HPLC-based measurement of glycated hemoglobin using dried blood spots collected under adverse field conditions

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Abstract

Glycated hemoglobin (HbA1c) measured using high performance liquid chromatography (HPLC) assays with venous blood and dried blood spots (DBS) are compared for 143 paired samples collected in Aceh, Indonesia. Relative to gold-standard venous-blood values, DBS-based values reported by the HPLC are systematically upward biased for HbA1c<8% and the fraction diabetic (HbA1c≥6.5%) is overstated almost five-fold. Inspection of chromatograms from DBS assays indicates the % glycosylated calculated by the HPLC excludes part of the hemoglobin A which is misidentified as a hemoglobin variant. Taking this into account, unbiased DBS-based values are computed using data from the machine-generated chromatograms. When the DBS are collected in a clinic-like setting, under controlled humidity/temperature conditions, the recalculated values are almost identical to venous-based values. When DBS are collected under field conditions, the recalculated values are unbiased, but only about half the HbA1c values are measured reliably, calling into question the validity of the other half. The results suggest that collection conditions, particularly humidity, affect the quality of the DBS-based measures. Cross-validating DBS-based HbA1c values with venous samples collected under exactly the same environmental conditions is a prudent investment in population-based studies.

Keywords: Dried blood spots, HbA1c, HPLC

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1. Introduction

An epidemic of non-communicable diseases (NCDs) is following closely behind the epidemic of obesity spreading across the globe. In low and middle income countries, elevated levels of metabolic dysregulation as indicated by adverse lipid levels and blood glucose levels are fast becoming major public health problems. Unlike obesity rates, however, where alarming increases have been well-documented based on anthropometric measures collected in large-scale population-representative surveys, there is limited evidence on the prevalence of blood-based indicators of metabolic dysregulation in low and middle income countries. This information gap largely reflects a paucity of reliable data on biomarkers collected in large-scale population-representative studies, although the value of collecting this information both for improving human health and science is likely to be very high (Crimmins and Seeman 2004), particularly in low resource settings where life expectancy is low, large fractions of the population have limited access to health care and under-diagnosis of treatable conditions such as hypertension and diabetes is widespread (Frankenberg and Thomas 2002).

Successful collection and analysis of venous blood in population-based surveys is complicated because samples need to be maintained under well-controlled conditions that are challenging to assure in resource-poor contexts. In recent years, population-based studies have collected blood using minimally-invasive finger sticks—a straightforward procedure that is well-accepted by study subjects. Assays are then conducted either in situ with point of care tests (POCTs) or blood spots are created on filter paper, dried in situ and transported to a laboratory for assaying later (McDade et al. 2007, McDade 2014). Whereas POCTs and dried blood spots (DBS) have transformed understanding of important global health challenges such as HIV, malaria and anemia, the impact of these technologies on metabolic-related NCDs has been more muted (Samuelsson et al. 2015).

Glycated hemoglobin (HbA1c) is an ideal candidate for inclusion in population-based studies. Estimates put the worldwide prevalence of diabetes mellitus (HbA1c$\geq$6.5%) at over 422 million people—a number that has increased about four-fold over the last 35 years (NCD Risk Factor Collaboration 2016). Whereas interpretation of blood glucose levels is complicated in the absence of fasting, HbA1c is not affected by fasting because it is an indicator of the average plasma glucose concentration in the blood over the prior three months. During the three to four month life of a red blood cell, glucose molecules bond with hemoglobin molecules to form

glycated hemoglobin, A1c, which when isolated from other types of hemoglobin provides an indicator of the amount of glucose in the blood over the life of the cell.

Several established methods to measure HbA1c in DBS have been validated against venous blood. A recent meta-analysis of validations of HbA1c measured in paired DBS and venous samples concludes that the two are in close agreement when measured with high performance liquid chromatography (HPLC) and turbidimetric inhibition immunoassay methods (Fokkema et al. 2009, Affan et al. 2014, Mastronadi et al. 2015 and Miller et al. 2015). These studies have typically been conducted with blood samples collected under clinic-like conditions. Almost no evidence speaks to the performance of DBS measures when the samples are collected in population-representative field studies.

This study contributes to filling that gap by comparing HPLC-based HbA1c measures from DBS with venous blood measures for paired samples collected from subjects in Aceh, Indonesia, a low-resource setting characterized by high temperature and humidity. HPLC-reported values for DBS systematically overstate HbA1c in the blood by about 13%. This is a far larger overstatement than has been documented in the literature and result in a 500% overstatement of diabetes prevalence rates. Estimation of HbA1c, the percentage of hemoglobin A (HbA) that is glycated hemoglobin, depends on estimates of the numerator and the denominator. The HPLC produces graphs of the concentrations of hemoglobin variants and inspection of the chromatograms for the DBS establishes that the calculations of HbA1c percentage reported by the HPLC fail to capture part of the HbA area that likely belongs in the denominator. The first contribution of this research is that, by taking the missing HbA area in the DBS chromatograms into account, we establish the recalculated DBS HbA1c values are unbiased.

The study is designed to compare the validity of HbA1c measures using DBS collected in a controlled or clinic-type setting with DBS collected in a field-type setting where temperatures and humidity are more variable and, on average, higher. We show that HbA1c values in DBS collected in either setting suffer from the upward bias described above. That bias is removed when the missing HbA is taken into account. Moreover, these recalculated HbA1c values in DBS collected in a clinic setting match the venous blood values extremely closely. Values in DBS collected under field conditions are measured with more error, the size of which is modest for about half the DBS but sufficiently large in the other DBS to render those values unreliable. Data

recorded on the chromatogram provide a means of identifying samples that yield substantially biased values. Investigation of environmental conditions in the field at the time of collection suggests humidity is implicated in reduced precision of the measures. Further research in which environmental conditions during DBS creation, drying and assaying are carefully measured has the potential to yield important new information about reliability and validity of DBS results.

Finally, we conclude that the routine collection during the study and under the same field conditions of paired venous and DBS samples to cross-validate the HbA1c values is likely to be a good investment. Such data will assure the validity of the DBS-based values, provide an early warning about measurement problems and help insure against unforeseen problems encountered in the study.

2. Methods and data

We recruited 143 subjects recruited from three communities in Aceh, Indonesia, to participate in a study designed to evaluate different methods for the measurement of biomarkers related to four indicators of metabolic dysregulation: HbA1c, total cholesterol, HDL cholesterol and high sensitivity C-reactive protein, a marker of inflammation. This research focusses on HbA1c. Field work was conducted in two phases.

The first phase was conducted at our collaborating internationally-certified laboratory, Laboratorium Klinik Prodia, in Banda Aceh in February, 2016. 40 subjects presented at the lab, where a trained phlebotomist drew venous blood into two EDTA collection tubes. Using one of the tubes, Prodia assayed the samples the same day to measure HbA1c using ion exchange chromatography on a Bio-Rad D-10 System which is a fully automated HPLC system for the measurement of HbA1c and HbA2. The second tube was used to create four blood spots on Whatman 903 protein saver filter paper cards. The cards were air dried in the lab overnight, placed in plastic bags with desiccant packs and stored in a freezer at -30°C. Blood from the second tube was also used to measure HbA1c using the Afinion AS100 point of care test (POCT) system. We refer to this first assessment as our "controlled setting" since the lab was air-conditioned: the room temperature was kept around 22°C and relative humidity around 55% during the collection and drying periods as measured every ten minutes by Inkbird temperature and humidity loggers.

The second assessment was conducted in two communities outside Banda Aceh with 103 subjects. Identical procedures were followed in each community that mimic the procedures used in the clinic setting. Subjects presented at a community center where a trained phlebotomist drew two vials of blood. One tube was stored on ice packs; the second tube was used to create blood spots that were air dried overnight in the community center, placed in plastic bags with desiccant packs and transported to Banda Aceh within 24 hours where they were stored in freezers at -30C. All venous samples were transported to Prodia where they were assayed within 24 hours. We refer to this assessment as conducted under "field conditions" since air conditioning was limited. According to the loggers, temperatures were typically above 24°C and humidity was 70% or higher much of the time. In a study that collects DBS in the home, the environmental conditions are likely to be substantially more variable. We interpret contrasts between the controlled and field settings as indicative of the direction of differences likely to be observed in settings with less temperature and humidity control than typical clinic settings (e.g., no air conditioning).

After the second field sample assessment was completed, all the DBS were transported on dry ice to the Herningtyas laboratory at the Universitas Gadjah Mada, Yogyakarta, Indonesia where they were stored in freezers at -30°C until the DBS were assayed for HbA1c in January 2017. These assays were also conducted on a Bio-Rad D-10 HPLC system. All DBS protocols were validated against the same system in the Biomarker lab at the University of Washington (Herningtyas et al. 2018) using DBS created by the USC/UCLA Biodemography Center for cross-validation of biomarker measurement in studies across the globe (Crimmins et al. 2014).

Statistical analyses begin with a comparison of estimated moments of the distributions of the DBS values of HbA1c with the venous blood values from the Prodia lab. Paired samples are directly compared using bivariable linear regression models and the discrepancies between them are summarized using Bland –Altman plots. Multivariable regression models investigate whether temperature and humidity can explain the discrepancies.

3. Results

This subsection describes the distributions of the DBS-based and venous-based measures of HbA1c. Comparisons of paired samples for the same respondent indicate substantial, systematic upward bias in the DBS results. Hypotheses that might explain these differences are examined. Inspection of the chromatograms from the DBS analyses suggests an explanation and

possible correction which, when implemented, corrects for upward bias. After the correction the DBS and venous blood values are no longer systematically different. We then investigate whether the environmental conditions at the time of measurement are associated with the quality of the DBS measures.

A. DBS measures as reported by BioRad D-10

Results of measuring HbA1c for all 143 study subjects are summarized in panel A of Table 1. According to the values based on venous blood in the first column of the table, The average level of HbA1c is 5.72% based on venous blood (column 1) and 6.47% based on DBS analyses reported by the Bio-Rad D-10 (column 2). Since the same patterns describe the sub-samples collected under controlled (panel B) and field (panel C) conditions, we focus on the entire sample in this sub-section. The lower and upper quartiles of the study subjects lie between 5.0 and 5.7% according to the venous-based values but according to the DBS, the 25th percentile of the distribution is at the 75th percentile of the venous values; specifically the lower and upper quartiles are at 5.7% and 7.0%, respectively. The entire distribution of HbA1c is shifted to the right in the DBS-based values relative to the venous values (panel A of Appendix Figure 1). This shift is reflected in the fraction for whom measures indicate a diabetes diagnosis (HbA1c$\geq$6.5%). According to the venous-based analyses, 12% of the study subjects are diabetic but 5 times as many, 59%, are diabetic according to the DBS-based analyses. Assuming the venous values are correct, only about half the study subjects are accurately assigned to the correct side of the 6.5% divide by the DBS values. Not only are the DBS-based values substantially and significantly higher than the venous values but the upward bias relative to the venous values is clinically significant.

This upward bias is apparent in panel A of Figure 1 which displays the relationship between the venous values (on the x-axis) and DBS values reported by the HPLC (on the y-axis). The dashed 45° line represents equality of the two measures and the solid line is the estimated regression of the DBS values on the venous values. That regression is reported in the first column of Appendix Table 1a. Not only is it clear that the DBS values are upward biased for all values of HbA1c below 9% (which accounts for 95% of the subjects) but there is also substantial error in the DBS-based measures as summarized by the R^2 for the regression which is 0.73.

(Restricting attention to the 90% of subjects whose HbA1c is measured as below 7.5% by venous blood, the R^2 is only 0.36 as shown in column 2 of Appendix Table 1a.)

The DBS protocols and results from the Herningtyas lab have been carefully validated (Herningtyas et al. 2018), leaving open the possibility that the gold standard venous-based results are wrong. Panel B of Figure 1 displays the relationship between HbA1c measured for the same 143 subjects at the time the blood was drawn using an Afinion AS100 POCT and the venous-based values. The concordance is extremely high, with the R^2 being 0.97, and there is no evidence of significant bias. We conclude that the venous-based values are correct. (This concordance is extremely high for both the clinic and field samples.)

The DBS values in Panel A of Figure 1 are based on assays conducted in January 2017. It is possible that there were problems with those assays. All DBS were re-assayed at the Herningtyas lab in February 2017 to verify the original values. The correlation between the two DBS measures is 0.98 indicating very high test-retest validity and rigorous adherence to assay protocols. Results from the re-assay, in Panel C of Figure 1, are similar to those from the first assay.

DBS can be compromised in a field setting in various ways; they may have too little blood (we used venous blood to create optimally-sized and virtually identical spots); they may have been dried incorrectly (we dried them overnight as recommended); they may have been transported or stored inappropriately (we stored the DBS in plastic bags with desiccant packs in freezers and transported them on dry ice). It is possible to provide a direct assessment of whether the DBS had been compromised: the DBS used to measure HbA1c were also used to measure high sensitivity C-Reactive Protein (hsCRP) using an ELISA method with an FDA-cleared enzyme immunoassay kit manufactured by Percipio Biosciences. The DBS and venous-based values for hsCRP are displayed in panel D of Figure 1: they are very close indicating the quality of the DBS is sufficient to reliably measure hsCRP.

It has been established that, relative to venous-based assays, HbA1c values from DBS are typically upward biased (Little et al. 1985). However, the bias described in the literature is very small in comparison with the large bias documented in this study. Since venous or DBS assay implementation problems or DBS quality do not appear to be implicated, we turn next to investigate whether the bias is related to the environmental conditions under which the DBS were collected.

Recall that the first phase of data collection was conducted in a clinic-like setting with temperature and humidity well-controlled by air conditioning, whereas the second phase was conducted under field-like conditions (albeit with some air conditioning). Figure 2 separates subjects by the setting in which they were measured. In the controlled setting (panel A1), the DBS and venous values of HbA1c are very highly correlated (R^2=0.98) but the DBS values remain upward biased for the vast majority of subjects (Appendix Table 1b, column 1). Even conditioning on HbA1c<7.5%, the correlation between the DBS and venous values is very high (R^2=0.93, Appendix Table 1b, column 3). This suggests that it should be possible to re-construct HbA1c values by making an intercept and slope adjustment as suggested by the regression estimates in the figures and in accordance with Mastronadi et al. (2015).

The picture is more mixed for the DBS collected in the field setting. On the one hand, the bias is smaller than in the controlled setting, although it remains statistically significant for all cases whose HbA1c is measured using venous blood as below 7.5%. On the other hand, the DBS values are measured with more error in the field setting: the R^2 for all cases is 0.73 and only 0.36 for cases below 7.5% (Appendix Table 1b, columns 2 and 4, respectively).

B. Recalculated HbA1c values from DBS

The HPLC separates hemoglobin moieties detected in the DBS by charge. The samples are injected into a tube (chromatographic column) and interact with a solvent so that the various hemoglobins are released from the column at a specific time, the retention time. The concentration of the hemoglobin component is measured as it is released and these concentrations are plotted against the retention time to produce a chromatogram. Two examples, using DBS from this study, are displayed in Appendix Figure 2. The HbA1c concentration is given by the area under the A1c curve which is shaded. The example in panel A is from one of the control cards used to validate the protocols and follows the expected pattern. The HPLC reports estimates of the area under each of the Hb curves identified during the assay. The ratio of the area under the A1c curve to the total area of all curves up to and including Ao (but excluding fetal Hb, denoted F in the chromatogram) is adjusted for the instrument-specific calibration curve to yield the estimate of 4.9% attributed to HbA1c.

The example in panel B is drawn from the samples used in this study. It exhibits an unexpected peak to the right of Ao, identified as the Variant-Window, an observation pointed out

by Egier et al. (2011). The HPLC excludes this variant from the computation of the denominator to calculate the %HbA1c, which is reported as 10%. If the peak attributed to the Variant-Window had a slightly higher retention rate, it would likely have been identified as sickle Hb which is not thought to occur in this population (Piel et al. 2010) and so would be very surprising in this context. It is reasonable to assume that characteristics of the reliquified blood from the DBS caused the HPLC to identify the peak at the shoulder of Ao as an Hb-variant when, in fact, it is part of the Ao density function. Under that assumption, we have recalculated A1c as a percentage of total Hb, taking into account the instrument-specific calibration curve. Because the denominator is larger than that used in the value reported by the HPLC, the recalculated value is 1.4 percentage points smaller at 8.6%.[1]

Recalculated values of HbA1c from all of our validation samples are reported in column 3 of Table 1.[2] The upward bias in values reported by the HPLC relative to the venous values is fully addressed by the recalculations that correct the samples with pseudo-Hb variants: not only are the mean values very close, but the 25 and 75 percentiles of the distributions are also very similar. Moreover, 13% of the recalculated cases indicate diabetes in comparison with 12% of the venous values, and in 97% of the cases diabetes indications are the same for the recalculated and the venous values. The similarity of the distributions is illustrated in Appendix Figure 1 which displays nonparametric estimates of the distributions of the venous, recalculated and reported HbA1c values. The larger denominators in the recalculated values, which arise from including chromatographic areas that are ignored in the reported calculations, result in a shift of the recalculated distribution of HbA1c to the left of the reported values, which in turn makes it very close to the distribution of the venous values (Appendix Figure 1a).

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[1] Recalculated %HbA1c = ({ γ_0 + [(area under HbA1c curve)/(total area)] γ_1 } – δ_0)/ δ_1. where γ_0 and γ_1 are from the HPLC calibration curve and δ_0 and δ_1 are based on a regression that relates the HPLC estimates to whole blood equivalents based on data from the UW Biomarker lab. Specifically, we use: γ_0= -0.00615, γ_1 =1.439167, δ_0=0.0193, δ_1=0.6722.

[2] It is standard practice to verify values of HbA1c that are below 4% and above 14%; this protocol was followed for the HbA1c values reported by the HPLC but not for the recalculated values since they were not known at the time the assays were conducted (no reported or recalculated HbA1c values were above 14%). However, we have exploited the fact that all of the DBS were assayed twice to replicate the protocol. We examined all recalculated values below 4.5% in the first run; there are 12 cases and all but 2 are recorded as higher in the second run with 6 recording a value higher than 4.5%. For these cases, we have replaced the first run value with the second run value. One of the two values that is not higher in the second run is 3.8% in both runs; if this had been observed at the time of the assay, the DBS would have been inspected more carefully for problems. For the sake of comparability across samples, the case is included in the analyses. However, none of our conclusions is affected if this case is dropped.

All DBS-based HbA1c values reported in this paper follow standard practice and make regression-based adjustments to create whole-blood equivalent values. However, it is important to underscore that in comparison with the adjustments described above that correct the sample with pseudo-Hb variants, the regression-based adjustments are small. They account, for example, for 0.03 percentage points of the total adjustment of 0.83 percentage points in the shift at the mean for all samples in panel A of Table 1. Put another way, the adjustment for the variants accounts for over 96% of the difference in the means.

Results distinguishing DBS collected in the controlled and field settings are presented in panels B and C of Table 1, respectively. Whereas the gap between the distributions of the venous and reported DBS values is larger for DBS collected in the controlled setting relative to the field setting, the differences in the gap between the venous and recalculated distributions are small.

Although the similarity of the recalculated DBS and venous distributions is reassuring, it is nevertheless important to assess how well the recalculated DBS values line up with the venous values for each of the paired samples. They match extremely well, as shown in the lower panels of Figure 2. The data for DBS collected in the controlled setting, on the left hand side of the figure, indicate that not only has the bias in DBS values been removed by the recalculations but all the recalculated DBS and venous values are extremely close as indicated by their hugging the 45° line. This is summarized by the R^2 which is 0.98. There is a suggestion in the figure that at high values of HbA1c, DBS-based recalculated values are downward biased. This is consistent with the estimated regression line, in column 5 of Appendix Table 1b: the estimated slope is significantly less than one and the estimated intercept is significantly greater than zero. However, restricting attention to the 90% of the study subjects for which the venous-based values of HbA1c are less than 7.5%, the regression estimates of the slope and intercept are extremely close to and not statistically different from unity and zero, respectively (column 7), respectively confirming that the recalculated DBS-based values are too low at high levels of HbA1c and the overall regression is pivoted by those values. Although the difference between the recalculated and venous-based values of HbA1c is not clinically important, the results suggest a non-linear transformation of the DBS-based reported values may perform even better.

Results for DBS collected in the field setting, shown in the lower right hand panel of Figure 2, also indicate no bias in the recalculated values. However, there is a good deal of error in those values and, as a result, while the regression slope and intercept are far from unity and

zero, respectively, the estimates are poorly determined and neither estimate is statistically significantly different from its target value. (For example, in the model using values of HbA1c<7.5% in column 8 of Appendix Table 1b, the slope is 0.85 and the intercept is 0.64. The reduced precision is reflected in the large standard errors and the small R^2 which is 0.46. As shown in column 6 of the table, the R^2 for the entire field setting sample is only 0.85.)

In sum, with the chromatogram data produced by the HPLC, it is straightforward to recalculate the values of HbA1c and those values match closely with the venous-based values, although the quality of the match varies with the environmental conditions under which the DBS were collected. It would be convenient if an equation could be developed to translate reported to recalculated values. Appendix Figure 3 provides direct evidence on the likely success of this approach. The figures display the reported HbA1c values on the x axes and the recalculated values on the y axes along with a nonparametric regression estimate of the relationship between the two. In the controlled setting (panel A), the relationship is essentially linear and extremely well determined: a linear function will translate the reported values to values that are almost identical to the recalculated values. Unfortunately, this is not the case in the field setting (panel B): the function is not linear and the relationship displays a good deal of dispersion around the estimated function. In this context, the chromatograms are critical: without them, it is not possible to construct unbiased recalculated values from the reported DBS-based HPLC values.

C. Evaluation of chromatograms

It is useful to inspect the chromatograms more closely in order to assess whether any of the DBS can be identified as compromised. While several potential criteria exist, in this case, retention time turns out to be most informative. Specifically, drawing on retention times from the controlled setting DBS, we identify results as standard if the time HbA1c integration reaches its peak is between 0.80 and 0.87 minutes, if it returns to baseline by 1.75 minutes, if total integration time for HbA1c is less than 1.1 minutes and total time for the chromatogram is less than 2.5 minutes. Of the DBS collected under controlled settings, 2.5% (i.e. one card) was outside these limits and identified as substandard; of the DBS collected under field conditions, 46% were identified as substandard (47 cards).

Separating the DBS by these criteria is key. Figure 3 displays the relationship between the venous-based and recalculated DBS-based values of HbA1c for the standard DBS (in panel

A) and substandard DBS (in panel B). The regressions underlying the estimated lines are in panel C of Appendix Table 1b. The venous and recalculated DBS HbA1c values are extremely close for the standard DBS and the values are tightly clustered around the regression line (R^2=0.95 for all samples and 0.81 restricting to the venous-based HbA1c values<7.5%). There is much more error (or "noise") for the substandard DBS: the R^2 is 0.68 for all substandard DBS and only 0.26 for those DBS for which the venous-based values of HbA1c<7.5%.

D. Summarizing key results

Thus far, we have established that the reported DBS-based HbA1c values from the Bio-Rad D-10 are biased upwards relative to the venous-based values but, with the chromatograms, it is straightforward to recalculate the DBS-based values. The main conclusions from analyses of those data are summarized well by the Bland-Altman plots in Figure 4 (Bland and Altman 1986). The zero abscissa is in red, the mean difference between the recalculated DBS-based and venous-based values is represented by the horizontal short dashed line and 1.96 times the standard deviation of the difference is represented by the longer dashed lines.

Recalculated values from DBS collected in the controlled setting (in panel A) match the venous-based values very closely, all but one lie within the standard deviation bars and the extreme value is at a high HbA1c value, indicating the recalculated DBS values are downward biased at these levels as noted above. Panel B of the figure displays the Bland-Altman plots for the DBS collected under field conditions. The plot for the DBS judged to be of good quality after inspection of the chromatograms, in panel B1, is remarkably similar to the plot in panel A for DBS collected in the controlled setting apart from a small downward bias.

Results from the DBS collected in the field but judged substandard based on the chromatograms are not as satisfactory (panel B2). While there is no evidence of bias in the recalculated DBS-based estimates for this sample, the values are extremely noisy as indicated by the wide standard deviation bars. This poses a problem for clinical applications. However, in large-scale population-based studies, the costs of noise in the estimates may be mitigated by measuring HbA1c for larger samples under the assumption that there are no subject-specific characteristics that are correlated with the direction or magnitude of the noise. While that would seem a reasonable working assumption, the question is important and warrants systematic interrogation.

E. Environmental factors that predict DBS rejections

This study was not designed to identify characteristics that are correlated with noise in the DBS-based estimates of HbA1c. However, the data can provide evidence on the extent to which environmental conditions at the time the DBS were collected predict whether or not the chromatogram is judged standard or sub-standard. Given the results in Figures 3 and 4, this provides one window into the extent of noise in the DBS-based HbA1c measures.

We focus on temperature and relative humidity variation and restrict attention to DBS collected in our field settings (since only one DBS was rejected in the controlled conditions). It is important to re-iterate that because we conducted the study in community centers with good ventilation, environmental conditions were likely less extreme than would otherwise be the case. There is very little variation in the amount of time that DBS were dried, although the conditions under which they were dried vary with the time of day the blood was collected from study subjects. There is effectively no difference in the handling and storage of DBS after collection.

Table 2 reports results of multivariable regression models that test whether the percentage of DBS collected in the field setting that are judged substandard are predicted by environmental factors measured at the time of blood collection. The first model, which includes humidity and temperature entered linearly, indicates substandard rates rise significantly with humidity but not with temperature. As shown in the second model, the effects of humidity are approximately linear and significant when humidity exceeds 70%. In a preliminary bivariate model, we found substandard rates are significantly higher only when temperature exceeds 24C but, as the second model shows, there is no independent effect of temperature after controlling humidity. These results are graphically summarized in Figure 5. We find no evidence that time of day when the DBS were collected predicts substandard rates, suggesting that collection in the cooler evening does not materially impact the DBS from the perspective of measurement of HbA1c.

These results have two important implications for the design of studies that collect DBS in adverse field settings. First, the relative humidity at the time DBS are collected (and probably the conditions during drying) affect the quality of HbA1c measurements extracted from the blood on these spots. Second, when humidity levels are high, DBS-based HbA1c values are very noisy. It would be prudent to collect information on humidity and temperature at the time DBS

are collected and dried in the field and it is possible that the noise in the DBS-based values may be mitigated by adjusting for environmental factors when calculating HbA1c.

F. Estimation of HbA1c when chromatograms are not available

We have established that when chromatograms are not available, it is difficult to generate the recalculated values of HbA1c using a general-purpose regression model. However, if HbA1c is measured for a random subsample of study subjects using both DBS and venous blood, it may be possible to use those paired observations to estimate a function that translates the reported DBS-based measurement to venous blood equivalents for the entire sample. To investigate the feasibility of this approach, half the sample values from this study were randomly selected to estimate the model:

$$\text{lnVenous} = \beta_0 + \beta_1 \, \text{lnDBS} + \beta_2 \, \text{lnDBS}^2 + \varepsilon \qquad [1]$$

where lnVenous is the logarithm of the venous-based HbA1c values and lnDBS is the logarithm of the values that are reported by the BioRad D-10. The quadratic term is included to capture the fact that DBS-based values tend to be downward biased at high values of HbA1c. Each of the estimated coefficients, including the quadratic term, is statistically significant and the R^2 for the model is 0.84.

The results from [1] were used to predict whole blood equivalents for the other half of the sample using the reported DBS values. The distributions of the venous-based values, reported DBS-based values and the predicted values from regression [1] for this half-sample are displayed in panel B of Appendix Figure 1. The distribution of the predicted values matches the distribution of the venous-based values very well. First, the centers of the distributions of the venous-based and predicted values for this half-sample are extremely close: they are 5.78% and 5.73%, respectively. The mean of the recalculated DBS values is very similar, 5.70% while the mean of the reported DBS values is much higher, 6.43%. Second, the shape of the distribution of the predicted values mimics the venous-based values. In contrast, the shape of the distribution of the reported values does not mimic the venous-based values. This is an important point: shifting the center of the distribution of the reported DBS values to match the mean of the venous-based distribution (or the expected mean for the population based on external data) will not yield HbA1c estimates that match the distribution of the true HbA1c values.

Of course, the regression model [1] cannot address the noise that is inherent in the HbA1c values derived from DBS that were collected in field conditions and, especially, the DBS that were rejected after examination of the chromatograms. To wit, in a regression relating the predicted values for the half-sample not used to estimate [1] to the lab values, the R^2 is 0.72 which is very similar to the value for the reported values from DBS collected in the field setting in Figure 2 panel B1. The only way to reduce the noise in these measures is to isolate the sources of the noise and adjust study protocols or build an empirical model that adjusts for that noise.

These results suggest that a sample of paired venous and DBS samples collected under the field conditions in the study can assure that estimates of HbA1c from the study replicate the distribution in the underlying study population even in the absence of chromatograms needed to recalculate DBS-based values. Our results indicate that building a venous-blood validation study for a subsample of study subjects into the protocol for the collection of DBS in a challenging field setting is likely to be a good investment. The paired samples not only provide an important check on the quality of the measurements but also provide insurance in situations like the example of HPLC-based measures of HbA1c described above. Moreover, as noted by Gregg et al. (2014), a validated cross-walk between DBS and venous blood values collected from each wave of a study will facilitate longitudinal analyses of DBS-based results.

3. Conclusions

Using venous blood drawn from study subjects in a challenging field setting, we have compared the HbA1c values measured using venous samples assayed in a Bio-Rad D-10 HPLC at a validated lab with the HbA1c values from DBS created from the blood samples at the same time and assayed in a different lab, also using a Bio-Rad D-10. We have three main conclusions.

First, the reported values of HbA1c from HPLC analyses of DBS are substantially upward biased for all values of HbA1c that lie below 7.5%. These cases account for over 90% of the sample in this study. The reason is that the values reported by the HPLC exclude part of the area under the Hb curves that should be included in the denominator of the calculation of the percentage HbA1c. Since this exclusion shrinks the denominator, the HbA1c:HbA ratio is systematically upward biased. This bias is substantively large and clinically important in our study setting using the Bio-Rad D-10. For example, whereas 12% of the study subjects presented with HbA1c$\geq$6.5% according to the venous-based assays, this percentage was 59% based on the

reported results from the DBS assays. We show that with data from the chromatogram produced by the HPLC, it is straightforward to recalculate the percentage HbA1c and those values closely match the paired venous-based values. For example, 13% of these cases present with HbA1c$\geq$6.5% and 97% of the classifications are the same in the venous-based and recalculated DBS-based values. Not only is the recalculation simple, it is also easy to automate.

A limitation of this study is that it is not clear why the Bio-Rad D-10 reported values are upward biased. It is possible that creating DBS from venous blood collected in EDTA tubes compromised the measures although anti-coagulants do not affect HbA1c measures using venous blood or in POCTs). It is also possible that handling procedures affected the DBS, although we followed standard protocols for drying and storing DBS and we have measured hsCRP with great accuracy from the same DBS. It may be that lab procedures are implicated; with two plates of assays, we are unable to investigate this issue in any depth. However, we provide evidence that the environmental conditions under which the DBS are collected affect the quality of the HbA1c values. Our second conclusion is that the conditions at time of collection affect both the reported and recalculated HbA1c values. While collection of DBS in well-controlled conditions yields (recalculated) HbA1c values that closely match the paired venous values, collection in humid conditions results in HbA1c values from DBS that are very noisy and are not likely to be reliable for clinical purposes. A limitation of this study is that it is too early to tell whether this noise is random, which would affect the precision with which associations are estimated but would not bias association studies. There is a need for systematic assessments of the roles that temperature and humidity play in order to develop improved protocols for the collection and analysis of DBS that will be used to measure HbA1c. Rigorous evaluations of collection methods that control temperature and humidity in the field in a cost-effective way would be of substantial value.

Third, in addition to validating field collection and laboratory protocols, studies conducted in challenging field conditions are likely to benefit from also validating HbA1c results from DBS assays against an established measure of HbA1c using paired samples collected under the actual field conditions of the study for a sample of subjects. Incorporating this type of validation protocol into field-based biomarker measurement studies will not only provide a reliable mechanism to translate DBS values to serum or whole blood equivalent values but also provide insurance against the types of problems that have arisen with the reported DBS-based HbA1c values in this study. With the paired field-conditions measures of HbA1c, it is possible to

adjust the DBS values even in the absence of chromatograms for each subject. Whereas this project used a reference lab for this validation, a POCT device, such as the Afinion AS100 used in this study, may be a logistically less demanding alternative for in-field validation.

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Fig 1. DBS, venous and POCT measures

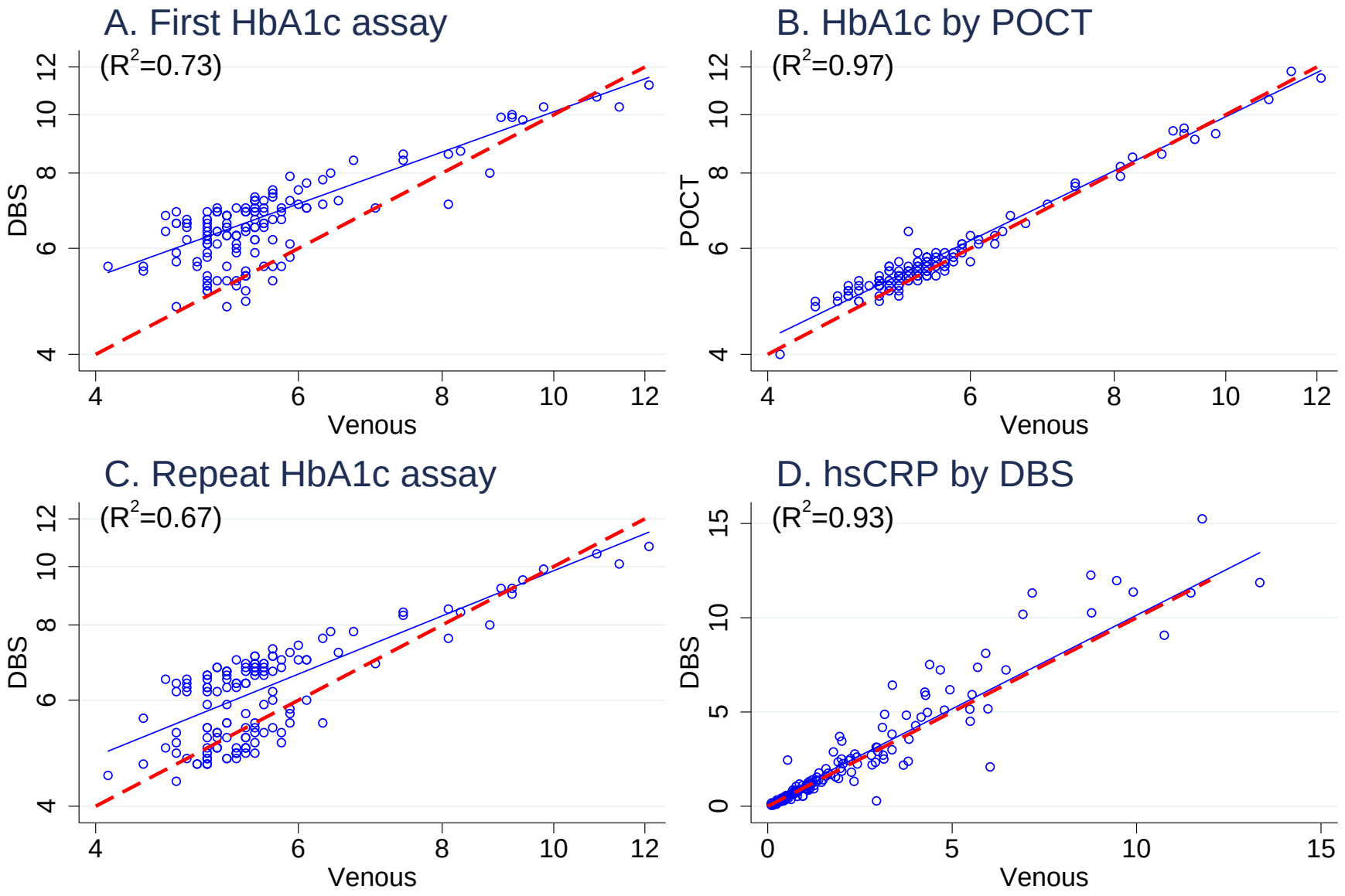


Fig 2. Venous, reported DBS and recalculated DBS values

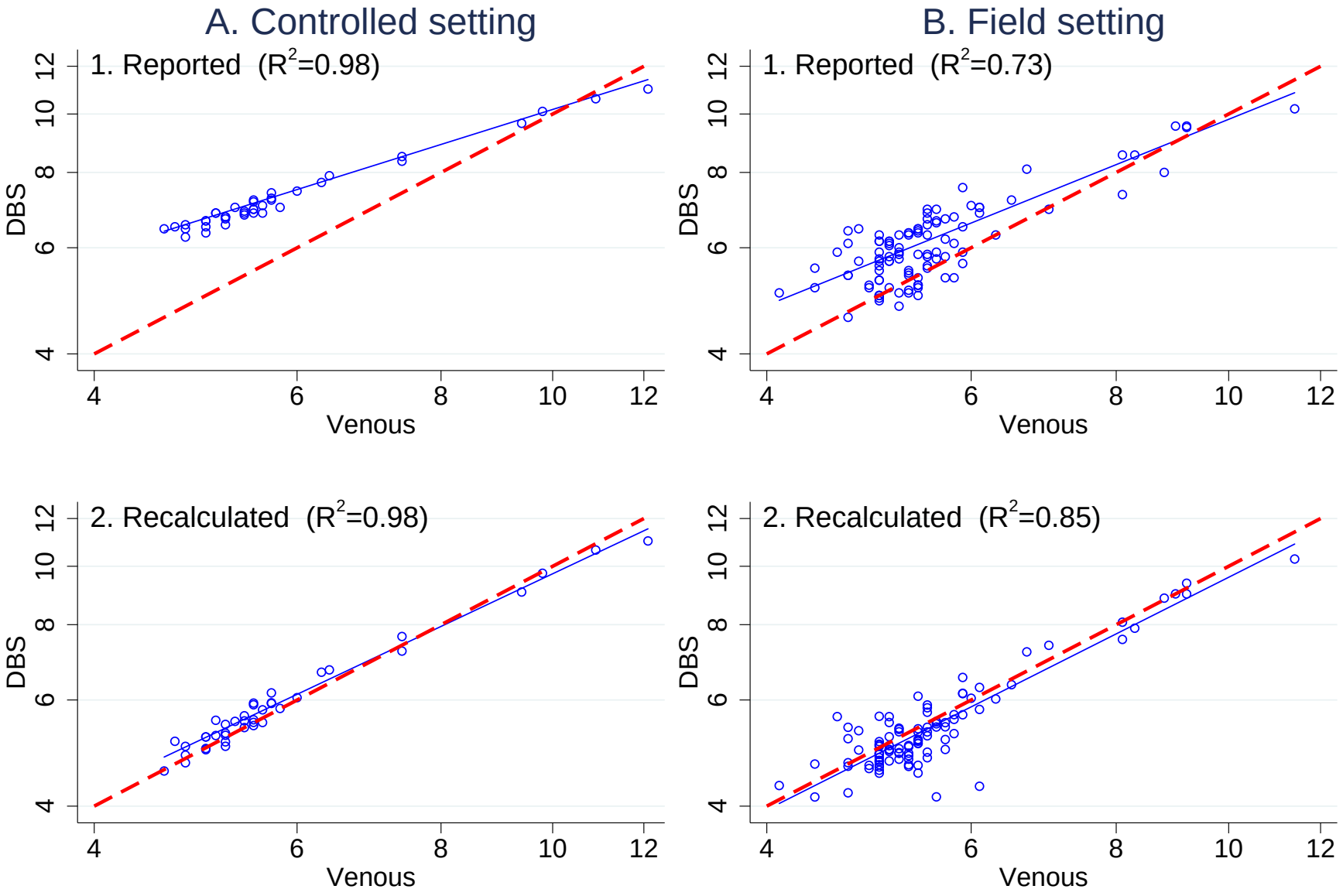


Fig 3. Recalculated DBS and Lab values from field setting

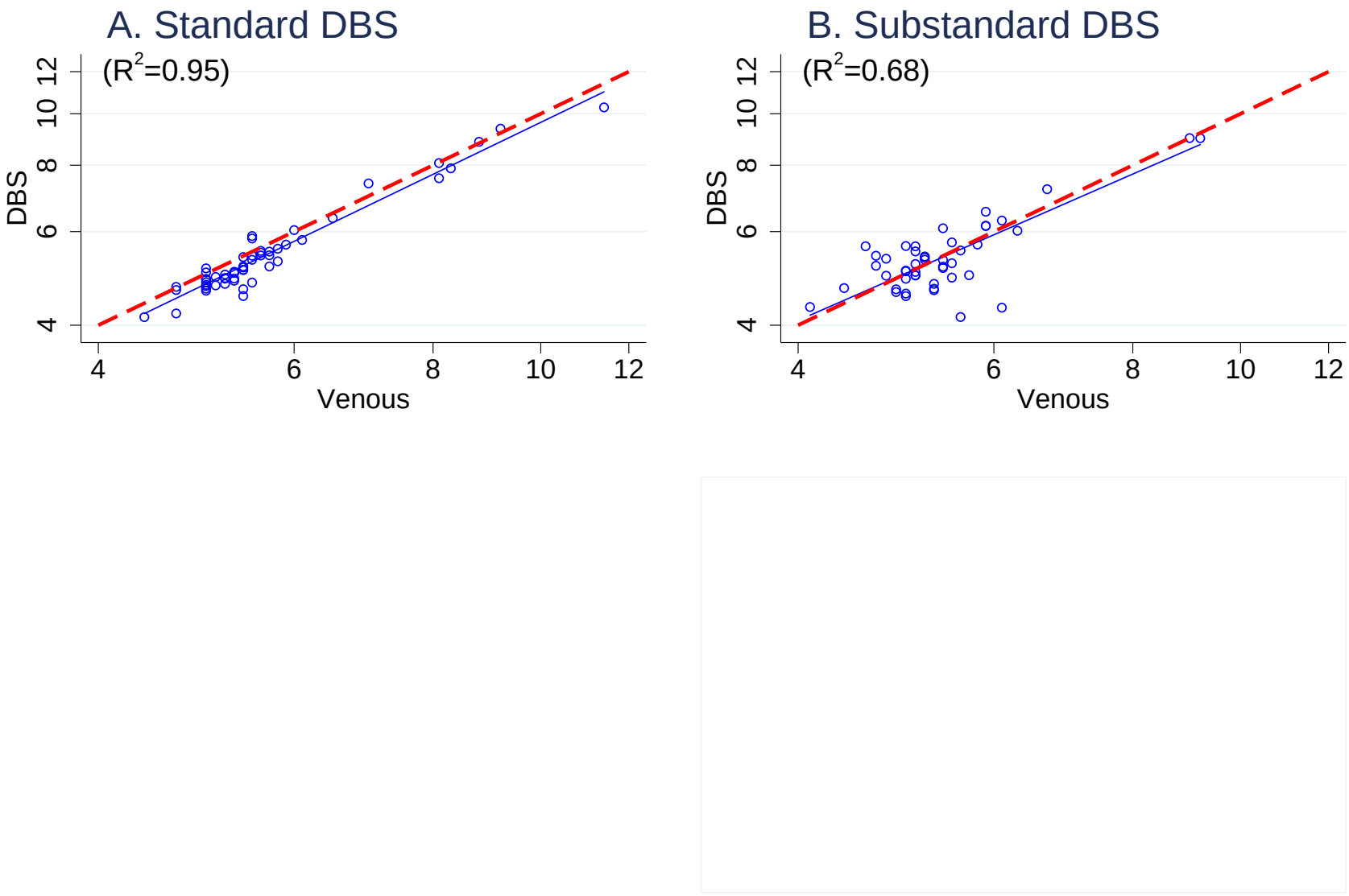
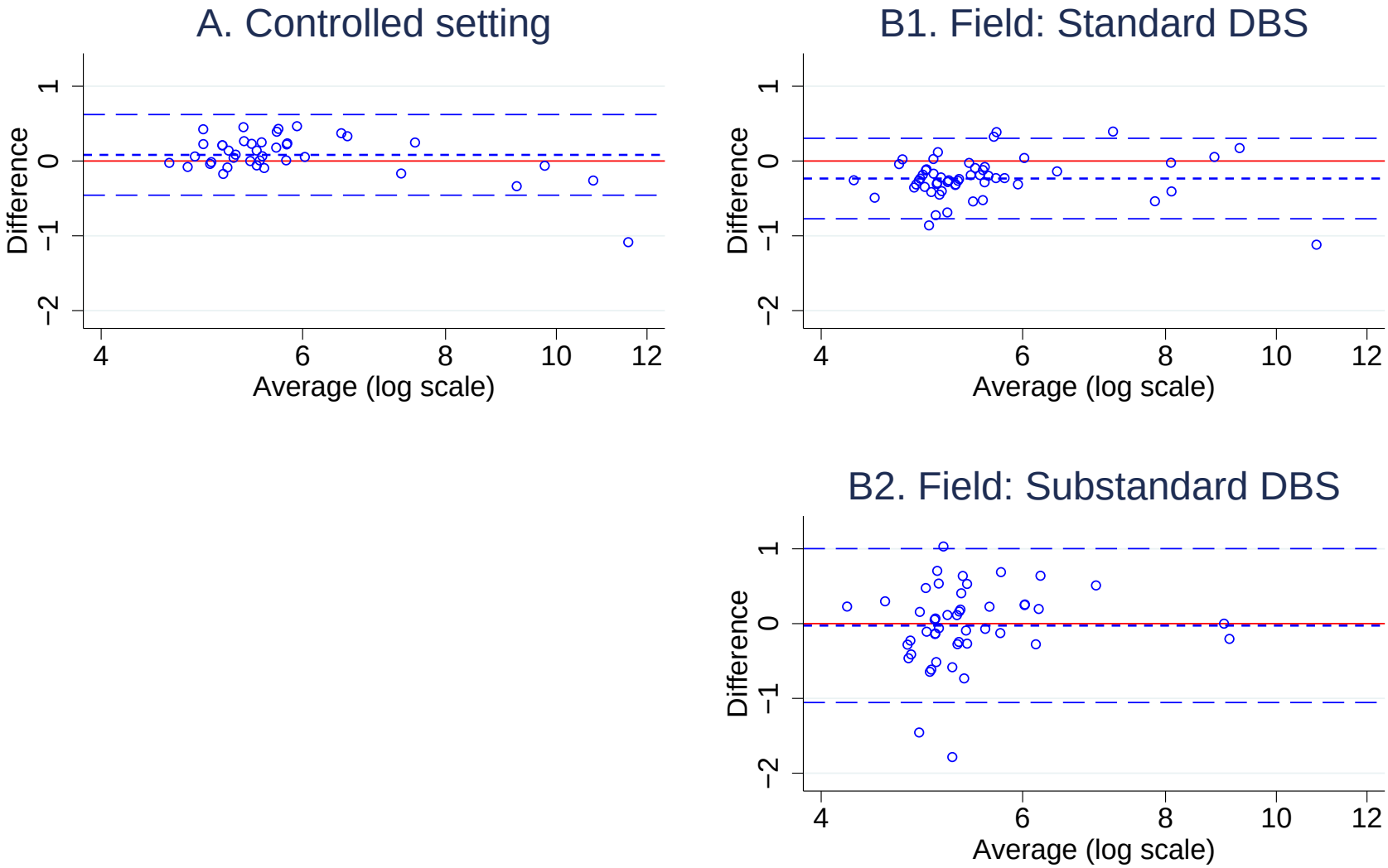
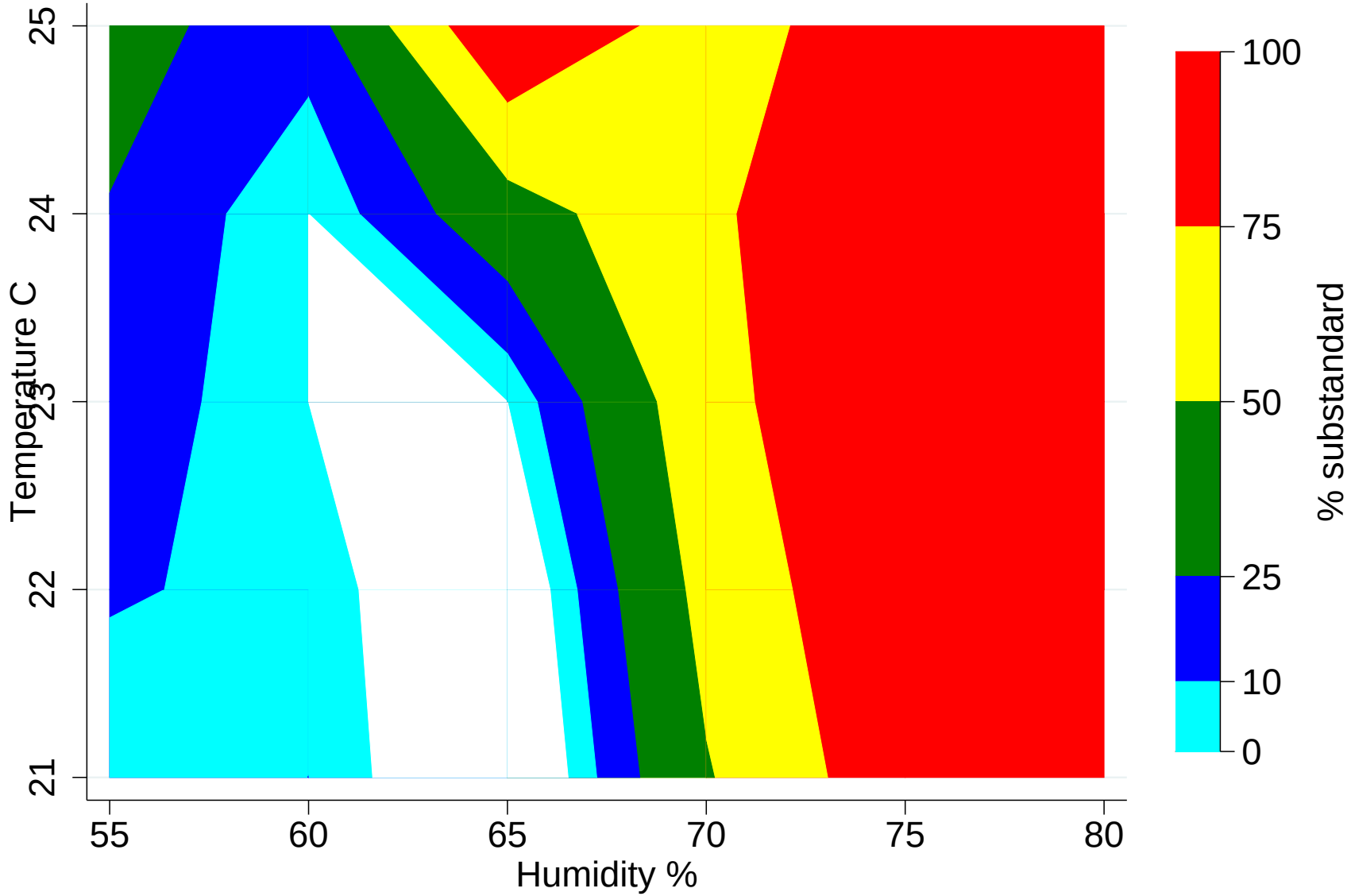


Fig 4. Bland−Altman plots: Recalculated DBS and Lab values

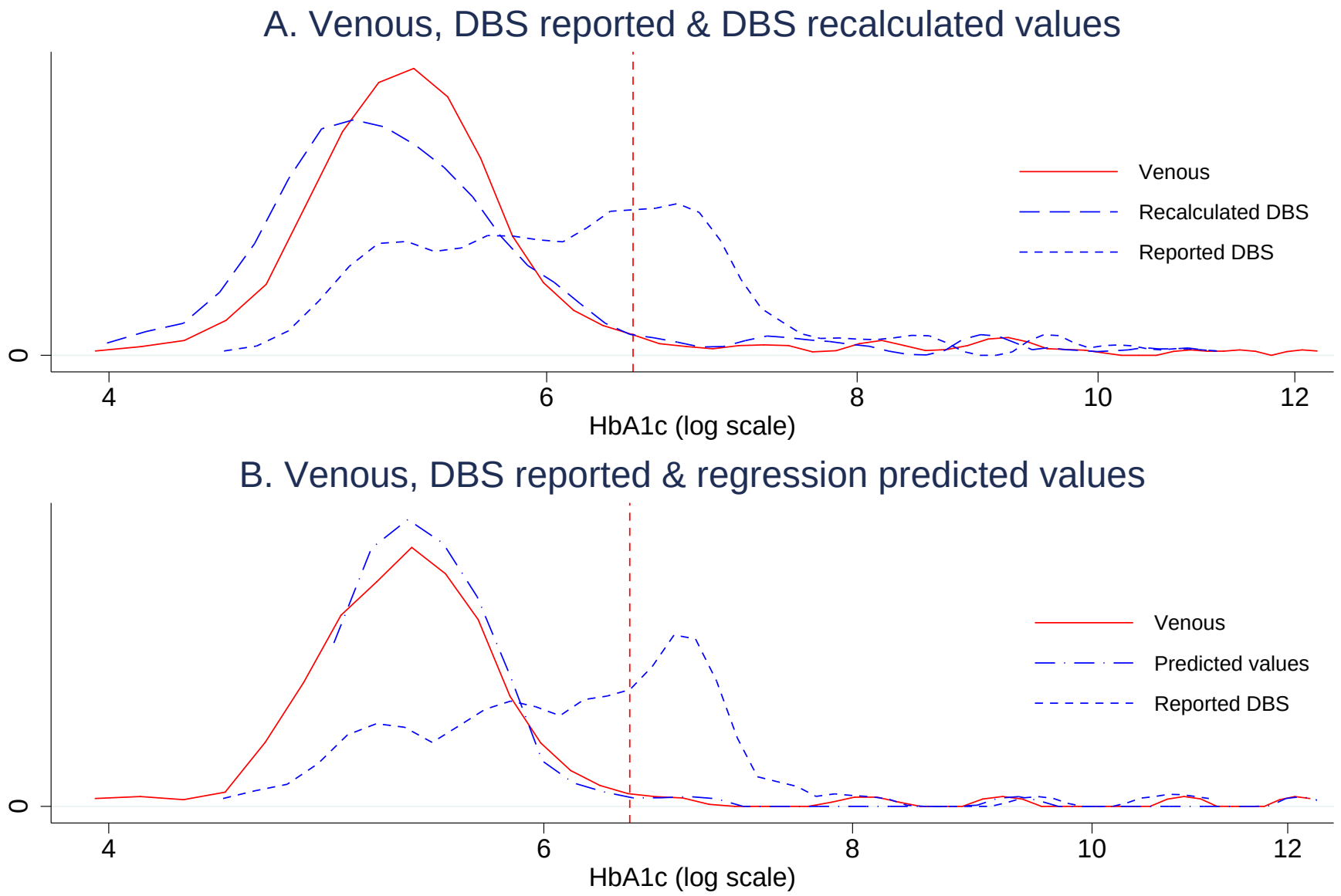


Note: Difference=Recalculated DBS−Venous value

Fig 5. % substandard DBS by humidity and temperature

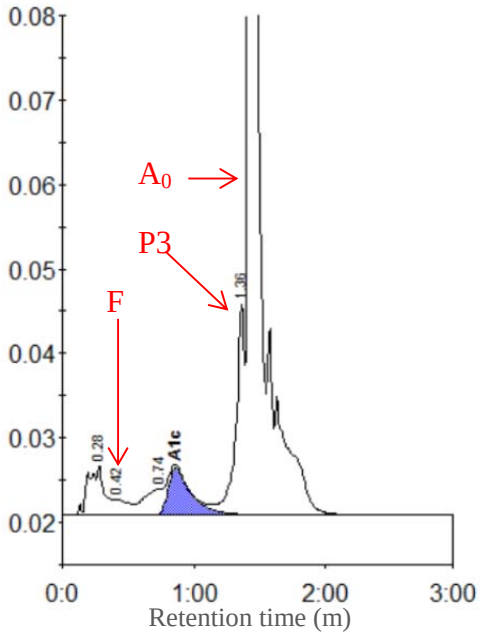


Appendix Fig 1. Distributions of HbA1c %



Appendix Fig 2: Sample chromatograms from DBS

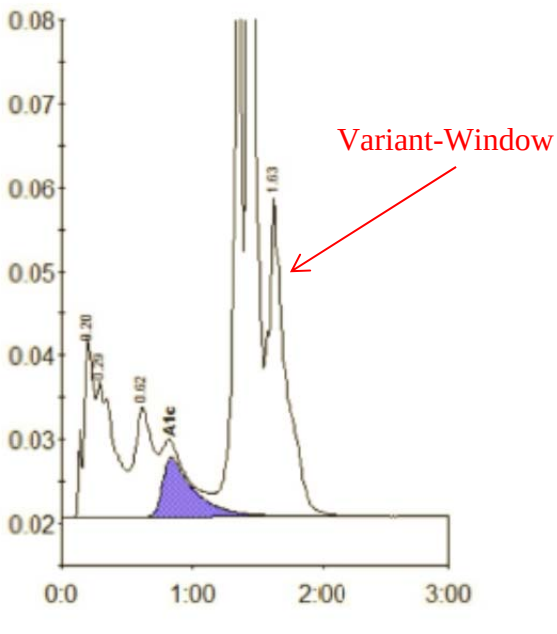
A. Well-determined example



Peak table

Peak	R.time	Height	Area	Area %
A1b	0.28	5960	52031	2.9
F	0.42	1892	14055	0.8
LA1c/CHb-1	0.74	3044	31473	1.7
A1c	0.86	5575	68225	4.9
P3	1.36	24926	111745	6.2
A0	1.42	414461	1527441	84.6
Total Area:	1804971			
A1c	4.9%			

B. Example with problem Hb variant



Peak table

Peak	R.time	Height	Area	Area %
A1a	0.20	21380	115863	5.9
A1b	0.29	16006	153686	7.9
LA1c/CHb-1	0.62	13016	128495	6.6
A1c	0.84	7086	120006	10.0
P3	1.36	78154	342514	17.5
A0	1.44	201748	791851	40.6
Variant-Window	1.63	38262	300091	15.4
Total Area:	1952505			
A1c	10%			

Appendix Fig 3. Reported and recalculated HbA1c %

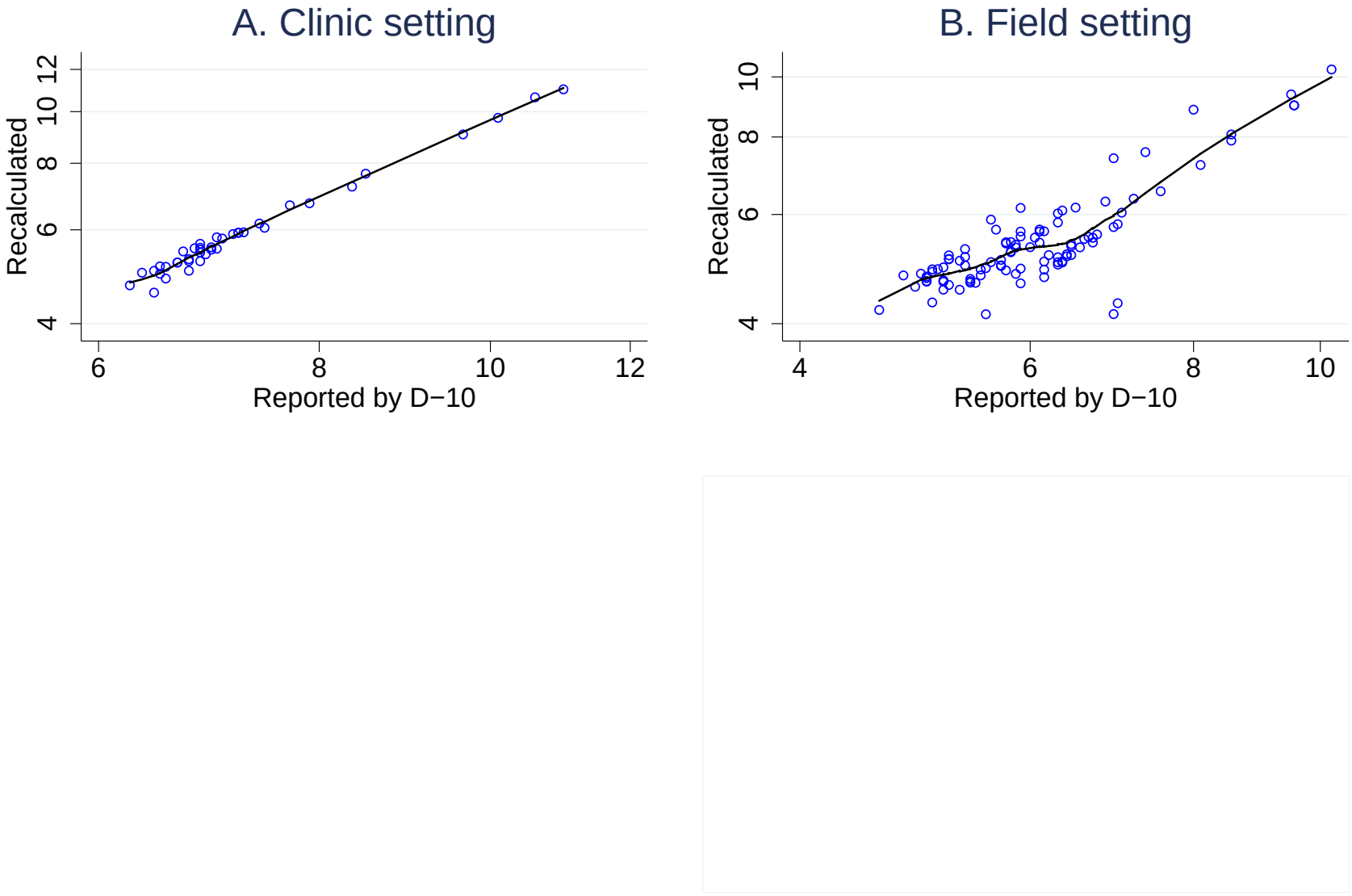


Table 1. Distribution of HbA1c values
Venous, reported DBS and recalulated DBS values

		A. All measures			B. Collected in controlled setting			C. Collected in field setting		
		Venous values [1]	DBS reported [2]	DBS recalculated [3]	Venous values [4]	DBS reported [5]	DBS recalculated [6]	Venous values [7]	DBS reported [8]	DBS recalculated [9]
% HbA1c										
Mean		5.72	6.47	5.63	5.97	7.31	6.05	5.62	6.14	5.46
Std error		0.11	0.10	0.11	0.27	0.18	0.24	0.11	0.11	0.11
25%ile		5.0	5.7	4.9	5.1	6.7	5.2	5.0	5.4	4.8
75%ile		5.7	7.0	5.8	5.8	7.3	6.0	5.7	6.5	5.6
HbA1c >=6.5%										
% of cases		12	59	13	15	93	20	11	46	11
Congruence of values										
Lab>=6.5%	% agree		41	86		8	80		54	88
	% disagree		47	2		78	5		35	1
Lab < 6.5%	% agree		12	11		15	15		11	10
	% disagree		0	1		0	0		0	1
Total	% agree		53	97		23	95		65	98
	% disagree		47	3		78	5		35	2
% DBS sub-standard							2.5			46.1
Sample size		143	143	143	40	40	40	103	103	103

Table 2. Percentage of DBS that are substandard and environmental characteristics Relationships with humidity and temperature at time of collection of DBS

		% DBS that are substandard (field setting only)	
	[1]	[2]	[3]
Humidity (%)	5.73		
	[1.37]		
Temperature (C)	-3.91		
	[6.60]		
Relative humidity (<60% reference)			
60-64.9%		13.69	15.72
		[12.38]	[12.22]
65-69.9%		26.15	31.33
		[19.40]	[19.11]
70-74.9%		54.27	62.82
		[21.87]	[22.06]
>=75%		69.09	76.29
		[28.57]	[28.23]
Temperature (<21C reference)			
21C			
22C			
23C			
>=24C		20.91	20.51
		[19.15]	[18.72]
Time of day			1.84
			[1.79]
Intercept	-237.00	10.00	-17.92
	[83.73]	[7.27]	[25.19]
R^2	0.42	0.40	0.43
Observations		103	

Standard errors in brackets below coefficient estimates.

Appendix Table 1a. Relationship between DBS-based values and Lab values

Linear regression estimates and standard errors

	A. Reported DBS (by D-10)		B. Recalculated DBS	
	All values	Lab<7.5%	All values	Lab<7.5%
	[1]	[2]	[3]	[4]
Venous value	0.78	0.92	0.93	0.94
	[0.04]	[0.11]	[0.03]	[0.07]
Constant	1.99	1.29	0.32	0.24
	[0.24]	[0.58]	[0.15]	[0.37]
R^2	0.73	0.36	0.90	0.59
Observations	143	131	143	131

Standard errors in brackets

Appendix Table 1b. Relationship between DBS-based values and Lab values

Linear regression estimates and standard errors by collection conditions

	A. Reported DBS				B. Recalculated DBS				C. Recalculated DBS (Field setting)			
	All values		Lab<7.5%		All values		Lab<7.5%		All values		Lab<7.5%	
	Controlled setting	Field setting	Controlled setting	Field setting	Controlled setting	Field setting	Controlled setting	Field setting	Field-Standard	Field-Substd	Field-Standard	Field-Substd
	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]
Venous value	0.67	0.81	0.78	0.86	0.89	0.94	1.01	0.85	0.97	0.90	1.09	0.67
	[0.02]	[0.05]	[0.04]	[0.12]	[0.02]	[0.04]	[0.05]	[0.10]	[0.03]	[0.09]	[0.08]	[0.17]
Constant	3.31	1.57	2.72	1.33	0.71	0.20	0.07	0.64	-0.06	0.50	-0.73	1.69
	[0.10]	[0.28]	[0.20]	[0.64]	[0.12]	[0.22]	[0.27]	[0.52]	[0.17]	[0.51]	[0.42]	[0.90]
R^2	0.98	0.73	0.93	0.36	0.98	0.85	0.92	0.46	0.95	0.68	0.81	0.26
Observations	40	103	36	95	40	103	36	95	55	48	49	46

Standard errors in brackets