Journal of Immunological Methods xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

The importance of sample collection when using single cytokine levels and systemic cytokine profiles as biomarkers — a comparative study of serum versus plasma samples

Tor Henrik Anderson Tvedt [a,*], Kristin Paulsen Rye [b], Håkon Reikvam [a], Annette K. Brenner [a], Øystein Bruserud [a,b]

[a] Section of Hematology, Department of Medicine, Haukeland University Hospital, Norway
[b] Institute of Clinical Science, Section of Hematology, University of Bergen, Norway

ARTICLE INFO

Article history:
Received 4 September 2014
Received in revised form 20 January 2015
Accepted 21 January 2015
Available online xxxx

Keywords:
Cytokine
Adhesion molecules
Matrix metalloproteinases
Serum
Plasma
Ticagrelor
Bivalirudin

ABSTRACT

Background: Cytokines, soluble adhesion molecules and metalloproteinases can be detected in human serum or plasma samples. Such systemic levels are widely used as biomarkers in epidemiological and clinical studies.
Methods: We prepared serum samples and three types of plasma samples (EDTA, heparin, citric acid) from 20 healthy individuals. The levels of 31 cytokines, four soluble adhesion molecules and eight matrix metalloproteinases were analyzed by Luminex technology.
Results: Most mediators showed detectable levels in both plasma and serum. Several mediators that can be released by platelets showed increased serum levels, especially CCL5 and CD40L, but for the other mediators the serum levels did not correlate with peripheral blood platelet counts and for these last mediators serum and plasma levels often showed strong correlations. The use of bivalirudin for anticoagulation significantly increased and citric acid combined with platelet inhibitors (ticagrelor, acetylsalicylic acid plus prostaglandin E_2) did not alter plasma levels of platelet-store mediators compared with citric acid alone. The impact of sample preparation differed between mediators; for many mediators strong correlations were seen between serum and plasma levels even when absolute levels differed. Soluble adhesion molecule levels showed only minor differences between samples. Unsupervised hierarchical clustering suggested that the effect of sampling/preparation was strongest for serum and heparin plasma samples.
Conclusion: Careful standardization of sample preparation is usually necessary when analyzing systemic mediator levels, and differences caused by sample preparation should be considered as a possible explanation if studies show conflicting results.

Abbreviations: OOR<, out of range below; OOR>, out of range above; CV%, coefficient of variation; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; MMP, matrix metalloproteinase; ICAM, intracellular adhesion molecule; VCAM, vascular cell adhesion molecule; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; bFGF, basic fibroblast growth factor; TNF, tumor necrosis factor; INF, interferon; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; HGF, hepatocyte growth factor; TPO, thrombopoietin; CD40L, CD40 ligand; CCL, CC motif chemokine ligand; CXCL, CXC motif chemokine ligand; ASA, acetylsalicylic acid; PGE_2, prostaglandin E_2.

* Corresponding author at: Section of Hematology, Department of Medicine, Haukeland University Hospital, N-5021 Bergen, Norway. Tel.: +47 55 97 29 69.
E-mail address: Tor.Henrik.Anderson.Tvedt@helse-bergen.no (T.H.A. Tvedt).

1. Introduction

Cytokines are soluble mediators that are important for communication between cells and they have function as regulators of a wide range of cellular functions including proliferation, differentiation and survival. The cytokine network is also important for regulation and coordination of complex biological processes like angiogenesis, immune responses and inflammation. The cytokines thereby become important for the maintenance of the normal physiological

http://dx.doi.org/10.1016/j.jim.2015.01.006
0022-1759/

status and in the development of several human disorders, including autoimmunity, carcinogenesis and activation of coagulation (Melve et al., 2011; Reikvam et al., 2012; Bruserud, 2013). However, the effects of the cytokine network are further modulated by other soluble mediators; these interacting mediators can be soluble cytokine receptors, biologically active soluble adhesion molecules, matrix metalloproteinases (MMPs), Tissue inhibitors of MMPs (TIMPS) and soluble heat shock proteins (HSPs) (Bruserud, 1997; Hatfield et al., 2010; Fredly et al., 2012; Reikvam et al., 2013).

Serum or plasma prepared from peripheral blood samples is easily available from patients, and such samples are often collected as a part of clinical studies and stored in biobanks. Several studies have shown that evaluation of broad serum/plasma mediator profiles including cytokines, MMPs and soluble adhesion molecules can be a valuable scientific tool and may even give clinically useful information (Reikvam et al., 2013). Such broad profiling has been made possible with the development of Multiplex immunoassays that measure a large number of soluble mediators at an acceptable cost per sample in small sample volumes. However, several mediators are stored in peripheral blood cells and may then be released during the ex vivo handling of samples, whereas other mediators may be shed from the cells due to the presence of extracellular proteases (De Jongh et al., 1997). The knowledge about these processes is limited, and it is thereby difficult to judge the importance of different preparation methods. The goal of the present study was therefore to compare the soluble mediator profiles in healthy individuals for serum and plasma samples prepared with different anticoagulants (i.e. heparin, EDTA and citrate).

2. Material and methods

2.1. Blood sampling and preparation of serum and plasma samples

Blood samples were collected from 20 healthy volunteers including eleven females and nine males; the median age being 37 years (range 26–56 years). For each individual four tubes were drawn, one tube with clot activator for serum preparation (BD Vacutainer® Blood Collection Tubes; Becton, Dickinson, Franklin Lakes, New Jersey, US; product no. 367825) and three tubes for plasma preparation containing different anticoagulants, i.e. EDTA (BD Vacutainer®, product no 367835), heparin (BD Vacutainer®, product no. 366667) and citrate (BD Vacutainer®, product no. 363083), respectively. For each type of sample we used tubes with the same batch number for all individuals. Peripheral venous blood samples were collected. Blood for preparation of serum was collected onto tubes with coagulation-activating reagents. Serum samples were allowed to coagulate for 120 min at room temperature before centrifugation (1300*g* for 10 min) and subsequent serum collection. Plasma samples were centrifuged at 2000*g* for 15 min at room temperature; centrifugation then started within 30 min from sampling. Samples were finally distributed into cryotubes and then frozen immediately for storage at −80 °C.

We also compared the use of bivalirudin (provided as the drug Angiox, Hälsa Pharma, Lübeck, Germany) versus citric acid as anticoagulant for the preparation of plasma samples, and we compared the levels of platelet-derived mediators for plasma prepared from peripheral blood samples anticoagulated with (i) citric acid alone (Greiner bio-one Vacuette® blood collection tubes; Kremsmünster, Austria; product no. 454332), (ii) citric acid plus the platelet-inhibitory agents sodium salicylic acid (ASA; Merck, Whitehouse Station, New Jersey, US) and prostaglandin E_2 (PGE_2; Merck), and (iii) citric acid plus the platelet inhibitor ticagrelor (Selleckchem, Boston, Massachusetts, US). Bivalirudin was dissolved in saline (Greiner bio-one Vacuette®; product no. 454241) and ticagrelor in 5% ethanol and 95% saline, whereas ASA was dissolved in 0.1 M sodium bicarbonate and PGE_2 in 70% ethanol (Foss et al., 2001; Bexborn et al., 2009; Nylander et al., 2013). All reagents were added into the tubes immediately before blood sampling. The final concentrations were bivalirudin 50 μg/mL (Bexborn et al., 2009), ASA 1 mM (Foss et al., 2001), PGE_2 1 μM (Foss et al., 2001) and ticagrelor 15 μM (Nylander et al., 2013). The cell donors for these experiments were eight healthy blood donors (two women and six men (aged 35 –69 years)). The samples were centrifuged at 2000*g* for 10 min, and stored at 4 °C prior to analysis within 24 h after sampling. The plasma levels of CXCL5, VEGF and MMP-9 were determined by ELISA analyses for these samples (R&D Systems; Abingdon, UK).

2.2. Analysis of serum/plasma levels

Cytokine levels were determined by Luminex analyses (R&D Systems) and included (i) the interleukins IL-1alpha, IL-1beta, IL1-RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-10, IL-12, IL-13 and IL-17; (ii) the chemokines CCL2, CCL3, CCL4, CCL5, CCL11, CXCL5, CXCL10 and CXCL11; (iii) the growth factors bFGF, G-CSF, GM-CSF, VEGF, TPO, EGF, HGF and Leptin; (iv) the immunomodulatory cytokines IFN-gamma, CD40L and TNF-alpha; (v) the matrix metalloproteinases (MMPs) MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, and MMP-13; and (vi) the adhesion molecules ICAM-1, VCAM-1, E-selectin and P-selectin. The intra-assay variation (i.e. the variation between duplicates was generally <10%). In our study we investigated the influence of sample preparation on measured cytokine levels, i.e. we compared differences between mediator levels in various samples from the same individual; to avoid the influence of inter-assay variations all samples from the same individual were analyzed in the same assay.

2.3. Statistical and bioinformatical analyses

The data were analyzed with IBM SPSS Statistics version 21 and Graphpad Prism version 5. The Wilcoxon's signed rank test was used to compare paired samples and the Mann–Whitney-U test to compare the different groups. Spearman's correlation was used for correlation analysis; an r-value > 0.80 was then considered as a high degree of correlation and p-values < 0.05 were regarded as statistically significant. The Chi-square test was used to compare categorized data.

Coefficient of variation (CV% defined as standard deviation (SD) × 100 relative to the corresponding mean) was calculated for all samples/mediators when the corresponding median level exceeded the lowest standard. For CV% calculation OOR< was set to 0.64 similar to what has been recommended by others (Wong et al., 2008).

For bioinformatical analyses cytokine values flagged as OOR< were replaced with 90% of the lowest observed value, while values flagged as OOR> were replaced by 110% of

Please cite this article as: Tvedt, T.H.A., et al., The importance of sample collection when using single cytokine levels and systemic cytokine profiles as biomarkers — a comparative..., J. Immunol. Methods (2015), http://dx.doi.org/10.1016/j.jim.2015.01.006

the highest observed value. Values were normalized to the calculated geometrical mean and log (2) transformed and median normalized before an unsupervised hierarchical clustering analysis was performed using the Euclidian distance measurement; complete linkage analysis was performed using the J-Express 2009 analysis suite (MolMine AS, Bergen, Norway). Unsupervised hierarchical clustering was performed with Pearson's correlation as distance measure and average weighted linkage (Hosnijeh et al., 2010).

3. Results

3.1. Characterization of the sample donors

All our donors had normal hemoglobin levels, and the peripheral blood platelet and the total leukocyte counts as well as the relative and absolute levels of peripheral blood leukocyte subsets were all normal. The donors were all healthy, did not use any medication and showed no clinical signs of intercurrent disease. Their CRP levels were normal at the time of sampling.

3.2. Levels of soluble mediators in serum and various types of plasma samples — a comparison of median level, variation range and coefficience of variance

The levels of TNF-alpha, INF-gamma, bFGF, IL-1alpha, IL-1beta, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-17, MMP-12 and MMP-13 either showed undetectable levels or equally low values for the majority of donors (>80%) for all four sampling methods. There was a considerable variation in serum/plasma levels between the healthy individuals for the other mediators. Fig. 1 shows vertical scatterplots for the meditators EGF, VEGF, CD40L, P-selectin, TPO, MMP-1, MMP-8 and MMP9 as examples for mediators where serum levels are significantly higher than the corresponding plasma levels. The Supplementary Table 1 lists the median level, range and coefficient of variation (CV%) for each mediator; the table in addition gives the fraction of samples with a measured concentration within the range of the corresponding standard curve and the number of samples reported with no technical error. Twenty-nine mediators had a median level within the range of the standard curve and serum samples showed the highest median concentration for all these except for 4 mediators. The exceptional mediators were CXCL10 and CXCL11 that showed the highest median concentration in heparin plasma, and Leptin and MMP-2 that showed the highest median concentration in EDTA plasma. No mediator had highest median concentration in citric acid plasma.

EGF and VEGF concentrations were very low and close to undetectable in all types of plasma whereas high levels were observed in serum samples. For CD40L, MMP-1, MMP-8, MMP-9, TPO and P-selectin, the median serum levels were at least two times higher than the corresponding median concentration in any type of plasma, whereas the median concentration of CCL11 in heparin plasma was six times higher than for any other sample type. Finally, all MMP-7 EDTA measurements were lower than the lowest standard concentration.

The CV% was calculated for each mediator and preparation method, and the samples with the lowest CV% differed between mediators:

- Serum samples showed the lowest CV% for CD40L, IL-1RA, CCL5, CXCL5, CXCL8, CXCL10, E-selectin, P-selectin, MMP-2, MMP-7 and MMP-8.
- Heparin samples showed the lowest CV% for CCL2, CCL11, TPO, HGF and VCAM-1.
- EDTA samples showed the lowest CV% for Leptin, MMP-1, MMP-3 and MMP-9.
- Citric acid samples showed the lowest CV% only for ICAM-1.

Thus, none of our four sample preparation methods were associated with a generally low CV% for all mediators when investigating healthy individuals.

3.3. The contribution of ex vivo platelet release to serum levels of soluble mediators

Platelets can release a wide range of soluble mediators, including both cytokines and soluble adhesion molecules (Bruserud, 2013). For these mediators ex vivo release during sample coagulation/preparation may thus contribute to the serum levels and thereby contribute to the differences between sample types (Fig. 1). We therefore investigate whether serum levels of soluble mediators showed any correlation with peripheral blood platelet counts (Table 1), but significant correlations were only detected for P-selectin (all four preparation methods), sCD40 (heparin, citric acid), CXCL5 (citric acid), EGF (EDTA) and VEGF (EDTA) but not for any other mediators. CCL5 serum and plasma samples showed similar high levels exceeding the highest standard; CCL5 was therefore not included in any further analysis because we regard these high levels to be caused by platelet release during ex vivo handling of the samples (Apelseth et al., 2010).

3.4. Comparison of mediator levels in different sample categories — the influence of sample preparation on mediator levels differs between various mediators

We compared the mediator levels in serum and different types of plasma by (i) analysis of differences between measured levels (Wilcoxon's test), and (ii) by calculating the correlation coefficients (Spearman's rank correlation) for each of the following mediators: CCL2, CCL3, CCL4 CCL5, CCL11, CXCL5 CXCL11, TPO, Leptin, CD40L, IL-1RA, VCAM-1, ICAM-1, E-selectin, P-selectin, MMP-1, MMP-2, MMP-3, MMP-7, MMP-8 and MMP-9. The overall results are summarized in Fig. 2. Based on these observations the following conclusions can be drawn:

- For IL1-RA and CCL3 there were no or only minor differences between all six comparisons, i.e. there were strong correlations for all comparisons (Spearman's rank correlation) and no or only minor differences when comparing different samples (Wilcoxon's test).
- For another group of mediators there were considerable differences when comparing the levels for the four different sampling strategies, but despite this there were strong correlations between the levels for at least five out of the six combinations. This means that even though the measured levels differ, the same variation between

Please cite this article as: Tvedt, T.H.A., et al., The importance of sample collection when using single cytokine levels and systemic cytokine profiles as biomarkers — a comparative..., J. Immunol. Methods (2015), http://dx.doi.org/10.1016/j.jim.2015.01.006

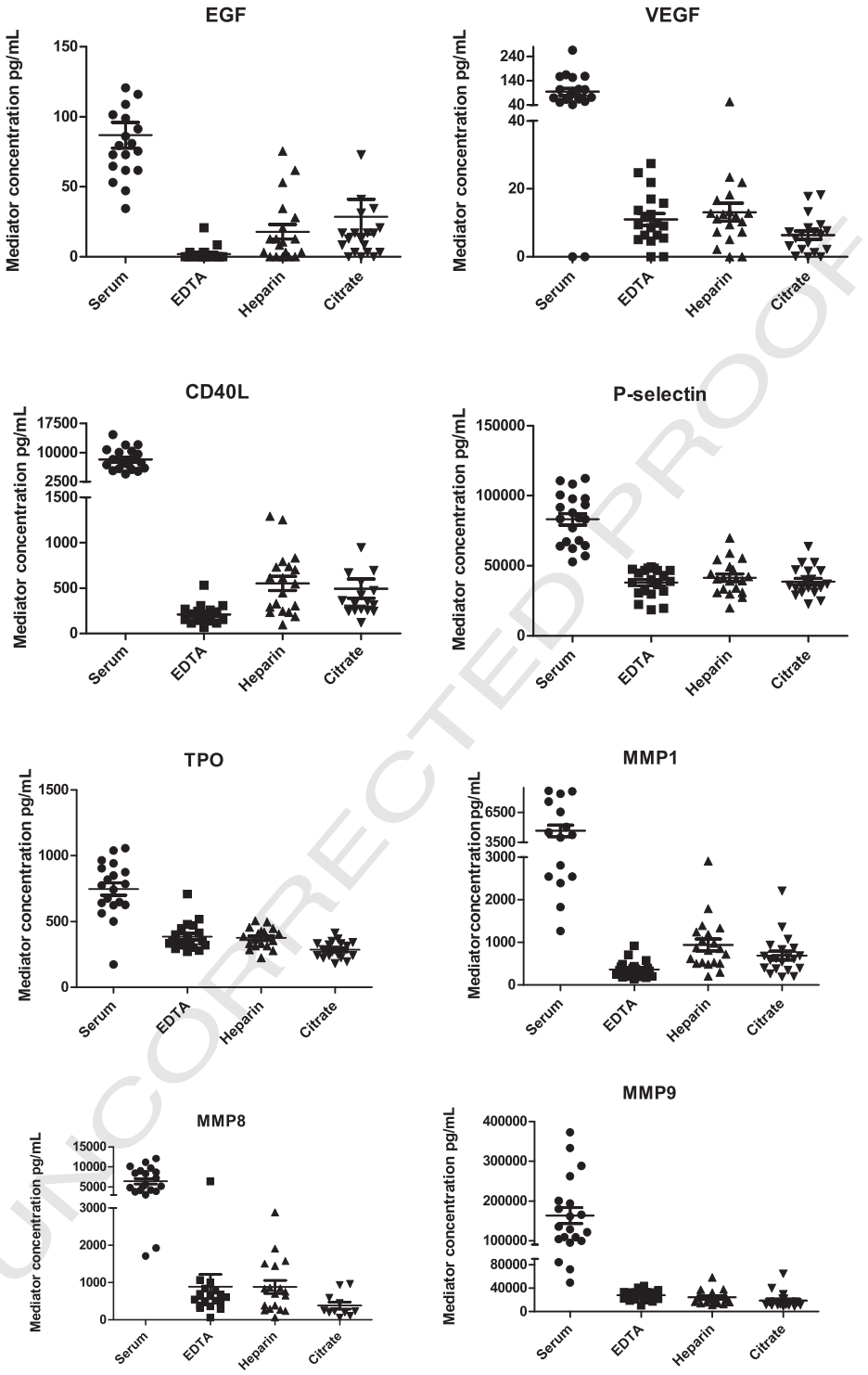


Fig. 1. Serum levels are significantly higher than the corresponding plasma levels for certain mediators. The figure presents the overall results for EGF, VEGF, CD40L, P-selectin, TPO, MMP-1, MMP-8 and MMP-9 investigated in blood samples derived from 20 healthy individuals.

individuals can be detected in all or most samples as demonstrated by the significant correlations. This was seen for all the soluble adhesion molecules (VCAM-1, ICAM-1, E-selectin and P-selectin) as well as for CCL2, TPO, Leptin, MMP-2 and MMP-3. For CCL2, CCL4 and MMP-1 the levels also differed between samples but strong correlations were seen only for 4 of the 6 combinations.

Please cite this article as: Tvedt, T.H.A., et al., The importance of sample collection when using single cytokine levels and systemic cytokine profiles as biomarkers — a comparative..., J. Immunol. Methods (2015), http://dx.doi.org/10.1016/j.jim.2015.01.006

Table 1
Correlation matrices for different soluble mediators released by platelets and platelet count. Significant values ($p < 0.05$) are marked with bold.

Mediator		r-Value	p-Value	Mediator		r-Value	p-Value	Mediator		r-Value	p-Value
CCL2	Serum	−0.265	0.273	**IL1RA**	Serum	−0.168	0.505	**MMP8**	Serum	0.168	0.478
	EDTA	−0.298	0.215		EDTA	0.085	0.731		EDTA	0.08	0.752
	Heparin	−0.341	0.152		Heparin	0.068	0.781		Heparin	0.21	0.402
	Citrate	−0.046	0.847		Citrate	0.092	0.707		Citrate	−0.261	0.438
CCL3	Serum	0.312	0.18	**EGF**	Serum	0.421	0.073	**MMP9**	Serum	−0.061	0.798
	EDTA	0.3	0.212		**EDTA**	**0.523**	**0.022**		EDTA	−0.049	0.837
	Heparin	0.433	0.064		Heparin	0.425	0.07		Heparin	0.391	0.088
	Citrate	0.212	0.383		Citrate	0.377	0.102		Citrate	0.186	0.432
CCL4	Serum	0.338	0.157	**HGF**	Serum	0.321	0.181	**CD40L**	Serum	0.439	0.06
	EDTA	0.35	0.142		EDTA	0.007	0.978		EDTA	0.413	0.088
	Heparin	0.297	0.217		Heparin	0.112	0.659		**Heparin**	**0.498**	**0.03**
	Citrate	0.422	0.064		Citrate	0.157	0.534		**Citrate**	**0.491**	**0.038**
CXCL5	Serum	0.002	0.994	**P-selectin**	**Serum**	**0.583**	**0.007**				
	EDTA	0.263	0.263		**EDTA**	**0.484**	**0.031**				
	Heparin	0.253	0.281		**Heparin**	**0.487**	**0.029**				
	Citrate	**0.589**	**0.006**		**Citrate**	**0.557**	**0.011**				
CXCL8	Serum	−0.44	0.052	**MMP1**	Serum	−0.145	0.543				
	EDTA	−0.26	0.282		EDTA	−0.089	0.709				
	Heparin	−0.09	0.715		Heparin	0.102	0.669				
	Citrate	−0.134	0.586		Citrate	0.181	0.444				

- For CCL5, MMP-8 and MMP-9 there were no significant correlations when comparing the levels in various samples.
- For the last four mediators we observed several significant differences between the levels in various samples, and significant correlations between different samples were seen only for a minority of the six combinations.

We therefore conclude that the influence of sample preparation on systemic mediator levels varies among soluble mediators. For a majority of mediators there are considerable differences between the levels measured in different samples, i.e. the levels are dependent on the sample preparation. Despite this difference in absolute levels, there are often significant correlations between samples so that the same variation between individuals can be detected independent of the preparation.

3.5. Alternative methods for preparation of plasma samples: citric acid combined with platelet inhibitors or bivalirudin alone as anticoagulant

We investigated whether the plasma levels of platelet-derived mediators could be decreased by adding platelet-inhibitory agents together with citric acid during peripheral blood sampling. Control samples without platelet inhibitors were prepared by either saline and ethanol (ticagrelor) or bicarbonate and ethanol (ASA, PGE_2) without the platelet inhibitors to the sampling tubes at the same volumes as for test tubes containing PGE_2/ASA/platelet inhibitor. Peripheral blood samples were collected from eight healthy blood donors. We examined the levels of CXCL5, VEGF and MMP-9 which all can be derived from peripheral blood platelets (Sheu et al., 2004; Kalvegren et al., 2011; Bruserud, 2013). Control cultures showed detectable CXCL5 levels for all eight donors, detectable MMP-9 for seven donors but detectable VEGF only for one/two donors, respectively. The presence of both ASA/PGE_2 and ticagrelor during sampling had only minor and divergent effects and differences did not reach statistical significance neither for CXCL5 nor MMP-9, and VEGF levels were not altered either. Briefly, ASA/PGE_2 had divergent effects both for CXCL5 (range 61–181% of control) and MMP8 (range 98–134% of control); the same was true for ticagrelor (CXCL5 53–121% and MMP9 47–202% of corresponding controls, respectively).

We compared the use of citric acid and bivalirudin as anticoagulants for plasma preparation. Samples prepared by using bivalirudin showed increased levels compared with the corresponding controls: (i) for CXCL5 a minor decrease was seen for one exceptional sample (73% of control) whereas for the other 7 a 2.0–10.3 fold increase was seen; (ii) for MMP9 one exceptional sample showed a decrease (91% of control) whereas the other seven samples showed a 1.6–7.5 fold increase; and (iii) for VEGF an increase to detectable levels was seen for 4 patients.

Thus, the presence of platelet inhibitors has only minor effects on the levels of platelet-derived inhibitors in plasma samples, whereas anticoagulation by bivalirudin is associated with increased levels of several platelet-derived mediators in the samples. This is true for soluble mediators stored both in platelet alpha granules (MMP-9, VEGF) and dense bodies (CXCL5) (Sheu et al., 2004; Bruserud, 2013).

3.6. Unsupervised hierarchical cluster analysis of systemic mediator levels

We did a hierarchical cluster analysis that included serum and plasma levels for soluble adhesion molecules (ICAM-1, VCAM-1, E-selectin, P-selectin), matrix metalloproteinases (MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9) and several chemokines (CCL2, CCL3, CCL4, CCL5, CCL11, CXCL5, CXCL11) as well as other cytokines (TPO, Leptin, EGF, VEGF, IL-1RA, CD40L, HGF, G-CSF). These mediators were selected because they showed detectable systemic levels for a majority of samples within the range of the standard curve. This analysis resulted in two main clusters, and the lower main cluster could be further

Please cite this article as: Tvedt, T.H.A., et al., The importance of sample collection when using single cytokine levels and systemic cytokine profiles as biomarkers — a comparative..., J. Immunol. Methods (2015), http://dx.doi.org/10.1016/j.jim.2015.01.006

Upper

IL1-RA			
	Serum	EDTA	Heparin
EDTA	0.775*		
Heparin	0.818*	0.882*	
Citrate	0.933*	0.892*	0.905*

CD40 Ligand			
	Serum	EDTA	Heparin
EDTA	0.579*		
Heparin	0.521*	0.25	
Citrat	0.35	0.24	0.535*

CCL2			
	Serum	EDTA	Heparin
EDTA	0.38		
Heparin	0.584*	0.921*	
Citrate	0.38	0.952*	0.918*

CCL3			
	Serum	EDTA	Heparin
EDTA	0.568*		
Heparin	0.607*	0.825*	
Citrate	0.582*	0.600*	0.523*

CCL4			
	Serum	EDTA	Heparin
EDTA	0.824*		
Heparin	0.754*	0.822*	
Citrat	0.27	0.39	0.464*

CCL5			
	Serum	EDTA	Heparin
EDTA	0.01		
Heparin	-0.26	0.13	
Citrat	0.03	0.06	0.21

CCL11			
	Serum	EDTA	Heparin
EDTA	0.815*		
Heparin	0.850*	0.690*	
Citrate	0.849*	0.932*	0.641*

CXCL5			
	Serum	EDTA	Heparin
EDTA	0.533*		
Heparin	0.43	0.523*	
Citrate	0.12	0.520*	0.28

CXCL10			
	Serum	EDTA	Heparin
EDTA	0.686*		
Heparin	0.844*	0.710*	
Citrat	0.785*	0.831*	0.829*

CXCL11			
	Serum	EDTA	Heparin
EDTA	0.555*		
Heparn	0.34	0.14	
Citrate	0.512*	0.41	0.27

VEGF			
	Serum	EDTA	Heparin
EDTA	0.451		
Heparin	0.589*	0.775*	
Citrat	0.487*	0.749*	0.722*

EGF			
	Serum	EDTA	Heparin
EDTA	0.368		
Heparin	0.492*	0.545*	
Citrat	0.279	0.086	0.06

Lower

TPO			
	Serum	EDTA	Heparin
EDAT	0.473*		
Heparin	0.512*	0.828*	
Citrate	0.487*	0.630*	0.732*

Leptin			
	Serum	EDTA	Heparin
EDTA	0.992*		
Heparin	0.875*	0.992*	
Citrate	0.988*	0.981*	0,971*

VCAM1			
	Serum	EDTA	Heparin
EDTA	0.931*		
Heparin	0.952*	0.904*	
Citrate	0.880*	0.827*	0.830*

ICAM1			
	Serum	EDTA	Heparn
EDAT	0.965*		
Heparin	0.964*	0.982*	
Citrate	0.917*	0.884*	0.884*

E-selectin			
	Serum	EDTA	Heparin
EDTA	0.976*		
Heparin	0.974*	0.964*	
Citrate	0.798*	0.761*	0,743*

P-selectin			
	Serum	EDTA	Heparin
EDTA	0.826*		
Heparin	0.737*	0.774*	
Citrate	0.786*	0.820*	0.802*

MMP1			
	Serum	EDTA	Heparin
EDAT	0.568*		
Heparin	0.708*	0.570*	
Citrate	0.332	0.186	0.526*

MMP2			
	Serum	EDTA	Heparin
EDTA	0.412		
Heparin	0.603*	0.483*	
Citrate	0.583*	0.517*	0.758*

MMP3			
	Serum	EDTA	Heparin
EDTA	0.986*		
Heparin	0.952*	0.953*	
Citrate	0.937*	0.934*	0.941*

MMP7			
	Serum	EDTA	Heparin
EDAT			
Heparin	0.663*		
Citrate	0.900*		0.654*

MMP8			
	Serum	EDTA	Heparin
EDTA	0.277		
Heparin	0.480*	0.384	
Citrate	0.301	-0.288	0.268

MMP9			
	Serum	EDTA	Heparin
EDTA	0.283		
Heparin	0.559*	0.402	
Citrate	-0.105	0.383	0.421

Fig. 2. Comparison between various samples with regard to differences in mediator levels and correlation of levels between samples — an overview of the results for 24 soluble mediators. Four different samples (serum and plasma prepared by addition of EDTA, heparin or citric acid) were examined for each individual and 6 different combinations were therefore compared. The color of each cell indicates whether the statistical comparison of mediator levels in the two corresponding sample sets showed statistically significant differences (Wilcoxon's signed rank test; green box, $p < 0.05$) or not (red box, $p > 0.05$). The value in each cell represents the r-value for the corresponding mediator and preparation method. Significant r-value ($p < 0.05$) is marked with asterisk. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

divided into three new subclusters (Fig. 3). Each of these four clusters showed specific characteristics:

- Upper cluster. This cluster included all 20 serum samples together with one citric acid sample.
- Upper middle and lower middle clusters. These two clusters included the majority of EDTA and citric acid samples; the upper middle cluster then included eleven citric acid samples versus eight EDTA samples whereas the lower middle cluster included eleven EDTA versus six citric acid samples. However, EDTA and citric acid samples from the same individual tended to cluster close to each other; six such pairs were found in the upper middle cluster, five pairs in the lower middle and one exceptional pair in the lower cluster.
- Lower cluster. This cluster included 17 out of the 20 heparin samples together with 1 EDTA/citric acid pairs and one additional citric acid sample.

The non-random localization of serum samples in the upper cluster and heparin samples in the lower cluster reached statistical significance (Chi-square test, p-value < 0.01), and the samples in the upper cluster (i.e. mainly serum samples) showed significantly higher levels of MMP-8, MMP-9, CD40L, CCL4, CCL5, EGF, VEGF and CXCL5 compared with the other

Please cite this article as: Tvedt, T.H.A., et al., The importance of sample collection when using single cytokine levels and systemic cytokine profiles as biomarkers — a comparative..., J. Immunol. Methods (2015), http://dx.doi.org/10.1016/j.jim.2015.01.006

clusters. The lower cluster included most of the heparin samples, but only CCL11 and CXCL11 levels were significantly higher for samples in the lower cluster compared with all other samples. Thus, the close localization of serum and heparin samples in this analysis suggests that the sample preparation has a relatively strong influence on the measured levels for these two sampling strategies; for serum samples the close clustering seems to be determined by a relatively large group of mediators, whereas the clustering of heparin samples is determined mainly by the overall profile, and only CCL11 and CXCL11 differed significantly from the other samples. On the other hand, the tendency for pairwise localization of EDTA and citric acid samples in the upper and lower middle clusters suggests that individual characteristics of the sample donors are relatively more important for these samples than the sampling methods.

4. Discussion

Microbead technology has made it possible to measure multiple mediators simultaneously in small sample volumes. This makes cytokine profiling attractive for the analysis of complex biological processes. Serum and different types of plasma are easily obtainable, and such sampling has been included in several clinical and epidemiological studies during the last decades. It will therefore be important to know how sample preparation affects the measured levels of soluble mediators, e.g. direct or indirect effects of additives in collection tubes, or differences between serum and various types of plasma. Such information will be essential when comparing the results from different studies and may explain apparently conflicting results.

Several studies have previously shown that serum samples have higher levels of several mediators compared to different types of plasma samples (Wong et al., 2008; Biancotto et al., 2012; Krishnan et al., 2014). This is confirmed in the present study, and the increased serum levels are also reflected in our cluster analysis where serum samples group together in a common main cluster. The most likely explanation for this is platelet release during ex vivo sample preparation or activation of immunocompetent cells by the coagulation (Jung et al., 2008; Kalvegren et al., 2011). A difference is especially seen for certain platelet-derived mediators that show high serum levels whereas their levels in all types of plasma are low or undetectable (i.e. CD40L, VEGF and EGF). However, our study suggests that the impact of platelet release differs among mediators; for several mediators known to be released by activated platelets there was no correlation between serum levels and platelet counts in peripheral blood, but rather significant correlations between serum and plasma levels. This observation suggests that for these mediators the platelet release has a minor effect on the serum levels, and one would then expect serum and plasma samples to reflect the same variation between individuals. Finally, for certain mediators we could only detect significant correlations between platelet counts and plasma levels but not serum levels. This last observation suggests that the importance of ex vivo release should not be considered only for serum samples but also for plasma samples at least for these mediators. One possibility to reduce the problem of platelet release is to use plasma and to add platelet-inhibitory agents at the time of sampling (Foss et al., 2001), but this approach is less suitable for large-scale blood sampling and the agents may in addition have effects on the leukocytes (Bruserud and Lundin, 1987).

All our donors had normal hemoglobin levels, and the peripheral blood platelet and total peripheral blood leukocyte counts as well as the relative and absolute levels of leukocyte subsets were all normal. The impact of sampling procedures may increase if individuals with leukocytosis or thrombocytosis are studied, whereas the impact of this ex vivo platelet release would probably decrease if thrombocytopenic patients are studied. However, the possible problem of platelet release has to be addressed in all studies independent of the peripheral blood platelet counts.

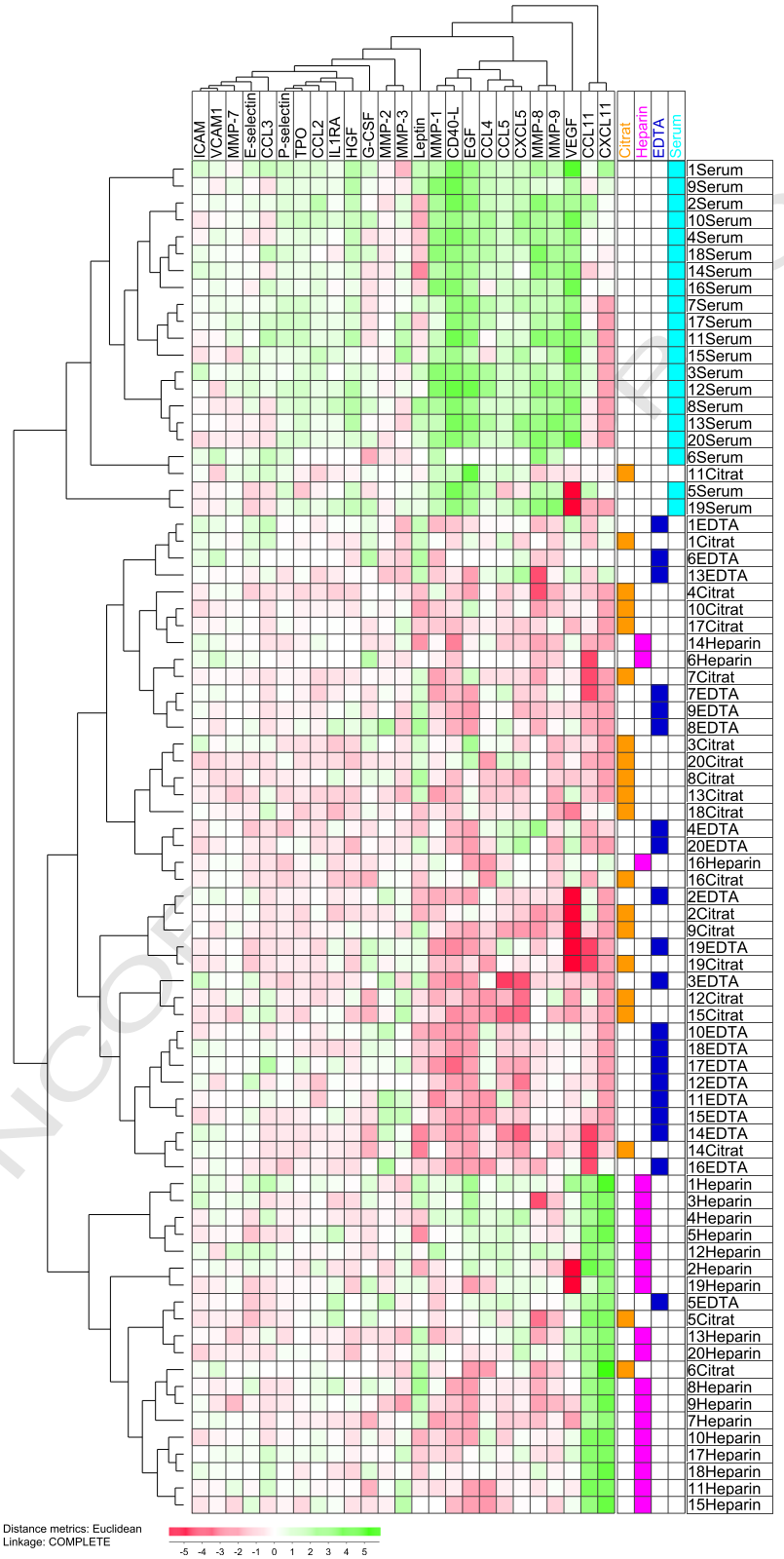
Previous studies have shown that serum mediator levels may depend on sample preparation and whether blood is collected onto tubes with or without coagulation-activating reagents (Hosnijeh et al., 2010; Biancotto et al., 2012), but it should be emphasized that this difference has been investigated only for a limited number of mediators. We only investigated serum samples that were collected onto tubes with coagulation-activating agents and this may explain the relatively high levels of several mediators in our study. Additional studies are needed to clarify whether tubes with or without coagulation-activated agents should be preferred.

Due to the effect of platelet release on mediator levels the use of EDTA plasma has been recommended in certain previous studies (Krishnan et al., 2014). However, others have reported that EDTA is unsuitable due to platelet adhesion and aggregation that might lead to reduced mediator levels (Biancotto et al., 2012; Patil et al., 2013), and for certain mediators (e.g. MMP-7) the presence of EDTA makes the detection impossible. Rather, citrate plasma has been recommended as the best compromise for analysis of matrix metalloproteinases and TIMPs (Mannello, 2008). Our present study additionally showed that certain mediators had increased levels in EDTA plasma; this was true both for Leptin and MMP-2 which showed significantly increased levels. Furthermore, our clustering analysis showed that EDTA and citric acid samples from the same individual usually clustered together in the same main cluster (Fig. 3, upper and lower middle clusters), and these two main clusters included more than 90% of the EDTA and citric acid samples. Thus, the distribution of these samples in our cluster analysis is not random and indicates that EDTA and citric acid influence mediator levels in a similar way.

We investigated whether the plasma levels of platelet-derived mediators could be altered by the presence of platelet-inhibitory agents (ASA/PGE_2 or ticagrelor) or by using an alternative anticoagulant (bivalirudin). We used the same concentrations of ASA/PGE_2 and bivalirudin as used in previous sampling studies (Foss et al., 2001; Bexborn et al., 2009), whereas ticagrelor was used at a concentration known to cause platelet inhibition (Nylander et al., 2013). However, the presence of ASA/PGE_2 or ticagrelor together with citric acid had only minor effects on the levels of platelet-released mediators, while increased levels were seen for bivalirudin compared with citric acid. Thus, our present results do not support the use of these strategies to minimize the release of soluble mediators from platelets during plasma preparation.

Previous studies have demonstrated that the release of MMPs by platelets may depend on the activation signal (Kalvegren et al., 2011). Thus, the contribution of ex vivo platelet release to

Please cite this article as: Tvedt, T.H.A., et al., The importance of sample collection when using single cytokine levels and systemic cytokine profiles as biomarkers — a comparative..., J. Immunol. Methods (2015), http://dx.doi.org/10.1016/j.jim.2015.01.006



Please cite this article as: Tvedt, T.H.A., et al., The importance of sample collection when using single cytokine levels and systemic cytokine profiles as biomarkers — a comparative..., J. Immunol. Methods (2015), http://dx.doi.org/10.1016/j.jim.2015.01.006

serum/plasma levels may thus differ between various mediators and also between serum and various types of plasma.

Most heparin samples (17 out of 20 samples) clustered together; this observation suggests that the levels in heparin plasma are influenced by the sampling/preparation procedure. For some of the mediators (CCL11, CXCL11) the levels were significantly higher in heparin plasma compared with the other samples. On the other hand, heparin may also reduce measured mediator levels by increased ex vivo adsorption (Fujita et al., 2002), and heparin may even have effects on immunocompetent cells (Bruserud and Lundin, 1987). Altered cytokine release or cytokine binding/adsorption during ex vivo handling may therefore explain why heparin samples differ from the other samples.

The impact of sampling and ex vivo handling thus differed between mediators (Fig. 2). However, the soluble adhesion molecules differed from the other mediators as their levels only showed minor differences between samples, indicating that sample preparation did not have a major impact on the measured levels (Fig. 2). Previous studies have also addressed the question whether sampling and preparation will affect the measurements of systemic mediator levels (Fatas et al., 2008; Wong et al., 2008; Hosnijeh et al., 2010; Biancotto et al., 2012; Patil et al., 2013; Krishnan et al., 2014), but none of these studies included the soluble adhesion molecules ICAM-1, VCAM-1, P-selectin and E-selectin.

Several direct inhibitors of coagulation do not rely on calcium depletion or antithrombin effects, including hirudin and dabigatran. Both these agents inhibit thrombin directly and do not rely on antithrombin; for this reason their off-target effects are probably minimal. Although hirudin interacts late in the coagulation cascade, it has previously been used in whole-blood models that required minimal effects on calcium depletion, heparin or coagulation activation (Bexborn et al., 2009). The possible use of these agents for plasma preparation and biobanking should be further investigated.

The experience from acute myeloid leukemia illustrates that the examination of how analysis of mediator profiles may become useful compared to analysis of single mediators both for evaluation during treatment, for prognostic evaluation and for the diagnosis of complications following intensive treatment (Reikvam et al., 2013). However, our present studies show that measured levels for several mediators will depend on sample preparation. Existing biobanks have often included only one sample type, and for seriously ill patients the available blood sample volumes for biobanking may be limited and preparation of only one type of sample is possible. The optimal type of sample may then differ between mediators. A previous study suggested that citrate plasma seems to be the best compromise for analysis of matrix metalloproteinases and TIMPs (Mannello, 2008), but this may not be true for other mediators. Careful standardization of detailed methodological descriptions will therefore be essential.

Our study demonstrates that mediator levels may differ between plasma and serum samples from the same individuals, and such differences in sample preparation may explain different observations in different studies. When a correlation between levels in serum and plasma samples is seen, a possible solution might be the conversion of values from one type of samples to an alternative type. However, as pointed previously (Jung and Wu, 2010) in their example with MMP-9, it is difficult to convert data in such a way, and calculations based on correlation analyses alone are not sufficient. In our opinion such transformation of data for one type of sample in order to allow for a direct comparison with another type of sample should be avoided or only be done with great care.

Scientific studies of soluble mediators in patients will often require a broad initial screening (often based on biobank material) before mediators are selected for large-scale clinical studies, e.g. more than 100 mediators in certain AML studies (Reikvam et al., 2013). The sample volume and the types of samples (i.e. plasma or serum) will often be limited, and in real life one often has to compromise with regard to sample type in such initial screenings. However, a careful selection of the optimal sample preparation has to be a part of the additional studies when a limited number of mediators are selected for scientific evaluation of defined soluble mediator signatures in clinical practice.

Our present study shows that the systemic (serum or plasma) levels of several soluble mediators depend on sample preparation, but this impact differs between mediators. These effects of sampling have to be considered when comparing observations from different studies. Our results therefore emphasize the importance of carefully standardized sampling procedures, and detailed methodological descriptions should be included in future presentations of scientific results. This will be essential to allow comparison of results from different studies and to consider whether differences in sample preparation can explain divergent results.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jim.2015.01.006.

Acknowledgments

The study was supported by the Norwegian Cancer Society. Q10

Addendum Q11

Tor Henrik Tvedt performed all statistical analysis, Øystein Bruserud designed the study, Kristin Paulsen performed the Luminex assay analysis and Håkon Reikvam performed the bioinformatical analyses. Annette K. Brenner performed the experiments with platelet inhibitors and hirudin as anticoagulant. Øystein Bruserud and Tor Henrik Tvedt wrote the manuscript; all authors approved the final version.

Fig. 3. Unsupervised hierarchical cluster analysis of soluble mediator levels in serum and plasma prepared with three different anticoagulants. Concentrations of 25 mediators were determined using the Luminex technology for 20 healthy controls, and levels were compared in serum and in plasma prepared with three different anticoagulants, i.e. heparin, EDTA and citric acid. The concentrations were normalized and log (2) transformed before an unsupervised hierarchical clustering with Euclidian distance measurement with complete linkage was performed. The results are presented as dendrograms and a heat map for visualization and interpretation. Red indicates low and green high values. The mediators are indicated at the top of the figure and the individuals and sample techniques are shown on the right in the figure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Please cite this article as: Tvedt, T.H.A., et al., The importance of sample collection when using single cytokine levels and systemic cytokine profiles as biomarkers — a comparative..., J. Immunol. Methods (2015), http://dx.doi.org/10.1016/j.jim.2015.01.006

References

Apelseth, T.O., Bruserud, O., Wentzel-Larsen, T., Hervig, T., 2010. Therapeutic efficacy of platelet transfusion in patients with acute leukemia: an evaluation of methods. Transfusion 50, 766.

Bexborn, F., Engberg, A.E., Sandholm, K., Mollnes, T.E., Hong, J., Nilsson Ekdahl, K., 2009. Hirudin versus heparin for use in whole blood in vitro biocompatibility models. J. Biomed. Mater. Res. A 89, 951.

Biancotto, A., Feng, X., Langweiler, M., Young, N.S., McCoy, J.P., 2012. Effect of anticoagulants on multiplexed measurement of cytokine/chemokines in healthy subjects. Cytokine 60, 438.

Bruserud, O., 1997. Serum levels of soluble ICAM-1 in acute leukemia patients with chemotherapy-induced neutropenia. Leukemia 11, 1596.

Bruserud, O., 2013. Bidirectional crosstalk between platelets and monocytes initiated by Toll-like receptor: an important step in the early defense against fungal infections? Platelets 24, 85.

Bruserud, O., Lundin, K., 1987. The effect of drugs used in anticoagulation therapy on T lymphocyte activation in vitro I. Heparin inhibits activation by soluble antigen or allogeneic cells but not by phytohaemagglutinin. J. Clin. Lab. Immunol. 23, 117.

De Jongh, R., Vranken, J., Vundelinckx, G., Bosmans, E., Maes, M., Heylen, R., 1997. The effects of anticoagulation and processing on assays of IL-6, sIL-6R, sIL-2R and soluble transferrin receptor. Cytokine 9, 696.

Fatas, M., Franquelo, P., Franquelo, R., 2008. Anomalous flotation of separator gel: density or viscosity? Clin. Chem. 54, 771.

Foss, B., Abrahamsen, J.F., Bruserud, O., 2001. Peripheral blood progenitor cell grafts contain high levels of platelet-secreted mediators. Transfusion 41, 1431.

Fredly, H., Reikvam, H., Gjertsen, B.T., Bruserud, O., 2012. Disease-stabilizing treatment with all-trans retinoic acid and valproic acid in acute myeloid leukemia: serum hsp70 and hsp90 levels and serum cytokine profiles are determined by the disease, patient age, and anti-leukemic treatment. Am. J. Hematol. 87, 368.

Fujita, M., Ishihara, M., Ono, K., Hattori, H., Kurita, A., Shimizu, M., Mitsumaru, A., Segawa, D., Hinokiyama, K., Kusama, Y., Kikuchi, M., Maehara, T., 2002. Adsorption of inflammatory cytokines using a heparin-coated extracorporeal circuit. Artif. Organs 26, 1020.

Hatfield, K.J., Reikvam, H., Bruserud, O., 2010. The crosstalk between the matrix metalloprotease system and the chemokine network in acute myeloid leukemia. Curr. Med. Chem. 17, 4448.

Hosnijeh, F.S., Krop, E.J., Portengen, L., Rabkin, C.S., Linseisen, J., Vineis, P., Vermeulen, R., 2010. Stability and reproducibility of simultaneously detected plasma and serum cytokine levels in asymptomatic subjects. Biomarkers 15, 140.

Jung, K., Wu, C.Y., 2010. Methodological weakness in using correlation coefficients for assessing the interchangeability of analyte data between samples collected under different sampling conditions — the example of matrix metalloproteinase 9 determined in serum and plasma samples. Clin. Chem. Lab. Med. 48, 733.

Jung, K., Klotzek, S., Stephan, C., Mannello, F., Lein, M., 2008. Impact of blood sampling on the circulating matrix metalloproteinases 1, 2, 3, 7, 8, and 9. Clin. Chem. 54, 772.

Kalvegren, H., Jonsson, S., Jonasson, L., 2011. Release of matrix metalloproteinases-1 and -2, but not -9, from activated platelets measured by enzyme-linked immunosorbent assay. Platelets 22, 572.

Krishnan, V.V., Ravindran, R., Wun, T., Luciw, P.A., Khan, I.H., Janatpour, K., 2014. Multiplexed measurements of immunomodulator levels in peripheral blood of healthy subjects: effects of analytical variables based on anticoagulants, age, and gender. Cytometry B Clin. Cytom. Q12

Mannello, F., 2008. Serum or plasma samples? The "Cinderella" role of blood collection procedures: preanalytical methodological issues influence the release and activity of circulating matrix metalloproteinases and their tissue inhibitors, hampering diagnostic trueness and leading to misinterpretation. Arterioscler. Thromb. Vasc. Biol. 28, 611.

Melve, G.K., Ersvssr, E., Kittang, A.O., Bruserud, O., 2011. The chemokine system in allogeneic stem-cell transplantation: a possible therapeutic target? Expert. Rev. Hematol. 4, 563.

Nylander, S., Femia, E.A., Scavone, M., Berntsson, P., Asztely, A.K., Nelander, K., Lofgren, L., Nilsson, R.G., Cattaneo, M., 2013. Ticagrelor inhibits human platelet aggregation via adenosine in addition to P2Y12 antagonism. J. Thromb. Haemost. 11, 1867.

Patil, R., Shukre, S., Paranjape, R., Thakar, M., 2013. Heparin and EDTA anticoagulants differentially affect the plasma cytokine levels in humans. Scand. J. Clin. Lab. Invest. 73, 452.

Reikvam, H., Hatfield, K.J., Fredly, H., Nepstad, I., Mosevoll, K.A., Bruserud, O., 2012. The angioregulatory cytokine network in human acute myeloid leukemia — from leukemogenesis via remission induction to stem cell transplantation. Eur. Cytokine Netw. 23, 140.

Reikvam, H., Fredly, H., Kittang, A.O., Bruserud, O., 2013. The possible diagnostic and prognostic use of systemic chemokine profiles in clinical medicine-the experience in acute myeloid leukemia from disease development and diagnosis via conventional chemotherapy to allogeneic stem cell transplantation. Toxins 5, 336.

Sheu, J.R., Fong, T.H., Liu, C.M., Shen, M.Y., Chen, T.L., Chang, Y., Lu, M.S., Hsiao, G., 2004. Expression of matrix metalloproteinase-9 in human platelets: regulation of platelet activation in in vitro and in vivo studies. Br. J. Pharmacol. 143, 193.

Wong, H.L., Pfeiffer, R.M., Fears, T.R., Vermeulen, R., Ji, S., Rabkin, C.S., 2008. Reproducibility and correlations of multiplex cytokine levels in asymptomatic persons. Cancer Epidemiol. Biomarkers Prev. 17, 3450.

Please cite this article as: Tvedt, T.H.A., et al., The importance of sample collection when using single cytokine levels and systemic cytokine profiles as biomarkers — a comparative..., J. Immunol. Methods (2015), http://dx.doi.org/10.1016/j.jim.2015.01.006