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# The use of multiplex platforms for absolute and relative protein quantification of clinical material

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ABSTRACT

When introducing multiplex platforms to measure protein content in precious clinical material there is an increased risk of cross reactivity, loss of sensitivity as well as accuracy. In this paper, four multiplex platforms and one singleplex platform were compared by running pre- and post-treatment plasma samples from CML patients. We found a variation of absolute protein concentrations between platforms. For some of the analytes and platforms, relative differences between pre- and post-treatment samples correlated. We conclude that absolute concentrations measured by different platforms should be compared with caution and comparing relative differences could be more accurate.



## Significance

Today many research groups select a multiplex platform for their clinical research investigations based on the current availability of a certain machine in-house. Clinical material is usually scars and can rarely be used to explore intra- or inter assay variations with regard to absolute or relative protein concentrations; still the material as well as the assay can affect the results obtained. Herein we had an unique possibility to obtain and analyze clinical material from CML patients pre- and post TKI therapy to assess inflammatory patterns using a plethora of assays in order to investigate how absolute and relative protein concentrations correlate between these platforms.

## 1. Introduction

The possibility of multiplex protein analysis in complex samples such as human plasma or serum is becoming increasingly important and the multiplexed platforms are used in for example clinical diagnostics; biomarker validation; measurement of changes in protein abundance and modeling

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networks, and measurement of pathways in physiological and disease states [1]. Multiplex protein quantification sets new and high demands on specificity of the detection reagents as well as buffers and other reagents used, as various targets may require different conditions for optimal quantification [2]. Consequently, multiplex assays require high levels of validation to ensure that the targets are detected optimally and with high specificity. Vignali has defined seven key parameters for creating a perfect multiplexed assay including specificity, sensitivity, simplicity, reliability, multiplexing abilities, cost and time [3].

Dependent on the application of the assay, some of these parameters may be more important than others. Additionally, certain patient material may affect assay results, requiring both the platform producer and the user to carefully evaluate data output with respect to the selected material. In this paper, we have investigated four different multiplex platforms for protein measurements and compared the results to results obtained from the golden standard of soluble protein quantification, the ELISA. Three of the multiplex platforms investigated (BioPlex, Meso Scale Discovery and Myriad RBM) as well as the ELISAs determine the absolute concentration of protein in the samples, while one platform (Somalogic) is designed as a discovery platform and measures only relative concentrations of protein.

The three multiplex platforms used for quantitative determination of proteins are, similar to ELISA, immunoassays where analytes are "sandwiched" between a capture- and a detection antibody before detection [2]. In a common singleplex ELISA the detection antibody is usually conjugated to an enzyme, that after the addition of enzyme substrate catalyses a reaction leading to color development in the microtiter plate. The intensity of the color is measured by spectrophotometry and corresponds to the amount of the specific protein to be detected in the unknown sample [4]. Another method, developed by Meso Scale Discovery, applies electrochemiluminescence to quantify the proteins in a microtiter plate [5]. This assay can be multiplexed since capture antibodies specific for different targets can be bound to distinct spots in the bottom of a microtiter plate. In the microsphere-based technology developed by Luminex Corporation, the Fc-parts of the capture antibodies are bound to groups of fluorescent microspheres. Each group of microspheres has slightly different fluorescence intensity and is covered with antibodies recognizing a distinct protein. The detection antibody is coupled to a fluorescent molecule to enable detection [2]. With the Luminex method two systems to quantify the level of protein are available. One of the systems, applied by Myriad RBM in this paper, is flow-based [6]. The other microsphere-based system utilizes magnetic fluorescent beads and is used by the BioPlex kit in this paper [7]. The methods described in this paper have different abilities of multiplexing, Meso Scale Discovery kits are available with up to 10 different analytes [8] while the Luminex technology can assay up to 50 analytes with the magnetic beads and up to 500 analytes with the flow technology [9].

Somalogic has developed a multiplex method for relative protein quantification of up to over 1100 analytes in one sample [10]. This technique is based on aptamer binding. Aptamers are folded, single-stranded, anionic oligonucleotides that can bind proteins with high specificity and affinity. Somalogic has developed Slow Off-rate Modified Aptamers called SOMAmers, these are modified aptamers that have a slower dissociation rate of the aptamer from its target protein compared to normal aptamers [11].

In this study, characteristics of different multiplex protein detection platforms and their ability to detect various proteins were compared. Plasma from patients with chronic myeloid leukemia (CML) before and after tyrosine kinase inhibitor (TKI) treatment was investigated in the study. We show that the absolute protein concentration varied when measured by the different platforms. However, fold changes in protein concentration after TKI treatment correlated for certain, but not all, analytes on some platforms.

## 2. Materials and methods

### 2.1. *Patient samples, control sample and sample preparation*

Frozen acid citrate plasma samples from patients with CML at baseline (before treatment) and after three months of treatment with imatinib (Gleevec, Novartis Pharmaceuticals, Basel, Switzerland) or dasatinib (Spyrcel, Bristol-Myers Squibb, New York, USA) were obtained from Helsinki University Central Hospital. The study was conducted in accordance with the Helsinki Declaration and was approved by the Regional Research Ethics Committee. All patients gave their written informed consent. Frozen samples were shipped to Uppsala, thawed on ice and subsequently vortexed. Aliquots of appropriate volumes for each analysis were pipetted into different tubes and refrozen at −70 °C. In each run a control sample containing chicken plasma was included.

### 2.2. *BioPlex Pro Human Cytokine 27-plex Panel*

Frozen plasma samples were thawed in 37 °C water bath and put on ice directly after thawing. Samples were centrifuged at 10,000 × *g* for 10 min prior to analysis to remove cell debris and aggregates. BioPlex Pro Human Cytokine 27-plex Panel (Bio-Rad Laboratories, Hercules CA, USA) analysis with MagPlex beads was performed in a flat bottom microtiter plate according to the manufacture's instructions. Briefly, samples were diluted 1:4 in sample diluent. Standard was reconstituted and diluted in a fourfold dilution series. Antibody coupled capture beads were prepared and plated. The bead solution was vortexed before addition to each well. Plate was washed, all wash steps were performed manually. First, wash solution was added to the plate that was subsequently covered with sealing tape. The plate was incubated on a shaker for 30 s at 1100 rpm and then for 1.5 min at 300 rpm. The plate was taken off the shaker and was incubated on a magnet for 1 min before the supernatant was discarded. After washing, diluted samples and standards were added in duplicates to the beads in the wells. The plate was incubated on a shaker and after incubation and wash, detection antibodies were added to each well. The plate was again incubated on a shaker and after another washing step, streptavidin-phycoerythin solution was added to the wells. After a last incubation step, beads

were resuspended in assay buffer and the plate was read with a MagPix (Luminex Corporation) using the xPONENT software (Luminex Corporation, Austin, TX, USA). The results were analyzed using the xPONENT software. The absolute concentrations of the samples were determined by construction of a standard curve for each analyte. Points in the standard curve with recovery below 70% or above 130% were invalidated and curve fit was made with a weighted 5PL method. In total, a volume of 30 μl undiluted sample was used for running duplicate in this assay.

### 2.3. *Interferon gamma (IFNγ) and monocyte chemotactic protein-1 (MCP-1) ELISA*

ELISAs for IFNγ and MCP-1 (BioLegend, San Jose, CA, USA) were run according to the manufacturers' instructions. Briefly, all components including frozen patient samples and control chicken plasma were brought to room temperature. Patient samples were spun at 10,000 × *g* for 5 min where after they were diluted in assay buffer. Samples were diluted 1:2 for the IFNγ ELISA and 1:4 for the MCP-1 ELISA. Standards were diluted in a twofold dilution series. Standard and samples were added in duplicates to the wells containing capture antibodies. For duplicate wells, the total volume of undiluted sample used for MCP-1 and IFNγ ELISAs was 30 μl and 55 μl, respectively. After incubation and wash, detection antibody was added. The plate was incubated and after another wash Avidin-HRP solution was added to the wells. After incubation, the plate was washed and substrate solution was added to the wells. Following a last incubation step, stop solution was added to the plate and the absorbance was read at 450 nm with the Emax ELISA reader (Molecular Devices LLC, Sunnyvale, CA, USA). The 570 nm absorbance was subtracted from the 450 nm value. SoftMax 2.35 software (Molecular Devices) was used for construction of standard curves and determination of protein concentration in unknown samples. Curve fitting was done with a 4PL algorithm according to the manufacturer's instructions.

### 2.4. *Human proinflammatory 9-plex Ultra-Sensitive kit*

Cryopreserved plasma and control samples as well as all kit components from the Human proinflammatory 9-plex Ultra-Sensitive kit (Meso Scale Discovery, Rockville, MD, USA) were brought to room temperature before analysis. The analysis was performed according to the instructions from the manufacturer. Reverse pipetting was applied in all pipetting steps to avoid bubbles. Briefly, the antibody coated plate was incubated with diluent for 30 min on a shaker (800 rpm). The calibrator containing known concentrations of all analytes to be analyzed was diluted in a fourfold dilution series to create a standard curve. Patient plasma samples were spun at 10,000 × *g* for 5 min to remove cell debris and aggregates. Duplicates of diluted calibrator and undiluted plasma samples were loaded on the plate and the plate was incubated on a shaker for two hours. After washing, labeled detection antibodies were pipetted to the wells and the plate was incubated for another 2 h. After incubation the plate was washed and read buffer was added to the plate just before reading in the SECTOR Imager 2400 (Meso Scale Discovery) using the MSD discovery workbench Software (Meso Scale Discovery). Curve fitting was done in the same software using a 4PL fit with $1/y^2$ weighting according to the manufacturer's instructions and concentrations were determined from the standard curves. For running duplicates in this assay, a total volume of 50 μl undiluted sample was used.

### 2.5. *Multi-analyte profiling (MAP) technology by Myriad RBM*

Tubes containing 100 μl frozen aliquots from all patient samples and chicken plasma control sample were shipped to Myriad RBM (Salt Lake City, UT, USA). Analysis of the custom human MAP containing 44 different analytes was run according to the laboratory standard operating procedures that involved automated pipetting. Samples were run in singlet.

### 2.6. *SomaScan by Somalogic*

Tubes containing 100 μl frozen aliquots from all patient samples and chicken plasma control sample were shipped to Somalogic (Boulder, CO, USA). 1129 analytes were analyzed in the SomaScan according to the laboratory standard operating procedures. Samples were run in singlet. Data were analyzed using the SomaSuite software (Somalogic).

### 2.7. *Data analysis*

The percentage of samples in which protein concentrations within the limits of quantification could be detected was depicted as % detectable samples for each analyte. After curve fitting and determination of absolute and relative concentrations in respective software, mean- and max fold changes as well as pooled CV-values were calculated. The mean fold change was calculated as the ratio between the mean concentration at three months time point and the mean concentration at baseline. To determine the maximum (max) fold change, fold changes for each individual patient was first calculated. Pooled coefficient of variation (CV) values were calculated by the formula:

$$CV_{pool} = \sqrt{\left[\frac{(CV_1^2 + CV_2^2, \cdots, CV_n^2)}{n}\right]}$$

where $CV_1$, $CV_2$, etc. represent CV-values obtained from each sample, and $n$ is the number of samples for which the pooled CV is calculated.

Fold changes were only calculated for samples where both of the duplicates were within the limits of quantification and CV-values were only included in the pooled CV-value if both of the duplicates were within the limits of quantification. To be able to compare measurements of protein concentrations in chicken plasma between all platforms, the ratio between protein concentration in chicken plasma and the protein concentration in the patient sample with the lowest detectable concentration of that particular analyte was calculated.

### 2.8. Statistical analysis

For correlation of relative differences between different platforms, the non-parametric Spearman correlation was applied. To test whether or not fold changes were different between platforms, the non-parametric unpaired Kruskal Wallis test and Dunn' post test was used when more than two platforms were compared. When two platforms were compared, Wilcoxon matched-pairs signed rank test was applied.

## 3. Results

### 3.1. Platform characteristics

In Table 1 characteristics of the different platforms are seen. The specificity of the assays has been tested by most manufacturers, but information of which analytes that have been tested for cross-reactivity was only available from the ELISA manufacturer. The cost per data point was highest for the ELISAs and lower for the multiplexed platforms. The Somalogic service had a high cost, but since a lot of analytes were investigated at the same time the cost per data point was much lower than for the other platforms. Protocols and instructions from manufacturers were easy to follow except for the BioPlex protocol that was sometimes problematic to interpret. The time consumed for running the different protocols in-house was between six and eight hours per kit.

### 3.2. The sensitivity of detection varied between platforms and the analyte investigated

As shown in Table 2 and Supplementary Tables 1–5 the ability to detect concentrations of investigated analytes in the samples, depicted as % detectable samples, varied between the different platforms. Samples with concentrations within the indicated limits of detection were considered detectable. MCP-1 concentrations could be detected in most samples on all different platforms investigated. IFNγ levels above background was detected in all samples after analysis with the BioPlex and Somalogic platforms but only in a few samples using the ELISA, Meso Scale Discovery and Myriad RBM platforms. For IL-8, the Meso Scale Discovery platform was more sensitive than BioPlex and Myriad RBM as protein concentrations could be measured in all samples. For the Somalogic platform, no limit of quantification or detection was reported, hence, here we report that all analytes could be detected in all samples.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euprot.2014.02.002.

### 3.3. CV values were acceptable for most analytes on all platforms investigated

For the BioPlex, ELISA and Meso Scale Discovery duplicates were run, hence, accuracy of these platforms could be reported by the CV-value. From all CV-values within one run, a pooled CV-value was calculated for each analyte. As seen in Table 2 and Supplementary Tables 1–3 the pooled CV-values were acceptable, <15% for all analytes but three. The ELISA platform had pooled CV-values of 2.81–13.69% (Supplementary Table 2) and Meso Scale Discovery had pooled CV-values of 6.24–14.87% (Supplementary Table 3). For the BioPlex platform all pooled CV-values but three were between 0.77 and 11.53%. The IL-8 assay had a pooled CV-value of 13.86%, the IL-1ra assay had a pooled CV-value of 32.38% and the G-CSF assay had a pooled CV-value of 22.11% (Supplementary Table 1). Somalogic reports a median %CV-value of 5.1% in plasma, this value was based on a reproducibility experiments spanning 12 individuals, 3 assay plates, and different operators, however, a CV-value for our specific run was not reported.

### 3.4. The relative changes in protein concentration after treatment varied between the platforms

Since the absolute protein concentration was not measured by all platforms the relative difference in concentration between the two time points was used to compare the performances of the different platforms (Table 2). A mean fold change greater than 1 corresponds to increased mean concentration after TKI treatment and a mean fold change of less than 1 corresponds to decreased concentration after TKI treatment. For the analytes IL-8, IL-6, VEGF and sE-selectin, a decrease in mean concentration after treatment was measured by all platforms measuring these analytes. For the analytes MMP-3 and PDGF-BB, an increase in mean concentration after treatment was seen with all methods. ELISA and Myriad RBM detected an increase in IFNγ concentration after treatment while BioPlex, Meso Scale Discovery and Somalogic all detected decreasing levels. For MCP-1, all platforms but Somalogic detected increased concentrations with treatment. Myriad RBM detected an increase in sCD40L after treatment while Somalogic detected a decrease. In Fig. 1, fold changes after TKI treatment measured by the different platforms are shown for the individual patients. For MCP-1, VEGF and sE-selectin (Fig. 1B, E and F) all platforms measuring theses analytes detected similar fold changes. For other analytes such as IFNγ, IL-8, IL-6 and sCD40L (Fig. 1A, C, D and H) there was a large variation in fold changes measured. For IL-8 the median fold change was significantly different when comparing the Meso Scale Discovery and the Somalogic platforms. For PDGF-BB the fold changes followed the same pattern in both the BioPlex and the Somalogic platforms. The magnitudes of the fold changes, however, were significantly different (Fig. 1I). In the Somalogic platform 430 (38%) of the proteins were significantly different expressed between the two time points, only two of these proteins displayed elevated levels after treatment (data not shown). A company representative informed us that this high number of proteins with decreased concentration may be due to unspecific binding in samples taken before treatment.

### 3.5. Relative differences in protein concentrations correlated between the different platforms for some analytes

Direct correlations between relative differences in protein concentration measured on different platforms were made to investigate if an increase/decrease in one method gave the same result in another method (Figs. 2 and 3). The relative

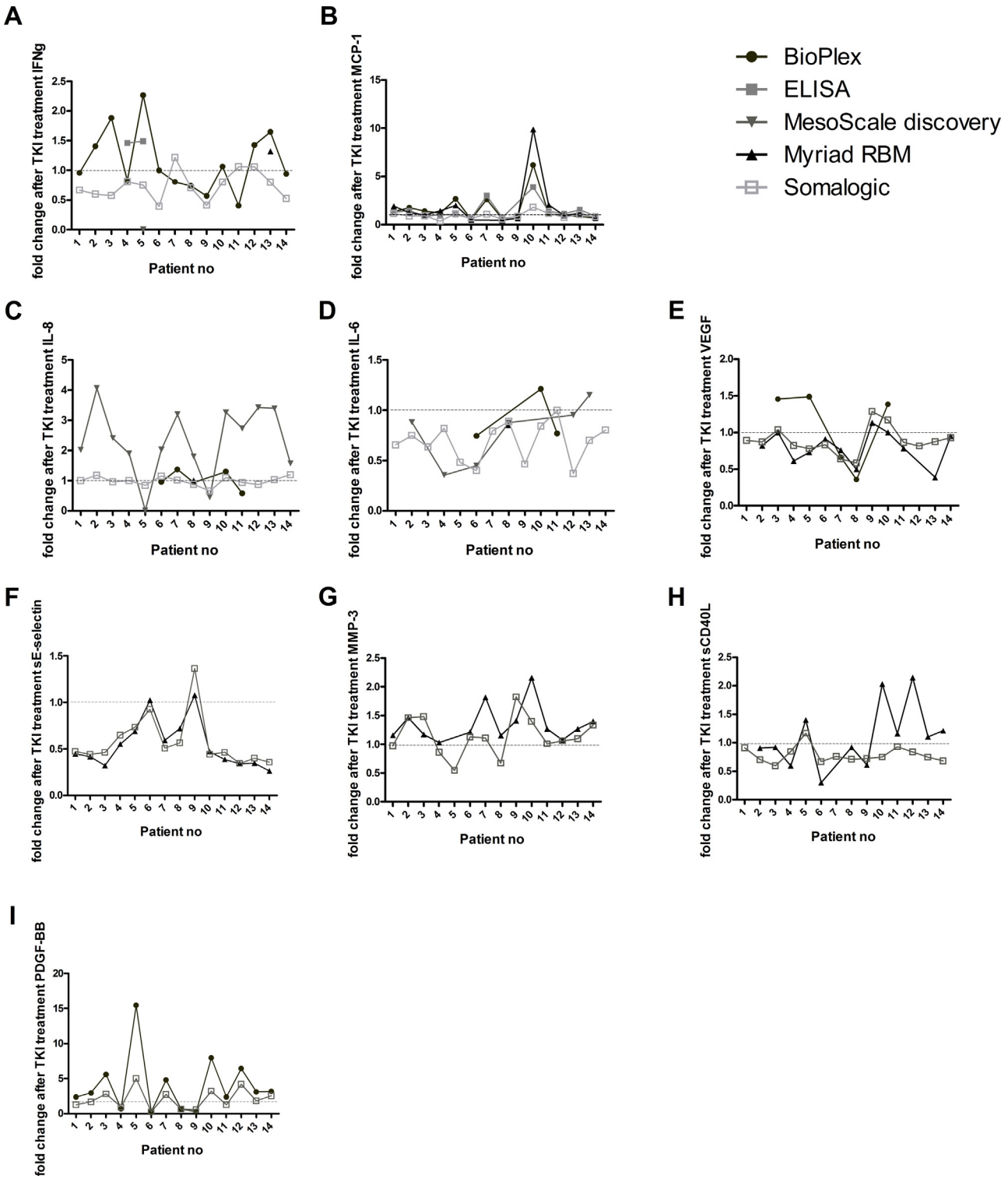


**Fig. 1 – Relative differences in protein concentrations of individual patients. The relative differences in protein concentration were calculated as described in Section 2. The relative differences measured by the various platforms for the individual patients are shown. Dots below the dotted line indicate a decrease in concentration after treatment and dots above the dotted line symbolize an increase after treatment. The different platforms are represented by a distinct shape. Relative differences for the proteins IFNγ (A), MCP-1 (B), IL-8 (C), IL-6 (D), VEGF (E), sE-selectin (F), MMP-3 (G), sCD40L (H), PDGF-BB (I) are shown.**

**Table 1 – Brief description of the various platforms tested.**

| | ELISA | BioPlex | Meso Scale Discovery | Myriad RBM | Somalogic |
|---|---|---|---|---|---|
| Specificity | Cross-reactivity has been tested for various recombinant cytokines/chemokines | Specificity has been tested, however, no information about which analytes that have been tested is available | No information | Validation has been performed as defined by principles by the Clinical Laboratory Standards Institute (USA) | No information |
| Simplicity | Protocol was easy to follow | Partly confusing protocol | Protocol was easy to follow | Instructions were easy to follow | Instuctions were easy to follow |
| No. of analytes analyzed | 1/kit | 27 | 9 | 44 | 1129 |
| Cost (USD)/kit | 560[a] | 6700[a] | 1728[a] | 5940[b] | 31000[b] |
| Cost (USD)/data point | 9.00 | 4.00 | 3.10 | 4.35 | 0.89 |
| Time | ca 6 h/kit | ca 1 working day | ca 7 h | 24 days from sending samples to receiving report | 38 days from sending samples to receiving first results |
| In house/Service | In house | In house | In house | Service | Service |

[a] Costs for personnel and analysis equipment not included.
[b] Freight not included.

difference in MCP-1 concentration was significantly correlated in all methods that measured this analyte (Fig. 2A). Also the relative differences in sE-selectin (Fig. 2B) and MMP-3 (Fig. 2C) concentrations measured by Myriad RBM correlated to the differences measured on the Somalogic platform. Relative PDGF-BB concentration differences measured by Somalogic correlated to the differences measured by BioPlex (Fig. 2D). For VEGF the relative differences correlated significantly when measured by Somalogic and Myriad RBM but not when Myriad RBM was compared to BioPlex measurements (Fig. 3A). The IL-8 relative difference in concentration measured by Meso Scale Discovery did not correlate to the difference measured by BioPlex or Somalogic (Fig. 3B). Spearman *r*-values and *p*-values are indicated in respective graph.

### 3.6. *Absolute protein concentration differed between the platforms*

The BioPlex, ELISA, Meso Scale Discovery and Myriad RBM platforms all determined the absolute concentration of an analyte in a sample with help of standard curves. Hence, for some of the analytes, the absolute concentration could be

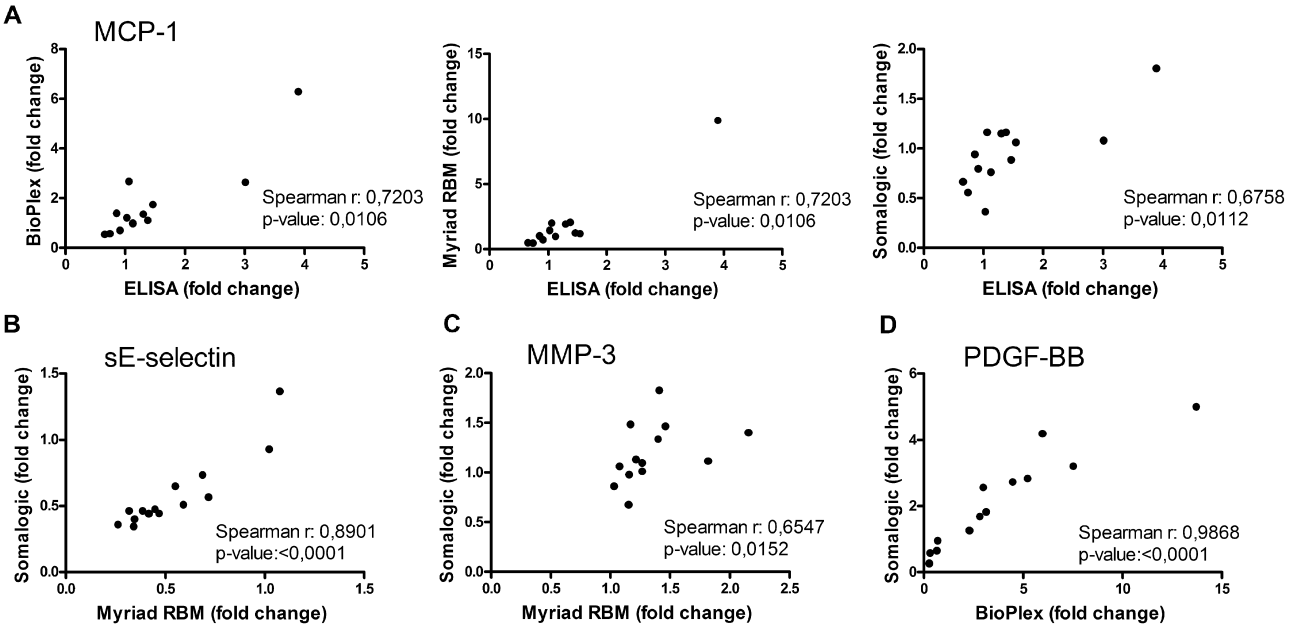


**Fig. 2 – Correlations of relative differences between different platforms. (A) Correlation of relative differences of MCP-1 measured by four different platforms. (B and C) Correlation of relative differences in sE-selectin (B) and MMP-3 (C) concentration measured by Somalogic and Myriad RBM. (D) Correlation of relative differences in PDGF-BB concentration measured by Somalogic and BioPlex. Statistically significant correlations were determined with the non-parametric Spearman correlation. Spearman *r*-value and *p*-values are indicated in the graphs.**

**Table 2 – Analyte characteristics for the different platforms analyzed.**

| Analyte | Standard curve range | Detectable samples (%) | Mean fold change | Max fold change | Pooled CV (%) |
|---|---|---|---|---|---|
| **IFNγ** | | | | | |
| BioPlex | 1.87–30,646 pg/ml | 100 | 0.99 | 2.25 | 11.53 |
| ELISA | 15.6–1000 pg/ml | 14 | 1.47 | 1.49 | 2.81 |
| Meso Scale Discovery | 0.61–10,000 pg/ml | 32 | 0.02 | 0.004 | 10.06 |
| Myriad RBM | Not provided | 11 | 1.32 | 1.32 | – |
| Somalogic | No standard curve | 100 | 0.70 | 1.22 | – |
| **MCP-1** | | | | | |
| BioPlex | 1.45–23,735 pg/ml | 96 | 1.24 | 6.28 | 7.02 |
| ELISA | 7.8–500 pg/ml | 93 | 1.23 | 3.90 | 13.69 |
| Myriad RBM | Not provided | 96 | 1.23 | 9.88 | – |
| Somalogic | No standard curve | 100 | 0.82 | 1.80 | – |
| **IL-8** | | | | | |
| BioPlex | 1.71–27,965 pg/ml | 54 | 0.98 | 1.37 | 31.86 |
| Meso Scale Discovery | 0.61–10,000 pg/ml | 100 | 0.18 | 4.13 | 11.92 |
| Myriad RBM | Not provided | 29 | 0.95 | 1.00 | – |
| Somalogic | No standard curve | 100 | 0.98 | 1.19 | – |
| **IL-6** | | | | | |
| BioPlex | 1.18–19,412 pg/ml | 43 | 0.93 | 1.21 | 9.15 |
| Meso Scale Discovery | 0.61–10,000 pg/ml | 54 | 0.75 | 1.17 | 13.96 |
| Myriad RBM | Not provided | 7 | 0.85 | 0.85 | – |
| Somalogic | No standard curve | 100 | 0.47 | 1.00 | – |
| **VEGF** | | | | | |
| BioPlex | 1.79–29,354 pg/ml | 54 | 0.80 | 1.48 | 5.01 |
| Myriad RBM | Not provided | 93 | 0.70 | 1.13 | – |
| Somalogic | No standard curve | 100 | 0.86 | 1.29 | – |
| **sE-selectin** | | | | | |
| Myriad RBM | Not provided | 100 | 0.45 | 1.08 | – |
| Somalogic | No standard curve | 100 | 0.50 | 1.36 | – |
| **MMP-3** | | | | | |
| Myriad RBM | Not provided | 100 | 1.28 | 2.16 | – |
| Somalogic | No standard curve | 100 | 1.10 | 1.83 | – |
| **sCD40L** | | | | | |
| Myriad RBM | Not provided | 93 | 1.06 | 2.03 | – |
| Somalogic | No standard curve | 100 | 0.80 | 1.17 | – |
| **PDGFbb** | | | | | |
| BioPlex | 1.56–25,637 pg/ml | 100 | 2.16 | 13.71 | 6.53 |
| Somalogic | No standard curve | 100 | 1.61 | 5.00 | – |

compared between the platforms. The concentration measured by one platform did not correspond to the concentration measured by all other platforms for any of the analytes investigated (Fig. 4). For MCP-1 similar patterns of protein concentration was seen after measurement by the three different methods. In the ELISA and Myriad RBM methods, the concentrations measured were in about the same range with exception of a few samples. The concentrations measured by the BioPlex platform were lower than the concentrations measured by the two other platforms (Fig. 4B). IL-8 was detected in all samples on the Meso Scale Discovery platform, but only in a few samples on the BioPlex and Myriad RBM platforms. The concentrations, however, were higher in most of the detected samples of the BioPlex and Myriad RBM platforms (Fig. 4C). Both Meso Scale Discovery and Myriad RBM measured very high concentrations of IL-6 in samples from one patient both before and after treatment. The BioPlex method did not detect high levels of IL-6 in the same samples (Fig. 4D).

### 3.7. *Unspecific binding of protein in chicken plasma*

To compare the amount of unspecific binding to proteins present in plasma from other species, a chicken plasma sample was run on all platforms. To be able to compare the amount of protein measured in chicken plasma by the different platforms, protein concentration in chicken plasma was divided with the concentration measured in the patient sample with lowest detected concentration for the specific analyte. As seen in Fig. 5, BioPlex (Fig. 5A), ELISA (Fig. 5B), Myriad RBM (Fig. 5C) and Somalogic (Fig. 5D) all measured concentrations of at least one analyte in the chicken plasma. In the BioPlex assay the concentration of 18.5% (5/27) of the analytes were higher in the chicken plasma than in the sample with lowest detected concentration of that specific analyte (ratio > 1). Corresponding values for ELISA and Somalogic were 100% (2/2) and 76% (858/1129), respectively. The Somalogic platform detected concentrations of analytes in chicken plasma that was more than ten times higher than the lowest detected concentration in a

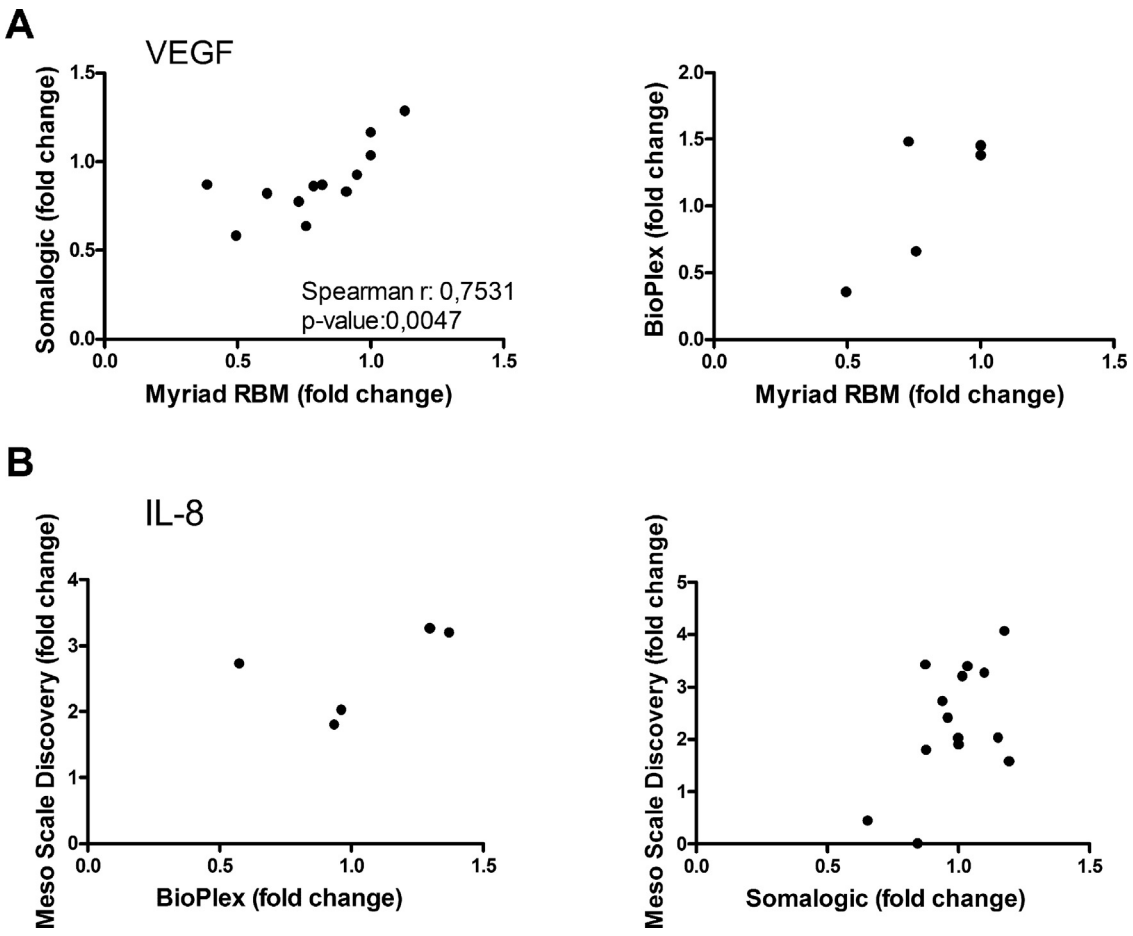


**Fig. 3 – Correlating and non-correlating relative differences on different platforms. Correlation of relative VEGF (A) and IL-8 (B) differences on different platforms. Statistically significant correlation was determined with the non-parametric Spearman correlation. Spearman *r*-value and *p*-value are indicated in the graph.**

sample (ratio > 10) for 27.6% (312/1129) of the analytes (Fig. 5D), a ratio higher than ten was not seen for any of the other platforms. Meso Scale Discovery did not detect concentration in chicken plasma of any of the analytes investigated on this platform.

## 4. Discussion

Multiplex techniques are widely used and are becoming increasingly popular for the quantification of proteins in human plasma as a small amount of sample can result in a great number of analytes analyzed. The challenge with multiplex assays is that targets with various properties should be recognized in the same assay. In contrast, singleplex assays buffers and other reagents can be specifically tailored to detect a single target. Moreover, when the numbers of targets increase, the risk of unspecificity increases. Hence, to be able to compare protein quantification data obtained by different multiplex platforms, the platforms must be compared with respect to accuracy, sensitivity and robustness. Various studies have compared two or more multiplex methods for protein quantification [12–17].

The present study aimed at comparing multiplex assays performed in our laboratory with assays performed at companies offering multiplex services through analysis of the same patient plasma samples pre- and post-therapy. The platforms compared have different characteristics and would probably be used for different applications. One apparent difference is the detection reagent. BioPlex, ELISA, Meso Scale Discovery and Myriad RBM are all using antibodies to detect proteins while the Somalogic platform uses modified aptamers as binders of the proteins to detect. Aptamers are binders reported to have many advantages compared to antibodies [18]. In our study, however, a high background was seen when samples taken before TKI treatment were analyzed by the Somalogic platform. The samples analyzed were from patient with CML before therapy, i.e. patients with a high tumor-burden in their blood. It is possible that the high tumor burden leads to an increase in protein content and/or secretion of "sticky" products into plasma which could lead to unspecific interactions with the SOMAmers. The high background in pre-treatment samples resulting in a decrease in protein concentration in most post-treatment samples was not seen in any of the other platforms. Moreover, higher background was also seen in chicken plasma when using the

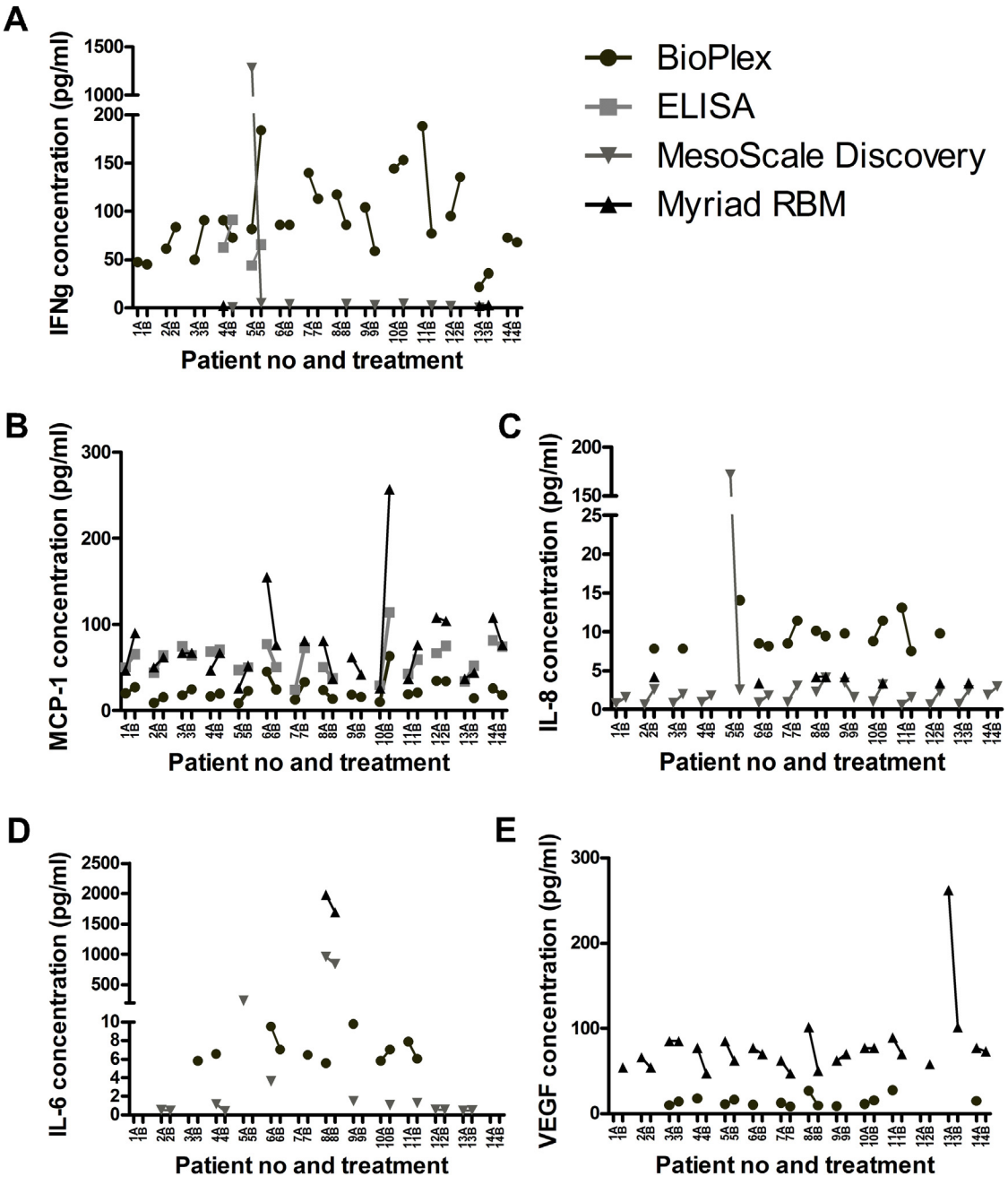


**Fig. 4 – Comparison of absolute concentrations of various analytes measured on different platforms. The absolute concentration of IFNγ (A), MCP-1 (B), IL-8 (C), IL-6 (D) and VEGF (E) as measured by different platforms at baseline (time point A) and after three months of treatment (time point B). Each platform is represented by a distinct shape. Only samples with concentrations above detection limit are shown in the figure.**

Somalogic platform, compared to the other platforms, further indicating unspecificity in the assay. It should be noted that the concentration of proteins in chicken plasma was related to the concentration in the patient sample with lowest detected concentration. Hence, the difference calculated depends on the concentration of analyte in the patient samples. The data generated from the pre- and post therapy patient material on the Somalogic platform stresses the fact that the user, and the companies providing the platforms, must increase their knowledge in how different diseases may influence assay results. The provider could possibly validate the assays using patient material or at least inform the user of problems that may arise when using complex human material.

Since not all platforms investigated measured absolute protein concentration, the relative change in protein concentration between the two sampling time points was used for comparison. Analytes that had detectable levels of protein in more than one platform were compared. The sensitivity of the different assays varied and this was most apparent when investigating IFNγ, IL-8 and IL-6. The BioPlex assay measured

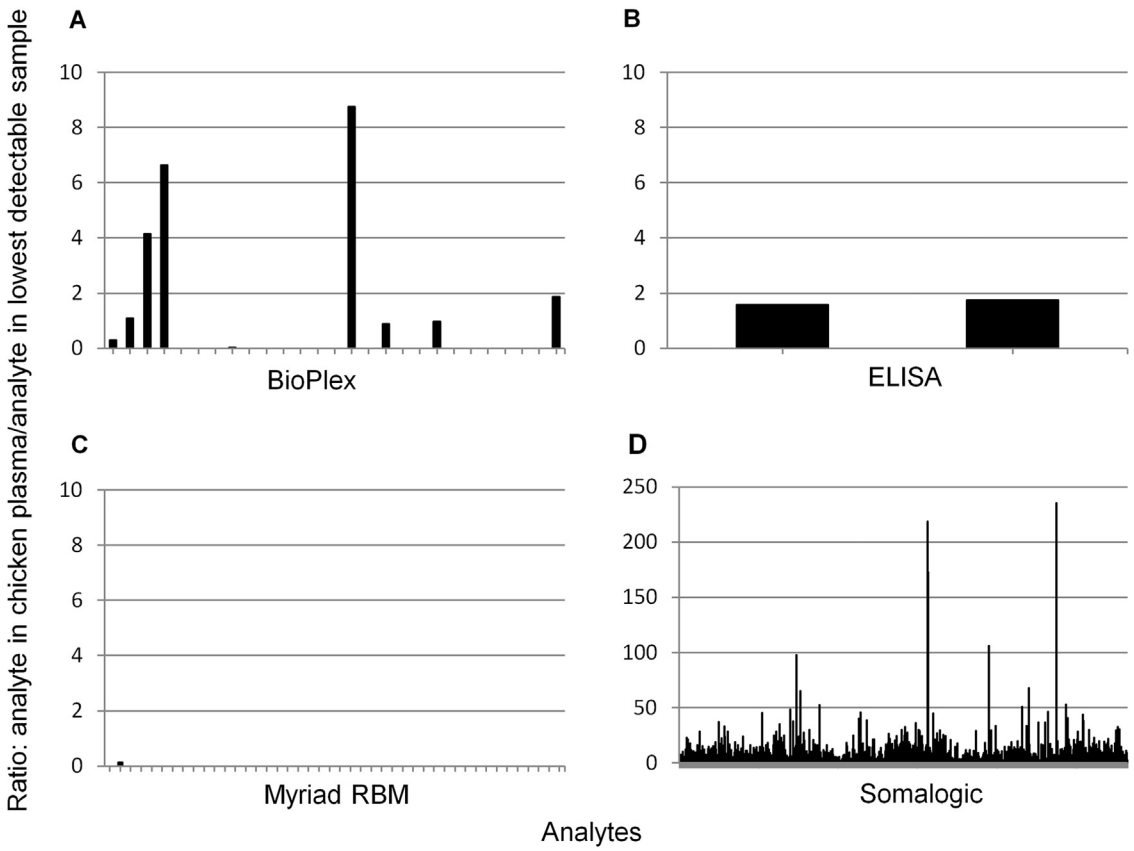


**Fig. 5 – Detection of protein in chicken plasma. The amount of protein binding in chicken plasma by the different platforms was determined by calculating the ratio of the analyte amount detected in chicken plasma and the analyte amount detected in the lowest detectable patient sample. The ratio is shown on the y-axis. Each bar on the x-axis represents one analyte that was detected in the chicken plasma by the platform. For ticks on the x-axis without a bar, that analyte could not be detected in the chicken plasma on that platform. Proteins detected in the chicken plasma are shown for BioPlex (A), ELISA (B), Myriad RBM (C) and Somalogic (D).**

IFNγ concentrations in all samples, while it was only detected in a few samples in the ELISA and Meso Scale Discovery assays. The ELISA is so far the golden standard for protein quantification, and the protocol only needs to be optimized with respect to a very limited numbers of antibodies. If IFNγ concentration measured by ELISA was considered as the "true" concentration, the BioPlex platform appeared to overestimate the concentration for most samples. This may be because of unspecific binding of antibodies.

The intra-assay accuracy was acceptable [19] (pooled CV-values <15%) for all but three analytes where CV-values could be calculated. In our study no inter-assay CV-values were determined since every assay was run only ones. Others, however, have reported lot dependent differences for the BioPlex kit [14].

Even though the mean fold change in concentration as well as the absolute concentration varied among different platforms in our study, the fold changes for some of the analytes correlated between some platforms. This has also been shown by others comparing multiplex platforms with other multi- or singleplex platforms [12,13,20]. Different multiplex methods use different antibodies for protein detection, and the proteins used for the creation of a standard curve can also vary between the platforms. This may be one reason for obtaining different absolute concentrations but similar fold changes when measuring the same analyte by different platforms.

## 5. Conclusions

Taken together, our results show that absolute concentrations measured by different multiplex platforms and/or laboratories should be compared with caution and controls with known concentrations not provided by the assay manufacturer should be run among the samples to ensure appropriate concentration measurements. Which multiplex assay to use should be chosen depending on application and the demands on the assay. The relative concentrations can be compared for some of the analytes measured by some of the platforms. Before performing protein concentration comparison studies, such as comparing certain analytes in patients included in different clinical trials, thorough validation [21] of the assay should be performed to assure that the results are truly comparable. Lastly, users as well as platform providers must be cautious about the choice of platform when evaluating invaluable patient-derived material. Preferably, pre- and

post-therapy samples should be assessed with or without spiked proteins on a variety of different platforms before hand to ensure that the disease stage does not bias the results. The pre-screening validation should then lead to the final choice of platform for screening of the complete set of patient material.

## Conflicts of intrest

The authors declare no conflicts of interest except for Dr. Loskog that is the CEO of Lokon Pharma AB, a scientific advisor at NEXTTOBE AB and both Dr Loskog and Dr Mangsbo have a royalty agreement with Alligator Bioscience AB. None of these declared issues have an economical conflict with the results present in the current manuscript.

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