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ORIGINAL ARTICLE

Evaluation of the New Cyclosporine and Tacrolimus Automated Electrochemiluminescence Immunoassays under Field Conditions

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SUMMARY

Background: **Careful monitoring of the post-transplantation immunosuppressant drugs (ISDs) cyclosporine (CsA) and tacrolimus (TAC) in whole blood is essential to prevent adverse drug events. Immunoassays represent the most widely used methodology for therapeutic drug monitoring. In this study, the technical performance of the new automated electrochemiluminescence immunoassays (ECLIAs) for CsA and TAC measurement were assessed under field conditions.**

Methods: **Residual whole blood samples from patients undergoing CsA or TAC therapy following organ transplant were used to evaluate the assays at six independent laboratories across four countries. Experiments included within-run imprecision using PreciControl ISD controls and recovery of commercial external quality assurance (EQA) scheme samples. Both assays were compared with liquid chromatography-tandem mass spectrometry (LC-MS/MS), using methods routinely employed at each investigational site, as well as with an equivalent commercial chemiluminescent microparticle immunoassay (CMIA) and enzyme multiplied immunoassay (EMIT).**

Results: **Within-run imprecision testing gave coefficients of variation of ≤ 5% in the > 90.0 - 2000 ng/mL range for the CsA ECLIA and ≤ 4.2% in the 3.5 - 12 ng/mL range and ≤ 4.9% in the > 12 - 40 ng/mL range for the TAC ECLIA. EQA sample recovery by ECLIA gave a mean bias of 6.9% for CsA and 4.9% for TAC versus the spiked concentration or the mean LC-MS/MS value. Deming regression analysis of ECLIA method comparison to LC-MS/MS for all sites yielded a slope of 1.22, intercept 8.43 ng/mL and r = 0.97 for CsA and a slope of 1.22, intercept -0.51 ng/mL and r = 0.96 for TAC. Comparison with CMIA yielded a slope of 0.87, intercept 5.51 ng/mL and r = 0.97 for CsA and a slope of 0.98, intercept 0.12 ng/mL and r = 0.97 for TAC. Comparison with EMIT yielded a slope of 1.23, intercept -8.74 ng/mL and r = 0.96 for CsA.**

Conclusions: **The CsA and TAC ECLIA compare favorably with existing commercial immunoassays and with LC-MS/MS. They represent modern generation assays that meet the demands of monitoring drug concentrations in current immunosuppressive regimens. This study also highlights the importance of standardizing protocols and LC-MS/MS methods to give improved comparability between ISD assays.**

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INTRODUCTION

Cyclosporine A (CsA) was the first calcineurin inhibitor (CNI) to be introduced as an immunosuppressant drug in the 1980s. Over the following years CsA became the cornerstone for preventing rejection in transplantation medicine [1].

In the early 1990s, the discovery of the second generation CNI, tacrolimus (TAC), further enhanced the therapeutic success of organ transplantation [2]. TAC has subsequently replaced CsA as the first-choice immunosuppressant. However, as the only immunosuppressant approved for the treatment of bone-marrow graft-versus-host disease, CsA remains an important drug in transplantation medicine. This is especially true given the uncertainty over the higher incidence of diabetes in patients receiving TAC therapy as compared with CsA [3,4].

Following the introduction of TAC, therapeutic drug monitoring (TDM) was recommended. CNIs are critical dose drugs with a narrow therapeutic index between adequate immunosuppression and adverse drug events. Therefore, the toxicity profile of CNIs is a continued concern and can lead to poor patient outcome [2-5]. Drug levels also exhibit a high degree of inter- and intra-patient variability, and there is a risk of poor or non-compliance due to lifelong reliance on the drug [5]. Treatment strategies often involve combining CNIs with other drugs and significant drug-drug interactions can occur [6]. As a result, effective TDM is extremely important in CNI patients.

The need for tight control of immunosuppressive drug levels to maintain each patient's drug exposure within a narrow therapeutic window has made it extremely important for laboratories to provide rapid and precise drug concentration measurements [5].

The majority of clinical laboratories measure CNIs by semi-automated and automated immunoassays [7]. This is supported by recent surveys, which suggest that 60 - 80% of participating laboratories are using an immunoassay [8,9]. However, quantification of CNI in blood using liquid chromatography mass spectrometry (LC-MS) and liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) is becoming increasingly common [5]. An advantage of using LC-MS/MS is the favorable analytical specificity and sensitivity over existing immunoassays, as well as multiplex testing capabilities. However, implementation of LC-MS/MS into routine clinical laboratories remains a challenge, and the lack of acceptance may reflect the initial high instrument costs, lack of automation, and need for specialized staff training [10].

The use of immunoassays offers flexibility in terms of operation and timeframe as well as being relatively easy to incorporate into existing automated systems and laboratory workflow [10]. However, many of the currently available assays have limited analytical performance and can be susceptible to interference from cross-reacting drugs, molecules or metabolites, resulting in overestimation of the drug concentration [11,12]. Only a few of the existing immunoassays offer the requisite sensitivity, accuracy, and precision [13-15]. Therefore, the need remains for alternative, robust and time-efficient assays for CsA and TAC to fulfill the therapeutic monitoring needs of the clinical routine laboratory.

The new Elecsys® automated electrochemiluminescence immunoassays (ECLIA) (Roche Diagnostics GmbH, Mannheim, Germany) for the quantification of CsA or TAC in whole blood have been developed to be implemented on an existing commercial analyzer, the cobas®e analyzer (Roche Diagnostics GmbH, Rotkreuz, Switzerland). Both assays have been shown to be fit for purpose for TDM in transplantation medicine in previous analytical evaluations [8,9].

The aim of this study was to assess the technical performance of both the CsA and TAC ECLIA under field conditions, as well as to generate descriptive data comparing these assays to LC-MS/MS and routine methods in specific clinical cohorts. These cohorts consist of transplant patients receiving CNI therapy preventing organ rejection.

MATERIALS AND METHODS

A multicenter evaluation of the Elecsys® CsA and TAC ECLIA involved six independent laboratories from four countries (UK, Italy, Germany, and Spain), although not all investigations were performed by all sites. Only remnant leftover samples from routine testing were used and waivers were obtained from the responsible ethics committees where applicable. All investigational sites conducted the study in accordance with the Declaration of Helsinki (as amended in Tokyo, Venice, and Hong Kong), or country-specific regulations and followed the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Good Clinical Practice guidelines.

ECLIA

The ECLIA assays, for use on the cobas e analyzer, use the principle of electrochemiluminescence for detection and measurement of analyte in samples [16]. The ECLIA measuring principle uses a ruthenium-labelled complex and biotinylated analyte-specific antibodies, which are combined with pre-treated samples and recovered with streptavidin microparticles.

Measurements were performed according to the manufacturer's instructions. Briefly, the material to be measured (calibrators, quality control [QC] material or patient whole-blood specimen) was equilibrated to room

temperature (20 - 25°C) and mixed gently but thoroughly to re-suspend erythrocytes. 300 µL of this material, for both CsA and TAC analysis, was then combined with 300 µL of Elecsys® ISD sample pre-treatment reagent (Roche Diagnostics GmbH, Mannheim, Germany), in a single pre-treatment step, and vortexed for ≥ 10 seconds. Samples were subsequently centrifuged for 4 minutes at ≥ 10,000 g and the supernatant was transferred to an appropriate vial and capped before being loaded onto the system. Pre-treated samples were stored in closed tubes for up to 4 hours at 20 - 25°C.

The TAC and CsA ECLIA were calibrated using their respective CalSet (Roche Diagnostics GmbH, Mannheim, Germany), each with two concentration levels. Calibrators were reconstituted according to the manufacturer's instructions and stored in 300 µL aliquots at 2 - 8°C for 7 days or below -15°C for 28 days. CalSet level 1 and 2 were processed with pre-treatment reagent alongside patient samples (the same for both CsA and TAC immunoassays). Calibration was performed once per reagent lot and as required for maintaining QC values within acceptable limits. The lot calibration stability period was 28 days.

The QC to measure appropriate function of the instrument was PreciControl ISD (PC ISD) and was provided by Roche Diagnostics, GmbH (Mannheim, Germany). QC samples were prepared and stored in a similar manner to calibrators. In order to validate each run, controls were measured prior to the investigation of patient samples and sample measurements were only valid if the corresponding control material fell within the target range. The limit of detection of the CsA and TAC ECLIA assays are 30 ng/mL and 0.5 ng/mL, respectively.

LC-MS/MS and comparator assays

Each site tested the CsA and/or TAC ECLIA and compared them with either LC-MS/MS measurements or at least one comparator assay. Details of the comparator assays used by each site are given in Table 1. Sample measurement using chemiluminescent microparticle immunoassay (CMIA) on the Abbott Architect platform (Abbott Laboratories, Abbott Park, IL, USA) and enzyme multiplied immunoassay (EMIT) on the Integra 800 platform (Roche Diagnostics GmbH, Mannheim, Germany) were performed according to the manufacturers' instructions and according to site-specific standard operating procedures. The investigational sites utilizing LC-MS/MS performed measurements according to the protocols developed and routinely used at the specific site.

Samples

EDTA whole-blood samples from patients who had received a heart, kidney or liver transplant under CsA or TAC therapy were provided based on the availability at the different sites. Details of the cohorts tested at the different sites and the number of samples contributed are given in Table 1. Samples were "remnant" samples, i.e., leftover from other sampling. Samples to be tested within 8 hours were stored at room temperature (18 - 25°C). Alternatively, samples to be tested within 7 days were stored refrigerated at 2 - 8°C. Where longer storage was required, samples were frozen below -20°C. Samples not available on site were shipped frozen on dry ice in a thermally insulated container and stored at below -20°C until analysis. Samples were measured within 6 months after collection. Aliquoted samples did not undergo more than one freeze/thaw cycle. Frozen samples were thawed and mixed thoroughly before use. Samples were never centrifuged.

Assay imprecision

The appropriate function of the instrument and the reagent handling was verified by performing a within-run precision experiment using PC ISD controls at three concentration levels. 21 aliquots were prepared from each level with 300 µL per aliquot. Following system calibration, a single run was performed with 21 replicates from each sample material.

The acceptance criteria for CsA ECLIA assay imprecision were set at a concentration of 30.0 - 90.0 ng/mL: ≤ ± 7.00 ng/mL (SD); at a concentration of > 90.0 - 2000 ng/mL: ≤ 8% (coefficient of variation [CV]). The acceptance criteria for TAC ECLIA assay imprecision were set at a concentration of 0.5 to 3.5 ng/mL: ≤ ± 0.25 ng/mL (SD); at a concentration of > 3.5 - 12 ng/mL: ≤ 5% (CV); and at a concentration of > 12 - 40 ng/mL: ≤ 6% (CV).

External quality assurance (EQA) scheme

EQA samples from the International Proficiency Testing (IPT) scheme organized by Analytical Services International (ASI Ltd, London, UK) were measured at all investigational sites by ECLIA, LC-MS/MS, CMIA or EMIT. The scheme includes samples that are either spiked to a known concentration ("spiked" samples) or pooled patient samples. All instruments were calibrated before the experimental run. Samples were measured in triplicate in a single analytical run for each sample material. For each EQA sample, bias relative to the spiked value for spiked samples, or to the mean LC-MS/MS value from the overall EQA group results (as displayed on the IPT reports) for pooled samples, was calculated for the ECLIA, LC-MS/MS, CMIA, and EMIT method means.

Method comparison

The method comparison experiment was performed at all investigational sites, comparing the ECLIA on cobas e 411, e 601, e 602 and modular analytics platforms with either LC-MS/MS or an alternative comparator immunoassay (depending on the site) for CsA or TAC. Remnant samples from patient cohorts for heart, liver or kidney were measured with one replicate per method. More than 60 samples per tested cohort and a minimum of 120 samples over all cohorts were measured for each assay. Comparisons were calculated using weighted

Deming regression.
Results were rated against the following criteria. CsA ECLIA versus LC-MS/MS: Slope 1.00 ± 0.20; intercept ≤ ± 30.0 ng/mL; Pearson's correlation $r \geq 0.9$; SD for Deming on ECLIA CV = 10%. CsA ECLIA versus CMIA: Slope 1.00 ± 0.20; intercept ≤ ± 30.0 ng/mL; Pearson's correlation $r \geq 0.9$; SD for Deming on CMIA = 15% and ECLIA CV = 10%. TAC ECLIA versus LC-MS/MS: Slope 1.00 ± 0.20; intercept ≤ ± 0.5 ng/mL; Pearson's correlation $r > 0.9$; SD for Deming on ECLIA CV = 5%. TAC ECLIA versus CMIA: Slope 1.00 ± 0.20; intercept ≤ ± 0.5 ng/mL; Pearson's correlation $r > 0.9$, SD for Deming on ECLIA CV = 7% and CMIA CV = 10%. To gain further information on method comparability, Bland-Altman difference plots were evaluated.

Discrepant results
Discrepant results were resolved by comparing them with the respective results obtained with LC-MS/MS. All results displaying a greater than 40% difference to LC-MS/MS were considered discrepant. If sufficient sample volume remained, the sample was re-tested in triplicate using all analyzers. Where insufficient sample volume was available, testing was repeated in triplicate using the assay which showed the discrepant value. Where sample volumes were very low, single or duplicate testing was used.

Statistical analysis
Statistical analysis was performed using the Windows-based Computer Aided Evaluation (WinCAEv) data capture software to calculate slope, intercept, and Pearson's *r*.

RESULTS

Assay imprecision
Results for within-run precision of the ECLIA are given in Table 2. For the CsA ECLIA all sample concentrations > 90.0 - 2000 ng/mL had a within-run CV ≤ 5% (within the acceptance criteria). For the TAC ECLIA all sample concentrations between 3.5 - 12 ng/mL had a within-run CV ≤ 4.2%, and all sample concentrations between 12 - 40 ng/mL had a within-run CV ≤ 4.9% (within acceptance criteria).

EQA scheme
Results from the EQA sample measurements are outlined in Table 3. The mean of all sites is presented for the ECLIA. For all other methods tested, the results for each site are displayed separately. ECLIA yielded a mean bias of 6.9% and 4.9% for CsA and TAC samples, respectively, across all sites. For CsA samples, a mean bias of 3.1% was found for LC-MS/MS at the Newcastle site and a mean bias of -27.3% at the Bad Oeynhausen site. A mean bias of 2.3% and -17.5% was found using CMIA and EMIT, respectively. For TAC samples, a mean bias of -2.3% was found for LC-MS/MS at the Newcastle site and -13.6% at the Bad Oeynhausen site. CMIA yielded a mean bias of 1.2%, 7.9%, and 14.7% at the Milan, Dortmund, and Bilbao sites, respectively.

Method comparison
Comparability of CsA and TAC ECLIA results with LC-MS/MS and alternative routine assays were investigated for specimens derived from heart, liver, and kidney recipients using weighted Deming regression analysis.

Comparison of CsA assays
Figure 1A shows the weighted Deming regression of the CsA LC-MS/MS measurements versus ECLIA for 264 samples from all sites and cohorts (Bad Oeynhausen, n = 123; Newcastle, n = 120; Peterborough, n = 21). Assay-specific results were combined for the evaluation. The slope value for all transplant patients was 1.22 (95% CI: 1.18 - 1.26), the intercept was 8.43 ng/mL (95% CI: 5.09 - 11.8 ng/mL) and Pearson's correlation *r* was 0.97. The expected results were met at the Newcastle site (slope 1.16, 95% CI: 1.11 - 1.21; intercept -0.74 ng/mL, 95% CI -7.52 - 6.05; Pearson's correlation $r = 0.99$). However, the slope from samples measured at the Bad Oeynhausen site did not meet the expected results (slope 1.39, 95% CI: 1.31 - 1.46; intercept 4.14 ng/mL, 95% CI -1.49 - 9.77; Pearson's correlation $r = 0.97$). In addition, only 21 samples could be measured at the Peterborough site due to sample unavailability. Therefore, the method comparison over all sites did not quite meet the expected criteria due to the high slope. Pearson's correlation *r* was very similar for the global comparison and the three individual patient cohorts (heart = 0.97, liver = 0.99, and kidney > 0.9) (Supplementary Figure 1). The bias between the ECLIA and LC-MS/MS methods for CsA is demonstrated in the Bland-Altman difference plot shown for each site in Figure 1B. The mean bias at the Newcastle site was 16.3% (± 3 SD: -17.6 - 50.2%), compared with 44.2% (± 3 SD: -16.7 - 105.2%) at Bad Oeynhausen and 46.5% (± 3 SD: -1.2 - 94.3%) at Peterborough.
Results of the comparison between CsA ECLIA and EMIT for all the available cohorts are shown in Figure 2A (Dortmund, n = 126). The slope value was 1.23 (95% CI: 1.14 - 1.32), intercept -8.74 ng/mL (95% CI: -17.6 - 0.13 ng/mL) and Pearson's correlation *r* was 0.96. The comparison between CsA ECLIA and CMIA for all cohorts is shown in Figure 2B (Milan, n = 128). The slope value was 0.87 (95% CI: 0.82 - 0.91), intercept 5.51 ng/mL (95% CI: -1.52 - 12.5 ng/mL) and Pearson's correlation *r* was 0.97. These results were well within the pre-defined expected ranges. Pearson's correlation *r* was the same for the global comparison and the individual patient cohorts (Supplementary Figure 2). The Bland-Altman difference plots for CsA ECLIA versus EMIT and CMIA are shown in Figure 2C and 2D. Comparison of ECLIA and EMIT gave an

Table 1. Overview of competitor assays used and samples tested at each of the study centers.

Laboratory	Instrument	Method	Cohort	Number of samples contributed		
				TAC	CsA	Total
Bad Oeynhausen (Germany)	cobas e 411 LC-MS/MS	ECLIA in house	Heart	121	123	244
Dortmund (Germany)	cobas e 602 Integra 800 (CsA) Abbott Architect (TAC)	ECLIA EMIT CMIA	Kidney	125	126	251
Newcastle (UK)	E170 LC-MS/MS	ECLIA in house	Heart, liver, kidney	120	120	240
Peterborough (UK)	cobas e 602 LC-MS/MS (Addenbrookes Hospital, Cambridge)	ECLIA in house	Liver, kidney	117	21	138
Milan (Italy)	cobas e 411 Abbott Architect	ECLIA CMIA	Liver, kidney	128	128	256
Bilbao (Spain)	cobas e 601 Abbott Architect	ECLIA CMIA	Liver, kidney	124	0	124

CsA - cyclosporine, CMIA - chemiluminescent microparticle immunoassay, ECLIA - electrochemiluminescence immunoassay, EMIT - enzyme multiplied immunoassay technique, TAC - tacrolimus.

average bias of 15.3% and 3 SD limits of -28.6% and 59.2%. Comparison of ECLIA and CMIA gave an average bias of -9.0% with 3 SD limits of -54.5% and 36.5%.

Comparison of TAC assays

Results of the method comparison between LC-MS/MS measurements versus ECLIA for TAC from all sites and cohorts are shown in Figure 3A. Assay specific results were combined for the evaluation. 120 samples were measured at the Peterborough site; however three showed results below the technical limit. The weighted Deming regression analysis using 358 paired data points (Bad Oeynhausen, n = 121; Newcastle, n = 120; Peterborough, n = 117) revealed a slope value for all transplant patients of 1.22 (95% CI: 1.17 - 1.26), the intercept was -0.51 ng/mL (95% CI: -0.74 - -0.28 ng/mL) and Pearson's correlation *r* was 0.96. The expected results were met for one site, Newcastle (slope 1.06, 95% CI: 1.02 - 1.10; intercept -0.46 ng/mL, 95% CI -0.70 - -0.23; Pearson's correlation *r* = 0.99). The method comparison from Bad Oeynhausen and Peterborough did not meet the expected results. The slope for all sites did not meet the expected results. Pearson's correlation *r* was very similar for the global comparison and the three patient cohorts (heart = 0.97, liver = 0.96, and kidney = 0.96) (Supplementary Figure 3). The Bland-Altman difference plot for TAC ECLIA versus LC-MS/MS for all sites is shown in Figure 3B. The mean bias observed at the Newcastle site was -1.3% (± 3 SD: -28.8 - 26.1%) compared with 20.7% (± 3 SD: -18.5 - 60.0%) at Bad Oeynhausen and 23.6% (± 3 SD: -23.9 - 71.0%) at Peterborough.

Figure 4A shows the weighted Deming regression analysis method comparison of TAC ECLIA and CMIA for all cohorts using 377 paired data points (Bilbao, n = 124; Dortmund, n = 125; Milan, n = 128). The slope value was 0.98 (95% CI: 0.96 - 1.0), intercept 0.12 ng/mL (95% CI: 0.01 - 0.22 ng/mL) and Pearson's correlation *r* was 0.97. All results were well within the defined expected ranges. Pearson's correlation *r* was similar for the individual patient cohorts (liver = 0.99 and kidney = 0.97) (Supplementary Figure 4). The Bland-Altman difference plot for TAC ECLIA versus CMIA for all sites is shown in Figure 4B. The mean bias observed at the Bilbao site was -2.2% (± 3 SD: -29.3 - 24.8%) compared with 8.6% (± 3 SD: -21.3 - 38.5%) at Dortmund and -3.7% (± 3 SD: -30.8 - 23.4%) at Milan.

DISCUSSION

In this study, the technical performance of the new CsA and TAC ECLIAs were investigated at six independent study sites. Good assay precision and close correlation with LC-MS/MS and other routine methods was observed. All comparison data fell within the acceptance criteria for within-run precision.

Excellent correlation was observed between CsA ECLIA and EMIT as well as between both CsA and TAC ECLIA and CMIA. For the ECLIA versus CMIA method comparison calculated results were well within the defined expected ranges, including at each individual site and for all transplant types, and there were no significant differences between the individual cohorts. Therefore, both the CsA and TAC ECLIA compare fa-

Table 2. Imprecision of the ECLIA assay.

	CsA			TAC		
	Mean, ng/mL	SD, ng/mL	Within-run CV, %	Mean, ng/mL	SD, ng/mL	Within-run CV, %
Bad Oeynhausen						
ISD L1	71.09	2.71	3.8	2.70	0.13	4.9
ISD L2	337.71	7.93	2.3	11.69	0.46	3.9
ISD L3	1310.76	39.37	3.0	23.28	1.13	4.9
Dortmund						
ISD L1	77.07	2.04	2.6	2.66	0.06	2.1
ISD L2	369.95	9.01	2.4	11.06	0.27	2.4
ISD L3	1462.05	36.27	2.5	21.33	0.35	1.6
Bilbao						
ISD L1	82.03	2.72	3.3	2.56	0.15	5.7
ISD L2	346.51	17.23	5.0	10.11	0.42	4.2
ISD L3	1307.76	31.06	2.4	19.68	0.41	2.1
Newcastle						
ISD L1	75.24	3.04	4.0	2.54	0.08	3.2
ISD L2	357.50	8.30	2.3	10.66	0.24	2.3
ISD L3	1307.05	22.61	1.7	20.18	0.44	2.2
Peterborough						
ISD L1	79.23	1.68	2.1	3.02	0.07	2.2
ISD L2	373.15	10.39	2.8	11.55	0.22	1.9
ISD L3	1435.95	39.25	2.7	21.57	0.44	2.0
Milan						
ISD L1	82.53	3.69	4.5	3.15	0.10	3.3
ISD L2	342.24	5.87	1.7	10.95	0.25	2.3
ISD L3	1274.14	56.83	4.5	20.29	0.64	3.2

Results from measurements at all centers are shown.
Target concentration for CsA, PreciControl ISD level 1: ~80 ng/mL, PreciControl ISD level 2: ~300 ng/mL, PreciControl ISD level 3: ~1080 ng/mL.
Target concentration for TAC, PreciControl ISD level 1: ~2.40 ng/mL, PreciControl ISD level 2: ~9.40 ng/mL, PreciControl ISD level 3: ~17.30 ng/mL.
CsA - cyclosporine, CV - coefficient of variation, ISD - immunosuppressant drug, SD - standard deviation, TAC - tacrolimus.

vorably with currently available immunoassays.

For the CsA ECLIA and LC-MS/MS method comparison, combined data analyzed by weighted Deming regression and Pearson's correlation revealed values close to, but just outside the expected range. Close correlation was found between the CsA ECLIA and LC-MS/MS, as previously observed [9]. However, the slope value was slightly higher than the expected value of 1.00 ± 0.20. Method comparison at the Newcastle site showed excellent agreement with the LC-MS/MS method. However, results obtained from the Bad Oeynhausen site displayed a high slope and the small number of samples that were obtained from the Peterborough site also showed a trend for a slope that deviated from the expected range. Therefore, the presence of a slope just outside the expected criteria in the method comparison for all sites occurred as a result of the high slope from these two sites. Close correlation was also found between TAC ECLIA and LC-MS/MS, as previously demonstrated [8]. However, as for the CsA assay, the method comparison over all sites was just outside the expected results due to a high slope from two of the sites. ECLIA showed excellent comparison to the LC-MS/MS method at the Newcastle site; at the Bad Oeynhausen and Peterborough sites, however, the slope was again marginally higher than the expected value.

Data from the EQA scheme demonstrated very different results in sample recovery between the Newcastle and Bad Oeynhausen sites. The mean bias for measurement of CsA by LC-MS/MS at the Bad Oeynhausen site was

Table 3. Mean bias between method-specific CsA and TAC concentrations found with the EQA samples.

CsA							
	IPT sample number	Spiked samples				Pooled samples	
		340A	342C	344A	345B	346B	348B
IPT	Spiked conc, ng/mL	1000	800	400	80	*Pooled*	*Pooled*
	Mean LC-MS/MS results, ng/mL	979	789	376	79.5	43.3	118
LC-MS/MS	Newcastle						
	Result, ng/mL	1044	847	402	85.5	43.9	118
	Bias, %	4.4	5.8	0.4	6.9	1.4	-0.1
	Bad Oeynhausen						
	Result, ng/mL	863	461	229	47.3	41.0	96
	Bias, %	-13.7	-42.4	-42.7	-40.8	-5.3	-18.9
CMIA	Milan						
	Result, ng/mL	1053.6	823.7	407.8	81.3	42.8	121.2
	Bias, %	5.4	3.0	2.0	1.6	-1.2	2.7
EMIT	Dortmund						
	Result, ng/mL	862	699	345	69.0	25.3	107
	Bias, %	-13.8	-12.6	-13.7	-13.7	-41.6	-9.4
ECLIA	All sites						
	Mean results, ng/mL	1050	856	396	87	45	138
	SD	42.0	33.0	12.0	3.0	3.0	4.0
	Bias, %	5.0	7.1	-1.1	8.5	4.8	16.9
TAC							
	IPT sample number	Spiked samples				Pooled samples	
		208C	210A	211B	214B	206A	212B
IPT	Spiked conc, ng/mL	2.0	12	20	10	*Pooled*	*Pooled*
	Mean LC-MS/MS results, ng/mL	2.0	11.9	19.1	9.2	6.0	7.3
LC-MS/MS	Newcastle						
	Result, ng/mL	1.97	12.0	18.9	9.23	6.0	7.37
	Bias, %	-1.7	0.0	-5.5	-7.7	0.0	0.9
	Bad Oeynhausen						
	Result, ng/mL	1.97	8.63	12.53	8.43	6.30	7.03
	Bias, %	-1.7	-28.1	-37.3	-15.7	5.0	-3.7
CMIA	Milan						
	Result, ng/mL	1.83	12.67	19.97	9.73	6.43	7.73
	Bias, %	-8.4	5.6	-0.2	-2.7	7.2	5.9
	Dortmund						
	Result, ng/mL	2.10	12.70	20.60	10.50	6.57	8.70
	Bias, %	5.0	5.8	3.0	5.0	9.5	19.2
	Bilbao						
	Result, ng/mL	1.83	14.17	22.50	11.7	7.40	9.17
	Bias, %	-8.4	18.1	12.5	17.0	23.3	25.6
ECLIA	All sites						
	Mean results, ng/mL	2.05	12.70	20.56	10.06	6.49	7.98
	SD	0.07	0.16	0.30	0.29	0.16	0.16
	Bias, %	2.6	5.8	2.8	0.6	8.2	9.2

Values shown represent the mean of a given method's measurement results. In the case of ECLIA this is from all participating laboratories. For methods with equal to, or smaller than three investigational sites, the results for each site are displayed separately.
CMIA - chemiluminescent microparticle immunoassay, CsA - cyclosporine, ECLIA - electrochemiluminescence immunoassay, EMIT - enzyme multiplied immunoassay technique, EQA - external quality assurance, LC-MS/MS - liquid chromatography-tandem mass spectrometry, IPT - international proficiency testing, SD - standard deviation, TAC - tacrolimus.

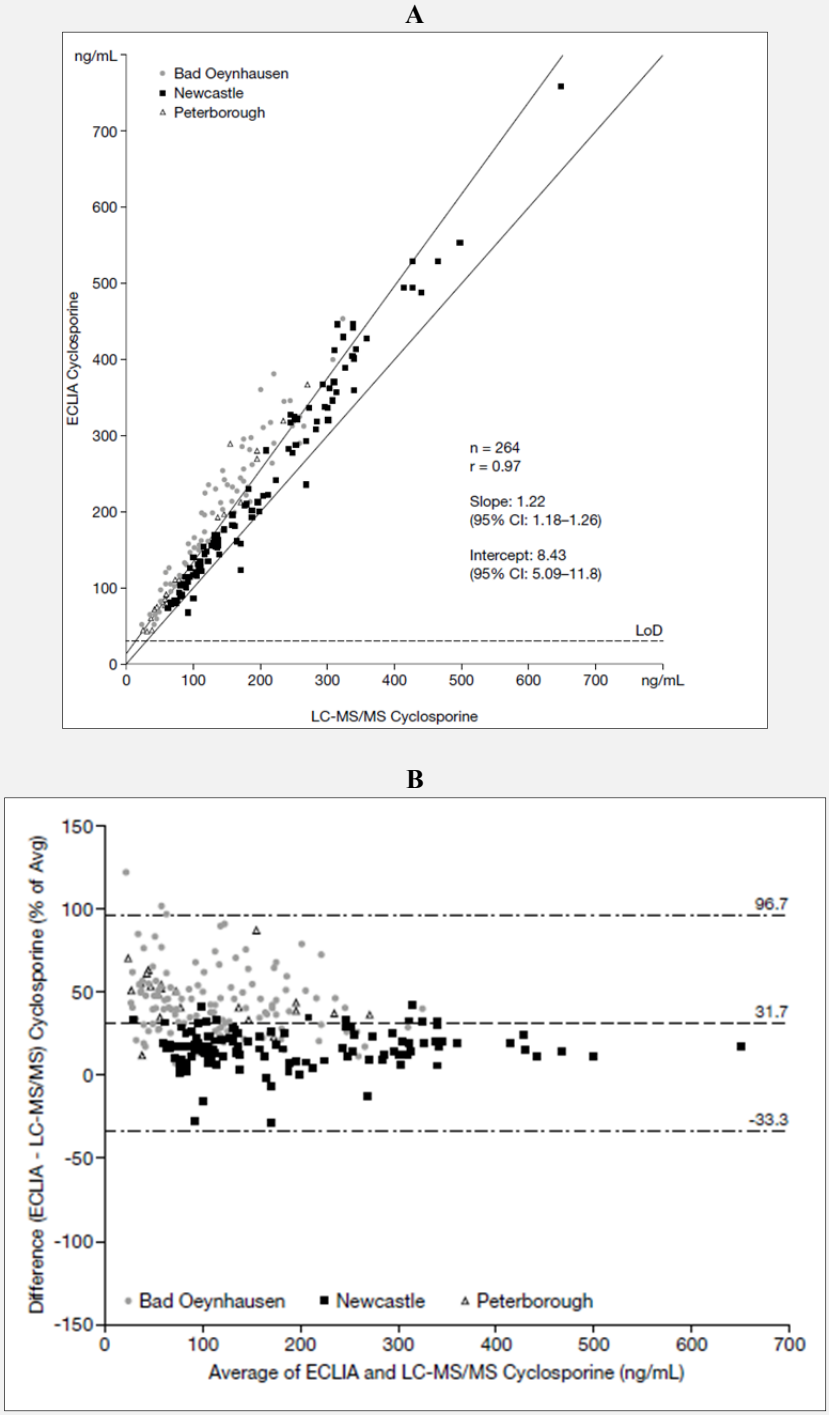

Figure 1. Method comparison of CsA assays with weighted Deming regression (A) and Bland-Altman analysis (B) of ECLIA versus LC-MS/MS at all centers.

Regression parameters and 95% CI are indicated within the plot. Results from individual centers are indicated using the different symbols. The dashed line in the regression plots parallel to the x axis represents the limit of detection (LoD) of the CsA ECLIA measuring range (30 ng/mL). The Bland-Altman plot is displayed using the average of the two methods on the x axis and the normalized percentage difference on the y axis. The mean bias across all sites and ± 3 SD range are indicated with dashed lines.

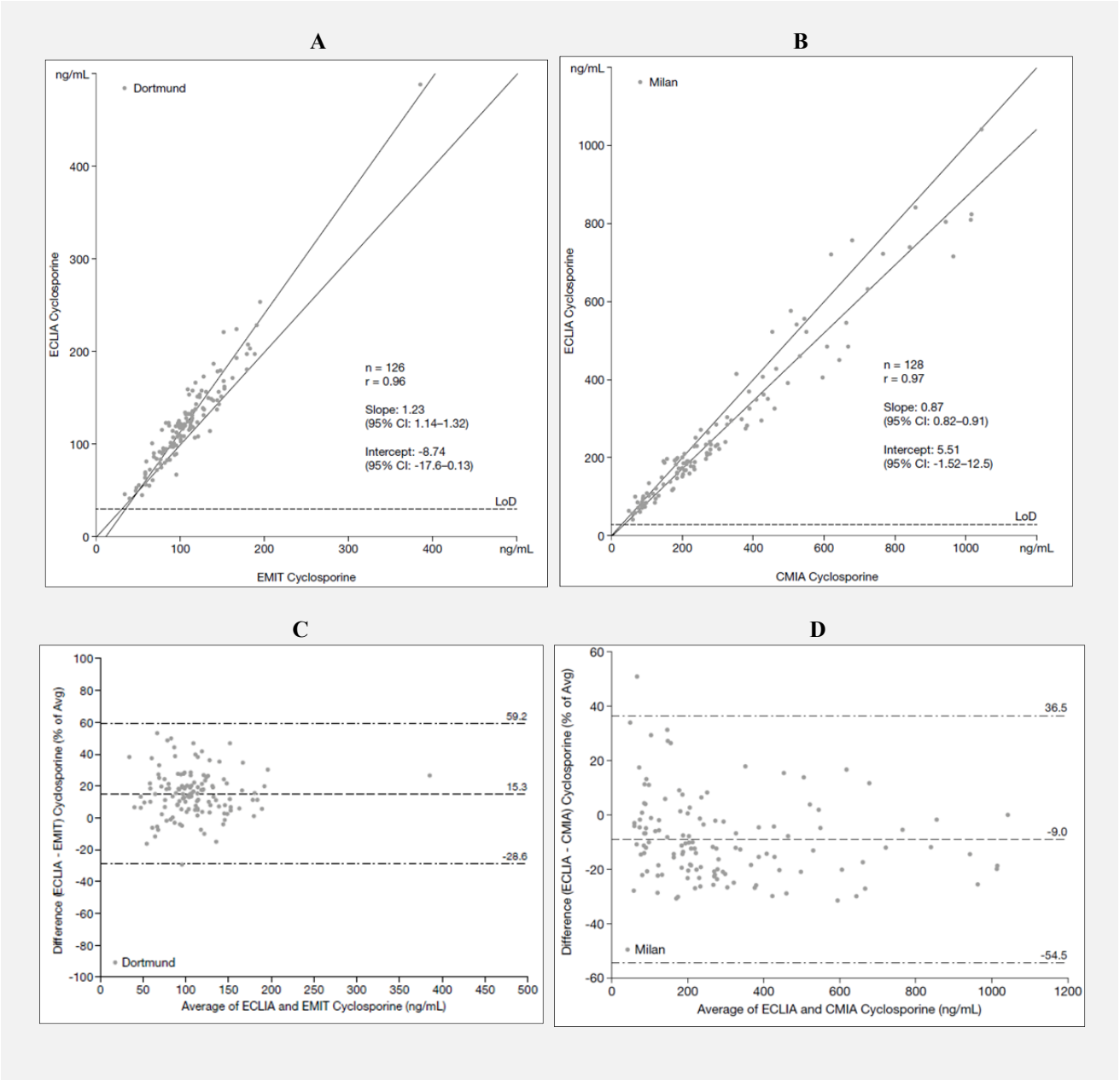

Figure 2. Method comparison of CsA assays with weighted Deming regression and Bland-Altman analysis of ECLIA versus either EMIT (A and C, respectively) or CMIA (B and D, respectively).

Regression parameters and 95% CI are indicated within the plots. The dashed line in the regression plots parallel to the x axis represents the limit of detection (LoD) of the CsA ECLIA measuring range (30 ng/mL). Bland-Altman plots are displayed using the average of the two methods on the x axis and the normalized percentage difference on the y axis. Bias and ± 3 SD range are indicated with dashed lines.

-27.3%, whereas samples analyzed by LC-MS/MS at the Newcastle site showed a bias of only 3.1%. A similar result was found when comparing EQA results for TAC measured by LC-MS/MS with those analyzed at Bad Oeynhausen displaying a mean bias of -13.6% compared with -2.3% at the Newcastle site (Table 3). This suggests that the negative bias demonstrated at the Bad Oeynhausen site has significantly skewed the data when LC-MS/MS is compared with ECLIA for all sites.

The cause of this site-specific discrepancy is likely to be a result of differences in standardization of the LC-MS/MS instruments as well as deviation in extraction and separation of the sample calibrators and controls. The three sites comparing ECLIA with LC-MS/MS used a different LC-MS/MS method with different sample pretreatment and assay conditions. For example, use of a different column and/or spectrometer set-up can significantly influence the sample recovery. These differences

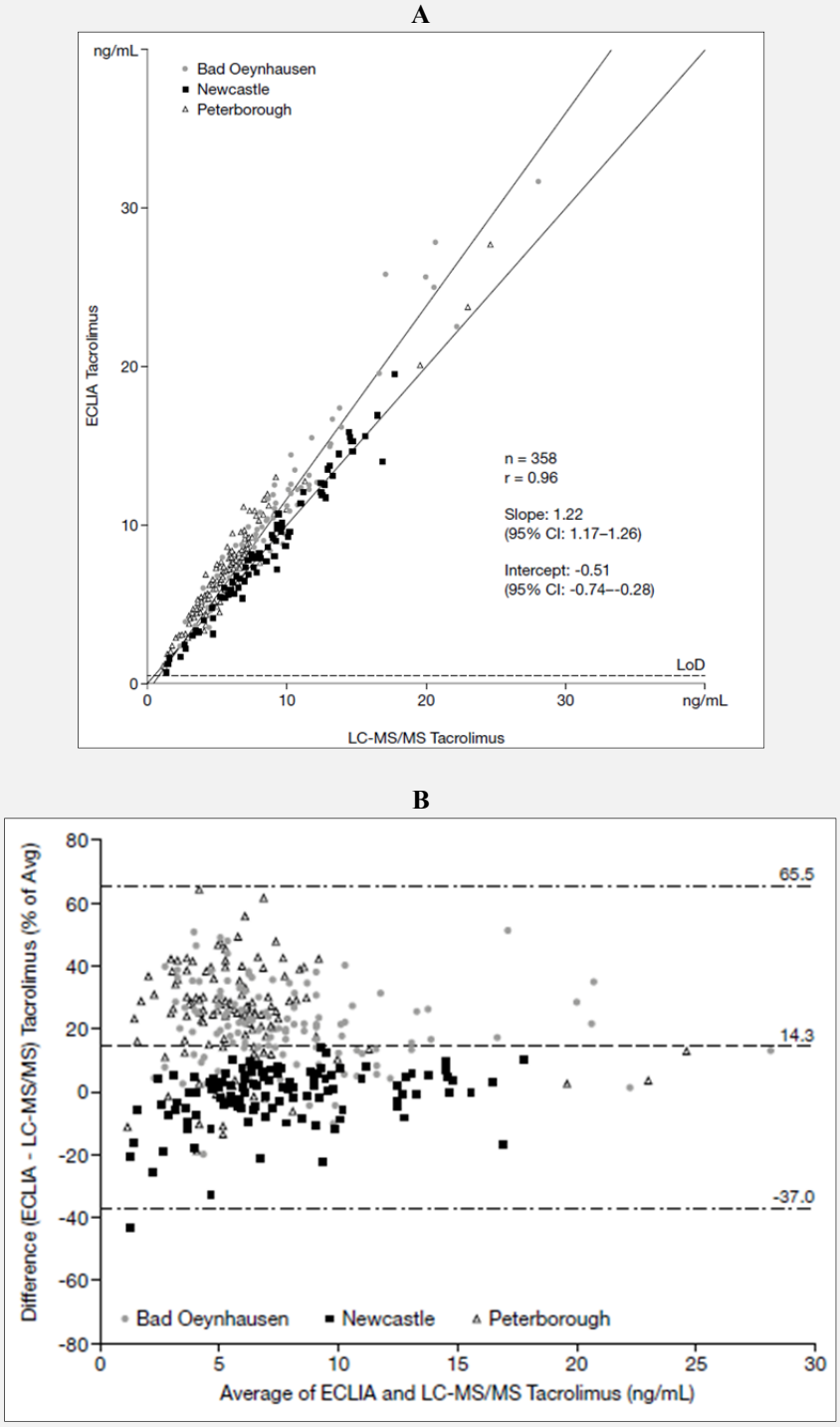

Figure 3. Method comparison of TAC assays with weighted Deming regression (A) and Bland-Altman analysis (B) of ECLIA versus LC-MS/MS at all centers.

Regression parameters and 95% CI are indicated within the plot. Results from individual centers are indicated using the different symbols. The dashed line in the regression plots parallel to the x axis represents the limit of detection (LoD) of the TAC ECLIA measuring range (0.5 ng/mL). The Bland-Altman plot is displayed using the average of the two methods on the x axis and the normalized percentage difference on the y axis. The mean bias across all sites and ± 3 SD range are indicated with dashed lines.

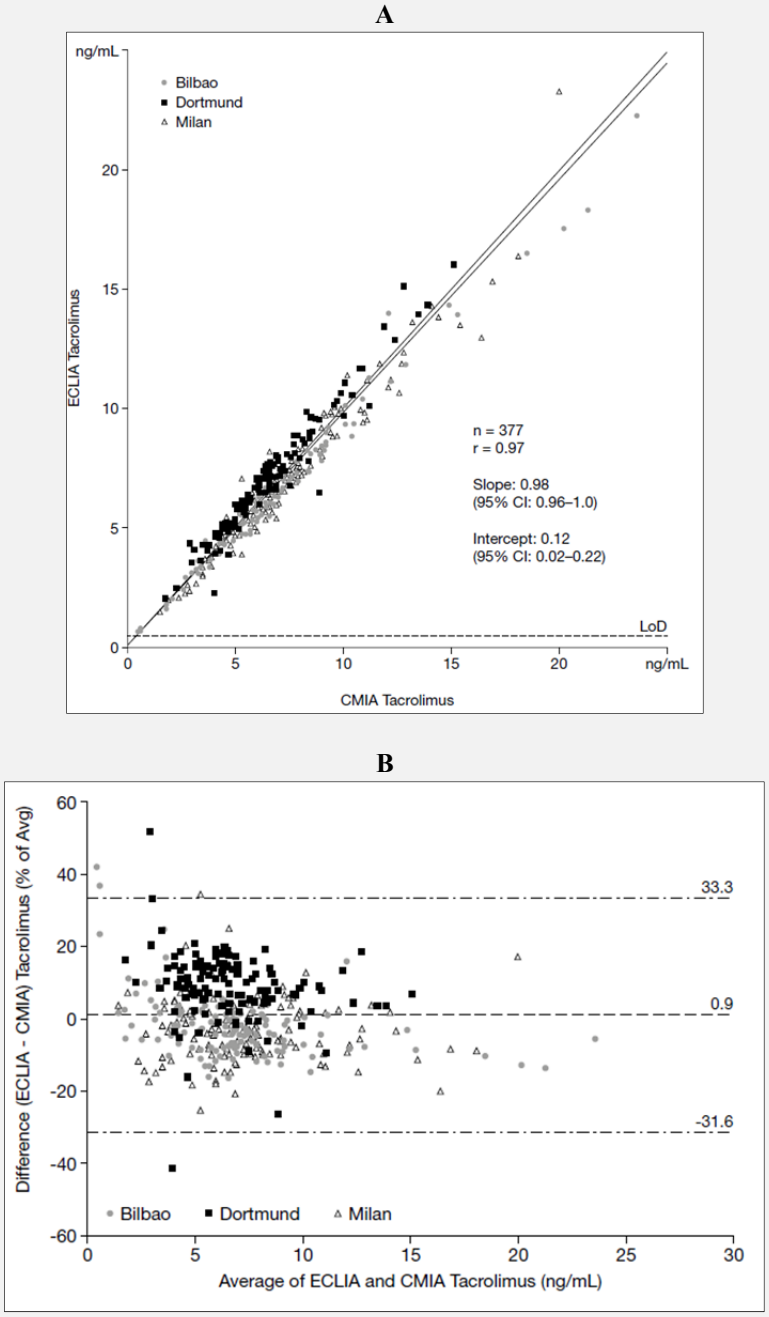

Figure 4. Method comparison of TAC assays with weighted Deming regression (A) and Bland-Altman analysis (B) of ECLIA versus CMIA.

Regression parameters and 95% CI are indicated within the plot. Results from individual centers are indicated using the different symbols. The dashed line in the regression plots parallel to the x axis represents the limit of detection (LoD) of the TAC ECLIA measuring range (0.5 ng/mL). The Bland-Altman plot is displayed using the average of the two methods on the x axis and the normalized percentage difference on the y axis. The mean bias across all sites and ± 3 SD range are indicated with dashed lines.

observed in LC-MS/MS indicate a need for standardization of these methods in terms of calibration and sample extraction. This standardization is already offered by immunoassays, with the lot number of ECLIA reagent, calibrators, and QC materials being identical at all sites, allowing comparability between laboratories.

Most available immunoassays for CsA and TAC monitoring are characterized by a certain extent of cross-re-

activity with therapeutically active metabolites, although this is largely accepted in clinical practice [17]. Due to its high level of analytical specificity, LC-MS/MS does not detect these molecules, which might explain some of the discrepancies observed.

Cross-reactivity with metabolites commonly found in whole blood was evaluated by the manufacturers during ECLIA development for both CNIs. The only reported cross-reactivity from TAC metabolites for the TAC ECLIA occurred with the active metabolite, M-II (~70%) [18]. The cross-reactivity observed here is lower than that demonstrated for the CMIA (94%). Cross-reactivity with pharmacologically inactive TAC metabolites was reported for CMIA (~45%) and antibody-conjugated magnetic immunoassay (ACMIA) (~18%); however, ECLIA is not affected [15,19]. The CsA ECLIA also has significantly reduced CsA metabolite interference relative to other commonly used immunoassays, with a cross-reactivity of 6% for AM9, 2% for AM1 and AM4n, and no detectable cross-reactivity for AM1c, AM1c9, and AM19 [20,21]. Conversely, CMIA detected all tested metabolites [21]. Given that the cross-reactivity of both CNI ECLIAs has been shown to be low, the difference in LC-MS/MS method standardization is the most likely cause of the observed discrepancies in results. It is interesting to see from this study that, despite the intrinsic difference in their methodology, comparability between LC-MS/MS and the ECLIA was still good.

Both the CsA and TAC ECLIA have demonstrated excellent overall performance in previous analytical evaluations, and offer high precision and compare favorably with LC-MS/MS and other routine assays under field conditions [8,9], which is consistent with other comparisons of routinely used immunoassays versus LC-MS/MS [22,23].

The ECLIAs were found to be easy to handle, with a calibration curve of only two points providing a significant time-saving compared to the seven-point calibration most commonly applied for LC-MS/MS assays. This ease of use was found despite the inclusion of a manual protein precipitation step before automated analysis. This extra step ensures removal of endogenous antibodies, which can cause interference, and utilizes a single pre-treatment reagent and handling procedure for both CsA and TAC assays, thereby reducing the risk of operator handling errors and contributing to the ease of use of the assays. Endogenous antibodies have been shown to interfere with several immunoassays, particularly the ACMIA method, which does not include a protein precipitation step, causing false-positive results [17,24-27]. As a result, removal of endogenous antibodies by protein precipitation gives more accurate results and contributes to better patient safety [24,25]. CMIA has been shown to be less susceptible to the interferences seen with ACMIA [28], and in this study, ECLIA shows excellent concordance with CMIA.

The need for standardized, accurate, and precise measurement of immunosuppressive drugs presents a major challenge for clinical laboratories. Evaluation of the technologies available should be based on their medical value and the benefits they offer to patients. In the case of TDM, due to the necessity of maintaining therapeutic values within a narrow therapeutic range, analytical accuracy, and excellent precision as well as rapid sample turnaround time represent the highest priorities [10]. The simplicity of automated and semi-automated immunoassays provides ease of use and superior operational flexibility to LC-MS/MS, which is extremely important in the clinical setting, allowing measurement of multiple factors, including hormones and serologic factors alongside TDM [10]. Immunoassays also offer greater throughput than LC-MS/MS and the fact that automated systems do not rely on specialized operators removes potential source of human error and site-to-site or period-to-period variability. The improved workflow offered by immunoassay technology could have many implications, accelerating clinical decision making through a reduction in turnaround time, as well as reducing the overall costs [10]. This could lead to earlier and more informed treatment decisions that help to maximize therapy efficacy [10]. Many of the currently available immunoassays, however, lack the required analytical performance that is provided by the LC-MS/MS. Given that the majority of clinical laboratories currently still measure CNIs using an immunoassay [8,9], the observation from this study, that the new CNI ECLIAs are not only superior to LC-MS/MS in terms of workflow but also match it in terms of performance, is very important.

CONCLUSION

The data presented here show that the ECLIA represents a robust and promising alternative for the quantitative determination of CsA and TAC in human whole blood that not only offers a reduced laboratory workload, but also offers the required technical performance to meet the demands of TDM in current immunosuppressive regimens. This will aid in the management of heart, liver, and kidney transplant patients.

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Declaration of Interest:
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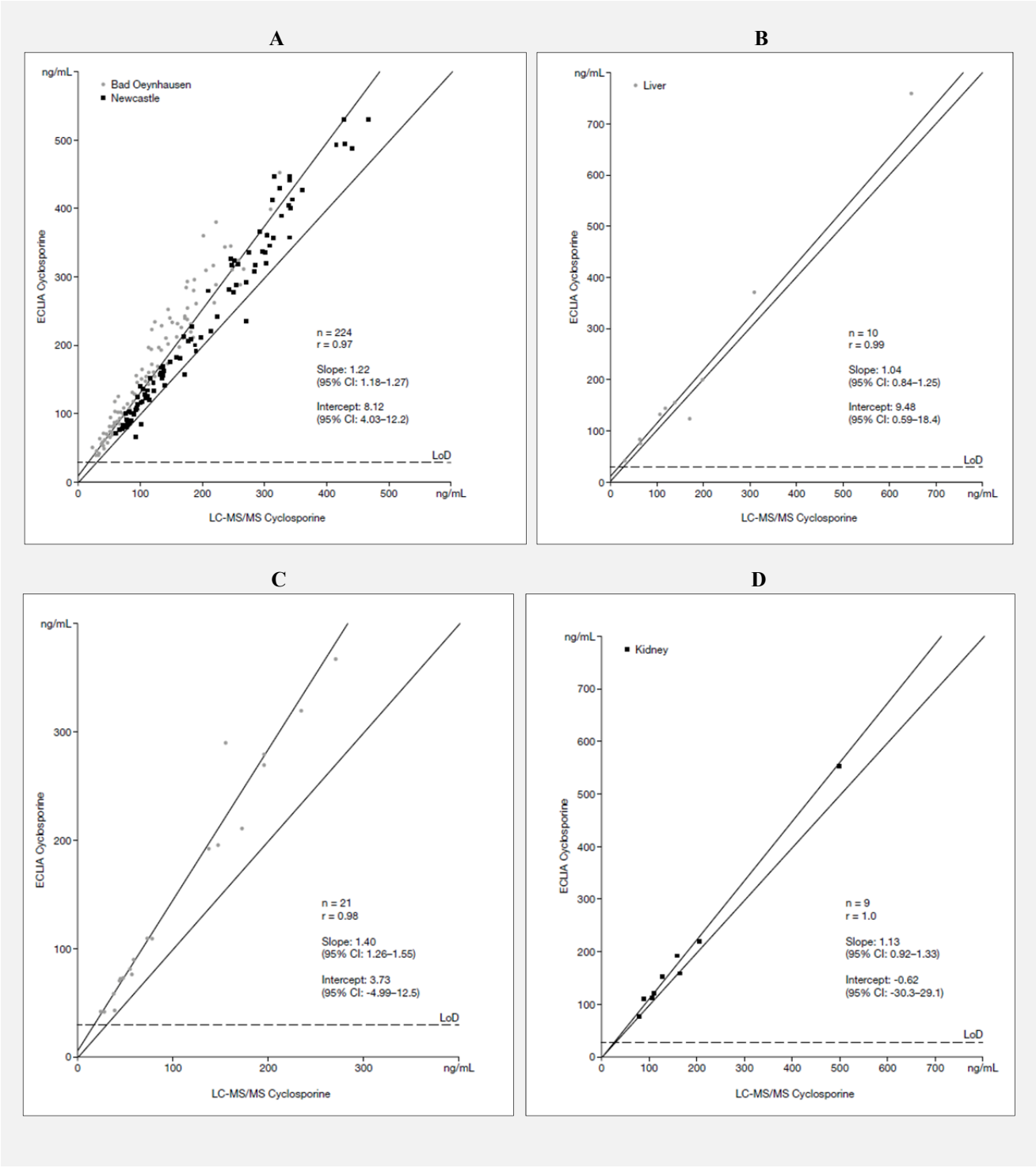
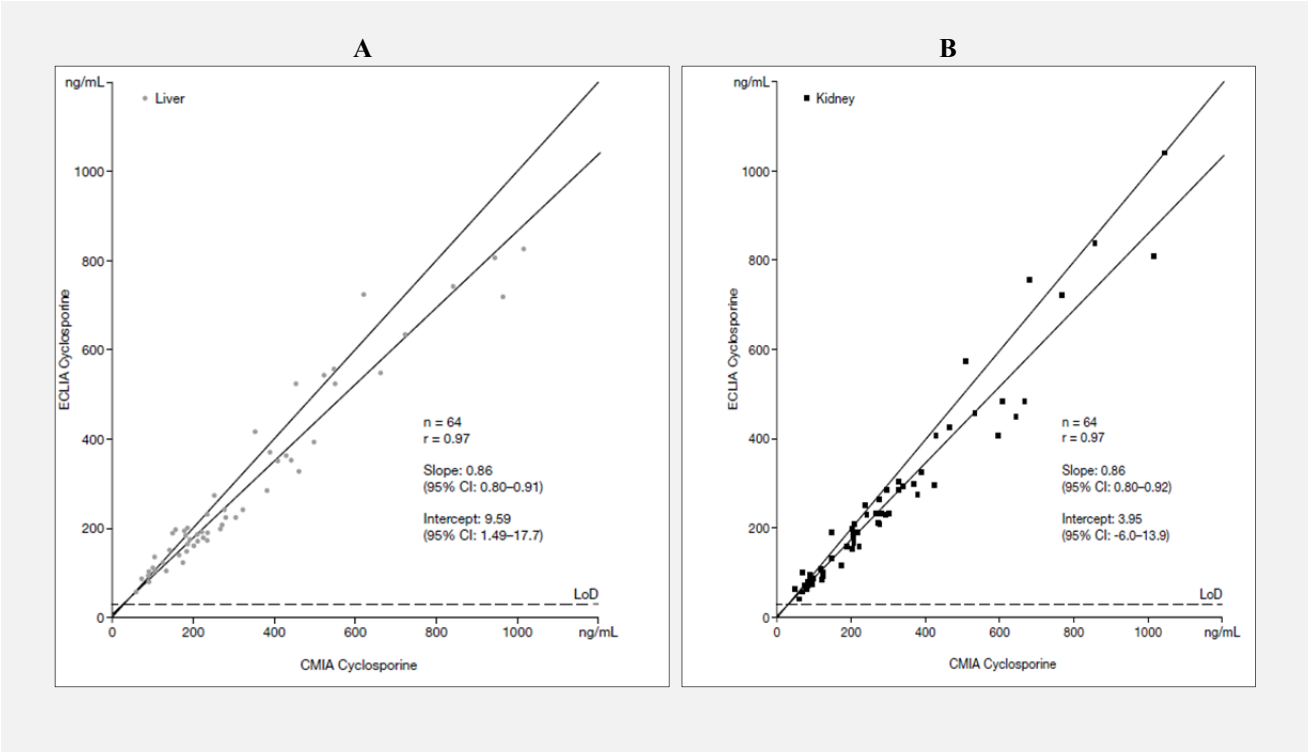
SUPPLEMENTARY DATA



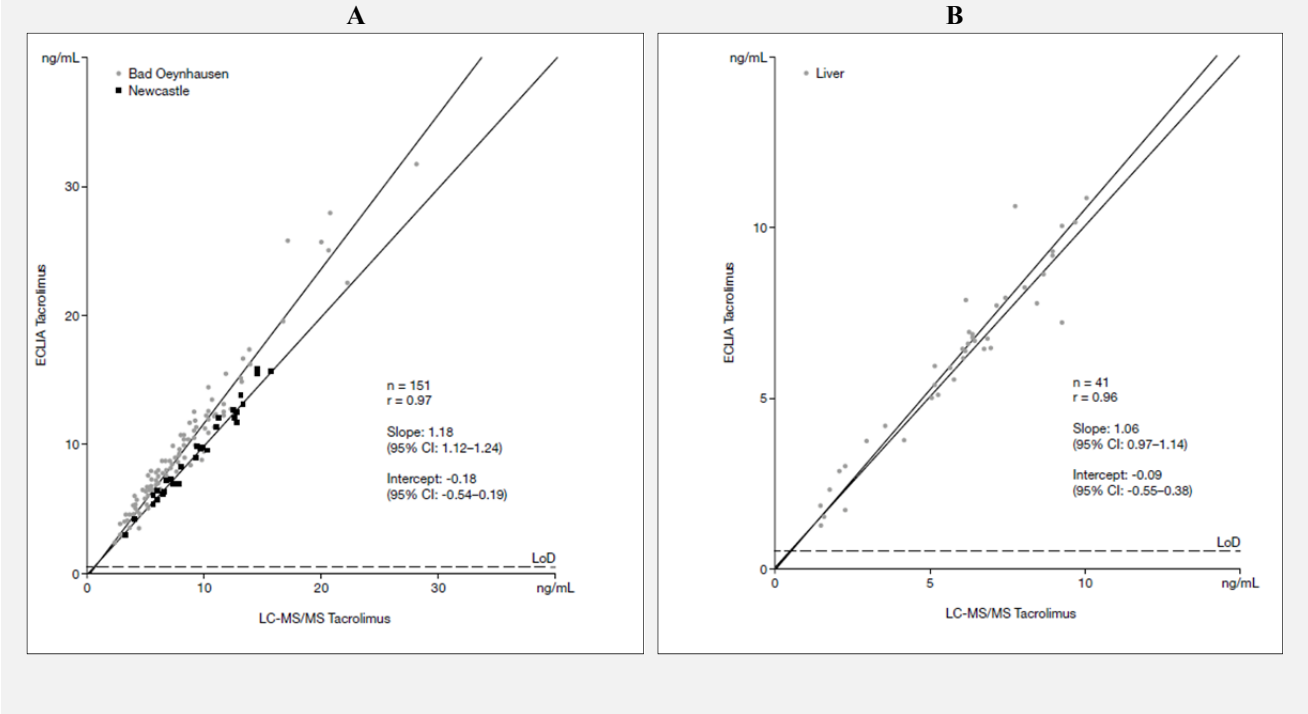
Supplementary Figure 1. Method comparison of CsA assays with weighted Deming regression for ECLIA versus LC-MS/MS for heart (A) liver (B) and kidney (Peterborough = C, Newcastle = D).

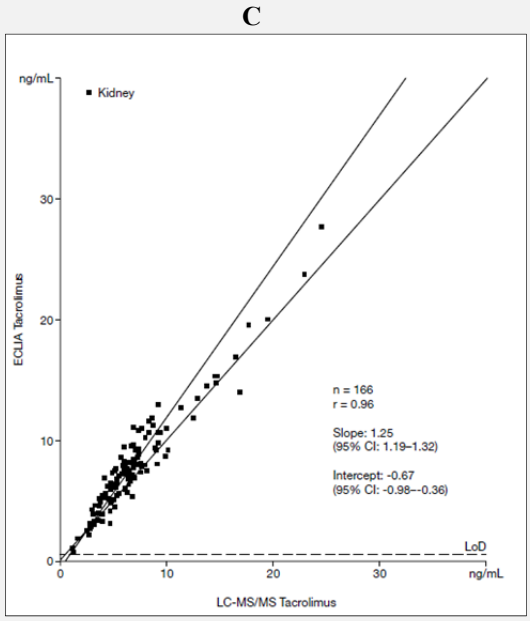
Regression parameters and 95% CI are indicated within the plot. The dashed line in the regression plots parallel to the *x* axis represents the limit of detection (LoD) of the CsA ECLIA measuring range (30 ng/mL).



Supplementary Figure 2. Method comparison of CsA assays with weighted Deming regression for ECLIA versus CMIA for liver (A) and kidney (B).

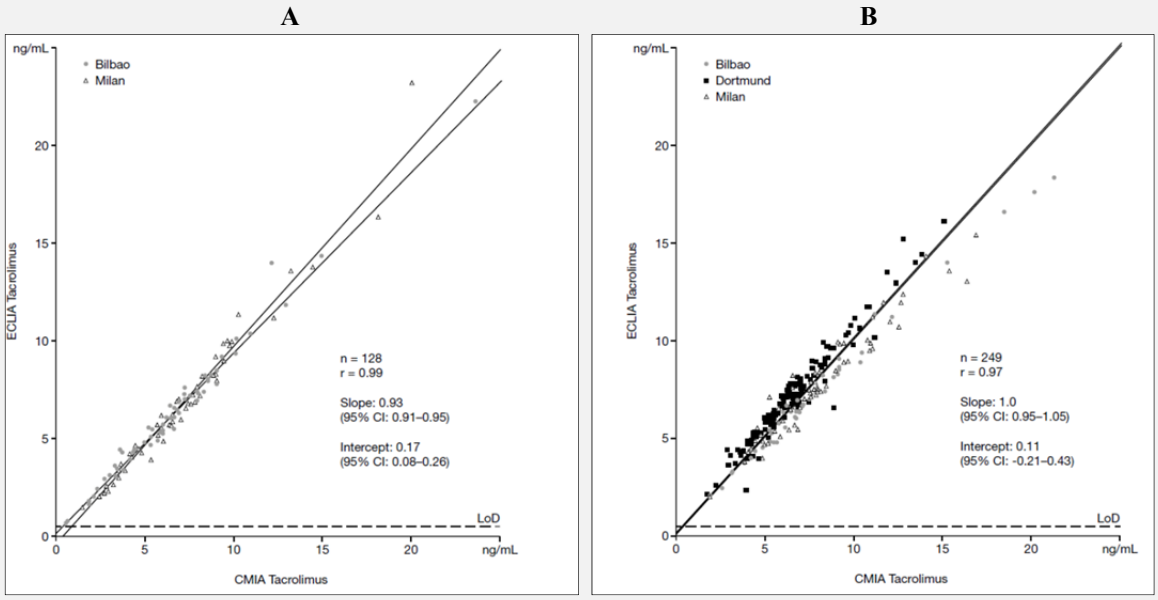
Regression parameters and 95% CI are indicated within the plot. The dashed line in the regression plots parallel to the *x* axis represents the limit of detection (LoD) of the CsA ECLIA measuring range (30 ng/mL).





Supplementary Figure 3. Method comparison of TAC assays with weighted Deming regression for ECLIA versus LC-MS/MS for heart (A), liver (B) and kidney (C).

Regression parameters and 95% CI are indicated within the plot. The dashed line in the regression plots parallel to the *x* axis represents the limit of detection (LoD) of the TAC ECLIA measuring range (0.5 ng/mL).



Supplementary Figure 4. Method comparison of TAC assays with weighted Deming regression for ECLIA versus CMIA for liver (A) and kidney (B).

Regression parameters and 95% CI are indicated within the plot. The dashed line in the regression plots parallel to the *x* axis represents the limit of detection (LoD) of the TAC ECLIA measuring range (0.5 ng/mL).