RESEARCH REPORT

Master of Medicine in Chemical Pathology

University of the Witwatersrand

The measurement of immunosuppressive drugs by mass spectrometry and immunoassay in a South African transplant setting

Submitted by:

Dr Amy Strydom (MBChB)

Student number: 731021

Supervisors:

Dr Sean Currin (MBChB, FCPath (SA) Chem, MMed (Wits))

Chemical pathologist, Charlotte Maxeke Johannesburg Academic Hospital

NHLS & University of the Witwatersrand

Dr Doreen Jacob (MBChB, FCPath SA Chem, MMed (Wits))

Chemical pathologist, Helen Joseph Hospital

NHLS & University of the Witwatersrand

Dr Taryn Pillay (MBChB, FCPath (SA) Chem)

Head of Department, Chris Hani Baragwanath Academic Hospital

NHLS & University of the Witwatersrand

Table of Contents

1) PLAGIARISM DECLARATION

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Amy Strydom (Student number: 731021) am a student

registered for the degree of MMed Chemical Pathology in the academic year 2025.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature: _____ Date: 24/02/2025

2) LETTER SIGNED BY CO-AUTHORS

Amy Strydom
Department of Chemical Pathology
National Health Laboratory Services
University of the Witwatersrand
7 York Rd, Parktown 2193, Johannesburg, South Africa
21 November 2024

To whom it may concern

Subject: Confirmation of First Authorship

I, Amy Strydom (student number: 731021), confirm that I am the first author of submitted article titled *"The measurement of immunosuppressive drugs by mass spectrometry and immunoassay in the South African Transplant setting"* published in *Practical Laboratory Medicine* in November 2024 (DOI: doi.org/10.1016/j.plabm.2024.e00440).

This research forms part of the requirements for the MMed in Chemical Pathology degree, and I have significantly contributed to the study's conception, design, data analysis, and manuscript preparation. While several co-authors supervised me on this project, my role as the first author reflects my primary contributions in conducting the research and preparing the manuscript for publication.

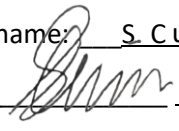
Thank you for your attention. Please find acknowledgement from the co-authors of this manuscript that the above information is correct and true below.

Sincerely
Amy Strydom

Signature: 

Date: 21/11/2024

Supervisor name: S. C urrin

Signature: 

Date: 27/11/2024

Supervisor name: Doreen Jacob

Signature: 

Date: 29/11/2024

Supervisor name: Dr Taryn Pillay

Signature: 

Date: 29/11/24

3) ABSTRACT

Abstract

Objectives: Liquid chromatography tandem mass spectrometry (LCMS/MS) is the gold standard for measurement of immunosuppressive drugs (ISDs), but is technically demanding and less accessible in resource-limited countries. Immunoassays can also measure ISD concentrations, but may be limited by cross-reactivity. We evaluated the performance of the Roche electrochemiluminescence immunoassay (ECLIA) for cyclosporine, everolimus and sirolimus against LCMS/MS in an African population for the first time.

Methods: Bias for ECLIA was estimated by comparing ECLIA-measured ISD concentrations to those obtained by LCMS/MS in 42, 43 and 47 patient samples for cyclosporine, everolimus and sirolimus, respectively. Precision was assessed by performing replicate measurements of quality control materials.

Results: Deming regression analysis for all ISDs showed strong correlation between ECLIA and LCMS/MS with a Pearson's r of > 0.94. The slopes for cyclosporine, everolimus and sirolimus were 0.94 [95% CI: 0.87 – 1.03], 1.35 [95% CI: 1.23 – 1.44] and 0.96 [95% CI: 0.85 – 1.15] with y-intercepts of 31.60 µg/L [95% CI: 2.02 – 57.63], 0.23 µg/L [95% CI: -0.21 – 0.72] and 2.61 µg/L [95% CI: 1.30 – 3.56], respectively. Difference plots showed a median bias of 2.07% [95% CI: -1.42 – 6.99%], 41.2% [95% CI: 34.9 – 51.8%] and 34.9% [95% CI: 28.4 – 47.3%] for cyclosporine, everolimus and sirolimus, respectively.

Conclusions: The cyclosporine ECLIA yielded results comparable to LCMS/MS while poorly comparable results were obtained for everolimus and sirolimus, which may be explained by ISD metabolite cross-reactivity, amongst other factors. The poor comparability, although not unique, is noteworthy and the clinical consequences of these differences require further investigation.

4) INTRODUCTION

Introduction

Immunosuppression, achieved with the use of immunosuppressive drugs (ISDs), plays a vital role in preventing solid organ rejection post-transplantation [1]. In the 1980s, the introduction of cyclosporine revolutionised post-transplant care, with reported improvements in graft survival rates of more than 80% compared to older drug regimens [2]. Further developments, including the introduction of newer drugs with fewer adverse effects, such as tacrolimus, everolimus and sirolimus has allowed for improved one-year graft survival of more than 90% in most transplant centres [3–5]. In the South African tertiary public hospital setting, despite limited resources, reported 1-year graft survival rates for adult patients vary between 89.4% to 91.7% for renal transplants [6,7] and 72% to 81% for liver transplants [8,9]. While the reasons for graft rejection are multi-factorial, some of the major modifiable risk factors leading to decreased survival include non-adherence and inadequate circulating concentrations of ISDs [10,11].

Due to risk of toxicity, as well as the risk of organ rejection from sub-therapeutic ISD concentrations, numerous groups have published guidelines and consensus reports mandating therapeutic drug monitoring (TDM) for patients on ISDs (ISD-TDM) [11–14]. The gold standard method for measurement of ISDs is liquid-chromatography tandem mass spectrometry (LCMS/MS) [14,15]. This method provides good analytical sensitivity and has been shown to be highly specific for the measurement of ISDs [16]. LCMS/MS is, however, costly to run, requires highly skilled staff, has complex method validation, limited automation and little manufacturer support [15,16]. Immunoassay methods are also used for ISD-TDM and address some of the limitations of LCMS/MS by being easily implementable, having less stringent skill requirements from staff, and the potential for shorter turn-around times

as part of a 24/7 laboratory service [17]. The main limitation of the use of immunoassay methods is decreased specificity due to cross-reactivity [18].

Currently, there are no published studies assessing immunoassays for ISD-TDM in an African population [19–35]. Drug metabolism of ISDs and the drug metabolites formed may vary depending on the pharmacogenomic profile of a population [36–38]. The presence of various single nucleotide polymorphisms (SNPs) in the Cytochrome p450 (CYP 450) system are known to cause clinically relevant alterations in drug metabolism in different populations [39]. African populations, known to have greater genetic variation than other populations, have been shown to house CYP 450 SNPs of serious clinical consequence [40,41]. SNPs in the genes encoding for CYP3A4 and CYP3A5, the major enzymes involved in the metabolism of cyclosporine, everolimus and sirolimus, have been identified in the South African population, but the clinical implications of these findings are yet to be explored [41,42]. A South African study that investigated the presence of various CYP3A5 SNPs in 43 transplant patients reported differences in ISD dosing requirements based on the presence of these SNPs [43].

The implications of variable pharmacokinetics and the inherent cross-reactivity of drug metabolites for immunoassay-based measurements make the verification of an immunoassay's analytical performance within that patient population a necessity. In this study, the first to be performed in an African population, the performance of the Roche Electrochemiluminescence Immunoassay (ECLIA) for cyclosporine, everolimus and sirolimus was evaluated against the gold standard LCMS/MS method through a method comparison experiment to determine its suitability in an African population.

5) METHODS

Methods

Setting

The study took place at the University of Witwatersrand and National Health Laboratory Service (NHLS) Laboratory in Johannesburg, South Africa. The laboratory services the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) and surrounding areas. Ethical approval was obtained for the study from the Health Research Ethics Committee of the University of the Witwatersrand (M230642).

Sample Collection

For the method comparison, a total of 50 remnant whole blood EDTA samples with concentrations of ISDs spanning the analytical range of the immunoassay and medical decision limits were collected for each drug over 3 months. Each whole blood sample was aliquoted into a 2.0 mL Provetta cryo-vial and stored at -80° C for less than 6 months (as per manufacturer recommendation and stability studies [44–46]) until analysis. The frozen samples were thawed for 45 minutes and gently mixed to ensure homogeneity before aliquoting each whole blood sample into two Eppendorfs for repeat analysis by LCMS/MS and then ECLIA measurement within 2 hours of LCMS/MS analysis. For the precision study, Roche Diagnostics quality control (QC) materials (PreciControl® ISD and PreciControl® Everolimus) were used.

Method Comparison

Analysis by ECLIA

For the measurement of cyclosporine, everolimus and sirolimus, the samples require pre-treatment using 300 µL of Elecsys® ISD sample pre-treatment solution, followed by centrifugation for 4 minutes at 10 000 g. Calibration was performed using Elecsys® CalSet

calibrators and QC was within acceptable limits. Analysis of 150 µL of the supranatant of these samples was performed using the Roche Cobas e602 module as per the manufacturer's instructions. Measurement by this assay is based on a competitive principle between the drugs and a ruthenium-labelled analogue. After a voltage is applied, an electrochemiluminescent signal is used to determine the drug concentration [44].

All samples were analysed within both the uncapped and capped stability period of 30 minutes and 4 hours respectively, after the pre-treatment step as per manufacturer instructions.

Analysis by LCMS/MS

Analysis by LCMS/MS took place in an ISO 15189 accredited laboratory that participates in the Randox International Quality Assessment (RIQAS) external quality assessment scheme for ISDs. Analysis of cyclosporine, everolimus and sirolimus by LCMS/MS was performed on an ExionLC™ AC Series coupled to a Sciex® QTRAP 4500 tandem mass spectrometer. Chromsystems® calibrator standards were used to perform a 6-point calibration to allow for quantitation of each drug. Analysis of QC using Whole Blood Chromsystems MassCheck® control materials (levels 1-4) was within acceptable limits.

Each aliquot of sample was processed with the addition of Chromsystems® Immunosuppressant deuterated internal standards (Cyclo-d12, Evero-d4, Siro-d3), cell lysis and protein precipitation using a haemolysate (Zinc Sulphate) and acetonitrile, followed by centrifugation at 10 000 g for 5 minutes. The pre-treated samples were separated by gradient reverse phase chromatography using a Waters® ACQUITY UPLC BEH-phenyl column - mobile phase A (5mM ammonium acetate and 0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). Electrospray ionization (ESI) was operated in positive mode. The tandem mass spectrometer is operated in multiple reaction

monitoring (MRM) for the detection and quantitation of each drug using precursor and product ion m/z mass transitions. Quantitation of each drug was performed using QuanLynx software and a six-point standard curve.

Precision Study

For 4 days, two levels of QC materials were analysed 3 times per day for each drug for the ECLIA method on the Roche Cobas® e603 instrument. For each analysis, a separate 300 µL aliquot of reconstituted QC material was subjected to the same pre-treatment procedure as the patient samples using Elecsys® ISD sample pre-treatment solution as described previously.

Statistical Analysis

Statistical analysis was performed using R statistical software [47]. For the method comparison, results of the LCMS/MS analysis in which there was a >15% difference between the initial LCMS/MS analysis and the single repeat LCMS/MS analysis were excluded from the data set which left 42, 43 and 47 data points for the method comparisons of cyclosporine, everolimus and sirolimus, respectively. The single repeat LCMS/MS result was used for the method comparison. Deming regression analysis was performed for each drug with confidence intervals (CI) deter-mined using the Bootstrap (quantile) method. Difference plots were constructed to assess bias. The median bias and its confidence intervals were calculated using the Wilcoxon signed rank test.

The within-run and within-laboratory variability estimates were calculated using published precision estimation formulae [48]. The laboratory's analytical coefficient of variation (CVa)

for the LCMS/MS method and the CVa data from the precision study were used to calculate the error variance ratios (δ) for the Deming regressions.

6) RESULTS

Results

Information about the collected samples is summarised in Table 1.

Table 1: Demographic Data

Cyclosporine	
Age	39 [31 - 59]
Sex (% male)	55%
LCMS/MS	333.35 µg/L [150.4 – 710.6]
ECLIA	342.6 µg/L [162.5 – 714.2]
Everolimus	
Age	35 [20 – 51]
Sex (% male)	60%
LCMS/MS	5.11 µg/L [2.5 – 6.9]
ECLIA	6.78 µg/L [3.6 – 9.2]
Sirolimus	
Age	44 [39.5 – 59.5]
Sex (% male)	57%
LCMS/MS	6.77 µg/L [5.4 – 8.9]
ECLIA	9.49 µg/L [7.5 – 10.7]
All data are median and inter-quartile range (IQR) or percent (%)	

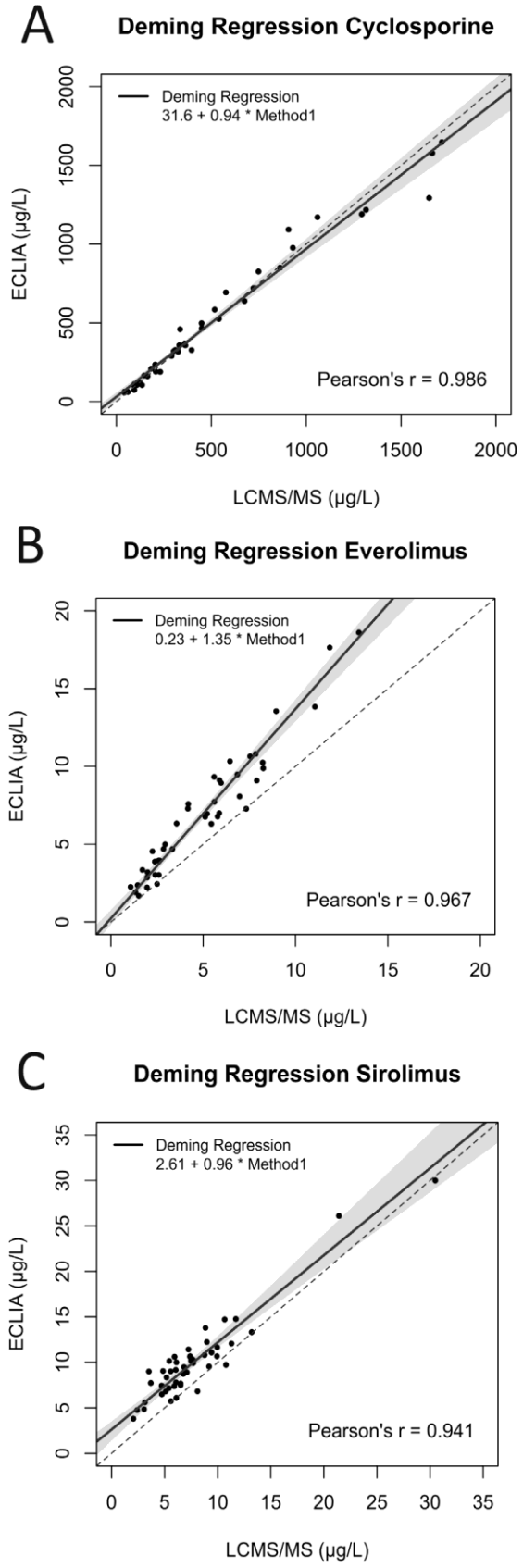
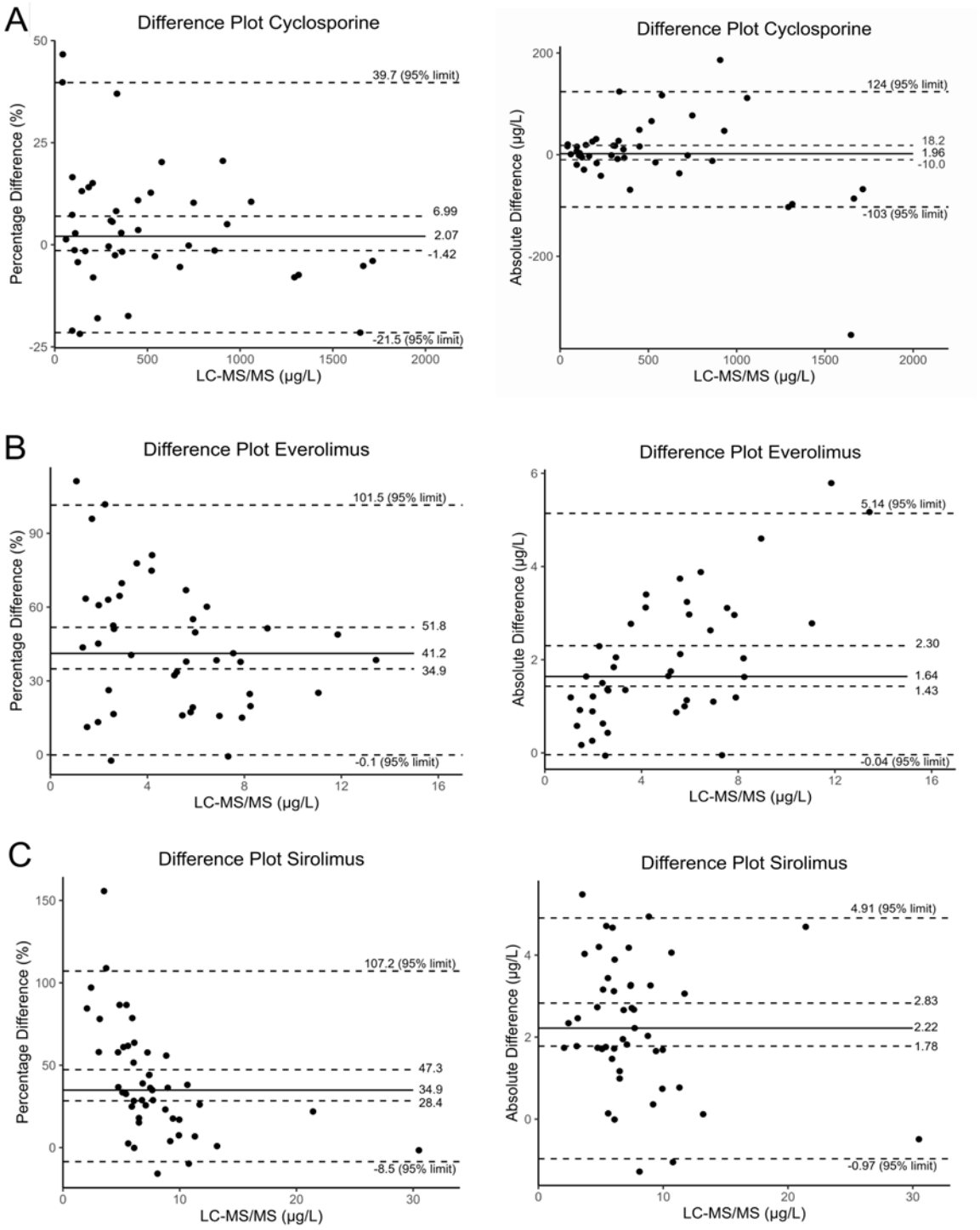


Figure 1: Deming regression analyses for (A) cyclosporine, (B) everolimus, (C) sirolimus. The dashed line is the identity (1:1) line and the grey shaded area represents the 95% confidence intervals for each drug

Method Comparison and Estimation of Bias:

As illustrated in Figure 1, Deming regression analysis (δ = 0.46) for cyclosporine yielded a slope of 0.94 [95% CI: 0.87 – 1.03], a y-intercept of 31.60 µg/L [95% CI: 2.02 – 57.63] and a Pearson's r value of 0.986 [95% CI: 0.973 – 0.992]. For everolimus, Deming regression analysis (δ = 0.76) yielded a slope of 1.35 [95% CI: 1.23 – 1.44], a y-intercept of 0.23 µg/L [95% CI: -0.21 – 0.72] and a Pearson's r value of 0.967 [95% CI: 0.939 – 0.982]. For sirolimus, Deming regression analysis (δ = 0.51) yielded a slope of 0.96 [95% CI: 0.85 – 1.15], a y-intercept of 2.61 ug/L [95% CI: 1.30 – 3.56] and a Pearson's r value of 0.941 [95% CI: 0.896 – 0.967].

Difference plots (Figure 2) for the cyclosporine method comparison revealed a median absolute difference of 1.96 µg/L [95% CI: -10.0 µg/L - 18.2 µg/L], corresponding to a median percentage difference of 2.07% [95% CI: -1.42% - 6.99%]. For everolimus, difference plots showed a median absolute difference of 1.64 µg/L [95% CI: 1.43 µg/L - 2.30 µg/L] and a median percentage difference of 41.2% [95% CI: 34.9% - 51.8%], while sirolimus showed a median absolute difference of 2.22 µg/L [95% CI: 1.78 µg/L - 2.83 µg/L] and a median percentage difference of 34.9% [95% CI: 28.4% - 47.3%].



There were a total of 12 misclassifications (27.9%, p = 0.001) for the ECLIA method compared to LC-MS/MS for everolimus (Table 2) and 9 misclassifications (19.2%, p = 0.008) for sirolimus, while no samples were misclassified for cyclosporine. For everolimus and sirolimus, the ECLIA method classified more samples as supra-therapeutic and fewer as sub-therapeutic when compared to LC-MS/MS.

Table 2: Classification of samples per therapeutic category for ECLIA and LC-MS/MS

Drug	Method	Sub-therapeutic*	Therapeutic*	Supra-therapeutic*	Total disagreement (ECLIA vs LC-MS/MS)
Cyclosporine	ECLIA	8	17	17	0 (0%)
	LC-MS/MS	8	17	17	
Everolimus	ECLIA	12	17	14	12 (27.9%)§
	LC-MS/MS	17	19	7	
Sirolimus	ECLIA	1	39	7	9 (19.2%)§
	LC-MS/MS	6	38	3	

*Based on therapeutic steady-state trough concentration range of 100- 400 µg/L for cyclosporine, 3 - 8 µg/L for everolimus, 4 - 12 µg/L for sirolimus in keeping with local recommendations [13, 44]
§ p-value < 0.05 using McNemar's test

Precision

The within-run and within-laboratory precision estimates (Table 3) for cyclosporine, everolimus and sirolimus were <10% at both clinically relevant concentrations of QC material.

Table 2: Within-run and within-laboratory precision estimates (CVa) of cyclosporine, everolimus and sirolimus

Drug and QC Level		Within-run Precision	Within-lab Precision
Cyclosporine	84 µg/L	3.02%	2.76%
	272 µg/L	5.98%	6.40%
Everolimus	2.60 µg/L	5.32%	5.71%
	10.10 µg/L	9.60%	9.93%
Sirolimus	2.74 µg/L	5.98%	6,68%
	9.13 µg/L	4.65%	3,83%

7) DISCUSSION

Discussion

This study, based in South Africa, is the first to evaluate the analytical performance of an immunoassay for TDM-ISD in an African population through a method comparison between the Roche Elecsys® ECLIA method and the gold standard for TDM-ISD, LCMS/MS. We found that the Roche Elecsys® ECLIA immunoassay and LCMS/MS methodologies were comparable for cyclosporine but not for everolimus and sirolimus.

There are a number of published method comparison studies for ISD immunoassays [19–35]. In general, literature on the performance of cyclosporine, everolimus and sirolimus immunoassays is inconsistent [19–35,49] compared to tacrolimus [19–25]. Cyclosporine immunoassays, previously plagued by extensive cross-reactivity of drug metabolites [49], have shown improved analytical performance since the introduction of assays utilising monoclonal antibodies [25–28]. For everolimus measurement, some studies reported adequate performance [25,32], while others noted biased and inconsistent results with the potential for clinical misinterpretation [30,31]. Similarly, method comparison studies for sirolimus immunoassays reported varied bias estimates, but overall were assessed as being fit for use [25,32–35]. Due to cost constraints, we omitted the evaluation of tacrolimus from our study, as there is an extensive body of literature reporting acceptable analytical performance by immunoassay for this drug [19–25].

Our study demonstrates strong correlation (Pearson's $r > 0.94$) between the Roche ECLIA method and LCMS/MS for cyclosporine, everolimus and sirolimus. Cyclosporine demonstrated a clinically acceptable bias of 2.1% which is comparable to other studies [25,27–29]. Everolimus, however, demonstrated positive proportional bias with a slope of 1.35 on regression analysis, as well as a large positive bias of 41.2%. Similar proportional

biases for everolimus were reported by Lee et al. (slope 1.22) and Verstraete et al. (slope 1.21) using the Roche ECLIA assay [31,32]. Furthermore, Shipkova et al. reported a bias of 34.2% between the Roche Everolimus ECLIA method and LCMS/MS [30], while Verstraete et al reported a bias of 26.5% to 36.5% [31]. For sirolimus by ECLIA, we observed a median positive bias of +2.22 µg/L, which echoed the findings of Lee et al who reported a mean bias of +2 µg/L for the Roche ECLIA method compared to LCMS/MS in an Asian population [32]. In contrast, Stove et al reported a mean bias of 0.41 µg/L compared to LCMS/MS in a study performed in a European population [34]. Our findings are consistent with published literature and highlight the importance of performing method comparison studies across population groups, given the impact of pharmacogenomics on drug metabolite formation.

Bias has important clinical implications, especially when a single therapeutic target is used, regardless of the measurement methodology. For method comparisons, the International Association for Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) recommends a slope of < ±10% and a linear regression intercept that does not differ appreciably from zero compared to the reference method. An allowable bias of <5.8% is also discussed in the context of a hypothetical total allowable error (TEa) of 15% [15]. In our study, the bias for both everolimus and sirolimus exceeded two of these parameters. A clinically significant bias has the potential to cause dosing discrepancies and poses risks to transplant patients who may be monitored at different laboratories that use different methodologies for ISD-TDM. Guidance on the adoption or establishment of method-specific therapeutic ranges, which is currently not provided by IATDMCT and IFCC guidelines [14,15], should be clearly outlined.

There are various explanations for the observed bias. There is currently no certified reference material or reference method for cyclosporine, everolimus and sirolimus, which limits calibrator traceability, resulting in possible calibration bias [15]. Secondly, cross-reactivity from drug metabolites is of particular importance for the measurement of these drugs [18]. The Roche ECLIA for everolimus has a reported cross-reactivity for 24-hydroxy everolimus, 25-hydroxy everolimus and 40-phosphatidylcholine everolimus of 21.3%, 15.4% and 109.3% respectively [44]. For sirolimus, the reported cross reactivity of 12-hydroxy sirolimus, 27-O-Desmethyl sirolimus and 39-O-Desmethyl sirolimus is 10.4%, 65.0% and 108.6% respectively [44]. Importantly, the immunosuppressive activity of these metabolites for both drugs is not yet fully elucidated but is reported to be less than the parent drugs [15,18]. Cyclosporine metabolism yields many metabolites (AM1, AM9, AM4N, AM19, AM1c) of varying immunosuppressive activity [14,18]. The Roche ECLIA method for cyclosporine reports non-detectable cross-reactivity from AM19, AM1c and AM1c9, 2% cross-reactivity for AM1 and AM4N and 6% cross-reactivity for AM9 [44]. The efforts to decrease cross-reactivity in newer immunoassays are evident in our results for cyclosporine.

Imprecision needs to be considered along with bias for any measurement procedure. The calculated imprecision of the assay is acceptable according to IATDMCT APS which recommend a minimum CVa of <10 % for immunoassay-based methods [15]. Our imprecision estimates for the Roche ECLIA method are comparable to CVa estimates in other studies [23–25,27–35]. Another component of precision which should be considered is the variability among different clinical samples. The wide scatter of biases in our difference plots can partially be explained by heterogeneity among individuals [43] and the resulting

variation of ISD metabolites [44]. This heterogeneity or imprecision between individuals means that a consistent level of bias is difficult to specify in our population.

Lastly, the practical aspects of these methods must be considered. Laboratories in resource-limited settings lack widespread access to LCMS/MS [50], necessitating the use of more accessible methods like ECLIA. Both ECLIA and LCMS/MS for ISD measurement require manual sample preparation, including cell lysis and extraction, increasing the workload compared to general automated immunoassay analysis. However, ECLIA is more convenient with fewer pre-treatment steps and less stringent staff requirements than LCMS/MS making it better suited for resource-limited laboratories, such as those in sub-Saharan Africa.

This study has several strengths. It is novel in that it is the first method comparison study for ISD to be conducted in an African population. Secondly, remnant patient samples, which allow for a better assessment of the effect of variable metabolites, were used in this study as opposed to spiked samples. Furthermore, analysis of samples by ECLIA was conducted within 2 hours of LCMS/MS analysis for all samples and there were duplicate LCMS/MS measurements for all samples. Finally, this study has a few limitations. Most samples for sirolimus and everolimus were within the therapeutic range with very few samples in the toxic range which limited assessment of correlation and concentration-dependent bias at higher concentrations. The concentrations assessed in our study do, however, represent those commonly encountered in clinical practice. Secondly, the study made use of anonymised remnant samples and therefore stratification of results into different transplant types (a recommendation by the IATDMCT) as well as consideration of factors such as time after transplantation, differences in therapeutic regimens and differences in ethnicity was

not possible and would have resulted in underpowered findings. The tacrolimus immunoassay was not assessed in this study due to financial constraints. Acceptable analytical performance in the African population cannot be assumed and should be assessed in future studies.

In conclusion, this study, the first to be conducted in an African population, demonstrated significant bias between ECLIA and LCMS/MS for everolimus and sirolimus measurement. In contrast, the cyclosporine immunoassay demonstrated comparability with LCMS/MS in this patient population. The lack of comparability between these methods for everolimus and sirolimus measurement, highlighted by this study and other literature, is noteworthy. Although not unique to ISDs, this problem is perhaps under-recognised in the transplant community and the clinical consequence of these differences requires further investigation and possibly patient outcome studies to explore the potential risks posed by this non-comparability.

Research funding: Funding for LCMS/MS analysis of patient samples was provided by NHLS K-Project Funding (PR2346632). Roche Diagnostics supplied the ECLIA reagent kits and the other consumables required for analysis by ECLIA.

Ethical approval: The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (Clearance number: M230642).

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APPENDIX A: Published Article

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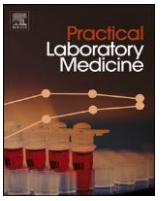

Registered Report Stage II

The measurement of immunosuppressive drugs by mass spectrometry and immunoassay in a South African transplant setting

Amy Strydom *, Doreen Jacob, Taryn Pillay, Refeletse Malahlela, Sean Currin

Department of Chemical Pathology, National Health Laboratory Service and University of the Witwatersrand, Johannesburg, South Africa

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ABSTRACT

Objectives: Liquid chromatography tandem mass spectrometry (LC-MS/MS) is the gold standard for measurement of immunosuppressive drugs (ISDs), but is technically demanding and less accessible in resource-limited countries. Immunoassays can also measure ISD concentrations, but may be limited by cross-reactivity. We evaluated the performance of the Roche electro- chemiluminescence immunoassay (ECLIA) for cyclosporine, everolimus and sirolimus against LC- MS/MS in an African population for the first time.

Methods: Bias for ECLIA was estimated by comparing ECLIA-measured ISD concentrations to those obtained by LC-MS/MS in 42, 43 and 47 patient samples for cyclosporine, everolimus and sirolimus, respectively. Precision was assessed by performing replicate measurements of quality control materials.

Results: Deming regression analysis for all ISDs showed strong correlation between ECLIA and LC-MS/MS with a Pearson's r of >0.94. The slopes for cyclosporine, everolimus and sirolimus were 0.94 [95 % CI: 0.87–1.03], 1.35 [95 % CI: 1.23–1.44] and 0.96 [95 % CI: 0.85–1.15] with y-intercepts of 31.60 µg/L [95 % CI: 2.02–57.63], 0.23 µg/L [95 % CI: 0.21 – 0.72] and 2.61 µg/L [95 % CI: 1.30–3.56], respectively. Difference plots showed a median bias of 2.07 % [95 % CI: 1.42 – 6.99 %], 41.2 % [95 % CI: 34.9–51.8 %] and 34.9 % [95 % CI: 28.4–47.3 %] for cyclosporine, everolimus and sirolimus, respectively.

Conclusions: The cyclosporine ECLIA yielded results comparable to LC-MS/MS while poorly comparable results were obtained for everolimus and sirolimus, which may be explained by ISD metabolite cross-reactivity, amongst other factors. The poor comparability, although not unique, is noteworthy and the clinical consequences of these differences require further investigation.

1. Introduction

Immunosuppression, achieved with the use of immunosuppressive drugs (ISDs), plays a vital role in preventing solid organ rejection post-transplantation [1]. In the 1980s, the introduction of cyclosporine revolutionised post-transplant care, with reported improvements in graft survival rates of more than 80 % compared to older drug regimens [2]. Further developments, including the introduction of newer drugs with fewer adverse effects, such as tacrolimus, everolimus and sirolimus has allowed for improved

* Corresponding author. Department of Chemical Pathology, National Health Laboratory Services, University of the Witwatersrand, 7 York Rd, Parktown, 2193, Johannesburg, South Africa.
E-mail address: amy.strydom@wits.ac.za (A. Strydom).

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one-year graft survival of more than 90 % in most transplant centres [3–5]. In the South African tertiary public hospital setting, despite limited resources, reported 1-year graft survival rates for adult patients vary between 89.4 % and 91.7 % for renal transplants [6,7] and 72 %–81 % for liver transplants [8,9]. While the reasons for graft rejection are multi-factorial, some of the major modifiable risk factors leading to decreased survival include non-adherence and inadequate circulating concentrations of ISDs [10,11].

Due to risk of toxicity, as well as the risk of organ rejection from sub-therapeutic ISD concentrations, numerous groups have published guidelines and consensus reports mandating therapeutic drug monitoring (TDM) for patients on ISDs (ISD-TDM) [11–14]. The gold standard method for measurement of ISDs is liquid-chromatography tandem mass spectrometry (LC-MS/MS) [14,15]. This method provides good analytical sensitivity and has been shown to be highly specific for the measurement of ISDs [16]. LC-MS/MS is, however, costly to run, requires highly skilled staff, has complex method validation, limited automation and little manufacturer support [15,16]. Immunoassay methods are also used for ISD-TDM and address some of the limitations of LC-MS/MS by being easily implementable, having less stringent skill requirements from staff, and the potential for shorter turn-around times as part of a 24/7 laboratory service [17]. The main limitation of the use of immunoassay methods is decreased specificity due to cross-reactivity [18].

Currently, there are no published studies assessing immunoassays for ISD-TDM in an African population [19–35]. Drug metabolism of ISDs and the drug metabolites formed may vary depending on the pharmacogenomic profile of a population [36–38]. The presence of various single nucleotide polymorphisms (SNPs) in the cytochrome P450 (CYP450) system are known to cause clinically relevant alterations in drug metabolism in different populations [39]. African populations, known to have greater genetic variation than other populations, have been shown to house CYP450 SNPs of serious clinical consequence [40,41]. SNPs in the genes encoding for CYP3A4 and CYP3A5, the major enzymes involved in the metabolism of cyclosporine, everolimus and sirolimus, have been identified in the South African population, but the clinical implications of these findings are yet to be explored [41,42]. A South African study that investigated the presence of various CYP3A5 SNPs in 43 transplant patients reported differences in ISD dosing requirements based on the presence of these SNPs [43].

The implications of variable pharmacokinetics and the inherent cross-reactivity of drug metabolites for immunoassay-based measurements make the verification of an immunoassay's analytical performance within that patient population a necessity. In this study, the first to be performed in an African population, the performance of the Roche Electrochemiluminescence Immunoassay (ECLIA) for cyclosporine, everolimus and sirolimus was evaluated against the gold standard LC-MS/MS method through a method comparison experiment to determine its suitability in an African population.

2. Methods

2.1. Setting

The study took place at the University of Witwatersrand and National Health Laboratory Service (NHLS) Laboratory in Johannesburg, South Africa. The laboratory services the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) and surrounding areas. Ethical approval was obtained for the study from the Health Research Ethics Committee of the University of the Witwatersrand (M230642).

2.2. Sample collection

For the method comparison, a total of 50 remnant whole blood EDTA samples, with concentrations of ISDs spanning the analytical range of the immunoassay and medical decision limits, were collected for each drug over 3 months. Each whole blood sample was aliquoted into a 2.0 mL Provetta cryo-vial and stored at −80 °C for less than 6 months (as per manufacturer recommendation and stability studies [44–46]) until analysis. The frozen samples were thawed for 45 min and gently mixed to ensure homogeneity. Each whole blood sample was then aliquoted into two Eppendorf tubes for repeat analysis by LC-MS/MS, followed by ECLIA measurement within 2 h of the LC-MS/MS analysis. For the precision study, Roche Diagnostics quality control (QC) materials (PreciControl® ISD and PreciControl® Everolimus) were used.

2.3. Method comparison

2.3.1. Analysis by ECLIA

For the measurement of cyclosporine, everolimus and sirolimus, the samples require pre-treatment using 300 µL of Elecsys® ISD sample pre-treatment solution, followed by centrifugation for 4 min at 10 000 g. Calibration was performed using Elecsys® CalSet calibrators and QC was within acceptable limits. Analysis of 150 µL of the supranatant of these samples was performed using the Roche Cobas e602 module as per the manufacturer's instructions. Measurement by this assay is based on a competitive principle between the drugs and a ruthenium-labelled analogue. After a voltage is applied, an electrochemiluminescent signal is used to determine the drug concentration [44].

All samples were analysed within both the uncapped and capped stability period of 30 min and 4 h respectively, after the pre-treatment step as per manufacturer instructions.

2.3.2. Analysis by LC-MS/MS

Analysis by LC-MS/MS took place in an ISO 15189 accredited laboratory that participates in the Randox International Quality Assessment (RIQAS) external quality assessment scheme for ISDs. Analysis of cyclosporine, everolimus and sirolimus by LC-MS/MS

was performed on an ExionLC™ AC Series coupled to a Sciex® QTRAP 4500 tandem mass spectrometer. Chromsystems® calibrator standards were used to perform a 6-point calibration to allow for quantitation of each drug. Analysis of QC using Whole Blood Chromsystems MassCheck® control materials (levels 1–4) was within acceptable limits.

Each aliquot of sample was processed with the addition of Chromsystems® Immunosuppressant deuterated internal standards (Cyclo-d12, Evero-d4, Siro-d3), cell lysis and protein precipitation using a haemolysate (Zinc Sulphate) and acetonitrile, followed by centrifugation at 10 000 g for 5 min. The pre-treated samples were separated by gradient reverse phase chromatography using a Waters® ACQUITY UPLC BEH-phenyl column - mobile phase A (5 mM ammonium acetate and 0.1 % formic acid in water) and mobile phase B (0.1 % formic acid in acetonitrile). Electrospray ionization (ESI) was operated in positive mode. The tandem mass spectrometer was operated in multiple reaction monitoring (MRM) for the detection and quantitation of each drug using precursor and product ion *m/z* mass transitions. Quantitation of each drug was performed using QuanLynx software and a six-point standard curve.

2.4. Precision study

For 4 days, two levels of QC materials were analysed 3 times per day for each drug for the ECLIA method on the Roche Cobas® e603 instrument. For each analysis, a separate 300 µL aliquot of reconstituted QC material was subjected to the same pre-treatment procedure as the patient samples using Elecsys® ISD sample pre-treatment solution as described previously.

2.5. Statistical analysis

Statistical analysis was performed using R statistical software [47]. For the method comparison, results of the LC-MS/MS analysis in which there was a >15 % difference between the initial LC-MS/MS analysis and the single repeat LC-MS/MS analysis were excluded from the data set, which left 42, 43 and 47 data points for the method comparisons of cyclosporine, everolimus and sirolimus, respectively. The single repeat LC-MS/MS result was used for the method comparison. Deming regression analysis was performed for each drug with confidence intervals (CI) determined using the Bootstrap (quantile) method. Difference plots were constructed to assess bias. The median bias and its confidence intervals were calculated using the Wilcoxon signed rank test. The two methods were further evaluated by determining how many samples would be misclassified as sub-therapeutic, therapeutic or supra-therapeutic when comparing LC-MS/MS to ECLIA. The McNemar test was used to assess the significance of discordant classification between the two methods.

The within-run and within-laboratory variability estimates were calculated using published precision estimation formulae [48]. The laboratory's analytical coefficient of variation (CVa) for the LC-MS/MS method and the CVa data from the precision study were used to calculate the error variance ratios (δ) for the Deming regressions.

3. Results

Information about the collected samples is summarised in Table 1.

3.1. Method comparison and estimation of bias

As illustrated in Fig. 1, Deming regression analysis (δ = 0.46) for cyclosporine yielded a slope of 0.94 [95 % CI: 0.87–1.03], a y-intercept of 31.60 µg/L [95 % CI: 2.02–57.63] and a Pearson's r value of 0.986 [95 % CI: 0.973–0.992]. For everolimus, Deming regression analysis (δ = 0.76) yielded a slope of 1.35 [95 % CI: 1.23–1.44], a y-intercept of 0.23 µg/L [95 % CI: 0.21 – 0.72] and a Pearson's r value of 0.967 [95 % CI: 0.939–0.982]. For sirolimus, Deming regression analysis (δ = 0.51) yielded a slope of 0.96 [95 %

Table 1
Demographic data.

Cyclosporine	
Age	39 [31–59]
Sex (% male)	55 %
LC-MS/MS	333.4 µg/L [150.4–710.6]
ECLIA	342.6 µg/L [162.5–714.2]
Everolimus	
Age	35 [20–51]
Sex (% male)	60 %
LC-MS/MS	5.1 µg/L [2.5–6.9]
ECLIA	6.8 µg/L [3.6–9.2]
Sirolimus	
Age	44 [39.5–59.5]
Sex (% male)	57 %
LC-MS/MS	6.8 µg/L [5.4–8.9]
ECLIA	9.5 µg/L [7.5–10.7]

All data are presented as median and inter-quartile range (IQR) or as percentages (%).

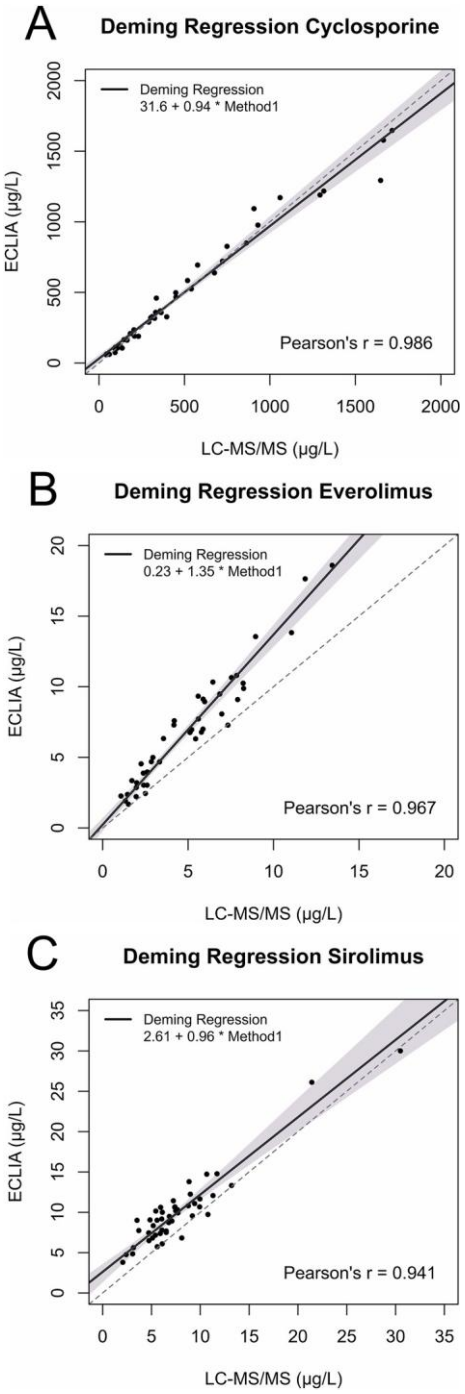

Fig. 1. Deming regression analyses for (A) cyclosporine, (B) everolimus, (C) sirolimus. The dashed line is the identity (1:1) line and the grey shaded area represents the 95 % confidence interval for each drug.

CI: 0.85–1.15], a y-intercept of 2.61 µg/L [95 % CI: 1.30–3.56] and a Pearson's r value of 0.941 [95 % CI: 0.896–0.967].

Difference plots (Fig. 2) for the cyclosporine method comparison revealed a median absolute difference of 1.96 µg/L [95 % CI: 10.0 µg/L - 18.2 µg/L], corresponding to a median percentage difference of 2.07 % [95 % CI: 1.42 %–6.99 %]. For everolimus, difference plots showed a median absolute difference of 1.64 µg/L [95 % CI: 1.43 µg/L - 2.30 µg/L] and a median percentage difference of 41.2 % [95 % CI: 34.9 %–51.8 %], while sirolimus showed a median absolute difference of 2.22 µg/L [95 % CI: 1.78 µg/L - 2.83 µg/L] and a median percentage difference of 34.9 % [95 % CI: 28.4 %–47.3 %].

There was a total of 12 misclassifications (27.9 %, p = 0.001) for the ECLIA method compared to LC-MS/MS for everolimus

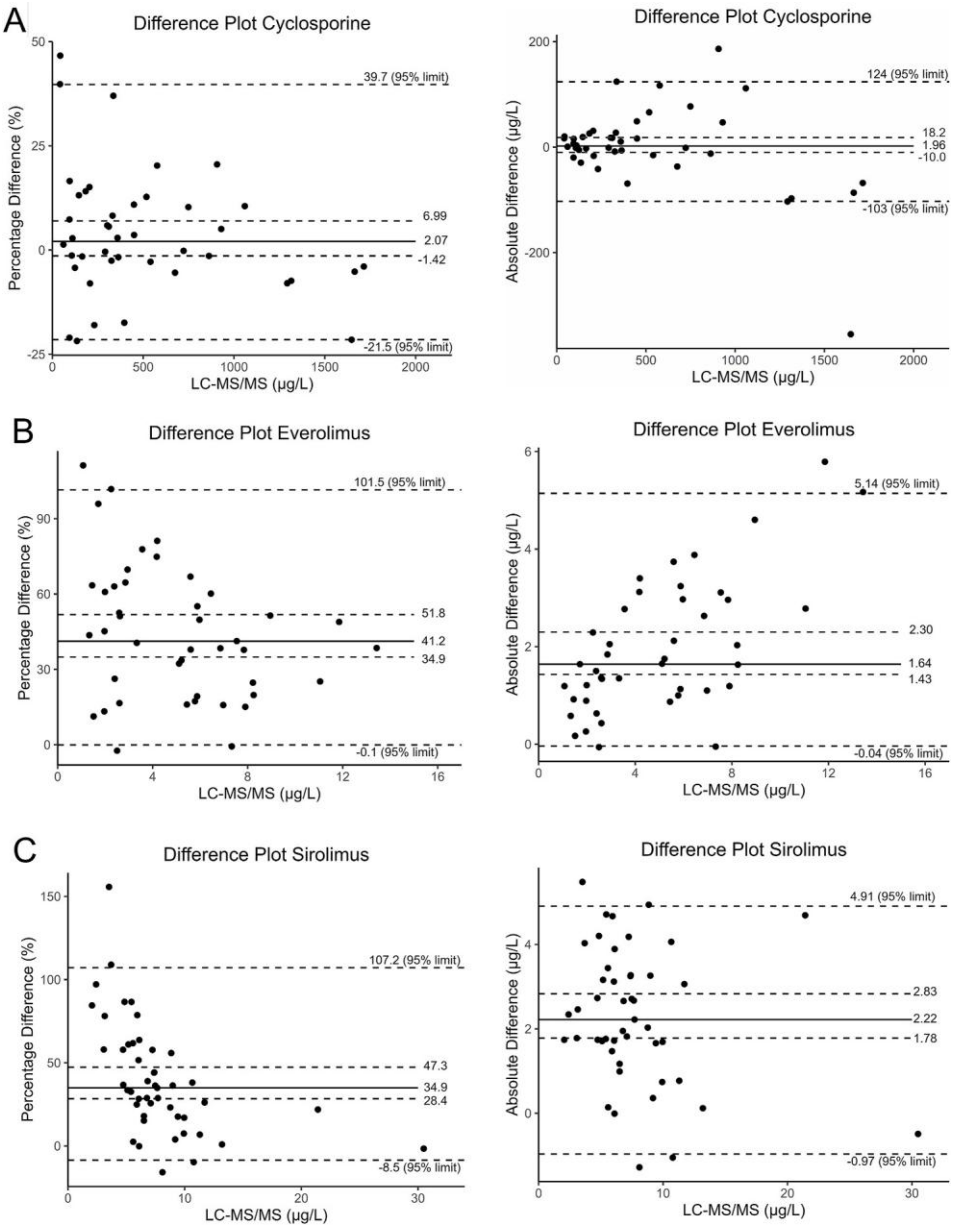

Fig. 2. Difference plots (% difference and absolute difference for (A) cyclosporine, (B) everolimus and (C) sirolimus. The black solid line indicates the median value. The inner dashed lines indicate the confidence interval for the median difference. The outer dashed lines indicate the 95 % limits of the percentage and absolute difference data sets for each drug.

(Table 2) and 9 misclassifications (19.2 %, $p = 0.008$) for sirolimus, while no samples were misclassified for cyclosporine. For everolimus and sirolimus, the ECLIA method classified more samples as supra-therapeutic and fewer as sub-therapeutic when compared to LC-MS/MS.

3.2. Precision

The within-run and within-laboratory precision estimates (Table 3) for cyclosporine, everolimus and sirolimus were <10 % at both clinically relevant concentrations of QC material.

4. Discussion

This study, based in South Africa, is the first to evaluate the analytical performance of an immunoassay for TDM-ISD in an African

Table 2
Classification of samples per therapeutic category for ECLIA and LC-MS/MS.

Drug	Method	Sub-therapeutic[a]	Therapeutic[a]	Supra-therapeutic[a]	Total disagreement (ECLIA vs LC-MS/MS)
Cyclosporine	ECLIA	8	17	17	0 (0 %)
	LC-MS/MS	8	17	17	
Everolimus	ECLIA	12	17	14	12 (27.9 %)[b]
	LC-MS/MS	17	19	7	
Sirolimus	ECLIA	1	39	7	9 (19.2 %)[b]
	LC-MS/MS	6	38	3	

[a] Based on therapeutic steady-state trough concentration range of 100–400 μg/L for cyclosporine, 3–8 μg/L for everolimus, 4–12 μg/L for sirolimus in keeping with local recommendations [13,44].

[b] p-value <0.05 using McNemar's test.

Table 3
Within-run and within-laboratory precision estimates (CVa) of cyclosporine, everolimus and sirolimus by ECLIA.

Drug and QC Level		Within-run Precision	Within-lab Precision
Cyclosporine	84 μg/L	3.02 %	2.76 %
	272 μg/L	5.98 %	6.40 %
Everolimus	2.60 μg/L	5.32 %	5.71 %
	10.10 μg/L	9.60 %	9.93 %
Sirolimus	2.74 μg/L	5.98 %	6.68 %
	9.13 μg/L	4.65 %	3.83 %

population through a method comparison between the Roche Elecsys® ECLIA method and the gold standard for TDM-ISD, LC-MS/MS. We found that the Roche Elecsys® ECLIA immunoassay and LC-MS/MS methodologies were comparable for cyclosporine, but not for everolimus and sirolimus.

There are a number of published method comparison studies for ISD immunoassays [19–35]. In general, literature on the performance of cyclosporine, everolimus and sirolimus immunoassays is inconsistent [19–35,49] compared to tacrolimus [19–25]. Cyclosporine immunoassays, previously plagued by extensive cross-reactivity of drug metabolites [49], have shown improved analytical performance since the introduction of assays utilising monoclonal antibodies [25–28]. For everolimus measurement, some studies reported adequate performance [25,32], while others noted biased and inconsistent results with the potential for clinical misinterpretation [30,31]. Similarly, method comparison studies for sirolimus immunoassays reported varied bias estimates, but overall were assessed as being fit for use [25,32–35]. Due to cost constraints, we omitted the evaluation of tacrolimus from our study, as there is an extensive body of literature reporting acceptable analytical performance by immunoassay for this drug [19–25].

Our study demonstrates strong correlation (Pearson's $r > 0.94$) between the Roche ECLIA method and LC-MS/MS for cyclosporine, everolimus and sirolimus. Cyclosporine demonstrated a clinically acceptable bias of 2.1 % which is comparable to other studies [25,27–29]. Everolimus, however, demonstrated positive proportional bias with a slope of 1.35 on regression analysis, as well as a large positive bias of 41.2 %. Similar proportional biases for everolimus were reported by Lee et al. (slope 1.22) and Verstraete et al. (slope 1.21) using the Roche ECLIA assay [31,32]. Furthermore, Shipkova et al. reported a bias of 34.2 % between the Roche Everolimus ECLIA method and LC-MS/MS [30], while Verstraete et al. reported a bias of 26.5 %–36.5 % [31]. For sirolimus by ECLIA, we observed a median positive bias of +2.22 μg/L, which echoed the findings of Lee et al. who reported a mean bias of +2 μg/L for the Roche ECLIA method compared to LC-MS/MS in an Asian population [32]. In contrast, Stove et al. reported a mean bias of 0.41 μg/L compared to LC-MS/MS in a study performed in a European population [34]. Our findings are consistent with published literature and highlight the importance of performing method comparison studies across population groups, given the impact of pharmacogenomics on drug metabolite formation.

Bias has important clinical implications, especially when a single therapeutic target is used, regardless of the measurement methodology. For method comparisons, the International Association for Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) recommends a slope of $< \pm 10$ % and a linear regression intercept that does not differ appreciably from zero compared to the reference method. An allowable bias of <5.8 % is also discussed in the context of a hypothetical total allowable error (TEa) of 15 % [15]. In our study, the bias for both everolimus and sirolimus exceeded two of these parameters. Moreover, the positive bias observed with the ECLIA method for everolimus and sirolimus resulted in a clinical misclassification rate of 27.9 % and 19.2 %, respectively, when compared to LC-MS/MS. Such misclassifications, which affect the distribution between sub-therapeutic, therapeutic and supra-therapeutic concentrations, pose risks for transplant patients, potentially leading to incorrect dose adjustments [10,11]. As a result, a patient's dose may be dependent on whether they are being monitoring with an LC-MS/MS or ECLIA method for everolimus and sirolimus. This carries significant clinical implications and warrants further investigation, including potential outcome studies going forward. Guidance on the adoption or establishment of method-specific therapeutic ranges, which is currently not provided by IATDMCT and IFCC guidelines [14,15], may be necessary and should be clearly outlined.

There are various explanations for the observed bias. There is currently no certified reference material or reference method for cyclosporine, everolimus and sirolimus, which limits calibrator traceability, resulting in possible calibration bias [15]. Secondly, cross-reactivity from drug metabolites is of particular importance for the measurement of these drugs [18]. The Roche ECLIA for

everolimus has a reported cross-reactivity of 21.3 % for 24-hydroxy everolimus, 15.4 % for 25-hydroxy everolimus and 109.3 % for 40-phosphatidylcholine everolimus [44]. For sirolimus, the reported cross reactivity of 12-hydroxy sirolimus, 27-O-Desmethyl sirolimus and 39-O-Desmethyl sirolimus is 10.4 %, 65.0 % and 108.6 %, respectively [44]. Importantly, the immunosuppressive activity of these metabolites for both drugs is not yet fully elucidated, but is reported to be less than the parent drugs [15,18]. Cyclosporine metabolism yields many metabolites (AM1, AM9, AM4N, AM19, AM1c) of varying immunosuppressive activity [14,18]. The Roche ECLIA method for cyclosporine reports non-detectable cross-reactivity from AM19, AM1c and AM1c9, 2 % cross-reactivity for AM1 and AM4N and 6 % cross-reactivity for AM9 [44]. The efforts to decrease cross-reactivity in newer immunoassays are evident in our results for cyclosporine.

Imprecision needs to be considered along with bias for any measurement procedure. The calculated imprecision of the assay is acceptable according to IATDMCT analytical performance specifications, which recommend a minimum CVa of <10 % for immunoassay-based methods [15]. Our imprecision estimates for the Roche ECLIA method are comparable to CVa estimates in other studies [23–25,27–35]. Another component of precision which should be considered is the variability among different clinical samples. The wide scatter of biases in our difference plots can partially be explained by heterogeneity among individuals [43] and the resulting variation of ISD metabolites [44]. This heterogeneity or imprecision between individuals means that a consistent level of bias is difficult to specify in our population.

Lastly, the practical aspects of these methods must be considered. Laboratories in resource-limited settings lack widespread access to LC-MS/MS [50], necessitating the use of more accessible methods like ECLIA. Both ECLIA and LC-MS/MS for ISD measurement require manual sample preparation, including cell lysis and extraction, increasing the workload compared to general automated immunoassay analysis. However, ECLIA is more convenient with fewer pre-treatment steps and less stringent staff requirements than LC-MS/MS making it better suited for resource-limited laboratories, such as those in sub-Saharan Africa.

This study has several strengths. It is novel in that it is the first method comparison study for ISD to be conducted in an African population. Secondly, remnant patient samples, which allow for a better assessment of the effect of variable metabolites, were used in this study as opposed to spiked samples. Furthermore, analysis of samples by ECLIA was conducted within 2 h of LC-MS/MS analysis for all samples and there were duplicate LC-MS/MS measurements for all samples. Finally, this study has a few limitations. Most samples for sirolimus and everolimus were within the therapeutic range with very few samples in the toxic range which limited assessment of correlation and concentration-dependent bias at higher concentrations. The concentrations assessed in our study do, however, represent those commonly encountered in clinical practice. Secondly, the study made use of anonymised remnant samples and therefore stratification of results into different transplant types (a recommendation by the IATDMCT) as well as consideration of factors such as time after transplantation, differences in therapeutic regimens and differences in ethnicity was not possible and would have resulted in underpowered findings. The tacrolimus immunoassay was not assessed in this study due to financial constraints. Acceptable analytical performance in the African population cannot be assumed and should be assessed in future studies.

In conclusion, this study, the first to be conducted in an African population, demonstrated significant bias between ECLIA and LC-MS/MS for everolimus and sirolimus measurement. In contrast, the cyclosporine immunoassay demonstrated comparability with LC-MS/MS in this patient population. The lack of comparability between these methods for everolimus and sirolimus measurement, highlighted by this study and other literature, is noteworthy. Although not unique to ISDs, this problem is perhaps under-recognised in the transplant community and the clinical consequence of these differences requires further investigation and possibly patient outcome studies to explore the potential risks posed by this non-comparability.

CRediT authorship contribution statement

Amy Strydom: Writing – review & editing, Writing – original draft, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization, Visualization. **Doreen Jacob:** Writing – review & editing, Supervision, Methodology. **Taryn Pillay:** Writing – review & editing, Supervision. **Refeletse Malahlela:** Project administration. **Sean Currin:** Writing – review & editing, Supervision, Conceptualization, Project administration, Funding acquisition, Methodology.

Ethical approval

The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (Clearance number: M230642).

Research funding

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The authors report that financial support was provided by the National Health Laboratory Service and Roche Diagnostics. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported on this paper.

Data availability

Data will be made available on request.

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APPENDIX B: Ethics Clearance Certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Dr Amy Strydom

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M230642 MED23-03-118

NAME: (**Principal Investigator**)	Dr Amy Strydom
DEPARTMENT:	Chemical Pathology National Health Laboratory Service
DEGREE:	Master of Medicine in Chemical Pathology
PROJECT TITLE:	*A Method Comparison Between Liquid Chromatography Tandem Mass Spectrometry and Chemiluminescent Immunoassay for the Measurement of Immunosuppressive Drugs*
DATE CONSIDERED:	30/06/2023
DECISION:	Approved unconditionally
CONDITIONS:	
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - Nicoleen.Potgieter@wits.ac.za
SUPERVISOR:	Dr S. Currin, Dr T. Pillay and Dr D. Jacob
APPROVED BY:	Professor Paul Ruff, Chairperson, HREC (Medical)
DATE OF APPROVAL:	27/11/2023 **EXPIRY DATE:** 27/11/2028

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions.Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. I **agree to submit a yearly progress report.** The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **June** and will therefore be due in the month of **June** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

28/11/2023
Date

APPENDIX C: Accepted Protocol

UNIVERSITY OF THE WITWATERSRAND

FACULTY OF HEALTH SCIENCES

Research Protocol

A Method Comparison Between LC-MS/MS and Chemiluminescent Immunoassay for the Measurement of Immunosuppressive Drugs

In partial fulfilment of the requirements for the degree Master of Medicine: Chemical Pathology

Submitted by:

Dr Amy Strydom (MBChB)

Student number: 731021

Supervisors:

Dr Sean Currin (MBChB, FCPath (SA) Chem, MMed (Wits))

Chemical pathologist, Charlotte Maxeke Johannesburg Academic Hospital

NHLS & University of the Witwatersrand

Dr Doreen Jacob (MBChB, FCPath SA Chem, MMed (Wits))

Chemical pathologist, Helen Joseph Hospital

NHLS & University of the Witwatersrand

Dr Taryn Pillay (MBChB, FCPath (SA) Chem)

Head of Department, Chris Hani Baragwanath Academic Hospital

NHLS & University of the Witwatersrand

INTRODUCTION

Immunosuppression, achieved with the use of immunosuppressive drugs (ISDs), plays an important role in preventing solid organ rejection post-transplantation and in the treatment of some autoimmune disorders through their immune-modulatory effects (1–4). There have been significant developments in the field of immunosuppressive therapies since the advent of modern-day solid organ transplantation in the 1950s (2). This procedure, once considered 'experimental', is now accepted as the gold standard for treatment of end-organ disease (2,5). Currently, 1-year patient and graft survival surpasses 80% for both kidney and liver transplantation, but improving the prognosis for these patients over the long term still poses several challenges (5–7). While the reasons for graft rejection are multi-factorial, some of the major modifiable risk factors leading to decreased survival involve inadequate dosing of ISDs (5,8).

Commonly used ISDs post-transplantation include cyclosporine, tacrolimus, everolimus and sirolimus. The immunosuppression and improved graft survival offered by ISDs need to be balanced with the potential toxicities that these drugs pose (13,14). Due to the risk of these toxicities, as well as the risk of potential organ rejection should circulating concentrations be inadequate, it is necessary to monitor the concentrations of these drugs so that clinicians can establish a basis for the adjustment of doses prescribed and to ensure that therapeutic concentration ranges are obtained (13).

Therapeutic Drug Monitoring (TDM) involves the measurement of drug concentrations in biological fluids and offers an approach to personalised medicine that allows clinicians to individualise drug regimens and adjust dosage or dosing intervals to achieve systemic drug concentrations that are associated with good clinical outcomes, and to minimise the risk of toxicities associated with the drugs being used (13,15). TDM in patients on ISDs (ISD-TDM) is warranted because of the narrow therapeutic

index of these drugs, the clear association between drug concentration and clinical effect, the presence of large intra-individual and inter-individual differences in pharmacokinetic and pharmacodynamic profiles, potential drug-drug interactions, and for the monitoring of compliance to medication (13,16). The measurement of trough concentrations of these drugs is supported by numerous groups that recommend ISD-TDM and there are several published guidelines and consensus reports on the topic (17–19).

The gold standard and recommended method for measurement of these ISDs is liquid-chromatography tandem mass spectrometry (LC-MS/MS) (14). This method provides high analytical sensitivity which is important in ISD-TDM as the therapeutic doses in patients post-transplantation are low (µg/L) (14,16,20). LC-MS/MS is specific for the drugs measured and does not cross-react with other ISDs or drug metabolites (14). LC-MS/MS is however costly to run, requires highly skilled and well-trained staff, has complex method validation, limited automation and little manufacturer support (14,16). Immunoassay methods can also be used for ISD-TDM and address some of the limitations of LC-MS/MS by being easy to use, having less stringent skill requirements from staff and shorter turn-around times (14,16). Clinicians require timeous results to improve quality of care and to prevent patient harm in cases of suspected toxicity, dose titrations, out-patient adherence checking and dose-adjustments (21). The main limitation of the use of immunoassay methods is decreased specificity due to cross-reactivity (16).

There are few published studies that evaluate the analytical performance of the electrochemiluminescence assays (ECLIA) for ISD-TDM. Fung et al. assessed the performance of the Roche e411 ECLIA compared to the Abbott ARCHITECT i2000 chemiluminescent microparticle immunoassay (CMIA) and then used a small subset

of samples (n=20) to compare LC-MS/MS to the ECLIA method for measurement of cyclosporine, tacrolimus and sirolimus (22). The study found that the ECLIA method yielded acceptable performance that was comparable to the LC-MS/MS method, with superior precision for cyclosporine measurement and superior functional sensitivity for tacrolimus and sirolimus measurement compared to the CMIA method (22). Similar findings were obtained in a study by Vogeser et al. that compared the Roche ECLIA method to LC-MS/MS for cyclosporine A measurement (23). Shipkova et al. performed a similar study for tacrolimus measurement using a Roche ECLIA method which also found that the assay was fit for purpose in terms of precision, accuracy and functional sensitivity (24). This study included analysis of frozen remnant samples that were analysed within 6 months of freezing, but without remeasuring the samples using LC-MS/MS prior to analysis by immunoassay (24). To the best of our knowledge, there are no studies comparing the measurement of all four discussed ISDs using the Roche ECLIA method and LC-MS/MS. Additionally, none of these studies have been performed on an African population. Laboratories in developing countries may not have widespread access to LC-MS/MS, necessitating the use of more accessible methods that offer analytic capabilities that are in line with those offered by the gold standard (15,25). This study, based in Southern Africa, aims to determine if measurement of cyclosporine, tacrolimus, everolimus and sirolimus using an ECLIA method is suitable for ISD-TDM.

STUDY OBJECTIVES

- To determine the precision of the Roche e602 ECLIA method for cyclosporine, tacrolimus, everolimus and sirolimus measurement

- To determine the bias of the Roche e602 ECLIA method compared to the gold standard (LCMS-MS) for ISD-TDM of cyclosporine, tacrolimus, everolimus and sirolimus.
- To determine if the Roche e602 ECLIA is suitable for its intended use by comparing the determined accuracy and precision with standards recommended by the International Association of Therapeutic Drug Monitoring and Clinical Toxicology Immunosuppressive Drug Scientific Committee (IATDMCT) (26).

METHODS:

Study design:

Prospective analytical study

Study setting:

The study will take place at the NHLS laboratory at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH).

Study population:

Remnant samples from patients that are receiving one or more of cyclosporine, tacrolimus, everolimus and sirolimus for whom drug levels were requested by the treating clinician will be used to determine bias. Quality Control (QC) materials will be used to determine precision.

Sample size:

A total of 30 measurements (15 measurements each of 2 concentrations of QC material) will be obtained to determine precision and 40 remnant patient samples per drug will be analysed to determine bias. A total of 15 measurements per QC material concentration is required to determine precision for the ECLIA assay (27,28). A

minimum of 40 measurements per analyte spanning the analytical range of the assay is required to determine the bias of the method for a method comparison study (29,30).

Procedure:

The method validation will be based on the CLSI EP 15 and CLSI EP 09 documents to determine precision and accuracy respectively (27,30)

The Roche ECLIA method for all four of the drugs in this study requires a pre-treatment step of all samples in which 300µL of sample is treated with ISD sample pre-treatment reagent which acts as a haemolysate to allow for extraction of each drug from erythrocytes (31–34). After centrifugation for 4 minutes at ≥10 000 g, an aliquot of the yielded supranatant is used for analysis (31–34). The ECLIA methodology makes use of a competition principle with the analyte concentration being read off a calibration curve based on the signal detected after the electrochemiluminescence reaction (31–34). For the LC-MS/MS method, the sample is aliquoted into the tubes used for analysis and as part of the sample preparation, a haemolysate, as well as deuterated internal standards for each of the four drugs, is added prior to analysis. The sample is introduced into the analyser and mobile phases of varying polarities are used to allow for a gradient elution from a UPLC BEH-phenyl column. The mass spectrometer utilises a multiple reaction monitoring (MRM) technique for the detection and quantitation of each of the four drugs.

Precision study:

Quality control materials of two concentrations will be analysed three times per day for five days for the precision study with the results recorded daily.

Bias study:

The samples analysed using LCMS-MS that are selected for the study must have concentrations that span the analytical range of the immunoassay method and medical

decision limits. Table 1 below indicates the ranges required. Ten samples are required at minimum per range group below.

Drug	Range 1 (µg/L)	Range 2 (µg/L)	Range 3 (µg/L)	Range 4 (µg/L)
Cyclosporine	30 – 150	150 – 400	400 – 600	600 – 2000
Tacrolimus	0.5 – 4	4 – 8	8 – 12	12 – 40
Everolimus	0.5 – 4	4 – 8	8 – 12	12 – 30
Sirolimus	0.5 – 4	4 – 8	6 – 12	12 – 30

Table *1*: Ranges of samples required for the method comparison to determine bias

Results from the initial routine LC-MS/MS analysis will be captured on a spreadsheet using a unique identifier. Remnant samples of sufficient volume (500µL) and with suitable results obtained by LC-MS/MS will be placed in cryo-tubes and frozen at -80° Celsius. The remnant patient samples will be thawed prior to ECLIA analysis and an aliquot of the remaining sample re-analysed by LC-MS/MS to ensure that no deterioration, although not anticipated, has occurred during storage after the first LC-MS/MS measurement.

DATA ANALYSIS:

For the precision study, the mean, standard deviation (SD) and analytical coefficient of variation will be calculated for the different data sets. ANOVA will be performed on the data to assess if the differences between intra-day and inter-day variability is statistically significant. The bias of the ECLIA method will be assessed by comparing results to the reference method with a linear regression, as well as a difference plot. A Deming regression will be used to determine the correlation co-efficient for each drug if the data is parametric. If the data is non-parametric, a Passing Bablok

regression will be used. The regression will provide information on systematic error present in the measurement system using ECLIA compared to LCMS-MS, including proportional and constant error based on the slope and y-intercept generated by the regression respectively. For the difference plot, the values obtained by the reference method will be plotted on the x-axis. The absolute difference between the two methods will be plotted on the y-axis if error is independent of concentration and constant. Alternatively, if the error is concentration-dependent, the percentage difference will be plotted on the y-axis. If the data is parametric, the agreement limits for the plot will be at ± 1.96 SD from the mean (95% limits). If non-parametric, the median can be used with the 2.5^{th} and 97.5^{th} centiles forming the agreement limits. The statistical analysis of the data will be performed using Stata. The imprecision and bias observed will be compared to the analytical performance recommendations stated in the guidelines most recently published by IATDMCT (26).

RESEARCH OUTPUTS:

- The research report forms part of the requirements for the degree Master of Medicine in Chemical Pathology
- The findings of the research will provide information about the suitability of the Roche e602 ECLIA method for ISD-TDM
- Publication of the report in a peer-review journal

ETHICAL CONSIDERATIONS:

Ethics approval will be applied for from the Human Research Ethics Committee of the University of the Witwatersrand. Remnant samples will be used in the study, and no patient details or identifiers will be used. The data obtained will be stored securely on

a password-protected desktop and the relevant permission to perform the study in the laboratory will be obtained.

TIMING:

	Mar 2023	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan 2024	Feb
Literature Review												
Writing Protocol												
Protocol Assessment												
Ethics Application												
Sample collection												
Sample Analysis												
Data Analysis												
Writing Report												

FUNDING:

Manufacturer to sponsor costs of ECLIA reagent, control materials, calibrators.

		Cyclo	Tacro	Evero	Siro	Total
SPONSORED COSTS						
Reagent	100 tests per kit	R10638.22	R10488.39	R4155.61	R9910.81	R35193.03
Control Material	3 x 3 mL	R4860.09	R4860.09	R4105.38	R4860.09	R18685.65
Calibrators	3 x 1 mL	R6075.09	R6075.09	R6750.77	R5132.03	R24032.98
TOTAL SPONSORED COSTS						**R77911.67**
COSTS REQUIRING FUNDING						
LCMS-MS prior to ECLIA Analysis	50 x unit cost R 216.81	R10840.50	R10840.50	R10840.50	R10840.50	R43362
Cryo-tubes	500 x 1.8 mL tubes	R1625	R1625	R1625	R1625	R6500
Cryo-boxes	1 box per drug	R450	R450	R450	R450	R1800
Publication		R40000				R40000
TOTAL COSTS REQUIRING FUNDING						**R91662**

An application for the required funding will be submitted to the Academic Affairs and Research Management System for NHLS K-funding.

PROBLEMS:

A potential limitation of the study is that the samples will need to be batched before analysis by immunoassay, rendering them subject to deterioration. Some of the ISDs are more commonly requested than others which might make accumulating sufficient results spanning all four of the ranges difficult. In our experience, sirolimus is less commonly requested by clinicians and we therefore anticipate that there may be a challenge in collecting sufficient sample numbers with concentrations suitable for determination of bias. Another potential challenge is that it may be difficult to collect

enough remnant samples of sufficient volume (i.e., 500 µL) across the measuring ranges required for each drug over the sample collection period. Due to financial constraints, the number of measurements for the precision component is limited to being less than those stated by CLSI EP15 guidelines and therefore the statistical power of the data is reduced. The samples should ideally be run in duplicate by the candidate method for CLSI EP09. Due to financial constraints and limited reagent, only a single measurement will be obtained for estimation of bias.

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APPENDIX D: Turnitin Report

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by Amy Strydom

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