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REGULAR ARTICLE

Immunoassay and antibody microarray analysis of the HUPO Plasma Proteome Project reference specimens: Systematic variation between sample types and calibration of mass spectrometry data

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Four different immunoassay and antibody microarray methods performed at four different sites were used to measure the levels of a broad range of proteins ($N = 323$ assays; 39, 88, 168, and 28 assays at the respective sites; 237 unique analytes) in the human serum and plasma reference specimens distributed by the Plasma Proteome Project (PPP) of the HUPO. The methods provided a means to (1) assess the level of systematic variation in protein abundances associated with blood preparation methods (serum, citrate-anticoagulated-plasma, EDTA-anticoagulated-plasma, or heparin-anticoagulated-plasma) and (2) evaluate the dependence on concentration of MS-based protein identifications from data sets using the HUPO specimens. Some proteins, particularly cytokines, had highly variable concentrations between the different sample preparations, suggesting specific effects of certain anticoagulants on the stability or availability of these proteins. The linkage of antibody-based measurements from 66 different analytes with the combined MS/MS data from 18 different laboratories showed that protein detection and the quality of MS data increased with analyte concentration. The conclusions from these initial analyses are that the optimal blood preparation method is variable between analytes and that the discovery of blood proteins by MS can be extended to concentrations below the ng/mL range under certain circumstances. Continued developments in antibody-based methods will further advance the scientific goals of the PPP.

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Abbreviations: BDAA, BD Asian-American specimen set; **BDAF**, BD African-American specimen set; **BDCA**, Caucasian-American specimen set; **CAMS**, Chinese Academy of Medical Sciences; **DB**, Dade Behring; **GNF**, Genomics Institute of the Novartis Foundation; **IPI**, international protein index; **IRM**, international reference material; **MSI**, Molecular Staging; **PPP**, Plasma Proteome Project; **RLS**, resonance light scattering; **TC-RCA**, two-color rolling circle amplification; **VARI**, Van Andel Research Institute

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1 Introduction

Antibody-based analytical methods can provide quantitative, reproducible, and sensitive measurements of specific analytes. These capabilities are valuable both for routine clinical analysis and for the high-throughput exploration of hypotheses regarding specific proteins. The multiplexing of antibody-based assays through the use of planar microarrays [1–6] and bead arrays has opened up new research opportunities. The various formats of antibody microarrays and the applications and relative merits of each are reviewed elsewhere [7]. Several goals of the Plasma Proteome Project (PPP) of the HUPO can be advanced through the use of antibody-based methods.

One of the major goals of the pilot phase of the PPP was to determine the effects of the blood preparation method on the quality of proteomic data. Blood may be prepared as serum (the soluble portion of clotted blood) or as plasma (the soluble portion of anticoagulated blood), and various anticoagulants may be used to make plasma. Before attempting a large-scale study of the human plasma proteome, it is necessary to determine if the preparation method introduces systematic alterations to the levels of all proteins or specific proteins, or whether certain preparation methods are desirable or not for certain applications. Antibody-based methods are well suited to study that question, since the levels of multiple proteins may be precisely and accurately measured in multiple samples. An additional valuable use of antibody-based methods for the PPP is to provide complementary information to the broad-based discovery capabilities of separations and MS methods.

The exploration of these topics was facilitated by the assembly of human serum and plasma reference specimens by BD Diagnostics (Franklin Lakes, NJ), the National Institute for Biological Standards and Control (NIBSC, UK), and the Chinese Academy of Medical Sciences (CAMS, Beijing) [8]. Blood samples, each pooled from a male and female donor, were prepared in four ways: as serum, as plasma anticoagulated with sodium citrate, as plasma anticoagulated with K-EDTA, and as plasma anticoagulated with lithium heparin. Four different laboratories used antibody-based methods to analyze the reference specimens, with each laboratory using a distinct method. The combined data sets were used to investigate the level of systematic variation in protein levels introduced by the preparation methods and to gain further insight into the suitability of the various methods for proteomic analyses. We evaluated the following: evidence for bias in the concentrations of all the proteins in general; evidence for protein-specific alterations in concentration as a function of preparation method; and the relationship between the detectability of proteins by MS and their concentrations in plasma or serum.

2 Materials and methods

2.1 Reference specimens

Reference specimens were prepared by BD Diagnostics, NIBSC, and CAMS [8]. BD prepared three different specimens (designated BDAA, BDAF, and BDCA), each a pool from a male and female donor, in four different ways – as serum, as plasma anticoagulated with sodium citrate, as plasma anticoagulated with K-EDTA, and as plasma anticoagulated with lithium heparin – resulting in 12 different samples. CAMS prepared one specimen, pooled from a male and a female donor, with the four methods. The NIBSC made available its Thrombosis and Hemostasis standard, a lyophilized citrate-anticoagulated-plasma [8]. The samples were shipped frozen on dry ice to the four sites. The receiving sites were not blinded to the sample types. Dade Behring (DB) received all the specimens, Van Andel Research Institute (VARI) received the three BD specimen sets, and the later participants Genomics Institute of the Novartis Foundation (GNF) and Molecular Staging (MSI) received the BDAA, BDAF, CAMS, and NIBSC and the BDAA, BDAF, and NIBSC specimen sets, respectively.

2.2 DB immunoassays

DB immunoassays (see Supplemental Table 1, http://www.vai.org/vari/labs/haab.asp) were performed on a Behring Nephelometer (BN) II (2.2/D, serial no. 330135) and on a Dimension (DIM) RxL (serial no. 970933-AX) from DB (Deerfield, IL) with the HUPO PPP specimens [8]. Most tests performed are approved by the Food and Drug Administration (FDA) only for serum samples, as outlined in the manufacturer data sheets. Tests for ferritin (FERR), soluble transferrin receptor (sTfR), cardiac troponin I (cTNI), and

Table 1. Number of individual assays with consistent maxima or minima in each preparation type for each data set. Each column gives the number of proteins for a given preparation method that showed a maximum (top) or minimum (bottom) value in that preparation method for every sample and every replicate. Total number of assays in each data set is given in the right column

	Data set	Citrate	EDTA	Heparin	Serum	Total	Total assays
Maxima	DB	0	0	2	10	12	33
	GNF	1	13	3	4	21	88
	MSI	0	10	0	9	19	168
	VARI	0	1	4	1	3	28
	Total	1	24	9	24	55	317
Minima	DB	24	0	0	1	25	33
	GNF	4	1	0	3	8	88
	MSI	0	1	2	1	4	168
	VARI	3	0	0	0	3	28
	Total	31	2	2	5	40	317

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myoglobin (MYO) on the Dimension system are also approved for heparinized plasma. Tests for C-reactive protein (CRP), IgE, β2-microglobulin, and MYO on the BN system are also approved for EDTA and heparinized plasma. The creatine kinase MB (mass assay, MMB), human chorionic gonadotropin (HCG), and thyroid stimulating hormone (TSH) assays are FDA-approved for use in serum, EDTA-plasma, and heparin-plasma samples. The fibrinogen, plasminogen, antithrombin III, and fibronectin tests are approved only for EDTA- and citrate-plasma samples, not for serum. In case test formats were not compatible with a sample type (*e.g.*, fibrinogen in serum), data were not considered.

Two assay systems were used at DB: the BN and the Dimension methods. Both are rapid, specific, precise, and accurate [9–11]. For each analysis, appropriate Dade Behring standards, calibrators, and controls were utilized, along with a PSA control from Bio-Rad Laboratories (Hercules, CA). These standards are based on highly purified proteins and/or common international reference materials (IRMs) [12–14]. BN systems are dedicated protein analyzers that apply either antiserum or particle-enhanced immunonephelometric quantitation of analytes [10, 15]. Proteins in the human sample form immune complexes with specific particle-bound or antiserum antibodies. These complexes scatter a beam of light, with intensity proportional to the relevant protein concentration. Dimension methods on routine clinical analyzers are enzyme immunoassays based on the "sandwich" principle. A sample incubated with chromium dioxide particles coated with an mAb and a conjugate reagent labeled mAb specific for the protein to be analyzed forms a particle/protein/conjugate sandwich. Unbound conjugate is removed by magnetic separation and washing. The sandwich bound conjugate enzyme triggers an amplification cascade, which produces a colored product [9].

2.3 Antibody arrays at GNF

2.3.1 Antibodies, reagents, microarray printing, and platform

Antibodies and antigens (Supplemental Table 1) were purchased from various vendors. Resonance light scattering particles (RLS) refer to colloid gold particles coated with an antibiotin antibody [16, 17] purchased from Genicon Sciences, now Invitrogen (Carlsbad, CA). A total of 88 sandwich immunoassays were assembled and optimized in two antibody array panels, panels A and B (Brinker *et al.*, in preparation).

2.3.2 Microarray layout and processing

Forty-eight identical antibody microarrays with up to 48 different capture antibodies were printed onto single glass microscope slides. Four such slides were mounted onto a slide holder effectively generating a microtiter plate with 384 spacing and an antibody microarray at the bottom of each well. On each slide, eight of the wells were incubated with standard mixtures of purified antigens in diluent, resulting in an eight-point standard titration curve that was used to quantify the analyte concentrations in each sample. The 40 remaining wells *per* slide were incubated with four dilutions (2-, 20-, 200-, and 200 000-fold) of ten samples. The diluent used throughout contained Roche "Complete" protease inhibitor cocktail at one tablet *per* 50 mL. After incubation for 1 h, all arrays were washed; a mixture of biotinylated detection antibodies was applied for 1 h, followed by washing. In a final 1 h incubation, RLS gold particles were applied to the arrays. Excess material was removed by washing. Slides were dipped twice into 50 mL deionized water and spun dry before coating with "RLS archiving" solution. For further details see Saviranta *et al.*, [18].

2.3.3 Array imaging and data analysis

Microarray slides stained with RLS particles were imaged at a resolution of 10 μm with a 16 bit CCD camera-based scanner (Invitrogen) and images analyzed with ArrayVision, version 8.0 (Imaging Research, St. Catharine's, Canada). Median-trimmed mean signal values for each spot on a slide were imported into EXCEL. For each slide, standard curves for each analyte were generated by four-parameter logistic fitting. Unknown sample concentrations were calculated using the corresponding signal values, the curve fitting parameters, and the dilution factors. An average concentration (derived from the three replicate spots) was calculated for each of the dilutions. To obtain a single concentration value, the program automatically chose the lowest dilution that gave a signal in the dynamic range of the assay. We performed one four-slide experiment for each of the two antibody array panels. For each of the four HUPO reference specimen preparations, three aliquots of the Asian-American, African-American, and Chinese samples and one aliquot of the NIBSC citrate-plasma reference sample were incubated on the same slide and measured against the same set of standard curves.

2.4 Antibody microarrays at MSI

2.4.1 Chip manufacture

A Teflon mask was applied to each slide creating 16 individual sample wells with 0.65 cm diameter. Prior to printing, glass slides were cleaned and derivatized with 3-cyanopropyltriethoxysilane. Panels of 25–37 capture antibodies were spotted in quadruplicate into each sample well using a Perkin-Elmer SpotArray Enterprise noncontact arrayer equipped with piezoelectric tips, delivering ~350 pL for each 120 μm antibody spot. Antibodies were applied at a concentration of 0.5 mg/mL at defined positions within each of the six production chips. Each well of a slide was

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printed with a single array type, containing panels of 26, 27, 26, 37, 25, and 28 antibodies, respectively, for chips 1–6. (See Supplemental Table 1 for a complete listing of antibodies surveyed.)

2.4.2 Rolling circle amplification (RCA) immunoassay

The manual RCA microarray immunoassay reported previously [4] was modified to optimize performance on an automated platform (Protedyne BioCube). Incubation times were increased from 30 to 45 min for two of the assay steps (RCA signal amplification and detector incubation), and the number and volume of washes between steps increased from 2 to 4–5 and from 20 to 30 μL, respectively. Slides were scanned using an LS200 scanner (TECAN). Scanned images were analyzed using proprietary software. The fluorescence intensity was analyzed for each sample analyte with the resulting mean intensity measurements converted into concentration values.

2.4.3 Conversion of mean fluorescent intensity to concentration

Preparations of standardized multiplex analyte titration series were manufactured using recombinant analytes diluted in buffer covering the range from 12 pg/mL to 81 ng/mL at 14 discrete points plus zero analyte buffer blanks. These titration points were distributed among the 16 available wells on three control slides. The standard titrations, designed to overlap the linear range of detection for each individual analyte, were used to generate standard curves from which sample analyte concentrations were determined. A four-point standard titration was run on every slide for normalization and quality control purposes. The four wells, designated "anchor point" controls, were derived from the standard 14-point titration series run on separate control slides to generate standard curves for each analyte. Anchor point controls contained a cocktail of all cytokines corresponding to the printed capture antibodies for a given array. The anchor points were prepared at four concentrations that fell within the linear range of detection for each analyte. Individual sample values were normalized using linear regression of the anchor points to reduce assay imprecision observed among replicates. Fluorescence intensities of the four spot replicates for each analyte within an anchor point well were averaged on a logarithmic (base two) scale to generate within-slide titration curves. Linear regression coefficients (slope and intercept) were calculated between individual titration curves from each slide to generate an "average" titration curve. Calculated slope and intercept were used to transform averaged analyte values for each sample well. Data normalization was performed on the data set after removal of outliers.

2.5 Antibody microarrays at VARI

2.5.1 Fabrication of antibody microarrays

Antibodies (Supplemental Table 1) prepared at ~500 μg/mL in 1× PBS were printed in microarrays on the surfaces of NC-coated microscope slides (FAST™ slides, Schleicher & Schuell) using a custom-built contact arrayer.

2.5.2 Serum labeling

The 12 PPP reference specimens were received from BD. An aliquot from each of 12 serum samples was divided into a portion to be labeled with NHS-digoxigenin and a portion to be labeled with NHS-biotin (Molecular Probes). The digoxigenin-labeled samples were pooled, and equal amounts of the pool were transferred to each of the biotin-labeled samples. Each labeled protein solution was supplemented with nonfat milk to a final concentration of 3%, Tween-20 to a final concentration of 0.1%, and 1× PBS to yield a final serum dilution of 1:100.

2.5.3 Processing of antibody microarrays

One hundred microliters of each labeled serum sample mix was incubated on a microarray with gentle rocking at room temperature for 2 h. After washing, the arrays were detected by two-color RCA (TC-RCA) (see [19] for experimental details). The biotin-labeled proteins were detected with green fluorescence; the digoxigenin-labeled proteins were detected with red fluorescence.

2.5.4 Analysis

The microarrays were scanned (ScanArray; Perkin-Elmer Life Sciences) for fluorescence using laser excitation at 543 and 633 nm; GenePix 5.0 (Axon Laboratories) was used to quantify the images. For spots with fluorescence signal surpassing an intensity threshold in both color channels [3], the ratio of background-subtracted median sample-specific fluorescence to background-subtracted median reference-specific channel fluorescence was calculated, and ratios from replicate antibody measurements within the same array were averaged.

2.6 Retrieval and matching of IPI numbers for the analytes

International protein index (IPI) accession numbers were obtained for each analyte in the quantitative assays of this study using two search methods. In the first search, the analyte names were subjected to an internet search to retrieve the proper protein names. The analyte names were then used to generate Sequence Retrieval System (LION Bioscience, Heidelberg, Germany) queries of the IPI database [20] using the SRS server at EBI (http://srs.ebi.ac.uk).

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The search parameters were as follows: protein name is in IPI AllText and OrganismName is Human. The data returned were Accession Number(s) and EntryName. The returned name from the IPI database was compared with the input analyte name, and records with the names not matching were discarded. IPI numbers corresponding to precursor forms of proteins were retained.

In the second search, the list of protein names against which the antibodies were raised was searched against the Human Protein Reference Database to identify all possible alternate names. These alternate names were further verified using the OMIM and Swiss-Prot databases. The IPI database was then searched using these names, and all IPI IDs, which corresponded to the protein name in question, were assigned to it. Each sequence corresponding to each IPI ID was further verified by conducting a BLASTP against the nr data set. The outputs were manually analyzed, and LocusLink identifiers were assigned to each sequence and cross-checked with those assigned in the IPI database. Alternate IPI IDs, as specified in the IPI data set, were also assigned so as to give all possible identifiers for each protein. Protein name and all alternate names were used to query the HUGO gene nomenclature committee's database, and the results verified using LocusLink identifiers. This allowed annotation of all entries with their gene name and gene symbol.

3 Results

3.1 Antibody-based measurements of the HUPO reference specimens

The PPP reference specimens were distributed to four different laboratories for immunoassay or antibody microarray analysis. Each of the four sites used a distinct technology for analyzing the specimens. The 39 immunoassays performed on DB clinical analyzers were based on immunonephelometric methods (33 tests) and sandwich-like enzyme immunoassays (6 tests) that use antibody-coated magnetic chromium dioxide particles. The GNF measured 88 different serum proteins using microarray-based sandwich assays detected by RLS. MSI used antibody microarrays to target 168 different proteins, mostly cytokines, using sandwich assays and detection by RCA [4, 21]. VARI measured 28 different serum proteins using TC-RCA detection on antibody microarrays [19]. The antibodies used by each site are listed in Supplemental Table 1. Each site independently designed their own experiments based on individual resources and experience, and the targeted proteins varied significantly between sites. The complete data sets are available at http://www.vai.org/vari/labs/haab.asp.

Two of the sites (MSI and GNF) ran the samples in triplicate, one in duplicate (VARI), and one had duplicate measurements for four of the samples (DB). The reproducibility of the replicate data is a good indicator of data quality. Replicate measurements showed good reproducibility for each data set, as depicted by the correlations of the different antibody measurements for the same sample in two separate experiments (Fig. 1). The average correlation coefficients between the different antibody measurements from replicate experiments were 0.99 for the DB set, 0.95 for the GNF set, 0.94 for the MSI set, and 0.96 for the VARI set. These high average correlations indicate that each data set is highly internally consistent.

Two of the data sets (GNF, MSI) used standard curves of purified antigens to calibrate the data and to calculate the concentrations of each of the measured proteins. DB analyzers used reference materials (standards, controls, and calibrators) that are based on IRMs and purified antigens for calibration and the determination of the concentrations of the analytes. The measured concentrations cover a broad range, from several mg/mL to below 1 pg/mL (Fig. 2). The GNF and MSI data sets, focusing on cytokine detection, account for most of the low-abundance measurements, while the DB and VARI sets focused on common mid-to-high-abundance serum proteins. Some overlap existed between the sets: six analytes were common between DB and GNF, three were common between DB and MSI, 11 were common between DB and VARI, 57 were common between GNF and MSI, 10 were common between GNF and VARI, and nine were common between MSI and VARI.

While the precision between replicates within each data set is good (Fig. 1), occasionally large differences were observed between platforms in the measured concentrations of common analytes. Of the 57 common analytes between GNF and MSI, seven were measured more than ten-fold higher at GNF and eight were measured more than ten-fold higher at MSI. These deviations between assays in the measurement of common analytes can be seen in Fig. 2. Supplemental Table 2 provides the average measured concentrations of the analytes that were measured at more than one site. Interlaboratory variation is not uncommon and may be due to differences in the specificities of the antibodies used, the sample storage and treatment methods, and the calibration methods. The full exploration of the sources of variation between the laboratories was beyond the scope of this study, yet the existence of the occasional variation highlights the need for methods for calibration and validation across laboratories and platforms.

3.2 Systematic variation between the preparation methods of the PPP reference specimens

We investigated whether the blood preparation methods (serum, citrate-plasma, EDTA-plasma, heparin-plasma) introduced systematic bias into the abundances of all the proteins in general. A systematic bias in concentration would be evidenced by a consistent shift in the concentrations of analytes in one preparation method relative to the other methods. The protein abundances were compared between the samples that were prepared from the same starting material, *i.e.*, we compared the four preparations within the

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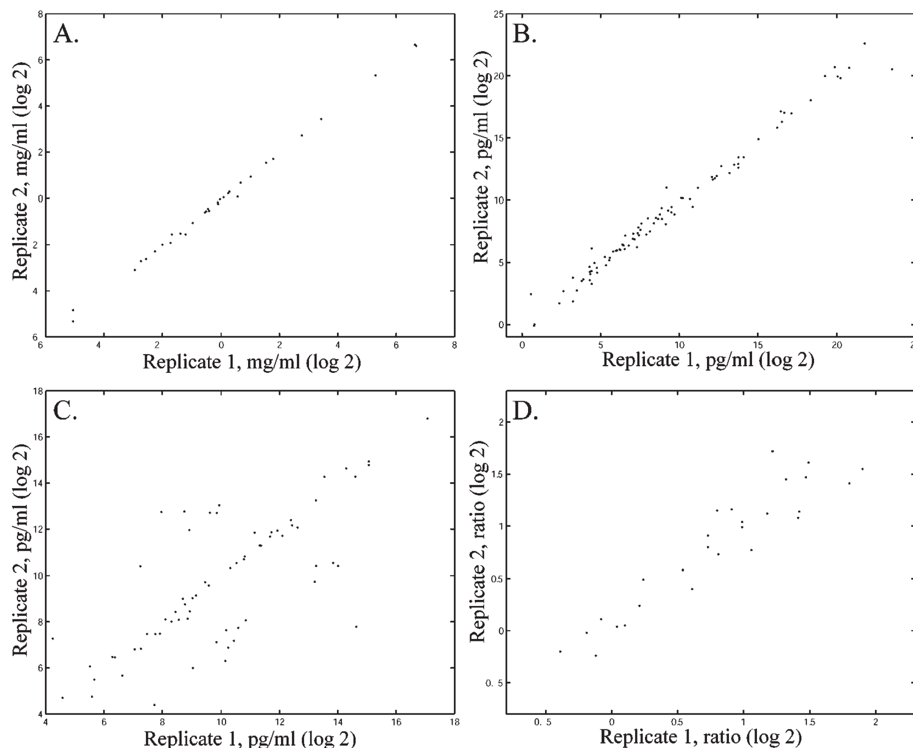

Figure 1. Correlations between replicate measurements of one sample. Duplicate antibody measurements from a plasma sample were plotted against each other. Scatter plots from each of the four data sets are shown: (A) DB; (B) GNF; (C) MSI; (D) VARI. Correlations for each of the plots were 0.99, 0.95, 0.94, and 0.96, respectively. Plots are shown for the samples: (A) CAMS, citrate-plasma; (B) BDAA, citrate-plasma; (C) BDAF, citrate-plasma; and (D) BDCA, heparin-plasma.

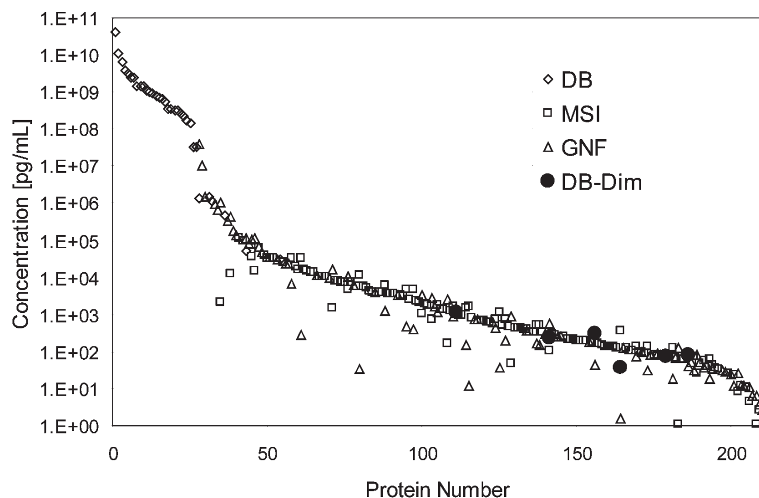

Figure 2. Concentration range of the proteins measured in these studies. Geometric mean concentration over all the samples is plotted for each of 295 quantitative assays for 231 unique proteins. Set consists of quantitative measurements from the DB BN system (33 analytes), the DB Dimension system (6 analytes), MSI antibody microarrays (168 analytes), and GNF antibody microarrays (88 analytes). For analytes measured by more than one laboratory, the geometric mean concentration derived by each laboratory is displayed.

BDAA specimen set, the four preparations within the BDAF specimen set, *etc*. For each preparation type (citrate-plasma, EDTA-plasma, *etc*.), the number of proteins that had a maximum concentration in that preparation was totaled. The number of proteins with minimum concentrations also was totaled for each preparation method. Those numbers were compared to the numbers of maxima or minima that would be expected by chance. Frequencies of maxima or minima much greater or lower than would be expected by chance could indicate systematic bias in the concentrations in a particular preparation method.

The results of that analysis are shown in Fig. 3. The proportion of proteins that had maxima (Fig. 3A) or minima (Fig. 3B) in each preparation type is indicated by the position on the *x*-axis of a different vertical line for each of the four data sets. The distribution of maxima and minima in each preparation method that would be expected by chance was calculated by permutation and is indicated by the histograms in each plot. As expected, the average frequency in the randomly permuted data is 0.25, since the maxima and minima are evenly distributed among the four preparation methods. All four data sets had a significantly lower frequency of

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Table 2. Concentrations and associated MS summary information. Information relating to 70 IPI numbers (66 unique analytes) that had a match between the analyte-derived lists and the MS-derived lists is presented. “Antibody name” = the name that was used in the searches for analyte-associated IPI numbers. “Name from analyte search” = the name in the IPI database that matched the antibody/analyte name. “Concentration” = the geometric mean concentration over all specimens as found by immunoassay or antibody microarray. “# Labs” = the number of laboratories (out of 18) that found a particular IPI number. “# Peptides” = the average number of different peptides found for that IPI number. “IPI set” = the analyte-associated list from which a match was found (see Section 2), either list 1, list 2, or both (1, 2, or B). In four instances, two different IPI numbers were associated with one analyte

Antibody name	Name from analyte search	Concentration, pg/mL	# Labs	# Peptides	Laboratory	IPI SET
Albumin	Albumin	4.0E + 10	17	201	DB	2
Transferrin	Transferrin	2.3E + 09	16	249	DB	2
Apolipoprotein A I	Apolipoprotein A-I	1.4E + 09	17	82	DB	2
α2-macroglobulin	Alpha-2-macroglobulin	1.4E + 09	17	211	DB	2
α1-antitrypsin	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	1.1E + 09	15	183	DB	2
C3c	Complement component 3	9.5E + 08	5	98	DB	2
Haptoglobin	Haptoglobin	8.8E + 08	18	113	DB	2
Hemopexin	Hemopexin	7.5E + 08	16	86	DB	2
Apolipoprotein B	Apolipoprotein B (including Ag(x) antigen)	7.2E + 08	13	328	DB	2
Fibrinogen	Fibrinogen, gamma polypeptide	6.7E + 08	16	66	DB	2
Fibrinogen	Fibrinogen, gamma polypeptide	6.7E + 08	12	51	DB	2
α1-acid-glycoprotein	Alpha-1-acid glycoprotein 2 precursor	6.1E + 08	14	24	DB	1
α1-acid-glycoprotein	Orosomucoid 1	6.1E + 08	16	45	DB	2
Antithrombin III	Serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1	3.2E + 08	17	70	DB	2
Apolipoprotein A-II	Apolipoprotein A-II	3.0E + 08	15	18	DB	2
Prealbumin	Transthyretin (prealbumin, amyloidosis type I)	2.6E + 08	17	27	DB	2
Ceruloplasmin	Ceruloplasmin (ferroxidase)	2.1E + 08	15	134	DB	2
C4	Complement C4 precursor [Contains: C4A anaphylatoxin]	1.7E + 08	17	157	DB	1
Plasminogen	Plasminogen	1.4E + 08	12	72	DB	2
Fibronectin	Fibronectin 1	1.1E + 08	1	86	DB	2
Apolipoprotein E	Apolipoprotein E	3.4E + 07	8	30	DB	2
vWF	Von Willebrand factor	1.3E + 06	2	46	GNF	2
β2Microglobulin	Beta 2-microglobulin protein	1.1E + 06	1	1	DB	1
β2Microglobulin	Beta-2-microglobulin	1.1E + 06	3	1	DB	2
sTfR	Transferrin receptor (p90, CD71)	5.8E + 05	1	2	DB	2
VAP-1	Amine oxidase, copper containing 3 (vascular adhesion protein 1)	1.2E + 05	2	6	MSI	2
Protein C	Mannose-binding lectin (protein C) 2, soluble (opsonic defect)	9.7E + 04	2	7	MSI	2
VCAM-I	Vascular cell adhesion molecule 1	9.4E + 04	3	9	MSI/GNF	2
TGFβ1	Transforming growth factor, beta 1 (Camurati-Engelmann disease)	7.5E + 04	2	2	GNF	2
IGF-BP3	Insulin-like growth factor binding protein 3	5.9E + 04	6	17	MSI/GNF	2
ICAM-1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	4.3E + 04	2	4	MSI/GNF	2
MMPg	Matrix metalloproteinase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	4.1E + 04	2	5	MSI/GNF	2
VE-cadherin	Cadherin 5, type 2, VE-cadherin (vascular epithelium)	3.0E + 04	3	11	MSI	2
M-CSF R	Colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog	2.6E + 04	3	11	MSI	2
L-Selectin	Selectin L (lymphocyte adhesion molecule 1)	1.7E + 04	5	10	MSI	2
ALCAM	Activated leukocyte cell adhesion molecule	1.6E + 04	2	5	MSI	2
IGFBP2	Insulin-like growth factor binding protein 2, 36 kDa	1.5E + 04	1	3	MSI	2
TIMP1	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)	1.4E + 04	1	3	MSI/GNF	2
EGF R1	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	1.1E + 04	3	3	GNF	2

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Table 2. Continued

Antibody name	Name from analyte search	Concentration, pg/mL	# Labs	# Peptides	Laboratory	IPI SET
MMP2	Matrix metalloproteinase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)	8.8E + 03	1	7	MSI/GNF	2
NAP-2	Nucleosome assembly protein 1-like 4	7.5E + 03	1	1	MSI	2
LIF Rα	Leukemia inhibitory factor receptor	5.0E + 03	2	4	MSI	2
PDGF-Rα	Platelet-derived growth factor receptor, alpha polypeptide	4.6E + 03	1	2	MSI	2
MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	2.6E + 03	1	1	MSI/GNF	2
FasL	Tumor necrosis factor (ligand) superfamily, member 6	1.5E + 03	1	2	MSI/GNF	2
NSE	Enolase 2 (gamma, neuronal)	1.4E + 03	1	1	GNF	2
MMP8	Matrix metalloproteinase 8 (neutrophil collagenase)	9.0E + 02	1	1	MSI/GNF	2
VEGF-D	C-fos induced growth factor (vascular endothelial growth factor D)	5.0E + 02	1	1	MSI/GNF	2
ENA-78	Chemokine (C-X-C motif) ligand 5	3.4E + 02	1	1	MSI	2
CD30	Tumor necrosis factor receptor superfamily, member 8	3.3E + 02	1	2	MSI/GNF	2
MPIF-1	Chemokine (C-C motif) ligand 23	3.2E + 02	1	1	MSI	2
GROb	Chemokine (C-X-C motif) ligand 2	3.0E + 02	1	1	MSI	2
BDNF	Brain-derived neurotrophic factor	3.0E + 02	1	1	MSI	2
AFP	Alpha-fetoprotein	2.9E + 02	2	2	MSI/GNF	2
IGF-IR	Insulin-like growth factor 1 receptor	2.4E + 02	1	1	MSI/GNF	2
Calcitonin	Calcitonin/calcitonin-related polypeptide, alpha	1.9E + 02	1	1	GNF	2
Calcitonin	Calcitonin gene-related peptide type 1 receptor precursor	1.9E + 02	1	1	GNF	1
FGFβ	Fibroblast growth factor-20	1.6E + 02	1	1	GNF	1
IL-10Rβ	Interleukin 10 receptor, beta	1.5E + 02	1	1	MSI	2
Angp2	Angiopoietin 2	9.7E + 01	1	1	GNF	2
MCP-1	Splice isoform A of P15529	7.6E + 01	1	1	MSI/GNF	1
SCF	KIT ligand	5.9E + 01	1	1	MSI/GNF	2
IFNγ	Interferon, gamma	5.4E + 01	1	1	MSI/GNF	2
OSM	Oncostatin M	4.8E + 01	1	1	MSI	2
IL1α	Interleukin 1, alpha	4.5E + 01	1	1	MSI/GNF	2
TNFα	Tumor necrosis factor (TNF superfamily, member 2)	3.7E + 01	2	1	MSI/GNF	2
AR	Androgen receptor (dihydrotestosterone receptor; testicular feminization; spinal and bulbar muscular atrophy; Kennedy disease)	2.6E + 01	1	1	MSI/GNF	2
I-TAC	Chemokine (C-X-C motif) ligand 11	2.3E + 01	1	1	MSI	2
CGB	Chorionic gonadotropin, beta polypeptide	1.9E + 01	1	1	MSI/GNF	2
IL7	Interleukin 7	7.0E + 00	1	1	MSI/GNF	2

maxima in citrate-plasma (Fig. 3A, top left), well below what is expected by chance. The GNF and MSI sets showed a high frequency of maxima in the EDTA-plasma samples (Fig. 3A, top right); the VARI measurements were often highest in heparin-plasma (Fig. 3A, lower left), and the DB measurements were frequently highest in the serum samples (Fig. 3A, lower right). For the minimum values, all methods showed a significantly frequent occurrence of minima for the citrate samples (Fig. 3B, top left), and the DB data were very seldom lowest using heparin-plasma or serum samples (Fig. 3B, lower left and lower right). The other frequencies are close to what might be expected by chance. These analyses show evidence for general biases in protein concentrations as a result of blood preparation method.

We examined the magnitudes of concentration differences between the sample types. For each protein, the concentration in each preparation method was divided by the maximum concentration found in that specimen set. For example, if a protein had a concentration of 100 pg/mL in citrate-plasma and 200 pg/mL in serum, citrate-plasma was given a 0.5 and serum was given a 1.0. The median concentration ratios for each preparation method are shown in Fig. 4 for each of the four data sets. Each data set shows the citrate-plasma preparation with the lowest average abundances, from about 85% of the maximum values (DB) to about 40% of the values (GNF and MSI). Consistent with the results from Fig. 3, serum had the highest concentrations in the DB set, EDTA-plasma in the MSI and GNF sets, and heparin-plasma in the VARI set. The variation between preparation methods is similar between the DB and VARI sets and between the GNF and MSI sets, and the GNF and MSI sets had broader variation in the relative abundances (larger error bars) than the other two sets. These relationships could be related to the similarity between the groups in

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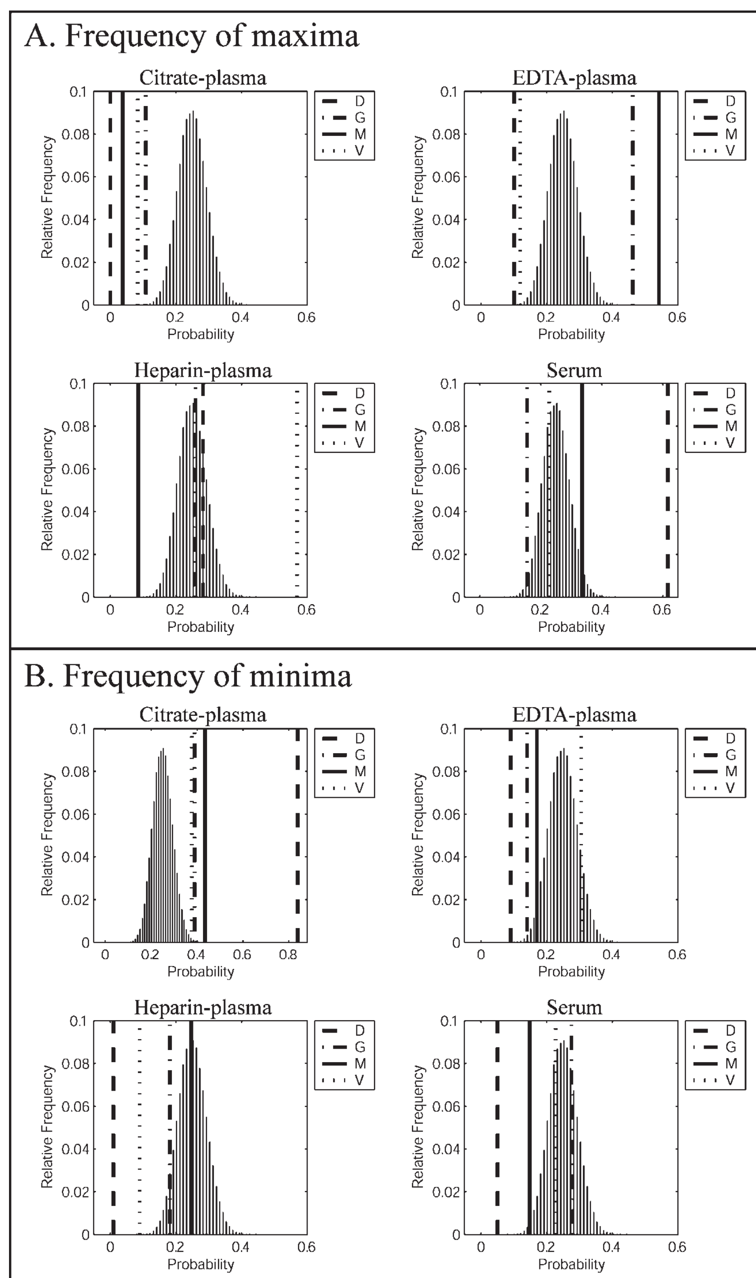

Figure 3. Frequency of maxima (A) and minima (B) in each of the four preparation methods: citrate-plasma (upper left), EDTA-plasma (upper right), heparin-plasma (lower left), and serum (lower right). Position on the *x*-axis for the vertical lines in each plot indicate the frequency of maxima (A) or minima (B) in a given preparation method. Each line represents one of the four data sets, with the identities given in the legend: DB = dashed line, GNF = dotted/dashed line, MSI = solid line, VARI = dotted line. Distribution of randomly-occurring frequencies also is included in each graph.

the proteins measured; GNF and MSI measured mostly cytokines, while VARI and DB measured higher-abundance serum proteins.

3.3 Consistent alterations in specific protein abundances

We then examined whether specific proteins, as opposed to all the proteins in general, were consistently highest or lowest in a certain preparation type. Evidence for such a bias would be indicated by multiple specimen sets showing agreement in the alteration of the concentration of a specific protein, *e.g.*, if all three of the BD specimen sets showed a certain protein higher in a certain preparation method. We identified the proteins that always gave a highest value in one particular preparation method, in every specimen set, and in every replicate experiment. Such biases toward a particular preparation method are more than 99% likely not to have occurred by chance, as determined by a permutation test similar to that described above. A summary of these results is shown in Table 1. Many proteins were always highest in serum or in EDTA-plasma, particularly in the DB and GNF

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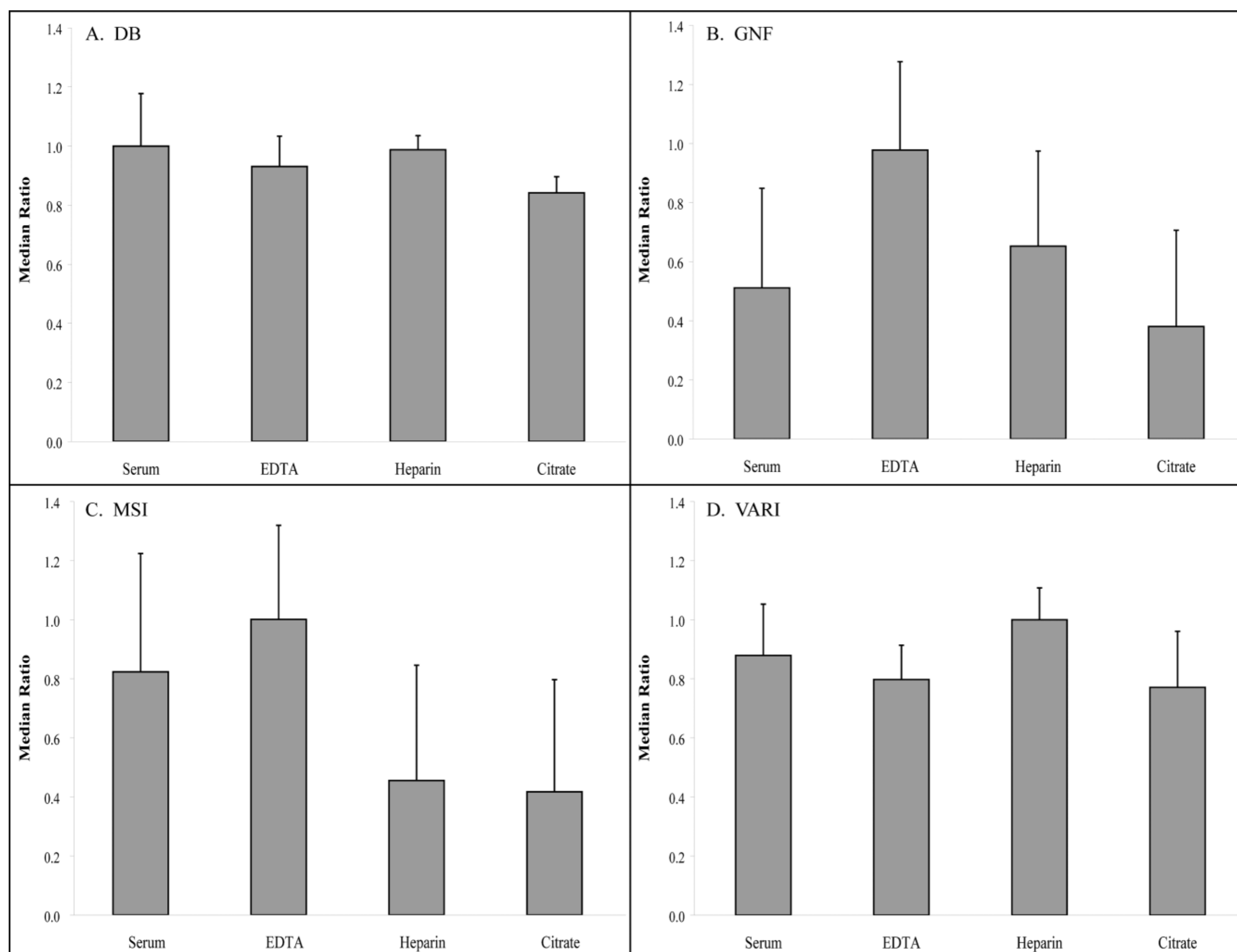

Figure 4. Median relative concentration ratios in each sample type. Concentration of each protein in each preparation type was divided by the concentration of the preparation type that was highest for a given sample. Median relative change in concentration is depicted for each preparation type from the (A) DB, (B) GNF, (C) MSI, and (D) VARI data sets. Error bars represent the SD in relative concentration change over all the proteins.

sets, respectively. Twenty-four proteins were always lowest in citrate-plasma in the DB set. These specific biases tend to follow the trends seen in the overall concentrations shown in Figs. 3, 4, but occasionally proteins are altered counter to those trends. For example, in the GNF set, all the four preparation methods had proteins that were consistently elevated. A complete list of the proteins that seem to have concentrations systematically affected by preparation method, along with the magnitudes of the alterations, is provided in the Supplemental Table 3. The magnitude of the difference between preparation methods was usually below three-fold, but some proteins had much larger alterations (a ten-fold change or more) in certain preparation methods. The most consistent differences were in the DB set; the 24 proteins that were always lowest in citrate-plasma ranged from 73 to 88% of the maximum values, and the ten proteins that were always highest in serum ranged from 138 to 119% of the minimum values.

The bias for a particular preparation method in a specific protein is visually depicted in Fig. 5 for two representative proteins from each data set. The replicate measurements from each sample were plotted with respect to preparation method, with the solid lines representing the averages between the replicates. In each case shown, one preparation method is consistently highest in every sample and every replicate. EDTA-plasma, heparin-plasma, and serum each have examples in which the concentrations seem to be systematically elevated in one preparation method. Independently-collected ELISA data are plotted along with the microarray data for hemoglobin (Fig. 5G). The concordance between the microarray ELISA measurements are very good for each sample (0.94 over all the samples), validating the accuracy of the microarray measurements and the fact that the hemoglobin concentrations are highest in EDTA-plasma for these samples.

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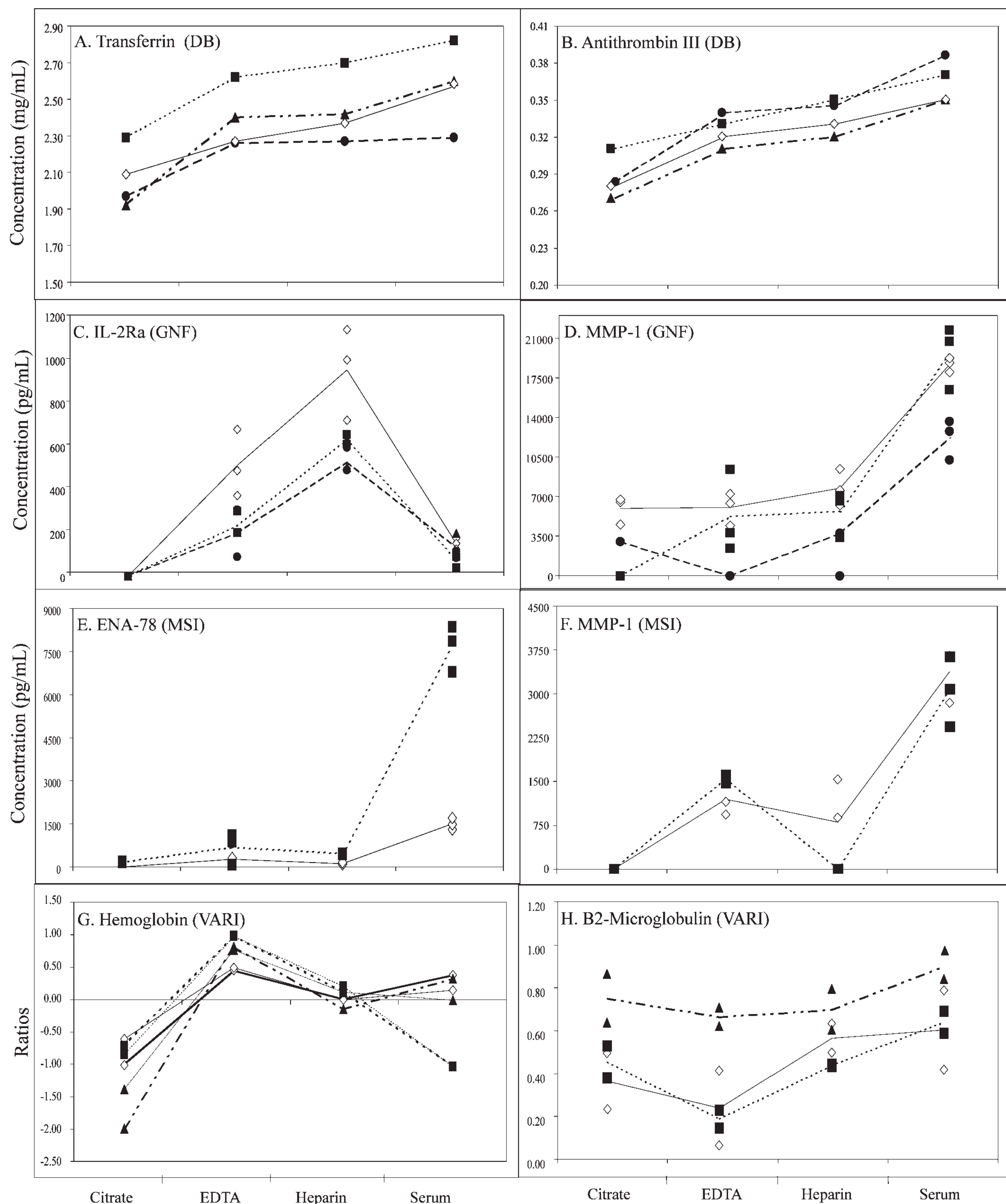

Figure 5. Variation in the concentration of individual proteins across different preparation methods (see text for basis of selecting these proteins). Analyte and data set (in parentheses) are indicated in each plot. Replicate data from two to four different samples are plotted with respect to preparation method. Individual values for each sample are shown by the following: BD sample 1: open diamonds; BD sample 2: solid squares; BD sample 3: solid triangles; and CAMS: solid circles. Averages of the replicate data are shown by a solid line for BDAA, a dotted line for BDAF, two dots and a dash for BDCA, and a dashed line for the CAMS specimen. Graph G includes ELISA data that has been normalized to the same scale as the microarray data, represented by darker versions of the corresponding lines for the microarray data.

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3.4 Linkage of MS data and antibody-based measurements

Another valuable use of these data for the PPP was to investigate relationships between the quantitative antibody-based measurements and the MS information derived from other work within the PPP. Based on the informatics integration methods (see Adamski *et al.*, this issue), 9504 unique IPI proteins were included in the combined data (see http://www.bioinformatics.med.umich.edu/app1/test/). The link between the MS data and the antibody-based measurements was made through IPI numbers. Two different search methods were used to find IPI numbers that corresponded to the analytes measured in the quantitative antibody-based assays (see Section 2), generating two lists of analyte-associated IPI numbers. Seventy IPI numbers that were common between these lists and the MS summary data were identified and are presented in Table 2. In four cases, two IPI numbers were associated with the same analyte name. Table 2 also gives the average concentration (the geometric mean over all samples, including the NIBSC sample, and all data sets) of each analyte, the number of laboratories (out of 18) finding that IPI number, and the average number of peptides found for that IPI number. The relationships between the MS summary data and the average concentrations were examined (Fig. 6). Figure 6A shows that individual laboratories made identifications in the 10–10 000 pg/mL range, with multiple laboratories finding the same IPI numbers above that range. Only single peptide identifications were made below around 200 pg/mL, with a steadily increasing average number of peptides above that (Fig. 6B). Both metrics increased steadily with concentration. The lack of data points in the 1–100 μg/mL range is primarily due to the low number of immunoassay and antibody microarray measurements in that range, as shown in Fig. 2.

4 Discussion

The analysis of the HUPO PPP reference specimens by antibody-based methods provided a useful complement to the other studies of the PPP. This work examined the use of immunoassays and antibody microarray methods to investigate the systematic variation of specific proteins between the PPP's reference specimens' sample preparation methods and to provide insights into the concentration-dependence of protein discovery by MS methods. The use of four distinct methods from four independent laboratories gave a broad view of the capabilities of antibody-based methods. Each of the four data sets had highly internally reproducible data, as shown by the high average correlations between replicate data, although the values did not always agree in the measurements of common analytes. The occasional lack of concordance between the sets underscores the importance of the use of common IRMs for cross validation and calibration between laboratories and methods. An international reference standard for 15 abundant serum proteins, CRM 470 [14], has been developed; its use has significantly reduced interlaboratory variation in many protein assays in European quality assurance programs [22]. Of note, DB analyzers used standards, calibrators, and controls based on common IRMs that are generally applied in clinical chemistry. Antibody microarray measurements have not yet achieved the precision standard of clinical analyzers.

We investigated two aspects of the effect of sample preparation on protein concentration: systematic alterations of all proteins in general and consistent alterations in the concentrations of specific proteins. The most common general systematic alteration was a reduction of protein concentrations in the citrate-plasma preparation. This effect is attributable to the dilution of the plasma fraction of whole blood by the sodium citrate solution [23] and by the osmotic with-

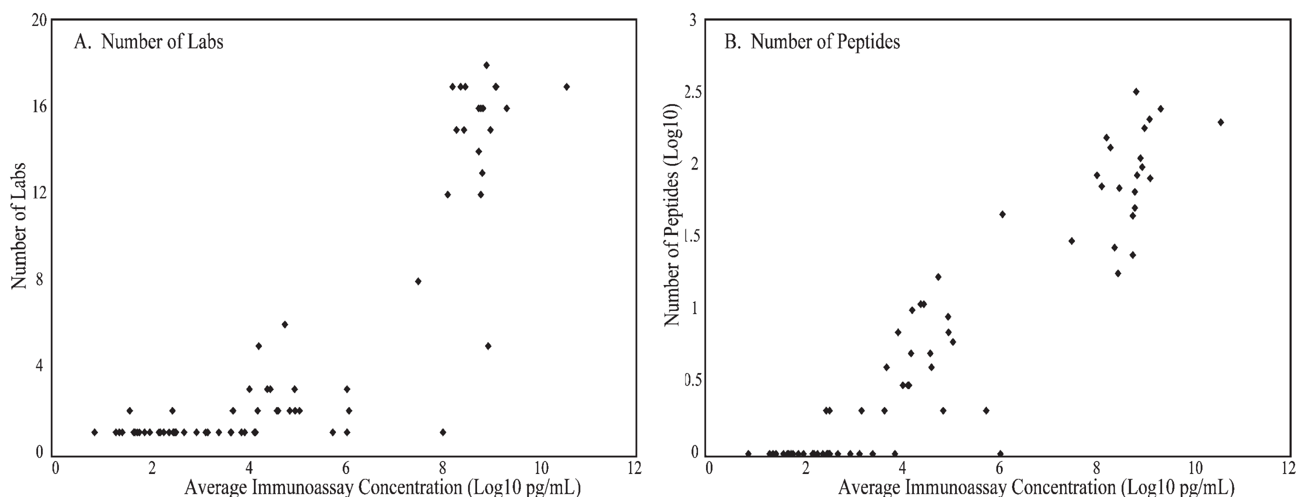

Figure 6. MS summary data with respect to concentrations measured by immunoassays and antibody microarrays. Concentration is pg/mL (log base 10). (A) Number of laboratories finding a given protein, (B) number of peptides for each protein identification.

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drawal of water from blood cells caused by the high salt concentration in the anticoagulant. When whole blood at a hematocrit of 0.4–0.5 is mixed with sodium citrate solution at a ratio of 9:1, the dilution of citrated plasma will be 15–19.5% (10% dilution from the citrate solution plus additional dilution from osmosis) [23]. The concentration reduction in citrate-plasma was the most consistent in the DB data and was explainable by the dilution factor, with 14 of the 17 consistently reduced proteins lower than the serum preparation by less than 20%. We might note that most of the DB analyses were not approved for use with citrate-plasma. The other data sets showed less consistent alterations in the citrate-plasma concentrations, perhaps due to lower precision in the measurements or other sources of variation besides dilution, as discussed below. Of great importance for proteomics analyses, the dilution in citrate-plasma did not seem to affect protein identification in PPP analyses using various fractionation and MS methods, as the citrate-plasma specimens gave similar numbers of proteins identified relative to the other specimen types and similar detection of low-abundance immunoassayed proteins (see Simpson *et al.*, and Omenn *et al.*, this issue).

The preparation method that generally gave the highest protein concentrations varied among the four data sets. The GNF and MSI sets had higher protein abundances in the EDTA-plasma preparation, the DB set had higher abundances in the serum preparation, and the VARI set had highest values in the serum and heparin-plasma. The GNF and MSI sets focused on cytokine detection, and the relatively higher concentration of the cytokines in EDTA-plasma could indicate a protective effect of EDTA on cytokine stability, perhaps through EDTA's role as a protease inhibitor. The more abundant, common serum proteins measured in the other two sets could be less susceptible to protease activity and therefore not necessarily higher in the EDTA-plasma preparation. Other sources of variation in concentration could be the anticoagulant-induced release of certain analytes by lymphocytes, such as the release of tumor M2-PK in heparin-plasma but not in EDTA-plasma [24], interference in certain assays by anticoagulants, or variability in protease activity or protein stability due to the presence or absence of certain anticoagulants.

The analysis of specific proteins showed that certain proteins were always highest or always lowest in certain preparation methods. The fact that some of these alterations were counter to the overall trends noted above shows that blood preparation methods can have variable effects on specific proteins or antibodies. Anticoagulants may in some cases specifically interact with certain proteins or specifically affect the stability of certain proteins. Such effects have been seen in previous studies. In one study, the levels of several hormones were either elevated or reduced between matched serum and EDTA-plasma and between matched serum and citrate-plasma samples [25]. Another study showed that parathyroid hormone is more stable in EDTA-plasma than in serum [26]. The levels of the cytokines IL-6, TNF-α, and leptin were found to be highly variable in citrate-anticoagulated- and heparin-anticoagulated-plasma but not in EDTA-anticoagulated-plasma or serum [27]. In some cases, an anticoagulant might actually bind to specific proteins. For example, EDTA binds to hemoglobin [28], which might be related to the observed consistent elevation of the hemoglobin measurements in the EDTA-plasma samples.

Based on the above observations, it is clear that comparisons between samples are only accurate when using samples that were collected with precisely the same method. Which preparation method to use in every case, however, is less obvious. No single preparation method is optimal for every analyte – the use of certain anticoagulants may interfere with some assays, and the activation of the clotting cascade may be detrimental for other assays. Therefore, the development of assays for individual proteins needs to be evaluated and optimized on a case-by-case basis. The information contained in Supplemental Table 3 could be used as a starting point for identifying potential anticoagulant-protein interactions that could affect an assay. Although assays for individual proteins must be individually optimized, it would be advantageous to use a single preparation method for proteomics methods and highly-multiplexed assays. Additional studies with an appropriate number of samples of each blood preparation method have to be performed to address the optimal blood preparation method for proteomics and highly-multiplexed studies, perhaps focusing on the consistency and stability of analytes rather than simply on concentration.

The final part of this study investigated the use of the antibody measurements to determine the concentration dependence of MS protein identification, using summary data from 18 different laboratories. A clear dependence on concentration was observed for both the number of laboratories finding certain proteins and the number of peptides found for each protein. It is encouraging that a precipitous decline in identifications at lower concentrations was not observed, but rather a steady decrease through most of the concentration range. Although the likelihood of identifying a protein and the quality of the identifications drop significantly for lower-abundance analytes, identifications were still made in the pg/mL range. Continued refinements and improvements in the technologies should make the identification of low-abundance proteins more common.

These studies demonstrate the benefits of high-throughput, high-precision, and high-sensitivity antibody-based analytical methods. We identified general and specific alterations in the protein concentrations that are related to the blood preparation method. In general, it appears that many cytokines are more stable in EDTA-plasma, specific interactions may occur in some cases with each anticoagulant, and a general dilution occurs with the use of citrate as an anticoagulant. The antibody-based methods also were useful for providing insights in the performance of MS-based protein identifications, showing that low concentration protein identifications are less frequent but still possible. In the

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continuing projects of the PPP, immunoassays and antibody microarrays will be useful in further studying these and other topics, such as characterizing the variation of many proteins in large populations of samples. Calibration using certified reference standards will be needed to reduce variation between laboratories and platforms.

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