Application of a Fully Automated Dried Blood Spot Method for Therapeutic Drug Monitoring of Immunosuppressants

Another Step Toward Implementation of Dried Blood Spot Analysis

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• ***Context.***—**The follow-up of patients under lifelong immunosuppressant therapy is pivotal to prevent allograft rejection after transplant. Part of the difficulties associated with routine monitoring of immunosuppressant concentrations can be alleviated by home sampling using dried blood spots (DBSs).**

Objective.—**To evaluate the applicability of a DBS method for the determination of immunosuppressants in venous blood samples, making use of an automated extraction platform.**

Design.—**Paired venous DBSs and whole blood samples were analyzed for tacrolimus (n = 162), sirolimus (n = 47), everolimus (n = 45), and cyclosporin A (n = 61) with liquid chromatography coupled to tandem mass spectrometry, using fully automated extraction for DBSs. Agreement between the automated DBS and whole blood method was assessed by using Bland-Altman comparison. Both an analytical and a clinical acceptance limit were predefined at more than 67% of all paired samples within 20% of the mean of both samples and more than 80% of all paired samples within 20% of the whole blood concentration, respectively.**

Results.—**An impact of the hematocrit (hct) on DBS quantitation was observed for all analytes, which could be alleviated for all analytes by using a hct conversion formula based on a tacrolimus data subset: [$DBS_{corrected}$] = [$DBS_{measured}$]/(1.6305 – 1.559*hct). After correction, both analytical and clinical acceptance criteria were met for all analytes.**

Conclusions.—**Automated DBS analysis shows great potential for routine therapeutic drug monitoring of immunosuppressants, avoiding any manual sample handling.**

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The calcineurin inhibitors tacrolimus and cyclosporin A and the mTOR inhibitors sirolimus and everolimus have been applied in the past decades in transplant care to prevent rejection. Because of the optimization of immunosuppressant therapy, adjusted 1-year graft survival rates of renal transplants have increased to up to 97% for living donors in 2017.[1] Despite this improvement, lifelong monitoring of the blood levels via therapeutic drug monitoring (TDM) remains one of the key aspects of the follow-up of these patients. The main reason for the necessity of TDM for these drugs is their narrow therapeutic range and large variation in interindividual and intraindividual pharmacokinetics. The need for lifelong monitoring puts quite a burden on the patient, as this requires regular traveling to the hospital to have their blood samples drawn and analyzed. This can partially be overcome by the use of dried blood microsampling, as this approach enables sampling in the home context, with samples being sent via regular postal services to the laboratory. The current COVID-19 pandemic has taught us the value of this approach, as for vulnerable patients it would be very beneficial to avoid regular hospital visits for routine blood draws.

Different methods for the analysis of immunosuppressants from dried blood spots (DBSs) have been successfully developed and clinically applied.[2–5] However, although the benefits from a patient-centric point of view are largely accepted, more efforts are still needed to enable implemen-

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tation in a routine clinical laboratory, as automated methods are still lacking. To overcome this bottleneck for implementation, we previously set up and validated a fully automated DBS-based method[6] to overcome the labor-intensive manual handling associated with the extraction of dried blood microsamples. Such an automated setup fits better in the increasingly automated workflow of many clinical laboratories. The use of automated extraction is also beneficial from a sensitivity point of view. A main difference is that when using manual extraction, typically only a part of the extract can be injected onto the liquid chromatography tandem mass spectrometry (LC-MS/MS) system, whereas when using automated extraction the full extract is transferred. Another barrier for implementation that can be identified is the lack of standardization in the field. As a next step, standardization of microsampling methods should be considered to a reference method in whole blood. Recently, such a reference method was established by Taibon et al.[7]

This study aims to demonstrate the clinical applicability of our methodology by applying it on a large set of venous patient samples to assess the agreement with venous EDTA-anticoagulated whole blood. To this end, we applied 2 fully validated LC-MS/MS methods for the analysis of DBSs (fully automated extraction) and venous whole blood samples (manual extraction), using venous leftover samples from patients treated with tacrolimus, sirolimus, everolimus, or cyclosporin A. Demonstrating concordance between results obtained by fully automated DBS analysis and conventional liquid blood analysis is pivotal before proceeding to a next stage, in which capillary samples will be analyzed in a home-sampling study to confirm clinical applicability for patients.

MATERIALS AND METHODS

Chemicals, Stock Solutions, and Calibrators and Quality Controls

Tacrolimus, sirolimus, everolimus, cyclosporin A Cerilliant stock solutions (1 mg/mL in acetonitrile) were purchased from Sigma-Aldrich (Diegem, Belgium). Ammonium formate, zinc sulfate, and 2-propanol were also obtained from Sigma-Aldrich. The internal standards tacrolimus-^{13}Cd4, sirolimus-^{13}Cd3, and cyclosporin-d12 were purchased from Alsachim (Illkirch Graffenstaden, France), while everolimus-d4 was from TRC (Toronto, Canada). LC-MS–grade acetonitrile was obtained from Biosolve (Valkenswaard, the Netherlands). Formic acid and LC-MS–grade methanol were purchased from Chem-Lab (Zedelgem, Belgium). Ultrapure water was produced by a Millipore purification system (Merck Millipore, Overijse, Belgium). Desiccant packages were obtained from Sigma-Aldrich (10-g Minipax absorbent packets). PE 226 DBS cards were purchased from Perkin Elmer (Waltham, Massachusetts). Based on the commercially purchased solutions, stock and working solutions were generated for all 4 analytes. On the day before analysis, aliquoted working solutions for the calibrators and quality controls (QCs) were thawed and spiked to whole blood (for the analysis of DBSs). The spiking volume was 2%, and the spiked blood was incubated on a roller for 1 hour before the generation of DBSs or the extraction of whole blood, as also outlined in Deprez et al.[6] Calibrators with nominal concentrations of approximately 1, 2, 3, 5, 10, 20, 30, 40, and 50 ng/mL for tacrolimus, sirolimus, and everolimus and 20, 40, 60, 100, 300, 600, 900, 1200, and 1500 ng/mL for cyclosporin A were prepared, as described earlier.[6]

Sample Collection and Analysis

The use of blood from healthy volunteers (for the generation of calibrators and QCs) and of leftover blood samples from patients was approved by the ethics committee of Ghent University Hospital (Ghent, Belgium) (EC-BC 7324 and EC-BC 7241). The hematocrit (hct) of blank blood from a healthy female volunteer was adapted to 0.36 L/L (by the addition of a specific amount of plasma) for the generation of calibrators and QCs. The adapted hct (L/L) was measured by using a Sysmex XN-10 hematology analyzer (Sysmex Corporation, Kobe, Japan) and ranged from 0.35 to 0.37 (median, 0.36; with a single exception of 0.33 and 0.40) across the different analysis batches. Venous whole blood patient samples (EDTA-anticoagulated) were collected at Ghent University Hospital. To generate DBSs, 25 μL of venous whole blood was pipetted onto the PE 226 DBS card. After sample collection was completed, the DBS cards were dried for at least 24 hours at room temperature (around 20°C) before analysis. After overnight drying, they were put in a plastic bag containing desiccant (10-g Minipax absorbent packet per plastic bag). A 4-mm-diameter area was clamped during the automated extraction procedure by using a DBS-MS 500 (Supplemental Figure 1, see Supplemental Digital Content 1, containing 4 figures and 2 tables, at https://meridian.allenpress.com/aplm in the July 2023 table of contents). The whole blood and DBS extraction and analysis procedures were performed as described previously.[6] For all whole blood samples, 25 μL of the extract was injected onto a Shimadzu Prominence HPLC system (Shimadzu, Brussels, Belgium), coupled to a SCIEX QTRAP 5500 mass spectrometer (SCIEX, Framingham, Massachusetts). The Shimadzu setup included a CBM-20A system controller, 2 LC-20AD pumps, a DGU-20A5R degasser, a SIL-20ACHT autosampler, and a CTO-20AC column oven containing a Kinetex 2.6-μm Phenylhexyl 50 × 2.1-mm column (Phenomenex, Torrance, California), equipped with the corresponding guard column, maintained at 55°C. LC and MS settings were described previously.[6]

Method Performance and Incurred Sample Reanalysis

Pivotal to the methodologic comparison of venous DBS and liquid whole blood is the demonstration that the performance of both methods on real samples is similar to the performance as observed in the validation.[6] We therefore first performed a (re)analysis of patient samples to estimate the imprecision on duplicate analysis of patient samples. A total of 162 tacrolimus, 47 sirolimus, 45 everolimus, and 61 cyclosporin A paired venous whole blood and DBS samples were obtained and analyzed via LC-MS/MS in different batches, using a previously validated method for whole blood and a fully automated extraction method for DBS. Reanalysis in independent batches was performed for 39 tacrolimus, 45 sirolimus, 43 (42 for whole blood) everolimus, and 59 (56 for whole blood) cyclosporin A patient samples (both blood and DBS) to evaluate incurred sample reanalysis (ISR). For the DBS samples, replicate spots ($n = 4$) were made on the same DBS card (2 of 4 spots were used for the original measurements, and the remaining 2 spots were used for reanalysis). The 2 replicate spots for ISR were frozen at −20°C together with an additional calibration curve and QCs (per batch) after drying for at least 24 hours and after analysis of the first 2 spots. For the ISR of DBS patient samples, DBS cards were thawed batchwise before reanalysis and analyzed with both the frozen (as outlined above) and a fresh calibration curve. Analyses of patient samples were performed in duplicate (ie, 2 remaining spots on the same card). Also the paired whole blood samples were subject to incurred reanalysis, in duplicates. Both patient samples and calibrators and QCs were stored in the meantime (between the first and second analysis) at 4°C until extraction for reanalysis. Reanalysis was performed within 8 days after the first analysis, as stability of all immunosuppressants at 4°C is ensured for at least 14 days.[8–11] The acceptance criterion was based on European Medicines Agency (EMA) guidelines on bioanalytical method validation, as at least two-thirds of the samples should have a deviation less than 20%.[12] The deviation was calculated as the (mean of the original duplicate measurements − mean of the incurred duplicate measurements)/average value of both. Based on the (original and incurred) duplicate measurements of both DBS and whole

blood samples, the method imprecision could be estimated. The coefficient of variation (CV) on both original and incurred duplicate measurements (for DBS and whole blood) was calculated from the formula for standard deviation (SD) as proposed by Ekins in 1983.[13,14] $SD = \sqrt{\frac{\sum_{i=1}^{n}(A_i - A'_i)^2}{2n}}$, with $A_i - A'_i$ representing the difference between the duplicates and *n*, the number of duplicates. To obtain the imprecision, expressed as CV (%), the SD was divided by the mean of all duplicates. Additionally, based on ANOVA (analysis of variance), the interday and total imprecision (summed intraday and interday) were calculated from the result of the duplicate measurements on 2 different days ($n = 2 \times 2$). Further clarification about the setup of the analyses can be found in Supplemental Figure 2. Here, the different analyses and comparisons are displayed. All individual data obtained from the measurements of each patient sample, together with the detailed calculations, are summarized in a Supplementary Data File (see Supplemental Digital Content File 2).

Setup and Application of a Hematocrit Correction Model for Tacrolimus

To demonstrate method applicability, a comparative study was performed for all analytes between venous blood samples collected on PE 226 DBS cards (extracted with an automated setup) and the corresponding venous liquid EDTA-anticoagulated whole blood. Before clinical application of DBS methods, several aspects need to be verified. After a successful analytical validation including the evaluation of DBS-specific parameters,[6] a clinical validation is required.[15] According to recent guidelines on the validation of DBS-based methods for TDM purposes, this requires 2 steps. First, a comparison of venous DBS and venous whole blood is needed to evaluate if a dried matrix gives the same result as the standard matrix, in this case liquid venous EDTA-anticoagulated whole blood. In a second step, capillary DBSs are to be compared with both venous DBSs and venous whole blood, to detect potential capillary-venous differences.[15] Demonstrating equivalence between dried and liquid blood is important to rule out a methodologic (dried versus liquid blood) difference.[15] In this study we aimed at evaluating the first part (ie, venous DBS and venous whole blood comparison) needed for a clinical validation.

As limits of acceptance, we defined an analytical and clinical acceptance criterion. The limit of analytical acceptance was based on the EMA criterion for ISR such that at least 67% of all paired samples should be within 20% of the mean of both samples.[12] Limits of clinical acceptance were set such that at least 80% of all paired samples should be within 20% of the whole blood concentration of the sample. For tacrolimus, 162 venous DBS patient samples were analyzed in conjunction with the paired venous whole blood samples. For sirolimus, everolimus, and cyclosporin A, 47, 45, and 61 venous DBS patient samples were analyzed, respectively. The number of samples included for each analyte is in line with the proportional determination of these analytes at Ghent University Hospital, with tacrolimus being the immunosuppressant for which TDM is by far most frequently performed. The number of samples included in this study additionally fulfills the Clinical and Laboratory Standards Institute guideline, as a sample size of at least 40 sample pairs for method comparison is recommended.[16] Concentrations of tacrolimus, sirolimus, everolimus, and cyclosporin A were determined in venous DBSs and whole blood, both in duplicate. To assess the effect of the hct on DBS quantitation, for all leftover patient samples the hct was determined by using a Sysmex XN-10 hematology analyzer (Sysmex Corporation) (approved by the ethics committee of Ghent University Hospital EC BC-7241).

During method validation, the relative internal standard–compensated recovery appeared to be hct-dependent: at lower hct values a higher relative recovery was observed, while at higher hct values a lower relative recovery was present.[6] A similar trend was observed for the recovery of real venous DBS patient samples. This phenomenon can be explained by a difference in extractability of the DBS with different hcts rather than a hct-area bias. If only an area bias would be present, an adverse effect would be seen because of a different spreading onto filter paper. Here, we believe that the area bias is overruled by the extraction effect (which has the opposite direction of the area bias). On the basis of the validation data, we concluded that the validated hct range was from 0.29 to 0.52 L/L. In the validation manuscript, we additionally concluded that for real samples outside the validated recovery range, an hct correction would need to be applied. Based on these results, patient samples for all analytes across the complete hct range were included, as our aim was to get a full insight into how the hct affects quantification from DBS when using automated DBS extraction under our experimental conditions. The hct (L/L) of the patient samples ranged from 0.189 to 0.497 for tacrolimus, from 0.174 to 0.520 for sirolimus, from 0.234 to 0.577 for everolimus, and from 0.204 to 0.461 for cyclosporin A. In line with the data obtained during method validation, a clear effect of the hct on DBS quantitation was present for all analytes. Consequently, a correction algorithm was set up for the DBS results, based on the hct value of the liquid whole blood. To set up this correction formula, the data set of 162 tacrolimus samples was randomly divided into 2 subsets of 81 samples: a reference set and a test set. The reference set was used to set up the correction formula, while the test set was used to evaluate the validity of the correction factor. Randomization of the samples was done with the built-in Matlab function "datasample." For a first random set of 81 samples (reference set), the mean DBS concentration (of duplicate measurements) and the mean whole blood concentration (of duplicate measurements) were calculated. The percentage difference was calculated as (mean DBS concentration − mean whole blood concentration)/mean whole blood concentration. This percentage difference was plotted against the hct of the samples, and linear regression analysis was performed. Based on the regression equation ($y = ax + b$), a corrected mean DBS concentration for both the reference set and test set was calculated as $DBS_{corrected} = DBS_{measured}/[(a*hct) + b + 1]$. The deduction of the aforementioned formula, including a step-by-step description, is outlined in Supplemental Figure 3.

To assess the robustness of the model, the approach described above was performed for 10 000 iterations, with each time a random division of the data set into a reference set of 81 samples to set up the model, with the remaining set of 81 samples to validate the model (test set). For each iteration, the corrected mean DBS concentration could be calculated for that specific reference set. Hence, for each of the 10 000 iterations, the percentage difference between the corrected mean DBS concentration and the mean whole blood concentration could be obtained for each of the 81 samples of the reference set. A histogram was plotted where the number of iterations was displayed against the percentage of the 81 samples meeting the clinical acceptance criterion (<20% difference with whole blood).

To verify the validity of the model, for each of the 10 000 iterations, the remaining 81 samples (comprising the test sets) were used to independently confirm the validity of the model. Also here, for each iteration, the corrected mean DBS concentration was obtained from the correction formula deduced from the regression analysis, as outlined above. As was done for the reference sets, a histogram was set up displaying the number of iterations as a function of the percentage of the samples meeting the clinical acceptance criterion.

Based on the regression equation of each iteration, an "overall" regression analysis could eventually be deduced from the mean of all slopes and intercepts. This converged, with increasing iterations, to the regression equation if all 162 samples would have been used to set up a correction model. As a "final" regression equation ($y = a_{final}x + b_{final}$), the latter approach was used to calculate the corrected DBS concentrations for all analytes.

Results of Incurred Sample Reanalysis for Each Analyte in Dried Blood Spots (DBSs) and Whole Blood and Method Imprecision Based on Duplicate Measurements

	First Analysis		Incurred Sample Reanalysis		Interday Variation		Total Variation		Validation Data[a]	
	No. of Samples	CV, %	No. of Samples	CV, %	No. of Samples	CV, %	No. of Samples	CV, %	Within Run, %	Total Imprecision, %
Tacrolimus										
DBSs	162	9.6	39	10.7	39	Neg value	39	11.7	6.7–9.3	8.9–11.4
Whole blood	162	7.1	39	7.3	39	9.7	39	12.2	3.7–7.8	6.7–9.7
Sirolimus										
DBSs	47	6.7	45	9.2	45	5.6	45	9.8	7.2–12.5	13.4–19.1
Whole blood	47	7.5	45	5.7	45	6.0	45	9.1	3.4–10.1	3.4–16.8
Everolimus										
DBSs	45	6.8	43	8.3	43	5.7	43	9.5	7.8–9.6	7.8–16.2
Whole blood	45	9.3	42	6.7	42	6.1	42	10.2	6.0–9.9	7.8–11.2
Cyclosporin A										
DBSs	60[b]	5.7	56[c]	7.9	56[c]	5.3	56[c]	8.8	2.6–5.9	3.9–10.2
Whole blood	60[b]	6.2	55[b]	7.5	55[b]	6.4	55[b]	9.3	2.9–8.7	4.7–8.7

Abbreviations: CV, coefficient of variation; LLOQ, lower limit of quantification; Neg, negative.

[a] Validation data as determined in Deprez et al.[6] *J Chromatogr A*. 2021;1653:462430.
[b] One sample below LLOQ.
[c] One outlier was excluded from data analysis via Grubbs test, 2 samples below LLOQ.

Application of the Tacrolimus Hematocrit Correction Model on Sirolimus, Everolimus, and Cyclosporin A Patient Samples

To verify whether a generic correction model was valid for all analytes, we also applied the final slope and intercept ($y = a_{final}x + b_{final}$), calculated from the tacrolimus data set, to calculate corrected mean DBS concentrations for the other immunosuppressants included in the method.

To assess the agreement between both methods (corrected DBS and whole blood) and estimate the bias, Bland-Altman difference plots were made for sirolimus, everolimus, and cyclosporin A paired DBS and whole blood samples as well as for tacrolimus, using Matlab software.

RESULTS AND DISCUSSION

Method Performance and Incurred Sample Reanalysis

Incurred sample reanalysis is recommended by the EMA guideline to assess the spread of individual patient results and was performed in this study for both DBS and whole blood. As both the first and ISR analyses were performed in duplicate, these data could be used to assess the variation of both methods and independently verify, using authentic patient samples, the imprecision of the method found during the method validation.[6]

For tacrolimus, sirolimus, and everolimus, 39, 45, and 43 samples (42 for whole blood), respectively, were reanalyzed in both DBSs and whole blood on a second day. For cyclosporin A, 59 DBS samples (with 2 below the lower limit of quantification [LLOQ] and 1 outlier, 56 samples were included in data analysis) and 56 whole blood samples (with 1 below LLOQ, 55 samples were included in data analysis) were subject to incurred reanalysis. The deviation between the first and second analysis was calculated according to the EMA guideline,[12] and for all analytes the criterion of at least 67% of the samples having a deviation of less than 20% was amply met (Supplemental Table 1). The DBS results were evaluated against both a frozen and fresh calibration curve, as shown in Supplemental Table 1. The intraday imprecision of both the DBS and whole blood method was calculated by using the duplicate measurements performed in the original analysis and the reanalysis. These CVs, being within 11%, were of the same order of magnitude as in the method validation (Table). For cyclosporin A, a single deviating value in the incurred DBS results could be identified as an outlier via Grubbs test and was therefore excluded from data analysis. These data also allowed evaluation of interday precision (via ANOVA analysis), with the CV (%) for all analytes being below 10%, and a total imprecision (intraday + interday precision), which is in line with the total imprecision observed during method validation (Table). The detailed validation data (last column of the Table), as previously described by Deprez et al,[6] are shown in Supplemental Table 2. Overall, these data demonstrate that, using real patient samples, an adequate and similar performance in terms of precision was achieved as in the original method validation.

Setup of Hematocrit Correction Model for Tacrolimus

As also observed during method validation,[6] we found a hct-dependent recovery for all analytes, with a clear underestimation of the concentration in DBSs for high hct values and an overestimation of the concentration in DBSs for low hct samples. Because of the presence of this hct effect, agreement between DBS and whole blood did not meet the acceptance criteria. This is exemplified for tacrolimus in Figure 1, which plots the percentage difference between DBS (mean of duplicates) and whole blood (mean of duplicates) as a function of the hct for all 162 tacrolimus samples.

A reference set, defined by randomly selecting 81 of the 162 tacrolimus samples, was used to subsequently perform a linear regression analysis, as shown in Figure 2, A. Based on the regression equation, corrected DBS concentrations could be calculated according to the following formula: Corrected DBS = Measured DBS/[(a*hct) + b + 1], with a = −1.297 and b = 0.517, eventually yielding $DBS_{corrected} = DBS_{measured}/$

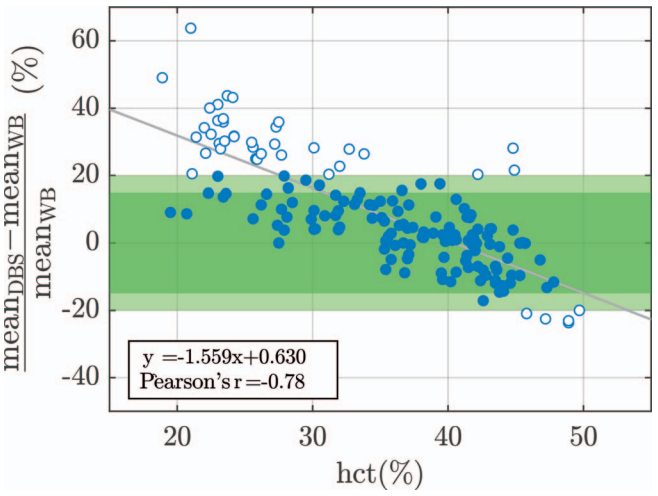

Figure 1. *Hematocrit (hct) effect for tacrolimus displayed as the percentage difference between the mean dried blood spot ($mean_{DBS}$) concentration and the mean whole blood ($mean_{WB}$) concentration as a function of the hct of the sample (n = 162). The clinical acceptance criterion (20% difference of the whole blood concentration) is represented by the light green–filled area.*

[–1.297*hct + 1.517], as also outlined in Supplemental Figure 3. After applying this correction formula to all 81 data points from the reference set, the percentage difference with whole blood was again plotted against the hct (Figure 2, B). As expected, the hct bias was nullified, with 78 of 81 samples (96%) meeting the clinical acceptance criterion (before correction, this was 62 of 81 [77%]). Figure 2, C and D, shows the linear regression analysis for the 81 remaining samples (test set), before and after correction of the DBS concentration, using the above-mentioned formula that was generated from the independent reference set. While before application of the formula, only 57 of 81 test samples (70%) met the clinical acceptance limit, this increased to 80 of 81 (99%) after application of the formula (Figure 2, D). The clinical acceptance limit is indicated by the light green–filled area displayed in Figure 2. In Supplemental Digital Content 2 we included in the "tacrolimus" worksheet, in column D, which samples were attributed to the reference set (Figure 2, A and B) and which samples to the test set (C and D) by randomization.

To assess the robustness of the applied correction methodology, we repeated the above-mentioned approach 10 000 times, with each time 81 reference samples being randomly drawn out of the data set of 162 samples. The histogram displayed in Figure 3, A, displays the number of iterations for which the result for the reference samples met the clinical acceptance criterion of 20% difference between corrected DBS and whole blood concentrations. The median and minimum percentage of samples meeting the clinical acceptance criterion was 98% and 94%, respectively. This implies that in all iterations at least 80% of the samples had a (corrected) mean DBS concentration that differed less than 20% from the mean whole blood concentration.

The (10 000) models generated above were also applied on the corresponding independent test samples (each time comprising 81 samples), for each of these samples yielding corrected mean DBS concentrations, based on the respective correction formulas from each regression analysis. Similarly as above, a histogram was plotted, taking into account the clinical acceptance criterion, as depicted in Figure 3, B. Also here, the median percentage of samples meeting the clinical acceptance criterion was 98%, and in all iterations at least 93% of the (corrected) mean DBS concentration differed less than 20% from the corresponding mean whole blood concentration. Since this is the result of repeated (10 000 times) random selection from the complete data set, this represents a "worst case scenario," with in no instance less than 80% of the samples meeting the acceptance criterion. In conclusion, we can deduce that, irrespective of the random selection of (81) samples from the data set to serve as a "reference set" to set up a correction formula, the clinical acceptance criterion will anyhow be met for tacrolimus. The overall mean formula for all iterations (n = 10 000) was $y = -1.559x + 0.63xx$, with the third and fourth decimals of the b factor varying depending on the data selection (each time the 10 000 iterations will be performed, these numbers will slightly change because of variation). This formula corresponds with the formula that would be obtained if all 162 tacrolimus data points would be used to set up the model via linear regression analysis: $y = -1.559x + 0.6305$. The latter formula was considered the final correction model, with $a_{final} = -1.559$ and $b_{final} = 0.6305$ (ie, the linear regression equation when all 162 tacrolimus samples are used). Using this final correction factor, 98% (158 of 162) of the samples met the clinical acceptance criterion.

Application of Model on Sirolimus, Everolimus, and Cyclosporin A Patient Samples

We next examined whether the above-mentioned "final" correction formula for tacrolimus would also allow adequate correction of the mean DBS concentration for the other analytes (Figure 4, A through F). Figure 4, A, C, and E, depict the uncorrected data for sirolimus, everolimus, and cyclosporin A, while in Figure 4, B, D, and F, corrected mean DBS concentrations (according to the correction formula outlined above, with $a_{final} = -1.559$ and $b_{final} = 0.6305$) were used to plot the percentage difference between DBS and whole blood concentrations as a function of the hct. Two assessments can be made from these plots: first, whether the correction could alleviate the hct effect; and second, whether the data met the analytical and/or clinical acceptance criteria.

The setup algorithm was successful in eliminating the hct bias for all analytes, since the slopes (in Figure 4, B, D, and F) were not significantly different from zero. Zero was included in the 95% CI around the slope of the linear regression analysis of sirolimus, everolimus, and cyclosporin A. The regression analyses before and after application of the correction factor can be found in Figure 4. In this Figure, the light green–filled areas indicate the maximal allowed deviation between DBS and whole blood of 20%. The constant bias that can be observed in Figure 4, D, is extensively discussed below. For volumetric microsampling-based methods (using Mitra or HemaXis), mostly no hct effect is found.[17–19] Nevertheless, in some instances a minor, nonsignificant hct trend can still be observed.[20] Also when performing manual subpunched DBS analysis, correction factors are often needed to improve the agreement between whole blood and DBS results.[21] Other conventional DBS methods are capable of minimizing the hct effect within the acceptable limits, hence no correction is needed.[2,3,22]

After correction, the analytical acceptance criterion was met for all analytes, with 89% (42 of 47), 87% (39 of 45), and 95% (57 of 60, as 1 sample was below the LLOQ) for

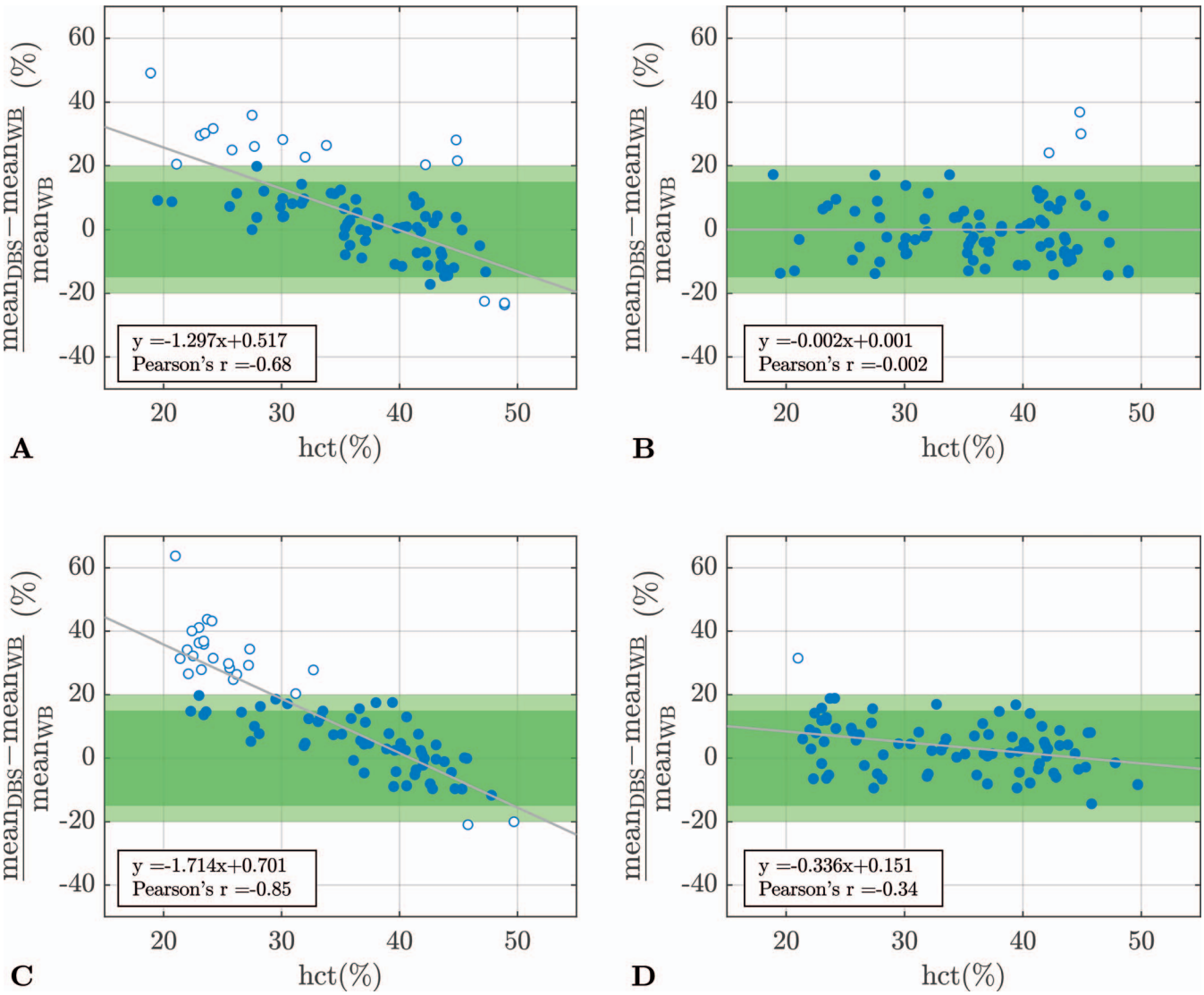

Figure 2. *Linear regression analysis for a random subset of 81 tacrolimus samples (reference set) of the percentage difference between the mean dried blood spot (mean$_{DBS}$) concentration and the mean whole blood (mean$_{WB}$) concentration as a function of the hematocrit (hct) of the sample (n =81). Uncorrected data are displayed in (A). B, Percentage difference after correction of the DBS concentration. C and D, The remaining 81 samples to verify the model (test set) before (C) and after (D) correction using the regression analysis from (A). The clinical acceptance criterion (20% difference of the whole blood concentration) is represented by the light green–filled areas.*

sirolimus, everolimus, and cyclosporin A, respectively. Additionally, after correction, the clinical acceptance criterion was met for cyclosporin A for 97% (58 of 60) of the samples. Also for sirolimus and everolimus, for which, respectively, 91% (43 of 47) and 84% (38 of 45) of the corrected DBS concentrations lay within 20% of the corresponding whole blood concentrations, the minimum of 80% of samples complying with this criterion was achieved. It should be mentioned, though, that Veenhof et al[23] proposed a limit of clinical relevance (as set by a multidisciplinary team) at a range of 85% to 115% around the ratio of the paired DBS and whole blood samples for at least 80% of the samples (indicated by the darker green–filled areas in Figures 1, 2, and 4). For sirolimus and everolimus this more stringent criterion would not be met for our data set (for sirolimus because of the larger total error [TE], for everolimus because of the larger TE in combination with a remaining bias after correction, as outlined below). However, this criterion is hardly used in practice for LC-MS/MS-based microsampling methods.[17–19,24] In a clinical validation study by the same group (Veenhof et al[23]), 77.3% and 61.5% of the concentrations of sirolimus and everolimus derived from DBS samples lay within 15% of those determined in whole blood samples, also not meeting their predefined criterion of 80% because of the spread on the data.[23] In the article by Vethe et al,[18] a lower within-run CV (%) was found for their Mitra-based tacrolimus method. However, looking at the agreement of capillary Mitra with whole blood, for 6.9% of the samples the absolute deviation was above 20%, which is in line with our tacrolimus results. In other articles, by Zwart et al,[17] Tron et al,[19] and Paniagua-Gonzalez et al,[24] also applying volumetric microsampling for tacrolimus determination, a similar precision and agreement with whole blood (in terms of the percentage of samples deviating ≤20% from the whole blood concentration), compared to our method, was

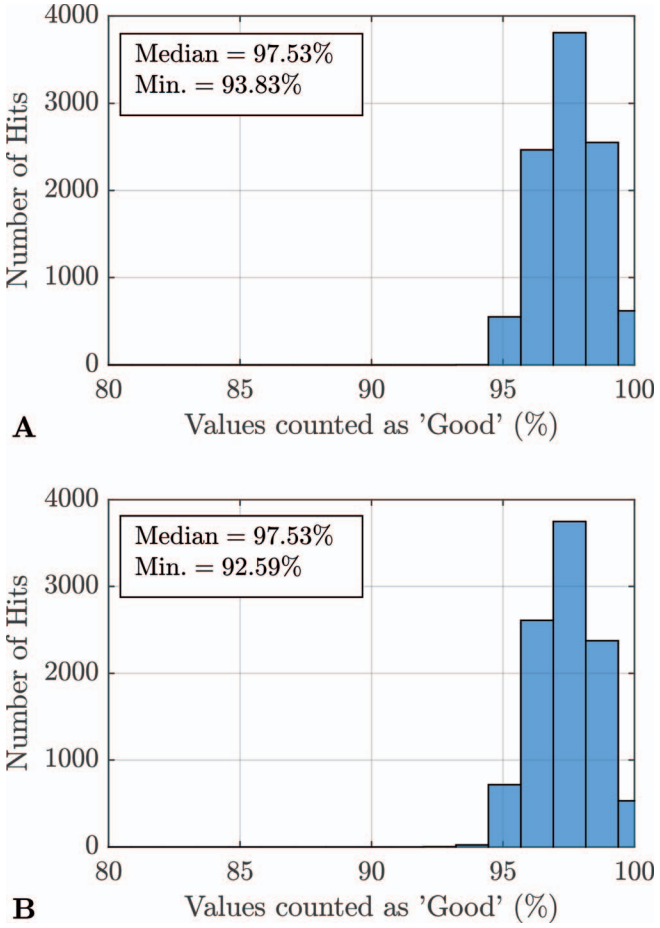

Figure 3. *Histograms depicting the number of iterations plotted against the percentage of the 81 samples meeting the clinical acceptance criterion (<20% difference with whole blood) after correction of the dried blood spot concentration (and the median and minimum [Min.]). A, Depiction of the "reference" sets to set up the model. B, Depiction of the "test" sets, each time containing the remaining 81 samples (out of 162) that were used to verify the models from (A).*

obtained. Nevertheless, for the method by Paniagua-Gonzalez et al,[24] a good agreement could only be obtained after transformation of the Mitra data (before, on average, a 22% lower Mitra concentration was observed). For manual DBS-based methods,[21,25–27] in some instances an overestimation of the tacrolimus concentration in DBSs is observed,[21] imposing the need of a correction factor. In conclusion, our data, obtained by fully automated DBS analysis, can be considered compliant with the current state of the art. In a recently published method by Bressán et al,[27] volumetric application of 10 µL onto Whatman 903 paper was performed.[27] Here, a perfect agreement was reported for all immunosuppressants (tacrolimus, sirolimus, everolimus, and cyclosporin A) between whole blood and venous DBS, with less than 5% of the samples exceeding a difference of 15%. Despite the clear improvement in agreement by the volumetric application of blood on DBS, the requirement for volumetric application is a clear limitation when envisaging application of this method in a home-sampling context.

This work is inevitably also associated with some limitations. A limitation of our method might be that no methodologic agreement was established of our whole blood method against a reference method for immunosuppressant determination using LC-MS/MS. Recently, a reference method was established by Taibon et al.[7] This could serve as an anchor point to verify the trueness of our method in the future. The method by Taibon et al[7] includes a longer extraction time of whole blood as compared to our method. However, we additionally verified the influence of a prolonged extraction time to our method (20 minutes and 1 hour instead of 5 minutes), and this did not yield higher analyte to IS ratio signals (Supplemental Figure 4). Moreover, verification of our whole blood method using 7 external QCs (from both Recipe and Chromsystems) yielded acceptable results (ie, within 11% absolute mean bias, n = 3).

Bland-Altman plots depicting the difference between corrected DBS concentrations and whole blood concentrations as a function of the mean of these concentrations are shown in Figure 5, A through D. For tacrolimus (Figure 5, A), Bland-Altman analysis revealed a mean bias of –0.35% (95% CI, –1.72% to 1.03%). For sirolimus (Figure 5, B), everolimus (Figure 5, C), and cyclosporin A (Figure 5, D), a mean bias of –1.24% (95% CI, –5.01% to 2.55%), 4.88% (95% CI, 1.42%–8.33%), and –1.16% (95% CI, –3.74% to 1.42%), respectively, was found. The 95% CI of the mean differences encompassed zero for all analytes, except for everolimus, for which a consistent positive bias for the DBS results appeared to be present after correction. This indicates that after hct correction of the DBS concentrations, no significant differences between venous DBS and venous whole blood were found for tacrolimus, sirolimus, and cyclosporin A. For everolimus, the mean (statistically significant) bias of approximately 5% suggests that the conversion formula to correct for the hct effect, which was set up on the basis of the tacrolimus data set, is not ideally suited for this analyte. Seeking for an explanation for the slightly different hct effect present for everolimus in comparison with tacrolimus, we compared the average hct values of the data set for each analyte, as this can cause a slight shift in the regression analysis. For everolimus, on average, a higher hct (L/L) of the samples, 0.38 (median, 0.40), compared to 0.35 (median, 0.36) for tacrolimus, could be found. However, this cannot fully explain the remaining bias for everolimus, as for cyclosporin A the mean hct (L/L) of 0.31 (median, 0.29) was equally different in the other direction. The mean hct (L/L) for the sirolimus data set was 0.37 (median, 0.38), closest to the mean hct of the tacrolimus data set. We conclude that a hct correction factor specifically dedicated to everolimus could be used in the future to yield even better results for everolimus than the ones obtained here—an independent data set of everolimus samples could be used to generate such an everolimus-specific hct correction factor.

Despite the complete correction for the effect of the hct on DBS quantitation for sirolimus, and the achievement of the clinical acceptance criterion, a wider spread of the data on the Bland-Altman plot can be observed. Also here, we performed an in-depth analysis to search for the root cause. An explanation for the wider spread can be found in the larger TE of the method. Bland-Altman comparisons showed limits of agreement with a span of 34.7%, 50.5%, 45.1%, and 38.9%, for tacrolimus, sirolimus, everolimus, and cyclosporin A, respectively. These results can be expected from our validation data.[6] A TE (= absolute $\text{bias}_{\text{mean}} + 2 \times \text{CV}_{\text{mean}}$) of 17% and 23% on tacrolimus measurements in

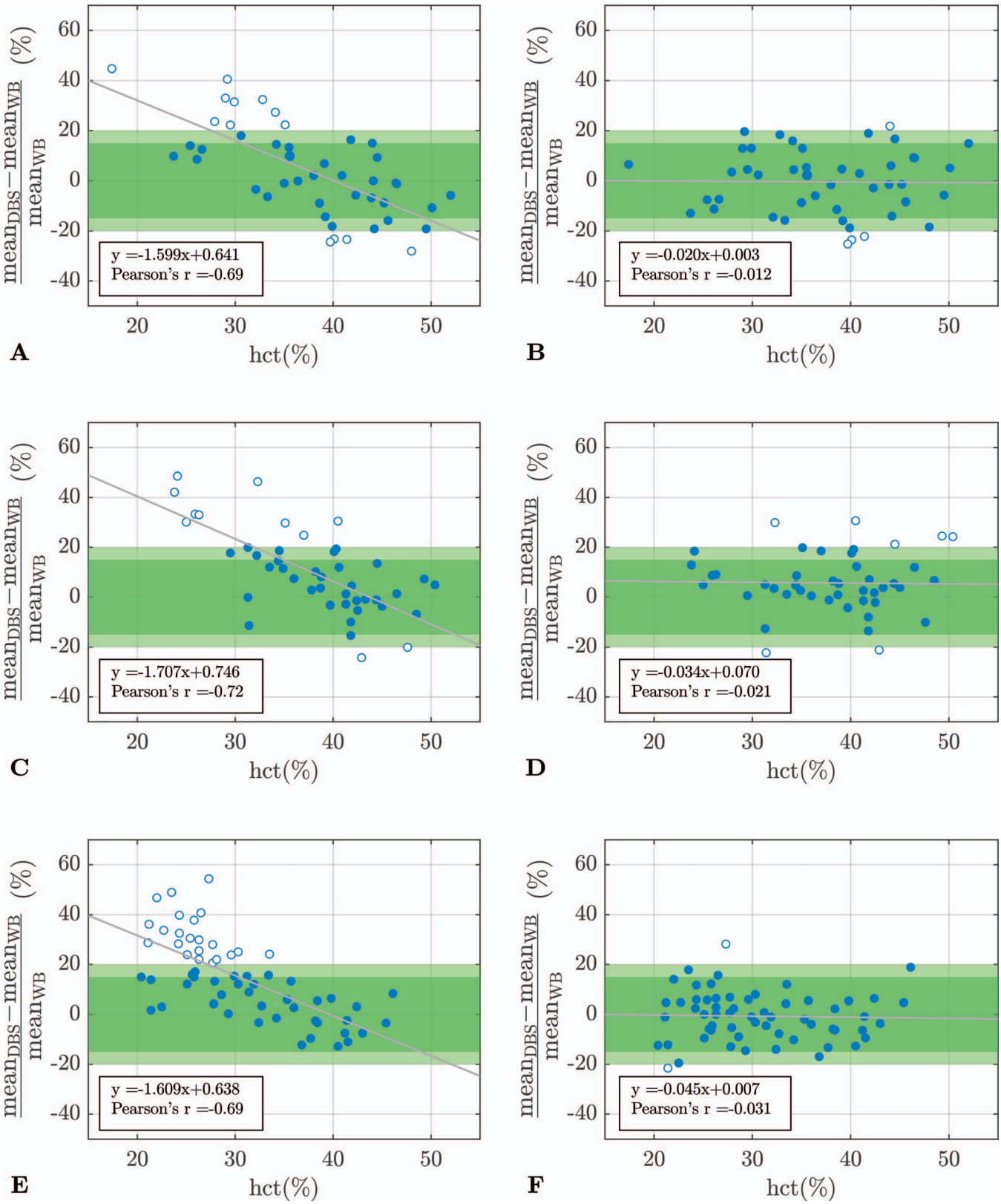

Figure 4. *Linear regression analysis for sirolimus (A) (n = 47), everolimus (C) (n = 45), and cyclosporin A (E) (n = 60) of the percentage difference between the mean dried blood spot ($mean_{DBS}$) concentration and the mean whole blood ($mean_{WB}$) concentration as a function of the hematocrit (hct) of the sample, before correction based on the hct (A, C, and E) and after correction (B, D and F). The clinical acceptance criterion (20% difference of the whole blood concentration) is represented by the light green–filled areas. In (D), a remaining bias for everolimus of 4.9% can be observed.*

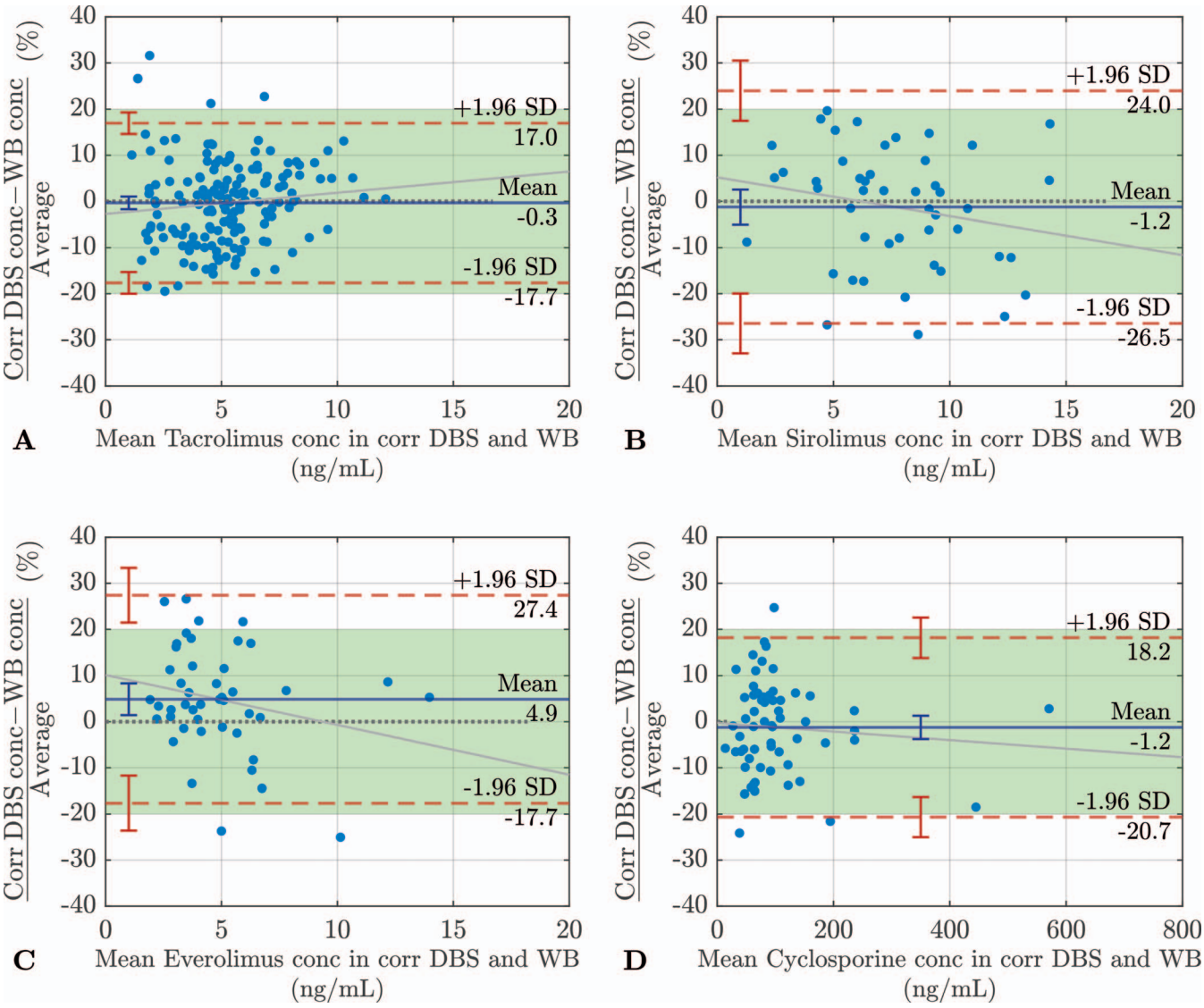

Figure 5. *Bland-Altman plots for tacrolimus (A) (n = 162), sirolimus (B) (n = 47), everolimus (C) (n = 45), and cyclosporin A (D) (n = 60). Mean differences between corrected dried blood spot concentrations (corr DBS_{conc}) and whole blood concentrations (WB_{conc}) are represented by full blue lines, and limits of agreement are represented by broken red lines. The analytical acceptance criterion (20% difference of the average) is represented by the green-filled areas.*

liquid blood and DBS samples, respectively, could be derived. For sirolimus the TE was 28% and 32%, for everolimus 28% and 30%, and for cyclosporin A 19% and 18% for the liquid blood and DBS methods, respectively. This degree of variation is in line with what another group, also using LC-MS/MS analysis, has recently reported for the determination of sirolimus and everolimus in DBS.[23] However, these TEs are not meeting the proposed TE limit by Seger et al[28] of 15%. When aiming at implementing these methods in clinical practice, further methodologic improvements are recommended to further reduce the TE of both the whole blood and DBS methods. The trendlines (gray) displayed in the Bland-Altman plots give the impression that a concentration-dependent deviation is present for each analyte. However, for all analytes, zero was included in the 95% CI of the slope and intercept, except for everolimus where the intercept was statistically different from 0 (as outlined above).

A disadvantage of the applied correction algorithm is that knowledge of the hct is required. Fortunately, multiple approaches are available to predict the hct from a DBS, such as potassium measurement or noncontact approaches including near-infrared spectroscopy or reflectance spectroscopy, as described in literature.[29–36] This would avoid the need for hct determination of whole blood samples based on impedance methodology. Additionally, the automated extraction unit (DBS MS-500) is to be expanded with a built-in reflectance spectroscopy–based hct prediction module,[29,35,37] allowing seamless integration of hct measurements in the fully automated system.

CONCLUSIONS

In conclusion, in this study, a critical evaluation of the applicability of a DBS method making use of an automated extraction platform (coupled to liquid chromatography–tandem mass spectrometry) for the quantification of 4 immunosuppressants (tacrolimus, sirolimus, everolimus,

and cyclosporin A) in venous patient samples was performed. We showed that, provided a correction formula was applied to correct for the impact of the hct, automated DBS analysis shows great potential for routine TDM of immunosuppressants, avoiding any manual sample handling (apart from inserting a card into a rack).

For all analytes, the hct biased the DBS results obtained after automated extraction of venous PE 226 DBS samples. By setting up an hct correction factor, this hct effect could be overcome for all analytes. For tacrolimus, sirolimus, and cyclosporin A, the hct-corrected data met both the clinical and the analytical criteria. While the correction was able to eliminate the hct effect for everolimus, and the acceptance criteria were met, an additional study will be necessary to determine whether an "everolimus-tailored" correction factor may yield even better agreement between DBS and whole blood results, as a significant bias between whole blood and corrected DBS was still present when using the correction formula based on tacrolimus data.

To our knowledge, we are the first to demonstrate the successful applicability of an automated extraction method for immunosuppressants from venous DBS. In this study, venous DBSs were generated in a controlled laboratory setting. In the future, as a second part of the clinical validation of the DBS-based method, capillary finger prick samples will need to demonstrate the applicability of the method in a real-life setting.

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