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Effect of anticoagulants on 162 circulating immune related proteins in healthy subjects

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ABSTRACT

Diagnosis of complex disease and response to treatment is often associated with multiple indicators, both clinical and laboratorial. With the use of biomarkers, various mechanisms have been unraveled which can lead to better and faster diagnosis, predicting and monitoring of response to treatment and new drug development. With the introduction of multiplex technology for immunoassays and the growing awareness of the role of immune-monitoring during new therapeutic interventions it is now possible to test large numbers of soluble mediators in small sample volumes. However, standardization of sample collection and laboratory assessments remains suboptimal.

We developed a multiplex immunoassay for detection of 162 immune related proteins in human serum and plasma. The assay was split in panels depending on natural occurring concentrations with a maximum of 60 proteins. The aim of this study was to evaluate precision, accuracy, reproducibility and stability of proteins when repeated freeze–thaw cycles are performed of this in-house developed panel, as well as assessing the protein signature in plasma and serum using various anticoagulants.

Intra-assay variance of each mediator was < 10%. Inter-assay variance ranged between 1.6 and 37% with an average of 12.2%. Recoveries were similar for all mediators (mean 99.8 ± 2.6%) with a range between 89–107%. Next we measured all mediators in serum, EDTA plasma and sodium heparin plasma of 43 healthy control donors. Of these markers only 19 showed similar expression profiles in the 3 different matrixes. Only 5 mediators were effected by multiple freeze-thawing cycles. Principal component analysis revealed different coagulants cluster separately and that sodium heparin shows the most consistent profile.

Abbreviations: IL, interleukin; CC, CC-chemokine (beta chemokine); CXC, CXC-chemokine (alpha chemokine); XC, C-chemokine (gamma chemokine); TNF, tumor necrosis factor; IFN, interferon; LIGHT, homologous to lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for binding to herpesvirus entry mediator; APRIL, A Proliferating-Inducing Ligand; TWEAK, TNF related weak inducer of apoptosis; MIF, Macrophage Migration Inhibitory Factor; LIF, Leukemia Inhibitory Factor; OSM, Oncostatin M; CHI3L1/YKL-40, Chitinase-3-like protein; TSLP, Thymic stromal lymphopoietin; LAP, Latency-Associated Peptide of Transforming Growth Factor beta 1 (TGF beta 1); MIC-1/GDF15, Macrophage Inhibitory Cytokine-1; OPG, Osteoprotegerin; OPN, Osteopontin; Dkk1, Dickkopf related protein 1; PAI-1, Plasminogen Activator Inhibitor 1; RBP4, Retinol binding protein 4; FABP4, Fatty acid binding protein-4; TPO, Thrombopoietin; SAA-1, Serum amyloid A1; DPP-IV, Dipeptidyl peptidase IV; G-CSF, Granulocyte Colony-Stimulating Factor; M−CSF, Macrophage Colony-Stimulating Factor; GM−CSF, Granulocyte-Macrophage Colony-Stimulating Factor; SCF, Stem Cell Factor; HGF, Hepatocyte Growth Factor; EGF, Epidermal growth factor; FGF-basic, Fibroblast Growth Factor-basic; NGF, Nerve Growth Factor; BDNF, Brain-Derived Neurotrophic Factor; PlGF, Placenta Growth Factor; CTGF, connective tissue growth factor; VEGF, Vascular Endothelial Growth Factor; ICAM−1, Intercellular Adhesion Molecule 1; VCAM−1, Vascular Cell Adhesion Molecule-1; sCD14, soluble Monocyte differentiation antigen CD14; sCD40L, Soluble CD40-Ligand; sCD163, Scavenger receptor cysteine-rich type 1 protein; MMP, Matrix Metalloproteinase; TIMP-1, Tissue Inhibitor of Metalloproteinases 1; TREM−1, Triggering receptor expressed on myeloid cells 1; PD-1, Programmed Death 1; Fas, Fibroblast-associated; Fas-L, Fibroblast-associated Ligand; LAIR-1, leukocyte associated Ig like receptor 1; IL-18BPa, Interleukin-18-binding protein; IL-1R, IL-1 receptor; TACI, transmembrane activator and calcium-modulator and cyclophilin ligand interactor; Cyst C, Cystatin C; SLPI, Secretory Leukocyte Protease Inhibitor; Tie-2, Tyrosine kinase with Ig and EGF homology domains 2; C5a, Complement 5a; KIM−1, Kidney Injury Molecule-1; ACE, Angiotensin-Converting Enzyme; hs-CRP, high sensitive C-Reactive Protein; THBS-1, Thrombospondin-1; PDGF-BB, Platelet-derived growth factor – BB; HPE, High Performance Elisa buffer

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1. Introduction

Inflammation comprises of a series of coordinated responses to tissue impairment either caused by pathogens or from physical agents such as trauma or radiation. Chronic inflammation in tissue is sustained by activation of both the innate (neutrophils and macrophages) and the adaptive immune system (T and B cells), most commonly via cytokines. As a result of chronic inflammation destruction of tissue may contribute to development and progression of autoimmune disease [1]. Diagnosis of these complex diseases and response to treatment is often associated with multiple indicators, both clinical and laboratorial. With the use of biomarkers, which are measurable indicators used to distinguish precisely, reproducibly and objectively either a normal biological state from a pathological state, or the response to a specific therapeutic intervention [2], important insights into various immune mechanism have been unraveled. This knowledge can lead to new drug development, better diagnosis, and predict and monitor response to treatment. This approach has led to precision medicine in the form of immunotherapies which have seen exceptional advances throughout the past decade for both autoimmune diseases and cancer [3]. These treatments are designed to elicit or amplify an immune response for cancer treatment or reduce and suppress immune reactivity for autoimmune disorders. In addition to this, there is growing awareness of the role of immune-monitoring during these kinds of interventions. The aftermath of the TGN1412 phase I clinical trial in 2006 revealed that the life threatening events were related to up regulation of immune modulatory proteins such as cytokines and chemokines [4,5]. Subsequently, there is a growing need for rapid, accurate, sensitive and reproducible technology. With the introduction of multiplex technology for immunoassays it has been possible to test large numbers of soluble mediators in small sample volumes, with the evident benefits such as reduction of sample volume, but also turnaround time and cost [6–8].

Although standardization has been prominent in day to day clinical practice, standardization of sample collection and laboratory assessments remains suboptimal. Inconsistency in sample collection can affect the results of biological assays and thus several characteristics require thorough evaluation and standardization [9]. This standardization is not limited to assay validity and reproducibility but also pre-analytical treatment and appropriate specimen types. The aim of the study was to evaluate precision, accuracy and reproducibility of an in-house developed panel of 162 immune related markers, including cytokines, chemokines, growth factors, soluble receptors, and metabolic markers. This marker set was chosen based on their potential role in immune related diseases. In addition we explored the expression of these proteins across three different sample types (serum, EDTA plasma and heparin plasma) as well as the effect of freeze-thawing cycles and expression profile in males and females

2. Material and methods

2.1. Serum and plasma collection

Blood samples were collected from 43 healthy anonymous adult volunteers (11 males and 32 females, mean age 42.6 years, range 25–61 years) using the following blood collection tubes: normal clotting tube (SST II Advance, BD Biosciences) for serum, sodium heparin and EDTA tubes for plasma (all BD Biosciences). All samples were collected in the morning, and were kept at room temperature until further processing. Within 4 h after venepuncture all samples were centrifuged and cell free plasma and serum was stored at -80°C until further analysis. All samples obtained were approved for collection by the medical ethics committee of the University Medical Center Utrecht (protocol 07–125/C). Informed consent was obtained from each individual who donated blood samples.

2.2. Protein production in whole blood culture

From 8 donors protein production was induced using heparinized whole blood sample which was stimulated with a combination of 100 ng/ml lippopolysacharide (LPS, Sigma Aldrich, Zwijndrecht, the Netherlands) and 7 μg/ml phytohemagglutinin (PHA, Murex Biotech, Dartford, United Kindom). To prevent any dilution effects 10 μl of stimulus was added to 1 ml whole blood and cultured for 24 and 48 h at 37 °C in 5% CO_2. After culture and centrifugation cell free plasma samples were pooled and frozen at −80 °C until further analysis.

2.3. Pre-analytical preparation

Before analysis all thawed samples were centrifuged through 0.22 μm spin-X filtration columns (Corning, Corning NY USA) to remove debris. Non-specific (heterophillic) antibodies, which may interfere with the assay, were blocked using Heteroblock (Omega Biologicals, Bozeman, MT, USA) as previously described [10,11]. If applicable, samples were diluted in high performance elisa buffer (HPE buffer, Sanquin, Amsterdam The Netherlands).

2.4. Multiplex immunoassay

All 162 coating, biotin labeled detecting antibodies and recombinant proteins were purchased from various commercial sources (supplemental table 1). Magnetic carboxylated polysterene microspheres were purchased from Luminex (Austin, TX, USA). Covalently coupling of the capture antibodies was performed as previously described (50 μg/ml antibody per 6.25×10^6 microspheres [12]. Calibration curves from recombinant proteins were constructed using two-fold dilution steps in serum diluent (Bio-Techne, Abington, United Kingdom). Positive control (biotin coated) and negative control (mouse IL-6, BD Biosciences) microspheres were taken along in each sample as previously described [13]. In house assay procedures were as previously described [12,14,15]. In short, after pre-analytic treatment 50 μl sample was incubated with 10 μl microsphere suspension (500 per mediator) for 1 h. After automated washing (sheath fluid, 0.5% Tween-20, 0.01%NaN_3), 25 μl secondary antibody cocktail (8 μg/ml each) was added and incubated for 1 additional hour and thereafter washed. Next 25 μl of streptavidin R-phycoerythrin (BD biosciences, 25 ng/well) was added and incubated for 20 min. After washing, samples were measured in 100 μl HPE buffer. All incubation steps were performed at room temperature protected from light and with continuous shaking. Acquisition of data was performed using a FlexMAP3D system (Bio-Rad) using xPonent 4.1 software (Luminex). Data analysis was performed using Bioplex manager 6.1.1 (Bio-Rad). All assays were performed at the ISO9001:2008 certified multiplex core facility of the laboratory of translational immunology of the university medical center Utrecht.

2.4.1. Dynamic range standard curve

The assay dynamic ranges were defined by the concentration ranges of the calibration curves covered. To optimize dynamic ranges, we titrated all protein calibrator series (13 points) to a maximum fluorescence intensity of at least 30.000 (FlexMap3D System, Luminex Austin TX USA).

2.4.2. Cross-reactivity

To determine assay cross-reactivity we tested the response of microspheres to single recombinant proteins. Single recombinant proteins were dissolved in HPE buffer and tested at concentrations of 4 times the highest calibration point. Percentage of cross-reactivity was calculated as the ratio of fluorescence intensity in response to a single recombinant protein compared with the maximum fluorescence intensity.

2.4.3. Assay reproducibility

To assess assay reproducibility, we measured sodium heparin