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Effects of spot size on biomarker levels of field-collected dried blood spots: A new algorithm for exact dried blood spot size measurement

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Abstract

Objectives: The quality of blood values analyzed from survey-collected dried blood spot (DBS) samples is affected by fieldwork conditions, particularly spot size. We offer an image-based algorithm that accurately measures the area of field-collected DBS and we investigate the impact of spot size on the analyzed blood marker values.

Methods: SHARE, a pan-European study, collected 24 000 DBS samples in 12 countries in its sixth wave. Our new algorithm uses photographs of the DBS samples to calculate the number of pixels of the blood-covered area to measure the spot sizes accurately. We ran regression models to examine the association of spot size and seven DBS analytes. We then compared the application of our new spot-size measures to common spot-size estimation.

Results: Using automated spot-size measurement, we found that spot size has a significant effect on all markers. Smaller spots are associated with lower measured levels, except for HbA1c, for which we observe a negative effect. Our precisely measured spot sizes explain substantially more variance of DBS analytes compared to commonly used spot-size estimation.

Conclusion: The new algorithm accurately measures the size of field-collected DBS in an automated way. This methodology can be applied to surveys even with very large numbers of observations. The measured spot sizes improve the accuracy of conversion formulae that translate blood marker values derived from DBS into venous blood values. The significance of the spot-size effects on biomarkers in DBS should also incentivize the improvement of fieldwork training and monitoring.

1 | INTRODUCTION

Internationally harmonized social surveys with a broad set of social, economic, psychological, and health characteristics of respondents have an important place in health research since they put health outcomes in the context of country-specific cultural and institutional environments, among them the coverage and efficacy of health care systems. One example is the Survey of Health, Ageing and Retirement in Europe (SHARE), a research infrastructure for studying the effects of health, social, economic, and environmental policies over the life-course of European citizens and beyond. From 2004 until today, 530 000 in-depth interviews with 140 000 people aged 50 years or older from 28 European countries and Israel have been conducted (Börsch-Supan et al., 2013; Börsch-Supan, 2021). A recent development is to improve the health measurement in such international social surveys by including blood-based biomarkers in order to overcome the subjectivity of self-assessed health measures, which tend to generate country-specific differences purely due to differential response behavior (Holland & Wainer, 1993).

Blood biomarkers are measured conventionally in serum or plasma obtained from venous blood samples (VBS). For large-scale international surveys, however, taking VBS is often prohibitively expensive and administratively difficult since ethical and data protection requirements vary greatly across countries. Hence, such surveys rely increasingly on minimally invasive microsampling methods. The collection of dried blood spots (DBS) is the most well-known among them (Brindle et al., 2010; Crimmins et al., 2014; McDade, 2014; McDade et al., 2007; Weir, 2008). In its Wave 6 in 2015, SHARE collected analyzable DBS samples with up to five blood spots from each of the about 24 000 respondents in 12 SHARE countries at their place of residence in order to complement self-reported with objective health data.

DBS samples enable the collection of a few drops of whole blood from a simple finger prick in a non-clinical environment, performed by well-trained interviewers or through self-administration by the respondent (Edelbroek et al., 2009). The blood is applied to filter paper for drying, whereupon DBS can be shipped under ambient conditions and can be bio-banked for storage and retrospective analyses. A non-volumetric blood sample (a single hanging drop of blood) is applied to the filter paper. For subsequent analyses, though, a volumetric amount of blood can be derived from a fully saturated portion of the DBS by punching a disc of fixed diameter, known as a punch (Freeman et al., 2018; Hannon & Therrell, 2014; Lim, 2018; McDade, 2014; McDade et al., 2007).

Despite these advantages, DBS have their own demands. The small quantities of blood collected require highly sensitive analytical instrumentation and conversion of measurement to plasma or serum values (standard equivalents). Standard procedures for DBS collection have been originally developed for newborn screening usually performed in clinics (Mei et al., 2010; Li & Lee, 2014; Freeman et al., 2018). In a non-clinical setting like a population survey, DBS are inevitably exposed to varying fieldwork conditions such as outside temperature, humidity, and shipping time (Adam et al., 2011; Hannon et al., 2013; Parsons et al., 2020). In addition, sample quality may be affected by shortened drying times, missing humidity protection, or collection of smaller than optimal blood volumes. In fact, of the more than 60 000 blood spots collected in SHARE, more than 57% had a diameter of less than 0.7 cm, which is considered suboptimal for chemical analysis, and almost 40% below 0.6 cm. There are many reasons, why SHARE and other surveys (e.g., Crimmins et al., 2020; Peck et al., 2009) experienced difficulties in collecting sufficiently large blood spots by the interviewers. A leading explanation is that interviewers did not wait until a large enough spot had formed and fallen on the filter card, but deposited a smaller blood volume still on the finger. This is particularly relevant for large population surveys where data collection is outsourced to national survey agencies, which employ lay interviewers that are trained to take DBS but cannot be monitored perfectly in the field.

In order to assess the influence of field conditions on biomarker values, SHARE conducted a post-field validation experiment with a non-SHARE donor sample, simulating the identified fieldwork conditions in the laboratory, creating a data set with 3420 different outcomes. (Börsch-Supan et al., 2021). The results of the laboratory experiment showed that the environmental factors, as well as sampling differences, influence the quality of the DBS and the therein-measured biomarker levels, although not all analytes suffer in the same way from these conditions. Moreover, we found that donor characteristics had no discernable influence on the relationship between environmental factors and biomarker values. These results are in accordance with the findings of the Health and Retirement Study (HRS; e.g., Crimmins et al., 2020) and other studies (Edelbroek et al., 2009; Freeman et al., 2018; Lim, 2018). A major finding of these experiments was the importance of spot size. Börsch-Supan et al. (2021) then used the validation results to generate formulae which convert the DBS values into venous blood values for a given set of fieldwork conditions (e.g., temperature, humidity protection, spot size). For these formulae to be applied to the survey-collected

DBS, information on the fieldwork conditions must be provided. SHARE had installed various monitoring mechanisms to this effect (Börsch-Supan et al., 2020).

This study concludes the installation of monitoring mechanisms by establishing a new algorithm that precisely measures the blood-covered area of a spot for each DBS in an automated way. For this approach, we took advantage of the fact that all SHARE DBS samples selected for analyses were photographed during the punching process. This unique approach allows for spot-size measurement of large amounts of DBS after collection. Previously published studies measured the applied blood volume by weighing a punched disc or used radioisotopic methods (De Vries et al., 2013; Hewawasam et al., 2018). Others estimated the diameter of the entire spot with (electronic) calipers, photographic blood-area determination (Denniff & Spooner, 2010; Hall et al., 2015; Moat et al., 2020; Peck et al., 2009; Vu et al., 2011), or divided the spots into different size categories (Crimmins et al., 2020). Neither of these methods is suitable for the spot-size determination of huge amounts of field-collected DBS in large surveys like SHARE.

We investigate the impact of, and the large variations in, spot size on the marker-value levels of different biomarkers assayed from the DBS samples, based on the about 24 000 field-collected SHARE DBS samples. We show that spot size is associated with the raw biomarker values, while controlling for other environmental and sampling factors. We also compare the exact measures made by the new algorithm to human-eye estimation, guided by the preprinted circles on the collection card, by dichotomization of the spots depending on their sizes. Based on our findings we conclude that (i) spot size measures should be considered when working with biomarker data derived from fieldwork-collected, smaller than optimal DBS samples and (ii) an exact measurement of spot size is superior to mere size estimation.

Spot size matters because the concentration of a marker may correlate with the size of the entire blood spot even if the chemical analysis is based on a punch that has a well-defined volume. One reason is that different volumes spread differently across the paper, influencing the concentration of a marker contained in the punch. George and Moat (2016) report that the application of smaller volumes creates proportionally larger spots; this results in significantly lower analyses results for many markers. Due to chromatographic effects, some markers do not spread evenly across the spot. In particular, red blood cells (RBC) and associated markers accumulate at the periphery as has been confirmed by several studies (Cernik, 1974; Moat et al., 2020; Parsons et al., 2020; El-Hajjar et al., 2007; Holub et al., 2006). The spreading of the blood is also influenced by the volume percentage of RBCs, the hematocrit, a variable that cannot be controlled when collecting DBS on filter paper. Not all analytes are affected by these issues to the same degree. Analytes associated with red cells may be less influenced by hematocrit than are serum analytes (O'Broin, 1993; Vu et al., 2011). Previous studies assessing the impact of DBS size on analyte concentrations have used venous heparin- or EDTA-treated blood, both anticoagulants (Moat et al., 2020). When comparing the results of those experiments to the results based on field-collected capillary blood, it should be considered that clotting or other biological factors might influence the spreading of blood components (O'Broin, 1993). Moreover, the analysis result of a particular blood marker may vary depending on the punch site, i.e., whether the punch was taken in the center or at the perimeter of a blood spot (chromatographic effect). Perimeter punches tend to have higher analysis values for some analytes (Moat et al., 2020; Parsons et al., 2020).

2 | DATA AND METHODS

We use a dataset that includes interview data from SHARE Wave 6, information on fieldwork conditions and sample quality related to respondent-matched DBS samples collected during the same wave, and biomarker values derived from these DBS samples. Among sample quality measures, the size of blood spots used for laboratory analysis plays an important role. Our new algorithm to measure them is described in Section 2.4, followed by a subsection on our statistical methods.

2.1 | Respondent characteristics from interview data

We collected about 24 000 blood samples in 12 out of the 20 countries participating in SHARE Wave 6. Samples were probability-based and representative of the population age 50 and older. Countries were selected by ease of ethical approval. All respondents without medical obstacles were asked to participate in the blood collection. Consent rate was 77%, ranging from 39% in Greece and 68% in Italy on the low end to 89% in Slovenia and 91% in Sweden on the high end. This resulted in the following country samples: Belgium (3690), Denmark (2861), Estonia (3683), France (578), Germany (3147), Greece (807), Israel (1073), Italy (2175), Slovenia (2242), Spain (1827), Sweden (3013), and Switzerland (2123). Sampling process and data cleaning steps are described in detail in Börsch-Supan et al. (2020) and http://www.share-project.org/data-documentation/waves-overview/wave-6.html.

The data set includes the following respondent characteristics from the SHARE Wave 6 interview data (Börsch-Supan et al., 2013; Börsch-Supan, 2020; Malter & Börsch-Supan, 2017): Age at the interview measured in years; gender as a dummy for female sex; body mass index (BMI, kg/m^2) calculated as the ratio from self-reported weight to height; education as a categorical variable with levels high (aggregated ISCED 1997 levels 5–6), middle (ISCED 1997 3–4), and low (ISCED 1997 0–2). The respondents' health status is included as self-reported general health, again with levels high (excellent, very good), middle (good), and low (fair, poor). For both variables low is coded as 1, while medium and high are coded as 2 and 3, respectively. Additionally, the dataset includes a country indicator.

2.2 | DBS sample collection, sample quality, and fieldwork conditions

We used harmonized collection protocols and DBS collection kits, thoroughly trained our interviewers for DBS collection, and implemented interviewer and sample monitoring throughout the fieldwork. SHARE DBS filter cards (Ahlstrom 226 filter paper) provided five preprinted circles of 1 cm in diameter for blood-droplet collection. The interviewers were instructed to fill up as many circles as possible. Respondents were not asked to be fasted. All DBS samples were sent to the SHARE biobank in Odense, Denmark, where they were visually inspected. Number, size, and quality of the blood spots were documented before storage in freezers at −23°C until analyzed. The biobank also controlled for, and reported on, missing desiccant (influencing humidity protection) and spot discoloration (indicating that a wet DBS was packaged). Among other impacts on the samples beyond our control were malfunctioning national postal systems, with consequences on shipment time, and unusually high temperatures encountered during shipment. The implementation and monitoring of the DBS collection in SHARE Wave 6 has been described in detail in Börsch-Supan et al. (2020). The mean values, standard deviation, and range for the fieldwork conditions that were measured during fieldwork are shown in Table 1 followed by a description of the collection process.

Drying time (minutes): Interviewers were instructed to let the blood sample dry until the end of the interview but at least 15 min. This followed the protocol that was used at that time by the HRS (Crimmins et al., 2015). As an estimation of the drying time, we used the interview length between the blood spot module, programmed in computer-assisted personal interviewing (CAPI), and the end of the interview (or the time until the end of the interview session if the interview was conducted in more than one session on several days).

TABLE 1 Mean values and range of fieldwork conditions in SHARE wave 6

	Mean (*SD*)	Range
Drying time (min)	22.16 (14.19)	1–83
Shipment time (days)	5.64 (4.55)	1–30
Outside temperature (°C)	13.99 (8.22)	0–35
Open bag (yes/no)	0.11 (0.31)	0–1
Desiccant missing (yes/no)	0.02 (0.13)	0–1

Humidity protection during shipment: The DBS were shipped in a plastic bag with a sachet containing molecular sieve as desiccant, which absorbs humidity faster than Silica gel. The indicator for humidity protection consisted of two dummy variables: one for a not tightly closed plastic bag containing the DBS sample and one for a missing desiccant inside the bag. Information on complete or missing components was registered for each sample on arrival at the biobank.

Shipment time (days) was measured as the time between sample collection (date noted on card) and arrival at the biobank, where the card with the DBS sample was stored frozen until laboratory analysis.

Outside temperature (°C) was estimated by the interviewer at the time of blood collection. The estimate was entered into the CAPI. During Wave 6 pretest, we double checked these estimates by using long-term temperature trackers and found no substantive deviations (Börsch-Supan et al., 2020). Since high temperatures were particularly prevalent in Israel, we added long-term temperature trackers (WarmMark Long Run indicator 31°C up to 168 h) to all DBS collected there.

Spot size (cm^2) is not presented in Table 1 as it could not be measured during fieldwork. Instead, it was determined from images available for each spot, see Section 2.4 below. As the blood-covered area is proportional to the volume of blood contained in a DBS, the area of the blood spot can be used as a proxy for the blood volume.

Implausible values for drying time (234 obs.), shipment time (235 obs.), and outside temperature (183 obs.) are set to missing.

2.3 | Biomarker analysis

The SHARE DBS samples (Börsch-Supan, 2021) have been analyzed for 17 biomarkers. Seven routine blood biomarkers: high-density lipoprotein (HDL), total hemoglobin (tHb), glycated hemoglobin (HbA1c), total cholesterol (TC), triglycerides (TG), C-reactive protein (CRP),

and Cystatin C (CysC) were analyzed at the Department of Laboratory Medicine, University of Washington, Seattle, USA. Ten proteins: the cyto-, chemo-, and neurokines IL-8, IL12/23, IL-16, IL-18, MCP1, and BDNF; the growth factors EGF and VEGF; and the apolipoproteins ApoE4 and Clusterin (APOJ) were assayed at the Statens Serum Institut, Copenhagen, Denmark. This paper focuses on the first seven markers. Laboratory assays were performed according to published techniques as described previously in Börsch-Supan et al. (2021).

The DBS samples were analyzed in two randomly drawn batches: The first batch contained about 8000 observations, analyzed in 2018; the second batch with almost 16 000 observations was analyzed in 2020/21.

For laboratory processing, small circular discs (the punches) with a given diameter of 3.2 mm are cut out of a blood spot area, which is completely covered and fully soaked with blood to ensure a fixed blood volume. A freely falling blood drop of ca. 50 µl creates a spot of ca. 1 cm in diameter. In such a DBS, the volume contained in this small cylinder of a punch is calculated to be 3.2–3.4 µl. Based on CLSI standard, this volume is calculated to be 3.42 µl at 55% hematocrit (Chace et al. 2014 in: Li & Lee, 2014). The punching of all SHARE DBS samples was performed at Statens Serum Institut. For the analysis of all 17 SHARE markers, five punches per DBS sample are necessary. HDL, tHb, HbA1c (together referred to as A-marker set) can be analyzed from the same eluate of two punches. One additional punch is necessary for TC, TG, CRP, CysC (the B-marker set). Two further punches are needed to analyze the cytokine and other markers (the C-marker set) in a multiplex immunoassay. A sample with blood of insufficient quality (e.g., smeared or overlapping spots) still can be analyzed for HbA1c from one punch taken from such an otherwise unsuitable spot. HbA1c is a relative measure, the percentage of glycated from total hemoglobin, and should therefore be concentration-independent except for variations of biomarker concentration within the blood spot as described at the introductory section of this paper.

The amount of blood material (number and size of the blood spots) contained in a sample restricts the number of biomarkers that can be analyzed. Not all SHARE DBS samples contained enough blood material for five punches and, hence, not all biomarkers could be analyzed in all samples. We introduced a priority scheme determining the marker sets to be assayed for each number of possible punches in a DBS sample (see Table 2). The chosen priority aimed to maximize the number of biomarker information for each of the marker sets, with a priority for HbA1c and the biomarkers of the B-marker set.

Since A and C markers need two punches for analysis, they could come from different spots. In these cases, we used the average size of the two spots. If there was no suitable spot available for the analysis of all A markers, we used one punch only and restricted the analysis to HbA1c, referring to it as A* marker. Knowledge of the punching scheme is therefore important. For each spot, the dates and the times of punching are stored in the file names of the images. It allows us to establish a chronological order of punches for a sample and to match each punch (and its size) to the respective marker set.

Mean and standard deviation of the raw DBS values for the SHARE DBS sample are presented in Table 3. These raw values are not comparable to standard blood values derived from routine laboratory assays of venous blood draws. This has several reasons. First, the raw values have not been converted to venous standard values according to the conversion formulae (Börsch-Supan et al., 2021). Second, they are based on a non-fasting state, which is particularly important for the interpretation of triglyceride and total cholesterol values. For TG (76 obs.) and CRP (52 obs.) we removed physiologically not meaningful values. The rightmost columns show the intra- and inter-assay variability measured as coefficient of variation (CV).

2.4 | Automated measurement of spot size

We developed a new algorithm (AMoSS, Automated Measurement of Spot Size) that enables us to calculate the spot size of each DBS by an automated approach for large sets of images. In addition, the algorithm counts the number of punches on each spot and for each sample. The algorithm used the punching scheme (presented in Table 2) to link every punch uniquely, and thus the spot and calculated spot size with the corresponding marker set for which the punch was analyzed in the laboratory.

For the measurement of spot size, we used images that have been taken from each spot immediately before punching the small discs for laboratory analysis (semi-automated robot punching system Panthera Puncher 9, Perkin Elmer, Waltham, MA, USA. The camera is an integrated part of the puncher and is located adjacent to the punch head. It takes photos from above at an almost perpendicular angle; the deviation from a perfectly right angle is so small that it can be safely ignored. We did not control for the angle. Punching—and hence photographing—is performed from the rear side of the filter card as this reveals the fully soaked area of a spot. As some samples needed to be punched from several spots, several images for one sample may exist. This amounted to almost 64 000 images for around 24 000 DBS samples. We used the images to establish the algorithm that precisely measures the pixels of the blood-covered area of the spots and counts the number of

TABLE 2 Priority scheme for DBS sample punching

Possible # punches per DBS sample	Punch 1	Punch 2	Punch 3	Punch 4	Punch 5
5 (and more)	C	C	A	A	B
4	C	C	A*	B	
3	A	A	B		
2	A*	B			
1	B or A* *(depending on spot quality)*				

Note: For each quantity of possible punches in a sample, punches for a fixed choice of marker sets have been made in a fixed order; A, B, C refer to the marker sets described in the text, A* refers to a single punch derived from an otherwise unsuitable spot, which is only analyzed for HbA1c.
Abbreviation: DBS, dried blood spot.

TABLE 3 Mean values and range of biomarkers based on DBS in SHARE wave 6

	Observations	Mean (standard deviation)	Range	Intra-assay CV (%)	Inter-assay CV (%)
HDL cholesterol (mg/dl)	14 719	100.11 (23.66)	15–234	7.41	11.54
Total hemoglobin (g/dl)	14 719	13.50 (2.36)	5.3–25.9	5.16	7.36
HbA1c (%)	14 090	6.59 (0.79)	4.2–16.35	1.32	2.39
Total cholesterol (mg/dl)	22 760	312.47 (78.59)	2.0–718	6.18	8.88
Triglycerides (mg/dl)	22 611	229.31 (109.12)	40–996	5.30	13.70
C-reactive protein (mg/L)	22 381	1.82 (3.64)	0–49.98	4.19	11.88
Cystatin C (mg/L)	22 760	1.08 (0.35)	0.25–4.78	3.67	7.04

Note: Raw DBS values before conversion to standard venous equivalents.
Abbreviation: DBS, dried blood spot.

punches on the spot that are virtually marked in color (green) by the camera of the punching machine (see Figure 1).

AMoSS is programmed in R (R Core Team, 2020), using "EBImage" (Pau et al., 2010) for image manipulation. The procedure that was applied to AMoSS is described in detail in Figure 1. All functions used within the algorithm are automatically executed on the data. AMoSS needs the path to the folder with the stored images of the spots to use as input. The output is a dataset with the image ID, the size of the spots, and the number of punches, both for each spot and for the sample.

In order to count the number of pixels of each spot, we convert the images to raster objects. Raster objects save the data in pixels rather than arrays, as is the case for images. The size of the spots is calculated by the number of black pixels in relation to the known total number of pixels in the image.

$$\text{spot size} = \frac{\text{black pixels}}{\text{total pixels}} \times \text{image area}$$

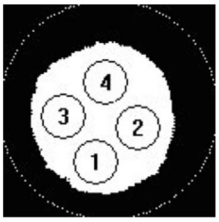
AMoSS provides the exact area of the DBS in the images (in cm^2).

The algorithm detects if the image area does not cover the entire spot. This is tested before the previously described algorithm is applied. For this, we use the unprocessed black and white images. We apply a median filter to remove noise outside the spot area. We then use the fill function. If the black spot is fully surrounded by white background, then the image area covers the entire spot and we fill the whole spot with white color. However, if the spot touches the edge of the image, it remains visible in its black color. We then count the objects in the image. If the spot has been turned white, the white background remains the only object on the image. Otherwise more than one object remains in the image. Such an image is then cropped again with an aspect ratio that is sufficiently large to cover the entire spot. Finally, spot size is determined according to the larger dimensions of the image.

To determine the number of punches in each DBS, AMoSS uses the original images showing the punches marked in green. As we know the diameter of one punch and the total number of pixels in the image, we can calculate that a single punch contains 600 pixels. Again, we convert the images to raster objects. Since all pixels in the green punching outline share the same color value, we can determine the total number of green pixels in the image. We divide the total number of green pixels by 600 to obtain the number of punches for each spot in the sample.

1. Prior to applying the algorithm, all images are cropped to the same format. In order to calculate the exact spot size, we need to know the length of the edges of the images. Using the known diameter of one punch (3.2 mm), we calculate an image area of 1.4971 cm^2. We also know the total number of pixels in the image is 131x131. This information is necessary to be able to determine the proportion of area covered by the DBS.

2. To determine the spot area, a second set of images is generated converting the colored images to black and white. Thus, it is easier to remove noise from the images and erase any marks from the spot area. After conversion, only the outlines and number of punches remain on the otherwise black DBS.



3. For further image manipulation, we produce the negative image, which turns the spot white and the surroundings black.

4. We apply a fill function. This function fills patches in an object that are surrounded by pixels with a certain integer value. This function fills all outlines on the DBS area with white pixels.

5. We remove further noise by smoothing the image using a median filter. The function scans each pixel and replaces the pixel value by the median of neighboring pixel values. This removes the outline of the pre-printed circle on the black background. Any further noise that could be present on the images is also removed.

6. In a final step, we convert the images back to the original color. The resulting images show the clean DBS area in black pixels only and the surroundings in white.

FIGURE 1 Procedure applied for image manipulation using automated measurement of spot size

2.5 | Statistical analysis

We ran OLS-regressions using robust standard errors to assess the influence of fieldwork conditions and sample quality, especially spot size, on biomarker values. The raw values of the seven biomarkers (Table 3) were used as dependent variables. As independent variables, we used the fieldwork conditions described in Section 2.2. We controlled for several respondent characteristics in order to separate biomedical effects depending on age, sex, BMI, education, and self-reported health (see Section 2.1). We included a country indicator to account for unobservable country heterogeneity. Observations with missing values were excluded from our

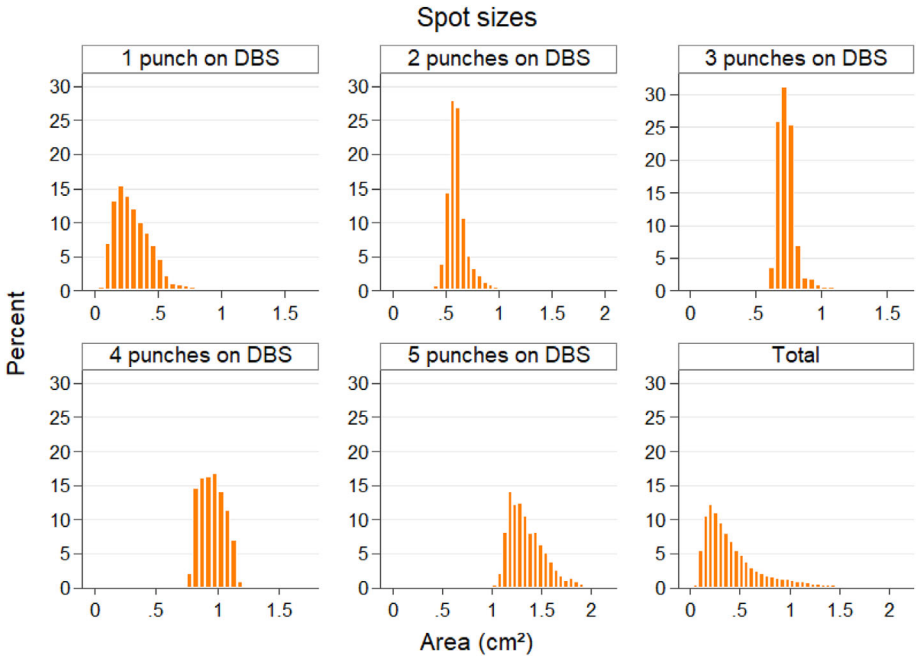


FIGURE 2 Spot size distribution for the dried blood spot (DBS) collected in SHARE Wave 6 by number of punches. Figure shows distribution of spot-size areas for the DBS depending on the number of punches that could be obtained from each spot. "Total" shows the distribution of sizes for all spots in the sample.

TABLE 4 Mean values and range of spot size in SHARE wave 6

		Diameter (cm)			Area (cm^2)		
	N	**Mean (*SD*)**	**Median**	**Range**	**Mean (*SD*)**	**Median**	**Range**
All spots	61.446	0.71 (0.24)	0.66	0.19–1.63	0.44 (0.31)	0.34	0.03–2.08
A markers	15.059	0.90 (0.28)	0.89	0.32–1.63	0.70 (0.41)	0.63	0.08–2.08
A* markers	6.906	0.52 (0.19)	0.48	0.19–1.22	0.24 (0.19)	0.18	0.03–1.17
B markers	23.086	0.77 (0.27)	0.70	0.31–1.63	0.52 (0.38)	0.39	0.07–2.08
C markers	15.573	0.90 (0.28)	0.91	0.34–1.63	0.70 (0.40)	0.65	0.09–2.08

analysis. The number of observations included in each regression varies depending on the biomarker (see Table 3). Since the two batches of observations were chemically analyzed in different years (see Section 2.3), we controlled for the potential impact of storage on the marker values by a dummy variable indicating the batch number in all regressions. Statistical analyses were conducted in Stata (StataCorp., 2015) and R (R Core Team (2020).

In order to test the explanatory power of spot-size measurement and emphasize the advantage provided by AMoSS, we ran different regression models for each marker. They differ by the measure of spot size. In Model 1, we used the continuous measure calculated by AMoSS. To accommodate a non-linear relationship between the biomarker levels and the area, the continuous area measure entered the regression by a set of cubic splines (Burden et al., 1997). Following Harrell's (Harrell Jr., 2001) suggestions, we used five knots placed at the 0.05, 0.275, 0.5, 0.725, and 0.95 quantiles. For Model 2, we built a dichotomized measure that distinguishes between smaller and larger spots. Following the approach of the HRS, where 1 cm in diameter is used as a threshold (Crimmins et al., 2020), we use the corresponding spot area (0.785 cm^2) as a threshold. This results in a share of larger spots of 41% for A markers and 19% for B markers, respectively. While dichotomization in the studies cited by Crimmins et al. (2020) is based on eyesight, we based the dichotomization of our spot sizes on the continuous measures calculated by AMoSS. As a robustness check, we also tested eight different threshold values roughly corresponding to the diameter values listed in the first column of Table 5. Finally, Model 3 does not include any spot size information and serves as baseline comparison to assess the role of spot size in explaining marker values.

We applied three widespread methods to select the best fitting model: adjusted R^2, Akaike information criterion (AIC, Akaike, 1998), and Vuong test (Vuong, 1989). The Vuong test is based on the likelihood-ratio principle.

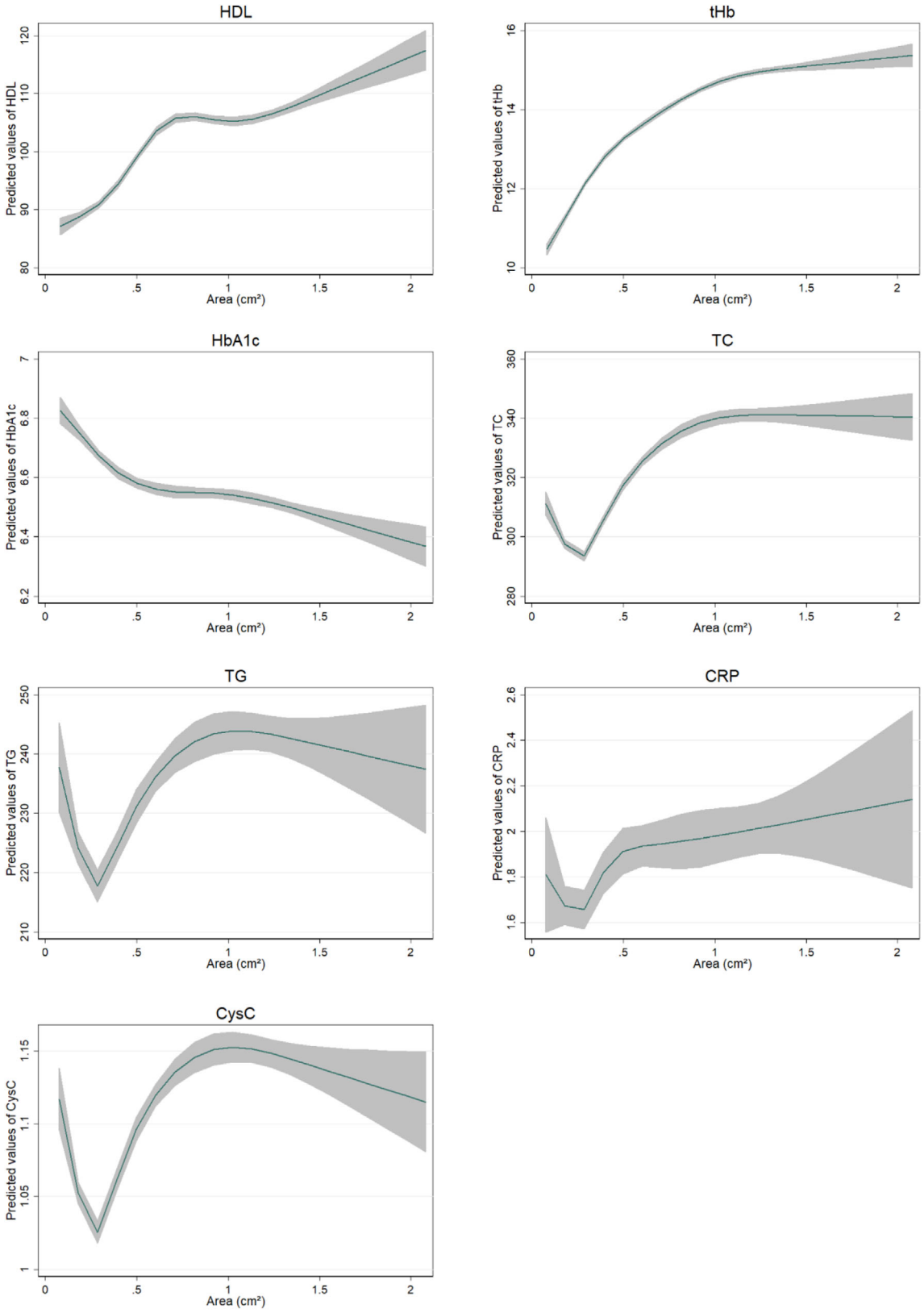


FIGURE 3 Effects of spot size on biomarker analytes with 95% confidence interval

It compares two competing models to find the one that is closest to the true distribution of the data. We can attribute all differences between our models to the spot-size measures because they are otherwise identical.

3 | RESULTS

Figure 2 shows the distribution of the spot area that was calculated using AMoSS per each number of punches

that could be obtained from one DBS. The larger the spots, the more punches could be collected. For most DBS spots, only one punch could be taken. We observe the smallest spots for DBS that yielded only one punch. The distribution of spot sizes across all spots ("Total" in Figure 2) is right skewed, which is mainly driven by the spots with only one punch.

Table 4 provides the mean values, standard deviation (*SD*), and range of the spot areas that have been measured using AMoSS for all markers and separately for A, A*, B, and C markers, resp. The selection process based on the punching scheme (Table 2) leads to systematically lower spot sizes for B markers as compared to A and C markers, while A* markers are particularly small by definition.

In Figure 3, we show the effects of the spot size on the respective biomarker levels as measured by the cubic splines in Model 1.

The effect of spot size is highly significant for all analytes and shows non-linear relationships. For HDL and tHb, we see that larger areas are monotonously associated with a higher biomarker level. Towards the ends of the larger area values, the confidence intervals get wider due to the lower number of observations in this area. This is true for all markers but to a different extent. The relationship between spot size and analyte value is reversed for HbA1c; we see a significant and monotonous negative association.

The effects show a different pattern for the B-marker set. We do not find monotonicity but rather oscillations depending on the spot's size. For small areas up to 0.2–0.3 cm^2, we observe a significantly negative association for TC, TG, CRP, and CysC. For area values larger than 0.3 cm^2, the association is significantly positive. For TG and CysC, we find a negative association for areas larger than 1 cm^2. However, we again observe wider confidence intervals for these area values.

Since spot size may have a more intuitive meaning when measured as a diameter rather than an area or volume, Table 5 presents the percentage effect of spot size on analyte values relative to a spot diameter of 1 cm. This translation is based on the assumption of a perfect circle, hence area $= \pi/4$*diameter squared.

Analyte values are seriously underestimated for small spot diameters (0.7 cm or less, which encompasses 57.1% of the DBS collected in SHARE) except for HbA1c, where analyte values from small spot sizes are overestimated relative to 1-cm spots. These underestimates are statistically significant. They are also substantially larger than the intra-assay variation (Table 3) except for HbA1c. For

TABLE 5 Effect of spot size on analyte values relative to a spot diameter of 1 cm, based on Model 1

Diameter	HDL	tHb	HbA1c	TC	TG	CRP	CysC
1.3	2.04%***	5.63%***	−0.75%***	1.97%***	0.29%	4.48%	0.00%
1.2	0.33%	4.23%***	−0.29%*	1.67%***	0.62%	2.88%	0.44%
1.1	−0.28%	2.46%***	−0.08%	1.11%***	0.62%	1.34%	0.53%
1	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
0.9	−1.18%***	−3.17%***	0.09%	−2.09%***	−1.74%**	0.62%	−1.57%***
0.8	−5.45%***	−6.69%***	0.46%***	−4.93%***	−4.42%***	1.44%	−4.11%***
0.7	−11.13%***	−10.56%***	1.19%***	−8.82%***	−7.61%***	6.28%*	−7.43%***
0.6	−14.54%***	−15.14%***	2.03%***	−12.11%***	−9.57%***	14.82%***	−10.14%***
0.5	−15.59%***	−19.72%***	2.84%***	−11.81%***	−8.14%***	15.44%***	−8.92%***

Note: Asterisks measure whether the marker value of a given diameter differs significantly from the marker value obtained in a blood spot with a diameter of 1 cm.
*$p < .1$. **$p < .05$. ***$p < .01$.

TABLE 6 Effect of small spot size on analyte values, based on dichotomous measurement of spot size (Model 2)

Thres-hold	HDL	tHb	HbA1c	TC	TG	CRP	CysC
<1 cm	10.76***	2.071***	−0.116***	33.81***	17.31***	0.222***	0.0773***
	(0.363)	(0.0337)	(0.00936)	(1.195)	(1.737)	(0.0630)	(0.00543)
<0.8 cm	13.56***	2.249***	−0.139***	32.38***	16.29***	0.252***	0.0808***
	(0.338)	(0.0332)	(0.00946)	(0.920)	(1.463)	(0.0516)	(0.00437)

Note: Asterisks measure significance of the dichotomous spot-size indictor. *$p < .1$, **$p < .05$, ***$p < .01$.

spot diameters less than 0.6 cm, which encompasses 39.3% of the DBS collected in SHARE, they are also larger than the inter-assay variation except for HbA1c and TG.

Model 2 uses a dummy variable for spot-size information (spot size smaller than 1.0 cm based on Crimmins et al. (2020) and 0.8 cm as a robustness check). The effect of spot size is highly significant in both threshold specifications of this model. Except from HbA1c, we find positive associations between size and analyte values (Table 6).

The effects of fieldwork conditions other than spot size and the effects of respondent characteristics on the biomarker levels are presented in Table 7. Since the effects for the fieldwork conditions and respondent characteristics remain similar regardless which spot-size information is used in the regression model, Table 7 only displays the results from Model 1.

We find significant associations for the other fieldwork conditions. However, they differ across analytes. The respondent characteristics show expected and explainable values.

Tables 8 and 9 depict adjusted R^2, AIC, and Vuong tests to indicate the explanatory power, the goodness of fit and the statistical difference between the three models. The adjusted R^2 from Model 1 using continuous spot size with spline interpolation always explains more variance of the analyte values than the other models. However, the magnitude of the difference between the models varies by marker. For tHb, we observe the largest difference between the adjusted R^2-value for Model 1 and Models 2 and 3, respectively. The smallest difference can be found for CRP. Further, Model 1 always has the lowest AIC values for all markers. This suggests that the

TABLE 7 Effects of fieldwork conditions and respondent characteristics on the biomarker levels in Model 1

Variables	HDL	tHb	HbA1c	TC	TG	CRP	CysC
Female	6.691***	−0.988***	−0.0271***	16.41***	0.572	0.0475	−0.0186***
	(0.338)	(0.032)	(0.009)	(0.845)	(1.434)	(0.0493)	(0.004)
Age	−0.171***	−0.0201***	0.00601***	−0.702***	−0.362***	0.0174***	0.0130***
	(0.0182)	(0.0017)	(0.0005)	(0.0458)	(0.0766)	(0.00270)	(0.0002)
Education level (Middle)	0.951**	−0.000264	−0.0295**	1.754	3.352*	−0.101	−0.0158***
	(0.433)	(0.040)	(0.012)	(1.083)	(1.846)	(0.0647)	(0.006)
Education level (High)	2.489***	−0.0128	−0.0517***	3.564***	−2.388	−0.163**	−0.0244***
	(0.484)	(0.044)	(0.013)	(1.193)	(1.976)	(0.0712)	(0.006)
BMI score	−0.792***	0.0328***	0.0218***	−1.106***	3.363***	0.0931***	0.00909***
	(0.038)	(0.003)	(0.001)	(0.090)	(0.156)	(0.00669)	(0.001)
Drying time	0.0454***	0.0011	0.000383	0.0392	0.138***	−0.000668	0.000299**
	(0.0118)	(0.0011)	(0.0003)	(0.0299)	(0.0530)	(0.00183)	(0.0002)
Outside temperature	−0.00582	−0.0237***	0.00231***	0.201***	0.0373	−0.00442	−0.000645**
	(0.0249)	(0.0022)	(0.0007)	(0.0608)	(0.105)	(0.00351)	(0.0003)
Open bag	−2.349***	−0.036	0.0206	−3.914***	0.469	−0.0869	−0.0240***
	(0.589)	(0.054)	(0.017)	(1.463)	(2.599)	(0.0747)	(0.007)
Desiccant missing	−7.758***	0.218*	−0.149***	−14.08***	7.534	0.189	−0.00211
	(1.297)	(0.120)	(0.036)	(3.055)	(5.603)	(0.228)	(0.016)
Shipment time	−0.557***	−0.0485***	0.0290***	−1.106***	−0.0697	−0.0103*	−0.00616***
	(0.0475)	(0.0042)	(0.0029)	(0.1080)	(0.174)	(0.00616)	(0.0005)
Health (Medium)	1.206***	0.144***	−0.0630***	4.848***	2.970*	−0.568***	−0.0803***
	(0.414)	(0.038)	(0.012)	(1.032)	(1.755)	(0.0611)	(0.005)
Health (Good)	2.720***	0.253***	−0.107***	11.39***	−4.056*	−0.730***	−0.104***
	(0.498)	(0.045)	(0.014)	(1.217)	(2.098)	(0.0695)	(0.006)
Observations	14 719	14 719	14 090	22 760	22 611	22 381	22 760
Variables	0.271	0.388	0.539	0.363	0.067	0.030	0.234

Note: Robust standard errors in parentheses.

*$p < .1$. **$p < .05$. ***$p < .01$.

TABLE 8 Model selection measures (adj. R^2 and AIC) to evaluate the advantage of continuous spot size

	Model 1		Model 2		Model 3	
	Adj. R^2	AIC	Adj. R^2	AIC	Adj. R^2	AIC
HDL	0.269	130 315	0.227	131 134	0.180	132 018
tHb	0.387	59 845	0.292	61 975	0.113	65 286
HbA1c	0.538	22 317	0.533	22 474	0.528	22 622
TC	0.363	253 039	0.343	253 728	0.315	254 662
TG	0.066	274 858	0.063	274 922	0.059	275 011
CRP	0.029	120 726	0.028	120 739	0.028	120 750
CysC	0.233	10 566	0.225	10 807	0.217	11 020

Note: Model 1 uses continuous spot size and is compared to models using dichotomized spot size (Model 2) or no spot-size information at all (Model 3).

TABLE 9 Vuong test to evaluate the advantage of continuous spot size

	Model 1 vs. model 2	Model 1 vs. model 3	Model 2 vs. model 3
HDL	0.000***	0.000***	0.000***
tHb	0.000***	0.000***	0.000***
HbA1c	0.000***	0.000***	0.000***
TC	0.000***	0.000***	0.000***
TG	0.002***	0.000***	0.000***
CRP	0.200	0.450	0.710
CysC	0.000***	0.000***	0.000***

predictive accuracy can be increased using the continuous spot size.

The Vuong test statistic indicates which model is closer to the true distribution of the data. We find significant differences between Model 1 and Model 2 for all markers except CRP. Furthermore, Table 9 documents significant differences between Model 1 and Model 3 for all markers except CRP. When comparing Model 2 and Model 3, we find significant differences for all markers except CRP.

4 | DISCUSSION

We developed a new algorithm (AMoSS, Automated Measurement of Spot Size) that enables us to calculate the exact spot size of each DBS in an automated way for large sets of DBS images. Using the results of this automated spot-size measurement, we found that spot size has a significant non-linear relationship with all markers. Some markers, however, are more sensitive to spot size than others. Further, our model comparisons show that more variance of the raw marker values can be explained using the exact measured spot sizes provided by AMoSS and included in the model as splines. CRP is the only marker for which we do not find significant differences between Model 1 and both Models 2 and 3.

An important lesson learned is the non-linear relationship between spot size and biomarkers. Using the new spot size measure that we calculated with AMoSS and using cubic spline interpolation, we are able to account for this non-linearity. This is obviously not possible when only the dichotomized information about spot size is available. Nonetheless, Table 9 shows that it is always better to include any spot-size information rather than none at all, except for CRP. This can be seen when comparing the adjusted R^2, AIC values, and Vuong test for Models 2 and 3.

Spot size can be substantially influenced by the interviewer's ability to collect the blood samples as trained. The interviewers had been instructed, but could not be monitored, to wait until a sufficiently large blood drop has formed and fallen on the filter paper filling the preprinted circle. As a robustness check, we controlled for the unobservable heterogeneity among interviewers by adding interviewer fixed effects to Models 1–3. We still find the same effects for the variables in the regression models and the same significant differences between the models that differ by spot-size information.

Our robustness checks also show that the commonly used threshold of 1 cm to distinguish small and large spots is not necessarily the most suitable dichotomization. Using the same model selection methods as before to test different thresholds, we were able to demonstrate

that 0.8 cm (0.503 cm^2 in area) instead of 1 cm significantly improved the measures of goodness of fit. This is consistent with Moat et al. (2020), who found that spots with less than 0.8 cm in diameter created a bias in the analytical test results. In general, we suggest that dichotomization should not be conducted in a data-driven manner, but ideally prior to analysis and based on theoretical considerations.

We tested the same models (Model 1–Model 3) using non-linear (quadratic) specifications for the continuous covariates. We find the same effects for spot size and the same significant differences between the models.

Despite these improvements, our methods cannot eliminate yet other uncertainties linked to spot size: Paper properties, which are beyond user control, can cause changes of spot size and punch volume. Inhomogeneity in the paper may influence the lateral spreading and cause uneven distribution of the blood or irregularities in thickness change the assumed blood volume in the punch (Mei et al., 2001; Hall et al., 2015; Moat et al., 2020; Ren et al., 2010). Lateral spreading is also influenced by the viscosity of the blood determined through the proportion of blood cells in a sample, the hematocrit, which is a donor characteristic. As mentioned earlier, when collecting drops of blood on filter paper, the hematocrit cannot be measured. Denniff and Spooner (2010) evaluate the relationship between hematocrit and DBS area; the bias is within acceptable limits (<10%) for normal adult hematocrit values (36%–50%), when comparing the area to blood samples of 45% hematocrit. Li (Li & Lee, 2014) consider the impact of hematocrit as negligible when measured quality-control sample concentrations vary within ±15% of the normal values.

Many SHARE DBS sample punches were taken close to the perimeter of the spot, not always keeping a distance of 1–2 mm from the periphery. Biomarker concentration there may differ slightly from that in the center. This, together with unobserved hematocrit may have influenced the assay results of the markers HbA1c and tHb, which are associated with red blood cells. In order to evaluate the influence of the punch location on the marker values in field-collected DBS samples, a further algorithm could be written, which measures the shortest distance between the outline of the punch and the perimeter of the blood spot. This is subject of our future research.

5 | CONCLUSIONS

This study examined how field conditions and sample quality, in particular spot size, influence the marker values measured from filter-paper based DBS samples collected in a survey. Börsch-Supan et al. (2021) have demonstrated in a validation experiment that correcting for these factors substantially improves the accuracy of biomarker data collected under difficult field conditions. Conversion formulae such as developed in that paper allow us to infer values that we would have obtained under optimal field conditions.

This study shows the necessity to carefully adjust for one particularly important fieldwork condition, namely the size of the blood spot on the filter paper if spot sizes vary as considerably as they tend to do in large-scale population studies. In these cases, it is important to measure it accurately. Our new algorithm presented here provides precise post-collection spot-size measurement by exploiting the images that are generated during the punching process of DBS.

The new spot-size information improves the accuracy of conversion formulae that translate blood-marker values analyzed from SHARE DBS into standard blood equivalents. This increases the quality of marker values gained from field-collected DBS and makes the data more reliable and user-friendly. Therefore, the data dissemination of DBS biomarkers should include a measure of spot size if one suspects that fieldwork conditions were less than optimal. For large-scale surveys, taking images from the DBS during the punching process is a feasible method to conduct spot-size measurement. Refinements of the image analyses—such as the location of punches within the spot—can improve conversion formulae even further.

However, it is of course better to minimize the large variance in the data that is generated by suboptimal fieldwork conditions by thorough measures of ex-ante prevention instead of applying statistical methods of ex-post correction. Concerning spot size, it is important to conduct extensive interviewer training and ascertain quality control during fieldwork because average spot size can be influenced considerably by interviewers. In particular, measures should be taken to ensure that interviewers will collect sufficiently large blood spots that entirely fill the pre-printed circle. Training and monitoring measures are needed to enforce a greater homogeneity across interviewers and to reduce the variance of spot size that has been observed in large population studies. These efforts, though costly and possibly not perfect, will improve the raw data collected at baseline such that ex-post adjustments are needed to a lesser extent.

AUTHOR CONTRIBUTIONS

Rebecca Groh: Conceptualization; formal analysis; methodology; building the algorithm AMoSS; writing-original draft, writing review, and editing. **Luzia M. Weiss:** Conceptualization; formal analysis; methodology. **Martina Börsch-Supan:** Conceptualization; writing-original draft, writing review, and editing. **Axel**

Börsch-Supan: Reviewing and revising drafts; formal analysis, writing review, and editing.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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