UC Davis
UC Davis Previously Published Works

Title
Multiplexed measurements of immunomodulator levels in peripheral blood of healthy subjects: Effects of analytical variables based on anticoagulants, age, and gender

Permalink
https://escholarship.org/uc/item/5f4168zr

Journal
Cytometry Part B Clinical Cytometry, 86(6)

ISSN
1552-4949

Authors
Krishnan, VV
Ravindran, Resmi
Wun, Ted
et al.

Publication Date
2014-11-01

DOI
10.1002/cyto.b.21147

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Cytometry Part B (Clinical Cytometry) 86B:426–435 (2014)

Original Article

Multiplexed Measurements of Immunomodulator Levels in Peripheral Blood of Healthy Subjects: Effects of Analytical Variables Based on Anticoagulants, Age, and Gender

V. V. Krishnan,[1,2,3] Resmi Ravindran,[2] Ted Wun,[4,5] Paul A. Luciw,[2,3] Imran H. Khan,[2,3]* and Kim Janatpour[3]

[1]Department of Chemistry, California State University, Fresno, California 93740

[2]Center for Comparative Medicine, University of California Davis, Davis, California 95616

[3]Department of Pathology and Laboratory Medicine, University of California School of Medicine, Davis, California 95616

[4]Department of Internal Medicine, Division of Hematology/Oncology, University of California Davis, Sacramento, California

[5]Section of Hematology and Oncology, Veterans Administration Northern California Health Care System, Sacramento, California

Multiplex microbead immunoassay (MMIA) is a powerful technology for a wide range of biomedical and clinical applications. It is important to study the normal concentration ranges of immunomodulators under different sample preparation conditions and age groups of subjects in order to more precisely determine their reference values for use in assessing alterations of their levels in disease. The aim of this study was to determine the plasma concentrations of immunomodulators (cytokines, chemokines, and growth factors) in the peripheral blood from healthy subjects by the use of a large multiplex panel, and to determine the effects of different anticoagulants, age, and gender on the immunomodulator levels. In addition, the assay precision for these biomarker analytes was determined. Plasma samples from 107 healthy subjects, aged 18 to 85 years, were collected in three different anticoagulants (sodium citrate, EDTA, Heparin); corresponding serum samples were also obtained. Multiplex microbead immunoassays were performed for measuring a total of 23 analytes including chemokines, cytokines, and growth factors (IL-1β, IL-1ra, IL-2, IL-6, IL-7, IL-8, IL-12 p70, IL-17, IFN-γ, IP-10, MCP-1, PDGF-BB, RANTES, TNF-α, IL-1a, IL-16, HGF, MIG, TNF-β, PDGF-ABBB, EGF, Flt-3 Ligand, VEGF). For these analytes, our results showed that the anticoagulant affected the concentration measurements and the coefficients of variation. However, the relative levels of the analytes (profiles) of samples collected in a particular anticoagulant are consistent. The analytes IL-1β, IL-7, Flt-3 Ligand, and IL-12p70 show the largest variation (up to fourfold) between the age groups. In addition, no statistically significant differences in the level of the analytes were found between the sexes. © 2013 International Clinical Cytometry Society

Key terms: multiplex microbead; luminex; cytokines; chemokines; anticoagulant; normal range

V. V. Krishnan and Resmi Ravindran contributed equally to this work.

Present address of Kim Janatpour: Genoptix Medical Laboratory, 2110 Rutherford Road Carlsbad, CA, 92008.

*Correspondence to: Imran H. Khan, Comparative Medicine, University of California Davis and Department of Pathology and Laboratory Medicine, University of California School of Medicine, Davis, CA 95616, USA. E-mail: ihkhan@ucdavis.edu or kjanatpour@hotmail.com

Grant sponsor: Department of Pathology and Laboratory Medicine's Proteomics and Informatics Initiative at UC Davis Medical Center.

Grant sponsor: Department of Pathology Informatics, Initiatives and NIH (to V.V.K.); Grant numbers: P20 MD 002732 and P20 CA 138025.

Grant sponsor: Part of this work was supported by NIH to Dr. Ralph Green, Department of Pathology, UCDMC; Grant number: R01 HL083276.

Grant sponsor: Mentored Clinical Research Training Program, UC Davis CTSC (to K.J.); Grant number: UL1 TR000002.

Received 12 September 2013; Revised 4 November 2013; Accepted 2 December 2013

Published online 10 December 2013 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/cyto.b.21147

© 2013 International Clinical Cytometry Society

How to cite this article: Krishnan VV, Ravindran R, Wun T, Luciw PA, Khan IH, and Janatpour K. Multiplexed Measurements of Immunomodulator Levels in Peripheral Blood of Healthy Subjects: Effects of Analytical Variables Based on Anticoagulants, Age, and Gender. Cytometry Part B 2014; 86B: 426–435.

A wide variety of cytokines and chemokines are important modulators of immune response pathways (1–7). These proteins are critical regulators of normal immune responses, inflammatory reactions and cell proliferation, and play key roles in chronic conditions such as heart disease, diabetes, and cancer, as well as acute conditions, such as infections (8–14).

These immunologic mediators have traditionally been measured using enzyme-linked immunosorbent assay (ELISA) methods. However, these methods are costly, time consuming, requiring a relatively large sample size, and can only measure one analyte per assay kit. The Luminex multiplex immunoassay platform system is a highly efficient fluorescent (or magnetic) bead-based capture/detection sandwich immunoassay that allows for the efficient measurements of multiple analytes simultaneously in a single reaction vessel with small sample volumes (14–19).

In the field of immunoassay diagnostics there are gaps in knowledge that are barriers to full implementation of novel multiplex bead-based assay formats. What is the effect of anticoagulants on the precision and the concentrations of the analytes measured? Does age or sex affect the baseline levels of immune modulators in healthy subjects? The fundamental premise of biomarker identification is based on how discriminatory a specific analyte is for a specific disease or condition compared to a control group: ideally defined as a group of healthy subjects. In addition, the precision of these measurements is critical. Analytical variables, such as sample matrix effects, condition of storage (short or long term), type of specimen (plasma or serum) as well as choice of anticoagulants have been evaluated in the literature (9,10,15,20–22). This includes a recent study by Biancotto et al. (20) which analyzed 72 analytes in serum and plasma from 11 healthy donors as a function of anticoagulants, sodium heparin, acid citrate dextrose and EDTA.

In this study, we evaluated the precision and variation due to sample conditions of 23 serological markers. These include pro-inflammatory and anti-inflammatory cytokines, several receptors, chemokines, growth, and angiogenic factors. We measured the levels of these analytes in 107 healthy volunteers across a broad age range. The larger number of healthy subjects and well-defined subgroups in this study compared to earlier studies and a multivariate analysis approach add value to understanding the effect of analytical variables.

We show significant anticoagulant and age dependent differences in analytes concentrations, but the relative concentrations of the various analytes remain similar for a given anticoagulant. Ranges did not significantly differ between men and women, yet significant differences were noted due to age. These results should inform future studies using multiplex-bead technology to measure levels of these analytes as potential biomarkers for health, and disease states.

MATERIALS AND METHODS

Study Design

Two independent studies were designed: to determine (a) precision of the measured analytes (Fig. 1) and (b) variation in the measured analytes as a function of anticoagulants, sex, and age (Fig. 2). Study subjects were healthy volunteer blood donors, ages of 18 to 85, recruited from Delta Blood Bank (Stockton, CA). The study was IRB approved and all subjects gave signed, informed consent. Twenty milliliters of peripheral venous blood was drawn into vacutainer tubes containing three different anticoagulants (citrate, EDTA, or Heparin) and without anticoagulant (serum) from the tubing used for blood donation. Samples were drawn, centrifuged immediately after blood draw, and plasma/serum was aliquotted and stored at −80°C.

For the precision study (Fig. 1), three samples from each age group (randomly selected two males and one female) were assayed three times in each plate and repeated three times (PL1, PL2, and PL3). This procedure was conducted for each of the samples collected in three anticoagulants and sera, leading to a total of 27 measurements per sample condition.

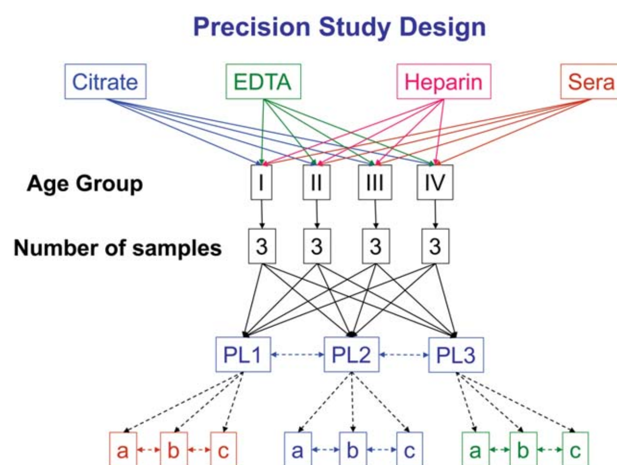

Fig. 1. Study design for the precision of multiplex microbead assay. Data from three healthy subjects from each of the four age groups are repeated in three independent plates, three times each, leading to 27 replicates for each sample and a total of 108 samples per anticoagulants. Age groups I, II, III, and IV are defined to be 18 to 30, 31 to 50, 51 to 64, and 65 to 85 years old, respectively.

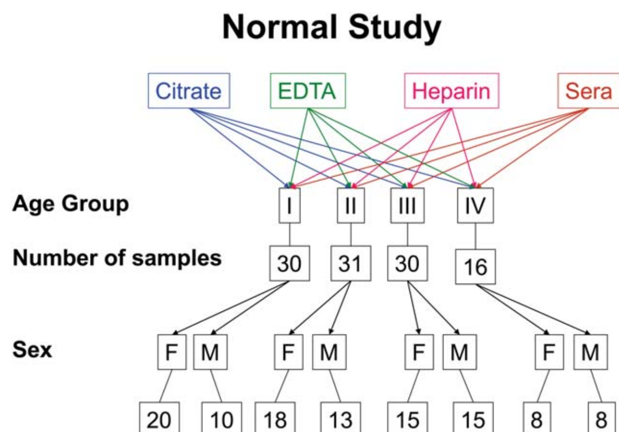

FIG. 2. Study design to determine the effect of anticoagulants and the variation related to age and sex in the multiplex microbead assay. Each age group is represented by approximately 30 samples (except for age group IV) and divided into subgroups according to sex, leading to a total of 107 samples per anticoagulant. Age groups I, II, III, and IV are defined to be 18 to 30, 31 to 50, 51 to 64, and 65 to 85 years old, respectively.

To determine the effect of age, sex and sample collection conditions, 107 individuals were used per preanalytical sample collection (Fig. 2). Four age groups were defined as Group I (age range 18–30, with 20 females and 10 males), Group II (age range 31–50, 18 females and 13 males), Group III (age range 51–64, 15 females and males each), and Group IV (age range 65–85, 8 females and males each). All samples (precision and normal study) were assorted on the same plates sharing the wells to minimize laboratory batch effects.

Experimental Measurements

We measured a total of 23 cytokines, chemokines and growth factors using the Luminex multiplex bead-based technology; (IL-1β, IL-1ra, IL-2, IL-6, IL-7, IL-8, IL-12 p70, IL-17, IFN-γ, IP-10, MCP-1, PDGF-BB, RANTES, TNF-α, IL-1a, IL-16, HGF, MIG, TNF-β) (Bio-Rad, Hercules, CA) and PDGF-ABBB, EGF, Flt-3 Ligand, VEGF (Millipore, Billerica, MA). The assays were performed in accordance with manufacturers' protocols and methods which have been reported previously (23). One plasma sample spiked with the analytes was measured in triplicate and used to determine the instrument coefficient of variation (CV). One sample was also spiked with the standards provided by the assay manufacturer and measured in triplicate at the same conditions and spike recovery was not evaluated.

Concentrations (pg/ml) of different analytes in the samples were determined by generating standard curves with the multiplex assay system. MasterPlex QT software for quantitation was used for five point curves fitting to generate standard curves and analyze data according to the manufacturer's instructions (MiraiBio, Alameda, CA). The analyte concentrations were transformed to log2 scale for further analysis to accommodate the dynamic range in the concentration values.

Statistical Methods

A linear model fit was determined for each analyte using the LIMMA package using R, and lists of analytes with the most evidence of differential levels between the groups (men vs. women; age group, sample collection type) were obtained. Significant analytes were selected by a two-step process. First, the initial data set consisted of measurement of all the analytes for which a signal was detected for at least one feature (e.g., age group) for one subject. Second, the data from all the subjects were combined into a single data set. The resulting combined data set consisted of analytes that exhibited modulation for at least one subject for at least one of the features tested. Differential measurements in patient samples across the age groups points (group I vs. groups II, III, and IV) were detected by an F test. P-values for different analytes were transformed to compensate for multiple comparisons using the False Discovery Rate (FDR) adjustment for multiple comparisons using the Benjamini-Hochberg procedure (24,25). Fold changes were derived from multivariate statistical analysis. This analysis allowed a comparison of more than one statistical variable in an age group and therefore increases the statistical dimensionality of the data to get a meaningful value for fold change and adjusted p-values for multiple comparisons.

Hierarchical cluster analysis was performed using a Euclidean distance metric without standardization to identify natural grouping of analyte profiles among the samples. To adjust for the large dynamic range between different microbead sets, the data were further scaled between the different analytes to give a relative intensity scale ranging from −2 to 2 and the clustered results were presented as dendograms and heat maps. All the analyses were performed with the Bioconductor and R software package (26).

RESULTS

Coefficient of Variation of the Analytes

The first objective of this study was to determine the coefficient of variation (CV) of the analytes. Table 1 lists the measured CVs of all the analytes in the multiplex assay along with that for the spiked sample. The CVs of 17 of the 23 analytes was measureable in the assay, with the exception of IL-8, IL-17, TNF-β, EGF, and VEGF for all the sample collection types (Table 1). CVs of IL-1β, IL-1ra, IFG-γ, IP-10, MCP-1, PDGF-BB, IL-16, HGF, MIG, and PDGF-ABBB were measurable in all the sample conditions. Citrate sample showed CVs ranging from 4.6% (MCP1) —25.1% (RANTES); EDTA samples showed CVs ranging from 4.7% (PDGF-ABBB)—21.2% (IL-6); heparin had a range of 6.5% (MIG)—26.7% (IL-1a) and serum samples had a range of 6.1% (MCP-1)—56.2% (IL-1ra). Samples collected in heparin showed generally higher CVs than the other sample types. The CV values of the raw MFI data (before conversion to concentration via standard curves) are in the same range as that calculated based on concentrations. The spiked sample had a range

Table 1
Coefficient of Variation of all the Measured Analytes

Proteins	Citrate (%)	EDTA (%)	Heparin (%)	Serum (%)	Spiked (%)
IL-1β	8.2	11.2	17.0	8.0	6.0
IL-1ra	12.6	16.0	20.8	13.3	4.7
IL-2	13.7	12.6	NA	12.6	3.6
IL-6	7.8	21.2	NA	10.6	4.5
IL-7	7.6	11.6	NA	9.9	4.4
IL-8	NA		NA	NA	3.4
IL-12 p70	12.7	11.4	NA	13.2	3.6
IL-17	NA	NA	NA	NA	5.9
IFN-γ	5.5	10.5	16.4	10.3	3.2
IP-10	5.3	6.9	7.8	6.4	2.8
MCP-1	4.6	7.0	7.4	6.1	4.9
PDGF-BB	6.1	10.5	12.5	11.3	2.8
RANTES	25.1	NA	NA	NA	24.0
TNF-α	7.8	12.8	NA	10.2	5.3
IL-1a	NA	NA	26.7	56.2	2.2
IL-16	9.8	12.9	6.8	9.1	4.5
HGF	8.8	11.5	8.2	6.5	3.9
MIG	7.1	9.9	6.5	7.2	1.7
TNF-β	NA	NA	NA	NA	6.6
PDGF-ABBB	5.6	4.7	8.1	6.2	4.5
EGF	NA	NA	NA	NA	4.2
Flt-3 Ligand	12.2	NA	NA	NA	3.9
VEGF	NA	NA	NA	NA	NA

NA: not available due to measurements below the limit of detection.

of 1.7% to 6.6% for the 22 analytes, except for RANTES with a CV of 24%.

Analyte Concentrations

Twenty-two of the 23 analytes were detected across all the samples and in all age groups (Table 2). IFN-γ was the lowest detected (in average of 30% samples across all age groups) and therefore was not used in further analysis. IL-12p70, MCP-1, TNF-α, HGF, MIG, TNF-β, PDGF-ABBB, and VEGF were measured 100% of the time, and except for IL-1β (68% average), the remainder of the analytes were detected in >75% of all subjects. The concentrations varied considerably among the analytes (Fig. 1, Table 2) and this observation is consistent with the ranges measured in other studies (20). With reference to the mean value of a particular analyte across the different sample conditions, (age group differences vide supra), IL-2, EGF, and IL-6 showed the lowest difference (within the CV) across all the sample collection conditions, whereas PDGF-BB, RANTES, and PDGF-ABBB showed the largest differences. For these three analytes, samples collected in heparin showed the lowest average concentration, whereas the serum had the highest and this trend was observed for other analytes as well (Table 1). For the analytes in the mid-range of concentrations, MIG and IP-10 showed the largest variations, with MIG having average high concentration in both samples collected in heparin and in sera, with much lower values for samples collected in citrate and EDTA.

Age Dependent Modulation of Analytes

Multivariate statistics were used to identify the analytes that showed significant changes in their levels across the age groups. The fold changes were calculated with reference to age group I so as to determine whether a particular analyte changed (either positively or negatively) with respect to the lowest age group. Table 3 lists the analytes that showed significant changes when compared, and Figure 3 highlights the results for IL-1β, IL-7, and TNF-α. Analytes were selected such that the fold changes with respect to group I is 1.5 times greater and a P value <0.05, in at least one of the comparisons (age groups I vs. II or III or IV) in all the sample conditions. The overall trend of the age dependent variations of the cytokines was similar in all sample conditions. IL-1β, IL-7, IL-12p70, and MIG were significantly higher with increase in age, while Flt-3 Ligand was lower. Variations with age were more prominent in the serum samples in comparison with others (Fig. 3, Table 3). It is important to note that none of the analytes showed significant differences between men and women in each age group and in all the sample conditions.

Interaction Between Age of the Subject and Anticoagulant

To determine the overall age dependent variation of all the analytes and the respective trends in the pre-experimental sample conditions, a hierarchical cluster analysis was performed (Fig. 4). The clustering analysis, using all the analytes (except IFN-γ) clearly classified the sample conditions (horizontal dendograms, Fig. 4); heparin and serum samples profiles were different from each other while the profiles in citrate and EDTA were similar. In addition, the analytes group into three major clusters (vertical dendograms, Fig. 4), represented by A, B and C. Analytes in group A (IL-1α, IL-16, MIG, MCP-1,

Table 2
Concentrations (pg/ml) of the Cytokines, Chemokines, and Growth Factors for the Different Age Groups

a. Samples Collected in Sodium Citrate

	Sodium Citrate											
	I			II			III			IV		
Proteins	Mean	SD	%	Mean	SD	%	Mean	SD	%	Mean	SD	%
IL-1β	2.6	1.4	100	5.2	3.0	100	10.5	2.8	100	5.5	0.8	100
IL-1ra	189.3	114.8	100	217.7	74.7	100	275.6	126.1	100	173.9	62.6	100
IL-2	15.5	8.9	100	12.9	9.2	100	15.1	15.3	100	8.9	3.6	100
IL-6	12.1	3.6	100	12.5	3.9	100	15.7	11.5	100	8.4	2.4	100
IL-7	5.2	2.1	100	9.9	4.4	100	14.4	4.7	100	8.0	1.9	100
IL-8	7.3	4.8	97	5.9	3.8	66	4.2	1.9	68	1.0	1.4	25
IL-12 p70	7.6	4.6	94	15.5	6.5	100	21.5	11.0	100	12.2	4.5	100
IL-17	22.4	17.2	58	26.8	18.6	63	18.6	11.0	61	10.0	10.8	38
IFN-γ	223.8	121.3	100	241.5	87.2	100	235.0	94.5	100	158.6	37.8	100
IP-10	510.1	454.6	100	376.6	141.3	100	641.2	521.7	100	753.9	434.5	100
MCP-1	34.7	21.9	100	35.3	7.5	100	42.1	18.3	100	34.1	7.6	100
PDGF-BB	7,975.3	5,509.3	100	4,717.7	2,460.7	100	5,155.8	2,651.1	100	3,566.8	1,035.4	100
RANTES	5,556.6	11,992.9	90	19,565.3	18,223.3	100	14,047.2	12,538.4	100	27,804.1	35,681.9	100
TNF-α	82.6	12.8	100	62.4	32.8	100	82.8	46.4	100	66.8	32.8	100
IL-1a	1.4	0.7	45	1.2	0.5	100	1.1	0.8	74	0.6	0.7	100
IL-16	96.4	44.4	100	120.9	58.9	100	120.6	85.7	100	114.6	26.0	100
HGF	209.2	99.2	100	186.9	50.3	100	243.8	100.2	100	248.8	67.6	100
MIG	346.8	582.3	100	186.2	72.9	100	482.9	344.9	100	639.6	460.9	100
TNF-β	1.2	1.6	45	1.5	0.9	63	2.8	3.8	42	2.3	2.1	44
PDGF-ABBB	1,365.4	626.7	100	990.0	526.2	100	1,038.6	490.1	100	844.8	541.1	100
EGF	57.8	31.1	97	38.1	22.9	100	40.1	22.5	100	33.9	10.5	94
Flt-3 Ligand	139.0	150.8	94	69.7	25.7	97	37.4	30.0	94	43.7	19.6	94
VEGF	20.3	34.7	52	9.7	7.0	19	10.0		3			0

b. Samples Collected in EDTA

	EDTA											
	I			II			III			IV		
Proteins	Mean	SD	%	Mean	SD	%	Mean	SD	%	Mean	SD	%
IL-1β	2.1	1.5	100	4.4	3.6	97	12.1	9.7	100	4.9	2.1	100
IL-1ra	165.1	107.7	100	167.6	98.6	100	211.8	148.8	100	105.9	68.6	100
IL-2	10.5	8.0	100	11.3	11.0	97	20.1	38.8	100	8.1	5.6	94
IL-6	9.5	3.7	100	10.7	4.8	100	18.3	27.2	100	8.4	4.1	100
IL-7	3.1	2.3	94	8.8	4.6	84	12.4	5.6	100	6.2	4.1	100
IL-8	3.8	3.9	94	5.6	4.9	53	4.4	2.3	65	6.0	7.1	13
IL-12 p70	5.1	3.9	77	11.4	7.0	100	19.3	17.0	100	7.4	5.1	100
IL-17	10.4	8.5	39	34.2	28.5	38	15.5	14.3	71	23.7	38.4	19
IFN-γ	179.9	95.2	100	210.7	122.3	100	230.1	124.3	100	133.3	80.6	100
IP-10	553.7	609.2	100	431.6	216.4	100	800.9	696.8	100	1,081.5	780.4	100
MCP-1	28.1	16.1	100	32.0	9.5	100	42.1	27.6	100	31.1	8.6	100
PDGF-BB	4,307.2	3,212.7	100	3,406.8	2,080.0	100	4,814.1	2,879.5	100	3,659.6	3,330.4	100
RANTES	6,041.8	16,109.7	100	27,638.4	36,532.4	100	19,651.0	27,450.9	100	58,770.2	76,641.1	81
TNF-α	69.0	12.3	100	54.3	27.3	97	93.5	72.5	100	70.0	42.9	94
IL-1a	1.1	0.6	45	1.1	0.9	100	1.9	3.4	74	0.6	0.7	100
IL-16	117.0	49.3	100	164.0	114.6	100	181.3	153.9	100	211.9	179.4	100
HGF	239.1	132.7	100	229.2	73.0	100	322.0	155.3	100	338.8	105.7	100
MIG	361.8	625.9	100	225.5	114.4	100	576.5	465.5	100	802.4	565.7	100
TNF-β	1.1	2.2	42	1.6	1.0	63	2.8	3.7	52	2.2	2.4	56
PDGF-ABBB	497.1	321.6	100	386.8	365.7	100	397.7	263.4	100	338.6	282.3	100
EGF	37.1	18.2	100	36.9	24.8	100	48.6	20.0	100	39.6	19.2	100
Flt-3 Ligand	128.0	94.4	94	78.5	31.9	94	38.8	13.6	100	37.7	13.1	100
VEGF	33.7	64.7	97	15.7	9.5	91	25.6	17.4	61	14.5	11.9	50

(Continued)

and TNF-β) were elevated in samples collected in heparin, whereas lowered in both citrate and EDTA collected samples. In contrast, the analytes in group B (RANTES, IP-10, HGF, IL-8, IL-17, and PDGF-BB) were higher in the sera, but relatively low in all the other three conditions. The third larger group of analytes designated as C, showed low levels in the heparin but higher levels in citrate, EDTA, and serum. The effect of

Table 2
(Continued)

c. Samples Collected in Heparin

	Heparin											
	I			II			III			IV		
Proteins	Mean	SD	%	Mean	SD	%	Mean	SD	%	Mean	SD	%
IL-1β	1.4	1.7	100	2.1	2.2	38	6.5	15.0	100	1.5	0.5	100
IL-1ra	128.1	114.3	100	93.6	91.6	100	397.8	1,437.9	65	48.3	39.2	100
IL-2	8.2	11.1	84	13.7	38.3	47	19.4	86.3	74	5.8	6.8	38
IL-6	11.8	7.4	100	7.1	4.6	100	17.3	66.2	97	5.1	2.4	100
IL-7	1.1	0.4	71	1.9	2.3	72	2.1	1.1	100	0.6	0.5	88
IL-8	10.7	6.0	97	8.2	6.8	94	6.9	2.6	87	5.5	3.7	81
IL-12 p70	2.8	2.9	42	5.0	6.1	59	20.6	74.8	81	3.2	4.5	56
IL-17	8.9	11.3	35	20.1	14.4	59	36.6	33.4	87	18.3	18.3	75
IFN-γ	86.8	64.4	100	59.1	42.4	100	148.8	648.9	100	24.9	43.8	100
IP-10	530.0	643.8	100	374.1	230.1	100	708.3	446.8	100	839.2	528.2	100
MCP-1	88.3	54.9	100	101.3	39.7	100	143.2	124.9	100	140.1	81.9	100
PDGF-BB	890.8	823.8	100	923.5	696.0	100	1,487.3	1,230.0	100	714.9	270.4	100
RANTES	9,442.0	5,063.4	65	13,065.5	14,439.9	100	15,903.9	16,320.7	94	12,340.9	8,787.1	75
TNF-α	40.1	15.4	100	24.9	18.2	66	31.6	95.8	87	14.1	9.0	50
IL-1a	1.5	1.1	61	2.3	1.2	100	2.6	2.0	100	7.6	18.1	100
IL-16	243.8	112.7	100	273.3	119.9	100	399.3	243.6	100	502.6	404.7	100
HGF	221.8	126.2	100	258.0	72.6	100	434.4	177.8	100	443.3	102.5	100
MIG	643.4	962.5	100	408.7	191.9	100	1,200.3	744.2	100	1,533.4	1,149.4	100
TNF-β	3.9	4.2	97	4.8	2.6	97	7.7	9.2	100	6.7	6.6	100
PDGF-ABBB	742.2	589.3	97	599.5	590.5	100	924.5	1,277.1	84	450.2	120.9	100
EGF	52.1	47.6	90	27.0	14.5	100	47.6	102.5	97	28.3	36.9	100
Flt-3 Ligand	157.4	198.4	84	64.9	34.6	97	64.6	138.5	90	80.7	44.2	81
VEGF	17.4	14.6	32			0			0			0

d. Serum Samples

	Sera											
	I			II			III			IV		
Proteins	Mean	SD	%	Mean	SD	%	Mean	SD	%	Mean	SD	%
IL-1β	2.9	5.1	100	4.2	3.3	100	9.5	3.8	100	4.6	0.6	100
IL-1ra	200.2	102.6	100	136.8	50.9	100	140.7	223.2	100	70.0	24.3	100
IL-2	16.0	10.5	100	9.6	9.3	97	12.9	25.8	100	6.5	4.1	94
IL-6	12.5	6.0	100	11.1	3.4	100	14.1	22.3	97	7.6	2.4	100
IL-7	6.2	4.0	100	13.1	10.0	97	16.7	6.6	100	10.0	2.8	100
IL-8	17.0	9.4	100	16.6	13.4	100	13.2	7.1	90	6.1	3.6	100
IL-12 p70	4.1	5.3	77	8.6	7.2	100	12.4	18.9	100	4.9	2.3	100
IL-17	37.7	24.4	97	54.7	35.6	97	74.3	45.4	97	39.3	21.4	100
IFN-γ	204.7	90.3	100	211.5	80.8	100	216.7	230.9	100	126.3	35.1	100
IP-10	1,196.8	1,468.6	100	728.3	386.2	100	1,166.5	751.5	100	1,385.7	824.8	100
MCP-1	59.2	36.5	100	66.7	27.0	100	80.5	52.7	100	65.4	25.3	100
PDGF-BB	61,733.4	171,671.9	100	13,481.9	6,975.6	97	17,365.3	11,540.4	100	13,777.4	9,211.9	100
RANTES	21,267.1	9,852.6	39	180,597.9	381,846.0	81	33,016.4	33,560.3	87			0
TNF-α	76.1	13.2	100	50.0	30.8	100	60.1	39.5	97	54.7	20.1	88
IL-1a	1.6	1.0	48	1.4	0.5	100	1.2	0.8	81	1.3	0.8	100
IL-16	146.2	61.6	100	177.4	76.5	100	177.5	103.8	100	152.9	45.1	100
HGF	506.7	208.7	100	535.5	171.7	100	650.7	265.0	100	561.6	143.6	100
MIG	660.7	1,027.9	100	435.8	206.8	100	1,150.2	694.9	100	1,516.9	1,160.0	100
TNF-β	2.1	2.8	81	3.2	1.6	78	3.8	4.5	74	3.6	2.5	63
PDGF-ABBB	2,388.2	1,072.6	100	2,592.3	1,216.8	100	2,840.3	1,639.1	100	2,822.0	1,268.7	100
EGF	42.5	35.8	84	43.6	32.1	94	33.7	32.5	94	25.2	21.9	88
Flt-3 Ligand	111.4	143.7	94	63.8	29.6	84	48.2	52.2	74	59.1	28.0	75
VEGF	14.3	17.5	32	6.4	2.8	25	4.0		6	10.0		6

I, II, III, IV represent age groups 18 to 30, 31 to 50, 51 to 64, and 65 to 85 years old, respectively; SD: standard deviation; %: percentage of samples that have measureable concentrations above the limit of detection.

age on the individual analytes was also sequentially related within the group (in the order I, II, III, and IV for heparin and serum). It is interesting to note that Flt-3 Ligand, which showed an increase in the levels upon aging, did not cluster with any one of the groups.

Table 3
Fold Changes of Selected Analytes Showing Age-Dependent Changes

	Citrate			EDTA		
Analytes	II vs. I	III vs. I	VI vs. I	II vs. I	III vs. I	VI vs. I
IL-1β	(1.54)	(3.12)*	(1.83)	(1.54)	(4.20)*	(1.96)*
IL-7	(1.53)*	(2.36)*	(1.43)	(1.90)*	(3.10)*	(1.44)
Flt-3 Ligand	1.34	2.62*	1.80	1.66	2.69*	2.67*
IL-12p70	(1.74)*	(2.49)*	(1.51)	(1.52)	(2.91)*	(1.20)
MIG	1.30	(1.53)	(2.24)*	1.30	(1.50)	(2.53)*
TNFα	1.58	1.19	1.50	1.50	(1.11)	1.25
IL-17	(1.51)	1.03	2.35	(2.51)	(1.28)	1.70
IL-2	1.49	1.14	1.67	1.33	(1.41)	1.41
IFNγ	(1.01)	(1.12)	1.25	1.04	(1.29)	1.43
IL-1ra	1.01	(1.39)	1.01	1.24	(1.19)	1.75
IL-8	1.20	1.69	3.47	(1.49)	(1.35)	(1.23)

	Heparin			Sera		
Analytes	II vs. I	III vs. I	VI vs. I	II vs. I	III vs. I	VI vs. I
IL-1β	(1.41)	(2.62)*	(1.04)	(1.38)	(3.52)*	(1.82)
IL-7	(1.22)	(1.48)	1.18	(1.59)*	(2.56)*	(1.43)
Flt-3 Ligand	1.86*	3.04*	1.27	1.33	2.04*	1.08
IL-12p70	(1.22)	(1.61)	1.26	(1.31)	(2.31)*	(1.05)
MIG	1.17	(2.01)*	(2.74)*	1.10	(2.02)*	(2.76)*
TNF-α	2.13*	2.56*	2.65*	1.80*	1.41	1.49
IL-17	(3.16)*	(6.31)*	(2.87)	(1.34)	(2.39)*	1.07
IL-2	1.10	3.83*	(1.69)	1.95*	1.44	2.68*
IFNγ	1.40	2.11*	4.22*	1.04	(1.02)	1.61
IL-1ra	1.25	1.40	2.36*	1.57	1.81*	2.75*
IL-8	1.66	1.46	2.28*	1.17	(1.06)	2.67*

I, II, III, IV represent age groups 18 to 30, 31 to 50, 51 to 64, and 65 to 85 years old, respectively. Fold changes within parenthesis represent down regulation, with *representing a P value <0.05 in that comparison (FDR adjusted for multiple comparison).

DISCUSSION

Variation effects on the quantitative determination of the concentration of 23 different peripheral blood analytes of healthy subjects collected in three different anticoagulants and sera were measured. Because the group of individuals was large ($n = 107$), we were able to address additional issues, related to the possible effect that age and sex of the subjects might have on the biomarker analyte levels.

There are complex changes in the immune system associated with age. These changes include reduction in response and modulation in regulatory and immune effector cells leading to changes in expression of various chemokines and cytokines (27–37). Shurin et al. (37) investigated the effect of age on 30 different serum biomarkers involved in pro- and anti-inflammatory responses. These measurements were performed in healthy subjects between 40 and 80 years old. The overall trends in our measurements for experiments performed in sera are similar (Table 2, d).

In a recent study, Biancotto et al. (20) have analyzed the role of anticoagulants (sodium heparin, acid citrate dextrose and EDTA) on the measurement of 72 analytes from 11 healthy donors. A total of 21 analytes overlapped with the 50 different cytokine/chemokine/growth factors analyzed by Biancotto et al. (20), while we have performed an additional two growth factors (PDGF-AABB and Flt-3 Ligand) in this studies. Our results are similar to the results of Biancotto et al. (20) for IL-8, IP-10, and PDGF-BB (more elevated in sera), and MCP-1 and IL-16 (more elevated in heparin). In contrast, VEGF showed significant differences between measurements in serum and all types of plasma, regardless of anticoagulant in the earlier study, we did not find it to be significantly different between the experimental conditions. In this study we have approximately 10 times larger sample size (107 vs. 11) and therefore a much better statistical power is expected.

In this study we have used Luminex multiplex bead-based technology, with the antibody kits purchased from Bio-Rad or Millipore (Materials and Methods). Earlier studies have compared the performance of multiplex microbead assays between various vendors and it was noted that for some analytes the consistency between the vendors is large (23,38–40). Though our conclusions are broadly applicable, specific care must be envisaged when other kits or vendors are used.

A limited number of studies have focused on the effect of anticoagulants used for sample collection in quantitative measurement of cytokine/chemokine levels in healthy subjects. In one of the earliest investigations on this issue, Thavasu et al. (10) suggested that the optimal conditions for blood collection for consistent immunoassay measurements of circulating recombinant cytokines

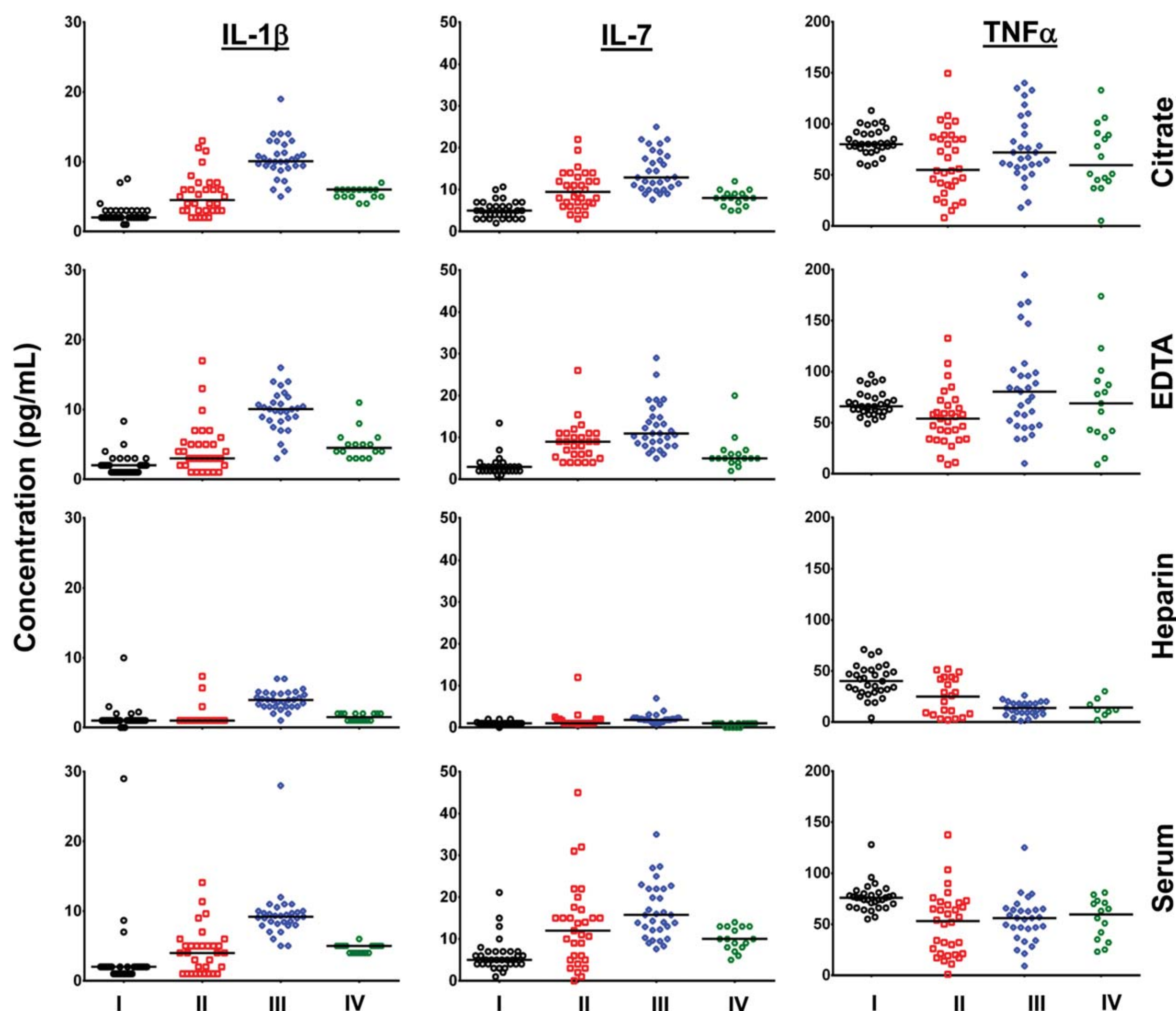

Fig. 3. Age dependent effects on the analytes in healthy subjects. Median dot plots of IL-1β (left panels), IL-7 (central panels), and TNF-α (right panels) grouped into age groups I (18–30 years, black circles), II (31–50 years, red squares), III (51–64 years, blue diamonds), and IV (65–85 years, green hexagons). The choice of anticoagulants (citrate, EDTA, or heparin) and serum is marked on the right. Symbols represent individual measurements in pg/ml and the horizontal bars are the respective median values of each group.

are rapid processing at 4°C of EDTA anticoagulated blood. In a model of porcine blood cells, it was noted that heparin and EDTA differentially affected cytokine mRNA levels (41). In FXa-activated human monocytes, Ben-Hadj-Khalifa et al. noted the anticoagulant effect on production of the anti-inflammatory cytokine IL-10 suggested cross-talk between inflammation and coagulation (42). Preparation of serum includes removal of fibrinogen, platelets, and other circulating proteins from the plasma that could influence the presence or detection of an analyte on such a sample. Effects on coagulation and fibrinolysis appear to be mediated by different cytokines, IL-6 being most relevant for activation of coagulation and TNF playing a pivotal role in the induction of fibrinolytic responses. Also, during the coagulation cascade, cellular activation can release inflammatory mediators, that may affect cytokine levels. The reason for the differences in cytokine concentrations observed between the plasmas collected in different anticoagulants is not known. EDTA and citrate both sequester calcium to prevent coagulation, whereas heparin acts by binding to antithrombin. Cluster analysis (Fig. 4) supports the known facts that the mode of action of citrate and EDTA inhibit anticoagulation are more similarly than heparin or sera.

Cytokines, chemokines, and certain growth factors play an integral role in the regulation, coordination and persistence of the inflammatory process. Knowledge of the level of cytokine and chemokine concentrations in healthy subjects is necessary to determine if levels are aberrant in disease. Furthermore, the choice of cut-off level between "normal" and "elevated" or "decreased" needs to be carefully considered. The development of bead-based multiplex technology has made possible the simultaneous measurement of many molecules in very

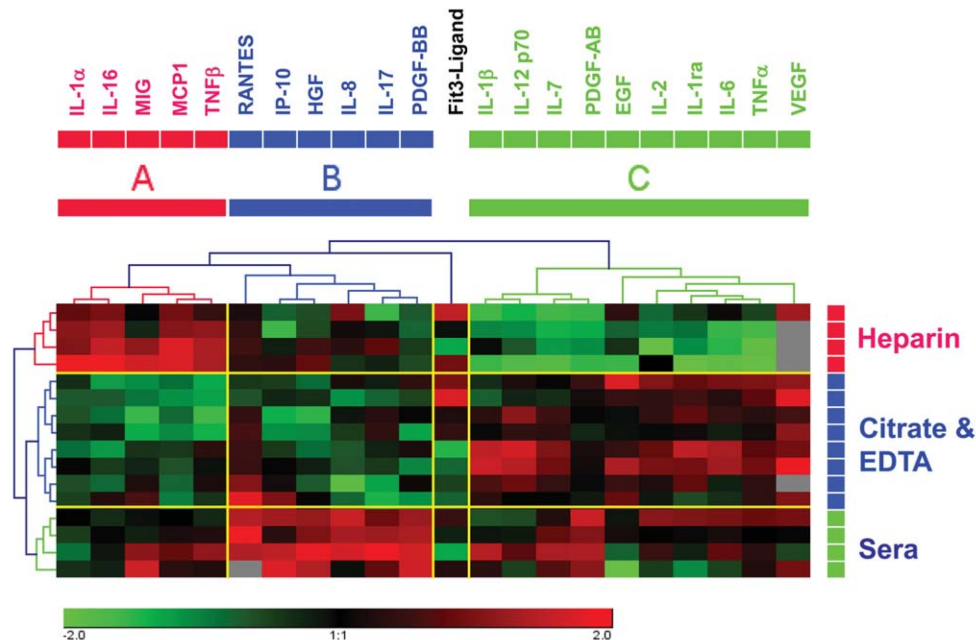

Fig. 4. Hierarchical cluster analysis of the combined effect of age and choice of samples conditions: The clustering processes generated natural groupings sample conditions in the form of dendograms (left color coded) and are presented as heat maps. Intensity scales are shown in arbitrary units shown by the scale at the bottom of the panel ranging from green to red in a relative scale. Names of analytes above each heat map represent various analytes and those on the right (color coded) represent sample conditions. The analytes are grouped into three clusters based on the profiles and the sample conditions are grouped together as well.

small amounts of sample. With the use of this technology, sample pooling can be avoided and individual information from each subject can be obtained. The utility of these measurements will depend on rigorous attention to detail in the collection and processing of samples in addition to assay precision. Our results emphasize the need to consider experimental design carefully when a study requires measurement of biomarkers in the peripheral blood. Cross comparison of levels or profiles of biomarker analytes performed in different anticoagulants or a combination of plasma and serum should be avoided. MMIA has the advantages in terms of efficiency of performance, precision and accuracy to measure multiple analytes, on par with ELISA. Though individual research groups might have a choice of anticoagulant, our group finds that EDTA provides consistent results across multitude of applications.

ACKNOWLEDGMENTS

The authors thank Dr. R. Green and Dr. J. Miller for many discussions. Thanks to Daniel Vang for technical assistance. Thanks to Dr. Spindler, the staff and blood donors at Delta Blood Bank, Stockton CA. The work was performed under UC Davis Medical Center IRB, "Analysis of soluble blood analytes in blood donors" (# 200614863).

LITERATURE CITED

1. Finkelman FD, Shea-Donohue T, Goldhill J, Sullivan CA, Morris SC, Madden KB, Gause WC, Urban JF Jr. Cytokine regulation of host defense against parasitic gastrointestinal nematodes: Lessons from studies with rodent models. Annu Rev Immunol 1997;15:505–533.
2. Hutchinson KL, Villinger F, Miranda ME, Ksiazek TG, Peters CJ, Rollin PE. Multiplex analysis of cytokines in the blood of cynomolgus macaques naturally infected with Ebola virus (Reston serotype). J Med Virol 2001;65:561–566.
3. Strieter RM, Belperio JA, Keane MP. Cytokines in innate host defense in the lung. J Clin Invest 2002;109:699–705.
4. White ES, Strieter RM, Arenberg DA. Chemokines as therapeutic targets in non-small cell lung cancer. Curr Med Chem Anticancer Agents 2002;2:403–417.
5. Holtzman MJ, Byers DE, Benoit LA, Battaile JT, You Y, Agapov E, Park C, Grayson MH, Kim EY, Patel AC. Immune pathways for translating viral infection into chronic airway disease. Adv Immunol 2009;102:245–276.
6. Wong MM, Fish EN. Chemokines: Attractive mediators of the immune response. Semin Immunol 2003;15:5–14.
7. Zeytun A, Chaudhary A, Pardington P, Cary R, Gupta G. Induction of cytokines and chemokines by Toll-like receptor signaling: strategies for control of inflammation. Crit Rev Immunol 2010;30:53–67.
8. Gu Y, Zeleniuch-Jacquotte A, Linkov F, Koenig KL, Liu M, Velikokhatnaya L, Shore RE, Marrangoni A, Toniolo P, Lokshin AE, et al. Reproducibility of serum cytokines and growth factors. Cytokine 2009;45:44–49.
9. Elshal MF, McCoy JP. Multiplex bead array assays: Performance evaluation and comparison of sensitivity to ELISA. Methods 2006;38:317–323.
10. Thavasu PW, Longhurst S, Joel SP, Slevin ML, Balkwill FR. Measuring cytokine levels in blood. Importance of anticoagulants, processing, and storage conditions. J Immunol Methods 1992;153:115–124.
11. Khan IH, Ravindran R, Krishnan VV, Awan IN, Rizvi SK, Saqib MA, Shahzad MI, Tahseen S, Ireton G, Goulding CW, et al. Plasma antibody profiles as diagnostic biomarkers for tuberculosis. Clin Vaccine Immunol 2011;18:2148–2153.
12. Campbell M, Lie WR, Zhao J, Hayes D, Mistry J, Kung HJ, Luciw PA, Khan IH. Multiplex analysis of Src family kinase signaling by microbead suspension arrays. Assay Drug Dev Technol 2010;8:488–496.
13. Ravindran R, Khan IH, Krishnan VV, Ziman M, Kendall LV, Frasier JM, Bates R, Griffey SM, Fahey JR, Luciw PA. Validation of multiplex microbead immunoassay for simultaneous serodetection of multiple infectious agents in laboratory mouse. J Immunol Methods 2010; 363:51–59.

14. Krishhan VV, Khan IH, Luciw PA. Multiplexed microbead immunoassays by flow cytometry for molecular profiling: Basic concepts and proteomics applications. Crit Rev Biotechnol 2009;29:29–43.
15. Skogstrand K, Thorsen P, Norgaard-Pedersen B, Schendel DE, Sorensen LC, Hougaard DM. Simultaneous measurement of 25 inflammatory markers and neurotrophins in neonatal dried blood spots by immunoassay with xMAP technology. Clin Chem 2005;51:1854–1866.
16. Wong HL, Pfeiffer RM, Fears TR, Vermeulen R, Ji S, Rabkin CS. Reproducibility and correlations of multiplex cytokine levels in asymptomatic persons. Cancer Epidemiol Biomarkers Prev 2008;17:3450–3456.
17. Skogstrand K. Multiplex assays of inflammatory markers, a description of methods and discussion of precautions—Our experience through the last ten years. Methods 2012;56:204–212.
18. Dupuy AM, Kuster N, Lizard G, Ragot K, Lehmann S, Gallix B, Cristol JP. Performance evaluation of human cytokines profiles obtained by various multiplexed-based technologies underlines a need for standardization. Clin Chem Lab Med 2013:1–9.
19. Belabani C, Rajasekharan S, Poupon V, Johnson T, Bar-Or A. A condensed performance-validation strategy for multiplex detection kits used in studies of human clinical samples. J Immunol Methods 2013;387:1–10.
20. Biancotto A, Feng X, Langweiler M, Young NS, McCoy JP. Effect of anticoagulants on multiplexed measurement of cytokine/chemokines in healthy subjects. Cytokine 2012;60:438–446.
21. Ray CA, Bowsher RR, Smith WC, Devanarayan V, Willey MB, Brandt JT, Dean RA. Development, validation, and implementation of a multiplex immunoassay for the simultaneous determination of five cytokines in human serum. J Pharm Biomed Anal 2005;36:1037–1044.
22. Hosnijeh FS, Krop EJ, Portengen L, Rabkin CS, Linseisen J, Vineis P, Vermeulen R. Stability and reproducibility of simultaneously detected plasma and serum cytokine levels in asymptomatic subjects. Biomarkers 2010;15:140–148.
23. Khan IH, Krishnan VV, Ziman M, Janatpour K, Wun T, Luciw PA, Tuscano J. A comparison of multiplex suspension array large-panel kits for profiling cytokines and chemokines in rheumatoid arthritis patients. Cytometry Part B Clin Cytom 2009;76B:159–168.
24. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. Behav Brain Res 2001;125:279–284.
25. Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Stat Soc Ser 1995;57:289–300.
26. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 2004;5:R80.
27. Gomez CR, Nomellini V, Faunce DE, Kovacs EJ. Innate immunity and aging. Exp Gerontol 2008;43:718–728.
28. Nomellini V, Gomez CR, Kovacs EJ. Aging and impairment of innate immunity. Contrib Microbiol 2008;15:188–205.
29. Solana R, Pawelec G, Tarazona R. Aging and innate immunity. Immunity 2006;24:491–494.
30. Albright JW, Bream JH, Bere EW, Young HA, Winkler-Pickett R, Ortaldo JR. Aging of innate immunity: functional comparisons of NK/LAK cells obtained from bulk cultures of young and aged mouse spleen cells in high concentrations of interleukin-2. Exp Gerontol 2004;39:73–82.
31. Linton PJ, Dorshkind K. Age-related changes in lymphocyte development and function. Nat Immunol 2004;5:133–139.
32. Lloberas J, Celada A. Effect of aging on macrophage function. Exp Gerontol 2002;37:1325–1331.
33. Valente SA, Fallon WF Jr, Alexander TS, Tomas ER, Evancho-Chapman MM, Schmidt SP, Gorski R, Pizov O, DeFine L, Clark AJ. Immunologic function in the elderly after injury–The neutrophil and innate immunity. J Trauma 2009;67:968–974.
34. Schroder AK, Rink L. Neutrophil immunity of the elderly. Mech Ageing Dev 2003;124:419–425.
35. Bruunsgaard H, Pedersen M, Pedersen BK. Aging and proinflammatory cytokines. Curr Opin Hematol 2001;8:131–136.
36. Marik PE, Zaloga GP. The effect of aging on circulating levels of proinflammatory cytokines during septic shock. Norasept II Study Investigators. J Am Geriatr Soc 2001;49:5–9.
37. Shurin GV, Yurkovetsky ZR, Chatta GS, Tourkova IL, Shurin MR, Lokshin AE. Dynamic alteration of soluble serum biomarkers in healthy aging. Cytokine 2007;39:123–129.
38. Colombo MB, Haworth SE, Poli F, Nocco A, Puglisi G, Innocente A, Serafini M, Messa P, Scalamogna M. Luminex technology for anti-HLA antibody screening: evaluation of performance and of impact on laboratory routine. Cytometry Part B Clin Cytom 2007;72B:465–471.
39. Dupuy AM, Kuster N, Lizard G, Ragot K, Lehmann S, Gallix B, Cristol JP. Performance evaluation of human cytokines profiles obtained by various multiplexed-based technologies underlines a need for standardization. Clin Chem Lab Med 2013;51:1385–1393.
40. Khan SS, Smith MS, Reda D, Suffredini AF, McCoy JP Jr. Multiplex bead array assays for detection of soluble cytokines: comparisons of sensitivity and quantitative values among kits from multiple manufacturers. Cytometry Part B Clin Cytom 2004;61B:35–39.
41. Duvigneau JC, Sipos W, Hartl RT, Bayer M, Moldzio R, Stevenson L, Adair B, Gemeiner M. Heparin and EDTA as anticoagulant differentially affect cytokine mRNA level of cultured porcine blood cells. J Immunol Methods 2007;324:38–47.
42. Ben-Hadj-Khalifa S, Nguyen P, Mahjoub T, Hezard N. Anticoagulant properties of the anti-inflammatory cytokine IL-10 in a factor Xa-activated human monocyte model. Eur Cytokine Netw 2012;23: 87–92.