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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

Table 1
Assay performance characteristics.

	Assay working range		Assay sensitivity	Assay Precision		Assay recovery
Mediator	LLOQ(pg/ml)	ULOQ(pg/ml)	LLOD(pg/ml)	Intra-assay CV(%)	Inter-assay CV(%)	Recovery (%)
IL-1RA	8.4	20639	2.5	4	11.5	100.1
IL-1a	2.1	5051	0.6	4.3	4.5	100.5
IL-1b	1.2	2041	0.4	3.4	3.5	100.8
IL-2	4.2	9980	1.3	3.3	5.7	99.5
IL-3	5	20064	1.5	8.4	11.9	97.7
IL-4	0.6	2527	0.2	6.7	8.4	99.2
IL-5	2.9	5064	0.9	8.1	11.2	99.4
IL-6	2.8	10020	0.9	4.4	11	100
IL-7	2.6	5150	0.8	4.4	10.2	101
IL-9	2.5	10140	0.8	2.8	4.9	100.5
IL-10	3.4	10081	1	6	6.8	99.6
IL-11	8	10018	2.4	7.7	12.7	100.6
IL-12p70	6.6	19405	2	8.1	15.9	101.3
IL-13	6	4980	1.8	5.8	10.1	99.1
IL-15	3	5049	0.9	5	7.9	99.7
IL-17	5.7	5049	1.7	5.1	7.8	99.6
IL-17F	18	22312	5.4	5.8	14.8	93.8
IL-18	1.3	4986	0.4	5.1	8.8	99.7
IL-20	84	95784	25	5.5	15.5	100.1
IL-21	16	42008	4.8	6.6	8.2	100.4
IL-22	3.7	10409	1.1	5.1	14.4	101.4
IL-23p19	29	80433	8.9	5.7	9.4	101.3
IL-25/IL-17E	164	103398	49	6.9	10.6	100.3
IL-26	75	199162	22	6	14.9	95.3
IL-27	3.3	9348	1	6.7	13.7	101.5
IL-29	21	20223	6.3	8.2	11.9	101.6
IL-31	24	19987	7.3	5.5	6.4	99.9
IL-33	11	19798	3.3	6.6	7.7	99.8
IL-37	88	88535	27	4.2	9.8	98.7
TNF-a	2.2	4764	0.7	3	7.6	99.6
TNF-b	3	10258	0.9	4.6	12.5	101.8
IFN-a	1.4	2535	0.4	3.3	7.1	100.4
IFN-b[#]	19	13871	5.8	6.7	12.2	100.6
IFN-g	8.8	10217	2.6	4.7	10.8	100.3
LIGHT	8.1	20399	2.4	9.9	10.6	99.9
APRIL	6.1	19028	1.8	2.5	5.6	96.5
TWEAK	10	20711	3	4	16.8	101
MIF	30	20711	9	4.5	10.9	100.9
LIF	9.6	10083	2.9	7.4	10.1	101.2
OSM	6.6	9993	2	8.8	9.4	95.7
CHI3L1/YKL-40	25	37664	7.5	4.3	10.6	103.7
TSLP	2.1	1245	0.6	7.1	14.7	98.2
LAP/TGF-b	4.4	9846	1.3	6.3	6.9	101.1
MIC-1/GDF-15	2.1	2484	0.6	2.7	14.6	95.7
CCL1/I-309	4.3	4942	1.3	5.8	12.6	98.6
CCL2/MCP-1	4.2	5258	1.3	2.3	11.9	100.4
CCL3/MIP-1a	33	18997	9.9	8.6	8.6	98.7
CCL4/MIP-1b	2.7	8929	0.8	4.3	12.7	101
CCL5/RANTES	4.2	4556	1.2	2.9	10.5	101.6
CCL7/MCP-3	11	9391	3.3	4.7	13.8	101.8
CCL8/MCP-2	5.5	6445	1.7	5.8	18	99.3
CCL11/Eotaxin	10	5071	3	5	12.3	101.3
CCL13/MCP-4	17	49860	5	6.4	10.9	101
CCL17/TARC	2.2	4472	0.7	2	8.5	101
CCL18/PARC	12	43402	3.7	1.6	1.6	101.1
CCL19/MIP-3b	3.2	5427	1	9.6	11.4	99.8
CCL20/MIP-3a	1.2	4909	0.4	2.6	14.6	99.3
CCL22/MDC	4.8	4501	1.4	2.6	8	100.5
CCL23/MPIF	2.2	4956	0.6	4.9	18.9	107.8
CCL25/TECK	9.7	20838	2.9	5.6	12.9	93.7
CCL26/Eotaxin-3	3.4	4876	1	5.5	13.7	100.6
CCL27/C-TACK	5	10364	1.5	3.5	8.9	98.7
CCL28/MEC	29	42925	8.8	5.4	16.9	103.6
CXCL1/GRO-a	11	16658	3.2	4.3	15.1	98.3
CXCL4/PF4	3.6	10176	1.1	3.5	6.2	99.5
CXCL5/ENA-78	3.1	10189	0.9	3.5	14	99.4
CXCL6/GCP-2	6.6	19553	2	5.7	18.5	95.5
CXCL7/NAP-2	8.3	19808	2.5	2.5	8.4	105.3
CXCL8/IL-8	4.5	8428	1.3	5.8	13.7	101.7
CXCL9/MIG	4.2	5055	1.2	7.1	12.9	101.4
CXCL10/IP-10	2.5	4916	0.8	2.3	14.7	99.7
CXCL11/I-TAC	2	4836	0.6	2.8	10.7	100.2

(*continued on next page*)

Table 1 (*continued*)

	Assay working range		Assay sensitivity	Assay Precision		Assay recovery
CXCL12/SDF-1a	5.2	5043	1.6	4.6	8.2	93.8
CXCL13/BLC	2.3	4617	0.7	5.4	11.7	99
CXCL14/MIP2g/BRAK	26	49147	7.8	6.9	17	104.5
XCL-1	17	83627	5.1	7.3	11	96.8
OPG	15	39091	4.5	ND	ND	95.7
OPN	49	191064	14	2.7	5.6	102
Scleronstin/SOST	23	40591	7	9	15.3	105.3
Dkk1	7.9	40419	2.4	4.9	7.2	103.8
Adiponectin	272	209885	81	6.5	18.7	99.9
Adipsin	4.9	19646	1.5	7.2	9.9	101.7
Apelin	386	1052341	116	5.4	6.6	96.3
Leptin	16	51790	4.8	4.3	14.6	98.7
Chemerin	135	334763	40	4.1	14.8	99.3
Resistin	6.8	19368	2	3.6	20.8	95.7
PAI-1	807	2163130	242	5.1	17.6	103.1
RBP4	923	1994298	276	4.3	9.9	99.8
FABP-4	80	196086	24	3.3	6.4	101.9
TPO	366	536050	110	4	13.9	100.1
SAA-1	1187	523914	356	4.6	8.3	91.7
DPP-IV/sCD26	18	19457	5.7	2.3	5.9	95.8
G-CSF	40	36880	12	8	9.3	98.5
M-CSF	15	10589	4.6	3.7	9.5	101.9
GM-CSF	15	48011	4.6	7.5	15.1	97.2
SCF	4.5	20188	1.3	7.5	12.9	100.7
HGF	3.6	10172	1.1	7	13.4	98.9
EGF	5.2	10646	1.6	5.1	17	101.5
Amphiregulin	20	4988	5.9	6.2	15.3	100.3
FGF basic	16	80726	4.7	6.8	13.3	99.8
NGF	3.6	10008	1.1	4.9	13.3	98.8
BDNF	5.5	5505	1.7	6.2	13.2	99.5
PlGF	2.7	5034	0.8	6.2	19.7	97.6
CTGF	1818	5374	525	7.9	15	99.1
VEGF-A	4.2	10204	1.2	4.2	14.2	102.1
Lipocalin-2/NGAL	14	10805	4.2	2.6	19.4	95.4
ICAM	32	51799	9.6	5.1	9.2	99.3
VCAM	17	104638	5.1	3.1	13.1	101.4
sCD14	127	219484	38	2.6	2.8	100
CD40L/CD154	7.2	9059	2.2	4.2	19.7	97.6
sCD163	86	167323	27	7	14.7	100.7
MMP-1	7.8	37523	2.3	4	12.6	95.1
MMP-3	5.6	19921	1.7	2.9	17	99.3
MMP-8	11	56539	3.2	2.9	34.6	101.9
MMP-9	203	517791	61	3	5.4	102.7
S100A8	85	400927	26	3.8	21.6	95.7
TIMP-1	21	20280	6.3	2.9	4.5	103.1
TREM-1	4.4	19794	1.3	6.6	7.3	100
Cathepsin A	244	420824	73	3.5	10	95.7
Cathepsin B	40	103666	12	4.9	11.5	99.3
Cathepsin L	3.9	19241	1.2	5.2	16.8	105.3
Cathepsin S	6.1	19895	1.8	2.9	6.2	99.7
PD-1	9.1	50661	2.7	6.8	14.4	100.5
Periostin/OSF-2	1441	164027	432	2.2	9.1	95.9
FAS	24	102096	7.2	3.2	11.6	102.9
FAS-L	17	47277	5.2	6.9	14.5	101.6
LAIR-1	364	50440	109	7.6	11.1	100.3
IL-18Bpa	28	100665	8.6	3.1	15.4	101.9
IL-1R1	5.4	19976	1.6	7.1	2	100.6
IL-1R2	4.2	19981	1.2	5.7	16.9	102.5
IL-1R4/ST-2	1.2	4979	0.6	5.9	26.9	89.9
TNF-R1	8.9	20265	2.7	3.4	7.1	101
TNF-R2	1.4	10169	0.4	3.7	8	99.1
TACI	16	50272	4.8	5.8	12.8	102.7
sIL-2Ra	47	100033	14	5.7	15.4	99.9
sIL-6R	30	20022	8.9	3	18.6	103.1
IL-7R alpha	547	103935	164	4	7.2	100.9
VEGF-R1/FLT1	105	180537	31	6.6	20.2	97.8
sSCF-R	125	98222	37	3.8	17.9	102.6
erythropoetin-R	27	79487	8.2	6.1	23.5	98.3
Galectin-1	29	99076	8.8	3.3	11.9	101
Galectin-3	45	91537	14	5.7	11.3	102
Galectin-7	53	13147	16	9.7	24.3	97.3
Galectin-9	21	50427	6.2	5.9	7.9	99.3
P-selectin	629	676643	188	2.9	7.1	98.6
E-selectin	146	555334	44	3.8	11.5	102.2
Cyst C	147	288428	44	2.2	8.6	101.2

(*continued on next page*)

Table 1 (*continued*)

	Assay working range		Assay sensitivity	Assay Precision		Assay recovery
SLPI	2.9	9519	0.9	0.9	1.9	95.4
Elastase	14	43095	4.3	6.4	14.7	97.6
ElafinTrappin-2	6	4563	1.8	5.8	7	100.4
Angiopoietin 1	9.6	19936	2.9	2.6	15.1	103.1
Angiopoietin 2	20	39248	6.1	4.1	6.4	99.1
Tie-2	15	80188	4.5	4	19.5	102.7
C5a	3.4	19428	1	2.1	13.2	98.3
BDCA3/thrombomodulin	68	205351	43	4.7	11	97.3
THBS-1	5842	773689	1753	6.7	17.3	103.7
PDGF-BB	4.6	10081	1.4	3.4	13.9	97.1
Endoglin	3.9	19894	1.2	3.7	12.3	99
ACE	29	49370	8.6	2.9	6.7	101.7
KIM-1/TIM-1	4.1	10958	1.2	5.6	17.3	99.9
hs-CRP	39	42692	12	1.9	12.5	103.1
Granzyme B	41	24982	12	6	16.8	105.3

Assay working range for each mediator was assessed by calculating the lower limit of detection (LLOD) and quantification (LLOD) as well as the upper limit of quantitation (ULOQ) by running 30 samples over 3 consecutive runs, all expressed as pg/ml, except #IFN-b U/ml. Intra- and inter-assay variation expressed as coefficient of variation (VC = SD/mean). Recovery expressed as percentage was calculated by spiking known amounts of each mediator in heat treated plasma samples.

plasma samples of 8 stimulated and 8 unstimulated healthy controls (see Section 2.2). Each sample was measured in triplicate and at three different time points to assess the intra-assay and inter-assay variation (n = 9). Intra-assay variation is calculated as the mean CV of the triplicates. For reproducibility of the assay the inter-assay variation is determined as the mean CV for 3 consecutive runs. The acceptance criteria for intra- and inter-assay variation are respectively < 10% and < 15% [16].

2.4.4. Freeze-Thaw cycles

The effect of freeze-thawing cycles on the stability of proteins is tested by repeatedly freezing and thawing of four stimulated healthy control plasma's (see Section 2.2). These samples underwent three freeze-thawing cycles. Within each cycle samples were snap-frozen in liquid nitrogen, and subsequently placed on dry ice. Next samples were thawed in hand warm water. The first thawing cycle is set as baseline and a deviation of > 10% from baseline in the following cycles was marked as affected.

2.4.5. Detection limits

The lower limit of detection (LLOD) was calculated using estimates of the detection limit based on instrument and sample preparation variables. We assessed the limit of detection (LLOD) and quantification (LLOQ) for all mediators by calculating the average blank MFI (n = 30 over 3 consecutive runs), MFI standard deviation (SD), and 2∗SD. LLOD's were determined by interpolation of the mean blank MFI plus 2∗SD in the 5-parameter logistic standard curves. The LLOQ was calculated as 3∗LLOD. The upper limit of quantitation (ULOQ) was calculated from mean-3 SD of 10 replicates of highest standard point [17].

2.4.6. Linearity of the assay

To assess assay linearity, four samples were spiked with recombinant in protein calibrator and serial dilutions were made with HPE buffer In addition four stimulated plasma samples were also diluted in HPE buffer. Neat sample measurements (baseline) were set at 100%. For high (natural occurring) concentrations of various proteins, pre-dilutions of 1:10, 1:100 and 1:1000 were made to produce samples within the dynamic range of the assay, set on sodium heparin plasma.

2.5. Statistical analysis

All results are expressed as mean of the observed concentration ± SD For calculation of freeze–thaw cycles baseline values were set at 100% and follow-up points were related to this baseline value. Difference between the three different blood collection tubes are analyzed using a one-way ANOVA. A probability (p) value less than 0.05 was considered significantly different. Statistical analysis were performed using the statistical package for social sciences (SPSS) software version 21.0.0 (IBM). For comparison of the different matrices unsupervised heat maps were generated by normalizing all data between minimum (bleu) and maximum (red) for each individual marker using Omniviz 6.1.2.0 (Instem Scientific) and a unsupervised principal component analysis (PCA) was performed using R (version 3.3.2, The R Foundation for Statistical Computing, Vienna, Austria). Graphical visualization of the first and second principal component was plotted using the ggplot2 package.

3. Results

3.1. Assay development and validation

To develop this assay all recombinant protein–antibody combinations were optimized in single bead assays before stepwise expanding the assay with 5 mediators each time into in a multiplex panel. When more than 65 targets were mixed together in any given order we observed that the peak fluorescence intensity of the highest standard curve points were reduced and that the sensitivity was lost at the lower part. Therefore all further assays were performed with a maximum of a 60-plex assay, unless otherwise indicated.

In order to investigate potential cross-reactivity, a full mixture of microspheres and detection antibodies was incubated in the presence of a single protein at a concentration of at least 4 times the highest point used for creating standard curves. At this concentration no cross-reaction was observed with exception of 3 mediators, CCL2 was detected by CCL11 (4.4 ± 1.3%) and CCL13 (10.6 ± 3.1%), MMP1 was detected by MMP8 (5.8 ± 1.7%), and CCL3 was detected by CCL4 (49.1 ± 7.8%). Confirmatory assays were performed using single potential cross-reacting mediators at a concentrations used to create standard curves. Using this lower concentration, cross-reaction of CCL11, CCL13 and MMP8 was not detectable. Cross-reaction between CCL3 and CCL4 was still present, though significantly reduced to 9.3 ± 4.1%.

The LLOD and LLOQ were calculated for each individual mediator and showed that the majority of mediators could be measured in their low physiological range (Table 1). Intra- and inter-assay variance, expressed as coefficient of variation (CV), was calculated using 8 plasma samples (either stimulated or unstimulated). Intra-assay variance, measured in triplicate over 3 different time points were all < 10% with an average of 5.0% (Table 1). Unexpectedly OPG could not be assessed as all microspheres coagulated together in stimulated sodium heparin plasma, though this phenomena was not observed in normal biological

samples. Inter-assay variance was assed using the same control samples run over 3 different time points. Variance ranged between 1.6 and 34.6% with an average of 12.1% (Table 1). Thirty-seven mediators expressed a CV > 15% of which 7 expressed a CV > 20% (resistin 20.8%, MMP8 34.6%, S100A8 21.6%, IL-1R4/ST-2 26.9%, VEGF R1 20.2%, erythropoetin-R 23.5%, and galectin-7 24.3%).

Recovery was only assessed by spiking known amount of recombinant proteins in heat treated serum samples (n = 10). After a subtraction of their corresponding unspiked sample, recovery was calculated. Recoveries were similar for all mediators (mean 99.8 ± 2.6% Table 1) with a range between 89.9% and 107.8%.

Before assessing linearity of dilution, a trial run was performed with 5 sodium heparin plasma samples to set out dilutions for proteins which natural concentrations are above ULOQ or proteins which display a prozone effect, resulting in false negative values or inaccurately low results. Several proteins required pre-dilution (1:10, 1:100 or 1:1000) before assessing linearity of dilution in spike in plasma (supplemental table 2). Overall recovery after linear dilutions in the various matrices used is 101 ± 4% with the largest variation in complex matrices with increased dilutions (1:1 range 91–106%, 1:2 range 86–110%, 1:4 range 87–111% 1:8 range 85–119%, supplemental table 2).

3.2. Protein stability upon freeze-thawing

Next we questioned how stability of the various proteins was influenced by freeze-thawing cycles. (Next we questioned the influence of freeze-thawing cycles on the stability of the various proteins) Therefore four stimulated sodium heparin plasma samples underwent three freeze–thaw cycles. No deviation, defined as < 10% difference of baseline, in protein expression was observed in 157 mediators up to 3 cycle (overall recovery 1st cycle 102.3%, 2nd cycle 102.0%, 3rd cycle 102.8%). Four proteins were affected after 1 cycle (recovery IL-29 121 ± 50%, Galectin-7 54 ± 20%, OPG 112 ± 8%, p-selectin 119 ± 7%), and CCL5 was affected after 2 freeze-thawing cycles (recovery 114 ± 12%).

3.3. Effect of anticoagulant on immune protein profiling

To assess the impact of anticoagulant, serum, EDTA plasma and sodium heparin plasma was collected from 43 healthy donors and analyzed for all 162 markers, using the various dilutions as described above (for details see supplemental table 2). Considerable variation consisted between serum, EDTA and heparin plasma in detectable protein levels (Fig. 1). Sixty-six markers were not detectable in various individuals in the different matrices of which in some cases, various mediators, were not detected in more than 75% of the population (EDTA plasma n = 1, serum n = 6 and sodium heparin plasma n = 22, Fig. 1). Overall heparin plasma yielded 81.6% of data above LLOD, serum 88.6% and EDTA plasma 96.5%.

Only 19 markers showed similar expression profiles without any significant differences between the three different matrixes (IL-33, CCL11, CCL19, CCL23, CXCL12, adiponectin, adipsin, apelin, leptin, chemerin, leptin, PAI-1, FABP4, TPO, SAA-1, cathepsin-L, PD-1, TNF-R1, TACI and hs-CRP supplemental table 3). All other mediators displayed significant different levels between the various matrices used, of which EDTA plasma and heparin plasma 29 mediators (18%) alike EDTA plasma and serum 58 mediators (36%) alike, and serum and heparin plasma display similarity for 59 markers (37%) in expression profile (Fig. 2A, supplemental table 3). To visualize the differences in protein signature between the anticoagulants, data was plotted in a heat map (Fig. 2A). Due to the use of similar dilutions factors, CXCL4 and Elastase could only be partially assessed. CXLC4 was above ULOQ in serum as well as the majority of EDTA plasmas, whereas Elastase was above ULOQ in sodium heparin plasma (Fig. 2A).

To underscore the differences between the anticoagulants, we performed a principal component analysis which is visualized in Fig. 2B. This analysis shows that the different coagulants cluster separately and that sodium heparin shows the most consistent profile compared to serum and EDTA plasma, of which the latter one shows the most variability among the individuals (Fig. 2B).

3.4. Difference between sexes

Although this population of healthy control donors consists of predominately females (f/m ratio 3:1) there are significant differences in expression profiles between sexes in the various blood drawing tubes. As expected a significant higher expression of adiponectin and leptin was present in females regardless of anti-coagulant ($p < .001$ for all). Furthermore males expressed a significant higher level of MMP3 in serum and sodium heparin plasma (both $p < .001$) and CCL28 in serum and EDTA plasma (both $p < .01$). Individual differences were found in serum for CD40L $p < .01$. TWEAK ($p < .01$) and IL-7Ra ($p < .05$) in EDTA plasma and NGF ($p < .01$), HGF ($p < .01$), sIL-2Ra ($p < .05$) and endoglin ($p < .05$) in sodium heparin plasma.

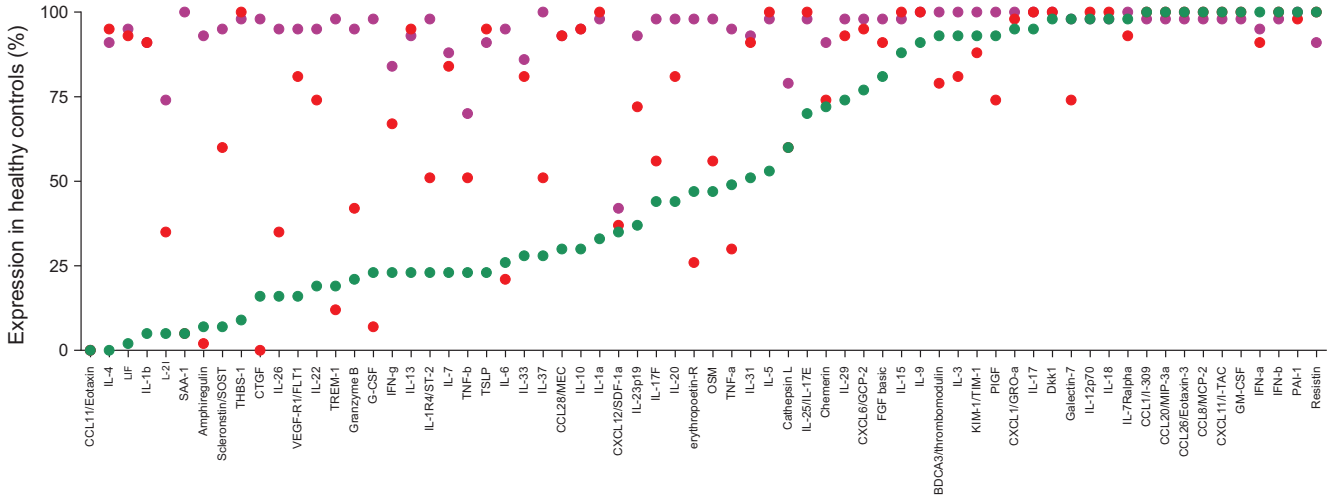

Fig. 1. Differences between presence of mediators in serum, EDTA plasma and heparin plasma. Protein profiles were measured in serum (red) EDTA plasma (purple) and Sodium heparin plasma (green) of 43 healthy controls. Shown are 66 mediators that did not have a full spectrum of detectable protein profile in the various anticoagulants. The presence of each individual mediator above the LLOD isshown as percentage of expression. In sodium heparin plasma 22 mediators are undetectable in 75% of healthy controls, whereas respectively n = 1 and n = 6 mediators for EDTA plasma and serum could not be detected. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

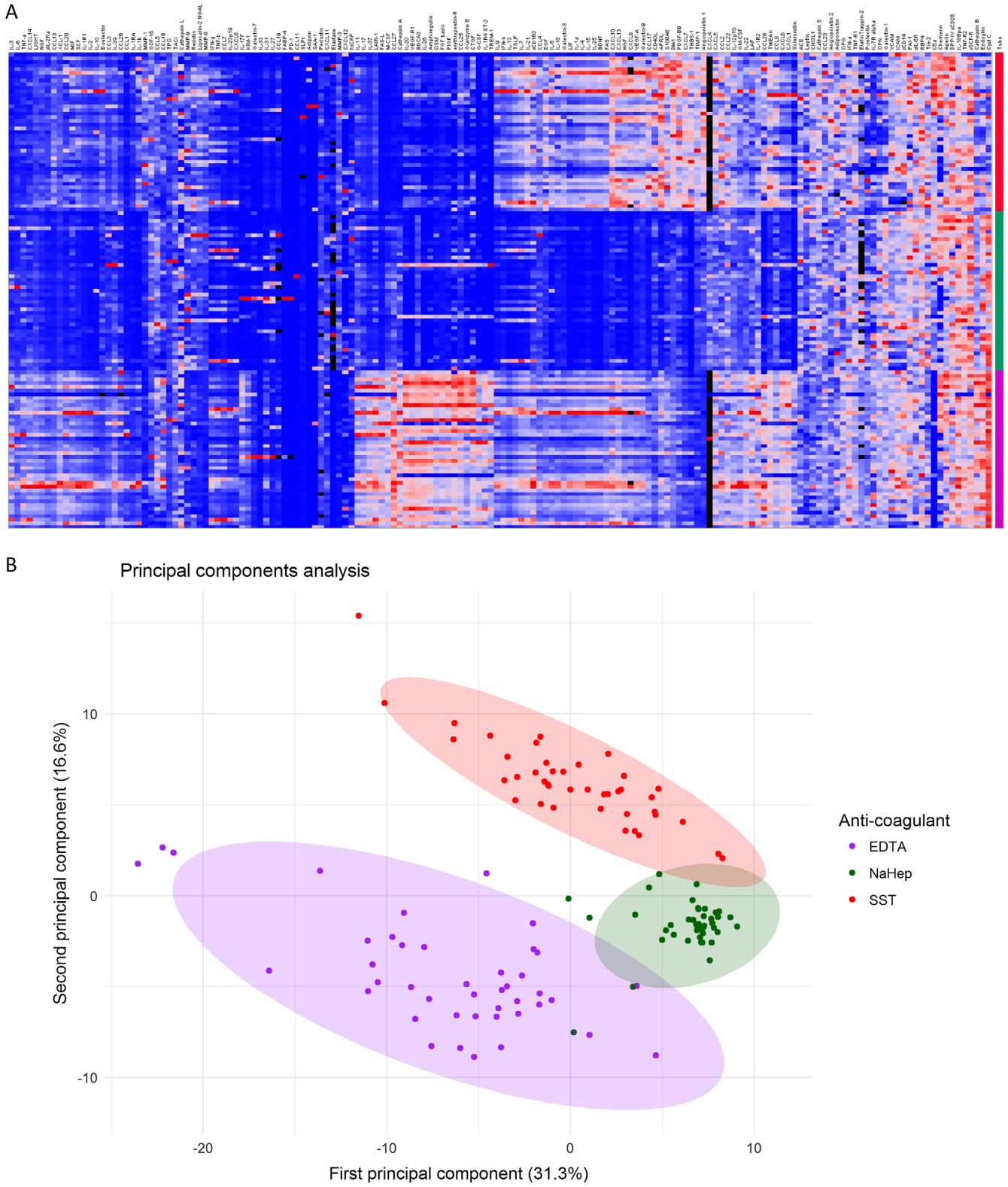

Fig. 2. Protein expression patterns in serum, sodium heparin plasma and EDTA plasma. 2A) Expression patterns were plotted as heat map showing all various markers in serum (red), sodium heparin plasma (green) and EDTA plasma (purple) of healthy individuals (n = 43). Data was normalized per individual marker (bleu; lowest expression, red; highest expression, black; > ULOQ using similar dilutions). 2B Next PCA was applied on the data set, showing that the various anticoagulant cluster together and the sodium heparin plasma (NaHep/green) displays the lowest variability between the healthy control population compared to serum (SST/red)) and EDTA plasma (EDTA/purple). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Observed concentrations of all mediators in serum, sodium heparin and EDTA plasma.

		Serum	range		EDTA	range		Na HEP	range	
Mediator		mean	min	max	mean	min	max	mean	min	max
IL-1RA	pg/ml	449	133	1975	641	63	4452	155	77	413
IL-1a	pg/ml	19	1.2	61	19	0.4	65	1.0	<	10
IL-1b	pg/ml	2.6	<	9.4	2.3	<	12	<	<	1.0
IL-2	pg/ml	1.4	<	6.0	2.6	<	10	<	<	2.4
IL-3	pg/ml	4.1	<	17	38	5.6	105	11	<	72
IL-4	pg/ml	1.5	<	4.4	1.5	<	4.8	<	<	0.3
IL-5	pg/ml	24	2.7	59	23	<	73	3.4	<	31
IL-6	pg/ml	0.8	<	4.1	2.1	<	7.0	1.0	<	8.7
IL-7	pg/ml	2.0	<	6.0	2.1	<	5.2	<	<	5.6
IL-9	pg/ml	20	2.2	81	23	1.1	75	3.4	<	13
IL-10	pg/ml	7.5	<	44	8.4	<	31	1.5	<	23
IL-11	pg/ml	27	5.8	92	78	4.0	179	31	6.6	107
IL-12p70	pg/ml	12	<	24	13	<	34	7.0	<	13
IL-13	pg/ml	11	<	46	9.9	<	30	3.1	<	32
IL-15	pg/ml	15	2.6	42	14	<	40	1.8	<	6.1
IL-17	pg/ml	10	4.6	18	37	2.6	84	11	3.6	48
IL-17F	pg/ml	34	<	206	443	<	1017	71	<	1596
IL-18	pg/ml	15	1.1	32	16	0.4	51	2.7	0.5	6.4
IL-20	ng/ml	1.1	<	5.4	6.3	<	10	0.4	<	7.6
IL-21	pg/ml	1816	286	4126	1536	164	3162	395	68	2472
IL-22	pg/ml	1.5	<	2.8	2.6	<	5.7	<	<	3.3
IL-23p19	pg/ml	31	2.4	291	63	4.5	455	39	1.1	407
IL-25/IL-17E	pg/ml	1463	210	4079	1409	48	4247	65	29	154
IL-26	ng/ml	2.8	<	27	45	<	75	2.6	<	55
IL-27	pg/ml	214	21	2062	405	60	5724	791	51	4603
IL-29	pg/ml	12	<	30	20	<	49	11	<	63
IL-31	pg/ml	28	<	302	69	<	378	46	<	481
IL-33	pg/ml	12	<	148	24	<	635	27	<	458
IL-37	pg/ml	27	<	59	181	<	366	23	<	112
TNF-a	pg/ml	0.7	<	2.9	2.6	<	7.3	1.0	<	3.7
TNF-b	pg/ml	1.4	<	6.3	2.2	<	9.4	1.2	<	8.9
IFN-a	pg/ml	1.5	<	4.2	2.1	<	4.0	1.7	0.5	4.1
IFN-b	U/ml	81	15	354	99	<	255	31	6.4	155
IFN-g	pg/ml	5.5	<	30	7.0	<	25	3.0	<	26
LIGHT	pg/ml	14.1	5.9	27	30	<	92	12	5.1	39
APRIL	pg/ml	2458	939	3623	1200	183	2446	212	69	1459
TWEAK	ng/nl	11	<	25	12	<	17	2.6	<	12
MIF	pg/ml	153	19	557	240	10	703	67	18	186
LIF	pg/ml	33	<	115	35	<	120	1.5	<	12
OSM	pg/ml	3.1	<	14	34	<	70	4.0	<	40
CHI3L1/YKL-40	ng/ml	25	7.2	51	16	4.7	46	21	8.6	56
TSLP	pg/ml	3.1	0.3	10	2.8	0.1	7.2	0.8	0.0	6.8
LAP/TGF-b	ng/ml	3.3	0.04	12	64	0.2	13	2.8	0.02	7.9
MIC-1/GDF-15	pg/ml	566	209	1813	402	81	2047	508	164	1533
CCL1/I-309	pg/ml	20	5.0	71	113	<	693	13	4.6	26
CCL2/MCP-1	pg/ml	214	56	395	171	10	423	93	27	197
CCL3/MIP-1a	pg/ml	463	132	1614	688	70	1658	448	49	3216
CCL4/MIP-1b	pg/ml	348	119	764	331	22	805	199	83	1305
CCL5/RANTES	ng/ml	42	6.0	96	62	8.3	146	57	7.5	204
CCL7/MCP-3	pg/ml	138	24	896	221	53	1066	165	34	1044
CCL8/MCP-2	pg/ml	44	<	111	39	<	65	18	<	55
CCL11/Eotaxin	pg/ml	<	<	<	<	<	<	<	<	<
CCL13/MCP-4	pg/ml	101	39	214	108	10	412	26	14	42
CCL17/TARC	pg/ml	120	18	258	61	4.5	115	9.9	4.1	23
CCL18/PARC	ng/ml	77	8.6	294	144	8.4	470	64	2.9	38
CCL19/MIP-3b	pg/ml	206.0	2.7	1826	205	28	1130	180	4.2	2862
CCL20/MIP-3a	pg/ml	50	4.9	157	98	0.8	274	12	3.9	36
CCL22/MDC	pg/ml	882	335	2051	824	69	1573	623	203	1530
CCL23/MPIF	pg/ml	314	5.1	960	293	120	677	283	3.5	907
CCL25/TECK	pg/ml	625	<	2182	2378	238	4336	432	15	3046
CCL26/Eotaxin-3	pg/ml	83	7.9	254	184	22	720	76	12	321
CCL27/C-TACK	pg/ml	89	34	207	2067	95	2972	469	94	1043
CCL28/MEC	pg/ml	412	<	1075	547	<	1094	53	<	895
CXCL1/GRO-a	pg/ml	261	<	961	530	31	1072	87	<	643
CXCL4/PF4[#]	pg/ml	>	>	>	>	69	>	1448	358	3826
CXCL5/ENA-78	ng/ml	1.1	0.3	2.4	1.0	0.04	2.2	0.5	0.2	1.1
CXCL6/GCP-2	pg/ml	266	<	1045	510	<	848	75	<	585
CXCL7/NAP-2	µg/ml	10	6.7	19	3.2	1.5	7.5	1.7	0.8	3.3
CXCL8/IL-8	pg/ml	391	56	3952	486	24	3006	271	27	3026
CXCL9/MIG	pg/ml	1800	264	4076	1341	62	3724	64	32	153
CXCL10/IP-10	pg/ml	4420	1346	5991	3125	119	7225	651	219	2098
CXCL11/I-TAC	pg/ml	94	4.8	364	217	71	1094	292	11	1842
CXCL12/SDF-1a	pg/ml	228	<	2816	198	<	2263	210	<	2598

(*continued on next page*)

Table 2 (*continued*)

		Serum	range		EDTA	range		Na HEP	range	
CXCL13/BLC	pg/ml	743	59	1296	603	20	1432	74	8.3	526
CXCL14/MIP2g/BRAK	pg/ml	857	358	1917	2255	326	6594	992	301	2872
XCL-1	pg/ml	228	90	409	468	23	1275	117	67	213
OPG	pg/ml	2009	440	4742	1938	718	2912	1265	151	3607
OPN	ng/ml	13	1.8	56	25	15	40	26	5.1	63
Scleronstin/SOST	pg/ml	145	<	1242	384	<	750	16	<	484
Dkk1	pg/ml	504	315	841	191	56	310	105	<	199
Adiponectin	µg/ml	111	20	267	103	15	204	109	18	281
Adipsin	ng/ml	2.6	1.7	3.5	2.7	1.7	3.6	2.6	1.5	3.5
Apelin	pg/ml	119	<	2664	<	<	<	<	<	<
Leptin	ng/ml	3.9	1.7	9.4	2.7	1.6	4.9	3.1	1.7	6.2
Chemerin	ng/ml	464	<	106	49	<	112	43	<	94
Resistin	ng/ml	3.6	<	11	1.3	<	5.9	2.7	0.09	14
PAI-1	µg/ml	0.6	0.1	13	0.5	1.2	11	0.6	0.1	13
RBP4	µg/ml	18	13	23	15	9.0	24	17	12	210
FABP-4	ng/ml	11	1.6	39	89	11	198	22	8.1	88
TPO	ng/ml	170	104	641	164	101	653	164	93	658
SAA-1	ng/ml	9.6	0.0	289	9.0	0.0	221	7.3	0.0	187
DPP-IV/sCD26	ng/ml	845	323	1269	768	270	1111	813	299	1262
G-CSF	pg/ml	2.8	<	53	546	<	1271	37	<	556
M-CSF	pg/ml	79	50	13	1139	135	2504	301	70	664
GM-CSF	pg/ml	146	43	50	197	5.2	513	80	35	181
SCF	pg/ml	217	33	743	314	5.8	997	57	17	211
HGF	pg/ml	3193	261	6062	2154	72	6442	327	58	1745
EGF	pg/ml	151	23	390	112	4.3	306	36	8.8	82
Amphiregulin	pg/ml	0.9	<	6.2	17	<	30	1.5	<	20.0
FGF basic	pg/ml	14	<	78	117	5.4	222	11	<	141
NGF	pg/ml	102	21	213	204	6.1	713	59	18	222
BDNF	pg/ml	2197	301	6225	2077	60	6299	65	30	179
PlGF	pg/ml	45	<	313	472	11	912	51	<	494
CTGF	pg/ml	67	<	209	302	<	539	49	<	330
VEGF-A	pg/ml	281	163	6473	2093	53	6645	381	52	1196
Lipocalin-2/NGAL	ng/ml	149	64	305	53	23	94	112	31	531
ICAM	ng/ml	541	100	1187	607	123	1117	510	134	1105
VCAM	ng/ml	1331	210	3231	1486	222	3317	1223	213	2663
sCD14	ng/ml	599	25	1186	665	68	1131	538	71	1307
CD40L/CD154	pg/ml	1254	117	2652	530	214	1143	360	74	792
sCD163	ng/ml	85	14	216	92	7.0	221	40	13	184
MMP-1	ng/ml	24	3.2	85	6.6	1.5	41	6.7	2.0	22
MMP-3	ng/ml	17	7.4	86	3.1	0.9	17	15	1.5	>
MMP-8	pg/ml	2118	999	4606	918	634	1620	1622	624	7715
MMP-9	ng/ml	1988	100	8296	920	271	6352	1879	261	8153
S100A8	ng/ml	5.9	2.2	12.5	4.0	1.0	8.6	1.5	0.4	5.2
TIMP-1	ng/ml	136	85	265	61	41	122	66	40	160
TREM-1	pg/ml	1.5	<	36	680	<	1601	50	<	1660
Cathepsin A	pg/ml	584	<	1441	12871	2427	25177	3421	472	13204
Cathepsin B	ng/ml	9.9	<	36	22	6.1	36	11	2.9	24
Cathepsin L	ng/ml	1.6	<	6.0	1.7	0.0	5.3	1.4	<	4.9
Cathepsin S	ng/ml	8.2	4.8	15	5.5	2.8	8.2	6.8	3.0	12
PD-1	ng/ml	7.8	0.4	123	8.0	0.6	47	223	0.4	9311
Periostin /OSF-2	ng/ml	135	22	977	162	87	381	174	59	664
FAS	ng/ml	8.1	1.3	22	8.5	0.6	21	1.5	0.5	3.7
FAS-L	pg/ml	28	15	53	65	45	1338	49	18	153
LAIR-1	ng/ml	3.1	1.5	7.0	11	1.4	19	2.4	0.6	8.8
IL-18Bpa	ng/ml	1.8	0.1	2.8	2.1	0.08	3.3	2.0	0.08	3.3
IL-1R1	ng/ml	0.7	0.2	3.6	1.2	0.06	4.2	0.2	0.08	0.8
IL-1R2	ng/ml	1.1	0.3	2.9	2.9	0.2	6.0	1.7	0.4	3.3
IL-1R4/ST-2	pg/ml	16	<	135	217	<	474	9.4	<	200
TNF-R1	ng/ml	3.4	0.7	7.5	2.9	0.4	5.3	3.1	1.3	5.4
TNF-R2	ng/ml	1.1	0.2	1.6	1.0	0.05	1.7	1.3	0.5	2.1
TACI	ng/ml	2.3	14	5.6	2.4	1.3	5.5	2.3	1.2	6.3
sIL-2Ra	pg/ml	309	109	811	574	32	2414	151	68	851
sIL-6R	ng/ml	20	13	31	18	11	28	20	14	31
IL-7R alpha	ng/ml	16	0.0	80	46	1.1	120	33	0.0	118
VEGF-R1/FLT1	ng/ml	1.7	0.0	8.3	11	0.0	180	0.5	0.0	10
sSCF-R	ng/ml	260	1.8	39	25	1.8	38	28	3.5	42
erythropoetin-R	pg/ml	63	0.0	667	778	0.0	1304	141	0.0	1037
Galectin-1	ng/ml	8.8	<	23	11	<	16	15	<	30
Galectin-3	ng/ml	11	<	344	8.9	<	25	2.1	<	5.1
Galectin-7	pg/ml	273	<	5956	1946	13	21206	1414	<	18363
Galectin-9	ng/ml	26	<	59	18	<	49	4.4	<	7.8
P-selectin	ng/ml	35	12	54	23	3.9	61	23	11	40
E-selectin	ng/ml	29	8.5	1166	21	9.0	65	15	4.3	37
Cyst C	ng/ml	36	0.9	105	99	1.9	117	78	15	113
SLPI	ng/ml	18	0.5	45	5.0	1.4	13	10	1.3	33

(*continued on next page*)

Table 2 (*continued*)

		Serum	range		EDTA	range		Na HEP	range	
Elastase	ng/ml	115	53	165	9.1	0.2	27	217	0.6	1712
ElafinTrappin-2	ng/ml	2.0	0.4	6.1	2.1	0.0	3.1	3.8	0.1	8.4
Angiopoietin 1	ng/ml	32	3.7	59	5.0	0.3	16	8.6	1.1	20
Angiopoietin 2	ng/ml	2.1	0.4	4.9	1.2	0.2	2.7	1.2	0.1	2.9
Tie-2	ng/ml	1.5	0.7	2.8	1.1	0.4	2.2	1.3	0.4	2.5
C5a	ng/ml	56	22	101	22	0.5	31	38	0.8	100
BDCA3/thrombomodulin	pg/ml	1284	0.0	3545	7520	948	12390	1757	0.0	8133
THBS-1	µg/ml	99	25	198	22	0.0	108	1.0	0.0	22
PDGF-BB	ng/ml	10	4.3	18	4.1	1.7	9.9	2.9	1.0	5.7
Endoglin	pg/ml	584.3	39.9	1299.6	1135.9	537.0	1719.3	1004.9	270.6	1656.8
ACE	ng/ml	195	76	439	158	63	366	183	76	421
KIM-1/TIM-1	pg/ml	181	<	1072	1270	6.1	5754	299	<	6664
hs-CRP	ng/ml	1889	77	14218	1463	67	11377	1768	75	14075
Granzyme B	pg/ml	19	<	115	141	<	312	19	<	187

Observed concentrations (mean value and range) in serum sodium heparin plasma and EDTA plasma of 43 healthy controls. < = < LLOD, > = > ULOQ. #; CXCL4 was run in similar dilutions set on found concentrations in sodium heparin plasma (see supplemental table 2) which resulted in > ULOQ for all EDTA plasma and Serum samples.

4. Discussion

With the use of ELISA, the classical gold standard immunoassay, target discovery of immune modulatory switch points have been developed in a variety of autoimmune diseases and cancer. This has led to the introduction of anti-cytokine therapy by administration of anti-IFN antibodies in rheumatoid arthritis in 1974 [18]. Since then, many effective immunotherapeutic treatments using fluorescent bead based assays have been developed replacing the classical ELISA [19,20]. With the use of this multiplex technology, discovery and monitoring of soluble biomarkers for complex disease phenotypes can be easily achieved, which can be beneficial for diagnosis, immune-monitoring for treatment, disease progression, discovery of potential new drug targets and personalized medicine. Although to date surrogate protein markers for complex diseases are constrained, a multi biomarker disease activity test for rheumatoid arthritis has been developed and implemented in day to day use [10,21]. The growing interest in multiplex technology also reveals the need for standardization and validation for both technology and sample handling [8,9]. Since 1990 regulatory agencies such as the Food and Drug Administration (FDA) and European Medicines Agency (EMEA) require stringent validation assays for bioanalytical methods relevant to either drug registration or modifications, which involve accuracy, precision and reproducibility [16]. In this validation study, we show that with use of an open structure bead based technology the majority of immune related markers can be measured reliably in complex matrices such as serum and plasma with low assays variation, good reproducibility from well-to-well, and day to day (Table 1). Less than 5%, cross reactivity was observed between two structurally related chemokines, namely CCL3 and CCL4. However, it should be noted that the cross-reaction observed of these recombinant proteins are at concentrations that exceeds physiological concentrations observed in plasma and serum, and thus, thereby reducing the chance of cross-reactivity in physiological samples.

To reduce variation due to the matrix effects, it is important to process the samples as quickly as possible by separating the plasma or the serum from cellular components and block interference during the assay of heterophilic antibodies [8,11,15,22]. Especially the later one gives rise to difficulties in immunoassays, resulting in false positive values [23]. As part of FDA criteria for assay validation this issue needs to be addressed, and therefore all samples were blocked using a commercially available blocking agent. We are aware that in many day-to-day clinical settings, and especially in multi-institutional trials, the handling procedures reflects the unavoidable limitations inherent in transferring patient samples from a clinic to a central laboratory capable of standardized processing. Although temperature and delay of sample handling will affect biomarker concentration [8,24], due standardization this effect can be minimized [9]. In our setting, samples were processed in a very well controlled environment, resulting in storage of all samples at −80 °C within 4 h after collection, though when longer processing times are expected samples can be placed on ice to keep the biomarker profile stable [8,15,25].

In this study we observed remarkably stable expression of the majority of proteins after 2 freeze-thaw cycles. Again, as these experiments were performed in a controlled environment, it could well be that, with prolonged time (several years) between various freeze-thawing cycles, protein levels will be affected.

In line with others, we demonstrate that the concentration of soluble mediators measured is depended on type of anticoagulant used [7,22,26–28]. We measured proteins signatures in various sequentially taken blood samples from healthy individuals, which were prepared by either spontaneous clotting for serum and two different methods of anticoagulation to create plasma, commonly used in day to day clinical practice. The coagulation cascade is a complex biochemical process which involves activation of platelets and other cellular components, resulting in release of various proteins. On the contrary, several proteins (such as fibrinogen) are used or secluded during the coagulation process. This results in differential expression patterns that are comparable to various plasma types (Fig. 2), which mainly involves typical platelets related markers such as IL-6, CCL5, CXCL4, CXCL8 and VEGF-A [15,26–28]. To rule out the effect of platelets, plasma might be better to mirror the protein profile *in vivo*. EDTA plasma is generated by chelating calcium, whereas heparin exerts its effect by binding to antithrombin III [29]. By chelating calcium, EDTA results in hypocalcemia which induces a cellular stress reaction resulting in increased levels of IL-1RA, TNFa, G-CSF and M-CSF (Table 2) [30,31]. Indeed various studies, including this one, these observations are noted, most strikingly for G-CSF in our dataset which a 200 fold higher in EDTA plasma (Table 2) compared to serum and heparin plasma [28].

In this study heparin plasma yields the most undetectable cases in our healthy cohort compared to serum and EDTA plasma (Fig. 1), however, principal component analysis shows that sodium heparin samples show the least variability in this cohort. This is in line with the study of Wong et al. and Hosnijeh et al. [26,32] but in contrast with other studies in which heparin yields the highest values [6,28]. The difference between these studies is the use of either lithium or sodium heparin. Indeed it has been shown that lithium induces a strong inflammatory response via NFκB activation, which results in the release of various immune related proteins (such as IL-6, and TNFa) from a variety of circulating cells [33,34]. Remarkably OPG could not be detected in sodium heparin stimulated blood. One explanation could be that sodium heparin can form micro precipitates which impacts the sample performance [35].

Overall in this cohort more than 80% of all proteins detected are significantly different in the various plasma and serum samples. The

proteins which express similar values across the various biological sample types are mainly the acute phase reactants such as CRP and SAA-1, and the various adipokines, such as leptin and adiponectin. Interestingly, regardless of the anti-coagulant used, the latter ones are, as expected, different between males and females. However, some other unrelated markers are differentially expressed between males and females, which is important to note especially in cohorts were one of the sexes is dominant, such as various autoimmune diseases. Although these differences are depending on which anticoagulant is used it underscoring the effect of anticoagulant used. In conclusion, xMAP technology offers the opportunity to develop and validate in-house assays for a wide variety of mediators to be tested in a single sample. Acceptable well to well and day to day reproducibility are obtained in complex matrices such as serum and plasma. However among other exogenous factors such as assay handling protocols, the expression of mediators is under influence of anti-coagulant used. Our data indicates that sodium heparin plasma might be the favorable matrix to be used for this technology as it shows the most consistent protein profile, with the least differences between males and females compared to serum and EDTA plasma. In addition our data indicates that up to two freeze thawing cycles will have minimal effect on the biomarker profiles.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2017.10.021.

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