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Survival Time to Biopsy-Proven Acute Rejection and Tacrolimus Adverse Drug Reactions in Pediatric Liver Transplantation

Natalia Riva, PhD; Marcelo Dip, MD; Esteban Halac, MD; Paulo Cáceres Guido, PharmD; Jean Baptiste Woillard, PhD; Nieves Licciardone, BSc; Debora Chan, BSc; Jefferson Buendía, PhD; Daniela Borgnia, BSc; Andrea Bosaleh, MD; María Teresa de Davila, MD; Oscar Imventarza, MD; Paula Schaiquevich, PhD.

Unit of Clinical Pharmacokinetics (Dr. Riva, PharmD Cáceres Guido, Dr. Schaiquevich); Liver Transplant Service (Dr. Dip, Dr. Halac, Dr. Imventarza); Laboratory (Licciardone); Microbiology Service (Borgnia); Pathology Service (Dr. Bosaleh, Dr. Teresa de Davila), Hospital de Pediatría JP Garrahan, Buenos Aires, Argentina.

Department of Pharmacology and Toxicology, Centre Hospitalier Universitaire à Limoges, Limoges, France (Dr. Woillard).

Basic Science-Mathematics, Universidad Tecnológica Nacional, Buenos Aires, Argentina (BSc Chan).

Pharmacology Department, University of Buenos Aires, Argentina (Dr. Buendia).

National Scientific and Technical Research Council, CONICET, Buenos Aires, Argentina (Dr. Schaiquevich).

Corresponding author

Paula Schaiquevich

Unit of Clinical Pharmacokinetics, Hospital de Pediatría J.P. Garrahan,

Combate de los Pozos 1881, C1245AAL, Buenos Aires, Argentina.

Email: paula.schaiquevich@gmail.com

Tel.: 54-11-4122-6000 (ext 6532).

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Abstract

Background: Despite advances in surgical procedures and the optimization of immunosuppressive therapies in pediatric liver transplantation, acute rejection (AR) and serious adverse drug reaction (ADR) to tacrolimus still contribute to morbidity and mortality. Identifying risk factors of safety and efficacy parameters may help in optimizing individual immunosuppressive therapies. This study aimed to identify peritransplant predictors of AR and factors related to the risk of ADR to tacrolimus in a large Latin-American cohort of pediatric liver transplant patients.

Methods: We performed a retrospective cohort study in a pediatric liver transplant population (n=72). Peritransplant variables were collected retrospectively including demographic, clinical, laboratory parameters, genomic (CYP3A5 donor and recipients polymorphism) and tacrolimus trough concentrations (C0) over a 2-year follow-up period. Variability in tacrolimus C0 was calculated using %CV and tortuosity. ADR and AR-free survival rates were calculated by using the Kaplan-Meier method, and risk factors were identified by multivariate Cox regression models.

Results: Cox proportional hazard models identified that high tortuosity in tacrolimus C0 was associated with an 80% increased risk of AR (Hazard ratio, HR, 1.80; 95% Confidence Interval (CI), 1.01-3.22; $p<0.05$), while steroid in maintenance doses decreased this risk (HR, 0.56; 95% CI, 0.31-0.99; $p<0.05$). Forty-six patients experienced at least one ADR including hypomagnesemia, nephrotoxicity, hypertension, malignancies, and tremor as a first event. Multivariate analysis showed that C0 values 10 days before the event (HR, 1.25; 95% CI, 1.21-1.39; $p<0.0001$) and CYP3A5 expresser recipients (HR, 2.05; 95% CI, 1.03-4.06; $p<0.05$) were independent predictors of ADR.

Conclusions: Tacrolimus C0 values, its variability, and CYP3A5 polymorphisms were identified as risk factors of AR and tacrolimus ADR. This knowledge may help to control and reduce their incidence in pediatric liver transplant patients. Prospective studies are important to validate these results.

Keywords: tacrolimus; adverse drug reactions; acute rejection; multivariate analysis; pediatric liver transplant.

Introduction

Optimal immunosuppressive therapy is a delicate balance between transplant rejection and associated adverse drug reactions (ADR)[1,2]. Tacrolimus has become the cornerstone in immunosuppression and is currently combined with mycophenolate mofetil and steroids, with or without the addition of an induction agent, to avoid acute rejection (AR) in pediatric patients with liver transplantation[3]. Notably, underexposure to tacrolimus may result in low immunosuppression leading to AR. On the contrary, tacrolimus overexposure puts patients at risk for life-threatening toxicity including severe infections, hypertension, renal dysfunction, post-transplant lymphoproliferative disease (PTLD), neurotoxicity, and diabetes [4-6]. Monitoring the safety of medicines, including a thorough analysis of reported ADR plays a role in defining pediatric medicines development[7, 8]. ADRs cause significant patient morbidity and mortality counteracting the improvements in transplant surgical procedures [4, 9]. Thus, therapeutic drug monitoring (TDM) is routinely performed for adjusting tacrolimus individual requirements to account for the high variability in its pharmacokinetics [10, 11].

Despite close individual monitoring, ADR and AR episodes are still detrimental to the patient´s quality of life. Limited information is available in the literature regarding the causes of inter-individual variability in tacrolimus pharmacodynamics in pediatric patients, and particularly in Latin-American liver transplant patients [12, 13]. Specifically, identification of risk factors for AR and ADR to tacrolimus may help in reducing the frequency of complications after liver transplantation, and hence minimize the risk of graft loss, non-compliance, and death [14]. Peritransplant factors including demographic, clinical, laboratory parameters, CYP3A5 genotype and tacrolimus exposure have been reported to impact immunosuppressive treatment efficacy and toxicity [15-18]. However,

findings have been inconsistent, and specifically, no genetic markers reliably predict the development of AR or tacrolimus ADR in pediatric liver transplant patients [18].

In this context, we conducted a combined analysis to describe the factors affecting both outcomes in pediatric liver transplant patients, which may help in optimizing individual immunosuppressive therapies, and ultimately, prolong patient and graft survival. For all mentioned, the aim of this study was to identify peritransplant predictors of AR and factors related to the risk of tacrolimus ADR in pediatric liver transplant patients.

Materials and Methods

The present study is a retrospective, single-center cohort study that was conducted at Hospital de Pediatría JP Garrahan (Buenos Aires, Argentina). The study was approved by the institutional review board (Protocol #740). Written informed consent was obtained from parents or guardians.

Study population

The present study was originally intended to evaluate a new immunosuppressive protocol in our hospital including the utilization of induction therapy to minimize the administration of steroids in pediatric patients who received the first liver transplantation. This study is comprised of patients included in a previous report[13]. Briefly, all children less than 18 years old at the time of transplant that received the first liver allograft at Hospital de Pediatría JP Garrahan (Buenos Aires, Argentina) between January 2010 and July 2012 were included. Hospital JP Garrahan is a tertiary-care center for pediatric patients with complex diseases and the leading center for pediatric liver transplant in Argentina. Exclusion criteria consisted of patients with less than 1

month of survival, re-transplant, combined or multi-visceral transplants and patients without appropriate follow-up or non-compliant patients as previously defined[13]. Follow-up information was collected for 2 years after transplantation for each included patient. All data were collected from the medical records, and a centralized database with restricted access was generated. Eligible patients received a unique identification number.

Immunosuppression

Immunosuppression consisted of a low-dose tacrolimus scheme and induction therapy as part of an immunosuppressive protocol implemented in 2010 as described elsewhere[19-22]. Tacrolimus (0.1 mg/kg/day) was initiated 24 hours after reperfusion, administered in monotherapy, in association with steroids and/or with antimetabolites. Mycophenolate mofetil (MMF) was added in those cases in which tacrolimus reduction was necessary, at a dose of 20-40 mg/kg/day [23]. Induction therapy was provided depending on the availability of basiliximab at the clinical center; it was administered at 10 to 20 mg doses at days 0 and 4 after transplantation. Concomitant drugs during maintenance treatment (30 days post transplantation) were sulfamethoxazole-trimethoprim, magnesium supplements, omeprazole (in all patients), acyclovir, and additional antibiotics, as needed.

Cases of biopsy-proven AR were treated with steroid pulse therapy consisting of 3-day methylprednisolone (10 mg/kg/day,i.v.) and 30%-50% increased dose of tacrolimus followed by weekly controls [21]. For oral steroid maintenance treatment, prednisone dose was administered and decreased to 1.25 mg/kg/day, at the discretion of the treating physician [21, 24].

Outcomes and variables

Data were collected retrospectively from the medical records, and the events of interest included biopsy-proven AR informed by histopathology and tacrolimus-related ADR. AR was listed as occurrence of acute cellular rejection requiring specific treatment at any time after transplant. Liver biopsies in the study population were performed in the context of clinical suspicion of AR. Rejection was suspected when a 50% or greater increase in liver enzyme activities was observed and was graded as mild, moderate, or severe depending on the Banff classification severity [25].

Adverse events are defined as all the events observed during drug exposure, whereas ADR imply a causal relationship to the drugs[26]. The most frequent and severe ADR to tacrolimus were recorded, including hypomagnesemia, nephrotoxicity, hypertension, PTLD, and tremor [4, 6, 27, 28]. We registered tacrolimus ADR in line with previous definitions[6, 13], in agreement with the Medical Dictionary for Regulatory activities (MedDRA)[29, 30] and organized according to the System Organ Class (SOC) classification and preferred terms. Before searches were performed, a detailed medical understanding of the ADR was conducted. Clinician's judgment remains the first and indispensable step to identify and assess an ADR. Therefore, ADRs were discussed in the weekly multidisciplinary meetings of the Department of Liver Transplantation, and ADR diagnoses were confirmed by the physician in charge after excluding other clinical or pharmacological causes and drug–drug interactions with azoles, macrolides, and calcium channel blockers (nifedipine) or anticonvulsants (phenobarbital and phenytoin). ADRs were evaluated using the Adverse Drug Reaction Probability Scale (Naranjo) [31]. Incidence of ADR and AR was calculated as the ratio between the number of first cases (ADR or biopsy-proven AR) and the initial population exposed to tacrolimus.

Several peritransplant and post-transplant variables were studied including *demographic characteristics*: age, weight at transplant, sex, and primary diagnosis; *transplant features*: type of graft (partial graft from a living or deceased donor vs. a whole graft from a deceased donor), type of donor (deceased vs. living donor), and days post transplantation; *biochemical values*: magnesemia, hemoglobin, hematocrit, albumin, serum creatinine, uremia, total bilirubin, liver function tests as Aspartate Aminotransferase (AST), Alanine transaminase (ALT), alkaline phosphatase (ALP), and Gamma-glutamyl transpeptidase (GGT) activities; *clinical status*: Epstein bar virus (EBV) and cytomegalovirus (CMV) infections, sepsis, and death; *genotyping*: CYP3A5*3 polymorphism in donors and recipients[32]. In addition, we registered concomitant immunosuppressive agents such as induction administration with basiliximab, steroid maintenance (at least 30 consecutive days), azathioprine, MMF, and sirolimus. Other concomitant drugs registered were azoles, macrolides, anticonvulsants, and calcium channel blockers.

Regarding tacrolimus pharmacology**,** the dose and the dose-normalized trough concentrations (C0/D) were evaluated. Both the median value of tacrolimus trough concentrations in the 7 to 10-day window time prior to the occurrence of an ADR or AR and the median value during the last month of follow-up for those patients who did not present an event of interest were calculated. Furthermore, different measures of tacrolimus C0 variability were obtained and described as follows:

a) Tortuosity was defined as the ratio between the lengths of the observed values and the straight line that joined the initial and final observation obtained from the collected C0. The value expressed as the median of tortuosity (tortuosity = 1.10) was used as the

cutoff point to categorize the population in high tortuosity (≥1.10) and low tortuosity (<1.10). Tortuosity has been broadly used to represent variability in clinical studies [33,34]. b) Percent coefficient of variation (%CV).

Tacrolimus monitoring and CYP3A5 genotyping

Tacrolimus trough concentrations were quantified using the chemiluminescent microparticle immunoassay (Architect® Abbott, Chicago, IL, USA). Whole-blood quality controls (Lyphochek® Whole Blood Immunosuppressant, Bio-Rad, Irvine, CA, USA) were daily assessed for assay acceptance. In addition, specimens were routinely assessed as part of an international proficiency testing program for the external quality control of tacrolimus[35]. Total imprecision was less than 8% and quality control values lied in the range of +/- 2 SD. Subsequently, tacrolimus doses were adjusted according to trough blood levels, liver and kidney function, and EBV/CMV viral load. Tacrolimus C0 target levels in the first 6 months were 7-8 ng/ml, during the next 6 months 5-7 ng/ml, and 5 ng/ml after the first year post transplantation.

CYP3A5 (CYP3A5*1/*1, CYP3A5*1/3 or *3/*3 genotypes) polymorphisms were assessed in post-transplant liver biopsies (donor tissue) from transplant recipients. Genomic DNA extraction from blood and formalin-fixed, paraffin-embedded (FFPE) samples was carried out by QIAamp DNA Blood Mini and QIAamp DNA FFPE kits (QIAGEN, Hilden, Germany). CYP3A5*3 polymorphism was detected by PCR using specific primers and direct sequencing (ABi3500, Applied Biosystems, Foster City, CA, USA).

Sample size

The minimum sample size to detect the difference between groups of patients with and without AR or tacrolimus ADR was estimated with 80% power, a significance level of 0.05, a 20% effect size based on clinical criteria, and a proportion of 0.6 of patients with AR or ADR. Thus, the minimum sample size would be 50 patients with at least 25 developing an AR or developing a confirmed tacrolimus ADR.

Statistical analysis

The influence of factors on the first development of AR and the first development of ADR was studied using univariate unadjusted Kaplan-Meier log-rank test. Factors significant at a p-value of 0.2 in the univariate analysis, clinical relevance, and biological plausibility were tested in the multivariate model. Multivariate Cox proportional-hazards regression models were obtained by a stepwise forward approach followed by a backward elimination procedure to obtain those risk factors that were significant at a p-value of <0.05. Hazard ratios (HRs) <1 and >1 were considered as significant protective and risk factors, respectively. We tested age as a potential confounding factor in the multivariate model. Moreover, interactions between variables in multivariate analyses were tested using the Chi Square Test.

The proportionality criteria of the final models were verified using the Martingale residue method.

To determine the predictive power of the variables, receiver-operating characteristic (ROC) curves were developed. The area under the receiving operating characteristic curve (AUROC) was considered a useful predictor at values greater than 0.7. The

sensitivity and specificity were defined with the cutoff value that showed the highest sensitivity with the lowest "1-specificity" values.

Hardy–Weinberg equilibrium was assessed using the Fisher exact test in the "SNPassoc" package (R package version 1.9-2)[36]. The most probable CYP3A5 haplotype in each DNA sample was inferred using the haplo.stat R package (R package version 1.7.7[36].

Statistical analysis and graphs were performed using RStudio Version 0.99.486 – © 2009-2015, Inc.[36].

Results

In total, 89 patients were considered for inclusion. Patients were excluded due to a survival shorter than 1 month (n=5), unavailable medical records (n=4), re-transplantation during the first month post-surgery (n=2), and non-adherence as previously defined (n=6) (**Figure 1**). Therefore, 72 patients were finally included in the analysis. The demographics, laboratory parameters, and clinical characteristics of the included patients are shown in **Table 1**.

From the total study population, 56 recipients and 58 donors were genotyped for CYP3A5 polymorphisms as 16 and 14 genotyping data from recipients and donors, respectively, were missed due to limited amount of DNA or not available FFPE liver tissue (**Figure S1, http://links.lww.com/TDM/A245**). Distribution of genotype of CYP3A5 by recipient-donor combination is depicted in **Table S1, http://links.lww.com/TDM/A245**. The genotype frequencies of the CYP3A5 polymorphism did not deviate from Hardy–Weinberg equilibrium ($p>0.5$).

Tacrolimus ADR and AR experienced by the included patients as first event related to their time of presentation are shown in **Table 2**. The most frequent ADR were hypomagnesemia and nephrotoxicity, which mainly developed during the first month after liver transplantation. The observed ADR incidence was comparable to that previously reported by others, also in pediatric liver transplant patients [27]. In addition, the 12-month AR-free survival in the study group was 41.4% (95% CI, 30.1-53.1%), comparable to that reported in a pediatric liver transplant population in North America[37].

Factors linked with AR

A total of 47 AR episodes were registered in the follow-up period. Of the potential risk factors for rejection analyzed in the unadjusted univariate model, factors significant at a p-value of 0.2 and clinically and biologically plausible were tacrolimus C0 in the 7 to 10-day window before the onset of AR, tacrolimus C0 high tortuosity (≥1.10), and concomitant administration of steroids in maintenance doses (**Table S2, http://links.lww.com/TDM/A245).** Unadjusted Kaplan-Meier curves for AR-free survival according to the use of steroids and tacrolimus C0 tortuosity are depicted in **Figure 2A and 2B,** respectively. Nonetheless, no significant relationship between the risk of AR and patient/donor CYP3A5 genotype, %CV in tacrolimus C0 levels, patient age at transplant, body weight, and induction treatment with basiliximab could be identified (p>0.05).

The final multivariate Cox model showed significant associations between AR and the administration of steroids in maintenance doses (yes *vs* no: HR, 0.56; 95% CI, 0.31-0.99; p=0.049) and the tacrolimus concentration variability expressed as high tortuosity

(high tortuosity *vs* low tortuosity: HR, 1.80, 95% CI, 1.01-3.22; p=0.046) (**Table 3**). When controlling for age as a potential confounder, the relationship between steroids and tortuosity with development of AR remained essentially the same as the one observed in the final model.

Finally, the incidence rate of AR in the time post transplantation was depicted in **Figure 3A** according to the different scenarios presented as follows:

a) Without steroids and low tortuosity

b) Without steroids and high tortuosity

c) With steroids and low tortuosity

d) With steroids and high tortuosity

Steroids reduced the AR risk, while tortuosity increased it. The most unfavorable situation (b) presented an almost doubled incidence rate of AR compared to the most favored group (c) during the first 3 months after transplantation (**Figure 3A**). Although there was no significant difference in AR incidence in the first 3 months post transplantation, there is a clear trend in the effect of steroids and tortuosity on AR. For all the groups, the incidence of AR declines after the first 3 months. Moreover, no significant difference in AR rate among groups was observed between 3 and 24 months post transplantation.

Factors related to tacrolimus ADR

A total of 46 ADR as first event were observed during the study period. Of the potential risk factors for ADR analyzed in the univariate model, significant associations were observed with tacrolimus C0 in the 7 to 10-day window before the onset of the ADR, recipient CYP3A5 polymorphism, %CV of tacrolimus C0, patient age, concomitant

administration of immunosuppressive drugs, patient body weight, and high tortuosity (**Table S2, http://links.lww.com/TDM/A245**). All significant factors identified in the univariate analysis increased the risk of the development of ADR except for the use of concomitant immunosuppressive drugs, such as MMF, azathioprine, or sirolimus, which reduced the risk (**Table S2, http://links.lww.com/TDM/A245**).

Factors that were independent predictors of tacrolimus ADR identified and retained on the multivariate analysis included tacrolimus C0 in the 7 to 10-day window prior to the event (HR, 1.25; 95% CI, 1.12-1.39; p<0.0001) and the recipient polymorphism of the CYP3A5 (expressers *vs* non-expressers: HR, 2.05; 95% CI, 1.03-4.06; p=0.041) (**Table 3**). When controlling for age as a potential confounder, the relationship between recipient CYP3A5 expression and tacrolimus exposure with development of ADR remained essentially the same as the one observed in the final model.

Taking into account the association between tacrolimus C0 values and the incidence of tacrolimus ADR, a threshold was estimated using ROC analysis. Interestingly, a value of tacrolimus C0 in the 7 to 10 days prior to the event higher than 7 ng/ml best described the population at risk of ADR (AUROC=0.80; 95% CI, 0.69-0.91) (**Figure 4A**).

ADR-free survival was significantly lower in the CYP3A5 expresser group as depicted in the unadjusted Kaplan-Meier curve (**Figure 4B)**, which was supposed to receive higher tacrolimus doses secondary to a higher clearance. In relation to this finding, recipients CYP3A5 expressers required a median (range) tacrolimus dose 33% (4-56) higher than non-expressers depending on the post-transplant period. In **Figure 5A and 5B**, tacrolimus doses and dose-normalized tacrolimus C0 of CYP3A5 expressers (CYP3A5*1/*1 and CYP3A5*1/*3 patients) and CYP3A5 non-expressers

(CYP3A5*3/*3) are shown as geometric means with 95% CI for each period after liver transplantation up to 2-year follow-up. As expected, tacrolimus doses were significantly higher in recipient CYP3A5 expressers than non-expressers in most time periods ($p<0.05$), and therefore, a higher requirement is associated with a higher risk of ADR. Tacrolimus C0 in the 7 to 10-day window prior to the ADR was categorized as high exposure (≥7ng/ml) and low exposure (<7ng/ml) to tacrolimus based on the median value. Therefore, according to the expression of recipient CYP3A5 and tacrolimus exposure, the incidence rate of ADR was depicted for each of the four possible situations (**Figure 3B**):

a) Non-expresser recipients and low exposure

b) Non-expresser recipients and high exposure

c) Expresser recipients and low exposure

d) Expresser recipients and high exposure

Although there was no significant difference in the incidence rate among the groups, there is a clear trend in the effect of CYP3A5 and tacrolimus C0 concentrations on tacrolimus ADR. Both factors increased the risk of ADR development; therefore, the most unfavorable situation (d) presented almost five times the incidence of ADR compared to the most favorable group (a) during the first 3 months post liver transplantation as shown in **Figure 3B**. Detailed ADR are depicted in **Table S3, http://links.lww.com/TDM/A245**.

Discussion

The present analysis represents the largest study of factors associated with the development of AR and tacrolimus ADR in pediatric liver transplant patients in Latin America. Besides the identification of significant factors associated with AR and ADR, several important observations have been made in this study that could contribute to prevent these events, which increase morbidity and mortality of pediatric liver transplant patients.

Long-term evidence to date in children with liver transplantation suggests that tacrolimus is effective in preventing acute and chronic rejection[4]. AR is a common complication, occurring in as many as half of the pediatric liver transplant patients within the first six postoperative weeks [38, 39]. Therefore, identifying risk factors in association with AR is important to control its incidence and to increase AR-free survival.

Risk factors for AR

In our study, steroids have a significant negative association with AR, while high variability in tacrolimus trough concentrations presented a positive association with AR. Specifically, patients who received immunosuppressive therapy with steroids secondary to renal impairment, elevation of hepatic enzymes or other medical conditions, showed a significantly decreased risk of AR with respect to patients without steroid therapy. Since 1980, oral prednisone and prednisolone constitute the backbone of most induction and maintenance immunosuppressive regimens in solid organ transplantation to avoid AR[40,41]. Steroid exposure has been shown to determine therapy efficacy, as evidenced by corticosteroid withdrawal studies where transplant rejection became more likely in

patients in whom prednisolone/prednisone was minimized or removed [42, 43]. New protocols of immunosuppression in pediatric transplant patients consist of corticosteroid withdrawal or even complete avoidance of its administration in an effort to reduce the potential risk of adverse events, namely reduced growth rate related to long-term steroid therapy[21, 44]. Despite many studies presenting a comparable AR-free survival rate between adult liver transplant patients with steroids and with steroid minimization or avoidance without basiliximab, scarce studies in pediatric liver transplant patients are available [21, 40, 44] and further studies are needed to determine the final role of steroids in this clinical setting.

Fluctuations in tacrolimus blood concentrations over time may result in both excessive and insufficient immunosuppression [16, 45]. In the included cohort of patients, tacrolimus trough level variability expressed as tortuosity was a risk factor for the development of AR. Our results are consistent with previous reports that showed an association between high standard deviation values in tacrolimus C0, the increased risk of AR and graft failure in pediatric solid organ transplant patients[46]. Moreover, in a pediatric renal transplant population, it was shown that patients with late AR presented higher percent coefficient variation (%CV) of tacrolimus C0 levels than those free of the event. Interestingly, tortuosity is proposed by our group as a new parameter to describe the variability in C0 levels related to the incidence of AR.

The high AR rate described by our study was intensively discussed by the transplant team at our institution. Previous results reported by Ng et al. [37] in an American pediatric liver transplant population also described a comparable AR incidence. Nonetheless, we wanted to evaluate the factors that may explain this high rate. It has been previously reported a significant association between the risk of AR and the administration of

induction treatment [15, 17, 44, 47], age and primary diagnosis [15] in pediatric and adult liver transplant patients. These factors were analyzed in our study population, but no significant association was found. The final multivariate model included in the present analysis retained administration of steroids as a protective factor and tacrolimus trough concentrations variability as a risk factor for AR. By identifying these both covariates as significantly related to AR development, currently the multidisciplinary team of liver transplantation at our hospital is actively working on controlling tacrolimus variability and optimizing immunosuppressive therapy so as to prevent AR. Further studies are being carried out for reassessment of the rate of AR in accordance with all the variables considered in this study, with special emphasis on steroids administration and tacrolimus variability.

Some of the risk factors associated with AR reported elsewhere were not found to be significant in our multivariate analysis. In consistency with the results presented here, other authors did not observe an association between the expression of CYP3A5 and biopsy-proven AR [48].

Conflicting data with regard to the association between tacrolimus blood concentrations and AR in adult liver transplant patients have been reported [49, 50]. In our study, tacrolimus C0 levels in the 7 to 10-day window before the onset of AR was not retained in the final model, in line with previous results [51].

Immunosuppression with interleukin-2 receptor antagonist antibodies is accepted in adults, and its first use in pediatric solid organ transplantation has yielded remarkable results[17, 44]. The incidence of AR has been shown to be significantly lower in pediatric liver transplant recipients receiving induction therapy compared to those free of basiliximab [22]. Nonetheless, it was not possible to demonstrate a significant protective

effect of basiliximab against the development of AR in the present study population. A possible explanation may be related to differences between the immunosuppressive regimen used by others and ours [17, 22]. Altogether, the administration of induction therapy should be reconsidered as part of the immunosuppressive protocol in our hospital, and further studies are encouraged to be performed to confirm the role of basiliximab.

Organ shortage has become a problem that has triggered the development of innovative surgical techniques, such as the split liver method and the use of living donors to try to alleviate this problem and expand donor supply. Our study showed no significant association between the type of donor (deceased *vs*. live related) or type of graft (complete *vs*. technical variant) and AR-free survival, which means that donor supply possibilities are wider.

Risk factors for tacrolimus ADR

The potent immunosuppression provided by tacrolimus and its specific side effects influences the long-term patient and graft survival [1]. The most frequent ADR including nephrotoxicity and hypomagnesemia developed during the first month after liver transplantation. A positive association was observed between recipient CYP3A5 expression and tacrolimus C0 levels in the 7 to 10-day window with the incidence of ADR in line with previous reports [27, 52].

There is controversy regarding the relationship between CYP3A5 genotype and the risk of tacrolimus ADR. Interestingly, some authors have not found a significant association between CYP3A5 genotype and nephrotoxicity [53] or even found a lower risk associated with the CYP3A5*1 allele [54]. On the other hand, others described a higher risk of

449 histologically confirmed chronic tacrolimus nephrotoxicity in CYP3A5*1 carriers than
450 in non-expressers [55]. CYP3A5 expressers may produce more metabolites than non-
451 expressers with nephrotoxic effects, increasing the incidence of tacrolimus ADR events
452 as observed in our study [52, 55]. Specifically, CYP3A5 expression augments intestinal,
453 renal, and liver tacrolimus clearance and reduces its bioavailability. In CYP3A5
454 expressers, higher doses of tacrolimus are required to achieve blood concentrations
455 within the therapeutic range. In consequence, as described by others, dose-normalized
456 tacrolimus C0 levels were significantly lower in patients expressing CYP3A5 (either
457 donor or recipient) than non-expressing transplant patients [48]. In agreement with data in
458 pediatric renal transplant patients [56], we found that CYP3A5 recipient expressers require
459 a median dose 33% higher than non-expressers to obtain an adequate trough
460 concentration.
461 Most frequent ADR described in this study were related to tacrolimus C0 concentrations
462 7 to 10 days prior to those endpoints as reported by others [49, 57] reinforcing the role of
463 TDM in the individualization of immunosuppressive therapy. Based on ROC analysis,
464 tacrolimus levels higher than 7 ng/ml predict the development of ADR, a close value to
465 that previously reported by Staatz et al., who suggested a target tacrolimus C0 of 6
466 ng/ml to minimize toxicity [27].
467 Some of the risk factors that have been cited in the literature were not found to be
468 significant in our multivariate analysis but were observed as significant factors
469 identified in the univariate analysis and are worth discussing. Previously, we observed
470 that younger patients (under 1.3 years old) presented lower risk of ADR than older
471 patients. Young children have a lower tacrolimus bioavailability secondary to a higher
472 hepatic drug metabolism and an increased intestinal first-pass metabolism that decreases

with age [58, 59]. Despite age not being included in the final multivariate model, as we informed before [13], younger children have lower incidence of ADR in our study and this should be considered in future studies in order to confirm the effect of this factor on tacrolimus ADR.

We observed that concomitant immunosuppressive drugs were negatively associated with the occurrence of ADR, and this is in accordance with a synergistic immunosuppressive effect, which allows lower tacrolimus dosages.

Finally, there is a good agreement in the trend between incidence of AR, ADR and significant risk and protective factors retained in the present Cox-proportional hazard model. This information is important in designing programs toward management of tacrolimus ADR and AR.

Our study has certain limitations. First, this study has the same limitations that apply to all retrospective descriptive studies and has to be acknowledged. Second, the area under the curve (AUC) of tacrolimus blood concentration *vs*. time is the best marker of systemic exposure to tacrolimus[60]. However, in pediatrics, tacrolimus therapeutic monitoring is based on monitoring trough concentrations (C0)[11]. Third, regarding the analytical assay for tacrolimus quantitation in blood samples, we have to acknowledge the limitation of working with an immunoassay due to cross-reactions with tacrolimus metabolites. Mass spectrometric methods are only available in a limited number of private clinical centers as shown in a national survey conducted by our group, and thus, immunoassays play a major role in the routine analysis of immunosuppressant drugs in our country[61]. Despite previous reports showed a significant correlation between chemiluminescent microparticle immunoassay (CMIA) and liquid chromatography tandem mass spectrometry (LC-MS/MS) methods, the substantial cross-reactivity of

CMIA with active and non-active tacrolimus metabolites may account for a positive bias of the immunoassay method to LC-MS/MS. The lack of analytical specificity of CMIA may be of particular importance in transplant patients with liver dysfunction (e.g., severe cholestasis) that have shown an over-proportional increase in the concentration of certain metabolites with respect to the tacrolimus trough concentrations[62]. Thus, our results must be interpreted with caution. We also have to acknowledge that Naranjo algorithm is not specific for immunosuppressive therapy and transplant patients. There are multiple methods for assessing the causality of suspected ADR[63]. Although all published algorithms are operational and relatively easy to use, none has been universally accepted as a gold standard [63, 64] due to well-known limitations[63, 65 -67]. However, Naranjo algorithm remains commonly used for the cause-effect assessment of suspected ADR in case reports and observational studies perhaps due to its relatively simple application [68-71]. We also acknowledge that despite all efforts, some tacrolimus ADR could have been missed or rejected due to misinformation. For instance, diabetes post transplantation was not confirmed by our clinical reports. Finally, the brand of tacrolimus (Prograf®, Astellas Laboratory, Ireland or Tacrolimus Sandoz®, India) that patients received after July 2013 depended on the provision of the National Organ Procurement Program. This Program supplies, free of charge, to those patients who are in need, with the immunosuppressant drug product depending on the winning bid established by the government. Although we do not have the reliable registry of the brand that patients received, tacrolimus TDM in pediatric transplant patients undergoing immunosuppressant substitution is essential to ensure safety and efficacy of the immunosuppressive treatment, as previously reported for this population[72]. All patients had close clinical and pharmacological monitoring of

tacrolimus, with dosage adaptations when required to ensure similar exposure if substitution occurred.

Conclusion

In conclusion, the present study is the first that identifies factors related to the safety and efficacy of immunosuppressive treatment with tacrolimus in the largest cohort of pediatric liver transplant patients in Latin America. Since tacrolimus remains as the cornerstone of immunosuppressive treatments, the optimization of the therapy with this calcineurin inhibitor is of great importance [1]. Therefore, Cox models were built to explain the development of AR and ADR using predictor variables. Further studies in larger cohorts of pediatric patients should validate the present observations.

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Figure legends

Figure 1. Flow diagram detailing inclusion and exclusion criteria for pediatric liver transplant patients.

Figure 2. Unadjusted Kaplan-Meier curves for acute rejection-free survival according to (A) the use of steroids and (B) tortuosity in tacrolimus trough levels.

Figure 3. Incidence of acute rejection according to the administration of steroids and the tortuosity (A) and incidence of adverse drug reactions according to the recipient CYP3A5 expression and tacrolimus C0 values (B).

Abbreviations (A) ■ without steroids and high tortuosity; ▼ with steroids and high tortuosity; ● without steroids and low tortuosity; ▲ with steroids and low tortuosity; (B) ▼ recipient CYP3A5 expressers and tacrolimus C0 ≥7 ng/ml; ■ recipient CYP3A5 non-expressers and C0 ≥7 ng/ml; ▲ recipient CYP3A5 expressers and C0 <7 ng/ml; ●: recipient CYP3A5 non-expressers and C0 <7 ng/ml.

Figure 4. Receiver operating characteristic (ROC) curve for tacrolimus C0 values (A) and Kaplan-Meier curve for tacrolimus drug reaction-free survival according to the recipient CYP3A5 genotype (B).

Figure 5. Dose (A) and dose-normalized tacrolimus trough concentrations (B) of CYP3A5 expressers (CYP3A5*1/*1 and CYP3A5*1/*3 individuals) and non-expressers (CYP3A5*3/*3).

Abbreviations. C0: tacrolimus trough concentrations (ng/ml).

Values are expressed as geometric means. The error bars represent the corresponding 95% confidence intervals. *p<0.05

Table 1. Demographics and relevant medical history (n=72)

Characteristic/Parameters	**Results**
Total subjects	72
Age (years)[a]	5.3 (5.4)
Gender (females/males)	45/27
Weight (kg)[a]	21.0 (18.9)
Type of donor (deceased/living)	53/19
Follow-up time (months)[a]	20.1 (7.8)
Graft type: complete/technical variant	26/46
Primary diagnosis	**Number (%)**
Biliary atresia	29 (40.3)
Fulminant liver failure	14 (19.4)
Cholestaticcirrhosis[b]	7 (9.7)
Hepatic cirrhosis: autoimmune and cryptogenic	12 (16.6)
Malignancies[c]	7 (9.7)
Metabolic diseases: Metabolic Liver Failure	3 (4.2)
Immunosuppressive therapy	**Number (%)**
Basiliximab	52 (72)
Tacrolimus	72 (100)
Prednisone (1.25-3.75 mg/kg/day)	40 (56)
MycophenolateMofetil (20-40 mg/kg/day)	34 (47)
Azathioprine	6 (8)
Sirolimus	5 (7)

Death	5 (7)
Liver function and blood parameters	**Mean (SD)**
AST (UI/L)	92.9 (176.5)
ALT (UI/L)	127.0 (171.8)
GGT (UI/L)	233.1 (280.2)
Total bilirubin (mg/dl)	2.1 (4.6)
Direct bilirubin (mg/dl)	1.8 (4.1)
Albumin (g/dl)	3.6 (0.7)
Hematocrit (%)	31.7 (4.9)
Renal function	
Serum Creatinine (mg/dl)	0.4 (0.2)
Urea (mg/dl)	40.4 (24.4)

[a] Data are expressed as mean (standard deviation).

[b] Including Alagille syndrome, congenital hepatic fibrosis, sclerosing cholangitis.

[c] Including hepatoblastoma and hepatocellular carcinoma.

Table 2. Tacrolimus adverse drug reactions and acute rejection incidence with the observed time of presentation in the study population

Adverse drug reactions	**Incidence (%)**	**Time of presentation, days Median (range)**
All first adverse drug reactions	46 (64)	26 (4-540)
Hypomagnesemia	29 (40.3)	26 (6-187)
Nephrotoxicity	11 (15.3)	24 (9-301)
Hypertension	3 (4.2)	37 (20-99)
PTLD	2 (2.8)	403 (267-540)
Tremor	1 (1.4)	4
Acute rejection	47 (65.3)	43 (2-582)

Abbreviations: PTLD, post-transplant lymphoproliferative disease

Table 3. Multivariate analysis of risk factors for acute rejection and adverse drug reactions

	Acute Rejection (n=47)	
Factor	**Hazard Ratio (95% CI)**	**p**
Steroids (Yes *vs* No)	0.56 (0.31-0.99)	0.049
Tortuosity (high *vs* low)	1.80 (1.01-3.22)	0.046
	Adverse drug reactions (n=46)	
Factor	**Hazard Ratio (95% CI)**	**p**
CYP3A5 R (expressers *vs* non-expressers)	2.05 (1.03-4.06)	0.041
C0 (ng/ml)	1.25 (1.12-1.39)	<0.0001

Abbreviations. CI: confidence interval;CYP3A5 R: recipient CYP3A5 polymorphism; C0: median tacrolimus C0 in the 7 to 10-day windowprior to the occurrence of the adverse drug reaction or the median value during the last month of follow-up for those patients who did not present an event of interest; high tortuosity: tortuosity ≥1.10; low tortuosity: tortuosity <1.10.

Figure 1

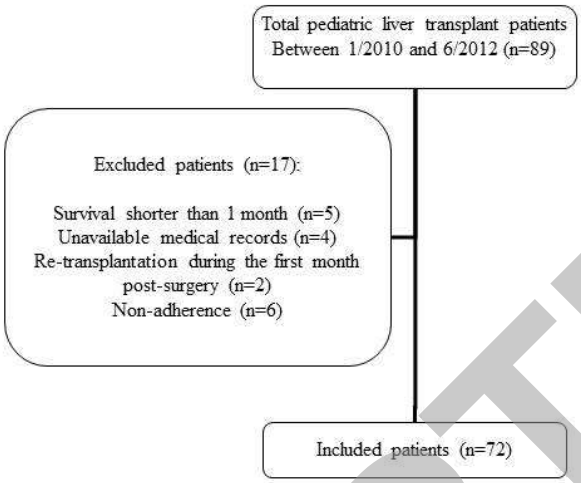

Figure 2

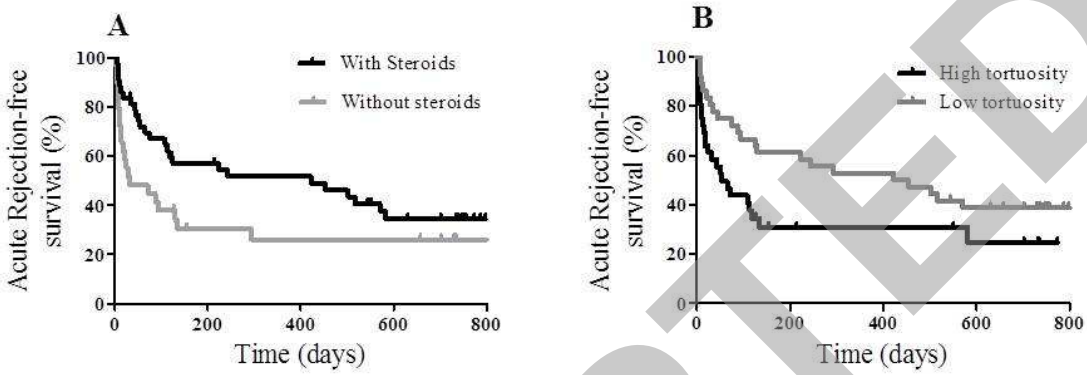

Figure 3

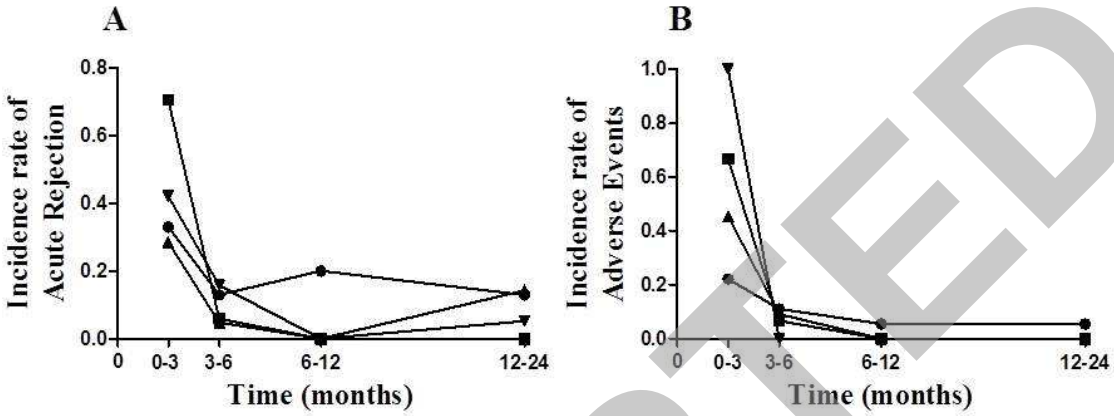

Figure 4

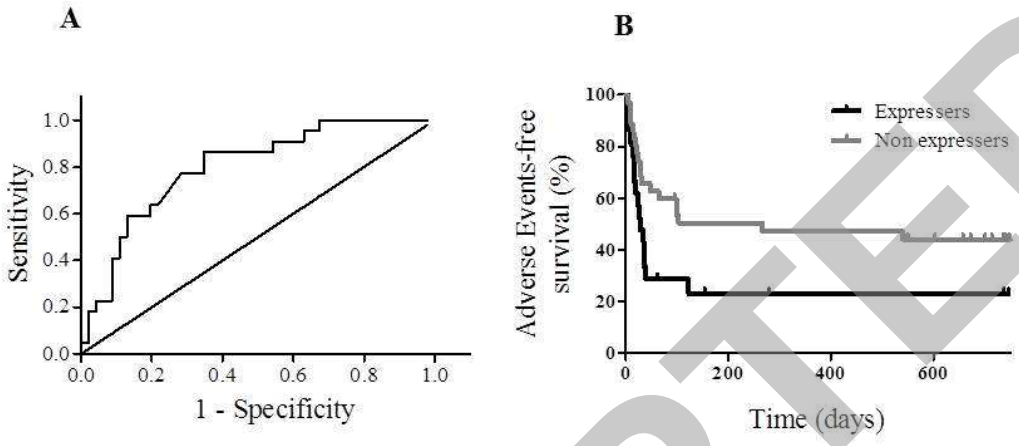

Figure 5