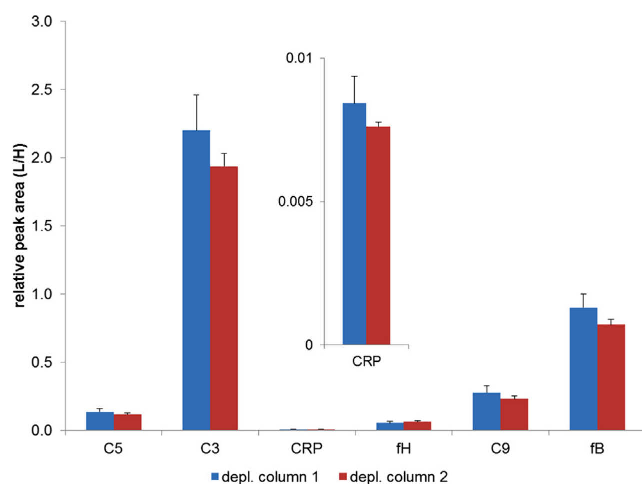

Fig. 2 – Comparison of the performance of two different depletion cartridges.

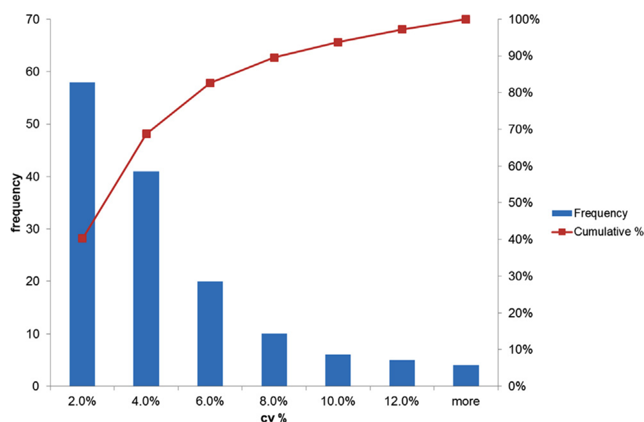

Fig. 3 – Illustration of the technical reproducibility of the MRM assay.

was 0.925 for CRP, 0.442 for CFH and −0.053 for complement C3 (Suppl. Fig. 1).

4. Discussion

The targets of the assay herein described are identical to the assay we reported earlier [28]. However, due to different instrumentation the assay parameters, such as transition selection, collision energy, and cycle time, were newly optimized. The triple quadrupole instrument allows us to monitor more transitions without losing the appropriate amount of data points across the peak and in addition, provides wider dynamic range and improved sensitivity. The reproducibility of the MRM assay performed on the TSQ Vantage instrument was considerably better than that in the

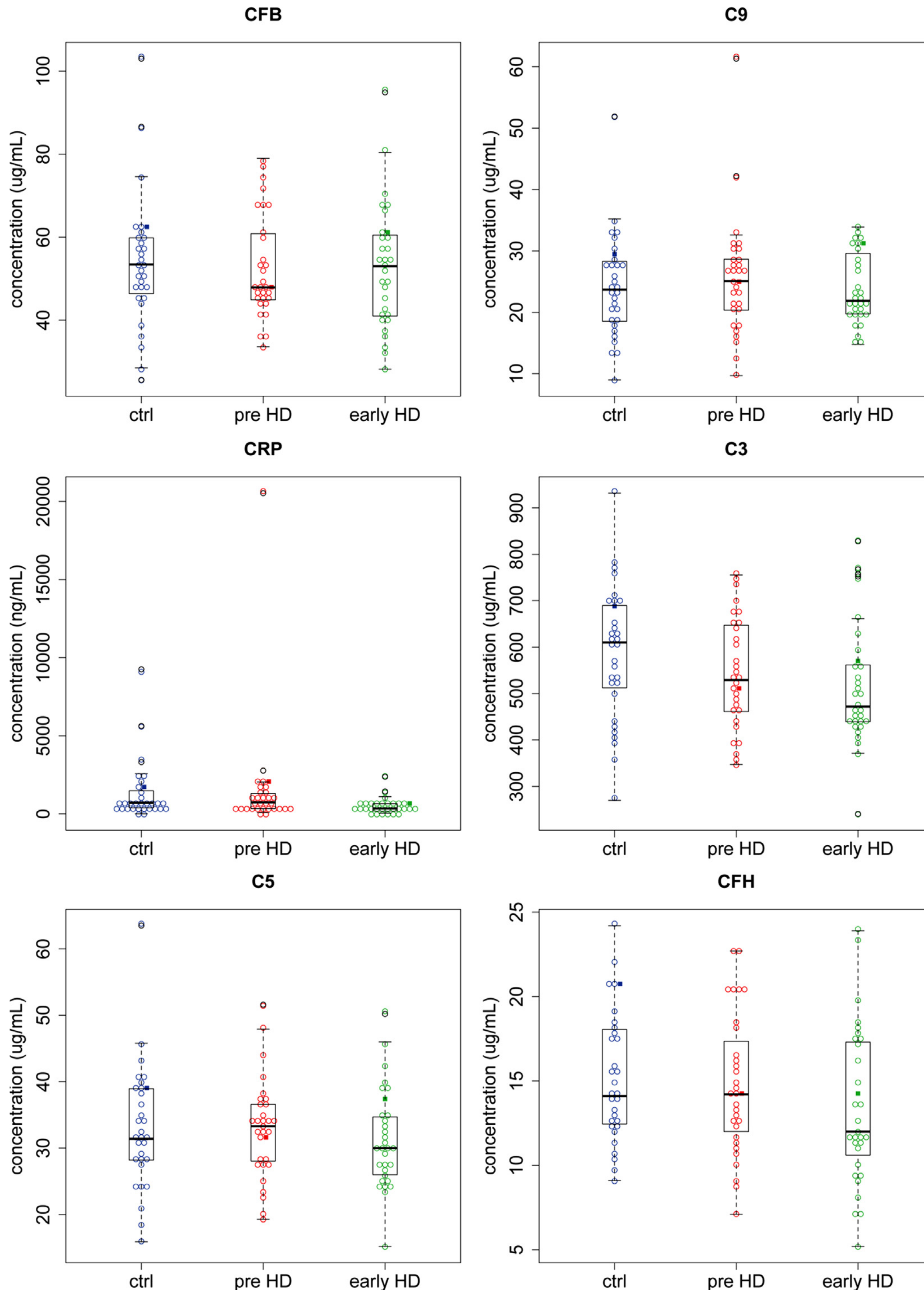

Fig. 4 – Box-plot illustration of the levels of the six inflammatory markers measured in the three patient groups. A circle represents each individual sample and the levels of the pooled samples are illustrated as squares.

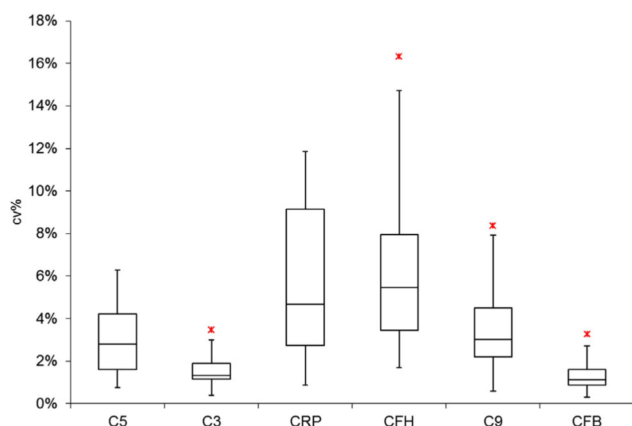

Fig. 5 – Box-plot illustration of the reproducibility of the assay based on triplicate measurements.

previous assay executed on a linear ion trap instrument. The precision of the assay was dependent on protein concentration and on the concentration of the spiked internal standard. The relative standard deviation of the triplicate measurements was much lower when the concentration ratio of the endogenous compound and the corresponding internal standard was close to 1, as illustrated in Fig. 5. This observation agrees well with the finding of Borchers' group [29]. The best precision was achieved by the measurements of C3 and CFB, where the average endogenous/standard ratios were kept at 2.3 and 0.5, respectively, while the poorest relative standard deviations were measured for CFH and CRP with average endogenous/standard ratios of 0.08 and 0.04, respectively.

The removal of the most abundant proteins from plasma increased the preparation time and moderate and low abundant proteins might be lost in this step due to non-specific binding. However, depletion could contribute to protecting the chromatographic system from overload, minimizing the matrix interferences and improve the analytical reproducibility. Our previous results showed that the depletion step did not significantly increase the variation of the sample preparation but in addition improved the measurement precision [30].

Although increasing number of studies have demonstrated the role of the complement system in neurodegenerative diseases and reported up- and down-regulated complement system members based on comparative proteomics analysis of different patient groups [22,23], we were not able to justify these findings by the investigation of two different subject cohorts [24]. These contradictory results may be attributed to different patient material handling, heterogeneity of the patient cohort or the various measurement procedures. Parallel to the mass spectrometry based quantification additional inflammatory markers were measured in the same sample cohort using antibody-based assays (Luminex assays). None of the examined markers showed correlation with disease progression as it was reported in our recent publication [24]. Three of the markers were measured with both antibody and MRM assays in this study but interestingly, only the CRP values showed good correlation between the two techniques. CRP is a relatively small molecule, in comparison with C3 and CFH that have many different epitopes, against which antibodies are available with various specificities. A possible explanation for the poor correlation in-between the two quantification techniques may be that the Luminex assay detects different forms of the protein, or has different affinity to different forms than the MRM assay.

5. Conclusions

In the present paper we reported on a quantitative mass spectrometry assay developed for the measurement of complement components and C-reactive protein. We presented a highly reproducible nanoLC–MRM-MS platform that allows absolute quantification of 6 target proteins using the stable isotope dilution strategy. The assay had a dynamic range with at least 4 orders of magnitude and the LOQs of the various targets were in the attomolar range. The assay was successfully utilized for the analysis of a HD patient cohort.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euprot.2014.02.003.

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