Evaluation of 92 cardiovascular proteins in dried blood spots collected under field-conditions

Off-the-shelf affinity-based multiplexed assays work well, allowing for simplified sample collection

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Abstract
Workplace-collected blood spots deposited on filter paper were analysed with multiplexed affinity-based protein assays and found to be suitable for proteomics analysis. The protein extension assay (PEA) was used to characterize 92 proteins using 1.2 mm punches in repeated samples collected from 20 workers. Overall, 97.8% of the samples and 91.3% of the analysed proteins passed quality control. Both within and between spot correlations using six replicates from the same individual were above 0.99, suggesting that comparable levels are obtained from multiple punches from the same spot and from consecutive spots. Protein levels from dried blood and wet serum from the same individuals were compared and the majority of the analysed proteins were found to be significantly correlated. These results open up for simplified sample collection of blood in field conditions for proteomic analysis, but also highlight that not all proteins can be robustly measured from dried whole blood.

KEYWORDS
affinity based proteomics, dried blood spots, sample collection

INTRODUCTION

Blood-based protein biomarker tests are routinely used for screening and diagnosis of a wide range of diseases. They are also used for monitoring of health and risks factors in work environments where workers are exposed to hazardous substances. These tests are usually based on plasma or serum extracted from whole blood samples taken at health care facilities by trained personnel. Screening based on dried blood spots (DBS) is regularly used in new-born and have been so for over 5 decades.1,1 Today, however, DBS is not used clinically for screening, diagnosis or monitoring in adult populations. In the recent decade, several studies have showed that outstanding issues of DBS sampling in adults can be resolved, and have identified several benefits over conventional phlebotomy including reduced need for medically trained personnel, facilitated handling and easier storage of the samples.2,3 Standard protocols for analysing blood from DBS involve punching out circular discs with dried blood from the paper and resolving the blood in a buffer prior to analysis. The European Bioanalysis Forum have published recommendations for each step from sample collection, transportation, extraction and storage.4 The punched discs are generally 3.2 or 6 mm in diameter meaning that 1–2 punches have typically been taken from a single DBS. Depending on the starting volume needed for the assay, this has put limits to the number of assays that could be carried out from the same sample or blood drop. Previous investigations...
have shown that a large number of analytes can be accurately measured from DBS. The majority (67%) are small molecules, nucleic acids and trace elements (e.g., metals).\[^{15}\] Investigations using high-throughput methods characterizing large molecules (e.g., proteins) have though been lacking, primarily due to technological restraints of the small spotted volume. Recent development of high-throughput antibody-based methods for protein measurements has now overcome some of these difficulties. The protein extension assay (PEA) allows for quantification of 92 proteins from 1 \(\mu\)L of starting wet-volume or a 1.2 mm punched disc from a dried sample.\[^{6,7}\] By using 1.2-mm punches, four to 19 times more punches can theoretically be taken from the same surface as compared to 3.2- or 6-mm punches, allowing for a large number of analytes to be measured from a single sample.

Detailed studies on inter- and intra-spot variability in measurable protein abundance levels in DBS have also been lacking. Here, we have used a commercially available assay measuring 92 cardiovascular protein biomarkers to evaluate the robustness of the obtained measurements from DBS. We have also compared the results obtained from DBS with sera collected from the same individuals.

### MATERIAL AND METHODS

#### Samples

A total of 23 male non-smoking construction workers working at seven different workplaces in Stockholm county, Sweden were sampled twice using finger-prick. The first sampling took place at the workplace once in March-June 2019 during a normal work period (sampling took place in the morning of Tuesday-Thursday). A second sampling took place in the morning at the Clinic of Occupational and Environmental Medicine, Stockholm County, directly after the participants’ summer vacation (July-September 2019) immediately before resuming work. The dried blood samples were taken by a trained nurse at the workplace and at the clinic using the same protocol. First, the finger of the participant was wiped off with chlorhexidine, air-dried, and then the third or the fourth digit (either on left or right hand) was pricked with a lancet and capillary blood was allowed to drip onto a filter paper (Whatman protein saver #903, Sigma Aldrich) with pre-marked circles. The leftmost circle was filled with the first drop. Then, two to three circles were filled from the right to the left. A blank circle was left between drop one and two. The reason for this was that the first drop was excluded from all analysis and should be easily distinguished from the drops available for analysis. The filter-papers were then allowed to dry in a closed plastic box from the sampling until the nurse came back to the clinic (usually 6 h). The collection papers were put in plastic bags, sealed and stored at \(-20^\circ\text{C}\). This study is approved by the local ethics committee at Karolinska Institutet (No. 2019-00208). Twenty of the 23 workers (Table 1) were selected for proteomics analysis. Two individuals were excluded based on BMI above 37, one individual was excluded based on very high “snus” (moist powder smokeless tobacco product) usage (21 boxes per week). For 19 of the 20 samples (95%) collected at the first time point, three usable drops were collected and for one sample, two usable drops were collected. At the second time point, three usable drops were collected for all 20 samples (100%).

For the study participants, venous blood samples were also collected at the workplace and at the clinic by a trained nurse. The blood samples were, after 15 min in room temperature, separated by centrifugation for 10 min, and plasma and serum were stored at \(-20^\circ\text{C}\). At the end of the same week (mostly Thursdays), the samples were frozen at \(-80^\circ\text{C}\).

#### Preparation from DBS and PEA analysis

In order to minimize batch-effects and possible variance introduced by mixing dry and wet samples across several plates, two separate runs were done. The first contained dried samples only and the second a mix of dried samples and their corresponding wet serum samples. The first plate was run in January 2020 and the second in April 2020 at the Clinical Biomarker Facility, Uppsala University, Uppsala, Sweden. The facility is a certified analysis lab by the manufacturer of the PEA. The commercial PEA comes with 92 pre-selected proteins with expected measurability in human serum or plasma. The protein selection is done by the manufacturer and based on previous function and disease association in the literature.

In the first run, a total of 88 DBS-samples was analysed in one plate. From each analysed DBS, 1.2 mm diameter punches were taken close to the centre of the spot with a Uni-Core micropuncher (General Electric) (Figure 1C and D). In the second run, 40 of the DBS-samples, their corresponding serum samples and additional replicates of serum samples were analysed together in one plate. One punch or 1 \(\mu\)L serum were mixed with 3 \(\mu\)L incubation mix containing probes (consisting of paired antibodies labelled with unique corresponding DNA oligonucleotides) in microtiter plate wells and analysed according to the standard protocol of Olink Proteomics commercialized Target 96 Cardiovascular Disease (CVD) II panel. To ascertain similar probe concentrations and reaction volumes in wet and dry samples, 1 \(\mu\)L negative control was added to compensate for the missing volume in the DBS samples during the first step.

The PEA is an affinity-based assay and for each protein, a pair of oligonucleotide-labelled antibody probes binds to the targeted protein. If the two probes are in close proximity, a PCR target sequence is formed by a proximity-dependent DNA polymerization event and the resulting sequence is subsequently detected and quantified using

### Table 1

| Unit | Mean | StdDev | Median | MAD\[^{c}\] | Range |
|------|------|--------|--------|-------------|--------|
| Age\[^{a}\] year | 38.9 | 13.1 | 35.4 | 14.8 | 20–61 |
| BMI \(\text{kg/m}^2\) | 26.4 | 4.1 | 25.9 | 3.9 | 19.2–36.2 |
| Snusb box/week | 4.9 | 2.4 | 5.0 | 3.0 | 1–7.5 |

\[^{a}\]Reported in whole years only.

\[^{b}\]Numbers are based on the 7 (35%) participants that self-reported using snus (moist powder smokeless tobacco product).

\[^{c}\]Median absolute deviation.
real-time PCR. The resulting abundance levels are given in NPX (Normalized Protein eXpression) on a log2-scale. Four internal controls are used in the PEA to assess the performance of the assay itself and individual samples. The quality control (QC) then consists of two stages.

First, the standard deviation of the internal controls on each plate is required to be below 0.2 NPX. Second, each sample is required to deviate (absolute median deviation) less than 0.3 NPX compared to the assays’ internal control samples. The PEA defines a lower detection limit (LOD) for each protein (defined as three standard deviation above noise level) at run-time based on the negative controls that are included in each run. All measurements below this limit were removed from further analysis. Each protein was further required to have measurements in at least 90% of the samples.

In the first run, two (2) of the DBS samples and eight (8) proteins did not pass initial quality control performed by the facility and were removed from further analysis. The eight proteins were: Interleukin-4 receptor alpha (IL-4RA) with 88.4% of measurements above LOD, SLAM family member 7 (SLAMF7, 73.3% above LOD), Gastrotropin (GT, 68.6%), Fibroblast growth factor 23 (FGF-23, 67.4%), Interleukin 6 (IL-6, 62.8%), Interleukin 17D (IL-17D, 8.1%), Carbonic anhydrase 5A (CA5A, 3.5%) and Natriuretic peptides B (BNP, 2.3%). In the second run, the DBS and serum samples were QC:ed separately as the ingoing concentrations were expected to deviate significantly. Four (4) of the DBS-samples did not fulfil the QC-requirements and one (1) of the corresponding serum samples and were removed from further analysis. On the protein level, seven (7) proteins did not meet requirement in the DBS-proportion (FGF-23 (85.3% above LOD, SLAFM7 (75.0%), IL6 (75%), GT (66.7%), IL-17D (55.6%) CA5A (30.6%) and BNP (5.6%)) and three (3) in the serum samples (Integrin Subunit Beta 1 Binding Protein 2 (ITGB1BP2, 2.2% above LOD), BNP (34.8%) and Poly [ADP-ribose] polymerase 1 (PARP-1, 76.1%)) and were removed from further analysis. After the separate QC-stages for dried and wet samples of the second run, 83 proteins in 35 samples were kept in common for further analysis.

A complete list of all proteins on the CVD II panel together with QC-parameters used here is provided in Table S1, and summarized in a flowchart in Figure S1.

**Figure 1** Overview of position of punches. (A) For all 20 individuals, punches were taken from spots 2 and 3. (B) For one individual, a total of six punches were taken, duplicate punches from spots 2, 3 and 4. (C) Actual card with one punch taken from spot 2 and one for spot 3. The diameters of the dashed circles are ½ inch, circa 12.7 mm, and the diameter of the punches are 1.2 mm. (D) Actual card with a total of six punches, two from each used spot.

**Statistical methods**

All analyses were carried out using R[8] (version 3.5.3). Coefficients of variance (CV) were calculated based on the NPX-values and defined as the standard deviation over the mean. The %CV as stated in the text is the CV multiplied with 100. Correlations between observations were calculated using the Pearson correlation with two-sided P-values as given by the “cor.test” function in R. P-values for differences in distributions of values between spots were calculated using the Wilcoxon-ranked based test. All linear regression models were fitted using the "lm" function and estimates of explained variance were taken directly from the returned model. Statistical significance of the fitted models was calculated using the “anova” function. Confidence intervals surrounding the fitted models were calculated based on 100 equally spaced points over the observed range using the “predict” function with the “interval”-parameter set to “confidence”. Adjustment for multiple hypothesis testing was done using strict Bonferroni adjustment.

**RESULTS**

DBS collected under field-conditions are suitable for analysis with the protein extension assay

We aimed at collecting four drops of blood from each participant where the first drop of blood should not be used for analysis as it has been suggested to contain a higher proportion of tissue fluids.[9] In 95% (19/20) of the samples taken before the summer holiday at least three usable spots were collected. Among the samples taken after the summer holidays, at least three usable spots were collected for 100% (20/20) of the participants. A total of 88 dried samples from these 20 individuals were first analysed using the protein extension assay (PEA), Cardiovascular II panel (Table S1). All samples were analysed in duplicates from both before and after the summer holiday with punches taken from spot 2 and spot 3 from all individuals (Figure 1A and C). For one individual, a set of four additional punches was taken from before and
after the summer holiday resulting in two punches from spots 2, 3 and 4, respectively (Figure 1B and D). Eighty six of the 88 analysed samples (97.8%) passed the internal QC criteria defined by the manufacturer. The two samples not passing QC was from two different individuals among the 19 samples with duplicated measurements. Requiring at least 90% of PEA measurements above LOD for each of the proteins, 84 (91.3%) of the proteins passed QC and were included in downstream analysis. In a second analysis with PEA, paired serum and dried whole blood from 20 samples were analyzed before and after the summer holiday. In that analysis, 83 (90.2%) proteins in 35 individuals with measurements in both wet and dried samples passed QC. According to the manufacturer of the assay, at least 90% of the protein on the Cardiovascular II panel should be detectable in 75% of healthy individuals. Although a stricter requirement on the individual levels was used here, the expected number of proteins was detected from both wet and dried samples.

**Individual protein abundance levels are robust within and between spots**

Using the sample with multiple punches from the same dried spots (Figure 1B and D), we first calculated intra-spot correlation and variability. Comparing the observed NPX-levels for the 84 proteins between punches within the three spots, all estimated correlations coefficients were above $R^2 = 0.9957$ with nominal $P$-values $<6.6 \times 10^{-99}$ (Figure 2A, Table S2). The largest absolute difference within a spot was found for Serpin A12 (SERPINA12), which in one
spot differed by 1.27 NPX. The mean absolute difference for all proteins in punches within the same spot ranged from 0.086 to 0.18 NPX (Table S2) with a trend towards lower differences with increasing protein abundance levels (Figure 2B, Table S2, nominal P-values ranging from $1.8 \times 10^{-3}$ to 0.89). We then calculated %CV for pairs of punches from the same spots for all 84 proteins (Table S3). The mean %CVs from punches within spots ranged from 0.43 to 14.2 (Figure S2a, Table S3) and when compared to the intra-assay %CV in plasma samples as reported by the manufacturer (ranged from 3 to 21), 13.1% (11 of 84) of the assays were found to have higher %CV in analysis from DBS compared to plasma (Figure S2b). The protein with the highest increase in %CV (1.7 times higher) was found for Polymeric immunoglobulin receptor (PIgR) with 5.1 in DBS compared to three reported by the manufacturer in plasma. The highest %CV overall in DBS was found for Fibroblast growth factor 21 (FGF-21), SERPINA12 and Renin with 14.2, 13.3 and 12.5, respectively compared to 12, 10, and 8 as reported for Fibroblast growth factor 21 (FGF-21), SERPINA12 and Renin with manufacturer in plasma. The highest %CV overall in DBS was found for Proline and Arginine Rich End Leucine Rich Repeat Protein (Prelp) with 8.2 times higher %CV in the samples collected before the summer holiday compared to samples collected after the summer holiday (Figure S2ef), while only one protein lactoylglutathione lyase (GLO1) were reported for trained personnel and can be collected by individuals or patients.

We proceeded with calculating inter-spot correlation and variability using the same data as above. When comparing all individual punches from a spot to all other individual punches from another spot, we found all correlation coefficients to be above $R^2 = 0.99$ with nominal P-values $< 1.0 \times 10^{-83}$ (Figure 2C, Table S2). As above, the largest absolute difference between any pair of punches between spots was found for SERPINA12 (1.50 NPX). Between spots, the mean absolute difference for all proteins ranged from 0.10 to 0.30 NPX (Table S2) with a trend towards more similar distribution of differences between comparison with increasing protein abundance levels (Figure 2D, Table S2, nominal P-values ranging from $3.2 \times 10^{-3}$ to 0.95). We then calculated %CV for pairs of punches from the different spots for all 84 proteins (Table S3). Between spots, the mean %CVs across pairs of punches ranged from 1.2 to 31.8 (Table S3, Figure S2c). Compared to the intra-assay %CV in plasma samples as reported by the manufacturer 12 of the 84 (14.3%) of the assays were found to have higher %CV in analysis from DBS compared to plasma (Figure S2d). Four proteins were found to have over 1.5 times the %CV in plasma reported by the manufacturer; SERPINA12, FGF-21, Decorin (DCN) and Leptin with 31.7, 19.5, 14.5, and 9.4 compared to 10, 12, 7, and 6, respectively. SERPINA12, FGF-21 and DCN were also the three proteins with the highest %CV overall in the DBS analysis.

When comparing point estimates of %CVs for all punches across all spots (data used above), 50 proteins were found to have 1.5 times larger %CV values among samples collected before the summer holiday compared to samples collected after the summer holiday (Figure S2ef), while only one protein lactoylglutathione lyase (GLO1) were found to have at least 1.5 times the %CV among samples collected after the summer holiday compared to samples collected before. The largest increase among “before” samples compared to “after” was found for “Proline And Arginine Rich End Leucine Rich Repeat Protein” (Prelp) with 8.2 times higher %CV in the samples collected before compared to after the summer holidays. The same protein also displayed one of the largest increases of mean protein levels in the “before” samples compared to “after” with a mean increase of 0.68 NPX. Overall (Figure S3), we found a moderate, significant correlation ($R = 0.45, P < 3.3 \times 10^{-4}$) between changes in mean values and changes in %CV between the two sampling occasions with change in mean explaining 19% of the observed variance in changes in %CV.

Finally, we looked at the total distribution of protein abundance levels between spots and found no statistical differences between spot 2 and 3 for any of the 84 proteins (all nominal P-values $> 0.16$, Table S4). This was the same both for spots collected before the participants’ summer holiday and after (Table S4). We also tried modelling the abundance levels as a function of spot number and found no statistically significant proportion of the variance in any the protein abundance levels explained by the spot number (all P-values $> 0.21$, Table S4).

The majority of protein abundance levels in DBS covaries with serum levels

Thirty-five (35) of the 40 samples (87.5%) and 83 of the 92 proteins (90.2%) with both DBS and serum measurements jointly passed QC and were used to investigate the relationship between observed abundance levels in the two sample types. Although both measurements are reported in NPX, the absolute levels were not comparable between the two sample types due to the separate pre-QC normalization. The relationship between the levels were though comparable and since all datapoints passing QC lied in the expected linear proportion of all ingoing assays’ measuring ranges, linear modelling and correlations could be used. Figure 3A illustrates the relation between the observed values in DBS (y-axis) and serum (x-axis) with the 83 proteins shown in different colors. We also fitted linear models using the serum data as input and the DBS data as response (shown as solid lines in Figure 3A). For 62 of the 83 proteins (74.7%, mean [range] % variance explained $= 46.9 [9.2, 94.9]$) the explained proportion of variance in the responses was nominally significant (ANOVA, $P < 0.05$) with 45 (54.2%, mean [range] % variance explained $= 58.5 [28.5, 94.9]$) remaining significant after strict adjustment for multiple hypothesis testing (Bonferroni). The significance of the models is shown in Figure 3B. We then calculated Spearman’s correlation coefficients (Figure 3C) for the two samples types and found significant correlations for 60 of the 83 proteins (72.2%, mean [range] $R^2 = 0.45 [0.12, 0.93]$) out of which 39 (47.0, mean [range] $R^2 = 0.58 [0.32, 0.93]$) remained significant strict adjustment for multiple hypothesis testing (Bonferroni). Model parameters and correlation coefficients for all 83 proteins are reported in Table S5.

DISCUSSION

DBS offers flexibility and convenience in sampling and handling

With health-care systems already under pressure, methods and practices that can aid and simplify how samples are collected and analysed are essential for maintaining and increasing current standard. Collection of dried blood spots (DBS) through finger-prick relieves the need for trained personnel and can be collected by individuals or patients.
themselves at home or at workplaces. The collected dried samples can even be sent through ordinary mail and does not need specialized transportation. A study published in 2009 investigated the willingness of participants \( (N = 2048) \) to collect blood as DBS using finger prick and performed laboratory analysis of four analytes in the collected blood from adults in the US.\(^{[10]}\) The study found that 84.5% of the participants were willing to deposit blood on filter paper from finger prick, and that usable samples for downstream analysis using 6 mm punches where obtained in 99% of the cases. More recently, in a study monitoring therapeutic drug use in immunocompromised patients, blood collected from finger prick and stored as DBS was compared to routine phlebotomy in clinical settings.\(^{[11]}\) In that study, the analyses carried out from DBS samples were found to be equal to the results found in analysing venous blood with 60% of the patients preferring the DBS sampling method compared to phlebotomy, which was only preferred by 15%; 25% of the patients had no preferred method. In addition, a significantly lower perception of pain was reported during DBS sampling compared to routine blood collection. Samples collected at health care facilities or under laboratory conditions can differ from those collected at, for example, worksites or by self-sampling at home due to technical aspects but also from environmental influence. The dried format also alleviates the need for specialized transportation or handling of the collected samples. The very small amount of blood that is deposited has been an absolute restriction on the total number of assays that can be performed using the DBS format, especially for larger molecules such as proteins. However, recent advances for high-throughput ultrasensitive technologies such as the protein extension assay (PEA) have made it possible to investigate a large number of proteins from a single sample with up to 92 proteins being characterized from a 1.2 mm punched disc. With more responsibilities being transferred to the individual, self-sampling of blood for, for example, screening or diagnostics would be an attractive and cost-efficient addition to site-based healthcare.

Previous studies\(^{[7,12–14]}\) have demonstrated the possibility of characterizing protein abundance levels from DBS, both for fewer analytes in large sample sets and for a higher number of proteins from smaller samples sets constructed under laboratory conditions. The recent work by Eshghi and colleagues\(^{[12]}\) reports encouraging results: absolute concentrations were determined for over 200 proteins by mass spectrometry. In that study, 50 μL of blood drawn using finger

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**FIGURE 3** Comparison of wet and dry measurements for 83 proteins. (A) Scatterplot of datapoints obtained from DBS (y-axis) and wet serum (x-axis). Individual measurements in spots coloured by protein. Coloured lines represent linear models with DBS values as response and serum values as input. (B) Colour-coded representation of the significance of the variance explained in the response based on the input for the linear models in (A). Black lines represent non-significant models, yellow lines nominally significant \((P < 0.05)\) and red lines significance after adjustment for multiple hypothesis testing (Bonferroni, \(q < 0.05/83 = 6.0 \times 10^{-4}\)). (C) Histogram of correlation coefficients (Spearman’s R) for DBS compared to serum. Colours represent significance of the correlation, coded as in B)
prick technique was pipetted onto spots of the same type of paper cards used here and then five 6 mm punches (one from each spot) was used for analysis. Importantly, that study also showed that for the vast majority of the studied proteins (190 of 245, or 78%) the detected concentrations remained stable for up to two months of storage of the DBS in room temperature. Previous investigations have addressed the long-term effects of storage of DBS at either -4°C or -24°C for up to 40 years. After 10 years of storage, the median of protein measured abundance levels were 80% and 93%, respectively of those observed after 0.5 years of storage. From that study, it is clear that while some proteins remain stable for decades, other display significantly changes with temperature-dependent half-lives in the range of 10 to 50 years. Overall, 66% or 24% of the proteins in stored DBS while some proteins remain stable for decades, other display significantly changes in measurable levels. This is comparable to another study investigating the effects in plasma stored at -70°C for over 20 years where 17% of the examined proteins were significantly affected by storage time.

What are the major differences in DBS compared to wet samples?

Although high correlations in protein concentrations have been observed between dried whole blood, dried plasma and wet plasma, absolute levels have been reported to be quite different. The study by Miller and colleagues measured leptin (LEP) from 63 matched DBS and plasma samples and found a correlation coefficient of \( R = 0.976 \) but with mean concentrations of 3.49 and 7.16 ng/mL, respectively. Here, a similar correlation coefficient between DBS and serum \( R = 0.99 \) was obtained. Miller concluded that this difference in concentration was due to dilution effects of erythrocytes in whole blood compared to plasma.

The fraction of erythrocytes in whole blood, often reported as the volume fraction in whole blood (hematocrit, Hct) have been shown to affect molecular measurements from DBS in two major ways. Firstly, the viscosity of the deposited blood is dependent on the Hct, which can influence the thickness of the DBS itself across a single spot. A non-uniform thickness of the DBS results in that a larger volume of blood is obtained when a punch is taken closer to the centre of the DBS compare to the punches closer to the edge. Secondly, previous studies have shown co-variance between measurands and Hct. This has not been extensively studied for larger molecules such as proteins, but one study specifically compared prostate-specific antigen (PSA) to Hct in DBS. In that study, PSA measurements varied 10%–18% in the extremes \( Hct 0.24–0.61 \) of the monitored Hct interval compared to a reference interval \( (0.45) \). Apart from Hct, a large variety of additional factors influence circulating proteins levels. One such factor is individual age, and comparing a reference interval of individuals aged 45–55 years with individuals aged 23–27 and 67–82 with 425 proteins measured with PEA in the Northern Swedish Health Population Study revealed that about 20% of the studied proteins displayed similar variation sizes to that of reported for Hct. For a common lifestyle marker such as body mass index (BMI), comparing a reference interval of 18.5–24.9 to <18.5 and 25–29.9 kg/m² resulted in similar variation sizes for BMI as that reported for Hct in about 10% of the proteins in the study. This shows that Hct can be an important co-variate similar to other anthropometrical or lifestyle-related factors in proteomic studies from DBS and needs to be evaluated in larger future studies.

In our analysis, comparing levels from matched DBS and serum samples, six (6) proteins had very high correlation coefficients (Spearman's \( R > 0.9 \)). These were hydroxyacid oxidase 1 (HAOX1), FGF-21, chymotrypsin C (CTRC), vascular endothelial growth factor D (VEGF-D), Fatty acid-binding protein 2 (FABP2) and LEP. The first four \( (4) \) of these had over 90% of the observed variance in DBS explained by the linear models. One explanation to the high correlations between serum and DBS could be that these proteins are expressed in specific tissues and secreted into the blood stream. Such expression pattern would not be influenced by inclusion of the red blood cell proteome, although the concentrations would differ. Since the selection of the proteins on the assay largely is based on previous association with cardiovascular diseases, and therefore not random, and since the number of total proteins is small, investigations into, for example, comparing the correlation patterns with overrepresentation of Gene Ontology terms or enriched pathways is not possible. A brief search in the Human Protein Atlas \( (\text{http://www.proteinatlas.org}, \text{accessed October 2020}) \); however, suggests that HAOX1, FGF-21 is predominately expressed in liver, CTRC in pancreas, FABP2 in small intestine and duodenum, while VEGF-D and LEP is expressed in several different tissue types including blood for VEGF-D. This shows that the highly correlated values between DBS and serum is not exclusive to secreted tissue-specific expression but can be observed among proteins expressed also in multiple tissues.

Technical issues are to be considered with DBS

The off-the-shelf commercially available PEA is not validated for dried whole blood but for wet plasma and serum. According to the manufacturer, at least 90% of the proteins of the Cardiovascular II panel used here are expected to be readily detected in EDTA plasma in healthy donors. Here, 91.3% of the proteins analysed and 97.8% of the included DBS samples passed QC. Although this demonstrates the technical application of PEA to DBS collected under field-conditions, we did observe considerably higher %CV for some of the proteins, both within spots and between spots, compared to those reported by the manufacturer for plasma. For some proteins we also observed substantially different %CV among the samples that were collected at the workplace compared to those collected at the clinic just after the summer holiday. We also saw that this increase in variance correlated with an increase in absolute values, which could explain the observation. The PEA has an analytical measuring range that is defined by the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ). The lower limit of detection is determined at run time for each analysis with PEA while the upper limit needs to be determined using dilution series, which is in general not done for a normal analysis with PEA.
The behaviour of the assay is such that while concentrations below the LLOQ will not yield a measurement, concentrations higher than the ULOQ will be read as lower than their actual value because of an excess of antigen compared to the available amount of antibody probes (the so-called hook effect). The manufacturer of the assay provides calibration curves for each analyte in NPX for concentrations in plasma, but since the NPX is runtime specific, the obtained values cannot be compared directly between runs or when different sample sources are used. However, for PRELP, which had the largest increase in %CV between samples sets here, according to the manufacturers’ website (www.olink.com, accessed March 2020) the ULOQ lies around 9 NPX. In our data, 26% of measurements in the "before"-data lies within 90% of the ULOQ as compared to only 2% in the "after"-data. As with all affinity-based assays, PEA data have a sigmoid relationship with the true protein concentration in a sample. Data closer to the extremes, therefore, have an increased risk of being in the non-linear phase of the curve, hence a difference of 1 NPX no longer corresponds to a doubled protein concentration. Although speculative, this could explain the higher degree of uncertainty in the data collected during the work period, compared to the data collected immediately after the summer holiday, where a higher degree of observed lower values would actually correspond to even higher concentrations close to the linear spectra of measurability of PEA. Even though we did see an increase in the %CV’s from samples collected after the summer holidays at a clinic compared to those observed when the samples were collected at the worksite, the majority of proteins from both collection points had %CV lower than those reported in plasma by the manufacturer supporting the use of DBS as reliable collection methods under field conditions.

Strengths and limitations of the current study

Some limitations with the current study should be acknowledged. Firstly, the sample size—only 20 individuals—is small. A larger sample set would give higher statistical power to detect expected associations with the available covariates but also better point estimates of correlations and %CV. All individuals are men, so no sex-dependent differences in terms of detected proteins can be carried out. The decision to include only men was based partly on the distribution of sexes on the collaborating workplaces but also on the fact that fewer confounding variables are preferred with a smaller sample size. This is also why only non-smokers were included in the current study. We relied on the linear range in the assays defined by the manufacturer for wet samples, and did not have the resources to define individual lower or upper limits of detection for the proteins analysed here using DBS, which would have been the gold standard. Lastly, Hct was not measured in the samples and direct comparisons between observed protein levels and Hct could, therefore, not be performed.

The strengths of this study include a controlled study design with wet and dry samples collected at two timepoints in the presence of a trained nurse ensuring identical pre-analytical handling of the samples and adherence to sampling instructions. A trained nurse collected the venous blood samples used in the comparison of wet and dry samples. Furthermore, all DBS samples were prepared by the same individual and analysed in duplicates with one sample analysed in six biological replicates per timepoint, ensuring adequate underlying data for estimates of within- and between spot variation. All compared samples were also run at the same time in a single chip minimizing any assay variance such as plate effects. Biological variance was also minimized as only non-smoking males were included in the study.

Conclusions and prospects

Our conclusion is that PEA works well with DBS collected under field conditions and that obtained concentrations from consecutive spots are comparable. Certain proteins do, however, show large differences in measurability in wet as compared to dry samples. Although high correlations were observed, the absolute concentrations obtained from dried whole blood are expected to be different from plasma or serum and caution is advised in relation to the linearity of the PEA within the observed concentration ranges. Looking forward, the work reported here could be expanded to safe and efficient sample collections for immediate and longitudinal studies of exposure to hazardous substances in work environments. A first sample collection of both venous blood and an instructed, supervised collection of DBS allows for baseline comparative data for the specific intended assay, and also for continued non-supervised DBS sample collection for several additional time-points. These additional samples could be taken at fixed intervals or when work conditions or exposure change and could be analysed together with baseline data when the study is completed.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

Conceptualization (KB, SE), Formal analysis (SE), Funding acquisition (KB, SE), Investigation (EA, JSe, JSv, KG, MÅ), Methodology (KB, SE), Resources (EA, JSe, JSv, KB, KG, MÅ, SE), Software (SE), Visualization (SE), Writing – original draft (SE), Writing – review & editing (EA, JSe, JSv, KB, KG, MÅ, SE).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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