Journal of Health Care Communications
ISSN: 2472-1654

Open access Research Article

Reaching the Remote: Dried Blood Spot Analysis for Disease Diagnosis on a Protein Microarray Platform

Metoboroghene O. Mowoe[1*], Tristan Rensburg[2], Hisham Ali[2], Joshua Gqada[2], Urda Kotze[2], Marc Bernon[2], Bradley Africa[2], Eduard Jonas[2], Jonathan M. Blackburn[1]

[1]*Department of Integrative Biomedical Sciences, University of Cape Town, Cape Town, South Africa*
[2]*Department of General Surgery, University of Cape Town, Cape Town, South Africa*

ABSTRACT

Context: Cancer remains one of the leading causes of death globally with an estimated 19.3 million cases and 10 million mortalities in 2020. In Africa and Asia, where remoteness is prevalent, access to healthcare facilities is limited, providing a significant barrier to effective screening and early detection of cancers in at risk groups and thus, incomplete registries.

Objectives: Here, we utilised low resource, low cost dried blood spots (DBS) based sample collection coupled with robust, protein microarray technology to enable quantitative, multiplexed measurements of diagnostic auto antibody biomarkers of disease, in minimal sample volumes. I

Methods: Specifically, we describe the development of a DBS extraction and elution method from low cost, home-made blood cards. We then show that DBS stored at room temperature (25℃, RT) for up to 15 d yield comparable autoantibody signatures to autologous serum samples stored at -80℃ and those from samples prepared on a commercially available blood card. We further conducted a pilot study, comparing total IgG and three previously identified autoantibodies up regulated in pancreatic cancer (PC), in DBS from 11 PC patients stored at RT for up to 15 d.

Results: We found comparable protein profiles across commercially developed blood cards and our low cost, in house kit with no significant difference in autoantibody profiles over 15 d ($p>0.05$).

Conclusion: Such low cost, DBS based sample collection methods, combined with regular, RT courier shipments and ultrasensitive protein microarray based detection in a remote laboratory, thus have the potential to facilitate future, unbiased, large scale serosurveys and serological diagnostic testing within remote, rural communities.

Keywords: Dried blood spots; Diagnosis; Cancer; Remote; Communities; Rural populations

INTRODUCTION

Geographic variations in cancer incidence, and consequentially survival, are partly due inequity and inequality in terms of global access to healthcare and the quality of registries in remote, rural populations [1,2]. Thus, reported disease incidence rates are often inversely correlated to rural population proportions in different regions (**Figure 1**), despite a worse health status in remote, rural areas than urban areas [3,4]. This implies dramatic under reporting in such areas due to limited access to screening and is most prominent in middle and low income countries (LMICs), most of which exist on the African and Asian continents.

Limited access to diagnostic tools, funding for diagnostic and laboratory services and specialists, combined with patient related barriers to follow through with diagnostic referrals, impede disease diagnosis in these communities [5]. To overcome diagnostic limitations, dried blood spots (DBS) have been used as an analytical matrix in the clinical setting for over half a century, primarily for disease screening of neonates [6-8]. They are

Received:	01-June-2022	**Manuscript No:**	IPJHCC-22-13720
Editor assigned:	03-June-2022	**PreQC No:**	IPJHCC-22-13720(PQ)
Reviewed:	17-June-2022	**QC No:**	IPJHCC-22-13720
Revised:	22-June-2022	**Manuscript No:**	IPJHCC-22-13720(R)
Published:	29-June-2022	**DOI:**	10.36846/IPJHCC-7.6.70026

Corresponding author Metoboroghene O Mowoe, Department of Integrative Biomedical Sciences, University of Cape Town, Cape Town, South Africa, E-mail: m_mowoe@yahoo.co.uk

Citation Mowoe MO, Rensburg T, Ali H, Gqada J, Kotze U, et al. (2022) Reaching the Remote: Dried Blood Spot Analysis for Disease Diagnosis on a Protein Microarray Plaform. J Healthc Commun. 7:70026

minimally invasive, require little to no training and storage infrastructure, and obviate the risk associated with needles and syringes used in venous blood collection. However, the minimal recovered sample volumes can create technical challenges for downstream protein biomarker measurements that lack the massive signal amplification of PCR methods.

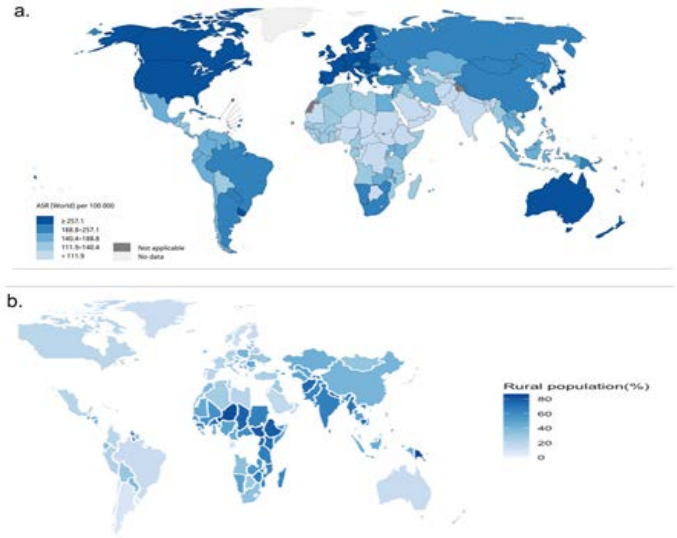


Figure 1: Maps of global cancer incidence and rural population percentage a.) Estimated age-standardized global incidence rates for all cancers, including both sexes and ages in 2020 (produced in http://gco.iarc.fr/today/home) [22], b.) Percentage of global rural population (data derived from https://data.worldbank.org/indicator/SP.RUR.TOTL.ZS?end=2019&start=1960&view=map) and plotted using ggplot [26] in R.

In cancers, autoantibodies (Aabs) have gained recent popularity as candidate biomarkers since they exhibit increased levels during the early stages of disease and can potentially predict disease progression and treatment outcomes [9]. Increases in Aab concentration may be detectable months to years before clinical symptom presentation [10-13] and other biomarkers are measurable, making them ideal diagnostic biomarker candidates. However, it is increasingly clear that multiplexed panels of Aab biomarkers are required to provide clinically useful early diagnostic performance [14], which in turn poses technological challenges in multiplexing classic ELISA measurements, both in terms of sample volumes required and assay costs.

Protein microarrays, in principle, can solve these problems by providing a miniaturised, highly multiplexed, ultra-sensitive ELISA-like assay format and have been used, successfully, to monitor disease activity [15] and discover novel autoantibody biomarkers [16] in serum. Moreover, previous studies have shown that therapeutic antibody titres measured in DBS are comparable to those measured in serum and plasma [17,18], suggesting that the combination of DBS based sample collection with protein microarray based autoantibody detection might enable screening of at risk remote populations for early cancer biomarkers. Here, we describe the development and validation of a robust protein microarray based method for the quantitation of autoantibody profiles in DBS from patients with chronic pancreatitis (CP) and pancreatic cancer (PC).

MATERIALS AND METHODS

Sample Collection and Dried Blood Spots

Inclusion criteria for method development and validation were blood samples from one CP patient prior to resective surgery and 11 randomly selected, late stage PC patients, respectively (**Table 1**). This study was approved by the University of CapeTown Human Research Ethics Committee (HREC 559-2018). Written informed consent was obtained from individuals for whom study samples were derived.

Table 1: Demographic and Clinical Characteristics of Patients from which Dried Blood Spots were extracted

Variable	Disease cohort	
	Chronic pancreatitis (n=1)	Pancreatic cancer (n=11)
Age (y)	49.0 ± 5.00	61.82 ± 11.37
	Sex (n)	
Male	1	4
Female	-	7
	Race (n)	
Black	1	4
Coloured	-	5
Other	-	2

In-house DBS cards: Here, 50 µL of whole blood from all 12 patients was pipetted unto Whatman™ filter paper 1 (150 mm; Cat#1001150) and allowed to dry for 1 h, at room temperature (23, RT). The filter paper was then placed in resealable plastic bags with a drierite desiccant (#737828-454G, Sigma-Aldrich) and stored in the dark, until ready for further use.

Commercial DBS cards: ArrayIT blood cards were used to compare and validate the results from our low cost, homemade cards. Following collection, 50 µL of whole blood from the CP patient was pipetted unto ArrayIT dried blood cards (#ABC, ArrayIT Corporation) and allowed to separate into red blood cell components and serum for 5 min. The blood cards were then left to dry at RT for 5 min, according to the manufacturers' instructions, placed in the anti-static shipping envelopes provided, and stored in the dark, until ready for further use.

Serum control: Following DBS collection, whole blood was centrifuged at 1300 × g for 13 mins, and serum was isolated and stored at -80℃ until ready for further use.

Sample Extraction

In house Whatman™ DBS cards: Based on previous DBS methods and the requirements for microarray assays, a method of serum extraction from DBS was developed. On days 1, 5, 10, and 15, a 5 mm disc was excised from blood spots on the Whatman™ filter paper using a disposable punch (#MT3336, Integra Miltex). The filter discs were handled with fine tipped forceps to prevent contamination. Each disc was soaked in 250 µL Phosphate Buffered Saline containing 0.1% Tween 20 (PBST) in a 24 well plate and incubated at RT on a shaker for 60 min × 100 rpm. Subsequently, the eluent was placed in 1.5 mL microfuge tubes and centrifuged at 14000 g × 10 min to sediment cell debris, after which each supernatant was transferred to a

clean tube.

Commercial ArrayIT DBS cards: To validate our in house Whatman™ filter paper method, we extracted serum from ArrayIT blood cards based on the manufacturers' instructions on days 1, 5, 10, and 15. A 5 mm disc was excised from the serum portion of the blood card, presumed to contain antibodies, antigens, and other serum proteins. The disc was wet with 10 µL of PBST, placed in 1.5 mL microfuge tubes, and allowed to rehydrate for 30 mins at RT. Subsequently, the disc was centrifuged at 14 000 g × 1 min to elute the serum into the collection tube. The disc was then rewet with 10 µL of PBST and re-centrifuged to elute any remaining bound proteins. The eluents were combined, bringing the final volume to 20 µL.

Microarray Analysis

To determine the optimal dilution factor of the DBS eluents from each card for the assays, a microarray assay including 5 control spots of known con

centrations was performed (**Figure 2**). We found that, similar to the serum control, a 1:800 dilution of ArrayIT eluent produced an optimal.

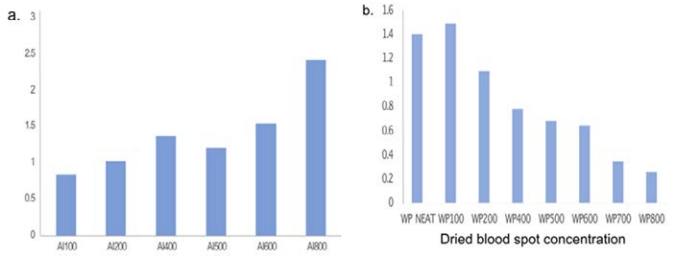


Figure 2: Protein expression to determine optimal dilution for a.) ArrayIT and b.) Whatmann dried blood spot eluents for subsequent microarray assays. *Ai(n) – ArrayIT dried blood card (dilution factor); W ()– Whatmann filter paper dried blood card (dilution factor).

Method development: For the microarray assays, Sengenics IMMUNOMETM arrays pre-printed with 1622 proteins on each array were used. Prior to the assays, the slides were removed from their storage solution and washed 3 × 5 min in PBST and 1 × 5 min in ddH2O. The serum and eluents from each method were diluted based on the dot blot assay and slides were incubated with sample on an orbital shaker at 100 rpm for 60 min in a light protected slide processing dish to prevent photobleaching. Subsequently, slides were washed 3 × 5 min and rinsed 1 × 5 min in PBST and ddH20, respectively. Slides were then incubated in 20 µg/ml of detection antibody (Alexa-fluor 647 coupled goat anti-human IgG (H+L); Cat#A21445, ThermoFischer Scientific) for 30 min on an orbital shaker at 100 rpm for 30 min. Again, slides were washed in PBST and ddH20, respectively, and then dried via centrifugation at 1300 RCF × 3 min at RT. Dried slides were scanned according to pre-set parameters and saved as TIFF files, which were used for data extraction downstream.

Validation: The assay process was replicated on 11 PC patients using a custom made chip including 3 proteins that had previously been identified (unpublished data) to be upregulated in PC patients (MAGEA5, MART.1, NY.CO.45) and a total anti-human IgG control.

Statistical Analysis

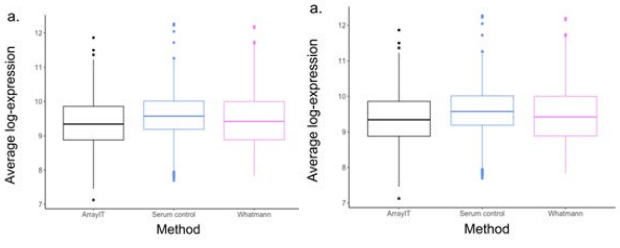
The microarray image data was extracted using Mapix (v 8.5.0) and the Sengenics IMMUNOMETM gal file, and median foreground and background intensities were read into RStudio (v.4.0) for pre-processing using the Pro-MAP single channel microarray analysis pipeline script (developed by MOM). Briefly, non-specific binding, array data that was not significantly different to surrounding background (defined as spot intensities <2SD of the median background) were filtered out. Data was then normexp background corrected and cyclic loess normalized prior to downstream analysis. A one way analysis of variance (ANOVA) was used to compare the average log expression intensity of proteins from the three sample collection methods (serum, ArrayIT, and Whatman™ DBS). Subsequently, a limma linear model using calculated array weights, was fit to the normalized microarray data to fully model the systematic part of the data and determine variability between the groups using the limma package in R [19]. To determine variability in the data based on the comparisons of interest, we extracted contrasts matrices. In this way, we were able to determine if there were differences between: 1. eluents from ArrayIT and Whatman™ DBS methods, and 2. eluents from the two DBS methods on Day 1, 5, 10, and 15. Subsequently, an empirical Bayes method was used to moderate the standard errors of the estimated log fold changes.

Finding no differences between methods or across days we then compared Aab profiles of our Whatman™ method over 15 d. To determine variability in Aab profiles of the 11 PC patients over 15 d for validation of our findings, a limma time course analysis was run. The probability level of $p<0.01$ was determined as differentially expressed. All statistical analyses were conducted using R (version 3.6.0) and all result images were created using the ggplot2 package in R [20]. Power analysis for an effect size=0.8, yielded a power=0.805.

RESULTS

Comparison of Serum control to dried blood card methods

Median log expression intensity of proteins from the CP serum control (9.67 ± 0.745) was slightly higher than that of the corresponding ArrayIT (9.60 ± 0.834) and Whatman™ (9.63 ± 0.675) eluents. However, there was no significant difference between the average log expression of the serum control versus ArrayIT ($t=-2.820$, and $p=0.013$) and Whatman™ eluents ($t=-1.421$, $p=0.330$) ($F=3.977$, $p>0.01$) (**Figure 3a**). Furthermore, the MA plot of the protein profiles from all three samples on day 1 showed normalized log fold change values (M) close to zero (**Figure 3b**). A density plot of the serum control and two DBS methods showed a shift of the peak density to lower average log-expression values for the two DBS methods (**Figure 3c**), implying that the DBS samples had lower non-specific background signals.



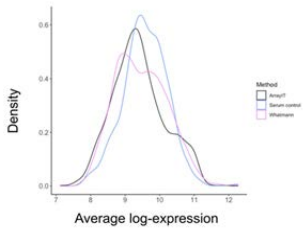


Figure 3: Comparison of average log protein expression from chronic pancreatitis serum control, ArrayIT and Whatman™ eluent samples assayed on the immunome array. a.) Boxplot comparison median average log intensities found in serum sample and ArrayIT and Whatman™ eluent samples b.) MA plot obtained comparing the three extraction methods. M represents the expression intensity of serum versus the average of the two other samples c.) Smoothed empirical densities for average protein log intensities in serum control and DBS arrays.

Comparison of Protein Expression of ArrayIT and Whatman™ Eluent Arrays and Over Time

There was no significant difference between immunome protein expression of eluents from the ArrayIT and Whatman™ arrays (adj. P>0.248) over all time points measured (**Figures 4a and 4b**).

Concurrently, MA plot showed M values close to zero, but slightly higher than the comparison of serum control to both DBS eluents (**Figure 4b**). Furthermore, we found that there was a slight but non-significant decrease in average log expression over time (adj. P>0.721) (**Figure 4c**). Furthermore, there was a slight decline in the abundance of protein expression over time (**Figure 4d**).

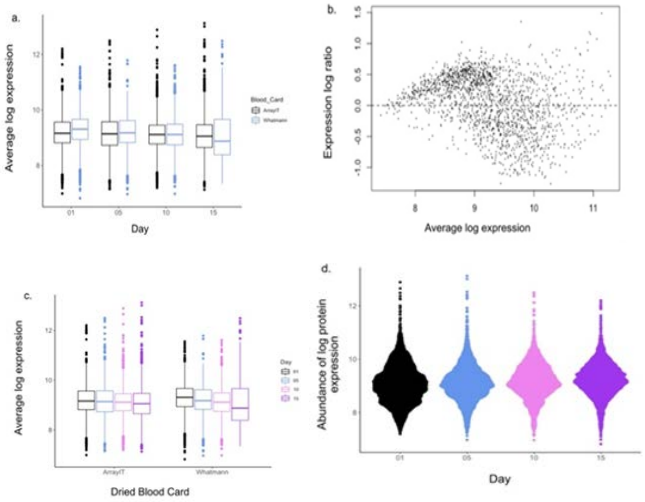


Figure 4: Comparison of average log protein expression of the two dried blood spot eluents from CP patient assayed on the immunome arrays, over time. a.) Boxplot comparing average log expression of ArrayIT and Whatman™ DBS protein arrays b.) MA plot obtained comparing the two DBS methods c.) Boxplot comparing protein expression over time in both DBS array types d.) Bee swarm plot visualizing distribution of relative maintained signal for the 1622 proteins analysed compared over 15 d.

Validation of Whatman™ DBS Method on Custom Microarray

Multiple linear regressions: We ran a multiple linear regression analysis to determine if any factors other than time had an effect on protein expression intensity in the 11 PC patients assayed on the custom DBS array. We found no effects of age, race, or gender on protein intensity (F=0.962, p=0.493).

Time course analysis: A limma time course analysis was run to compare protein expression intensities of the 3 proteins and IgG controls assayed over 15 d in 11 PC patients. We found no proteins differentially expressed in any of the time comparisons (**Figure 5a**). Furthermore, we found no significant difference in expression intensity of any of the proteins over 15 d (p<0.01) (**Figure 5b**). However, as previously observed we found a reduction, albeit non-significant, in protein abundance over time (**Figure 5c**).

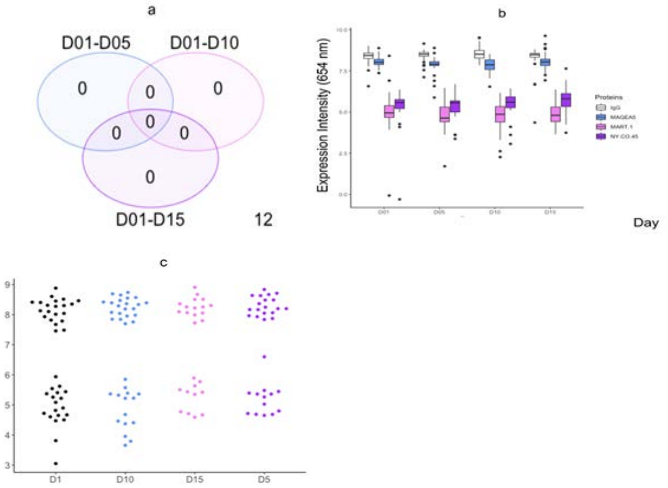


Figure 5: Comparison of protein intensity expression and abundance from day 0 – 15 in 11 pancreatic cancer patients assayed on the custom DBS array. a.) Venn diagram showing number of proteins significantly different in each of the comparisons of days eluted, b.) Boxplot comparing expression intensity of each of the 4 proteins over 15 d, c.) Bee swarm plot visualizing distribution of relative maintained signal for the 4 proteins analysed compared over 15 d.

DISCUSSION

Cancer is an enormous global burden that is predicted to increase with the growth and ageing of populations [21,22]. Unfortunately, economically disadvantaged countries face a disproportionally high burden of infection related cancers compared to their developed counterparts. Fortunately, the cancer burden can be largely mitigated by early disease detection [23]. Serological testing plays an important role in early screening and diagnosis in several disease areas and the identification of more effective biomarkers for early detection. Serum autoantibody profiles can indicate the presence of diseased cells months to years prior to symptom presentation [10-13]. This is especially useful in rare malignancies, such as PC, known to clinically present at an advanced stage in most patients. Large serosurveys may be key to discovering early detection tools, especially in populations with large rural, remote communities, where access to the associated technologies remains limiting.

This study was performed to quantitatively compare results derived from serum obtained from venous blood collection under routine conditions and blood dried on two different DBS cards, commercial (Array IT) and homemade (Whatman™ no 1). Several studies have shown a correlation with data from venous blood samples and blood dried on Whatman™ 903 substrates [24]. However, Whatman™ no 1 differs from 903 in that the latter is made specifically for protein retention, but the former is more easily accessible largely due to cost and usefulness for various other laboratory experiments. Our use of the latter was influenced by the need for a more cost effective method

of blood collection for primarily low income communities. We developed a method of extracting eluent sample from blood cards made using Whatman™ no 1, eliminating the need for phlebotomies and reducing the sample collection burden on stressed healthcare systems, especially in LMICs.

The results presented here show that eluent extracted from these blood cards up to 15 d following blood collection yielded results comparable to those from serum isolated from whole blood following venous blood collection. In addition, Whatman™ eluents yielded profiles comparable to those of Array IT blood cards, thus representing a low cost alternative to currently available commercial blood cards which typically cost ~USD10/sample. Notably, protein expression intensities from the serum control sample remained higher, albeit non-significantly, compared to DBS eluents, but simultaneously, the DBS samples appeared to present with a lower background, perhaps due to permanent absorption of larger macromolecules and complement factors on to the membranes.

The dried blood spot modality has the potential for wide scale screening of diseases such as cancers and autoimmune, infectious, and cardiovascular diseases where this is necessary to drastically reduce fatalities. The widespread use of DBS in the past has been impeded by small sample volumes and low target analyte concentrations requiring a sensitive and specific assay for detection and quantification. The use of protein microarray methods for DBS eluent testing has the unique advantage of requiring minimal volumes (1 µl) per assay, without compromising the ultra-sensitivity (pg/ml detection range) of the resultant assays [25]. Thus, DBS samples can be used for multiple testing or to support further analyses. Future studies will investigate the feasibility of a self-collection kit for at home finger prick DBS collection using the method developed here to elute and test samples that show comparable analytical performance to venepuncture derived blood samples [26].

CONCLUSION

Overall, this study could pave the way to large scale serosurveys in remote populations by utilizing simple, low cost DBS sample collection, combined with RT courier shipments to a centralized testing laboratory and miniaturized, protein microarray based quantitative, multiplexed biomarker detection, thereby increasing the effectiveness of screening campaigns and early diagnosis, and the accuracy of global cancer registries.

AUTHOR CONTRIBUTIONS

Conceptualization: JMB, MOM; Methodology, Validation, Formal analysis, and Investigation: MOM; Resources: MOM, JMB, EJ, UK; Data curation: MOM, TR, HA, JG, UK, MB, BA, EJ; Writings Original Draft: MOM; Writing Review and Editing: MOM, JMB, EJ; Visualization: MOM; Supervision: JMB, EJ; Project administration: MOM; Funding acquisition: JMB, EJ. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

REFERENCES

1. Valsecchi MG, Steliarova-Foucher E (2008) Cancer registration in developing countries: Luxury or necessity?. Lancet Oncol. 9(2):159-167.
2. Parkin DM, Bray F, Ferlay J, Pisani P (2005) Global cancer statistics, 2002. CA: A Cancer J Clinicians. 55(2):74-108.
3. Strasser R (2003) Rural health around the world: Challenges and solutions*. Fam Prac. 20(4):457-463.
4. Krümmel EM (2009) The circumpolar inuit health summit: A summary. Int J Circumpolar Health. 68(5):509-518.
5. Anticona Huaynate CF, Monica JPT, Malena C, Holger MM, Richard O, et al. (2015) Diagnostics barriers and innovations in rural areas: Insights from junior medical doctors on the frontlines of rural care in Peru. BMC Health Serv Res. 15(1):454.
6. Guthrie R, Susi A (1963) A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. Pediatr. 32(3):338.
7. Eyles DW, Ruth M, Cameron A, Pauline K, Thomas B, et al. (2010) The utility of neonatal dried blood spots for the assessment of neonatal vitamin D status. Pediatr Perinatal Epidemiol. 24(3):303-308.
8. Crossley JR, Smith PA, Edgar BW, Gluckman PD, Elliott RB (1981) Neonatal screening for cystic fibrosis, using immunoreactive trypsin assay in dried blood spots. Clinica Chimica Acta. 113(2):111-121.
9. Patel AJ, Tan TM, Richter AG, Naidu B, Blackburn JM, et al. (2022) A highly predictive autoantibody-based biomarker panel for prognosis in early-stage NSCLC with potential therapeutic implications. Br J Cancer. 126(2):238-246.
10. Anderson KS, LaBaer J (2005) The sentinel within: Exploiting the immune system for cancer biomarkers. J Proteome Res. 4(4):1123-1133.
11. Lernmark A (2001) Series Introduction: Autoimmune diseases: Are markers ready for prediction?. J Clin Invest. 108(8):1091-1096.
12. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Gregory JD, et al. (2003) Development of autoantibodies before the clinical onset of systemic lupus erythematosus. N Engl J Med. 349(16): 1526-1533.
13. Henriksson G (2014) Presymptomatic autoantibodies in Sjögren's syndrome: What significance do they hold for the clinic?. Exp Rev of Clin Immunol. 10(7):815-817.
14. Zaenker P, Ziman MR (2013) Serologic autoantibodies as diagnostic cancer biomarkers:A review. Cancer Epidemiol Biomarkers Prev. 22(12): 2161-81.
15. Robinson WH, Fontoura P, Lee BJ, Neuman de Vegvar HE, Tom J, et al. (2003) Protein microarrays guide tolerizing DNA vaccine treatment of autoimmune encephalomyelitis . Nat Biotechnol. 21(9):1033-1039.
16. Price JV et al. (2013) Protein microarray analysis reveals BAFF-binding autoantibodies in systemic lupus erythematosus. J Clin Invest. 123(12):5135-45.
17. Prince PJ, Matsuda KC, Retter M, Scott G (2010) Assessment of DBS technology for the detection of therapeutic antibodies. Bioanal. 2(8):1449-1460.
18. Kaendler K, Warren A, Lloyd P, Sims J, Sickert D (2013) Evaluation of dried blood spots for the quantification of therapeutic monoclonal antibodies and detection of anti-drug antibodies. Bioanal. 5(5):613-622.
19. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, et al. (2015)

Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 43(7):e47.

20. Wickham H (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.
21. Omran AR (2005) The epidemiologic transition: A theory of the epidemiology of population change.1971. Milbank Q. 83(4):731-757.
22. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, et al. (2021) Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians.
23. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, et al. (2015) Global cancer statistics, 2012. CA: A Cancer Journal for Clinicians. 65(2):87-108.
24. Moretti M, Freni F, Tomaciello I, Vignali C, Groppi A, et al. (2019) Determination of benzodiazepines in blood and in dried blood spots collected from post-mortem samples and evaluation of the stability over a three-month period. Drug Test Anal. 11(9):1403-1411.
25. Beeton-Kempen N, Duarte J, Shoko A, Serufuri JM, John T, et al. (2014) Development of a novel, quantitative protein microarray platform for the multiplexed serological analysis of autoantibodies to cancer-testis antigens. Int J Cancer. 135(8):1842-1851.
26. Hadley W (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.