

Variability in whole blood tacrolimus concentrations after oral and continuous intravenous administration in the early post-lung transplantation period: a retrospective multicenter cohort study

BACKGROUND: A relationship between high tacrolimus variability after lung transplantation (LTx) and an increased risk for rejection of the allograft or nephrotoxicity is increasingly suggested in literature. The highly variable bioavailability of tacrolimus after oral administration is thought to play a predominant role. In the clinical practice, there is no consensus on the optimal administration route, early after LTx, to obtain a reduced variability.

OBJECTIVE: The purpose is to investigate the effect of continuous intravenous and oral administration on tacrolimus variability early after lung transplantation.

METHODS: In this retrospective study, 224 patients received intermittent oral administration of tacrolimus and 298 received continuous intravenous administration. Intra-patient variability (IPV) and the time within the therapeutic range (TTR) were calculated using daily tacrolimus whole blood concentrations from the first 14 days after LTx. Linear regression was used to investigate the effect of the administration route on variability.

RESULTS: The mean IPV in the intravenous group, weighted for the number of samples available per patient, was $29.2\% \pm 10.9$ compared to $31.7\% \pm 10.5$ in the oral group ($p < 0.001$). After adjusting for effect modifiers, the mean IPV in the intravenous group was 20.2% and 7.8% higher in the oral group ($p < 0.001$). Univariate analysis showed that TTR was 30.7% (18.7-41.1) and 22.1% (13.0-30.8) in the intravenous and oral group, respectively ($p < 0.001$). After adjustment, TTR was 14.4% lower in the oral group (13.3%) than in the intravenous group (27.7%), ($p < 0.001$).

CONCLUSION: The variability in tacrolimus concentrations, measured as IPV and TTR, is higher when tacrolimus is administered orally in the first 14 days after LTx in comparison to continuous intravenous infusion, with a switch to oral administration once the patient is more stable.

Abbreviations: CI, confidence interval; CLAD, chronic lung allograft dysfunction; C_{max}, Highest tacrolimus concentration; C_{min}, Lowest tacrolimus concentration; CV, coefficient of variance; ECLS, extra corporeal life support; eGFR, estimated Glomerular Filtration Rate; EHR, electronic health record; ICU, intensive care unit; IPV, intra-patient variability; IQR interquartile range; LC-MS/MS, liquid chromatography-Mass spectrometry; LTx, lung transplantation; RDP, research data platform; SD, standard deviation; SOFA, sequential organ failure assessment; TR, therapeutic range; TTR, time within therapeutic range; UMCG University Medical Center Groningen; UMCU, University Medical Center Utrecht.

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Introduction

There is increasing literature suggesting a relationship between high variability of tacrolimus pharmacokinetics and early post-transplantation toxicity, potentially leading to an increased risk of morbidity and mortality (1). For instance, a higher incidence of rejection was seen in liver transplant recipients with a higher variability (2). In kidney transplant recipients, the early post-transplantation variability was associated with rejection of the allograft on the long term (3,4), but not with acute rejection at 6 months post-transplantation (3,5). In the 6-12 months after lung transplantation, a high tacrolimus standard deviation independently increased the risk for chronic lung allograft dysfunction (CLAD) and death by 46% and 27% respectively (6). However, results for lung transplantations, as well as heart transplantations, are still ambiguous. (7,8).

Tacrolimus is known to have a narrow therapeutic range, making therapeutic drug monitoring essential. Reaching the therapeutic range shortly after lung transplantation (LTx) is challenging because recipients are clinically unstable, mainly due to inflammation, severe bleeding and organ dysfunction (e.g., gut dysmotility)(9). These factors may contribute to a high variability in tacrolimus exposure within recipients, which, in turn, may lead to worse outcomes such as the development of rejection of the allograft or nephrotoxicity (10,11). Variability in bioavailability is expected to be contributing the most to the development of a poor outcome (9,12). The overall conclusion of a systematic review described this association between a high variability in tacrolimus on the one hand and acute rejection and mortality on the other hand in solid organ transplants as well (1).

Although intravenous administration of tacrolimus theoretically bypasses the highly variable absorption step and may diminish the variation in bioavailability as a contributing factor to a high variability of tacrolimus blood levels (9), there is no consensus on the best route of administration in the early post-transplantation period. The oral and the intravenous route are both used in clinical practice to administer tacrolimus shortly after LTx. Currently, no information on different tacrolimus administration routes in the early post-LTx period, and their effects on within-patient variability, is available.

Within-patient variability is a measure of variation in tacrolimus (trough) concentrations in an individual patient over a certain time (in which the dose was not altered) (1) and is frequently expressed as standard deviation (SD), coefficient of variation (CV), intra-patient variability (IPV) and (percentage of) time within the therapeutic range (TTR). These are different parameters to describe the variability early after transplantation. In this period, the blood concentrations may differ due to changes in pharmacokinetics as well as changes in dose, making interpretation of the variability difficult. Measuring the variability by establishing SD or CV does not take into account the significant outliers in concentration. SD may be a good prognosticator ≥ 6 months for CLAD after lung transplantation, but seems a poor prognosticator within 6 months (6). To measure variability more reliably, IPV could be used, as each single concentration is included in this equation as well as dose alterations. Alongside the IPV, the time within the therapeutic range is of relevance as well. A patient may show high IPV within the therapeutic range or low IPV outside of the therapeutic range. The latter still has an increased risk for rejection or nephrotoxicity despite the low IPV. Thus, it has been suggested to combine measures of variability to improve the predictive value (1). Additionally, time within the therapeutic range could have a stronger clinical relevance while the therapeutic range is the clinical target. In lung transplant recipients, every 10% increase in TTR has been inversely related to high-grade acute cellular rejection, CLAD, mortality, and the infection rate at 1 year post-transplant (13). Conversely, another study in lung transplantations failed to find an association between TTR and acute rejection (14).

The purpose of this retrospective cohort study is to investigate the effect of continuous intravenous and oral administration on tacrolimus variability, measured as IPV and TTR, in lung transplant patients in the early post-transplantation period.

Methods

Setting

In the Netherlands, lung transplantations are performed in three different academic hospitals, two of which are the University Medical Center Utrecht (UMCU) and the University Medical Center Groningen (UMCG). The UMCU used Metavision (Ite Medical, Tiel) on the Intensive Care Unit (ICU) and HiX[®] (Chipsoft BV, Amsterdam) on the clinical ward as its Electronic Health Record (EHR) system in the study period. The UMCG used Metavision until 2017 and Epic (Epic System Corp., Verona, Wisconsin, USA) afterwards. Data-extraction from the EHR was made available through the Research Data Platform (RDP). The full study protocol can be found in Appendix 1. The deviations from the protocol are presented in Appendix 2. The Research Ethics Committee of the UMCU confirmed that the Medical Research Involving Human Subjects Act was not applicable to this study and a waiver was granted (no. 22-510/DB).

Post-transplantation regimen

The protocol in the UMCU (15) was to commence oral tacrolimus administration on the first day after LTx, initially 0.07 mg/kg twice daily, followed by dose adjustments based on trough concentrations. Exceptionally, in case oral administration was not possible, a few tacrolimus doses were given as an intermittent intravenous injection. The target of trough concentrations for oral administration was between 12-15 µg/L for the entire study period. The UMCG (16) administered tacrolimus through continuous intravenous infusion, starting 12 hours after perfusion of the transplanted lung(s), in a dose of 0.01 mg/kg/24 hours until the recipient experienced sufficient bowel movement with defecation. Afterwards, the route of administration was switched to oral 0.1 mg/kg/day divided into two doses. A therapeutic range (TR) from 13-15 µg/L was aimed for during the first week after LTx and 10-15 µg/L during the second week.

Additional immunosuppressive medication consisted of mycophenolate-mofetil, basiliximab and (methyl)prednisone for both hospitals. The remaining regimen consisted of antibiotics, prophylactic anticoagulants and analgesics. The regimens were comparable with few small differences (e.g., (methyl)prednisone dose as part of triple therapy, time of mycophenolate-mofetil initiation and anticoagulation prophylaxis, see appendix 3 and 4) that were deemed irrelevant for the outcomes. Hence, the two post-transplantation protocols of the UMCU and UMCG were similar. The main difference was the administration route of tacrolimus initiated directly after transplantation and the therapeutic range that was aimed for.

Patient selection

The expected sample size consisted of approximately 600 patients. Patients were eligible for inclusion if they had undergone a lung transplantation between January 2010 and January 2020 in either the UMCU or the UMCG. Information from the first lung transplantation was included in the analysis only. Patients aged 18 years or older at the time of LTx were included if they had received tacrolimus orally or as continuous intravenous infusion in the early post-LTx phase; the first 14 days after LTx. Exclusion took place if a patient had less than three tacrolimus concentrations available in the early post-LTx period, for instance due to early mortality, because this is the minimum amount of concentrations necessary to calculate IPV accurately (8). If multiple concentrations or times of sample taking were missing, patients were also not included in the analysis. In the UMCU, patients transplanted before

June 1st 2011 were excluded, as explained in the following section. Another exclusion criterion was the objection to the use of their medical data for scientific research.

Tacrolimus concentrations

In the first 14 days after LTx, the whole blood tacrolimus (trough) concentrations were measured daily, just before the following tacrolimus dose, and reported in µg/L. The time of sample taking was respective to the start of surgery for the oral group and to the reperfusion of the transplanted allograft in the intravenous group. Tacrolimus concentrations were analyzed with Liquid Chromatography-Mass spectrometry (LC-MS/MS) in both hospitals in the study period, with a measurement uncertainty of 15% for both centers. There was an exception for the UMCU patients transplanted before June 1st 2011, because the analysis method of the UMCU was switched from immunoassay to LC-MS/MS on this date. Since the accuracy between those methods differs, patients transplanted before June 1st 2011 were excluded.

Design

This study entails a retrospective, multicenter, observational cohort study. Baseline characteristics from the pre-operative screening were gathered along with data from the first two weeks after LTx.

As mentioned before, the therapeutic range at which was targeted, differed between the hospitals.

If a concentration was reported below the detection limit (<2.0, <1.3 or <1.0 µg/L), a comparison was made between the time of the first administration and the time that that specific sample was taken. In case the sample was taken before the first administration, the reported concentration was substituted with 0. When the sample was taken after the first administration, the concentration was converted to 0.9 µg/L.

Furthermore, in some cases two different concentrations were reported for the same patient at the same time. If the percentage difference between those concentrations exceeded 15% and could therefore not be explained by the measurement uncertainty, both concentrations were excluded. Otherwise, the average was included in the analysis.

As peak levels in intermittent oral dosing are unintentionally wrong measurements that may bias the variability, potential peak concentrations were identified if the following two conditions were true: [1] the difference between the potential peak concentration and the preceding and following concentration was minimally 7 µg/L and [2] the two preceding, as well as the two following concentrations showed a difference of maximally 3 µg/L. A sensitivity analysis was performed to define the optimal limits for identification. All potential peak levels were investigated by comparing the time of dosing and time of blood collection. If the unusually high concentration could not be explained by aforementioned timing or clinical factors in the EHR (i.e., elevation of the tacrolimus dose, initiation of a CYP3A4- or P-gp-inhibitor, diarrhea or packed red cells therapy), it was considered a peak concentration and excluded from the calculation of variability. This analysis was not performed for the intravenous group, as information on intravenous dosing was lacking. Theoretically there are no peak levels during continuous intravenous dosing, even though there may have been peak concentrations after the switch to oral.

Outcomes

Primary endpoint

The primary outcome was the variability, defined as the IPV and the percentage of time within the therapeutic range (TTR).

The IPV was calculated as following: (8)

$$IPV \% = \frac{1}{T} \times \sum_{t=1}^T \frac{abs(X_t - X)}{X} \times 100$$

In this equation, X was the mean of all available concentrations in the first 14 days after LTx. X_t was each individual concentration within the period mentioned and T was the number of available tacrolimus concentrations. In this study, IPV was not corrected for dose, since the primary aim was to investigate the differences in variability and not necessarily the cause of variability. Doses were adjusted according to the measured concentrations, hence it is part of the protocol.

For each patient, the times beneath, within and above the therapeutic range were calculated using Linear Interpolation to determine the time that the lower or upper limit was crossed, as first reported by Rosendaal *et al.*(17). Figure 1 is a visual representation of the time in- and outside of the oral therapeutic range.

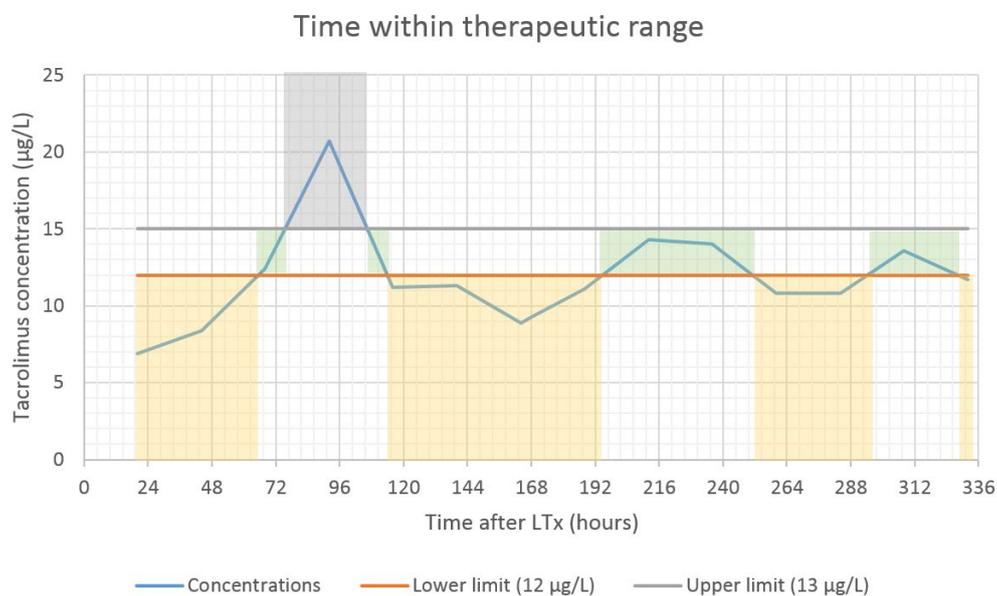


Figure 1: Representation of the calculation of the time in- and outside of the oral therapeutic range of tacrolimus. The green, grey and yellow areas represent the time within, above and beneath the therapeutic range, respectively.

Secondary endpoint

The secondary outcomes were length of stay and mortality at the ICU and during hospital admission.

Covariates

Information on baseline characteristics, such as sex, age and comorbidities was registered during the pre-operative screening. Other data that were gathered consisted of primary lung disease, the type of LTx (i.e., unilateral or bilateral), the need for Extra Corporeal Life Support (ECLS) and the need for dialysis. This information was needed to test for confounding bias and effect modification. Pulmonary hypertension as a pre-LTx comorbidity was not included in the analysis because there was a difference between the centers in method to determine whether pulmonary hypertension was diagnosed or not.

Statistical analysis

Main analysis

The analyses were performed on an 'Intention to treat' basis. Thus, patients from the UMCU who had received few tacrolimus administrations through intermittent intravenous bolus injections were still

included in the oral group. The independent samples t-test was used to evaluate the difference in mean IPV, mean TTR and length of stay at the ICU and in the hospital. To investigate the difference in mortality, the Chi-square test was used.

First, univariate linear regression was performed, with IPV and TTR as separate dependent variables and administration route as independent variable. Subsequently, covariates that had a significant asymmetrical distribution between the groups were added to the multiple linear regression analysis. The effect of statistically significant confounders was tested. If the percentage difference between the unadjusted and adjusted B was $\geq 10\%$, the confounder was included in the model and adjusted for. Interaction terms were introduced to investigate effect modification. A variable was regarded as effect modifier if the p-value of the interaction term was < 0.05 . Significant effect modifiers were included in the multiple linear regression model.

The differences in means (B), 95% Confidence Interval (CI) and the p-value were reported. A p-value < 0.05 for two-tailed tests was considered statistically significant. IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, N.Y., USA) was used to perform all statistical analyses.

Sub analysis

In a sub analysis, the variability in the first week in each center was compared to the variability in the second week in the same center. Moreover, the variability in week 1 was compared between the two centers, as well as the variability in week 2.

Sensitivity analyses

Alongside the main analysis, two sensitivity analyses were performed. In one, the variability results were gathered while applying the same therapeutic range for both hospitals. A lower limit of $12 \mu\text{g/L}$ was applied for the first week and this changed to $10 \mu\text{g/L}$ in the second week. The upper limit remained $15 \mu\text{g/L}$ throughout both weeks. In the other, we investigated how many concentrations were (in)accurately identified as potential peak concentrations if the two preceding and following concentrations maximally differed $2 \mu\text{g/L}$, in order to establish the most accurate definition of peak concentration identification.

Results

Study population

As can be seen in figure 2, 584 patients were eligible for inclusion, of which 270 were transplanted in the UMCU and 314 in the UMCG. 11 patients had undergone re-transplantation and the data from the second LTx were excluded from the analysis. 12 patients had died before start of tacrolimus treatment and 2 patients lacked information on tacrolimus concentrations. 7 patients were excluded because they had less than three whole blood tacrolimus concentrations available within the first 14 days after LTx. Finally, the 30 UMCU patients whose concentrations were measured with immunoassay instead of LC-MS/MS were excluded, leaving 522 patients available for inclusion in the analysis.

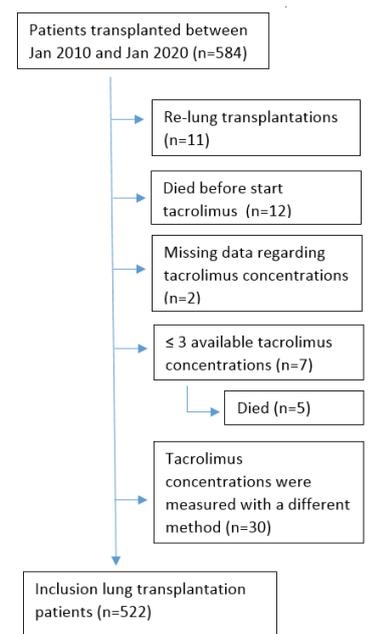


Figure 2: Overview of exclusion criteria and the number of included patients.

In total, from these 522 patients, 5817 whole blood tacrolimus concentrations in the first 14 days after LTx were gathered, as presented in figure 3. 105 concentrations (1.8%) were beneath the detection limit. 76 of these concentrations were substituted by 0 and excluded from analysis because these measurements were performed before the first dose of tacrolimus. The algorithm to detect serum peak levels revealed 23 concentrations from the UMCU population as potential peak concentrations. After individual analysis of these 23 potential peak concentrations, 5 were judged as actual peak concentrations and were discarded. Finally, there were 31 cases in which two different concentrations were reported for the same time. The average had to be taken for 30 concentrations and 2 concentrations were eliminated because the difference exceeded 15%. In the end, 5704 concentrations (98.1%) were available for the calculation of variability.

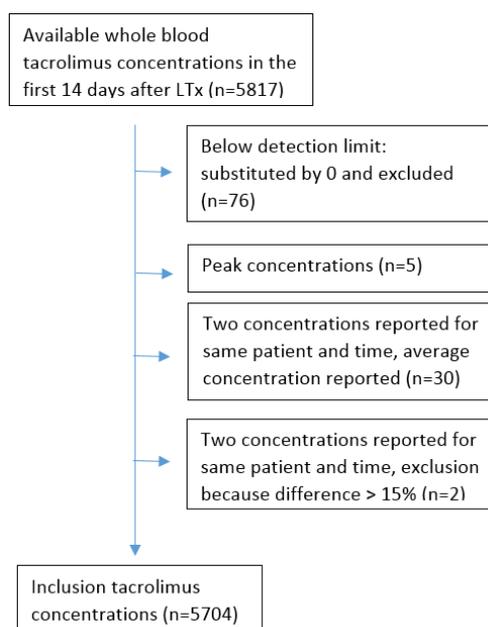


Figure 3: Overview of exclusion criteria and the number of included tacrolimus concentrations.

Table 1 describes the baseline patient characteristics at the time of lung transplantation. The baseline characteristics of the oral and intravenous group are similar. The proportion of patients with obstructive airway disease and disease of pulmonary circulation is somewhat larger in the intravenous group, whereas the proportion of suppurative and restrictive lung diseases is larger in the oral group. Moreover, ECLS was more necessary in the intravenous group than in the oral group. The need for ECLS and dialysis are both seen as a measure of disease severity and were correlated ($p < 0.001$). As a result, a combined variable was created in which the need for ECLS and/or dialysis was recorded, in order to investigate an intervening effect of disease severity on the outcome parameters.

Table 1: Baseline lung transplant patient characteristics as determined at the time of lung transplantation.

Characteristic	Oral, n (%)	Intravenous, n (%)	p-value
	224 (42.9)	298 (57.1)	
Female sex	107 (47.8)	143 (48.0)	0.961
Age (years), median (IQR)	55 (43-61)	55 (46-61)	0.747
Pre-LTx comorbidities			
PH	11 (4.9)	33 (11.0)	0.012
DM	36 (16.1)	44 (14.8)	0.706
RI	13 (5.8)	19 (6.4)	0.787
Baseline SCr (µmol/L), median (IQR)	66.0 (53.0-79.8)	66.0 (51.0-80.3)	0.892
Baseline CKD-EPI eGFR (mL/min/1.73m ²), mean ± SD	99.3 ± 23.0	99.1 ± 24.9	0.922
Primary lung disease			<0.001
Obstructive airway disease	73 (32.6)	151 (50.7)	
Disease of pulmonary circulation	6 (2.7)	33 (11.1)	
Suppurative lung diseases	50 (22.3)	38 (12.8)	
Restrictive lung diseases	95 (42.4)	71 (23.8)	
Other diagnosis	0 (0.0)	5 (1.7)	
Re-transplant	6 (2.7)	5 (1.7)	0.761
Type of LTx			0.151
Left	13 (5.8)	10 (3.4)	
Right	20 (8.9)	16 (5.4)	
Bilateral	191 (85.3)	271 (90.9)	
Heart-Lung	0 (0.0)	1 (0.3)	
CVVH or dialysis in the first 14 days after LTx	31 (13.8)	45 (15.1)	0.686
ECLS in the first 14 days after LTx	60 (26.8)	124 (41.6)	<0.001
<small>CKD-EPI, eGFR Chronic Kidney Disease Epidemiology Collaboration estimated glomerular filtration rate; CVVH, Continuous VenoVenous Haemofiltration; DM, Diabetes Mellitus; ECLS, Extra Corporeal Life Support; IQR, interquartile range; LTx, lung transplantation; SCr, Serum Creatinine; SD, standard deviation; PH, Pulmonary Hypertension; RI, Renal Insufficiency.</small>			

Variability

Table 2 presents the primary comparison in variability parameters between the oral and intravenous groups. The results are also presented while weighted for the number of samples per patient.

The weighted mean IPV was significantly higher in the oral group (31.7% ± 10.5) compared to the intravenous group (29.2% ± 10.9) (p<0.001). The weighted median TTR is 22.1% (13.0-30.8) for the oral group and 30.7% (18.7-41.1) for the intravenous group (p<0.001). The percentage of time above the therapeutic range is statistically significantly larger in the intravenous group than in the oral group; 34.3% ± 22.6 versus 26.0% ± 16.8 (p<0.001). In addition, the time to reach the therapeutic range is higher for the intravenous group, namely 78 hours compared to 73 hours in the oral group (p=0.046). The average tacrolimus concentration was similar for both groups, just like the minimum and maximum concentration.

Table 2: Parameters of variability, represented for the oral and intravenous group and displayed weighted for number of samples. Oral therapeutic range: 12-15 µg/L. Intravenous therapeutic range: 13-15 µg/L in week 1 and 10-15 µg/L in week 2.

Variability parameter	Oral, n (%)	Intravenous, n (%)	p-value	Oral: weighted for number of samples, n (%)	Intravenous: weighted for number of samples, n (%)	p-value
	224 (42.9)	298 (57.1)		2586 (45.3)	3118 (54.7)	
IPV (%), mean ± SD	31.5 ± 10.4	29.2 ± 11.0	0.017	31.7 ± 10.5	29.2 ± 10.9	<0.001
median (IQR)	30.2 (24.5-37.2)	27.4 (22.0-34.3)		30.4 (24.9-37.4)	27.2 (21.8-34.3)	
<i>Min-max</i>	7.3-72.9	7.9-70.5		7.3-72.9	7.9-70.5	
Time beneath TR (hours), mean ± SD	137.4 ± 58.9	90.7 ± 52.5	<0.001	139.1 ± 57.6	93.3 ± 52.2	<0.001
Percentage beneath TR (%), mean ± SD	50.1 ± 20.9	34.5 ± 19.6	<0.001	50.3 ± 20.5	35.0 ± 19.2	<0.001
Time within TR (hours), median (IQR)	60.3 (33.1-85.5)	77.9 (46.4-109.6)	<0.001	61.4 (34.2-85.5)	81.7 (50.0-110.5)	<0.001
Percentage within TR (%), median (IQR)	22.1 (13.0-30.9)	29.8 (17.8-41.0)	<0.001	22.1 (13.0-30.8)	30.7 (18.7-41.1)	<0.001
Time above TR (hours), mean ± SD	70.1 ± 45.9	91.6 ± 60.3	<0.001	71.4 ± 45.8	90.8 ± 59.6	<0.001
Percentage above TR (%), mean ± SD	26.0 ± 17.1	35.1 ± 23.2	<0.001	26.0 ± 16.8	34.3 ± 22.6	<0.001
First time TR was reached (hours), median (IQR)*	73.0 (54.4-110.5)	78.3 (59.0-112.1)	0.572	73.0 (55.0-115.0)	78.3 (59.1-113.0)	0.046
Average tacrolimus concentration (µg/L), median (IQR)	12.4 (11.2-14.1)	13.0 (11.7-14.7)	0.001	12.4 (11.3-14.1)	12.9 (11.7-14.6)	<0.001
Cmin (µg/L), median (IQR)	5.8 (3.0-7.7)	5.6 (3.7-7.5)	0.708	5.7 (3.0-7.6)	5.5 (3.7-7.4)	0.101
<i>Min-max</i>	0.9-13.3	0.9-23		0.9-13.3	0.9-23	
Cmax (µg/L), median (IQR)	21.4 (17.7-26.4)	20.6 (17.6-25.2)	0.309	21.6 (17.8-26.5)	20.5 (17.6-25.2)	<0.001
<i>Min-max</i>	11.4-80.1	10.3-100.1		11.4-80.1	10.3-100.1	
Difference between Cmax and Cmin, median (IQR)	15.7 (12.7-21.1)	15.1 (11.2-19.8)	0.139	16.0 (12.9-21.3)	15.1 (11.2-19.8)	<0.001

Cmax, Highest tacrolimus concentration; Cmin, Lowest tacrolimus concentration; IPV, intra-patient variability; IQR, interquartile range; SD, standard deviation; TR, therapeutic range.

*The time post-LTx at which a concentration was measured was determined differently between groups. For the oral group, the start of surgery was taken as the reference time for the time at which blood concentrations were taken. For the intravenous group, reperfusion of the lung allograft was used.

The results from the univariate and multiple linear regression with IPV as the outcome can be consulted in table 3. The data were weighted for number of available tacrolimus samples per patient. In the univariate model, the mean IPV was 2.5% [95% CI 2.0-3.1] higher for the oral group compared to the intravenous group ($p < 0.001$).

After adding the covariates that were unevenly distributed (i.e., primary lung disease and ECLS) between the two groups into the model, only ECLS returned significantly ($p = 0.003$). However, ECLS did not change $B \geq 10\%$. The interaction term was significant for the following variables: sex, primary lung disease, baseline estimated Glomerular Filtration Rate (eGFR) and the combined variable for ECLS and dialysis. These were incorporated in the multiple linear regression model. This led to an adjusted difference in IPV of 7.8% [95% CI 5.3-10.4]; the IPV in the oral group was 7.8% higher than the IPV in the intravenous group. The model was statistically significant ($p < 0.001$).

Table 3: The unadjusted and adjusted results for intra-patient variability (IPV).

		B	95% CI		p-value
			Lower	Upper	
Unadjusted	<i>Intravenous (Ref.)</i>	29.2%	Ref.	Ref.	Ref.
	<i>Oral</i>	31.7%	31.3	32.1	<0.001
	<i>Mean oral IPV compared to mean intravenous IPV</i>	+2.5%	2.0	3.1	<0.001
Adjusted	<i>Intravenous (Ref.)</i>	20.2%	Ref.	Ref.	Ref.
	<i>Oral</i>	28.0%	26.1	30.0	<0.001
	<i>Mean oral IPV compared to mean intravenous IPV</i>	+7.8%	5.3	10.4	<0.001

CI, Confidence Interval; B, linear regression coefficient; ECLS, Extra Corporeal Life Support; Ref., reference.

The results stratified by the need for ECLS and/or dialysis are presented in table 4. For the patients that did not need ECLS and/or dialysis in the first 14 days after LTx, there was no difference in IPV ($p = 0.053$). In the patients that were in need of ECLS and/or dialysis, the IPV in the oral group was 7.3% higher than the IPV in the intravenous group ($p < 0.001$).

Table 4: The results for IPV stratified for the need for ECLS and/or dialysis.

		B	95% CI		p-value
			Lower	Upper	
No ECLS and/or dialysis	<i>Intravenous (Ref.)</i>	31.0%	Ref.	Ref.	Ref.
	<i>Oral</i>	30.3%	29.8	30.9	0.053
	<i>Mean oral IPV compared to mean intravenous IPV</i>	-0.7%	-1.5	0.009	0.053
ECLS and/or dialysis	<i>Intravenous (Ref.)</i>	27.0%	Ref.	Ref.	Ref.
	<i>Oral</i>	34.3%	33.6	35.0	<0.001
	<i>Mean oral IPV compared to mean intravenous IPV</i>	+7.3%	6.4	8.1	<0.001

CI, Confidence Interval; B, linear regression coefficient; ECLS, Extra Corporeal Life Support; Ref., reference.

Table 5 displays the unadjusted and adjusted results for the percentage of time within the therapeutic range. The unadjusted difference in mean TTR was -7.1% [95% CI -7.9 - -6.2]; the mean TTR in the oral

group was 7.1% lower than in the intravenous group. This difference was statistically significant as well ($p < 0.001$).

Subsequently, primary lung disease and ECLS were inserted into the regression model. Both turned out to be significant confounding factors (both $p < 0.001$) but neither of them changed $B \geq 10\%$. The interaction term was significant for age, sex, primary lung disease and baseline eGFR. Hence, these remained in the model. The adjusted difference in TTR was -14.4% [95% CI 6.8-22.1], ($p < 0.001$). Ergo, the time within the therapeutic range was 14.4% higher in the intravenous group compared to the oral group.

Table 5: The unadjusted and adjusted results for percentage of time within the therapeutic range (TTR).

		B	95% CI		p-value
			Lower	Upper	
Unadjusted	<i>Intravenous (Ref.)</i>	30.7%	Ref.	Ref.	Ref.
	<i>Oral</i>	23.7%	23.1	24.3	<0.001
	<i>Mean oral TTR compared to mean intravenous TTR</i>	-7.1%	-7.9	-6.2	<0.001
Adjusted	<i>Intravenous (Ref.)</i>	27.7%	Ref.	Ref.	Ref.
	<i>Oral</i>	13.3%	7.4	19.3	<0.001
	<i>Mean oral TTR compared to mean intravenous TTR</i>	-14.4%	-22.1	-6.8	<0.001

CI, Confidence Interval; B, linear regression coefficient; ECLS, Extra Corporeal Life Support; Ref., reference.

Secondary outcomes

The median length of stay on the ICU did not differ significantly between the oral and the intravenous group, 7 days (4-17) and 6 days (3-18), respectively ($p = 0.641$). In contrast, the patients in the intravenous group had a significantly prolonged hospital admission; a median of 35 (25-54) days compared to 28 (21-44) days in the oral group ($p < 0.001$).

Mortality during admission to the ICU was similar for both groups: 17 patients (7.6%) in the oral group died and 16 (5.4%) in the intravenous group ($p = 0.828$). During hospital admission, 20 patients (8.9%) in the oral group and 25 patients (8.4%) in the intravenous group died ($p = 0.302$).

Sub analysis

Week 1 versus week 2

Appendix 5 shows that the TTR in the intravenous group increased from 12.9% (5.3-23.5) in the first week to 47.5% (26.0-66.5) in the second week ($p < 0.001$). Accordingly, the percentage of time beneath the TR of the intravenous group changed drastically from 53.1% (31.2-75.8) in the first week post-LTx to 1.8% (0.0-30.7) in the second week ($p < 0.001$). In the oral group, the TTR also increased significantly ($p < 0.001$), although less drastically. The TTR in the first week was 18.3% (7.8-32.3) and increased to 22.8% (10.2-38.2) in the second week after LTx. The percentage of time beneath the therapeutic range did not change significantly; from 46.0% (25.0-73.3) in the first week to 48.0% (24.8-70.0) in the second week ($p = 0.080$). For both groups, the IPV is larger in the first week than in the second week ($p < 0.001$).

Sensitivity analyses

Same therapeutic range

As can be seen in appendix 6, the percentage of time beneath the TR decreases for both groups if the same range is applied. This change was more evident for the oral group (50.3% \pm 20.5 in the main

analysis versus $38.5\% \pm 19.3$ in the sensitivity analyses). Accordingly, the TTR increases for both groups as well. We did not observe a difference in percentage of time within the therapeutic range between the oral group ($35.5\% \pm 15.8$) and intravenous group ($35.0\% \pm 17.6$) anymore ($p=0.23$). In the main analysis, the intravenous group reaches the therapeutic range for the first time after a median of 78 hours (59-113), while the oral group reaches it after 73 hours (55-115). However, once the same therapeutic range is applied, the time at which the oral group reaches the range remains the same but the intravenous group now reaches the TR earlier than the oral group, after 69 hours (53-101).

Discussion

Administering tacrolimus orally directly after LTx leads to a higher variability in tacrolimus concentrations than continuous intravenous administration. In the univariate analysis, we found IPV to be statistically significantly higher and the time within the therapeutic range to be significantly lower in the group that was treated with oral tacrolimus in comparison to the continuous intravenous group. After adjustment for effect modifiers, the effect of the administration route on IPV and TTR became larger and the models remained statistically significant. Between the two centers, there was no statistically significant difference in the mortality or the length of ICU stay. The intravenous group remained within the hospital longer than the oral group, but this was not directly explainable. Perhaps the distance to the hospital played an essential role, but this was not further investigated.

The higher variability in the oral group may be primarily caused by a highly variable bioavailability, as was shown by Sikma *et al.* (2020); the relative bioavailability of tacrolimus may vary up to 55% shortly after transplantation. (9) This may be due to gut (dys)motility or an abnormal blood supply to the gastro-intestinal tract or liver in these clinically unstable patients, resulting in unpredictable uptake of tacrolimus. Intravenous administration bypasses the duodenum where tacrolimus is predominantly absorbed. Next to lower IPV, the intravenous group also showed that the time within the therapeutic range was significantly higher than in the oral group. A possible explanation is that the therapeutic range of the intravenous group was $2 \mu\text{g/L}$ wider than that of the oral group in the second week. In contrast, the therapeutic range of the intravenous group is stricter in the first week. The first days are crucial to determine the optimal dose, and the strict therapeutic range may be more difficult to reach in the first week due to instability and dose finding. In summary, our findings are expected to be related to instability in the first 2 weeks after LTx.

This study provides new information on the relationship between the administration route of tacrolimus in the early post-LTx period and the variability in whole blood tacrolimus concentrations. Investigating the variability after oral tacrolimus administration, Ensor *et al.* (2018) observed a median TTR of 20.7% for a therapeutic range between $12\text{-}15 \mu\text{g/L}$ in the first 6 months and $10\text{-}12 \mu\text{g/L}$ up till one year after lung transplantation (13). In our oral population, the TTR was 22.1% when a range of $12\text{-}15 \mu\text{g/L}$ was applied. In another study in lung transplant recipients, a TTR of $46.8\% \pm 17.6$ was observed, using a therapeutic range of $10\text{-}15 \mu\text{g/L}$ for oral administration as well (14). This literature shows that there is no indisputable therapeutic range for tacrolimus after LTx.

The higher IPV in the first week could be explained by the fact that patients are generally less stable, experience more organ dysfunction, are in higher need for extracorporeal support and the ongoing search for the optimal dose. Mentioned factors are less prominent in the second week post-LTx. Contrary to our expectations, the intravenous group needed more time until the therapeutic range was reached, as seen in the main analysis. The explanation may be that the intravenous range is stricter than the oral range in the first week but wider in the second week, which might have affected the time within TR. By comparing the variability while the therapeutic range was identical for both groups, we were able to determine the effects of the different therapeutic ranges on the results regarding

variability (see Appendix 6). Indeed, once the same therapeutic range was applied, we observed that the intravenous group reached the therapeutic range earlier than the oral group. When the same therapeutic range was applied, the TTR increased in both groups, compared to the main analysis. Remarkably, there was no significant difference in the first time at which the therapeutic range was reached between the two groups anymore. However, identical therapeutic ranges for different routes of administration are not completely justifiable either. With continuous intravenous dosing, the therapeutic range should be higher than the therapeutic range for trough concentrations in oral dosing, in order to obtain an exposure similar to that of intermittent dosing. The therapeutic range after lung transplantation is still a matter of debate in the clinical practice.

The fact that two large cohorts were used added to the strength of this study. Moreover, the lung transplantation protocols were similar and tacrolimus concentrations were determined with LC-MS/MS. The latter is in contrast to Gallagher *et al.* (2015) and Kao *et al.* (2021), who analyzed the tacrolimus levels using immunoassay, which is known to have cross-reactivity with tacrolimus metabolites (6,14). This potentially leads to a higher variability and makes interpretation more difficult. Therefore, we excluded the concentrations measured with immunoassay. Whereas we focused on variability between two administration routes, previously mentioned studies have focused on the effects of variability, often defined as CV or SD and thereby ignoring outliers, on clinical outcomes such as rejection, allograft dysfunction and nephrotoxicity, within a cohort with only one administration route (6,14).

For more information on accurately defining peak concentration identification, see Appendix 7.

Despite above mentioned strengths, there are limitations as well.

Initially, information on drug-drug interactions with tacrolimus was supposed to be incorporated in the multiple linear regression model. Unfortunately this information was not available for the intravenous population in time, although it very well may have affected the variability. The same is true for the Sequential Organ Failure Assessment (SOFA) score. This is a disease burden measure and was expected to potentially contribute to the results. Other factors that were expected to contribute, but were not obtained due to the retrospective design, were the dynamical state and intestinal function. Alas, we were not able to investigate their effect on variability. Regardless, we did have information on ECLS and dialysis, which are indirect measures of disease severity. We were able to investigate if these were confounders or effect modifiers and include them in the multiple linear regression model.

Secondly, even though we attempted to exclude tacrolimus peak concentrations, due to the retrospective nature of this study, we cannot be certain that we excluded all misclassified 'supratherapeutic trough' concentrations, which could have led to a variability higher than the variability in reality. In addition, we also cannot be certain that we did not accidentally exclude an actual supratherapeutic trough concentration, which may have led to a lower variability. This was only relevant for the oral group and once patients in the intravenous group had switched to oral administration.

Thirdly, at the time of writing we did not have information regarding the administration of tacrolimus (i.e., dose and time) for the intravenous group. Consequently, we were not able to determine the length of intravenous administration nor exclude peak concentrations once those patients had switched to oral administration. It was also not possible to correct for changing doses before calculating IPV. Doses are often adjusted in the early post-transplantation period and this may explain a part of the observed IPV. With regard to the time of administration, we would have preferred to take the time of first dose as the starting point, to relate the time at which concentrations were measured

to. Consequently, we had to choose a different starting point, which was determined differently for each group. For the oral group, the start of the surgery was taken as the reference time whereas the reperfusion of the lung(s) was used for the intravenous group. This only affected the first time the therapeutic range was reached. If corrected for, the time until the oral therapeutic range is reached would become smaller than reported in table 2.

Regarding the limitations, we are positive that these cannot be the sole explanation for our findings. Therefore, we are confident that the results can be deemed reliable.

The first results suggest that continuous intravenous administration in the early post-LTx period leads to more stability within therapeutic range and seems to be superior to oral administration. However, not all variability can be explained by the route of administration solely. Other potentially contributing factors, such as tacrolimus dose adjustments, should be investigated more closely. For future perspective, the individual tacrolimus concentrations should be corrected for the preceding dose before calculation of IPV. Besides, it is crucial to investigate the clinical effects of the differences in variability. It would be relevant to determine the occurrence of short-term nephrotoxicity and rejection in relation to tacrolimus variability. This would help in the search for the optimal tacrolimus dosing strategy early after lung transplantation.

Conclusion

The variability in tacrolimus concentrations, measured as IPV and TTR, is higher when tacrolimus is administered orally in the first 14 days after LTx in comparison to continuous intravenous infusion, with a switch to oral administration once the patient is more stable.

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Appendices

Appendix 1 Crixus protocol

Variability in whole blood tacrolimus concentrations and its effect on rejection and nephrotoxicity after oral and continuous intravenous administration in lung transplant recipients in the early post-transplantation period: a retrospective multicenter cohort study

Version 2.2 April 2022

Short title: Crixus	
Version: 2.2	
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Summary

Rationale

Tacrolimus is a vital immunosuppressant used after lung transplantation (LTx). Tacrolimus whole blood concentrations vary highly, especially early after transplantation due to affected drug metabolism and high variability in bioavailability in the post-surgical phase. High variability in tacrolimus whole blood concentration increases the risk for allograft rejection and drug toxicity. A common tacrolimus-related adverse event is nephrotoxicity. Tacrolimus is administered orally on the intensive care unit in the University Medical Center Utrecht (UMCU) and administered as continuous intravenous infusion on the intensive care unit in the University Medical Center Groningen (UMCG). Continuous intravenous administration may decrease the variability in whole blood concentrations, and hence the incidence and subsequently the risk of rejection and nephrotoxicity. The central research question is: "What is the difference in variability, with regard to the therapeutic window, in whole blood tacrolimus concentrations after oral and intravenous administration in the first two weeks after lung transplantation?".

Hypothesis

There's a difference in variability and time within therapeutic range of whole blood tacrolimus concentrations between oral administration and continuous intravenous infusion in the first two weeks after lung transplantation.

Objective

Primary objective: To determine the difference in variability in tacrolimus whole blood concentrations, and related to this the time and percentage beneath, in and above the therapeutic range, after oral administration versus continuous intravenous administration in the first two weeks after lung-transplantation, hereafter referred to as 'the early post-transplantation period'.

Secondary objectives:

- To determine the risk of rejection of the lung allograft, after oral administration versus continuous intravenous administration of tacrolimus, in the first three months after lung transplantation.
- To determine the risk of nephrotoxicity after oral administration versus continuous intravenous administration of tacrolimus in lung transplant recipients.
- To determine the post-LTx length of stay at the ICU after oral administration versus continuous intravenous administration of tacrolimus in lung transplant recipients.
- To determine the post-LTx length of stay in the hospital after oral administration versus continuous intravenous administration of tacrolimus in lung transplant recipients.
- To determine the mortality during hospital admission after oral administration versus continuous intravenous administration of tacrolimus.

Study design

This is a retrospective observational cohort study

Inclusion criteria

- Having undergone a lung transplantation between January 2010 and January 2020, performed in either the UMCU or the UMCG.

- Having received tacrolimus orally or as continuous infusion in the first 14 days after lung transplantation.
- Age \geq 18 years

Exclusion criteria

- Less than three whole blood tacrolimus concentrations available within the first two weeks after LTx.
- Objection to use medical data.
- Partial oral and partial intravenous administration of tacrolimus in UMCU patients. Exceptionally, a one-time only intravenous administration of tacrolimus will be accepted because of the possibility that mistakes were made in the validation of these doses, and it is not guaranteed that these administrations were, in fact, intravenous.

Study population

All lung transplant recipients between January 2010 and December 31st 2019 in the UMCU (oral administration) and in the UMCG (continuous intravenous administration).

Main study parameters/endpoints

Primary endpoint:

- Variability in whole blood tacrolimus concentrations
- Time and percentage beneath therapeutic range of tacrolimus
- Time and percentage within therapeutic range of tacrolimus
- Time and percentage above therapeutic range of tacrolimus

Secondary endpoints

- The occurrence of rejection of the lung allograft in the first three months after transplantation
- The occurrence of nephrotoxicity
- The post-LTx Intensive Care Unit (ICU) length of stay
- The post-LTx hospital length of stay
- Mortality during hospital admission

Nature and extent of the burden associated with participation, benefit and group relatedness:

None, as this is a retrospective study.

1. Introduction and rationale

Tacrolimus, a calcineurin inhibitor (CNI), is an essential drug in preventing rejection after lung transplantation (LTx). Because of the small therapeutic range and the high variability in pharmacokinetics, therapeutic drug monitoring is pivotal to dose effectively¹. Low tacrolimus levels increase the risk of rejection and high tacrolimus levels cause toxicity, importantly nephrotoxicity, affecting both the short- and long-term outcome of lung transplant patients^{2,3}.

It is important to obtain tacrolimus trough blood levels within the therapeutic range early after transplantation⁴. The high variability of tacrolimus pharmacokinetics early after lung transplantation is largely due to high variability in bioavailability¹. To decrease this variability, tacrolimus may be administered intravenously. This study aims to investigate the differences in variability of whole blood tacrolimus concentrations between oral and continuous intravenous administration, also with respect to the therapeutic range, in order to investigate the preferred method of administration in the early phase after lung transplantation.

2. Objectives

Primary Objective

To determine the difference in variability in tacrolimus whole blood concentrations, and related to this the time and percentage beneath, in and above the therapeutic range, after oral administration versus continuous intravenous administration in the early post-transplantation period.

Secondary Objective(s)

- To determine the difference in risk of rejection of the lung allograft, after oral administration versus continuous intravenous administration of tacrolimus, in the first three months after transplantation.
- To determine the difference in risk of nephrotoxicity after oral administration versus continuous intravenous administration of tacrolimus in lung transplant recipients.
- To determine the difference in post-LTx length of stay at the Intensive Care Unit (ICU) after oral administration versus continuous intravenous administration of tacrolimus in lung transplant recipients.
- To determine the difference in post-LTx length of stay in the hospital after oral administration versus continuous intravenous administration of tacrolimus in lung transplant recipients.
- To determine mortality during hospital admission after oral administration versus continuous intravenous administration of tacrolimus.

3. Study design

This is a retrospective, multi-center cohort study, including all lung transplantation recipients transplanted in the University Medical Center Utrecht (UMCU) and University Medical Center Groningen (UMCG) from January 2010 up to December 31st 2020, who have been administered tacrolimus orally (UMCU) or intravenously (UMCG). Apart from the route of administration of tacrolimus in the early post-transplantation period, the treatment protocols of both hospitals are comparable, with only few differences that are deemed negligible for the outcomes. For the oral and intravenous group, whole blood tacrolimus concentrations from the first 14 days after lung transplantation (the early post-transplantation period) will be used to determine variability, regardless of the timing of the switch from intravenous to oral administration. This period of 14 days was chosen because in most cases, patients from the UMCG will switch from intravenous to oral administration

within this time. That makes the two groups incomparable after 14 days. In addition, the purpose is to specifically investigate the effect of the early-postoperative period on the fluctuations in tacrolimus concentration. Looking at the concentrations after 14 days would not give adequate results for this purpose.

For the oral group, the tacrolimus trough blood concentrations (samples taken within two hours of the next oral administration) will be used to determine the variability. It is aimed to exclude tacrolimus peak whole blood concentrations because these will lead to a distortion of the results. Whether a concentration is a peak or trough will be judged manually as follows: if a tacrolimus concentration increases by $\geq 7 \mu\text{g/L}$ compared to the previous concentration, and the two concentrations before and after this elevated concentration have been roughly steady (within a range of $2 \mu\text{g/L}$), we will be triggered to delve into the patient's file in order to deduce whether it concerns a peak or a trough concentration. The factors that are taken into account are changes in tacrolimus dose, the start of an agent that increases the tacrolimus concentration, the occurrence of diarrhea or packed cells administration. Caution must be taken during the exclusion of potential peak concentrations. The unjustified exclusion of concentrations will have detrimental effects on the outcomes. As a result, each triggered concentration should be reviewed carefully by at least two researchers. For the continuous intravenous group, the concentrations are rather constant, if no dose adjustment has taken place. These samples were also taken daily, around the same time. At some point in time, the route of administration for these patients is changed from intravenous to oral. From that moment on, their concentrations have to be judged in the same manner to exclude peak concentrations.

Nephrotoxicity may occur as an adverse event of tacrolimus use.⁵ There are two types of CNI-induced nephrotoxicity described in literature. Firstly, acute CNI nephrotoxicity may be observed due to functional (i.e., vascular) changes in the kidneys and the accompanying decreased Glomerular Renal Function (GFR) has shown to recover in time. Secondly, chronic CNI nephrotoxicity (i.e., tubular and glomerular injury, followed up by fibrosis of the kidney) may be a result of long-term CNI administration when irreversible deterioration in renal function is seen. In clinical practice, renal function is determined through the estimated Glomerular Filtration Rate (eGFR). For the calculation of eGFR, two equations are generally in use: the Modification of Diet in Renal Disease (MDRD) equation and the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation. The latter equation also incorporates information on ethnicity, which is generally not recorded in the patient's medical file. Hence, ethnicity is omitted from the equation. In lung transplant patients, the CKD-EPIcreatinine (CKD-EPI_{Cr}) equation has shown to perform better than the MDRD equation. As a result, the UMCU has switched from the MDRD to the CKD-EPI equation on October 7th 2013. For patients transplanted before this date, the renal function has to be recalculated using the CKD-EPI equation. The UMCU has switched from the MDRD equation to the CKD-EPI equation on January 16th 2015. For patients transplanted before this date, the renal function has to be recalculated using the CKD-EPI equation. Frankly, the CKD-EPI equation which also incorporates cystatin C, along with creatinine, has proven to be the best estimator of renal function in critically ill patients⁶. Unfortunately, cystatin C was not routinely monitored in either center throughout the study period.

In addition, acute CNI nephrotoxicity, defined as Acute Kidney Injury (AKI) is a predictor for a faster decreasing GFR in the first year after transplantation, which may be a measure for the development of Chronic Kidney Disease (CKD)⁷. CKD is diagnosed as such when a patient has been clinically stable for at least 3 months and an eGFR $< 60 \text{ mL/min/1.73m}^2$ is seen for > 3 months⁸. For this study, it is not desirable to look at the eGFR > 3 months after lung transplantation because a decreased eGFR after 3 months can also be attributed to other factors such as cumulative tacrolimus exposure or other nephrotoxic drugs. In summary, the renal function at 3 months after LTx will be taken along in the analysis because this is likely to be correlated to worse long-term outcomes.

The follow-up period will end 3 months after lung transplantation, because after these 3 months the occurring outcomes (nephrotoxicity for instance) may also be the result of other factors, such as the cumulative tacrolimus dose.

4. Study population

4.1 Population (base)

The source population consists of lung transplant recipients in the UMCU and UMCG. Because this is a retrospective study, the 'recruitment' of participants has already taken place. Therefore, all recipients eligible according to the in- and exclusion criteria define the total study population.

The protocol after lung transplantation is comparable between the UMCU and the UMCG, as can be seen in detail in section 5.2. The main difference between the two protocols is the route of administration of tacrolimus.

4.1.1 Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

- Having undergone a lung transplantation between January 2010 and December 31st 2020
- Having received tacrolimus orally or as continuous infusion in the first 14 days after lung transplantation
- Age \geq 18 years

4.1.2 Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- Less than three whole blood tacrolimus concentrations available within the first 14 days after LTx. Other studies in which variability was calculated have set a limit to minimally three available concentrations⁹. If no more than two concentrations are available, the variability cannot be calculated accurately.
- Objection to use medical data
- Partial oral and partial intravenous administration of tacrolimus in UMCU patients. Exceptionally, a one-time only intravenous administration of tacrolimus will be accepted because of the possibility that mistakes were made in the validation of these doses, and it is not guaranteed that these administrations were, in fact, intravenous.

4.2 Sample size calculation

Each year approximately 30 and 25 lung transplantations are performed in the UMCG and the UMCU, respectively. Therefore, in our study period of 10 years, approximately 600 patients should be available for inclusion.

5. Methods

5.1 Study parameters/endpoints

5.1.1 Main study parameter/endpoint

Primary endpoint:

- Variability in whole blood tacrolimus concentrations
- Time and percentage that the tacrolimus concentration is beneath the therapeutic range
- Time and percentage that the tacrolimus concentration is within the therapeutic range
- Time and percentage that the tacrolimus concentration is above the therapeutic range

5.1.2 Secondary study parameters/endpoints (if applicable)

1. The occurrence of rejection of the lung allograft within first three months after transplantation.
2. The occurrence of nephrotoxicity

3. The post-LTx ICU length of stay
4. The post-LTx hospital length of stay
5. Mortality during hospital admission

5.1.3 Definitions of outcomes

- Early post-transplantation period = first two weeks after lung transplantation.
- Tacrolimus trough concentration = the blood sample that was taken within two hours before the next oral tacrolimus administration. Whether or not a concentration is a trough concentration (for the oral group) will be determined using the time of tacrolimus administrations and the time that the blood sample was taken. Therefore, for each dose and each concentration, the time of administration or sampling needs to be available as well. In both the UMCU and UMCG, the tacrolimus concentration is determined as a whole blood concentration and not as an unbound concentration. Even though the latter would be more desirable, because it is better related to tacrolimus efficacy and toxicity, the unbound concentration is difficult to determine properly. The bound and unbound concentrations may vary easily within the tube because of external factors, such as movement.
- Variability^{9,10,11} = Intra-patient variability (IPV):

$$IPV\% = \frac{1}{T} \sum_{t=1}^T \frac{\text{abs}(X_t - \bar{X})}{\bar{X}} \times 100,$$

For this equation, the values have the following meanings:

X is the mean C₀/D of all available samples in the first 14 days after lung transplantation;

X_t is an individual value of C₀/D measured in the period mentioned;

and T is the number of all available values for an individual patient. Variability will be determined using daily tacrolimus trough concentrations from the first 14 days after lung transplantation.

- Oral therapeutic range = tacrolimus trough concentrations ranging from 12-15 µg/L in week 1 and 2 post-LTx, as seen in the clinical practice.
- Intravenous therapeutic range = tacrolimus concentrations ranging from 13-15 µg/L during the first week post-LTx, and ranging from 10-15 µg/L from in week 2 post-LTx¹².
- Time beneath therapeutic range = a sum of the amount of time the tacrolimus concentrations of a patient are beneath the therapeutic range, counting from the first available concentration. A line will be drawn between two concentrations. For example, one might be beneath the therapeutic range and the other might be in the therapeutic range. The time beneath the therapeutic range, in this example, is regarded as the time until the line crosses the lower limit of the therapeutic range¹³. For this calculation, the trough concentrations that are taken once daily will still be used. So, for a patient in the oral study group, the real time beneath/in/above the therapeutic range might be different. The aim of this study is not to look at the pharmacokinetics of tacrolimus. We will investigate the variability (in tacrolimus trough concentrations) with regard to the therapeutic range.

The lower limit of the therapeutic range differs between week 1 and 2 after transplantation for the intravenous study group. Therefore, the time beneath the therapeutic range will be calculated separately for week 1 and 2 and this will be added up.

- Percentage beneath therapeutic range = the total amount of time the tacrolimus concentrations of a patient are beneath the therapeutic range, compared to the total amount of time in the first two weeks after LTx (14 days*24 hours = 336 hours) and expressed as a percentage.
- Time within therapeutic range = a sum of the amount of time the tacrolimus concentrations are within the therapeutic range, counting from the first available concentration. A line will be drawn between all the available concentrations. For example, one might be in the therapeutic range and the other might be beneath the therapeutic range. The time in the therapeutic range, in this example, is regarded as the time until the line crosses the lower limit of the therapeutic range¹³. The lower limit of the therapeutic range differs between week 1 and 2

after transplantation for the intravenous study group. Therefore, the time in the therapeutic range will be calculated separately for week 1 and 2 and this will be added up.

- Percentage within therapeutic range = the total amount of time the tacrolimus concentrations of a patient are in the therapeutic range, compared to the total amount of time in the first two weeks after LTx (14 days*24 hours = 336 hours) and expressed as a percentage.
- Time above therapeutic range = a sum of the amount of time the tacrolimus concentrations of a patient are above the therapeutic range, counting from the first available concentration. A line will be drawn between two concentrations. For example, one might be above the therapeutic range and the other might be in the therapeutic range. The time above the therapeutic range, in this example, is regarded as the time until the line crosses the upper limit of the therapeutic range¹³. The upper limit of the therapeutic range does not differ between week 1 and 2, nor between the oral and intravenous group. Therefore, the time above the therapeutic range will be calculated collectively for week 1 and 2.
- Percentage above therapeutic range = the total amount of time the tacrolimus concentrations of a patient are above the therapeutic range, compared to the total amount of time in the first two weeks after LTx (14 days*24 hours = 336 hours) and expressed as a percentage.
- Efficacy = whether or not rejection has occurred (surrogate endpoint).
- Rejection = surrogate endpoint for efficacy of tacrolimus. This is diagnosed based on clinical grounds and the judgement of clinically experienced lung transplant doctors and will be defined as a prescription of methylprednisolone (1000 mg during three days) as this is the treatment specific to rejection.
- Nephrotoxicity = the occurrence of acute kidney injury, acute kidney disease (AKD) or the need for Continuous Renal Replacement Therapy (CRRT) or haemodialysis at three months after LTx. If either one of these has occurred in a patient, it is regarded that the outcome 'nephrotoxicity' has occurred. Subsequently, the kind of nephrotoxicity, the stages of severity (see table 1) and whether or not recovery of the renal function has occurred, will be documented.
- Acute kidney injury (AKI) = clinically decreased renal function (defined as eGFR), calculated using the CKD-EPIcr equation. This equation also takes ethnicity into account. As we do not have information on this, this factor (*1.159 for black patients) is replaced by 1. AKI is defined, according to international standards (KDIGO criteria), as an increase in serum creatinine (SCr) $\geq 50\%$ within 7 days, compared to baseline, or an increase in serum creatinine by $\geq 26.5 \mu\text{mol/L}$ within 48 hours, compared to baseline^{8,14}.
- Acute Kidney Disease (AKD) = if the GFR $< 60 \text{ mL/min/1.73m}^2$ or GFR decrease of $\geq 35\%$ compared to baseline or an increase of serum creatinine $> 50\%$ compared to baseline^{8,14}. Whether AKD has occurred or not, will be determined at 2 weeks, 1 month and 3 months after LTx.
- Post-LTx length of stay at ICU = from the date of LTx-admission to the ICU until the date of discharge from the ICU.
- Post-LTx length of hospital stay = from the date of LTx-admission to the hospital until the date of discharge from the hospital.
- Mortality during hospital admission = death in the critical period after LTx, during admission to the hospital.

Table 1: Definition of the different AKI stages.

Stage	AKI ¹⁴
0	No change or SCr increase <26.5 µmol/L and no urinary output criteria
1	Increase of SCr by ≥ 26.5 µmol/L for ≤48 hours or ≥ 150% for ≤ 7 days and/or urinary output <0.5ml/kg/h for >6 hours
2	Increase of SCr by > 200% and/or urinary output < 0.5 ml/kg/h for 12 hours
3	Increase of SCr by > 300% (≥ 353.6 µmol/L with an acute increase of ≥ 44.2 µmol/L) and/or urinary output <0.3 ml/kg/h for >24 hours
4	Continuous VenoVenous Hemofiltration (CVVH)

5.1.4 Other study parameters (if applicable)

The following section gives more information as to how certain variables are defined. See table 3 in section 6.1 for an extensive list of all necessary study parameters.

5.1.5 Definition of other variables

- Chronic cardiac insufficiency = general term for the following diagnoses: heart failure, cardiac insufficiency, left ventricle dysfunction, right ventricle dysfunction, reduced ejection fraction.
- SOFA score = sequential organ failure assessment score, indicates how many organs are affected due to illness and indirectly determines the severity of illness. See table 2 for the definition of the scores. For each organ, a score of 0-4 can be attributed and the sum of the 6 systems in table 2 will make up the total SOFA score.

Table 2: Definition of the SOFA scores ¹⁵

SOFA score	0	1	2	3	4
Respiration					
PaO ₂ /FIO ₂ (mmHg) (kPa)	> 400 > 5.3)	301–400 (4.1–5.3)	201–300 (2.8–4.0)	101–200 (1.4–2.7)	≤ 100 ≤ 1.3)
Coagulation					
Platelets (x10 ³ /mm ³)	> 150	101–150	51–100	21–50	≤ 20
Liver					
Bilirubin (mg/dl) (µmol/l)	< 1.2 < 20)	1.2–1.9 (20–32)	2.0–5.9 (33–101)	6.0–11.9 (102–204)	≥ 12.0 ≥ 204)
Cardiovascular					
Hypotension	No hypotension	MAP < 70 mmHg	Dopamine ≤ 5 or dobutamine (any dose)*	Dopamine > 5	Dopamine > 15
Central nervous system					
Glasgow coma score	15	13–14	10–12	6–9	< 6
Renal					
Creatinine (mg/dl) (µmol/l) or urine output	< 1.2 < 110)	1.2–1.9 (110–170)	2.0–3.4 (171–299)	3.5–4.9 (300–440) < 500 ml/day	> 5.0 > 440) < 200 ml/day

* adrenergic agents administered for at least 1 h (doses given are in µg/kg/min)

- Use of pharmaceutical agents that decrease the tacrolimus concentration: CYP3A4-inducers (i.e., phenytoin, rifampicin, carbamazepine and phenobarbital¹⁶), Pgp-inducers (i.e. rifampicin), caspofungin, flucloxacillin and sevelamer¹⁷. Use of CYP3A4-inducers in the two weeks before and after lung transplantation is of relevance. The use of the other agents, that decrease tacrolimus concentrations, in the first two weeks after transplantation is of relevance.

- Use of pharmaceutical agents that increase the tacrolimus concentration: CYP3A4-inhibitors (i.e., clarithromycin, erythromycin, itraconazole, ketoconazole, posaconazole, voriconazole¹⁶, isavuconazole, fluconazole and theophylline), Pgp-inhibitors (i.e., amiodarone, azithromycin, diltiazem, ticagrelor, verapamil and nifedipine)^{17,18}. These agents could possibly be prescribed during admission to the ICU, and because of the half-life, the pre-LTx use of these agents is also of relevance. Potentially nephrotoxic agents: amikacin, gentamicin, tobramycin, amphotericin B, co-trimoxazole > 960 mg/day, vancomycin, Angiotensin Converting Enzyme (ACE) inhibitors, angiotensin receptor blockers (ARB).
Even though (val)ganciclovir and (val)acyclovir are potentially nephrotoxic as well, they are standard of post-LTx care for both the UMCU and UMCG and therefore will not be taken along in the determination of the amount of potentially nephrotoxic agents in use.
- Reason for LTx = defined according to the official and international ET classification. A = obstructive airway disease; B = disease of pulmonary circulation; C = suppurative lung diseases; D = restrictive lung diseases; E = retransplant.
- Dialysis = Continuous VenoVenous Hemofiltration (CVVH) or Intermittent HemoDialysis (IHD)
- ECMO = Extracorporeal Membrane Oxygenation, which is further specified as either Veno-Arterial (VA) or VenoVenous (VV).

5.2 Study procedures

As this study is a retrospective study, there will be no active procedures or tests to obtain information on the study parameters. The Electronic Patient Files, that are documented in HiX and in MetaVision for the UMCU and Epic for the UMCG, will be consulted and the defined study parameters will be extracted from these files. On top of that, some information on the UMCU-patients will be gathered from a list of LTx-patients that was drawn up by the pulmonology department of the UMCU. These data were collected manually from HiX.

Subjects will not be asked to actively undergo procedures. There will be no additional medical interventions for the purpose of this study, and as a result the physical and psychological integrity of the subjects will not be violated. Thus, this study is not subject to the WMO legislation.

Hereby, we would like to give an overview on the types of data that have been collected before, during and after transplantation.

Before transplantation, there was a screening. Amongst others, baseline serum creatinine (and the times of measurement) and whether dialysis was in use at this time were determined during the screening. The serum creatinine and body weight closest to, but measured before, LTx will be needed. Sex was known from the moment the patient was registered at the hospital. Pre-existent comorbidities, i.e., diabetes mellitus, hypertension, heart failure and renal insufficiency, were also established at the screening, as were use of ACEi or ARB, use of agents that decrease the tacrolimus concentration, reason for transplantation and type of transplantation. In addition, smoking status in the medical history was determined (indirectly through previous cotinine serum levels in the patients' history).

Data that were documented at the time of transplantation were age, year of LTx and the hospital at which the transplantation took place.

The information that was gathered within the first 14 days after transplantation was: determination of the highest SOFA score, the use of potentially nephrotoxic agents, use of agents that decrease or increase the tacrolimus concentration, post-transplantation Extra Corporal Membrane Oxygenation (ECMO) support, daily haematocrit values, route of administration of tacrolimus and length of intravenous tacrolimus administration. Moreover, every individual dose of tacrolimus (+ time after LTx in hours) and daily whole blood tacrolimus concentrations (+ time after LTx in hours) were gathered in

these two weeks. From the latter, it will be determined which concentration was the highest and which was the lowest. In addition, the time that tacrolimus concentrations were beneath, in or above the therapeutic range will be calculated with this information. Daily serum creatinine (+ time after LTx in hours) and the need for dialysis (CVVH or IHD) for each serum creatinine value were documented. Using this, eGFR will be calculated. Next to this, urinary output was measured daily, in case the catheter was still in place, within the 2 weeks after LTx. As a result, the occurrence of AKI (within 7 days after LTx) and the occurrence of AKD (at week 2 after LTx), were determined. Whether rejection of the lung allograft occurred or not, was also established in this period, through prescription of methylprednisolone 1000 mg for three days.

At one month after transplantation, the following information was collected: serum creatinine and eGFR, and consequently the occurrence of AKD. There was a margin for the assessment of serum creatinine: the measurement closest to the 'one month after transplantation'-point, but within a range of one week before or after 'one month after transplantation' will be used,. The need for dialysis was also documented at one month after transplantation. Whether rejection of the lung allograft occurred or not was also established in this period.

At three months after transplantation, information was gathered on: serum creatinine and eGFR, and as a result the (predicted) occurrence of CKD. There was a margin of 10 days for the assessment of serum creatinine and eGFR around the 'Three months after transplantation'-point. The need for dialysis and the length of CVVH or IHD, the length of mechanical ventilation and the rejection of the lung allograft were also documented at this point. The post-LTx length of ICU stay, post-LTx length of hospital stay, the reason for ICU-admission and the number of transplantation-related re-admissions to the ICU was determined throughout the whole follow-up period. Finally, mortality was recorded; whether it occurred at the ICU or at any moment during hospital admission, was recorded for ICU and hospital admission separately.

The tacrolimus whole blood concentrations for the oral group were determined as followed: Tacrolimus was administered twice daily. The blood sample to determine a whole blood tacrolimus (trough) concentration was taken maximally 2 hours before the next dose. This means that the sample was taken approximately 10-12 hours after the previous oral dose. For the intravenous group the blood samples were taken once a day, each day at the same time.

5.2.1 Standard of care after lung transplantation

The standard medical treatment after lung transplantation (regarded as day 0), to prevent rejection, is comparable in both medical centers (UMCU¹⁶ and UMCG¹²) and consists of the following:

1. Tacrolimus
 - UMCU: oral administration. The administration is started on the day after LTx (day 1). The start dose is: 0.07 mg/kg twice daily through a gastro-intestinal tube or orally. Dose adjustments are done based on whole blood trough levels.
 - UMCG: intravenous administration. 0.01 mg/kg/24 hours, continuous infusion. When motility of the gut has returned, the mode of administration is switched to oral dosing (0.1 mg/kg/day, divided over two administrations)
2. Mycophenolate-mofetil
 - UMCU: day 1-3: twice daily 1,5 grams orally. Day 4-week 4: twice daily 1 gram orally.
 - UMCG: 1000 mg orally before transplantation. Then postoperatively 2 times daily 1000 mg intravenously or orally, start < 8 hours after transplantation.
3. Basiliximab day 0 and 4 20 grams intravenously. This is true for both centers.
4. Prednisolone
 - UMCU: The UMCU administers (methyl)prednisone: 500 mg intravenously before reperfusion of each transplanted lung and 100 mg intravenously 6 hours after reperfusion. Day 0-3: 4 times

daily 25 mg intravenously. This is followed by 30 mg orally, once daily, for 4 days. After this, a phasing out scheme is followed: 25 mg per day for a week and subsequently 20 mg per day for a week.

- UMCG: The UMCG administers (methyl)prednisone: 500 mg intravenously before reperfusion of each transplanted lung. Then 3 times daily 125 mg intravenously in the first 24 hours postoperatively. This is followed by prednisolone once daily 0.5 mg/kg intravenously or orally during day 2-7 after lung transplantation. Subsequently, once daily 0.25 mg/kg orally until month 6 after lung transplantation.

The remaining standard medical treatment consists of antibiotics, anticoagulants and analgesics:

5. Ganciclovir, acyclovir or valaciclovir
 - UMCU: if CMV +/-, acyclovir 5 mg/kg per 12 hours, intravenously for 5 days, then switch to orally. In all other cases, ganciclovir twice daily 5 mg/kg intravenously for 3-5 days, then switch to orally.
 - UMCG: if CMV +/-, valaciclovir twice daily 500 mg orally, starting 48 hours after LTx. In all other cases, start ganciclovir once daily 5 mg/kg intravenously 24 hours after LTx.
6. Co-trimoxazole:
 - UMCU: 480 mg 3 times per week, starting 48 hours after LTx.
 - UMCG: 960 mg every other day, starting 48 hours after LTx.
7. Amphotericin B: spray 4 times daily 5 mg, from admission to the ICU until extubation. Followed by amphotericin oral suspension during first 3 months after LTx.
8. Antibiotics. Patients are administered SDD 4 times daily in both centers. Therefore, there will not be a difference with regards to SDD between the oral and intravenous group, and adding this as a study parameter is not necessary.
9. Pantoprazole
10. Prophylaxis with anticoagulants if necessary
11. Analgesics

5.3 Withdrawal of individual subjects

Because of the retrospective study design, all data have previously been gathered in the clinical setting. Patients hospitalised in the UMCG have signed an informed consent form (see section 7.2) for their data being used in scientific research. Due to the large study population (> 500 participants), the limited time available for this study and the effort it would take to obtain informed consent of each patient in hindsight, the exception for informed consent is applicable to this study, and patients are not asked to sign an informed consent form in retrospect. Active withdrawal of patients is not applicable.

5.3.1 Specific criteria for withdrawal (if applicable)

N/A due to the retrospective study design.

5.3.2 Replacement of individual subjects after withdrawal

N/A

5.3.3 Follow-up of subjects withdrawn from treatment

N/A

6. Statistical analysis

6.1 Statistical information study parameters

Table 3 shows the statistical information of all study parameters.

Table 3: Statistical information on study parameters.

Parameter	Categorical or continuous	Quantitative or qualitative	Calculation of parameter
Pseudonymised patient ID	Continuous	Qualitative	n/a
Hospital	Categorical: UMCU or UMCG	Qualitative	n/a
Age at time of LTx	Continuous	Quantitative	(Date of transplantation) – (date of birth)
Sex	Categorical: Female or male	Qualitative	n/a
Body weight at time of LTx	Continuous	Quantitative	n/a
Pre-LTx Diabetes Mellitus (diagnosis of DM in the medical history)	Categorical: Yes or no	Qualitative	n/a
Pre-LTx hypertension (diagnosis of hypertension in the medical history)	Categorical: Yes or no	Qualitative	n/a
Baseline Serum Creatinine	Continuous	Quantitative	mg/dL --> $\mu\text{mol/L}$: *88.4 $\mu\text{mol/L}$ --> mg/dL: *0.0113
Time of Baseline Serum Creatinine	Continuous	Qualitative	(Date&time of latest serum creatinine before LTx) – (Date&time of LTx) = - x hours
Baseline eGFR ¹⁹	Continuous	Quantitative	$e\text{GFR} = 141 * \min(\text{SCr}/k)^\alpha * \max(\text{SCr}/k, 1)^{-1.209} * 0.993^{\text{age}} * 1.018$ [if female] [†]
Pre-existent renal insufficiency	Categorical: Yes or no	Qualitative	Determined based on baseline eGFR. eGFR < 60 mL/min/1.73m ² → pre-existent renal insufficiency
Smoking status in medical history	Categorical: Former smoker Non-smoker	Qualitative	n/a
Use of potentially nephrotoxic agents in first 14 days after LTx	Continuous	Quantitative	Sum of nephrotoxic agents in use
Use of agents that increase the tacrolimus concentration in first 14 days after LTx	Categorical: Yes or no	Qualitative	n/a

Use of agents that decrease the tacrolimus concentration in first 14 days after LTx	Categorical: Yes or no	Qualitative	n/a
Reason of admission to the ICU in the 3 months after LTx	Categorical	Qualitative	n/a
Number of LTx-related re-admissions to ICU in 3 months after LTx	Continuous	Quantitative	If Reason of admission is Re-LTx or transplantation-related → regarded as transplantation-related re-admission to ICU
ECMO support in first two weeks after LTx	Categorical: VV, VA or no	Qualitative	n/a
Highest SOFA score in first 14 days after LTx	Categorical: 5 6 7 8 ≥9	Qualitative	n/a
Daily haematocrit values within the first 14 days after LTx	Continuous	Quantitative	n/a
Length of CVVH within first 3 months after LTx	Continuous	Quantitative	(Last day of CVVH) – (first day of CVVH)
Length of IHD within first 3 months after LTx	Continuous	Quantitative	(Last day of IHD) – (first day of IHD)
Length of mechanical ventilation in the first 3 months after LTx	Continuous	Quantitative	(Last day of mechanical ventilation) – (first day of mechanical ventilation)
Year of LTx	Continuous	Qualitative	n/a
Reason for LTx	Categorical: A = obstructive airway disease B = disease of pulmonary circulation C = suppurative lung diseases D = restrictive lung diseases E = retransplant	Qualitative	n/a
Type of LTx	Categorical: Single	Qualitative	n/a

	Double Heart-lung transplantation Re-LTx		
Minutes post-LTx start of tacrolimus	Continuous	Qualitative	(Date&time start tacrolimus) – (date&time LTx)
Tacrolimus route of administration at start	Categorical: Oral Intravenous	Qualitative	n/a
Length (days) of intravenous administration of tacrolimus in first 14 days after LTx	Continuous	Quantitative	(Date&time after LTx at which switch from IV to oral was made) - (date&time start tacrolimus administration)
Every individual dose (twice daily for oral) of tacrolimus in week 1 and 2 after LTx + hours after LTx per administration	Continuous + continuous	Quantitative + quantitative	n/a (Date&time of tacrolimus dose x) – (date&time of LTx)
Daily tacrolimus whole blood concentrations in first 14 days after LTx + hours after LTx per concentration	Continuous	Quantitative	(Date&Time that blood sample x was taken) – (date&time of LTx)
Tacrolimus trough concentration for each tacrolimus concentration	Categorical: Yes or no	Qualitative	(Time of tacrolimus concentration x) – (time of tacrolimus dose x) If outcome ≥ 10 hours --> trough concentration
Highest tacrolimus concentration in the first 14 days after LTx	Continuous	Quantitative	n/a
Lowest tacrolimus concentration in the first 14 days after LTx	Continuous	Quantitative	n/a
Difference between highest and lowest tacrolimus concentration in the first 14 days after LTx	Continuous	Qualitative	(Highest tacrolimus concentration – lowest tacrolimus concentration)
Intra-patient variability (IPV) of tacrolimus in first two weeks after LTx	Continuous	Quantitative	$IPV\% = \frac{1}{T} \sum_{t=1}^T \frac{abs(X_t - \bar{X})}{\bar{X}} \times 100,$

Time beneath therapeutic range of tacrolimus	Continuous	Quantitative	Sum of different time intervals spent beneath the therapeutic range: < 12 µg/L for oral group in week 1 and 2 < 13 µg/L for IV group in week 1 < 10 µg/L for IV group in week 2
Percentage of time beneath therapeutic range of tacrolimus	Continuous	Quantitative	(Time beneath therapeutic range)/(336 hours)*100%
Time within therapeutic range of tacrolimus	Continuous	Quantitative	Sum of different time intervals spent in the therapeutic range: 12-15 µg/L for oral group in week 1 and 2 13-15 µg/L for IV group in week 1 10-15 µg/L for IV group in week 2
Percentage of time within therapeutic range of tacrolimus	Continuous	Quantitative	(Time within therapeutic range)/(336 hours)*100%
Time above therapeutic range of tacrolimus	Continuous	Quantitative	Sum of different time intervals spent above the therapeutic range: > 15 µg/L for oral group and IV group in week 1 and 2
Percentage of time above therapeutic range of tacrolimus	Continuous	Quantitative	(Time above therapeutic range)/(336 hours)*100%
Highest tacrolimus concentration within first 14 days after LTx	Continuous	Quantitative	n/a
Lowest tacrolimus concentration within first 14 days after LTx	Continuous	Quantitative	n/a
Difference between highest and lowest concentration	Continuous	Quantitative	(Highest concentration in first 14 days after LTx) – (Lowest concentration in first 14 days after LTx)
Prescription of methylprednisolone 1000 mg for three days in first three months after LTx	Categorical: Yes or no	Qualitative	n/a
Rejection in first three months after LTx	Categorical: Yes or no	Qualitative	Determine whether rejection has occurred based on prescription of methylprednisolone 1000 mg for three days
Daily serum creatinine in first 14 days after LTx and at 1 and 3 months	Continuous	Quantitative	n/a

Time (in hours for first two weeks, in days for month 1 and month 3) after LTx of daily serum creatinine measurements	Continuous	Qualitative	(Date&time of SCr measurement) – (Date&time of LTx)
Dialysis for every SCr measurement in first 14 days after LTx and at 1 and 3 months	Categorical: No CVVH IHD	Qualitative	n/a
Daily urine output in first 14 days after LTx	Continuous	Quantitative	n/a
Every eGFR ¹⁹ in first 14 days after LTx and at 1 and 3 months	Continuous	Quantitative	$eGFR = 141 * \min(Scr/k)^\alpha * \max(Scr/k, 1)^{-1.209} * 0.993^{age} * 1.018$ [if female] [†]
AKI in first 7 days after LTx	Categorical: Yes or no	Qualitative	((Highest SCr _{0-7days})- (SCr _{baseline}))/(SCr _{baseline})*100% ≥ 50% OR SCr _{0-48hours} – SCr _{baseline} ≥ 26.5 μmol/L OR Oliguria for ≥ 6 hours
AKI stages	Categorical: Stage 1 Stage 2 Stage 3 Stage 4	Qualitative	See table 1
AKD at 2 weeks after LTx	Categorical: Yes or no	Qualitative	GFR < 60 mL/min/1.73m ² at 2 weeks after LTx OR ((GFR _{t=2weeks})- (GFR _{baseline}))/(GFR _{baseline})*100% ≥ -35% OR ((SCr _{t=2weeks})- (SCr _{baseline}))/(SCr _{baseline})*100% ≥ 50%
AKD at 1 month after LTx	Categorical: Yes or no	Qualitative	GFR < 60 mL/min/1.73m ² at 1 month after LTx OR ((GFR _{t=1month})- (GFR _{baseline}))/(GFR _{baseline})*100% ≥ -35% OR ((SCr _{t=1month})- (SCr _{baseline}))/(SCr _{baseline})*100% ≥ 50%
AKD at 3 months	Categorical: Yes or no	Qualitative	GFR < 60 mL/min/1.73m ² at 3 months after LTx OR

			$\left(\frac{\text{GFR}_{t=3\text{months}} - \text{GFR}_{\text{baseline}}}{\text{GFR}_{\text{baseline}}}\right) * 100\% \geq -35\%$ OR $\left(\frac{\text{SCr}_{t=3\text{months}} - \text{SCr}_{\text{baseline}}}{\text{SCr}_{\text{baseline}}}\right) * 100\% \geq 50\%$
Post-LTx length of hospital stay	Continuous	Quantitative	(Date of discharge from hospital) – (date of admission to hospital)
Post-LTx length of stay on ICU	Continuous	Quantitative	(Date of discharge from ICU) – (date of admission to ICU)
Mortality during ICU admission	Categorical: Yes or no	Qualitative	n/a
Mortality during hospital admission	Categorical: Yes or no	Qualitative	n/a
† SCr = serum creatinine in $\mu\text{mol/L}$. $\kappa = 61.9$ for females, 79.6 for males $\alpha = -0.329$ for females, -0.411 for males min = minimal of SCr/κ or 1 max = maximum of SCr/κ or 1			

6.2 Statistical analysis

Statistical analysis will be performed using SPSS. Results are considered significant if p is below 0.05 for two-tailed tests.

6.2.1 Descriptive statistics

Medians and means (including interquartile ranges (IQRs) and standard deviations (SDs) will be used to display the patient characteristics of the study population. Incidences are presented for the occurrence of rejection and nephrotoxicity (AKI, AKD, CKD) in the oral and intravenous population. Percentages will be used to express variability and the time within and outside of the therapeutic range. The table with baseline patient characteristics will also give an overview on the proportions of patients for certain covariates.

6.2.2 Potential confounders and effect modifiers

Univariate analysis will be performed to identify potential confounders. First of all, the association between covariates and the exposure (oral or intravenous administration in the early post-transplantation period) will be analysed with logistic regression.²⁰ ANCOVA will be used to investigate the association between covariates and the primary outcomes (variability and the time and percentage beneath/in/above the therapeutic range). Logistic regression will also be used to investigate the association between covariates and the secondary outcomes (rejection and nephrotoxicity). When a covariate shows a statistically significant association ($p < 0.1$) with both the exposure and the outcomes, it will be regarded as a confounder and it will be corrected for in the multiple analysis.

The statistical significance of effect modifiers will be researched with an interaction term test. Based on the identification of effect modifiers, results will be presented in strata.

6.2.3 Missing data

In case of missing data, multiple solutions can be initiated, depending on the kind of data that is missing. In case there is no information on comorbidities, the assumption is made that the patient does not have comorbidities. The same is true for missing information on the use of nephrotoxic agents, the

need for dialysis (CVVH or IHD) or ECMO support, use of methylprednisolone 1000 mg for three days and mortality during hospital admission. If there is no information available on these parameters, it is assumed there was no use of nephrotoxic agents or methylprednisolone, no need for dialysis or ECMO support and no mortality.

Patients are analysed separately if the following data are missing: length of intravenous administration (for the UMCG patients), route of administration (if it cannot be deduced from the hospital in which the transplantation took place), dose(s) of tacrolimus or if there are less than three serum creatinine values in the early post-transplantation period. Moreover, if a patient has less than three whole blood tacrolimus concentrations, the variability cannot be calculated accurately. Consequently, the patient will be excluded. If data are missing on the time of tacrolimus dose or time of tacrolimus concentration, that dose or concentration cannot be used in the analysis because that information is needed to determine if the concentration is a trough concentration. If the information is unavailable and the dose or concentration wouldn't be excluded, it might give a much higher variability and the results would not be an appropriate answer to the research question.

Single imputation will be used in the case of missing data on body weight and SOFA score.

We will need information on the day and time pre-LTx at which this serum creatinine or eGFR was determined. If information on the date of start of tacrolimus is missing, it is assumed that tacrolimus will start at the day after the lung transplantation.

6.2.4 Primary study parameter(s)

In case the data are normally distributed, the independent samples t-test will be used to investigate the (statistical significance of the) difference in the mean variability and mean time and percentage beneath/in/above the therapeutic range between the oral versus intravenous group. However, in case of non-normal distribution, the Mann-Whitney U test will be used. The identified confounders will be added to the multiple analysis, using stepwise forward entry. This will eventually lead to an adjusted Odds Ratio (OR), 95% Confidence Interval (95% CI) and p-value.

6.2.5 Secondary study parameter(s)

To evaluate the secondary objectives, the following steps will be taken.

Firstly, the Chi-square test will be used to investigate the difference between the oral and intravenous group for the occurrence of rejection and nephrotoxicity. The independent samples t-test will be used once again to determine the difference in mean post-LTx length of ICU stay and mean post-LTx length of hospital stay between the oral and intravenous group. Finally, the Chi-square test will also be used to study the difference in mortality between the oral and intravenous group.

In order to adjust for the identified confounders, a multiple analysis will be performed for each secondary objective and the confounders will be added to the model with stepwise forward entry. This will result in adjusted OR's, 95% CI and p-values for each secondary objective.

6.2.6 Sensitivity analyses

In the main analysis, a concentration is regarded as a potential peak concentration if the difference between this concentration and the preceding and following concentration is 7 µg/L or larger and the two preceding and two following concentrations are rather consistent, ergo; do not differ more than 2 µg/L from each other. For this sensitivity analysis, the difference between a potential peak concentration and the preceding and following concentration is first changed to 6 µg/L and later to 5 µg/L. The purpose of this sensitivity analysis is to see how many potential peak concentrations will be identified and how this differs from the main analysis. Another sensitivity analysis includes changing

the range, in which the two preceding and two following concentrations are allowed to differ from each other, from 2 µg/L to 3 µg/L. This will be done to investigate if the identification and exclusion of the tacrolimus peak concentration is adequate. Consequently, variability will be determined twice: (1) with all available concentrations, (2) with the identified peak concentrations excluded.

Finally, a sensitivity analysis will be performed, including the patients with missing data. Through this intention-to-treat analysis, it is investigated if the missing data, type of transplantation for example, significantly change the results (for the primary and secondary outcomes) found in the main analysis.

7. Ethical considerations

7.1 Regulation statement

This study will be conducted according to the 'Code of conduct for medical research' and in accordance with the EU GDPR.

7.2 Recruitment and consent

As this entails retrospective research, and all the data have already been gathered for medical purposes, the participating patients have not given explicit informed consent for this study. However, at the time of transplantation, participating patients have been informed by the supervising doctors on what happens with the medical data and patients from the UMCG have signed for informed consent to transfer their medical information for research purposes. This means a 'no-objection' construction is applicable. Upon treatment initiation in the UMCU, patients have to give their approval or objection to the use of data for research purposes. Subsequently, the 'register of objection' will be checked. Explicit informed consent does not need to be retrieved in hindsight because of the large study population (see section 5.3) and due to the fact that a large part of the population might not be alive anymore at the time of research.

As there is no active patient involvement anymore, the attachment of the patient information letter and informed consent is not applicable.

8. Administrative aspects and publication

8.1 Handling and storage of data and documents

The data which could make identification of subjects possible, e.g., patient ID, will be pseudonymised in order to make indirect identification of patient data impossible. Instead of date of transplantation, we will use year of transplantation and for other variables we will directly use the time with respect to lung transplantation instead of calculating the time variables ourselves. The key will be saved by the coordinating investigator, Dr. M.A. Sikma, on a disk at the 'Nationaal Vergiftigingen Informatie Centrum' (NVIC). The data will be saved into the Research Structure Folder and the ICT-system of the UMCU will automatically generate back-ups. All involved investigators will have access to the data. The UMCG will deliver their secured data and will not have direct access to the data of the UMCU. Data will be kept for at least 15 years. For more information on the handling and storage of data and documents, please consult the Data Management Plan.

8.2 Amendments

Amendments are changes made to the research after an ethical committee gave an advice non-WMO. Any change that may cause the investigation to fall within the scope of the WMO is submitted to the ethical committee that gave the non-WMO advice. Furthermore, deviations from the protocol will be mentioned in the manuscript in the 'Deviations and limitations' section in the Discussion.

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Appendix 2 Deviations from protocol

Throughout the research period, we have come across a few aspects in the study protocol that needed adjustments. These deviations will be described in this section.

First and foremost, due to the limited time of this project, we were only able to investigate the primary outcome, variability, and the secondary outcomes length of stay and mortality. The clinically relevant outcomes rejection and nephrotoxicity were not yet investigated. Hence, there was no need to look at the information at 1 month and 3 months after LTx yet.

Secondly, we did not adjust the tacrolimus concentrations for dose. Changes in doses may be an explanatory factor for high variability. However, the changes in dosage are necessary in the early post-transplantation phase, in order to obtain concentrations inside of the therapeutic range. Hence, the dose adjustments are part of the protocol. To add to that, initially we were only interested in the difference in variability and we were not investigating potential causes yet.

Thirdly, we did not exclude patients from the UMCU with partial oral and partial intravenous administration, as we had previously planned. The reason for it was that intermitting intravenous injection is not comparable to continuous intravenous injection. It is still part of the UMCU protocol to administer intravenously if oral administration is no option. With regard to the intention-to-treat nature of this study, it was decided to include these patients anyway.

Previously, we had not investigated if other tacrolimus detection methods could have been used in the study period. Since we only found out later that immunoassay was used in the UMCU until June 1st 2011, the decision to exclude patients transplanted before this date was made later on, during the research.

In the end, only intra-patient variability (IPV) and time/percentage within range were regarded as primary outcomes. The information on time/percentage outside of the range was presented descriptively only.

We decided to use another method (the method in which the two preceding and two following concentrations could maximally differ 3 µg/L instead of 2 µg/L) to identify peak concentrations once the sensitivity analyses were performed, since we found more potential peak concentrations and also identified slightly more actual peak concentrations. Hence, it was concluded that this method was more extensive.

Unfortunately, we were not able to receive all requested variables in time. Prominent variables that were still missing at the time of writing were: bodyweight, the use of drugs that have an interaction with tacrolimus, SOFA score, on which specific days dialysis was present and the use of ACE-inhibitors and ARB's. Additionally, extraction of the comorbidity hypertension was impossible. Eventually, we had to let go of the smoking status as well, as the smoking status was not determined in the same way for the two groups and a lot of data on the smoking status from the oral group was missing because these patients originated from another hospital (St. Antonius Hospital).

Finally, the statistical analysis and the identification of confounders as described in the protocol was not how it was actually performed. At the time of writing the protocol, the statistical analysis was not yet completely determined. The statistical analyses were clarified before actually performing the analyses. Next to that, two additional sensitivity analyses were added to test the robustness of the results regarding variability.

Appendix 3 Post-lung transplantation protocol UMCU

Immunosuppressive treatment:

1. Tacrolimus (Prograf): start twice daily 0.07 mg/kg orally, then adjustment of doses depending on the measured trough concentrations
2. Mycophenolate-mofetil (CellCept): Day 1-3 twice daily 1.5 gram orally. From day 4 until week 4 twice daily 1 gram orally
3. Prednisone: 100 mg intravenously 6 hours after reperfusion. On day 0-3 4 times daily 25 mg intravenously. Afterwards, a phasing-out schedule is followed
4. Basiliximab (Simulect): after reperfusion of the lung administration of 20 mg intravenously. On day 4 post-LTx another intravenous 20 mg administration
5. Solumedrol treatment in case of acute rejection

Infection prophylaxis:

- CMV negative donor/acceptor: acyclovir 5 mg/kg intravenously for 12 hours, followed by valacyclovir 500 mg twice daily orally.
CMV positive donor/acceptor: ganciclovir twice daily 5 mg/kg intravenously, followed by valganciclovir once daily 900 mg orally.
- Co-trimoxazole: 480 mg orally three times per week
- Ganciclovir (twice daily 5 mg/kg intravenously) or acyclovir (5 mg/kg intravenously for every 12 hours) or valacyclovir (twice daily 500 mg)
- Amphotericin B: three times daily 5 mg for 6 weeks or until discharge
- SDD: 4 times daily and 4 days pre-operatively mupirocin three times daily in both nostrils
- Piperacillin/tazobactam: 3 times daily 4.5 gram intravenously

Analgesics:

- Acetaminophen: 4 times daily 1 gram rectally
- Remifentanyl is administered intravenously or morphine through continuous intravenous infusion or bupivacaine/morphine epidurally

Thrombosis prophylaxis:

- Dalteparin: once daily 2500 or 5000 IU, depending on the body weight

Other medication:

- Ventolin/acetylic cysteine spray 4 times daily
- Pantoprazole once daily 40 mg intravenously during mechanical ventilation
- Esomeprazole twice daily 20 mg orally after extubation
- Calcium regulating medication
- Movicolon: 4 times daily one sachet

Appendix 4 Post-lung transplantation protocol UMCU

Immunosuppressive treatment:

1. Tacrolimus: 0.01 mg/kg/24 hours through continuous intravenous infusion, start 12 hours after reperfusion of the first lung allograft. Switch to twice daily orally (0.1 mg/kg/day, separated into two administrations) when sufficient gut motility has returned
2. Mycophenolate-mofetil: 1000 mg orally before transplantation. Twice daily 1000 mg intravenously or orally postoperatively, start < 8 hours after transplantation
3. (methyl)prednisolone: 500 mg intravenously before reperfusion of each transplanted lung. Then 3 times daily 125 mg intravenously in the first 24 hours postoperatively. Administration of prednisolone once daily 0.5 mg/kg intravenously or orally during day 2-day 7 post-LTx. Once daily 0.25 mg/kg orally during the first 6 months after LTx
4. Basiliximab: 20 mg intravenously on day 0 and 20 mg intravenously on day 4

Infection prophylaxis:

- Ganciclovir (once daily 5 mg/kg intravenously, start 24 hours postoperatively, later switch to valganciclovir once daily 900 mg orally) or valacyclovir (twice daily 500 mg orally, start 48 hours post-LTx)
- SDD: 4 times daily
- Co-trimoxazole: 960 mg every other day. Start 48 hours postoperatively
- Amphotericin B sprays: 4 times daily 5 mg until extubation
- Antibiotics according to advised scheme or otherwise ceftazidim 3 times daily 2 grams intravenously during the first 4 days

Analgesics:

- Morphine intravenously
- Acetaminophen: 4 times daily 1 gram rectally

Thrombosis prophylaxis:

- Fraxiparin: once daily 2850 IU subcutaneously

Other medication:

- Pantoprazole: once daily 40 mg intravenously

Appendix 5 Sub analysis week 1 versus week 2

Table 4: Sub analysis in which the results for variability are compared for week 1 and week 2 after LTx within each group (oral and intravenous administration). The results are weighted for number of samples available per patient.

Variability parameter	Oral: weighted for number of samples, n (%)			Intravenous: weighted for number of samples, n (%)		
	Week 1 range 12-15	Week 2 range 12-15	p-value	Week 1 range 13-15	Week 2 range 10-15	p-value
	1260 (49.5)	1287 (50.5)		1786 (63.2)	1040 (36.8)	
IPV (%), median (IQR)	29.9 (22.9-38.8)	21.0 (14.9-29.5)	<0.001	27.9 (20.7-36.6)	18.4 (12.7-25.1)	<0.001
Time beneath TR (hours), median (IQR)	51.4 (29.3-89.3)	65.4 (32.6-99.8)	<0.001	63.6 (38.5-97.5)	2.5 (0.0-39.4)	<0.001
Percentage beneath TR (%), median (IQR)	46.0 (25.0-73.3)	48.0 (24.8-70.0)	0.080	53.1 (31.2-75.8)	1.8 (0.0-30.7)	<0.001
Time within TR (hours), median (IQR)	20.8 (8.9-38.1)	30.8 (14.1-52.2)	<0.001	16.2 (6.4-30.2)	55.4 (31.4-84.7)	<0.001
Percentage within TR (%), median (IQR)	18.3 (7.8-32.2)	22.8 (10.2-38.2)	<0.001	12.9 (5.3-23.5)	47.5 (26.0-66.5)	<0.001
Time above TR (hours), mean ± SD	32.9 ± 31.4	36.5 ± 34.9	0.005	37.2 ± 34.0	42.4 ± 38.9	<0.001
Percentage above TR (%), mean ± SD	50.2 ± 30.7	47.8 ± 30.0	0.022	29.4 ± 26.5	35.4 ± 32.2	<0.001
Number of samples, mean ± SD	5.7 ± 0.7	6.4 ± 0.9	<0.001	6.2 ± 0.9	5.7 ± 0.9	<0.001
Average concentration (µg/L), median (IQR)	11.8 (9.8-14.5)	12.7 (11.0-14.4)	<0.001	12.1 (10.5-14.3)	13.7 (11.7-15.7)	<0.001
First time TR is reached (hours), mean ± SD	72.5 ± 28.1*	n.v.t.	n.v.t.	79.0 ± 31.4	n.v.t.	n.v.t.
Cmin (µg/L), median (IQR)	6.2 (3.0-8.6)	8.4 (6.7-10.1)	<0.001	5.6 (3.6-7.6)	9.8 (8.0-11.5)	<0.001
Cmax (µg/L), median (IQR)	18.4 (14.2-23.6)	17.7 (14.9-22.5)	0.745	17.7 (15.0-21.8)	18.1 (15.2-22.2)	0.108
Difference Cmax-Cmin (µg/L), median (IQR)	12.6 (8.3-16.8)	8.9 (6.3-13.1)	<0.001	11.9 (8.7-16.2)	8.0 (5.0-12.1)	<0.001
Total duration (hours), median (IQR)	120.0 (120.0-122.0)	144.0 (142.0-144.0)	<0.001	121.5 (120.0-144.0)	120.0 (119.5-123.2)	<0.001

Cmax, Highest tacrolimus concentration; Cmin, Lowest tacrolimus concentration; IPV, intra-patient variability; IQR, interquartile range; SD, standard deviation; TR, therapeutic range.
 *The time post-LTx at which a concentration was measured was determined differently between groups. For the oral group, the start of surgery was taken as the reference time for the time blood concentrations were taken. For the intravenous group, reperfusion of the lung allograft was used.

Appendix 6 Sensitivity analysis identical therapeutic range

Table 5: Sensitivity analysis in which for both groups the upper limit was 15 µg/L throughout the two weeks after LTx, the lower limit was 12 µg/L in the first week and changed to 10 µg/L in the second week. The results were weighted for number of samples available per patient.

Variability parameter	Oral: weighted for number of samples, n (%)	Intravenous: weighted for number of samples, n (%)	p-value
	2586 (45.3)	3118 (54.7)	
IPV (%), mean ± SD	31.7 ± 10.5	29.2 ± 10.9	<0.001
Time beneath TR (hours), mean ± SD	106.4 ± 54.2	82.1 ± 51.2	<0.001
Percentage beneath TR (%), mean ± SD	38.5 ± 19.3	30.7 ± 18.8	<0.001
Time within TR (hours), mean ± SD	98.0 ± 44.7	93.5 ± 47.7	<0.001
Percentage within TR (%), mean ± SD	35.5 ± 15.8	35.0 ± 17.6	0.230
Time above TR (hours), mean ± SD	71.4 ± 45.8	90.8 ± 59.6	<0.001
Percentage above TR (%), mean ± SD	26.0 ± 16.8	34.3 ± 22.6	<0.001
Number of samples, median (IQR)	12 (11-13)	11 (9-12)	<0.001
Average concentration (µg/L), median (IQR)	12.4 (11.3-14.1)	12.9 (11.7-14.6)	<0.001
First time TR is reached (hours), median (IQR)	73.0 (55.0-115.0)*	68.9 (53.0-100.7)	<0.001
Cmin (µg/L), mean ± SD	5.4 ± 2.7	5.6 ± 2.6	0.007
Cmax (µg/L), median (IQR)	21.6 (17.8-26.5)	20.5 (17.6-25.2)	<0.001
Difference Cmax-Cmin (µg/L), median (IQR)	16.0 (12.9-21.3)	15.1 (11.2-19.8)	<0.001
Total duration (hours), median (IQR)	288.0 (266.0-290.0)	265.5 (263.8-288.0)	<0.001
Cmax, Highest tacrolimus concentration; Cmin, Lowest tacrolimus concentration; IPV, intra-patient variability; IQR, interquartile range; SD, standard deviation; TR, therapeutic range.			
*The time post-LTx at which a concentration was measured was determined differently between groups. For the oral group, the start of surgery was taken as the reference time for the time blood concentrations were taken. For the intravenous group, reperfusion of the lung allograft was used.			

Appendix 7 Definition peak concentration identification

In this sensitivity analysis, we tested six different definitions to identify potential peak concentrations. These are presented down below, along with the number of potential peak concentrations they identified.

1. The difference between the potential peak concentration had to be minimally 7 $\mu\text{g/L}$ and the two preceding and two following concentrations were not allowed to differ more than 2 $\mu\text{g/L}$ from each other. *11 potential peak concentrations were identified.*
2. The difference between the potential peak concentration had to be minimally 6 $\mu\text{g/L}$ and the two preceding and two following concentrations were not allowed to differ more than 2 $\mu\text{g/L}$ from each other. *11 potential peak concentrations were identified.*
3. The difference between the potential peak concentration had to be minimally 5 $\mu\text{g/L}$ and the two preceding and two following concentrations were not allowed to differ more than 2 $\mu\text{g/L}$ from each other. *13 potential peak concentrations were identified.*
4. The difference between the potential peak concentration had to be minimally 7 $\mu\text{g/L}$ and the two preceding and two following concentrations were not allowed to differ more than 3 $\mu\text{g/L}$ from each other. *19 potential peak concentrations were identified.*
5. The difference between the potential peak concentration had to be minimally 6 $\mu\text{g/L}$ and the two preceding and two following concentrations were not allowed to differ more than 3 $\mu\text{g/L}$ from each other. *20 potential peak concentrations were identified.*
6. The difference between the potential peak concentration had to be minimally 5 $\mu\text{g/L}$ and the two preceding and two following concentrations were not allowed to differ more than 3 $\mu\text{g/L}$ from each other. *29 potential peak concentrations were identified.*

In the end, method 1 and 4 were deemed most relevant. All identified concentrations from both methods were judged with information from the Electronic Health Record Data. Factors that were investigated were the time of administration, compared to the time of sample drawing, dose adjustments, start of medications that may alter the tacrolimus concentration, packed cells therapy or diarrhea. From the 11 potential peak concentrations that were identified in method 1, 3 were regarded as actual peak concentrations. From the 19 potential peak concentrations that were identified in method 4, 5 concentrations were regarded as actual peak concentrations.

Apparently, we did miss a few actual peak concentrations with method 1, hence we chose method 4 to detect and exclude peak concentrations. A visual representation of this method is depicted in figure 1.

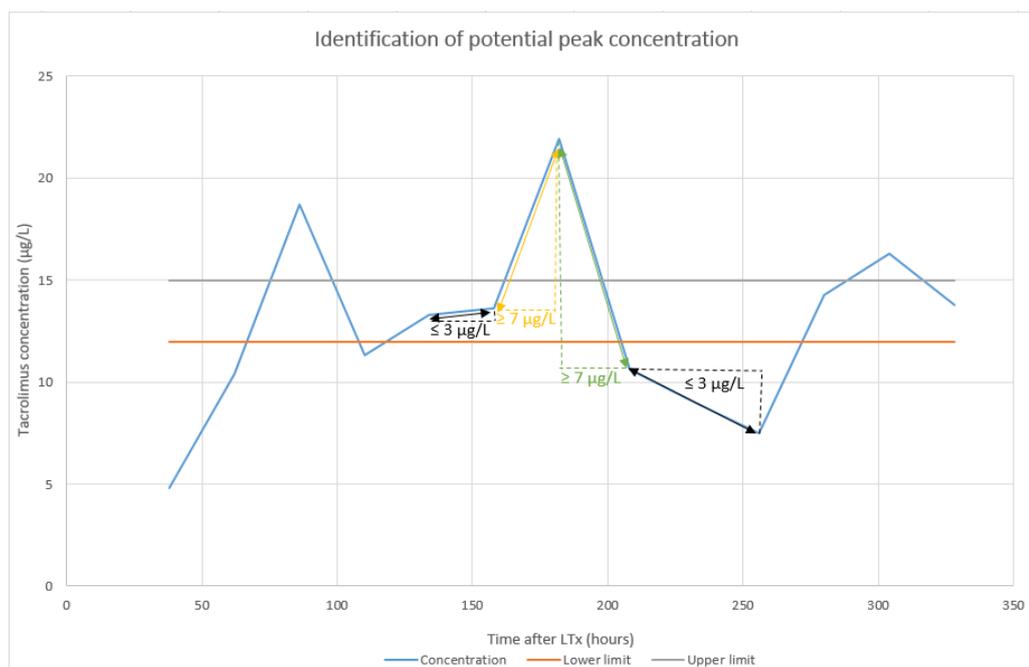


Figure 2: A visual representation of the identification of peak concentrations.