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The clinical validation of a dried blood spot method for simultaneous measurement of cyclosporine A, tacrolimus, creatinine, and hematocrit

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1. Introduction

The immunosuppressive drugs cyclosporine A (CsA) and tacrolimus are used after transplantation to prevent an organ rejection or graft-versus-host disease. The drugs have a narrow therapeutic range, and a large inter-patient variability in their pharmacokinetics [1–4]. Consequently, transplant recipients are at risk for drug under- and over-exposure, which is in turn associated with allograft rejection and drug-related toxicity. To avoid these adverse clinical outcomes, immunosuppressive drug exposure is monitored frequently after transplantation *via* venous blood sampling, and doses are adjusted accordingly, a practice known as therapeutic drug monitoring (TDM) [1,4–7].

A more recently developed method that can be used for TDM is dried blood spotting (DBS) [8,9]. With this method, a drop of capillary blood, taken from the finger by a finger prick, is collected on a filter paper, from which immunosuppressive drug concentrations can be measured. One advantage of DBS-sampling is that patients could perform the blood sampling at home, which could reduce the number of hospital visits. Moreover, DBS sampling at home makes it easier to draw blood at a specific time point (*i.e.* pre-dose concentrations) or at multiple time points after dose administration (*i.e.* an area under the curve; AUC). Finally, the finger prick is less invasive than a venipuncture and only a small amount of blood is required.

However, before DBS can be implemented in clinical practice, several technical and practical issues need to be solved [10]. DBS drug concentration measurement may be affected by hematocrit [8,11,12]. Patients with a higher hematocrit may have more viscous blood and generate smaller drops, that will distribute less on DBS filter paper. Also, the extraction of the immunosuppressive drugs from the DBS paper may be affected by hematocrit [8,11,12]. Therefore, it might be necessary to correct the laboratory results from the DBS method for the hematocrit. Other factors may also affect drug measurement with DBS [10], such as the differences in sample location (venous versus capillary), and the use of anticoagulant drugs, although these effects have not been characterized well. Finally, as CsA and tacrolimus are nephrotoxic, monitoring of the kidney function is of clinical importance [13]. Up until recently, it

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was not possible to measure kidney function and immunosuppressive drug concentrations from one and the same DBS spot, which formed a barrier for clinical application [14,15]. To optimize the precision of DBS for the measurement of immunosuppressive drug concentrations and kidney function, a correction factor for hematocrit, the effect of the DBS filter paper, and potential differences in sampling location, should be evaluated, and the DBS method should be clinically validated [10].

The aim of this study was to clinically validate a DBS method for CsA, tacrolimus, and creatinine, using a liquid chromatography-tandem mass spectrometry method (LC-MS/MS). Moreover, the need for a hematocrit correction, the effect of the use of anti-coagulant agents, the effect of the DBS filter paper and potential differences in sampling location were evaluated.

2. Methods

2.1. Patient population

Patients were eligible for participation if they were at least 18 years old and received either tacrolimus or CsA as part of their immunosuppressive treatment, or if serum creatinine or hematocrit was measured as part of their routine clinical care. All patients that were screened for eligibility received a solid organ or stem cell transplantation. Patients were excluded from the analysis if they took their medication before blood sampling (*i.e.* if a peak concentration was measured), if no or not enough blood was available for the standard concentration measurement, if no sufficient DBS spot was available for DBS concentration measurement (*i.e.* if the DBS spot was too small or the DBS spot was not acceptable), or if the drug concentration was under the limit of quantification (LOQ).

2.2. Study design

Patients were screened for eligibility and asked to give written informed consent before a scheduled visit to the outpatient clinic. A minimum of 40 patients was included per immunosuppressive drug, which was in accordance with the guidelines for clinical validation of the Official International Association for Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) [10].

Blood sampling consisted of one venipuncture, which was drawn as part of standard clinical practice, and one finger prick. For this analysis, a total of four blood samples was collected and analyzed (Supplementary Figure S1): 1) a DBS sample obtained by the finger prick [“]$_{\text{DBS-finger}}$, 2) a whole-blood sample (microtainer) obtained by the finger prick ([“]$_{\text{WB-finger}}$), 3) a DBS sample from venous blood obtained by conventional venipuncture ([“]$_{\text{DBS-venous}}$), and 4) a whole-blood sample obtained by conventional venipuncture (standard clinical practice; [“]$_{\text{WB-venous}}$). Preferably, the time between the finger prick and the venipuncture was as short as possible. However, due to the COVID-19 restrictions, it was not possible to draw the blood samples simultaneously. To assess whether the time between the finger prick and venipuncture was associated with differences in concentrations measured in the samples, the time of both the finger prick and the venipuncture were registered.

Finally, all patients filled in a questionnaire, in which the patients were asked to rate the clearness of the instructions for DBS (“poor”, “moderate”, “sufficient”, “good”), to rate the pain during a venipuncture and a finger prick (scale 0–10), and to give their preference for one of the sampling methods.

2.3. Laboratory analyses

The validation of the methods was based on the Food and Drug Administration guidelines for bioanalytical validations [16]. Details on the analytical validation of the whole-blood and DBS method for tacrolimus, CsA and creatinine can be found in Table 1 and Supplementary Data S1. In short, the DBS method for tacrolimus, CsA and creatinine and the whole-blood method for tacrolimus and CsA were analytically validated on a Waters Acquity UPLC-MS/MS system (Waters Corp., Milford, MA, USA). The creatinine concentration after venous sampling was analyzed in serum on a Cobas8000 system (Roche Diagnostics, Basel, Switzerland). The bias and precision of this method are 3.7% and 2.0%, respectively. Hematocrit in DBS was measured according to a validated method using near-infrared spectroscopy (NIR; Shimadzu, Den Bosch, The Netherlands) with a FlexIR NIR Fiber Optic Accessory probe (Pike Technologies, Madison, WI, USA) [17,18]. Hematocrit analysis of the venous whole-blood samples was performed on a Sysmex XN-1000 hemocytomery analyzer (Sysmex Corporation, Kobe, Japan). The bias and precision of this method are −1.9% and 1.9% respectively.

A DBS extraction recovery experiment was performed for tacrolimus, CsA and creatinine (Supplementary Data S1). For tacrolimus, the average recovery was 0.93 (range 0.75 – 1.16), for CsA, the average recovery was 0.86 (range 0.81 – 0.95), and for creatinine the average recovery was 0.86 (range 0.77 – 0.93). For all compounds, the IS-compensated recovery of each sample was plotted against its hematocrit, with R^2 of 0.38 for tacrolimus, 0.02 for CsA, and 0.72 for creatinine. These findings demonstrate the need for evaluation of a correction factor and hematocrit effect in the clinical validation study.

Table 1
Results analytical validation.

	Intraday		Interday	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
CsA (range 15.0 – 1200.0 µg/L)				
Whole-blood				
Low	-10.9	2.5	3.8	11.6
Middle	0.9	2.6	2.3	2.4
High	-0.9	3.9	3.3	3.4
DBS				
Low	-4.2	5.0	7.2	8.3
Middle	7.2	4.3	9.4	11.8
High	4.4	4.0	6.6	7.9
Tacrolimus (range 2.0 – 35.0 µg/L)				
Whole-blood				
Low	-5.1	5.5	5.6	7.6
Middle	-5.1	2.2	3.6	6.2
High	-5.4	1.9	2.3	5.9
DBS				
Low	13.1	2.4	5.1	14.0
Middle	14.6	2.4	9.4	17.4
High	13.0	2.8	8.2	15.4
Creatinine (range 18 – 1071 µmol/L)				
DBS				
Low	1.3	4.1	6.2	6.4
Middle	1.8	3.0	3.7	4.1
High	8.0	1.4	3.5	8.7
Hematocrit				
Whole-blood				
L1	-3.1		1.9	
L2	-4.2		1.9	
DBS*				
Low	3.0	2.4	9.1	2.1
Middle	-0.8	3.6	-0.7	3.8
High	-3.5	1.9	-3.2	3.0

* van de Velde et al. 2021.

2.4. Study endpoints

The aim of this study was to clinically validate a DBS method for the measurement of CsA, tacrolimus, creatinine concentrations and hematocrit.

The primary study endpoint was the agreement and bias between finger prick DBS concentrations and conventional concentrations of the immunosuppressive drugs, creatinine, and hematocrit. Secondary study endpoints were 1) whether a correction factor for the DBS method could improve the agreement and bias between [“]$_{\text{DBS-finger}}$ and [“]$_{\text{WB-venous}}$, 2) the effect of the DBS filter paper on drug and creatinine concentration measurement, 3) the effect of the location of blood sampling (venous *vs.* capillary) on drug and creatinine concentration measurement, 4) the effect of anticoagulant use on drug and creatinine concentration measurement, and 5) the patients' experience with the DBS method.

2.5. Statistical analysis

Statistical analyses were performed using R (Version 3.5.3) and GraphPad Prism (Version 5.00). Continuous variables were described as median with interquartile range (IQR). Categorical variables were described as number of cases with a proportion.

The correlation between the [“]$_{\text{DBS-finger}}$ (before and after applying the conversion formulas) and conventional [“]$_{\text{WB-venous}}$ was calculated with Passing-Bablok regression. The Bland-Altman method was used to evaluate potential bias by testing the agreement between the two methods. According to the guideline on bioanalytical validation by the European Medicine Agency, the difference between DBS and venous concentrations should be less than 20% of the mean of the two concentrations for at least 67% of the samples [10,19]. However, in the Erasmus MC we aim to reach stricter criteria for clinical validation: the difference between DBS and venous concentrations should be less than 15%, of the mean of the two concentrations for 95% of the samples.

It was evaluated whether a conversion formula could optimize the agreement and minimize systematic differences between the standard and the DBS measurements. Because systemic differences could have multiple causes and shapes, different conversion formulas were evaluated. To evaluate whether hematocrit correction was required, correction factors with and without hematocrit correction were evaluated. Moreover, as the hematocrit effect might depend on the height of the concentration of the analyte, a conversion formula including an interaction term between the hematocrit and the concentration of the analyte was evaluated. To determine whether correction for hematocrit was needed, uncorrected DBS hematocrit values measured with NIR ([Ht]$_{\text{DBS-finger}}$) were used. The following conversion formulas were applied: 0) No conversion formula, 1) Correction for the linear regression slope (with the regression line forced through 0), 2) Correction for the Deming regression slope, 3) Correction for the Deming regression slope and intercept, 4) Correction for hematocrit using simple linear regression with the estimated concentration as dependent variable and the DBS concentrations and hematocrit as independent variables, 5) Correction for hematocrit using linear regression with the estimated concentration as dependent variable and the DBS concentrations and hematocrit as independent variables and with an interaction term between DBS concentrations and hematocrit. The results of the Passing-Bablok regression and the Bland-Altman analysis were compared before and after correction for all conversion formulas that were applied. The bias based on the Passing-Bablok regression was considered significant when the 95% CI of the intercept does not include 0 or when the 95% CI of the slope does not include 1. Moreover, the agreement and the distribution of the corrected DBS concentrations and the standard whole-blood or serum concentrations were evaluated graphically. For the Bland-Altman analysis, the proportion of samples within the 20% and 15% Limits of Agreement (LOA) and the mean absolute bias were evaluated. Moreover the distribution of the bias was evaluated graphically. If results of these analyses were similar for multiple correction formulas, the simplest correction formula was chosen.

Moreover, the effect of the timing of blood sampling and the effect of using anticoagulant agents was investigated. Correlations between non-parametric continuous variables were calculated using Spearman's correlation coefficient. The Mann-Whitney *U* test was used to evaluate potential differences in non-parametric continuous variables between two groups.

2.6. Ethical considerations

The present study was performed in accordance with the principles of the Declaration of Helsinki (7th revision, October 2013, approved by the 64th WMA General Assembly, Fortaleza, Brazil) and the Medical Research Involving Human Subjects Act (WMO). In addition, study procedures were performed in accordance with the ethical standards of the institutional research committee of the Erasmus MC (Erasmus MC Medical Ethical Review Board, number 2018-027). Written informed consent was obtained from all patients prior to inclusion.

3. Results

In this study, a total of 180 patients was included. The inclusion per analyte depended on whether creatinine and hematocrit were measured as part of a patient's standard clinical care and which immunosuppressive drug the patients used. (CsA, n = 41; tacrolimus, n = 57; creatinine, n = 180; hematocrit, n = 180). Of these patients, 177 were included in the final analysis (CsA, n = 30; tacrolimus, n = 37; creatinine, n = 176; hematocrit, n = 170). Patients were excluded from the final analysis if they took their medication before blood sampling (*i.e.* if a peak concentration was measured; CsA, n = 0; tacrolimus, n = 6), if there was no venous blood or no sufficient DBS spot (*i.e.* if the DBS spot was too small or the DBS spot was not acceptable) available to determine immunosuppressive drug or creatinine concentrations or hematocrit values (CsA, n = 4; tacrolimus, n = 6; creatinine, n = 4; hematocrit, n = 10), if the drug concentration was outside the limits of quantification (CsA, n = 1; tacrolimus, n = 1), or if the time between DBS and venous blood sampling for CsA and tacrolimus concentration measurement was more than 45 min (CsA, n = 6; tacrolimus, n = 7; Supplementary Figure S2).

3.1. Baseline characteristics

Table 2 presents the baseline characteristics of the 177 included patients stratified by analyte. The majority of patients was male (n = 101; 57.1%). The median age at the time of blood sampling was 62.0 years (IQR 50.0–68.0). Out of the 177 patients, 89 (50.2%) received a kidney transplant, 11 (6.2%) received a liver transplant, 8 (4.5%) received a heart transplant, 17 (9.6%) received a lung transplant, 48 (27.1%) a stem cell transplant and 4 (2.3%) a combined transplantation. The measured immunosuppressive drug, and creatinine concentrations, and hematocrit values are summarized in Supplementary Table S1.

3.2. The robustness of DBS

3.2.1. Time between the finger prick and the venipuncture

As immunosuppressive drug concentrations depend on the time after dose ingestion, we evaluated whether the difference between the measured immunosuppressive drug concentrations correlated with the time between the blood sampling methods. Because this effect is larger for peak concentrations compared to pre-dose concentrations, these samples were already excluded. The median time between the finger prick and the venipuncture was 18.5 min (range 6–93) for CsA and 23 min (range 7–133) for tacrolimus. The time between the finger prick and the venipuncture significantly correlated with biases (difference/mean (%)) in [“]$_{\text{DBS-finger}}$ and [“]$_{\text{WB-venous}}$ for both CsA and tacrolimus (Spearman's correlation coefficients of −0.36 and −0.35 respectively;

Table 2
Baseline characteristics.

	Cyclosporine A (n = 30)	Tacrolimus (n = 37)	Creatinine (n = 176)	Hematocrit (n = 170)
Gender				
Male / Female	17 (56.7%) / 13 (43.3%)	22 (59.4%) / 15 (40.5%)	101 (57.4%) / 75 (42.6%)	96 (56.5%) / 74 (43.5%)
Age (years)	59.0 (IQR 46.3 – 67.0)	58.0 (IQR 50.0 – 69.0)	62.0 (IQR 50.0 – 68.0)	62.0 (IQR 50.0 – 68.0)
Bodyweight (kg)	82.5 (IQR 67.9 – 87.2)	80.0 (IQR 67.2 – 87.8)	78.0 (IQR 67.0 – 86.9)	78.0 (IQR67.0 – 87.5)
Height (cm)	175.0 (IQR 168.5 – 183.0)	172.0 (IQR 167.8 – 177.0)	174.0 (IQR 167.0 – 181.5)	175.0 (IQR 167 .0– 181.6)
Hematocrit (L/L)	0.36 (range 0.29 – 0.41)	0.36 (range 0.29 – 0.47)	0.37 (range 0.24 – 0.47)	0.37 (range 0.24 – 0.47)
Type of transplantation				
Kidney	5 (16.7%)	36 (97.3%)	88 (50.0%)	85 (50%)
Liver	0	0	11 (6.3%)	11 (6.5%)
Lung	0	1 (2.7%)	17 (9.7%)	16 (9.4%)
Heart	4 (13.3%)	0	8 (4.5%)	7 (4.1%)
Stem cell	20 (66.7%)	0	48 (27.3%)	47 (27.6%)
Heart-lung	1 (2.8%)	0	1 (0.6%)	1 (0.6%)
Heart-kidney	0	0	2 (1.1%)	2 (1.2%)
Liver-kidney	0	0	1 (0.6%)	1 (0.6%)
Anticoagulant drugs	16 (53.3%)	17 (45.9%)	79 (44.9%)	75 (44.1%)
DOAC	0	1	3	3
LMWH	3	0	6	6
TAI	12	10	48	45
VKA	0	5	17	17
TAI + VKA	0	0	2	2
LMWH + DOAC	1	0	1	1
TAI + DOAC	0	1	1	1
TAI + LMWH	0	0	1	0

DOAC, Direct oral anticoagulants; LMWH, Low molecular weight heparin; TAI, Thrombocyte aggregation inhibitors; VKA, Vitamin K antagonist.

all: p less than 0.05; Supplementary Figure S3).

To minimize an effect of the time between DBS and venous blood sampling on our correction formulas, the samples with more than 45 min between DBS and venous blood sampling were excluded for the final analysis (CsA, n = 6; tacrolimus n = 7; Supplementary Figure S2).

3.2.2. The effect of anticoagulant use

The effect of the use of anticoagulant agents on the DBS measurements was evaluated. Out of the 177 included patients, 79 (44.6%) patients used anticoagulants (Table 2). The biases (difference/mean (%)) between $[“]_{DBS\text{-}finger}$ and $[“]_{WB\text{-}venous}$ were not significantly different between patients using and not using anticoagulant agents for CsA, tacrolimus, and hematocrit (p greater than 0.05 using the Mann Whitney *U* test; Supplementary Table S2). For creatinine the bias was smaller among anticoagulant users compared to the bias in non-users (medians −8.0% *vs.* −12.4% respectively, p = 0.048 using the Mann Whitney *U* test; Supplementary Table S2).

3.2.3. The effect of the DBS filter paper and sample location

The effect of the DBS filter paper and the location of blood sampling on the measurement of drug, creatinine concentrations, and hematocrit was evaluated. For the effect of the DBS filter paper, the agreement between DBS and whole-blood concentrations was evaluated (*i.e.* $[“]_{DBS\text{-}finger}$ *vs.* $[“]_{WB\text{-}finger}$ and $[“]_{DBS\text{-}venous}$ *vs.* $[“]_{WB\text{-}venous}$; Supplementary Figure S4C and D and Table S3). For the effect of the sample location, the agreement between samples drawn by a fingerprick and samples drawn by venipuncture, with the same method, was evaluated (*i.e.* $[“]_{DBS\text{-}finger}$ *vs.* $[“]_{DBS\text{-}venous}$ and $[“]_{WB\text{-}finger}$ *vs.* $[“]_{WB\text{-}venous}$; Supplementary Figure S4B and F and Table S3). For the majority of the samples, the drug concentrations measured using DBS were slightly different from drug concentrations measured in whole-blood. These results indicated a small systematic bias regarding the effect of the filter paper. No effect of the sample location was observed for CsA, tacrolimus and creatinine, as concentrations of samples obtained by a finger prick were similar to concentrations obtained by venous blood sampling. For hematocrit, a relatively large bias is observed when comparing the location of blood sampling (slope 0.675 for $[Ht]_{DBS\text{-}finger} \sim [Ht]_{WB\text{-}venous}$ and slope 0.724 for $[Ht]_{DBS\text{-}finger} \sim [Ht]_{DBS\text{-}venous}$), whereas this bias was smaller when comparing $[Ht]_{DBS\text{-}venous}$ and $[Ht]_{WB\text{-}venous}$ (0.946) (Supplementary Figure 4.4. and Table S3).

3.3. Clinical validation

Table 3 shows the results of the Passing-Bablok regression of DBS concentrations obtained by a finger prick ($[“]_{DBS\text{-}finger}$) on whole-blood concentrations obtained by venipuncture ($[“]_{WB\text{-}venous}$) before and after applying a correction formula.

3.3.1. Cyclosporine A

A total of 30 patients were included for the validation of the DBS method for CsA. Passing-Bablok regression indicated a systematic bias for CsA, with $[CsA]_{DBS\text{-}finger}$ being lower than $[CsA]_{WB\text{-}venous}$ (Passing-Bablok intercept −1.630, 95%-CI −11.309–7.739; slope 0.828, 95%-CI 0.770–0.959). Without correction, 53% of the CsA measurements were within the 20% LOA and 37% of the CsA measurements were within the 15% LOA (Table 4). The following correction formula, based on the linear regression with interaction for hematocrit and DBS concentrations, was found for cyclosporine: $[CsA]_{corrected} = -85.332 + 0.820 * [CsA]_{DBS\text{-}finger} + 232.761 * [Ht]_{DBS\text{-}finger} + 1.106 * [CsA]_{DBS\text{-}finger} * [Ht]_{DBS\text{-}finger}$, with a Passing-Bablok intercept of −1.655 (95% CI −12.468–11.288) and a slope of 1.000 (95% CI 0.927–1.103; Table 3; Fig. 1A; Supplementary Figure S5A). Using this correction formula, 97% of the CsA measurements were within the 20% LOA and 90% of the CsA measurements were within the 15% LOA (Table 4; Fig. 2A). The mean bias was −0.0013 μg/L (95% CI −36.525 – 36.522).

3.3.2. Tacrolimus

A total of 37 patients were included for the validation of the DBS method for tacrolimus. Passing-Bablok regression indicated a small systematic bias (Passing-Bablok intercept −1.895, 95%-CI −3.289 – −0.935; slope 1.228, 95%-CI 1.038–1.465). Without correction, 68% of the tacrolimus measurements were within the 20% LOA and 59% of the tacrolimus measurements were within the 15% LOA (Table 4). A correction formula that included a hematocrit correction was found for tacrolimus: $[Tac]_{corrected} = 2.584 + 0.805 * [Tac]_{DBS\text{-}finger} - 2.443 * [Ht]_{DBS\text{-}finger}$, with a Passing-Bablok intercept of 0.059 (95% CI −0.917–0.835)

Table 3
Passing-Bablok regression before and after applying correction formulas.

	[“]$_{DBS\text{-}finger}$ ~	Slope	95% CI	Intercept	95% CI
Cyclosporine A					
–	$[CsA]_{WB\text{-}venous}$	0.828	0.770 – 0.959	−1.630	−11.309 – 7.739
*Correction formula**	$[CsA]_{corrected} = -85.332 + 0.820 * [CsA]_{DBS\text{-}finger} + 232.761*[Ht]_{DBS\text{-}finger} + 1.106*[CsA]_{DBS\text{-}finger} * [Ht]_{DBS\text{-}finger}$	1.000	0.927 – 1.103	−1.655	−12.468 – 11.288
Tacrolimus					
–	$[Tac]_{WB\text{-}venous}$	1.228	1.038 – 1.465	−1.895	−3.289 – −0.935
*Correction formula***	$[Tac]_{corrected} = 2.584 + 0.805 * [Tac]_{DBS\text{-}finger} - 2.443* [Ht]_{DBS\text{-}finger}$	1.002	0.848 – 1.184	0.059	−0.917 – 0.835
Creatinine					
–	$[Crt]_{WB\text{-}venous}$	0.857	0.815 – 0.907	5.141	−0.372 – 10.226
*Correction formula****	$[Crt]_{corrected} = ([Crt]_{DBS\text{-}finger} - 1.547)/0.900$	0.954	0.908 – 1.010	3.721	−2.555 – 9.482
Hematocrit					
–	$[Ht]_{WB\text{-}venous}$	0.675	0.586 – 0.780	0.112	0.074 – 0.146
*Correction formula**	$[Ht]_{corrected} = ([Ht]_{DBS\text{-}finger}\text{-}0.130)/0.634$	1.139	0.978 – 1.307	−0.056	−0.114 – 0.003

* Correction for hematocrit using linear regression with interaction with DBS concentrations and hematocrit as independent variables.
** Correction for hematocrit using simple linear regression with DBS concentrations and hematocrit as independent variables.
*** Correction for the deming slope & intercept Crt, creatinine; CsA, cyclosporine A; DBS, dried blood spot; Ht, hematocrit; Tac, tacrolimus; WB, whole-blood.

Table 4
Bland-Altman results for DBS measurements after a fingerprick.

	Cyclosporine A (n = 30)	Tacrolimus (n = 37)	Creatinine (n = 176)	Hematocrit (n = 170)
No correction factor				
Within 20% LOA	16 (53%)	25 (68%)	149 (85%)	158 (93%)
Within 15% LOA	11 (37%)	22 (59%)	112 (64%)	145 (85%)
Absolute bias	-30.327 μg/L	-0.580 μg/L	-12.4486 μmol/L	-0.0046 L/L
95% CI bias	-85.234 – 24.579	-2.233 – 1.073	-45.952 – 21.055	-0.081 – 0.0718
With correction factor				
Within 20% LOA	29 (97%)*	36 (97%)**	162 (92%)***	150 (88%)***
Within 15% LOA	27 (90%)*	35 (95%)**	149 (85%)***	131 (77%)***
Absolute bias	-0.0013 μg/L	-0.0016 μg/L	0.0377 μmol/L	-0.0001 L/L
95% CI bias	-36.525 – 36.522	-1.417 – 1.421	-33.542 – 33.618	-0.088 – 0.088

LOA = limits of agreement (difference/mean).
* Correction for hematocrit using linear regression with interaction with DBS concentrations and hematocrit as independent variables.
** Correction for hematocrit using simple linear regression with DBS concentrations and hematocrit as independent variables.
*** Correction for the Deming slope & intercept.

and a slope 1.002 (95% CI 0.848–1.184; Table 3; Fig. 1B; Supplementary Figure S5B). Using this correction formula, 97% of the tacrolimus measurements were within the 20% LOA and 95% of the tacrolimus measurements were within the 15% LOA (Table 4; Fig. 2B). The mean bias was −0.0016 μg/L (95% CI −1.417–1.421).

3.3.3. Creatinine

For creatinine (n = 176), Passing-Bablok regression indicated a small constant bias, with $[Crt]_{DBS\text{-}finger}$ being lower than $[Crt]_{WB\text{-}venous}$ (Passing-Bablok intercept 5.141, 95%-CI −0.372–10.226; slope 0.857, 95%-CI 0.815–0.907). The following correction formula, based on the Deming slope and intercept, was found for creatinine: $[Crt]_{corrected} = ([Crt]_{DBS\text{-}finger} - 1.547)/0.900$, with a Passing-Bablok intercept of 3.721 (95%-CI −2.555–9.482) and a slope of 0.954 (95%-CI 0.908–1.010; Table 3; Fig. 1C). Using this correction formula, 92% of the creatinine measurements were within the 20% LOA and 85% of the measurements were within the 15% LOA (Table 4; Fig. 2C). The mean bias was 0.038 μmol/L (95% CI –33.542–33.618).

3.3.4. Hematocrit

Finally for hematocrit (n = 170), Passing-Bablok regression indicated a systematic bias between hematocrit values measured with NIR spectroscopy from DBS cards versus the standard method in whole-blood (Passing-Bablok intercept 0.112, 95%-CI 0.074–0.146; slope 0.675, 95%-CI 0.586–0.780). The following correction formula, based on the Deming slope and intercept was found: $[Ht]_{corrected} = ([Ht]_{DBS\text{-}finger}\text{-}0.130)/0.634$, with a Passing-Bablok intercept of −0.056 (95%-CI −0.114– 0.003) and a slope of 1.139 (95%-CI 0.978–1.307; Table 3; Fig. 1D). Using this correction formula, 88% of the hematocrit measurements were within the 20% LOA and 77% of the measurements were within the 15% LOA (Table 4; Fig. 2D). The mean bias was −0.0001 L/L (95% CI −0.088–0.088).

3.4. The patients' experience with DBS

The questionnaire on a patient's experience with the DBS method was completed by 175 participants. The clearness of the instructions was considered good by most patients (poor, n = 0 (0%); moderate, n = 1 (0.6%); sufficient, n = 24 (13.7%); good, n = 148 (84.6%); missing, n = 2 (1.1%)). The median pain score of the venipuncture (1.0; IQR 0.0–3.0; range 0.0–9.0) was comparable to the median pains score of finger prick (1.0; IQR 0.0–2.0; range 0.0–5.0). Most patients preferred a finger prick over a venipuncture (n = 88; 50.3%), some patients preferred a venipuncture over a finger prick (n = 16; 9.1%) and 70 patients had no preference (40.0%).

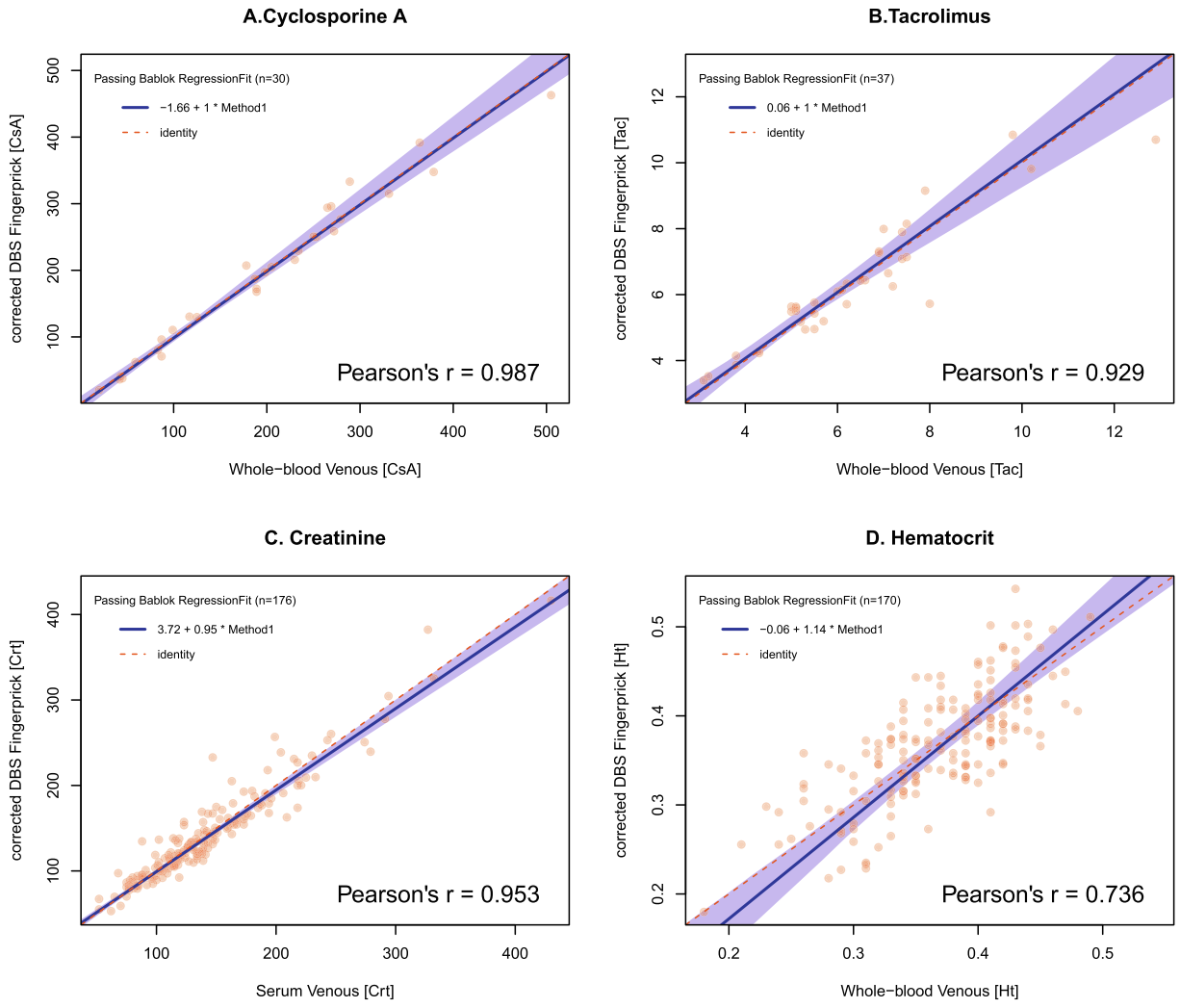

Fig. 1. Passing-Bablok intercept and slope of (A) Cyclosporine A, corrected for hematocrit using linear regression with interaction: $[CsA]_{corrected}$ = -85.332 + 0.820 * $[CsA]_{DBS\text{-}finger}$ + 232.761*$[Ht]_{DBS\text{-}finger}$ + 1.106*$[CsA]_{DBS\text{-}finger}$ *$[Ht]_{DBS\text{-}finger}$; (B) Tacrolimus, corrected for hematocrit using simple linear regression: $[Tac]_{corrected}$ = 2.584 + 0.805 * $[Tac]_{DBS\text{-}finger}$ −2.443* $[Ht]_{DBS\text{-}finger}$; (C) Creatinine; corrected for the Deming slope and intercept: $[Crt]_{corrected}$ = ($[Crt]_{DBS\text{-}finger}$ − 1.547)/0.900; (D) Hematocrit; corrected for the Deming slope and intercept: $[Ht]_{corrected}$ = ($[Ht]_{DBS\text{-}finger}$-0.130)/0.634.

4. Discussion

In this study, a DBS sampling method was clinically validated for simultaneous measurement of CsA, tacrolimus, and creatinine concentrations and hematocrit. For its use in clinical practice correction formulas should be used, to correct among others for hematocrit.

The robustness of the DBS measurement was investigated by evaluating the effect of the location of blood sampling (capillary blood from the finger versus venous blood from the arm), anticoagulant use, and the time between venous and DBS sampling. First, the sample location appeared to have no effect on the measurements of the analytes CsA, tacrolimus, and creatinine, as concentrations in capillary blood drawn by a finger prick were similar to concentrations measured in blood samples drawn by venipuncture. However, a relatively large bias was observed when comparing the hematocrit samples obtained by a fingerprick versus venipuncture, whereas this bias was smaller when comparing two venous hematocrit values (DBS versus whole-blood samples). Secondly, no large biases were observed in the analytical validation of hematocrit measurement using NIR spectroscopy [17]. Therefore, we hypothesize is caused by differences in blood composition when blood is obtained by a fingerprick versus a venipuncture (*i.e.* wound fluid). Second, the effect of anticoagulant use on the DBS measurement was evaluated. Only for creatinine, the median bias in anticoagulant users was 4% lower compared to the bias in non-users. This difference in bias remained after applying the correction formula. However, the difference in bias between non-users and users was small and the bias was distributed around zero, and therefore not considered clinically relevant. Moreover, if anticoagulant use would affect DBS sampling, for example via an effect on the spreading of the blood over the filter paper, we would expect a different direction of the effect and we would expect to see similar results for the other analytes. Therefore, we believe this finding is an incidental finding or caused by confounding effects. Third, the time between the finger prick and the venipuncture is preferably as little as possible in a validation study, especially for drug concentration measurement. However, due to the COVID-19 restrictions in our hospital and laboratory, it was not possible to draw the blood samples simultaneously. For both CsA and tacrolimus, we found a significant correlation between the time between venipuncture and DBS sampling and the drug concentrations. To minimize this time effect on the results of this clinical validation and the correction factors, samples with more than 45 min between DBS and venous blood sampling were excluded from further analysis.

The present study showed small systematic differences between $[\text{“}]_{DBS\text{-}finger}$ and $[\text{“}]_{WB\text{-}venous}$ with, in general, DBS drug concentrations being lower than whole-blood concentrations. The application of a correction factor improved the agreement and bias for all analytes. After

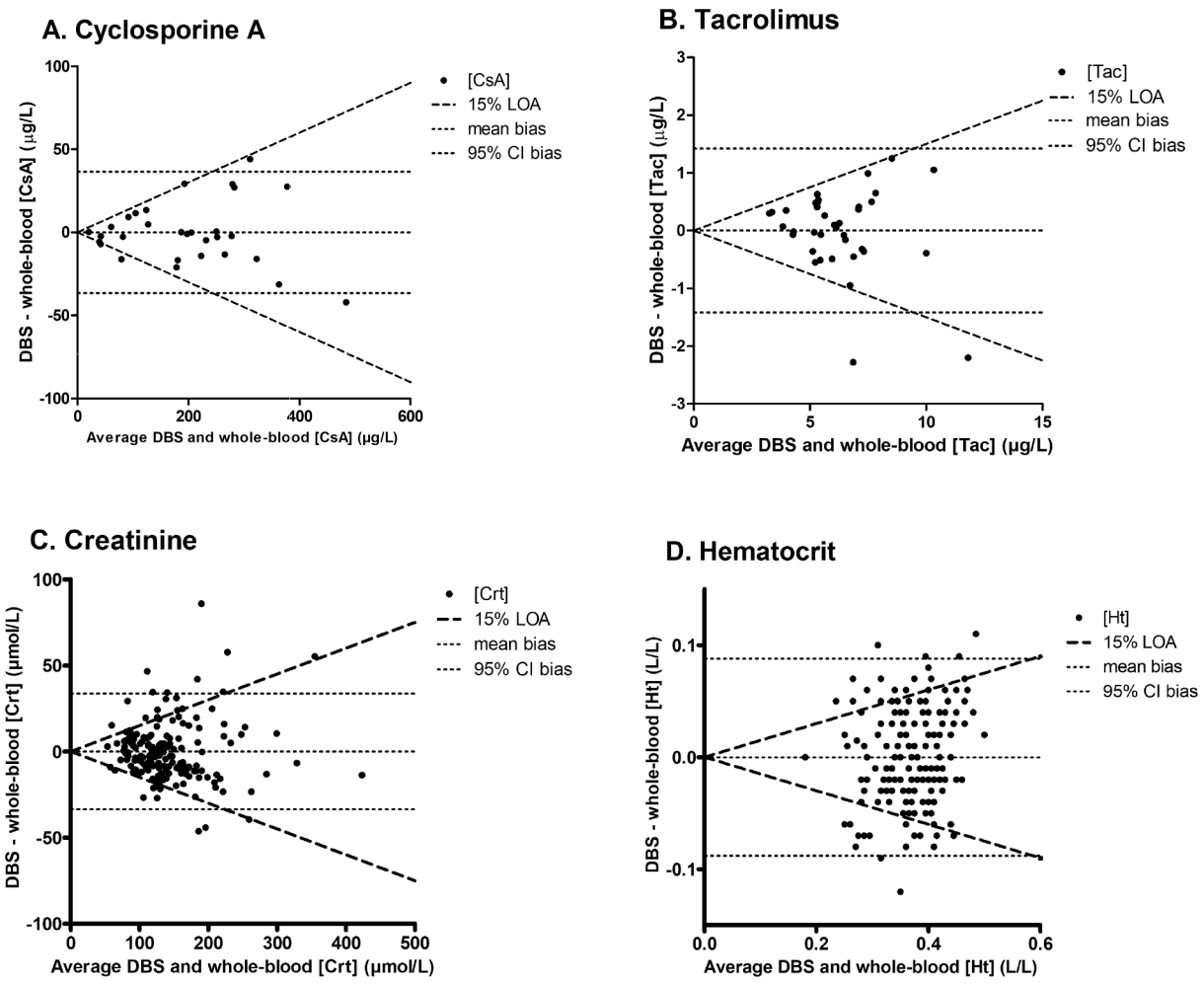

Fig. 2. Bland-Altman plot for [“]$_{\text{DBS-finger}}$ of (A) Cyclosporine A, corrected for hematocrit using linear regression with interaction: $[\text{CsA}]_{\text{corrected}} = -85.332 + 0.820 * [\text{CsA}]_{\text{DBS-finger}} + 232.761 * [\text{Ht}]_{\text{DBS-finger}} + 1.106 * [\text{CsA}]_{\text{DBS-finger}} * [\text{Ht}]_{\text{DBS-finger}}$; (B) Tacrolimus, corrected for hematocrit using simple linear regression: $[\text{Tac}]_{\text{corrected}} = 2.584 + 0.805 * [\text{Tac}]_{\text{DBS-finger}} - 2.443 * [\text{Ht}]_{\text{DBS-finger}}$; (C) Creatinine; corrected for the Deming slope and intercept: $[\text{Crt}]_{\text{corrected}} = ([\text{Crt}]_{\text{DBS-finger}} - 1.547)/0.900$; (D) Hematocrit; corrected for the Deming slope and intercept: $[\text{Ht}]_{\text{corrected}} = ([\text{Ht}]_{\text{DBS-finger}} - 0.130)/0.634$. LOA, Limits of agreement, difference/mean; Bias, absolute difference; 95% CI bias, difference ± 1.96 standard deviation of the difference.

applying a correction factor, the DBS method fulfilled the requirements for clinical validation, as described in the IATDMCT guidelines (>67% of the samples <20% LOA), for CsA, tacrolimus, creatinine and hematocrit measurement [10,19]. As in clinical practice bias over 20% can have clinical consequences, more precise results are required, and therefore our center has stricter criteria (95% of the samples within the 15% LOA). For tacrolimus, the DBS method also fulfilled these stricter criteria. For CsA (90%) and creatinine (85%), the DBS method was close to meet these stricter criteria. As the method easily fulfills the official requirements for clinical validation, the method could be used for creatinine and CsA monitoring in clinical practice. However, one should consider a little higher variability compared to venous blood sampling in the measurements using DBS when interpreting the results.

The small systematic differences between [“]$_{\text{DBS-finger}}$ and [“]$_{\text{WB-venous}}$ might be explained by the effect of the DBS filter card. The extraction recovery rate of the blood from the filter paper is not 100%, and consequently lower concentrations can be measured in the DBS samples, although one would expect this was corrected for using DBS calibrators. In recent years, multiple research groups validated a DBS method for CsA, tacrolimus, and creatinine measurement [8,14,20–26], of which only few performed a clinical validation [8,14,20]. In line with the present study, Hinchliffe *et al.* observed in a clinical validation, a negative bias for tacrolimus, and a non-significant positive bias for CsA DBS measurement in respectively 42 and 46 solid organ transplant recipients [20]. Also, Veenhof *et al.* and Koster *et al.* observed small, but non-significant systematic differences between DBS and whole-blood concentrations for CsA, and tacrolimus [8,14]. Therefore, no correction formula was considered necessary and it was not evaluated whether a correction formula could improve the agreement and bias. Also, other analytical and clinical validation studies did not observe significant systematic differences and did not apply a correction factor [8,21,24,25,27,28]. For creatinine, Veenhof *et al.* observed lower DBS concentrations compared to venous concentrations, for which a correction factor was applied [14]. Differences between studies may be explained by differences in DBS filter papers that were used, study design (analytical *vs.* clinical validation) and analytical methods. Therefore it is important to extensively validate the method before implementing DBS in clinical practice according to the official guidelines [10,19].

Hematocrit is a factor that can affect DBS measurements in different ways. Hematocrit can affect the extraction recovery rate of the sample from the DBS paper, as well as the spreading of the blood over the DBS filter paper (*i.e.* area bias) [10–12,29]. In the present study a correction factor that included hematocrit, improved bias and agreement between [“]$_{\text{DBS-finger}}$ and [“]$_{\text{WB-venous}}$ for both CsA and tacrolimus. Remarkably, the direction of the hematocrit correction was different for the analytes, indicating that the main effect of hematocrit on DBS measurements may differ per analyte. For CsA, $[\text{CsA}]_{\text{DBS-finger}}$ were corrected to higher

concentrations with a higher hematocrit and higher CsA concentrations. These findings indicate that the recovery of the blood from the filter paper is lower with increasing hematocrit values, and that this hematocrit effect also depends on the height of a patient's CsA concentration. This is supported by previous studies, which observed an association between extraction recovery rates and hematocrit values for different analytes [23,30,31]. However, this is not supported by the findings of our analytical validation, in which the hematocrit effect on the CsA recovery rate appeared low. In contrast, for tacrolimus we would have expected an effect of hematocrit on recovery based on the analytical validation. However, in the clinical validation, $[Tac]_{DBS\text{-}finger}$ were corrected to lower concentrations with higher hematocrit. Although it should be noted that the hematocrit effect for tacrolimus was small, this finding might be explained by the viscosity of the blood and it's spreading over the DBS filter paper. Patients with a higher hematocrit have more viscous blood and generate smaller drops that might be less distributed on the DBS paper [32]. Consequently, higher DBS concentrations are measured in samples with higher hematocrit values. An explanation for the differences in hematocrit effect for CsA and tacrolimus is that hematocrit has a combined effect on DBS measurement and that which hematocrit effect is most important can differ per analyte. Another explanation for the correction factors that were found in the present study could be that there is no causal relationship between hematocrit and DBS measurement, but that there is an unknown factor that affects the DBS measurements of both hematocrit and other analytes, and thereby leads to inclusion of hematocrit as correction factor. These different findings for the hematocrit effect in the clinical validation and the analytical validation support the need for clinical validation studies. Previous studies also reported on hematocrit effects in DBS sampling for CsA, creatinine, and tacrolimus [8,22,23,33]. In an analytical validation of a DBS method for creatinine, hematocrit correction improved the agreement between DBS and whole-blood samples [33]. In an analytical validation performed by Koster *et al.* positive biases were observed for increasing hematocrit values for both CsA and tacrolimus [8]. In line with the present results they observed a hematocrit and concentration dependent bias for CsA measurement with DBS. After hematocrit correction the biases were within the 15% LOA for CsA and tacrolimus. However the authors stated that the hematocrit effect was caused by a chromatographic effect rather than an extraction effect, as no effect on extraction recovery was observed. In a clinical validation they observed a non-significant positive bias and no hematocrit correction was needed [8]. In another study by Koster *et al.*[22] a DBS method for the measurement of tacrolimus and CsA concentrations was clinically validated, without the need for hematocrit correction.

In this study, the DBS method was clinically validated for the simultaneous measurement of CsA, tacrolimus and creatinine, and hence the method can be used in clinical practice. The main advantage of using a DBS method is that the blood sampling can be performed at home. This allows blood sampling at a specific time point (pre-dose concentrations), which reduces the variability in the concentration measurement caused by the timing of dose ingestion and blood sampling. Blood sampling at home also enables blood sampling at multiple time points (AUCs), which is the gold standard for TDM of tacrolimus. Moreover, most patients prefer a finger prick over venous blood sampling. However, when implementing DBS in clinical practice one should consider that home sampling requires appropriate training of patients, healthcare physicians, and laboratory technicians. Without the right instructions and training, mistakes in DBS blood sampling and analysis are easily made. This can in turn lead to a high proportion of samples that are of insufficient quality for analysis or can affect the validity of your results [34–36]. However, by providing clear instructions and by training the patients for DBS sampling during hospitalization, we expect little problems with at-home sampling. Another consideration is that, based on the acceptance criteria, for DBS tacrolimus measurement the LLOQ is 2 ng/mL, whereas for the whole-blood tacrolimus concentration measurement the LLOQ is 1.0 ng/mL. Therefore, for patients in which a very low tacrolimus concentration is expected, the whole-blood method might be favorable.

A limitation of this study is that the venipuncture and the DBS sample were not drawn simultaneously due to COVID-19 restrictions. This might have caused differences in drug concentrations that were not caused by the method of measurement, but by a "real" difference in concentrations. However, as mentioned above, this time effect was minimized by excluding samples with more than 45 min between DBS and venous blood sampling. Moreover, the order of DBS sampling and venipuncture was different per patient, and therefore a dilution of the time effect is expected. Another limitation is that we had to exclude some samples from the final analysis for several reasons, among which peak concentration measurement and time effects. Therefore, for CsA and tacrolimus we did not reach the recommended number of samples for a clinical validation [10].

Conclusion

A DBS sampling method was clinically validated for simultaneous measurement of CsA, and tacrolimus and creatinine with the use of correction formulas and can therefore be used in clinical practice.

Declarations

The other authors have no conflicts of interest to disclose.

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Conflicts of interest

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CRediT authorship contribution statement

Marith I. Francke: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Project administration. **Bart van Domburg:** Methodology, Validation, Investigation, Writing – original draft. **Samah Bouarfa:** Investigation, Data curation, Writing – review & editing. **Daan van de Velde:** Investigation, Writing – review & editing. **Merel E. Hellemons:** Resources, Writing – review & editing. **Olivier C. Manintveld:** Resources, Writing – review & editing. **Suzanne Last-Koopmans:** Resources, Writing – review & editing. **Midas B. Mulder:** Resources, Writing – review & editing. **Dennis A. Hesselink:** Conceptualization, Resources, Writing – review & editing, Supervision. **Brenda C.M. de Winter:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data is available upon reasonable request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2022.08.014.

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