Multi-plex analysis of inflammatory cytokines in human blood, breath condensate, and urine matrices

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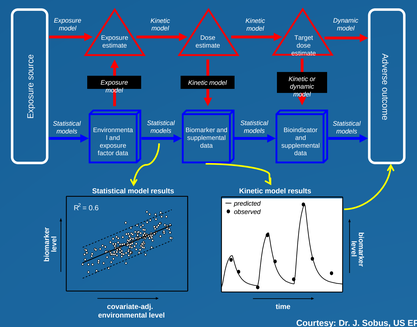
Abstract

Scientific evidence suggests that inflammation is associated with human health effects and health endpoints, yet most studies have focused on human populations that are already considered "unhealthy". As such, it is pertinent to measure inflammatory biomarkers in human biological media with an appropriate sensitivity and specificity for an environmental or low-level exposure. To this end, we used Meso Scale Discovery's (MSD) MUTIL-SPOT Human T_H1/T_H2 Cytokine10-Plex Assay Ultra-Sensitive Kit (Gaithersburg, MD) to evaluate the use of this methodology for the investigation of interleukins (1β, 2, 4, 5, 8, 10, 12p70 and 13), IFN- γ, and tumor necrosis factor-α (TNF- α) in human plasma, exhaled breath condensate and urine. We have found that the MSD instrumentation and the T_H1/T_H2 cytokine 10-plex method have sufficient sensitivity and specificity for assessing the target biomarkers in a nominally healthy population. The ten cytokines in the panel had limits of detection ranging from 0.054-2.3 pg/mL, method blank coefficient of variations (CVs) that remained below 10% for >98% of the results, and average between and within plate CVs of 17.3 and 14.6, respectively. Our results demonstrate that the methodology performs suitably to identify low-level inflammatory responses in human blood, exhaled breath condensate and urine in "normal" healthy subjects.

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 extremely low concentrations of probative chemicals.**
- **Observing such responses or perturbations in human systems biology is extremely important as they provide direct empirical evidence of the initiation or progression of an adverse outcome pathway (AOP) understanding the pathways and their initiating events is of critical value in ultimately preventing disease.**
- **It is valuable to develop methods that can quantitatively measure inflammatory biomarkers in human biological media with an appropriate sensitivity and specificity for assessing environmental or low-level exposures that would serve as preclinical indicators of disease risk along the pathway from inflammation to ultimate health endpoints.**
- **Having an early set of indicators will help the public health and medical communities to intervene before irreversible disease states are reached.**



Courtesy: Dr. J. Sobus, US EPA

Methods

Sample Collection

Blood Plasma

- **Blood samples were collected over four, three-day periods, from healthy human volunteers in 10mL Vacutainer® (Becton, Dickinson and Company, Franklin Lakes, New Jersey) collection tubes containing EDTA.**
- **Tubes were centrifuged at ≤1300 RCF, the separated blood fractions were aliquotted into individual 2mL polypropylene storage vials, and then frozen at ≤-80° C until analysis.**

Exhaled Breath Condensate

- **Exhaled breath condensate (EBC) samples were collected using a RTube™ (Respiratory Research, Inc., Austin, Texas) exhaled breath condensate collector.**
- **Participants were asked to breathe for 10 minutes into the RTube using a normal breathing pattern.**
- **After collection, the RTube was removed from the sleeve and allowed to thaw. The volume of the sample was measured, placed in a 2mL polypropylene storage vial, and then frozen at ≤-80° C until analysis.**

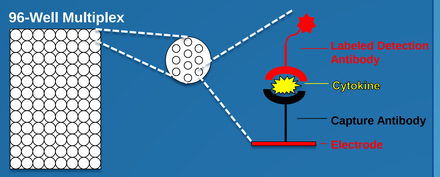
Urine

- **Urine samples were collected over four, three-day periods, with each period being separated by 10 days.**
- **Participants were provided with portable thermoelectric coolers containing temperature data loggers (Easy Log EL-USB-LITE or EL-USB-1, Lascar Electronic, Ltd) to store their urine samples.**
- **Every urine void over each three-day period for the respective study participant was collected in an individual 1L polypropylene container.**
- **Each sample was aliquotted into 8mL polypropylene vials and frozen at ≤-80° C as they were received at the EPA Humans Studies Facility.**

Analysis

- **The ten cytokines of interest to this study were analyzed using a multiplex electrochemiluminescent immunoassay system and a Meso Scale Discovery (MSD) SECTOR Imager 2400 (Meso Scale Discovery, Gaithersburg, MD).**
- **The panel, Human T_H1/T_H2 10-plex Ultra-Sensitive Kit, was designed by MSD to analyze the following human biomarkers, per well: interleukins (1β, 2, 4, 5, 8, 10, 12p70 and 13), IFN- γ, and TNF- α.**

96-Well Multiplex



All biological specimens were collected after informed consent from healthy human adult volunteers at the U.S. EPA Human Studies Facility (HSF) in Chapel Hill, NC. 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 (IRB Study #: 09-1344).

Results

Objective 1

Can the method detect and adequately quantitate our cytokines of interest in plasma, exhaled breath condensate, and urine?

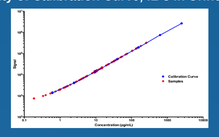
Are modifications necessary to increase detections?

Percentage of Samples Greater than the LOQ

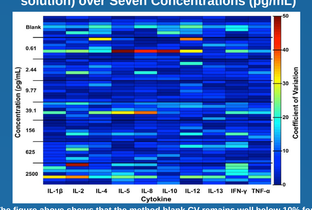
Cytokine	Blood[1]	EBC[2]	Urine[3]	EBC-W/O[4]	EBC-W/[5]
IL-1β	77.5	87.0	92.6	42.9	57.1
IL-2	92.5	95.7	100	28.6	85.7
IL-4	45.0	85.9	37.0	100	100
IL-5	85.0	85.9	81.5	85.7	100
IL-8	95.0	92.4	88.9	100	85.7
IL-10	90.0	83.7	92.6	100	100
IL-12	90.0	90.2	96.3	57.1	28.6
IL-13	55.0	7.60	44.4	42.9	71.4
IFN-γ	80.0	83.7	96.3	100	85.7
TNF-α	97.5	88.0	100	85.7	71.4

1:n=40, 2:n=40, 3:n=27, 4:n=10 samples without carrier, 5:n=10 samples with carrier

Linearity of Calibration Curve, IL-8 in Urine Samples



Coefficient of Variations for Method Blanks(PBS/BSA solution) over Seven Concentrations (pg/mL)



The figure above shows that the method blank CV remains well below 10% for each cytokine, and at every concentration, for >98% of the results

Coefficient of Variations for "Real World" Samples*

	Plasma		Exhaled Breath Condensate		Urine	
Cytokine	Between Plate[1]	Within Plate[2]	Between Plate[1]	Within Plate[2]	Between Plate[1]	Within Plate[2]
IL-1β	20.0	19.5	18.7	17.6	31.4	10.2
IL-2	15.8	14.6	19.1	25.3	16.4	10.8
IL-4	25.7	10.1	24	12	29.7	11.8
IL-5	18.1	10.7	18.3	17.3	8.37	7.76
IL-8	11.9	21.7	45.7	21.2	21	10.6
IL-10	3.20	24.2	8.88	9.03	26.8	9.29
IL-12 p70	15.8	20.4	11.5	10.7	8.14	8.27
IL-13	14.2	20.1	12.9	13.2	24.1	9.45
IFN-γ	14.9	49.1	13.4	16.6	10.8	8.35
TNF-α	19.0	34	28.3	25.9	10.1	12.5
Average	15.9	22.4	20.1	16.9	18.7	9.90

1:n=25, 2:n=16, 3:n=26, 4:n=17, 5:n=17, 6:n=20

Objective 2

What are the methodological parameters of each cytokine for our "real world" samples?

Descriptive Statistics (pg/mL) and Select Percentiles for Cytokines in Blood Plasma (n=291)

Cytokine	%<LOQ	Min	5%	25%	50%	75%	95%	Max
TNF-α	0	0.146	1.18	1.43	1.69	2.50	4.30	8.77
IL-8	0.344	<LOQ	1.14	1.54	2.01	2.65	4.04	41.7
IL-12 p70	1.03	<LOQ	0.203	0.264	0.345	0.560	1.27	1.81
IL-10	2.06	<LOQ	0.464	0.699	1.239	1.644	2.55	7.21
IL-5	6.53	<LOQ	<LOQ	0.115	0.211	0.346	0.689	20.4
IL-4	8.59	<LOQ	<LOQ	0.229	0.284	0.373	2.033	2.89
IFN-γ	22.0	<LOQ	<LOQ	0.0841	0.279	0.540	1.07	4.55
IL-13	27.1	<LOQ	<LOQ	<LOQ	1.56	2.09	3.01	4.38
IL-1β	27.5	<LOQ	<LOQ	<LOQ	0.248	1.59	3.06	40.3
IL-2	29.9	<LOQ	<LOQ	<LOQ	0.0912	0.165	0.342	1.04

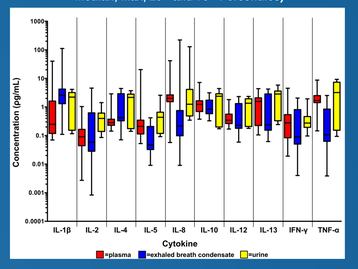
Descriptive Statistics (pg/mL) and Select Percentiles for Cytokines in EBC (n=253)

Cytokine	%<LOQ	Min	5%	25%	50%	75%	95%	Max
IL-4	4.35	<LOQ	0.256	0.331	0.423	2.91	3.81	4.45
IL-10	10.7	<LOQ	<LOQ	0.584	0.839	1.72	2.28	3.15
IL-8	11.9	<LOQ	<LOQ	0.100	0.218	0.748	2.40	222
IL-1β	20.6	<LOQ	<LOQ	1.32	2.58	4.23	10.6	112
TNF-α	20.9	<LOQ	<LOQ	0.0632	0.107	0.842	1.26	2.53
IFN-γ	22.9	<LOQ	<LOQ	0.0513	0.0912	0.789	1.48	2.06
IL-12 p70	28.1	<LOQ	<LOQ	<LOQ	0.231	1.31	1.88	2.33
IL-5	28.9	<LOQ	<LOQ	<LOQ	0.0469	0.208	0.317	0.420
IL-13	30.0	<LOQ	<LOQ	<LOQ	0.237	2.86	3.59	4.26
IL-2	30.4	<LOQ	<LOQ	<LOQ	0.0595	0.621	1.11	4.59

Descriptive Statistics (pg/mL) and Select Percentiles for Cytokines in Urine (n=46)

Cytokine	%<LOQ	Min	5%	25%	50%	75%	95%	Max
IL-8	0	0.346	0.376	0.491	1.25	4.10	12.92	129
IL-1β	2.17	<LOQ	0.130	0.157	2.22	3.20	3.65	4.15
TNF-α	6.52	<LOQ	<LOQ	0.155	3.20	6.97	8.62	9.31
IL-4	8.70	<LOQ	<LOQ	0.176	2.17	2.79	3.56	3.73
IL-5	15.2	<LOQ	<LOQ	0.120	0.436	0.655	1.06	2.58
IL-2	17.4	<LOQ	<LOQ	0.141	0.399	0.607	0.864	1.43
IL-13	21.7	<LOQ	<LOQ	0.331	2.85	3.60	4.51	5.84
IFN-γ	23.9	<LOQ	<LOQ	0.196	0.274	0.455	0.780	1.93
IL-12 p70	26.1	<LOQ	<LOQ	<LOQ	1.36	1.93	2.14	2.35
IL-10	32.6	<LOQ	<LOQ	<LOQ	2.39	2.90	3.72	4.42

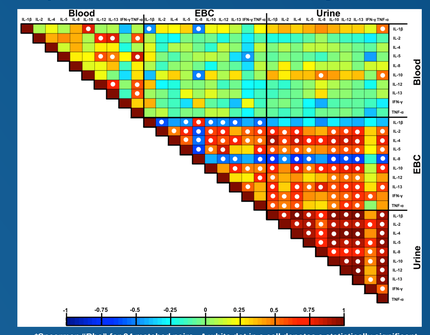
Inflammatory Cytokine Box Plots for 3 Biological Media (Min, Median, Max, 25th and 75th Percentiles)*



Objective 3

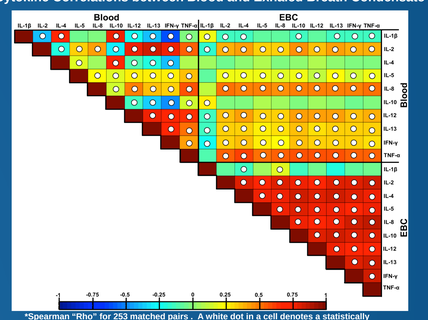
Are there any relationships between the three biological media?

Cytokine Correlations between the Three Biological Media*



***Spearman "Rho" for 24 matched pairs. A white dot in a cell denotes a statistically significant (p<0.05) positive or negative correlation.**

Cytokine Correlations between Blood and Exhaled Breath Condensate*



***Spearman "Rho" for 253 matched pairs . A white dot in a cell denotes a statistically significant (p<0.05) positive or negative correlation.**

Conclusions and Future Work

- We have demonstrated that the MSD instrumentation and the T_H1/T_H2 cytokine 10-plex plates have sufficient sensitivity and specificity for assessing the target biomarkers in a nominally healthy population.
- Our results show that the methodology performs suitably to identify low-level or chronic inflammatory status in human blood, exhaled breath condensate, and urine samples from nominally healthy subjects.
- This work represents the first successful "within- and between-subject" cross-media evaluation of ultra trace-level inflammatory markers.
- We anticipate that the information presented here will lay the groundwork for evaluating the external effects of environmental contaminants on human biology and provide evidence for *in vivo* initiating events that could be implemented for *in vitro*, high-throughput toxicity testing.

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