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Influences of Blood Sample Processing on Low–Molecular-Weight Proteome Identified by Surface-Enhanced Laser Desorption/Ionization Mass Spectrometry

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Background: **Profiling approaches in proteomics, such as surface-enhanced laser desorption/ionization (SELDI) mass spectrometry, are used in disease marker discovery. The aim of this study was to investigate the potential influence of selected preanalytical factors on the results obtained.**

Methods: **Plasma samples anticoagulated with EDTA, citrate, or heparin, and serum samples from healthy volunteers were profiled by SELDI on CM10, immobilized metal affinity capture (IMAC) array with copper, and H50 chip surfaces. Using linear mixed-effects models, we examined the influence of elapsed time between venipuncture and sample separation (immediate to 24 h) and the type of serum tube used (Greiner Vacuette activator, gel serum separator, or plain tubes). We analyzed purified platelets, as well as platelet-poor and platelet-rich plasma samples treated with calcium and/or thrombin to determine the platelet contribution, directly or via the clotting process, to the profiles generated. We then used cluster analysis to identify samples with similar peak profiles.**

Results: **Different plasma types and sera could be distinguished on the basis of cluster analyses of their spectral profiles. Elapsed time between venipuncture and separation of plasma and serum from blood samples altered the profiles obtained, particularly for serum samples and particularly on IMAC chips. The type of serum collection tube also affected the profiles because of differences in clotting time. In vitro manipulation of platelets revealed that specific peaks in IMAC profiles of serum appeared to be derived directly from platelets. Several other peaks, including some of those exhibiting time-dependent changes, arose during the clotting process.**

Conclusion: **Preanalytical variables, such as sample handling, can markedly influence results.**

Disease processes are associated with substantial interindividual heterogeneity attributable to multiple genetic and epigenetic influences. Consequently, parallel analysis of multiple genes or proteins is a logical approach for obtaining individualized information when searching for potential new disease markers for use in diagnosis, prognosis, and disease monitoring. This approach has already been effective in the use of DNA microarrays. In breast cancer, for example, profiling 25 000 genes globally during the discovery phase led to the generation of disease-specific arrays containing smaller numbers (<100) of the most informative genes *(1)*, simplifying assay and data analysis.

Proteomics-based studies essentially enable the examination of the functional "end-units", thus complementing other methods that cannot predict quantity or form (and, hence, function) because of transcriptional, translational, and posttranslational modifications and epigenetic influences *(2)*. Blood is an ideal source of markers because it is easily accessible and reflects secondary systemic changes, as well as containing proteins/fragments shed by diseased tissue. Analysis of the fluid phase, plasma or serum, is a challenge however, because of the huge dynamic range of protein abundance and forms, with some carried

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by proteins such as albumin *(3–5)*. Promising techniques, such as 2-dimensional polyacrylamide gel electrophoresis after immunodepletion of the most abundant proteins, and multidimensional approaches such as liquid chromatography with tandem mass spectrometry, offer increased coverage and detection of not only classic blood proteins but also receptors, transcription factors, and even cytokines *(3, 4)*.

The use of mass spectrometry to generate mass profiles of protein/fragment mixtures in clinical samples is increasing, leading to identification of disease-associated proteins and profiles or molecular signatures for differentiation between groups in computational models. For example, lung cancer patients were stratified into 2 prognostic groups on the basis of tissue-sample profiling by classic matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)[3] mass spectrometry *(6)*. A variant of MALDI-TOF that has been more extensively used is surface-enhanced laser desorption/ionization (SELDI) TOF mass spectrometry, in which sample chips of differing surface chemistries selectively bind proteins, e.g., cations, in a sample before mass profiling *(7)*. The potential of SELDI-TOF is illustrated by the many studies with biologically and clinically relevant results, including identification of α-defensins as the agents in CD8 T-cell–derived anti-HIV1 activity *(8)*; profiling of different β-amyloid forms in Alzheimer disease *(9, 10)*; and in combination with various computer algorithms, the generation of impressive predictive diagnostic tests in many studies encompassing a range of diseases, such as ovarian *(11)*, prostate *(12)*, and breast *(13)* cancers as well as trypanosomiasis *(14)* and alcoholism *(15)*.

Such multiplex profiling and modeling approaches are promising, but concerns have been raised about their long-term robustness, the possible contributions of nonbiological variation to the results, and the need for better quality control (QC) *(16–20)*. Few studies have examined the effects of preanalytical procedures, such as chip preparation, matrix application *(21, 22)*, and sample processing [urine *(20, 23)* and blood *(24)*], but determining their relative influence on the results is essential, particularly when so little is known about this region of the proteome (<15 kDa). We have used SELDI, although the principle applies equally to other profiling approaches, to examine the effects of sample processing issues, such as time, tube, and anticoagulant type, on serum and plasma profiles. Understanding the influence of such factors is important in minimizing background experimental variation that may mask biological variation or be interpreted erroneously as disease specific.

[3] Nonstandard abbreviations: MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; SELDI, surface-enhanced laser desorption/ionization; QC, quality control; IMAC, immobilized metal affinity capture; PPP, platelet-poor plasma; PRP, platelet-rich plasma; TIC, total ion current; and S/N, signal-to-noise.

Materials and Methods

All chemicals were Analar grade and purchased from VWR Merck. ProteinChips, sinapinic acid, and calibrants were purchased from Ciphergen Biosystems. MilliQ water was used throughout.

SAMPLE COLLECTION

Venous blood samples were obtained from a total of 10 healthy volunteers (4 male, 6 female; age range, 23–54 years) after they had given informed consent. Volunteers were nonfasting, and the samples were all taken during the morning. The Vacuette® system (Greiner Bio-One) was used for venipuncture, and for tubes containing additives, blood was collected directly into the tubes up to the specified volumes. The specific tube types used as standard in the main parts of the study were all from Greiner Bio-One and were manufactured from polyethylene terephthalate (PET) plastic: they were Z/serum clot-activator tubes (cat. no. 456089; plain tubes coated with micronized silica particles), lithium heparin tubes (cat. no. 455084; coated with lithium heparin at 14 IU of heparin/mL of blood), citrate tubes [cat. no. 454327; containing 0.109 mol/L (3.2%) sodium citrate], and EDTA tubes (cat. no. 454286; coated with 1.8 mg of dipotassium EDTA/mL of blood). The tubes used in each part of the study are indicated in the relevant sections. All samples were maintained at room temperature (18–20 °C) before processing at the times indicated in each experiment with centrifugation at 2000*g* and 20 °C for 10 min unless otherwise specified. Serum and plasma (avoiding the fluid immediately above the buffy-coat layer) were aspirated, divided into aliquots, snap-frozen in liquid nitrogen, and stored at −80 °C overnight before analysis the following day.

EFFECTS OF PROCESSING TIME AND ANTICOAGULANT TYPE ON SERUM AND PLASMA

Approximately 30 mL of blood was taken from each of 3 volunteers directly into each of the 4 tube types [plain (activator), EDTA, citrate, and heparin tubes] and mixed by gentle inversion. Each of these samples was then divided further into 1.5-mL plain plastic pediatric tubes; thus, no further additives were introduced, and the correct concentrations of anticoagulants were maintained. As above, 1 tube of each type was centrifuged, and serum and plasma were collected at $t = 0$, 30 min, 1 h, 2 h, and 4 h (a total of 20 conditions per individual). For each individual, the samples were all analyzed as 1 experiment (with the 3 experiments spanning 2 months), randomized and in duplicate, using the 3 chip types, H50, CM10, and immobilized metal affinity capture array with copper surface (IMAC30-Cu), for 120 profiles per person. Samples from an additional 3 individuals were taken to investigate the effects of a 24-h vs a 1-h processing delay in the 4 types of tubes and were profiled with CM10 and IMAC30-Cu chips.

To investigate the possibility that the marked differences observed between serum and plasma samples on

IMAC chips were caused by the additives in the anticoagulant tubes, serum was added to each of the EDTA-, citrate-, and heparin-containing tubes to produce similar final concentrations of anticoagulant as in plasma samples. These samples were analyzed on IMAC30-Cu chips. To rule out effects of freezing on plasma and serum as the cause of these differences, 2 samples were examined fresh and after 1 cycle of freezing. In addition, 2 sets of serum and plasma samples were compared on chips at multiple dilutions (1 in 2.5 to 1 in 10).

effects of different clotting tube types on the serum profile with time

To investigate whether the type of activator tube influenced results and also to determine changes occurring during coagulation, we compared Greiner Vacuette tubes containing no additive (cat. no. 454001), a silica coating (cat. no. 456089), or gel serum separator with silica activator (cat. no. 454228). Blood samples (35–40 mL) from each of 3 individuals were collected into these 3 tube types (4 of each tube type per individual) and allowed to clot for 30 min, 1 h, 2 h, or 4 h before separation. The serum samples were then analyzed on IMAC30-Cu and CM10 chips.

determination of cellular and circulating protein contributions to serum and plasma profiles

Using samples from 6 individuals (3 for the full experiment and 3 for the cell-free part only), we examined the relative contributions to the serum profile of circulating proteins (or their derived fragments) and cell-derived proteins. For the full study, ~30 mL of blood from each individual was collected into 1 activator, 3 EDTA-containing, and 3 citrate-containing tubes. Serum samples, platelet-poor plasma (PPP), and platelet-rich plasma (PRP) were prepared from both citrate- and EDTA-anticoagulated blood *(25)*, and a serum mixture was also prepared from PPP and PRP after addition of calcium chloride, thrombin, or both (Jones Pharma) or water (for details of the methods, see the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue9/). Supernatants from platelets prepared from the PRP and treated with 20 mmol/L calcium chloride and 5 U/mL thrombin were also collected, and all samples were compared by use of IMAC30-Cu and CM10 chips.

SELDI analysis of samples

Matrix preparation and calibration were performed essentially as described previously [Ref. *(20)*; also see the online Data Supplement]. We checked the performance of the SELDI by running a daily QC serum sample on a CM10 chip. We processed the protein chips (8-spot) with a Biomek 2000 liquid-handling station. All samples were analyzed in duplicate, with each prepared individually and randomized throughout the chips for each experiment. A QC sample of pooled normal serum was also included on each chip and was assigned to spot A for the first chip, spot B for the second, and so forth to ensure representative coverage of each spot in the larger experiments. CM10, IMAC30-Cu, and H50 chips were loaded with 5 μL of sample diluted 1 in 10 in 50 mmol/L HEPES, pH 7.6 (CM10), or 0.1 mol/L sodium phosphate–0.5 mol/L NaCl, pH 7.0 (IMAC and H50), with appropriate chip pretreatment and washing (see the online Data Supplement). After being air dried, the chips were analyzed by SELDI, with acquisition up to 100 kDa, an optimal mass range of 3–15 kDa, a laser intensity of 180–190 (200–220 for >10 kDa), detector sensitivity of 9, and collection of 50 transients across the spot surface. At least 2 different production batches of some chip types were used with no apparent effect on reproducibility.

data analysis

Brief descriptions of the QC and biostatistical data analysis approaches appear below (see the online Data Supplement for detailed descriptions). Briefly, QC spectra were examined to identify any systematic errors, such as changes in instrument performance or spot bias. For each QC spectrum exported as raw data in ".csv" format, total ion current (TIC) was examined. After peak detection and alignment *(20)*, the maximum intensity value, the number of matched peaks between spectra, and the mean intensity deviation were examined to detect outliers. Each parameter was examined across the entire *m/z* range and in a series of subranges. Similar routines were used to determine replicate acceptability (Rogers et al., manuscript in preparation).

Peaks were detected by use of Ciphergen Express, Ver. 3.0. Spectrum alignments were checked and, if necessary, realigned by use of 3 to 4 dominant common peaks within each of the 2 regions (i.e., *m/z* 2000–10 000 and 10 000–20 000) of each chip type. Before peak cluster detection, baseline subtraction was used, but because of the large differences in sample and chip types, normalization of TIC between spectra was not used. For the lower mass range of 2–10 kDa, a noise setting of 2 sigma was used for determining noise only in the spectral mass region of interest. A signal-to-noise (S/N) ratio >3 was used for the first pass, and a S/N ratio >2 was used for cluster completion in the second pass, with a cluster mass setting of 0.1% (i.e., ±0.05%) and a requirement to be in a minimum of 10% of samples. For the 10- to 20-kDa regions, the higher-intensity laser runs were used at a noise setting of 3 sigma and the same S/N settings. The cluster mass setting was 0.2% (i.e., ±0.1%). Peak data were exported as ".csv" format files for subsequent statistical analysis.

To investigate the variations in SELDI-TOF determinations among different sample types and processing times, linear mixed-effects models were used, and a separate model was fitted for each peak, chip type, and mass range. In these models, the response variable was the

intensity value at 1 peak cluster. The effects of tube type (citrate, EDTA, heparin, or serum; a 4-level factor with citrate as baseline) and time (0, 0.5, 1, 2, and 4 h; a 5-level factor with time 0 as baseline) were modeled. We also included an interaction term for tube type and time, e.g., a term that models differences in the effect of time according to tube type. A random-effect term in the model described individual-specific effects, allowing for the correlation between peak intensities measured on the same individual. Only the levels of 0.5% ($P < 0.005$) and 0.1% ($P < 0.001$) were considered significant as an ad hoc measure to account for the large number of tests performed. Additionally, to investigate the nature of the changes in intensity observed in the changing peaks, we performed a simple trend test, with time defined as a continuous variable, coded in equal ordered intervals ($t = 0$ coded as 0; $t = 30$ coded as 1; $t = 60$ coded as 2; $t = 120$ coded as 3; and $t = 240$ coded as 4).

Hierarchical clustering was used to observe natural groupings in the data in this study, with a Euclidean distance measure and the Ward linkage agglomeration method *(26)*, based on peak heights first standardized by subtracting the mean and dividing by the standard deviation for that peak. The results of these measures of similarity between observations are displayed in a dendrogram. All analyses were undertaken in R *(27)*, using the lme() function in library(nlme) *(28)* for the linear mixed-effects models and hclust() for the cluster analysis.

All peaks listed in the *Results* section have been assigned mean *m/z* values to facilitate comparison between experiments in which detected peak masses may have varied within the limits of the mass cluster windows. These values were assigned based on analysis of 12 replicates on each chip type of a pooled sample, in 3 independently calibrated runs.

Results

Representative examples of the profiles of the different fluids and chip types are shown in Fig. 1A, and the marked similarities in gross profiles between individuals are shown in Fig. 1B. On CM10 chips, 147 different peaks were seen overall (84 in 2–10 kDa, 34 in >10–20 kDa, and 29 in >20–100 kDa), with 80%–90% common to both serum and plasma in the range <20 kDa. Marked differences were apparent, however, even between the 2 anticoagulants, citrate and EDTA, which both act by chelating calcium, as was clearly demonstrated by the perfect clustering of all samples from all individuals in the time-course experiments based on sample type (Fig. 1C). With IMAC30-Cu chips, the numbers of peaks detected overall were similar to those detected on CM10 chips, but many more differences were readily apparent between plasma and serum samples, both in terms of numbers of peaks detected (e.g., for the time-course study in the range 2–20 kDa, a total of 84 peaks in serum vs 70 in citrate-plasma) and their intensities (Fig. 1A). Several experiments investigating a possible explanation for the marked differences between the serum and plasma profiles on IMAC chips (IMAC-Cu or IMAC-Ni) failed to reveal any obvious analytical explanations, such as possible interfering effects of the anticoagulants on chip chemistry or selective in vitro degradation of plasma samples during freezing (data not shown). Differences between plasma profiles were less apparent than with serum, as reflected in the poorer clustering compared with CM10 (Fig. 1C). For H50 chips, many fewer peaks were seen (Fig. 1A); there was little difference between serum and plasma types and no evidence of clustering based on sample type (Fig. 1C). The cluster analysis was repeated with an uncentered Pearson similarity measure, and very similar results were obtained.

Examination by linear mixed-effects models of profiles generated after different periods of elapsed time from venipuncture to sample processing showed significant interaction effects at the 0.1% level ($P < 0.001$) between time and sample type for several peaks for the CM10 and IMAC30-Cu chips. We found no significant interactions when examining the H50 chip. Because of the interaction, we examined the effect of time in stratified models, one for each chip type and tube type, and the H50 chip type was included for completeness. This procedure was followed in all subsequent analyses.

With these stratified models, we observed changes in peaks, with the number of changes depending on sample type (serum > EDTA-plasma > heparin-plasma > citrate-plasma; see Table 1 and the online Data Supplement). Within 4 h after venipuncture, CM10 profiles of serum samples showed significant changes in 12 of 96 (12.5%) peaks in the range <20 kDa, whereas in the citrate samples, none of the 105 peaks changed significantly, in EDTA only 5 changed, and in heparin, 1 peak showed changes in the same mass range, when compared with $t = 0$ (Fig. 2; also see the online Data Supplement). There was no overlap between the serum and plasma samples in terms of the specific peaks affected. Only 9 peaks were affected on the H50 profiles, all of which were in serum samples; only very low intensity peaks were affected, and none were in the *m/z* region <20 kDa. In the same *m/z* range on IMAC-Cu chips, 2 of 70 peaks (2.8%) in the citrate samples changed significantly with time in the first 4 h, compared with 30 of 84 peaks (35.7%) in serum; only 5 and 8 peaks were affected in heparin and EDTA samples, respectively (Table 1 and Figs. 2 and 3). Many of the changes in the serum samples were readily apparent within 30 min of venipuncture (Table 1 and Fig. 3), whereas virtually all significant changes in the plasma samples did not occur until 4 h after venipuncture. Only 7 serum peaks changed significantly at 30 min after venipuncture, with only 2 of these changing further after 60 min (Table 2). It is also noteworthy that the majority of changes were increases in peak intensity, with the peak at *m/z* 2189.2 in serum a notable exception, and although many of the changes in serum peaked by 1 h and remained at a plateau for the 4-h period, some peaks (e.g.,

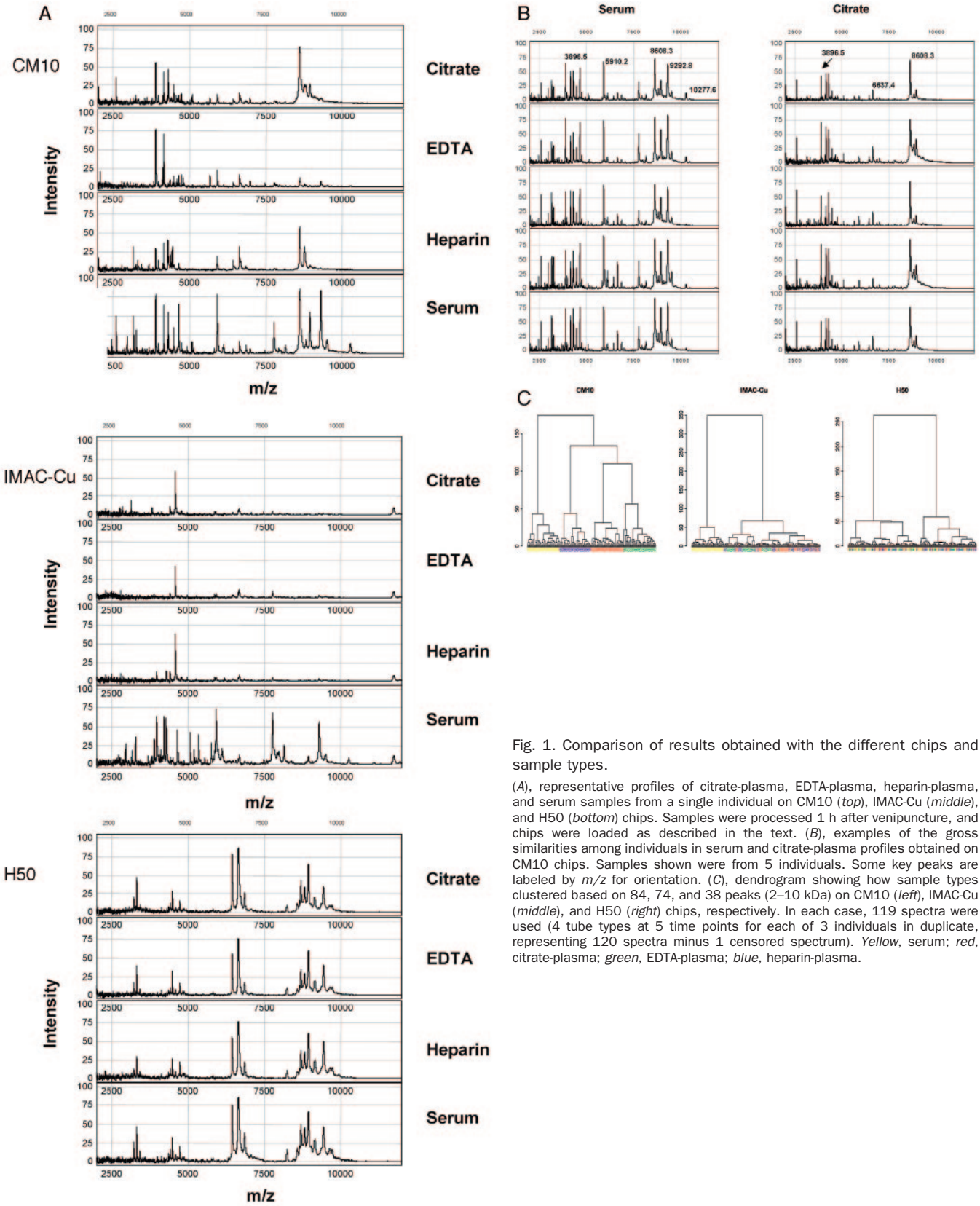

Fig. 1. Comparison of results obtained with the different chips and sample types.

(*A*), representative profiles of citrate-plasma, EDTA-plasma, heparin-plasma, and serum samples from a single individual on CM10 (*top*), IMAC-Cu (*middle*), and H50 (*bottom*) chips. Samples were processed 1 h after venipuncture, and chips were loaded as described in the text. (*B*), examples of the gross similarities among individuals in serum and citrate-plasma profiles obtained on CM10 chips. Samples shown were from 5 individuals. Some key peaks are labeled by *m/z* for orientation. (*C*), dendrogram showing how sample types clustered based on 84, 74, and 38 peaks (2–10 kDa) on CM10 (*left*), IMAC-Cu (*middle*), and H50 (*right*) chips, respectively. In each case, 119 spectra were used (4 tube types at 5 time points for each of 3 individuals in duplicate, representing 120 spectra minus 1 censored spectrum). *Yellow*, serum; *red*, citrate-plasma; *green*, EDTA-plasma; *blue*, heparin-plasma.

Table 1. Effects of tube type and time interval between venipuncture and sample processing on peak profiles generated.[a]

		Mean intensity					
Tube type	**Peak, *m/z***	***t* = 0**	***t* = 30 min**	***t* = 60 min**	***t* = 120 min**	***t* = 240 min**	***P*,[b] trend**
Citrate	3887.3	1.50	1.53	1.23	2.33	***3.95***	0.0009
EDTA	3887.3	8.56	12.00	14.49	20.60	***28.30***	0.0001
	3975.8	0.65	1.09	0.86	1.46	***1.99***	0.0004
	7766.9	25.57	29.45	37.17	39.82	***53.35***	0.0001
	7922.7	2.28	2.71	4.09	4.81	***10.46***	0.0005
	7973.9	3.20	4.14	5.46	6.78	***13.04***	0.0006
	8141.8	4.50	5.68	7.47	9.63	***17.12***	0.0004
Heparin	3959.5	3.35	5.85	5.72	12.62	28.97	0.0001
	3975.8	3.86	6.08	6.60	7.99	15.59	0.0001
	4285.9	6.07	8.84	12.83	12.97	26.35	0.0001
	4301.1	2.33	3.33	4.30	5.20	11.62	0.0001
Serum	2189.2	11.14	9.47	5.66	6.23	***4.33***	0.0001
	2666.9	2.36	9.65	***11.14***	***11.83***	***8.38***	0.0134
	2730.0	7.14	***9.56***	***11.70***	***16.78***	***17.79***	0.0001
	2885.5	3.94	3.23	6.05	***8.66***	***9.67***	0.0001
	2956.2	18.98	***42.58***	***37.20***	***42.35***	30.57	0.1425
	3195.7	2.94	***11.08***	7.21	7.35	5.44	0.8200
	3266.5	5.03	***17.35***	12.27	11.59	6.84	0.8049
	3277.9	20.60	28.33	***38.05***	***42.48***	***40.70***	0.0001
	3685.5	7.79	10.29	13.78	***20.74***	***19.50***	0.0001
	3776.7	2.58	3.94	***9.05***	***14.12***	***18.60***	0.0001
	3819.0	5.86	9.91	9.94	***14.60***	***15.70***	0.0001
	3959.5	36.21	48.12	***63.04***	***66.47***	***69.18***	0.0001
	4213.7	25.40	***45.71***	***55.87***	***60.43***	***55.50***	0.0001
	4285.9	43.27	52.50	***61.97***	***63.56***	***60.31***	0.0009
	4301.1	20.27	24.19	***35.58***	***40.08***	34.95	0.0006
	4476.5	5.59	6.27	8.56	10.69	***13.84***	0.0001
	5068.5	4.74	17.00	***33.60***	***41.18***	***39.53***	0.0001
	5196.7	6.00	***16.70***	***18.22***	10.75	2.96	0.2166
	5267.8	3.86	***8.77***	***8.85***	***10.97***	7.58	0.0307
	5340.2	19.47	***53.78***	***53.59***	***62.08***	***43.87***	0.0121
	5541.5	2.26	***9.66***	***9.31***	***12.41***	8.23	0.0178
	5810.1	4.90	***12.56***	11.71	***14.54***	9.30	0.1056
	5966.5	16.03	***37.88***	***35.64***	***40.21***	24.96	0.2408
	6113.7	15.27	***38.28***	***33.71***	***39.03***	25.78	0.1936
	8937.7	10.23	12.74	***20.40***	***22.42***	***26.46***	0.0001

[a] Results shown are for the *m/z* 2000–10 000 range on the IMAC-Cu chip; the *m/z* 10 000–20 000 range and CM10 and H50 chips also were analyzed (see the online Data Supplement). Peaks shown in bold italics are those changing significantly at the 0.1% level, i.e., $P < 0.001$, from the $t = 0$ time point with the mean intensity shown at each time point.

[b] *P* values from a test for trend over the 5 time points. Results are based on a total of 20 samples (4 tube types at 5 time points) analyzed in duplicate from each of 3 individuals on each chip type.

m/z 3266.5, 5196.7, 5966.5, and 6113.7) showed the possibility of a partial return toward original values (Fig. 3). The significance level of the test for trend is reported in the last column in Tables 1 and 2. Although some changes did not appear monotonic, in most cases there was significant evidence of a trend, with peak intensities in most cases increasing with time.

Because of the large differences between chip types and fluid types in relation to peak number, spectra were not routinely normalized with respect to TIC before analysis, but we examined the effect of normalizing by TIC, using the CM10 profiles from this experiment. Even within this single chip type, differences in TIC were apparent among sample types, but almost all peaks with a significant intensity change in our initial analysis, i.e., in the absence of normalization, showed nearly identical results after normalization, further substantiating the changes observed.

To further confirm changes with time and examine the degree of change with delayed sample processing, we compared CM10 and IMAC30 chip samples processed within 1 and 24 h (see the online Data Supplement). Substantial numbers of peaks changed, with both decreases and increases in intensity. In the citrate samples,

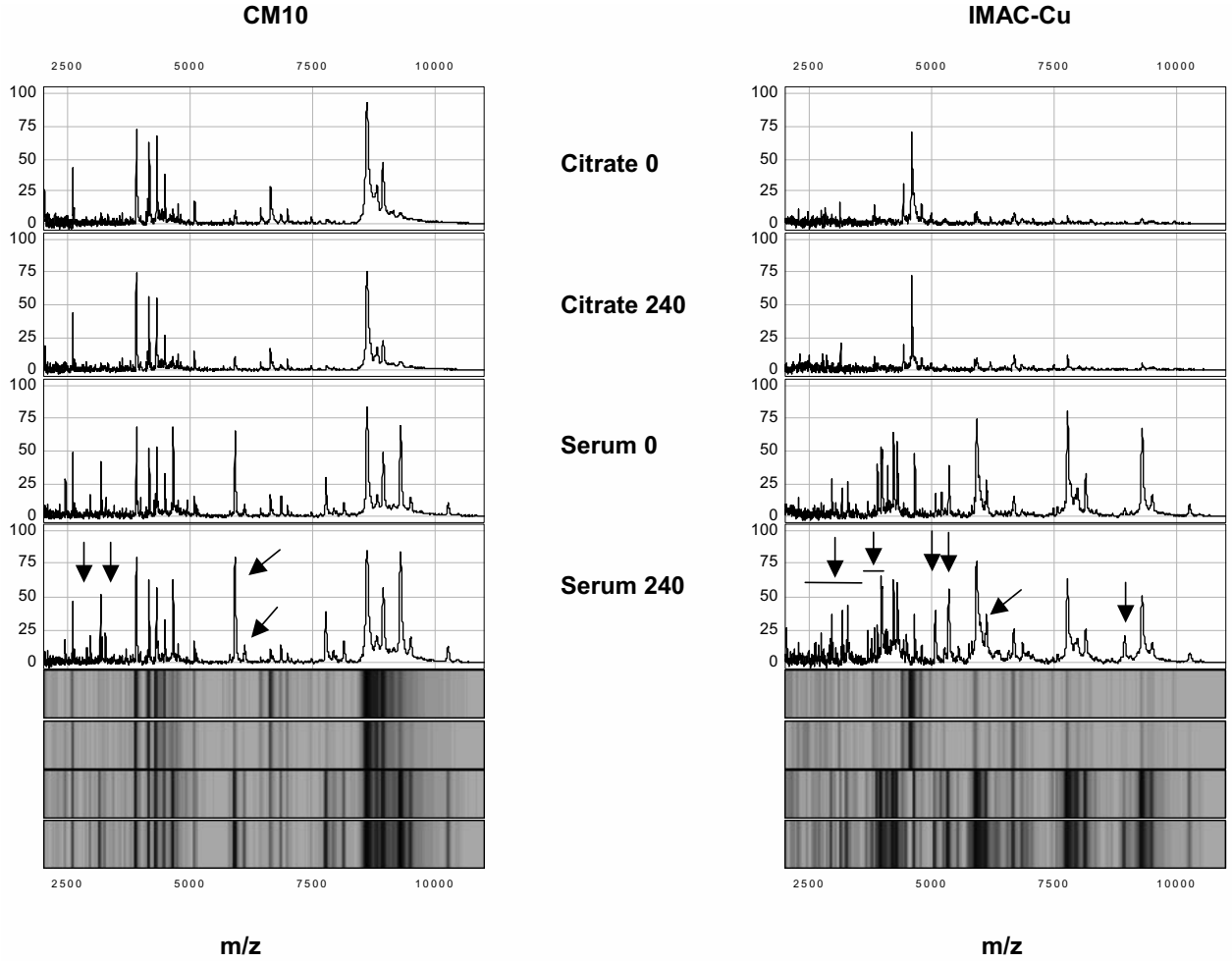

Fig. 2. Representative examples of serum and citrate-plasma profiles on CM10 (*left*) and IMAC30-Cu (*right*) chips, showing major peaks changing with time of processing.

Profiles are for citrate-plasma and serum samples at $t = 0$ and 4 h, with comparable views of the gel results *below* the profiles. Some of the more marked changes are indicated, including those for *m/z* 2888.3, 3268.6, 5910.2, and 6116.7 on CM10 chips and 2666.9–3277.9, 3685.5–3959.5, 5068.5, 5340.2, 6113.7, and 8937.7 on IMAC30-Cu chips (*arrows*).

very few changes were seen. A substantial increase in the proportion of peaks affected was apparent, particularly in the EDTA samples. For example, 24 peaks <20 kDa on the IMAC profile changed significantly over 24 h, compared with only 8 in the shorter time course. In confirmation of the shorter time-course experiments, in which changes in EDTA-plasma peaks did not appear to be significant until 4 h, 6 of the 8 peaks that had changed significantly at 4 h were significantly changed at 24 h, suggesting a set of changes in plasma that occur gradually with time. In similar confirmation of the earlier results, in which serum profile changes seemed to occur predominantly in the first 30–60 min, only 5 of the 30 serum peaks that changed in the short time period on the IMAC profile also changed significantly between 1 and 24 h; 3 of these peaks (*m/z* 2956.2, 5196.7, and 11097.0) appeared to be reversing the changes observed during the immediate postvenipuncture phase. As expected, given the predominant effect of clotting on the serum profile particularly evident on IMAC chips, we found little overlap between peaks affected by time in the plasma samples and those in the serum samples, although in the IMAC-Cu profiles of both citrate and heparin samples at 24 h, 3 of 5 peaks changing in each case were those that changed in serum samples in the short time course.

Comparison of the 3 types of serum tubes produced similar results in that no changes with time were apparent in the activator (silica particles) tube or the activator/gel serum separator tubes on the CM10 chip, and only 14 peaks changed on the IMAC chips, 4 of which were common to both gel and activator tube types and included the *m/z* 3776.7 and 5196.7 peaks shown in Table 2. The activator tubes were those used in the large time-course study above; hence, the results would be expected to be similar. The time comparison of 30 min rather than $t = 0$ was used, as in the plain tubes this was the minimum time for any clotting to be apparent. For the plain tubes, 43 peaks changed significantly between 30

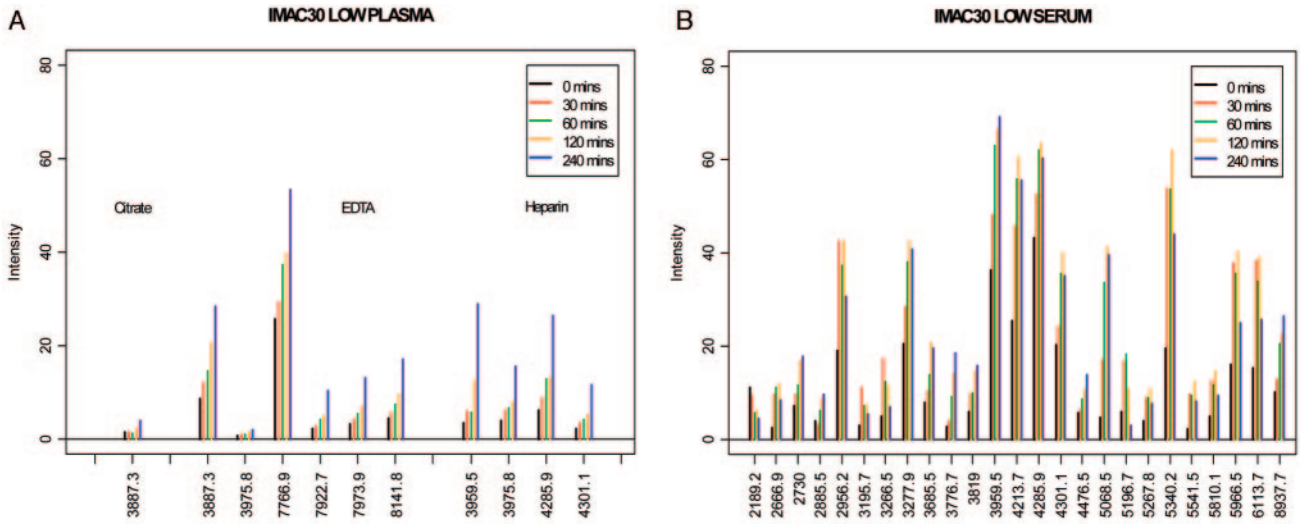

Fig. 3. Plots of changes in time of low-mass peaks in plasma (*A*) and serum (*B*) samples on IMAC chips.

Results are for those peaks that exhibited significant changes ($P < 0.001$) with time interval between venipuncture and sample processing compared with $t = 0$ (Table 1). The peak at *m/z* 3887.3 in the EDTA sample may represent the doubly charged peak of *m/z* 7766.9.

and 240 min (data not shown), including many of the peak changes in Table 1 but also additional changes, presumably reflecting the delayed clotting of this type of sample. Significant differences were apparent, however, in a small number of peaks between the gel and activator tubes. For example, at $t = 30$ min in the range below 10 kDa on the CM10 chip, peaks at *m/z* 3670.96 and 3826.69 were significantly higher in the gel tubes ($P < 0.01$). On the IMAC30 chip, peaks at *m/z* 3289.88 and 4297.76 were significantly lower in the gel tubes (see the online Data Supplement). These differences were not significant at later time points, however.

Analysis of the various serum samples generated from in vitro clotting of EDTA- or citrate-plasma in the presence or absence of platelets provided further information about the observed plasma/serum differences, which were particularly evident on the IMAC chips (Fig. 4). These results were reproducible between individuals in terms of the specific peaks observed to change. The previously observed differences between serum and plasma samples were reproduced in this study, with conversion of the citrated PPP to serum after activation with either calcium and/or thrombin leading to the emergence of some of the peaks characteristic of serum samples, particularly in the case of the IMAC profiles, but with little effect on the CM10 profiles. Conversion of PRP

Table 2. Effects of time interval between venipuncture and sample processing on CM10 and IMAC30 peak profiles of serum samples.[a]

A. Change from $t = 30$ min time point

		Mean intensity				
Chip	**Peak, *m/z***	**$t = 30$ min**	**$t = 60$ min**	**$t = 120$ min**	**$t = 240$ min**	**P,[b] trend**
CM10	7935.1	4.02	***7.77***	6.57	5.81	0.0838
IMAC30	2885.5	3.23	6.04	8.65	***9.67***	0.0002
	3776.7	3.94	9.05	***14.12***	***18.60***	0.0001
	3959.5	48.12	63.04	66.48	***69.18***	0.0006
	5068.5	17	33.6	***41.18***	***39.53***	0.0002
	5196.7	16.7	18.22	17.25	***2.96***	0.0001
	8937.7	12.74	20.41	22.43	***26.46***	0.0005

B. Change from $t = 60$ min time point

		Mean intensity			
Chip	**Peak, *m/z***	**$t = 60$ min**	**$t = 120$ min**	**$t = 240$ min**	**P,[b] trend**
IMAC30	3776.7	9.05	14.12	***18.60***	0.0003
	5196.7	18.22	10.75	***2.96***	0.0001

[a] Peaks shown in bold italics are those changing significantly at the 0.1% level, i.e., $P < 0.001$, from the $t = 30$ min (A) and $t = 60$ min (B) time points, with the mean intensity shown at each significant time point in the *m/z* 2000–20 000 range.

[b] P value from a test for trend over the 5 time points.

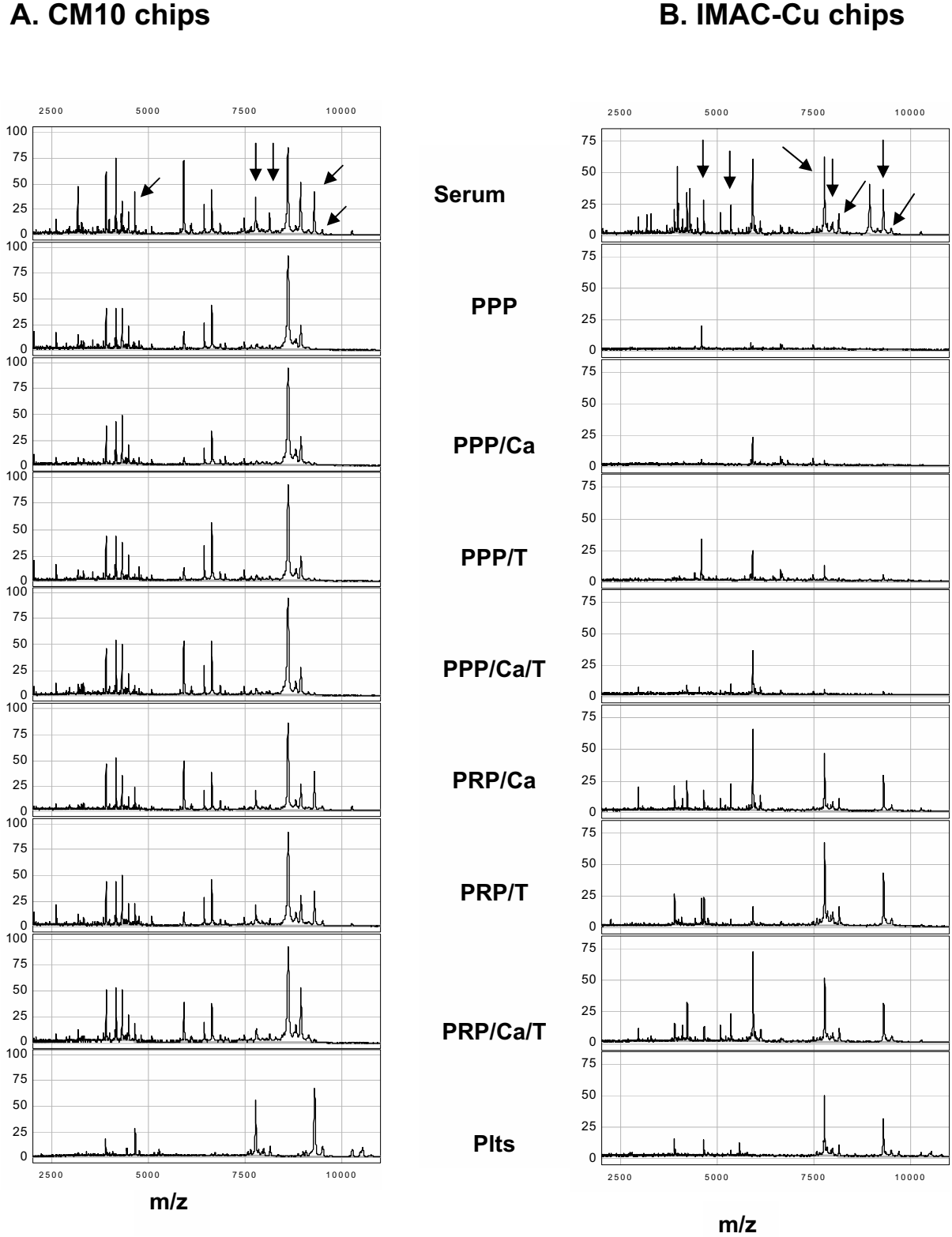

Fig. 4. Profiles of the peaks obtained in the *m/z* 2000–10 000 range on CM10 (*A*) and IMAC-Cu (*B*) chips for serum, citrated PPP, PRP, and supernatant from activated platelets (*Plts*).

For citrated PRP and PPP, the profiles are shown after addition of vehicle control, calcium (*Ca*), thrombin (*T*), or both calcium and thrombin (*Ca/T*). Some peaks in serum samples (*arrows*) correspond to the *m/z* values of the main peaks in platelet releasate. Examples of the peaks in the *m/z* 10 000–20 000 mass range that changed can be seen in the online Data Supplement.

produced similar changes, but additionally, many other peaks characteristic of serum were produced (Fig. 4). Comparison of the main peaks of platelet releasate with serum revealed peaks at *m/z* 4647.1, 5340.2, 7766.9, 7973.9, 8141.8, 9290.8, 9497.8, 10283.7, 11097.0, and 17052.2 in both that were largely or completely absent from plasma, implying a direct contribution of platelet-derived proteins to the serum profile, although this result would need to be confirmed by sequence analysis. Interestingly, 4 of these peaks were among those previously identified as increasing significantly in EDTA samples left at room temperature for 4 h after venipuncture, but they did not change in citrate samples (2 of the peaks did change by 24 h), supporting the acknowledged greater stability of platelets in citrate anticoagulant vs EDTA.

Only 2 of the platelet-derived peaks were among those previously found to change significantly in serum with time after venipuncture, implying early release during the clotting process. Most of these peaks (within the mass cluster range) were also identified on the CM10 chip. Although the peaks at *m/z* 7766.9 and 8141.8 were initially thought to occur similarly in plasma on this chip type, the observed peaks subsequently were found to be plasma peaks ~25 Da greater in size, leading to resolved double peaks in serum in each case. The differences between these sample types can be interpreted on the basis of the serum profile on IMAC chips, in particular those arising from circulating proteins such as those seen in the plasma samples but containing major components of proteins that arise from coagulation and not platelet dependent, proteins that arise directly from platelets, and proteins that arise during coagulation and are not directly derived from platelets but are secondary to platelet activation. It is also clear that some peaks in the profile are not likely attributable to these sources and may arise from other cells, such as neutrophils. Similar results were also observed in the EDTA samples handled similarly, although there were some very specific differences (data not shown) in comparison with citrate samples.

Discussion

Many studies have demonstrated that mass spectrometric protein profiling and pattern analysis approaches offer exciting opportunities in disease diagnostics. The majority of such studies to date have used SELDI as the analytical platform. Concerns about this approach, however, include the possibility that it is limited to analysis of only the most abundant proteins and consequently reflects changes occurring as secondary systemic effects rather than disease-related proteins per se; its reproducibility over time and between laboratories; and whether some of the results arise because of nonbiological variation *(16, 18, 19)*. Studies are now addressing these issues, with collaborative groups, such as the Early Detection Research Network—Prostate-SELDI Investigational Collaboration (ESPIC) sponsored by the National Cancer Institute/Early Detection Research Network, demonstrating the achievable extent of interlaboratory reproducibility of SELDI profiling and developing QC protocols *(29)*. In terms of comparability, our serum profiles generated on IMAC-Cu chips resemble those presented as examples by the ESPIC collaborative study *(29)*, and our mean *m/z* values of 5908.1, 7766.9, and 9290.8 are comparable to their values of 5906.5, 7768.6, and 9289.2 for their selected serum QC peaks. Our QC measures are currently being developed further, together with improved peak detection routines and improved methods of normalization, which will be particularly critical for smaller peaks and smaller changes. The impact of preanalytical variables, ranging from patient posture to sample timing and tube type, on the quality of laboratory results for many routine clinical measurements is well recognized *(30)*. Few studies, however, have examined the effect of preanalytical procedures, such as sample processing, on the profiles generated by mass spectrometry. This study provides important insights into handling and processing of blood samples. Although we have used SELDI as the analytical platform, these results could apply equally to other profiling approaches, regardless of whether the mass spectrometer is interfaced with ProteinChips, liquid chromatography, or capillary electrophoresis.

The analysis of low–molecular-weight proteins or peptide fragments is in its infancy, but thousands of peptides and proteins are believed to occur in human serum, many as fragments of much larger proteins and some bound to carrier proteins such as albumin *(3–5, 31, 32)*. MALDI-TOF analysis of hemofiltrates from patients undergoing dialysis for chronic renal failure revealed an estimated 5000 different peptides circulating in blood *(31)*. Many of these peptides represented multiple fragments of known proteins, but the extent to which these cleavage products are specific to this sample source vs being representative of peptides circulating in healthy individuals is not clear because most would be expected to be cleared via the kidney in individuals without renal disease.

Maintenance of hemostasis and the balance between coagulation and fibrinolysis is dynamic and usually tightly controlled *(33)*. This process is essentially a series of enzymatic reactions causing sequential activation and inactivation of proteases, and platelet activation causing clot formation; there thus is potential for a resulting release of peptides and protein fragments, whether from platelets directly (e.g., granule contents or cleaved platelet protease-activated receptors) or from the various proteins involved, such as fibrinogen peptides A and B cleaved from fibrinogen during conversion to fibrin. There are many examples of the effects of sample type on analytes measured by immunoassay. For example, vascular endothelial growth factor is found in much higher concentrations in serum than plasma because it is released from platelets during clotting *(25)*. Similarly, concentrations of neutrophil-derived lactoferrin and elastase are significantly higher in serum than plasma because of their release from neutrophils during sample clotting *(34)*. It is

not surprising that the serum and plasma profiles observed in our study are so markedly different, and the clear involvement of platelets as a source of some of the peaks can be demonstrated. This study also shows the potential utility of this approach in investigating changes and defects in coagulation events.

This preliminary study, based on a large number of samples obtained from a relatively small number of healthy volunteers, clearly illustrates the importance of sample handling, and the consistency of the results is demonstrated by their overlap in different parts of the study. Various sample-handling recommendations can be made on the basis of these results, but with the caveat that only a small window of the proteome has been examined and that some changes may be analyte specific. Consideration must also be given to the practicalities involved in sample collection. Many of the observed serum peaks, particularly on the IMAC-Cu chip, arose either directly from platelets or as products of events during coagulation, with most occurring within 30 min of venipuncture. For serum analysis, the recommendation would be to allow at least 30–60 min of clotting time before processing to minimize differences arising from the overriding effects of coagulation. This recommendation, however, is based on the use of activator tubes, which promote rapid clotting. Clearly, diseases associated with perturbations in clotting times and alterations in platelet number may also influence the profiles. It may be impossible to control for these influences, but with knowledge of the specific peaks affected, these effects can be monitored and may indeed yield useful disease information if specificity can be achieved. The importance of downstream validation of findings cannot be overemphasized.

Each of the anticoagulants used for preparation of plasma samples has inherent advantages and disadvantages. Although platelets are most stable in citrate anticoagulant, collecting tubes usually contain a liquid form that dilutes the plasma. The consequences of this dilution are affected by hematocrit and by optimal vs suboptimal filling of the tube. Heparinized samples also appear to be relatively stable, but heparin, which acts by binding to and enhancing the activity of antithrombin III, also binds a significant number of other proteins *(35)*, thus possibly interfering with or preventing their binding to chip surfaces. EDTA-treated blood is only slightly less stable, but over longer time periods, marked changes appear as elapsed time before centrifugation increases. Because the activity of many proteases requires metals, the chelating action of EDTA may be advantageous. Parathyroid hormone, for example, is thought to be most stable in EDTA tubes for this reason *(36)*. Complement activation may also be prevented or minimized in EDTA tubes, but the proteinase inhibitor Futhan (nafamostat mesilate) completely prevents complement activation and also inhibits thrombin, plasmin, and kallikreins *(37)* and thus may further stabilize samples. Whichever anticoagulant is used, plasma samples should be processed as soon as possible after venipuncture, ideally within the first hour, during which relatively few changes occur.

Other preanalytical aspects, such as centrifugation (speed, time, and temperature), storage time and temperature, and exposure to freeze–thaw cycles, are also likely to be important and require further investigation or, at the very least, consistency within studies. For example, if centrifugal speeds are too low and/or care is not taken in removing the plasma layer, contamination with platelets may occur and subsequently affect profiles. Similarly, we used 20 °C as the temperature of centrifugation, and all samples were left at room temperature after venipuncture; although protein stability generally is better at 4 °C, cold activation of platelets occurs. Some changes may occur with storage but will be analyte specific, such as tau protein in cerebrospinal fluid, which was stable for at least 6 freeze–thaw cycles, whereas amyloid β-42 showed decreases after 3 such cycles, with similar differences in stability depending on storage temperature *(38)*. Sample banks, often accumulated over many years, are valuable resources for investigation. With the extra sensitivity of mass spectrometry and other techniques, it is likely that even quite small changes, particularly those leading to degradation and breakdown into smaller fragments, may be detected.

The direct effect of tube additives such as silicones, surfactants, and plasticizers on MALDI-TOF analysis of samples was highlighted in a recent study *(39)*, which reported multiple interfering peaks consistent with polymeric compounds, particularly polyvinylpyrrolidone, in the region *m/z* 1000–3000 in some tube types. We did not find such effects, possibly because of the particular tubes we used, the different matrix, or the lack of binding of interfering factors to the chip surfaces used with SELDI. Similarly, exogenous factors, such as polymers derived from different brands of plastic blood tubes and specific anticoagulants, may cause ion suppression effects *(40)*, although such effects were very system-dependent. Clearly therefore, downstream analysis methods can also influence the choice of sample type, and ultimately, with more use of prefractionation and subsequent increased coverage of the proteome, the influence of preanalytical variables will become even more apparent.

Several profiling studies have demonstrated changes in acute-phase proteins. These markers generally have low specificity, but different forms have been described for some, such as N-terminally cleaved forms of serum amyloid α in renal cancer *(41)*, which may be useful clinically if validated. An earlier study used SELDI with gold chips to analyze the sera of patients with myocardial infarction and found several peaks from the C3f portion of the complement C3b α chain, α-fibrinogen peptide A, and peptides arising from these by progressive N-terminal deletion *(24)*. These peaks may represent activity of an endoproteinase followed by an N-terminal exopeptidase, i.e., an aminopeptidase, with profiles changing with time in plasma and serum. Such studies highlight the difficulty

of controlling for disease-specific variation in changes seen during sample processing, which may be less predictable than those shown consistently here in healthy volunteers. Many of the changes associated with disease may be secondary rather than direct effects of disease. In cancer, for example, fragments of proteins arising from increased protease activity, such as laminin, may be useful clinical markers *(42)*. However, it must be demonstrated that they are related to disease biology, are not artifacts of sample processing, and can be used with sufficient specificity.

The effect of disease on preanalytical changes may be impossible to completely eradicate, but awareness is important so that precautions can be taken to avoid bias introduced by inconsistent sample processing, which was recently suggested to be the most important "threat to validity" in marker research *(43)*. This together with issues such as study design and data analysis presents a major challenge for any of the "-omics" fields *(44)*. Future studies are needed if technologic breakthroughs are to fulfill their clinical promise. Many of the issues that need to be addressed apply to validation of routine clinical measurements and have recently been summarized *(45)*. Inclusion of more data regarding samples, processing, and storage in published studies is desirable so that sources of bias can be assessed.

A thoughtful overview of the mass spectrometric profiling approach has highlighted the promise in terms of sensitivity and resolution *(46)*, but many analytical and preanalytical issues remain to be addressed, whether for SELDI, the higher resolution hybrid quadrupole-TOF mass spectrometer fitted with the SELDI ProteinChip ion source *(47)*, or for other approaches. Methods under development involve extraction/fractionation before tryptic digestion and high-resolution MALDI profiling of the peptides produced *(48)*, as well as profiling of existing peptides with high-resolution MALDI after prefractionation based on reversed-phase magnetic particles and with subsequent data modeling *(32)*. The latter study rigorously examined the circumstances necessary for generation of reproducible and complex spectra, including laser conditions and matrix composition. Equally important is the development and evaluation of the optimal methods of data analysis, including new bioinformatic and biostatistical methods, each with their own specific attributes and drawbacks *(49)*. Mass spectrometric profiling is a promising method of marker discovery, but well-designed studies are critical to allow proper interpretation of the clinical utility of the results and identification of key variables.

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