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Short communication

Detection and quantitation of twenty-seven cytokines, chemokines and growth factors pre- and post-high abundance protein depletion in human plasma

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ABSTRACT

Cytokines, chemokines and growth factors (CCGFs) in human plasma are analyzed for identification of biomarkers. However concentrations of CCGFs are very low; it is difficult to identify and quantify low abundance proteins in the presence of the high abundance proteins (HAPs) unless HAPs are removed prior to analysis. However, there is a concern that the low abundance proteins such as CCGFs may also be removed during the HAP depletion process. In this study, we have examined whether or not depletion of the HAPs enhances detection of the CCGFs by immuno-assays. Top 14 HAPs were depleted from 10 healthy volunteers' plasma using MARS-14 immuno-depletion column and a total of 27 CCGFs were analyzed by bead-based multiplexed immuno-assay. All 27 CCGFs were detected in neat plasma (NP), 25 were detected in flow through fraction (FT) and 21 were detected in bound protein (BP) fraction. Concentrations of 22 CCGFs were significantly higher in NP compared to FT and BP. Only one CCGF had higher concentration in FT compared to NP. The remaining 2 CCGFs were not different between NP and FT. It was counter-productive for the detection of 24 CCGFs after HAP removal, primarily due to post-depletion protein precipitation and/or re-suspension of pellets.

Human plasma is a protein rich complex biofluid that represents a comprehensive snapshot of the processes occurred in the body [1]. Concentrations of cytokines, chemokines and growth factors (CCGFs) in plasma have often been correlated with disease progression and continue to be a source of candidate biomarkers for a range of conditions. A recent

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Table 1 – A summary of the depletion techniques and outcomes of high abundance protein depletion on downstream analysis.

Depletion method	Analysis method	No. of non-specific proteins depleted	Outcome of HAP depletion	Refs.
Aurum Serum Protein Minikit, ProteoExtract, Albumin/IgG, MARS, POROS Albumin/IgG Removal Kit	1-D & 2-D gels, MALDI MS/MS	Not analyzed	Increased number of protein spots appeared on 2-D gels, not all spots identified by MALDI MS/MS	[6]
Albumin depletion kits from Viva Science, Millipore, ABI, and MARS 6	1-D gels and 2-D DIGE gels, MALDI MS/MS	0	76% increase in spot detection in MARS columns depleted plasma compared to crude plasma	[7]
ProteoExtract Albumin/IgG removal Kit, MARS 6, Proteome Lab IgY-12	2-D gels, Antibody micro-arrays	115 spots on bound fraction of IgY-12	234 spots detected in IgY-12 depleted plasma compared to 159 spots in crude plasma in the pH range 4–7	[8]
MARS 6, Cibacron blue	2-D gels	Not analyzed	Modest increase in number of spots on 2-D gel in MARS 6 depleted plasma	[9]
Mimetic blue column	2-D gels, MALDI MS	Several plasma proteins	Visualization of new proteins due to removal of albumin and IgG	[10]
ProteoPrep20, combination of Proteo Extract and ProteoPrep20 depletion, low abundance protein enrichment	2-D LC MS/MS	Not analyzed	25% more proteins identified after ProteoPrep20 depletion compared to low abundance protein enrichment method	[11]
ProteoMiner (protein equalizer)	2-D gels	N/A	800 spots in protein equalized plasma compared to 115 spots in crude	[12]
MARS 7 and 14	Peptide IEF followed by LC MS/MS	23 low abundance	25% increase fractionated plasma compared to crude	[13]
Zoom IEF fractionation	2-D gels, LC MS/MS	N/A	Proteins were fractionated in solution in various narrow pH ranges for further analysis.	[14]
ProteoMiner (protein equalizer)	LC MS/MS	N/A	55% more proteins were identified in CPLL treated plasma compared to crude	[15]
MARS 14; ProteoMiner (protein equalizer)	2-D LC MS/MS	167 in MARS column	568, 497, and 1037 proteins were identified from neat serum, MARS-14 and PM equalized, respectively	[16]
ProteoMiner (protein equalizer); albumin and IgG depletion	2-D gels; MALDI MS	Not analyzed	157, 427, 557 spots detected on 2-D gels from crude, albumin and IgG depletion and ProteoMiner treatment, respectively	[17]
ProteoPrep and API (Abundant Protein Immunodepletion)	Peptide IPG-IEF, LC MS/MS	Not identified	38 new proteins were identified after ultra-depletion which were not identified in crude plasma	[18]
anti-albumin antibody affinity chromatography and SEC	RP-HPLC, 1-D SDS PAGE, MALDI and LC MS/MS	35 proteins	Not analyzed	[19]
IgY 12 micro beads	1-Dand 2-D gels, LC MS/MS	Not detected	Many protein spots previously masked by abundant proteins were revealed in the depleted plasma and improved resolution	[20]
Molecular mass fractionation	SELDI-TOF	Low molecular mass proteins bound to carriers	Total serum/plasma biomarker concentration is largely determined by the carrier protein clearance rate, not the unbound biomarker clearance rate.	[21]
MARS 6 and 14, Proteoprep 20	LC-MS/MS	45, 53 and 61 proteins in MARS 6, 14 & ProteoPrep20, respectively	Non-specific removal of low abundance proteins; un-bound proteins were not analyzed.	[22]
Montage Albumin Deplete Kit	Microarray immunoassay	Cytokines were depleted with albumin	Concentrations of spiked cytokines were reduced in albumin depleted plasma compared to un-depleted plasma.	[23]
Proteoprep 20	Antibody microarray	Not analyzed	86 proteins were detected with higher intensities and 110 with lower intensities after depletion	[24]

study has summarized a list of reports on CCGFs that showed various CCGFs were associated with various diseases, and observed in various human body fluids including plasma [2]. Although plasma is an ideal matrix for biomarker discovery, it is extremely challenging to detect, analyze and characterize the low abundance proteins such as CCGFs in the presence of the high abundance proteins (HAPs) using gels or mass spectrometry. Dynamic range of plasma proteins is over 10 orders of magnitude [3], for example, the top 9 HAPs correspond to 90% and the top 21 proteins correspond to 99% of total plasma proteins [4]. The HAPs (e.g., albumin, IgG, etc.) interfere with identification of the low abundance proteins and therefore, HAPS are removed prior to downstream analysis [5]. A large number of studies have shown that significant increase in total number of protein identification upon depletion of the HAP's using various proteomic technologies (Table 1), [6–17]. Recently, an innovative IgY based depletion technique has identified 38 new proteins that were not previously identified in either neat plasma or depleted by any other techniques [18]. Despite this, there is a concern that the low abundance proteins including CCGFs may also be inadvertently removed during the HAP depletion process. Several studies have demonstrated that large numbers of low abundance proteins were removed when albumin only was depleted from plasma (Table 1), [19–21]. Moreover, when multiple HAPs were depleted from plasma using an immunodepletion system, a total of 101 non-targeted proteins were also removed [22]. In another study it was observed that after albumin depletion from human plasma, recovery of spiked cytokines ranged from 2% to 100% depending on cytokine [23], analyzed by microarray immuno-assay. However, it is controversial whether or not one should remove HAPs prior to biomarker discovery from human plasma.

A recent report from our lab has demonstrated that a large number of CCGFs (48 CCGFs analyzed) can be efficiently detected and quantified from healthy human plasma, saliva and urine using a bead-based multiplexed immuno-assay system [2]. However, there is no report available to-date whether depletion of the HAPs would even enhance detection of the CCGFs further using the same multiplexed immuno-assay system. In this present study, we have determined the presence (at a detectable level, based on the lowest level of known concentration of the standard curve) or absence of 27 CCGFs from non-depleted plasma (i.e., neat plasma, NP), depleted plasma (i.e., flow-through, FT) and also fraction that contains removed HAPs (i.e., bound proteins, BP) and measured their concentrations.

Ten healthy volunteers (5 male and 5 female) were recruited for this study. Blood collected and samples were prepared as previously described [2]. The study was approved by the Macquarie University Human Ethics committee (reference: 5201100498). Prior to CCGF analyses, the top 14 HAPs (albumin, IgG, transferrin, haptoglobin, alpha 1-anti trypsin, IgA, fibrinogen, alpha2 macroglobulin, alpha1 acid-glycoprotein, IgM, apolipoprotein AI, apolipoprotein AII, complement C3, and transthyretin) were depleted from NP samples using a multiple affinity removal system (MARS-14) immuno-depletion column (Agilent, CA, USA) according to manufacturer's instructions. During the HAP depletion process, FT and BP fractions were collected and concentrated by acetone precipitation. Fractionated proteins (FT and BP), neat plasma and one of the two sets of serially diluted standards were also precipitated by cold acetone (1 in 9 parts) overnight at −20 °C and pelleted by centrifugation at 4000 × *g* for 60 min at 4 °C. Neat plasma and serially diluted standards were precipitated to observe the effect of protein precipitation and followed by re-solubilisation effect. The pelleted proteins form all the samples and standards were re-suspended in PBS (same volume as for the starting plasma volume prior to HAPs depletion).

A 27-plex bead-based kit (Bio-Rad, CA, USA) was used to measure concentrations of 27 CCGFs (listed in Table 2 and the full names are described in Supplementary Table 1) from precipitated (processed) and non-precipitated (un-processed) NP, precipitated FT and precipitated BP (50 μl in each well) according to manufacturer's instructions and also following previous report [2]. Data were acquired, analyzed and standard curves (Log(x)–Linear(y)) generated using the Bio-Plex Manager v6.0 software (Bio-Rad, CA, USA). For calculation of concentrations in non-precipitated NP non-precipitated standards, and for precipitated NP, FT and BP samples precipitated standards were used. Standard error, ANOVA and *t*-tests were calculated using Microsoft Excel 2010 for comparison between sample types (processed and un-processed NP, FT and BP). Any CCGFs that were detected in less than 3 subjects were excluded from statistical analysis.

Out of 27 CCGFs analyzed, all 27 CCGFs were detected in both precipitated and non-precipitated NP. Of these, almost all CCGFs were detected in all 10 subjects in NP (Table 2 and Supplementary Table 2). In FT, 25 CCGFs were detected and two CCGFs (IL-1ra and IL-12) were not detected at all. Most of the detected CCGFs in FT were present in at least 9 subjects (Supplementary Table 2). In BP, 21 CCGFs were detected and 6 CCGFs were not detected at all in any subject. In BP, CCGFs were not detected in all the subjects but varied from 1 to 10 subjects for each CCGF (Table 2 and Supplementary Table 2). There was no CCGF that was detected in BP but not detected in FT. Detection of the lowest number of CCGFs in BP compared to NP and FT was expected. In theory, BP should contain depleted HAPs only. However, in our study, a significant number of non-targeted CCGFs were detected in BP suggesting many CCGFs were depleted along with HAPs, a finding supported by other reports [19,22,23]. These non-specifically removed CCGFs are most likely due to direct interaction between the CCGFs and the targeted HAPs. For example, PDGF-bb (one of the CCGFs that was detected in the BP fraction) is a known interacting partner of α-2 macroglobulin [25], one of HAPs that was depleted in this study. It is also possible that CCGFs might have depleted as a complex. For example, VEGF, a growth factor, was detected in BP fraction is known to interact with vitronectin [26] and vitronectin was non-specifically removed during albumin depletion by an immuno-depletion column [19].

There were two CCGFs (IL-1ra and IL-12) detected in both precipitated and non-precipitated NP but not detected in either the FT or BP fractions. Even though concentrations of these two CCGFs were in the medium range, they should still have been detected in one of the fractions. However, it may be possible that during HAP depletion process and/or protein precipitation step (FT and BP fractions), concentrations of these two CCGFs were further diluted out. It has been reported

Table 2 – Average concentrations of CCGFs in neat plasma, neat plasma after acetone precipitation, flow through and bound protein fractions.

	Neat plasma without processing			Neat plasma after precipitation			Flow through			Bound protein		
CCGFs	Average (pg/ml)	SE	*n*	Average (pg/ml)	SE	*n*	Average (pg/ml)	SE	*n*	Average (pg/ml)	SE	*n*
IL-1b	2.07	0.31	10	0.61	0.07	10	0.54	0.04	10	0.25	0.05	10
IL-1ra	94.21	16.17	10	17.59	1.96	10	0	0	0	0	0	0
IL-2	11.22	2.92	10	2.82	0.87	8	4.31	0.38	9	4.21	0.27	7
IL-4	2.97	0.42	10	0.45	0.06	10	0.23	0.04	9	0	0	0
IL-5	5.95	0.98	10	0.40	0.12	4	0.19	0.04	8	0	0	0
IL-6	8.11	1.56	10	2.22	0.54	10	0.31	0.13	4	6.32	0	1
IL-7	6.29	0.97	10	25.73	2.96	10	0.70	0.13	10	0.2	0.02	10
IL-8	31.09	5.00	10	6.05	0.79	10	1.41	0.14	9	0.41	0.16	4
IL-9	6.13	1.29	9	0.45	0.14	9	0.51	0.09	8	0.30	0.14	5
IL-10	23.65	8.85	10	2.20	0.14	10	2.63	0.19	9	1.74	0.28	10
IL-12 (p70)	71.97	54.93	9	4.40	0.77	10	0	0	0	0	0	0
IL-13	13.65	4.14	10	8.84	1.64	10	0.33	0.03	10	0.18	0.02	10
IL-15	8.35	1.27	7	30.77	2.77	10	20.45	1.41	9	11.38	1.05	7
IL-17	42.91	6.91	10	4.63	0.84	10	8.81	2.21	9	0	0	0
Eotaxin	37.19	5.63	10	26.98	4.10	10	19.54	2.52	10	0	0	0
FGF b	27.9	3.48	9	15.21	2.00	10	13.36	0.69	9	11.44	1.59	7
G-CSF	73.17	12.51	10	8.12	2.00	9	4.47	0.47	9	1.16	0.29	5
GM-CSF	13.72	3.68	9	20.42	3.69	10	67.74	1.91	9	74	2.99	9
IFN-g	72.97	13.3	10	9.86	1.55	10	1.80	0.31	8	0.59	0	2
IP-10	252.74	55.06	10	81.40	19.06	10	85.71	21.88	9	3.54	0.76	7
MCP-1 (MCAF)	28.19	3.55	10	24.98	3.88	10	13.95	1.68	9	0.59	0.23	2
MIP-1a	2.49	0.34	10	1.10	0.14	10	0.61	0.03	10	0.44	0.05	10
PDGF-bb	169.48	58.75	10	120.36	50.57	10	290.02	148.52	9	16.37	9.49	7
MIP-1b	21.97	3.24	10	22.29	3.77	10	10.82	1.88	10	0.68	0.17	6
RANTES	1732.82	235.88	10	1739.24	207.80	10	997.10	155.22	10	14.96	6.68	7
TNF-a	43.92	7.72	10	3.77	0.53	10	3.58	0.43	9	1.22	0.37	6
VEGF	16.79	2.54	10	21.51	4.37	10	23.32	4.96	9	4.31	0.69	6

Concentrations of all the CCGFs for individual volunteer are shown in Supplementary Table 2. CCGFs = cytokines, chemokines and growth factors, SE = standard error, *n* = number of subjects.

that protein recovery was reduced due to protein precipitation [27,28].

Average concentrations of most of the CCGFs were significantly higher in un-processed NP compared to processed NP (after precipitation, centrifugation and re-suspension of pellet), FT and BP except for IL-7, IL-15, and GM-CSF (Table 3). This loss of recovery in processed NP indicates overall loss of recovery in FT is primarily due to precipitation of proteins and/or re-suspension of pellets. However, IL-7 was significantly higher ($P \leq 0.05$) in processed NP followed by un-processed NP, FT and BP, respectively. IL-15 was significantly higher ($P \leq 0.05$) in processed NP followed by FT, BP and un-processed NP. However, there was no significant difference between un-processed NP and BP for IL-15. GM-CSF was significantly higher ($P \leq 0.05$) in BP and FT followed by un-processed NP and processed NP. There was no significant difference between BP and FT, and processed or un-processed NP for GM-CSF. There was no significant difference between sample types for PDGF-bb (Table 3).

Our main purpose of this work was to observe whether depletion of HAPs from human plasma using an immuno-depletion column increases or decreases the detection of CCGFs using a bead based multi-plex immuno-assay system. High-performance liquid chromatography (HPLC)-based immuno-depleted samples cannot be analyzed without processing due to sample dilution and buffer system used for depletion. Sample processing includes concentration of samples either by precipitation or ultra-filtration, re-suspension of pellets and in some cases needs to perform an additional step, buffer exchange, for assay compatibility. This means post-depletion sample processing is associated with depletion process but not with neat samples. Our main comparison is to observe the difference between neat plasma (no sample processing is required for analysis) vs depleted plasma (depleted samples must go through sample processing prior to analysis) and the data are presented in this manuscript accordingly. It was observed that overall loss of recovery is associated with depletion process. However, to figure out the source of loss of recovery, we have processed in parallel neat plasma and also serially diluted standard, the same standard set that was used for calculation of concentrations of CCGFs for non-precipitated neat plasma. It was observed that there were some losses of recovery in precipitated neat plasma compared to non-precipitated plasma due to sample processing alone (acetone precipitation only, no HAP removal involved) and the losses were enhanced due to removal of HAPs. Data presented in the manuscript suggests that loss of recovery in FT is mainly due to combination of post-depletion precipitation of proteins and/or re-suspension of pellets, and non-specific removals of CCGFs (Table 3, Supplementary Table 3).

Table 3 – Concentration difference between neat plasma (NP), neat plasma after acetone precipitation (NP-ppt), flow through (FT) and bound protein (BP) fractions of detected CCGFs.

CCGFs	NP (pg/ml)	NP-ppt (pg/ml)	FT (pg/ml)	BP (pg/ml)	P-value (ANOVA)	F-value (ANOVA)	P-value (t-test)
IL-1b	2.07 a	0.61 b	0.54 b	0.25 c	5E−9	25.51	–
IL-1ra	94.21	17.59	0	0	–	–	1.12E−3
IL-2	11.22 a	2.82 b	4.31 b	4.21 b	7.15E−3	4.86	–
IL-4	2.97 a	0.45 b	0.23 c	0	3.38E−8	35.83	–
IL-5	5.95 a	0.4 b	0.19 b	0	2.49E−5	19.5	–
IL-6	8.11 a	2.22 b	0.31 c	0	6.14E−4	10.34	–
IL-7	6.29 b	25.73 a	0.7 c	0.2 d	5.31E−14	59.26	–
IL-8	31.09 a	6.05 b	1.41 c	0.41 d	7.33E−8	23.22	–
IL-9	6.13 a	0.45 b	0.51 b	0.3 b	5.31E−6	15.25	–
IL-10	23.65 a	2.2 b	2.63 b	1.74 b	2.85E−3	5.66	–
IL-12 (p70)	71.97	4.40	0	0	–	–	0.25
IL-13	13.65 a	8.84 a	0.33 b	0.18 c	1.51E−4	8.91	–
IL-15	8.35 c	30.77 a	20.45 b	11.38 c	1.83E−8	26.56	–
IL-17	42.91 a	4.63 b	8.81 b	0	1.21E−6	24.08	–
Eotaxin	37.19 a	26.98 a	19.54 b	0	0.02	4.29	–
FGF basic	27.90 a	15.21 b	13.36 b	11.44 b	5.21E−5	10.77	–
G-CSF	73.17 a	8.12 b	4.47 b	1.16 c	1.39E−7	21.77	–
GM-CSF	13.72 b	20.42 b	67.74 a	74 a	2.46E−16	95.34	–
IFN-g	72.97 a	9.86 b	1.8 c	0	2.69E−6	22.38	–
IP-10	252.74 a	81.4 b	85.71 b	3.54 c	1.46E−4	9.28	–
MCP-1 (MCAF)	28.19 a	24.98 a	13.95 b	0	0.01	5.03	–
MIP-1a	2.49 a	1.1 b	0.61 c	0.44 d	7.07E−9	24.79	–
PDGF-bb	169.48	120.36	290.02	16.37	0.22	1.56	–
MIP-1b	21.97 a	22.29 a	10.82 b	0.68 c	7.57E−5	10.14	–
RANTES	1732.82 a	1739.24 a	997.1 b	14.96 c	1.44E−6	15.89	–
TNF-a	43.92 a	3.77 b	3.58 b	1.22 c	5.39E−8	22.84	–
VEGF	16.79 a	21.51 a	23.32 a	4.31 b	0.02	3.83	–

Any CCGF detected in less than three subjects for each sample type (NP, NP-ppt, FT and BP) was excluded from statistical analysis. An ANOVA was performed when CCGFs were detected in all four or three sample types and *t*-test was used when detected in two sample types. When the overall *F* test was significant ($P \leq 0.05$), the mean values were compared with *t*-text. The letters at the end of the concentrations in each row represent the significant difference between the sample types. i.e., a: highest concentration, b: second highest, c: third highest and d: lowest. The same letters indicate the difference between the samples is not significant. CCGFs = cytokines, chemokines and growth factors.

We have observed an issue with the multiplex analysis system which is concentrations of various CCGFs is very different in plasma but they are analyzed simultaneously in a single assay. This is less than ideal because concentrations of majority of the CCGFs are very low but concentrations of some CCGFs very high. Ideally, for detection of some higher concentrations CCGFs plasma samples should be diluted out prior to analysis and for detection of the other lower concentrations CCGFs, plasma should be analyzed neat if not concentrated prior to analysis. But in real life, samples are analyzed either diluted or un-diluted for the detection of multiple CCGFs in a single assay due to high cost associated with optimization of assay conditions.

In conclusion, the highest numbers of CCGFs were detected in both un-processed and processed NP followed by FT and BP. Out of 27 CCGFs analyzed, detection level of GM-CSF only was improved by HAP depletion. Overall HAP depletion was counter-productive for the detection of 24 CCGFs primarily due to post-depletion precipitation of proteins and/or re-suspension of pellets, and also for non-specific removals of CCGFs. Although other reports showed more proteins were identified after HAP depletion using mass spectrometry or gel based proteomics, however, it appeared that antibody based analytical technique is capable of identifying similar or more proteins in neat plasma compared to HAP depleted plasma.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.euprot.2014.02.012.

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