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Stability and reproducibility of simultaneously detected plasma and serum cytokine levels in asymptomatic subjects

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Abstract

Blood levels of cyto- and chemokines might reflect immune deregulations which might be related to lymphomagenesis. Potential utility of stored blood samples of a prospective cohort was evaluated by the effect of different blood sample types and freeze-thaw cycles on analyte levels. Bead-based immunoassays were performed on two fresh samples (serum, citrate and heparin plasma) of 10 asymptomatic adults collected 14 days apart and on aliquots of the first samples which were put through one to three freeze-thaw cycles to measure 11 cytokines, four chemokines and two adhesion molecules. Median coefficients of variation (CVs) of the measured analytes were 20%, 24% and 32% in serum, citrate and heparin plasma, respectively. Strong correlations (rank correlation coefficient 0.74–0.98) were observed between sample types, although small differences in analyte levels were observed for most analytes. Freeze-thaw cycles did not markedly change analyte levels. Our study supports the use of this assay among asymptomatic subjects in epidemiological studies.

Keywords: *Cytokine; freeze-thaw cycle; reproducibility*

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Introduction

Cytokines are humoral signal molecules that are involved in cellular communications especially in immune response. T lymphocytes are the major source of cytokines with T-helper (Th) cells being the most prolific cytokine producers. Interleukin (IL)-2, interferon (IFN)-γ and tumour necrosis factor (TNF)-β produced by Th1 lymphocyte cells are involved in proinflammatory immune responses (cell-mediated immune response), whereas Th2 cells produce IL-4, IL-5, IL-9 and IL-13 which promote anti-inflammatory antibody-dependent immune responses (Lan et al. 2006). There are other types of T cells that can influence the expression and activation of Th cells, such as natural regulatory T cells, along with less common cytokine profiles such as the Th3 subset. Both regulatory T cells and Th3 cells produce the cytokine transforming growth factor (TGF)-β and IL-10. Recently, the characterization of another novel Th subtype, Th17, which has a potential function in the pathogenesis of autoimmune diseases and host defence has been described (Harrington et al. 2005, Steinman 2007). Cytokine signalling is characterized by considerable redundancy (several cytokines respectively elicit the same cellular response) and

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pleiotropy (each cytokine acts on multiple molecular targets) (Wong et al. 2008). Thus blood levels of multiple cytokines collectively might reflect subtle status of a deregulated immune response or a failure to modulate the immune response appropriately.

Recently, genetic studies have shown an association of the TNF G-308A polymorphism with an increased risk of non-Hodgkin's lymphoma (NHL), particularly diffuse large B-cell lymphoma (DLBCL), but not follicular lymphoma. The IL-10 T-3575A polymorphism was also associated with increased risk of NHL, particularly DLBCL. For individuals homozygous for the TNF-308A allele and carrying one or both IL-10-3575A alleles, risk of DLBCL was doubled. These reports offer important evidence linking immune function to lymphomagenesis among non-immunocompromised individuals (Rothman et al. 2006). Given the pivotal role of cytokines in immune function it is of interest to study prospectively if subtle deregulation of cytokine levels and/or their interrelations is related to the occurrence of NHL.

Plasma and serum cytokine concentrations can be quantified by standard enzyme-linked immunosorbent assay (ELISA). However for each cytokine a volume of 50–100 µl would be required resulting in a large volume demand if multiple analytes are to be quantified. In contrast, the Luminex xMAP platform (Luminex Corp., Austin, TX, USA) enables the quantification of up to a 100 different analytes in volumes of 25–50 µl (Vignali 2000, Kellar et al. 2001). The latter is of importance especially in prospective cohort studies as only small volumes are generally available.

To test the applicability of the Luminex xMAP system to quantify a suite of cytokines and chemokines in prospectively collected plasma or serum, we studied the reliability and levels of the assay in serum, sodium citrate plasma and heparinized plasma specimens from healthy donors. The choice of these specimen types was based on the available biological materials within the European Prospective Investigation into Cancer and Nutrition (EPIC). Type of media for blood fraction is considered an important issue in biomarker measurements. Wong et al. (2008) showed that plasma (heparin and acid citrate dextrose) measurements generally are not reflective of serum levels. On the other hand, Tworoger & Hankinson (2006) concluded that ethylenediamine tetra-acetic acid (EDTA) plasma is the best sample type for cytokines, although serum and heparin plasma were deemed acceptable as well. Effects of multiple freeze-thaw cycles on immune markers levels have been evaluated in several studies. According to Aziz et al. (1999a) no significant differences in mean value of examined cytokines and soluble activation markers in plasma and serum after ten freeze-thaw cycles have been shown, while another study reported that the levels of TNF-α increased significantly after three cycles (Flower et al. 2000). We studied the influence of freeze-thaw cycles on cytokines levels and their interrelations. The latter study was performed as often biological specimens are used for multiple molecular assays necessitating freeze-thaw cycles before the specimen can be tested.

Material and methods

Subjects and samples

Venous blood samples (serum, citrate and heparinized plasma) were collected from ten healthy individuals (age 20–40 years). After collection, the fresh samples were analysed within 2 h of collection for 11 cytokines (including proinflammatory, anti-inflammatory, Th1 and Th2 cytokines), four chemokines and two adhesion molecules. Samples were subsequently aliquoted and stored at -80°C. Subsets of the stored aliquots were put through one, two or three freeze-thaw cycles. Samples were thawed over night at 4°C and re-frozen the next day at -80°C. A second fresh sample was collected approximately 2 weeks after the initial blood draw. Again these samples were analysed within 2 h after blood draw at which time the stored freeze-thaw samples were re-analysed (Figure 1).

Cytokine measurements

All specimens were analysed for IL-1β, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IFN-γ, TNF-α, RANTES (regulated upon activation, normal T cell expressed and secreted), intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), eotaxin and IFN-inducible protein (IP)-10 in duplicate, using the Luminex multianalyte profiling technology (Lab-MAP™), according to the protocol described by de Jager et al. (2003) except that instead of a 1-h incubation, an overnight incubation at 4°C was used. Capture and detection antibodies and recombinant proteins were purchased from different commercial sources as described previously (de Jager et al. 2005). As a reporter; streptavidin-PE (BD Biosciences Pharmingen, San Diego, CA, USA) was used. Mean fluorescence intensities were calculated from duplicates for each sample. Standard curves were derived from recombinant protein standards with 12-fold dilutions. Detection limits were 1.22 pg ml^{-1} for IL-1β, IL-1α, IL-2, IL-5, IL-6, IL-8, IL-10, IL-13; 2.44 pg ml^{-1} for IL-12 and eotaxin and 24.4 pg

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ml^{-1} for ICAM and VCAM on both the first and second test days. However, limits of detection varied between the first and second test days for IL-4 (1.22 vs 0.24 pg ml^{-1}, respectively), IFN-γ (2.44 vs 1.22), TNF-α (4.88 vs 1.22), RANTES (1.22 vs 2.44) and IP-10 (2.44 vs 4.88). Cytokines measured in concentrations below their respective detection limit or above the maximum detectable range (RANTES 5000 pg ml^{-1}) were imputed based on a maximum likelihood estimation (MLE) procedure (Helsel 1990, 2005, Lubin et al. 2004). This procedure generates unbiased reproducibility statistics when at least 50% of measurements are above the limit of detection (LOD) and below the maximum detectable level (Lubin et al. 2004). Therefore, no statistics were calculated for analytes with more than 50% of measures below the LOD or above the maximum detectable level.

Statistical analysis

Cytokine concentrations were log-transformed as measured levels appeared to follow an approximate log-normal distribution. For each analyte reproducibility between duplicate samples by blood type (serum, citrate and heparin plasma) was evaluated by the coefficient of variation (CV, standard deviation / mean × 100). To estimate the effect of measurement error (based on duplicates) and intraindividual variance (based on two separate blood draws) on the ability to compare subjects, the intraclass correlation coefficient (ICC, interindividual variance / (inter- + intraindividual variance)) was calculated based on the two fresh samples.

Spearman's rank correlation coefficients (R_{sp}) were calculated to compare results from the different sample types (e.g. serum, citrate and heparin plasma) and between samples that had gone through different freeze-thaw cycles (e.g. fresh or one, two and three freeze-thaw cycles).

We also evaluated the effects of blood sample type, freeze-thaw cycles and test day (could also be interpreted as batch effect) on cytokines concentrations using a linear mixed-effects model:

$$\mathrm{Ln}(\gamma_{ij}) = \mu + \beta 1 \times \text{sample type} + \beta 2 \times \text{freeze - thaw cycle} + \beta 3 \times \text{test day} + \alpha_i + \varepsilon_{ij}$$

Where y_{ij} denotes the cytokine levels for subjects (i = 1... 10) on the j_{th} blood draw (j = 1, 2). The overall mean concentration of the first fresh sample in serum type is denoted by μ, α_i = normally distributed subject effect, ε_{ij} = normally distributed error term and the βs are regression coefficients. Sample type is citrate (1), heparin plasma (2) and serum (reference category (ref.)), freeze-thaw cycle is fresh (ref.), one, two or three freeze-thaw cycles and test day represents the first (ref.) or second test day.

Finally to construct the smallest, most informative set of markers and to study if these sets differed by blood sample type or freeze-thaw cycles we performed principal component analyses (PCA) using Varimax rotation. These PCA analyses were restricted to cytokines whose presumed targets are principally leukocytes and have function as immunomodulating agents (IL-6, IL-1β, IL-13, IL-5, IL-12, IL-1α, IL-10, IFN-γ, TNF-α, IL-2). The number of principal components (PCs) was determined with scree plots using a cut-off of 1 for the sorted Eigenvalues of the covariance matrix. The correlation between interindividual cytokines and each PC was assessed by factor loadings.

Statistical analyses were performed using SPSS software (v.11.5, SPSS Inc., Chicago, IL, USA) and SAS (v.9.1, SAS Institute, Cary, NC, USA). All *p*-values are two-sided, with $p < 0.05$ considered as statistically significant.

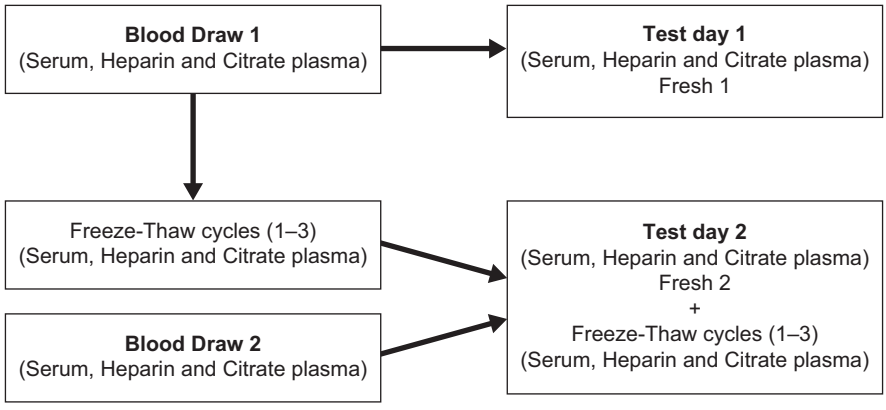

Figure 1. Protocol for evaluating the effects of freeze-thaw cycles, type of sample preparation and test day. Whole blood samples collected from asymptomatic adults ($n = 10$) for 2-week time interval.

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Results

In total 5100 cytokine measurements were successfully performed. We detected unexpected reproducible high levels (higher than mean + 2 SD based on log-transformed values) of most cytokines in one of the ten asymptomatic volunteers without any medical explanation. As these levels were not deemed representative for asymptomatic subjects in the general population we removed this person from the analyses. However, analyses including this subject would not have materially changed the results of the study. Serum and plasma ICAM levels were all above the maximum detectable levels in the assay and therefore results are not included. For the following analytes more or equal than 50% of the measurements were either below the LOD or above the maximum detectable level: IL-4 and RANTES (citrate); IL-1β, IL-4, IL-13, TNF-α and RANTES (heparin plasma); IL-4, IL-13, TNF-α and RANTES (serum). Therefore, no reproducibility statistics for these analytes are given. As IL-4 and RANTES were not detected in the majority of samples for all blood sample types this analyte was not included in any of the statistical analyses. IL-1β, IL-13 and TNF-α were retained in some of the statistical analyses as these analytes were detected in more than 50% for at least one blood sample type. Median CVs for duplicate measurements for all analytes were 20% (8–105), 24% (8–90) and 32% (8–98) in serum, citrate and heparin plasma, respectively (data not shown).

Descriptive statistics (geometric mean and standard deviation) of cytokines by type of blood samples are presented in Table 1. Geometric mean for the different cytokines, chemokines and adhesion molecules varied four orders of a magnitude from 0.4 to 3435, 0.3 to 5005 and 0.1 to 6786 pg ml^{-1}, for citrate, heparin and serum levels, respectively. Although absolute levels of analytes varied by sample type, overall levels were quite similar between the different blood sample types. Interestingly, the number of samples below the LOD seemed to be fewer for citrate than heparin plasma and serum.

Correlation analyses of selected protein markers between blood sample types

Correlation analyses between the different blood sample types are presented in Table 2. In general, strong correlations in analyte levels between serum and plasma blood types ($R_{sp=}$ 0.74–0.98) were observed except for IL-1β (0.14) for citrate versus serum samples. Results were similar when only values above the LOD were taken into account (data not shown).

Influence of storage time, type of blood samples and freeze-thaw cycles on cytokine levels

Results from the freeze-thaw experiments for citrate plasma are presented in Table 3. Results for serum and plasma heparin were essentially similar and therefore only the results for citrate are presented in detail. Overall levels were comparable between the samples that had gone through various freeze-thaw cycles (i.e. 1, 2 and 3), but differences were observed between

Table 1. Descriptive statistics of cytokine concentration by type.

			Citrate					Heparin					Serum				
	Subject	Sample	g. mean	g. SD	<LOD	>Range	ICC	g. mean	g. SD	<LOD	>Range	ICC	g. mean	g. SD	<LOD	>Range	ICC
IL-1β	9	18	3.58	2.2	1		0.39	NC	NC	14		NC	1.86	2.19	8		0.78
IL-1α	9	18	16.73	3.46			0.89	19.72	4.13	1		0.85	17.37	3.09			0.85
IL-2	9	18	32.83	3.27			0.79	34.71	3.43			0.80	41.29	3.72			0.89
IL-4	9	18	NC	NC	12		NC	NC	NC	13		NC	NC	NC	14		NC
IL-5	9	18	25.89	5.4	1		0.93	12.38	9.1	4		0.89	27.29	5.39			0.96
IL-6	9	18	8.12	2.42			0.74	2.26	3.44	8		0.55	3.82	3.56	5		0.73
IL-8	9	18	14.58	3.62			0.99	13.68	4.17			0.91	22.03	2.98			0.98
IL-10	9	18	40.1	2.98			0.90	48.94	3.33			0.83	43.8	2.98			0.90
IL-12	9	18	52.34	2.96			0.90	44.67	3.45			0.93	68.9	2.73			0.92
IL-13	9	18	1.45	2.03	8		0.79	NC	NC	13		NC	NC	NC	14		NC
IFN-γ	9	18	5.1	5.78	6		0.86	4.19	6.78	6		0.75	5.52	5.25	6		0.79
TNF-α	9	18	22.43	5.34	5		0.55	NC	NC	9		NC	NC	NC	10		NC
RANTES	9	18	NC	NC		11	NC	NC	NC		9	NC	NC	NC		17	NC
VCAM	9	18	271. 5	3.22			0.86	245.15	3.88			0.90	317.04	2.83			0.86
Eotaxin	9	18	124.99	1.54			0.83	292.5	1.29			0.77	170.01	1.43			0.76
IP-10	9	18	48.91	1.57			0.92	39.8	1.64			0.84	43.16	1.45			0.86

Geometric means (g. mean) and standard deviations (g. SD) calculated by means of two fresh samples; number of samples with cytokine value lower than limit of detection (<LOD) and maximum value (>Range); intraclass correlation (ICC) between two fresh samples based on log-transformed values; IL, interleukin; IFN, interferon; TNF, tumour necrosis factor; RANTES, regulated upon activation, normal T cell expressed and secreted; VCAM, vascular cell adhesion molecule; IP, interferon-inducible protein; NC, not calculated due to more or equal than 50% missing.

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cytokine levels in the fresh samples and in samples that had gone through at least one freeze-thaw cycle (median percentage of changes for all cytokines 22%, range –128 to 83). However, it is important to note that the first fresh sample was not analysed concurrently with the frozen samples which may account for part of the observed differences (i.e. batch or test-day effect). Nevertheless, high rank correlations between the test results of the fresh sample and of the samples that had gone through at least one freeze-thaw cycle were still observed for most cytokines, except for IL-1β, IL-6, IL-13 and TNF-α that had rank correlations less than 0.5 and IL-4 and RANTES for which we could not calculate any correlation statistics as more than 50% of the measurements were below 50%.

The effect of freeze-thaw cycles, blood sample type and test day were further explored in a linear mixed-effects model (Table 4). In this multiple regression model differences in cytokine levels between blood sample types were observed for all cytokines except for IFN-γ. Median ratio for citrate and heparin plasma versus serum, respectively, were 0.85 (range 0.23–4.26) and 0.83 (range 0.53–1.77). Freeze-thaw cycles did not significantly influence cytokine levels (except IL-1β, IL-13 and TNF-α), although there appeared to be a general tendency for lower levels with increasing number of freeze-thaw cycles. For some analytes (IL-1α, IL-10, IL-13, IFN-γ, VCAM and eotaxin) a significant effect of test day was found. No interaction between sample type and freeze-thaw cycles was observed (data not shown).

Table 2. Correlation of fresh samples by blood sample type.

	Subject	Sample	R_{sp} Citrate vs serum	R_{sp} Heparin vs serum	R_{sp} Citrate vs heparin
IL-1β	9	18	0.14	NC	NC
IL-1α	9	18	0.97	0.94	0.95
IL-2	9	18	0.97	0.97	0.98
IL-5	9	18	0.96	0.84	0.87
IL-6	9	18	0.79	0.81	0.82
IL-8	9	18	0.95	0.92	0.88
IL-10	9	18	0.96	0.93	0.93
IL-12	9	18	0.90	0.89	0.94
IL-13	9	18	NC	NC	NC
IFN-γ	9	18	0.95	0.98	0.96
TNF-α	9	18	NC	NC	NC
VCAM	9	18	0.98	0.96	0.98
Eotaxin	9	18	0.84	0.79	0.80
IP-10	9	18	0.95	0.91	0.85

R_{sp}, Spearman's correlation coefficients (rho) between serum and plasma levels of log-transformed cytokines including two fresh samples; IL, interleukin; IFN, interferon; TNF, tumour necrosis factor; VCAM, vascular cell adhesion molecule; IP, interferon-inducible protein; NC, not calculated.

Table 3. Correlations of up to three freeze-thaw cycles versus first fresh sample in citrate.

	Fresh 1		Freeze-thaw 1			Freeze-thaw 2			Freeze-thaw 3		
	g. mean	g. SD	g. mean	g. SD	R_{sp} vs. fresh	g. mean	g. SD	R_{sp} vs. fresh	g. mean	g. SD	R_{sp} vs. fresh
IL-1β	3.89	2.21	2.02	1.92	0.05	2.11	1.83	0.14	2.76	1.79	0.58
IL-1α	20.96	3.62	13.49	3.33	0.73	11.18	3.98	0.90	10.17	2.63	0.82
IL-2	32.23	4.1	33.63	2.96	0.90	33.15	2.94	0.90	31.42	2.51	0.93
IL-5	25.47	5.2	24.31	4.96	0.95	16.4	6.42	0.93	16.53	5.13	0.93
IL-6	8.76	2.6	5.22	2.69	0.57	4.65	2.47	0.42	4.85	2.71	0.50
IL-8	14.64	3.8	13.01	3.84	0.73	11.54	3.99	0.82	11.57	2.97	0.95
IL-10	44.33	3.27	33.67	3.21	0.93	29.69	3.42	0.97	26.41	3.04	0.83
IL-12	51.93	3.27	50.76	3.1	0.87	44.98	3.03	0.93	40.28	2.38	0.88
IL-13	1.19	2	1.34	1.89	0.33	1.17	2.08	0.43	1.5	1.89	0.57
IFN-γ	3.32	5.88	6.13	6.7	0.98	5.03	8.62	0.98	5.52	5.05	0.98
TNF-α	25.71	4.32	7.05	9.31	0.27	6.04	10.06	0.57	6.02	24.87	0.33
VCAM	357.96	3.16	215.54	3.3	0.97	187.45	3.55	1.00	173.83	3.03	1.00
Eotaxin	115.89	1.46	130.47	1.57	1.00	123	1.57	0.98	118.85	1.55	0.93
IP-10	49.49	1.53	53.4	1.46	0.90	53.94	1.61	0.90	51.61	1.65	0.90

R_{sp}, Spearman's correlation coefficient (rho); g. mean, geometric mean; g. SD, geometric standard deviation; IL, interleukin; IFN, interferon; TNF, tumour necrosis factor; VCAM, vascular cell adhesion molecule; IP, interferon-inducible protein; ($n = 9$).

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Table 4. Fixed-effect estimations of the effect of freeze-thaw cycles, sample types and test day based on a linear mixed-effects model.

		Freeze-thaw cycle			Sample type			Test day	
Cytokines (Ln)	Intercept		Coefficient[a]	*p*-Value[b]		Coefficient[c]	*p*-Value[b]	Coefficient[d]	*p*-Value[b]
IL-1β	0.55	1	-0.420[f]	0.01	1	0.677[e]	< 0.0001	0.057	0.73
		2	-0.529[e]		2	-0.519[e]			
		3	-0.223						
IL-1α	2.687		-0.021	0.06		-0.141	0.02	-0.442	< 0.0001
			-0.105			0.038			
			-0.216[f]						
IL-2	3.74		-0.007	0.990		-0.25[e]	0.001	0.027	0.76
			-0.015			-0.184[e]			
			-0.004						
IL-5	3.148		0.044	0.069		-0.029	< 0.0001	-0.1002	0.584
			-0.29			-0.63[e]			
			-0.35						
IL-6	1.467		-0.067	0.197		0.493[e]	< 0.0001	0.289	0.09
			-0.3			-0.274[f]			
			-0.27						
IL-8	3. 058		-0.015	0.379		-1.476[e]	< 0.0001	-0.065	0.41
			-0.105			-0.429[e]			
			-0.105						
IL-10	3.678		-0.0001	0.198		-0.185[e]	0.0005	-0.285	0.0008
			-0.067			0.068			
			-0.156						
IL-12	4.23		-0.009	0.23		-0.318[e]	< 0.0001	0.02	0.82
			-0.072			-0.372[e]			
			-0.167						
IL-13	0.113		-0.32[f]	0.008		0.45[e]	< 0.0001	0.453	0.0006
			-0.43[e]			0.157			
			-0.223						
IFN-γ	2.08		-0.134	0.198		-0.21[f]	0.123	0.79	< 0.0001
			-0.28			-0.113			
			-0.175						
TNF-α	1.06		-0.153[f]	0.009		1.45[e]	<0.0001	-0.49	0.31
			-0.578[e]			-0.18			
			-0.654						
VCAM	5.50		0.078	0.071		-0.226[e]	<0.0001	-0.54	<0.0001
			0.002			-0.238[e]			
			-0.09						
Eotaxin	5.2		-0.044	0.286		-0.31[e]	<0.0001	0.145	0.001
			-0.072			0.57[e]			
			-0.079						
IP-10	3.78		0.08	0.099		0.106[e]	<0.0001	0.035	0.374
			0.085			-0.083[e]			
			0.08						

Fixed-effect estimations of different blood sample types, freeze-thaw cycles and test day for individual log-transformed cytokines, results obtained by multiplex analyses ($n = 9$). [a]Freeze-thaw cycle 1, 2 and 3, reference category is fresh sample; [b]type III *p*-value; [c]citrate (1) and heparin (2), reference category is serum; [d]second test day; [e]*p*-value < 0.01; [f]*p*-value < 0.05. IL, interleukin; IFN, interferon; TNF, tumour necrosis factor; VCAM, vascular cell adhesion molecule; IP, interferon-inducible protein.

Within-subject variability in cytokine levels

The ICC was calculated for analytes with less than 50% missing values based on the results of the two fresh samples collected over a 2-week time period (Table 1). Overall ICCs were high indicating that the intraindividual variance was minimal as compared with the interindividual variance, except for IL-1β and TNF-α in citrate, and TNF-α, IL-6 and RANTES in heparin plasma.

Inter-relationship between immune markers

For citrate plasma, heparin plasma and serum three PCs were found which in the case of citrate plasma and

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Table 5. Principal component analysis using varimax rotation with Kaiser normalization using log-transformed cytokine concentrations.

	Citrate			Heparin			Serum		
	C.1	C.2	C.3	C.1	C.2	C.3	C.1	C.2	C.3
IL-6	0.96			0.91			0.75	-0.45	0.42
IL-1β	0.93				0.88[a]		0.87		
IL-13	0.91			0.59[a]	0.71[a]		0.89[a]		
IL-5	0.63		0.46	0.92			0.70		0.53
IL-12			0.92	0.71		0.59			0.94
IL-1α			0.90	0.63		0.66			0.91
IL-10			0.86	0.44	0.48	0.51			0.84
IFN-γ		0.89				0.91		0.85	
TNF-α		0.80			0.80			0.95[a]	
IL-2		0.76				0.92		0.89	

Factors with an Eigenvalue > 1 are displayed. The correlation between each cytokine and factors presented as factor loadings if above 0.4. Total variances explained by three components (Cs) are 84.8, 86.9 and 86.7 for citrate, heparin and serum, respectively. [a]More than 50% below the limit of detection. IL, interleukin; IFN, interferon; TNF, tumour necrosis factor.

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serum were comparable (Table 5). The first component consisted of IL-6, IL-1β, IL-13 and IL-5. The second component included IL-2, IFN-γ and TNF-α while the third component consisted of IL-12, IL-1α and IL-10. The PCs for heparin plasma differed in that IL-6, IL-5 and IL-12 were grouped in the first component, IL-1β, IL-13 and TNF-α in the second PC and IL-1α, IL-10, IFN-γ and IL-2 in the third component. PCA analyses on the samples that had gone through multiple freeze-thaw cycles rendered similar results (data not shown).

Discussion

Simultaneous detection of multiple cytokines could provide a comprehensive assessment of an individual immune status. As the multiple cytokine assay only requires a minimum amount of biological material, its application in nested case-control studies in prospective studies, such as EPIC seems feasible. However, many factors may influence the measurements of these immune cell factors including sample collection, processing and storage (Aziz et al. 1999a, b, Holland et al. 2003, de Jager et al. 2006, Tworoger & Hankinson 2006, Pfleger et al. 2008). Therefore, before such assays can be used successfully in prospective studies these factors need to be known (Vineis & Perera 2007). We therefore studied the reproducibility of the assay, explored differences between plasma and serum cytokine levels and studied the influence of freeze-thaw cycles on cytokines levels.

In this study we used a single MLE imputation method to impute cytokine levels below the LOD and above the maximum range. Statistical analyses based on multiple imputations did not change the results and therefore the statistical inferences based on the partly imputed data presented in this manuscript are robust. We did not re-analyse samples for which results were above the maximum range, by diluting the biological sample, as the purpose of the study was to explore the suitability of the multiplex bead assay to analyse a range of analytes in a single assay. Re-analysing the biological samples by diluting the sample would lead to an increase in the necessary sample volume which is often is not feasible in prospective cohort studies.

Median CV% for the analytes in serum, citrate and heparin plasma was between 20 and 32%. This laboratory variability was similar to another study reporting on cytokines multiplex assays in serum and/or plasma (Wong et al. 2008). Although these CV%s were higher than one would normally accept for a single analyte assay they are not likely to affect the ability to distinguish between individuals. This was clearly demonstrated in our study where the ICCs for most cytokines, based on two repeated blood samples collected 1–2 weeks apart and tested on two different days, were generally above 0.8. This indicated that besides laboratory error, temporal differences in individual cytokine levels were relatively minor as compared with differences between asymptomatic persons, at least within a 2-week period.

In several studies, comparisons have been made between serum and plasma immune markers; however the results have been conflicting (Tworoger & Hankinson 2006). In our study small but statistically significant differences in cytokine levels, except IFN-γ between the different blood sample types were detected. Furthermore, it was clear that for heparin plasma more cytokine measurements were below LOD or out of range making heparin plasma less suitable for multiplex cytokine analyses than citrate plasma or serum.

Rank correlations between measured analyte levels in different sample types were relatively high for most of the analytes, except for IL-1β. A reason for this low correlation is unknown but might be related to

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the relative high number of values below the LOD for serum samples. These results are different from those of a recent study that employed multiplex cytokine assays in samples from asymptomatic persons where large differences in cytokines levels between serum, heparin and citrate plasma were reported (Wong et al. 2008). It is unclear why our results differ from this study but differences in sampling processes and antibodies used might play a role. The latter might indicate that the choice of antibodies or commercial kits is crucial and that results obtained with different kits/antibodies might be difficult to compare, at least in absolute sense.

Assessment of the freeze-thaw stability is important in studies of biomarkers because of the use of previously thawed samples and/or repeated analysis for a failed run. Ray et al. (2005) reported that IL-1β for two cycles, TNF-α for three and IL-8 for four freeze-thaw cycles were stable. We found similar results in that no statistically significant differences were observed in analyte levels for up to three freeze-thaw cycles except for IL-1β, IL-13 and TNF-α. However, closer inspection of the results indicate that for most analytes the concentration levels were decreasing slightly by each freeze-thaw cycle as indicated by the increasing negative parameter estimates for each subsequent freeze-thaw cycle in the mixed-effects model. As such it seemed that freeze-thaw cycles did lead to some degradation of cytokines, chemokines and adhesion molecules but that this is a relative minor effect.

Cytokines have been classified to subgroups by their structural homology and by the kind of Th cells (Th1, Th2) they originate from. We examined the interrelationships among ten cytokines. Three out of the four cytokines in the first component are classically considered to be Th2 cytokines (IL-5, IL-13) or involved in Th2 regulation (IL-6). From the second component, IL-2 and IFN-γ are in agreement with the Th1 cytokines. IL-10 as a member of the newly introduced Th3 secreted cytokine group can be found in the third component (Table 5). These findings were relatively consistent with an earlier study (Wong et al. 2008) on different panel of cytokines that grouped IL-7, IL-10, IL-12 and IFN-γ and IL-4 and IL-6 together. Results of these two studies indeed indicate that based on a multiple cytokine measures a pattern can be distinguished that reflects the redundancy, synergism and antagonism present in the cytokine network that can be related to the more classical categorization of Th responses and could provide important insights in Th1/Th2/Th3 shifts in immune response. This observed pattern seemed to be similar for citrate plasma and serum and not to be affected by freeze-thaw cycles. Patterns observed in heparin plasma tended to be different.

Our study is one of the first to study comprehensively the influence of different blood sample types and freeze-thaw cycles on the reproducibility, levels and inter-relations of a suite of cytokines, chemokines and adhesion molecules. Furthermore, it has been shown that cytokine levels do not vary much in time within a subject (at least in a 2-week period). This latter is important if indeed single plasma or serum cytokine measurements are used to classify prospectively an individual's immune response. However, it would be of interest to repeat this latter experiment on a larger population and with a larger time interval between the two sample collections to test the temporal variance over a large period of time. In addition, due to the long storage times of prospective cohort samples, what we found can not be necessarily extrapolated to those cohorts. The influence of long-term freezing (years) should be further analysed. In conclusion, the results of our study tend to support the use of the Luminex-based cytokine assay among asymptomatic subjects in (prospective) epidemiological studies.

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