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Research paper

Pre-analytical effects of blood sampling and handling in quantitative immunoassays for rheumatoid arthritis ☆

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ABSTRACT

Variability in pre-analytical blood sampling and handling can significantly impact results obtained in quantitative immunoassays. Understanding the impact of these variables is critical for accurate quantification and validation of biomarker measurements. Particularly, in the design and execution of large clinical trials, even small differences in sample processing and handling can have dramatic effects in analytical reliability, results interpretation, trial management and outcome. The effects of two common blood sampling methods (serum vs. plasma) and two widely-used serum handling methods (on the clot with ambient temperature shipping, "traditional", vs. centrifuged with cold chain shipping, "protocol") on protein and autoantibody concentrations were examined. Matched serum and plasma samples were collected from 32 rheumatoid arthritis (RA) patients representing a wide range of disease activity status. Additionally, a set of matched serum samples with two sample handling methods was collected. One tube was processed per manufacturer's instructions and shipped overnight on cold packs (protocol). The matched tube, without prior centrifugation, was simultaneously shipped overnight at ambient temperatures (traditional). Upon delivery, the traditional tube was centrifuged. All samples were subsequently aliquoted and frozen prior to analysis of protein and autoantibody biomarkers. Median correlation between paired serum and plasma across all autoantibody assays was 0.99 (0.98–1.00) with a median % difference of −3.3 (−7.5 to 6.0). In contrast, observed protein biomarker concentrations were significantly affected by sample types, with median correlation of 0.99 (0.33–1.00) and a median % difference of −10 (−55 to 23). When the two serum collection/handling methods were compared, the median correlation between paired samples for autoantibodies was 0.99 (0.91–1.00) with a median difference of 4%. In contrast, significant increases were observed in protein biomarker concentrations among certain biomarkers in samples processed with the 'traditional' method. Autoantibody quantification appears robust to both sample type (plasma vs. serum) and pre-analytical sample collection/handling methods (protocol vs. traditional). In contrast, for non-antibody protein

Abbreviations: ACR, American College of Rheumatology; BD, Becton Dickinson; cAPOE, citrullinated apolipoprotein; cBig, citrullinated biglycan; cClu, citrullinated clusterin; CCP, cyclic citrullinated peptides cFibA1 and cFibA2, citrullinated fibrinogen; cH2B, citrullinated histone 2B; CRP, C-reactive protein; cFil, citrullinated filaggrin; EGF, epidermal growth factor; FCS, fetal calf serum; H2B, histone 2B; Ig, immunoglobulin; IL-6, interleukin 6; MBDA, multi-biomarker disease activity; MMP-1, matrix metalloproteinase 1; MMP-3, matrix metalloproteinase 3; MSD, Meso Scale Discovery; PBST, phosphate-buffered saline Tween-20; RA, rheumatoid arthritis; SAA, serum amyloid A; SST™, serum separator transport; TNF-RI, tumor necrosis factor receptor superfamily member 1A; VCAM-1, vascular cell adhesion molecule 1; VEGF-A, vascular endothelial growth factor A; YKL-40, human cartilage glycoprotein 39.

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biomarker concentrations, sample type had a significant impact; plasma samples generally exhibit decreased protein biomarker concentrations relative to serum. Similarly, sample handling significantly impacted the variability of protein biomarker concentrations. When biomarker concentrations are combined algorithmically into a single test score such as a multi-biomarker disease activity test for rheumatoid arthritis (MBDA), changes in protein biomarker concentrations may result in a bias of the score. These results illustrate the importance of characterizing pre-analytical methodology, sample type, sample processing and handling procedures for clinical testing in order to ensure test accuracy.

1. Introduction

Mutliplexed immunoassays that provide multiple, parallel protein measurements on the same specimen have become popular tools in biomarker discovery research and the measurement of protein biomarkers in clinical trials. By measuring several proteins from a single sample, multiplexed immunoassays offer the advantages of specimen conservation, high throughput analysis, and efficiency in terms of time and cost. Given the complexity of multiplexed immunoassays, rigorous investigation of pre-analytical requirements in addition to extensive validation of analytical performance is necessary to ensure the reliability and consistency of assay results (Ellington et al., 2009, 2010). An understanding of the pre-analytical requirements of multiplexed immunoassays is particularly important since studies have shown that the majority of variations and errors in protein biomarker measurements occur in the pre-analytical phase prior to specimen analysis (Rai and Vitzthum, 2006). Indeed, studies have shown that even small differences in pre-analytical variables, such as the processing or handling of a specimen, can dramatically affect the analytical reliability and reproducibility of multiplex immunoassays (Tuck et al., 2009).

The importance of pre-analytical variables has been recognized in the context of clinical trials. Multiplexed immunoassays for measurement of protein biomarkers have the potential to improve the value of clinical trials and can be integral to the design of a trial, and the development of well-defined protocols for sample collection and processing has been recommended in order to minimize the risk of inadvertently introducing subtle differences in sample handling that may affect study results (Dancey et al., 2010; Sturgeon et al., 2010). Given their relatively high cost, clinical trials aim to obtain as much information as possible. However, trials often involve more than one center and more than one specimen type may be collected (biological fluids, tissue, etc.), and hence a thorough understanding and characterization of the pre-analytical variables that impact assay performance are critical. These variables include the method of sample collection, the type of anticoagulants or preservatives that are used, the procedure used to process the sample, the time between collection and assay, and the storage conditions used during this interval (Gerszten et al., 2008). Ideally, these pre-analytical variables should be evaluated for each individual assay included in the multiplex assay (Wener, 2011).

Recently, multiplexed immunoassays have been introduced for the diagnosis and classification of rheumatoid arthritis (RA) (Hueber et al., 2005; Curtis et al., 2010; Chandra et al., 2011). RA is an inflammatory joint disease that involves complex interactions between multiple proteins in a number of tissues, including bone, cartilage and synovium (Graudal et al., 1998). The molecular pathophysiology of RA remains unclear, and patients with RA vary considerably in the course of disease and response to treatment (Scott and Steer, 2007). It has been shown that regular quantitative assessment of RA disease activity, termed tight control, is key to improving patient outcomes (Grigor et al., 2004; Goekoop-Ruiterman et al., 2005). Although several biomarkers that are predictive of RA disease activity have been identified, no single biomarker adequately reflects disease activity or response to RA therapy (van der Pouw Kraan et al., 2003; Hueber et al., 2007; Rioja et al., 2008; Chandra et al., 2011). Hence, the use of multiplexed immunoassays to simultaneously measure multiple biomarkers may provide a more comprehensive, objective measure of disease activity that could be used as a complement to other clinical measures of RA to improve patient outcomes.

The multi-biomarker disease activity (MBDA) test is a multiplexed immunoassay available through the CLIA-certified laboratory at Crescendo Bioscience (Vectra™ DA; Crescendo Bioscience™, South San Francisco, CA) that employs an algorithm based on the measurement of 12 protein biomarkers to provide a measure of disease activity for patients with RA (Curtis et al., 2010). As part of a comprehensive program to assess the performance of the MBDA test in clinical practice, other studies have evaluated the criterion and discriminant validity of the MBDA score (Curtis et al., 2010) as well as the analytical validity of the MBDA test with regards to precision, dynamic range, cross-reactivity, and effect of interfering substances (Eastman et al., 2010). In the present study, we examined the effect of pre-analytical variables related to the collecting, processing and handling of blood samples on the performance of the MBDA test and each of the protein biomarker immunoassays that comprise the MBDA test. Here, we report on the measurement of the protein biomarkers and MBDA score in serum versus plasma as well as in serum samples processed by two different methods. For comparison, we also evaluated the effects of these pre-analytical variables on the measurement of autoantibodies typically found in RA patients using custom immunoassays developed on the Meso Scale Discovery (MSD) platform. The data indicate that blood collection, processing, and handling methods had a significant impact on some non-antibody protein biomarker measurements, whereas autoantibody measurements appeared relatively robust to these pre-analytical variables. Changes in the protein biomarker concentrations from pre-analytical sample handling introduced a bias in the MBDA score. The results of this study illustrate the importance of characterizing pre-analytical variability to ensure the accuracy of protein biomarker tests, and confirm that standardized serum processing

and handling procedures for protein biomarker tests are critical to ensure the reliability of results obtained in clinical trials.

2. Materials and methods

2.1. Materials

2.1.1. Autoantibody biomarkers

The peptides derived from potential RA autoantigens used in this study are listed in Table 1. All peptides were synthesized by Biomer Technology (Pleasanton, CA) with a terminal biotin. Labeled secondary antibody against human IgG was from Meso Scale Discovery (MSD, Gaithersburg, MD).

2.1.2. Protein biomarkers

Sources for the capture antibodies, detection antibodies, and analyte standards used to measure the 12 protein biomarkers that comprise the MBDA test are listed in Table 2. All other reagents, with the exception of wash buffer components, were from MSD.

2.1.3. Multiplex standards for MBDA test

Prediluted multiplexed calibrator sets were prepared for each panel. Each standard curve consisted of 8 points spanning the full range of the assay, including an assay blank. Prediluted standards were prepared with recombinant proteins spiked into the appropriate sample diluent containing the equivalent serum concentration that is present in diluted samples. Prediluted standards were aliquoted into single-use vials and stored at −80 °C.

2.1.4. Assay quality controls for MBDA test

Prediluted, multiplexed quality control (QC) run control sets were used to monitor the execution of each assay run. If the observed biomarker concentrations of any QC run control fell outside of expected ranges, all samples on the failed assay plate were repeated.

Serum process controls were used to monitor the entire assay process, including sample dilution, at both the biomarker level and the score level. If any process control failed the MBDA score specifications, all samples on the plates which contained the failed process control were repeated.

For patient samples, the percent coefficient of variation (% CV) of the signals between the duplicate wells was calculated for each marker. If the % CV was above the biomarker specific acceptability limit (typically 20%), the concentration reported for that sample was deemed unreliable and was retested.

Table 2
Sources[a] of capture antibody, detection antibody and analyte standards.

Biomarker	Capture antibody	Detection antibody	Analyte standard
VCAM-1	R&D Systems	R&D Systems	R&D Systems
EGF	R&D Systems	R&D Systems	Peprotech
VEGF-A	Peprotech	R&D Systems	Peprotech
IL-6	R&D Systems	R&D Systems	Peprotech
TNF-R1	R&D Systems	R&D Systems	Peprotech
MMP-1	R&D Systems	R&D Systems	R&D Systems
MMP-3	R&D Systems	R&D Systems	R&D Systems
YKL-40	Quidel	Quidel	Peprotech
Leptin	R&D Systems	Hytest	Peprotech
Resistin	Antigenix America	R&D Systems	Peprotech
SAA	Anogen	Anogen	Peprotech
CRP	Hytest	Hytest	Peprotech

[a] Source locations: Anogen (Mississauga, Ontario, Canada); Antigenix America (Huntington Station, NY); Hytest (Turku, Finland); Peprotech (Rocky Hill, NJ); Quidel (San Diego, CA); R&D Systems (Minneapolis, MN).

2.1.5. Instrumentation

Microplates are read on the SECTOR Imager 6000 reader (MSD, Gaithersburg, MD), which uses an ultra-low noise charge-coupled device (CCD) camera with a custom-designed telecentric lenses to detect light emitted at ~620 nm upon electrochemical stimulation. Plate images are obtained in six sectors and data is subsequently acquisitioned into MSD Discovery Workbench Software.

2.2. Methods

2.2.1. Sample collection protocol: serum vs. plasma

Paired serum and plasma samples were collected from RA subjects who fulfilled the American College of Rheumatology (ACR) 1987 criteria (Arnett et al., 1988). All samples were collected under Investigational Review Board approved protocols with informed consent. To collect samples, 32 individuals with RA had matched serum and plasma samples drawn with Serum Separator Transport (SST™) Tubes and EDTA Vacutainer tubes from Becton Dickinson (BD, Franklin Lakes, NJ), respectively, from the same needle stick. Both the serum and plasma tubes were processed per manufacturer's instructions, aliquots prepared, frozen and subsequently tested in the MBDA protein biomarker and autoantibody biomarker assays.

2.2.2. Serum sample collection protocol: 'protocol' vs. 'traditional'

To evaluate serum collection and handling variables, serum samples were collected from 10 individuals who were diagnosed with RA based on ACR 1987 criteria (Arnett et al., 1988). All samples were collected under Investigational Review Board approved protocols with informed consent.

Table 1
Peptides used in the study.

Peptide ID	Protein source	Sequence	Citrullinated	Cyclized
cApoE	Apolipo E	A[CIT]LKSWFEPLVEDMQ[CIT]QWAG	+	+
cBig	Biglycan	EDLL[Cit]YSKLY[Cit]LGLGHNQI[Cit]	+	−
cH2B	Histone 2B	IMNSFVNDIFE[Cit]IAGEAS[Cit]L	+	+
cClu	Clusterin	HFS[Cit]ASSIIDELFQD[Cit]FFT[Cit]	+	+
cFibA1	Fibrinogen A	THSTK[CIT]GHAKS[CIT]PV[CIT]GIHTS	+	+
cFil	Filaggrin	TIHAHPGS[CIT][CIT]GGRHGYHH	+	+
H2B	Histone 2B	PEPVKSAPVPKKGSKKAIN	−	+
cFibA2	Fibrinogen A	NTKESSSHHPGIAEFPS[cit]GK	+	−

Matched sets of BD SST™ were used to draw blood. One set of tubes from each individual was incubated at ambient temperature for 30–45 min and then centrifuged for 10 min at a 1000 to 1300 RCF (g) per manufacturer's instructions. This was followed by overnight shipment in a temperature controlled (2–8 °C) package ("protocol"). A matched tube from each individual was simultaneously shipped overnight at ambient temperature while remaining on the clot (e.g. not centrifuged; "traditional"). Upon arrival, the traditional tube was centrifuged and all samples were aliquoted and frozen for analysis in the MBDA lower case protein biomarker and autoantibody biomarker immunoassays. The 10 matched sets of processed serum were run on two duplicate plates for each multiplexed panel (panels A, B, and C). Due to limited amount of samples available, only 7 matched sets were analyzed for autoantibody experiments.

2.2.3. Autoantibody biomarker measurements

The autoantibody biomarkers were evaluated with custom assays using the MSD platform. Briefly, eight peptides (Table 1) were immobilized onto streptavidin MSD plates. All incubations were applied with continuous shaking at 750 rpm and plates were washed 3 times between incubations with wash buffer (phosphate buffered saline + 0.05% Tween-20, PBST). After blocking with blocker A from MSD for 1 h, the plates were probed with 50 μL of samples that were diluted 1/50 in sample diluents supplemented with 10% fetal calf serum (FCS) and incubated for 90 min. SULFO-TAG conjugated secondary antibody against human immunoglobulin G (IgG, MSD, Gaithersburg, MD) was diluted 1/5000 and used to quantitatively measure the presence of each autoantibody. Electrochemiluminescence signal was quantified on the SECTOR Imager 6000 reader immediately after 150 μL of MSD Read Buffer T (containing surfactants and tripropylamine as a coreactant for light generation) was loaded in each well. Samples collected under different conditions were run in duplicate on one plate and raw signals were used for data analysis.

2.2.4. Protein biomarker measurements

The 12 protein biomarkers that constitute the MBDA test were measured using analyte-specific capture and detection antibodies. Briefly, multi-spot 96-well plates were coated with analyte-specific capture antibodies on three panels: panel A includes epidermal growth factor (EGF), interleukin-6 (IL-6), leptin, and vascular endothelial growth factor (VEGF-A); panel B includes C-reactive protein (CRP), serum amyloid A (SAA), and vascular cell adhesion protein 1 (VCAM-1); and panel C includes matrix metalloproteinase-1 (MMP-1), MMP-3, resistin, tumor necrosis factor receptor 1 (TNF-R1), and cartilage glycoprotein-39 (gp-39, also known as YKL-40). Dilutions for panels A, B and C were 1/2, 1/1000 and 1/20, respectively. Fifty microliters (for panels A and C) and 25 μL (for panel B) of standard, blank, control, or sample were added to the appropriate well in the 96-well plate. The plates were incubated for 120 min with continuous shaking at 750 rpm and then washed 3 times in PBST wash buffer. Twenty-five microliters of prediluted blends of SULFO-TAG conjugated detection antibodies was added to each well. Following incubation with the detection antibody blend for 60 min, plates were washed again, and upon adding 150 μL of read buffer, the electrochemiluminescence signal was quantified as in Section 2.2.3. MSD Discovery Workbench calculates the four-parameter logistic regression curve fits (Findlay and Dillard, 2007) for each standard curve and determines concentrations for all samples. The concentration of the samples was used for further comparison of results obtained with different sample collecting/handling processes.

2.3. Calculations

The MBDA algorithm was developed in a separate series of studies and clinically validated in an independent cohort (Curtis et al., 2010) using the DAS28-CRP as a gold standard (Prevoo et al., 1995; Inoue et al., 2007). Derivation and clinical validation of this algorithm are reported elsewhere (Curtis et al., 2010). The MBDA algorithm was developed by analyzing biomarker assays performed on samples from several observational studies (Verstappen et al., 2007; Karlson et al., 2008).

$$\begin{aligned}\text{PTJC} = \text{Prediction of Tender Joint Count} = &-26.72\\ &+3.243 * [\text{YKL} - 40]^{1/10} - 11.97 * [\text{EGF}]^{1/10}\\ &+15.72 * [\text{IL} - 6]^{1/10} + 0.4594 * [\text{Leptin}]^{1/10}\\ &+3.881 * [\text{SAA}]^{1/10} + 0.7388 * [\text{TNF} - \text{RI}]^{1/10}\\ &-0.2557 * [\text{VCAM} - 1]^{1/10} + 0.7003 * [\text{VEGF} - \text{A}]^{1/10}\end{aligned}$$

$$\begin{aligned}\text{PSJC} = \text{Prediction of Swollen Joint Count} = &-26.63\\ &+3.232 * [\text{YKL} - 40]^{1/10} - 11.93 * [\text{EGF}]^{1/10}\\ &+15.67 * [\text{IL} - 6]^{1/10} + 0.4578 * [\text{Leptin}]^{1/10}\\ &+3.868 * [\text{SAA}]^{1/10} + 0.7363 * [\text{TNF} - \text{RI}]^{1/10}\\ &-0.2548 * [\text{VCAM} - 1]^{1/10} + 0.6979 * [\text{VEGF} - \text{A}]^{1/10}\end{aligned}$$

$$\begin{aligned}\text{PPGS} = \text{Prediction of Patient Global Score} = &-13.489\\ &+5.474 * [\text{IL} - 6]^{1/10} + 0.486 * [\text{SAA}]^{1/10}\\ &+2.246 * [\text{MMP} - 1]^{1/10} + 1.684 * [\text{Leptin}]^{1/10}\\ &+4.14 * [\text{TNF} - \text{RI}]^{1/10} + 2.292 * [\text{VEGF} - \text{A}]^{1/10}\\ &-1.898 * [\text{EGF}]^{1/10} + 0.028 * [\text{MMP} - 3]^{1/10}\\ &-2.892 * [\text{VCAM} - 1]^{1/10} - 0.506 * [\text{Resistin}]^{1/10}\end{aligned}$$

$$\begin{aligned}\text{MBDA score} = \text{round}\Big(\max\Big(\min\Big(&\big(.56 * \text{sqrt}\big(\max(\text{PTJC}, 0)\big)\\ &+ .28 * \text{sqrt}\big(\max(\text{PSJC}, 0)\big)\big) + .14 * \text{PPGS}\\ &+ .36 * \log\big(\text{CRP}/10^6 + 1\big)\Big) * 10.53 + 1, 100\Big), 1\Big)\Big)\end{aligned}$$

All concentration values except that of CRP are $X^{1/10}$ transformed prior to use in the algorithm.

MBDA algorithm scores are integers from 1 to 100, with disease activity thresholds designed to be equivalent to thresholds from DAS28CRP:

- MBDA algorithm scores ≤29 are considered low disease activity.
- MBDA algorithm scores of 30 to ≤44 are considered moderate disease activity.
- MBDA algorithm scores >44 are considered high disease activity.

Table 3
Correlation and % change of marker measurements on paired serum/plasma samples.

	Slope[a]	r[b]	Median % change[c]
Autoantibody biomarkers			
cApoE	0.91	0.98	−8
cBig	1.02	0.98	−4
cH2B	1.00	0.99	6
cClu	0.89	1.00	−3
cFibA1	1.07	0.98	−3
cFil	0.97	0.99	2
H2B	1.05	0.99	−5
cFibA2	0.91	0.99	−5
Protein biomarkers			
VCAM-1	0.75	0.97	−19
EGF	0.21	0.33	−51
VEGF-A	0.34	0.87	−55
IL-6	0.93	1.00	−6
TNF-R1	1.03	0.99	−7
MMP-1	1.28	0.74	23
MMP-3	0.90	1.00	−9
YKL-40	1.13	0.99	−7
Leptin	1.21	0.99	6
Resistin	0.84	0.91	−18
SAA	1.02	1.00	−12
CRP	0.88	1.00	−12
MBDA	0.78	0.93	3

[a] Slope of the linear regression line.
[b] Correlation coefficient.
[c] Median % change = (plasma − serum) / serum.

3. Results

3.1. Comparison of plasma vs. serum samples

3.1.1. Autoantibody biomarker measurements in plasma and serum samples

All autoantibody assays exhibited less than 10% difference (median difference) between the two sample types (Table 3), well within the Food and Drug Administration suggested specification at ±15% for accuracy (FDA, 2001). All analyses for autoantibodies were calculated on raw signals in antibody biomarker measurements. An additional more stringent analysis compared the correlation coefficient and slope of linear regression. In comparison of plasma and serum matched sample sets, the correlation was 0.99 (0.98 to 1.00) with a slope of approximately 1.00, indicating little or no difference in quantitation of autoantibody signals in serum vs. plasma samples.

3.1.2. Protein biomarker concentration comparisons between plasma and serum samples

For protein biomarkers of matched plasma and serum samples, only 67% of the biomarkers were highly correlated achieving correlation coefficients of 0.95 (range 0.33–1.00) (Table 3). The protein concentrations had a systematic shift with the slope of most markers being less than 1.00, indicating serum concentrations were measured higher for most biomarkers. The plasma EGF concentrations were not correlated with matched serum EGF concentrations (correlation coefficient of 0.33).

As shown in Fig. 1A, 5 out of 12 protein biomarkers (VCAM-1, EGF, VEGF-A, MMP1 and resistin) had shifts >15% in the median % difference in concentration across the 32 patient samples. Aside from leptin and MMP-1, median protein concentrations in plasma were lower than those in serum. While the median change for MMP-1 showed significantly greater concentrations in plasma over serum (Fig. 1A), the individual subjects in this study provided mixed results, with 12 subjects' plasma showing lower MMP-1 concentrations and 20 subjects with greater MMP-1 concentrations.

3.2. Impact of serum sample handling: traditional vs. protocol

3.2.1. Serum sample handling for autoantibody assays

Autoantibody measurements between traditional and protocol samples showed median differences of generally less than 15% (Table 4). The autoantibody levels between these two sample handling methods were highly correlated, with a median correlation coefficient of 0.99 (0.91–1.00). Slopes of their linear regression curve across the 32 subjects were spread between 0.7 and 1.4.

3.2.2. Serum sample handling for protein biomarker measurements

When correlations of protein concentrations from matched sets of samples across the 32 subjects were calculated between traditional and protocol handling methods, only 7 of the 12 biomarkers achieved correlation coefficients ≥0.95 with a range of 0.05 to 1.00 (Table 4).

As shown in Fig. 1B, significant differences in biomarker concentrations, (>±15% median percent difference) between the two sample handling methods were seen in 67% (8/12) of the individual biomarkers measured. Of the markers with significant differences in the traditional samples, 7 biomarkers increased, while only leptin decreased. The EGF and IL-6 serum concentrations in samples handled with the traditional method increased as much as 40-fold, while VEGF-A and resistin concentrations also increased 2 to 4-fold.

3.3. MBDA and pre-analytical variables

The MBDA scores were evaluated across different pre-analytical variables. In Fig. 2A, a bias was observed when the difference of MBDA scores between plasma and serum was plotted against the MBDA scores of the serum samples. Samples with low serum MBDA scores had artificially inflated scores when plasma was used as a sample. While changes in the concentration of several biomarkers were observed in this subset of samples, e.g., EGF, VEGF-A, resistin, the largest and most consistent change associated with the elevated MBDA score was reduced concentrations of EGF which has a negative coefficient in the algorithm. In Fig. 2B, a similar bias was observed when the difference of MBDA scores between the traditional vs. protocol serum sample handling methods was evaluated relative to the MBDA score for the protocol method. Again, samples with low “protocol” MBDA scores were artificially inflated by the traditional method, but this time primarily as a result of the elevated concentration of IL-6. In both comparisons, samples with artificially deflated scores were observed at high MBDA scores. While changes in several of the biomarkers were observed in the samples with the deflated MBDA scores, elevated EGF concentrations were consistently observed.

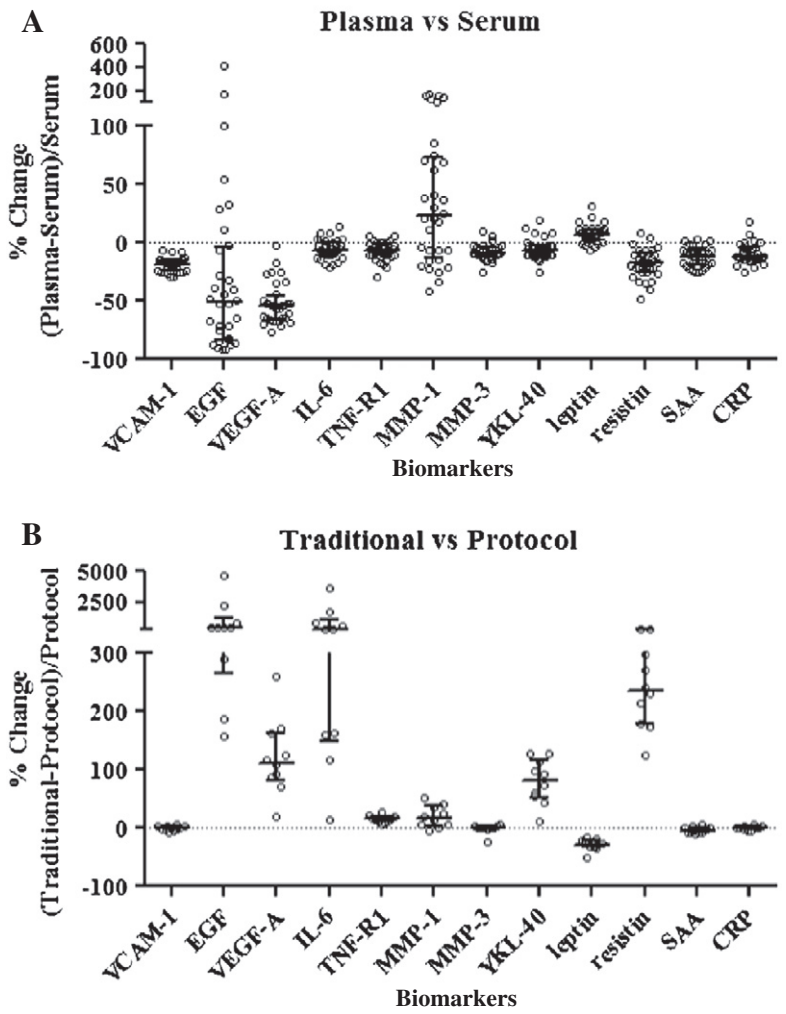

Fig. 1. Changes of biomarker concentrations in paired samples. A, Plasma versus serum samples. Percent changes were determined by using the difference of biomarker concentrations between plasma and serum samples divided by biomarker concentrations in serum samples. B, Traditional versus protocol sample handling. Percent change is the difference of biomarker concentration between the traditional and protocol methods relative to the concentration from the protocol method. Median and inter-quartile ranges are presented.

4. Discussion

This study investigated two types of pre-analytical variables that occur prior to the point of actual sample analysis: blood sampling methods (serum vs. plasma) and serum collection/handling methods (traditional vs. protocol). Although serum and plasma are both routinely collected samples and the composition is considered similar, this is the first study to the authors' knowledge where quantitative measurements of 12 proteins in a multiplexed platform and eight autoantibodies from matched samples are compared in a systematic way in rheumatoid arthritis subjects. We observed that effects of pre-analytical variables were remarkably different on protein biomarkers vs. antibody biomarkers. It is critical to collect samples under well-defined protocols for both biomarker discovery and validation studies, especially because even within a panel of multiplexed biomarker assays, different biomarkers were affected very differently by these pre-analytical variables.

Previous studies comparing plasma and serum have shown that the measurable levels of analytes may vary between the 2 sample types (Miles et al., 2004). Quantifications with two common RA autoantibody assays, anti-cyclic citrullinated peptides (CCP) and rheumatoid factor (RF) have been demonstrated equivalent with either serum or plasma (Rantapaa-Dahlqvist et al., 2003). When sample handling variables, such as sample type (e.g., serum vs. plasma), room temperature storage, heat treatment, hemolysis, and repetitive freeze–thaw cycles, were evaluated on the performance of immunoassay detection of antibodies against *Erysipelothrix rhusiopathiae* (Neumann and Bonistalli, 2009), no significant impact was found suggesting that immunoglobulin G antibody was stable in cases of common sample mishandling events. Autoantibodies are human immunoglobulins against an individual's own proteins and should present similar characteristics to antibodies against bacteria. In fact, our results confirmed that antibodies appear to be stable biomarkers that were not largely affected by pre-analytical variables.

Table 4
Correlation of marker measurements on paired traditional/protocol samples.

	Slope[a]	r[b]	Median % change[c]
Autoantibody biomarkers			
cApoE	0.80	0.99	−16
cBig	0.70	0.91	−4
cH2B	0.72	1.00	10
cClu	1.37	0.98	−6
cFibA1	1.07	0.99	2
cFil	0.76	0.99	−24
H2B	0.89	0.95	5
cFibA2	0.90	0.96	−4
Protein biomarkers			
VCAM-1	0.99	0.98	−1
EGF	1.70	0.72	416
VEGF-A	1.10	0.90	112
IL-6	0.27	0.05	329
TNF-R1	1.13	0.99	16
MMP-1	0.92	0.98	17
MMP-3	0.92	0.97	1
YKL-40	0.97	0.87	83
Leptin	0.71	0.98	−29
Resistin	2.69	0.89	235
SAA	0.91	0.94	−5
CRP	0.98	1.00	1
MBDA	0.42	0.65	1

[a] Slope of the linear regression line.
[b] Correlation coefficient.
[c] Median % change = (traditional − protocol) / protocol.

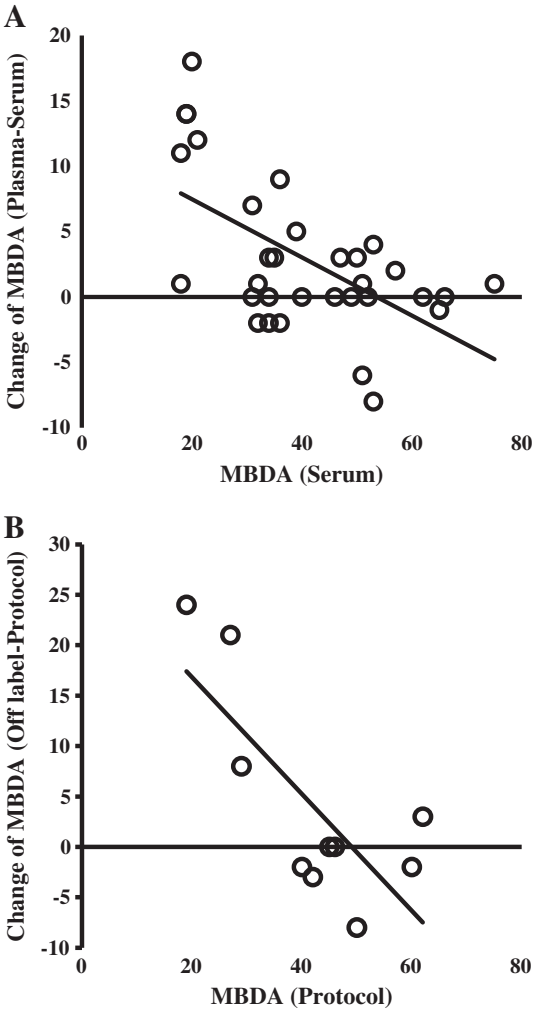

Fig. 2. Correlation of MBDA scores and changes in MBDA scores. A, Plasma versus serum samples. Changes were determined by using the difference of MBDA scores between plasma and serum samples. B, Traditional versus protocol samples. Changes were determined by using the difference of MBDA scores between plasma and serum samples.

The difference of autoantibody measurements in paired samples is largely within +/−15%. The impact of blood sampling (serum vs. plasma) was minimal for autoantibody quantification with correlation coefficients near 1.0.

For the non-antibody protein biomarker assays, the difference between plasma and serum concentrations was dependent on individual biological characteristics of the proteins. Concentrations of some protein biomarkers were lower in plasma than in serum, e.g., VEGF-A, EGF, VCAM-1 and resistin, while other protein biomarkers exhibited no significant change. For CRP, we have observed a correlation of 1.00 between plasma and serum samples, with median difference of 12%. This result agreed with previous studies when CRP was measured in matched plasma and serum samples in protein biomarker measurements (Miles et al., 2004). For MMP-1, however, we observed a wide range of concentration changes between RA subjects, with 60% demonstrating increased concentrations in plasma and 40% of RA subjects showing decreased concentrations. The CVs of all duplicate measurements were less than 10% (data not shown), so that assay variability is not likely contributing to the diverse results.

Protein biomarker concentrations are also greatly affected by post-collection sample handling methods. One can surmise that this is a result of blood cell lysis when samples had prolonged (>12 h) contact with blood cells at room temperature (traditional conditions). Not surprisingly, this sample handling method appeared to predominantly affect blood cell secreted proteins, such as EGF, VEGF-A, IL-6, YKL-40 and resistin. Among them, both EGF and IL-6 concentrations had a median increase of 3–4 fold, respectively. On the other hand, some proteins are not known to be secreted by blood cells. For example, VCAM-1 is expressed in endothelial cells (Osborn et al., 1989), both SAA (Uhlar and Whitehead, 1999) and CRP (Pepys and Hirschfield, 2003) are produced predominantly by the liver. All three proteins remained stable to the traditional sample handling.

As the pre-analytical sample handling has an impact on non-antibody protein concentrations, it would stand to reason that it may also impact the results of a multi-biomarker disease activity algorithm. The MBDA scores from samples that were obtained by different pre-analytical sample types and sample handling variables were evaluated. The use of plasma, as compared to serum, significantly impacted a large number of subjects' MBDA score, with changes from +18 to −8 MBDA units (Fig. 2A). The MBDA score obtained from serum handled by the traditional method also resulted in significant changes, −8 to +24 MBDA units (Fig. 2B), relative to the protocol method. With both pre-analytical variables, the magnitude of the change of MBDA scores was inversely correlated with the MBDA scores measured with serum samples.

5. Conclusions

Autoantibody biomarker measurements appear robust to blood collection and handling methods. In contrast, blood collection, processing and handling methods had a significant impact on measurable serum protein concentrations. Plasma samples generally exhibited decreased levels for the protein biomarkers assayed. The results of this study illustrate the importance of characterizing pre-analytical variability to ensure test accuracy for development, validation, and clinical testing with biomarker assays. This is especially critical when these assays are integrated in large clinical trials, where using standardized serum processing and handling procedures would be an essential part of the study design, directly affecting results interpretation and next phase of trials.

Disclosures

This work was funded by Crescendo Bioscience. Xiaoyan Zhao, Ferhan Qureshi, P. Scott Eastman, William C. Manning, Claire Alexander and Lyndal K. Hesterberg are employees of Crescendo Bioscience. Crescendo Bioscience owns patents relating to the MBDA test. Patent applications that include William H. Robinson have been filed by Stanford University for the use of autoantibody biomarkers in rheumatoid arthritis, and royalties have been received for these patents. In addition, licensing agreements between Stanford University and Crescendo Bioscience regarding the use of autoantibody biomarkers have been established.

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References

Arnett, F.C., Edworthy, S.M., Bloch, D.A., McShane, D.J., Fries, J.F., Cooper, N.S., Healey, L.A., Kaplan, S.R., Liang, M.H., Luthra, H.S., et al., 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 31, 315.

Chandra, P.E., Sokolove, J., Hipp, B.G., Lindstrom, T.M., Elder, J.T., Reveille, J.D., Eberl, H., Klause, U., Robinson, W.H., 2011. Novel multiplex technology for diagnostic characterization of rheumatoid arthritis. Arthritis Res. Ther. 13, R102.

Curtis, J.R., Haney, D., Van der Helm, A.H., Shen, Y., Knevel, R., Cavet, G., Dirven, L., 2010. Validation of a multi-biomarker test for rheumatoid arthritis (RA) disease activity (Vectra DA) in a multi-cohort study. Arthritis Rheum. 62 (Suppl. 10), 1782.

Dancey, J.E., Dobbin, K.K., Groshen, S., Jessup, J.M., Hruszkewycz, A.H., Koehler, M., Parchment, R., Ratain, M.J., Shankar, L.K., Stadler, W.M., True, L.D., Gravell, A., Grever, M.R., 2010. Guidelines for the development and incorporation of biomarker studies in early clinical trials of novel agents. Clin. Cancer Res. 16, 1745.

Eastman, S., Manning, B., Qureshi, F., Cavet, G., Haney, D., Shen, Y., Alexander, C., Fleischmann, R., Hesterberg, L.K., 2010. Assay development for precise measurement of disease activity serum biomarkers. Ann. Rheum. Dis. 69 (Suppl. 3), 170.

Ellington, A.A., Kullo, I.J., Bailey, K.R., Klee, G.G., 2009. Measurement and quality control issues in multiplex protein assays: a case study. Clin. Chem. 55, 1092.

Ellington, A.A., Kullo, I.J., Bailey, K.R., Klee, G.G., 2010. Antibody-based protein multiplex platforms: technical and operational challenges. Clin. Chem. 56, 186.

FDA, 2001. Guidance for Industry Bioanalytical Method Validation. Food and Drug Administration.

Findlay, J.W., Dillard, R.F., 2007. Appropriate calibration curve fitting in ligand binding assays. AAPS J. 9, E260.

Gerszten, R.E., Accurso, F., Bernard, G.R., Caprioli, R.M., Klee, E.W., Klee, G.G., Kullo, I., Laguna, T.A., Roth, F.P., Sabatine, M., Srinivas, P., Wang, T.J., Ware, L.B., 2008. Challenges in translating plasma proteomics from bench to bedside: update from the NHLBI Clinical Proteomics Programs. Am. J. Physiol. Lung Cell. Mol. Physiol. 295, L16.

Goekoop-Ruiterman, Y.P., de Vries-Bouwstra, J.K., Allaart, C.F., van Zeben, D., Kerstens, P.J., Hazes, J.M., Zwinderman, A.H., Ronday, H.K., Han, K.H., Westedt, M.L., Gerards, A.H., van Groenendael, J.H., Lems, W.F., van Krugten, M.V., Breedveld, F.C., Dijkmans, B.A., 2005. Clinical and radiographic outcomes of four different treatment strategies in patients with early rheumatoid arthritis (the BeSt study): a randomized, controlled trial. Arthritis Rheum. 52, 3381.

Graudal, N.A., Jurik, A.G., de Carvalho, A., Graudal, H.K., 1998. Radiographic progression in rheumatoid arthritis: a long-term prospective study of 109 patients. Arthritis Rheum. 41, 1470.

Grigor, C., Capell, H., Stirling, A., McMahon, A.D., Lock, P., Vallance, R., Kincaid, W., Porter, D., 2004. Effect of a treatment strategy of tight control for rheumatoid arthritis (the TICORA study): a single-blind randomised controlled trial. Lancet 364, 263.

Hueber, W., Kidd, B.A., Tomooka, B.H., Lee, B.J., Bruce, B., Fries, J.F., Sonderstrup, G., Monach, P., Drijfhout, J.W., van Venrooij, W.J., Utz, P.J., Genovese, M.C., Robinson, W.H., 2005. Antigen microarray profiling of autoantibodies in rheumatoid arthritis. Arthritis Rheum. 52, 2645.

Hueber, W., Tomooka, B.H., Zhao, X., Kidd, B.A., Drijfhout, J.W., Fries, J.F., van Venrooij, W.J., Metzger, A.L., Genovese, M.C., Robinson, W.H., 2007. Proteomic analysis of secreted proteins in early rheumatoid arthritis: anti-citrulline autoreactivity is associated with up regulation of proinflammatory cytokines. Ann. Rheum. Dis. 66, 712.

Inoue, E., Yamanaka, H., Hara, M., Tomatsu, T., Kamatani, N., 2007. Comparison of Disease Activity Score (DAS)28-erythrocyte sedimentation rate and DAS28-C-reactive protein threshold values. Ann. Rheum. Dis. 66, 407.

Karlson, E.W., Chibnik, L.B., Cui, J., Plenge, R.M., Glass, R.J., Maher, N.E., Parker, A., Roubenoff, R., Izmailova, E., Coblyn, J.S., Weinblatt, M.E., Shadick, N.A., 2008. Associations between human leukocyte antigen, PTPN22, CTLA4 genotypes and rheumatoid arthritis phenotypes of autoantibody status, age at diagnosis and erosions in a large cohort study. Ann. Rheum. Dis. 67, 358.

Miles, R.R., Roberts, R.F., Putnam, A.R., Roberts, W.L., 2004. Comparison of serum and heparinized plasma samples for measurement of chemistry analytes. Clin. Chem. 50, 1704.

Neumann, E.J., Bonistalli, K.N., 2009. Effect of blood sample handling post-collection on *Erysipelothrix rhusiopathiae* antibody titres. Vet. J. 180, 325.

Osborn, L., Hession, C., Tizard, R., Vassallo, C., Luhowskyj, S., Chi-Rosso, G., Lobb, R., 1989. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell 59, 1203.

Pepys, M.B., Hirschfield, G.M., 2003. C-reactive protein: a critical update. J. Clin. Invest. 111, 1805.

Prevoo, M.L., van't Hof, M.A., Kuper, H.H., van Leeuwen, M.A., van de Putte, L.B., van Riel, P.L., 1995. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum. 38, 44.

Rai, A.J., Vitzthum, F., 2006. Effects of preanalytical variables on peptide and protein measurements in human serum and plasma: implications for clinical proteomics. Expert Rev. Proteomics 3, 409.

Rantapaa-Dahlqvist, S., de Jong, B.A., Berglin, E., Hallmans, G., Wadell, G., Stenlund, H., Sundin, U., van Venrooij, W.J., 2003. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum. 48, 2741.

Rioja, I., Hughes, F.J., Sharp, C.H., Warnock, L.C., Montgomery, D.S., Akil, M., Wilson, A.G., Binks, M.H., Dickson, M.C., 2008. Potential novel biomarkers of disease activity in rheumatoid arthritis patients: CXCL13, CCL23, transforming growth factor alpha, tumor necrosis factor receptor superfamily member 9, and macrophage colony-stimulating factor. Arthritis Rheum. 58, 2257.

Scott, D.L., Steer, S., 2007. The course of established rheumatoid arthritis. Best Pract. Res. Clin. Rheumatol. 21, 943.

Sturgeon, C., Hill, R., Hortin, G.L., Thompson, D., 2010. Taking a new biomarker into routine use—a perspective from the routine clinical biochemistry laboratory. Proteomics Clin. Appl. 4, 892.

Tuck, M.K., Chan, D.W., Chia, D., Godwin, A.K., Grizzle, W.E., Krueger, K.E., Rom, W., Sanda, M., Sorbara, L., Stass, S., Wang, W., Brenner, D.E., 2009. Standard operating procedures for serum and plasma collection: early detection research network consensus statement standard operating procedure integration working group. J. Proteome Res. 8, 113.

Uhlar, C.M., Whitehead, A.S., 1999. Serum amyloid A, the major vertebrate acute-phase reactant. Eur. J. Biochem. 265, 501.

van der Pouw Kraan, T.C., van Gaalen, F.A., Kasperkovitz, P.V., Verbeet, N.L., Smeets, T.J., Kraan, M.C., Fero, M., Tak, P.P., Huizinga, T.W., Pieterman, E., Breedveld, F.C., Alizadeh, A.A., Verweij, C.L., 2003. Rheumatoid arthritis is a heterogeneous disease: evidence for differences in the activation of the STAT-1 pathway between rheumatoid tissues. Arthritis Rheum. 48, 2132.

Verstappen, S.M., Jacobs, J.W., van der Veen, M.J., Heurkens, A.H., Schenk, Y., ter Borg, E.J., Blaauw, A.A., Bijlsma, J.W., 2007. Intensive treatment with methotrexate in early rheumatoid arthritis: aiming for remission. Computer Assisted Management in Early Rheumatoid Arthritis (CAMERA, an open-label strategy trial). Ann. Rheum. Dis. 66, 1443.

Wener, M.H., 2011. Multiplex, megaplex, index, and complex: the present and future of laboratory diagnostics in rheumatology. Arthritis Res. Ther. 138, 134.