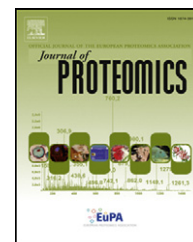


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Profiling post-centrifugation delay of serum and plasma with antibody bead arrays☆☆☆

Ulrika Qundos^a, Mun-Gwan Hong^a, Gunnel Tybring^b, Mark Divers^b, Jacob Odeberg^a, Mathias Uhlen^a, Peter Nilsson^a, Jochen M. Schwenk^{a,*}

^aScience for Life Laboratory, School of Biotechnology, KTH - Royal Institute of Technology, Box 1031, SE-171 21 Solna, Sweden

^bKI Biobank, Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Nobels väg 12 A, SE-171 77 Stockholm, Sweden

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ABSTRACT

Several biobanking initiatives have emerged to create extensive collections of specimen for biomedical studies and various analytical platforms. An affinity proteomic analysis with antibody suspension bead arrays was conducted to investigate the influence of the pre-analytical time and temperature conditions on blood derived samples. Serum and EDTA plasma prepared from 16 individuals was centrifuged and aliquots were kept either at 4 °C or in ambient temperature for 1 h and up to 36 h prior to first storage. Multiplexed protein profiles of post-centrifugation delay were generated in 384 biotinylated samples using 373 antibodies that targeted 343 unique proteins. Very few profiles were observed as significantly altered by the studied temperature and time intervals. Single binder and sandwich assays revealed decreasing levels of caldesmon 1 (CALD1) related to EDTA standard tubes and prolonged post-centrifugation delay of 36 h. Indications from changes in CALD1 levels require further confirmation in independent material, but the current data suggests that samples should preferentially be frozen during the day of collection when to be profiled with antibody arrays selected for this study.

Biological significance

Affinity-based profiling of serum and plasma by microarray assays can provide unique opportunities for the discovery of biomarkers. It is though often not known how differences in sample handling after collection influence the downstream analysis. By profiling three types of blood preparations for alterations in protein profiles with respect to time and temperature post centrifugation, we addressed an important component in the analysis and of such specimen. We believe that this analysis adds valuable information to be considered when biobanking blood derived samples.

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Abbreviations: FDR, false discovery rate; MFI, median fluorescence intensity; HPA, Human Protein Atlas; nMFI, normalized median fluorescence intensity; LMM, linear mixed effects model; CV, coefficient of variance.

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* Corresponding author. Tel.: +46 8 5248 1482; fax: +46 8 5248 1425.

E-mail address: jochen.schwenk@scilifelab.se (J.M. Schwenk).

1. Introduction

Over the recent years, biobanks have become more widely recognized as an essential and valuable resource in medical research. Prospective as well as retrospective and high quality samples are being collected within specific research projects, routine health care or population cohorts. They provide powerful tools for exploring the etiology of common complex diseases, better understanding of the interplay between genetic and lifestyle determinants and the development of new diagnostic biomarkers and analytical methods. Several large-scale population cohorts, such as UK Biobank [1], Lifelines in the Netherlands and HUNT in Norway, are pushing these frontiers further. As for all new prospective biobanks, these studies have standard operations in place as well as IT systems enabling a complete tracking of time and temperature from needle to freezer. However, when utilizing old sample collections, such as legacy samples, stored for decades most of these samples have varying standards and lack documentation on how samples have been collected, processed and stored [2,3]. Success in identifying novel biomarkers may well depend on variability in sampling handling conditions between or within biobanks rather than disease states. Uncertainty still remains to what extent and how vulnerable different methodological approaches, such as the emerging affinity proteomic technologies, are in this aspect for their ability to discover and verify biomarkers.

For the widely used analysis based on mass spectrometric determination of a target of interest, proteolytic activities or other molecular modifications are important factors that alter the experimental outcome, hence standardized collection and handling procedures for proteomics [4] or metabolomics [5] have become important factors in study designs. In an emerging alternative to mass spectrometric analysis of proteins, namely affinity proteomics, the use of reagents such as antibodies is about to be established for a proteome-wide scale [6–8]. Alongside these efforts, technological advances have been taking place, and regarding the analysis of serum and plasma, the use of microarray technologies and the like are often at the forefront [9]. Among the different types of protein microarrays, the use of beads to create arrays in suspension in combination with direct sample labeling offers an attractive platform to now screen larger sample collections with up to 384 [10] or even 1728 antibodies per sample [11]. These high throughput methodologies are well suited for unbiased discovery proteomics, as they allow the analysis of many samples at a time.

The purpose of this study was to investigate the extent to which the pre-analytical phase between centrifuge and freezer affects antibody-based plasma profiling. We analyzed protein profiles generated by highly multiplexed single-binder assays, and focused on serum and two types of plasma collection tubes. A set of 16 healthy volunteers donated blood at a single occasion and upon collection the obtained specimens were handled differently from the time of centrifugation until being frozen for storage. A linear mixed model was used to assess each protein profile with regard to time and/or temperature and alterations in variance across time per temperature were evaluated.

2. Methods and material

2.1. Sample collection

Blood samples were collected from 16 donors, 8 males and 8 females in the age range 24–60 years. The study participants were a group of volunteers, matched for age and gender and were not diagnosed with a disease such as cancer or cardiovascular disorder. The ethical review board in Stockholm, Sweden, with giving consent by each participant, approved this study. From each sample donor, a total volume of about 50-ml blood was drawn into two 9-ml EDTA-Plasma Preparation Tube (K₂EDTA-PPT blood collection tubes: BD cat. no. 362799), two 9-ml EDTA standard (K₂EDTA blood collection tubes: BD cat. no. 367525) and two 9-ml serum venepuncture collection tubes (Serum blood collection tubes: SST-tubes BD cat. no. 367953). The two sample types plasma (PL1) and serum (SER) were thus prepared in three types of primary containers (PED ± gel and SST), here described according to SPREC version 2.0 [12]. All samples were gently mixed for one min and the plasma samples were centrifuged immediately at 2000 *g* for 10 min while serum was centrifuged within 40 min (SPREC; pre-centrifugation delay A and centrifugation A). The sample collection tubes were placed either in room temperature or at 4 °C. Plasma and serum were then aliquoted in 100-μl fractions into 2D bar coded tubes (REMP tubes in plate, SRBR-96-300, Brooks Life Science System) in the time intervals 1, 3, 8 and 36 h after sample collection (SPREC; post-centrifugation delay A-J) and stored at –80 °C until analysis (SPREC; long-term storage Z) (Fig. 1). Twelve individuals were included in the first phase of the study, while 16 individuals were included for verification.

2.2. Antibodies

To build a 384-plex bead array, 364 affinity-purified rabbit antibodies were selected from the Human Protein Atlas (HPA) [6] and provided by Atlas Antibodies AB. Additionally, rabbit IgG (Jackson ImmunoResearch Laboratories), albumin-binding protein (Affibody AB), anti-albumin antibody (rabbit IgG, Dako), anti-KLK3 antibodies 1H12, 5A6, 8A6 (all mouse IgG, HyTest Ltd.), anti-CNDP1 antibody AF2489 (goat IgG, R&D Systems), and anti-CNDP1 3A6, 5B2, 8A3 (all mouse IgG, Atlas Antibodies AB) were utilized. As summarized in Supplementary Table 1, 373 out of 380 antibodies were unique and a total of 343 unique proteins were targeted. The antibodies were selected based on their protein targets classified as serum/plasma proteins, such as a signal peptide predicting protein secretion, as well as in house indications from other plasma biomarker projects, such as Western blot analysis.

2.3. Bead coupling

Antibodies were coupled to carboxylated beads (Luminex Corp.) according to the manufacturer's protocol and as described previously [10]. The coupling efficiency for each antibody was determined via R-phycoerythrin-labeled anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories,) and a 383-plex bead mixture, denoted antibody suspension bead array was created as described previously [13]. A second coupling of 20 out

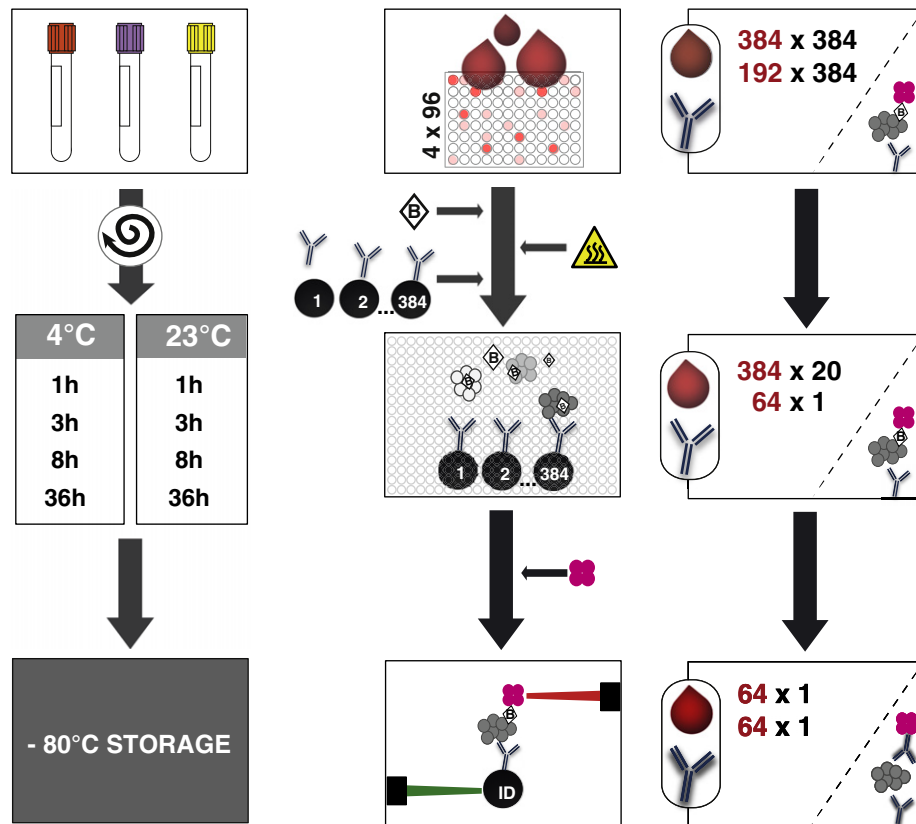


Fig. 1 – Study overview. The figure provides an overview of sample collection and processing, assay workflow for the antibody suspension bead array and the verification of the identified candidate protein. (Left panel) Blood samples from 16 individuals were collected and serum and plasma (SER SST and PL1 PED ± gel) prepared and exposed to 4 °C or an ambient temperature post-centrifugation for 1, 3, 8 and 36 h until final storage at –80 °C. (Center panel) Samples were transferred into a microtiter plate format according to a randomized layout prior to direct labeling and subsequent heat treatment. After an overnight incubation with the antibody suspension bead array and wash-step, a reporter molecule was added and finally a flow cytometer like instrument with a dual laser employed to detect captured proteins in conjunction with bead identity. (Right panel) The experimental strategy included profiling of samples with both a single binder and sandwich assay.

of 373 antibodies was performed to build a 20-plex bead array applied in the initial verification stage.

2.4. Sample labeling and assay procedure

Three hundred and eighty four serum and plasma samples (built from 288 unique samples) were labeled and analyzed in accordance with previous studies [14] with minor changes. At first, samples were thawed at RT, centrifuged for 10 min at 3000 rpm, and transferred into a microtiter plate (Abgene). The plates were centrifuged (1 min at 3000 rpm), and 3 µl of each sample was added to 22 µl of 1× PBS in four 96-well microtiter plates with a liquid handler, according to a randomized layout (TECAN, Freedom Evo 150). N-Hydroxysuccinimidyl ester of biotinoyl tetraoxapentadecanoic acid (Pierce) was then added at a 10-fold molar excess to yield an overall 1:10 sample dilution followed by a 2-h incubation at 4 °C. The molar excess was calculated based upon an approximate total protein content in blood (60 mg/ml) and theoretical, average molecular weight of 70 kDa. A 10-fold molar excess of biotin to protein in 1:10 diluted plasma was added in the labeling reaction. The reaction was stopped by the addition of a 250-fold molar excess of Tris-HCl, pH 8.0 over biotin and incubated for another 20 min at 4 °C prior

to a final storage at –80 °C. All samples were subsequently utilized without removing unincorporated biotin and diluted 1:50 (CyBio, CyBi@-Selma) in an assay buffer composed of 0.5% (w/v) polyvinyl alcohol and 0.8% (w/v) polyvinylpyrrolidone (Sigma) in 0.1% casein in PBS supplemented with 0.5 mg/ml rabbit IgG (Bethyl Laboratories). The samples were heat treated in a thermocycler at 56 °C or 23 °C for 30 min; and 23 °C for 15 min. Then, 45 µl was combined with 5 µl of the 384-plexed bead mixture in a 384-well flat bottomed half-area microtiter plate (Greiner), and incubation took place overnight on a shaker at an ambient temperature. Beads were washed in wells with 3 × 100 µl of PBST (1× PBS, pH 7.4, 0.1% Tween20) (BioTek, EL406) followed by 10 min with 50 µl of a stop solution containing 0.1% paraformaldehyde in PBS. Beads were washed, and 50 µl of 0.5 µg/ml R-phycoerythrin-labeled streptavidin (Invitrogen) in PBST was added and incubated for 20 min. Finally, beads were washed and measured in 100 µl of PBST (Luminex Corp., FlexMap3D).

2.5. Sandwich immunoassay

Crude, meaning neither diluted nor labeled plasma EDTA standard samples were diluted 1:10 in assay buffer, combined

with a bead array and incubation took place for 1 h. HPA017330 was labeled with RPE conjugated Fab fragments anti-rabbit IgG according to manufacturer's protocol (Z-25355, Molecular Probes). Beads were washed and 50 μ l 0.1 μ g/ml labeled detection antibody was added and incubated for 30 min. Beads were washed followed by 10-min incubation with stop solution. Finally, beads were washed and measured in 100 μ l of PBST (Luminex Corp., LX200).

2.6. Study design

A total of 288 unique samples from 12 individuals (6 females + 6 males) and of three plasma or serum preparation types, exposed to 4 °C or an ambient temperature with a post-centrifugation delay of 1, 3, 8 and 36 h prior to freezing were selected for the first discovery phase. The shorter time intervals 1, 3 and 8 h were focused upon and the most extreme time interval 36 h selected as an endpoint. All samples were then randomized and distributed across four 96-well plates for sample labeling and heat treatment and transferred to one 384-well plate for the remainder of the assay procedure. In addition, 24 sample pools were created based on sample preparation type and post centrifugation delay (time and temperature) and used to assess the performance of the assay in relation to the variability between the conditions. Thus 384 wells were analyzed. To assess reproducibility, 192 samples were reanalyzed in a second, independent experiment. In the verification phase, primary findings were verified by recoupling antibodies of interest creating a second bead array utilized in the reanalysis of the 384 samples. For further verification of a finding, all 16 individuals' plasma EDTA standard samples exposed to both temperatures for 3 or 36 h post-centrifugation were analyzed ($n = 64$) with a 20-plex single binder assay and secondly a sandwich immunoassay. In order to judge the selectivity of the anti-CALD1 antibody HPA017330, 24 plasma samples were analyzed with a 20-plex bead array including HPA017330 for target capture and labeled HPA017330 for target detection.

2.7. Data analysis

For each bead identity a minimal of 20 events was used as a threshold to qualify the median fluorescence intensity (MFI) for further analysis. The MFI values were processed per sample preparation type. The first step was a distance based outlier removal based on z score distribution, with z -value $>1.5 \times$ interquartile range per sample group and antibody as cut-off. It was followed by probabilistic quotient normalization [15] for each sample plate as previously described [16]. The deviation between the plates in a blood sample preparation type was adjusted using multidimensional MA-normalization assuming that the mean of samples within a plate is same for all plates (nMFI) [17]. Finally, the pre-processed values were log-transformed. The intensity differences across sample groups divided by handling temperature and time were tested with linear mixed effects model (LMM) [18], in which two models estimating the fixed effects of 1) time and 2) the interaction of time and temperature and both variables were compared for each antibody. The time was the converted integers from 1 to 4 for 1, 3, 8, or 36 h, respectively in order to decrease the dominant effect of 36-h group. For both models, the

donor ID is modeled as a random effect accounting for the dependence of samples of the same human subject. Among the two, the model with higher significance was selected. The homogeneity of variance across groups separated by exposed time in each handling temperature was tested with Bartlett's method [19]. The overall variability within repeated analysis of certain condition combination was calculated as the median of the coefficient of variation (CV, ratio of standard deviation and mean) for each protein profile.

Fold changes between samples were calculated from mean intensities per antibody and log-2 transformed for the comparison of blood sample preparation types serum and plasma. We used false-discovery rate (FDR) corrected p -values from Wilcoxon rank-sum test to determine sample preparation dependant protein profiles and FDR from LMM and Bartlett's test to determine which profiles were altered due to handling conditions. For single-plexed experimental verification, unadjusted p -values from Wilcoxon rank-sum test were used. All data processing and analysis were conducted using the R environment [20].

3. Results

The study was designed to determine whether differences in time and temperature during the period after centrifugation and until the first freezing of samples would influence protein profiles in serum and plasma samples. Serum and two types of plasma preparations were collected from twelve individuals and processed in accordance to the scheme shown in Fig. 1. Here, the collected specimens were placed at either room temperature or +4 °C for 1, 3, 8, or 36 h after centrifugation. All samples were then frozen at –80 °C and thawed directly prior to the processing involving direct sample labeling. Suspension bead arrays were used to create protein profiles for 373 antibodies in 96 samples per sample preparation type.

At first, the distributions of profile intensity for the three sample preparation types, collected in serum, EDTA standard plasma and EDTA plasma preparation tubes (PPT) were compared using the median value. While the intensity spread was found to be narrower and of higher intensity for serum samples than the two plasma samples (Fig. 2A–C), the two plasma sample types revealed concordant intensity distributions. The variability in protein profiles was assessed for the three preparation types, each represented by eight sample pools that were created based on conditions of the post-centrifugation delay period. As the analysis of triplicated samples in Fig. 2D shows, the variability was not changing neither for any of the investigated centrifuge-to-freezer condition nor for the sample preparation types. When comparing coefficients of variation (CV) collected for all antibodies and sample preparation types, there was a consistently higher CV between different samples than within sample replicates. Sample profiles from replicated measurements of 192 samples correlated with a median $r = 0.95$ (0.8–1.0) (Supplementary Fig. 1A). We then used 373 HPA antibodies targeting 343 unique proteins to discriminate between serum and plasma EDTA PPT or plasma EDTA standard tube samples. As already indicated above, a majority (86%) of the protein profiles in serum were at a higher intensity level than both plasma types (322 out of 373 antibodies). As listed in Table 1, the

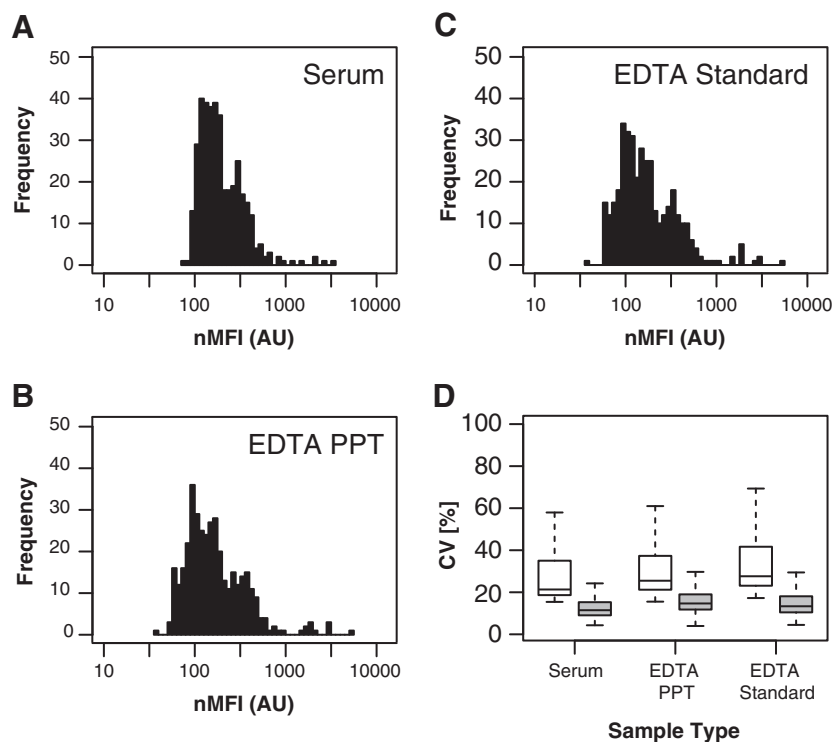


Fig. 2 – Global data analysis. (A–C) The distributions of median intensity per antibody and preparation types (A) serum, (B) EDTA PPT and (C) EDTA standard are shown. (D) The variances in antibody profiles determined within a sample post-centrifugation condition are shown across replicates (gray boxes) and across different samples (white boxes) for the three preparation types. Sample replicates were derived from pools of 24 samples and the variation was calculated from the coefficients of variation (CV).

most prominent differences – judged by differentially detected profiles' intensity fold change (absolute value) and FDR – were however linked to plasma and exemplified by two antibodies directed towards the known plasma protein fibrinogen (FGB). A subset of the antibody selection ($n = 18$) had previously been included in an analysis comparing serum and plasma with antibody suspension bead arrays [21] and besides two, all antibody-generated profiles were concordant with respect to sample type associated intensity difference and a statistical significance level $FDR < 1 \times 10^{-02}$. In addition, the two plasma preparation types used in the study

were compared. Here only one antibody, which targeted caldesmon 1 (HPA017330), was found to reveal a differential profile with higher intensity levels in EDTA standard compared to EDTA PPT plasma preparation.

We then investigated the effect of differential temperature and time during the post-centrifugation delay period on protein profiles. Among the 343 unique proteins selected for this study, it was found that the most susceptible profile to a centrifuge-to-freezer condition, indicated by a compromised intensity profile, was for HPA017330 and decreased after the 8-h time interval (Fig. 3). Its target protein is caldesmon 1 (CALD1) and in the

Table 1 – Sample type discriminating profiles. Univariate Wilcoxon rank-sum tests were performed to compare profiles from the sample preparation types serum, plasma EDTA PPT and plasma EDTA standard. Ten antibodies (targeting 9 proteins) with an absolute fold change ≥ 1.3 discriminated between plasma and serum with $FDR \leq 1 \times 10^{-24}$. Six of the nine proteins are classified as a secreted protein.

Antibody ID	Gene name	Uniprot ID	Serum vs. EDTA PPT		Serum vs. EDTA standard		EDTA PPT vs. EDTA Standard	
			Fold change	q-value	Fold change	q-value	Fold change	q-value
HPA001901	FGB	P02675	–3.6	1.2E-27	–3.5	4.8E-28	0.1	4.1E-01
HPA001900	FGB	P02675	–3.5	1.8E-27	–3.5	3.8E-29	0.0	8.6E-01
HPA008356	RET	P07949	–3.3	4.0E-29	–3.3	5.3E-30	0.0	7.4E-01
HPA016004	CHST8	Q9H2A9	–3.2	5.1E-29	–3.2	3.7E-30	0.0	9.1E-01
HPA035596	TRH	P20396	–2.5	8.2E-29	–2.5	5.5E-30	0.0	9.9E-01
HPA030867	ADAM12	O43184	–2.5	3.7E-29	–2.5	9.5E-31	–0.1	9.1E-01
HPA008549	SLC22A2	O15244	–1.7	7.0E-28	–1.7	7.7E-30	–0.1	7.4E-01
HPA017737	PI3	P19957	1.4	4.9E-24	1.4	6.0E-25	0.0	5.2E-01
HPA008791	MYBL1	P10243	1.7	3.4E-29	1.7	4.0E-30	0.0	9.7E-01
HPA003873	LTB4R	Q15722	2.2	1.2E-28	2.0	1.1E-29	–0.1	3.8E-01

analysis using a linear model for time effect HPA017330 revealed a significant decrease ($FDR = 3 \times 10^{-03}$, slope = -0.14) in plasma EDTA standard samples (Fig. 3A). The trend was verified in a repeated experiment using the same samples and $FDR = 8 \times 10^{-03}$ was achieved. For EDTA PPT collection tubes a similar, but statistically not significant trend was observed (LMM $p = 6 \times 10^{-03}$, $FDR = 8 \times 10^{-01}$, slope = -0.07). Further to this, four individuals were added to the analyses to supplement the previously employed samples from 12 individuals in order to be profiled for CALD1. Samples exposed to 4 °C or an ambient temperature were combined to represent 3-h or 36-h exposure time. First, the single binder assay revealed a difference between the two groups of $p = 1 \times 10^{-03}$ (unadjusted). Then, a dual binder sandwich assay (Fig. 3C) was conducted and revealed a p -value = 6×10^{-03} to confirm that significantly lowered CALD1 levels were found in samples left for 36 h compared to 3 h. The latter assay was repeated as well and the two intensity profiles correlated with Pearson's correlation coefficient $r = 0.93$ (Fig. 3D). Furthermore, these sandwich immunoassays were performed together with 19 other antibodies to evaluate the HPA017330 as detection antibody. While anti-CALD1 revealed MFI levels of ± 150 AU, all other captured antibodies showed 3-fold lower MFI levels ($MFI \pm 50$ AU; Supplementary Fig. 2). This supports the previous observation that the antibody

HPA017330 detected the protein caldesmon 1 in the single binder assay.

To identify whether the comparison of the profiles generated each time interval changed due to sample exposure time, inter-individual variation within each time interval was assessed using a Bartlett's test. We found 20 out of 373 HPA antibodies to reveal differential, non-homogenous inter-individual variation ($FDR < 1 \times 10^{-02}$). In the verification phase, 12 out of these 20 antibodies were included, however none of the previously observed exposure time-dependent variation was found to be significant (cut-off = LMM $FDR < 1 \times 10^{-02}$; Supplementary Table 2).

4. Discussion

In the presented study, affinity purified polyclonal antibodies were used to protein profile biotinylated serum and plasma samples to identify changes due to pre-analytical sample handling with respect to temperature and time prior to first storage. From 373 antibodies that targeted 343 unique proteins, only one antibody showed significant changes over prolonged exposure times during post-centrifugation delay. Even though the longest used exposure interval of 36 h may exceed those times commonly used in serum and plasma sampling handling

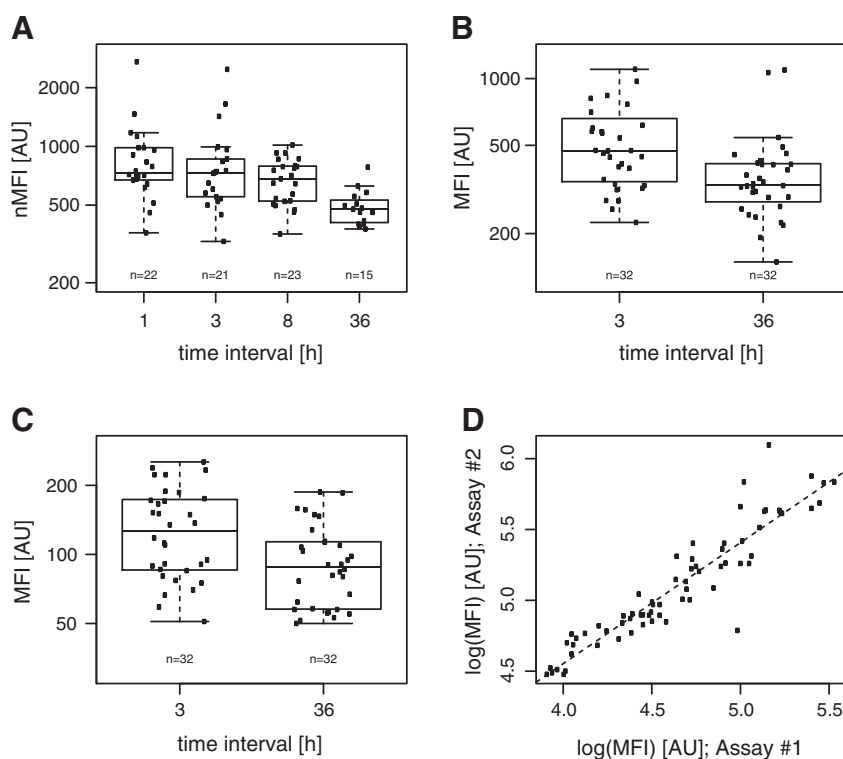


Fig. 3 – Profile variance across handling condition. The protein profile most susceptible to be compromised by post centrifugation delay conditions was caldesmon 1 (CALD1). Data from plasma EDTA standard samples exposed to room temperature or 4 °C respectively for 1, 3, 8 and 36 h prior to final storage at -80 °C are shown. (A) A linear mixed-effects model (model: $nMFI \sim \text{time} + \text{donor}$) identified antibody HPA017330 targeting CALD1 with $FDR = 3 \times 10^{-03}$. (B) Further confirmation of the finding was achieved by targeting CALD1 in an increased number of samples exposed to either temperature for 3 or 36 h (unadjusted p -value = 1×10^{-03}). (C) A sandwich immunoassay was conducted with HPA017330 as capture and detection antibody to confirm a decrease of the CALD1 profile over time (unadjusted p -value = 6×10^{-03}). (D) The sandwich immunoassay was repeated and the two intensity profiles correlated with Pearson's correlation coefficient $r = 0.93$.

procedures, shipping samples at an ambient temperature from sites of collection to central storage locations may still represent an economically and logistically interesting scenario worth considering.

There is a great value in biobanked samples providing contemporary biomarker discovery efforts with constantly growing numbers of specimen. However, various standards and lack of documentation on how samples have been collected and processed is a general weakness of all biobanks, which limits the possibility to utilize its' full potential. Since this variation potentially could have a great impact on the results of downstream analysis there is a need to define a minimum level of standard operation procedures for samples and for information linked to the sample. In order to utilize the full potential of archived samples in medical research and to combine these with newly collected material it is crucial to have information about pre-analytical factors that might compromise the sample integrity. Alternatively, it would be necessary to identify proteins that are significantly influenced by variation in sample handling that can qualify as marker candidates of biobank sample integrity. With proteins in mind, alteration in profiles could be derived by proteolytic degradation, complex formation, precipitation, or even denaturation. Hemolysis, degree of coagulation or coagulation inhibition as well as the depletion of hematopoietic cells also influence the integrity of samples. Ideally, changes in protein profiles should be determined to estimate the extent of degradation and whether a certain archived biobank material can be used in a particular analysis method. The results we presented for CALD1 in this study cohort may provide first indications of such a scenario. And also that it could be possible to find other protein profiles that are linked to variations in sample handling in all pre-analytical steps from needle to freezers, such as pre-centrifugation or post-centrifugation delay.

In this study, we for instance observed that 86% of the targeted proteins were detected at a higher intensity level in serum than in either plasma preparation type. This observation may be supported by an affinity proteomics study, where a longer storage time before centrifugation was associated with increased levels of inflammatory markers in serum, more so than plasma [22]. Furthermore, mass spectrometry has previously identified differential protein profiles of serum and plasma as well as serum or plasma collection tube types [23]. The profile for an antibody raised against caldesmon 1 (CALD1) was sensitive to handling conditions in EDTA standard plasma, both using the discovery driven single antibody assay as well as sandwich assays. The same but not significant trend was observed in profiles generated in EDTA PPT collection tubes. The plasma PPT and serum tubes contain a gel plug that physically separates cells from plasma during centrifugation to facilitate improved handling. Our current observation is that it could be likely that such a plug might have prevented leakage of proteins from blood cells and thereby might have minimized the effect. For the presented study, it is also noteworthy to mention that even though samples from only a small number of individuals were included, all samples were collected at one location and handled by a few people. This reduced the impact of having different locations and operators but suggested to extend later studies of this design across several collection sites and personal. A success factor for biobanks is therefore to use standardized protocol and full documentation of all steps in the pre-analytical chain at locations of collection,

distribution and storage, as well as documenting the subsequent sample handling history at sites of analysis [24,25].

The method applied in the presented study is based on direct labeling of the proteins in the samples followed by a heat treatment step to retrieve epitopes [26] and the analysis in single binder assays. Even though no analytical detection limits were available for the included antibodies, the semi-quantitative information obtained from the comparative analysis still allows revealing putative changes in protein profiles. While the discovery of only one profile could potentially also indicate a lack in sensitivity or specificity, similar findings were made in a previous study, which employed a different class of affinity reagents (aptamers) and investigated the impact based on pre- and post-sample centrifugation handling times [27]. Concordant results were also recently described for mass spectrometric methods, where the *a priori* selection of targets of interest is not necessarily generally required [4,28]. In the presented study, affinity-purified polyclonal antibodies were applied for protein profiling. These antibodies show oligo-epitopic target recognition [29] and their ability to detect a respective protein may for this reason be partially less sensitive to protein degradation than an analysis based on defined peptides. This could on the other hand also be viewed as an advantage of these antibodies when screening for differential protein profiles in across many samples in a comparison of healthy and diseased individuals. As also mentioned above, protein denaturation or aggregation might occur during sample handling and this is however expected to effect protein detectability, as the accessibility of the target epitope(s) is reduced. We have observed this in another context of a previous study, where intensity levels of most antibodies decreased when samples had been heated to 96 °C prior to analysis [26].

The protein identified as being susceptible to prolonged post-centrifugation delay was caldesmon 1 (CALD1). The protein exists in distinct isoforms and is expressed as a high molecular weight variant (h-CALD1) predominantly in smooth muscle. The low molecular weight isoforms lack a central repeated domain of h-CALD1 but are expressed in non-muscle tissues and cells and have previously been described as glioma marker in serum [30]. CALD1 binds Ca(2+)-calmodulin, actin, tropomyosin, myosin, and phospholipids and has an essential role in regulating smooth muscle and non-muscle actomyosin interactions as well as cell motility functions [31]. The anti-CALD1 antibody HPA017330 had been raised against a 132-residue long fragment, matched to most splice variants and all isoforms (Supplementary Fig. 3). It stained cytoplasmic expression in smooth muscle, myoepithelial cells, endothelium, ovarian and endometrial stroma in immunohistochemistry and subcellular staining was localized to actin filaments and the plasma membrane [<http://www.proteinatlas.org/ENSG00000122786>]. Other anti-CALD1 antibodies have confirmed these staining patterns but did not qualify for plasma analysis due to concentration or formulation. Further evidence for the single binder CALD1 profile was achieved using a dual binder assay, where HPA017330 was used for both capture and detection. A current, yet speculative explanation for the alteration of the CALD1 profiles is that the prolonged incubation in EDTA leads to extensive calcium ion chelation where Ca-binding proteins and their associated binding partners may precipitate. Nevertheless, additional experimental evidence using mass spectrometry or

other means of detection, and even many more samples as well as an optimized immunoassay enabling an improved discriminative power would be needed to establish more the current indications from CALD1 decrease in EDTA plasma could become a useful indicator for handling variability for already biobanked samples as well as for samples being collected.

Biobanks aim to serve as a dynamic resource for research today and long into the future perhaps with technologies we cannot yet predict. Many samples will though/however be stored for decades and in some biobanks risk repeated freeze-thaw-cycles, for example during rounds of aliquotation and withdrawal. At this point our study does not provide evidence if certain processing conditions prior to sample freezing could trigger processes that lead to more substantial changes in profiles than we observed. Even though this goes beyond the scope of our study as we only looked at samples being stored for a short period and transferred in one shipped, sample profiles from long-term storage and freeze/thaw cycles should also be investigated. Nevertheless, the made observations should be considered when aiming at improving study design for antibody array analysis or other methodologies that aim to conduct biomarker discovery studies in serum and plasma.

In conclusion, our study shows that the protein profiles generated in directly labeled serum and plasma and by antibody bead arrays appeared generally not compromised by time and temperature conditions post-centrifugation and prior to storage. This is in good agreement with observations made for mass spectrometry [4] and alternative protein scaffolds [27]. The presented work therefore provides a perspective on post centrifugation delay in the analysis of blood-derived samples when choosing multiplexed and affinity based assays to profile proteins.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2013.04.020>.

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