Analysis of Tacrolimus Concentration in Bile: A Patient-Specific Approach to the Early Detection of Acute Rejection After Liver Transplantation

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Article

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Abstract: Introduction: Tacrolimus (TAC) stands out as one of the most widely utilized immunosuppressive drugs in the prevention of rejection following solid organ transplantation. In case of liver transplantation (LTx), the biochemical pathway for drug metabolism and excretion is an attribute of the transplanted organ, the primary focus of immunological and functional monitoring. Consequently, the different reduced hepatic functions in every patient may lead to diminished TAC catabolism and excretion, resulting in a reduced biliary TAC concentration (TACbile) and a consequent elevated blood TAC concentration (TACblood). Our study aims to create a personalized parameter for early the hepatic rejection after LTx by analyzing the hepatic excretion rate of TAC through the evaluation of TACbile and TACblood. **Methods:** This is a prospective single center observational cohort study. The protocol was registered in Clinicaltrial.gov (code NCT03882164). **Results:** The endpoints analyzed in the protocol will be the assessment of the predictive ability of TACbile as a marker of early liver rejection and the assessment of a relationship between the reduction in Tacrolimus biliary excretion and Early Allograft Dysfunction (EAD). **Conclusions:** The development of a personalized rejection predictive system could significantly improve the clinical outcome after LTx.

Keywords: liver transplantation; tacrolimus; bile; rejection; early allograft dysfunction

1. Introduction

Tacrolimus (TAC) stands out as one of the most widely utilized immunosuppressive drugs in the prevention of rejection following solid organ transplantation. Discovered in 1987, it found its inaugural application in Italy in 1991 for anti-rejection purposes post-liver transplantation (LTx). Pharmacokinetic studies involving both healthy volunteers and transplant patients have elucidated that TAC is rapidly absorbed following oral administration, reaching peak plasma concentration within 1-2 hours. Subsequently, it is predominantly found in peripheral blood bound to erythrocytes and undergoes hepatic metabolism via CYP3A4, with 96% of elimination occurring through bile.

The primary activity of TAC involves binding to a membrane protein (FKBP), thereby reducing the production of inflammatory cytokines through calcineurin inhibition.

Due to TAC's ubiquitous activity, overdose-induced side effects primarily affect the gastrointestinal system (nausea, vomiting, diarrhea, cramps), urinary system (nephrotoxicity,

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hypomagnesemia, hyperkalemia), and nervous system (seizures, blurred vision, itching, and headaches).

The narrow therapeutic index of TAC necessitates therapeutic drug monitoring (TDM), particularly in the initial days post-transplantation or in cases of clinical-laboratory suspicion of under- or overdosage. The most widely employed TDM technique involves assessing TAC concentration in peripheral blood (TACblood) before and after oral intake .

Despite optimal TDM management, rejection remains a prevailing challenge, with rates of up to 20% in biopsy-confirmed cases undergoing LTx. Clinically and laboratoristically, rejection may initially manifest as early allograft dysfunction (EAD) and ultimately as structural hepatic damage .

In the context of TAC utilization post-LTx, it is noteworthy that the biochemical pathway for drug metabolism and excretion is an attribute of the transplanted organ, the primary focus of immunological and functional monitoring. Consequently, reduced hepatic function may lead to diminished TAC catabolism and excretion, resulting in an elevated TACblood.

To comprehensively analyze TAC excretion, it is imperative to evaluate bile TAC concentration (TACbile) through post-LTx bile sampling.

The Kehr T-tube is an external biliary drainage positioned in the common bile duct to secure biliary anastomosis and assess the quantity and quality of bile produced by the liver graft. It is especially utilized in cases of LTx from marginal donors or in Donation after Circulatory Death (DCD). Such LTx instances pose a higher risk of post-transplant complications, including an increased rejection rate. Therefore, in these patients, detecting functional dysfunction before graft damage development becomes even more desirable.

Our study aims to create a patient-centred model to predict early the hepatic rejection after LTx by analyzing the hepatic excretion rate of TAC. Furthermore, we aim to assess the correlation between hepatic excretion rate and the onset of EAD. Considering the importance of achieving therapeutic efficacy in the early post-LTx phase, which is generally associated with a higher risk of organ rejection, our study focused on the first 10 days after LTx.

2. Materials and Methods

2.1. Study Design

This is a prospective single center observational cohort study. The protocol was registered in Clinicaltrial.gov (code NCT03882164).

2.2. Population

All patients undergo LTx after retrieval from a deceased donor, with preservation of the inferior vena cava (with double caval anastomosis according to Starzl or with cavotomy for “piggyback” caval anastomosis).

When the end-to-end hepato-choledochal biliary anastomosis is created, a Kehr T-tube is inserted, left open until the 10th postoperative day, then clamped and removed approximately 4 months after LTx.

All patients receive similar pharmacological therapies during and after LTx.

2.3. Inclusion Criteria

- Patient undergoing liver transplantation (in 1st post-operative day, POD);
- Age >18 years;
- Intraoperative placement of a Kehr T-tube in the bile duct;
- Immunosuppressive therapy with TAC;
- Normal functioning of the Kehr T-tube (with corresponding bile drainage).

2.4. Exclusion Criteria

- Age <18 years;

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- Liver transplantation not recent (beyond 1st POD);
- Liver transplantation without the placement of a Kehr T-tube;
- Immunosuppressive therapy with an active ingredient other than Tacrolimus;
- Malfunction of the Kehr T-tube.

2.5. Immunosuppresive Protocol

The standard immunosuppression regimen involves the use of Tacrolimus and Mycophenolate mofetil from the day of transplant, associated with a short course of corticosteroids for induction.

The administration of Tacrolimus begins with a dosage of approximately 0.1 mg/kg/day divided into two daily doses. Subsequently, this dosage was adapted depending on the TACblood, to obtain a TACblood value between 7 and 9 ng/mL.

Anti-rejection treatment consisted of high doses of methylprednisolone (1 g/day for 3 days).

2.6. Microbiologic Surveillance

Antibiotic prophylaxis is administered for approximately 7 days, depending on the recent medical history of the donor and recipient: in patients at low risk of infection, Piperacillin/Tazobactam is used at a dosage in line with the patient's renal function; in case of anamnestic positivity to specific microorganisms, antibiotic therapy is adapted to the clinical and microbiological results. In the case of a donor with positive antibodies for Cytomegalovirus (CMV), surveillance was performed through the search for viral DNA with Polymerase Chain Reaction (PCR) and possible treatment with Ganciclovir (5 mg/kg/day every 12 hours, adapted to renal function) in the recipient with a negative anamnestic for CMV infection.

2.7. Post-LTx Complications Surveillance

All patients underwent daily clinical, biochemical, and ultrasound examinations. In case of worsening of the patient's laboratory conditions, a clinical diagnosis of rejection was made. The biochemical evaluation was performed using a score that takes into account the progressive increase of total bilirubin and eosinophils in peripheral blood, the increase of the absolute eosinophil count above 600/mm3, and the progressive decrease of platelets between the 5th and 7th post-transplant day. Each element was assigned a score of 1. Treatment was started in patients with clinically relevant rejection, defined by a biochemical score >2. If the haemo-coagulative and clinical picture made the risk of peri-procedural complications acceptable, these patients underwent percutaneous ultrasound/CT-guided liver biopsy. In this way, a histological diagnosis of acute liver graft rejection was made. Transplant pathologists read biopsies, and rejection was classified as mild, moderate, and severe.

2.8. Tacrolimus Sampling

A peripheral blood sample is used for daily monitoring of Tacrolimus for clinical purposes. It is stored in a tube with ethylenediaminetetraacetic acid (EDTA) at room temperature for a few hours before analysis.

In conjunction with the blood sampling, a sample of approximately 5 ml of bile was collected in a Falcon-type tube from the Kehr T-tube for experimental purposes. The sample is stored at a temperature of 4°C if analyzed within 72 hours of sampling. Otherwise, it is stored at -80°C and analyzed within 5 days of sampling. The validity and validation of this protocol are currently being evaluated in a series of patients undergoing liver transplantation with the placement of a Kehr T-tube. Both biological samples are collected before the morning administration of Tacrolimus.

The same method is used during the entire study period.

For clinical reasons, blood samples are labeled with the patient's details. Bile samples, on the other hand, are identified with a code arbitrarily chosen by the collector. Therefore, TACbile dosages are performed in a single-blind fashion.

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The TACblood and TACbile values for each patient included in the study are calculated from the 1st to the 10th postoperative day.

The analysis of the drug concentration is described in the dedicated paragraph.

2.9. Statistical Plan

The sample will be described in terms of its clinical and demographic characteristics using descriptive statistical techniques. Specifically, quantitative variables will be represented by the following measures: minimum, maximum, range, mean, and standard deviation. Qualitative variables will be presented with tables of absolute frequencies and percentages. The normality of continuous variables will be assessed using the Kolmogorov-Smirnov test.

The primary objective will be achieved by conducting logistic regression. For secondary objectives, a longitudinal data analysis will be performed. In particular, the statistical analysis will be conducted in two phases. In the first phase, a polynomial mixed model will be used to estimate the baseline trend for Tacrolimus biliary excretion data and to obtain an estimate of the residual variance (Root Mean Square Error - RMSE of the differences between observed and expected Tacrolimus biliary excretion values). In the second stage, the residual variance values obtained in the first phase will be used to estimate the risk of developing liver failure. This will be done through the Cox proportional hazards regression model. Longitudinal baseline trajectories were estimated using multilevel longitudinal modeling. P values are based on the two-sided likelihood ratio test with a significance level of 5%, and 95% confidence intervals are based on the Wald statistic. Data lost during follow-up will be censored. Data processing and analysis were performed using Analyst (version 1.7) and Multiquant (version 3.0.2) softwares (Waters Corporation, Milford, MA, USA). The analyses will be performed using the statistical software SPSS 25.

2.10. Sample Size Determination

Considering the reported literature prevalence of early rejection in liver transplantation (ranging from 10% to 30%) and EAD (ranging from 19% to 33%), a sample size of at least 55 patients is deemed necessary. In this sample, approximately 10 to 32 early rejection events are observed. This sample size is deemed sufficient for conducting logistic regression on the last measurement of Tacrolimus biliary excretion (independent variable) and the Early Allograft Dysfunction (EAD) event (dependent dichotomous variable).

2.11. Consent Form

In accordance with Good Clinical Practice (GCP) guidelines and in compliance with the Declaration of Helsinki and current guidelines for observational studies, every patient will be informed about the procedures carried out within the scope of the scientific work. The patient can freely choose whether to participate by signing an informed consent form. A specific certification declaring participation in the scientific study will be included in the current hospitalization medical record. At any time, the patient can withdraw their consent to participate in the study. In the case of refusal to participate in the study, the patient will be assured the same clinical care provided to all patients.

At the end of the study, the patient will have access to the study results.

2.12. Ethics

This research protocol was approved by Fondazione Policlinico Universitario Agostino Gemelli IRCCS Ethics Committee (reference number: 0052054/20, study ID: 3733).

3. Results

3.1. Laboratory Analysis

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A 5 cc sample of bile is collected in the morning from the Kehr tube before the oral administration of Tacrolimus, concurrently with the blood draw for the evaluation of TACblood. The bile sample is stored at 4°C and analyzed on the same day. Daily sampling is systematically performed within the first ten days post-LTx.

The sample preparation for the quantification of immunosuppressant drugs in bile was performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using MassTox Immunosuppressants Testing kit (Chromsystems Instruments & Chemicals GmbH, Munich, Germany).

The kit was used according to the manufacturer instructions with some modifications to adapt to the new matrix. Analytical method validation, necessary for the different matrix used, was performed according to international guidelines.

The sample preparation was carried out according to the manufacturer instructions and 200µL aliquot of the supernatant was transferred into a glass vial for the injection to UPLC-MS/MS.

The chromatographic method was modified to make it more suitable for the bile matrix.

The UPLC separation was performed using a BEH Amide 1.7 µm, 2.1 × 50 mm column (Waters Corporation, Milford, MA, USA) operating at a flow rate of 400 µL/min with a gradient of mobile phase A (H2O containing 0.1% formic acid and 2 mM ammonium acetate) and mobile phase B (methanol containing 0.1% formic acid and 2 mM ammonium formate). The oven temperature was set at 30 °C. The injection volume was 20 µL, and the total run time, including 1 minute for equilibration of column, was 5 minutes.

The ESI source operates in positive mode, with a capillary voltage of 1.7 kV and a desolvation temperature of 350 °C. The source of the gas was set as follows: desolvation gas flow at 1000 L/Hr and cone gas flow at 50 L/Hr.

The quantification was achieved by plotting peak area ratio (standard/stable isotopically labeled IS standards) versus nominal concentration.

The concentration was calculated by the regression analysis of standards over the concentration range of the calibration curve.

3.2. Instrumentation and Analytical Parameters

The LC-MS/MS system consisted of an Acquity UPLC and autosampler Acquity I-Class (Waters corporation, Milford, MA, USA) system interfaced with a triple quadrupole mass spectrometer (Xevo TQS-Micro, Waters, Milford, MA, USA) equipped with an electrospray ion source.

3.3. Chemicals and Reagents

6Plus1 Multilevel Urine Calibrator SET, MassCheck, Internal Standard (consisting in isotopically labeled compounds for each analyte), precipitation and extraction reagents were purchased from Chromsystems Instruments & Chemicals GmbH (Munich, Germany).

Water, Methanol and Formic Acid (LC-MS grade) were purchased from Biosolve (Biosolve Chimie, Dieuze, France), ammonium acetate was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, German).

3.4. Primary Endpoint

The primary endpoint of the study is the assessment of the predictive ability of TACbile as a marker of early liver rejection.

TAC was administered to all patients according to local protocols on immunosuppressive therapy management. The first administration occurs on the 1st POD after LTx. The TAC formulation used involves oral administration every 12 hours (at 8 AM and 8 PM) at a dosage correlated with Tacrolimus blood levels analyzed through Therapeutic Drug Monitoring (TDM).

TDM is analyzed daily in clinical practice to assess the therapeutic index and prevent potential overdose toxicity. Additionally, daily lab tests and radiological assessments are conducted to

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evaluate the development of post-LTx complications. In case of clinical or laboratory suspicion of rejection, a percutaneous liver biopsy is performed to obtain a histological diagnosis of rejection.

Bile collected from the Kehr T-tube is sampled in the morning, before the oral administration of Tacrolimus, and concurrently with the blood withdrawal for TDM. The hepatic excretion index of Tacrolimus, obtained through the correlation between Tacrolimus blood levels and Tacrolimus bile levels, is compared with clinical and laboratory data.

3.5. Secondary Endpoint

The secondary endpoint regards the assessment of a relationship between the reduction in Tacrolimus biliary excretion and Early Allograft Dysfunction (EAD). EAD is defined according to the Olthoff criteria by the presence of one or more of the following: INR >1.6 on day 7; bilirubin >10 mg/dL on day 7; Alanine aminotransferase (ALT) > 2000 IU/L within the first 7 days. Clinical and laboratory assessment, conducted daily as per the protocol, allows for the diagnosis of EAD.

In this case, the excretory capacity of Tacrolimus used for the primary endpoint is employed to establish a correlation with the onset of EAD.

4. Discussion

We are conducting this study to validate the analysis of TACbile in the early diagnosis of hepatic rejection after LTx to avoid to the patients both the complications associated with graft rejection and the damages caused by pharmacological treatment of rejection.

Acute rejection after LTx causes structural hepatocellular injury that can result in persistent functional deficits. Additionally, patients with acute rejection experience worse post-LTx outcomes, even in the long term.

No factors predicting an increased risk of graft rejection have been reliably identified, nor are there non-invasive parameters detectable in the post-transplant period that can consistently indicate immune-mediated damage to the transplanted organ. Due to the limited effectiveness of laboratory parameters, the current gold standard for diagnosing rejection is histological evaluation following biopsy sampling of the graft. Although this procedure is minimally invasive and performed under ultrasound guidance, it may be somewhat unsafe peri-transplant due to the persistence of thrombocytopenia or coagulopathy associated with inadequate liver graft function.

For this reason, it is crucial to minimize the risk of rejection with proper immunosuppressive therapy. The most commonly used immunosuppressive drug is Tacrolimus due to its effectiveness at lower dosages than other available immunosuppressants. However, it does carry renal and neurological side effects and increases the risk of developing diabetes mellitus. Therefore, Tacrolimus blood concentrations are monitored daily in the early days after transplantation to maintain a therapeutic range.

Tacrolimus pharmacokinetics are characterized by an almost entirely biliary elimination, following various independent chemical reactions (demethylation, hydroxylation, conjugation of a drug molecule with uridine phosphate). All catabolic pathways involve the hepatic cytochrome P450 3A4 isoform. In our study, we analyzed the concentration of TACbile collected from the T-tube implanted in the bile duct during LTx.

TDM of tacrolimus in blood is standard practice to minimize the risk of acute rejection while avoiding toxicities. Subtherapeutic blood levels are strongly associated with an increased risk of rejection episodes, as they may fail to adequately suppress the immune response. During the early post-transplant period, when the risk of rejection is highest, tacrolimus blood concentrations are typically maintained in a higher therapeutic range (e.g., 8–12 ng/mL). Over time, as the risk diminishes, a lower range (e.g., 5–8 ng/mL) suffices for most patients.

However, maintaining tacrolimus within this therapeutic window can be challenging due to interindividual variability in its metabolism and pharmacokinetics. Genetic factors, such as polymorphisms in the CYP3A5 gene, significantly influence tacrolimus metabolism. For instance, individuals with the CYP3A5*1 allele metabolize tacrolimus more rapidly and often require higher

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doses to achieve target blood levels. Additionally, drug interactions, liver function, and patient adherence to therapy contribute to fluctuations in blood tacrolimus levels, impacting rejection risk.

While blood concentrations of tacrolimus are well-studied, its concentration in bile remains an emerging area of interest. Tacrolimus is excreted into bile as part of its enterohepatic circulation, and bile concentrations may serve as an indicator of local drug exposure within the biliary tract and liver. This is particularly relevant in liver transplantation, as bile ducts are susceptible to immune-mediated damage during acute rejection.

Studies have suggested that bile concentrations of tacrolimus could correlate with its immunosuppressive effects at the graft site. Low bile concentrations may indicate insufficient local exposure to the drug, potentially leaving the bile ducts and hepatic parenchyma vulnerable to immune attack. Conversely, adequate bile levels might enhance local immunosuppression, complementing systemic effects.

The relationship between tacrolimus blood and bile concentrations is not linear and varies based on individual pharmacokinetics and liver function. Some patients with therapeutic blood levels may still experience acute rejection, potentially due to suboptimal bile concentrations. This disconnect highlights the limitations of relying solely on blood TDM and suggests that bile monitoring might provide additional insights .

Factors affecting tacrolimus distribution to bile include hepatic enzyme activity, bile production, and the integrity of the bile duct system. For example, ischemic injury to the bile ducts during transplantation can impair tacrolimus transport into bile, potentially increasing the risk of localized rejection despite adequate systemic drug levels.

Emerging research suggests that combined monitoring of tacrolimus in blood and bile could enhance predictive accuracy for rejection risk. High blood levels without corresponding bile concentrations may indicate an issue with drug distribution, while low levels in both compartments may signal inadequate dosing.

Optimizing tacrolimus therapy in liver transplantation requires a nuanced approach that considers both systemic and local drug exposure. Incorporating bile concentration monitoring into routine practice could help identify patients at risk of rejection who may otherwise appear adequately immunosuppressed based on blood levels alone.

In our study, we analyzed the concentration of TACbile collected from the T-tube implanted in the bile duct during LTx.

The Kehr T-tube, a T-shaped medical device commonly used in biliary surgery, is typically made of silicone or rubber. It is inserted into the common bile duct, with the vertical limb providing external drainage through the abdominal wall and the horizontal limbs positioned inside the bile duct to maintain patency. In liver transplantation, the T-tube supports the newly constructed bile duct anastomosis, particularly in cases where biliary anatomy poses a challenge or risk of postoperative complications. Neverthless, its use has become more selective in modern practice due to evolving surgical techniques and technologies.

Bile duct reconstruction during liver transplantation is one of the most critical and complex aspects of the surgery. The biliary anastomosis connects the donor's bile duct to the recipient's bile duct or bowel. This process can be prone to complications. Dislodgement or obstruction of the T-tube can compromise biliary drainage and necessitate additional procedures. Bile leaks could develop at the insertion site of the –tube, requiring further intervention. The externalized portion of the T-tube can act as a conduit for bacteria, increasing the risk of cholangitis or other infections. A prolonged use of the T-tube can sometimes lead to scarring and strictures at the site of insertion. strictures, and infections.

Anyway, the T-tube also demonstrated benefits from a technical and diagnostic point of view. The T-tube permits flushing of the bile ducts to address sludge or small stones, which are relatively common after LTx. By draining bile externally, the T-tube reduces pressure on the anastomosis and minimizes the likelihood of leaks and strictures. A T-tube is often used to reduce the risk of such complications by decompressing the biliary system, providing external drainage to prevent bile

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buildup, and allowing easy access for diagnostic and therapeutic interventions, such as cholangiography or flushing to clear obstructions. The T-tube allows easy access to the bile for biochemical analysis which helps identify bile duct strictures or other complications.

In our feasibility analysis, the bile was collected from the T-tube to analyze the TACbile. Subsequently, we put in correlation TACblood and TACbile, demonstrating that the concentrations of tacrolimus did not follow a linear trend. Furthermore, the variability of the two values was correlated with the post-LTx clinical course.

Our work builds upon this observation, assuming that the inconsistent difference between TACbile and TACblood correlates with the liver's varying ability to metabolize and eliminate Tacrolimus, depending on pathological processes such as acute rejection, which can alter liver function.

Acute rejection is a significant complication following LTx, characterized by the recipient's immune system attacking the transplanted organ. Despite advances in immunosuppressive therapies and surgical techniques, acute rejection remains a critical challenge that can impact graft function and patient outcomes. When promptly treated, acute rejection episodes are often reversible, with minimal impact on long-term graft survival. However, recurrent or severe rejection can lead to chronic rejection, characterized by progressive fibrosis and graft dysfunction.

After LTx, the recipient's immune system recognizes donor antigens as foreign, triggering an immune cascade. This activation results in the proliferation of cytotoxic T-cells and the release of pro-inflammatory cytokines, leading to direct damage to the liver's hepatocytes and bile ducts.

Acute rejection often manifests within the first three months post-LTx, though it occurs mainly in the first week after the LTx at any time. The initial manifestations are mainly laboratory and include elevated liver enzymes (ALT, AST), increased bilirubin, and alkaline phosphatase levels, suggesting hepatic dysfunction. A higher concentration of eosinophils can demonstrate a cellular activation.

Histological examination remains the gold standard for diagnosing acute rejection. Key findings include lymphocytic infiltration of the portal tracts, bile duct damage, and endothelialitis (inflammation of the blood vessels). Anyway, clinical signs and symptoms are often non-specific, making early diagnosis challenging.

To further amplify the difference between TACbile and TACblood, an index based on the ratio of TACblood to TACbile, called the "Blood-Bile Ratio of Tacrolimus" (BBRT), was developed.

The BBRT may expand the yet limited armamentarium in the post-LTx period for the patient-specific non-invasive diagnosis of acute rejection.

5. Conclusions

Rejection after LTx is a widespread and difficult-to-prevent complication, especially in the first seven days after surgery. In case of rejection, pharmacological treatment has significant side effects. The development of a personalized approach to predict the rejection can significantly improve the clinical outcomes after LTx.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and the principles of the Good Clinical Practice (GCP) guidelines. The study was approved by Fondazione Policlinico Universitario Agostino Gemelli IRCCS Ethics Committee (reference number: 0052054/20, study ID: 3733, date: December 23th 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study at the time of LTx. All data were made anonymous.

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