Analysis of inflammatory cytokines in human blood, breath condensate, and urine using a multiplex immunoassay platform

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ABSTRACT

A change in the expression of cytokines in human biological media indicates an inflammatory response to external stressors and reflects an early step along the adverse outcome pathway (AOP) for various health endpoints. To characterize and interpret this inflammatory response, methodology was developed for measuring a suite of 10 different cytokines in human blood, exhaled breath condensate (EBC), and urine using an electrochemiluminescent multiplex Th1/Th2 cytokine immunoassay platform. Measurement distributions and correlations for eight interleukins (IL) (1β, 2, 4, 5, 8, 10, 12p70 and 13), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) were evaluated using 90 blood plasma, 77 EBC, and 400 urine samples collected from nominally healthy adults subjects in North Carolina in 2008-2012. The *in vivo* results show that there is sufficient sensitivity for characterizing all 10 cytokines at levels of 0.05-0.10 ρg/ml with a dynamic range up to 100 ng/ml across all three of these biological media. The measured *in vivo* results also show that the duplicate analysis of blood, EBC and urine samples have average estimated fold ranges of 2.21, 3.49, and 2.50, respectively, which are similar to the mean estimated fold range (2.88) for the lowest concentration (0.610ρg/ml) from a series of spiked control samples; the cytokine method can be used for all three biological media. Nine out of the 10 cytokines measured in EBC were highly correlated within one another with

Spearman ρ coefficients ranging from 0.679 to 0.852, while the cytokines measured in blood had a mix of negative and positive correlations, ranging from -0.620 to 0.836. Nearly all of the correlations between EBC and blood were positive. This work also represents the first successful within- and between-person evaluation of ultra trace-level inflammatory markers in blood, EBC, and urine.

INTRODUCTION

Random intermittent environmental exposures or chronic low-level exposures can produce subtle biological responses in humans that are generally difficult to quantify due to the complexity of biological matrices and the variability in responses to environmental exposures within and between individuals (Pleil, 2009, Pleil and Sobus, 2013). Observation of such responses or perturbations in humans, however, is necessary to provide direct empirical evidence of the initiation or progression of an adverse outcome pathway (AOP) (Ankley et al., 2010, Vinken, 2013, Pleil and Sheldon, 2011). An AOP, in the context of human environmental exposure and risk assessment, can be described as the process by which an exogenous environmental stressor interacts with a receptor at the molecular level, thus initiating a sequence of events that can eventually lead to an adverse health effect (Ankley et al., 2010). Understanding the linkages between environmental exposures and adverse health effects requires quantitative knowledge of exposure, the interaction of exposure and molecular targets, and the progression of key events along the AOP. Biomarkers measurements are perhaps the best way to simultaneously evaluate each of these events and processes, and to link *in vitro* experimental results with *in vivo* outcomes and systemic effects (NRC, 2012, Pleil et al., 2012, Pleil, 2012). Therefore, the discovery and identification of meaningful biomarkers and appropriate analytical methodologies for quantifying these biomarkers in targeted studies are important for understanding exposure-health effect linkages (Pleil and Stiegel, 2013).

The human inflammatory response has been studied in detail as an initial biological response to all types of external influences, including physical injury, psychological stress, environmental chemicals, bacteria, and viruses, and thus plays an important part in exposure-response mechanisms or AOPs (Koh et al., 2008, Dybing et al., 2004, Duramad et al., 2007, Selgrade et al., 2006, Spatari et al., 2013, Nakamura et al., 2014). Scientific evidence suggests that inflammation has been directly associated with a large number of adverse health outcomes, including cancer, cardiovascular disease, and autoimmune disease (Elenkov and Chrousos, 1999, Murase et al., 2013, Campbell, 2004, Kampa and Castanas, 2008, Goldbergova et al., 2012). Furthermore, research has shown that inflammation related to environmental exposures can create or exacerbate cardiopulmonary health effects such as asthma and chronic obstructive pulmonary disease (COPD) (Devlin et al., 2012, Devlin et al., 1991, Salvi et al., 1999, Pope et al., 2004). To date, most studies of inflammatory responses to environmental exposure focus on human populations that are already considered "unhealthy" or "at risk" so ultra-sensitive methods for quantifying inflammation have been considered unnecessary.

Current *in vitro* and *in vivo* research investigating links between inflammatory responses and health effects often focus on a very selective group of proteins, primarily chosen based on the anticipated location of the inflammatory response (Candela et al., 1998, Bonisch et al., 2012, Ghoniem et al., 2011, Robroeks et al., 2006). Cytokines are a large group of intercellular signaling proteins that play an important role in the innate and the adaptive human immune response to stressors. They have a vast range of responsibilities, including: recruitment of cells of the immune systems in response to an exogenous/endogenous antigen, cell growth and differentiation, and initiating or suppressing an inflammatory response (Chung and Barnes, 1999). The majority of research into pro- and anti-inflammatory cytokines focuses on their

79 expression from T helper cell Type 1 (Th1), T helper cell Type 2 (Th2), and macrophages
80 (Chung and Barnes, 1999, Boonpiyathad et al., 2013, Cannas et al., 2010, Cousins et al., 2002,
81 Elenkov and Chrousos, 1999, Sack et al., 2006). Unlike hormones, which are loosely
82 categorized into the other class of signaling molecules in the body, cytokines are usually
83 expressed in localized areas and are rarely stored, meaning that their expression is exclusively
84 linked as a response to specific stimuli.

85 In the past, and still to some extent, the choice of which cytokine to study was often
86 limited by methodological constraints, including: lack of specificity due to competitive antibody
87 binding, time/cost constraints associated with the need to run multiple singleplex ELISAs for
88 different biomarkers, and matrix interferences from the selected biological media (blood,
89 cerebrospinal fluid, urine, etc.) (Elshal and McCoy, 2006, Malekzadeh et al., 2012). The
90 development of very specific antibodies and multiplex and bead-based analytical techniques
91 have resolved issues with specificity but assay robustness has typically limited the use of the
92 analytical technique to one biological medium (Bomert et al., 2011, Chowdhury et al., 2009,
93 Tighe et al., 2013, van Bussel et al., 2013).

94 In human-based health effects research, blood is considered the "gold standard"
95 biological fluid of choice. Consequently, there is considerably less knowledge of cytokine
96 expression in other human biological media, especially when it pertains to environmental
97 exposures. In this study, 10 cytokines (interleukins [1β, 2, 4, 5, 8, 10, 12p70 and 13], interferon-
98 γ (IFN- γ), and tumor necrosis factor-α (TNF- α)) are investigated in three human biological
99 media: plasma, EBC, and urine. These cytokines are primarily produced by Th1 cells, Th2 cells,
100 and macrophages and are associated with initiating (IL-1β, IL-2, IL-8, IL-12p70, IFN- γ, and
101 TNF- α) or suppressing (IL-4, IL-5, IL-10, IL-13) an inflammatory response (Chung and Barnes,

1999). The majority of literature values for "control" samples in case-control studies show that each individual cytokine, as sampled in blood, EBC, or urine, are either near or below the methodological limit of detection (~1-10 ρg/mL) or are below this nominal value and are generally not reported (Sanchez Perez et al., 2006, Ostrowski et al., 1998, Jacobs et al., 2001, Matsunaga et al., 2006). An analytical method that can simultaneously measure each cytokine in plasma, EBC, and urine at a level of detection sensitive enough to address subtle biological changes clearly would enhance the prospect of successfully linking them to health effects. This study uses an existing ultra-sensitive multiplex (Human Th1/Th2 Cytokine 10-Plex Assay) kit from Meso Scale Discovery (Gaithersburg, MD) that is designed for human plasma/serum and applies it to blood, EBC and urine samples from a nominally healthy group of adult volunteers (Bastarache et al., 2014). The work presented here is novel as it describes analytical methods, measurement distributions, and correlations across 10 cytokines in three different biological media. All of these steps are critical for evaluating chemical interactions at a cellular level, resulting biological responses, and potential downstream health effects. The cytokine analyses were completed in distinct phases to address the following objectives:

1. Determine whether cytokine markers can be detected in human biological samples from nominally healthy volunteers, and whether method modifications are needed to improve detection.

2. Examine cytokine measurement reproducibility for each of the biological media.

3. Describe cytokine measurement distributions, and investigate pairwise correlations within and across biological media.

METHODS

2.1 Sample Collection

All biological specimens were collected with informed consent from healthy human adult volunteers at the United States Environmental Protection Agency's (US EPA) Human Studies Facility in Chapel Hill, North Carolina. The study protocol and procedures were reviewed and

approved by the University of North Carolina at Chapel Hill's Institutional Review Board and the EPA's Human Subjects Approving Official. A total of 90 whole blood samples and 77 exhaled breath condensate samples were collected from 15 adult volunteers (ages 23-37 years old) participating in an environmental exposure assessment study conducted at the Human Studies Facility in Chapel Hill, NC (IRB Study #: 09-1344). This was a broad human exposure study (of diesel exhaust and ozone) and so for the methods development effort, only a subset of pre-exposure and sham (clean air samples) was used. Urine samples were also collected from this cohort but only a limited number (n=18, from 4 subjects) were useful for the direct comparisons to blood and EBC due to logistics; sample selection was limited to the "pre-exposure" arm of the study and by the (unpredictable) timing of urine collection. As such, this data set was supplemented with a larger and more comprehensive sample set of an earlier study that included 382 spot urine samples collected from 29 adult volunteers, ages 19-50 years old, in 2009-2011 (IRB Study #: 09-0741). The addition of these samples allowed for a more comprehensive investigation of the methodological parameters and distributions of cytokines in urine.

We note that all of the samples (blood, breath, and urine) used for this work were treated as temporally independent because they were collected at widely disparate times (from pre-exposures and shams that were weeks apart for the 15-subject study and randomly stratified urine samples from the 29-subject study which took place over 1-year).

2.2 Blood

Six blood samples were collected per subject, in 10mL Vacutainer® (Becton, Dickinson and Company, Franklin Lakes, New Jersey) collection tubes containing EDTA. The tubes were centrifuged at ≥1300 RCF, and the separated blood fractions were aliquotted into individual 2mL

polypropylene - vials (Corning Incorporated, Corning, NY), and then frozen at ≤-80°C until analysis.

2.3 Exhaled Breath Condensate

Six EBC samples per subject were collected using a RTube™ (Respiratory Research, Inc., Austin, Texas) exhaled breath condensate collector using the method described in Pleil et al. (2008). After collection, the volume of each sample was measured, placed in a 2mL polypropylene vial (Corning Incorporated, Corning, NY), and then frozen at ≤-80°C until analysis.

2.4 Urine

A total of 400 spot urine samples were collected from 33 adults across the two studies. (Study 1: 18 samples from 4 subjects; Study 2: 382 samples from 29 subjects). For Study 1, samples were collected pre-, post- and 24-hr post exposure when physiologically available, and selected to match pre-exposure blood and breath samples. For Study 2, samples were collected over a period of 1 year, for 50 subjects (each individual sampled within a 6-week period), resulting in a total ~3,000 samples from which the 382 samples were randomly selected. Urine voids were individually collected in individual 1L high-density polyethylene containers (Chase Scientific Glass, Inc., Rockwood, TN). An 8mL-aliquot of each sample was transferred into a 10ml polypropylene vial (Corning Incorporated, Corning, NY) and immediately frozen at ≤-20°C until analysis. The random selection procedures served to minimize temporal auto-correlation.

2.5 Analysis

The ten cytokines of interest to this study were analyzed using a Meso Scale Discovery (MSD) multiplex electrochemiluminescent immunoassay system and SECTOR Imager 2400. The panel, Human Th1/Th2 10-plex Ultra-Sensitive Kit, was designed by MSD to analyze the following human biomarkers in each well: interleukins 1β, 2, 4, 5, 8, 10, 12p70 and 13; IFN- γ;

and TNF- α. The analysis method is described in more detail elsewhere, but briefly it proceeded as follows (Meso Scale Discovery, 2012). The 96-well plates were supplied by MSD in a prepared format and were ready to use without further preparation. Reagents for the assay were prepared according to the given procedure in the MSD literature. Prior to analysis, all samples were removed from -80°C storage, thawed in an ice bath for 30-45 minutes, and then vortexed for five seconds to achieve uniform consistency.

The calibration standards for the immunoassay were supplied in a single mix at 2500 ρg/mL and required a series of 4-fold dilutions to achieve an 8-point standard curve. Twenty-five μL of a proprietary diluent were added to each of the 96-well plates, and the plates were sealed and incubated at room temperature (approximately 22.2°C) for 30 minutes at 1000 rpm. Next, duplicate 25 μL aliquots of each calibration standard (a total of 16 wells) and 80 individual samples were then added to the plate. The 96-well plate was sealed and incubated at room temperature for two hours at 1000 rpm. Then, the plate was washed three times with a phosphate buffered saline-0.05% Tween (PBS-T) solution. Twenty-five uL of a detection antibody solution was added into each of the 96 wells followed by sealing and incubating the plate for an additional two hours at 1000rpm. The plate was washed three times with PBS-T and 150 μL of Read Buffer was added to each well. Finally, the plate was analyzed on a MSD SECTOR Imager 2400.

2.6 Methods Development

The methods development work proceeded in three stages. First, human blood plasma samples (n=13) and spiked method blanks (n=84, 12 repeated spikes at each of the 7 concentrations) were analyzed using the existing MSD methodology. The results from this analysis were a set of methodological parameter estimates (lower limit of quantitation [LLOQ], linear range, within- and between-plate variance, etc.) that were then used as a comparison

dataset for the other two biological media. LLOQs were defined as three times the standard deviation of the "zero" method blanks on a specific plate per each cytokine. Each 96-well plate that was analyzed had a different LLOQ for the respective cytokine. As such, the mean and standard error of the LLOQ were calculated for each cytokine and the % <LLOQ was then determined using the plate-specific LLOQ values.

In the second stage of our analysis, a selection of EBC (n=24) and urine (n=18) samples was analyzed using the existing method to determine if the immunochemistry was compatible between different biological media, to assure that the samples were pre-processed appropriately and fit into the associated concentrations of the calibration curve, and to determine if the MSD SECTOR Imager 2400 could identify the 10 target cytokines. Success in this stage was measured by the presence or lack of cytokines in EBC and urine. In a separate experiment, we analyzed an additional 77 plasma samples, 53 EBC samples, and 382 urine samples; these measurements were combined with those from the earlier experiment to allow a comprehensive statistical analysis.

2.8 Statistical Analysis

2.8.1 Model Development

The MSD DISCOVERY WORKBENCH® analysis software was used to estimate the concentration data for each target cytokine. These data are based on an internally calculated signal-concentration 4-paramter logistic calibration curve. When a sample's signal is below the instrument derived LLOQ the software will frequently report the signal but not a calculated concentration. We developed a series of 5-parameter logistic (5pl) models for each target cytokine to interpret these low-end signal values (Richards, 1959). Fitting the empirical data with this approach allowed us to extend the original calibration curve to make robust estimates between "zero" and the nominal (instrument derived) LLOQ.

2.8.2 Calibration and Sensitivity

The 5pl models were constructed for each cytokine per individual plate using the calibration standards from that analytical run, and then applied to the study samples on that respective plate (GraphPad Prism version 6.0, GraphPad Software, La Jolla, CA). Each LLOQ for a specific cytokine, in an individual analysis, was established during the model construction. Sample detections comparing the original calibration model to the 5pl model were also calculated during this stage using the initial set of 13, 24, and 18 blood, EBC, and urine samples, respectively. The cytokine distribution percentiles for each biological media were estimated using the Proc UNIVARIATE procedure of the SAS statistical software package version 9.3 (SAS Institute, Cary, NC, USA).

2.8.3 Correlations and Variance Estimates

An α=0.05 criterion was used as the significance level for all statistical tests. Spearman correlation coefficients were calculated in GraphPad for each cytokine measured in both blood and EBC, but not urine, given that the majority of the urine sample-set was derived from another sample cohort. Cytokine measurement distributions across all three biological media were evaluated via visual inspection of quantile-quantile plots and Shapiro-Wilks tests for normality (SAS Proc UNIVARIATE). All data were right-skewed and were natural log-transformed prior to further analysis to satisfy normality assumptions. This is a standard statistical approach for environmental and biological measurements (Pleil et al., 2014).

Two different sets of linear mixed-effect models (SAS Proc MIXED) were used to investigate within- and between-plate variation across the spiked calibration samples (balanced dataset), and within- and between-person variation across the repeated study samples (Rappaport and Kupper, 2008). Given that the same sample was run multiple times on a single plate and multiple times on different plates, the partitioning of spiked sample variance into these two

groupings helps determine if samples run on different plates are comparable to one another. More within-plate than between-plate variance shows that measurement variance can be treated as random error and that samples run on different plates are comparable.

$$Y_{cij} = \ln(X_{cij}) = \mu_{Y_c} + b_{ci} + \varepsilon_{cij} \quad (1.1)$$

The model used for spiked samples is given in equation 1.1, where c = spiked concentration of 0.610, 2.44, 9.80, 39.1, 156, 625, or 2500 ρg/mL; i = plate 1, 2, 3, 4, 5, 6, or 7 for the c^{th} concentration; j = measurement 1 or 2, on the i^{th} plate, for the c^{th} concentration. Here, X_{cij} represents the concentration of a cytokine (ρg/mL) from the j^{th} measurement, on the i^{th} plate, at the c^{th} concentration, and Y_{cij} is the natural logarithm of the measurement X_{cij}. The coefficients μ_{Y_h}, b_{ci}, and ε_{cij} represent, respectively, the true (logged) mean cytokine level at the c^{th} concentration, the random effect of the i^{th} plate at the c^{th} concentration, and the random-error effect of the j^{th} measurement, on the i^{th} plate, at the c^{th} concentration. It is assumed that b_{ci} and ε_{cij} are independent random variables with means of 0 and variances of $\sigma^2_{bY_c}$ and $\sigma^2_{wY_c}$, representing the between- and within-plate variance, respectively, at each concentration, c.

Duplicate study samples (n= 28, 18, and 54 duplicates for blood, EBC and urine, respectively) were investigated using SAS Proc NESTED to estimate the proportion of measurement error that could be attributed to biological matrix (i.e. blood, EBC or urine) effects; matrix effects were not examined using spiked samples, since spikes were prepared using a PBS/BSA solution.

$$Y_{Bij} = \ln(X_{Bij}) = \mu_{Y_B} + b_{Bi} + \varepsilon_{Bij} \quad (1.2)$$

Equation 1.2, similar to equation 1.1 but with "B" biological medium instead of "c" concentration, was used to evaluate the study samples, specifically: B = biological medium 1 (blood), 2 (EBC), or 3 (urine); i = 1, 2, …, n_B individuals for the Bth biological medium; and j =

1, 2, …, n_{Bi} measurements of a particular cytokine from the i^{th} individual, for the B^{th} biological medium. Here, it is assumed that b_{Bi} and ε_{Bij} are independent random variables with means of 0, and $\sigma^2_{bY_B}$ and $\sigma^2_{wY_B}$ represent the between- and within-person variance, respectively, for each of the biological media, B. Partitioning of the variance within- and between- persons helps determine whether the variance across the distribution for a specific cytokine (and a specific biological medium) is more closely related to an individual or to the group.

Restricted maximum likelihood estimates of variance components (between- and within-plate for spiked samples, and between- and within-person for study samples) were used to calculate estimated 95% fold-ranges and intraclass correlation coefficients (ICC). Estimated fold-ranges ($\hat{R}_{0.95}$) were calculated for each variance component result from the spiked calibration sample analysis (using Equation 2.1), duplicate study sample analysis (using Equation 2.2), and repeated study sample analysis (using Equation 2.3). The fold-range estimates used here are a scale-independent way of comparing the central 95% of the measurement data, thus making it easier to compare estimates across analytes and media (Rappaport and Kupper, 2008).

$$_{Y}\hat{R}_{0.95c} = e^{3.92\sqrt{\hat{\sigma}^2_{bY_c}+\hat{\sigma}^2_{wY_c}}} \quad (2.1)$$

For the spiked calibration samples, Equation 2.1 was used, where, $_{Y}\hat{R}_{0.95c}$ is the estimated overall 95% fold-range and $\hat{\sigma}^2_{bY_c}$ and $\hat{\sigma}^2_{wY_c}$ represent the estimated between- and within-plate variance components for each spiked concentration, *c*.

$$_{w}\hat{R}_{0.95B} = e^{3.92\sqrt{\hat{\sigma}^2_{wY_B}}} \quad (2.2)$$

For the duplicate study samples, Equation 2.2 was used, where, $_{w}\hat{R}_{0.95B}$ is the estimated within sample 95% fold-range and $\hat{\sigma}^2_{wY_B}$ represents the estimated within-person variance component for

each biological medium. Here, "duplicate" is defined as the same sample that was analyzed two different times.

$$_{Y}\hat{R}_{0.95B} = e^{3.92\sqrt{\hat{\sigma}^2_{bY_B}+\hat{\sigma}^2_{wY_B}}} \quad (2.3)$$

For the repeated study samples, Equation 2.3 was used, where, $_{Y}\hat{R}_{0.95B}$ is the estimated overall 95% fold-range and $\hat{\sigma}^2_{bY_B}$ and $\hat{\sigma}^2_{wY_B}$represent the estimated between- and within-person variance components for each biological medium.

Estimated ICCs ($\hat{\rho}$) were calculated for the spiked calibration samples (Equation 2.4) and the study samples (Equation 2.5) using the following formulas:

$$\hat{\rho}_C = \frac{\hat{\sigma}^2_{bY_C}}{\hat{\sigma}^2_{bY_C}+\hat{\sigma}^2_{wY_C}} \quad (2.4)$$

$$\hat{\rho}_B = \frac{\hat{\sigma}^2_{bY_B}}{\hat{\sigma}^2_{bY_B}+\hat{\sigma}^2_{wY_B}} \quad (2.5)$$

MATLAB version 8.2 (R2013b, Mathworks, Natick, MA) and GraphPad were used for graphical representations.

RESULTS

Objective 1- Detection of cytokines in three biological media

Table 1 shows the percentage of individual study sample cytokine levels that are above the respective LLOQs using either the instrument-derived software ("Original") or a 5pl model ("New). The majority of the cytokines in each biological medium have ≥ 80% of samples that are above the LLOQ when using the original instrument output. Results show increases in the percentage of samples above the LLOQ using the 5pl model, thus confirming that there is an improvement in overall system sensitivity. The EBC samples have less of an increase in the percentage of samples above the LLOQ when compared to the results from the blood or urine samples. Investigating this difference shows that a large percentage of the EBC samples had responses similar to those of the blank calibration points; this is not unexpected as EBC is a very dilute biological fluid. Overall, the use of the 5pl model increased the percentage of samples

above the LLOQ by 9.24%, 7.92%, and 9.39% for the blood, EBC and urine samples, respectively.

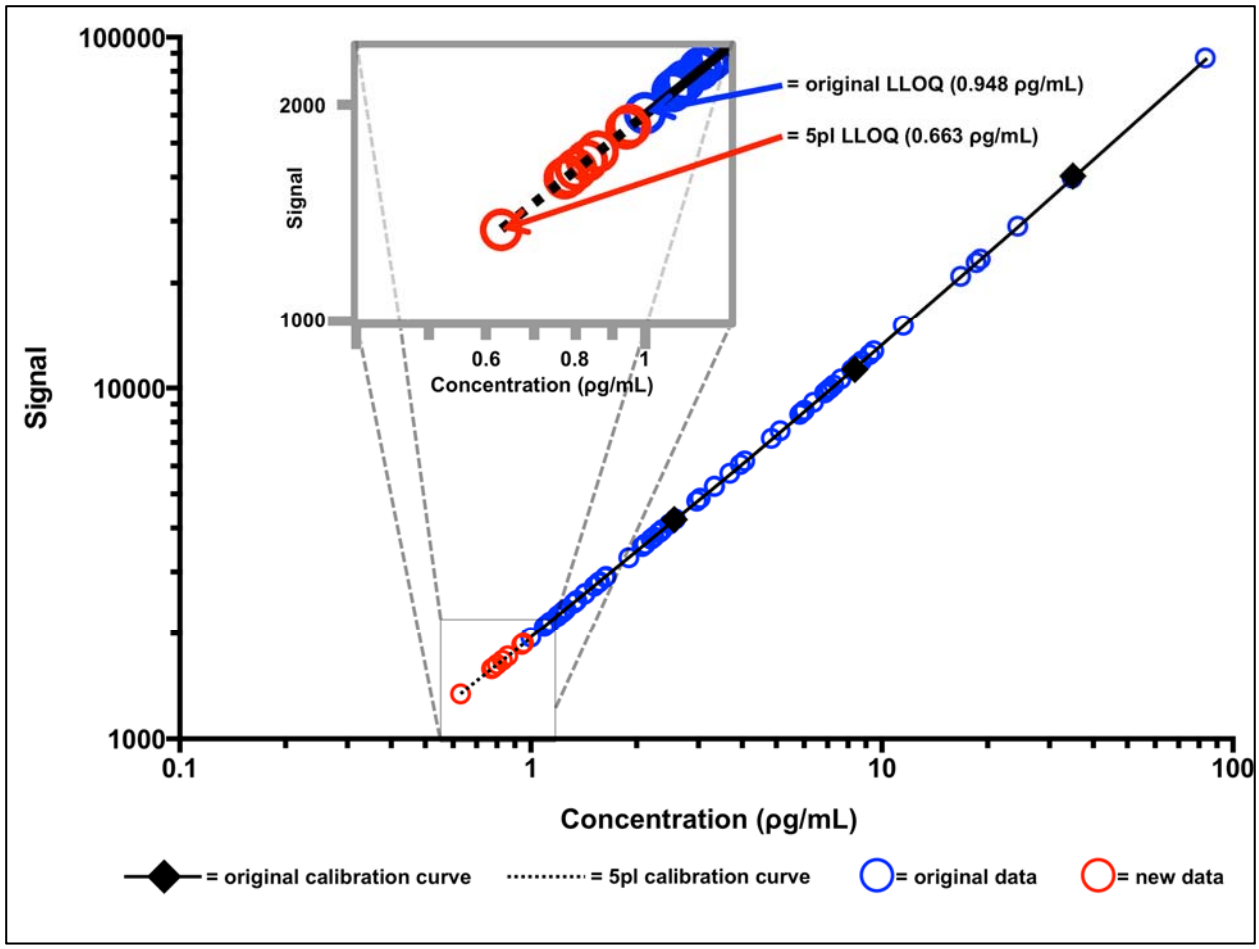
Table 1: Comparison of the Percentage of Samples above the nominal LLOQs between the Original Output and the "New" 5pl Model

	Blood[1]		EBC[2]		Urine[3]	
Cytokine	Original	New	Original	New	Original	New
IL-1β	100	100	87.5	95.8	100	100
IL-2	92.3	100	87.5	95.8	100	100
IL-4	69.2	100	79.2	91.7	83.9	100
IL-5	92.3	100	79.2	83.3	83.3	100
IL-8	100	100	83.3	100	88.9	100
IL-10	92.3	100	87.5	87.5	94.4	100
IL-12p70	100	100	75.0	83.3	100	100
IL-13	69.2	100	8.30	16.7	55.6	100
IFN-γ	92.3	100	75.0	79.2	100	100
TNF-α	100	100	83.3	91.7	100	100

1:n=13, 2:n=24, 3:n=18

Figure 1 displays the total range and a "zoomed-in" view of the lower end of the calibration curve, highlighting the additional quantitative data gained by using the 5pl model. The blue circles are calculated sample concentrations based on the original output and the red circles are additional sample concentrations achieved through the use of the 5pl model. This figure is a typical example of the range of values for nominally healthy adult subjects. In this specific example, eight additional sample concentrations were calculated based on the use of the 5pl model.

Figure 1: IL-8 Calibration Curve for Urine Displaying the Additional Sample Concentrations Gained from using a 5pl Model.



Objective 2- Cytokine methodological parameters

2.1 Overall fold-range ($_{Y}\hat{R}_{0.95c}$) and ICC ($\hat{\rho}_c$) estimates, based on repeated measurements of spiked calibration samples, are given for individual cytokines in Table 2. Here, the fold range estimates contain the central 95% of all cytokine measurements for the spiked samples at a given concentration level and biological media. The ICCs display the portion of total measurement variance that was observed between plates. These results were achieved using a prepared standard solution, and unlike blood, EBC or urine, with their associated proteins, cells, etc., should be indicative of a "best case" scenario where matrix-interferences play a minimal role in cytokine quantitation.

Table 2: Estimated 95% Fold-Ranges ($_{Y}\hat{R}_{0.95c}$) and Intraclass Correlation Coefficients ($\hat{\rho}_c$) for repeated measures of spiked calibration samples.

	Concentration (ρg/mL)[a]							
Cytokine	0.61	2.44	9.77	39.1	156	625	2500	Average
IL-1β	3.77 (0.00)	1.52 (0.00)	1.20 (0.00)	1.10 (0.00)	1.33 (0.00)	1.23 (0.281)	1.40 (0.00)	1.65 (0.040)
IL-2	2.28 (0.00)	1.46 (0.00)	1.30 (0.045)	1.12 (0.00)	1.24 (0.105)	1.16 (0.269)	1.57 (0.00)	1.45 (0.060)
IL-4	3.19 (0.00)	1.69 (0.195)	1.50 (0.044)	1.26 (0.00)	1.44 (0.230)	1.14 (0.100)	1.38 (0.00)	1.66 (0.081)
IL-5	1.21 (0.219)	1.24 (0.018)	1.23 (0.031)	1.09 (0.048)	1.27 (0.00)	1.13 (0.354)	1.13 (0.00)	1.19 (0.096)
IL-8	2.08 (0.00)	1.37 (0.00)	1.28 (0.216)	1.11 (0.294)	1.31 (0.00)	1.16 (0.180)	1.34 (0.00)	1.38 (0.099)
IL-10	2.19 (0.361)	1.48 (0.00)	1.25 (0.357)	1.13 (0.434)	1.42 (0.00)	1.12 (0.318)	1.66 (0.00)	1.46 (0.210)
IL-12 p70	2.10 (0.00)	1.21 (0.135)	1.41 (0.00)	1.26 (0.00)	1.31 (0.030)	1.27 (0.380)	1.40 (0.00)	1.42 (0.078)
IL-13	5.72 (0.00)	3.61 (0.00)	1.46 (0.132)	1.20 (0.211)	1.32 (0.042)	1.25 (0.285)	1.25 (0.00)	2.26 (0.096)
INF- γ	3.53 (0.358)	1.32 (0.00)	1.19 (0.341)	1.15 (0.00)	1.35 (0.00)	1.17 (0.262)	1.31 (0.00)	1.57 (0.137)
TNF-α	2.77 (0.225)	1.45 (0.00)	1.35 (0.370)	1.18 (0.410)	1.29 (0.00)	1.17 (0.151)	1.35 (0.00)	1.51 (0.165)
Average	2.88 (0.116)	1.64 (0.035)	1.32 (0.153)	1.16 (0.40)	1.33 (0.041)	1.18 (0.258)	1.38 (0.00)	

a: ${}_{Y}\hat{R}_{0.95c}(\hat{\rho}_c)$, all parameters were estimated using natural logged cytokine concentrations.

On average, the central 95% of measurements were observed within a 1.55 fold-range across the 10 cytokines and seven spiked concentrations. Fold range estimates generally increased as the spiked concentration decreased, with the lowest spiked concentration having the largest overall FRs. The average ${}_{Y}\hat{R}_{0.95c}$ for the 10 cytokines at 0.610ρg/mL is 2.88, where the average fold ranges for the other six concentrations, in increasing concentration from 2.44-2500ρg/mL, are 1.64, 1.32, 1.16, 1.33, 1.18 and 1.38, respectively.

ICC estimates of 0.00 to 0.380 suggest that the majority of measurement variance (i.e., 62-100%) was observed within-plate rather than between-plate. The ICC estimates do not display an increasing or decreasing trend across the spiked concentrations. Furthermore, results from the mixed models show no significant random plate effects across all cytokines and

concentrations ($p \geq 0.18$). Taken together, these results confirm that the variation in measurements between plates can be treated as random error, and that samples analyzed across different plate are directly comparable.

2.2 Table 3 displays mean (±standard error) and fold-range ($_{w}\hat{R}_{0.95\beta}$) estimates associated with all duplicate measurements of cytokines in blood, EBC, and urine. Fold-range estimates based on biological media measurements were similar to those of the lowest spiked concentration (0.610 ρg/mL [see Section 2.1]). The fold range estimates ranged from 1.36 to 4.29 for blood, from 2.12 to 4.96 for EBC, and from 0.935 to 38.7 for urine, while the lowest spiked concentration had fold ranges of 1.21 to 5.72. IL-13 had the largest fold-range for the blood and EBC samples, while IL-4 had the largest fold-range for the urine samples. These results were expected given that the majority of the sample concentrations (not shown) lie within a concentration range spanning from the LLOQ-1.00ρg/mL for the respective cytokines.

Table 3: Descriptive Statistics and Estimated Fold-Ranges for Duplicate Samples

	Blood		EBC		Urine	
	μ ± SEM[a]	$_{w}\hat{R}_{0.95B}$[b]	μ ± SEM[a]	$_{w}\hat{R}_{0.95B}$[b]	μ ± SEM[a]	$_{w}\hat{R}_{0.95B}$[b]
IL-1β	1.51(0.515)	3.95	5.26(1.29)	2.82	1.74(0.226)	3.33
IL-2	0.127(0.012)	2.50	0.340(0.051)	4.96	0.935(0.208)	1.97
IL-4	0.380(0.047)	1.36	1.27(0.159)	3.39	1.54(0.046)	5.25
IL-5	0.639(0.256)	2.03	0.106(0.0121)	2.40	0.444(0.043)	2.41
IL-8	2.60(0.450)	1.50	3.67(2.89)	2.27	38.7(9.27)	2.10
IL-10	1.24(0.063)	1.56	1.12(0.082)	2.12	1.83(0.325)	2.30
IL-12 p70	0.448(0.028)	1.45	0.612(0.074)	3.35	1.20(0.032)	2.09
IL-13	1.32(0.108)	4.29	1.38(0.163)	4.77	2.03(0.045)	1.58
INF- γ	0.469(0.059)	1.75	0.386(0.051)	4.45	0.945(0.050)	2.28
TNF-α	2.14(0.118)	1.82	0.433(0.058)	4.34	0.974(0.058)	1.73

a: μ ± SEM (mean ± standard error of the mean), b: estimated within-sample fold range, parameters were estimated using natural logged cytokine concentrations.

Objective 3- Study Sample Cytokine Measurement Distributions and Correlations

3.1- Descriptive statistics are given in Table 4a, b, and c for cytokines measured in blood plasma (n=90), EBC (n=77), and urine (n=400) samples, respectively; individual cytokines are sorted based on the percentage of samples above the LLOQ.

Table 4a: Descriptive Statistics (ρg/mL) for Cytokines in Plasma

Cytokine	LLOQ[a]	%>LLOQ	Min	5%	25%	50%	75%	95%	Max
IL-8	0.056 (0.038)	100	0.980	1.15	1.50	1.97	2.67	3.86	41.7
TNF-α	0.064 (0.038)	100	1.06	1.29	1.44	1.73	2.49	3.73	8.77
IL-10	0.224 (0.060)	99.0	<LLOQ	0.486	0.746	1.26	1.59	2.31	3.23
IL-12 p70	0.197 (0.028)	95.2	<LLOQ	0.207	0.257	0.339	0.582	0.830	1.62
IL-5	0.055 (0.038)	94.3	<LLOQ	<LLOQ	0.115	0.208	0.373	0.881	20.4
IL-4	0.228 (0.117)	81.0	<LLOQ	<LLOQ	0.220	0.277	0.347	0.569	2.59
INF- γ	0.163 (0.104)	80.1	<LLOQ	<LLOQ	0.085	0.305	0.649	1.51	3.01
IL-13	1.34 (0.845)	78.1	<LLOQ	<LLOQ	0.220	1.62	2.04	2.75	4.38
IL-2	0.087 (0.068)	78.1	<LLOQ	<LLOQ	0.047	0.093	0.189	0.262	0.633
IL-1β	0.511 (0.717)	73.3	<LLOQ	<LLOQ	<LLOQ	0.160	1.56	3.38	40.3

a: mean (standard deviation)

Table 4b: Descriptive Statistics (ρg/mL) for Cytokines in Exhaled Breath Condensate

Cytokine	LLOQ[a]	%>LLOQ	Min	5%	25%	50%	75%	95%	Max
IL-10	0.143 (0.062)	93.5	<LLOQ	<LLOQ	0.581	0.737	1.78	2.38	3.15
IL-8	0.369 (0.115)	92.2	<LLOQ	<LLOQ	0.151	0.245	0.807	2.27	222
IL-4	0.238 (0.107)	90.9	<LLOQ	<LLOQ	0.323	0.411	2.92	3.82	4.16
IL-5	1.32 (0.365)	89.6	<LLOQ	<LLOQ	0.032	0.047	0.181	0.312	0.368
TNF-α	0.032 (0.023)	87.0	<LLOQ	<LLOQ	0.075	0.120	0.804	1.27	2.53
IFN-γ	0.035 (0.015)	81.8	<LLOQ	<LLOQ	0.058	0.099	0.582	1.22	1.62
IL-12 p70	0.172 (0.063)	72.7	<LLOQ	<LLOQ	<LLOQ	0.220	1.33	1.81	2.08
IL-1β	0.666 (0.706)	71.4	<LLOQ	<LLOQ	<LLOQ	2.90	5.27	13.2	94.8
IL-2	0.187 (0.126)	71.4	<LLOQ	<LLOQ	<LLOQ	0.061	0.659	1.06	2.31
IL-13	0.388 (0.274)	55.8	<LLOQ	<LLOQ	<LLOQ	0.244	2.86	3.64	4.26

a: mean (standard deviation)

Table 4c: Descriptive Statistics (ρg/mL) for Cytokines in Urine

Cytokine	LLOQ[a]	%>LLOQ	Min	5%	25%	50%	75%	95%	Max
IL-2	0.161 (0.160)	96.5	<LLOQ	0.050	0.188	0.394	0.613	1.36	62.3
IL-1β	0.177 (0.084)	95.0	<LLOQ	<LLOQ	0.207	0.693	2.26	24.4	382
IL-4	0.130 (0.053)	94.5	<LLOQ	<LLOQ	0.027	0.070	0.997	3.27	3.73
IL-8	0.116 (0.050)	93.8	<LLOQ	<LLOQ	2.82	9.74	29.4	162	2270
IL-13	0.462 (0.238)	89.0	<LLOQ	<LLOQ	0.903	2.15	3.82	7.15	33.2
IL-10	0.216 (0.101)	88.7	<LLOQ	<LLOQ	0.125	0.379	1.77	3.63	141

IFN-γ	0.317 (0.254)	85.0	<LLOQ	<LLOQ	0.130	0.311	0.883	8.28	12.7
TNF-α	0.412 (0.240)	84.2	<LLOQ	<LLOQ	0.126	0.256	0.595	2.90	13.5
IL-5	0.234 (0.230)	73.0	<LLOQ	<LLOQ	<LLOQ	0.102	0.263	0.745	4.66
IL-12 p70	0.338 (0.217)	72.2	<LLOQ	<LLOQ	<LLOQ	0.308	0.750	1.73	2.35

a: mean (standard deviation)

Plasma samples had a greater percentage of samples (87.9%) that were above the LLOQ when compared to the EBC (80.6%) and urine (87.2%) samples. IL-4, IL-5, IL-8, IL-10, and TNF-α had the largest %>LLOQ in both plasma and EBC. IL-12p70 also had a large %>LLOQ in plasma, but was low in EBC and urine. IL-1β, IL-2, and IL-13 had the smallest %>LLOQ in the blood and EBC samples. The urine samples were unique in that the %>LLOQ was largest for IL-1β and IL-2; these results are the exact opposite of those observed for blood and EBC. LLOQs for specific cytokines were usually similar across the three biological media (e.g. $LLOQ_{IL\text{-}2,blood} \approx LLOQ_{IL\text{-}2,EBC} \approx LLOQ_{IL\text{-}2,urine}$). However, LLOQs for IL-5, IL-8, IFN-γ and TNF-α varied considerably across the three media, by as much as an order of magnitude. These variations did not appear to appreciably affect the percentage of measurements above these values. For example, IL-8 in the blood, EBC and urine samples had mean LLOQs of 0.056ρg/mL, 0.369ρg/mL, and 0.116ρg/mL, respectively, but 100, 92.2, and 93.8 percent of samples above the LLOQ.

The cytokine concentrations were observed across wide ranges (Tables 4a, b, c), spanning 2-4 orders of magnitude (Min-Max) depending on the cytokine and the biological medium. Investigating individual cytokines across the different biological media shows that they have different concentration ranges across media. For example, IL-8 ranged from 0.980-41.7ρg/mL in blood, <LLOQ-222ρg/mL in EBC, and <LLOQ-2270ρg/mL in urine, demonstrating a wide range across the three media for this specific cytokine. In contrast, IL-12p70 had concentration ranges across the three biological media that were very similar to one another; IL-12p70 ranged from <LLOQ-1.62ρg/mL in blood, <LLOQ-2.08ρg/mL in EBC, and

<LLOQ-2.35ρg/mL in urine. There were also large ranges exhibited within biological media. For example, the maximum concentrations in blood ranged from 0.633ρg/mL for IL-2 to 41.7ρg/mL for IL-8. The maximum concentrations in the EBC samples ranged from 0.368ρg/mL for IL-5 to 222ρg/mL for IL-8, and the maximum concentrations in urine ranged from 2.35ρg/mL for IL-12p70 to 2270ρg/mL for IL-8.

3.2 Table 5 displays estimated variance components, fold-ranges, and ICCs based on measurements of plasma, EBC, and urine samples. Here, $\hat{\sigma}^2_{wY_B}$, $\hat{\sigma}^2_{bY_B}$, and $\hat{\sigma}^2_{Y_B}$ are the estimated within-subject, between-subject, and total variance components for the respective cytokine in each biological medium. $_w\hat{R}_{0.95B}$ is the estimated fold-range containing the central 95% of cytokine measurements for any given person in the sampled population. The parameter $_b\hat{R}_{0.95B}$ represents the estimated fold-range containing the central 95% of mean cytokine levels across all individuals in the sampled population. Finally, $_Y\hat{R}_{0.95B}$ represents the estimated overall fold-range containing the central 95% of all measurements for all individuals in the sampled population.

Table 5: Estimated Variance Components, Fold-ranges, and ICCs for the Study Samples

	Blood*				EBC*				Urine*			
Cytokine	$\hat{\sigma}^2_{wY_B}$ ($_w\hat{R}_{0.95}$B)	$\hat{\sigma}^2_{bY_B}$ ($_b\hat{R}_{0.95}$B)	$\hat{\sigma}^2_{Y_B}$ ($_Y\hat{R}_{0.95}$B)	$\hat{\rho}_B$	$\hat{\sigma}^2_{wY_B}$ ($_w\hat{R}_{0.95}$B)	$\hat{\sigma}^2_{bY_B}$ ($_b\hat{R}_{0.95}$B)	$\hat{\sigma}^2_{Y_B}$ ($_Y\hat{R}_{0.95}$B)	$\hat{\rho}_B$	$\hat{\sigma}^2_{wY_B}$ ($_w\hat{R}_{0.95}$B)	$\hat{\sigma}^2_{bY_B}$ ($_b\hat{R}_{0.95}$B)	$\hat{\sigma}^2_{Y_B}$ ($_Y\hat{R}_{0.95}$B)	$\hat{\rho}_B$
IL-1β	0.954 (46.0)	1.21 (74.7)	2.16 (320)	0.559	0.360 (10.5)	1.71 (170)	2.07 (283)	0.826	0.479 (15.1)	0.373 (11.0)	0.853 (37.4)	0.438
IL-2	0.329 (9.48)	0.492 (15.6)	0.821 (34.9)	0.599	0.608 (21.2)	2.72 (642)	3.33 (1280)	0.817	0.280 (7.95)	0.134 (4.21)	0.414 (12.5)	0.324
IL-4	0.263 (7.46)	0.012 (1.53)	0.275 (7.80)	0.043	0.065 (2.71)	1.06 (56.1)	1.12 (63.3)	0.942	0.910 (42.1)	0.372 (10.9)	1.28 (84.7)	0.290
IL-5	0.491 (15.6)	0.543 (18.0)	1.03 (53.8)	0.525	0.124 (3.97)	0.824 (35.1)	0.947 (45.4)	0.870	0.262 (7.43)	0.388 (11.5)	0.650 (23.6)	0.597
IL-8	0.169 (5.01)	0.080 (3.03)	0.249 (7.06)	0.321	0.745 (29.5)	1.82 (197)	2.56 (530)	0.709	1.33 (92.4)	1.81 (195)	3.14 (1040)	0.576
IL-10	0.101 (3.46)	0.146 (4.48)	0.247 (7.00)	0.593	0.064 (2.70)	0.303 (8.65)	0.367 (10.8)	0.825	0.525 (17.1)	0.219 (6.27)	0.744 (29.4)	0.295
IL-12 p70	0.095 (3.35)	0.155 (4.68)	0.250 (7.09)	0.620	0.083 (3.09)	0.973 (47.8)	1.06 (56.2)	0.921	0.213 (6.10)	0.126 (4.03)	0.339 (9.80)	0.372
IL-13	0.301 (8.60)	1.08 (58.2)	1.38 (99.4)	0.781	0.100 (3.45)	2.17 (320)	2.27 (365)	0.956	0.303 (8.65)	0.158 (4.74)	0.461 (14.3)	0.342
INF- γ	0.325 (9.36)	1.09 (60.2)	1.42 (107)	0.771	0.109 (3.65)	1.72 (170)	1.83 (200)	0.940	0.393 (11.7)	0.326 (9.36)	0.719 (27.7)	0.453
TNF-α	0.055 (2.50)	0.104 (3.54)	0.159 (4.77)	0.655	0.253 (7.18)	1.65 (155)	1.91 (224)	0.867	0.158 (4.75)	0.134 (4.19)	0.292 (8.32)	0.458

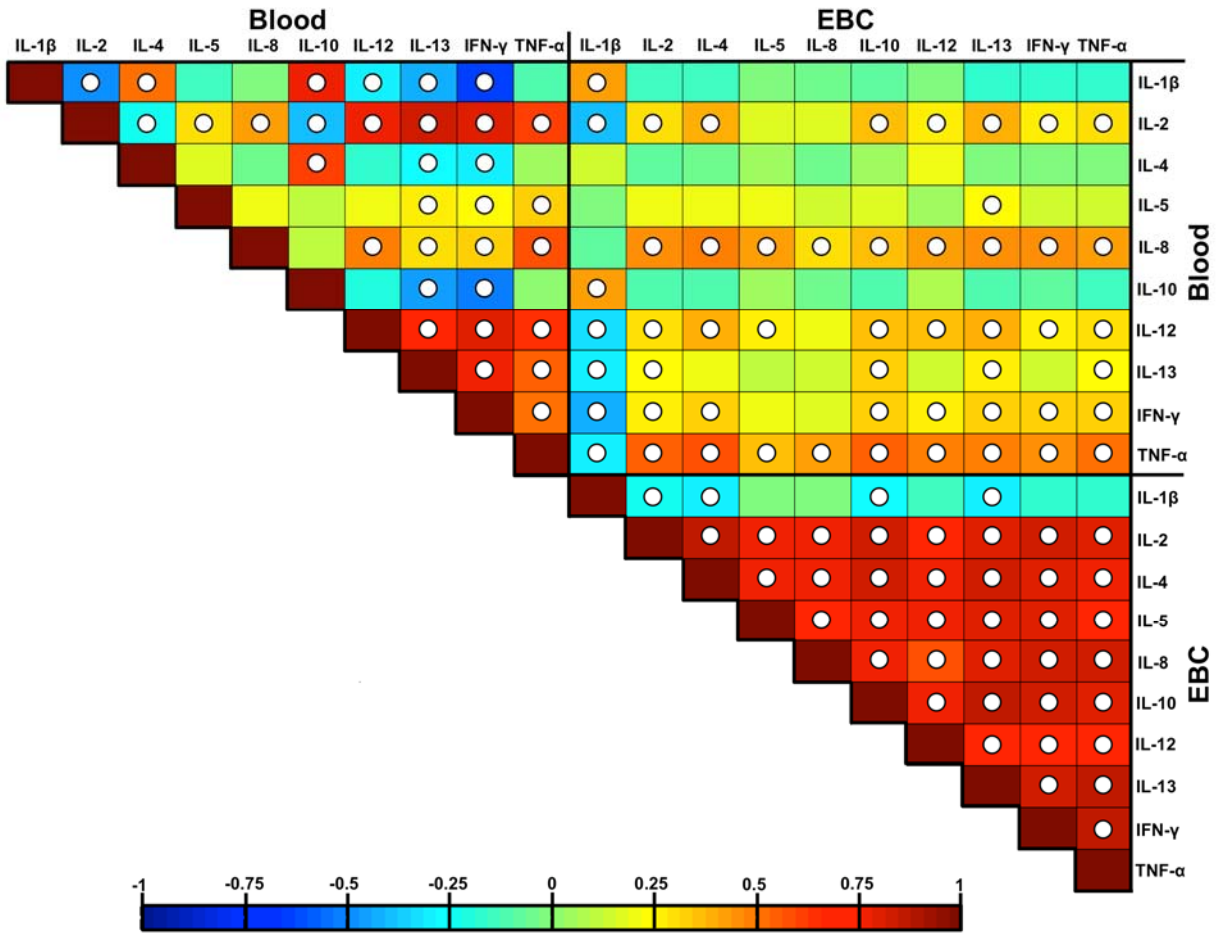
*All parameters were estimated using natural logged cytokine concentrations as per standard procedures (Pleil et al. 2014)

Total fold-range estimates (${}_{Y}\hat{R}_{0.95B}$) were between 4.77 and 320 for blood, 8.32 and 1040 for urine, and 10.8 and 1280 for EBC. These results suggest that total measurement variability was similar for urine and EBC, but somewhat smaller for blood. For the majority of the cytokines in blood (8/10 cytokines) and EBC (10/10 cytokines), ${}_{b}\hat{R}_{0.95B}$ was larger than ${}_{w}\hat{R}_{0.95B}$. However, the opposite trend was observed for 8 out of 10 urinary cytokines, where, fold-range estimates were larger within-person than between-person.

Large differences were also observed between the estimated within- and between-person fold ranges for specific cytokines across the three biological media. Considering results for IL-4, ${}_{w}\hat{R}_{0.95B}$ values were 7.46, 2.71, and 42.1, and ${}_{b}\hat{R}_{0.95B}$ values were 1.52, 56.1, and 10.9 for blood, EBC and urine, respectively. IL-4 is also interesting in that it is one of two cytokines in blood that had more estimated within-person variance than between-person variance. IL-2 is a more extreme example of large differences in FRs having ${}_{Y}\hat{R}_{0.95B}$ for blood, EBC and urine of 34.9, 1280, and 12.5, respectively.

3.3 Observations across biological media show that the estimated mean ICCs for the blood and EBC samples are 0.547 and 0.867, indicating that the majority of the variance in cytokine concentrations for these two biological media was observed between-people. Alternatively, the mean ICC across the urinary cytokines was 0.415 (i.e. more within-person than between-person variance). The results do not show a consistent trend across cytokines (i.e. an increase/decrease in within- or between- person variance) and media. This demonstrates that despite an overall across-media trend (more between-person variance for blood and EBC and within-person variance for urine), the actual source of variation is dependent not only on the medium but also

441 on the specific cytokine. These ICC results, and the accompanying variance results that
442 comprise the ICC, are consistent with the fold range result.
443 **3.4** The next step in this analysis was to investigate the relationship between the ten cytokines in
444 time-matched blood and EBC samples (n=77). We did not include urine samples in this analysis
445 due to the low numbers (n=18) of matches. These results show that all of the EBC cytokines,
446 except IL-1β, are positively correlated with one another, and that the blood samples have a mix
447 of positive and negative correlations (**Figure 2**). The Blood/EBC correlations have positive and
448 statistically significant correlations for most cytokines, except IL-1β, IL-4, IL-5, and IL-10.
449 Correlation coefficients ranged from -0.620 to 0.836 for the blood/blood comparisons, -0.045 to
450 0.852 for the EBC/EBC comparisons, and -0.236 to 0.600 for the blood/EBC comparisons. The
451 intensity of the colors in Figure 2 (i.e. the movement toward solid red for positive correlations
452 and solid blue for negative correlations) shows that there are stronger relationships within a
453 single media when compared to the relationships between the two media.
454 **Figure 2:** Cytokine Correlations (Spearman "Rho") between Blood Plasma and Exhaled Breath
455 Condensate.



* A white dot in a cell denotes a statistically significant ($p<0.05$) positive or negative correlation.

DISCUSSION

In this study, the main goal was to assess the feasibility of using an existing analytical method to identify target biomarkers of interest, simultaneously, in three different biological media. To our knowledge, this is the first time that blood, EBC, and urine samples from a healthy adult population have been analyzed using a Th1/Th2 cytokine suite. This work demonstrates a number of characteristics of human cytokine measurement and variability that have been previously unknown, and also extends the trace-level measurements further to encompass EBC and urine samples as a complement to blood/plasma data. These new observations can be categorized as follows:

Calibration

During initial tests, the ostensible calibration range of the instrument needed to be adjusted to address the low concentrations that were found in nominally healthy subjects. The instrumentation provides signal "count" numbers beyond the internal calibration default values, and so a set of equations were developed that could be used to interpolate such results back to a new LLOQ. The 5-parameter logistic model was chosen for these concentrations calculations primarily based on the range and shape (log-normally distributed) of the data, and the ability the model to interpolate low-end signals, averaging 8.85% more samples across the three biological media. Experience with the 5pl model showed that the LLOQ could be decreased even lower using calibration strategies (based on trial and error) wherein individual cytokines were tested at vanishingly low concentrations to assess the empirical quantitation limits.

Sensitivity and Method Precision

Our study results showed that the methodology based on the MSD immunochemistry instrumentation is well suited for measuring cytokines in three different biological media (plasma, EBC, and urine) at the extremely low concentrations expected in samples from the general population. This is particularly important for levels in exhaled breath condensate and urine, as these biological fluids represent "non-invasive" sampling pathways (in contrast to blood collection) and so are attractive for public health surveillance. The MSD instrument can achieve robust detection levels better than the nominal design criteria and the results show that the sensitivity requirements for the instrumentation are in the 0.01 ρg/ml range (see Tables 4a, b, and c). However, at some point the signal can no longer be considered distinct from noise and the experimental results usually had some measurements that were labeled "below LLOQ".

As shown in **Table 2**, the majority of the within- and between-plate variance for the spiked calibration samples lies within a plate, as such, any between-plate variance can be attributed to random error. This result was expected; the internal calibration standards on each

plate adjusted the samples accordingly so that samples analyzed on different 96-well plates could be compared to one another. The next step in the analysis, the introduction of the three biological media through the duplicate sample analysis, showed that in most cases there was an increase in variability. Once again, this was an expected outcome because the addition of a complex biological matrix often degrades measurement specificity and precision. The variation and fold-ranges remained similar to that of the lowest spiked concentration in Section 2.1, thus it was concluded that blood, EBC, and urine could be analyzed effectively and with the same amount of measurement variation as the "best case" scenario. In summary, these results showed that the method had the necessary sensitivity and precision to analyze "real world" samples from three different biological media.

Study Sample Cytokine Measurement Distributions and Correlations

As seen in Table 4a, b, and c, the MSD instrument is capable of developing cross-media cytokine data from nominally healthy human subjects. This is an important result because most previous work studied unhealthy patients where the inflammatory markers were already expected to be at high levels. Having access to robust "control" level statistics is necessary for the discovery of *in vivo* perturbations from the status quo. Furthermore, this allows us to make robust statistical comparisons from case-control studies without having to compare to "0", but to defensible low numbers for the control groups.

Given the available blood, EBC, and urine data, the final step of this analysis was to investigate intra-class and Spearman correlations between the different cytokines and across the three biological media. The expectation was that the majority of the ICCs would show more between-person variation than within-person variation, given that people live in their own "micro-environments" where they have their own exposures from food, water, air, behaviors, etc., and genetics that are different from one another. However, this was not the case for all

514 cytokines and all three media. The plasma results showed that the variance in the expression of
515 IL-4 and IL-8 are seemingly independent of the individual (i.e. everyone in the "group" looks
516 similar to one another) and all urinary cytokines except for IL-5 and IL-8 displayed this same
517 trend. There is an opposite trend for the remaining cytokines in blood and all ten cytokines in
518 EBC, where the results indicate that any variance is dependent on the individual. From these
519 results it is notable that the choice of biological medium plays a large role in the ICC, as
520 evidenced by the blood/EBC difference for IL-4 (0.043 and 0.942 for blood and breath,
521 respectively) and the EBC/urine difference for IL-4 (0.942 and 0.290 for EBC and urine,
522 respectively). It is also evident that individual differences, whether genetic, lifestyle, etc., likely
523 play an important role in these intra-class correlations.
524 Within a single medium, the pro-inflammatory cytokines (IL-1β, IL-2, IL-8, IL-12p70,
525 IFN- γ, and TNF- α) should have positive Spearman correlations with themselves and negative
526 Spearman correlations with the anti-inflammatory cytokines (IL-4, IL-5, IL-10, and IL-13).
527 However, this was not the case for IL-1β for the within-media EBC comparison in Figure 2.
528 This cytokine has a negative correlation with all other cytokines, including the cytokines in their
529 respective "inflammatory" classification. All other EBC cytokines co-vary sufficiently to
530 essentially provide the same information about a particular subject. However, the blood and
531 EBC cytokine correlations are mixed and seemingly do not show a pro/anti inflammatory or a
532 Th1 (IL-1β, IL-2, IL-12, IFN- γ, and TNF- α)/Th2 (IL-4, IL-5, IL-10, IL-13) "pattern". These
533 results could be indicative of a differential response/expression of cytokines by the individual
534 subjects in our study, but investigating these specific relationships is beyond the scope of this
535 paper.

The second overall outcome of the study showed that cytokine measurements in different media do not tell the same inflammatory story. Although this result was anticipated based on the biological media and expected location of inflammation, for example urinary cytokines are likely more closely linked to kidney/bladder function and EBC cytokines are more likely linked to respiratory inflammation, there was one noteworthy trend. Four of the five pro-inflammatory cytokines in blood had statistically significant correlations for the majority of the blood/EBC comparisons (Figure 2), showing that as they are expressed in one medium, they are distributed, or also expressed, in the other medium. It is expected that with further investigation these relationships can be solidified and the use of blood as a "gold standard" could shift in the direction of less-invasive breath collection techniques, even when trying to look at inflammation that is not respiratory/pulmonary related.

To note, these results were interpreted solely based on biological medium classification and not on the Th1/Th2 designation or an anti/pro inflammatory profile. This analysis has inherent limitations for assigning concrete relationships based on the progenitor cell and inflammatory classifications. The only goal here is to illustrate the within or between person variation for these cytokines and the three biological media; a more comprehensive analysis of the ten cytokines in this panel could potentially produce a pattern related to the Th1/Th2 profile or anti/pro inflammatory profile, but this is beyond the scope of this work.

Summary and Conclusions:

These results demonstrate that the method is sufficiently robust to address the complexity of matrix inferences that are often seen when searching for the same biomarker in three different biological media and that the cytokines measured in this study can be identified and quantified at ultra-trace levels. Furthermore, despite the fact that cytokines are often highly correlated within matrices, the between matrix results can be quite different. The presented data can be used to

guide future studies, and perhaps streamline new work, by focusing only on a few cytokines wherein the combination of sensitivity and correlation give optimal results at lower cost, or that a less invasive sampling medium (EBC or urine) could be substituted for blood collection. This is especially valuable for targeted studies wherein a pre-existing hypothesis is to be tested and there may be no need to measure a 10-plex suite when two or three cytokines with the best range, sensitivity, and precision would produce equivalent results.

Disclaimer

The United States Environmental Protection Agency through its Office of Research and Development has subjected this article to Agency administrative review and approved it for publication.

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