Research Article
Special Focus Issue – Immunoaffinity MS

Bioanalysis

Multiplexed longitudinal measurement of protein biomarkers in DBS using an automated SISCAPA workflow

Background: The use of DBS for quantitative protein biomarker measurement has been hindered by issues associated with blood hematocrit variations and lack of detection sensitivity, particularly when multiple biomarkers are measured. **Materials & methods:** An automated, multiplexed SISCAPA analysis was used to normalize blood volume variations in DBS and quantify proteins of varying abundance in longitudinal specimens. **Conclusion:** The results showed that after normalizing the spot-to-spot hematocrit variations, peptide surrogates of protein biomarkers could be accurately quantitated in DBS. This allowed the establishment of baselines for a variety of biomarkers in multiple individuals and enabled detection of changes over time, thus offering an effective solution for longitudinal personal monitoring of biomarkers relevant in health and disease.

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First draft submitted: 9 March 2016; Accepted for publication: 6 June 2016;
Published online: 15 July 2016

Keywords: DBS • longitudinal • monoclonal antibodies • MS • protein biomarkers • SISCAPA

Background

Personalized health monitoring by longitudinal measurement of blood-based protein biomarkers is a concept that has recently attracted much attention for its potential to impact health care. One profound example is a recent landmark study that has shown the value of longitudinal measurement of the biomarker cancer antigen 125 (CA125) for prediction of the occurrence of ovarian cancer [1]. As clearly demonstrated by this study, increases in CA125 from personal baseline levels were much more effective as diagnostic indicators than simple cutoff values based on average levels of the biomarker in the female population. Although the study demonstrates the potential of longitudinal monitoring, it remains difficult to perform such testing on a range of different biomarkers for a variety of diseases due to the expense of individual tests and the difficulty inherent in preparing and storing multiple samples over long time periods. Currently, the majority of clinical protein assays use specimens in liquid form (venous plasma or serum) and thus require a trained phlebotomist for collection. One attractive alternative is the use of DBS, which has many advantages over conventional plasma/sera sampling, including simplified sample collection procedures and increased stability that allows more efficient shipping and storage (all with greatly reduced costs), smaller blood volumes (allowing application to pediatric measurements) and the ability to obtain high quality samples from remote locations or from patients with limited mobility (e.g., confined to home). The ability to prepare samples without access to a phlebotomist allows patient-directed sampling at home or elsewhere, thus facilitating longitudinal sampling and increasing the probability for compliance, for example when periodic posttreatment sampling is required.

DBS have been in use for decades as a convenient tool for transport and storage of human blood samples [2]. However, the range of analytes that can be quantitated in DBS

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samples have been generally limited to metabolites, nucleic acids and some proteins (where drying can be shown not to impact the structure of epitopes detected in immunoassays). While DBS are widely used for PK and toxicologic applications [3] and more recently for the determination of drug concentrations [4,5], for many clinical applications accurate measurement of a broad range of protein targets is needed [6,7]. Most clinical-quality immunoassays are not multiplexed and require an amount of sample approximating that of an entire DBS punch, effectively limiting the utility of DBS for many clinical applications. Recent reports demonstrate that the protein content of DBS can be reliably extracted, digested and analyzed by targeted MS using multiple reaction monitoring (MRM) methodology [8]. Mass spectrometric assays using DBS are especially attractive because by digesting the proteins to peptides, generally with trypsin, and then measuring surrogate peptides that are unique to each protein ('proteotypic peptides') by MS, the problem of protein stability over time is alleviated. From the MS viewpoint, this approach has the effect of transforming the protein measurement problem into a small molecule quantitation problem, where isotope dilution methods are effective and well understood. However, high precision quantification of protein biomarkers in DBS samples remains challenging since most established MRM methods lack the sensitivity required to detect or measure many biomarkers in small sample sizes such as DBS. In addition, to measure biological variations in the levels of protein biomarkers within an individual over time (represented by CVi) the variation in the assay (CVa) needs to be ideally <0.5 CVi [9], which falls in the range of 2–10% total assay CV for most proteins, a target that is hard to reach by standard MRM assays performed using unenriched samples. One additional problem encountered with DBS is that sample-to sample hematocrit differences influence chromatography on the filter papers used for conventional sample collection, leading to volume differences between samples, and preventing accurate protein quantification. To resolve these issues, we have employed stable isotope standards and capture by antipeptide antibodies (SISCAPA) technology [10]. Using highly specific antipeptide antibodies with nanomolar affinities and more specifically, low off rates, the SISCAPA technology addresses the sensitivity limitations by enriching preselected target peptide(s) from the complex digested DBS matrix. In doing so, an eluate containing only the peptide target(s) of interest is injected into the mass spectrometer, thus allowing quantitation of low abundance biomarkers in a small sample size. Moreover, the purification step decreases the LC time [11,12], and in some cases eliminates completely the need for LC [13,14], hence increasing the sample throughput required for biomarker validation and for application in the clinical laboratory. Using an automated workflow, CVs (CVa) of less than 5% are achievable for multiplexed SISCAPA assays regardless of protein size or abundance [12], thus facilitating accurate quantification of biomarkers and determination of deviations from individual biological baselines.

SISCAPA technology has been used in clinical laboratories for several years for measurement of thyroglobulin, a biomarker used to monitor thyroid cancer [15,16]. This and other SISCAPA assays use liquid plasma samples of varying volume. To use this technology in DBS, assay sensitivity often has to be increased and the spot-to-spot variability due to hematocrit differences and their effects on chromatography of the blood upon contact with the filter paper must be addressed. To correct for spot-to-spot hematocrit and volume variations, we present a strategy in which a panel of 'normalization proteins' are used to determine the volume of plasma being analyzed in any given DBS sample. Using this strategy we were able to improve the normalization of protein measurements in DBS and to use our workflow to longitudinally monitor a range of clinically important biomarkers in 14 individuals.

Materials & methods

Peptides & monoclonal antibodies

General methods for proteotypic peptide selection, synthesis and quantification and selection of high affinity antipeptide rabbit monoclonal antibodies (RabMAbs) have been described previously [12,17,18]. Antibodies specific for the peptide targets selected for this study (Table 1) were from the SISCAPA Assay Technologies Ltd commercial catalog. Stable isotope labeled peptide internal standards were synthesized and quantified (by amino acid analysis) by New England Peptide (MA, USA).

Automated SISCAPA workflow

We developed an 'addition only' digestion method to enable facile, efficient and reproducible tryptic digestion of human blood both in liquid format and in DBS. After addition of measured quantities of stable isotope labeled versions of the target peptides as internal standards (SIS), the digested samples were then subjected to the SISCAPA process in which the target peptides and cognate internal peptide standards are simultaneously enriched using a panel of high affinity antibodies followed by an extensive washing procedure to remove the nonspecific background peptides. The bound peptides were then acid eluted from the antibody and analyzed by the mass spectrometer. The digestion and SISCAPA protocols were automated using an Agilent Bravo Liquid Handling Platform (Agilent Technolo-

Table 1. List of peptide targets, corresponding SIS peptide spike levels and antibody amounts per well.

Short name	Target	Peptide	SIS (fmol)	mAb (µg)
Alb	Albumin	LVNEVTEFAK	500,000	0.5
Apo A-I	Apolipoprotein A-I	ATEHLSTLSEK	50,000	0.5
Apo B	Apolipoprotein B	FPEVDVLTK	30,000	0.5
Apo E C-term	Apolipoprotein E	VQAAVGTSAAPVPSDNH	10,000	0.5
ATIII	Antithrombin III	VAEGTQVLELPFK	30,000	0.5
C3	Complement C3	IHWESASLLR	20,000	0.5
CRP	C-reactive protein	ESDTSYVSLK	500	0.5
Cystatin C	Cystatin C	ALDFAVGEYNK	500	0.5
Fib-G	Fibrinogen γ-chain	YEASILTHDSSIR	150,000	0.5
HbA	Hemoglobin β-chain	VHLTPEEK	1,000,000	3
Hp	Haptoglobin β-chain	VTSIQDWVQK	100,000	0.5
Hx	Hemopexin	NFPSPVDAAFR	100,000	0.5
IGF-1	Insulin-like growth factor-1	GFYFNKPTGYGSSSR	500	1
IgM	Immunoglobulin M	YAATSQVLLPSK	100,000	0.5
LBP	LPS binding protein	LAEGFPLPLLK	500	0.5
MBL	Mannose binding lectin	EEAFLGITDEK	1000	0.5
MPO	Myeloperoxidase	DYLPLVLGPTAMR	500	1
ORM1	α-1-acid glycoprotein	NWGLSVYADKPETTK	20,000	0.5
Pla	Plasminogen	LSSPAVITDK	15,000	0.5
SAA1	Serum amyloid A 1	GPGGVWAAEAISDAR	1000	0.5
TIMP1	Tissue inhibitor of metalloproteinases 1	GFQALGDAADIR	500	1
VWF	von Willebrand factor	HIVTFDGQNFK	500	0.5

gies, CA, USA), which facilitates the transfer of this workflow to other laboratories with the same or similar liquid handling robotic platforms. Details of the workflow and the automation specifications have been recently published [12]. DBS samples were delivered into 96-well plates using a PerkinElmer DBS Puncher (Cat. No. 1296–071; MA, USA) equipped with a 1/4 inch diameter puncher head.

LC–MS/MS

MRM transitions (Supplementary Table 1) and collision energies for each target peptide and its cognate SIS peptide were first determined by injecting 10 µl of 100 fmol/ µl peptide solutions (in 0.1% FA) on to the LC–MS/MS system. The transitions were optimized using the Skyline software (Dr. Mike MacCoss Lab, WA, USA). The LC–MS/MS platform consisted of a 1290 Infinity UHPLC coupled to a 6490 Triple quadrupole mass spectrometer (TQMS) using a JetStream interface (Agilent Technologies, CA, USA). A 20 µl aliquot of the final SISCAPA eluate for each sample was separated on a 2.1 mm × 50 mm 1.8-µm Zorbax 300 SB-C8 column (Agilent Technologies, Part No. 857750–906) with a flow rate of 0.6 ml/min. The target peptides were separated using a 10 min gradient with 0.1% formic acid (FA) in water as solvent A and 90% acetonitrile in 0.1% FA in water as solvent B. From initial conditions of 3% B, a gradient was developed to 8% B at 0.86 min, 13% B at 2.0–4.2 min, 16% B at 5.6–6.2 min, 18% B at 7.2 min, 70% B at 9.0 min then back to 3% B from 9.2 min to 10.0 min for column reequilibration. The gradient is summarized in Table 2.

The LC method was configured to effect overlapping injections with a needle wash between injections. Source conditions included drying gas at 200°C, sheath gas at 250°C and 11 l/min flow for both drying and sheath gases. The resolution of the Q1 was set at 0.7 full width half maximum and for Q3 the resolution was set to be 1.2 full width half maximum. MassHunter Workstation Software (Agilent) was used for both data acquisition and analysis: MassHunter LC/MS Data Acquisition for the 6400 series triple quadrupole (v. B.06.00) was used for data acquisition while MassHunter Quantitative Analysis (v. B.05.02)

Table 2. Summary of the mobile phase gradient.

Time (min)	Buffer A (0.1% FA)	Buffer B (90% ACN/0.1%FA)
0.00	97%	3%
0.86	92%	8%
2.00	87%	13%
4.20	87%	13%
5.60	84%	16%
6.20	84%	16%
7.20	82%	18%
9.00	30%	70%
9.20	97%	3%
10.00	97%	3%

was used to generate target peptide:SIS MRM peak area ratios (PAR). Statistical analysis and data visualization were carried out using the R language in R Studio except as noted.

Samples

This research was conducted according to the principles of the Declaration of Helsinki. DBS samples used in these studies (Supplementary Table 2) were self-collected using finger-prick blood spotted onto Whatman 903 cards by 14 volunteers (A–N) who provided informed consent. Two of these volunteers also contributed blood from which two replicate DBS sample sets ('*' and '+') were made to serve as internal controls. The filter paper cards were generally stored at 4C in the presence of desiccant except for brief periods at room temperature or at -20C. In cases where specimens had to be transported to the laboratory for processing, the guidelines provided by the Center for Disease Control and Prevention (CDC) for shipment of DBS specimens were used.

Results & discussion

We measured a SISCAPA multiplex panel consisting of one tryptic peptide from each of 22 clinically interesting proteins (Table 1) in 784 DBS samples self-collected by 14 volunteer subjects. The number of samples per subject ranged from 18 to 192, and spanned periods ranging from 107 to 2365 days (Supplementary Table 2). The samples were processed using an automated SISCAPA protocol in nine 96-well plates, each plate containing 88 subject samples and four replicate punches prepared from each of two DBS standards. Sample digestion and SIS peptide addition were performed on all samples in parallel over 2 days, followed by serial processing of each plate for SISCAPA enrichment and LC-MRM analysis over 9 days (1 plate per day).

Analyte dynamic range

The levels of labeled SIS peptides spiked into each sample were set to approximate levels of corresponding endogenous analyte peptides and ranged (Table 1) from 500 fmol (for IGF1, MPO, etc.) to 1,000,000 fmol (for HbA). Using these SIS levels to convert observed peak area ratios (PAR) to observed fmol, we measured levels of the target peptides ranging from approximately 83,000,000 fmol (HbA) down to <100 fmol representing a within sample dynamic range between analytes extending to >1,000,000-fold. While the levels of SIS peptides delivered into each sample were kept as constant as possible from well to well and plate to plate to retain method precision, the absolute amounts (based on amino acid analysis of SIS peptide stocks) dispensed are not sufficiently accurate to be able to provide 'absolute quantitation'. For this reason the subsequent statistical analysis of the samples is based on the measured peak area ratios without conversion to fmol scale.

Normalizing DBS sample amount

Given the well-known variation in blood content between apparently identical DBS punches, it is important to establish a method that allows normalization of the punches prior to comparisons. The mean and standard deviation of peak area ratios for each peptide in each subject were calculated over a subset of subject samples selected to represent baseline values. Baseline samples were identified by a 4-step procedure: (1) manual selection of samples showing no obvious large deviations; (2) calculation of average value and standard deviation (SD) of these manual baseline samples; (3) final reselection of baseline samples as those for which neither C-reactive protein (CRP) nor serum amyloid A (SAA) deviate from initial baseline by more than two standard deviations (SD) followed by (4) recalculation of each subject's baseline average value and SD. The proportion of samples selected as baseline by this method ranged from 26 to 83% across the 14 subjects. The individual sample PAR values were then divided by these subject-specific baseline average PAR values to place each peptide PAR on a normalized scale (centered on 1.0), and the reciprocal of this value taken as a scale factor (SF).

Since the primary variable to be normalized across DBS is the equivalent plasma volume, we explored alternative DBS sample normalization schemes using scale factors based on: an average SF over all the 22 measured peptides; albumin (the most abundant plasma protein); or an average SF over a subset of five proteins (albumin, C3, IgM, plasminogen and hemopexin) selected as being highly correlated in the control samples, relatively stable within each subject's baseline samples and generally varying in opposing directions

during major perturbations. Due to the extreme variations observed in inflammation proteins (primarily CRP and SAA over the course of infections in some subjects, approach 'A' yielded highly variable scale factors and was rejected. Similarly, the fact that albumin is itself a negative acute phase reactant, decreasing significantly in some infections, rendered approach 'B' ineffective in normalizing subjects' longitudinal values. Approach 'C', used in the subsequent analysis to scale all measured values except for HbA1c (which is measured as a ratio between two MRMs), relies on an average of the scale factors of 5 proteins (equally weighted, and each observed to be normally distributed with similar widths) to normalize each sample within subject. The average of these scale factors across all the samples was 1.006 with a CV of 10.3%, a level of variation consistent with the expected ±~10% variation in plasma volume for DBS punches.

After DBS scaling, the two standard samples (each run 36 times in total across the nine plates on 9 days) were used to examine the effectiveness of scaling. Scaling removes almost all of the systematic correlation among high abundance peptides due to sample volume variations as shown in Supplementary Figure 1 which compares peptide:peptide correlations over the 36 replicates of standard sample '*' DBS before and after scaling. Similarly, the scaling removes most of this correlation among the baseline samples of Subject M, while preserving (and in fact amplifying) the physiological correlations (here primarily inflammation-related) present in the set of all Subject M samples (both baseline and those collected during biomarker-altering events).

Assay precision in standard samples

Assay precision across the entire sample set and 9-day run duration was estimated as the average, for each peptide analyte, of the CV's across the two sets of 36 standard sample replicates. The average CV over all 22 peptides (excluding HbA1c which is a ratio of

Table 3. CV breakdown for multiplexed measurement of the 22 peptides.

Target	Average CV of raw PAR in standards (%)	Average CV of scaled PAR in standards (%)	Average CV of Scaled PAR in subject baseline samples (%)	Average CV of scaled PAR in all subject samples (%)	F-value
A1AG	11.30	8.50	11.20	22.80	34
Alb	7.20	2.10	4.70	6.60	247
ApoA1	7.90	4.70	9.10	11.20	272
ApoB	6.20	2.20	7.60	10.60	331
ApoE	7.20	5.20	12.50	14.20	791
ATIII	12.40	9.60	18.20	19.50	90
C3	6.20	1.80	7.30	9.70	254
CC	5.60	6.10	11.90	12.50	104
CRP	14.10	13.10	35.50	184.50	3
Fib	6.90	4.90	11.20	19.10	24
HbA	7.00	5.50	11.40	11.80	27
Hp	10.20	9.40	14.60	24.70	50
Hx	6.40	1.30	3.10	4.00	1195
IGF1	20.40	19.80	24.90	26.20	55
IgM	6.10	2.80	4.80	6.10	2305
LBP	7.60	6.40	12.00	36.40	19
MBL	8.10	7.70	13.20	17.70	619
MPO	11.70	12.80	23.30	35.30	7
Pla	6.70	2.20	3.90	5.10	324
SAA	10.90	8.80	26.30	228.40	2
TIMP1	9.50	8.00	11.40	13.90	67
vWF	18.10	18.00	21.20	24.30	61
HbA1c	6.50	6.50	9.10	9.80	109
Average	**9.40**	**7.30**	**13.60**	**33.80**	

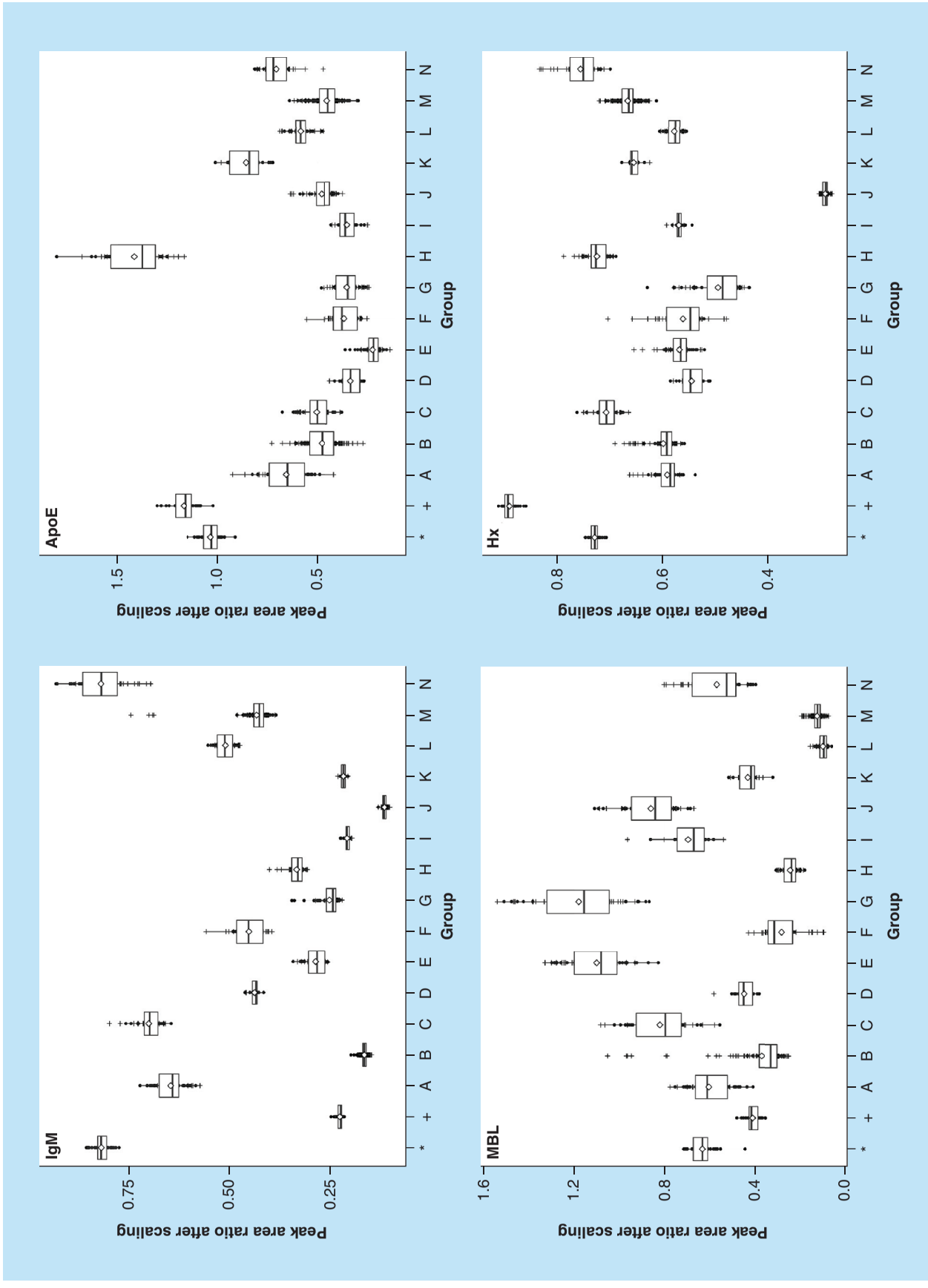

Figure 1. Abundances (scaled peak area ratios) of four proteins in serial DBS samples from 14 subjects (A–N) and two sets of replicate standard samples (* and +).

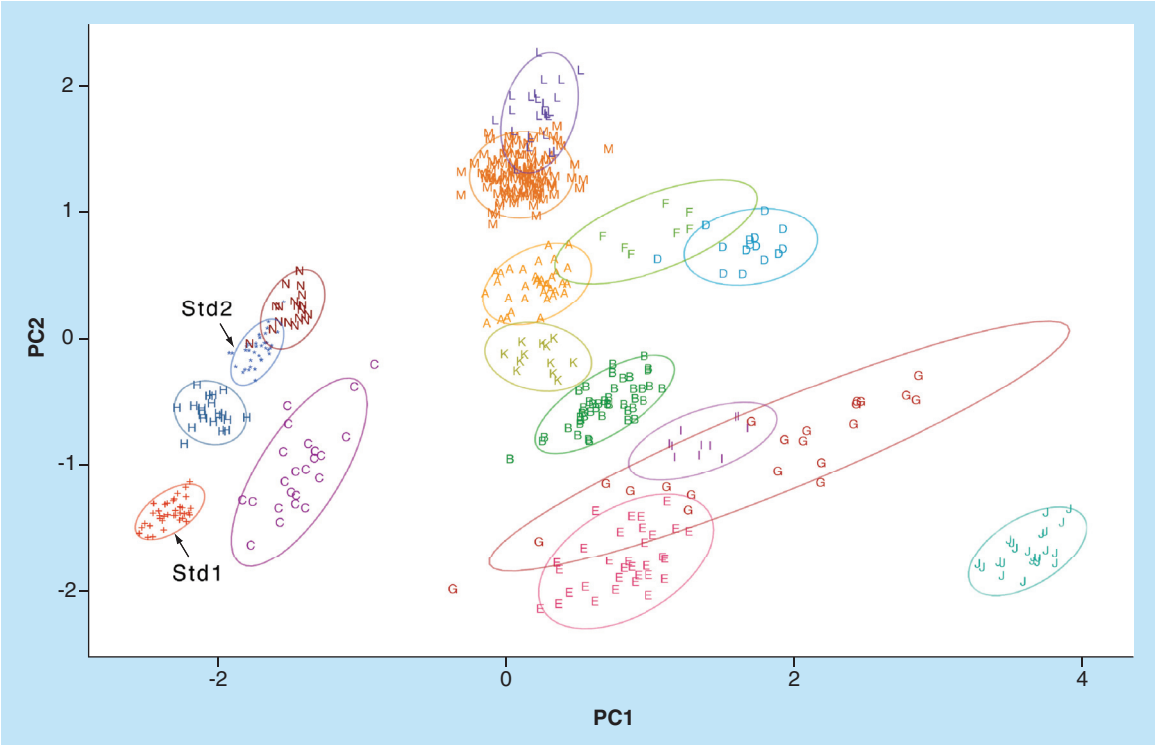

Figure 2. Plot of the first and second components of a principal components analysis of all DBS samples from 14 subjects (A–N) and replicate standard samples (* and +) using 5 proteins (IgM, Hx, ApoE Pla AND MBL).

glycated and nonglycated HbA peptides, and not subject to scaling between spots) decreased by 2.1% after scaling (Table 3). The five peptides used for scaling show the largest decreases in CV, as expected, while a few low abundance proteins showed little positive benefit from scaling.

Importantly, the CV for most assays across the replicate standard samples was substantially less than the average within-subject CVs of subjects' baseline samples, or the even greater average of CVs across all the subject samples. The assays are therefore sufficiently precise to measure biomarker variations in DBS.

Variation within & between subjects

A number of proteins showed striking differences between subjects. IgM, Hx, ApoE and MBL had the highest values of F (between-subject variability divided by within-subject variability; Table 3), and showed much larger differences between subjects than within each subject over time (Figure 1). Other proteins (e.g., LBP, A1AG, CRP, MPO) showed much larger variations within subjects over time than between subjects (Supplementary Figure 2). The subject-specific information in the abundances of proteins with large F values effectively provides a multidimensional fingerprint of each subject, which can be visualized in a principal component analysis (Figure 2) of IgM, Hx, ApoE, Pla and MBL in the baseline samples. The baseline samples collected from most subjects cluster tightly in this representation, demonstrating the stability of the protein pattern over the timescale of sample collection (>6 years for subject M) and the capability of the fingerprint to confirm the identity of individual subjects' samples. The replicate standard samples (symbols * and +) are the most tightly clustered groups, as expected since they should be identical apart from technical imprecision.

Personal baselines & CV's: changes on a scale of significance

All the analytes demonstrate subject-specific, that is, 'personal' baselines that are quite stable over time (e.g., the narrow distribution of IgM over the 192 samples from subject M in Figure 1), and, as noted above, many analytes exhibit within-subject variation much less than that between subjects (i.e., personal CV's less than group CV). We therefore employed a second stage of data normalization to 'personalize' the measured values: a subject's mean baseline PAR value for each peptide was subtracted from that peptide's scaled PAR values in all the subject's samples and the result was divided by the subject's baseline standard deviation for that peptide, resulting in values centered on zero and presented in units of personal standard deviations: that is, a scale of 'personal statistical signifi-

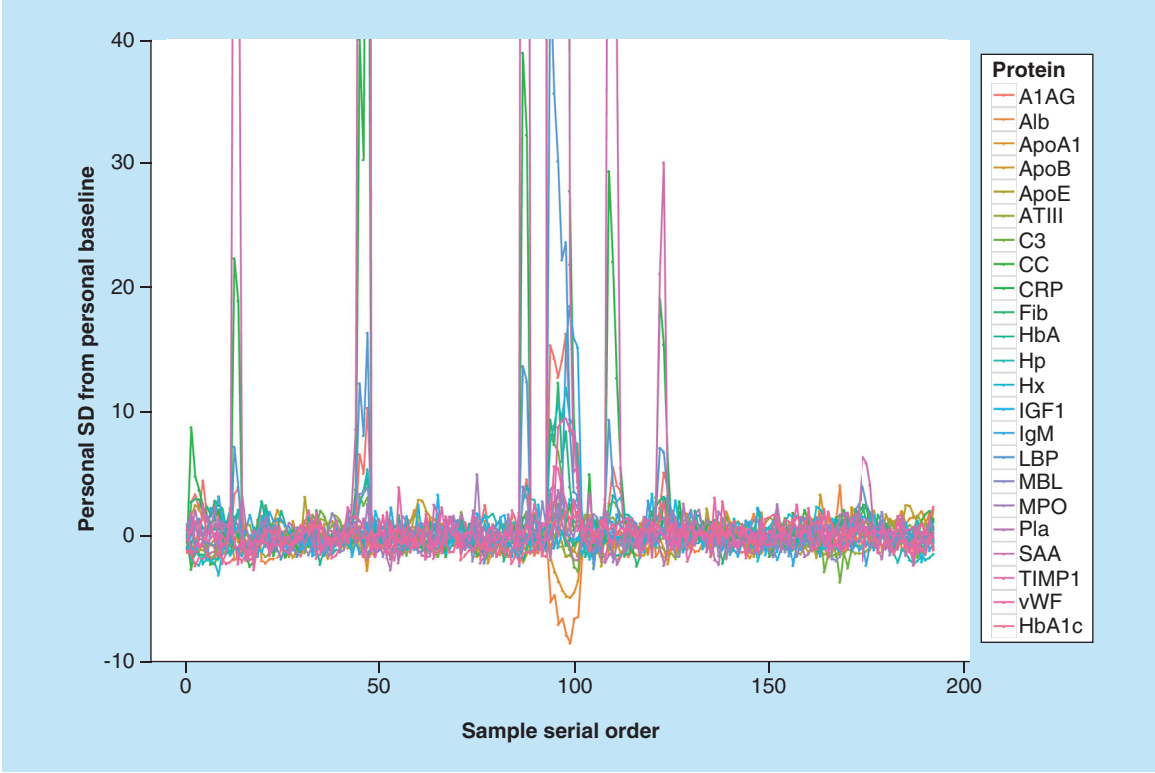

Figure 3. Abundance values for 22 proteins plus HbA1c measured in 192 serial DBS of one subject (M) plotted in units of personal standard deviations from personal mean (baseline).

cance'. In this format, Figure 3 summarizes values of 23 measurements (abundance of 22 proteins plus HbA1c value) over 192 serial DBS from subject M. Each measurement shows apparently random variation within a few SD of an extremely stable baseline for most of the samples, as expected, and six occurrences of large deviations from baseline in which SAA and CRP show elevations of 30–3000 SD from baseline (offscale in Figure 3). These events represent five self-reported upper respiratory infections ('colds') and one instance of pneumonia (samples 93–103) in which SAA and CRP are elevated by 3000 and 400 SD (750- and 10-fold in terms of PAR), respectively.

Magnitude of changes during nonbaseline events

Figure 4 presents details of some prominent 'nonbaseline' events in samples collected by four subjects (a kidney infection in B, two colds and pneumonia in M, a respiratory infection in N, and a full-term normal pregnancy during which two colds occurred in F) compared with prior and later baseline values. In each case the infections are marked by large elevations of SAA, CRP and LBP (largest to smallest) and persisting small reductions in albumin, all consistent with a simple acute phase response. Other acute phase proteins (Hp, Fib, A1AG) are also elevated but typically later in the evolution of the infection as is MBL except in subject M (who exhibits a very low level of this protein at baseline). IgM showed a significant elevation in the last stages of the major infection in subject M (18 SD from baseline, a 1.7-fold increase in amount), but was not significantly increased in the other infections. A series of large sustained changes occurred during normal pregnancy (subject F).

Changes in CRP and SAA are very strongly related (Figure 5), displaying a correlation over 3 or more orders of magnitude. A weaker but significant correlation persists in the baseline regions as well, which indicates that some of the apparently random variation within 2SD of baseline is a biologically driven signal.

Some proteins exhibit long-term variation on unexpected timescales. ATIII, a component of the coagulation system, shows an almost 3-fold variation across a 4-year period in subject M, best modeled as an annual sinusoidal function, peaking in late winter with troughs in late summer (Figure 6). This behavior is consistent with known seasonal variations in coagulation behavior thought to be related to ambient temperature.

Conclusion

We have measured the levels of 22 proteins and one posttranslationally modified protein (HbA1c) by SISCAPA-MS in a large cohort of DBS collected by 14 sub-

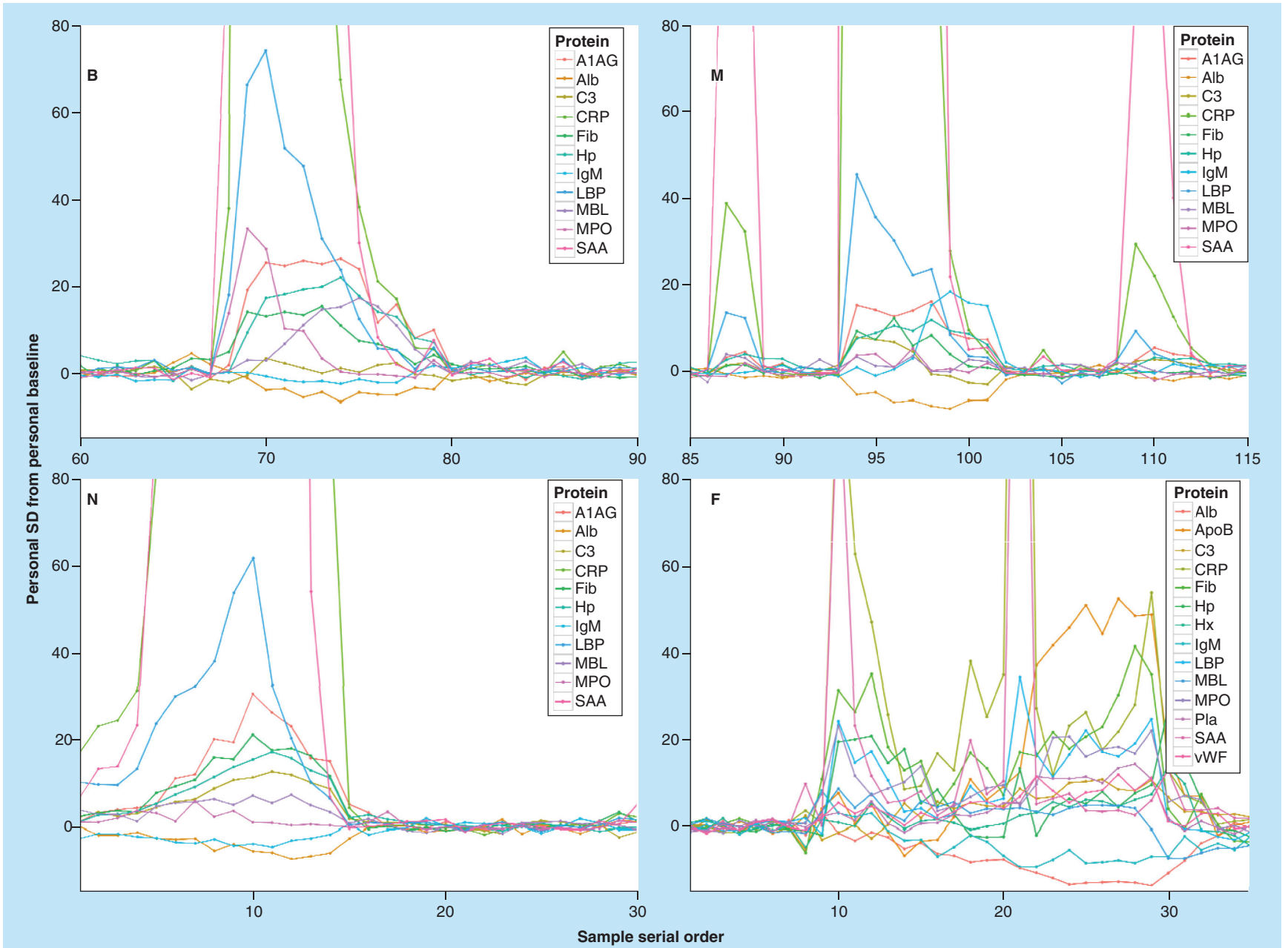

Figure 4. Events departing from baseline values for selected proteins in serial DBS samples from 4 subjects, including kidney infection (B), respiratory infections (M, N) and normal pregnancy (F).

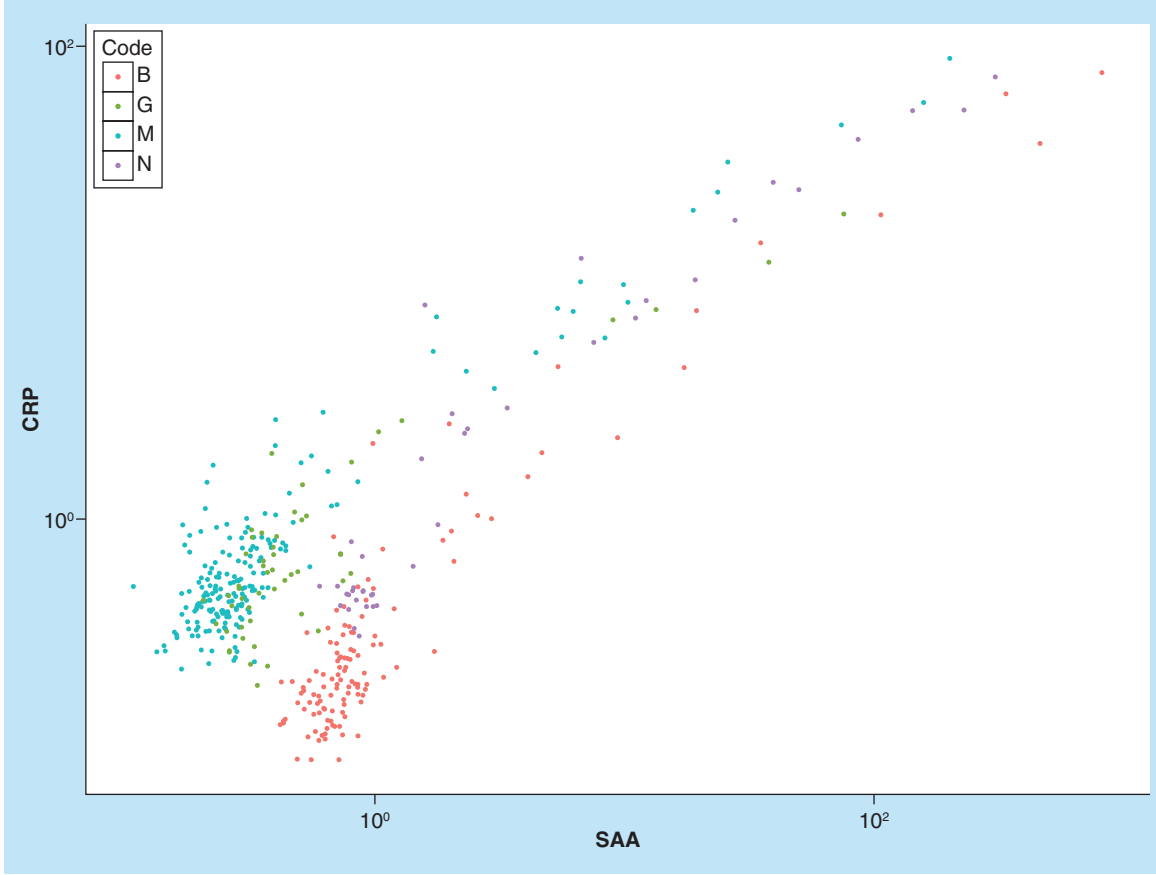

Figure 5. Relationship between abundances of CRP and SAA in serial DBS from four subjects (B, G, M, N).

jects over periods as long as 6.5 years. The results indicate that these proteins are remarkably stable in DBS collected by an individual over long periods, and that precise measurements of these can be obtained through mass spectrometric quantitation of proteotypic peptides liberated by tryptic digestion and subsequently enriched using monoclonal antipeptide antibodies (SISCAPA).

Variations in the individuals' protein levels over time were generally small in comparison to the differences between individuals, implying that comparing an individual's biomarker levels against his or her own baseline values would yield much more sensitive detection of biologically relevant physiological differences than comparison against a population distribution (i.e., the conventional clinical 'reference interval'). Using longitudinally collected DBS samples, we could 'personalize' the protein measurement scales by instead presenting results in terms that relate directly to statistical significance for an individual: that is, in terms of personal standard deviations from a personal baseline value for each protein measured. Personal baseline values and SD were computed from a subset of samples selected as showing no sign of inflammation, or other 'nonnormal' manifestations of disease, and represent a constant series of personal physical parameters that can be helpful in identifying samples from the same individual.

In contrast to the general constancy of protein measurements in baseline 'healthy' samples, we observed very large departures (in terms of SD) from baseline values due to infections, including both major and minor events, normal pregnancy and in the case of ATIII, season of the year. These represent biological signals clearly distinguishable from physiological, preanalytical and measurement workflow sources of noise.

The automated DBS-SISCAPA workflow presented in this manuscript enables precise relative quantitation of surrogate, proteotypic peptides measured against an added internal standard. The stability of normal baseline levels for various analytes in serial DBS over time, and the strong and mutually reinforcing signals observed in 'nonbaseline' events, indicate that this approach can be used to accurately track protein levels longitudinally. As such, the methodology provides a foundation based upon which a variety of research and clinical applications can be developed, though for clinical applications rigorous validation of the method according to current clinical guidelines is required.

The results demonstrate that personal baselines can be established based on precise relative quantitation of

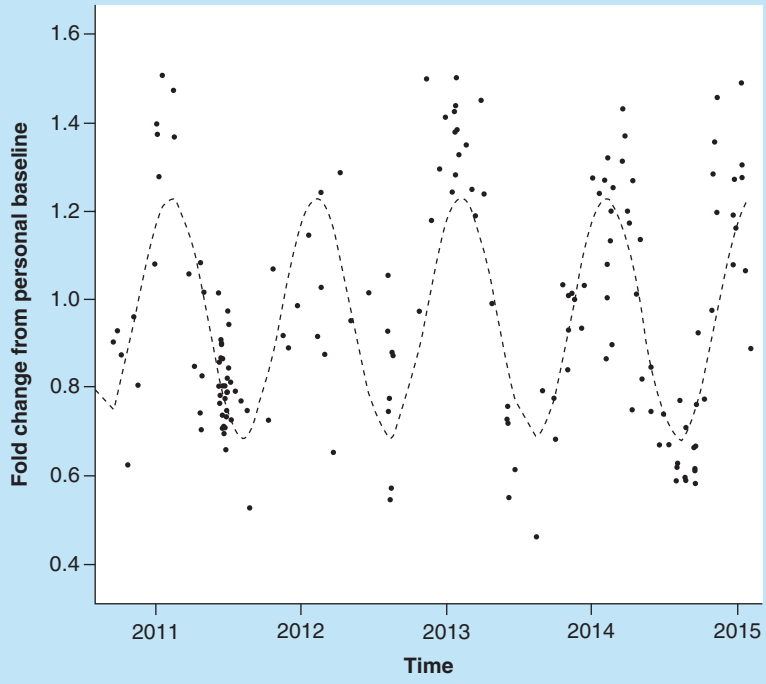

Figure 6. Values of ATIII in subject M plotted as a function of time, with a best fit (dashed line) to annual sinusoidal variation.

surrogate peptide analytes. However, by using one or more standard samples containing known amounts of target analytes (i.e., external standards, or calibrators) processed in parallel with test samples, the method can also be used for measurement of biomarkers on an absolute scale. This approach is similar to the long-accepted strategy of external calibration used in immunoassays and is limited by the availability of properly quantitated reference material for different proteins.

While samples collected over a long period of time and analyzed together showed good precision, we have not yet directly demonstrated that the same sample processed months or years apart will give reproducible values. This important study is beyond the scope of this manuscript; however, we believe the issue will be effectively addressed using the same external calibrator approach applied in current clinical immunoassay tests. It is also important to note that the SISCAPA technology, because of the upfront digestion step and measurement of surrogate peptides, is not susceptible to variations observed in immunoassay methods due to protein structural stability issues (e.g., denaturation) as a function of time, temperature, etc.

Future perspective

The benefits of longitudinal data analysis in diagnostic applications are enormous. Important biomarker changes can be detected earlier and more accurately by comparing test values with a patient's own 'normal' baseline and variance than with the much broader and less accurate parameters derived from a broad population of very different people (the classical population 'reference interval'). These benefits have been extremely difficult to realize in large part because the collection of serial samples by phlebotomy has been expensive and inconvenient, while the biomarker measurements themselves are expensive and require significant amounts of sample. DBS technology, even in the simple form of classical Whatman 903 filter paper cards, could enable longitudinal clinical applications provided that robust biomarker measurements can be made using them. In this manuscript we present a summary of an initial investigation of longitudinal DBS collected over spans of various intervals and show that such measurements can be made accurately and efficiently using peptide-enrichment MS (DBS-SISCAPA-MRM). Given recent increases in the sensitivity of mass spectrometers, it is likely that virtually all of the 115 protein biomarkers cleared to date by the US FDA for clinical use in plasma [19] are measurable in DBS or will be in the near future, illustrating the generality of the approach.

Our approach enables a number of scenarios yielding significant potential patient benefit. Tests for cancer recurrence (e.g., using CA125, thyroglobulin, PSA, CEA and alpha-fetoprotein) are typically recommended twice per year and are clearly more informative when interpreted as serial values [1]. Many tests used to detect and manage chronic disease risk, such as

measurement of HbA1c (diabetes) or lipoproteins, cholesterol and CRP (cardiovascular disease) can likewise be made more convenient (increasing compliance) and less expensive through a combination of DBS sample collection with cost–efficient multiplex measurement technologies. Finally, the ability to collect more frequent samples and measure more analytes at lowered cost translates into a significant improvement in the power of pharmaceutical clinical trials. These developments can improve patient outcomes while lowering overall healthcare cost, and so justify the regulatory, educational and technology development efforts required to bring them into routine use.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/bio-2016-0059

Financial & competing interests disclosure

The authors are owners and employees of SISCAPA Assay Technologies Ltd., a company commercializing proprietary protein measurement technology using peptide affinity enrichment coupled with mass spectrometry. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Open access

Executive summary

Background

- The use of DBS for quantitative protein biomarker measurement has been hindered by issues associated with blood hematocrit variations and lack of detection sensitivity, particularly when multiple biomarkers are measured.

Materials & methods

- Methods of normalizing for variations in DBS sample content were developed based on a panel of five minimally varying proteins.
- A total of 22 proteins of clinical interest spanning a >1,000,000-fold abundance range were measured in 784 serial DBS samples collected by 14 subjects over periods as long as 6.5 years.

Results & discussion

- Mass spectrometric quantitation of target-specific proteotypic peptides liberated by tryptic digestion of DBS samples and subsequent SISCAPA enrichment provided precise measurements with stable baselines.
- Low levels of within-subject variation of many proteins in baseline samples allowed computation of personal baseline levels and estimated standard deviations for each protein in each subject and presentation of results on a scale of SD from baseline: that is, personal statistical significance.
- Events such as infections and pregnancy captured in the serial DBS samples caused changes ranging up to 3000 SD from baseline for acute phase proteins SAA and CRP, with significant changes observed in a majority of the markers measured.

Conclusion

- Variations in the individuals' protein levels over time were generally small in comparison to the differences between individuals, implying that comparing an individual's biomarker levels against his or her own baseline values would yield much more sensitive detection of biologically relevant physiological differences than comparison against a population distribution (i.e., the conventional clinical 'reference interval').

Future perspective

- DBS samples can be used to accurately track protein levels over extended time periods in a variety of research applications and ultimately in the clinical laboratory.

References

Papers of special note have been highlighted as:
•• of considerable interest

1 Jacobs IJ, Menon U, Ryan A *et al.* Ovarian cancer screening and mortality in the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS): a randomised controlled trial. *Lancet* 387(10022), 945–956 (2015).

•• **Demonstrates the clinical utility of longitudinal monitoring of a protein biomarker (CA-125).**

2 Guthrie R, Susi A. A simple phenylalanine method for

detecting phenylketonuria in large populations of newborn infants. *Pediatrics* 32 (3), 338–343 (1963).

3 Discenza L, Obermeier MT, Westhouse R, Olah TV, D'Arienzo CJ. A bioanalytical strategy utilizing dried blood spot sampling and LC–MS/MS in discovery toxicology studies. *Bioanalysis* 4 (9),1057–1064 (2012).

4 Spooner N. A dried blood spot update: still an important bioanalytical technique? *Bioanalysis* 5 (8), 879–883 (2013).

5 Timmerman P, White S, Cobb Z, de Vries R, Thomas E, van Baar B. Update of the EBF recommendation for the use of DBS in regulated bioanalysis integrating the conclusions from the EBF DBS-microsampling consortium. *Bioanalysis* 5 (17), 2129–2136 (2013).

6 Anderson NL. The role of multiple proteomic platforms in a pipeline for new diagnostics. *Mol. Cell Proteomics* 4, 1441–1444 (2005).

7 Parker CE, Pearson TW, Anderson NL, Borchers CH. Mass spectrometry based clinical proteomics – a review and prospective. *Analyst* 135, 1830–1838 (2010).

8 Chambers AG, Percy AJ, Yang J, Borchers CH. Multiple reaction monitoring enables precise quantification of 97 proteins in dried blood spots. *Mol. Cell Proteomics.* 14 (11), 3094–3104 (2015).

9 Ricós C *et al.* Within-subject biological variation in disease: collated data and clinical consequences. *Ann. Clin. Biochem.* 44, 343–352 (2007).

•• **Summarizes the biological variations in levels of clinically important protein biomarkers within an individual over time (CVi).**

10 Anderson NL, Anderson NG, Haines LR, Hardie DB, Olafson RW, Pearson TW. Mass spectrometric quantitation of peptides and proteins using stable isotope standards and capture by anti-peptide antibodies (SISCAPA). *J. Proteome Res.* 3, 235–244 (2004).

•• **The original SISCAPA publication.**

11 Miller CA, Pope ME, Razavi M, Pearson TW, Anderson NL. Optimization of a rapid chromatographic method for a multiplexed SISCAPA assay. *J. Am. Soc. Mass Spectrosc.* 23, 147 (2012).

12 Razavi M, Leigh Anderson N, Pope ME, Yip R, Pearson TW. High precision quantification of human plasma proteins using the automated SISCAPA Immuno-MS workflow. *N. Biotechnol.* doi: 10.1016/j.nbt.12.008 (2016) (Epub ahead of print).

•• **The automated SISCAPA workflow.**

13 Razavi M, Frick LE, LaMarr WA *et al.* High throughput SISCAPA quantitation of peptides from human plasma digests by ultrafast, liquid-chromatography-free mass spectrometry. *J. Proteome Res.* 11, 5642–5649 (2012).

14 Razavi M, Johnson LD, Lum JJ, Kruppa G, Anderson NL, Pearson TW. Quantification of a proteotypic peptide from protein C inhibitor by liquid chromatography-free SISCAPA-MALDI mass spectrometry: application to identification of recurrence of prostate cancer. *Clin. Chem.* 59 (10), 1514–1522 (2013).

15 Kushnir MM, Rockwood AL, Roberts WL, Abraham D, Hoofnagle AN, Meikle AW. Measurement of thyroglobulin by liquid chromatography-tandem mass spectrometry in serum and plasma in the presence of antithyroglobulin auto-antibodies. *Clin. Chem.* 59, 982–990 (2013).

16 Netzel BC, Grebe SK, Carranza Leon BG *et al.* Thyroglobulin (Tg) testing revisited: Tg assays, TgAb assays, and correlation of results with clinical outcomes. *Clin. Endocrinol. Metab.* 100 (8), E1074–83 (2015).

17 Pope ME, Soste MV, Eyford BA, Anderson NL, Pearson TW. Anti-peptide antibody screening: selection of high affinity monoclonal reagents by a refined surface plasmon resonance technique. *J. Immunol. Methods.* 341, 86–96 (2009).

18 Razavi M, Pope ME, Soste MV *et al*. MALDI immunoscreening (MiSCREEN): a method for selection of anti-peptide monoclonal antibodies for use in immunoproteomics. *J. Immunol. Methods.* 364, 50–64 (2011).

19 Anderson NL. The clinical plasma proteome: a survey of clinical assays for proteins in plasma and serum. *Clin. Chem.* 56, 177–185 (2010).