Indian Journal of Experimental Biology
Vol. 61, January 2023, pp. 7-13
DOI: 10.56042/ijeb.v61i01.53135

Simultaneous measurement of HbA1C and Hemoglobin by Turbidimetric inhibition immunoassay from a single punch of dried blood spot samples

Rohini Bingewar, Rajashri Wagholikar, Varsha Kale & Ashwini Shete*
ICMR-National AIDS Research Institute, Bhosari, Pune, Maharashtra, India

Received 02 August 2021; *revised 29 September 2022*

Hemoglobin and glycosylated hemoglobin (HbA1C) are frequently monitored health indicators in population based studies for information about the status of nutrition and diabetes control. We present here possibly for the first time the findings of simultaneous estimation of Hemoglobin and HbA1C on Dried blood spot (DBS) samples by a single test. Validation was done by turbidimetric inhibition immunoassay (TINIA) using Roche Integra 400 plus instrument. Paired whole blood and DBS samples were tested for HbA1C estimation by Integra 400 plus. Total hemoglobin values obtained during HbA1C estimation were compared with hemoglobin values estimated by Coulter AcT 5 Diff CP Hematology counter. Agreement in HbA1C and hemoglobin values between paired whole blood and DBS samples was found to be high with R^2 values of 0.994 and 0.9349, respectively. Intra- and inter- assay precision was found to be within 10% for both parameters. Values obtained after assaying DBS samples prepared by spotting proficiency samples on Whatman 903 protein saver cards demonstrated acceptable standard deviation indices resulting in successful participation in EQAS programs for both these parameters. The results reveal the potential of TINIA for simultaneous estimation of hemoglobin and HbA1C from a single punch of the DBS samples.

Keywords: Anemia, Blood sugar, Diabetes, TINIA (Turbidimetric inhibition immunoassay)

Dried blood spot (DBS) samples are widely accepted as an alternative to conventional blood collection techniques and have been validated for several biomarkers[1,2]. As they require a small volume of blood, they can be prepared from finger pricks greatly reducing efforts and expenses spent at the time of collection. The low volume also reduces risk of transmission of blood borne pathogens through these samples significantly[3]. Since they require minimum space, a large number of samples can be stored in a compact space easily. Due to these advantages, they are often the preferred samples in population-based research[2]. Large-scale community based studies are required to be conducted for estimating incidences or prevalence of the conditions of global public health importance as well as for identifying different risk factors associated with them for developing various intervention strategies. It is also necessary to conduct such studies for assessing effectiveness of intervention strategies implemented for their control. Anemia and diabetes mellitus are among the most important global health problems as they affect most of the countries worldwide.

*Correspondence:
E-Mail: ashete@nariindia.org

Anemia is diagnosed and its severity is assessed by measuring hemoglobin concentrations[4].Hemoglobin is a commonly assessed biomarker in many community based studies being an indicator of nutrition and health of a population[5]. Diagnosis of diabetes can be done using glycosylated hemoglobin (HbA1C) estimation as per the recommendations of WHO expert consultation. It indicates glycemic control over the previous 3-4 months rather than informing about the short-term control[6,7]. An HbA1c of 6.5% is recommended as the cut point for diagnosing diabetes by WHO[8]. Further, HbA1C can be used to identify pre-diabetic condition helping to introduce intervention strategies even before the actual disease is developed[9]. It is also used for monitoring glycemic control in patients on antidiabetic medications[10]. HbA1c represents the fraction of hemoglobin which non-enzymatically binds to glucose in the bloodstream specifically at the N-terminal valine of its β chain[11]. Estimation of HbA1C involves estimation of total hemoglobin as well as glycosylated hemoglobin to calculate its percentage. Some of the platforms used for its estimation require a separate system to estimate total hemoglobin levels based on which HbA1C percentages are calculated[12]. Many of

the tests routinely done nowadays for HbA1C do not require separate hemoglobin estimation.

Considering importance of estimation of these markers at population level, here, we considered validating these markers on DBS which are the preferred samples for population based studies. Reports on validation of HbA1C estimation using DBS samples by Tina-quant are available. Additionally, we conducted a study to determine if the total hemoglobin values assessed during the estimation of HbA1C levels by Tina-quant Turbidimetric inhibition immunoassay (TINIA) in DBS samples correlate with the hemoglobin estimated by the gold standard routine diagnostic method used in our laboratory.

Methodology

Equipment

Cobas Integra 400 plus Biochemistry analyzer (Roche Diagnostics, Switzerland) and Coulter Act 5 diff analyzer (Beckman Coulter, USA) was used for HbA1Cand hemoglobin estimation. A Shaker incubator (Heidolph Instruments, Germany) was used for sample preparation.

Reagents and kits

Tina-quant Hemoglobin A1c Gen.3 Cassettes (Roche Diagnostics, Switzerland) were used for HbA1C estimation in whole blood and dried blood spots samples. COBAS INTEGRA Hemolyzing Reagent Gen.2 and Hemolyzing Reagent for Tina-quant HbA1c (Roche Diagnostics, Switzerland) were used for hemolyzing whole blood and DBS samples, respectively. Internal quality controls and calibrators manufactured by Roche Diagnostics were used for quality control of HbA1C estimation. Hemoglobin was estimated in the whole blood samples by Coulter AcT 5 Diff CP Hematology counter using calibrators and reagents supplied by the same manufacturer while internal quality control materials used were manufactured by Biorad, USA. The laboratory also participated in an External quality assurance program run by Christian Medical College (CMC), Vellore, India for HbA1C estimation and that by All India Institute of Medical Sciences (AIIMS), New Delhi, India for hemoglobin estimation.

Preparation of controls for HbA1c and haemoglobin estimations

Around 50 µL Liquid internal and external quality controls were spotted on predefined circles of the Whatman 903 protein saver card to prepare DBS controls. Two level Control Norm and Control Path manufactured by Roche Diagnostics were used for preparing DBS controls for HbA1c testing. Three level of Low, Normal and High controls manufactured by Biorad, USA were used for preparing Control DBS cards for hemoglobin estimation. The Spots were allowed to dry overnight and stored at –20°C after complete drying into a zip lock bag along with desiccants and humidity indicator card. Liquid external quality control samples were treated similarly to prepare DBS controls. The controls were processed in the same manner as that of the DBS samples to estimate hemoglobin and HbA1C by Tina-quant method.

Sampling

Whole blood samples were collected by venipuncture in EDTA vacutainer tubes from the study participants after obtaining informed consent from them. The study protocol was approved by ICMR-NARI Ethics Committee (Protocol No.: NARI-EC/2017-12). Hemoglobin was assessed from fresh whole blood using a hematology analyzer and HbA1C was assessed by Cobas Integra 400 plus using the manufacturer's instructions. Dried blood spot (DBS) samples were prepared by placing 50 µL blood samples on predefined circles of the Whatman 903 protein saver card. The cards were allowed to dry overnight at room temperature (20-25°C). The individual dried blood spot card was placed into a zip lock bag along with desiccants and humidity indicator card and stored at –20°C.

Processing of DBS samples for HbA1C estimation by Cobas Integra 400 plus

Single 3.2 mm DBS punch was eluted in 250 µL of hemolyzing reagent followed by shaking at 1250 rpm for 2 h at room temperature. The samples were properly mixed by pipetting several times till the DBS discs became completely white and the eluted sample was transferred to a sample cup for HbA1c estimation.

Stability study

The stability of HbA1C and Hemoglobin estimations in DBS samples was assessed by comparing the results of measurements on DBS samples stored at –20°C with those stored at ambient temperature (20-30°C) for 7 and 15 days.

Statistical analysis

Simple linear regression and Bland Altman analysis were done using GraphPad prism to assess

the agreement of HbA1C and hemoglobin measurements between the two types of samples. Internal DBS controls prepared for hemoglobin and HbA1C estimations were tested in replicates on a single and multiple days to assess intra and inter-assay precision. Co-efficient of variation (CV) was calculated by dividing standard deviation of control values by their mean after testing them in replicates. Data are presented as mean (±SD), and the accepted level of significance was P <0.05.The standard deviation Index (SDI) for proficiency samples was reported by EQAS providers.

Results

Comparison of HbA1c values in whole blood and DBS samples by Cobas Integra 400 plus

HbA1c values were compared in a total of 37 paired whole blood and DBS samples. HbA1c values ranged from 4.41-15.16% (Median: 6.06%) in venous blood as measured by Cobas Integra 400 plus, which was used as the gold standard. HbA1c values on DBS ranged from 5.06 to 15.16% (Median: 6.2%) when measured by Cobas Integra 400 plus. The values correlated with each other with R^2 of 0.994 (P <0.0001) when assessed by simple linear regression analysis (Fig. 1A). Bland Altman's analysis showed a bias of 0.126% with 95% CI of –0.29 to 0.54% (Fig. 1B).

Comparison of Hemoglobin concentrations obtained in DBS samples by Cobas Integra 400 plus with Coulter readings

Hemoglobin concentrations were compared in a total of 37 paired whole blood and DBS samples. Hemoglobin concentrations ranged from 6.2-20.2 g/dL (Median: 12.8 g/dL) as measured by Coulter analyzer, which was used as the gold standard. Hemoglobin concentrations in DBS ranged from 4.5 to 20.4 g/dL (Median: 12.4 g/dL) when measured by Cobas Integra 400 plus. The values correlated with each other with R^2 of 0.934 (P <0.0001) when assessed by simple linear regression analysis (Fig. 1C). Bland Altman's analysis showed a bias of –0.356 g/dL with a 95% CI of -2.33 to 1.62 g/dL (Fig. 1D).

Internal and external quality control

Internal quality control was assessed by calculating intra and inter-assay precision. Intra-assay precision was calculated by running two levels of DBS controls 5 times in a single run. The coefficient of variation was found to be 1.6 and 0.72% for normal and pathological HbA1C controls, respectively. % CV for hemoglobin was found to be 1.4 and 6.04 for normal and low controls. Inter-assay precision was estimated by running the controls over 20 runs. Inter-assay precision was 2.92 and 2.13 for normal and pathological HbA1C controls and 7.20 and 4.91 for normal and low hemoglobin controls, respectively. The laboratory passed successfully in all round of

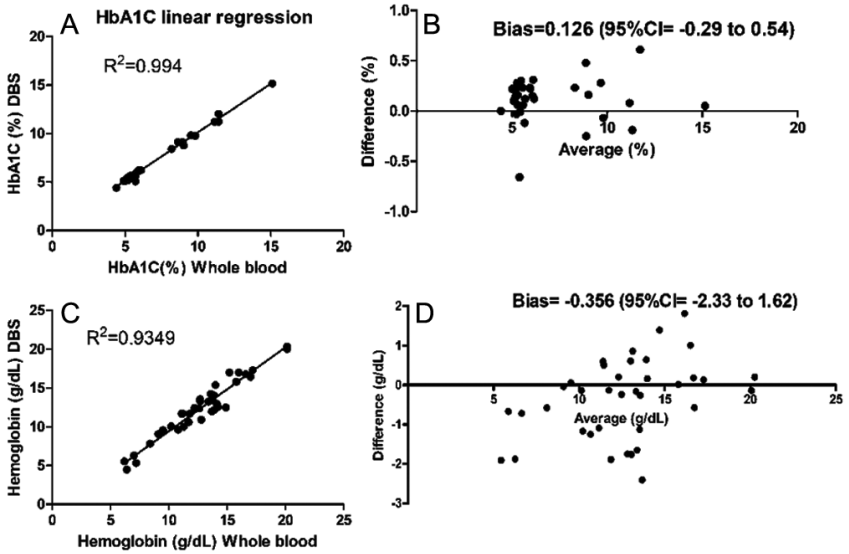

Fig. 1 — Agreement of HbA1C and hemoglobin values estimated using whole blood and DBS samples. (A) Linear regression of whole blood (X-axis) and DBS (Y-axis) HbA1C values estimated by Integra 400 plus analyzer showing R^2 value; (B) Bland Altman plot of the regression analysis showing bias between whole blood and DBS HbA1C values. The graph is plotted with average of the two values on X-axis and difference between them on Y-axis; (C) Linear regression of whole blood (X-axis) hemoglobin values assessed by hematology analyzer and DBS (Y-axis) hemoglobin values estimated by Integra 400 plus analyzer showing R^2 value; and (D) Bland Altman plot of the regression analysis showing bias between whole blood and DBS hemoglobin values. The graph is plotted with average of the two values on X-axis and difference between them on Y-axis.

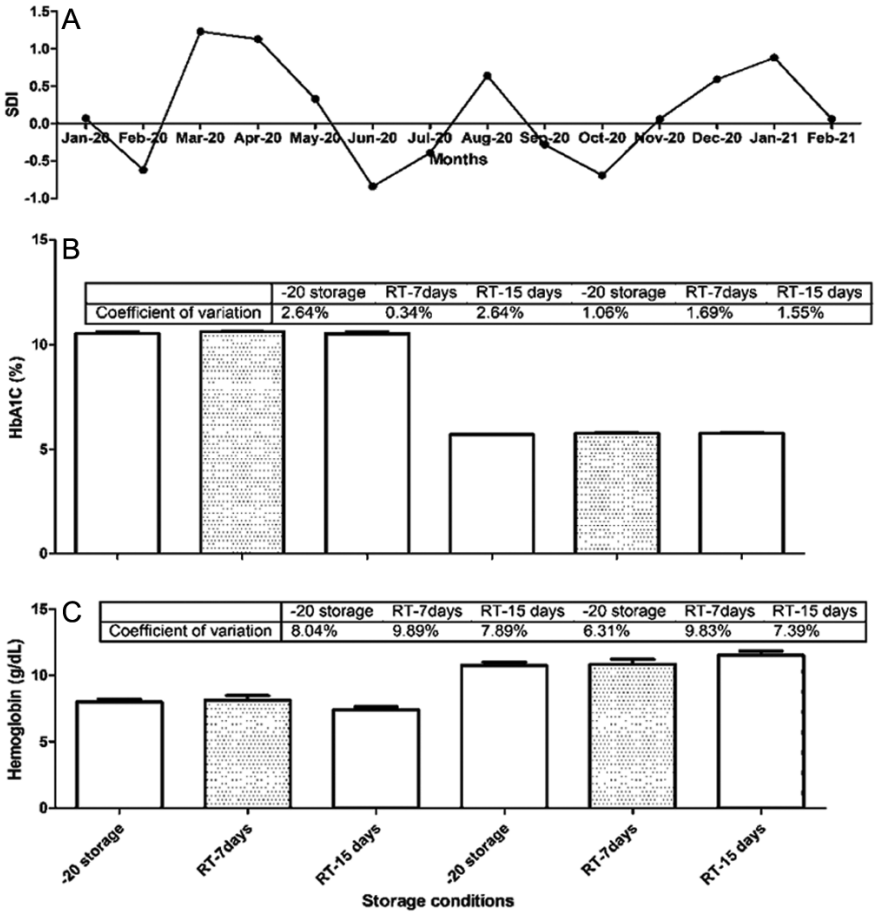

Fig. 2 — EQAS results and stability studies. (A) The graph shows standard deviation indices (SDI) plotted on Y-axis for proficiency samples after preparing DBS cards and processed during January 2020 to February 2021 as shown on X-axis; (B & C) The bar graphs show results of the stability study performed on two samples stored at –20°C, room temperature for 7 and 15 days after overnight drying. Storage conditions are plotted on X-axis. HbA1C (B) and hemoglobin (C) values are plotted on Y-axis. The figure also shows coefficient of variation for HbA1C and hemoglobin values of these samples.

EQAS conducted for HbA1C over a period of one year with SDIs ranging from –0.84 to 1.23 as shown in Fig. 2A. EQAS panel for hemoglobin estimation was processed twice. The laboratory passed successfully with SDI of –0.66 and 1.48 in these two rounds.

Stability study

The stability of hemoglobin and HbA1C was determined in DBS samples by storing the samples at ambient temperature (20-30°C) for 7 and 15 days and comparing the values with those stored at –20°C after overnight drying at the ambient temperature. There was no significant difference in both HbA1C and Hemoglobin values in DBS samples stored at the ambient temperature up to 15 days as compared to those stored at –20°C. The coefficient of variation was below 10% for all the samples as shown in Fig. 2 B & C.

Table 1 — Linearity results for HbA1C and hemoglobin (Hb)

Ref. val. (%)		Mean		% Accuracy		% CV	
HbA1C	Hb	HbA1C	Hb	HbA1C	Hb	HbA1C	Hb
15.11	20.5	14.5325	23.362	3.821972	–12.2507	0.812974	3.331256
11.42	17.3	11.944	19.398	–4.58844	–10.8155	0.660837	6.550557
9.83	14.5	10.362	15.276	–5.412	–5.07986	0.464837	8.308358
8.19	11.8	8.376	12.74	–2.27106	–7.37834	0.705472	5.410606
5.26	9.2	5.676	9.22	–7.90875	–0.21692	0.617889	4.801111
4.46	4.9	4.724	5.046	–5.91928	–2.89338	1.455864	4.651036

Linearity

Good linearity was observed over the range of 4.46 to 15.11%for HbA1C and4.9 to 20.5 g/dL for hemoglobin. R^2 values were 0.99and 0.98 for HbA1C and hemoglobin, respectively. Accuracy, as calculated by percent variation from the reference value, was within 15%, and %CV indicating precision was within 10% for both the parameters (Table 1). R^2 values observed in the linearity experiments using 'in house' controls were more robust than those observed

for actual samples as reported in the previous sections indicating influence of certain factors while collection and transportation of samples on accuracy of the results.

Discussion

Hemoglobin as well as HbA1C estimations are important for diagnosing two important global public health problems namely anemia and diabetes mellitus, respectively. Low to middle income countries are more severely affected by anemia due to higher prevalence of nutritional deficiencies as well as different infections and infestations[13]. It is responsible for increased morbidity and mortality associated with its severity[14-17] in different groups of populations. WHO has defined cut off values based on prevalence of anemia for classifying its public health significance recognizing its public health importance[18,19]. Thus, estimation of hemoglobin levels for determining prevalence and severity of anemia at population levels is important. Similarly, global health significance of diabetes mellitus cannot be under rated as it is responsible for one of the top 10 causes of death in adults[20] with its ever increasing incidence globally[21].

HbA1C estimation is an attractive alternative approach for diagnosing diabetes especially in population based studies as it doesn't require fasting or postprandial conditions for sample collection and has higher reproducibility and convenience over estimation of blood glucose levels[22-24]. HbA1C estimation involves measurement of percentage of total hemoglobin which is glycosylated using different platforms such as immunoassay, ion-exchange or boronate affinity high-performance liquid chromatography (HPLC), and enzymatic assays[25]. There have been several studies on the validation of HbA1C estimation on DBS samples using some of these platforms[26-29]. Similarly, successful validation of hemoglobin estimation in DBS samples by different colorimetric methods using Drabkin's or Sulfolyser reagents[30,31] has been reported. However, validation reports on simultaneous hemoglobin and HbA1C measurements in a single test are lacking. We used Tina-quant Hemoglobin A1c Gen.3 reagent with Roche Integra 400 plus system for performing HbA1C estimation. It detects hemoglobin and HbA1c simultaneously without requiring any additional reagents. It measures liberated hemoglobin after hemolysis of the blood samples bichromatically during the preincubation phase of the HbA1C after converting it into a derivative having a characteristic absorption spectrum[32].

We observed a high level of agreement between whole blood and DBS HbA1C values by Tina-quant(R) II Turbidimetric inhibition immunoassay as was also reported previously for this method[33]. We also evaluated whether the total hemoglobin values estimated by Tina-quant(R) II Turbidimetric inhibition immunoassay as a part of HbA1C estimation were in agreement with the values obtained through Coulter hematology analyzer which was used for routine hematology investigations in the laboratory. The hematological investigations were NABL accredited and passed successfully in the Biorad EQA program. Total hemoglobin values obtained through Integra 400 plus analyzer on DBS samples correlated highly with those estimated by the Coulter counter using whole blood samples. Since the lower limit of detection of hemoglobin by Integra 400 plus is 3.99 g/dL, it did not report the values which were below the detection limit. Inter and intra-assay coefficient of variation for HbA1C and hemoglobin estimations were found to be within 10% indicating good precision of the assay.

Sample transportation may be a problem in some of the hard to reach areas causing delays in timely storage and analysis of samples in community-based studies. Hence it is important to assess stability of the analytes of interest at room temperature. DBS samples have been shown to have a robust stability as number of analytes have been shown to be stable up to days to even years at room temperature[34-36]. Hence, they can be transported at room temperature in light weight packages without cold chain maintenance requirements. Analytes in DBS samples stored at low temperatures were also shown to be more stable than their respective plasma samples[37] indicating their utility in long term storage. HbA1C estimation on DBS samples was shown to be relatively insensitive to shipping and weather conditions[38]. We also found that along with HbA1C values, hemoglobin values were stable even after storing the samples at room temperature for 15 days. We assessed the stability in just two samples having two different levels of the analytes as a few other publications had also reported the stability in limited number samples with different ranges of the analytes[31,33]. However, further studies are required on more number of samples to prove stability of both these analytes.

HbA1C estimation is likely to be affected by hemoglobin levels as it represents the ratio of

glycosylated Hemoglobin to total Hemoglobin[39]. Presence of anemia has been shown to influence HbA1c levels in many studies suggesting need for correcting anemia before the levels are used for diagnosing diabetes and also for monitoring glycemic control in known diabetic patients[40,41]. Hence, it is important to interpret raised HbA1C levels in the presence of anemia even in population based studies. Hence, simultaneous measurement of HbA1C and hemoglobin is important in order to avoid erroneous interpretations.

Conclusion

We report here, possibly for the first time, validation results of hemoglobin estimation in dried blood spot (DBS) samples by the TINA-quant HbA1c method using Integra 400 plus analyzer. The methodology used for estimation showed good agreement of DBS values with their paired whole blood values for hemoglobin as well as HbA1C. Results of precision, stability and linearity studies also stressed utility of this method for measurement of these markers in DBS samples. Simultaneous measurement of the two important markers viz. HbA1C and hemoglobin by single test gains importance as it saves time, labour, cost as well as the precious samples collected in large-scale community based studies.

Ethical approval and consent to participate

The study was approved by Ethics committees of ICMR-National AIDS Research Institute (Protocol number: NARI-EC/2017-12). Written informed consent was obtained from the participants before enrolling them in the study.

Conflict of interest

Authors declare no competing interests.

References

1 McDade TW, Development and validation of assay protocols for use with dried blood spot samples. *Am J Hum Biol*, 26 (2014) 1.

2 McDade TW, Williams S & Snodgrass JJ, What a drop can do: dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. *Demography*, 44 (2007) 899.

3 Parker SP & Cubitt WD, The use of the dried blood spot sample in epidemiological studies. *J Clin Pathol*, 52 (1999), 633.

4 WHO Report *Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity*. (World Health Organization, Geneva), 2011.

5 Silva CL, Lima-Costa MF, Firmo JO & Peixoto SV, Hemoglobin level in older adults and the association with nutritional status and use of health services: the Bambui Project. *Cad Saude Publica*, 28 (2012) 2085.

6 Mallya HN VB, Shenoy RP & Bhatkalkar SN, Correlation of serum fructosamine, erythrocyte Na+-K+ ATPase and glutathione peroxidase with HbA1c levels. *Indian J Biochem Biophys*, 57 (2020) 451.

7 Chehregosha H, Khamseh ME, Malek M, Hosseinpanah F & Ismail-Beigi F, A View Beyond HbA1c: Role of Continuous Glucose Monitoring. *Diabetes Ther*, 10 (2019) 853.

8 WHO Report, *Use of Glycated Haemoglobin* (*HbA1c*) *in the Diagnosis of Diabetes Mellitus.* (World Health Organization, Geneva), WHO/NMH/ CHP/CPM/11.1 2011.

9 Tuso P, Prediabetes and lifestyle modification: time to prevent a preventable disease. *Perm J*, 18 (2014) 88.

10 Qaseem A, Wilt TJ, Kansagara D, Horwitch C, Barry MJ, Forciea MA, Clinical Guidelines Committee of the American College of Physicians, Fitterman N, Balzer K, Boyd C, Humphrey LL, Iorio A, Lin J, Maroto M, McLean R, Mustafa R & Tufte J, Hemoglobin A1c Targets for Glycemic Control With Pharmacologic Therapy for Nonpregnant Adults With Type 2 Diabetes Mellitus: A Guidance Statement Update From the American College of Physicians. *Ann Intern Med*, 168 (2018) 569.

11 Sherwani SI, Khan HA, Ekhzaimy A, Masood A & Sakharkar MK, Significance of HbA1c Test in Diagnosis and Prognosis of Diabetic Patients. *Biomark Insights*, 11 (2016) 95.

12 Weykamp C, HbA1c: A Review of Analytical and Clinical Aspects. *Ann Lab Med.,* 33 (2013) 393.

13 Safiri S, Kolahi AA, Noori M, Nejadghaderi SA, Karamzad N, Bragazzi NL, Sullman MJM, Abdollahi M, Collins GS, Kaufman JS & Grieger JA, Burden of anemia and its underlying causes in 204 countries and territories, 1990-2019: results from the Global Burden of Disease Study 2019. *J Hematol Oncol*, 14 (2021) 185.

14 Karoopongse E, Srinonprasert V, Chalermsri C & Aekplakorn W, Prevalence of anemia and association with mortality in community-dwelling elderly in Thailand. *Sci Rep*, 12 (2022) 7084.

15 Guinn NR, Cooter ML, Villalpando C & Weiskopf RB, Severe anemia associated with increased risk of death and myocardial ischemia in patients declining blood transfusion. *Transfusion*, 58 (2018) 2290.

16 Chami N, Hau DK, Masoza TS, Smart LR, Kayange NM, Hokororo A, Ambrose EE, Moschovis PP, Wiens MO & Peck RN, Very severe anemia and one year mortality outcome after hospitalization in Tanzanian children: A prospective cohort study. *PLoS One*, 14 (2019) e0214563.

17 Daru J, Zamora J, Fernandez-Felix BM, Vogel J, Oladapo OT, Morisaki N, Tuncalp O, Torloni MR, Mittal S, Jayaratne K, Lumbiganon P, Togoobaatar G, Thangaratinam S & Khan KS, Risk of maternal mortality in women with severe anaemia during pregnancy and post partum: a multilevel analysis. *Lancet Glob Health*, 6 (2018) e548.

18 WHO *Nutritional Anaemias: Tools for Effective Prevention and Control* (World Health Organization, Geneva), 2017.

19 Williams AM, Addo OY, Grosse SD, Kassebaum NJ, Rankin Z, Ballesteros KE, Olsen HE, Sharma AJ, Jefferds ME & Mei Z, Data needed to respond appropriately to anemia when it is a public health problem. *Ann N Y Acad Sci*, 1450 (2019) 268.

20 WHO, Diabetes: Key facts. *https://www.who.int/news-room/fact-sheets/detail/diabetes*, (World Health Organization, Geneva), 2020.

21 Liu J, Ren ZH, Qiang H, Wu J, Shen M, Zhang L & Lyu J, Trends in the incidence of diabetes mellitus: results from the Global Burden of Disease Study 2017 and implications for diabetes mellitus prevention. *BMC Public Health*, 20 (2020) 1415.

22 Florkowski C, HbA1c as a Diagnostic Test for Diabetes Mellitus - Reviewing the Evidence. *Clin Biochem Rev*, 34 (2013) 75.

23 Selvin E, Crainiceanu CM, Brancati FL & Coresh J, Short-term variability in measures of glycemia and implications for the classification of diabetes. *Arch Intern Med*, 167 (2007) 1545.

24 International Expert C, International Expert Committee report on the role of the A1C assay in the diagnosis of diabetes. *Diabetes Care*, 32 (2009) 1327.

25 Karami A & Baradaran A, Comparative evaluation of three different methods for HbA1c measurement with High-performance liquid chromatography in diabetic patients. *Adv Biomed Res*., 3 (2014) 94.

26 Maleska A, Hirtz C, Casteleyn E, Villard O, Ducos J, Avignon A, Sultan A & Lehmann S, Comparison of HbA1c detection in whole blood and dried blood spots using an automated ion-exchange HPLC system. *Bioanalysis*, 9 (2017) 427.

27 Mastronardi CA, Whittle B, Tunningley R, Neeman T & Paz-Filho G, The use of dried blood spot sampling for the measurement of HbA1c: a cross-sectional study. *BMC Clin Pathol*, 15 (2015) 13.

28 Waal Ed, Driver K & Warner J, Measurement of HbA1c from dried blood spot samples with the Abbott architect enzymatic HbA1c method. *Pathology*, 51 (2019) S109.

29 Thomas D, Seeman T, Potter A, Hu P, Crimmins E, Herningtyas EH, Sumantri C & Frankenberg E, HPLC-based Measurement of Glycated Hemoglobin using Dried Blood Spots Collected under Adverse Field Conditions. *Biodemography Soc Biol*, 64 (2018) 43.

30 Gupta A, Kapil U, Ramakrishnan L, Khenduja P, Yadav CP, Sofi NY & Khandelwal R, Validity of Estimation of Haemoglobin Content in Dried Blood Spot Samples. *Indian J Hematol Blood Transfus*, 33 (2017) 565.

31 Richardson G, Marshall D & Keevil BG, Prediction of haematocrit in dried blood spots from the measurement of haemoglobin using commercially available sodium lauryl sulphate. *Ann Clin Biochem*, 55 (2018) 363.

32 Cobas, A1C-3 kit insert: Tina-quant Hemoglobin A1c Gen.3 -Whole blood and Hemolysate Application. *file:///C:/Users/NARI%20DBS/Downloads/5336180190%20%20HBA1C%20TQ%20Gen.3,%20cobas%20c%20111%20(1).pdf* (Roche Diagnostics International AG, Switzerland) 2020.

33 Affan ET, Devarsetty P, Chow CK & Neal BC, Comparability of HbA1c and lipids measured with dried blood spot versus venous samples: a systematic review and meta-analysis. *BMC Clinical Pathology,* 14 (2014) 21.

34 Lehmann S, Delaby C, Vialaret J, Ducos J & Hirtz C, Current and future use of "dried blood spot" analyses in clinical chemistry. *Clin Chem Lab Med*, 51 (2013) 1897.

35 Heath AK, Williamson EJ, Ebeling PR, Kvaskoff D, Eyles DW & English DR, Measurements of 25-hydroxyvitamin D concentrations in archived dried blood spots are reliable and accurately reflect those in plasma. *J Clin Endocrinol Metab*, 99 (2014) 3319.

36 Wong P, Pham R, Whitely C, Soto M, Salyers K, James C & Bruenner BA, Application of automated serial blood sampling and dried blood spot technique with liquid chromatography-tandem mass spectrometry for pharmacokinetic studies in mice. *J Pharm Biomed Anal*, 56 (2011) 604.

37 D'Arienzo CJ, Ji QC, Discenza L, Cornelius G, Hynes J, Cornelius L, Santella JB & Olah T, DBS sampling can be used to stabilize prodrugs in drug discovery rodent studies without the addition of esterase inhibitors. *Bioanalysis*, 2 (2010) 1415.

38 Crimmins EM, Zhang YS, Kim JK, Frochen S, Kang H, Shim H, Ailshire J, Potter A, Cofferen J & Faul J, Dried blood spots: Effects of less than optimal collection, shipping time, heat, and humidity. *Am J Hum Biol*, 32 (2020) e23390.

39 Sakamoto N, Hu H, Nanri A, Mizoue T, Eguchi M, Kochi T, Nakagawa T, Honda T, Yamamoto S, Ogasawara T, Sasaki N, Nishihara A, Imai T, Miyamoto T, Yamamoto M, Okazaki H, Tomita K, Uehara A, Hori A, Shimizu M, Murakami T, Kuwahara K, Fukunaga A, Kabe I, Sone T & Dohi S, Associations of anemia and hemoglobin with hemoglobin A1c among non-diabetic workers in Japan. *J Diabetes Investig*, 11 (2020) 719.

40 Katwal PC, Jirjees S, Htun ZM, Aldawudi I & Khan S, The Effect of Anemia and the Goal of Optimal HbA1c Control in Diabetes and Non-Diabetes. *Cureus*, 12 (2020) e8431.

41 Segun Adeoye SA, Irina Erlikh, Sylvester Sarfraz, Tomas Borda & Lap Yeung, Anemia and Hemoglobin A1c level: Is there a case for redefining reference ranges and therapeutic goals? *British Journal of Medical Practitioners*, 7 (2014) a706.