

**TOWARD INDIVIDUALIZATION REGIMEN OF
TACROLIMUS IN PEDIATRIC LIVER
TRANSPLANTATION**

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Pour Eléa...

« On ne force pas une curiosité, on l'éveille »

Daniel Pennac

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LIST OF ABBREVIATIONS

6-MP	6-mercaptopurine
AAG	Alpha 1-acid glycoprotein
ABCB1	ATP-binding cassette subfamily B member 1
ACMIA	Antibody-conjugated magnetic immunoassay
ACR	Acute cellular rejection
ADCC	Antibody-dependent mediated cytotoxicity
AIC	Akaïke information criteria
ALG	Antilymphocyte globulin
ALT	Alanine amino transferase
AMR	Antibody mediated rejection
AP-1	Activator protein 1
APCs	Antigen presenting cells
AST	Aspartate amino transferase
ATG	Antithymocyte globulin
AUC	Area under the concentration curve
AZA	Azathioprine
BIC	Bayesian information criteria
BOV	Between occasions variability
Bregs	Regulatory B cells
BSV	Between subject variability
BW	Body weight
C_0 or C_{min}	Trough blood levels
C_0/D	Dose-corrected trough concentration

C_0/Dose	Tac trough concentration/weight-adjusted Tac daily dose ratio
CaMK	Calcium-calmodulin dependent kinase
CEDIA	Cloned enzyme donor immunoassay
CI	Confidence interval
CKD	Chronic kidney disease
CL/F	Apparent oral clearance
CL_H	Hepatic clearance drug
CL_{int}	Intrinsic clearance
C_{max}	Peak plasma/blood concentration
CMIA	Chemiluminescence microparticle immunoassay
CNI	Calcineurin inhibitor
C_{PRED}	Predicted concentrations
CR	Chronic rejection
CR	Complement receptor
CTL	Cytotoxic T lymphocytes
CV	Coefficient of variation
CWRES	Conditional weighted residuals
CyA	Cyclosporine A
CYP	Cytochromes P450
DAG	Diacylglycerol
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DHODH	Dihydroorotate dehydrogenase
DSA	Donor specific antibodies
DTH	Delayed type hypersensitivity
EBEs	Empirical Bayes Estimates

ECLIA	Electrochemiluminescence immunoassay
E_H	Hepatic extraction ratio
ELISA	Enzyme-linked immunosorbent assay
EMIT	Enzyme multiplied immunoassay technique
ε	Residual variability
ER	Endoplasmic reticulum
EVL	Everolimus
F	Oral bioavailability
F(ab)2	Variable antigen binding region
Fc	Fragment cristallizable
FKBPs	FK506-binding proteins
Fu	Unbound fraction
GFR	Calculated glomerular filtration rate
GGT	Gamma glutamyl transferase
GW	Graft weight
η	Random effects
HLA	Human leucocyte antigen
HPLC	High performance liquid chromatography
HPLC-MS	High performance liquid chromatography with single mass spectrometry
HPLC-MS/MS	High performance liquid chromatography with tandem mass spectrometry
ICAMS	Intercellular adhesion molecules
IFN γ	Interferon γ
IIV	Interindividual variability
IMPDH	Inosine monophosphate dehydrogenase

IPRED	Individually predicted concentrations
IS	Immunosuppression
IV	Intravenous
Ka	Absorption rate constant
KCs	Kupffer cells
KT	Kidney transplantation
LAR	Late acute rejection
LC-MSMS	Liquid chromatography tandem mass spectrometry
LD	Linkage disequilibrium
LDLT	Living donor liver transplantation
LOQ	Limit of quantification
LRT	Likelihood ratio test
LT	Liver transplantation
mAb	Monoclonal antibody
MAC	Membrane attack complex
MAP	Mitogen activated protein
MEIA	Microparticle enzyme immunoassay
MHC	Major complex of histocompatibility
MMF	Mycophenolate mofetil
MoMFs	Bone marrow monocyte-derived macrophages
MPA	Mycophenolate acid
mTOR	mammalian Target of rapamycin
NFATc	cytoplasmic Nuclear factor of activated T cells
NFATn	Nuclear factor of activated T cells
NFκB	Nuclear Factor κ Light Chain enhancer of activated B cells

NK	Natural killer
NO	Nitric oxide
NONMEM	Non-linear mixed effects modelling
NPDE	Normalized Prediction Distribution Error
OBS	Observed concentrations
OFV	Objective function value
P-gp	P-glycoprotein
PBMCs	Peripheral blood mononuclear cells
PBPK	Physiologically based pharmacokinetic
PD	Pharmacodynamics
PG	Pharmacogenetics
PI(4,5) P2	Phosphatidylinositol 4,5 biphosphate
PI3K	Phosphoinositide-3-kinase
PK	Pharmacokinetics
PKC θ	Phosphokinase C θ
PLC γ 1	Phospholipase γ 1
PLDLT	Pediatric living donor liver transplantation
PLT	Pediatric liver transplantation
POD	Post-operative days
POP PK	Population pharmacokinetics
PPRED	Population predicted
PPRs	Patterns recognition receptors
PTLD	Post transplant lymphoproliferative disease
Q _H	Hepatic blood flow
QMS	Quantitative Microsphere System
RAI	Rejection activity index

RCT	Randomized controlled trial
RLT	Reduced size liver transplantation
Scr	Serum creatinine
SD	Standard deviation
SE	Standard estimates
SLT	Split liver transplantation
SNP	Single nucleotide polymorphism
SRL	Sirolimus
$t_{1/2\beta}$	Terminal elimination half-life
Tac	Tacrolimus
TBI	Total body irradiation
TCR	T Cell receptor
TDM	Therapeutic drug monitoring
TGF β	Transforming growth factor β
θ	Fixed effects
TNF	Tumor necrosis factor
Tregs	regulatory T cells
V/F	Apparent volume of compartment
VCAMS	Vascular adhesion molecules
Vd	Volume of distribution
VPC	Visual Predictive Check
WRES	Weight residuals

PART I. GENERAL INTRODUCTION

Chapter 1. STATE OF THE ART IN PEDIATRIC LIVER TRANSPLANTATION

1.1 From liver surgery to pediatric orthotopic liver transplantation

The first man who evoked the concept of liver transplantation (LT) in the literature was the French surgeon Jean-Louis Lortat-Jacob in 1952, in his report on right hepatectomy in oncologic diseases. He knew that, with knowledge of anatomy and the ability of liver to regenerate, large hepatic volume could be safely removed. He mentioned the possibility to extend the indication of hepatectomy to other liver diseases. Thus, he anticipated not only the possibility of LT, but also the possibility of reduced LT.^{1,2} A few years later, Welch experimented the first heterotopic whole LT in dog in 1955,³ while Moore realized the first canine orthotopic LT in 1958.⁴ This was followed in the early 1960s by the development and research of orthotopic LT in animals by two main schools. The first one, in Boston, directed by Moore, with his fellow Sir Roy Calne, focused on the technical aspect of LT without adjuvant treatment. The second school, guided by Thomas Starzl, moved from Chicago to Denver in 1960 to develop, with international experts, many areas of LT such as graft conservation, technical aspects, coagulation, hemodynamics and immunosuppression (IS).⁵ The first clinical attempt of LT was done by Starzl, in Denver on March 1st of 1963 in a 3 year-old child with biliary atresia (BA). Unfortunately, the team was confronted with many difficulties such as adherences from previous surgeries,

bleeding from pressured small veins and absence of clotting. Finally, the child died during the intervention, because of intractable hemorrhage.⁶ This event was followed by a few other attempts of adult LT between 1963 and 1966 in Denver, Boston and Paris, which all resulted into patients' death from few hours to 23 days after surgery.⁷ After some time spent on research about IS and preservation techniques, trials started again in 1967 in Denver, with a series of 8 pediatric patients. Four of them died from infectious complication (liver gangrene and sepsis) between 2 and 6 months, 2 patients died from recurrence of liver cancer after 1 year, 1 died because of chronic rejection and only one had a long-term survival. At that time, IS regimen was the same as used in kidney transplantation and consisted in azathioprine, prednisone and anti-lymphocyte globulin while acute rejection was treated with graft radiotherapy and actinomycin C.⁷

Between 1963 and 1976, 111 LT had been performed in America with 1-year survival being only 28% because of technical and mechanical complications (embolisms, thrombosis, graft damage, bile duct reconstruction events) and of acute rejections.⁸ Subsequently to the growing experience of LT in America, other transplantation centers developed worldwide. In Europe, Sir Roy Calne made the first attempt of LT in a 10-months old child with BA, on June 6, 1968 in Cambridge but the patient died during surgery. Three years later, on March 17, 1971, Pr Jean-Bernard Otte realized the first successful LT in Europe in a 17 months old child with BA in Leuven. The child survived the operation, experienced acute rejection that could be treated with corticosteroids but unexpectedly

died from acute hemorrhage during liver biopsy.⁶ The LT program was interrupted until 1984.

Between 1968 and 1979, the Cambridge team experienced also discouraging initial results, as was observed in Denver, with only three survivors out of the 35 transplanted patients. However, due to medical and technical improvements, overall patient survival rapidly increased both in America and UK. In Denver, the 1-year survival rate even doubled between 1976 and 1977, with 62% and 41% in adult and pediatric transplanted patients respectively.⁸

Besides the great impact of acquired experience and surgical advances, the greatest improvement in long-term patient survival occurred in the early 1980s, after the introduction of Cyclosporine A in 1979. Indeed, Sir Roy Calne, who experienced very promising results with its usage in kidney transplant patients, employed it in two liver transplant recipients.⁹ Soon after, a trial was conducted in the early 1980s in liver transplanted recipients, which showed 83% of patient survival after 1 year.¹⁰ In 1981, Starzl moved to Pittsburgh and his transplant program extended rapidly with 265 transplanted pediatric patients until 1986. During the same period, our institution realized several pediatric transplantations under the supervision of Pr Otte. At the end of 1986, a meeting of the 8 main pediatric transplant centers (Boston, Brussels, Cambridge, Dallas, Hannover, Minneapolis, Pittsburgh, Los Angeles) showed very good results of LT with a long-term patient survival (1 year) of 57-83% since the global use of cyclosporine.⁶ At that time, some centers did not consider small babies for LT because of organ shortage and greater

technical problems in children less than one year, leading to poor outcomes, which was not the case in our institution.^{11, 12}

1.2 Facing with organ shortage

With these very encouraging results, indications for LT quickly widened and the pool of adults and pediatric liver transplant candidates expanded so much that it led soon to organ shortage, especially in children due to the scarcity of pediatric cadaveric donors. Mortality while being on the waiting list reached up to 50%.¹³

To overcome this problem, the Reduced Size LT (RLT) was first introduced in Europe by Bismuth and Houssin and became soon a widely practiced technique.¹⁴ The concept of RLT was to perform liver resection on a back table, in order to adapt the size of the graft to the size of the child recipient, the rest of the liver tissue being discarded. Despite excellent results with a 1-year patient survival of nearly 80%, this technique was soon criticized because it didn't increase the total pool of recipients and thus became out of practice.⁵

To solve the main critic of RLT, Pichlmayr, in Germany, described and introduced first, in 1988, a new technique: the Split LT (SLT).¹⁵ This consist of dividing (*ex vivo* and later *in situ*) the liver graft of a cadaveric donor in two parts that can be ultimately transplanted in two patients, most often the right lobe going to an adult while the left lobe or segment is going to a child recipient.¹⁶ Initial results were deceiving in America with pediatric and adult survival of only 67% and 20% respectively.¹⁶ But the first European results showed comparable results as those observed in whole LT.^{16, 17} The method

was validated at the end of the 1990s. With its wide application, SLT had a real benefit on the waiting list and pediatric transplantation outcome.¹⁶ However, because of the heavy logistic that this technique requires, some institutions were reluctant to implement SLT as a routine.⁶

Despite the technical innovations to face the organ shortage, this latter still progressed both in USA and Europe, while Japan could not have access to cadaveric donors due to legal issues.¹⁸

Because of the great success of RLT and SLT and the feasibility of doing *in situ* liver reduction, the idea to perform living donor LT (LDLT) arise. This concept was already mentioned by Smith and experimentally performed in dogs at the end of the 1960s.^{19, 20} It became a reality in human when Raia made the two first attempts in 1989 in Brazil.²¹ After Strong in Australia,²² and Broelsch in Chicago,²³ LDLT extended worldwide, especially in Asia. At UCL, Pr Otte and his team made the first pediatric LDLT in 1993 and at the end of the 1990s, he reported actuarial patient survival of 91.8% and 89.6% at one and five years post-transplant.²⁴ These results were comparable in some other part of the world.^{25, 26} Introduction of LDLT raised particular ethical questions in the transplant community, such as donor coercion, morbidity and mortality. Therefore, the UCL team developed, such as the Chicago and other teams did some years before, an ethical protocol approved by the local ethical committee with the necessity of a signed informed consent.^{6, 27}

Living donation became a significant activity in many transplant centers over the world (11% of pediatric liver transplantations (PLT)

were LDLT between 2010 and 2012 in the US²⁸ and the principal source of pediatric liver graft in countries such as Japan²⁹ and Korea.³⁰

However, since donor potential mortality is still a matter of debate and available living donor not always accessible for each candidate, other strategies to further increase the pool of available organs have been implemented such as political campaigns to enhance post-mortem donation, the use of non-heart beating donors and ABO-incompatible liver transplantations.³¹⁻³³

1.3 Liver transplant management and current outcomes

PLT is now an established therapy for pediatric chronic and acute liver failure. Since the 2000s, excellent results in PLT have been achieved worldwide, with a 1-year patient and graft survival of more than 90% and 85%, respectively.^{34,29,35} These results are not only due to progresses in surgery and learning curve experience, but also due to the better management and care of the patients by the multidisciplinary team (pediatric hepatologists and infectiologists, transplant surgeons, pediatric nurses, pediatric intensivists and anesthesiologists, social workers, psychologists and transplant coordinators) during:

- 1) The pre-transplant assessment and the waiting time period, where the team may improve nutritional and immunizations status and provide educational, psychological and social support.^{6,36,37}

- 2) The perioperative setting, with a dedicated pediatric anesthesiology team.^{38,39}

3) The early and late post-operative care,⁴⁰ where the team may lead to a better surgical complications and pain management, more efficient immunosuppressive therapy (including introduction of Tacrolimus (Tac) and steroid withdrawal) with less toxicity and adverse effects and finally an optimization of growth and development.^{6, 36}

In conclusion, during those last 50 years, technical advances, medical progresses and growing experience allowed the PLT to become an efficient and secure therapy for children with liver disease. Consequences of such a success, was the emergence of organ shortage that was partly solved by the emergence of LDLT. In the next chapter, I will present the results of the 250 first pediatric LDLT at Université Catholique de Louvain where main indications for LT and management of patients and donors will be presented.

**Chapter 2. LIVING DONOR LIVER TRANSPLANTATION IN
CHILDREN: SURGICAL AND IMMUNOLOGICAL RESULTS IN 250
RECIPIENTS AT UNIVERSITE CATHOLIQUE DE LOUVAIN**

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2.1 Context and hypotheses of research

In this retrospective study, we aimed to review the overall results of the 250 first pediatric living donor liver transplantations at UCL, including patient and graft survival as well as surgery and immunological complications. Moreover, we wanted to test the following hypotheses:

- The portoplasty technique is a safe and efficacious method to manage portal vein hypoplasia in LDLT recipients with biliary atresia (BA),
- If preceded by a protocol aiming at decreasing the isoagglutinin's levels, ABO incompatible living donor liver transplantation can be safely performed,
- Maternal donation leads to better graft tolerance.

We first showed that LDLT, including ABO-incompatible, was a safe and efficient therapy in children with liver disease since it provided an excellent patient and graft survival at 10 years of 93% and 90%, respectively. Then, we found a statistically higher frequency of portal vein hypoplasia in BA recipients when compared to non-BA patients (58,2% versus 7,2%, $p < 0.001$). In BA patients, portoplasty was associated with the lowest complication's incidence (4,5%). Finally, regarding the immunological outcomes, non-oncological indications for LT, paternal donation and cyclosporin as the primary immunosuppressive therapy were found as risk factors for acute rejection (AR). However, the sex effect on AR was only found in BA recipients.

Living Donor Liver Transplantation in Children Surgical and Immunological Results in 250 Recipients at Université Catholique de Louvain

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Objectives: To evaluate the outcome of pediatric living donor liver transplantation (LDLT) regarding portal vein (PV) reconstruction, ABO compatibility, and impact of maternal donation on graft acceptance.

Background: LDLT and ABO-mismatched transplantation constitute feasible options to alleviate organ shortage in children. Vascular complications of portal hypoplasia in biliary atresia (BA) and acute rejection (AR) are still major concerns in this field.

Methods: Data from 250 pediatric LDLT recipients, performed at Cliniques Universitaires Saint-Luc between July 1993 and June 2012, were collected retrospectively. Results were analyzed according to ABO matching and PV complications. Uni- and multivariate analyses were performed to study the impact of immunosuppression, sex matching, and maternal donation on AR rate.

Results: Overall, the 10-year patient survival rate was 93.2%. Neither patient or graft loss nor vascular rejection, nor hemolysis, was encountered in the ABO nonidentical patients ($n = 58$), provided pretransplant levels of relevant isoagglutinins were below 1/16. In BA recipients, the rate of PV complications was lower after portoplasty (4.6%) than after truncal PV anastomosis (9.8%) and to jump graft interposition (26.9%; $P = 0.027$). In parental donation, maternal grafts were associated with higher 1-year AR-free survival (55.2%) than paternal grafts (39.8%; $P = 0.041$), but only in BA patients.

Conclusions: LDLT, including ABO-mismatched transplantation, constitutes a safe and efficient therapy for liver failure in children. In BA patients with PV hypoplasia, portoplasty seems to constitute the best technique for PV reconstruction. Maternal donation might be a protective factor for AR.

Keywords: ABO compatibility, graft rejection, living donor liver transplantation, pediatrics, portoplasty

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The first human liver transplant was performed in a pediatric recipient in 1963. Since 30 years, liver transplantation (LT) is considered as a valid therapy for children with acute or chronic liver failure.¹ The shortage of deceased organ donors can be alleviated by using alternative techniques, including split livers. The concept of using the left liver lobe from postmortem adult donor for pediatric recipient has been further extended to living donor (LD) LT and was first reported by Raia et al² in 1989. Since 1993, our center has initiated an LDLT program in children under the leadership of Jean-Bernard Otte.³ The aim of this work was to review our experience in our first 250 LDLT cases and to analyze the following research hypotheses, all related to the context of living donation: (1) in LDLT recipients, portal vein (PV) hypoplasia associated with biliary atresia (BA) can be effectively managed by the portoplasty technique; (2) ABO-incompatible LD liver transplants can be safely performed using a specific protocol aiming at lowering isoagglutinins levels preoperatively; and (3) maternal donation is associated with a better graft acceptance.

PATIENTS AND METHODS

This retrospective, observational, single-center study was conducted after approval of the Committee on Human Experimentation of the institution. Clinical, surgical, and histological data of patients were retrieved from electronic medical files. Between July 1993 (initiation of our LDLT program) and June 2012, a total of 486 pediatric (younger than 16 years) liver transplants were performed at our center, including 250 (51.4%) children who received LD liver transplants. The sex ratio (male/female) was 1.1, the median age was 1.2 years (range, 0.3–14.3 years), and median body weight was 11.9 kg (range, 4.3–65 kg). The distribution of LT according to the type of liver graft (deceased or LD) is presented in Figure 1. The median follow-up of the survivors was 7.4 years (range, 0.4–19.2 years). In the 250 LDLT recipients, the pretransplant diagnoses were as follows: BA ($n = 153$; 61.2%);

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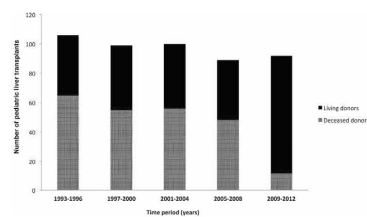


FIGURE 1. Distribution of 486 pediatric liver transplants at Cliniques Universitaires Saint-Luc, according to eras and type of liver graft donation (deceased or LDs).

hepatic malignancy ($n = 31$; 12.4%); progressive familial intrahepatic cholestasis ($n = 19$; 7.6%); metabolic disorders ($n = 18$; 7.2%); Alagille syndrome ($n = 7$; 2.8%); and other diagnoses ($n = 22$; 8.8%). In 3 different age subgroups of patients, the pretransplant diagnosis was distributed as shown in Table 1.

Presurgical Evaluation of LDs

The LDLT protocol was submitted and accepted by the Ethical Committee. The use of an extrafamilial LD was restricted to a special ethical consultation. LDs, with a median age of 33.8 years (range, 19.1–56.3 years), were mothers in 123 cases, fathers in 106 cases, uncles in 8 cases, aunts in 5 cases, grandmothers in 2 cases, grandfathers in 2 cases, a cousin in 1 case, and unrelated donors in 3 cases, one of them being a good Samaritan donor. Medical contraindications in the candidate LD were ruled out after assessment by an internist (“medical advocate” not belonging to the transplant team) with a detailed history and physical examination. All donors had neither significant medical history nor previous liver illness, which might have increased the operative risk or decreased the quality of the transplant. The median body mass index of the LD was 23.3 kg/m² (range, 16.2–31.7 kg/m²); among them, 14 donors had body mass index of 27 kg/m² or more. All donors were evaluated using routine blood testing, including blood cell count, coagulation profile with thrombophilic workup (protein C, protein S, antithrombin III, and activated protein C resistance), liver and kidney function tests, hepatitis B virus, hepatitis C virus, Epstein-Barr virus, cytomegalovirus serology, chest radiography, and electrocardiography. All donors had normal liver function tests, including cytolytic and cholestatic enzymes and bilirubin levels. Psychological assessment

was carried out by a psychologist or psychiatrist, ruling out any psychological disturbance or donor coercion. Preoperative Doppler ultrasonography was routinely performed to evaluate the liver echogenicity (exclusion of steatosis, detection of any focal parenchymal lesions) and vascular anatomy of hepatic veins (HVs) and hepatic artery (HA) and to exclude a possible intrahepatic PV anomalies. Before 2001, all potential donors underwent abdominal computed tomography (CT). In the first 85 cases, transvenous hepatic arteriography was also performed to rule out HA anomalies. Since 2001, “all in one” magnetic resonance imaging (MRI) allowing volumetry and vascular and biliary reconstructions replaced CT and angiography. Since 2010, for adolescent recipients ($n = 6$), the liver graft volume was estimated on the basis of angio-CT combined with cholangio-MRI; detailed liver volume and segmental vascular reconstruction was performed by Mevis software (MeVis Medical Solutions AG, Germany).⁴

Presurgical Evaluation of Recipients

This included clinical, growth, and nutritional assessment, liver and kidney function tests, metabolic profile, serological assays, eye fundus examination, electroencephalography, chest radiography, cardiac ultrasonography, and other specific investigations according to the etiology and presentation of liver disease. Doppler ultrasound examination of the abdomen provided the following information: (1) diameter of the PV and superior mesenteric vein (SMV); (2) direction and velocity of the portal flow; (3) HA resistivity index; and (4) spleen longitudinal measurement and presence of ascites. No abdominal CT scan was performed in a routine manner in the recipient.

Surgical Technique for LDs

Surgical technique for LDs at our center has been fully detailed in a previous publication.⁵ Since this latter article, some technical adaptations have been implemented: (1) although an epigastric inverted T-shaped incision extending to the xiphoid process was used initially, a midline laparotomy from xiphoid to umbilicus that allowed an adequate exposure has been performed for the last 150 cases. (2) The ductus venosus ligament was transected close to the left HV and subsequently used for hanging maneuver at the time of left bile duct transection. As the transection plane approached the left hepatic duct, a hanging maneuver was carried out between the ductus venosus ligament and the round ligament to suspend the pedicle of the left lobe. (3) From July 2006, in the last 109 LDLT patients, we routinely carried out retrograde HA perfusion, with the aim to flush intrahepatic arterial tree, including periductal arterial plexuses, through continuation of portal perfusion and finger clamping of the graft outflow from the HV.

TABLE 1. Distribution of Pretransplant Diagnoses in the Three Age Subgroups, Among 250 Pediatric LDLT Recipients, at Cliniques Universitaires Saint-Luc

Pretransplant Diagnosis	Age Subgroup			Total	P*
	<1 yr	1–5 yr	>5 yr		
BA	96 (85.7%)	50 (52.1%)	7 (16.7%)	153 (61.2%)	<0.001
Malignancy	6 (5.3%)	13 (13.6%)	12 (28.6%)	31 (12.4%)	
PFIC	4 (3.6%)	11 (11.5%)	4 (9.5%)	19 (7.6%)	
Metabolic diseases	2 (1.8%)	11 (11.5%)	5 (11.9%)	18 (7.2%)	
Alagille syndrome	0	6 (6.2%)	1 (2.4%)	7 (2.8%)	
Others	4 (3.6%)	5 (5.2%)	13 (30.9%)	22 (8.8%)	
Total	112	96	42	250	

* χ^2 test.

PFIC indicates progressive familial intrahepatic cholestasis.

Surgical Technique for Recipients

Surgical technique for recipients at our center has also been previously detailed in the publication of Darwish et al.⁵ but new techniques were implemented since this last article. Among the 31 patients with liver malignancy, hepatectomy entailed en bloc excision of the retrohepatic inferior vena cava (IVC) in 25 cases. The IVC has been replaced using the internal jugular vein (IJV) (n = 22), or the internal iliac vein (n = 1) harvested from the LD, or a UW-preserved ABO-compatible iliac vein graft from a deceased donor (n = 2). In case of PV hypoplasia, which was defined as PV diameter of 4 mm or less, further dissection of the SMV at the mesenteric pedicle was carried out to prepare the implantation of a venous jump graft in 30 cases (12%), allowing portal revascularization of the transplant.⁶ More recently, the dissection was extended downward to the confluence of the splenic vein and SMV for lateral portoplasty with donor inferior mesenteric vein (IMV) performed in 51 cases (20%). The lateral portoplasty, as shown in Figure 2, consisted in opening longitudinally the anterior wall of the recipient's PV and suturing the donor IMV patch to the recipient's PV with a side-to-side anastomosis using 7/0 absorbable monofilament running sutures to enlarge the PV diameter. Thereafter, anastomosis between this reconstructed recipient's PV and the graft portal stump was performed as a classical trunk anastomosis.⁶

In cases without portal hypoplasia, PV anastomosis was performed in an end-to-end fashion between the graft left PV and the main portal trunk of the recipient. In cases of PV hypoplasia (n = 81), a vascular interposition graft was used for the reconstruction of portal flow in the initial 30 cases, with these grafts being distributed as follows: preserved iliac vein grafts recovered from deceased donors (n = 20, including 2 from ABO-incompatible donors, maximal preservation time of 14 days), recipient IJV (n = 9), and an LD IMV (n = 1). These vascular grafts were anastomosed in the recipient either at the confluence of the superior mesenteric and splenic veins in an end-to-end fashion (n = 19) or to the SMV in an end-to-side fashion (n = 11). In the remaining more recent 51 cases, laterolateral portoplasty was carried out using donor IMV graft.⁶

The arterial anastomosis was carried out as end-to-end anastomosis between the graft left HA and the recipient HA, using magnifying loupes ($\times 5.5$) and interrupted 8/0 Prolene (Ethicon Ltd, Edinburgh, United Kingdom) sutures according to a semimicrosurgical technique in 249 cases and in 1 early case performed under surgical microscope $\times 10$ with 9/0 Prolene interrupted sutures. In 38 instances, the graft had 2 arteries. Among these, double arterial anastomosis was performed (n = 13); in the remaining 25 cases, the greater artery was reconstructed and the other ligated after ensuring a good backflow. One graft had 3 HAs of which only 1

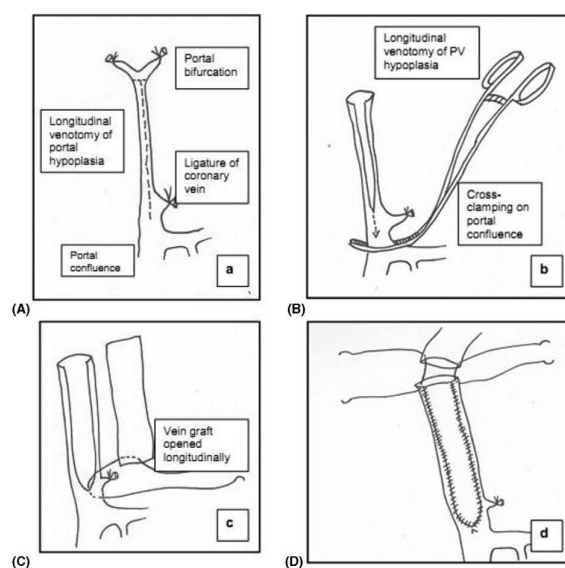


FIGURE 2. Portal anastomosis using the portoplasty technique in case of PV hypoplasia. A, Dissection of the PV down to the confluence of the superior mesenteric vein and the splenic vein ligature of the coronary vein. B, Longitudinal venotomy of the hypoplastic recipient's PV. C, The vein graft is opened longitudinally, and the vein patch is sutured to the recipient's PV with side-to-side anastomosis. D, The anastomosis between this reconstructed recipient's PV and the graft portal stump is done as a classical trunk anastomosis.

was reconstructed and the other 2 were ligated. In 4 patients, an arterial vascular conduit from an iliac artery graft recovered from a deceased donor was used to connect the graft left HA to the recipient infrarenal aorta. After completion of the vascular anastomoses, arterial and portal flow was checked using intraoperative Doppler ultrasonography (Aloka, SSD-2000, model IPC-1230 v; Mire-Mitaka-shi, Tokyo, Japan) and, if required, in case of poor flow, by extrahepatic flowmetry (HT107, medical volume flowmeter; Transonic System, Inc, Ithaca, NY).

Regarding the biliary drainage, the graft comprised a single duct (n=184), 2 ducts allowing a single anastomosis (n=11) or 2 ducts that required 2 separate anastomoses (n=48), 3 ducts anastomosed separately (n=4), 2 anastomoses for 3 ducts (2 together) (n=2), and 3 anastomoses for 4 ducts (n=1). In 246 patients, the biliary drainage was constructed using an existing or de novo Roux-en-Y jejunal loop of 30 to 50 cm and an end-to-side hepaticojejunostomy with interrupted 7/0 PDS sutures, without stenting. A duct-to-duct anastomosis was performed in 3 patients, and in a remaining patient, a duct-to-duodenum anastomosis was carried out in the context of a short bowel.

The abdominal wall was either closed directly (n=213) or closed temporarily using a prosthesis (n=37).⁷

Posttransplant Management of Pediatric Recipients

The general medical management of pediatric LT recipients at Cliniques Universitaires Saint-Luc has been described in detail in previous publications.^{8,9} Immunosuppression was given according to 5 main schemes: (1) cyclosporine (Sandimmun; Novartis Pharma) + steroids + azathioprine (Imuran; Aspen) (n=23); (2) tacrolimus (Prograf; Astellas) + steroids ± antimetabolites [azathioprine or mycophenolate mofetil (Cellcept; Roche)] (n=67); (3) tacrolimus + steroids + anti-CD25 monoclonal antibody [basiliximab (Simulect; Novartis Pharma)] ± antimetabolites (mycophenolate mofetil) (n=10); (4) steroid-free tacrolimus monotherapy ± antimetabolites (mycophenolate mofetil) (n=16); (5) steroid-free tacrolimus immunosuppression + anti-CD25 monoclonal antibody [basiliximab or daclizumab (Zenapax; Roche)] or lymphocyte-depleting agent (Thymoglobulin; Genzyme) ± mycophenolate mofetil (n=133). No immunosuppression was given to 1 patient who received, before LT, bone marrow transplantation from the same LD.¹⁰ Acute rejection (AR) was diagnosed in case of elevated liver enzymes and/or cholestasis with a biopsy-proven graft rejection (BANFF score)¹¹ and treatment by intravenous methylprednisolone.

Management of ABO Mismatch

Among the 250 LDLT recipients, 192 patients were ABO identical, 47 ABO-compatible, and 11 ABO-incompatible recipients with their donors. In the ABO-incompatible group, 6 patients had antidonor isoagglutinins (ADi) level of 1/16 or less and received a transplant without any special management and received post-transplant conventional immunosuppression (2 patients received Scheme 2, 1 patient received Scheme 3, and 3 patients received Scheme 5, as detailed earlier). Another 5 patients with ADi level above 1/16 were prepared for ADi reduction using anti-CD20 monoclonal antibody (375 mg/m², rituximab, MabThera; Roche), starting 2 weeks before LT (n=4), plasmapheresis (n=4), starting 6 days before LT in case of the elevated ADi level, and intravenous immunoglobulin (n=1) 1 day before LT to prevent resynthesis of ADi. The ADi level was checked at day 7, day 5, and day 1 before LT and day 2 after LT. All these 5 patients in the ABO-incompatible group received immunosuppression based on tacrolimus, monoclonal anti-CD25 antibody [Simulect on postoperative day (POD) 0 and POD 4], and steroids.

Statistical Methods

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 20.0. Data were expressed as mean (standard deviation) in case of normally distributed variables or as median (range) otherwise. Differences between groups were assessed by the χ^2 test and were considered statistically significant when $P < 0.05$. The Kaplan-Meier method was used to calculate patient survival, graft survival, PV complication-free survival, and acute rejection-free survival. Univariate analyses with the log rank test and multivariable stepwise Cox regression analyses were performed to determine the risk factors associated with AR. Covariates with a P value of less than 0.2 at the univariate analysis were entered in the multivariate Cox regression with a forward stepwise inclusion. P values of 0.05 or less were assumed to be statistically significant. Covariates tested as risk factors for AR were as follows: LT indications (oncological, nononcological), recipient sex, age of recipient (<6 months, >6 months), donor sex, sex mismatch (male to female vs male to male), ABO status (identical, compatible, incompatible), anti-CD25 induction, primary immunosuppression (cyclosporine, tacrolimus), steroid treatment or not, and antimetabolite treatment or not. Primary analysis was done on the whole population. Then, because of the absence of cyclosporine-treated patients since 1999, patients treated with cyclosporine were excluded from the analyses. Moreover, to assess the hypothesis of a putative benefit from maternal donation on graft acceptance, only recipients who received a parental (maternal or paternal) graft were included in the analyses. Finally, to test whether the effect of maternal donation was different between BA patients and patients with other primary diagnoses (non-BA), subanalyses were also conducted in BA and non-BA recipients.

RESULTS

Surgical Outcome for LDs

No donor mortality was encountered in this series. A detailed analysis of morbidity is described elsewhere (unpublished observation).

Overall Outcome for the 250 Pediatric Recipients

One-year, 5-year, and 10-year posttransplant patient survival rates were 96.0%, 93.9%, and 93.2%, respectively. Fifteen patients (6.0%) died during the follow-up period. Mortality was due to sepsis (n=3), including severe colitis with *Enterobacter* bacteremia (POD 18), respiratory tract infection associated with multiple cerebral abscesses (POD 133), and biliary sepsis with vancomycin-resistant enterococci (POD 500); delayed function of a small-for-size graft, complicated by PV thrombosis (PVT) (POD 32); severe cellular AR associated with cerebral thrombosis and gastrointestinal bleeding on POD 32 after retransplantation for PVT; cancer progression due to recurrent hepatoblastoma (n=3) (POD 158, POD 476, POD 772) and recurrent hepatocarcinoma (n=1) (POD 521); severe hepatitis (n=3), including adenovirus (POD 29), posttransplant fulminant hepatitis of unknown etiology (POD 36) and hepatitis B complicated by liver necrosis (POD 2490); posttransplant lymphoproliferative disease (n=2) (POD 224, POD 1246); and undetermined cause in 1 child who suddenly died outside the hospital after abdominal pain and vomiting (POD 233). One-year, 5-year, and 10-year posttransplant graft survival rates were 95.0%, 91.5%, and 90.0%, respectively. Seven patients (2.8%) were retransplanted with a deceased graft because of PVT (n=2) (POD 3; POD 22; this child died on POD 32 as mentioned later); HA thrombosis (HAT) (n=1) (POD 9); and chronic rejection (n=4) (POD 210, POD 538, POD 640, POD 1602). The 11 ABO-incompatible patients were alive with the first graft by the end of the follow-up. Neither graft-versus-host hemolysis

nor HAT, nor acute vascular rejection, was observed in the ABO nonidentical recipients. In this latter group, 3 children developed early biliary complications during the first posttransplant month, including 1 anastomotic biliary leak, 1 leak from a missed duct in the cut surface, and 1 anastomotic stricture. All of these 3 patients were reoperated on and surgically corrected. No case of diffuse intrahepatic biliary strictures was encountered among the ABO-incompatible patients.

HV Outflow Complications

The incidence of these complications was 0.8% (2/250 cases). HV thrombosis occurred in a 2-year-old recipient who received a transplant for multinodular hepatoblastoma with en bloc liver and retrohepatic IVC resection, followed by IVC reconstruction using donor left IJV. Venoplasty was carried out between 2 distantly separated HV ostia. On POD 1, this child developed significant ascites and Doppler ultrasonography revealed the absence of HV flow associated with reversed portal flow. The child was immediately reoperated on, and a new anastomosis was performed successfully using recipient IJV, connected between initially reconstructed retrohepatic IVC and the main left HV of the graft. In the second case, the child received a transplant for BA and developed HV stenosis on POD 102, which was treated by interventional radiology with balloon dilatation.

PV Complications

The overall rate of PV hypoplasia was 38.4% and, this rate was significantly higher in BA patients than in those with other diagnoses, as shown in Table 2. The 1-year and 5-year PV complication-free survival rates were 93.8% and 91.4%, respectively. The risk was higher in recipients with BA (18/153; 11.8%) than in those with other pre-LT diagnoses (4/97; 4.2%) ($P = 0.041$). Early PVT (within the first posttransplant month) was observed in 6 patients. Among them, 3 patients developed PVT on POD 1, 2 of them were immediately repaired using a venous jump graft between recipient SMV and the graft left PV; this venous graft being either a segment of the recipient left IJV in one case or a preserved iliac vein homograft recovered from a deceased donor in the other instance. The third patient with PVT on POD 1 required a retransplant on POD 3 due to hepatic insufficiency. One patient received a diagnosis of PVT on POD 7; immediate thrombectomy and revascularization allowed salvage of the graft. The 1 patient with PVT complicated by hepatic failure on POD 28 was registered on the emergency waiting list for retransplantation but he died 4 days later before a graft became available. The remaining patient with PVT associated with severe rejection on POD 12 was reoperated on and thrombectomy was carried out, which failed to restore sufficient portal flow. On POD 13, a meso-Rex shunt was performed, which subsequently thrombosed again. On POD 22, this child was retransplanted with a deceased liver graft but developed severe refractory cellular AR; because of thrombophilic status, this child developed cerebral thrombosis and on POD 32 severe diffuse gastrointestinal hemorrhage, which led to mortality.

The 16 patients who presented late PV complications (beyond 1 month post-LT) had either portal stenosis ($n = 6$) or late thrombosis ($n = 10$), with a median posttransplant interval at diagnosis of 473 days (range, 34–912 days). Fifteen patients were subsequently reoperated on by means of an autologous jump graft using the recipient IJV implanted between the recipient SMV and the graft left PV at the Rex recessus.¹² One patient with PVT discovered on POD 34 received systemic heparin infusion with recanalization of the vein and normalization of portal flow. Among these 16 patients with late PV complication, 7 (43.8%) had portal reconstruction at LT using a preserved iliac vein recovered from a deceased donor. Accordingly, the use of preserved iliac vein for PV reconstruction in case of PV hypoplasia resulted in PVT in 7 of 20 instances (35%). The rate of PV complications according to the type of PV reconstruction, among patients with and without BA, is also detailed in Table 2.

Arterial Complications

Among all 250 LDLT patients, HAT was encountered in 2 patients (0.8%) with single arterial anastomosis, with a 5-year HAT-free survival rate at 99.3%. In the first HAT case (1.6-year-old boy with fulminant hepatitis), early in our experience, single arterial anastomosis was done under microscope. This patient was immediately reoperated on POD 0 for HA reanastomosis with successful revascularization of the liver and salvage of the graft. On POD 7, the child was reoperated on again for PVT. Later on, he developed biliary anastomotic stricture, which was surgically reconstructed on POD 98. The second patient with HAT (0.4-year-old girl with BA) had semimicrosurgical single arterial anastomosis. The HAT was found on POD 7, and cadaveric aortic jump graft failed to reestablish the flow; therefore, the child was retransplanted on POD 9 with a deceased whole liver graft. Both HAT patients are alive by the end of the follow-up.

Biliary Complications

Biliary complications developed in 65 patients (26%). Forty patients had anastomotic strictures, 19 developed a biliary leak, and 3 each had diffuse strictures and cholelithiasis. All the anastomotic strictures were reoperated on, with an overall 80% success rate.¹³ None but one of the children who encountered biliary complications lost their graft, nor died; in this child having 2 ductal Roux-en-Y anastomoses, the anastomotic stricture was surgically reconstructed on POD 494 but subsequent cholangitis development due to vancomycin-resistant enterococci led to patient death on POD 500. No correlation was found between the number of biliary ducts to be reconstructed in the graft and the biliary complication rate.

Immunological Complications

The overall 1-year AR-free survival rate was 48.0%. As shown in Table 3, nononcological indication for LT, recipient age older than 6 months (although not significant), male donor, and cyclosporine-treated patients had a higher AR rate at univariate analysis. At multivariate Cox regression, nononcological indications for LT, male

TABLE 2. Distribution of PV Hypoplasia and PV Complications According to the Type of Portal Flow Reconstruction in Children With or Without BA

	BA (n=153)	Non-BA (n=97)	P*
PV hypoplasia, n (%)	89/153 (58.2%)	7/97 (7.2%)	<0.001
PV complications, n (%)	18/153 (11.8%)	4/97 (4.2%)	0.041
PV complications/truncal PV anastomosis, n (%)	8/82 (9.8%)	4/87 (4.6%)	
PV complications/venous jump graft, n (%)	7/26 (26.9%)	0/4 (0%)	0.027
PV complications/portoplasty, n (%)	3/45 (6.6%)	0/6 (0%)	

* χ^2 test.

TABLE 3. Results of the Univariate and Multivariate Cox Regression Modeling for AR in Pediatric LDLT Recipients (N=250)

Prognostic Factors	1-yr ARFS*	P†	Multivariate RR	95% CI	P‡
Liver transplantation indications					
Nononcological (n=219)	45.4%	0.029	2.19	1.11–4.43	0.018
Oncological (n=31)	68.1%				
Donor sex					
Male (n=120)	42.0%	0.043	1.62	1.14–2.31	0.007
Female (n=130)	53.5%				
Recipient age					
>6 mo (n=240)	47.0%	0.133	NS	NS	NS
<6 mo (n=10)	70.0%				
Immunosuppression schemes					
(1) CyA + Std (n=23)	28.2%	0.044	1.94	1.14–3.03	0.025
(2) Tac + Std (n=67)	56.2%	0.001§			0.014
(3) Tac + Std + Induction (n=10)	67.5%	0.047¶			NS
(4) Tac (n=16)	43.8%	0.182			NS
(5) Tac + induction (n=133)	45.5%	0.023+*			NS

*The Kaplan-Meier test.

†Log-rank test (univariate).

‡Cox regression.

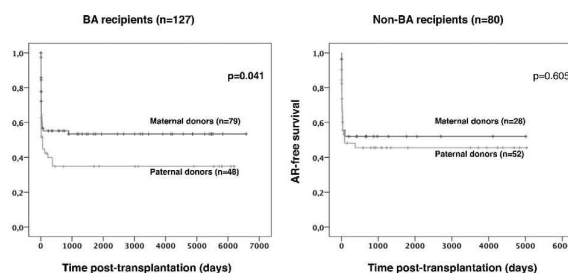
§(1) vs (2).

¶(1) vs (3).

|| (1) vs (4).

+ (1) vs (5).

ARFS indicates AR-free survival; CI, confidence interval; CyA, cyclosporine A; NS, not significant; RR, risk ratio; Std, steroids; Tac, tacrolimus.

**FIGURE 3.** AR-free survival rate among transplantation in children with BA and diagnoses other than BA (non-BA), according to paternal or maternal donation.

donor, and cyclosporine as the primary immunosuppressant were significant risk factors for AR (Table 3). The study of other covariates (cf statistical methods) did not show any significant impact on AR. Similar results were found when multivariate analysis was performed in recipients who received only parental graft donation (n=229), with a risk ratio for AR of 2.23, 1.60, and 1.88 for nononcological indications for LT, paternal donation, and cyclosporine as the primary immunosuppressant, respectively. After exclusion of all patients under cyclosporine-based immunosuppression (n=23) and of those with a nonparental graft (n=21), the influence of maternal versus paternal donation on the AR rate was studied in the subgroups of children with (n=127) and without (n=80) BA. In BA cases, paternal donation was associated with a significantly lower 1-year AR-free survival rate (39.8%) than in maternal donation (55.2%; $P=0.041$; Fig. 3); this was the only factor associated with the AR rate, with a risk ratio of 1.64 for AR at the multivariate analysis. No significant factor was associated with AR in the non-BA patients (NS). Among all LDLT recipients, 5 (2%) developed chronic

rejection, 4 of them being retransplanted with a deceased liver graft on POD 210, POD 538, POD 640, and POD 1602. All of these patients were alive at the end of follow-up.

DISCUSSION

LDLT using the left hepatic segments from adult LD constitutes a valid option to overcome the scarcity of deceased donor organs for children.¹ At our center, the pediatric LT program including the use of reduced and split hepatic grafts was started in 1984 and 1988, respectively, followed by the introduction of LDLT in 1993. The annual number of LDLT cases gradually increased from 9 in the initial year to 29 in the last year of our study (Fig. 1). In this group, LDs were selected very carefully and no mortality was encountered. The steps of donor operation have been progressively standardized with the aim to obtain as much length as possible at the division of the left PV and HA and to adequately identify any bile duct anatomical variations, using intraoperative cholangiography

performed after parenchymotomy. In a recently published study from the Registry of the Japanese Liver Transplantation Society that analyzed the results of the largest world cohort of LDLT pediatric patients, the 1-year, 5-year, and 10-year patient survival rates were 88.3%, 85.4%, and 82.8%, respectively.¹⁴ Our single-center series compares favorably with the corresponding figures of 96.0%, 93.9%, and 93.2%, respectively. The majority of children who underwent LD liver transplants had BA (61.2%); 85.7% of these recipients were younger than 1 year at LT (96/112). As evidenced by a previously published study from our center, BA was associated with PV hypoplasia in 56.0% of cases.⁶ In the present series, PV hypoplasia was encountered in 89 of 153 (58.2%) cases of BA patients versus 7 of 97 (7.2%) in non-BA patients. PV complications are reported as the most frequent vascular complication in pediatric LT patients with an incidence between 3% and 19%.^{6,15} Among all 22 PV complications in this study, 18 (81.8%) belonged to the BA group. These complications might be explained by secondary PV changes due to rapidly progressing sclerosis and fibrosis, previous Kasai procedure, and repeated attacks of cholangitis that could lead to PV inflammatory changes and hypoplasia.¹⁹ Our experience with PV reconstruction in case of PV hypoplasia can be divided into 2 periods, before and after 1998, according to the utilization of preserved iliac vein homografts from deceased donors in the early period.¹⁶ The use of such homografts provided early patency rates but turned out to be associated with an unacceptably high rate of late stenosis and subsequent thrombosis.¹⁷ In the whole series, a total of 20 preserved iliac vein vascular grafts were used, with 7 cases of late PVT (35.0%). This observation led us to avoid the use of preserved venous grafts after 1998. Most published reports emphasize the disadvantage of using a venous conduit for PV (n = 5): Millis et al¹⁸ reported a 9% incidence of complications for direct PV anastomosis compared with a 44% complication rate when venous cryopreserved conduits were used earlier in their experience. According to our results, the rate of PV complications observed in BA patients with a hypoplastic PV treated using an interposition venous jump graft was 26.9%. This complication rate was higher than the patients having the portoplasty technique using a patch of IMV from the LD (4.6%).

Since its first introduction by Starzl and Putnam¹⁹ in 1969, ABO-mismatch LT constitutes a feasible option to increase the donor pool up to nearly 25% to 35%.²⁰ In our series, 47 recipients were ABO compatible and 11 ABO-incompatible with their donors (non-identical: 30.2%). Previous reports have observed shortened graft survival after ABO-incompatible LT, with as low as a 20% graft survival rate.^{21–24} ABH antigens expressed on the HA, PV, capillaries, sinusoidal lining cells, and bile duct epithelium may react with relevant anti-A or anti-B isoagglutinins and trigger an antibody-mediated rejection, which, in turn, may jeopardize graft survival.²⁵ Isoagglutinins do not exist at birth and are produced during the first year of life through cross-immunization with intestinal bacterial antigens. In contrast with kidney transplantation, the impact of pretransplant ADI level is still not well defined in LT. However, the literature data suggest that higher titers of ADI may result in a higher risk of rejection.^{26,27} In ABO-incompatible transplants, ADI titer less than 1/16 is commonly associated with a minimal risk of acute humoral rejection.^{28,29} Therefore, the reduction of immediately pre-LT ADI level may result in longer graft survival. As presented in this study, in the ABO-incompatible group, 6 patients with median age of 0.7 year had an ADI level of 1/16 or less and underwent transplant without a special management, receiving conventional immunosuppression. When ADI levels are more than 1/16, there is no widely accepted guidelines on the type of pretransplant conditioning regimens that has to be used for ABO-incompatible recipients.³⁰ ABO-incompatible LDLT setting allows for planned preparation before surgery to reduce ADI levels using different

combination of therapeutic modalities including anti-CD20 monoclonal antibody administration, plasmapheresis, and intravenous immunoglobulins.^{31–33} Using this preconditioning approach, all of our ABO-incompatible patients were alive with the first graft by the end of the follow-up, with 1-year and 5-year patient and graft survival rates of 100%. The incidence of biliary complications was 28% compared with 26% in ABO-identical group (NS). The incidence of cellular AR in the ABO-incompatible and ABO-identical groups was 45% and 50%, respectively. Neither graft-versus-host hemolysis nor HAT, nor vascular AR, nor diffuse intrahepatic biliary strictures, was observed in the ABO nonidentical recipients. Our results additionally suggest that the proposed therapeutic strategy aiming at the reduction of pre-LT isoagglutinins level to 1/16 might provide a similar outcome as an ABO-identical liver transplant and constitutes a safe option to increase the LD pool.

Regarding AR, male donation and cyclosporine as the primary immunosuppressant were the only 2 factors associated with an increased risk, with a risk ratio for AR of 1.62 and 2.17, respectively. On the contrary, oncological indications for LT, when compared with all other indications, seemed to constitute a protective factor, with a risk ratio for AR of 0.46. Concerning this protection against AR, observed in recipients who received a transplant for oncological reasons (n = 30 hepatoblastomas; n = 1 hepatocarcinoma), our results are in line with those of Ruth et al.³⁴ who showed, in a retrospective matched control study, that children who benefited from LT for hepatoblastoma had lower rejection rate (50%) than children who received a transplant for BA (75%). We hypothesize that this is due to the effect of the chemotherapy received before transplantation. Further investigations are required to confirm these findings. The higher AR incidence rate in cyclosporine- versus tacrolimus-treated patients has already been shown in a randomized control trial of pediatric LT.³⁵ Thus, because of its higher immunosuppressive activity, tacrolimus has now become a cornerstone of the immunosuppressive regimen and cyclosporine is no longer prescribed at our center in *de novo* pediatric liver allograft recipients. Finally, the pejorative effect of male donation observed on the AR rate, or in other terms, the beneficial impact of *female* donation on AR rate is actually due to *maternal* donation. In contrast, in the 21 patients with nonparental living donation, this effect of donor sex was not found (data not shown). Moreover, in recipients with parental donation and who received tacrolimus as the primary immunosuppressant, this sex effect was only seen in patients who received a transplant for BA. A similar protective effect of maternal donation on AR has already been described in renal transplantation. Indeed, in 1998, Neu et al³⁶ described a longer time to first rejection in pediatric kidney recipients younger than 1 year and who received a maternal organ. More recently, maternal donation has also been associated in LT with lower graft failure and lower refractory rejection rates, but only in BA patients.³⁷ These authors attributed this positive effect to some degree of immunological acceptance toward noninherited maternal antigens (NIMA), via maternal microchimerism (MM). MM is a physiological phenomenon resulting from maternal to fetal cellular trafficking during pregnancy.³⁸ These maternal microchimeric cells (MMc) (hematopoietic or stem cells) can migrate and differentiate into different fetal organs (brain, liver, pancreas, heart, intestine, lung, muscle, skin, and spleen) and may persist a life-long time.^{39,40} The presence of these MMc can be associated with 2 different effects: (1) specific tolerance toward NIMA and (2) alloimmune reactivity. Actively acquired immunological tolerance toward foreign antigens after in utero exposure was first demonstrated in animal studies by Billingham et al⁴¹ in 1953. Since then, other studies in human solid-organ transplantation suggested the development of specific tolerance to NIMA through several immunological mechanisms, including T regulatory lymphocytes.^{42–44} Besides this tolerance effect toward NIMA, MM is also associated with alloimmune reaction because

MMC are found at higher rates in autoimmune diseases such as type I diabetes, neonatal lupus syndrome, juvenile idiopathic inflammatory myopathies, and BA.³⁸ In BA, different studies showed a higher rate of maternal cells in the livers of BA patients than in controls.^{45–48} Moreover, Muraji³⁹ showed that these MMC were predominantly CD45⁺CD8⁺ T lymphocytes and biliary epithelial cells (BECs), located in the portal area and sinusoids next to the damaged areas. However, the exact role of these MMC is unknown. Indeed, MMC could either be effector cells damaging host BECs such as in graft versus host disease (GvHD) or target cells such as in host versus graft disease (HvGD) or be bystanders.³⁹ In his last publication, Muraji³⁹ pushed forward the hypothesis of GvHD. Indeed, as better tolerance was observed with maternal livers of BA patients who received a transplant, and as only 50% of patients with BA will require early liver transplant due to progressing liver fibrosis, Muraji raised the possibility that only patients who were tolerant to their mother livers allowed maternal cells to persist and damage the host BECs, as in GvHD, with subsequent progressing fibrosis. In contrast, other patients who were not tolerant to their mother livers eliminated MMC preventing BA progression and early liver transplantation.³⁹ To sum up, maternal tolerance after fetal exposure to NIMA would lead, in BA patients, not only to some form of GvHD with disease progression but also to better maternal liver graft acceptance at the time of transplantation. Further prospective studies are required to confirm and elucidate this maternal effect hypothesis on AR.

CONCLUSIONS

LDLT, including ABO-mismatched transplantation, constitutes a safe and efficient therapy for liver failure in children. In BA patients with PV hypoplasia, portoplasty seems to constitute the best technique for PV reconstruction. Maternal donation might be a protective factor for AR.

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Chapter 3. GRAFT REJECTION AND IMMUNOSUPPRESSION IN PEDIATRIC LIVER TRANSPLANTATION

If excellent results in PLT and PLDLT have been achieved in terms of patient and graft survival with a minimal immunosuppressive regimen, acute liver rejection is still a major concern. In this chapter, we will describe how allograft rejection is promoted, the underlying mechanisms, and the different types of rejection that can be encountered in liver transplantation as well as the therapeutic approaches to prevent the graft from such an issue. Finally, we will briefly discuss the treatment strategies in case of acute rejection.

3.1 From liver transplant to graft rejection

Allograft rejection is an inflammatory reaction promoted by the transplantation itself, involving the activation of both the innate and adaptive immune system with allorecognition being the core of the process. In the absence of IS, acute rejection will lead to graft damage and destruction. This process takes place in a multistep manner: the initial inflammatory reaction, the allorecognition with activation and differentiation of T lymphocytes and the final effector phase.⁴¹⁻⁴⁴

3.1.1 Initiation of rejection

During the transplant procedure, the mobilisation of tissues, implantation and ischemic/reperfusion cause injuries to the graft with subsequent release of many damage-associated molecular patterns (DAMPs). These DAMPs are sensed by the pathogens –associated

patterns recognition receptors (PPRs), expressed by the innate immune cells of the graft. These cells will in turn enhance their transcription activity and produce proinflammatory cytokines (IL-1, IL-6, Tumor Necrosis Factor, Interferon (IFN)), chemoattractant cytokines and their receptors and expression of P-selectin on the endothelial cells. Finally, this reaction will stimulate the migration of donor antigen presenting cells (APCs), including dendritic cells (DCs), from the graft to the secondary lymphoid tissues and will attract innate immune cells such as macrophages, natural killer (NK) cells and neutrophils into the graft where they can cause damage to the donor cells. In lymphoid organs, APCs will activate the adaptive immune cells and recruit them to the graft. This early inflammatory reaction by the innate immune system is not antigen specific and always occurs after transplantation.^{41, 43, 44}

3.1.2 Allorecognition and T cell activation

The second step of rejection, allorecognition and activation, consists in the recipient T cell recognition of alloantigens, present in the donor graft, and the subsequent molecular reactions. This step takes place inside the secondary lymphoid organs (spleen and lymph nodes).

These alloantigens can be either major or minor.

Major alloantigens are represented by the Major Complex of Histocompatibility (MHC) type I and II or Human Leucocyte Antigen (HLA) in human.

Minor alloantigens are donor peptides presented by MHC class I or II.⁴¹⁻⁴³

Due to a very large polymorphism of the MHC, the likelihood that MHC disparities between donor and recipient exist is very high. Moreover, a significant percentage of the T cell repertoire is able to recognize a single MHC mismatch, therefore, a dynamic immune response is highly probable. The antigen-binding groove of MHC molecules contains a short peptide and it is that MHC-peptide complex that is recognized by the T cell receptor complex (TCR).⁴¹⁻⁴³ Alloantigen recognition can be achieved through 3 nonexclusive different pathways: ^{41, 43, 45}

The first one, the direct pathway, is the allorecognition due to the interaction of recipient TCR with intact allogeneic MHC-peptide complex, presented by donor APCs. The peptide associated by MHC class I will activate CD8+ cytotoxic T lymphocytes (CTL) while peptides presented by MHC class II will activate CD4+ helper T lymphocytes (CD4 Th). This pathway initiates a very powerful immune response because there is a high percentage of the T cell repertoire able to recognize allogeneic MHC. This can be explained by two hypotheses: 1) the high determinant density where T cells recognize the polymorphic residues on the MHC molecule, the bound peptide being accessory. As each MHC molecule present on an APC may activate an alloreactive T cell, this may lead to the activation of numerous alloreactive T cells. 2) The multiple binary complexes, where alloreactive T cells recognize the allogeneic MHC-peptide complex. As such, different bound peptides or self-peptides presented differently by donor MHC may produce different clones of alloreactive T lymphocytes. Direct allorecognition is specific to transplanted organs and predominates during the early post-

transplant period, where donor derived APCs migrate from the allograft to the secondary lymphoid tissues, eliciting the priming of naive T cells. This mechanism will decrease with time, because of a limited number of donor leucocytes initially present inside the graft.

The second one, the indirect pathway, occurs when a donor derived MHC-peptide has been processed and presented by a recipient's APC, via MHC class II, to CD4⁺ lymphocytes. Moreover, B lymphocytes are mainly stimulated to produce alloantibodies by CD4⁺ T lymphocytes activated through indirect allorecognition. As long as the allograft persists in the recipient, this mechanism can be used for antigen presentation and thus, becomes the predominant mechanism of long-term graft rejection.

Finally, more recent studies have proved the existence of the semi-direct pathway, where an intact donor MHC-peptide complex is captured and presented by host APC after cell-to-cell contact and transfer of MCH molecules and/or via exosomes. This pathway explains how a same and single recipient APC may activate both the direct (via MHC class I and CD8⁺ T lymphocytes) and indirect pathway (via MHC class II and CD4⁺ T lymphocytes).^{41, 43, 45, 46}

After the first antigen-specific T lymphocytes activation through MHC-peptide and TCR-CD3 interaction, a second signal is required to complete T cell activation and differentiation; otherwise, it leads to T cell anergy, deletion and antigen tolerance. This second signal is provided by the interaction between costimulatory molecules with their ligand. There are two types of costimulatory molecules. The B7 family including CD28 and CD152 (CTLA-4) on T lymphocytes, and Tumor Necrosis Factor (TNF) and TNF receptor family including

CD40 on APCs and B lymphocytes and its receptor, CD40L (CD154) on T lymphocytes. First, CD28 interacts with CD86, which is constitutively expressed on APCs and later with CD80, which is induced on APC, during the response. This interaction leads to lower the threshold of T cell activation, to enhance expression of IL-2 via increased stability of its mRNA, and to promote T cell proliferation. It also enhances expression of CD154 (CD40L) that interacts with CD 40, leading to APC activation and increasing the expression of costimulatory B7 family molecules, and thus amplifies T cell activation. At the same time, T cell activation upregulates the expression of CD152 that binds to CD80 and CD86 with 10-20 times higher affinity than CD28, and acts as a negative regulator of the immune response.^{41, 43, 47, 48}

Finally, the TCR-CD3/MHC-peptide interaction (signal 1) and costimulation (signal 2) will lead to recruitment of signalling molecules and the formation of clusters within the plasma membrane constituting immunological synapses. These synapses modulate T cell activation depending on interactions between plasma membrane and cytoskeleton, and coordinated by proteins movements inside lipid rafts.^{41, 43}

The formation of these synapses triggers different cellular downstream pathways including phosphorylation of different molecules and promotes the activation of phospholipase C γ 1 (PLC γ 1) which then hydrolyzes the membrane lipid phosphatidylinositol 4,5 biphosphate (PI (4,5) P2) producing the second messengers IP3 and diacylglycerol (DAG).^{41, 43, 49}

DAG results in the activation of two major pathways. The first one involves Ras- Mitogen Activated Protein (MAP) kinase pathway and leads to activation of the activator protein-1 (AP-1) transcription complex. The second one results in Phosphokinase C θ (PKC θ) activation. Once activated, PKC θ regulates Nuclear Factor Kappa Light Chain enhancer of activated B cells (NF κ B) activation by allowing its transport from cytosol to nucleus.^{41, 43, 49}

IP3 stimulates calcium efflux from endoplasmic reticulum (ER) to cytosol with secondary extracellular calcium influx. This increase in intracellular calcium levels results in the activation of calcium and calmodulin-dependent transcription factors, and signaling proteins, including the phosphatase calcineurin and the calcium-calmodulin-dependent kinase (CaMK). Once activated, calcineurin dephosphorylates the cytoplasmic nuclear factor of activated T cells (NFATc), leading to its translocation to the nucleus where it complexes with its nuclear counterpart (NFATn). In the nucleus, NFATs may form complexes with different transcription factors, including AP-1 that results in upregulation and production of various interleukins such as IL-2 and IL-2 receptor α -chain CD25. IL-2 and IL-15 will then act in an autocrine and paracrine way and deliver growth signals (signal 3) through activation of phosphoinositide-3-kinase (PI3K) and the target of rapamycin (mTOR) pathways, triggering cell cycle progression and thus a clonal expansion and differentiation of T cells. Lymphocyte expansion also requires the synthesis of purine and pyrimidine nucleotides through inosine

monophosphate dehydrogenase (IMPDH) and dihydroorotate dehydrogenase (DHODH) respectively.^{41, 43, 48, 49,}

3.1.3 T cell differentiation

After T cell activation, CD4⁺ MHC-II restricted T lymphocytes will differentiate into different T helper function with specific transcription factors and cytokines signatures, according to their environment and multiple factors such as the degree of ischemia-reperfusion, the degree of donor –recipient HLA mismatch, recipient immune status and immunosuppressive treatment as well as antigen load.

During T-cell activation, CD4⁺ lymphocytes may principally differentiate into Th1, Th2, Th9, TH17, Thf or Treg according to the level of inflammation in their microenvironment.^{41, 44, 50, 51}

When induced by IL-12, secreted by APCs, CD4⁺ lymphocytes differentiate into Th1. Th1 produce the transcription factor Tbet and release IFN γ , IL-2 and TNF α that activate NK cells, macrophages and other cell populations. It has a key role when driving the cellular immune response.

In contrast, in presence of IL-4, naïve T cell differentiate into Th2 cells secreting IL-4, IL-5 and IL-13. This will activate eosinophils and attract them to the graft. Th2 also induces humoral rejection by activation of B -lymphocytes producing plasma cells with clonal expansion and antibody release.^{41, 50}

Transforming growth factor β (TGF β), IL-21, IL-23, IL-6 and IL1 β drive T cell into Th17 with the release of IL-17 stimulating granulopoiesis and neutrophils migration to the graft as well as chemokines (CXCL1, CXCL2...).⁴¹

In presence of TGF β and IL-4, T cells may differentiate into Th9 cells that release IL-9 and activate mast cells.

Thf cells are found in lymph nodes and are the result of T cell differentiation in presence of IL-21 and IL-6. They release IL-21 and play a role in B cells maturation and antibody mediated rejection. Besides those subsets of T cells involved in immune response and graft rejection and in the absence of proinflammatory cytokines, TGF β may induce CD4⁺ T cells to differentiate in some regulatory T cells (Tregs) expressing FoxP3, involved in transplant tolerance. These Tregs have two origins: a natural thymic origin or a peripheral-induced origin, this latter occurring in the presence of an antigen and a permissive microenvironment.^{41, 43, 50, 52, 51}

The paradigm developed in the 1980s according to which Th1 pathway would have immunogenic effect contrarily to Th2 pathway that rather would promote tolerance is actually not correct. Indeed, both Th1 and Th2 have been shown to promote graft rejection. It seems now that the rejection versus tolerance rather depends on the balance between Th1 and Th17 cytopathic CD4⁺ cells and cytoprotective Treg cells.⁵¹

Besides the CD4 helper differentiation, CD8⁺ class I restricted T cells become effector cytotoxic T cells that can also be divided in two subsets Tc1 and Tc2 according to the type of cytokines they release.⁴¹

3.1.4 The effector phase of graft rejection

After being activated, leucocytes (activated B and T lymphocytes, macrophages, NK cells, neutrophils) must migrate from lymphoid organs to the site of inflammation, the liver allograft, and, more precisely, through its endothelium. As already mentioned in 3.1.1, this is achieved early after transplantation by the production and upregulation of chemokines that induce chemoattraction, adhesion molecules including selectins, integrins, and immunoglobulin superfamily members such as intercellular adhesion molecules (ICAMS) and vascular adhesion molecules (VCAMs).^{41, 43, 44} Activated leucocytes may then penetrate the graft and lead to its destruction by the combination of both innate and adaptive immune cells. Besides creating an inflammatory site and facilitating adaptive immune system activation, differentiation and action, the innate immune system may damage graft on its own but is not sufficient to elicit allograft rejection. Innate cells may either act as APCs and present alloantigen, bind alloantibodies, immune complexes or complement-coated cells with their complement receptor (CR). This latter process is known as opsonization and allows phagocytic cells to ingest damage donor tissue and clear antigen while increasing its presentation to T cells.^{41, 43, 44}

(a) The complement

Complement as a part of the innate immune system is involved in different ways in allograft rejection. It can be early activated by DAMPS, through the alternative and lectin pathways, participating thus in the initial inflammatory phase and ischemia-reperfusion

injuries.⁴¹ Later, once alloantibodies are produced by plasma cells and bound to alloantigen, they form immune complexes that may activate complement via the classical pathway. This leads to a cascade of reactions that cleaves C4 into C4a and C4b. This latter may be further degraded in small particles such as C4d, a marker of antibody-mediated rejection in kidney transplantation.^{41, 53} C3a and C4b may covalently bind to alloantibodies and target cells, leading these cells to be destructed by phagocytes with CR (opsonization), as mentioned above. Moreover, the cleavage of C3, C4 and C5 will induce the release of anaphylatoxins (C3a and C5a) that promote vasodilatation, chemoattraction of leucocytes, and stimulate T cell activation and proliferation. Finally, the elements generated through complement activation (C5b to C9) lead to the formation of the membrane attack complex (MAC) and induce cell lysis.^{41, 53}

(b) NK cells

NK cells are able to initiate a potent early innate immune response towards virus and bacterial infected cells. They may damage and kill them by the same manner than CD8+ cytotoxic T lymphocytes (see above). They detect and are activated by the “missing self”, cells without autologous class I MHC.^{41, 43} In the context of graft rejection, they potentiate the adaptive response, contributing to APC maturation, enhancing T cell response, and damaging cells but are neither necessary nor sufficient to mediate allograft rejection.⁵⁴ More recently, NK cells have also been involved in liver graft tolerance, but this role has to be elucidated.⁵⁵

(c) Macrophages

Macrophages are heterogeneous innate immune cells, widely spread into the body, which play also an important role in allograft rejection. Macrophages observed in the liver following injury may derive from different origins: resident liver macrophages or Kupffer cells (KCs) and bone marrow monocyte-derived macrophages (MoMFs).⁵⁶ KCs are located in the hepatic sinusoids.⁵⁷ They are renewed from local hepatic progenitors or are replaced by recipient MoMFs in the context of LT.⁵⁶ The main function of KCs and MoMFs is to kill and engulf microorganisms and pathogens, secrete cytokines and effect antigen presentation.⁵⁸ However, KCs seems to dominate the liver macrophage pool during homeostasis acting as a sentinel while MoMFs prevail in acute or chronic injury.^{56, 57} After being activated, KCs display the ability to differentiate into M1-like macrophages (classical) or M2-like macrophages (alternative) depending on the signals they receive from their environment.⁵⁷ Actually, the dichotomy between M1-like and M2-like macrophages is rather represented by a continuum, where boundaries are still unclear.^{56, 59}

M1 are activated by $\text{IFN}\gamma$ and $\text{TNF}\alpha$, released by Th1 cells and APCs. They can present antigen to naïve T cells, express costimulatory molecules (CD80 and CD86), release IL-12 and participate to the development of Th17.^{43, 44} They can produce oxygen reactive species and other enzymes able to damage vascular endothelium and parenchyma.⁴¹

On the other hand, M2 macrophages are considered as having immunoregulation function by producing large amount of anti-inflammatory cytokines such as IL-10 and TGF β . They are activated in response to IL-4 and IL-13 and involved in tissue repair.^{41, 44} Recently, KCs were also found to induce T lymphocyte apoptosis and play an important role in the regulation of liver transplantation tolerance.⁵⁸

(d) Neutrophils

Neutrophils are rapidly directed to the graft after transplantation and T cell activation, partly due to the secretion of Th 17. They participate in graft damage by the secretion of chemokines, the adhesion to vascular cells, and secretion of reaction oxygen species after release of myeloperoxidase and secretion of heparin binding proteins.^{42, 60}

(e) Eosinophils

Eosinophils are recruited and activated within the allograft by Th2 cells, through the production of IL-4, IL-5 and IL-13. Once activated, eosinophils release noxious substances such as major basic protein, eosinophil cationic protein and eosinophil peroxidase. The latter is highly toxic by promoting oxidative damage of tissue proteins. Therefore, eosinophils are able to lead to graft rejection via Th2 cells, even without the participation of CD8 and CD4 cytotoxic cells. The relevance of the Th2/eosinophils pathway should still be clarified but it is clinically admitted that blood hypereosinophilia precedes graft

rejection and that activated eosinophils are present in biopsies of solid organ allografts undergoing acute rejection.⁴²

(f) Cytotoxic CD8+ T lymphocytes

Once activated, cytotoxic CD8+ lymphocytes migrate to the graft. They recognize their target cells by their allogeneic class I MHC molecules and promote their death by different ways, including secretion of TNF α , the attack of membrane integrity by perforins and granzymes and Fas mediated apoptosis.^{42-44, 60}

(g) CD4 helper T cells

Specific alloantigen CD4 helper T cells and more particularly, the Th1 subset, contribute to allograft rejection by delayed type hypersensitivity (DTH), a non-specific effector mechanism. DTH response intervenes 48 to 72h after naïve T cells priming and is characterized by swelling, oedema and induration of allograft, due to increased vascular permeability and the presence of immune cells infiltrate comprising activated Th1 cells, macrophages and neutrophils.^{41, 42} Activated Th1 release proinflammatory cytokines such as IL-1, TNF α , and IFN γ that triggers macrophages to produce nitric oxide (NO), a highly reactive and cytotoxic compound at high concentrations. Cytokines also induce vasodilatation and oedema. TNF α leads to target cell apoptosis through caspase activation and the perforin/granzyme system.^{41, 42}

(h) B lymphocytes

B cells may play different roles in the alloimmune response. First, they can differentiate into antibody secreting plasma cells. Theses

alloantibodies are critical for the pathogenic immune response.⁴³ They are composed of two regions: the variable antigen-binding F(ab)2 region and the fragment crystallizable, Fc, region that can activate complement and mediates its effects through Fc receptor present on the surface of different immune cells. Binding of the Fc region to its receptor stimulates cell activation, phagocytosis, degranulation and cytotoxicity such as antibody-dependent cell mediated cytotoxicity (ADCC) in NK cells.⁶¹ Besides this antibody mediated immune response, B cells can present antigen to T cells, promote their activation and differentiation by costimulation pathways and the release of cytokines and are critical for T cell memory development.^{44, 61} Moreover, some B cells have been recently involved in the regulation of immune response, as studies found that B cell depletion lead to higher kidney allograft rejection rate.^{43, 44, 62, 63} These Regulatory B cells (Bregs) would mediate their inhibitory effect via secretion of IL-10 or even TGF β .⁶³ However, it seems that this tolerizing effect of B cells is present in kidney transplant only, but not in liver transplant patients.^{44, 64, 65}

(i) T and B cells memory

After the first antigen exposure, *de novo* memory T and B cells are generated. However, some pre-existing memory T and B cells may be encountered in two cases. The first one is due to previous antigen exposure in case of previous transplantations, blood transfusions or pregnancies. The other reason is because existing cross reactivity between memory T cells developed towards pathogens and graft alloantigens.^{44, 66} These *de novo* and pre-

existing memory cells are able to trigger a more powerful and rapid immune response in case of another presentation of the same antigen.⁴¹ This is due to a lower activation threshold and lower dependence of co-stimulation signals of memory cells.^{41, 44} Thus, memory cells may participate to acute and chronic allograft rejection. Moreover, they are particularly resistant to conventional IS, costimulation blockade and to the effect of Tregs, rendering allograft tolerance more difficult. Nevertheless, some studies pointed out that these cells need to migrate and infiltrate the allograft to initiate rejection and thus, blockade of their migration could be a promising therapy.⁶⁶

3.2 The different types of allograft rejection

Allograft rejection may occur through 4 different schemes:

3.2.1 The hyperacute rejection

Hyperacute rejection occurs very early, within minutes to hours post transplantation. This is a humoral reaction, caused by the presence in the recipient of pre-existing alloantibodies directed against antigens present in the allograft vessels (ABO, HLA). The binding of antigen triggers destruction of endothelial cells, intravascular thrombosis and haemorrhage and leads to graft infarcts and loss. This phenomenon is quite inexistent in the context of liver transplant as far as the donor and recipient ABO compatibility is respected and, in case of planned ABO incompatible LT, recipient pre-transplant conditioning as already mentioned in chapter II could prevent hyperacute rejection.³⁵ Moreover, contrarily to kidney or

heart transplantation, the liver is a very low immunogenic organ and HLA matching before LT is usually not performed since it has been shown in 1986 that it didn't statistically change graft outcomes.⁶⁷⁻⁶⁹

3.2.2 The acute cellular rejection

In the clinical setting, acute rejection may occur from day 5 to month 3 post LT.

Acute cellular rejection (ACR) is clinically suspected in case of fever, abdominal tenderness and ascites although clinical signs are not always present.⁷⁰ Besides or in case of no clinical signs, biological alterations constitute indirect evidence of ACR. Indeed, liver cytolysis with raised liver enzymes such as aspartate amino transferase (AST), alanine amino transferase (ALT), and signs of cholestasis with elevated gamma-glutamyl-transferase (GGT), alkaline phosphatase and bilirubin are often encountered.⁷⁰

Due to the low sensitivity and specificity of liver enzymes as markers of ACR, finding biomarkers of ACR is a growing area of research. Inflammatory cytokines are largely studied and if some of them (IL-4, IL-6, IL-15, $TNF\alpha$) have increased expression during ACR, they can't be differentiated from elevated levels due to infection.⁷¹ Recently, a high relative blood eosinophils count (> 10%) has been shown to be a good predictor for ACR at day 7 in PLDLT⁷² and even a good biomarker of late ACR.⁷³ Other assays and biomarkers such as Cylex ImmunoKnow assay, proteomics and donor-derived cell free DNA are potential and promising biomarkers but lack of specificity or large heterogeneity between studies are still

limitating problems. Therefore these techniques need more extensive and clinical research before being applied.^{71, 74, 75}

ACR can be suspected on clinical and biological signs, but the diagnosis is still biopsy-proven, based on histopathologic criteria. These criteria are met in an international grading system, the Banff schema, adopted by a panel of experts in LT in 1995 in Banff, in Canada.⁷⁰

Three typical histological features are encountered in ACR: 1) the presence of portal mixed cellular infiltrate composed by activated T lymphocytes, macrophages, eosinophils, neutrophils and plasma cells 2) typical bile ducts injuries and 3) portal and/or pericentral endothelitis. ACR requires at least the presence of two criteria. Each criterion is semi quantitatively scored from 1 to 3 according to the severity and extension. Each score is then added to the other ones and the final score of the Banff schema represents the Rejection Activity Index (RAI). This index allows for grading the severity of liver ACR. A RAI <2 corresponds to no ACR, 2 corresponds to indeterminate rejection, 3-4 to mild rejection, 5 to 6 to moderate rejection and 7 to 9 to severe rejection.⁷⁶ The RAI is supplementary information for clinician to decide whether it should treat rejection or not even though there is no clear-cut consensus on the therapeutic approach. Usually, a moderate or severe ACR is also accompanied by biological perturbations and beneficiate from additional treatment while the treatment is not modified when RAI<2.⁷⁰

Late acute rejection (LAR) may appear 3-6 months post-transplant, in case of immunosuppressive therapeutic changes or in case of non-compliance, a problematic frequently observed in

adolescents. Its incidence is between 8% and 30% in PLT.⁷⁷ The same characteristics as ACR may be found but with slight differences such as fewer blastic lymphocytes in infiltrates, less endothelitis, more lobular and interface activity. LAR may also present as isolated perivenular inflammation and dropout.⁷⁸ LAR predisposes to chronic rejection (CR) mainly when it is steroids resistant.⁷⁷

Treatment of acute cellular rejection in LT is a decision based on clinical, biological and histological evaluation. Depending on the severity, the treatment comprises either intravenous pulses of methylprednisolone followed by oral prednisone treatment for at least 6 months, or increase of standard IS or addition of new drugs.⁷⁶

3.2.3 The chronic rejection

CR usually happens either within the first-year post transplant or with late onset, after one year. It occurs either as the end stage of unresolved ACR or after multiple episodes of ACR, and rarely happens without any previous ACR.⁷⁹ CR progressively leads to ductopenia, arteries and veins loss and finally graft failure. CR incidence is reported to be 2.8% in Korea³⁰ and 5% in the North America experience⁸⁰ but is the third cause of graft failure after vascular complications and primary non-function in the Pittsburgh experience, representing 19% of indications for retransplantation.³⁴ It results most often from inadequate IS. A multi-variate analysis showed that risk factors for CR were: African-American recipients, autoimmune diseases, post-transplant lymphoproliferative disorder (PTLD) and multiple ACR episodes.⁸¹ CR is suspected in case of jaundice, pruritus, biological cholestasis, elevated liver enzymes, and hyperbilirubinemia. Histologically, images differ according to early or late CR, but diagnosis requires at least: 1) biliary epithelial senescence affecting the majority of bile duct with or without bile duct loss 2) cell foam obliterative arteriopathy and 3) bile duct loss in more than 50% of the portal tracts. In early stage, hepatocyte dropout and central perivenulitis are frequently observed as well as progressive perivenular fibrosis to veno-centric cirrhosis.⁷⁸ Treatment of CR consists of increasing basal IS or switching from cyclosporine to Tac or to add new immunosuppressive drug such as antimetabolite or mTOR inhibitor and retransplantation in case of failure.⁸²

3.2.4 The antibody-mediated rejection

As previously mentioned, preformed Donor Specific Antibodies (DSA) seemed, until recently, to be irrelevant in liver transplantation due to absence of hyperacute rejection and no evidence of adverse effect on graft outcomes.⁸³ Liver was thought to be Antibody Mediated Rejection (AMR) resistant for many reasons. First, because of its large size and its vast sinusoidal endothelial surface, able to absorb circulating antibodies. Then, because the liver secretes soluble HLA I that binds and inactivates antibodies that are cleared by Kupffer cells. Finally, due to its dual circulation and its capacity to regenerate after injury the liver is less sensitive to AMR injuries.⁸³⁻⁸⁵ However, recent studies in adult LT pointed out that preformed⁸⁶ and *de novo*⁸⁷ DSA decrease allograft survival. With advances in techniques trying to identify and quantify DSA in patients, the number of studies about acute AMR and chronic AMR is increasing. The concerned DSA are directed against HLA class II and are IgG isotype, with IgG3 reported as giving more often adverse consequences.^{88, 89} Acute AMR is now well recognized and suspected in case of allograft dysfunction with decreasing platelet and complement levels, circulating immune complexes in patient with preformed DSA and microvascular injury on the liver biopsy. Diagnostic criteria have even been published recently:⁸³

1) Presence of seric DSA, 2) Diffuse microvascular endothelial cell injury and microvasculitis, 3) strong and diffuse C4d deposition in tissue and 4) exclusion of potential other causes of injury.^{83, 85}

A certain overlap with ACR may occur with more than 70% of patients experiencing rejection having circulating DSA.⁸⁵ A panel of

experts even said that ideally the presence of DSA in liver transplant recipients should be tested before transplantation and monitored in positive patients 1-2 week after transplantation.⁸³ Chronic AMR starts also to appear as an entity in the literature.⁹⁰ Indeed, DSA have been associated with fibrosis in several studies, that seems to be perivenular and subsinusoidal.⁸³

The first study to point out this relationship was done in PLT where LT children with DSA had higher frequency of: bridging fibrosis and cirrhosis, diffuse/focal endothelial C4d staining and mild/indeterminate acute rejection than patients who were DSA negative.⁹¹ There was no clear evidence for a cause-effect relationship between DSA and liver graft fibrosis, a late outcome frequently encountered in PLT.^{85, 92} However, our group showed in 2016, in a multivariate analysis, that class II DSA (along with previous portal inflammation and recipient HLA-DRB1*03/04 genotype) significantly correlated with portal fibrosis.⁹³ When considering risk factors for development of DSA, class II HLA mismatching, inadequate IS, previous ACR, younger donor age and older recipient age are cited in the literature.^{83, 85} No clear therapeutic strategy is actually defined in the literature.⁸³ Thus, DSA development prevention by adequate IS seems the best option, until randomized controlled trials give new information.⁸³

We have just seen how and why organ rejection is an ineluctable outcome in allograft transplantation. Fortunately, chemical IS molecules have emerged to prevent such an issue. In this section, we will briefly review IS history, current regimen proposed in PLT and we will focus on the drug considered as the cornerstone of IS, Tac.

3.3 History of immunosuppression

In the early 1950s, IS for kidney transplantation was limited to total body irradiation (TBI) and was unsuccessful with a large mortality.⁹⁴ A noticeable improvement occurred with the introduction of 6 mercaptopurine (6-MP), a purine analogue, and its derivative, azathioprine (AZA), which was less toxic.⁹⁵ In the 1960s, Starzl introduced the next step with the use of steroids as rejection reversal agent first and then concomitantly to AZA. The immunosuppressive effects of corticosteroids are driven by the decrease in cytokine expression (IL-2, IL-1, IL-6, $\text{TNF}\alpha$, $\text{IFN}\gamma$), decrease of chemoattractant cytokines, poor antigen presentation by DC and APCs and poor proliferation of activated T lymphocytes.⁵ Other strategies such as splenectomy, thoracic duct drainage and thymectomy were of limited interest.⁹⁶⁻⁹⁸ The next step was the introduction of heterologous antilymphocyte globulin (ALG) in 1966,⁹⁹ which allowed the emergence of extra renal organ transplantation and was the precursor of subsequent various biological antibodies used in transplantation. After the discovery of the immune properties of Cyclosporine (CyA), a calcineurin inhibitor (CNI), in 1976 by Jean Borel,¹⁰⁰ its use in kidney and LT^{9, 10, 101} at the end of 1970s, as a maintenance immunosuppressive drug with concomitant corticosteroid therapy and sometimes azathioprine, led to a dramatic increase in graft survival with a 1 year actuarial survival of 80%¹⁰¹ and allowing the development of many LT centers. Most of transplanted patients worldwide benefited in the mid 1980 from a cyclosporine based immunosuppressive regimen.

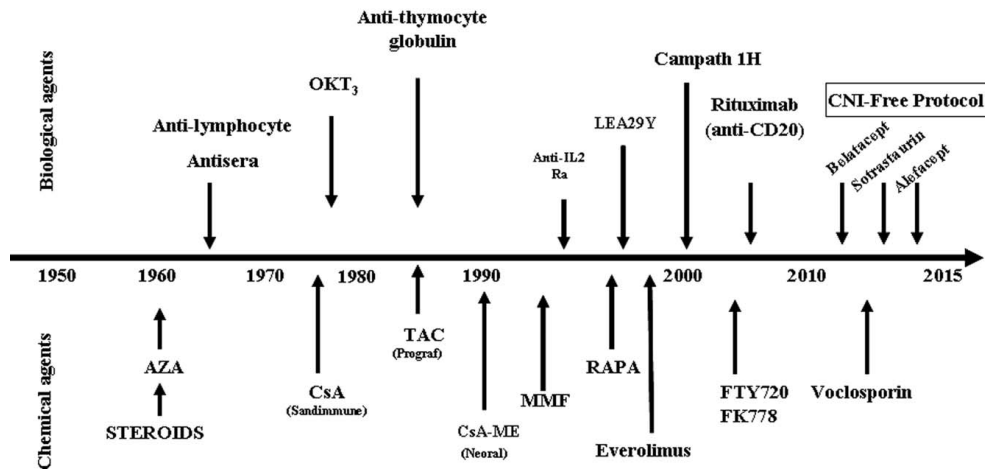


Figure 1: Timeline of immunosuppression. *From Selzner, 2010*¹⁰²

To go on the timeline of IS as represented in Figure 1, in 1981, Cosimi reported the first human trial of the use of mouse anti-human CD-3 monoclonal antibody (mAb), OKT3, a T lymphocytes depleting agent, to treat kidney acute rejection.¹⁰³ It was then employed to treat acute liver allograft rejection in liver transplant recipients or as induction agent to allow dose reduction of cyclosporine in case of renal impairment.¹⁰⁴

In PLT, McDiarmid showed that its prophylactic use didn't have any beneficial impact when compared to a CyA-AZA-prednisone regimen and thus was only recommended for steroid resistant rejection or in case of cyclosporine contraindication.¹⁰⁵ Due to considerable side-effects such as neutropenia and PTLT, its was progressively abandoned.

At the end of 1989, intractable liver allograft rejections were resolved by the introduction of another CNI, Tac, product of a soil

fungus, with immunosuppressive properties discovered in 1984 and firstly reported for its effect in 1987.¹⁰⁶

Due to its impressive capacity to salvage allografts from acute rejection in LT, but also in heart and kidney transplants, it was quickly used as a primary IS drug. In the early 1990s, the first trials realized in more than 200 transplanted patients where Tac replaced cyclosporine showed better actuarial patient survival, lower prednisone dose requirement, lower rejection rate and better quality of life.¹⁰⁷ The first study in PLT where Tac was introduced as a primary IS agent started in September 1989 in USA.¹⁰⁴ In 2004 a multicentric European trial comparing Tac and steroids versus CyA microemulsion + AZA + steroids in PLT showed a significant lower acute rejection rate and steroid resistant rejection rate in the group with Tac with no statistical difference in terms of patient survival and side effects. Progressively, Tac replaced CyA as the maintenance IS in LT and PLT worldwide.

In the following 20 years, several immunosuppressive drugs have been tested and used in clinical practice of kidney, heart and LT. Polyclonal horse and rabbit anti thymocyte globuline (ATG) have been used for steroid resistant rejection or induction of IS in kidney and liver transplantation. However, these lymphocytes depleting agents have been associated with higher incidence of PTLD in PLT¹⁰⁸ and have been replaced by the monoclonal humanized anti CD-25, IL-2R α , monoclonal antibody (Daclizumab) and the chimeric monoclonal antibody anti CD-25 (Basiliximab), as an induction therapy. Basiliximab was first reported in the literature as reducing incidence of acute liver allograft rejection.¹⁰⁹ Whereas Daclizumab is

not commercialized any longer, Basiliximab is now widely used as induction therapy in PLT and allows, with Tac, steroid-free IS regimen.^{110, 111} In order to reduce side effects attributed to high doses of CNI and/or steroids (hypertension, diabetes, infections, impaired growth...) while keeping efficient immune modulation, the new strategies were to combine immunosuppressive drugs with different mechanisms of action or even to avoid CNI and/or steroids by replacing them with new molecules. This is schematically represented in Figure 2.

In the 1990s, the introduction of mycophenolate mofetil (MMF) a prodrug hydrolyzed into active mycophenolate acid (MPA), in kidney transplantation showed benefit in prevention from early acute rejection when compared to AZA. MPA is a non-competitive reversible inhibitor of inosine monophosphate dehydrogenase, an important enzyme in the *de novo* guanine biosynthesis pathway. It inhibits preferentially the proliferation of activated B and T lymphocytes and has no renal toxicity.⁵ In pediatric liver transplantation, it has been successfully used for chronic or refractory rejection, in case of CNI toxicity, for CNI and corticosteroids sparing regimen. It is still employed, in some PLT centers, as baseline IS with CNI and steroids.^{112, 113}

Two inhibitors of the mTOR, a key enzyme in the cell cycle progression, prevent the cell cycle to go from G1 to S phase. Firstly, Sirolimus (SRL) was introduced in 1999 and proved its efficacy as rescue therapy for acute and chronic rejection and primary IS in pediatric transplantation. It also allowed CNI dose reduction^{114, 115} with renal function improvement.¹¹⁶ In adult *de novo* liver

transplantation studies, use of sirolimus with tacrolimus was associated with higher mortality, graft loss and hepatic artery thrombosis and was then not recommended by the company. However, such issues have not been reported in pediatric patients.¹¹⁷ Then, its derivative molecule, Everolimus (EVL), which benefits from a better bioavailability and shorter elimination half-life, has shown beneficial impact in pediatric kidney transplant in terms of renal function¹¹⁸ and promising effect as rescue therapy for chronic allograft dysfunction in PLT.¹¹⁹ Clinical trials of EVL with low doses of CNI in PLT were active and completed in 2016. Unfortunately, recruitment was stopped in December 2014 due to high rate of PTLD and high rate of related serious infections leading to hospitalization.¹²⁰

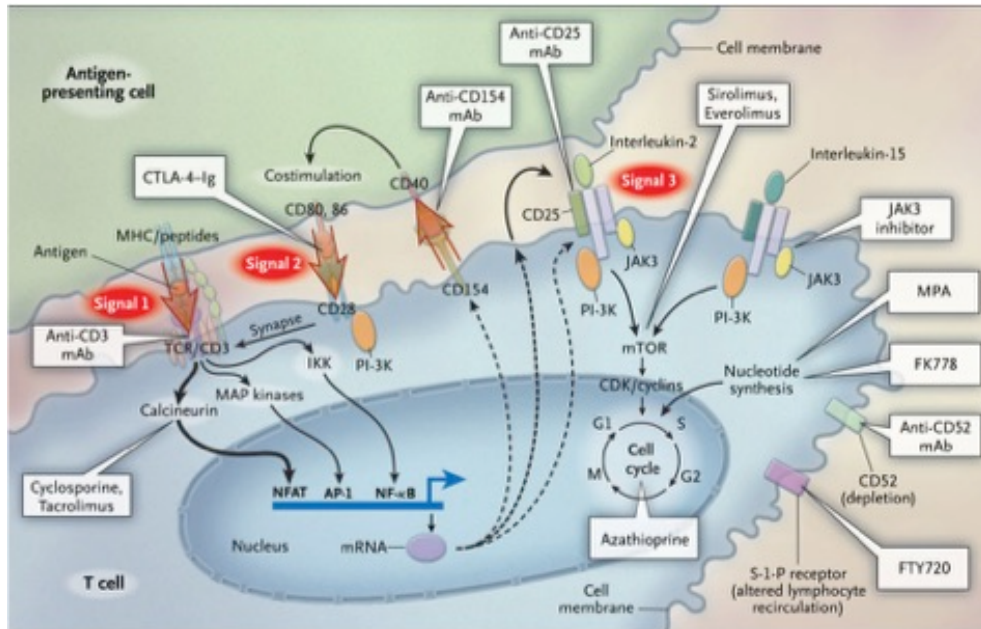


Figure 2: Mechanisms of action of the different immunosuppressive agents.

From Halloran, NEJM, 2004 ⁴⁸

3.4 Current strategies in pediatric liver transplantation

In those last years, the concerns about immunosuppressive treatments shifted. Patient and graft survival were no longer a limiting problem; therefore, questions about long-term outcomes of transplanted patients emerged, particularly those that could be linked to IS.

Among the main issues concerning pediatric transplantation outcomes and more specifically PLT, renal impairment due to CNI, reported to reach 30% of long term survivors,¹²¹ infections, PTLD and growth impairment are not uncommon.^{122, 123} In order to preserve renal function many strategies aiming at reducing CNI nephrotoxicity have emerged, utilizing different molecules. Currently, these are

based on induction with anti-CD-25 monoclonal antibodies or steroids and a low dose of Tac, that can be associated or not with either SRL or EVL or MMF.¹²⁴

In parallel, in order to reduce infections and growth impairment in children, the aim to withdraw or to totally avoid steroids in the immunosuppressive regimen appeared. This has been done in different centers including UCL with good results in terms of patient and graft survival and some benefit in terms of better growth and reduced infections.^{111, 125}

Finally, the ideal goal one would achieve is to reach liver graft tolerance. This is sometimes observed in patients in whom the treatment is stopped because of PTLD and who may stay many years without any IS treatment. But these cases are still rare, and the underlying mechanisms that explained it are not yet clearly established. At a lower level, the use of low dose maintenance IS therapy allows for proper tolerance or near tolerance, with limited IS adverse effects.^{126, 127}

In conclusion, we have presented the allograft rejection mechanisms and the different existing immunosuppressive molecules and strategies to avoid such an outcome. In the next chapter, we will focus more particularly on Tac, a drug that became a cornerstone in the immunosuppressive treatment in PLT.

Chapter 4. TACROLIMUS: A CORNERSTONE OF PEDIATRIC LIVER TRANSPLANT IMMUNOSUPPRESSION

4.1 History of Tacrolimus

After the discovery of immunosuppressive properties of CyA via inhibition of the production of IL-2, Japanese researchers from Fujisawa Pharmaceutical Ltd began looking for molecules with equivalent properties. In 1984, after two years spent at testing a wide variety of fermentation broths, they found a potent immunosuppressant produced by the strain of *Streptomyces tsukubaensis* N° 9993, a strain isolated from a soil sample from Toyosato-cho, Tsukuba-gun, Ibaraki prefecture in Japan. This immunosuppressant first designated as the “FR-900506 substance”¹²⁸ was then named FK-506, also called Fujimycin, and became in 1992, Tac.^{106, 129, 130} In 1986, Ochiai presented for the first time this new molecule at the 11th International Congress of the Transplantation society, but it was only in 1987 that the first reports about *in vitro* and *in vivo* actions appeared in the literature.^{106, 130} In these two papers, after a description of isolation and fermentation methods, Tac was tested *in vitro* and *in vivo* for its immunosuppressive effect and compared with prednisone and CyA. The authors showed that Tac had a 100 times higher *in vitro* activity than CyA by suppressing mouse mixed lymphocytes reaction, a representative of IL-2 dependent T cell growth. *In vivo*, Tac had 10 times and 3 times higher suppressant activity on humoral and cellular activity, respectively. Subsequently to these very promising results,

several preclinical *in vivo* studies were conducted in 1987-1988 in the USA and Europe, while at the same time the first enzyme-linked immunosorbent assay (ELISA) method to quantify Tac in plasmatic samples was developed.¹³¹ Soon, the question to determine if Tac had a potential clinical utility leads to a divergence between the USA (Pittsburgh), where studies in rats,¹³² dogs and primates¹³³ were encouraging and the UK (Cambridge), where unacceptable vascular toxicity was reported.¹³⁴ As this toxicity was not proved to be specific to Tac, the Pittsburgh team decided to realize the first clinical trials in 14 liver, kidney and pancreas transplanted patients, as a rescue therapy in 10 patients and as primary IS in 4, but without any prior human pharmacokinetic data available.¹³⁵ In this study, Tac was administered first by intravenous (IV) route and then by oral route. The pharmacokinetic data were obtained by measuring the plasmatic level of Tac. The first IV dose was 0.15 mg/kg followed by 0.075 mg/kg every 12h. For the oral route, the dose was 0.15 mg/kg/12h. The plasmatic trough levels were comprised between 0.2 to 7 ng/mL with an aim below 3 ng/mL. Patients also received steroids. The main signs of toxicity described in that study were transient nausea, vomiting and headaches. The authors showed that Tac allowed graft salvage in 7 out of the 10 patients and said that despite the impossibility to exclude nephrotoxicity, the evidence was that Tac was not nephrotoxic at a clinical range and did not cause hypertension. Moreover, they hypothesized that contrarily to CyA, Tac might have a wide therapeutic window.¹³⁵ In 1990, Venkataramanan from Pittsburgh published the first pharmacokinetic (PK) study about Tac and confirmed the previous results.¹³⁶ After this

first success, clinical studies using Tac as a first-line therapy were conducted in Pittsburgh in the spring of 1990 and showed for the first time that Tac could be nephrotoxic, cause hypertension and neurotoxicity but in an acceptable way.¹³⁷ However, they confirmed its efficacy with 1-year patient and graft survival of 92% and 87%, respectively.¹³⁸ At the same time, studies in PLT were conducted in Pittsburgh, at UCLA and at UCL in Belgium where Tac was prescribed either as a rescue or as primary therapy and gave very promising efficacy results. Moreover with only a few adverse events and the possibility to taper off steroid dose, Tac would be of particular interest in PLT.¹³⁹⁻¹⁴¹ The first US multicentric study published in 1994, including 5% of pediatric patients, showed that patient and graft survival were equivalent for both CyA and Tac but that Tac was more effective to prevent from acute graft rejection, from steroid resistant graft rejection and from refractory rejection, despite a higher incidence of adverse effects.¹⁴² The multicentric European study published the same year, without pediatric patient, was concordant with these results.¹⁴³ Tac was also studied in PLT as a conversion therapy and then was compared to CyA.¹⁴⁴⁻¹⁴⁶ Results showed a higher efficacy of Tac in term of rejection-free survival, lower dose of steroid required and absence of cosmetic damages such a gum hyperplasia or hypertrichosis. Tac became available on the Japan market in June 1993, in America in June 1994, and in UK in August 1994. The absence of difference in terms of 1-year patient and graft survival and the lower acute graft rejection incidence with Tac have been confirmed in a European multicentric trial in PLT, comparing Neoral-steroids-AZA to Tac-steroids.¹⁴⁷ Tac quickly

became the cornerstone of IS in PLT and other type of transplantation. Initially discovered by Fujisawa, it is now commercialized by Astellas Pharma, a society resulting from the fusion of Fujisawa and Yamanouchi in 2005.

4.2 Molecular structure and characteristics

The production of Tac, as initially reported, comprised two phases: the fermentation of *Streptomyces tsukubaensis* and the isolation procedure. Tac production starts during the first phase after 40h of incubation, when the mycelium reaches its stationary phase. Then, Tac is isolated and purified after being filtrated, separated by chromatography, evaporated and recrystallized.

Tac is a neutral macrolide lactone. It is characterized by a hemiketal-masked alpha beta diketoamide incorporated in a 23-member ring. It is hydrophobic and crystallizes as a colorless prism. Its molecular formula is $C_{44}H_9NO_{12} \cdot H_2O$ with a molecular weight of 822,05. At room temperature, it appears as a white crystalline powder and is stable for months, at a pH between 2 and 6, even at high temperature, high relative humidity or under fluorescent lamps. Tac is lipophilic and thus, soluble in solvents (like methanol, ethanol, acetonitrile) but insoluble in water. Its melting point is about 127-129°C and its maximal spectral absorbance in UV is 205 nm. Its molecular structure is represented in Figure 3. In blood, Tac seems not being stable long time at room temperature since a loss of 32.9% is observed after 24h.¹⁴⁸ This information comes from a recent study where Tac is measured by liquid chromatography tandem mass

spectrometry (LC-MSMS) and is somewhat different from previous studies where Tac, measured by immunoassays was stable at room temperature for 7 days. However, Tac is stable in blood sample conserved at +4°C for at least 48h when measured by LC-MSMS and at least for 2 weeks when measured by immunoassay. When frozen, Tac is stable for 1 month at -20°C and for 1 year at -70°C.^{148,}

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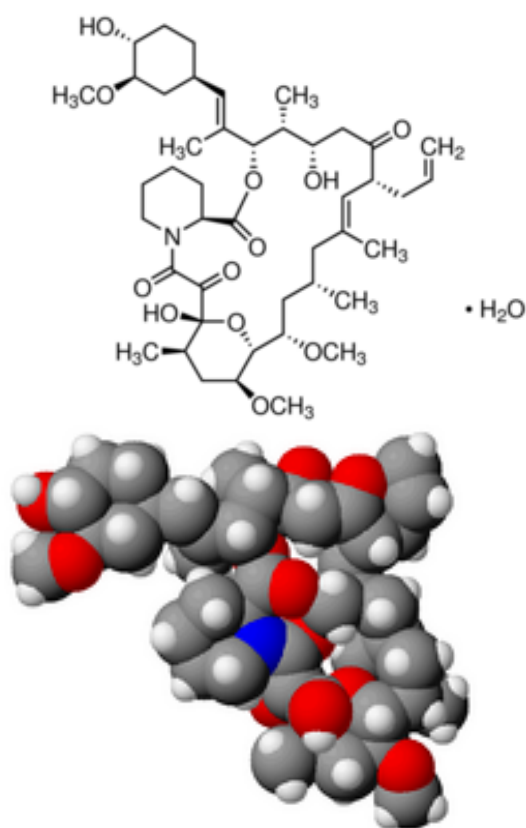


Figure 3: Tacrolimus molecule.

4.3 Mechanism of action

Tac exerts its main IS effect via altering signal transduction and finally IL-2 gene expression that occurs secondary to CD4+ helper lymphocytes activation as previously mentioned and illustrated in Figure 4.

Once entered the cell, Tac binds with high affinity to specific cellular proteins named immunophilins or FK506-binding proteins (FKBPs), and more specifically with FKBP12. Then, the complex Tac-FKBP12 interacts with the calcium-calmodulin dependent serine-threonine phosphatase, Calcineurin. This enzyme is actually a dimer of two subunits: Calcineurin A, a 59-kDa catalytic unit containing a calmodulin-binding domain and a region of autoregulation, and Calcineurin B, a 19-kDa calcium-binding regulatory unit. By interacting with the B and catalytic A subunits, Tac-FKBP12 inhibits the phosphatase activity of Calcineurin preventing translocation of the NFATc to the nucleus where it normally binds to other transcription factors such AP-1 and then to DNA to promote the transcription of IL-2 gene but also other genes (IL-3, IL-4, IL-10, IL-17, TNF alpha and IFN gamma).¹⁵⁰⁻¹⁵⁵ Besides NFAT and AP-1, Calcineurin is also involved in the activation of NFκB, another factor controlling transcription of numerous genes involved in the immune response, including IL-2.¹⁵¹ In addition, the indirect inhibition of Tac on IL-2 transcription has also been described through the MAPK and TGF-B1 pathway.¹⁵⁰ Finally, Tac effects are also reported to be due to some inhibition of nitric oxide synthase activation and to T lymphocytes apoptosis.¹⁵²

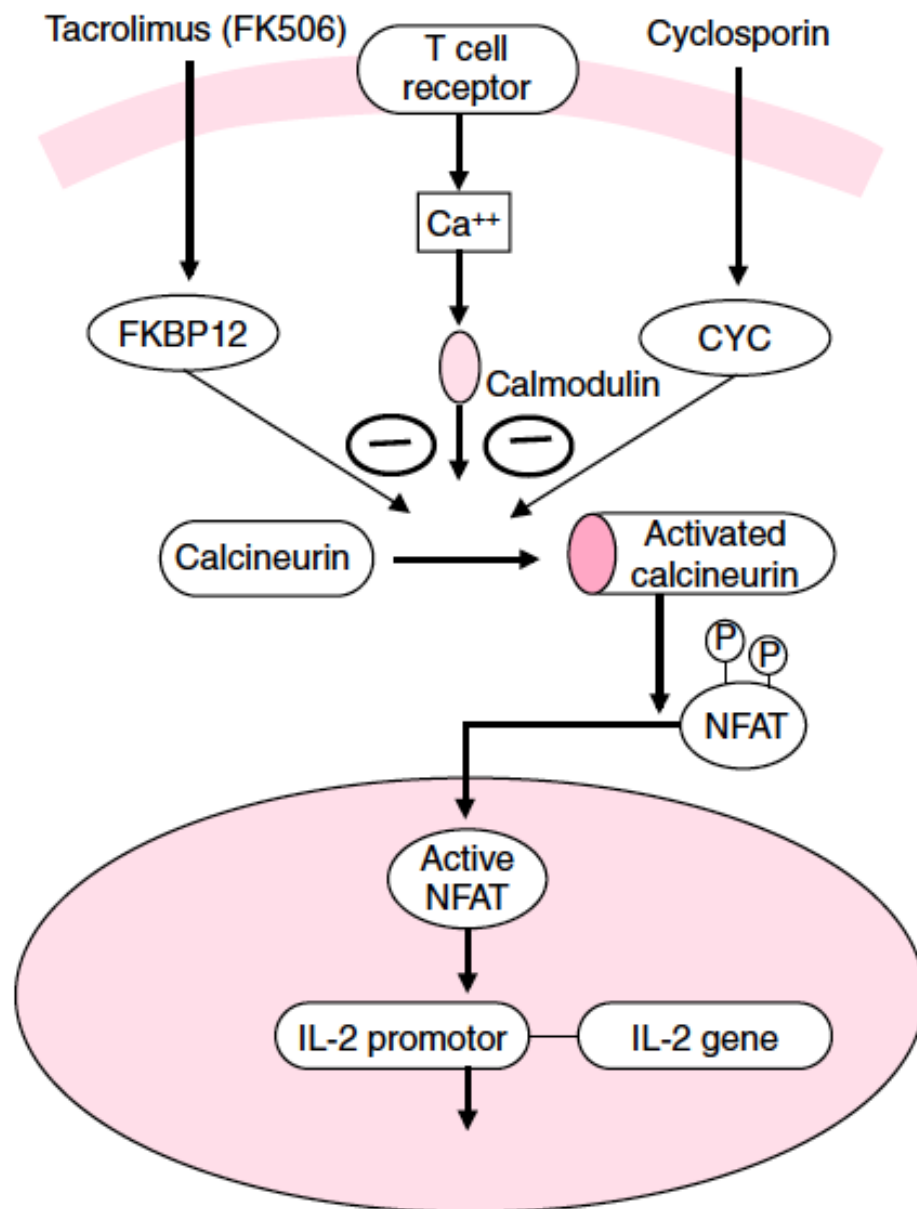


Figure 4: Mechanism of action of Tacrolimus. *Adapted from Scott 2003.* ¹⁵²

4.4 Pharmacokinetics and variability

After its administration, Tac undergoes absorption, distribution, metabolism and excretion. These 4 steps defining its PK exhibit a wide intra- and interpatient variability and are largely discussed in the following section with a particular emphasis on LT and pediatric recipients.¹⁵⁶ Figures 5 illustrate general Tac PK, absorption and first pass metabolism as well as distribution. Metabolism is illustrated in the next section in Figure 6.

4.4.1 Absorption and bioavailability

After oral administration Tac has a rapid but incomplete and variable absorption along the intestinal tract, from the duodenum to the colon.¹⁵⁷

The peak plasma/blood concentration $C_{\max}$ is reached between 0.5 h to 2 h after administration. However, some patients experience slow uptake yielding a flat absorption profile, and some show a secondary peak or a lag time (mean of 0.39 h, range 0 – 2 h).¹⁵⁸ Jusko reported a mean absorption rate constant (K_a) of 4.48 h^{-1} .¹⁵⁸ Tac Oral bioavailability (F), defined as the fraction of the Tac oral dose that reaches the arterial blood, is poor and highly variable (4-93%) with a mean of $25 \pm 10 \%$. There is no significant difference between children and adult liver transplanted patients, although lower oral Tac F of 19% has been reported in PLDLT.^{152, 156, 159-164}

Although this low oral Tac F may be partly explained by its hydrophobicity and the subsequent limited dissolution, it is mainly due to an extensive intestinal and hepatic first pass effect. Indeed, after reaching the intestinal lumen, Tac is passively absorbed through the phospholipid bilayer membrane into the enterocytes from where it can either reach the portal circulation, be effluxed back by a transporter expressed on brush border of the apical membrane of enterocytes, the P-glycoprotein (P-gp) or metabolized by cytochromes P450 (CYP) of the CYP3A subfamily.^{165, 166}

P-gp or ATP-binding cassette subfamily B member 1 (ABCB1), is an ATP- dependent efflux pump, encoded by the *ABCB1* gene on the human chromosome 7 q21.12 and widely distributed in the body (enterocytes, hepatocytes, renal proximal tubular cells, leucocytes, blood-brain barrier, blood-testis barrier and blood-placental barrier). Firstly, described in cancer cells, P-gp actively extrudes chemicals outside of the cells limiting both the efficacy and toxicity of numerous xenobiotics including Tac.^{167, 168} Besides being the substrate of drug transporters, Tac is also metabolized by the CYP enzymes. CYP are part of the oxidative phase I enzymes responsible for metabolism of endogenous compounds and more than 80% of xenobiotics.¹⁶⁹ Of all the CYP families present in the liver and in the intestine, the CYP3A subfamily accounts for almost 30-40% and 80% of the cytochromes, respectively.^{170, 171} The CYP3A subfamily is represented by four isoforms (and their 4 respective genes on the human chromosome 7 q22.1) that share at least 83% of aminoacids: CYP3A4, CYP3A5, and CYP3A7, and CYP3A43.^{170, 172-179} CYP3A4 is the predominant form in the adult liver and intestine with a very variable expression

between individuals, of 10- to 100-fold differences in liver and up to 30-fold differences in intestinal expression.^{162, 176, 179} CYP3A5 is also present in the fetal and adult liver and in the intestine.^{170, 178, 179} The presence of CYP3A5 enzymes which may account for up to 83% of total hepatic and intestinal CYP enzymes content, and 50% of the related metabolism, is mainly due to genetic control, as discussed later.^{176, 180} Expression level of hepatic CYP3A43 is very low and thus doesn't contribute to significant liver metabolism.^{181, 182} Finally, CYP3A7 known to be the fetal and new-born CYP3A isoform, decreases after birth giving rise to its adult isoform, CYP3A4. However, CYP3A7 is also present in adult liver and intestine, partly due to genetic variant.^{183, 184} Along the intestinal tract, CYP3A and P-gp are inversely expressed. Indeed, CYP3A content is maximal in the duodenum and progressively declines toward the ileum and colon whereas P-gp is principally expressed in the distal part of the intestine and in the colon, with a high variability of mRNA and protein expression for CYP3A and P-gp that are both regulated by the CAR and PXR transcriptional factors.¹⁸⁵⁻¹⁹⁰

4.4.2 Distribution

Animal studies showed that Tac is largely distributed to a wide variety of tissues: blood, lungs, heart, spleen, kidney, pancreas, brain, muscle and liver, but not found in the cerebrospinal fluid.^{136, 152, 160, 161} In whole blood, Tac is extensively bound into erythrocytes (83.2%, range 74.6–94.9%), followed by diluted plasma (16.1%, range 4.5–24.9%), and lymphocyte fraction (0.61%, range: 0.11–1.53%).¹⁹¹ There is a very wide and variable blood: plasma ratio of 4-

114 and a mean of 15, in transplant patients.^{152, 156, 160, 192-195} This ratio is concentration-dependent with lower ratio at higher Tac whole blood concentrations probably due to saturation of FKBP12.^{160, 193} In *ex vivo* conditions, Tac partitioning between plasma and red blood cells depends on Tac concentration, temperature of sample, hematocrit and the plasmatic concentration of proteins.^{152, 160, 192, 196, 197}

In plasma, Tac is highly bound to proteins (up to 99%) particularly to α 1-acid glycoprotein (AAG), albumin and globulins, and to a lesser extent, to lipoproteins.^{162, 191, 198 160} The Tac unbound blood fraction (*fu*), which is the active Tac fraction, is therefore very low (<1%). While hematocrit and red blood cell count significantly influence the fraction of Tac sequestered in erythrocytes, Tac unbound fraction inversely varies with hematocrit, albumin, α 1-acid glycoprotein and HDL-cholesterol concentrations in liver transplant recipients.^{191, 199} Studies in LT recipients found an apparent volume of distribution (*Vd*) based on blood concentrations about 0.9 to 1.5 L/kg in adults, that is much lower to *Vd* based on plasma concentrations (about 20 - 65L/kg), due to extensive Tac binding in to erythrocytes.^{156, 158, 160, 161, 200}

Although no specific data are available with Tac, pediatric patients are known to have higher red blood cell membrane permeability and larger unbound fraction of drugs in plasma due to lower quantities and affinity binding with proteins (albumin and α 1-acid glycoprotein). This higher *fu* allows drug to enter other compartments leading to a higher *Vd*.²⁰¹ Indeed, in PLT, *Vd* has been reported as 1.8 times higher when compared to adults with a

Vd up to 2.6 L/kg with IV route in PLT and up to 9 L/kg with oral administration.^{159, 202}

Tac crosses the placenta with an umbilical cord venous blood: maternal blood concentration ratio, representing fetal exposure, of 71% while mean umbilical cord venous plasma and unbound drug concentrations represent one fifth of the respective maternal concentrations. This is possibly due to higher Tac partitioning in fetal red blood cells, higher hematocrit values and action of placental P-gp.²⁰³ Tac is also transferred into breast milk but doesn't seem to reach significant daily exposure for the neonate with reported values between 0.06 and 0.14 µg/kg in fully breastfed babies.^{203, 204} While breastfeeding was not recommended in the past, recent studies concluded that mothers should not be discouraged to breastfeed while on Tac regimen.²⁰³⁻²⁰⁵

4.4.3 Metabolism and excretion

Tac is almost entirely eliminated by metabolism, principally in the liver, and to a lesser extent, in the intestine. It is therefore a class 2 drug of the Biopharmaceutics Drug Disposition Classification System.²⁰⁶ Tac undergoes metabolization by the CYP3A4, CYP3A5 and CYP3A7 enzymes, as previously mentioned. *In vitro* studies have shown that CYP3A5 is the predominant enzyme for metabolism of Tac, with CYP3A4 contributing, but having a lower efficiency for catalysis.^{165, 207} CYP3A7, has a much lower substrate affinity, reaction velocity and catalytic efficiency toward Tac than CYP3A4 and no clinical study has shown a significant effect on Tac metabolism yet.²⁰⁷ The main reactions Tac undergoes during

metabolism are demethylation and hydroxylation. To date, up to 15 metabolites have been identified.^{150, 160, 208, 209} The most prevalent one *in vitro* and *in vivo*, after Tac incubation with liver microsomes and when measured in whole blood of patients respectively, is 13-O-demethyl-Tac (M-I).^{162, 209, 210} This metabolite is approximately one-tenth as active as Tac, whereas a minor metabolite, 31-O-demethyl-Tac (MII), has been found to have similar immunosuppressive activity with Tac.^{166, 209} The remaining metabolites have weak or negligible pharmacological activity.¹⁶⁶

Based on plasma concentrations, systemic clearance of Tac is very high, with reported values in patients from 0.6 to 5.4 L/h/kg.^{156, 158, 160} However, the strong binding of Tac to erythrocytes limits its clearance from blood. Therefore, systemic blood clearance values in healthy volunteers and transplant patients are much lower, ranging from 0.03 to 0.09 L/h/kg.^{156, 160}

Thus, Tac is considered as a low-clearance drug with clearance about 3% of hepatic blood flow, on the basis of blood concentration data.^{162, 211}

The terminal elimination half-life ($t_{1/2\beta}$) of Tac is rather long (approximately 32 +/- 10 hours) in healthy volunteers but values between 3.5 to 40.5 hours (mean 12.1 hours) have been reported in transplant patients.^{156, 160} The high variability in reported $t_{1/2\beta}$ may be partly explained by the high interpatient variability in binding Tac to red blood cells.^{156, 160} Longer $t_{1/2\beta}$ have been reported in studies where patients and/or volunteers who received a single dose were studied for 72h after a dose rather than 12h.^{160, 162}

Concerning excretion, less than 1% of unchanged Tac is excreted into the urine, bile and faeces.^{156, 160, 211} More than 95% of Tac metabolites are eliminated into the bile. Finally, urinary excretion accounts for less than 3% of Tac elimination.²¹¹

In children, the CYP enzyme system is immature during the first months of life but after 6 to 12 months, reach an important metabolic activity, which may be even higher than the average adult activity.²¹² The mean total Tac body clearance in PLT patients was estimated to be 0.14 L/h/kg after intravenous administration, which is two times higher compared to adults. This higher clearance in children explains that they require a 2-fold Tac oral dose compared to adults to get the same systemic exposure. The reported mean $t_{1/2\beta}$ value in children is similar to the adult one and is around 12.4 hours.^{156, 159}

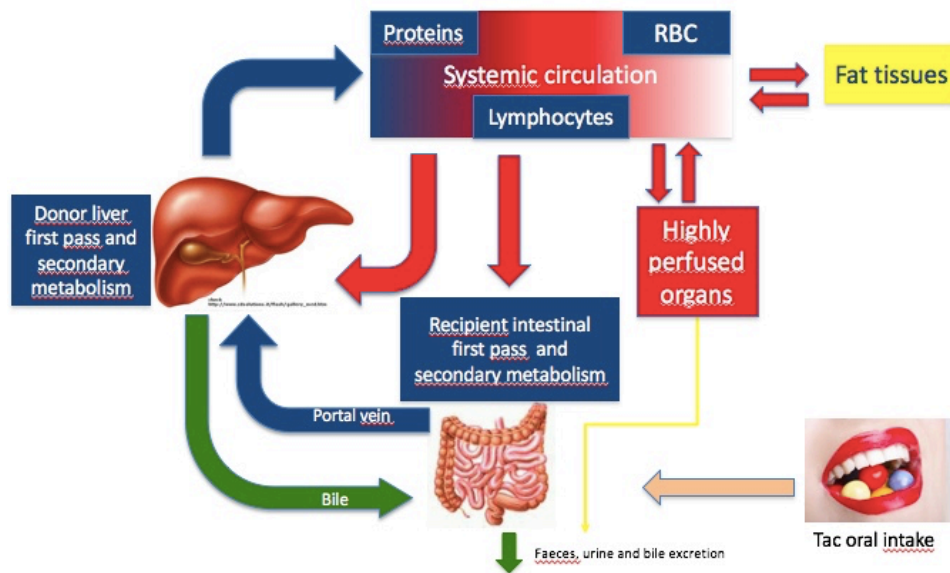


Figure 5.a: Schematic representation of Tac pharmacokinetics

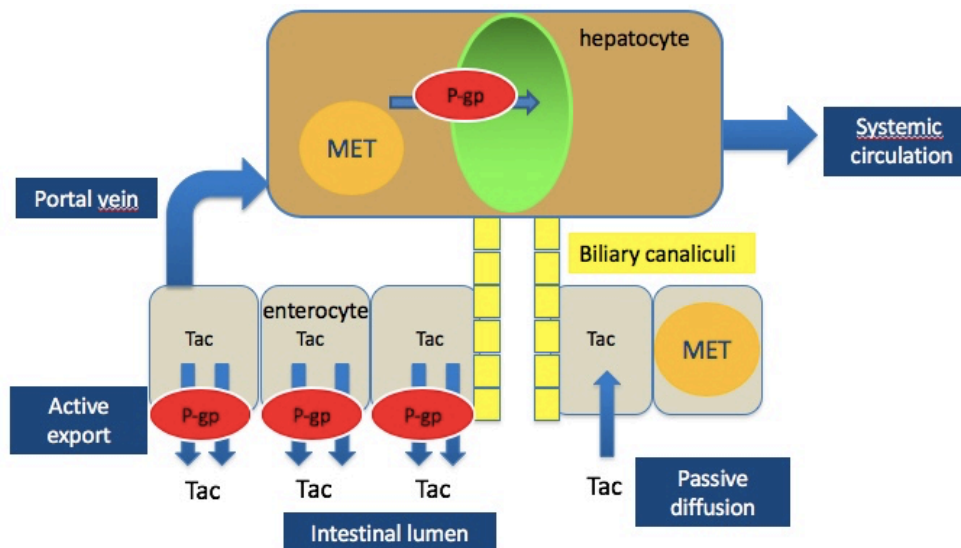


Figure 5.b: Schematic representation of Tac absorption and first pass metabolism. MET: metabolism; P-gp: P-glycoprotein

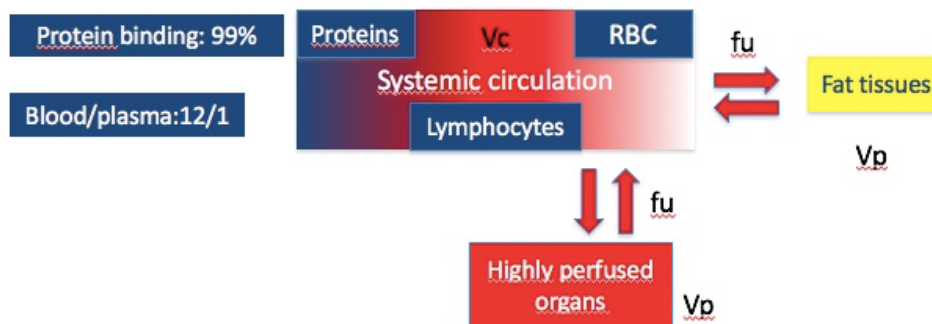


Figure 5.c: Schematic representation of Tac distribution.

fu: unbound fraction; Vc: volume of central compartment; Vp: volume of peripheral compartment

4.5 What could explain such a high Tac PK variability?

As stated earlier, if Tac has become a cornerstone of IS in clinical transplantation, its clinical use is partly limited by a wide intra- and inter- patient's PK variability. In this section, we will discuss about the main factors influencing Tac PK.

This subject has been extensively detailed in recent reviews.^{213, 214}

4.5.1 Age

As mentioned above, in clinical pediatric transplantation age showed an influence on Tac PK, with a 2 to 4-fold higher Tac doses, lower dose-corrected trough concentration (C_0/D) and higher Tac clearance in younger children.^{162, 215-221} Other studies found rather a

weight effect on Tac doses and or clearance. This effect could also be due to age given that weight is highly correlated with age. This difference in Tac PK according to age has been attributed to a relative higher liver weight/body weight ratio in younger children. This could be true in PLDLT where graft weight (GW)/body weight (BW) ratio can be particularly higher in children less than one year.

Another explanation could be that doses should be normalized by body surface area rather than weight due to allometry and as observed with cyclosporine in pediatric transplantation and with Tac in pediatric hematopoietic stem cell transplantation.²²²⁻²²⁴ However, normalizing Tac doses according to GW/BW has never been tested yet in pediatric liver transplantation.

4.5.2 Bile

Bile doesn't seem to have any influence on Tac absorption, contrarily to cyclosporine. However, biliary obstruction is reported to increase the concentration of Tac metabolites in the blood.^{162, 210, 225}

4.5.3 Drug interactions

Drug interaction occurs when a drug alters how the body absorbs, distributes, metabolizes, or excretes another drug.²²⁶ Moreover, besides PK effects, drug interaction may result in pharmacodynamic disturbances.²¹⁴ Tac is a substrate of intestinal and hepatic CYP3A enzymes and P-gp that have a very broad and large number of structurally divergent and overlapping substrates. Thus several medications prescribed in LT, may act as CYP3A/P-gp inhibitors and/or inducers, potentially interacting with Tac PK.²²⁶ The clinical relevance of such an interaction is all the more important as Tac has

a narrow therapeutic window and thus may greatly affect its efficacy and/or toxicity.

When considering CYP3A enzymes inhibition, 3 different pathways may intervene without exclusivity:²²⁷

- 1) Rapidly reversible competitive or non-competitive inhibition (e.g. azoles agents);
- 2) Slowly reversible through the formation of a slowly reversible complex (e.g. macrolides antibacterial agents);
- 3) Irreversible, mechanism-based ("suicide" inhibition) where CYP3A-mediated formed reactive metabolite(s) covalently binds to the enzyme in a fashion leading to its inactivation (e.g. 6', 7'-dihydroxybergamottin of grapefruit juice).

The major inhibitors of the CYP3A enzymes reported in the literature are:¹⁵²

- Calcium antagonists (diltiazem, nicardipine, nifedipine and verapamil),
- Imidazole antifungal agents (voriconazole, clotrimazole, fluconazole, itraconazole and ketoconazole),
- Macrolide antibacterial agents (clarithromycin and erythromycin),
- Prokinetic agents (cisapride and metoclopramide)
- Other drugs (bromocriptine, cimetidine, corticosteroids, cyclosporin, danazol and protease inhibitors) and grapefruit juice.

With regard to P-gp inhibition, three mechanisms may also be involved:²²⁸

- 1) Blocking drug binding site either competitively, non-competitively or allosterically;

- 2) Interfering with ATP hydrolysis;
- 3) Altering integrity of cell membrane lipids

Clinically important medications used in PLT inhibiting P-glycoprotein include azole antifungals, cyclosporin, calcium channel antagonists, and erythromycin.²²⁹

The CYP3A/P-gp inhibition seems to principally affect the oral Tac bioavailability rather than its clearance as shown by Floren in healthy volunteers who were administered Tac and ketoconazole with subsequent increase in Tac oral F without any significant change in clearance.^{229, 230} This intestinal CYP3A/P-gp inhibition may explain the discrepancies observed between clinical studies where the magnitude of the observed *in vivo* inhibition of Tac metabolism appeared to be much greater than predicted, based on the *in vitro* studies with human liver microsomes.²³¹ One of the possible explanations is that orally administered drugs reach much higher concentrations in enterocytes compared with hepatocytes and as absolute CYP3A content in the gut is lower than in the liver, so mechanism-based inhibitors will more easily deplete intestinal CYP3A4.²¹³ The clinical consequence is much higher Tac blood levels observed after oral co-administration of an inhibitor compared with its IV co-administration.

Therefore, all the inhibitors previously cited may increase the Tac whole-blood concentrations. Azoles are the strongest inhibitors of Tac metabolism. Fluconazole being the most known and frequently antifungal drug used in PLT, it may decrease Tac dose requirement by half in adult and PLT.^{221, 232} Moreover, this drug interaction seems

characterized by interpatient variability due to genetic variant as reported in adult kidney and PLT. This theme will be discussed in the pharmacogenetic section.^{221, 233}

Other medication frequently used as an antihypertension drug in PLT, is the calcium channel blocker nifedipine. Seifeldin described decreased daily and cumulative dosage requirement of Tac in LT patients under nifedipine.²³⁴ However, its effect in clinical PLT is unknown.

One should remind that all the drugs interfering with P-gp by changing the distribution of Tac in tissues and cells may also alter its toxicity and efficacy without a direct effect on whole-blood concentration.²²⁹

Whereas inhibition occurs almost immediately after that inhibitor has reached the CYP enzyme, induction is a slow and time-dependent regulatory process, mediated most of the time by gene transcription.²²⁹ Important inducers of CYP3A and P-gp that may be used in PLT are rifampicin, phenobarbital, carbamazepine, phenytoin and corticosteroids.^{235, 236}

Rifampicin is widely prescribed to cholestatic children before LT due to its effect against pruritus. Indeed, rifampicin induces hepatic 6(α)-hydroxylation of bile acids leading to an increase in bile acids available for glucuronidation and subsequent excretion via the kidneys.²³⁷ In healthy volunteers, co-administration of rifampicin significantly increased Tac clearance and decreased Tac bioavailability inducing both intestinal and hepatic metabolism of

Tac.²³⁸ This effect on Tac PK has been found significant in one study with PLT patients.²³⁹

Induction by corticosteroids is responsible for higher Tac exposure after corticosteroids withdrawal in adult kidney transplantation and higher Tac area under the curve (AUC) in pediatric kidney transplantation (KT). However conflicting results have been published.^{162, 240, 241} Although corticosteroids are increasingly avoided or early withdrawn in PLT, they still constitute the gold standard of acute graft rejection treatment and may thus have an impact on Tac PK in PLT.

Besides these interactions through metabolic enzymes and drug transporters, direct effect of medications on drug oral F have been reported through Tac adsorption or pH-mediated degradation with aluminum hydroxide gel, magnesium oxide and sodium bicarbonate.¹⁶⁰

Finally, co-administration of Tac with drugs known as causing renal dysfunction may enhance Tac nephrotoxicity. This is particularly the case with non-steroidal anti-inflammatories, aminoglycosides, cotrimoxazole, amphotericin B and acyclovir.²⁴²

4.5.4 Pharmacogenetics and ontogeny

(a) Definition

Pharmacogenetics has been defined while ago as « the study of variability in drug response due to heredity ». More recently, the term pharmacogenomics has been introduced and refers to all the genes

in the genome that may influence drug response. However, pharmacogenetics and pharmacogenomics are interchangeable.²⁴³

Pharmacogenomics is a cornerstone of personalized medicine that considers the unique clinical history and genetic background of each patient in order to improve health care, including prevention, diagnosis and treatment strategies.

Despite the different types of polymorphic markers, most sequence variations of DNA that retain attention in pharmacogenomics are single nucleotide polymorphisms (SNPs). A SNP is a single DNA base pair substitution that occurs at a frequency of 1% or greater in the population, possibly resulting in a different gene product and then a variant phenotype.²⁴⁴

Genetic polymorphisms of the Tac metabolizing enzymes, transporters and drug target/receptor may thus result in variations in Tac PK and/or pharmacodynamic (PD) after Tac administration. PD is defined as how the drug affects the body. In other words, PD describes the relationship between drug concentration at the site of action and the resulting effects (intensity and time course) as well as adverse effects. In the following paragraphs, we will focus on the pharmacogenetics of cytochromes (CYP3A4, CYP3A5 and CYP3A7) and transporters (ABCB1) involved in Tac inter-individual PK variability.

When dealing with pediatric pharmacogenomics, one should remind the intrinsic link with ontogeny, previously discussed. Indeed, developmental changes influence all the pharmacokinetics steps by affecting the level of expression and/or activity of enzymes and

transporters during childhood. Therefore, genotype-phenotype relationship may differ from adults to children.²⁴⁴

(b) Metabolizing enzymes

CYP3A4 has several genetic polymorphisms.²⁴⁵

While the wild-type allele is *CYP3A4*1A*, the two most studied *CYP3A4* SNPs are the *CYP3A4*1B* and *CYP3A4*22*. The first one involves an A to G transition at position -392 in the promoter region of the *CYP3A4* gene (rs2740574). The *CYP3A4*1B* allele has been associated with increased promoter activity *in vitro* and consequently with increased transcription.²⁴⁶ In a recent meta-analysis of KT patients, *CYP3A4*1B* have been associated with higher Tac dose requirement and lower Tac trough concentration/weight-adjusted Tac daily dose ratio (C_0/Dose).²⁴⁷ However, *CYP3A4*1B* is in linkage disequilibrium with *CYP3A5*-expressing allele *CYP3A5*1* in African Americans. Thus, its clinical significance in KT is controverted and is unknown in LT.

The second polymorphism, *CYP3A4*22* (rs35599367) involves a C to T transition at position 15389 located in the intron 6 of the *CYP3A4* gene. Discovered by Wang et al in 2011, this SNP led to a significant decrease in *CYP3A4* mRNA production and enzyme activity in human liver but not in the intestine.²⁴⁸ The same year, Elens et al. showed in adult KT that *CYP3A4*22* significantly altered Tac metabolism.²⁴⁹ Indeed, patients carrying the T allele (*CT* and *TT*) had a significant lower weight-adjusted daily Tac dose and a higher C_0/dose at 1 month when compared with the carriers of the wild-type

allele (CC).²⁴⁹ Similar results were found in pediatric heart transplant when Gijzen et al. showed that *CYP3A4**22 carriers needed 30% less Tac to reach similar target concentrations when compared to non-carriers.²⁵⁰ The effect of this SNP in PLT has not been tested yet.

The frequencies of the *CYP3A4* -392A>G and 15389C>T SNPs vary depending upon ethnicity. The *CYP3A4**1B allele is present in approximately 2-9.6% of Caucasians, 35-67% of Africans, 9.3-11% of Hispanics and 0% of Asians while the *CYP3A4**22 allele is present in about 5% of europeans, 3% of americans and less than 1% in south-asians and africans and 0% in east-asians.²⁵¹

CYP3A5 is also polymorphically expressed in human liver and intestine and harbours a very important functional SNP with a A to G transition at position 6986 in intron 3 of the *CYP3A5* gene (rs776746). This 6985 A>G SNP is referenced as *CYP3A5**3, the wild-type allele being *CYP3A5**1. The particularity of this SNP is that the wild-type allele occurs at a lower frequency than its variant *CYP3A5**3.¹⁸⁰ This *CYP3A5**3 allele confers low *CYP3A5* protein expression due to alternative mRNA splicing and truncation of the functional protein. Heterozygous and/or homozygous carriers of the *CYP3A5**1 wild-type allele encode high levels of full-length *CYP3A5* mRNA and express the same levels of functional *CYP3A5* protein (*CYP3A5* expressers). On the contrary, homozygous carriers of the *CYP3A5**3 variant allele produce very low or undetectable levels of functional *CYP3A5* protein (*CYP3A5* non-expressers). This was illustrated *in vitro* by Lin et al. who showed that livers with at least

one *CYP3A5*1* allele had a 2-fold higher CYP3A content than the *CYP3A5*3/*3* livers.¹⁷⁶ Since the study of Kuehl, several clinical studies in KT confirmed the large influence of CYP3A5 polymorphism on Tac PK. Indeed, most of the studies showed a significant higher Tac dose requirement in *CYP3A5*1* carriers to maintain the same target trough levels and a higher Tac clearance and lower AUC.²⁵²⁻²⁵⁴

In LT and PLT, this SNP seems also to significantly influence Tac PK. However, this is a special situation because both the donor liver and recipient intestine participate to Tac metabolism as illustrated in Figure 6 and the relative contribution of intestinal and liver CYP3A5 genotype is still unclear.

The frequency of the *CYP3A5*1* allele depends on ethnicity. It is present in about 5-15% of Caucasians, 45-73% of Afro-Americans, 25-35% of Asians and 25% of Mexicans.²⁵¹

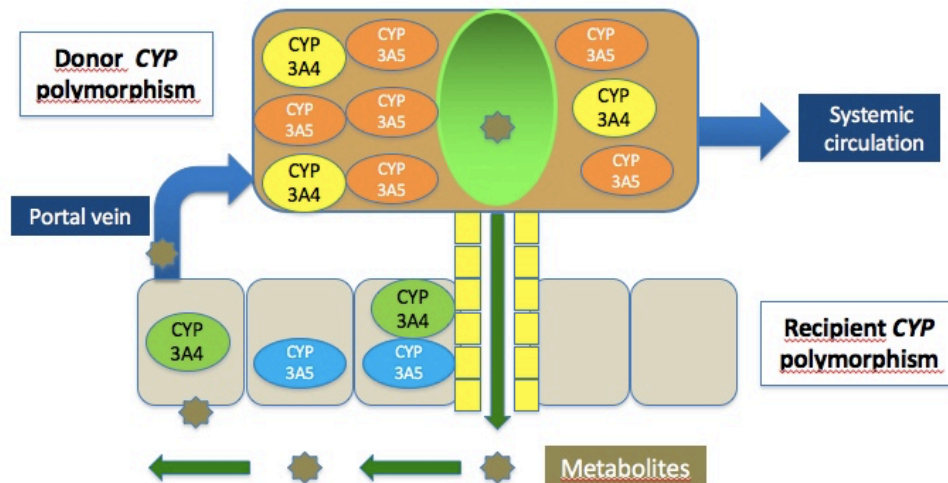


Figure 6: Schematic representation of the composite Tac metabolism including recipient intestinal and donor liver metabolism.

When working in pediatric transplantation, one should think of CYP3A7. Indeed, CYP3A7 is predominantly expressed in fetal liver and accounts for 30–50% of total cytochrome P450.²⁵⁵ Lacroix et al. showed that CYP3A4 accumulates during the first week of life, suggesting that the adult form (e.g. CYP3A4) progressively replace the fetal isoform (e.g. CYP3A7) during the early neonatal period.¹⁸⁴ Although CYP3A7 was first thought to be exclusively expressed in fetal liver, it was detected in 54–88% of adult livers based on mRNA content.^{182, 183} When based on isoform-specific substrate specificity, about 20% of adult livers express catalytically-active CYP3A7.¹⁸² CYP3A7 mRNA expression and protein expression in adult liver and intestine relates to genetic polymorphisms, with two third of high

expressors carrying the *CYP3A7*1C* allele and less frequently the *CYP3A7*1B*.^{183, 256} In intestine, high expression is exclusively due to the *CYP3A7*1C* allele. The *CYP3A7*1C* allele results from replacement of 60-bp of the *CYP3A7* promoter, the *CYP3A7*-Everted Repeat motif 6 (ER6) by the corresponding region of *CYP3A4* (*CYP3A4-ER6*) resulting in persistent *CYP3A7* protein expression in the adult liver.^{182, 183}

*CYP3A7*1C* allele occurs at frequencies of 3% in Caucasians and of 5% to 6% in African Americans.^{180, 183}

While *in vitro* studies showed a minor role of *CYP3A7* on Tac metabolism, *in vivo* studies are scarce and do not show any influence.^{207, 257} However, it has never been tested in PLT.

(c) Protein drug transporters

Besides cytochromes *CYP3A4/5*, *ABCB1*, also known as MDR1 or P-gp, an efflux transporter previously mentioned due to its role in Tac PK, is encoded by the *ABCB1* gene. This gene is highly polymorphically expressed with at least 62 coding SNPs identified.^{258, 259} Among all these SNPs, four of them are more extensively studied due to their putative and/or effective role in Tac PK. These SNPs are: rs1045642 (3435C>T in exon 26, Ile1145Ile) rs1128503 (1236C>T within exon 12, Gly412Gly) rs2032582 (2677G>T/A within exon 21, Ser893Ala/Thr) and rs2229109 (1199G>A within exon 11, Ser400Asn). While the *ABCB1* 3435C>T and 1236C>T are synonymous SNPs, meaning that the amino acid sequence is not

changed by the altered genetic code, *ABCB1* 2677G>T/A and 1199G>A are missense SNPs. The functional significance of these SNPs on *ABCB1* expression and function is still not completely elucidated.

The 3435C>T SNP may alter *ABCB1* mRNA stability, protein expression and folding as well as *ABCB1* insertion in the membrane and its substrate specificity. The underlying hypothesis is that the replacement of a frequent codon by a rare codon could affect the timing of cotranslational folding.^{260, 261} Hoffmeyer was the first to show an association between 3435C>T SNP and MDR1 expression and function. In his study, volunteers with the 3435TT genotype had significantly lower duodenal MDR-1 expression and higher digoxin plasma levels.²⁶² However, many other studies investigating the effects of this SNP as well as the 1236C>T and 2677G>T/A polymorphisms on Tac PK showed discordant results and if *ABCB1* variation has any effect on Tac PK, it may not be clinically relevant.^{251, 263-265} However, the main limitation of these studies is to consider the influence of SNPs independently. Indeed, the 1236C>T, 3435C>T, and 2677G>T/A are in strong linkage disequilibrium (LD).^{251, 258, 266} That means the allelic variants frequently occur together with the *ABCB1* 1236T-2677T-3435T (T-T-T) variant haplotype being present in about 32% of Caucasians, 5% of African Americans, 27% of Asian Americans and 35% of Mexican Americans.²⁵¹ Therefore synonymous SNPs may influence Tac PK due to either linkage disequilibrium (LD) with a non-synonymous SNP or by decreasing mRNA stability as for 3435C>T. This was illustrated by the study of Hawwa in 2009 in PLT where children had

significant correlation between the 2677G>T/A and 3435C>T polymorphisms and T-T-T haplotypes with higher Tac dose-adjusted pre-dose concentrations at various time points after drug initiation.²⁶⁷ These interesting results should be confirmed in larger pediatric population.

Finally, some researches on the 1199G>A polymorphism pointed out an effect on Tac PD. In 2007, Elens et al. genotyped 150 liver donors for 13 polymorphisms including the 1199G>A and compared Tac blood and liver concentrations. They found that the Tac hepatic concentration at day 7 on the last dose ratio was higher in livers carrying the 1199G>A mutation suggesting that the activity of P-gp towards Tac is decreased by this SNP.²⁵⁷ They found a similar effect for the 2677G>T/A SNP which could raise the existence of a putative LD between these two polymorphisms. Woodahl et al. excluded this in 2008 in a retrospective study of 121 patients with hematopoietic cell transplantation where LD between *ABCB1* SNPs was calculated. The 1199G>A SNP showed no significant LD with any of the three other *ABCB1* polymorphisms (1236C>T, 2677G>T/A, 3435C>T, $p < 0,05$).²⁶⁸ The same effect of 1199G>A on P-gp activity toward Tac was observed in 96 KT patients where carriers of the 1199G>A had 1,4-fold increased Tac concentration in PBMC at day 7 post transplantation when compared with the noncarriers ($p = 0,0014$).²⁶⁹ The same effect was also recently observed *in vitro*.²⁷⁰ However, none of these clinical studies demonstrated a direct correlation between *ABCB1* 1199G>A variation and Tac blood levels. To date, this allele has never been tested yet in PLT.

4.5.5 Time post transplantation

Population-pharmacokinetic (POP PK) studies in adult KT showed that Tac dose requirements decrease progressively after transplantation due to reduction in apparent oral clearance (CL/F). This phenomenon, also called Tac maturation, is attributed to decreasing CYP3A4 activity parallelly to steroid dose tapering and increasing hematocrit.^{213, 271, 272}

Although Pou et al. showed the same decrease of Tac CL/F in adult patients until 4 months post LT, POP PK adult and pediatric LT studies noticed a significant increase in CL/F during the first few weeks after LT.^{162, 273-277} This may be due to improvement in liver metabolic activity and/or induction of metabolic activity by concomitant steroid usage as well as changes in hematocrit.²⁷⁷ Thus, the exact effect of time on Tac PK in PLT is still unclear.

4.5.6 Gastric emptying and short-bowel

Kuypers *et al.* published in 2004 that in adult kidney transplant patients with delayed gastric emptying, Tac $C_{\max}$ was reached significantly more slowly ($t_{\max}$, 2 ± 1 h versus 1.48 ± 0.68 h; $p = 0.04$), without difference in the extent of Tac absorption. Thus, slow gastric emptying delays absorption without alterations in Tac exposure.²⁷⁸

Effective oral absorption of Tac has been reported in two LT children with short or absent small bowel and with jejunostomy.²⁷⁹⁻²⁸¹ If Tac absorption takes place predominantly in the small intestine, in case of short or absent small bowel, absorption seems to occur in the more distal part of the intestine and in the colon, where CYP3A enzymes are less expressed.¹⁶²

4.5.7 Non-adherence

Non-adherence to medications is a leading cause of morbidity and late acute rejection in children and adolescents who have had a transplant.^{280, 282, 283} A higher standard deviation (SD) and, therefore, more fluctuation in Tac blood levels over time seem to be indicative of erratic adherence. In pediatric liver and renal transplants, a higher variation in serum Tac levels, as measured by the SD or coefficient of variation (CV%) is associated with more frequent rejection episodes, respectively.^{282, 283} Therefore, looking at Tac blood level variation, monitoring SD or CV in transplanted adolescents might indeed become a gold standard for assessment of non-adherence.²⁸²

4.5.8 Gender

Clinical studies dealing about gender effects on the pharmacokinetic of Tac have been discordant. Whereas some of them showed that women had higher Tac clearance and dose requirement both in kidney and liver transplantation, several other studies did not show any differences.^{284, 285, 286} A recent study in adult KT showed a significant lower AUC in women after the first oral dose compared with men (55.2 ng.h/ml versus 94.4 ng.h/ml, respectively) but CYP3A5 genotypes were unknown.²⁸⁷ As it is clearly a well-described influent covariate of Tac PK in KT, a confounding factor cannot be excluded.

When considering uniquely the sex influence on CYP3A metabolism, a meta-analysis studying the gender effect on midazolam metabolism, published that women exhibited 16% higher weight-corrected midazolam oral clearance ($P < 0.001$) and 20%

higher systemic clearance ($P = 0.002$) than men whereas no sex-dependent difference in intestinal CYP3A activity was observed.²⁸⁸ *In vitro* studies described 2-fold higher hepatic CYP3A4 levels in female compared with male samples without any differences in P-gp expression.²⁸⁹ In 2005, Paine reported no sex difference in intestinal CYP3A4 expression but a 20% lower mean duodenal CYP3A4 content in post versus premenopausal women. No sex influence was also found on intestinal P-gp expression.²⁹⁰ This lack of consensus between clinical studies has been attributed to inadequate power to detect sex differences in drug clearance. Thus, although sex may influence CYP3A metabolism, it has no clinical relevance regarding Tac PK.

4.5.9 Nychthemeral rhythm

Chronopharmacology studies the differences in PK and PD of drugs according to their administration times.²⁹¹ Circadian rhythms are known to influence gastric pH, gastric emptying time, gastrointestinal transit time, organ blood flow, liver enzyme activity, renal function as well as transcription of numerous CYP enzymes.^{291, 292}

As these functions have a significant impact on the absorption, distribution, metabolism and excretion of drugs, circadian rhythms may influence drug PK. Several clinical studies in LT and KT showed circadian variation in the clinical pharmacokinetics of Tac with faster absorption, higher C_{max} and a greater AUC in patients after the morning dose compared with the evening dose.^{291, 293, 294} This

propriety explains why Advagraf®, a prolonged release formulation of Tac is prescribed once in the morning.

4.5.10 Renal function

Although renal clearance and excretion of Tac are negligible, chronic kidney disease may affect Tac disposition. Indeed, uremic toxins have been shown to interfere in animal studies with transcriptional activation, causing down-regulation of gene expression mediated by proinflammatory cytokines, and directly inhibiting the activity of the cytochrome P450s and drug transporters.²⁹⁵ In clinical transplantation, only two studies in hematopoietic stem cell transplantation and in liver transplantation showed influence of serum creatinine on Tac Cl/F.^{275, 296} One should remind that many factors affecting Tac PK may also affect renal function (e.g. sepsis, diarrhea) and that Tac is nephrotoxic.

4.5.11 Liver function

Liver function includes coagulation factors, albumin and plasmatic proteins synthesis, drug metabolism, bile production and excretion and therefore influences Tac bioavailability, distribution and elimination. Hepatic drug clearance (CL_H) is defined as the volume of blood from which the drug is removed completely by the liver per unit time. CL_H is a function of hepatic blood flow (Q_H) and the hepatic extraction ratio (E_H) of the drug:²⁹⁷

$$CL_H = Q_H \times E_H \quad \text{Equation 1}$$

E_H depends on liver blood flow, the intrinsic clearance of unbound drug (CL_{int}), and the fraction of unbound drug in blood (f_u). Therefore, the derived equation for CL_H is:

$$CL_H = Q_H \times \frac{f_u \times CL_{int}}{Q_H + f_u \times CL_{int}} \quad \text{Equation 2}$$

Because Tac is a low hepatic extraction ratio drug ($E_H < 0.3$), the hepatic clearance of Tac is mainly influenced by changes in blood/plasma binding and hepatic CL_{int} , with supposed increased f_u secondary to low albumin and hematocrit levels and the reduced hepatic CL_{int} in case of liver dysfunction. Several POP PK studies establishing a Tac PK model showed that either elevated liver enzymes (AST, ALT),^{217, 218, 274, 276, 285, 298} reflecting liver injury, signs of cholestasis such as GGT²¹⁷ and bilirubin levels,^{216, 275, 299} as well as albumin,^{274, 300, 301} international normalized ratio²⁹⁹ and factor V³⁰² influenced Tac clearance. In LT, liver dysfunction predominates in the early period after LT as well as during rejection episode. As stated earlier, time post transplantation is known to influence Tac clearance and Lock et al. showed that LT recipients developed Tac toxic levels when initial post-transplant function was poor as assessed by the LiMAX test.³⁰³ However, there is not yet a reliable biomarker of liver function that correlates well with the drug metabolizing capacity of the liver, especially in the pediatric population.

4.5.12 Hematocrit and plasmatic proteins

In blood, Tac is mainly distributed into red blood cells (RBC) and its equilibrium with plasma is slow. Plasmatic Tac is highly bound to AAG and albumin. Accordingly, changes in hematocrit and/or plasma protein concentrations could significantly influence Tac distribution, clearance and dose requirement but would not have a significant effect on the unbound fraction f_u . Indeed, a transient increase in Tac

f_u occurs when hematocrit decreases, but as Tac clearance is proportional to f_u , Tac f_u increase is immediately extracted by the liver resulting in an identical unbound concentration but a decreased whole blood concentration and higher daily dose requirement due to higher clearance. However, therapeutic effect remains the same as it relies on unbound Tac concentration. The best example of such a clinical situation is pregnancy. This was well illustrated in the article of Zheng where mean oral clearance (CL/F) based on whole blood Tac concentration was 39% higher during mid and late term pregnancy. Plasmatic and whole blood Tac unbound fraction increased by 91% and 100% respectively and were correlated with increase in Tac whole blood CL/F ($r^2 = 0.36$ and 0.47 respectively, $P < 0.01$). This higher clearance lead physicians to increase Tac dosages by 45% in pregnant transplant women in order to maintain Tac whole-blood trough concentrations in the therapeutic range. However, this led to increases in unbound Tac trough concentrations by 112% and unbound AUC by 173%.¹⁹⁹ Several clinical PK studies in LT found a higher clearance in case of lower hematocrit^{298, 300, 304} and/or albumin levels.^{274, 300, 304}

4.5.13 Food

The effect of food on the oral absorption of Tac appears to be dependent on its fat content and relative time of administration.^{156, 162, 213, 305, 306}

In a study with healthy volunteers, ingestion of food significantly reduced Tac oral rate and extent absorption as compared with the fasted state but did not influence its half-life time.³⁰⁵ Most studies

reported a decrease of 25-45% in AUC and an increased T_{max} when Tac is taken with a meal.²¹³

Oral Tac F appears to be lowest for high-fat meals compared with low-fat meals and fasted state. Bekersky investigated the effect of the timing of a meal on the rate and extent of Tac absorption.³⁰⁶ He compared four treatments:

(1) Fasting for 10 hours; (2) ingestion 1 hour before breakfast; (3) ingestion after breakfast; and (4) ingestion 1.5 hours after breakfast. Tac absorption in the fasting state had a significantly greater relative bioavailability than all other treatments. The AUC averaged 312, 276, 205 and 203 $\mu\text{g} \cdot \text{h/L}$ for treatments 1, 2, 3, and 4 respectively. Therefore, Tac should be administered in a fasted state, at least 1h before meal and 3h after meal, which is practically very difficult to observe for infants.

Food may also influence the oral Tac F by inhibiting or inducing the activity of CYP3A and/or ABCB1. The best-known food compound interacting with Tac PK is grapefruit juice, which can double bioavailability leading to elevated trough levels in LT.³⁰⁷

Grapefruit juice inhibits in a reversible and mechanism-based fashion, intestinal CYP3A4. These two mechanisms are at least due to the presence of a furanocoumarin, in grapefruit juice, the 6'7'-dihydroxybergamottin.³⁰⁸

Grapefruit juice also inhibits P-gp, at least *in vitro*.^{309, 310} Whether grapefruit juice inhibits intestinal P-gp activity *in vivo* has not been fully established, largely because an ideal P-gp probe substrate has not been identified.³¹¹ Besides inhibition, grapefruit juice also down-regulates CYP3A4 protein.³¹² The effect of grapefruit juice on

CYP3A4 lasts at least 10h after its ingestion possibly because mechanism-based inhibition results in rapid degradation of CYP3A4 and protein synthesis takes time.³¹² As grapefruit juice had no effect on systemic clearance of cyclosporine, its effect on CYP3A is restricted to the gut.

The same effect on Tac oral F has been attributed to pomelo, turmeric and ginger juice.³¹³

Thus, patients under Tac should be strongly recommended about avoiding grapefruit and pomelo juice. Let's just remind that Yee et al. in 1995 raised the possibility to use grapefruit juice as a mean to increase oral F and decrease the costs of cyclosporine treatment. This option could be mainly interesting in developing countries or to reduce the cost of a drug that has lost his patent. This was recently tested in a phase I trial where oncologic patients, treated with sirolimus alone, were compared to patients receiving sirolimus plus grapefruit juice or ketoconazole.³¹⁴

Contrarily, a KT women taking cranberries extracts for urinary tract infection prophylaxis had low Tac troughs levels despite dose increase that normalized when cranberries pills were stopped, letting suppose an induction of CYP3A/p-gp by cranberries.³¹⁵ This contrasts to *in vitro* and *in vivo* studies that showed either no effect or inhibition of cranberry juice on intestinal CYP3A.³¹⁶⁻³¹⁸

A well-known herbal component St. John's wort or hypericum perforatum, prescribed as antidepressant, induces Tac metabolism. Indeed, it significantly decreases Tac AUC while increasing apparent oral clearance through induction of CYP3A and P-glycoprotein.^{213, 229,}

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Finally, a new concern has been raised about potential interaction between cannabinoids and Tac metabolism due to CYP3A and P-gp inhibition.³²⁰⁻³²² With increasing marijuana consumption in adolescents, such an interaction should be evoked in case of unexpected high Tac trough levels in adolescents.

4.5.14 Generic formulations

After expiration of its drug patent in June 2009 in Europe, the monopoly marketing position of Prograf® by Astellas has been lost. Other companies were then allowed to enter the market with generic versions of the same active ingredient, leading to competition with the aim of costs savings.

Generic drug is identical-or bioequivalent-to a brand name drug in dosage form, safety, strength, route of administration, quality, performance characteristics and intended use.³²³ To be registered as equivalent to the innovator, bioequivalence studies, realized in healthy volunteers, are required to demonstrate that the 90 % confidence interval (CI) for the generic/innovator ratios of the AUC and the C_{max} is within the range of 80–125 % This acceptance range can be tightened to 90–111.11 % for narrow therapeutic index drugs such as Tac.³²⁴ However, there is no requirement to determine bioequivalence in the transplant population or to demonstrate efficacy, safety, and effects of co-prescribed medications. Several generic, twice-daily Tac formulations are now available on the market. In Belgium, there are two: Tacni (Teva) and Adoport (Sandoz).³²⁵ Most of the recent studies in adult LT using the Sandoz formulation as *de novo* IS therapy or as a switch to the brand

formulation showed no significant difference in terms of Tac exposure and outcomes. However, if manufacturers should demonstrate bioequivalence of their generic drug with the innovator drug, the demonstration of bioequivalence with the other generic formulations is not required. Moreover, there is little data on the effect of switching from one formulation to another on Tac exposure, a problem that could be encountered when pharmacists decide to switch a formulation by another one, without physicians' agreement. Finally, as generic formulations may have different shape and colour from the brand formulation, errors of administration could happen with subsequent significant variability of Tac PK with putative risks in terms of efficacy and toxicity.²²⁶ Therefore, switching between generic formulations should only be planned and supervised by transplant clinicians, with appropriate and close monitoring and follow-up. In case of unexpected Tac therapeutic drug monitoring (TDM) variability, rejection or toxicity, a possible switch of formulation should be sought for.

4.5.15 Inflammation

Various *in vitro* and *in vivo* animal and human studies showed an inhibitory effect of inflammation and disease on CYP3A-mediated drug metabolism.³²⁶⁻³²⁹ This is partly explained by CYP3A4 *mRNA* and protein down regulation caused by agents released during inflammation such as TNF, IL-6, IL-1, IFN, TGF beta and LPS. In critically-ill children, Vet showed that CRP and organ failure significantly reduced midazolam clearance, a surrogate marker of CYP3A-mediated drug metabolism.³³⁰ Although no clinical study in

pediatrics specifically studied the effect of inflammation on Tac clearance, one might think that inflammation could partially inhibit its metabolism leading to high Tac levels and toxicity.

4.5.16 Diarrhea

Several studies reported that severe diarrhea from infectious and non-infectious origin leads to increased Tac trough levels with possible subsequent toxicity.³³¹⁻³³⁴ Lemahieu et al. demonstrated that this was due to an increased oral bioavailability rather than decreased elimination.³³⁵ However, this effect was not observed in case of mild diarrhea (less than four stools per day).³³⁶ This results from two main mechanisms. Firstly, during diarrhea, intestinal transit time is shortened leading to a higher Tac delivery to the distal part of the small bowel and colon where CYP3A content is lower. Secondly, intestinal injury and inflammation also affect Tac disposition by altering the expression and activity of P-gp and CYP3A. Indeed, lower intestinal expression of CYP3A4 and P-gp was found in case of inflammation in most of the human studies.³³⁷⁻³⁴¹ Regarding P-gp activity, Lemahieu et al. showed that during diarrheal illness, its intestinal activity was reduced, possibly due to mucosal injury.³³⁵

Therefore, Tac C_0 should be measured and dose reduction should be considered in case of severe/chronic diarrhea.

4.5.17 Type of graft

In adult and pediatric liver transplantation, several studies compared Tac dose requirement and blood levels according to the type of graft: whole versus split graft.^{217, 218, 302} All these studies found a higher Tac clearance and dose requirement in whole graft

compared to split. This can be easily explained in adults by the higher mass in whole graft and thus higher CYP3 content. However, this explanation could not be used in PLT where Staatz and Willis showed that Tac CL/F was 7-fold lower with a split adult graft when compared to a whole child graft.²¹⁷ Staatz attributed this finding to a higher metabolic activity in children graft. This could be due to a relative higher CYP3A5 (found in 54% of children livers) content in children liver and/or the presence of CYP3A7.³⁴²

4.5.18 Ethnicity

Tac disposition varies widely according to ethnic origin of recipients in solid organ transplantation. It is well known that African-American patients require higher Tac doses than Asians, Mexican and Caucasian.^{162, 343, 344} This is due to a significant lower bioavailability and exposure in black patients.^{343, 345, 346} However, the first studies showing that ethnic effect did not perform genetic testing. Yet, it is known that the differences between ethnic groups regarding Tac disposition may largely result from racial differences in liver and intestinal CYP3A or P-glycoprotein expression and activity as mentioned above.

4.5.19 Galenic

Tac is available as an intravenous solution. It was the first formulation used after its introduction, totally replaced nowadays by the oral route with capsules (Prograf®, Astellas) of 0.5 mg, 1 mg and 5 mg. In 2007, a new once daily-prolonged release formulation (Advagraf®, 0.5 mg, 1 mg, 3 mg and 5 mg) received the European

marketing authorization (EMA). It is mainly used in adult kidney and LT, as *de novo* rejection prophylaxis or as a substitute for Prograf® or Cyclosporin and for treatment of rejection. It was introduced more recently in adolescent kidney and liver transplanted patients in order to reduce medical non-adherence.³⁴⁷⁻³⁴⁹ Specific granules for oral suspension (Modigraf®, Astellas, 0.2 mg and 1 mg) for children are now available since 2009 on the market (France) or as medical need program (Belgium).³⁵⁰ The advantage is to get better titration due to smaller increments of the dose in low-weight patients. However, one should be aware about an 18% increase of Tac AUC in healthy subjects after a single dose of Modigraf when compared to Prograf®.³⁵¹ Therefore, switching from one formulation to another should be carefully monitored. Other new formulations (sublingual, oily solutions, solid dispersions, liposomes...) are currently under investigation to improve oral delivery but will not be developed here.^{349, 352}

4.6 Pharmacodynamics and genetic variability

For most of the drugs including Tac, the concentration at the site of action is difficult to measure. Thus, plasma or whole blood concentrations are used as an alternative.³⁵³ However, since a few years, Tac concentration can be measured directly in peripheral blood mononuclear cells (PBMCs) of adult transplant patients and in transplanted organs (livers and kidneys).

The adverse events associated with Tac are nephrotoxicity, neurotoxicity, diabetogenesis, gastrointestinal disturbances,

hypertension, electrolytes disturbances, infections and malignant complications including post-transplant lymphoproliferative disease or PTLD associated to EBV infection.¹⁶²

Several adverse effects seem to be dose-related meaning that they appear or are more severe with high trough blood Tac concentrations. Consequently, dose reduction is indicated first.

Unfortunately, as for PK, there is a large inter- and intra- patient PD variability. That means even a therapeutic concentration of Tac may lead to adverse effects. We will develop in the next paragraphs the impact of the *CYP3A* and *ABCB1* genes on acute cellular rejection and nephrotoxicity.

4.6.1 Rejection

Acute cellular rejection in PLT is reported to occur in up to 60% of patients in Europe and 45% in the US.^{82, 354}

The direct site of Tac action is lymphocytes or PBMCs.

Capron et al. developed an analytical method to measure the Tac concentration in PBMCs.³⁵⁵ They also showed that clinical rejection was characterized by significant lower mean Tac PBMCs concentrations at day 5 and 7 post transplantation in adult LT and that intrahepatic Tac concentrations at day 7 correlated with Tac PBMCs concentration from day 5 post LT.³⁵⁶ The expression of the *ABCB1* transporter on lymphocytes is among the factors that may influence the Tac PBMCs concentrations. This expression can be impacted by genetic variants of the *ABCB1* gene. Indeed, Capron et al showed that *ABCB1* 1199G>A, 3435C>T and 2677G>A/T

polymorphisms appeared to reduce the P-gp activity with subsequent increased Tac PBMCs concentration in adult renal transplantation.²⁶⁹

Moreover, they found that patient with *CYP3A5**3/*3 genotype had significantly higher dose-adjusted trough PBMCs concentrations of Tac at day 7 and steady-state when compared to *CYP3A5**1/*3 and *CYP3A**1/*1 patients.²⁶⁹

However, when this thesis started, no genotype affecting *CYP3A5* or *ABCB1* was directly associated with rejection outcome in LT.

4.6.2 Nephrotoxicity

Renal impairment is an important cause of morbidity following PLT transplantation. In the most severe cases, renal dysfunction may lead to end-stage kidney disease requiring dialysis and/or transplantation.¹²² The underlying mechanism of Tac nephrotoxicity is glomerular afferent vascular constriction and shrinkage, which can lead in the long term to interstitial fibrosis and arterial hyalinosis.^{122, 357}

Besides the effect of Tac on renal hemodynamics and structure, Tac also leads to tubular dysfunction and electrolytes disturbances like hyperkalemia, hypomagnesemia and hyperchloremic metabolic acidosis (distal tubular acidosis).³⁵⁷

Estimating chronic kidney disease (CKD) (glomerular filtration rate GFR <90 ml/min/1.73 m²) after PLT may be difficult because of differences in GFR estimation or measurements and/or different definition of CKD.¹²² Most of the studies used serum creatinine level (*S_{CR}*) and the Schwartz formula to calculate GFR (cGFR).³⁵⁸ However, Scr is influenced by gender, fluid balance and muscle

mass and does not seem sensitive enough to detect CKD and leads to underestimation of its prevalence.³⁵⁸ Moreover, depending on the method of Scr measurements, the use of the modified Schwartz formula is recommended.³⁵⁹ Even if the gold standard is GFR measurement by inuline or iothexol clearance, this technique is difficult to implement in routine. The replacement of Scr by Cystatine C, a widely expressed protein not influenced by gender, height or body composition may be useful for the assessment of renal function.³⁶⁰

The prevalence of renal dysfunction in PLT was found to be 17.6% (GFR <90 mL/min/1.73 m²) at a mean of 5.2 years post transplantation (SPLIT data) and may be up to 22% and 25%.^{121, 358, 361}

Thus, a close monitoring of renal function is mandatory in PLT patients. Management strategies of renal dysfunction are early Tac minimization or withdrawal and switch to other immunosuppressive drugs such as MMF and/or mTOR inhibitors (sirolimus and everolimus).¹²²

If systemic exposure is linked to renal dysfunction, patients may exhibit a wide inter and intra- variability in the risk of developing CKD after PLT. Indeed, even a therapeutic range of Tac may lead to nephrotoxicity. This can be caused by underlying diseases (Alagille syndrome, hepatic fibrosis, inborn error of metabolism), concomitant use of nephrotoxic medications (aminoglycosides, acyclovir), and genetic polymorphisms of ABCB1 and CYP3A enzymes.^{357, 358, 361, 362}

Indeed, renal P-glycoprotein expression on the apical membrane of the renal tubules may play a role in regulating the nephrotoxicity of

calcineurin inhibitors.³⁶³ Therefore, *ABCB1* genetic polymorphisms may influence the renal Tac concentration.

In 2009, Hawwa et al showed in PLT a significant higher incidence of renal dysfunction in patients carrying either the *ABCB1* 3435T, or 2677T or 1236T alleles at 6 months post transplantation and in patients carrying the T-T-T haplotype at 6 months post transplantation.²⁶⁷ This was concordant with the results of study of Hebert in adult LT but other studies in adult KT and LT did not identify any association between *ABCB1* genotype and nephrotoxicity.³⁶⁴⁻³⁶⁶

Concerning the CYP3A5 enzymes, Kuypers showed in adult KT that carrying the CYP3A5*1 allele in the recipient was associated with higher nephrotoxicity.³⁶⁶ These results were in contradiction with other studies in adult KT.³⁶⁷ If donor genotype could influence intrarenal Tac metabolism in KT, Naesens did not find any effect of the donor and of recipient CYP3A genotype on nephrotoxicity.³⁶⁸ In adult LT, the recipient but not donor *CYP3A*1* allele was reported to be protective against renal impairment.³⁶⁹

No study in PLT showed an association between CYP3A genotype and kidney dysfunction.

Thus, it seems that the pharmacogenetic influence on Tac nephrotoxicity is still inconclusive.

Among other main side effects, hypertension is reported in 39% of patients, diabetes in 2.2% of patients, PTLD in 13.7% of patients.¹⁴⁷ Infections (bacterial, fungal and viral) are also very frequent and occur in up to 39-41% of patients in PLDLT during the

first 6 months.³⁷⁰ In the SPLIT study, infections represented up to 47% of causes of death.³⁵⁴

Besides having a narrow therapeutic window, we've just seen the wide intra-and inter-patient PK and PD variability, rendering adequate IS without adverse reactions difficult to achieve in daily practice. To overcome this problem, TDM of Tac is routinely implemented. It helps to reduce Tac PK variability in order to avoid both rejection and side effects. TDM use is supported by the existing correlation between blood Tac concentrations and clinical outcomes. We will see in the next section how TDM is currently performed and its limitations.

4.7 Therapeutic drug monitoring

4.7.1 Dose initiation

According to pediatric liver transplant team, first Tac dose is initiated on a bodyweight basis either from recommendations of the company (0.15-0.20 mg/kg/day in two doses, every twelve hours in the latest recommendations) or from team experience. Sometimes, the initial dose is empirically adapted according to covariates such as co-medications, age and the existence of adverse outcomes such as renal dysfunction (urine output less than 1 mL/kg/hour in children).³⁷¹

It is recommended by the company to orally initiate Tac no sooner than 6 hours after LT, by the mean of a PVC free nasogastric tube in patients unable to swallow, a frequent situation in the first days following PLT. Due to its mean half-life time value in liver

transplantation of around 12 h, Tac is administered twice a day and at least 1 h before and 3 h after meals to avoid food interaction as discussed earlier.

According to the first time of Tac intake (morning or evening dose) and the institution practices, the first Tac TDM is done after one or two doses.

4.7.2 Markers of exposure and target range

Historically, Tac trough blood levels C_0 or $C_{\min}$ has been used as a single time point for Tac TDM in solid organ transplantation because of its correlation with clinical outcomes. However, if higher Tac C_0 has been associated with increased risk of toxicity, there is less evidence regarding rejection.^{195, 372, 373, 374}

In 2009, experts have admitted in a consensus that the exposure between two consecutives doses, represented by AUC, is considered the best marker of drug exposure and has better correlations with rejection and toxicity.³⁷⁴ Moreover, the correlation between C_0 and AUC is controverted and whereas C_0 relies on both clearance and distribution volume, AUC only depends on clearance, overcoming the variability on distribution volume.^{375, 376} Currently, the accurate estimation of AUC by non-compartmental or compartmental estimation methods requires a high number of blood specimens (8 to 12). Thus, it is rather difficult to implement for financial, practical and ethical reasons especially in pediatric patients.^{374, 377} Therefore, AUC measurement is often restricted to clinical studies.³⁷⁵

Limited sampling strategies may overcome this limitation by allowing a good estimation of AUC with only a few samples from patients (2 or 4).³⁷⁶ AUC estimation can be done either by multiple linear regression or Bayesian estimation.³⁷⁷

The next question being to know the better Tac C_0 target, the first consensus conference on Tac TDM opted for a range between 5-20 ng/mL for whole blood Tac concentration in all transplanted patients. Later, the experts made recommendations according to the organ transplanted. With clinical experience and the introduction of induction therapies, a dramatic decrease in acute rejection was observed, allowing to reduce the target Tac whole blood down to 6-8 ng/mL in adult LT.^{374, 378}

However, the latest consensus conference suggested a 10-20 ng/mL during the first month post LT when risk of acute rejection is high, 5-15 ng/mL between month 1 and month 3 and then between 5 and 10 ng/mL.³⁷⁴ In daily practice, the target C_0 may vary from a center to another according to analytical methods, IS regimen, time post-transplant and risk of rejection and adverse outcomes. To illustrate this team-dependent variability regarding Tac C_0 target, we gave in Table 1 the desired target of 4 different teams worldwide.

COUNTRY	PERIOD	THERAPEUTIC RANGE
FRANCE ¹	Week 1 - Week 2	10 - 15 ng/mL
	> Week 2	8 - 12 ng/mL
CHINA ²	Month 1	10 - 15 ng/mL
	Month 2 - Month 6	8 - 12 ng/mL
	>Month 6	5 – 8 ng/mL
JAPAN ³	Week 1 - Week 2	10 - 12 ng/mL
	Week 3 - Week 4	10 ng/mL
	> Week 4	5-10 ng/mL
SWEDEN/AUSTRALIA ⁴	Month 1	10 – 20 ng/mL
	Month 3	5 – 15 ng/mL
	Month 12	2 – 10 ng/mL

Table 1: Tacrolimus C₀ target in different pediatric liver transplantation teams^{221, 276,}

379, 380

No pediatric study determined the optimal target range and values have been inferred from adult values.^{162, 374}

Regarding AUC, it is still uncertain what the correct target should be in LT and PLT. The best AUC range suggested in the literature referring adult KT was around 150 and 200 ng/h/mL. Therefore, future studies to determine the optimal target range AUC₀₋₁₂ in PLT are also warranted.^{162, 374}

4.7.3 Therapeutic drug monitoring: matrix and analytical assays

Because of the high concentration of Tac within erythrocytes, and impractical plasma or serum concentrations measurement, current worldwide data on Tac TDM are based on whole blood concentrations.³⁸¹

However, as lymphocyte is the site of action of Tac it seems logical that the Tac concentration at its target site is more relevant than in the whole blood to predict the efficacy of treatment. Immunological and chromatographic methods were recently developed to measure the Tac concentration inside the PBMCs.^{355, 382}

As mentioned above, Capron found that patients experiencing clinical rejection one week after transplantation had significantly lower Tac PBMC concentrations on day 7 after transplantation when compared to patients who did not experience rejection.³⁸³ However, Lemaitre et al/ did not find any correlation between Tac whole-blood concentrations, Tac PBMC concentrations, and intracellular CN activity.³⁸⁴

Three different types of analytical methods may be used to determine blood Tac concentrations and have been detailed by Alak: the immunoassays, chromatographic assays and bioassays. The bioassays (e.g. radioreceptor assay, pentamer formation assay) are mostly used in experimental approach but are poorly adapted to a routine TDM.³⁸⁵ In clinical practice, the most frequently used methods are immunoassays: enzyme-linked immunosorbent assays (ELISA

liquid phase extraction, Fujisawa and ELISA Pro-Trac, IncStar), microparticle enzyme-linked immunoassay, microparticle enzyme immunoassay (MEIA, Abbott Diagnostics, Chicago, IL), the enzyme multiplied immunoassay technique (EMIT, Dade Behring, Glasgow, DE), the antibody-conjugated magnetic immunoassay (ACMIA, Dade Behring-Siemens, Deerfield, IL), the cloned enzyme donor immunoassay (CEDIA, Microgenics, Fremont, CA), the chemiluminescence microparticle immunoassay (CMIA, Abbott Diagnostics) and the recent electrochemiluminescence immunoassay (ECLIA) developed by Roche Diagnostics and Quantitative Microsphere System (QMS™, Thermo-Fisher).^{381, 386}

Although immunoassays allow rapid results and may be easily implemented in laboratories, they could be biased by cross-reactivity with Tac metabolites or other drugs, as well as interactions with heterophilic antibodies or influence of endogenous factors such as hematocrit and albumin. Moreover, immunoassays may be limited by their analytical performance, particularly by their low sensitivity with a limit of quantification, LOQ, between 2 and 4 µg/L) and their calibration bias. Only CMIA and QMS™ have a functional sensitivity below 1 µg/L, and were reported to offer adequate accuracy and precision.^{374, 386}

Besides these immunologic methods, the high-performance liquid chromatography (HPLC) techniques, either with single mass spectrometric detection or with tandem mass spectrometry (HPLC-MS and HPLC-MS/MS respectively) were developed. Because these methods allow a more accurate and specific measurement of Tac and its metabolites and have robustness, their use rapidly expanded

worldwide from 2% of laboratories in 1999 to 53% in 2013.³⁸⁷ However, the choice of the assay method employed in a laboratory depends on several factors, including the expertise and equipment available within the laboratory, the cost of the technique and the volume of samples.

4.7.4 Dosage adaptation

In most of the centers worldwide, Tac TDM is daily performed just after transplantation due to high PK and PD variability during this period, especially in children due to developmental issues. It allows reducing the variability by adapting the dose individually according to the exposure marker, C_0 . The dose adaptation is then empirically done by physicians and depends mainly on their own experience, target concentrations, toxicity and rejection occurrence and introduction or withdrawal of co-medications. Many changes are thus required before C_0 reach the target range and be stable. Then, the frequency of TDM progressively decreases down to once a week to once a month.

There are several limitations of current Tac prescription and TDM in PLT practice. First, TDM is always done *a posteriori* and there is no anticipation yet in PLT regarding factors that may influence the first dose such as pharmacogenetic of CYP3A and ABCB1 enzymes of both the donor and recipients in the specific case of LT.

Secondly, the physician-based adaptation introduces another source of variability and is complicated in case of missed doses, non-steady-state conditions and/or concentration measured at non-

standard time points. Moreover, physicians frequently don't take into account the accumulation phenomenon that occurs with repeated oral administration. They want to reach as soon as possible the therapeutic range and frequently increase the Tac dose after the first TDM result, when C_0 is infratherapeutic. Indeed, time to reach steady-state concentrations is equal to 5 half-lives of the drug. That means even without any change in the posology, the C_0 will raise more or less rapidly and importantly during the first three days of Tac administration as illustrated in Figure 6. Increasing or decreasing dose too rapidly may expose to toxicity or rejection. Moreover, whether reaching the therapeutic range on day 1 after transplantation is a way to prevent from graft rejection is unknown.

Third, Tac TDM doesn't use the best marker of exposure AUC and current use of C_0 target range is derived from adult studies and not known in PLT.

Finally, less than 60% of patients are within the therapeutic range in adult KT during the 6 weeks post KT, and there is still a high rate of rejection and nephrotoxicity as previously mentioned.³⁸⁸

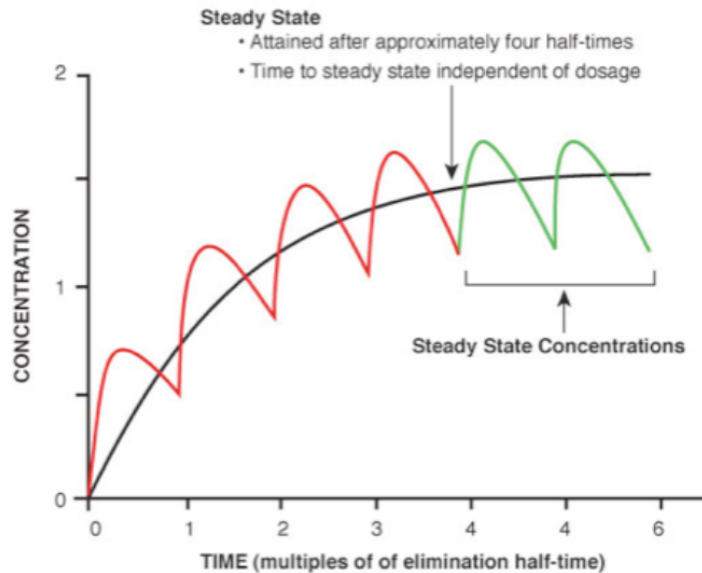


Figure 6: Drug accumulation after repeated administration. *From Tozer and Rowland.*³⁵³

To sum up, if Tac has become a cornerstone in PLT due to better results in terms of patient and graft survival, its clinical use is complicated due to high inter- and intraindividual PK and PD variability. Many individual factors may explain this variability. However, which of these factors are clinically significant in PLT is still debated and to what extent they influence Tac PK parameters is unknown. Thus, they are not taken into account and Tac prescription is still weight-based. TDM is then a useful tool to optimize Tac dosing although insufficient. Therefore, there is still a need for more advanced individualization of Tac prescription in PLT. This implies adjusting dosage regimen on individual characteristics and response.

4.8 Objectives of the thesis

The overall aim of this work is to better individualize Tac therapy in PLT. To do so, we plan:

- To build a POP PK model of Tac covering the first-year post transplant period in order to check the relevance and the delay of time effect on clearance in our cohort of PLT patients
- To build a robust POP PK model of Tac covering the initial period post-transplant where rejection is the highest and testing all the covariates known to influence Tac PK in our cohort of PLT patients.
- To build a robust POP PK model of Tac including donor and recipient genetic background in two cohorts of PLT patients from two different centers in order to be used in a PK-based TDM.

We will develop in the next chapter the concept of POP-PK and how it may help us to achieve this goal.

Chapter 5. POPULATION PHARMACOKINETICS MODELING AND BAYESIAN ESTIMATION

5.1 Toward individualized drug therapy

We've just seen that a weight-based Tac therapy is not adapted because of Tac high inter- and intra-individual PK and PD variability, influenced by many other factors than weight. Therefore, to select the appropriate personalized Tac dosage regimen, we should characterize the time course evolution of Tac concentration according to patient's characteristics.

To do so, the estimation/prediction of Tac PK parameters is first required (bioavailability, absorption rate, clearance and volume of distribution). When traditional two stages approach are used, determination of these parameters in a single individual requires multiple blood sampling during the dose interval. This is not always financially and ethically feasible in susceptible populations such as children or transplanted patients.

Another method is to use average PK parameters derived from PK studies performed on a small number of healthy volunteers or in highly selected patients. However, healthy volunteers don't reflect the target population, in our case liver transplanted children. Thus, the information on the critical PK variability that will occur during clinical use according to patient's characteristics (age, genotype, liver function) as well as pathophysiologic, environmental and drug related features, is obscured by these restrictions.³⁸⁹⁻³⁹¹ In contrast to these traditional PK methods, population pharmacokinetics defined as "*the*

*study of the sources and correlates of variability in the drug concentrations among individuals who are the target population receiving clinically relevant doses of a drug of interest*³⁸⁹ is then a powerful approach to encompass some or all of the following characteristics:

- It collects relevant PK information in patient's representative of the target population treated with the drug
- It seeks to identify and quantify sources of PK variability such as interindividual, intraindividual and interoccasion variability
- It tries to explain variability by identifying factors as mentioned above
- It aims to quantitatively estimate the magnitude of the unexplained part of the variability in the patient population.
- It may be used for sparse or extensive PK data.^{389, 391}

Population pharmacokinetic is a part of the science of quantitative pharmacology (pharmacometrics).³⁹² A POP PK model is indeed a pharmacostatistic model describing the relationship between dose, drug concentration and/or exposure and patient characteristics along with quantifying the unexplained pharmacokinetic variability and residual variability with the assumption that they follow specific distributions.^{390, 393} This modelisation can be achieved based on sparse drug concentrations obtained from the clinical settings and thus facilitate dose individualization.³⁹⁰

POP PK modeling was introduced a long time ago (1972) by Sheiner *et al.* and was initially developed to deal with sparse PK data collected during TDM. It is an important tool in drug development but is also used to enhance knowledge in PK of already used drugs.^{394,395}

5.2 Non - Linear Mixed Effect Modeling

Population PK parameters can be estimated either by fitting the combined data from all the individuals ignoring individual differences (“naïve pooled approach”) or by fitting each individual’s data separately first and then summarizing individual parameters estimates to generate mean (population) parameters (the “two stages approach”).^{395, 396} These methods have limitations. The Naïve pooled approach cannot estimate intersubject variability because estimate parameters are obtained assuming all data arise from a single individual. The “two stages method” requires rich sampled data with at least, for each individual, a minimum number of observations greater or equal to the number of PK parameter +1.^{377, 393} Moreover, if this approach can yield adequate estimates of mean parameters, it tends to overestimate interindividual variability since residual error is comprised in it.

In contrast, the Nonlinear Mixed Effects Modeling approach allows estimation of all parameters simultaneously, thus allowing patients with varying sample numbers and timing to contribute to the data.

In that approach, PK parameters become random variables and their distribution are estimated. “Nonlinear” refers to the fact that the dependent variable (e.g., concentration) is nonlinearly related to the model parameters and independent variables (e.g., time, dose).³⁹⁷ *Mixed effects* refer to all *effects* contributing to variability. There are of two: *Fixed* effects and *random* effects. While the first describe predictable effects on a variable (such as age, genetic polymorphism, time post transplantation...) the second refers to unpredictable variability. A population pharmacokinetic model normally consists of 3 interacting components:

- a fixed-effects structural model, to describe typical PK parameters of a drug in the population
- a random-effects statistical model, to describe variability of parameters
- a fixed-effects covariate model, to describe the effects of patients’ characteristics on PK parameters.³⁹⁰

POP PK modeling can be performed using different softwares such as: NONMEM® (NON-linear Mixed Effects Modeling, ICON development solutions), Phoenix® NLME™ (Pharsight), MONOLIX (Lixoft), KINETICA (Adept Scientific), S-ADAPT (University of Southern California, Biomedical simulation resources) and many others with free access.³⁹⁸ NONMEM®, developed by Sheiner and Beal in the late 70s, is a parametric POP PK software allowing model estimations and simulations that gained popularity in academic and pharmaceutical research.^{398, 399} NONMEM® is still widely used, and

considered as the gold standard software for such analysis and is the software employed in this work.^{400, 401} However, as NONMEM® has poor graphics, other programs are required such as R.⁴⁰² In NONMEM® POP PK modeling, fixed-effects and random-effects are represented respectively by THETAs (θ) and ETAs (η). THETA has the same value for every subject and is typically estimated from the data. ETA is a quantity describing the difference between an individual's parameter value and the population value.³⁹⁵

In the next section we will develop the model building. A NONMEM® model building is performed according to a predefined routine. The first step consists of generating accurate database. The database contains different information (patient number and characteristics, doses, concentrations, time) and should be checked to avoid data errors. The second step is to find the best base model fitting the data comprising the structural model and the statistic model. Then, potential covariates are tested stepwise and all statistical relevant covariates are included in a full model before being backward eliminated (see Figure 10) to obtain a final covariate model. The last step being validation of the model.³⁹⁷

5.3 The structural model

The structural model describes the drug absorption into the blood in case of extravascular dosing, and the kinetics following absorption of the drug.³⁹⁷

Analysis begins by the determination of the best pharmacokinetic mamillary compartmental model (central compartment with peripheral compartments connecting to it) fitting the data.^{390, 397}

A compartment is a virtual space in which the drug is instantly homogeneously distributed, then eliminated or exchanged with other compartments, with identical kinetics at all points in the compartment.

The number of compartments (e.g. one-, two- or three-compartment model) is determined and the model is preferentially parameterized as volumes V and clearance CL . Figure 7 shows one- and two compartments models.³⁹³

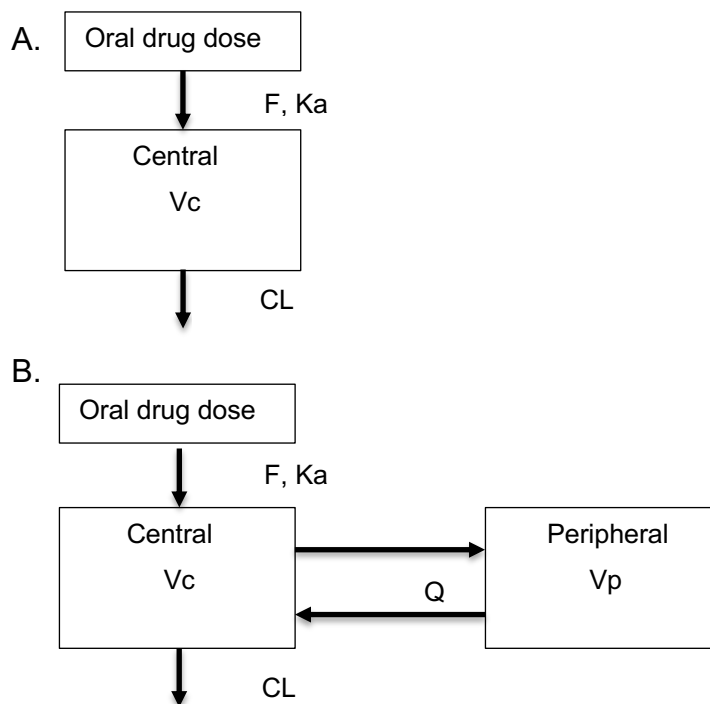


Figure 7: Compartmental PK modelisation of drug.

A. One compartment model. B. Two-compartment model.

F : bioavailability; K_a : absorption rate constant; CL : clearance; V_c : volume of the central compartment; V_p : volume of the peripheral compartment

Q : intercompartmental clearance

Considering the elimination phase, this can be either of the first order or zero order. First-order system implies an elimination rate proportional to concentration with a constant CL , leading to a doubling concentration in case of doubling dose. In contrast, zero-order is characterized by an elimination rate independent of concentration and leading to increased concentrations more than two-fold in case of doubling dose.

However, elimination usually moves progressively from a first-order to a zero-order state as concentrations increase, because of saturation of elimination pathways.³⁹⁷

Saturable elimination is represented by the sigmoidal Emax model illustrated by Equation 4 for a one compartment model.³⁹⁷

$$C = \frac{A}{V} \quad \text{Equation 3}$$

$$\frac{CL}{F} = \theta CL \left(1 + \frac{CL_{max} \cdot C}{CL_{50} + C} \right) \quad \text{Equation 4}$$

where A is the amount of drug in the central compartment, V is the volume of the central compartment, C is the concentration in the central compartment, CL_{max} and CL₅₀ are the maximum apparent clearance factor and the time delay to reach half the maximum apparent clearance, respectively.

The absorption profile for orally administered drugs (e.g. zero order or first order absorption with or without a lag time) is also determined.³⁹⁰

The absorption profile is characterized by two components: the oral bioavailability and the time course of the absorption rate. Absolute variability can only be estimated when concurrent extravascular (oral) and IV routes are available. When only extravascular data are available, it is impossible to estimate true CL and volume because F is unknown. Therefore, estimated parameters are a ratio of the unknown value of F: CL/F, V/F.

Extravascular absorption is classically represented with a first-order process using an absorption rate constant (K_a). This represents a passive absorption driven by the concentration gradient

between the absorption site and blood. If the appearance of drug in the blood is delayed for any reasons (gastric emptying), there is a need to add an absorption lag time.^{395, 397}

The choice of the best structural model relies on Likelihood ratio test (LRT), Akaike (AIC) or Bayesian information criteria (BIC) (see covariate model) and graphical examinations (residual versus time, residual versus predictions, observations versus predictions and precision of parameter estimation).³⁷⁷

5.4 The stochastic model

The statistical model describes variability around the structural model.³⁹⁷

There are three main types of variability:

- the interindividual variability (IIV)
- the between occasion variability (BOV)
- the residual variability.^{390, 395, 397}

5.4.1 The interindividual variability

IIV represents the differences in response to drugs between one patient and another;³⁵³ in other words, it is the difference between parameter values for a particular subject and the population value of the parameters. It is represented in Figure 8 and modeled in Equation 5^{390, 395}

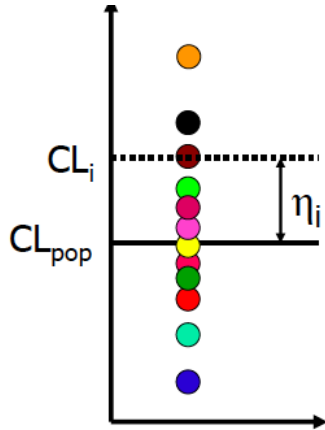


Figure 8: Intersubject variability of clearance in the population. *From Laffont*⁴⁰³

$$CL_i = CL_{pop} + \eta_i \quad \text{or} \quad CL_i = \theta_{CL} + \eta_i \quad \text{Equation 5}$$

where CL_i is the value of clearance in the i th individual, CL_{pop} or θ_{CL} is the parameter estimate of the typical value of clearance in the population and η_i is the individual deviation from the typical value. This can be extended for any other parameter such as V_d .

η_i is assumed to be normally or log normally distributed, centered around 0 and is described by its estimated variance, OMEGA squared (ω^2). Biological parameters are generally not normally distributed, because most of them can't go negative and are right skewed. However, the natural logarithm ($\ln$) of biological parameters is often approximately normally distributed.⁴⁰² Therefore, IIV in population pharmacokinetic models is described exponentially as follow (Equation 6)

$$CL_i = \theta_{CL} \times e^{\eta_i} \quad \text{Equation 6}$$

In order to express variability with the same scale as the parameter (e.g. L/h for clearance or L for volume of compartment), the variance of η_i 's is transformed to the apparent coefficient of variation (CV):

$CV (\%) = \sqrt{\exp(\omega^2) - 1} \times 100 \%$. For small variance (<30%), CV can be approximated by the root of ω^2 .^{390, 397}

5.4.2 The between occasion variability

Individual PK parameters may vary according time. Therefore, when patients are sampled at more than one occasion, BOV must be considered. If not, bias in both variance and structural parameters has been reported.⁴⁰⁴ BOV is cited as a component of IIV and is estimated according to

$$CL_{ik} = \theta_{CL} \times e^{\eta_i + \eta_k} \quad \text{Equation 7}$$

where e^{η_k} is the deviance between the individual parameter CL_i and the occasion-specific individual parameter CL_{ik} at the k th occasion. In this equation, η_k 's are assumed to be normally distributed, with mean zero and variance Π^2 .^{390, 397}

5.4.3 The residual variability

After taking IIV and BOV into account, the difference between the observed data for an individual and the model's prediction, represents residual variability or EPS (ϵ) as illustrated in Figure 9. EPS arises from multiple sources, including assay variability, errors

in sample time collection, errors in dosing time, intraindividual variability and model misspecification.

Models for residual error can be additive, proportional, mixt and exponential respectively as follow:

$$Y_{ij} = C_{predij} + \varepsilon \quad \text{Equation 8}$$

$$Y_{ij} = C_{predij} (1 + \varepsilon) \quad \text{Equation 9}$$

$$Y_{ij} = C_{predij} (1 + \varepsilon_1) + \varepsilon_2 \quad \text{Equation 10}$$

$$Y_{ij} = C_{predij} \cdot \exp(\varepsilon) \quad \text{Equation 11}$$

where Y_{ij} and C_{predij} are the model predicted concentrations with and without residual error, respectively, for the i th individual at the j th measurement. The error term ε is assumed to be normally distributed with mean zero and variance sigma squared (σ^2). In additive error, variance is constant and independent of the concentration level and has the same unity as the concentration. Proportional error components are estimated in percentages and allow the size of the residual error to increase in proportion to increasing concentration. The combined error model is commonly used because it describes a constant error model at low concentrations and a predominant proportional error model at high concentrations that reflects the assays variability.^{390, 397}

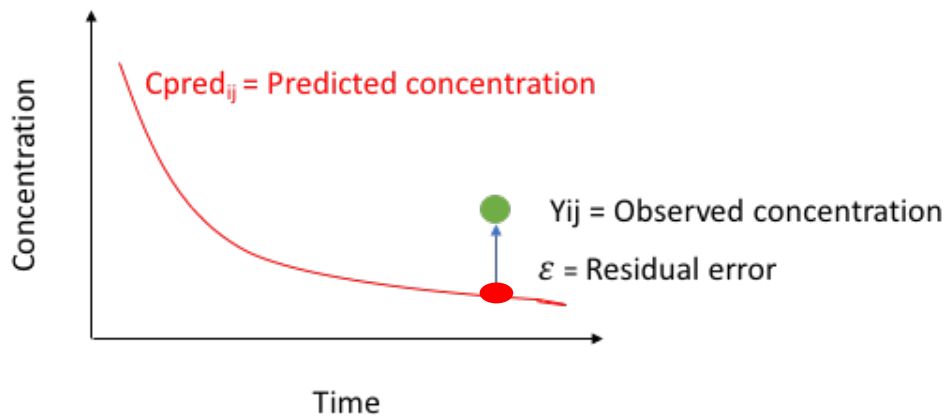


Figure 9: Residual variability (ε). ε is the deviance between observed concentration represented by green dot and predicted concentration represented by red dot. *From Laffont*⁴⁰³

In the recommendations, analyst should first refine the base structural model and then the residual error model.⁴⁰⁵

5.5 The covariate model

Covariates represent characteristics of patients that may explain a part of the PK variability.

- demographic variable (sex, age, weight)
- genetic variable (genotypes of *CYP3A* and *ABCB1*)
- timing variable (time post transplantation)
- laboratory variable (liver enzymes, creatinine) reflecting organ function or integrity
- drug interaction variable (presence of co-medication)
- analytical method (HPLC-MS, immunoassays)

When covariates are added to the structural model, they can reduce both BSV and unexplained variability and thus, let appear subgroups with specific dosage regimens.

Covariates are either continuous or discrete. Discrete covariates being dichotomous (e.g. sex) or polychotomous (e.g. race, genotype).^{377, 395}

The covariate model develops plausible mathematical relationship between covariates and parameters by incorporating the influence of covariate on theta, explaining a part of IIV.

For example, body size may explain a part of the drug clearance variability in the population. An indirect measure of body size such as total BW is modeled by using a biologically plausible mathematical function of the relationship between BW and clearance.

Continuous covariate effects can be incorporated into the population model using different functions: linear, exponential or power model as follow, respectively:^{377, 395}

$$CL = \theta + \theta_{WTcl} \times WT \quad \text{Equation 12}$$

$$CL = \theta \times \exp (\theta_{WTcl} \times WT) \quad \text{Equation 13}$$

$$CL = \theta \times (WT)^{\theta_{WTcl}} \quad \text{Equation 14}$$

Where θ_{WTcl} is the change in CL due to the weight effect.

Covariate parameter is typically centered or normalized to the mean values or normal value (70 kg for adult WT) for easier interpretation of the estimate of θ as follow:^{390, 395}

$$CL = \theta \times (WT - 70)^{\theta_{WTcl}} \text{ centered} \quad \text{Equation 15}$$

$$CL = \theta \times (WT/70)^{\theta_{WTcl}} \text{ normalized} \quad \text{Equation 16}$$

In case of pediatric patients, Holford justified the use of allometric functions to account for changes in CL as children mature based on weight.²²⁴

$$CL = \theta \times (WT/70)^{0.75} \quad \text{Equation 17}$$

For dichotomous covariates, the values of the covariate are usually set to 0 for the reference classification and 1 for the other classification. As for continuous covariates, common functions are used to describe dichotomous covariate effects.

Polychotomous covariates such as race, may be evaluated with a different factor for each classification against a reference value.³⁹⁷

Covariates tested are chosen according to properties of the drug. Indeed, for highly metabolized drugs, covariates such as weight, liver enzymes or genotype will be selected. Preliminary evaluation of covariate is made by regression-based technique, correlation analysis and graphically.

Covariate selection is usually based on objective function OBJ using the LRT and its derivative, the AIC and the Bayesian's information criterion BIC for nested models. The likelihood ratio test is restricted to comparison between *nested* models. Two models are nested if the alternative model including covariate is identical to the base model when the added parameter is fixed to 0. The AIC and BIC are described in equations 18 and 19:

$$\text{AIC} = -2\ln(L) + 2p \quad \text{Equation 18}$$

$$\text{BIC} = -2\ln(L) + p \times \ln(N) \quad \text{Equation 19}$$

Where L is the likelihood for an estimated model with p parameters and N is the sample size.

NONMEM does not output the likelihood directly. It rather calculates an objective function value (OFV) proportional to “ $-2 \times \log \text{likelihood}$ ”. Then models are compared with the difference between their OFV (ΔOFV). The OBJ is a reflect of how the model predictions fit the data (maximum likelihood = minimum OBJ = best fit).³⁹⁷ ΔOFV is χ^2 distributed and the contribution of an added parameter can be evaluated at a selected significance level, according to the χ^2 - distribution. A reduction in OFV of > 3.8 and > 6.6 are associated with significance levels of $p < 0.05$ and $p < 0.01$, respectively, with one degree of freedom (one added parameter). Covariates are tested stepwise until no other covariates improve the model. A full model is

then obtained. Finally, the significance of each covariate is evaluated by removing them sequentially (backward elimination).^{395, 406}

Finally, the model building may be represented by the flow chart in Figure 10:

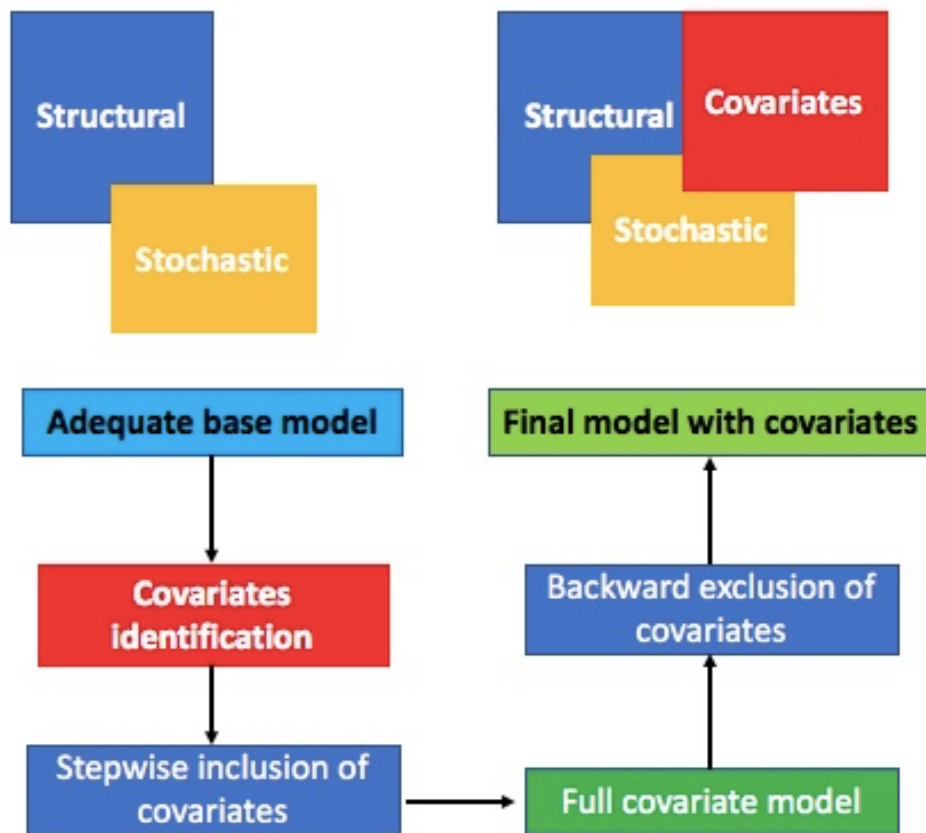


Figure 10: Flow chart of for building a final covariate model

To account with the residual variability (those not explained by fixed effects of covariates) individual PK parameters need to be estimated. In POP PK, concentrations measured in clinical settings, although sparse, may be used in conjunction with the typical pharmacokinetic parameters and their population distributions to estimate individual parameters with more reliability. This method allows dose individualization beyond the information obtained by covariates.³⁹⁰

5.6 Individualization of drug therapy with Bayesian estimation of individual PK parameters

The Bayesian estimation for individual parameters balances information from the population model (prior knowledge) with information obtained from the individual concentration measurements to obtain the most likely individual parameters. These estimates are also called Empirical Bayes Estimates (EBEs), *Maximum a Posteriori* Bayesian estimates or *posthoc* estimates, and are calculated by NONMEM.

Bayesian forecasting can be used as a tool for TDM, that uses population PK parameter estimates (e.g. mean population drug clearance), along with the expected associated variability and information about the patient (e.g. bodyweight), to predict initial drug concentrations according to specific doses as represented in figure 11. Measured drug concentrations are used as feedback as they become available. PK parameters for each patient become individualized and the influence of the population parameters decreases. Thus, it informs the clinician of the next appropriate dose

to maintain or reach the desired drug concentration.³⁹⁰ Indeed, knowing the individual parameters, AUC and thus adapted dose may be then determined according to target AUC

$$AUC_{\text{predicted}} = \frac{\text{Dose}}{CL_{\text{pred}}} \quad \text{Equation 20}$$

$$\text{Adapted dose} = \frac{\text{Current dose} \times \text{Target AUC}}{AUC_{\text{predicted}}} \quad \text{Equation 21}$$

The number of blood samples needed and the time to reach target drug concentrations can be reduced.²¹⁸

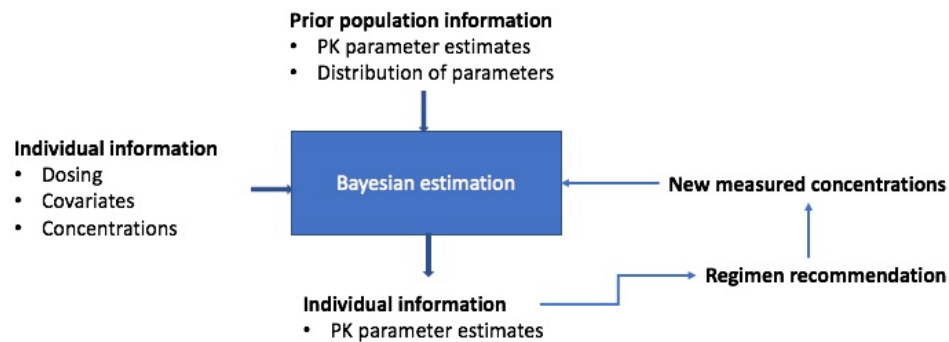


Figure 11: Schematic representation of Bayesian estimation of individual PK parameters. Adapted from Storset³⁹⁰

5.7 Model validation

After the statistical evaluation during model building, model should be further evaluated to be sure that it is appropriate for intended use. Models are better judged by their “fitness for purpose” rather than for being “right” or “true. “All models are wrong, some are useful” as stated Georges Box.⁴⁰⁷ Moreover, the final model should be the simplest for intended use according to the principle of

parsimony. Finally, the covariates should be clinically relevant to be included in the final model. A model can be validated either externally or internally.

External validation consists to compare the final model to an independent data set. It is the most reliable technique to validate a model. However, it is not always possible to perform.

Internal validation gathers different techniques such as:³⁹⁷

5.7.1 Parameters and standard estimates

To be validated, a model should have precise parameter estimates because it reflects the precision with which the covariate effect has been estimated. Precision may be either expressed as standard estimates (SE) or as confidence interval as shown in Equation 20. Usually, the acceptable level of precision is <30% SE for fixed effects and <50% SE for random effects but may depend on the size of the database. If the confidence interval includes the null value for a covariate, then the estimate of the covariate effect is unreliable.³⁹⁷

$$CI = \text{Parameter value} \pm 1.96 SE \quad \text{Equation 22}$$

5.7.2 Bootstrapping

Bootstrapping is a resampling technique that can be used as an alternative for estimating parameters precision. If the dataset contains n patients, bootstrapping consists in generating N replicate data sets of n individuals that are randomly drawn from the original database and that can be drawn multiple times or not drawn for each replicate. To adequately reflect the parameter distributions, many replicates (≥ 500 -1,000) are generated and evaluated using the final

model, and replicate parameter estimates are tabulated. The percentile bootstrap CI are constructed by taking the lower 2.5% and the upper 97.5% value of each parameter estimate from runs. If parameters are estimated with precision, again, IC should not include the null value.³⁹⁷

5.7.3 Data splitting

This technique consists of splitting the original dataset into a learning dataset and a validation dataset to test the predictive performance of a model. A random subset of the data (usually two-thirds) is used to build the model, while the remaining data is used for validation purposes.⁴⁰⁸

5.7.4 Graphical diagnostic plots

During the base and final model evaluation, the goodness of fit of the model is examined and the model assumptions are verified.

Goodness of fit is usually visually examined with graphics, the goodness of fit plots. The most informative graphics are plots of population predicted (PPRED) or individually predicted concentrations (IPRED) versus observed concentrations (OBS) with a line of identity and trend line presented in both linear and log-scale. Ideally, concentrations should fall randomly (without bias) and precisely around the line of unity. While a large spread around the line of unity in the plot of PPRED vs OBS is expected due to unexplained IIV, the IPRED versus OBS plot should be more precise because concentrations are predicted from the individual Bayesian parameter estimates.^{393, 409}

However, the information given by these plots is limited because they do not include time and provide little information about how to improve the model in case of bias.⁴¹⁰ Therefore, other traditional diagnostic plots are added in the model evaluation such as:

- Scatter plots of weight residuals (WRES) or conditional weighted residuals (CWRES) vs. predicted data with a zero line and trend line included.
- Scatter plots of WRES or CWRES vs. time with a zero line and trend line included.
- Absolute IWRESs vs. IPRED with a trend line included; a histogram or QQ-plot of weighted residual a zero line and trend line included.
- Individual plots that show how well the model describes the data at the individual level estimated individual parameters and individual plots of goodness of fit.³⁹³

However, since the two last decades, simulation based diagnostic methods since Visual Predictive Check (VPC), and Normalized Prediction Distribution Error (NPDE) became popular and accepted as the most informative plots.^{398, 411}

VPC is a graphical comparison of observations and simulated predictions based on model predictions including random effects. Practically, about 1000 simulations using the patient's dataset and the chosen model are run. Summary measures of the distribution of predictions and observations are compared visually. Typical summary measures are the median and an interval defined by the

lower 5% (or 2.5%) and upper 5% (or 2.5%) of the values.⁴¹² Then, analyst visually checks whether 90 % (or 95%) of the original observations fall within the 90 % (95%) prediction interval of the simulated concentrations.³⁹⁰ If so, the model describes the data well.⁴⁰⁹

NPDE generates a normalized reference distribution for each observation taking into account the correlation in residuals within a subject. NPDEs follow a standard normal distribution with mean 0 and unit variance and are uncorrelated within an individual. NPDE are more detailed in the methods section of article 1 and 2.^{393, 413}

Now that POP PK modeling concept and model building has been explained, let us review the existing POP PK models in LT.

5.8 Previous POP PK models in PLT

Several relevant POP PK models have been published in PLT before the beginning of this thesis and are summarized in table 2.

However, none of this model is satisfying for different reasons:

- they used a limited number of patients
- they were, either not tested on an independent group, or showed poor prediction when tested on independent group
- genotype for *CYP3A* and *ABCB1* was unknown and not included or was only available for recipient
- most of them didn't consider comedications
- the effect of GW/BW was not tested

-the length of study is either short, <3 months or not clearly delimited. Indeed, the post-operative days (POD) seem to be significant but has been tested in studies covering a limited time after transplantation. Thus, it is interesting to know whether clearance still rises after POD 50 or not. As growth is important during the first-year post transplant, and as using different models according to POD may be very difficult, the development of a unique model covering the first year may be useful.

Since the beginning of this work, other models have been published after 2012 and are added in Table 2.

Author	N	Age (years)*	Time post LT (days)	Disposition model	Covariates
Yasuhara et al.¹⁶⁴ 1995	55 (33/22)	4.2* 0.3-15	52	unknown	Cl: BW (allometry), POD V: BW (allometry)
Sam et al.²¹⁶ 2000	20 (16/4)	3.7* (1.1±13.9)	Not specified	1 compartment First order elimination	Cl: age V: BSA, F: BW, BIL
Staatz et al.²¹⁷ 2001	35	5.7* (0.5-16.6)	7-1464	1 compartment First order elimination	Cl/F: whole/split graft, age, AST, GGT
Garcia-Sanchez et al.³⁰¹ 2001	18	9.07 ¹ (0.3-16)	87	1 compartment First order elimination	Cl: BW (allometry), BIL, POD, ALT
Fukudo et al.²⁷⁶ 2006	130 (100/30)	1.2 ¹ (0.1-15)	50	1 compartment Time-varying elimination	Cl/F: BW (allometry), POD, AST, MDR1/CYP3A5 V: BW

Wallin et al.³⁸⁰ 2011	85 (73/12)	3.5 ¹ (0.4-16.9)	14	1 compartment Time-varying elimination	CI/F: POD, BW (allometry)
Jalil et al.⁴¹⁴ 2014	43	5* (0.65-17.56)	365	1 compartment First order elimination	CI/F: BW (allometry), POD, CYP3A5
Kassir et al.⁴¹⁵ 2014	30	7.3 ¹ (0.4-18.4)	At steady- state	2 compartments First order elimination	CI/F: BW (allometry) W: BW allometry
Yang et al.⁴¹⁶ 2015	52	1.8* (0.4-17.8)	1- unknown	1 compartment First order elimination	CI/F: POD, ALT BW: TP

Table 2: Previous population pharmacokinetic models of tacrolimus in pediatric liver transplantation.

Abbreviations are as follows: * Mean (range); ¹ Median (range); LT: liver transplantation; CL: clearance; CL/F: apparent clearance; BW: bodyweight; V: volume of distribution; BSA: body surface area; AST: aspartate aminotransferase; GGT: gamma-glutamyl transferase; BIL: bilirubin; MDR1: intestinal multi drug resistance protein type 1 mRNA level; CYP3A5: *cytochrome P450 A5*1* allele; POD: post-operative days; ALT: alanine aminotransferase; TP: total protein.

PART II. PERSONAL RESEARCH

**Chapter 1. POPULATION PHARMACOKINETICS ANALYSIS OF
TACROLIMUS IN THE FIRST YEAR AFTER PEDIATRIC LIVER
TRANSPLANTATION**

Published in European Journal of Clinical Pharmacology, 2013, 69, 1533-42

ADDENDUM

Page 1535, Table 1:

Liver transplant size (g)

Concomitant treatment with inhibitors of TAC metabolism (yes/no): 26/16

Concomitant treatment with inducers of TAC metabolism (yes/no): 3/39

Page 1539, Figure 2:

Fig. 2: Diagnostic plots for model validation

Top panel

Left histogram of normalized predictions of distribution errors (*NPDEs*)

Right *NPDEs* vs. time

Bottom panel

NPDEs vs. predictions

Population pharmacokinetic analysis of tacrolimus in the first year after pediatric liver transplantation

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Abstract

Purposes Tacrolimus (TAC) is the most widely used immunosuppressant for the prevention of acute rejection after solid organ transplantation. Its pharmacokinetics (PK) show considerable variability, making TAC a good candidate for therapeutic drug monitoring (TDM). The principal aim of the study was to describe the PK of TAC in pediatric patients during the first year after transplantation.

Methods Routine TDM trough levels of TAC were obtained from 42 pediatric liver allograft recipients during the first year after transplantation. A population PK model was developed using nonlinear mixed-effects modeling to describe TAC PK during this period and to explain the observed variability by means of patients' demographics, biochemical test results and physiological characteristics.

Results The PK of TAC were best described by a two-compartment model with first-order elimination. Apparent volumes of the central compartment, intercompartmental clearance and maximum blood clearance estimates were 253 L, 115 L/day and 314 L/day, respectively. The absorption first-order rate and volume of peripheral compartment were fixed to 4.5 h^{-1} and 100 L, respectively. While hematocrit levels, time after transplantation and bodyweight influenced TAC clearance, bodyweight was the only covariate retained on volume of distribution.

Conclusions We developed a TAC population PK model in pediatrics covering the first year after liver transplantation that may serve as a tool for TAC dose individualization as part of TDM.

Keywords Tacrolimus · Pediatrics · Liver transplantation · Population pharmacokinetics · Therapeutic drug monitoring

Introduction

Tacrolimus (TAC) is an immunosuppressive drug used to prevent graft rejection after solid organ transplantation. It acts by inhibiting calcineurin, an enzyme involved in the transcription and biosynthesis of the nuclear factor of activated T-cells, resulting in the inhibition of the life cycle of activated lymphocyte T-cells [1]. Even though there are no standard protocols for immunosuppressive therapy, most clinical centers implement a TAC-based immunosuppressive therapy after liver transplantation. TAC may be used in monotherapy with anti-CD25 induction or in combination with corticosteroids and/or mycophenolate mofetil according to the policy of the particular center [2].

The use of TAC is not always safe and effective, and TAC-related side-effects, such as nephrotoxicity and occurrence of acute rejection episodes, have been reported in adult and pediatric patients treated with the recommended doses of TAC (0.1–0.15 mg/kg/12 h) [2–5], sometimes up to 12 months post-transplantation [6]. This can at least partly be explained by the high pharmacokinetic (PK) variability of TAC [3].

The PK of TAC are characterized by a high unexplained variability, particularly in the earlier period after transplantation [3, 7]. Several factors, including time after transplantation, bodyweight, hematocrit, age, liver function tests, type of graft and genetic polymorphisms, contribute to this early variability [3, 7] in both pediatric [8–16] and adult

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liver transplantation [17–23]. As many pediatric patients are transplanted at a very young age (<1 year), subsequent rapid growth with the associated physiological modifications is to be expected during the first year, leading us to the question of whether such growth could influence the TAC PK even in the late period after surgery. TAC is therefore a good drug candidate for therapeutic drug monitoring (TDM). However, as currently performed, TDM in pediatric patients is mostly based on trough concentrations (C_0), and the latter are known to correlate poorly with clinical outcome (graft rejection and TAC toxicity). Moreover, C_0 -based therapeutic ranges in children are defined based on adult clinical data [24], with subsequent empirical adaptation of the doses based on these trough concentrations.

The area under the blood concentration–time curve (AUC) is expected to be a better marker of global systemic exposure to TAC, but the AUC is difficult to obtain using the classical PK approach for ethical reasons (high number blood samples required). The nonlinear mixed effects (NLME) modeling approach offers the possibility to simultaneously characterize the PK and the variability of highly variable drugs with good precision and accuracy. Moreover, relatively sparse data, such as routine TDM data, can be used for this purpose. It is also a powerful tool to explore the potential sources of variability and quantify their influence on different PK parameters [25].

The objectives of this study were: (1) to describe the PK of TAC after its oral administration in pediatric patients during the first year after liver transplantation, using a population PK modeling approach and (2) to identify and model the effect of demographic and clinical factors on TAC PK variability in this specific population, by using NLME modeling techniques.

Patients and methods

Patient characteristics and sampling

For the analysis we used retrospective routine TDM (whole-blood 12-h TAC C_0), demographic and clinical data from 42 pediatric de novo liver allograft patients from one Belgian university hospital (Cliniques Universitaires Saint Luc, Brussels). These patients had received a liver transplant between 2008 and 2011, were followed up to 1 year after transplantation and were given oral TAC (Prograf®) twice daily, i.e. at 8.00 a.m. and 8.00 p.m., beginning on the day of transplantation, with basiliximab (Simulect®) induction on day 0 and day 4. The initial dose of TAC was 0.1 mg/kg of bodyweight. Data (TAC doses, TDM, weight, biological values) were recorded every day during the 15 days post-transplantation, then twice a week until the end of the first month, once a week until the end of the third month, twice a

month until the end of the sixth month and monthly thereafter.

During hospitalization (at least 15 days following the transplantation), the TAC dose was adjusted daily according to trough levels obtained by TDM, with target levels of 5–15 ng/mL. Afterwards, the dose was adjusted monthly. All available routine TDM data on these patients were included in the analysis, beginning with the day after transplantation up to the patient experienced a rejection episode or, alternatively, until the end of the first year. Characteristics of the patients enrolled in the study are presented in Table 1.

For the determination of TAC concentrations, 1-mL blood samples were collected in EDTA tubes and immediately processed.

This study, in accordance with the Declaration of Helsinki, was approved by the hospital ethical committee.

Analytical methods

Blood TAC concentrations were determined from February 2008 to 2010 either by microparticle enzyme immunoassay (MEIA) performed on the IMx analyzer from Abbott Diagnostics (Wiesbaden, Germany) or by chemiluminescent microparticle immunoassay (CMIA) performed on the Architect analyzer from Abbott Diagnostics. The first method, which is linear from 3 to 30 ng/mL, was found to be precise on an interday basis, with a coefficient of variation (CV) of <11 % for all quality control (QC) samples tested. The second method was also linear from 3 to 30 ng/mL, with good precision (total CV <9 % for all QC samples). In the MEIA, the TAC metabolites 13-*O*-demethyl TAC (MI), 31-*O*-demethyl TAC (MII), 15-*O*-demethyl TAC (MIII) and 12-hydroxytacrolimus (MIV) are known to display cross-reactivity with the antibody (<1, 109, 90.5 and 8.8 %, respectively [26]). The cross-reactivity values for the CMIA were 94 and 45 % for MII and MIII, respectively, and under the limit of detection for both MI and MIV [27].

The laboratory successfully participates in the TAC International Proficiency Testing Scheme (Analytical Services International, London, UK).

Population PK analysis

Nonlinear mixed effects modeling was performed using NONMEM ver. 7.2 (double precision; Icon Development Solutions, Ellicott City, MD), the PsN-toolkit [28], a programming library for NLME modeling, and R (www.r-project.org), an S-PLUS-based aid with an interface containing graphical and statistical tools. The first-order conditional estimation approach with interaction between parameters was used throughout the entire modeling process of TAC blood concentrations. Different structural models were tested, including one-, two-

Table 1 Patients characteristics

Parameter	Median	Range
Gender ratio (Male/female) (<i>n</i>)	19/23	–
Age (years)	1.35	0.53–10.93
Donor age (years)	32.95	24.89–55.52
Donor/recipient age (years)	22.45	3.57–105.54
Body weight (kg)	10.20	5.5–31.4
Body height (cm)	79.0	63.6–128.5
Liver transplant size	252	119–572
Aspartate aminotransferase (AST) (U/L)	42	12–1533
Alanine aminotransferase (ALT) (U/L)	31	4–1301
AST/ALT	1.380	0.014–69.110
Gamma-glutamyltransferase (U/L)	24	4–392
Total bilirubin (mg/dL)	0.5	0.1–31.1
Direct bilirubin (mg/dL)	0.50	0.04–16.50
Alkaline phosphatase (U/L)	255	102–19375
Urea (mg/dL)	24	3–163
Plasma creatinine (mg/dL)	0.24	0.06–4.42
Calculated glomerular filtration rate (Schwartz formula) (mL/min/1.73 m ²)	133.93	11.92–604.75
Plasma protein (g/dL)	5.9	3.2–8.7
Serum protein (g/dL)	6.14	1.90–8.63
Plasma albumin (g/dL)	3.1	1.1–7.7
Serum albumin (g/dL)	3.69	2.21–7.10
Hematocrit (%)	29	18–39
Leucocytes (10 ³ /μL)	6.08	0.71–50.52
Eosinophils (10 ³ /μL)	0.3	0–3103.2
Platelets (10 ³ /μL)	226	5–735
Ischemic time (min)	32	26–56
Concomitant treatment with inhibitors of TAC metabolism (yes/no)	26/16	
Concomitant treatment with inhibitors of TAC metabolism (yes/no)	3/39	

TAC, Tacrolimus

and three-compartment models with first-order absorption rate. PK parameters were estimated by NONMEM using conventional equations in terms of the first-order absorption rate constant (K_a), clearance (CL), volumes of distribution (V) of the various compartments and intercompartmental clearance (Q). Since oral bioavailability (F) could not be determined, values for CL, V and Q correspond to the ratios CL/F, V/F and Q/F, respectively.

The interindividual variability (IIV) and interoccasion (IOV) variability in the PK parameters were modeled using an exponential model. Three different time points were considered during the analysis: (1) the hospitalization period (duration), (2) the first three months following hospitalization and (3) the 6 months thereafter. IIV and IOV terms were initially tested on all parameters. The value of a parameter in the i^{th} individual (P_i) was a function of the typical value of the parameter (θ) and of the individual deviation initially given by η_i , representing the interpatient variability for the i^{th} patient at the j^{th} occasion. The η and κ terms in the population were supposed to be symmetrically distributed,

zero-mean random variables with a variance that is estimated as part of the model estimation from Eq. 1:

$$P_i = \theta \times \exp(\eta_i + \kappa_i) \quad (1)$$

The η and κ terms were maintained in the structural model only when they improved the model based on the decrease of the Bayesian information criterion (BIC) computed as described below.

Additive, proportional and mixed error models were tested for the residual error as shown in Eqs. 2–4:

$$Y = \text{IPRED} + \varepsilon_{\text{add}} \quad (2)$$

$$Y = \text{IPRED} \times \varepsilon_{\text{add}} \quad (3)$$

$$Y = \text{IPRED} \times (1 + \varepsilon_{\text{prop}}) + \varepsilon_{\text{add}} \quad (4)$$

where Y represents the observed concentration, IPRED is the individual predicted concentration and ε_{add} and $\varepsilon_{\text{prop}}$ are

the additive and the proportional error terms on TAC concentrations, respectively. ε 's were supposed to be symmetrically distributed, zero-mean random variables with variance terms that are estimated as part of the population model-fitting process from Eqs. 2–4.

Model selection only referred to models for which the NONMEM minimization process was successful and was based on the following criteria: the BIC, the plausibility and the precision of parameter estimates and graphical analysis. The BIC was computed on the model objective function value (OFV) [29], the number of observations used during the modeling process (N_{obs}) and the number of parameters used (NPAR) as follows:

$$\text{BIC} = \text{OFV} + (\text{Ln}N_{\text{obs}} \times \text{NPAR}) \quad (5)$$

The models with the lowest BIC were evaluated further. The precision of the parameter estimates, expressed as the standard error of estimates, was generated by the covariance option within the NONMEM program. Goodness-of-fit plots including predictions and individual predictions versus observed concentrations, as well as conditional weighted residuals [30] (CWRES) versus predictions, and CWRES versus time after dose, were used for diagnostic purposes.

To explain interpatient and residual variability on PK parameters, relationships were investigated between these parameters and the following patient covariates: age, sex, race, weight, hematocrit, liver enzymes (amino transferase liver enzymes [Aspartate aminotransferase (AST) and alanine aminotransferase (ALT)], bilirubin, gamma glutamyl transferase (GGT), alkaline phosphatase), kidney function tests serum creatinine, serum urea, calculated glomerular filtration rate (cGFR) based on the new Schwartz formula [31], liver transplant/body weight ratio, co-administration of potential inducers or inhibitors of TAC metabolism, total plasma protein concentrations and analytical method. A difference of at least 3.84 of the OFV (chi-square p value ≤ 0.05) from the structural model OFV was considered to be statistically significant. Covariates that were continuous variables (age, weight, hematocrit, total plasma protein concentration, AST, ALT, GGT, serum creatinine, serum urea, cGFR) were centered to their median values and tested on the PK parameters in a nonlinear manner, such as in the following example:

$$\text{CL}/F = \theta_{\text{CL}} \times \left(\frac{\text{WT}}{\text{WT}_{\text{med}}} \right)^{\theta_{\text{WTonCL}}} \quad (6)$$

$$\text{CL}/F = \theta_{\text{CL}} \times \text{EXP} \left(\frac{\text{WT}}{\text{WT}_{\text{med}}} \times \theta_{\text{WTonCL}} \right) \quad (7)$$

where θ_{CL} is the population average apparent clearance and θ_{WTonCL} is the fractional change on the apparent clearance

due to the weight, WT is the patient bodyweight and WT_{med} is the median bodyweight in the dataset.

The influence of time variation on the apparent clearance was also assessed using an Emax model and the following equation:

$$\text{CL}/F = \theta_{\text{CL}} \times \left(1 + \frac{\text{CL}_{\text{MAX}} \times \text{TIME}}{\text{CL}_{50} + \text{TIME}} \right) \quad (8)$$

where CL_{MAX} and CL_{50} are the maximum apparent clearance factor and the time delay to reach half the maximum apparent clearance, respectively.

For the categorical covariates, such as sex or race, a change in a PK parameter (e.g. CL/F), was evaluated by the following type of equation:

$$\frac{\text{CL}}{F} = \begin{cases} \theta_{\text{CL}} & \text{in case of males} \\ \theta_{\text{CL}} \times \theta_{\text{fem}} & \text{in case of females} \end{cases} \quad (9)$$

where θ_{CL} is the population average apparent clearance for males, and θ_{fem} is the fractional change in apparent clearance in the case of females.

A full model was built using NONMEM that included all covariates showing a significant influence on PK parameters. A backward process was implemented to build the final model: only covariates which upon deletion generated an increase in OFV of >11 (chi-square p value ≤ 0.001) were retained in the final model.

Bootstrapping and simulations-based diagnostics were used to internally validate the final model. One thousand bootstraps were generated using the PsN toolkit and a confidence interval was built around the median of each parameter. Estimated values of each parameter obtained by the final model were compared to this confidence interval. Finally, the predictive performance of the model was evaluated using visual predictive check (VPC) and normalized predictions distribution errors (NPDE) [32]. The population PK model was used to simulate 2400 hypothetical patients. The distribution (median and 5th and 95th percentiles) of the simulated concentration–time curves was compared with the TAC concentration values in the original dataset. Regarding NPDEs, briefly, prediction discrepancies are obtained as the quantile of each observation within its predicted distribution. A model describes the data well when the predicted discrepancies are evenly distributed. Prediction distribution errors are obtained after de-correlation of both the observations and the simulated concentrations with respect to the empirical mean and variance obtained in the simulations. They are then normalized by using the inverse of the cumulative density function of the normal distribution with 0 mean and a variance of 1 [$N(0, 1)$]. Therefore, based on their construction, NPDEs follow the $N(0, 1)$ distribution and are uncorrelated within an individual [32]. Histograms

of NPDEs together with plots of NPDE versus time and NPDE versus predicted concentrations were generated and used as additional simulation-based diagnostics.

In order to subsequently perform a PK–pharmacodynamic (PK–PD) analysis and therefore determine the therapeutic window in pediatrics and to assess the influence of significant covariates on the apparent clearance, we performed an empirical Bayes estimation (by the POSTHOC and MAXEVAL=0 option of the NONMEM estimation subroutine) by using the developed POP PK model. Individual predicted values were generated for concentrations at different time points, apparent clearances and related area under the concentration–time curves. In addition, simulations of different scenarios were performed using final model parameter values.

Results

A two-compartment model with first-order elimination best fitted the observed concentrations. The following PK parameters were estimated: apparent volume of the central compartment (V_1/F), apparent intercompartmental clearance (Q/F) and apparent clearance (CL/F) (see Table 2). The absorption rate constant (K_a) and apparent volume of the peripheral compartment (V_2/F) were fixed based on literature data [33] and the sensitivity analysis during the modeling process, respectively.

The IIV terms were necessary to describe the variability in all of the PK parameters with the exception of the intercompartmental clearance for TAC. The removal of the IIV terms on these parameters was based on the BIC. As also shown in Table 2, an IOV term was only necessary for the clearance term. Mixed models were retained to describe the residual error.

The following covariates showed significant influence on TAC PK parameters: hematocrit, time after transplantation and bodyweight on CL/F and on volumes of distributions. These covariates were included in the model in a power manner, as shown in Eq. 6 for bodyweight. Time after the transplantation was imputed as a continuous covariate on CL/F using the following E-max model as shown in Eq. 8.

Table 2 summarizes the features of the final model. A good estimation of all the model parameters was obtained (standard error of estimates <50 % of estimates). The goodness of fit plots displayed in Figs. 1 and 2 also show that the model performed well in terms of fit to the data.

The validation of the final model by bootstrapping and VPC gave satisfactory results. Indeed, all parameter values were included in the 60–95 % confidence interval computed with the values derived from 1000 bootstraps. The distribution (2.5–97.5th percentiles) of the 1000 simulated concentration–time curves and the comparison with the observed TAC blood concentrations in the original dataset are shown in Fig. 3.

The overlap of the simulated and original distributions indicates the accuracy of the identified final model.

Bayesian estimation of apparent clearance and AUC using the final model parameters as prior information and patient trough levels enabled prediction of individual TAC CL/F and related AUC that could serve as a tool for AUC-based dose individualization as part of TDM program. Figure 4 shows the influence of significant covariates on empirical Bayes estimates of apparent clearance.

Discussion

In this study, a population PK analysis was performed to describe the PK behavior of TAC in the pediatric patient population the first year after transplantation. TAC PK are known to vary with body size and time after transplantation [9–11, 13–15]. Two previously published pediatric models described the influence of body size/bodyweight and time after transplantation on clearance, within a study period of 14 and 50 days post-transplantation [14, 15]. However, as already mentioned, physiological modifications, such as hematocrit level and those related to growth, are expected to occur during the first year post-transplantation in young transplanted patients, and as cellular rejection episodes may occur up to 12 months post-surgery, it is of interest to check whether these modifications may impact the TAC clearance after the early period, up to 1 year post-transplantation. In our model, the TAC clearance increases up to a steady-state at around 60 days post-surgery, which differs from the model described by Fukudo [14] where clearance was stable from post-surgery day 21 onwards. In contrast to the study by Wallin et al. [15], which focused on the early post-transplant period, hematocrit significantly influences the CL/F in our model, consistent with the TAC distribution, as discussed later. To the best of our knowledge, this is the first report on TAC PK covering a period of time as long as 1 year after transplantation. All previously published population PK studies in the pediatric patient population include data collected during shorter periods after transplantation [8–15], making the findings of our study quite interesting since they offer the possibility to use a unique tool for dose adjustment throughout a long period after transplantation. To do so, this model can, for example, be implemented in a software dedicated to Bayesian-based dosage individualization. In addition, given that the patients were included in the study from the first day after transplantation until the day they experienced a rejection episode, this model can also serve as a basis for a PK–PD analysis, with the ultimate goal to define a more appropriate concentration-based therapeutic window for TAC in pediatric patients. This subsequent analysis is beyond the scope of this report and will be included in another publication. Indeed, the currently used window is based on

Table 2 Final model characteristics

Parameters ^a (units)	Estimates [Bootstrap CI]	IIV/IOV [Bootstrap CI]
θ_{CL} (L/day)	0.001 [0.0007–0.0013]	0.3[0.27–0.32]/0.1[0.09–0.13]
θ_{T50} (days)	17.4 [12.5–21.6]	—
θ_{CLMAX}	314 [129–493]	—
θ_{Ka} (h ⁻¹)	4.5 []	—
θ_{V1} (L)	253 [193–326]	0.6[0.5–0.7]
θ_{V2} (L)	100 []	—
θ_Q (L/day)	115 [97–138]	—
$\theta_{wt \text{ on } V1}$	0.9 []	—
$\theta_{size/wt \text{ on } CL}$	0.12 [0.04–0.21]	—
$\theta_{Hct \text{ on } CL}$	0.85 [1.12–0.58]	—
ϵ_{prop}	0.02 [0.018–0.023]	NA
ϵ_{add} (ng/mL)	1.78 [1.63–1.95]	NA

CI, Confidence interval; IIV, interindividual variability; IOV, interoccasion variability; NA, not available

^a θ_{CL} , Typical value for baseline apparent clearance; θ_{CLMAX} , maximum time effect on apparent clearance; θ_{T50} , estimate of time delay to reach half the maximum apparent clearance value; θ_{Ka} , typical value for absorption rate constant; θ_{V1} , estimate of apparent volume of central compartment for a 10.2-kg (median) subject; $\theta_{wt \text{ on } V1}$, bodyweight effect on apparent volume of peripheral compartment; $\theta_{size/wt \text{ on } CL}$, liver transplant size/bodyweight ratio effect on apparent clearance; $\theta_{Hct \text{ on } CL}$, hematocrit effect on apparent clearance; θ_{V2} , typical value for apparent volume of peripheral compartment; θ_Q , typical value for apparent intercompartmental clearance; ϵ_{add} , additive residual error

adult data [24] even though it is well established that body size matters for TAC PK [3, 7, 10, 11, 13–15].

In most of previously published studies, only trough concentrations were used to develop population PK models, and TAC concentrations were modeled with a one-compartment model [3, 8–20, 22, 23]. However, given the physicochemical

properties of TAC and particularly its well-established accumulation in fat tissues, a two-compartment model should be more appropriate. The choice of a one-compartment model is therefore only justified by the lack of data in the distribution phase and by the (statistical) principle of parsimony on which model selection is based in most cases.

Fig. 1 Goodness of fit plots of the final population pharmacokinetic model. **Top panel** *Left* observed vs. population predicted concentrations, *right* observed vs. individual predicted concentrations. **Bottom panel** *Left* Conditional weighted residuals vs. predictions, *right* conditional weighted residuals vs. time

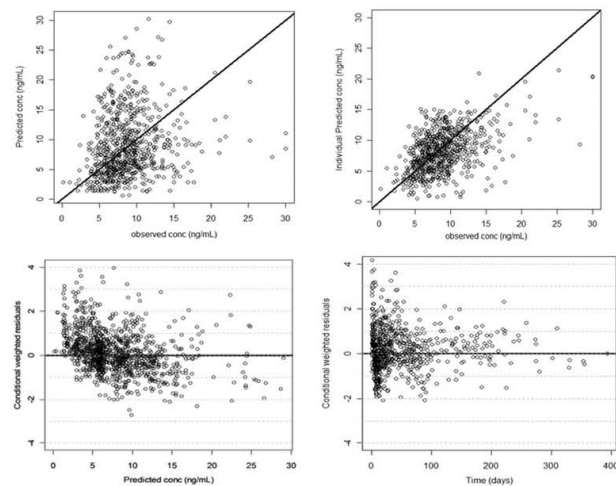
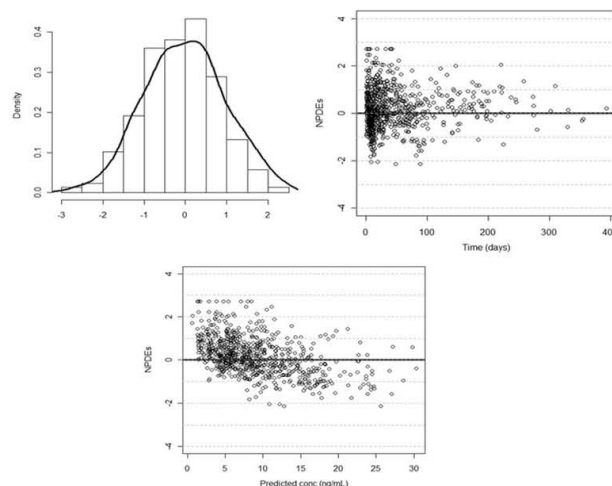


Fig. 2 Diagnostic plots for model validation. **Top panel** Left histogram of normalized predictions distribution errors (NPDEs), right NPDEs vs. predictions. **Bottom panel** NPDEs vs. time



Given the sparse sampling times, we were unable to describe the absorption and the early distribution phases. First-order processes were assumed, consistent with the passive diffusion that is the main TAC absorption process, and a fixed value of 4.5 h^{-1} was used for the first-order absorption rate process based on findings of a previous published study with more detailed sampling [10]. In our case, a one-compartment model had slightly worse fitting performances (a difference of

34 points in the OFV) with very close parameter estimates: when K_a was fixed to 4.5 h^{-1} , the typical values for apparent volume of distribution, time to reach apparent clearance and maximum apparent clearance were 301 L, 16.8 days and 361 L/day, respectively. Figure 1 shows the basic goodness of fit plots for the one-compartment model. Various models for elimination have been reported and tested during covariate model building, including first-order, time-independent and time-dependent Michaelis Menten elimination rates, but the unexplained final interindividual, interoccasion variability terms and even residual errors were sometimes rather high. A time-dependent first-order model was empirically chosen in this analysis based on the difference in the values of BIC as compared to that of the other models fitted to the data. This model has not been previously published for TAC but seems plausible given the range of concentrations explored and the expected recovery of the liver function over time in stable patients. Overall, distribution- and elimination-related parameter estimates fell in a range comparable to those reported previously. Interindividual variability components were needed to describe all estimated PK parameters, but the intercompartmental clearance and interoccasion variability components were necessary to describe TAC apparent clearance. This constitutes an insight in the favor of TAC TDM in pediatric patients during the first year after transplantation.

We tested various patient covariates on different parameters to explore whether they could explain the very large variability in our base model. The following covariates were found to

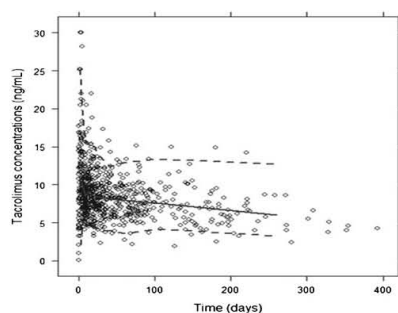


Fig. 3 Scatter plot visual predictive check from the final model. *Dash lines* Limits of the 95 % prediction intervals of simulated concentrations, *continuous line* median of simulated concentrations, *open circles* observed concentrations

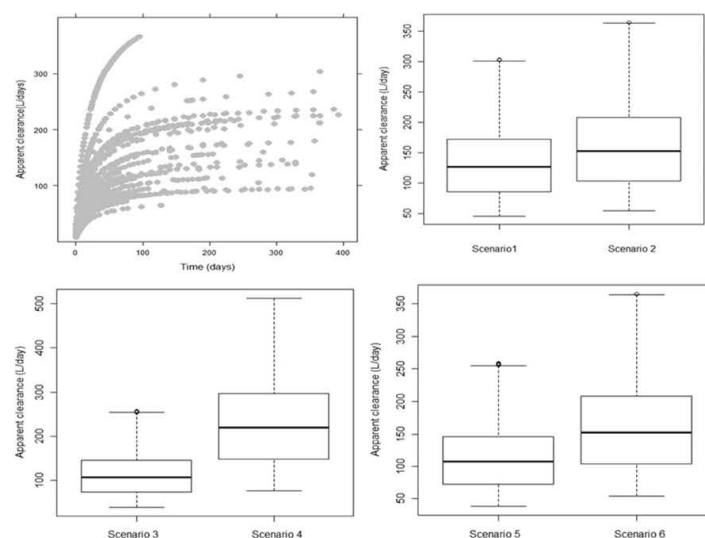


Fig. 4 Regression plot and box plots of 1000 simulated individual apparent clearances vs. significant covariates. **Top panel Left** Individual apparent clearances vs. time after transplantation for patients with a bodyweight of 10 kg and 30 % hematocrit levels, **right** individual apparent clearances vs. body weight; **scenario 1** patients with a 6-kg bodyweight and 30 % hematocrit levels, at day 50 after transplantation, **scenario 2** patients with 40-kg body weight and 30 % hematocrit levels, at day 50 after transplantation. **Bottom panel Left** individual apparent clearances vs.

hematocrit levels; **scenario 3** patients with 10-kg body weight and 18 % hematocrit levels, at day 50 after transplantation, **scenario 4** patients with 10-kg body weight and 45 % hematocrit levels, at day 50 after transplantation; **right** individual apparent clearances vs. liver transplant size/bodyweight ratio; **scenario 5** patients with liver transplant size/bodyweight ratio of 0.025 and 30 % hematocrit levels, at day 50 after transplantation, **scenario 6** patients with liver transplant size/bodyweight ratio of 0.056 and 30 % hematocrit levels, at day 50 after transplantation.

reduce the variability: liver transplant size/bodyweight ratio, time after transplantation and hematocrit levels for the apparent clearance and bodyweight for the apparent volumes of distribution. These findings are consistent with previously reported results on TAC PK in children [9, 11, 13, 15] and adults [17, 20, 23] and with known TAC PK characteristics such as high binding to red blood cells (which explains the negative influence of hematocrit levels on the apparent clearance) and high accumulation in fat tissues (which explains the influence of bodyweight on volume of distribution). Some of the covariates tested in our study and not retained in the final model based on used statistical criteria were found to be significant in population PK studies performed in adults. These include liver enzymes and creatinine clearance. These discrepancies can partly be explained by the lack of specificity of these covariates and/or their relatively low effect size. Finally, some covariates identified as influential on TAC PK in other studies [3, 7, 14, 16, 22] were not available for analysis in our study and therefore not tested; these include cytochrome P450 (CYP)

3A5 and ABCB1 genetic polymorphism. Indeed, previous pediatric studies in both kidney [34, 35] and liver [14] transplantation identified the expression of CYP3A5 as a significant covariate on TAC PK and showed that kidney transplant patients who expressed CYP3A5 (with a *CYP3A5**1/*3 or *CYP3A5**1/*1 genotype) and liver transplant recipients with a *CYP3A5**1-carrying graft liver had a significant higher apparent clearance than non-expressors and recipients with a non-*CYP3A5**1-carrying graft respectively. Moreover, in the particular case of liver transplantation, both the intestinal recipient and donor graft CYP3A5 expression may influence TAC metabolism in a sequential manner, as has been shown in liver transplant adults [36]. Therefore, we could expect a recipient and/or donor CYP3A5 effect on TAC PK in our cohort. For example, one patient in this study showed very high TAC clearance, leading us to hypothesize that this patient was a CYP3A5 intestinal expressor and/or received a *CYP3A5**1-carrying graft liver, which would explain his particular early and permanent high metabolism of TAC.

The missing genotype of *CYP3A5* is the principal limitation of this study. Moreover, as this study was conducted in pediatric living donor liver recipients under a TAC monotherapy regimen, the influence of cadaveric donor liver transplantation and the use of steroids and mycophenolate mofetil as a part of the immunosuppressive regimen were not tested. Consequently, we advise caution in applying our data to a cohort of patients with cadaveric donors or to hospitals with different internal practices.

When a linear PK for TAC is assumed, the Bayesian estimator based on the developed model for dose adjustment can be used when targeting an exposure in the middle of the therapeutic window, such as 180 ng h/mL for TAC [24]; in this case, Eq. 10 should be used for dose adjustment:

$$\text{Adjusted dose} = \frac{\text{Current dose} \times 180 \text{ ng h/mL}}{\text{AUC}_{\text{pred}}} \quad (10)$$

When there is a need for dose titration, the targeted exposure should be reached after a time delay of around 5 half-lives (2–4 days for TAC), which is the time delay needed to reach steady-state conditions. A new TDM should not be performed before this delay.

Currently, various computer programs are used for dose individualization based on patient concentrations. They very often use Bayesian estimators based on algorithms. These algorithms vary from one software to another, and they are based on empirical models developed in different settings and groups of patients and from studies of different durations. Their adequate use should only be based on interpolation, i.e. each algorithm should only be applicable in the specific cases of the patients and settings used to develop the specific model. At the present time, several models need to be used to perform dose adjustment in pediatric patients during the first year after transplantation. Our study was performed using data collected during the first year after transplantation; it therefore offers the possibility to develop a tool that can be incorporated in TDM-dedicated software, and this can be an attractive option in that the tool is based on one model.

Finally, it should be noted that two immunoassays have been used to quantify TAC in patient samples: MEIA and CMIA. Given that the present analysis was performed on routine TDM data, the analytical methods adopted were those used in the university hospital at the time of the study. TAC metabolites are known to display cross-reactivity with the antibodies used in both methods which could explain the slight underprediction of low concentrations with the developed final model. If it is now well established that liquid chromatography/tandem mass spectrometry is the method of choice to specifically quantify TAC in blood samples, it should also be noted that the laboratory successfully participates in the TAC International Proficiency Testing Scheme

(Analytical Services International, UK) at the time covered by this analysis.

Conclusion

A population PK model was developed for TAC in pediatric patients during the first year after liver transplantation. Hematocrit levels, liver/size bodyweight ratio and time after transplantation were retained as significant covariates for the apparent clearance, whereas bodyweight was found to significantly influence the apparent volumes of distribution.

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Conflict of interest statement The authors declare no conflict of interest related to this work.

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**Chapter 2. POPULATION PHARMACOKINETICS OF
TACROLIMUS EARLY AFTER PEDIATRIC LIVER
TRANSPLANTATION**

Published in Therapeutic Drug Monitoring, 2014, 36, 54-61

ADDENDUM

Page 55 of the PDF, TABLE 1

Urea (mg/dL)

Page 59 of the PDF, FIGURE 4

Diagnostic plots for internal model validation.

NPDEs versus predictions (A), NPDEs versus time (B), histogram of NPDEs (C), scatter plot visual predictive check from the final model (D). dots, observed concentrations; lines, median and 95 prediction interval.

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Only one of these models gave satisfactory results when fitted to our data (Table 3 and Fig. 5).

Population Pharmacokinetic Analysis of Tacrolimus Early After Pediatric Liver Transplantation

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Roger K. Verbeek, PhD,† and Pierre Wallemacq, PhD*

Background: Tacrolimus (TAC) pharmacokinetics (PKs) show considerable unexplained variability, particularly in the early period after transplantation. Therefore, TAC is a good candidate for therapeutic drug monitoring. The main objective of the present work was to propose a robust PK model for TAC in the early period after transplantation, with the final goal to provide practitioners with a tool for dose individualization in pediatric patients.

Methods: TAC concentration data were obtained from 82 pediatric liver allograft recipients during the first 2 weeks after transplantation. Previously published models, and a model recently developed by our group for pediatrics early after pediatric liver transplantation, were fitted to the data and their predictive performance compared with the performances of a model developed using the data from 82 pediatric patients.

Results: During the data-driven analysis, the PKs of TAC were best described by a 1-compartment model with time-varying first order elimination. Apparent volume of distribution and blood clearance estimates were 283 L and 10 L/h, respectively. The absorption was also considered to be a first order process, with a first order rate fixed to 4.45 hours. Parameters were estimated with good precision and accuracy. Although hematocrit levels, time after transplantation, liver weight, and body weight influenced the clearance, body weight was the only covariate retained on volume of central and peripheral compartments. Two of the 5 previous models showed acceptable predictive performances using the observed data.

Conclusions: Time after transplantation, body weight, and hematocrit levels were shown to influence TAC PK in the early pediatric post liver transplantation period and should be considered, besides therapeutic drug monitoring, by clinicians for the TAC posology prescription and adaptation.

Key Words: tacrolimus, population pharmacokinetics, pediatric liver transplantation, modeling, dose individualization

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The authors declare no conflicts of interest.

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INTRODUCTION

Tacrolimus (TAC) is a calcineurin inhibitor used to prevent graft rejection after solid organ transplantation. Calcineurin is an enzyme involved in the transcription and biosynthesis of the nuclear factor of activated T cells. Its inhibition by TAC further results in the inhibition of the life cycle of lymphocyte T cells.¹ In most clinical centers worldwide, a TAC-based immunosuppressive therapy is implemented after pediatric liver transplantation. Although TAC is sometimes used in monotherapy, combination with corticosteroids is still frequent.²

Occurrences of acute rejection episodes and TAC-related adverse effects, such as nephrotoxicity, have been reported in up to 50% of pediatric patients treated with standard doses of TAC (0.1–0.15 mg/kg per dose, twice a day), particularly in the early period after transplantation.^{2–5} This can be explained in part by the high variability of TAC pharmacokinetics (PKs).⁶ To solve this issue, trough concentrations C_0 are monitored in many centers and doses are empirically adjusted to target trough concentrations in the range of around 5–15 ng/mL, based on the reported rather acceptable correlation between trough concentrations and area under the concentration–time curve (AUC).^{3,6} However, TAC therapeutic drug monitoring (TDM) can be improved to increase the efficacy and safety of the treatment in this critical period. This can be achieved not only by a better choice of exposure marker and a better characterization of the therapeutic range but also by using appropriate tools, such as PK-based tools, to perform the TDM. Indeed, C_0 is known to correlate poorly with clinical outcome (graft rejection and TAC toxicity), and C_0 -based therapeutic ranges in children are defined based on adult clinical data.^{3,7}

AUC is expected to be a better marker of systemic exposure to TAC but is difficult to estimate routinely using classical PK approaches for ethical reasons (high number blood samples required). Nonlinear mixed effects modeling (NONMEM) approach combined with maximum a posteriori Bayesian estimation offers the possibility to characterize AUCs of highly variable drugs with good precision and accuracy from relatively sparse data, such as those collected in routine TDM. The NONMEM is also a powerful tool to explore the potential sources of variability and quantify their influence on different PK parameters.⁸

Four population PK models have been published for pediatric patients in this period. Two of them were developed using a limited number of patients and/or showed poor performance when tested on an independent group of subjects. The 2 others have not been tested on an independent

patient group.^{9–12} Moreover a population PK model has been developed recently by our group for pediatric patients during the first year after transplantation (V. Guy-Viterbo et al, Population pharmacokinetic analysis of TAC in the first year after pediatric liver transplantation, accepted for publication). It has not been externally validated either.

The main objective of this study was to propose a robust PK model for TAC early after pediatric liver transplantation, using a population modeling approach. This model could serve as a basis for PK-based TDM and for pharmacokinetic–pharmacodynamic (PK–PD) analysis, and ultimately, for the improvement of efficacy and safety profiles of TAC early after pediatric liver transplantation.

METHODS

Patient Characteristics and Sampling

Data used in the present analysis were retrospective routine TDM, demographic, and covariates data from 82 pediatric de novo liver allograft patients from a Belgian university hospital (Cliniques universitaires Saint Luc, Brussels, Belgium), transplanted between 2001 and 2011, who were given oral TAC (Prograf) twice daily, that is, at 8.00 AM and 8.00 PM from the day of transplantation with basiliximab (Simulect) induction on days 0 and 4. The initial posology of TAC was 0.1 mg/kg per dose, twice daily. The dose was daily adjusted according to trough concentration TDM, target levels being 8–10 ng/mL. All available routine TDM data for these patients were included from the day after transplantation until day 15. Characteristics of patients enrolled are presented in Table 1. For the determination of TAC concentrations,

1 mL blood samples were collected in EDTA tubes and stored at -80°C until analysis.

Analytical Methods (TAC Quantification)

TAC blood concentrations (a total of 1023 samples) were determined either by Microparticle Enzyme Immuno-Assay (MEIA) performed on the IMx analyzer from Abbott Diagnostics (Wiesbaden, Germany) or by Chemiluminescence Microparticle ImmunoAssay (CMIA) performed on the Architect analyzer (Abbott Diagnostics). The first method, linear from 3–30 ng/mL, was found to be precise on an inter-day basis: coefficient of variation <11% for all QC samples tested. The second method was linear from 3 to 30 ng/mL with good precision (total coefficient of variation <9% for all QC samples). Six hundred and nine (59.5%) and 414 (40.5%) samples were analyzed by MEIA and CMIA, respectively.

Population PK Analysis

NONMEM was performed by using NONMEM Version 7.1.2 (double precision; Icon Development Solutions, Ellicott City, MD) combined with PsN toolkit, a programming library for nonlinear mixed effects modeling, and R (www.r-project.org). The first order conditional estimation approach with interaction between parameters was used throughout the entire modeling process.

Model Building

A population PK model was developed from patient data using a data-driven approach. Different structural models were tested: 1- and 2-compartment, and 3-compartment models with zero or first order absorption, respectively. Michaelis–Menten and time-varying model were also tested for the elimination. PK parameters were estimated by NONMEM in terms of zero order (K_0) or first order absorption rate constant (K_{12}), clearance (CL), volumes of distribution (V) of the various compartments, and intercompartmental clearance (Q) using conventional equations. Because oral bioavailability (F) could not be determined, values for CL, V, and Q correspond to the ratios CL/F, V/F, and Q/F, respectively.

The interindividual variability (IIV) in the PK parameters was modeled using an exponential model, and all parameters were initially tested. The value of a parameter in the i th individual (P_i) was a function of the typical value of the parameter (θ), of the individual deviation initially given by η_i , representing the interpatient variability for the i th patient. The η terms in the population were supposed to be symmetrically distributed, zero-mean random variables with a variance that is estimated as part of the model estimation from equation 1:

$$P_i = \theta \times \exp(\eta_i). \quad (1)$$

The η terms were maintained in the structural model only when they improved the model fitting assessed by the decrease of the Bayesian information criterion (BIC) computed as described below.

Additive, proportional and mixed error models were tested for the residual error as shown in equations 2–4:

TABLE 1. Patients Characteristics (n = 82)

Patients Characteristics	Median (Range)
Ethnicity: white	82
Sex ratio (M/F)	41/41
Age at transplantation (yr)	0.96 (0.3–14.1)
Body weight (kg)	9.0 (5.3–66.8)
Body height (cm)	72.3 (58.0–164.0)
Body surface (m ²)	0.42 (0.3–1.74)
BMI (kg/m ²)	16.4 (13.1–24.8)
Creatinin clearance (mL/min)	146.6 (23.5–426.5)
Ureap (mg/dL)	18.0 (3–126)
Serum creatinin (μmol/L)	17.7 (5.3–199.8)
AST (U/L)	59.0 (11–1533)
ALT (U/L)	110 (13–1301)
GGT (U/L)	54.0 (6–1735)
Total bilirubin (mg/dL)	2.6 (0.1–31.1)
Direct bilirubin (mg/dL)	1.3 (0.1–15.4)
Albumin (g/dL)	3.0 (1.1–5.1)
Plasma proteins (g/dL)	4.8 (3.2–6.9)
Hematocrit (%)	28 (18–44)
Liver transplant size	255 (119–572)
Nifedipine or fluconazole comedication (yes/no)	51/81
Phenobarbital comedication	1/81
TAC whole-blood trough level (ng/mL)	9.3 (0.1–59.6)

$$Y = \text{IPRED} + \varepsilon_{\text{add}} \quad (2)$$

$$Y = \text{IPRED} \times (1 + \varepsilon_{\text{prop}}) \quad (3)$$

$$Y = \text{IPRED} \times (1 + \varepsilon_{\text{prop}}) + \varepsilon_{\text{add}}, \quad (4)$$

where Y represents the observed concentration, IPRED is the individual predicted concentration and ε_{add} and $\varepsilon_{\text{prop}}$ are the additive and the proportional error terms on TAC concentrations, respectively. The ε values were supposed to be symmetrically distributed, zero-mean random variables with variance terms that are estimated as part of the population model-fitting process from equations 2–4.

Model selection only referred to models for which the NONMEM minimization process was successful and was based on the following criteria: the BIC, the plausibility, and the precision of parameter estimates and graphical analysis. The BIC was computed on the model objective function value (OFV), the number of observations used during the modeling process (N_{obs}), and the number of parameters used (NPAR) as follows¹³:

$$\text{BIC} = \text{OFV} + (\text{Ln}N_{\text{obs}} \times \text{NPAR}). \quad (5)$$

The models with the lowest BIC were further evaluated. Precision of parameter estimates, expressed as standard error of estimates, was generated by the covariance option within the NONMEM program. Goodness-of-fit plots including predictions and individual predictions versus observed concentrations, as well as conditional weighted residuals¹⁴ versus predictions, and conditional weighted residuals versus time after dose, were used for diagnostic purposes.

To explain interpatient and residual variability of PK parameters, relationships were investigated between PK parameters and the following patient covariates: age, sex, race, weight, hematocrit, aminotransferase liver enzymes (AST and ALT), bilirubin, gamma glutamyl transferase (GGT), alkaline phosphatase, kidney function tests (serum creatinine, serum urea), estimated glomerular filtration rate (eGFR, based on the initial and updated Schwartz formula, with $k = 0.45$ for infants younger than 1 year, $k = 0.70$ for male adolescents, and $k = 0.41$ for others^{15,16}), coadministration of CYP3A inhibitors or inducers, liver transplant size–body weight ratio, total plasma protein concentration, and analytical method (MEIA or CMIA). Covariates that were continuous variables (age, weight, hematocrit, total plasma protein concentration, AST, ALT, GGT, serum creatinine, serum urea, eGFR, etc) were centered to their median values and tested on the PK parameters in a nonlinear manner using a power function:

$$\text{CL}/F = \theta_{\text{CL}} \times \left(\text{WT}/\text{WT}_{\text{med}} \right)^{\theta_{\text{WT on CL}}}, \quad (6)$$

where θ_{CL} is the population average apparent clearance and $\theta_{\text{WT on CL}}$ is the fractional change on the apparent clearance due to the weight, WT is the patient body weight, and WT_{med} is the median body weight in the dataset.

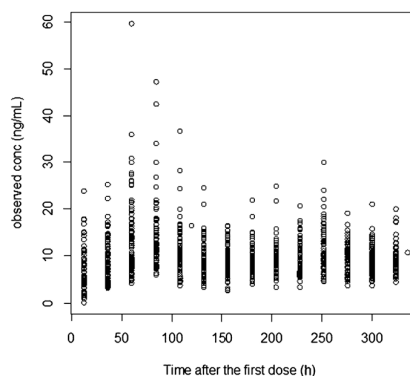


FIGURE 1. Time course of TAC blood concentrations.

For the categorical covariates, such as sex, race, a change in PK parameter, for example, CL/F, was evaluated by the following equation:

$$\text{CL}/F = \begin{cases} \theta_{\text{CL}} & \text{in case of males} \\ \theta_{\text{CL}} \times \theta_{\text{fem}} & \text{in case of females} \end{cases} \quad (7)$$

where θ_{CL} is the population average apparent clearance for males, and θ_{fem} is the fractional change in apparent clearance in case of females.

TABLE 2. Final Model Characteristics

Parameters (Units)	Estimates (Bootstraps 95% CI)	IIV, CV% (Bootstraps 95% CI)
θ_{BASCL} (L/h)	0.001	—
θ_{T50CL} (d)	3.97 (2.44–5.39)	26.7 (19–28)
θ_{CLMAX}	13.9 (6.5–22.8)	—
θ_{Ka} (h^{-1})	4.45	—
θ_{V} (L)	347 (269–500)	43.3 (19–53.6)
$\theta_{\text{wt on V}}$ (L/h)	0.44 (0.3–0.68)	—
$\theta_{\text{wt on CL}}$ (L/h)	0.21 (0.02–0.44)	—
$\theta_{\text{size on CL}}$ (L/h)	0.18 (0.09–0.37)	—
$\theta_{\text{inh on CL}}$ (L/h)	0.82 (0.70–0.92)	—
$\theta_{\text{HT on CL}}$ (L/h)	−0.04 (−0.37–0.01)	—
F	1	33 (30–37)
ε_{add} (ng/mL)	1.49 (1.41–1.58)	—
$\varepsilon_{\text{prop}}$	0.25 (0.22–0.27)	—

θ_{BASCL} , baseline apparent clearance for a 60-kg patient; θ_{T50CL} , Time to reach half the maximum apparent clearance for a 60-kg patient; θ_{CLMAX} , maximum apparent clearance for a 60-kg patient; θ_{Ka} , first order rate constant; θ_{V} , apparent volume of distribution for a 60-kg patient; $\theta_{\text{wt on V}}$, exponent of the allometric effect of body weight on the apparent clearance; $\theta_{\text{size on CL}}$, exponent of the allometric effect of body weight on the apparent volume of distribution; $\theta_{\text{inh on CL}}$, exponent of the allometric effect of liver transplant size–body weight ratio on the apparent clearance; $\theta_{\text{HT on CL}}$, exponent of the allometric effect of hematocrit levels on the apparent clearance; F, bioavailability factor; ε_{add} , additive residual error; $\varepsilon_{\text{prop}}$, proportional residual error.

CV, coefficient of variation.

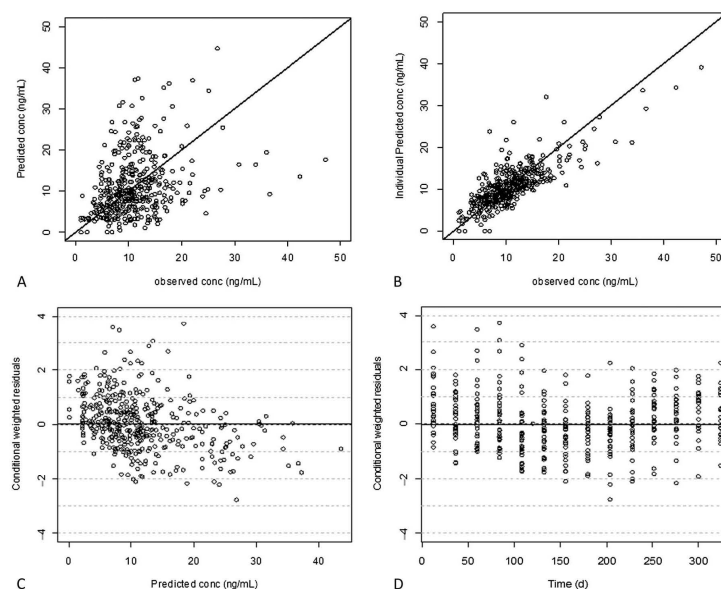


FIGURE 2. Goodness-of-fit plots of the final population PK model for data analyzed by MEIA. Observed versus predicted concentrations (A), observed versus individual predicted concentrations (B), conditional weighted residuals versus predictions (C), and conditional weighted residuals versus time (D).

The full model building approach was implemented: a full model was built using NONMEM including all covariates that had a potential to be clinically relevant on PK parameters and that were uncorrelated (coefficient of correlation < 0.3).

Model Validation

Bootstrapping and simulations-based diagnostics were used to internally validate the final model. One thousand bootstraps were generated using the PsN toolkit, and a confidence interval (CI) was built around the median of each parameter. Estimated values of each parameter obtained by the final model were compared with this CI. Finally, the predictive performance of the model was evaluated using visual predictive check and normalized prediction distribution errors (NPDEs).¹⁷ The population PK model was used to simulate 1000 hypothetical patients. The distribution (median and 5th and 95th percentiles) of the simulated concentration–time curves was compared with the TAC concentration values in the original dataset. Regarding NPDE, briefly, prediction discrepancies were obtained as the quantile of each observation within its

predicted distribution. A model was considered to properly describe the data when the predicted discrepancies were evenly distributed. Prediction distribution errors were obtained after decorrelation of both the observations and the simulated concentrations with respect to the empirical mean and variance obtained in the simulations. They were then normalized by using the inverse of the cumulative density function of normal distribution with zero-mean and a variance of 1 [$N(0, 1)$]. Therefore, by construction NPDE follow the $N(0, 1)$ distribution and are uncorrelated within an individual.¹⁷ Histograms of NPDE together with plots of NPDE versus time and NPDE versus predicted concentrations were generated and used as additional simulation-based diagnostics.

Assessment of Performances of Previously Published Models

Subsequently, the 4 previously published models,^{9–12} and the model developed recently by our group, were fitted to the data. Their parameter estimates were fixed. The predictive performances of these models were compared with the performances of the model developed using patient data.

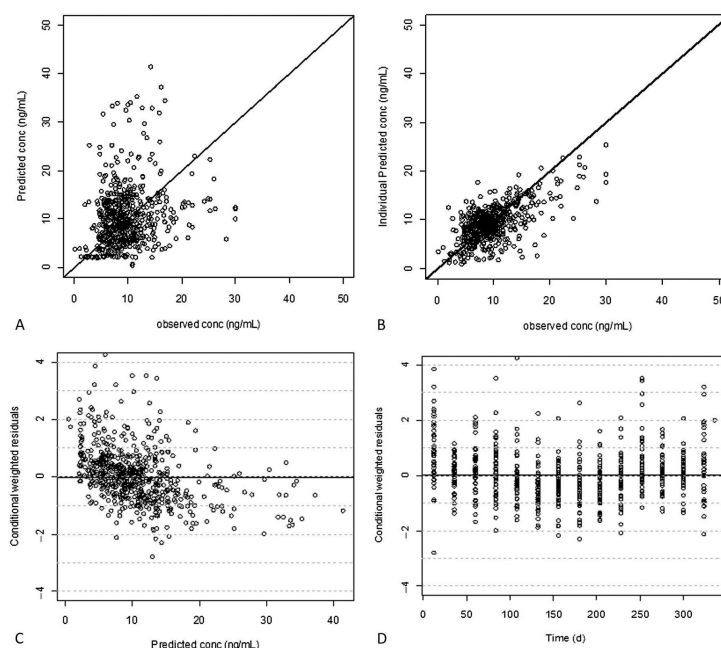


FIGURE 3. Goodness-of-fit plots of the final population PK model for data analyzed by CMIA. Observed versus predicted concentrations (A), observed versus individual predicted concentrations (B), conditional weighted residuals versus predictions (C), conditional weighted residuals versus time (D).

RESULTS

Figure 1 shows observed TAC whole-blood concentration–time profiles. A large variability was observed.

A 1-compartment model with time-varying first order elimination best fitted the observed concentrations. The following structural model was retained for the apparent clearance (equation 8):

$$CL = \text{BASCL} + (\text{CLMAX} \times \text{DAT} / \text{T50} + \text{DAT}), \quad (8)$$

where BASCL is the baseline apparent clearance, CLMAX is the maximum apparent clearance, DAT is the day after transplantation, and T50 is the time delay for the apparent clearance to reach half of its maximum value.

The following PK parameters were estimated: the first order absorption rate constant (K_a), apparent volume of the central compartment (V), and apparent clearance-related parameters (BASCL, T50, CLMAX). Absorption rate constant was fixed to 4.45 per hour based on literature data.¹⁸

IIV terms were necessary to describe the variability in V and CL . The removal of the IIV terms on these parameters was based on the BIC. A combined model was retained to describe the residual error. The following covariates showed significant influence on TAC PK parameters (ie, 95 CI around their estimates did not include 0): hematocrit, liver transplant size–body weight ratio, concomitant use of inhibitors, and body weight on CL/F and body weight on volume of distribution. Their effects were included in the model in a power manner for continuous covariates and in a multiplicative manner for the categorical covariate, as shown in equations 6 and 7, respectively. Table 2 summarizes the features of the final model developed using our patient data. Good estimation was obtained for all model parameters (standard error of estimates <50% of estimates). Basic goodness-of-fit plots are displayed in Figures 2 and 3 and show good fitting performances of the model to the data.

The internal validation of the final model by bootstrapping and visual predictive check gave satisfactory

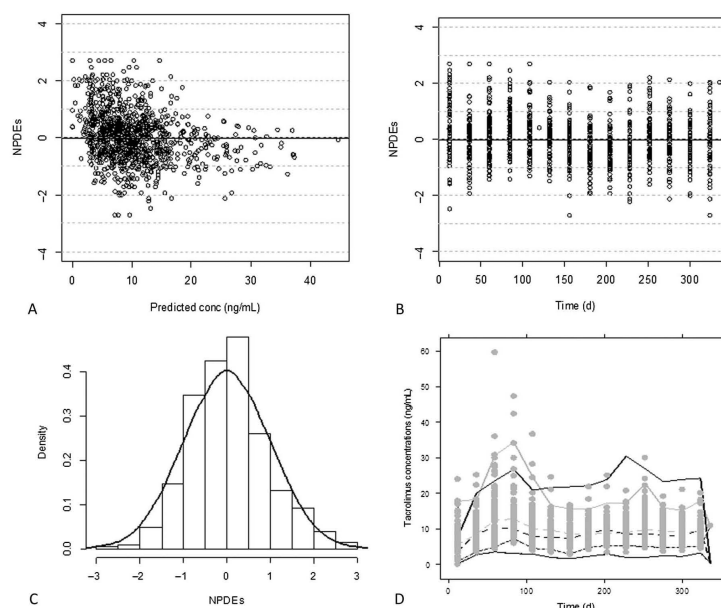


FIGURE 4. Diagnostic plots for internal model validation. NPDEs versus time (A), NPDEs versus predictions (B), histogram of NPDEs (C) scatter plot visual predictive check from the final model (D). dots, observed concentrations; lines, median and 95 prediction interval.

results. Indeed, all parameter values were included in the 95% CI computed with the values derived from 1000 bootstraps. The distribution (2.5th and 97.5th percentiles) of the 1000 simulated concentration–time curves and the comparison with the observed TAC blood concentrations in the original dataset are shown in Figure 4. The overlap of the simulated and original distributions indicates the accuracy of the identified final model.

Table 3 shows the features of the previous models and how they compare with the model developed using our patient data. The results of NPDEs generated after fitting each of the previously developed models to the observed data are shown in Figure 5. Satisfactory results were obtained consistently with 2 of these models; they had parameter estimates close to those of the model developed from raw data.

DISCUSSION

A population PK model was developed using a data-driven approach to describe the PK of TAC during the first 15 days after pediatric liver transplantation. This model could

form the basis for PK-PD analysis with the ultimate goal of increasing efficacy and safety of the use of TAC in this particular clinical setting.

Consistent with the previous studies in which trough concentrations were used to develop population PK models,

TABLE 3. Previous Models Performances

Model (Reference)	Disposition Model	Objective Function Value
Musuamba et al (data-driven analysis)	1-compartment model with time-varying elimination	3912
A (Sam et al ⁹)	1-compartment model with first order elimination	5685
B (Staat et al ¹⁰)	1-compartment model with first order elimination	4718
C (Fukudo et al ¹¹)	1-compartment model with time-varying elimination	4977
D (Wallin et al ¹²)	1-compartment model with time-varying elimination	4269
E (Guy-Viterbo et al, unpublished)	2-compartment model with time-varying elimination	4004

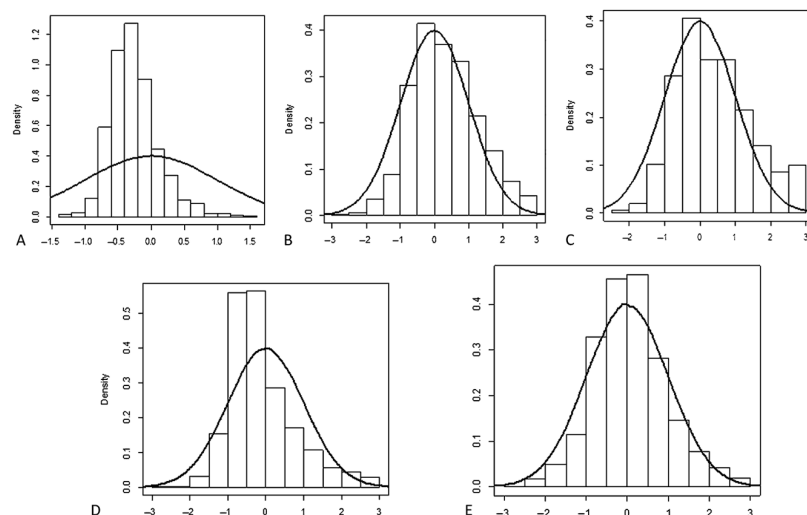


FIGURE 5. Diagnostic plots for external validation of previously published models. NPDEs generated after fitting to the observed data. (A): Sam et al⁹; (B): Staatz et al¹⁰; (C): Fukudo et al¹¹; (D): Wallin et al¹²; (E): Guy-Viterbo et al (model previously developed by our group).

the data-driven approach of the analysis led to the selection of a 1-compartment structural model.^{9-12,19-28} However, given the TAC physicochemical properties and particularly its well-established accumulation in fat tissues, a 2-compartment model should be more appropriate. The choice of the 1-compartment model is only justified by the lack of data in the distribution phase and by the (statistical) principle of parsimony on which model selection is based in most cases.

Given the sparse sampling times, it was not possible to describe the absorption phase during the present analysis, a first order process was assumed, consistent with passive diffusion which is the main TAC absorption process. A fixed value of 4.45 per hour was used for the first order absorption rate process based on the findings of a previously published study with more detailed samples.⁹⁻¹²

In this study, a time-dependent first order rate model was the best of the tested models to fit the data. This model is plausible given the expected recovering of the liver function over time. This is in agreement with findings in previously published models, including data collected within a sufficiently long time period to be able to characterize the time effect. Interindividual variability components were necessary to describe all estimated PK parameters, which is also consistent with results of the previously published models.

Several factors including time after transplantation, body weight, hematocrit, age, liver function tests, type of

graft, time after transplantation, and genetic polymorphisms have previously been reported to contribute to TAC PK variability, either in pediatric⁹⁻¹² or adult liver transplantation.¹⁹⁻²⁸ The following covariates were found to be significant in our model: body weight, time after transplantation, liver transplant size to body weight ratio, concomitant use of 2 inhibitors (nifedipine and fluconazole) and hematocrit levels for the apparent clearance, and body weight for the apparent volume of distribution. These findings are consistent with the previous results on TAC PK in children⁹⁻¹² and adults¹⁹⁻²⁸ and with known TAC PK properties such as high binding to red blood cells and high accumulation in fat tissues. Some of the covariates tested in the present study and not retained in the final model based on the statistical criteria used were found to be significant in population PK study performed in adults. They include liver function biomarkers and creatinine clearance. These discrepancies can be explained in part by the relatively low effect size of these covariates. Finally, some covariates identified as influential on TAC PK in other studies^{9-12,19-29} were not available and therefore were not tested during this analysis. They include CYP3A5 and ABCB1 genetic polymorphism.^{11,27}

Previously published population PK studies in pediatric patients early after liver transplantation⁹⁻¹² were also fitted to the data. Only one of these models gave satisfactory results when fitted to our data (Table 3 and Fig. 4). For 2 of these

models, this can be explained by the limited number of patients included in the analyses performed to develop the model. Poor predictive performances have already been reported for 2 of these models when fitted to an independent group of patients.

A model has been developed recently by our group for TDM of TAC in pediatric patients during the first year after transplantation.³⁰ This was a 2-compartment model with time-varying clearance. The predictive performances were good when tested in the present group of patients. This can therefore constitute per se an external validation of the earlier model developed by our group.

Finally, it should be noted that 2 immunoassays were used to quantify TAC in the patient samples: MEIA and CMIA. Given that the present analysis was performed on routine TDM data, the analytical methods were those used in the university hospital at the time of the study. By MEIA, TAC metabolites 13-O-demethyl TAC (MI), 31-O-demethyl TAC (MII), 15-O-demethyl TAC (MIII) and 12-hydroxytacrolimus (MIV) are known to display cross-reactivity with the antibody of <1%, 109%, 90.5%, and 8.8%, respectively.³¹ The cross-reactivity values for CMIA were 94% and 45% for MII and MIII, respectively, and <lowest limit of detection for both MI and MIV.³² If it is now well established that LCMS/MS is the method of choice to specifically quantify TAC in blood samples, it should also be noticed that the laboratory successfully participates in the TAC International Proficiency Testing Scheme (Analytical Services International, UK) at the time covered by these analyses.

CONCLUSIONS

A population PK model was developed for TAC in pediatric patients during the first 15 days after liver transplantation. The model was internally and externally validated, suggesting that it is suitable for PK-PD analyses and for the better characterization of the therapeutic window in this particular period and group of patients.

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**Chapter 3. INFLUENCE OF DONOR-RECIPIENT *CYP3A4/A5*
GENOTYPES, AGE AND FLUCONAZOLE ON TACROLIMUS
PHARMACOKINETICS IN PEDIATRIC LIVER
TRANSPLANTATION: A POPULATION APPROACH**

Published in Pharmacogenomics, 2014, 15, 1207-21

ADDENDUM

Page 1212 of the pdf, Table 1

Ethnicity:

Brussels:	Asian	0
	Caucasian	83
	Unknown	0



Influence of donor–recipient CYP3A4/5 genotypes, age and fluconazole on tacrolimus pharmacokinetics in pediatric liver transplantation: a population approach

Aim: To characterize the effect of donor and recipient CYP3A4, CYP3A5 and ABCB1 genotypes as well as relevant patient characteristics on tacrolimus pharmacokinetics in pediatric liver transplantation. **Patients & methods:** Data from 114 pediatric liver transplant recipients were retrospectively collected during the first 3 months following transplantation. Population pharmacokinetic analysis was performed using nonlinear mixed effects modeling, including characterization of influential covariates. **Results:** A two-compartment model with first order elimination best fitted the data. Estimates of apparent volume of the central compartment, intestinal clearance, hepatic clearance and intercompartmental clearance were 79 l, 0.01 l/h, 10.9 l/h and 105 l/h, respectively. Time post-transplantation, recipient age, donor CYP3A5 and CYP3A4 genotypes and fluconazole administration significantly influenced tacrolimus apparent clearance while bodyweight influenced volume of distribution. **Conclusion:** The proposed model displayed acceptable fitting performances and enabled identification of statistically significant and clinically relevant covariates on tacrolimus pharmacokinetics in the early pediatric post liver transplantation period.

Original submitted 12 December 2013; Revision submitted 7 May 2014

Keywords: age • fluconazole • pediatric liver transplantation • pharmacogenetics • tacrolimus

Background

Tacrolimus (Tac), a calcineurin inhibitor, is a cornerstone of the immunosuppressive regimen in solid organ transplantation. Nevertheless, Tac is difficult to handle owing to a narrow therapeutic window and its high pharmacokinetic (PK) and pharmacodynamic inter- and intra-patient variability [1]. To avoid graft rejection and side effects, such as nephrotoxicity, infection and post-transplant lymphoproliferative disease [1], Tac prescription benefits from therapeutic drug monitoring (TDM), currently based on the predose trough concentration (C_0). The dosing regimen is adapted according to TDM with a recommended C_0 target between 5 and 15 µg/l [2]. Despite this, a stable target C_0 is sometimes difficult to reach rapidly, and patients still experience rejection

and side effects [3]. Thus, it seems essential to identify covariates that influence the variability in drug exposure, helping clinicians, in addition to TDM, to adapt and anticipate posology.

First, age was shown to influence Tac PK with lower dose-corrected C_0 (C_0/D) in younger children [4–6]. However, it was hypothesized that the higher graft weight/bodyweight ratio (GW/BW) observed in younger children, which is known to influence Tac PK, is responsible for this age effect and that normalizing doses according to GW or GW/BW should reduce this difference [7,8]. This has never been tested in pediatric liver transplantation (PLT). Second, Tac is metabolized by CYP3A4 and CYP3A5 in the liver and in the intestine and is effluxed from the cells by the P-gp (coded for by the

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Future
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ABCB1 gene) [1]. SNPs may influence the activity of these enzymes and transporters. The *CYP3A5**3 allele, located in intron 3, which leads to a splicing defect with absence of protein, influences Tac PK in organ transplantation, leading to a lower dose requirement and lower Tac clearance (CL) for *CYP3A5* nonexpressors (*3/*3) compared with *CYP3A5* expressors (*1/*1 and *1/*3) [4,5,9–11]. Moreover, a novel SNP in the *CYP3A4* gene, *CYP3A4**22 (15389C>T), located in intron 6, was associated with a reduced expression of *CYP3A4* mRNA in the liver [12]. Additionally, a poor Tac metabolism in adult kidney transplantation and a decrease of Tac dose requirement by 30% in pediatric heart transplantation was observed when compared with the *CYP3A4**1/*1 (wild-type) genotype [13,14]. Its effect on Tac PK has never been tested in PLT. Although the effects of *ABCB1* genetic variants (C3435T and G2677T/A) on Tac PK are still debated, *ABCB1* G1199A, associated with reduced P-gp activity in adult liver transplantation, has never been tested in PLT [15,16]. Besides genetic factors and age, comedications could affect Tac disposition owing to PK drug–drug interactions. Fluconazole, a CYP3A inhibitor, has recently been shown to have no influence on Tac dose requirement in the case of a *CYP3A5* expressing graft [4]. However, whether the fluconazole effect on Tac PK is influenced by combined donor/recipient *CYP3A5* genotype is unknown. The potential influence of other frequently prescribed drugs such as nifedipine or proton pump inhibitors (PPIs) in PLT also remains uncertain [17]. Finally, time post-transplantation (TPT) and hematocrit could also have an effect on Tac PK as previously described [7,18].

The objective of this study was to assess the effects of recipient and donor pharmacogenetics as well as relevant patient characteristics on Tac PK (and therefore dose requirement) during the first 3 months following liver transplantation, given that this period is characterized by the highest rates both for PK variability and for risk of acute rejection. The tested genes were *CYP3A4*, *CYP3A5* and *ABCB1*, whereas patient characteristics included recipient age, comedications, GW/BW ratio, TPT, AST, ALT, GGT, ALP, total bilirubin, serum creatinine and hematocrit levels.

Patients & methods

Patients & study design

This was a retrospective, bicentric study, approved by the institutional review board of Cliniques Universitaires Saint-Luc in Brussels, Belgium and of Hôpital Necker in Paris, France. Written informed consent for recipient genetic testing was obtained from parents and from donors in the case of living donation.

All children (aged under 18 years) who received a *de novo* liver transplantation, from an ABO compatible (unless recipient was under 1 year of age with iso-hemagglutinins <1/32, [19]) living related (in Brussels) or deceased (in Paris) donor, under steroid-free Tac monotherapy and basiliximab induction were included in this retrospective study.

We used retrospective routine TDM (whole-blood C_0), demographic and clinical data from 114 *de novo* liver allograft patients transplanted between 2001 and 2011 in two pediatric transplant centers (n = 83, Cliniques Universitaires Saint Luc, Brussels, Belgium and n = 31, Hôpital Necker, Paris, France). Whole-blood Tac C_0 , Tac doses and biological data were collected daily from the day of transplantation up until day 14 and then at day 30, 60 and 90. Data collection was stopped in the case of a rejection episode, if the patient was diagnosed with liver cytolysis/cholestasis with histological signs (BANFF score) [20] and if they required additional treatment with methylprednisolone and/or mycophenolate mofetil. Biopsies were evaluated locally in each center by a pathologist who specialized in liver graft histology.

Patients were given oral Tac (Prograf®) from the day of transplantation, with basiliximab (Simulect®) intravenous induction on day 0 and day 4 (10 mg <35 kg, 20 mg >35 kg). The initial dose of Tac was 0.1 mg/kg/12 h in both centers and was then adjusted according to TDM. Target C_0 was 8–10 µg/l (day 1–60) then 6–8 µg/l (day 61–90) in Brussels, and 10–15 µg/l (day 1–15), 8–10 µg/l (day 15–30) and then 6–10 µg/l (day 31–90) in Paris.

Additional therapies frequently prescribed consisted of PPIs (omeprazole and esomeprazole) for gastric ulcer prevention, nifedipine for hypertension and fluconazole for fungal pre-emptive or curative therapy. They were registered at each time point as present or absent.

DNA was extracted from blood samples and/or buccal swabs from recipients and living donors and from formalin-fixed paraffin-embedded (FFPE) liver tissue obtained from reperfusion biopsies in the case of deceased donors. In recipients and in living donors, samples for DNA extraction were collected either during outpatient clinic consultation (Paris and Brussels) or during hospitalization for the 1-, 3- or 5-year post-transplantation follow-up (Brussels). Eighty-one samples from recipients and 76 samples from donors were collected for genotyping. Missing genotyping data were due to a limited amount of DNA in samples or to missing samples from patients and/or donors who were living abroad and who did not come back to Brussels during the study period.

Drug concentration measurement

For the analysis, we used a total of 1410 original Tac C_0 , determined by four different immunoassays: enzyme

multiplied immunoassay (EMIT), Dade Behring (DE, USA), Syva® (n = 79 C₀ from eight recipients); antibody-conjugated magnetic immunoassay (ACMIA), Siemens (IL, USA), RXL Dimension (n = 248 C₀ from 23 recipients); microparticle enzyme immunoassay (MEIA), Abbott (IL, USA), IMx (n = 479 C₀ from 37 recipients); and chemiluminescent immunoassay (CMIA), Abbott, ARCHITECT®, (n = 604 C₀ from 46 recipients). Linearity was observed for all the methods from 1.2–30 µg/l, 1–30 µg/l, 1.5–30 µg/l and 3–30 µg/l with EMIT [21], ACMIA [22], MEIA [23] and CMIA [23], respectively. All the methods were found to be precise on an interday basis with a coefficient of variation (CV) for all quality control samples tested below 14, 12, 11 and 7% for EMIT, ACMIA, MEIA and CMIA, respectively [2].

Interassay variability was considered in the model. However, due to possible variable calibration bias and metabolite cross-reactivities among the different immunoassays, we tested the concordance of the results in a second analysis where all the Tac C₀ were normalized on the HPLC-MS. To normalize all the concentrations on HPLC-MS values, we collected all the monthly Tac quality control data from the International Proficiency Testing Scheme (David Holt, Analytical Services International, UK) from 2001 to 2012. Only data from patient samples were included. Then, we calculated the coefficient of variation (CV%) existing between Tac concentrations measured by immunoassay and Tac concentrations measured by HPLC-MS: (mean value [immunoassay]–mean value [HPLC-MS])/mean value (HPLC-MS). We then calculated the mean CV for each year and immunoassay to obtain a correction factor. Finally, all C₀ were corrected by the specific year- and assay-dependent factor. Both laboratories in Brussels and Paris successfully participated in the UK Quality Assessment Scheme.

C₀ (µg/l), weighted-adjusted Tac 12 h doses (D₀/D_{0.12 h}, mg/kg/12 h) and C₀/D_{0.12 h} (µg/l per mg/kg/12 h) were used as previously described [6].

Genotyping analysis

Genomic DNA was isolated either from 200 µl of EDTA-treated whole blood by Qiasymphony® DNA mini kit (Qiagen, CA, USA), or by the MagAttract® DNA Mini M48 kit (Qiagen) in the case of buccal swabs. For FFPE liver tissue (six slides of 10 µm per donor), DNA was extracted by the MagAttract DNA Mini M48 kit (Qiagen). Because of the very small DNA quantity extracted from FFPE tissue (<1 ng/µl), a DNA preamplification by PCR was performed as previously described [24]. Genotyping for *CYP3A4**22, *CYP3A5**3 and *ABCB1* 1199G>A, 3435C>T and 2677G>A/T was performed as previously described [13,16].

Statistical analysis

Preliminary statistics

Preliminary statistical analysis was performed using IBM (IBM Corp., NY, USA) SPSS Statistics for Windows, Version 20.0. Mean ± standard deviation or median (range) were used to describe continuous variables whereas relative frequencies were used for categorical variables. In an initial step, correlations analyses were performed using Pearson χ^2 test for categorical covariates and Spearman's test for continuous covariates. Subsequently, effect of candidate covariates were tested separately, for each day post-transplantation, using either Mann–Whitney (n = 2 groups) or Kruskal–Wallis (n > 2 groups) tests, or linear regression analysis for continuous covariates. Significant and uncorrelated covariates from the preliminary tests were tested as fixed effects in a mixed model analysis (univariate) for log₁₀ transformed D_{0.12 h} and C₀/D_{0.12 h} with time post-transplant being considered as the repeated measurement, whereas analytical methods, recipient gender and transplant centers were entered as random variables to correct for the effect of these factors. Differences were considered statistically significant when p < 0.05. Hardy–Weinberg equilibrium was tested by the Pearson χ^2 test.

In these preliminary analyses, the effect of covariates on C₀/D_{0.12 h} was tested separately, considering C₀/D_{0.12 h} as a surrogate marker for Tac CL, as done in previously published studies, mainly for comparison purposes. However, in order to characterize Tac PK parameters and the influence (both in terms of statistical significance and clinical relevance) of all candidate covariates on Tac CL, the latter being more reliable than C₀/D_{0.12 h} for dose prediction and simulations, we performed a population PK (POP PK) analysis as described below.

Population PK model

Base model building

POP PK analysis for Tac blood concentrations was performed using nonlinear mixed effects modeling with NONMEM software, Version VII, Level 2.0, ICON Development Solutions, MD, USA. The first-order conditional estimation with $\eta - \epsilon$ interaction was employed for all model runs. Assessment of model adequacy and decisions about increasing model complexity was driven by the data and guided by goodness-of-fit criteria, including visual inspection of diagnostic scatter plots (e.g., observed vs predicted concentration and conditional-weighted residual and normalized distribution prediction errors vs time); successful convergence of the minimization routine with at least three significant digits in the parameter estimates; plausibility of parameter estimates; preci-

sion of parameter estimates; and correlation between model parameter estimates <0.95 (obtained from the correlation matrix of estimate in the NONMEM output). All parameter estimates were reported with a measure of estimation uncertainty, such as nonparametric bootstrap 95% CIs. An exploratory investigation of covariate-parameter relationships was also undertaken as part of the POP PK analysis. Parametric distributions were assumed for the random effects. As a starting point for this analysis, all interindividual error terms were described by an exponential error model or log-normal parameter distribution (Equation 1).

$$P_i = TVP \times \exp(\eta_i) \quad (1)$$

where P_i is the estimated parameter value for individual i . TVP is the typical population value (geometric mean) of the parameter and η_i are individual-specific interindividual random effects for individual i and parameter P and are assumed to be normally distributed: $N(0, \sigma^2)$ with covariances defined by the inter-individual covariance matrix. For PK observations in this analysis, the residual error model was initially described by a combined additive and proportional error model (Equation 2).

$$C_{ij} = C_{pij} \times (1 + \sigma_{pij}) + \sigma_{aij} \quad (2)$$

where C_{ij} is the j th measured observation (whole-blood Tac C_0) in an individual i , C_{pij} is the j th model predicted value (whole-blood Tac C_0) in an individual i , σ_{pij} and σ_{aij} are proportional and additive residual random errors, respectively, for an individual i and measurement j and are each assumed to be independently and normally distributed with 0 mean and variance equals σ^2 .

Covariate model building

Covariate model building for POP PK models has typically been conducted as a regression exercise characterized by varying degrees of prior scientific thought about inclusion of potential predictor variables. According to the likelihood ratio test, the difference in $2 \times \log$ likelihood from nested models was assumed to be asymptotically χ^2 distribution distributed with degrees of freedom equal to the difference in number of model parameters. A covariate modeling approach emphasizing parameter estimation rather than stepwise hypothesis testing was implemented for this POP PK analysis. First, predefined covariate-parameter relationships were identified based on exploratory graphics, scientific interest, mechanistic plausibility of prior knowledge, and a full model was constructed with care to avoid correlation or collinearity in predictors (covari-

ates with correlation coefficients >0.3 were not simultaneously included as potential predictors). Population parameters, including fixed-effects parameters (covariate coefficients and structural model parameters), and random-effects parameters were estimated. An exploratory assessment of any remaining trends was conducted by graphical inspection of all covariate effects (plots of maximum *a posteriori* Bayes estimates of individual random effects [η_i] and/or conditional-weighted residuals from the full model vs covariates). Inferences about clinical relevance of parameters were based on the resulting parameter estimates of the full model and measures of estimation precision (asymptotic standard errors, bootstrap 95% CIs or log-likelihood profile). No hypothesis testing was conducted. An attempt was made to incorporate known physiologic relationships into the covariate-parameter models. For example, the change in physiologic parameters as a function of patient age is empirically described by an allometric model (Equation 3).

$$P_i = TVP \times \left(\frac{age}{age_{med}} \right)^{\theta_{age}} \quad (3)$$

where P_i the parameter value in individual i , was described as a function of typical PK parameter value and of age for individual i (age), normalized by the median age (age_{med}). θ_{age} is an estimated parameter describing the allometric power function.

Categorical covariates were assessed as shown in Equation 4.

$$P_i = TVP \times (\theta_{COV})^{COV} \quad (4)$$

where P_i is the parameter value for an individual patient, TVP is the population average parameter, θ_{COV} is the parameter characterizing the covariate effect on the parameter, and COV is the covariate value in the dataset, which was coded as 0 or 1 for binary categorical variables.

Relationships were investigated between PK parameters and the following patient covariates: age, sex, race, donor and recipient *CYP3A4/5* and *ABCB1* (1199, 2677 and 3435) genetic polymorphisms, weight, hemoglobin, aminotransferase liver enzymes (AST and ALT), bilirubin, GGT, ALP, kidney function tests (serum creatinine, serum urea, calculated glomerular filtration rate based on the Schwartz formula [25]), GW/BW, coadministration of potential inducers or inhibitors of Tac metabolism, total plasma protein concentrations and analytical method. A difference of at least 3.84 of objective function value (OFV; χ^2 p-value ≤ 0.05) from the structural model OFV was considered statistically significant. Covariates that were continuous

variables (age, weight, hemato crit, total plasma protein concentration, AST, ALT, GGT, ALP, serum creatinine, serum urea, calculated glomerular filtration rate) were centered to their median values and tested on the PK parameters as previously shown.

Influence of time variation on the apparent liver clearance was also assessed using an Emax model using Equations 5 & 6:

$$HEPCL = TV_{HEPCL} \times \left(\frac{time}{T_{50} + time} \right) \quad (5)$$

with

$$CL_{TOT} = CL_{DIG} + CL_{HEP} \quad (6)$$

where CL_{HEP} is the hepatic clearance, CL_{DIG} is the intestinal clearance, and CL_{TOT} is the total clearance, T_{50} is the time delay after transplantation for recovery of half the maximum hepatic clearance.

Model evaluation

The final POP PK model was validated with a visual predictive check method. The basic premise is that a model and parameters derived from an observed data set should produce simulated data that are similar to the original observed data. Two hundred simulation replicates of the original data set were generated using the final POP PK model. Overlay plots of the observed concentrations versus time with the 95% prediction interval of the simulated dose normalized data were generated. The 95% prediction interval of the simulated data should cover 95% of the observed data. The precision of model parameters was investigated by performing a nonparametric bootstrap procedure.

Results

Population characteristics

One hundred and fourteen PLT recipients with a median age of 1.32 years (range: 0.15–15.84) were included. Patients from Paris were transplanted older, with a more advanced liver disease. Demographic and biological data are described in Table 1. The distribution of donor and recipient *CYP3A* and *ABCB1* genotypes are shown in Table 2. The Hardy–Weinberg equilibrium was respected for all genotypes (p -value > 0.5).

Analysis of correlations

Recipient age was found to significantly correlate with BW ($p = 0.903$; $p < 0.001$), body surface area (BSA; $p = 0.897$, $p < 0.001$) and GW/BW ($p = -0.859$; $p < 0.001$). Conversely, significant but less clinically relevant correlations were found between GW/BW and *CYP3A4**22T allele ($p = 0.115$; $p = 0.002$) donors,

with at least one *CYP3A4**22 T allele having a higher ratio. Significant correlation was also found between time post-transplantation and hematocrit ($p = -0.147$; $p < 0.001$).

Preliminary statistical analysis (linear model)

Age effect on Tac dose & trough levels

As shown in Figure 1, youngest children received significantly higher Tac D_{FW12h} ($p < 0.05$ except at day 4) and had the lowest Tac C_0/D_{FW12h} from day 2 to day 90 ($p < 0.01$, except at day 4). In order to test the confounding effect of the normalization factor for the administered dose, different normalization factors were subsequently used: BSA (calculated with the Mosteller formula, $mg/m^2/12h$), graft weight (GW, $mg/g/12h$) and GW/BW ($mg/\%/12h$). Then the effect of age on C_0/D_{12h} (with dose normalized either by BSA, GW or GW/BW) was tested. A positive age effect on C_0/D_{12h} was found when doses were normalized by BSA or by bodyweight (data not shown), whereas the age effect was negative when using GW/BW-adjusted doses: the mean Tac $C_0/D_{GW/BW12h}$ being 62.0 ± 52.7 , 54.7 ± 71.9 and $17.3 \pm 17.3 \mu g/l$ per $mg/\%/12h$ ($p < 0.001$), for children aged less than 1 year, between 1 and 5 years and above 5 years, respectively. This was expected given that children aged less than 1 year had higher GW/BW ratio when compared with older children ($4.05 \pm 0.19\%$ for children aged less than 1 year vs $2.42 \pm 0.14\%$ for children between 1 and 5 years vs $1.44 \pm 0.11\%$ for children above 5 years; $p < 0.001$). The age effect was not found when Tac doses were normalized by GW (not significant). In the linear mixed models analysis, age significantly increased $\log C_0/D_{FW12h}$ by a factor of 0.041 ($p < 0.001$) and GW/BW decreased $\log C_0/D_{FW12h}$ by a factor of -0.139 ($p < 0.001$).

Donor & recipient pharmacogenetic influence on Tac dose & trough levels

Concerning donor and recipient *ABCB1* 1199G>A and 3435C>T variants, no effect on Tac PK was observed throughout the study whereas the *ABCB1* 2677G>T/A polymorphism of the recipient had a significant effect at day 1 with higher C_0 and C_0/D_{FW12h} for mutated alleles (data not shown).

Patients with a graft carrying at least one *CYP3A4**22 T allele (CT or TT) tended to have higher Tac C_0 and C_0/D_{FW12h} from day 3 to 60 when compared with a *CYP3A4**1/*1 carrying graft, with significant differences for C_0 at day 3 and 10 ($22.4 \pm 16.9 \mu g/l$ vs 12.7 ± 6.6 ; $p = 0.035$ at day 3, and 14.9 ± 7.1 vs $8.9 \pm 3.4 \mu g/l$; $p = 0.015$ at day 10). When patients receiving fluconazole were excluded, the effect on C_0 at day 3 was higher and was also significant at day 30

Table 1. Patient demographics.				
	All	Brussels	Paris	p-value
Recipients, n	114	83	31	
Sex (male/female)	56/58	41/42	15/16	1.0
Age (years) [†] :	3.3 (3.9)	2.6 (3.5)	5.0 (4.6)	< 0.001 [‡]
– <1 year	48	44	4	0.001 [§]
– 1–5 years	41	24	17	
– >5 years	25	15	10	
Bodyweight (kg) [†]	14 (11.4)	12.2 (9.5)	18.9 (14.8)	< 0.0001 [‡]
Body height (cm) [†]	85 (26)	81 (23)	101 (31)	< 0.001 [‡]
BSA (m ²) [†]	0.56 (0.30)	0.50 (0.25)	0.73 (0.38)	< 0.0001 [‡]
Ethnicity:				
– Black		0	4	
– Caucasian		83	27	
Primary liver disease, n [¶]				0.281 [§]
Biliary atresia	65 (57%)	51 (61.4%)	14 (45.2%)	–
Alagille	3 (2.6%)	3 (3.6%)	0	–
PFIC	7 (6.1%)	4 (4.8%)	3 (9.7%)	–
Malignancy	16 (14%)	12 (14.5%)	4 (12.9%)	–
Metabolic	10 (8.8%)	6 (7.2%)	4 (12.9%)	–
Others	13 (11.4%)	7 (8.4%)	6 (19.4%)	–
Donors, n	114	83	31	–
Sex (male/female), n	54/58	39/44	15/14	–
Age (years) [†]	30.4 (10.3)	33.7 (7)	21.4 (12.2)	< 0.0001 [‡]
Ethnicity:				
– Asian		83	1	
– Caucasian		0	19	
– Unknown		0	11	
Graft size parameter				
Graft weight (g)/recipient bodyweight (g; % of bodyweight) [†]	2.99 (1.35)	3.08 (1.39)	2.44 (0.42)	0.154 [‡]
Liver & blood parameters				
AST (UI/L) [†]	336 (324)	250 (137)	630 (546)	0.000 [‡]
ALT (UI/L) [†]	449 (387)	373 (219)	723 (655)	0.011 [‡]
GGT (UI/L) [†]	52 (59)	44 (56)	85 (60)	0.000 [‡]
Total bilirubin (mg/dl) [†]	5.9 (1.7)	4.9 (2.9)	9.2 (6.1)	0.001 [‡]
Direct bilirubin (mg/dl) [†]	3.3 (3.3)	2.2 (1.6)	7.2 (4.6)	0.000 [‡]
Albumin (g/dl) [†]	2.8 (0.5)	2.7 (0.4)	2.8 (0.5)	0.657 [‡]
Hematocrit (%) [†]	28 (4)	28 (4)	28 (4)	0.527 [‡]
INR [†]	1.7 (0.5)	1.8 (0.5)	1.6 (0.5)	0.122 [‡]

Statistically significant results (p < 0.05) are highlighted in bold.
[†]Values are expressed as mean (standard deviation).
[‡]Mann–Whitney test.
[§]χ² test.
[¶]Values are expressed as frequency (percentage of the total).
[‡]Calculated based on Schwartz formula [25].
BSA: Body surface area, INR: International normalized ratio, PFIC: Progressive Familial Intrahepatic Cholestasis.

Table 1. Patient demographics (cont.)

	All	Brussels	Paris	p-value
Liver and blood parameters (cont.)				
Platelets (*1000/ μ l) [†]	123 (81)	135 (86)	82 (39)	0.004[‡]
Renal function				
Urea (mg/dl) [†]	25 (16)	26 (16)	24 (17)	0.710 [‡]
Creatininemia (mg/dl) [†]	0.32 (0.24)	0.27 (0.21)	0.41 (0.30)	0.025[‡]
Creatinine clearance (ml/min/1.73 m ²) ^{†*}	144 (68)	144 (67)	147 (71)	0.939 [‡]

Statistically significant results ($p < 0.05$) are highlighted in bold.

[†]Values are expressed as mean (standard deviation).

[‡]Mann–Whitney test.

[§] χ^2 test.

^{*}Values are expressed as frequency (percentage of the total).

^{*}Calculated based on Schwartz formula [25].

BSA: Body surface area; INR: International normalized ratio; PFIC: Progressive Familial Intrahepatic Cholestasis.

(26.5 ± 18.8 vs 12.6 ± 6.6 μ g/l; $p = 0.0087$ at day 3, and 14.8 ± 5.0 vs 8.9 ± 3.8 μ g/l; $p = 0.026$ at day 30). Seven of the eight patients with a graft carrying at least one *CYP3A4**22 T allele had one or more Tac $C_0 > 15$ μ g/l with a maximum of 59.1 μ g/l at day 1. However no effect on log Tac C_0/D_{BW12h} was observed for univariate mixed model analysis. There was no effect of recipient *CYP3A4* genotype on Tac PK either.

Donor *CYP3A5* expression was associated with a reduced C_0/D_{BW12h} from day 0 to 30 (significant at day 2 and 30; $p = 0.046$ and $p = 0.038$, respectively) when compared with donor nonexpression. Recipient *CYP3A5* expression was also associated with lower C_0/D_{BW12h} (significant at day 1; $p = 0.049$) when compared with nonexpression. During univariate mixed model analysis, both recipient *CYP3A5* and donor *CYP3A5* genotypes were found to influence log C_0/D_{BW12h} . Indeed, log C_0/D_{BW12h} was decreased by a factor of 0.215 ($p < 0.001$) in the case of donor *CYP3A5* expression and by 0.110 ($p = 0.001$) in the case of recipient *CYP3A5* expression.

Effect of comedications on Tac dose & trough levels

A total of 37 patients received fluconazole. As shown in Figure 2, fluconazole treated patients had significantly higher C_0/D_{BW12h} from day 2 to 30 ($p < 0.05$ except at day 3, 6, 10, 11, 13 and 14) compared with nontreated patients. When considering the effect of fluconazole according to donor *CYP3A5* genotype for univariate mixed model analysis, fluconazole increased log Tac C_0/D_{BW12h} by a factor of 0.378 ($p < 0.001$) and decreased log D_{BW12h} by a factor of -0.194 ($p = 0.001$) in the case of donor *CYP3A5* nonexpression. As shown in Figure 3, a different effect of fluconazole on log Tac C_0/D_{BW12h} and Tac D_{BW12h} was found in the case of donor *CYP3A5* expression. Indeed, in the case of donor *CYP3A5* expression, log C_0/D_{BW12h} was slightly

increased by a factor of 0.064 ($p = 0.454$) and log D_{BW12h} was decreased by a factor of 0.202 ($p = 0.279$). In *CYP3A5* expressor recipients with a *CYP3A5* non-expressor donor, log C_0/D_{BW12h} increased by 0.292 ($p < 0.001$) with concomitant intake of fluconazole. No significant effect of nifedipine and PPIs (omeprazole and esomeprazole) on Tac C_0/D_{BW12h} and Tac D_{BW12h} was found in this study.

Table 2. Allele frequencies for the *CYP3A4*, *CYP3A5* and *ABCB1* polymorphisms in donors and recipients

	Recipients, % (n)	Donors, % (n)
<i>CYP3A5</i>:	n = 80	n = 76
– *3/*3	80 (64)	85.5 (65)
– *1/*3	18.8 (15)	14.5 (11)
– *1/*1	1.3 (1)	0 (0)
<i>CYP3A4</i> 15389:	n = 81	n = 73
– CC	91.4 (74)	89 (65)
– CT	8.6 (7)	9.6 (7)
– TT	0 (0)	1.4 (1)
<i>ABCB1</i> 3435:	n = 80	n = 67
– CC	31.3 (25)	37.3 (25)
– CT	46.3 (37)	43.3 (29)
– TT	22.5 (18)	19.4 (13)
<i>ABCB1</i> 2677:	n = 80	n = 68
– GG	42.5 (34)	42.6 (29)
– GA/GT	43.8 (35)	47.1 (32)
– AA/TT	13.8 (11)	10.3 (7)
<i>ABCB1</i> 1199:	n = 81	n = 76
– GG	93.8 (76)	90.8 (69)
– GA	6.2 (5)	9.2 (7)
– AA	0.0 (0)	0.0 (0)

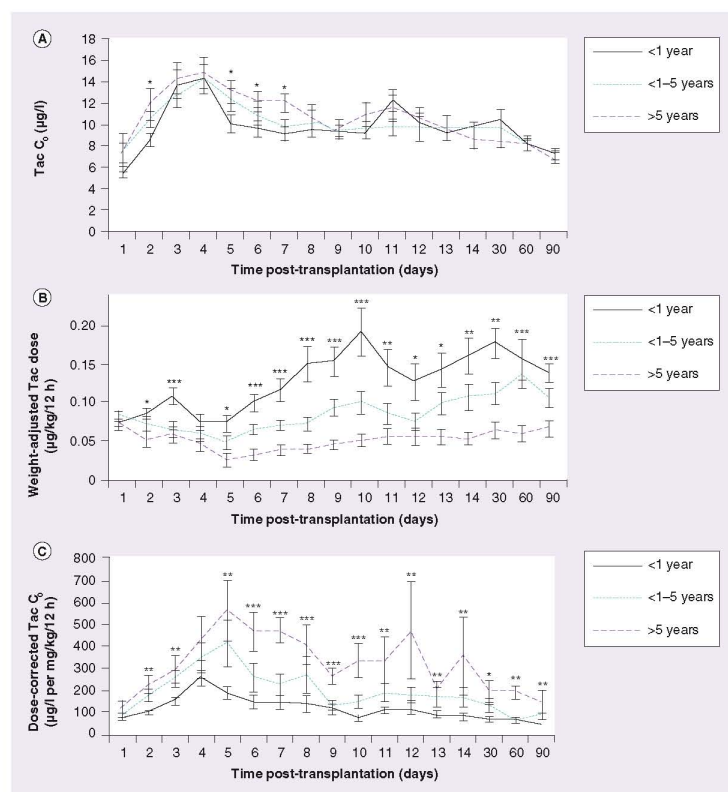


Figure 1. Effect of class of age on tacrolimus doses and trough concentrations. (A) Effect of class of age on mean Tac C_0 . Error bars represent standard error of the mean. (B) Effect of class of age on mean weight-adjusted Tac doses. Error bars represent standard error of the mean. (C) Effect of class of age on mean dose-corrected Tac C_0 . Error bars represent standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. C_0 : Trough concentration; Tac: Tacrolimus.

POP PK analysis

A two-compartment model with first-order elimination, best fitted the observed concentrations. As shown in Table 3, the following PK parameters were estimated: apparent volume of the central compartment (V_c/F), apparent intercompartmental clearance (Q/F) and apparent clearance (CL/F). Absorption rate constant (K_a) and apparent volume of the peripheral compartment

(V_p/F) were fixed, based on data from literature [10] and sensitivity analysis during the modeling process, respectively. Interindividual variability terms were necessary to describe the variability in clearance and volume of central compartment. As also shown in Table 3, a combined model was retained to describe the residual error.

The following covariates showed statistical significance and clinically relevant influence on Tac apparent

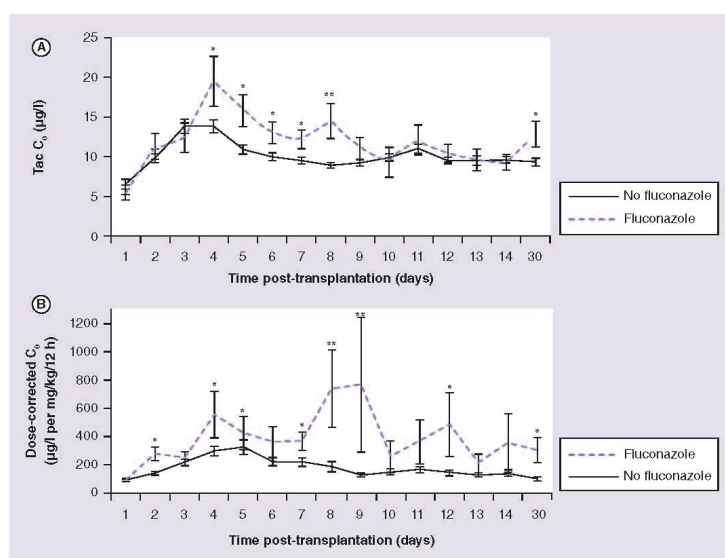


Figure 2. Effect of fluconazole on tacrolimus trough concentrations. (A) Effect of fluconazole treatment on mean Tac C₀ and mean dose-corrected Tac C₀. Error bars represent standard error of the mean. (B) Effect of fluconazole treatment on mean dose-corrected Tac C₀. Error bars represent standard error of the mean.

*p < 0.05; **p < 0.01.

C₀: Trough concentration; Tac: Tacrolimus.

hepatic clearance: time after transplantation, recipient age, donor *CYP3A5* expression, donor *CYP3A4**22 T allele and concomitant administration of fluconazole, according to donor *CYP3A5* expression. Bodyweight was found to significantly influence central volume of distribution. All the clinically relevant covariates were included in the model in a multiplicative manner as shown in the Equation 7:

$$CL_i = 0.01 + [10.9 \times \left(\frac{age}{1.3}\right)^{0.16} \times (1.3)^{DCYP3A5} \times (0.71)^{DCYP3A4} \times (0.7)^{FZLDe} \times (0.4)^{FDLne} \times \left(\frac{time}{144 + time}\right)] \quad (7)$$

where *CL_i* is the value for the total apparent clearance of the *i*th patient, *DCYP3A5* is 1 where donor is *CYP3A5* expressor (*CYP3A5**1/*3 or *CYP3A5**1/*1) and 0 otherwise, *DCYP3A4* is 1 where donor is a *CYP3A4**22 T allele carrier (*CYP3A4**1/*22 or *CYP3A4**22/*22) and 0 otherwise, *FZLDe* is 1 in the case of fluconazole treatment and donor *CYP3A5* expression and 0 other-

wise, *FZLDe* is 1 in the case of fluconazole treatment and donor *CYP3A5* nonexpression and 0 otherwise, and time is time post-transplantation in hours.

Recipient *CYP3A5* and *ABCB1* 2677 genotypes were found to be statistically significant but clinically not relevant on the apparent intestinal clearance: only 17 and 2% reduction of apparent intestinal clearance was seen for *CYP3A5* expressor recipients and for carriers of *ABCB1* 2677G>T/A mutation, respectively.

Table 3 summarizes the features of the final model. Good estimation of all the model parameters was obtained (standard error of estimates <40% of estimates). In addition, goodness of fit plots displayed in Figure 4 show good fitting performance of the model to the data. The validation of the final model by bootstrapping and visual predictive checks gave satisfactory results. Indeed, all the parameter values were included in the 60–95% CI computed with the values derived from 1000 bootstraps. When comparing the distribution (2.5th and 97.5th percentiles) of the 1000 simulated concentration–time curves with the observed Tac blood concentrations in the

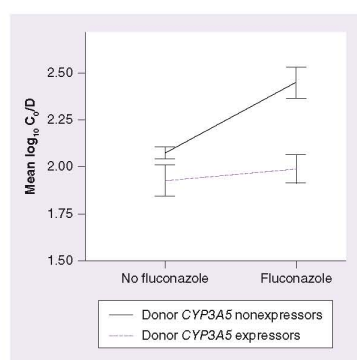


Figure 3. Impact of fluconazole treatment on mean log dose corrected trough concentrations according to donor CYP3A5 genotype. The solid line represents donor CYP3A5 nonexpressors and the dashed line represents expressors. Error bars represent standard deviation.

C₀/D: Dose-corrected trough concentration.

original dataset (not shown), the overlap of the simulated and original distributions indicates the accuracy of the identified final model.

Discussion

In this study, effects of covariates on Tac D_{BW12h} and C_0/D_{BW12h} and on Tac PK parameters were studied preliminarily using a linear mixed model and subsequently using a nonlinear mixed effects approach, the latter constituting the core of the analysis. As far as the POP PK analysis was concerned, a two-compartment model with linear absorption and elimination was used to describe the data and the apparent total clearance was in the range of previously reported values in PLT [10,26].

The results of this study were consistent with the previously reported significant higher Tac weight corrected dose requirement and lower C_0/D in young children receiving liver transplant [4–6]. The POP PK analysis indicated that this age effect on dose requirement and C_0/D_{BW12h} was the reflection of a nonlinear age effect (modeled by a power function) on Tac CL. We showed for the first time that this age effect on Tac C_0/D_{12h} was also apparent when doses were normalized by BSA and more apparent when doses were normalized by GW/BW. However, it should be noted that recipient age was highly correlated with both BSA and GW/BW, which could explain these findings. Indeed, GW was not correlated with age in PLT and the age effect disappeared with GW normalized doses during

our preliminary analysis. We therefore hypothesize that the age effect on Tac dose requirement is mainly due to the GW/BW ratio, the latter being higher in young recipients. Although hepatic CYP3A metabolism is reported to be increased in young children compared with adults, children still have lower average microsomal protein content (26 mg/g of liver in the neonatal period) when compared with adults (40 mg/g of liver). This paradox could be explained by a higher GW/BW ratio in children that counterbalances a lower microsomal content [27]. Other explanations for this age effect could be related either to a higher intestinal CYP3A4/5 mRNA expression in younger children [28] or to the presence of CYP3A7, the fetal CYP3A4 isoform, in children aged less than 6 months [29]. Therefore, despite a low *in vitro* activity toward Tac, CYP3A7 could, at least partly, contribute to Tac metabolism in children [30,31].

To the best of our knowledge, this work is the first study exploring and finding an effect of the CYP3A4*22 T allele in PLT. Our POP PK analysis showed that the presence of a CYP3A4*22 T allele in the donor significantly decreased Tac apparent clearance by 29%, which is consistent with previously described effects of this allele on Tac clearance in adult renal transplantation [32]. CYP3A4*22 T allele was also previously associated with lower Tac dose requirement at day 3 and during the first 14 days after pediatric heart transplantation [14]. Patients with graft carrying the CYP3A4*22 T allele, had significantly higher and supratherapeutic Tac C_0 at day 3; thus this allele could partly explain the high proportion (32%) of supratherapeutic C_0 observed at day 3. However, when C_0 (or C_0/D) is considered as the response variable, which was the case in these two studies, variability in drug accumulation rates until steady state is reached is a significant uncontrolled confounder and its ignorance constitutes a big limitation in the interpretability of these results. In line with the results of Wang, no effect of the recipient's intestinal CYP3A4*22 T allele on Tac PK was observed in the present analysis [12].

Donor CYP3A5 expression significantly decreased Tac C_0/D_{BW12h} and led to a 30% increase of Tac CL in the POP PK analysis. Such effect of the donor CYP3A5 genotype on Tac C_0/D and Tac CL was previously described in pediatric and adult liver transplantation [4,18,33]. Concerning the recipient CYP3A5 genotype, our results showed that patients carrying at least one CYP3A5*1 allele had a significantly lower log Tac C_0/D_{BW12h} and a higher intestinal clearance when compared with CYP3A5 nonexpressor recipients. Nevertheless, the POP PK analysis showed that this rise of clearance was not clinically relevant (17%), although statistically significant. Our results

Table 3. Final model characteristics.

Parameters (units)	Estimates	Bootstrap 95% CI
$\theta_{\text{DIG CL}}$ (l/h)	0.01	0.002–0.19
$\theta_{\text{DCYP3A5 expression on DIG CL}}$	1.17	1.04–1.65
$\theta_{\text{RABCB1 2677 GT/GA or TT/AA on DIG CL}}$	0.98	0.95–0.99
$\theta_{\text{t}_{50}}$ (days)	6	3.66–10.3
θ_{HEPCL} (l/h)	10.9	9.6–11.4
$\theta_{\text{K}_{\text{a}}}$ (h ⁻¹)	4.5 (–)	–
θ_{Vc} (l)	79	73–82
θ_{Vp} (l)	100 (–)	–
θ_{Q} (l/h)	105	97–168
$\theta_{\text{WT on Vc}}$	0.46	0.36–0.67
$\theta_{\text{AGE on CL}}$	0.16	0.11–0.23
$\theta_{\text{DCYP3A5 expression on HEP CL}}$	1.30	1.23–1.60
$\theta_{\text{DCYP3A4*22 on HEP CL}}$	0.71	0.53–0.87
$\theta_{\text{E2L on HEP CL (DCYP3A5 expression/DCYP3A5 nonexpression)}}$	0.7/0.4	0.5–0.8/0.2–0.6
IIV on CL_{TOT}	0.27	0.15–0.29
IIV on Vc	0.36	0.21–0.41
σ_{prop}	0.1	0.018–0.23
σ_{add} (μg/l)	2.38	1.63–3.95

– Parameter not estimated during analysis; σ_{add} : Additive residual error; σ_{prop} : Proportional residual error; $\theta_{\text{AGE on CL}}$: Liver transplant recipient's age effect on apparent clearance; $\theta_{\text{DCYP3A5 expression on HEP CL}}$: Donor CYP3A5 expression effect on apparent hepatic clearance; $\theta_{\text{DCYP3A4*22 on HEP CL}}$: Donor CYP3A4*22 (15389 C>T) polymorphism effect on apparent hepatic clearance; $\theta_{\text{E2L on HEP CL (DCYP3A5 expression/DCYP3A5 nonexpression)}}$: Effect of fluconazole on apparent hepatic clearance according to donor CYP3A5 expression; $\theta_{\text{K}_{\text{a}}}$: Typical value for absorption rate constant; θ_{Q} : Typical value for apparent intercompartmental clearance; $\theta_{\text{RABCB1 2677 GT/GA or TT/AA on DIG CL}}$: Effect of recipient's RABCB1 2677 G>T/A mutation on apparent intestinal clearance; $\theta_{\text{DCYP3A5 expression on DIG CL}}$: Effect of recipient's CYP3A5 expression on apparent intestinal clearance; θ_{Vc} : Estimate of time delay to reach half the maximum apparent clearance value; θ_{Vp} : Estimate of apparent volume of central compartment for a 10.2 kg (median) subject; $\theta_{\text{WT on Vc}}$: Typical value for apparent volume of peripheral compartment; $\theta_{\text{WT on Vc}}$: Bodyweight effect on apparent volume of central compartment; IIV on CL_{TOT} : Interindividual variability on apparent total clearance; IIV on Vc : Interindividual variability on apparent volume of central compartment.

contrast with the recently published study of Jalil *et al.* who showed in their POP PK analysis that apparent clearance increased 1.53-times in *CYP3A5* expressor recipients [10]. Notably, in that study, the donor *CYP3A5* genotype was unknown and patients received steroids. On the other hand, a meta-analysis published in 2013, comprising of adults and pediatric studies, failed to show any significant effect of recipient *CYP3A5* genotype on Tac $\text{C}_{\text{ss}}/\text{D}$ [34]. As shown in Equation 7, we hypothesize that, in our POP PK study, the effect of recipient *CYP3A5* genotype on intestinal clearance would reduce the Tac bioavailability and would be more apparent during the first days post-transplantation when the hepatic clearance is low. However, with the effect of time, this intestinal clearance is rapidly counterbalanced by the rising hepatic clearance, which explains the overall lack of clinical relevance for the recipient *CYP3A5* genotype. Thus, this covariate is not included in Equation 7. Other studies, using linear modeling in adult and

pediatric living donor liver transplantation, showed time dependent intestinal and liver metabolism with predominance of intestinal metabolism during the first week or during the first month and a switch to liver metabolism afterwards [18,35]. Initial liver metabolism inhibition during liver mass regeneration, as well as ischemia and reperfusion injuries, may explain delayed hepatic function and metabolism that recover progressively. It should be noted that the cutoff for clinical relevance (25% in our study) may be debated and could depend on the type of drug. Indeed, some studies used a cutoff of 20% for clinical relevance, which is close to our effect of 17% for the recipient *CYP3A5* genotype on Tac clearance [36]. As TDM is not available for first dose guidance, genotyping recipient for the *CYP3A5* gene could be interesting to reduce Interindividual variability in terms of intestinal clearance. However, recipient genotyping would be useful only for the first dose, since thereafter well performed TDM along with Bayesian

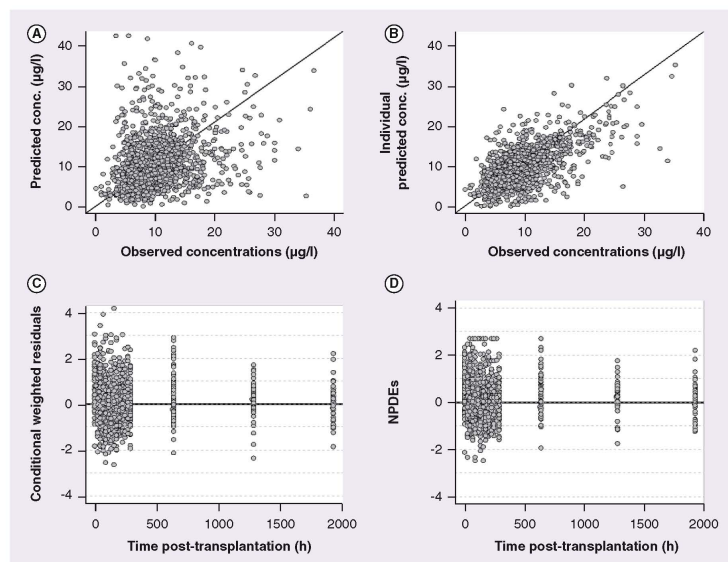


Figure 4. Goodness-of-fit plots of the final population pharmacokinetic model. (A) Observed versus population predicted concentrations; (B) observed versus predicted individual concentrations; (C) conditional weight residuals versus time post-transplantation; (D) NPDEs versus time post-transplantation. NPDE: Normalized prediction distribution error.

forecasting, according to Equation 7, would allow to rapidly correct a part of the PK variability. With the low prevalence of the *CYP3A5*1* allele in the Caucasian population, the high costs that genotyping would generate and the limited impact in terms of PK variability reduction it would provide, the benefit of systematic genotyping of recipient *CYP3A5* gene needs to be discussed. Further population PK–PD studies, focused on genotypes of interest in terms of efficacy and outcome, would help us to resolve this question.

It should be noted that some recipients' and donors' genetic polymorphisms status were missing in our patient cohort, due to logistical reasons. However, it should also be noted that 52 patients had complete data for genotypic polymorphism, which allowed us to perform the analysis in a reliable manner. Moreover, during the POP PK analysis, a sensitivity analysis was performed to test the influence of these missing values and the results showed that their influence was negligible.

Concomitant use of fluconazole led to lower Tac dose requirement and higher $\log C_0/D_{0-12h}$ in recipients when donors were *CYP3A5* expressors in comparison to nonexpressors as previously described [4]. We also showed that this effect was present and clinically relevant irrespective of recipient *CYP3A5* genotype. In the POP PK model, this effect was confirmed and the apparent Tac CL decreased by 60% in recipients with a *CYP3A5* nonexpressor donor but only by 30% in recipients with a *CYP3A5* expressor donor. No effect of either PPI or nifedipine was found.

Finally, as previously described in liver transplantation [18], we found that TPT significantly influenced C_0/D_{0-12h} as well as Tac hepatic CL, with a progressive rise in hepatic clearance with time. This effect was also observed in our previous POP PK model [7], where Tac CL increased until a steady state was reached at 60 days post-transplant. This is probably due to liver function recovery after transplantation. Other authors hypothesized that this positive effect of time post-transplantation could be explained by cytochrome induction due

to steroid treatment [10]. However, as our patients were not treated with steroids, this hypothesis alone cannot explain the positive time effect on Tac apparent CL.

The inclusion of genetic polymorphism covariates in the PK model, allowed a decrease of 20% in the variability of the clearance from 46 to 27%, which means that knowing donor genetic polymorphisms could be useful for reaching target concentrations more rapidly during TDM. As Tac dosing depends on the target exposure, which currently varies between centers, the latter should first be known. Subsequently, the results of our POP PK analysis can be used to identify starting dose and dosing adjustment (using Bayesian estimation) based on patient and donor characteristics. For example, if an average concentration of 10 µg/l is targeted the first week after transplantation, the following algorithms can be used to compute the first and the adjusted Tac doses (Equations 8–10):

$$\text{First dose (mg)} = \frac{0.01 \times 12 \times CL_i}{1 - \exp(-(CL_i/V_i) \times 12)} \quad (8)$$

$$V_i = 100 \times \left(\frac{WT}{10}\right) + 79 \times \left(\frac{WT}{10}\right)^{0.49} \quad (9)$$

with

$$\text{Adjusted dose (mg per 12 h)} = \frac{\text{administered dose} \times 0.01 \times 12}{\text{Bayesian estimated AUC}} \quad (10)$$

where V_i is the apparent total volume of distribution for the *i*th patient, WT is the patient's current

bodyweight, Bayesian estimated AUC is the patient's area under the concentration–time curve estimated using patient concentrations measured during TDM together with our model parameter estimates (particularly CL_i , the apparent clearance estimated using patient characteristics as shown in Equation 7).

This is the largest published Tac PK analysis in PLT that includes data regarding both living and deceased donor liver transplantation and that found a significant effect of donor *CYP3A5* and *CYP3A4* genotypes, recipient age, time and fluconazole on Tac CL (and $C_0/D_{BW12\text{ h}}$). This is also the first time the effect of donor *CYP3A4* genotype is shown in PLT. However, this study is limited by its retrospective design and some missing information on exposure to comedications. Moreover, TDM data was obtained via different immunoassays that introduced interassay variability and overestimation of Tac C_0 due to Tac metabolites. It is important to note that all the patients in this study were under Tac monotherapy with basiliximab induction, and extrapolation to patients receiving steroids and/or mycophenolate mofetil should be carried out very cautiously.

Conclusion

Owing to a significant effect on Tac PK in PLT, determination of donor *CYP3A5* and *CYP3A4* genotypes, prior to transplant, could help clinicians to identify patients at risk for Tac under- or over-exposure and to further improve dose adaptation in the case of concomitant fluconazole administration.

Future perspective

Validation of these results by prospective trials and introduction of this POP PK model in software

Executive summary

Effect of age on tacrolimus pharmacokinetics

- Young patients are characterized by a higher tacrolimus (Tac) apparent clearance and dose requirement. This may be explained by a higher graft weight/recipient weight ratio in young children.

Donor *CYP3A4* & *CYP3A5* genotypes are clinically relevant for Tac apparent clearance in pediatric liver transplantation

- Tac apparent clearance increased by 30% in the case of donor *CYP3A5* expression and decreased by 29% in the case of a *CYP3A4**22 T allele carrying graft.

Effect of fluconazole treatment on Tac pharmacokinetics

- Fluconazole leads to lower Tac apparent clearance and dose requirement. However, the magnitude of this effect depends on donor *CYP3A5* genotype, irrespective of recipient *CYP3A5* genotype.

Effect of time on Tac apparent clearance

- Time positively influences Tac apparent clearance during the first 3 months post-transplantation.

Conclusion

- This is the first population pharmacokinetics study in pediatric liver transplantation showing a significant effect of the donor *CYP3A4**22 T allele on Tac apparent clearance.
- The Tac pharmacokinetics model proposed in this study, along with Bayesian forecasting, could help clinicians to achieve target Tac concentrations more rapidly.
- Prospective trials are currently ongoing to validate these results.

for Bayesian forecasting will allow clinicians to adapt Tac doses for a more individualized immunosuppression protocol, with the aim of preventing graft rejection while avoiding acute and long-term side effects in a population with lifelong exposition to immunosuppressants.

Authors' contribution

V Guy-Viterbo participated in research design, data collection, data analysis and writing of the paper; H Baudet participated in data collection and writing of the paper; L Elens participated in data analysis; V Haufroid participated in research design and writing of the paper; F Lacaille, M Girard, D Debray and C Chardot participated in data collection and writing of the paper; R Reding and P Wallemacq participated in research design, data collection and writing of the paper; F Musuamba participated in data analysis and writing of the paper.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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PART III.GENERAL DISCUSSION

Chapter 1. GENERAL DISCUSSION AND PERSPECTIVES

1.1 General Discussion

Pediatric LT has become a safe, effective and standard of care therapy for children with end-stage, metabolic and oncologic liver disease. The story of PLT at Cliniques Universitaires Saint-Luc started a long time ago, in 1984 with Pr Otte. Since then, a dramatic improvement occurred with a 5-year patient and graft survival in patients with biliary atresia of 76 % and 64% respectively in the 1984-1992 period and of 98% and 95% in the 2003-2012 period (poster presentation Berlin 2012). This success, as observed worldwide, results from failures, researches, improvement and development of new surgical techniques, improvement in pre- and post-surgical care and improvement in immunosuppressive therapies. However, victim of its own success and progress, PLT soon faced to the limiting factor of organ shortage. Again, development and improvement of surgical techniques rendered LDLT possible, increasing the pool of PLT donors. At UCL, PLDLT quickly developed and became predominant in PLT.

The first step of this thesis was to review the results of the UCL experience in the first 250 PLDLT. In the first study, we confirmed very good short- and long-term patient- and graft survival. However, we pointed out a high acute rejection incidence with a one-year AR-free survival of 48% and also some adverse outcomes such as bacterial and viral infections as well as PTLD. All these issues are

linked more or less with IS regimen. At Saint-Luc, the current IS therapy is a steroid free Tac monotherapy with Basiliximab induction. This regimen has been introduced long time ago and was shown to be advantageous in terms of growth and infection rate when compared to steroid containing regimen.¹²⁵

However, Tac therapy is still not optimal and complicated by a very high inter- and intra- patient PK variability, which may be higher in children due to growth and maturation. Indeed, reaching and keeping therapeutic concentration may be difficult in some patients. Several published studies in children transplantation (see Table 2) found factors explaining the sources of Tac PK variability (PG, biological variable, comedications...) but were not all concordant. Thus, these factors are not currently taken into account and Tac dose initiation is still based on weight. Implementation of C_0 based Tac TDM partly helped clinicians to cope with this Tac PK variability but it is still insufficient and dose adaptation relies mostly on clinician's experience. Moreover, if C_0 correlates well with adverse outcomes, its correlation with acute rejection is still debated and AUC seems to be a better marker of Tac exposure.

The aim of this thesis was to find a way of changing Tac prescription and adaptation therapy in PLT from an old fashion to a newer individualized way.

To do so, we decided to conduct retrospective POP PK analyses, to identify the statistically significant and clinically relevant covariates explaining Tac PK variability and to quantify this variability. POP PK modeling is a particularly adapted tool in PLT PK studies since:

- it uses data of the population of interest

- data may be sparse (C_0)
- patients may contribute with varying samples number and timing
- a larger number of patients may counterbalance the sparse data per patient and improves understanding of PK variability
- covariates influencing PK variability may be identified and quantified
- as PK data are usually non-linear, non-linear mixed effects modeling gives usually less biases and more precise estimation of the parameter
- information obtained may be used for individualized dose prediction.^{393, 417}

The originality of the one year POP PK study part of this thesis was first to choose a two compartments model when using only C_0 by fixing the absorption constant and peripheral volume based on literature data. Indeed, most of the previous models relying on C_0 data reported one compartment model due to missing information on the absorption phase. However, we preferred to consider the physiochemical properties of Tac that highly accumulates in fat tissues over the statistical principle of parsimony, because it better reflects the real distribution of Tac in the body. It is confirmed by studies modeling complete PK profile data where a two-compartment model best fit the data.⁴¹⁵

Second originality of the paper was to study PK during the one-year post transplantation period compared to shorter periods in other studies. We confirmed that clearance increased with time up to 60 days after LT which was longer than in the studies of Fukudo and

Wallin.^{276, 277} Thus, this model may be applicable either in the immediate post-transplant period where PK variability is the highest but also during the long-term period at steady-state. It was also the first study showing an effect of the GW/BW ratio, which can be interesting in the settings of PLDLT where the ratio can be particularly high.

The second POP PK analysis focusing on the early period post transplantation (2 weeks), collected data from 82 patients. Although we developed a one compartment-based model, contrarily to the previous study, we also found an effect of time, hematocrit and GW/BW ratio on CL/F. In addition, comedications (inhibitors) showed to have a negative effect on CL/F, something that has been previously described in some adult and pediatric studies and that is intuitively known by clinicians since most of them reduce Tac doses when adding azole agents to a patient's therapeutic regimen. Finally, this study allowed us to externally validate our first model. The main limitations of these two first models were the missing information and testing of the influence of PG on Tac PK. Indeed, the *CYP3A5*3 SNP* is largely known to influence Tac PK in solid organ transplantation and thus is unavoidable to test. However, these models could still be used by clinicians when resources (technical or financial) are limited and/or when informed consent is not provided, preventing from obtaining PG information.

Aware of this important limitation and with the will to develop a robust POP PK model in PLT, we progressively collected PG information of recipients and donors during annual follow-up of our living donor liver transplanted patients. Moreover, we decided to

collect data from another center (Hôpital Necker, Paris, France) to enlarge the number of patients and to widen the scope of the model to cadaveric PLT. We finally provided a two-compartment model where time and age influenced clearance but also the use of fluconazole and the donor *CYP3A5* and *CYP3A4* genotypes.

This last work had the originality to perform at the same time a linear and non-linear regression analysis, as many previous studies on Tac PK did not use the POP PK methods and rather reported the effect of covariates on Tac C_0 and C_0/dose . However, as stated in the FDA recommendations, POP PK is now recognized as the most adequate tool to model PK parameters for the reasons exposed above.³⁸⁹

Moreover, to account for the interassay variability, we normalized the measured C_0 on the gold standard HPLC-MS methods by a correcting factor using data from the International Proficiency Testing Scheme. This is the first time that such corrections are reported in pediatric liver transplantation and may be useful when HPLC/MS or LC/MS methods are not available.

We finally provided a two-compartment model that is still today the more elaborate that has been published in PLT including clinical, biological and donor and recipient PG data. This model includes the effect of age on clearance in a power relationship (allometry), the effect of time with a sigmoid E_{max} model, a 30% increase and 29% decrease of Tac CL/F in case of donor *CYP3A5* expression and donor *CYP3A4*22* SNP, respectively and the inhibiting effect of fluconazole on Tac CL/F according to the *CYP3A5* status which confirmed previous study in PLT.²²¹

Not only providing a model we also proposed an algorithm for Tac dose initiation dedicated to clinicians. Such algorithms have been published by other teams, as nicely reviewed by Andrews et al, but as the model is more complete than the previous ones, we might hope a better performance.⁴¹⁸

In this work we have not consider the effect on Tac PK of the large quantities of fluid losses (peritoneal and/or pleural) and albumin replacements that some patients may have in the few weeks post LT. This is mainly due to lack of available information and it may constitute a limitation of our work. However, a previous study in adult LT showed no significant effect of ascites in Tac disposition.⁴¹⁹

Thus, with our work, we are soon able to individualize tacrolimus prescription in PLT. However, before being implemented, our last model and algorithm needs to be validated in an external group of patients and by a prospective trial. Such a trial is currently ongoing with two PLT centers in Paris and utilizes a limited sampling strategy. Since this method was shown to be highly correlated with AUC_{0-12h} , it may predict AUC_{0-12h} with more accuracy than when using C_0 .⁴²⁰

1.2 Perspectives

If Tac personalized regimen is particularly appealing in PLT, some questions/issues are still unanswered or debated and more research need to be achieved. Among the lot of possibilities, we may propose:

1. To enlarge the pharmacogenetic testing to:

- potentially other influent genes polymorphisms such as *CYP3A7*1C* and *POR*28*.

Adult carriers of the *CYP3A7*1C* allele were reported to have higher cyclosporine requirements in KT, while no effect was found in adult LT under Tac regimen.^{257, 421} A recent PK study in pediatric KT receiving Tac showed an opposite effect with a reduced clearance in the *CYP3A7*1C* carriers.⁴²²

Cytochrome P450 oxidoreductase (POR) interacts with CYP through electron donation. One of its genetic polymorphisms, *POR*28* (*rs1057868*; C>T), increases *in vivo* CYP3A activity towards Tac. Its effect on Tac PK in adult KT is controversial and has not been tested in PLT.^{142, 423, 424}

- consideration of *CYP3A5* and *CYP3A4* as clusters (poor metabolizer, intermediate or extensive metabolizers) as proposed in adult KT.^{425, 426}

- use alternative methods to find new potential effect of gene polymorphism such as a genome-wide association studies.⁴²⁷ This approach is used by the PersOnaliSing Immunosuppression To ImproVe Efficacy (POSITIVE) study in Canada in which the first aim is to identify genetic and host immune factors influencing IS dose requirements across different ages and transplant types in pediatric and young adults.⁴²⁸

2. To develop a PK-PD model in order to establish dose-concentration-response, based on our last model.

This would allow us to find the best AUC in PLT. Furthermore, we could also realize Physiologically based pharmacokinetic (PBPK) studies. Indeed, although built in a similar mathematical framework, PBPK models are parameterized using known physiology and include a larger number of compartments corresponding to the different organs or tissues in the body but also to nested compartments, such as different cell types within an organ or different organelles within a cell. These levels of hierarchical complexity allow modeling of specific metabolic pathways. These compartments are connected by flow rates that parallel the circulating blood system. PBPK models also provide estimates of PK parameters. Their mechanistic nature allows *in vitro-in vivo* extrapolations and permit rational scaling between organisms (animal to human) as well as developmental stages (adult to child). PBPK models have been used in several different capacities such as suggesting starting doses for children of different age groups, drug's behavior in case of liver disease, optimal design for clinical trial (sampling schedule, number of patients) and assessment of potential drug–drug interactions.^{429, 430}

3. To implement our last model in a suitable computer software developed to assist physicians in improving dose individualization.

Based on the model and Bayesian estimators, the software could propose a Tac initiation dose and dose adaptation based on the patients' demographic and clinical characteristics and, if available, previously measured drug concentrations. Computer-assisted dose individualization strategies have previously been shown to improve target achievement for Tac in adult LT.^{431, 432} If a clear and simple interface is provided, this method is easier to use for clinicians than dealing with PK algorithms.

4. To conduct prospective randomized-controlled study comparing the noninferiority/superiority of Model-Based versus weight-based Tac dosing with TDM for the following outcomes:

- time to target therapeutic concentration range achievement,
- incidence of acute graft rejection,
- incidence of renal failure, infection and PTLT.

Indeed, one randomized controlled trial (RCT) found earlier achievement of therapeutic drug concentration at the first steady state in adult KT patients receiving Tac dose based on their *CYP3A5* genotype when compared to patients receiving Tac dose based on weight, but they could not find any effect of genotyping based dosing on acute graft rejection neither on kidney failure. This lack of effect on graft rejection and renal failure was confirmed by a second RCT in adult KT. However, a recent meta-analysis by Rojas found a significant increased risk for transplant rejection in adult KT patients

expressing CYP3A5 (Rojas). Therefore, the Clinical Pharmacogenetic Implementation Consortium did not state for or against *CYP3A5* genotype guided Birdwell Tac dosing. This lack of effect on graft rejection was also observed in pediatric solid organ transplantation in 2018.⁴³³ Such issues should be tested to check whether earlier achievement of therapeutic concentrations leads to a reduced number of adverse outcomes in patients such as acute graft rejection, renal failure, infection and PTLD.

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