UNIVERSITY OF TURIN

Phd School in life and Health Sciences

Molecular Medicine

XXIX Cycle

Academic Years: 2014-2017

Title

Development of intracellular quantification methods for immunosuppressant drugs and applications to pharmacokinetic studies of pediatric patients

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Acknowledgments

*I would say **Thank you** to all the people that supported me during these long and incredible four years.*
Thanks to who supported my job and wiped my tears, who have taken my hand and said "everything will be perfect", who celebrated with me the successes, who gave me a smile when I was not enough happy to smile and who laughed with me till we had no air in the lungs.

*Thanks to my **mum** and **dad**, the sweetest and best parents that one would desire, who never lost a moment without telling me how much they love me: make me the woman I am.*

*Thanks to my "boss" **Prof. Antonio D'Avolio**, who gave me the chance to grow up, make experiences and believe in myself without losing the love in what I am doing.*

*Thanks to **Prof. Giovanni Di Perri**, who supported me till I was ready to spread my wings.*

*Thanks to **Jessica Cusato**, **Amedeo De Nicolò**, **Fabio Favata**, **Giovanna Fatiguso**, **Marco Simiele**, **Alessandra Ariaudo**, **Sarah Allegra**, **Valeria Avataneo** that shared with me the most of their days with jokes, smiling, hard work and also "food"!*

*Thanks to **Michele Pinon** and **Pier Luigi Calvo** who really made me understand how much love could be behind a pediatrician: when someone says " a doc must take care of his patients" must think about them!*

*Thanks to "my undergraduates" **Ivan Terribile** and **Clarissa Pisciotta**, who tolerated me as "teacher"!*

Thanks to my family that understood I was studying/working hard also during the Christmas holidays!
*And thanks to the longer list: **my friends**. Yes, I am a very lucky woman: I have no brothers or sisters from birth, but I have brothers and sisters of life.*

SUMMARY:

1. INTRODUCTION

1.1 Liver Transplant

In 1963 was done the first liver transplant of a children. Unfortunately, the children died for an unstoppable hemorrhage (Starzl, Esquivel, Gordon, & Todo, 1987). In the following 5 years was born the first immunosuppressor therapy. In 1967 was made the first successful liver transplantation in a 19 months old children (Starzl, 1978; Starzl et al., 1987) . Nevertheless the survival one year after transplant was lower than 30%. From 1979, with the therapy with cyclosporine the number of survivors after a transplantation arise (Otte, 2002).

In the last 20 years, the progress of surgical techniques and immune therapies the survival after one year after transplant became of 90%, with a good quality of life (Yazigi, 2013): pediatric liver transplantation has become a state-of-the-art operation with excellent success and limited mortality. Graft and patient survival have continued to improve as result of improvements in medical, surgical and anesthetic management, organ availability, immunosuppression, and identification and treatment of postoperative complication. Newer immunosuppression regimens have had a significant impact on graft and patients survival. Today the innovative surgical techniques give a chance of being transplanted also to the youngest children, reducing the higher mortality of transplanted children compared to adult patients.

Some years ago , the main problem was to find a liver transplant, but today, the main interest is in long-term follow-up, with prevention of immunosuppression-related complications and promotion of as normal growth as possible.

1.2 Therapeutic transplant indication

In Figure 1 and Table 1 are summarized the main indications for liver transplantation in pediatric population

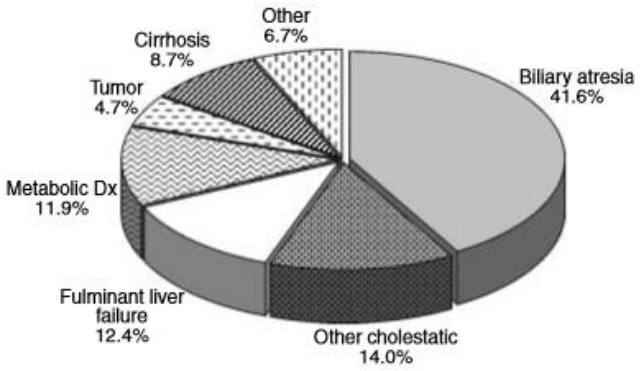


Figura 1 Main causes of hepatic transplant (McDiarmid, Anand, Lindblad, & Group, 2004).

Indications for Liver Transplantation	
Acute Liver Failure	**Metabolic Disorders Originating from the Liver**
Acute viral hepatitis	Hyperoxaluria
Drug or toxin induced hepatotoxicity	Amyloidosis
Acetaminophen overdose	Urea cycle defects
Autoimmune hepatitis	Branched chain amino acid disorders
Wilson's disease	Familial homozygous hypercholesterolemia
Cirrhosis from Chronic Liver Disease	**Malignancies**
Chronic viral hepatitis	Hepatocellular carcinoma
Alcoholic liver disease	Cholangiocarcinoma (limited)
Autoimmune hepatitis	Hepatoblastoma
Cholestatic liver disease	Fibrolamellar hepatocellular carcinoma
Wilson's disease	Metastatic neuroendocrine tumors
Hereditary and neonatal hemochromatosis	Hemangioendothelioma
Alpha-1-antitrypsin deficiency	**Miscellaneous**
Non-alcoholic steatohepatitis	Polycystic liver disease
Cryptogenic liver disease	Hereditary hemorrhagic telangiectasia
Budd-Chiari syndrome	Erythropoietic protoporphyria
Tyrosinemia	
Glycogen storage diseases	

Table 1: Indications for Liver Transplantation Evaluation: This table shows the major indications for liver transplantation in adults (O'Leary, Lepe, & Davis, 2008).

1.2.1 Cholestasis

Inherited syndromes of intrahepatic cholestasis and biliary atresia are the most common causes of chronic liver disease and the prime indication for liver transplantation in children

Estra-hepatic cholestasis: biliary atresia

Biliary atresia is the most common cause of cholestasis in neonates and the most common indication for pediatric liver transplantation: the incidence is 1 in 16000 live births (Engelmann et al., 2007). The clinical phenotype is produced by a fibrosing and inflammatory process that obstructs the lumen of extrahepatic bile ducts and disrupts the flow of bile into the duodenum. Bile duct abnormalities are also found within livers, typically with proliferation and plugging of the lumen by inspissated bile; variable degrees of portal inflammation, hepatocyte injury, and giant cell transformation coexist at diagnosis. Potential mechanisms involved in the pathogenesis of biliary atresia are reassumed in figure 2 (Santos, Choquette, & Bezerra, 2010).

Mechanism	Supporting data
Defect in morphogenesis	Development of jaundice soon after birth
	Coexistence of other embryologic abnormalities
	Abnormal remodeling of the "ductal plate"
	Inv mouse: model of biliary obstruction and situs inversus
	Polymorphisms in the *Jag1* gene
	Mutations in the *CFC1* gene (CRYPTIC protein) [37]
Defect in prenatal circulation	Vascular abnormalities
	Hepatic artery hyperplasia and hypertrophy
Toxin exposure	Time-space clustering of cases
Viral infection	Reovirus, rotavirus, CMV, HHV6, human papillomavirus detected in infants with biliary atresia
	Models of virus-induced injury to biliary tract in suckling mice
Immunologic dysregulation	Increased expression of intercellular adhesion molecules
	Infiltration of biliary structures by $CD4^+$, $CD8^+$, and NK [48••] lymphocytes and activated macrophages
	Prevention of experimental biliary atresia in mice by loss of α2β1 integrin, IFNγ, CD8+ cells, NK cells [46••,48••]
	Increased frequency of the HLA-B12 allele
	Expression of proinflammatory cytokines
	Oligoclonal expansion of lymphocytes
	Maternal chimerism [43]

Table 2: Pathogenesis of biliary atresia (CMV-cytomegalovirus, HHV-human herpesvirus, IFN-interferon, KK-natural killer) (Santos et al., 2010).

Intra-hepatic cholestasis

Intrahepatic cholestasis secondary to paucity of bile duct is an alteration of the anatomic integrity of the biliary tract, secondary to loss of key functions in organelles or the canalicular membrane. It may lead to severe cholestasis of infancy. In children, intrahepatic bile duct paucity may be **syndromic** (*Alagille's*) or **nonsyndromic** (e.g., *postviral, PSC*).

Alagille Syndrome

has a specific pattern of malformations. It is an autosomal dominant trait with cholestasis due to bile duct paucity, vascular and cardiac anomalies, ocular malformations, typical triangular face with broad forehead and butterfly-shaped vertebral arch (Engelmann et al., 2007).

Progressive familial intrahepatic cholestasis (PFIC)

is a chronic cholestasis syndrome that begins in infancy and usually progresses to cirrhosis within the first decade of life. Familial cholestasis syndromes can be divided in two groups. Patients with severe cholestasis and normal gamma glutamyl transferase (GGT) and patients with elevated GGT. In patients with PFIC, therapeutic options are still limited. Biliary diversion sometimes leads to a relief of the severe pruritus. If this operation fails, liver transplantation is the only option. Hepatocyte transplantation, gene therapy or specific targeted pharmacotherapy may represent alternative treatments in the future (Engelmann et al., 2007). Three types of PFIC have been identified and related to mutations in hepatocellular transport system genes involved in bile formation: **PFIC 1**, **PFIC 2** and **PFIC 3**. GGT activity is normal in PFIC1 and PFIC2 patients, but is elevated in PFIC3 patients. Both PFIC1 and PFIC2 are caused by impaired bile salt secretion due respectively to defects in ATP8B1 encoding the FIC1 protein, and in ABCB11 encoding the bile salt export pump protein (BSEP). Defects in ABCB4, encoding the multi-drug resistant 3 protein (MDR3), impair biliary phospholipid secretion resulting in PFIC3 (Davit-Spraul, Gonzales, Baussan, & Jacquemin, 2009).

The nonsyndromic form may result from infections in pregnancy (rubella, cytomegalie, hepatitis, chromosomal abnormalities (trisomy 18 and 21, monosomy X) or metabolic disorders such as cystic fibrosis or Zellweger's Syndrome.

	PFIC1 (Byler's disease)	PFIC2 (BSEP deficiency)	PFIC3 (MDR3 deficiency)
Transmission	autosomal recessive	autosomal recessive	autosomal recessive
Pruritus	severe	severe	moderate
Serum GGT activity	normal	normal	high
Ductular proliferation	absent	absent	present
Serum primary bile acid concentration	very high	very high	high
Bile composition	low primary bile acid concentration	very low primary bile acid concentration	low phospholipid concentration
Chromosomal locus	18q21-22	2q24	7q21
Gene/protein	*ATP8B1* FIC1	*ABCB11* BSEP	*ABCB4* MDR3
Hepatocyte location	canalicular membrane	canalicular membrane	canalicular membrane
Other sites of expression	Cholangiocytes Intestine, Pancreas	none	none
Functional defect	ATP-dependent aminophospholipid transport	ATP-dependent bile acid transport in bile	ATP-dependent phosphatidylcholine translocation in bile

Table 3: The main characteristics of PFIC (Davit-Spraul et al., 2009).

1.2.2 Metabolic diseases

Liver transplantation has become an accepted treatment for various hepatic-based metabolic disorders: liver-based metabolic diseases represent approximately 10% of pediatric liver transplants. Inborn errors of metabolism are caused by single enzyme defects that result in abnormalities in the synthesis or catabolism of proteins, carbohydrates, or fats.

Most are due to a defect in an enzyme or transport protein that alters a metabolic pathway. Two groups of inborn errors of metabolism can be distinguished: diseases that lead to structural liver damage with liver failure or cirrhosis, with or without injury to other tissues, such as **alpha-1-antitrypsin deficiency** (**A1ATD**), **Wilson's disease** and **cystic fibrosis** (**CF**), and diseases due to a metabolic defect expressed solely or predominantly in the liver but leading extrahepatic consequences such as **urea cycle disorders**, **Crigler-Najjar syndrome** and **hyperoxaluria** (Engelmann et al., 2007; Hansen & Horslen, 2008).

Structural liver damage disease

Gα-1 antitrypsin deficiency (A1ATD)

A1ATD is well known to adult physicians as a cause of chronic obstructive airway disease due to deficiency of the circulating protease inhibitor α-1 antitrypsin.
In pediatric populations, this condition is one of the more common causes of neonatal cholestasis, chronic liver disease, and liver failure.

Wilson's disease (WD)

The molecular basis of WD is now well understood, with mutations in the gene ATP7B being responsible for the failure of biliary excretion and incorporation of copper into ceruloplasmin. Pediatric presentations of WD are typically hepatic, including asymptomatic disease detected on routine physical examination, chronic hepatitis, cirrhosis, and fulminant hepatic failure.
Medical chelation therapy is highly effective and, therefore, only those patients who have progressive liver disease despite therapy should be considered for transplantation.

Cistic Fibrosis (CF)

Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene are responsible for the disease. CFTR functions as a chloride channel and may regulate other cellular transport pathways. The lungs and pancreas are the organs classically affected by CF, but liver disease has been increasingly recognized. Studies have shown that the lack of CFTR alters ductular chloride secretion, which results in viscous biliary secretions with subsequent biliary obstruction that leads to focal biliary fibrosis and ultimately cirrhosis.

Extrahepatic consequences disease

Disorders of urea cycle

The urea cycle is a series of biochemical reactions by which ammonia is detoxified and converted to the excretory product, urea. Only hepatocytes express all of the enzymes necessary for urea production. Defects result in an accumulation of nitrogenous waste, especially ammonia, which is highly neurotoxic. The 5 human diseases has been described as due to a deficiency of each of the enzymes involved in urea cicle. The disease names for the first 2 defects describe the enzyme defi-ciency, that is, carbamyl phosphate synthetase (CPS) deficiency and ornithine transcarbamylase (OTC) deficiency. The remaining 3 disorders are known by the characteristic metabolite detected in affected individuals, namely, citrullinemia, argininosuccinic aciduria (AS), and argininemia (AL). Urea cycle disorders are the primary causes of hyperammonemia in the neonatal period, but other organic acidemias can also present with severe hyperammonemia, and careful clinical and biochemical assessment is critical. Acute signs include anorexia, hypothermia, lethargy, irritability, vomiting, hyperventilation, and seizures.

Crigler-Najjar syndrome (CN)

CN syndrome is the result of defective bilirubin-UDPglucuronosyltransferase activity due to mutations in the gene uridine diphosphate glucuronosyltransferase 1. This results in unconjugated hyperbilirubinemia, which untreated can lead to kernicterus. This is a condition of severe neural injury associated with deep yellow staining of the basal ganglia, cerebellum, and bulbar nuclei. Manifestations include ataxia, athetosis, seizures, dysarthria, mental slowing, and lethargy. Treatment for CN1 includes

exchange transfusions to acutely reduce unconjugated bilirubin levels. Once serum bilirubin concentrations are acceptable, phototherapy is usually adequate to maintain them below critical levels. However, spending most of the day under phototherapy can severely affect quality of life. At present, LT is the only definitive treatment for CN1.

Primary hyperoxaluria type 1

Primary hyperoxaluria type 1 results from a deficiency of the peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT). The metabolic defect leads to excessive oxalate production, which injures the kidneys and accumulates in other tissues of the body. Renal damage results from deposition of calcium oxalate within the renal tubules or in the urinary tract as calculi. As renal function deteriorates, oxalate accumulates in other tissues; of particular importance is cardiac deposition, which leads to arrhythmias, heart block, and death. Extra-renal accumulation progresses rapidly once patients require dialysis because current forms of dialysis remove oxalate very inefficiently. AGT is expressed in hepatocytes, and therefore LT acts as enzyme replacement therapy. Urinary excretion of glycollate normalizes after LT, but oxaluria continues for a considerable time because of systemic accumulation of oxalate.

1.2.3 Acute liver failure

Acute liver failure (ALF) was relatively easy to recognize in the days before liver transplantation, because the diagnosis was based on end-stage disease manifestations such as profound coagulopathy, jaundice, encephalopathy and cerebral edema (Horslen, 2014). In Table 4 are reported the major causes of ALF in infants and children.

Viral	Hepatitis A Hepatitis B Hepatitis D Hepatitis E Herpes simplex Varicella zoster virus Epstein-Barr virus Cytomegalovirus Paramyxovirus Adenovirus Enterovirus Parvovirus B19 Severe acute respiratory syndrome Haemorrhagic fever viruses	Immune	Autoimmune hepatitis Haemophagocytic lymphohistiocytosis Neonatal haemochromatosis Autoimmune haemolytic anaemia with giant-cell hepatitis
		Toxic	Drugs/toxins/herbals *Amanita phalloides*
		Vascular	Budd-Chiari syndrome Veno-occlusive disease Ischaemic hepatitis/shock liver Post cardiac surgery Liver trauma
Bacterial	Septicaemia Leptospirosis *Salmonella typhi/S. paratyphi* *Bartonella* Rocky Mountain spotted fever	Neoplastic	Leukaemia Lymphoma Hepatocellular carcinoma
		Other	Reye's syndrome Hypothermia Heat stroke Massive liver resection Sickle cell anaemia
Metabolic	Hereditary fructose intolerance Urea cycle disorders Organic acidaemias Fatty acid oxidation defects Mitochondrial disorders Carnitine defects Wilson's disease Tyrosinaemia type 1 Niemann-Pick disease type C Acute fatty liver of pregnancy		

Table 4: Causes of ACF in children and infants (Horslen, 2014).

1.2.4 Primary liver Tumor

Primary malignant liver tumors make up just over 1% of all childhood cancers, with an incidence of approximately 1.0–1.5 per million children per year in the West.1 Hepatoblastoma and hepatocellular carcinoma account for the vast majority.

Epatoblastoma

Hepatoblastoma is the most common pediatric liver tumor and is usually diagnosed before five years of age. Treatment consists of a combination of chemotherapy and surgery, with the goal being attainment of complete local control by surgical resection and eradication of any extrahepatic disease. For patients whose tumors are too extensive to be conventionally resected, liver transplantation can be curative and remains the treatment of choice for eligible patients otherwise incurable by conventional resection (Trobaugh-Lotrario, Meyers, Tiao, & Feusner, 2016).

Hepatocellular carcinoma

Hepatocellular carcinoma accounts for about one-third of all primary paediatric malignant liver tumors in Western societies but a much higher proportion in countries where hepatitis B is endemic (Chen JC1 et al.1998). Hepatocellular carcinoma typically occurs in older children (10–14 years) and more commonly affects boys (Trobaugh-Lotrario et al., 2016).

Other primary malignant liver tumors

Liver transplantation has occasionally been performed for other primary malignant liver tumors in children, all of which are rare. Results have generally been poor.

Undifferentiated (Embryonal) Sarcoma

This is a highly malignant mesenchymal tumor which usually affects children aged between 5–10 years. Until recently, the prognosis was poor but long-term survival has now been reported after neoadjuvant multi-agent chemotherapy and partial hepatectomy.51,52 The Brussels group described two children who underwent OLT for an unresectable sarcoma; both died within 6 months of the transplant, one from tumor recurrence.46 Dower et al.31 reported a 6-year-old boy with a non-metastatic undifferentiated sarcoma which was successfully

treated by chemotherapy and transplantation; the key factor in this patient appears to have been the chemosensitivity of the tumor.

Hepatic Epithelioid HaemangioEndothelioma (HEHE)

HEHE is a slow growing malignant vascular tumor, distinct from haemangioendothelioma and angiosarcoma. It is most often encountered in young women. It may behave more aggressively in children.

Angiosarcoma

Angiosarcoma in children is often unresectable and lung metastases may be evident at presentation. Some cases represent malignant transformation of a pre-existing haemangioendothelioma. The treatment of this rare, high-grade malignancy has not been standardised. Occasional success after chemotherapy and partial hepatectomy has been reported.

1.2.5 Viral hepatitis

Viral hepatitis B and C are the cause of significant disease worldwide. Acute infection is more common with hepatitis B than C in childhood; but chronic asymptomatic infection leading to chronic liver disease and hepatocellular carcinoma is a considerable concern.

Hepatitis B

The main source of infection in childhood is perinatal transmission, which is effectively prevented using vaccination, antenatal screening and screening of blood products and organ donors. The vaccine is effective in 97% of newborn infants and lasts for 10-15 years. Following an acute infection, 90% will recover spontaneously; but approximately 1% of patients develop acute fulminant hepatitis requiring liver transplantation. Liver transplantation is an effective treatment for children with acute or chronic liver failure, but recurrence is high without prophylaxis (Kelly et. al, 2006).

Hepatitis C

The main route of transmission for hepatitis C was originally through infected blood products or organs; but now the most common source is vertical transmission which ranges from 2%-12% depending on maternal infectivity. Breast feeding is safe in mothers with low titres of hepatitis C RNA. The natural spontaneous clearance rate for hepatitis C is between 20% and 40% and is higher in children who have been parenterally infected compared to perinatal infection. It is a mild disease in children, but the indication for treatment is based on the future risk of cirrhosis and hepatocellular cancer. Liver transplantation for hepatitis C in children is rarely required, but 100% recurrence can be expected without prophylaxis (Kelly et. al, 2006).

1.3 The Transplant Operation

The first liver transplant was performed by Thomas Starzl, in 1963, on a 2-year-old child affected by biliary atresia. After this first case, and up to the early 1980s, the only technical option for pediatric liver transplantation was to transplant the whole liver of a donor with a weight as close as possible to that of the recipient. The development of techniques that allow surgeons to transplant portions of livers from adult donors has completely changed the fate of liver transplantation in pediatric patients.

Whole-liver transplantation

The procedure of whole-liver procurement in pediatric donors can be performed exactly as in adults. Whole-liver pediatric transplantation can be performed with two different techniques: the classic technique with inferior vena cava replacement, and the piggyback technique with preservation of the native inferior vena cava.

Reduced-size liver transplantation

This procedure consists in the procurement of the whole liver from an adult cadaver donor, which is reduced in its size on the back-table. In the original description, a right hepatectomy was performed on the back-table: the right lobe of the liver was discharged, while the left lobe, including the vena cava, was transplanted in a child. This technique allows surgeons to overcome differences in size between the donor and the recipient of up to four or five times. The development of this technique has led to almost total elimination of child mortality on the waiting list, through the utilization of an adult liver cadaver donor.

Living-related liver transplantation

The first description of the procedure in which segments 2 and 3 were procured from a living donor (the mother), and transplanted in a child affected by biliary duct atresia, dates back to 1988. The validity of this procedure is broadly recognized, and over 1200 cases have been performed worldwide, with a donor mortality and morbidity of approximately 0.2% and 10%, respectively.

Split-liver transplantation

Split-liver transplantation involves procuring a whole liver from a cadaver donor and dividing it into two sections along the round ligament, leaving the vascular structures for the two portions of hepatic parenchyma intact. Two partial organs are obtained from a single liver: the left lateral segment, which can be transplanted in a child, and the extended right liver, which can be transplanted into an adult (Spada, Riva, Maggiore, Cintorino, & Gridelli, 2009).

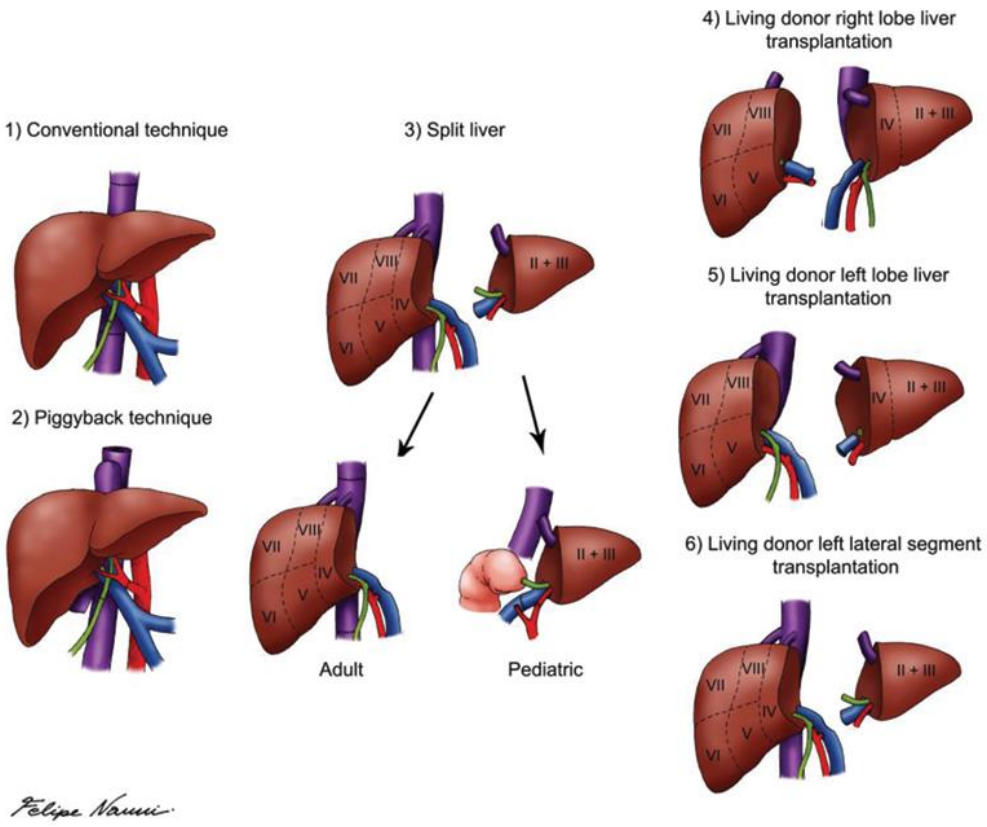


Figure 2: Examples of surgical techniques for liver transplantation (Wiederkehr J. C. , 2016).

1.4 Immunosuppression

The immune system recognizes graft as foreign and begins a destructive immune response mediated principally by the T-lymphocytes (Spada et al., 2009). In order to avoid destruction of the graft, immunosuppressive agents are required for induction and manteinance of immunosuppression and for the treatment of organ rejection (Moini, Schilsky, & Tichy, 2015).

Most immunosuppressive agents target T lymphocytes, which are primary mediators of the immune response and effectors of the rejection process. Current immunosuppression protocols usually include two or more agents to target different steps or mechanism of the immune response. The increase of multiple drugs not only increase the efficacy of the immunosuppression regimen but also often allows dose reduction of one or more of the drugs in an attempt to limit toxicity. Immunosuppression is usually heavier in the peri-operative period and early post-transplant (induction) when the risk of rejection is higher. Later, depending on graft function and tolerability, immunosuppressive doses are gradually reduced (maintenance) to levels adequate to prevent rejection and avoid toxicity. Although rare cases, the immunosuppression needs to be continued lifelong, inevitably exposing the recipient to the long term effects of chronic immunosuppression. Since there is no a single optimal immunosuppressive regimen, the delicate balance between effective prevention of rejection and avoidance of toxicity must be maintained (Girlanda, 2013).

1.5 TDM (Therapeutic Drug Monitoring)

Up to now, the most frequently used approach to face the toxic effects or the inefficacy of pharmacological treatments was the change of treatment suspension, the change of treatment or the “ad juvantibus” change in drug dosage. Recently, the better knowledge of the PK/PD and PG properties of drugs allowed the use of this information in order to guide the dose adjustment during therapy. In fact, since the concentration of the drug is rarely measurable in its site of activity, the Therapeutic Drug Monitoring (TDM) in plasma (or sometimes in blood) plays a crucial role: this is based on the determination of the plasma concentration of the drug in patients undergoing therapy, in order to monitor some factors that are classically difficult to control, such as the "compliance" of the patient and/or interactions between multiple drugs. It is moreover useful for particular conditions such as pregnancy. Although TDM is mainly performed on plasma samples, TDM is performed using different techniques, among which chromatography is considered the gold-standard. These technologies include techniques such as HPLC/UPLC (High/Ultra Performance Liquid Chromatography) and gas chromatography using different detectors: UV, PDA (Photodiode Array), fluorescence and mass spectrometers (standard, TQD or time-of-fly). The TDM, accompanied by PG testing, can be a powerful tool for treatment personalization and for treatment management. However, in order to correctly use TDM, several conditions are needed:

- Deep knowledge of PK properties, such as the dose-proportionality of drug concentrations, the drug halflife and the Tmax.
- Deep knowledge of PD properties, such as the Minimum Effective (or Inhibitory) Concentration (MEC or MIC, respectively) and the Maximum Toxic Concentration (MTC). Together, these define the “therapeutic range”. The effect and toxicity have to be concentration-dependent!
- Adequate technology to support the quantitative determination of drugs concentrations in biological matrix.
- Fully validated robust bioanalytical methods to quantify drugs.

1.6 Pharmacogenetics, Pharmacokinetics And Pharmacodynamics

The choice of the optimal dose to obtain the best results with fewer side effects is necessary for maintaining immunosuppression and avoiding the graft rejection. To solve these and many other aspects, over the years the following scientific disciplines have emerged:

Pharmacogenetics (PG)

study of genetic polymorphisms that may be involved in the kinetics of drugs or in the patients' response and, therefore, have a direct effect on the success of the therapy. The genetic typing of these polymorphisms may be useful in predicting the clinical outcome and/or the onset of toxic side effects in the patient before beginning therapy, in order to optimize it according to the individual characteristics of the patient.

Pharmacodinamics (PD)

PD is the study of the biochemical and physiological effects of drugs, as well as their mechanism of action. The main Pharmacodynamic parameters are:

MTC (Maximum Tolerated Concentration): maximum plasma concentration of the drug beyond which toxic side effects and/or non-tolerable toxicity occur.

MEC (Minimum Effective Concentration): plasma drug level below which therapeutic effects will not occur.

Pharmacokinetics (PK)

PK is the study of the absorption, distribution, metabolism and excretion of a drug (ADME system). These parameters will influence the concentrations of the drug in the different compartments of the body. The kinetic and dynamic aspects are closely related to each other, since the proportion of the effect depends on the concentration of the drug in the specific site of action (dose-effect relation). The main pharmacokinetic parameters are (Mehrotra, Lal, Puri, Madhusudanan, & Gupta, 2007):

AUC (Area Under the Curve): is represented by the area under the plasma concentration curve over time. It is a marker of global exposure.

Cmax (Maximum Concentration): the highest concentration reached by the plasma concentration-time curve, used as a drug toxicity marker. Its value must be within the therapeutic range (Figure 8).

Cmin (Minimum Concentration): minimum value reached by the curve between one dose and the next one. Once reached steady-state, state of equilibrium in which the amount of drug eliminated corresponds to the one introduced for each administration, it is important that the value of the Cmin doesn't fall below the value of the minimum effective concentration (MEC). In fact, the continued exposure to concentrations lower than the MEC brings a greater risk of resistant strains selection.

Ctrough (Trough Concentration): it is the plasma concentration of the drug immediately before the next dose. The value of the Cmin and Ctrough may not be equal, but they are generally close.

Vd (Volume of distribution): it indicates the ability of diffusion and penetration of the drug in various organs and tissues.

T1/2(half-life): indicates the time required to reduce by 50% the plasma concentration of the drug.

Cl (Clearance): volume of plasma that would contain the amount of drug excreted per minute or, alternatively, the volume of plasma that would have to lose all of the drug that it contains within a unit of time (usually 1 min) to account for an observed rate of drug elimination.

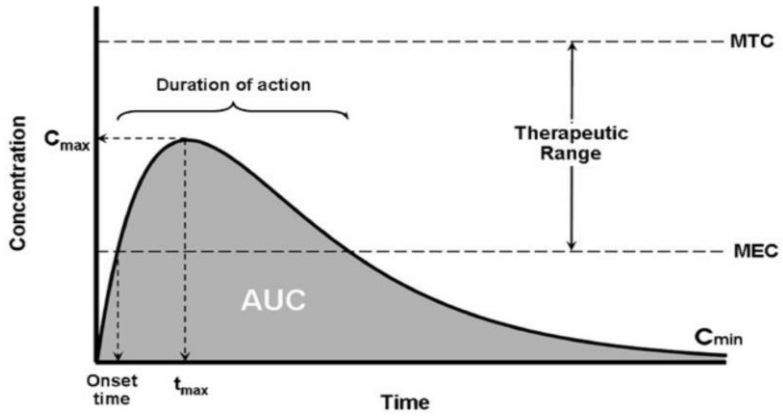


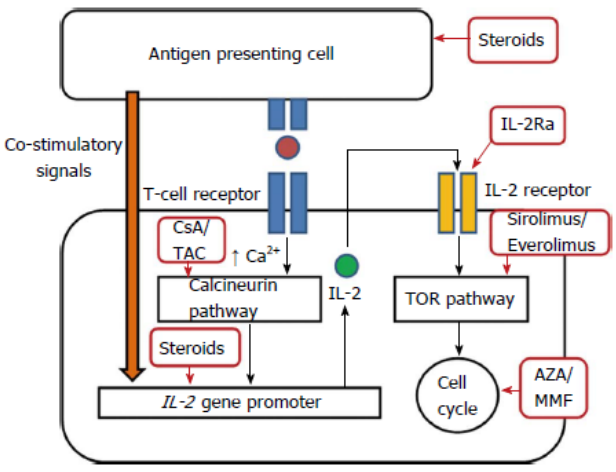
Figure 3: Area under the exposure curve and pharmacokinetics parameters

1.7 Immunosuppressors

In **Table 5** of Moini et al. are reassumed the main immunosuppressors used and their mechanism of action (Moini et al., 2015).

Agent	Classification	Indications	Dose
Methyl prednisolone (Medrol®), Prednisone or prednisolone[13,16,111]	Corticosteroids	Induction of immunosuppression, treatment of acute cellular rejection, Maintenance of immunosuppression	Variable according to the centers, the etiology of liver disease and history of rejections
Tacrolimus (Prograf®, Astagraf®)[53]	CNI	Maintenance of immunosuppression	Starting 0.1-0.15 mg/kg per day divided every 12 h and adjust to the desired trough level
Cyclosporine (Neoral®, Sandimmune®, Gengraf®)[52,55]	CNI	Maintenance of immunosuppression	Starting 10-15 mg/kg per day divided every 12 h and adjust to the desired (C2) level
Mycophenolate mofetil (Cellcept®, Myfortic®)[60]	Anti-metabolite	Maintenance of immunosuppression, treatment of rejection	Variable doses may be desired in any individual case
Azathioprine (Imuran®)[65]	Anti-metabolite	Maintenance of immunosuppression	Variable, maintenance dose may be 1.5-2.5 mg/kg per day, needs to be adjusted for adverse side effects
Sirolimus (Rapamune®)[68,68,71]	mTORI	Maintenance of immunosuppression, treatment of rejection, special interests for use in malignancies	Usual dosing is a 6 mg (or 3 mg/m^2) oral loading, followed by 2 mg/d (or 1 mg/m^2 per day) single dose, higher doses may be administered for individual cases[1]
Everolimus (Afinitor®)[68,69,72]	mTORI	Maintenance of immunosuppression, treatment of rejection, special interests for use in malignancies	Starting at 1 mg oral every twice a day and adjust to a trough level of 3-8 ng/mL[1]
[2]Muromonab-CD3 (OKT3)	T cell depleting monoclonal antibody	Induction of immunosuppression, treatment of steroid resistant rejection	Withdrawn from the market because of reduced use, no longer available since 2010
Alemtuzumab (campath-1H®)[44-46]	T cell depleting monoclonal antibody	Induction of immunosuppression	Variable between centers, a single dose of 30 mg may be used in operating room
ATG (Thymoglobulin®, ATGAM®)[27-30]	T cell depleting polyclonal antibody	Induction of immunosuppression, treatment of steroid resistant rejection	Variable between centers, For induction 1.5 mg/kg per day *iv* for 3 d and for treatment of rejection 1.5 mg/kg per day *iv* for 5-7 d of thymoglobulin may be used. For ATGAM a higher dose of 15 mg/kg per day is usually used
[2]Daclizumab (Zenapax®)[23,115]	IL-2Ra, monoclonal antibody	Induction of immunosuppression, treatment of steroid resistant rejection	For induction the first dose of 1 mg/kg is given within 24 h before Tx and 4 more doses are given after Tx with 2 wk intervals Withdrawn from the market because of reduced use, no longer available
Basiliximab (Simulect®)[23,113,114]	IL-2Ra, monoclonal antibody	Induction of immunosuppression, treatment of steroid resistant rejection	For induction a 20 mg *iv* dose is administered within 2 h prior to reperfusion and another 20 mg on days 4 post Tx

[1]Best to be started at least 30 d after transplantation; [2]Not manufacturing anymore. CNI: Calcineurin inhibitor; mTORI: Mammalian target of rapamycin inhibitor; iv: Intravenous; IL-2Ra: Interleukin-2 receptor antagonists; Tx:Transplantation



In **Figure 4** of Moini et al. are reassumed The cellular sites of action of the immunosuppressive agents commonly used in solid organ transplantation

AZA: Azathioprine; CsA: Cyclosporine; IL-2: Interleukin-2; IL-2Ra: Interleukin-2 receptor antagonist; TAC: Tacrolimus MMF: Mycophenolate mofetil; TOR: Target of rapamycin

1.7.1 Corticosteroids

Corticosteroids were the first drug for induction of immunosuppression since the first successful cases of solid organ transplantation, they are both effective for prevention and treatment of graft rejection. Their immunosuppressive mechanism is not fully clary yet, but is linked to the suppression of T cells, of antibody production and of synthesis of cytokines (like IL-2) and interferon-γ. The major problem of corticosteroids are their side effects: delirium, infections, hyperlipidemia, diabetes, obesity. There is also concern that higher doses of steroids increase the risk of disease recurrence of chronic hepatitis in liver transplanted patients.

1.7.2 Antibodies

Antibodies are used for inhibit or deplete recipient T-cells to decrease acute rejection episodes. The antibodies used for the immunosuppression are: Alemtuzumab, Thymoglobulin, ATGAM, Muromonab, Basiliximab, Daclizumab. The use of antibodies could help with reduction of dosage necessary of calcineurine inhibitors and corticosteroids, minimizing the adverse side effects related to these agents. The induction of immunosuppression with antibodies seems necessary in those patients with hepatitis C, diabete and hypertention, in whom corticosteroids therapy is not adeguate. No significant increase of side effect was observed in solid transplant recipients after induction with antibody; however, the cost of this kind of therapy is higher. The antibodies used for induction are divide in two groups: T-cell depleting and non-depleting. The risk of opportunistic infections (viral, fungal) is higher after T cell depleting, especially if prolonged, compared to the use of non-depleting agents. Alemtuzumab, Rituximab, Thymoglobulin, Muromonab are depleting agent that causes a profound depletion of total T cells but few studies exist on its use on children with a mechanism of action assumed in Figure 5 (Nguyen & Shapiro, 2014). Non-depleting antibodies (Basiliximab, Daclizumab) block lymphocyte function by binding to cell surface molecules involved in the regulation of cell function.

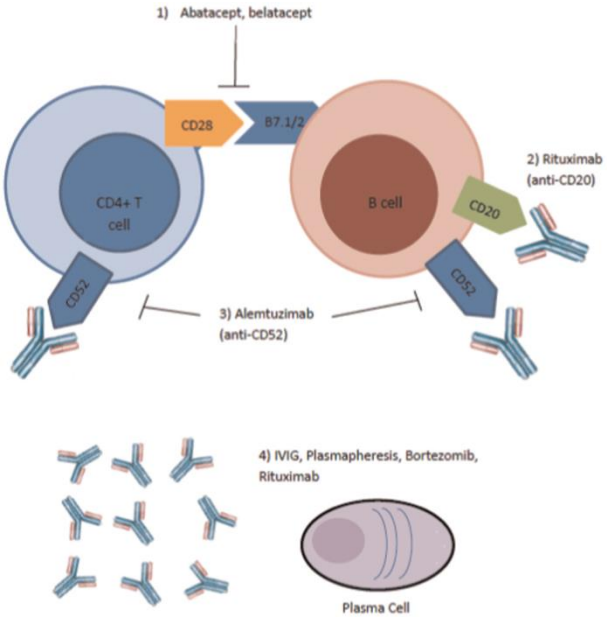


Figure 5: Abatacept, belatacept, and alemtuzimab inhibit binding at the sites of T and B cells (Nguyen & Shapiro, 2014)

1.7.3 Calcineurine Inhibitors (CNIs)

CNIs function as immunosuppressants by blocking T-cell activation by binding to specific receptor and blocking calcineurin, a calcium dependent phosphatase within T-cells (Moini et. al. 2015). Calcineurin inhibition indirectly blocks the transcription of cytokines, particularly IL-2, which regulate the proliferative T-cell response (Spada et al., 2009). CNIs inhibitors have similar side-effects: nephrotoxicity, neurotoxicity and hypertension. Most of these adverse effect are reversible after dose reduction or discontinuation of the drug. The main CNIs used for the maintenance of immunosuppression are: Cyclosporine and Tacrolimus.

Cyclosporine

Introduced for immunosuppression since 1970s and early 1980s, but is not the CNI of choice for liver transplant recipients; however in rare cases there might be a need to switch from tacrolimus to cyclosporine (Moini et al., 2015).
For the treatment of children with cyclosporine must be considered two important things: 1) cyclosporine bioavailability correlates with age, being lower in younger patients and 2) cyclosporine is metabolized in children at higher rate than adults, and appears to be inversely related to age (Spada et al., 2009).

Tacrolimus (TAC)

TAC (FK-506) inhibits, forming a complex with FKBP12, the activated serine threonine phosphatase, calcineurine, in T-limphocytes (Figure 6)

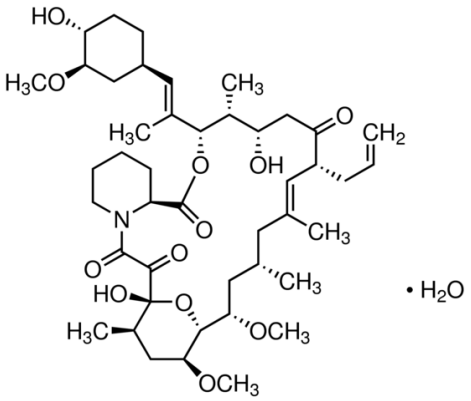


Figure 6: Tacrolimus chemical structure

The TAC activity is similar to that of Cyclosporine but more potent at the same concentration. At the molecular level, the effects of tacrolimus appear to be mediated by binding to a cytosolic protein (FKBP12) which is responsible for the intracellular accumulation of the compound. The FKBP12tacrolimus complex specifically and competitively binds to and inhibits calcineurin, leading to a calcium-dependent inhibition of T-cell signal transduction pathways, thereby preventing transcription of a discrete set of cytokine genes. Tacrolimus is a highly potent immunosuppressive agent and has proven activity in both in vitro and in vivo experiments. In particular, tacrolimus inhibits the formation of cytotoxic lymphocytes, which are mainly responsible for graft rejection. Tacrolimus suppresses T-cell activation and T-helper-cell dependent B-cell proliferation, as well as the formation of lymphokines (such as interleukins-2, -3, and γ-interferon) and the expression of the interleukin-2 receptor. It suppress T-cells activation and t-helper-cell-dependent interleukins 2 and 3, and interferon-γ, and the expression of interleukin-2 receptor.

Pharmacokinetic:

Adsorption and Distribution

TAC is adsorbed in the gastro-intestinal tract. Its bioavailability is low and variable, due to the first pass metabolism, P-glycoprotein mediated efflux and presence of food. TAC is highly bound by proteins (>98%) into red blood cells.

Metabolism and Excretion

Elimination was found to be slow from kidney, liver, spleen, lung and gastrointestinal tract. Toxicities have been associated with nervous system, kidney, heart and lymphoid organs. When TAC is administered intravenously or orally, it is metabolized to at least 9 metabolites. The major route of TAC elimination is fecal excretion (80-90%), only <2% is found in biliary excretion. When TAC is cleared by hepatic metabolism, principally via CYP3A4. The clearance of TAC is 2.25 L/h in healthy subject, 4.05 L/h in liver transplant patients. The t ½ (elimination half-life) in healthy subject is about of 43 hours. In liver transplanted pediatric patients the clearance is 2 times higher than those of adults and have an higher distribution volume of drugs, therefore an higher dosage is required for maintaining TAC levels into therapeutic efficacy range.

Pharmacology interactions:

Being a substrate of CYP3A4, TAC could be influenced by drugs that induce or inhibit this cytochrome, causing higher or lower plasmatic levels of TAC.

Inhibitors of CYP3A4

The main drugs that strongly inhibit CYP3A4 are: ketoconazole, fluconazole, Iitraconazole, voriconazole, erythromycin and HIV protease inhibitors. In addition, grapefruit juice induce higher TAC concentration.

Inducers of CYP3A4

The main drugs that induce this enzyme, causing the necessity of a higher dose of TAC, are: rifampicin, fenitoin and hypericin.

Other Drugs

Co-sub ministration of other drugs could cause toxicities: 1) amphotericin B and/or hybuprofen could cause the rise of nephrotoxicity 2) cyclosporine increase TAC half-life 3) aminoglicosides, gyrase inhibitors, vancomycin, cotrimoxazole,

Influence on TAC therapy	*Drugs*
Lower TAC concentration	Anti-acids, Carbamazepine, Dexamethasone, Phenobarbital, Phenytoin, Methylprednisolone, Rifampicin, Sirolimus, Sodium Bicarbonate
Synergic Nephrotoxicity	Aminoglycosides, Amphotericin B, Cisplatin, Ibuprofen, Diuretics,
Higher TAC concentrations	Cimetidine, Cisapride, Clarithromycin, Chloramphenicol, Clotrimazole, Danazole, Diltiazem, Etinil-estradiol, Erythromycin, Fluconazole, protease inhibitor, Itraconazole, Ketoconazole, Metoclopramide, Mibefradil, Nefazodone, Nicardipine, Niphedipine, Theophylline, Troleandromicin, Verapamil

Table 6: TAC drug interactions (Cattaneo D., 2005).

1.7.4 Antimetabolites

This group of drugs, composed by Azathiprine and Mycophenolate mofetil, is characterized by the inhibition of DNA and RNA synthesis and subsequent block of T-cells activation.

Azathioprine

Azathioprine is a derivative of 6-mercaptopurine and it decreases DNA and RNA synthesis and was used in the early era of transplantation in combination with corticosteroids. (Girlanda, 2013). After the introduction of more potent agents, such as tacrolimus, was replaced by Mycophenolate mofetil. Azathioprine major adverse side effect is related to the bone marrow suppression, its hematologic consequences and hepatotoxicity (Moini et al., 2015).

Mycophenolate mofetil (MMF)

MMF is used for the maintenance of immunosuppression and its active metabolite is mycophenolic acid (MPA) is a selective inhibitor of the inosine monophosphate dehydrogenase, an enzyme involved in de novo syntesis of purine. The resulting depletion of guanosine nucleotides causes the arrest of lymphocyte replication because they are unable to use the alternative pathway for nucleotide production.
Pharmacokinetic studies showed large inter-individual variations in mycophenolic acid concentrations (Spada et al., 2009). MMF is also an useful agent in combination with CNIs in immunosuppressive regimens. The major adverse side effects of MPA are hematologic and gastro-intesinal (Moini et al., 2015).

1.7.5 mTOR inhibitors

This group includes everolimus and sirolimus, two of the most recently introduced immunosuppressive agents in clinical transplantation, acting with a mechanism of action different from other immunosuppressants. They are inhibitors of the mammalian target of rapamycin.

Sirolimus

Sirolimus (also known as Rapamycin) was the first mTOR used in immunosuppression after transplantation (Figure 7).

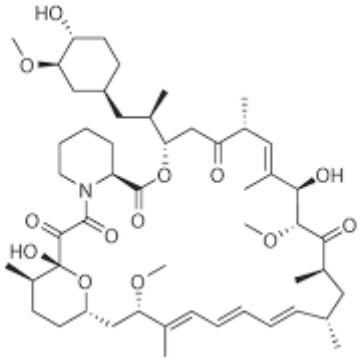


Figure 7: Sirolimus chemical structure

It is a bacterial macrolide antibiotic produced by Streptomyces isolated from a soil sample. Sirolimus binds the intracellular immunophilin FKB12, the same intracellular binding protein of Tacrolimus, but with a different mechanism of action: after binding, the complex sirolimus-FKB12 inhibits a complex called mammalian Target Of Rapamycin (mTOR). Inhibions of mTOR results in selectiv inhibitions of synthesis of new ribosomal proteins (Girlanda, 2013). This results in blockage of T-cells activation. The most relevant dose relates side effects of sirolimus are hyperlipidemia, thrombocytopenia and leukopenia (Spada et al., 2009).

Everolimus

Everolimus is a selective mTOR (mammalian target of rapamycin) inhibitor. Everolimus binds to the intracellular protein FKBP-12, forming a complex that inhibits mTOR complex-1 (mTORC1) activity.

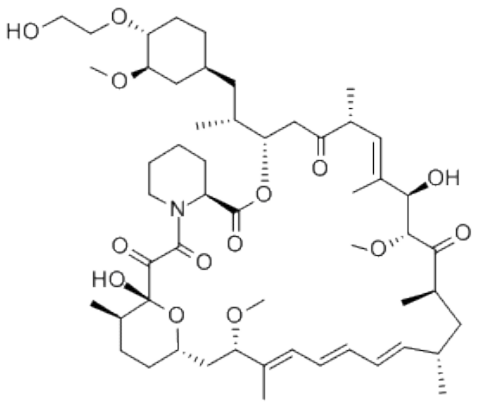


Figure 8: Everolimus chemical structure

Inhibition of the mTORC1 signaling pathway interferes with the translation and synthesis of proteins by reducing the activity of S6 ribosomal protein kinase (S6K1) and eukaryotic elongation factor 4E-binding protein (4EBP-1) that regulate proteins involved in the cell cycle, angiogenesis and glycolysis. S6K1is thought to phosphorylate the activation function domain 1 of the estrogen receptor, which is responsible for ligand-independent receptor activation. Everolimus reduces levels of vascular endothelial growth factor (VEGF), which potentiates tumor angiogenic processes. Everolimus is a potent inhibitor of the growth and proliferation of tumor cells, endothelial cells, fibroblasts and blood-vessel-associated smooth muscle cells and has been shown to reduce glycolysis in solid tumours in vitro and in vivo.

Pharmacokinetic:

Absorption and Distribution:

In patients with advanced solid tumors, peak everolimus concentrations (Cmax) are reached at a median time of 1 hour after daily administration of 5 and 10 mg everolimus under fasting conditions or with a light fat-free snack. Cmax is dose-proportional between 5 and 10 mg. Everolimus is a substrate and moderate inhibitor of PgP. Food effect In healthy subjects, high fat meals reduced systemic exposure to everolimus 10 mg (as measured by AUC) by 22% and the peak plasma concentration Cmax by 54%. Light fat meals reduced AUC by 32% and Cmax by 42%. Food,

however, had no apparent effect on the post absorption phase concentration-time profile. Plasma protein binding is approximately 74% both in healthy subjects and in patients with moderate hepatic impairment

Metabolism and Elimination.

EVE is a substrate of CYP3A4 and PgP. Following oral administration, EVE is the main circulating component in human blood. Six main metabolites of everolimus have been detected in human blood, including three monohydroxylated metabolites, two hydrolytic ring-opened products, and a phosphatidylcholine conjugate of everolimus. These metabolites were also identified in animal species used in toxicity studies, and showed approximately 100 times less activity than everolimus itself. Hence, everolimus is considered to contribute the majority of the overall pharmacological activity. The mean elimination half-life of everolimus is approximately 30 hours. Following the administration of a single dose of radiolabelled everolimus in conjunction with ciclosporin, 80% of the radioactivity was recovered from the faeces, while 5% was excreted in the urine.

Pharmacology Interactions

Co-administration with inhibitors and inducers of CYP3A4 and/or the multidrug efflux pump P-glycoprotein (PgP) should be avoided. If co-administration of a moderate CYP3A4 and/or PgP inhibitor or inducer cannot be avoided, dose adjustments of EVE can be taken into consideration. Concomitant treatment with potent CYP3A4 inhibitors result in dramatically increased plasma concentrations of everolimus. There are currently not sufficient data to allow dosing recommendations in this situation. Caution should be exercised when EVE is taken in combination with orally administered CYP3A4 substrates with a narrow therapeutic index due to the potential for drug interactions. If EVE is taken with orally administered CYP3A4 substrates with a narrow therapeutic index (e.g. pimozide, terfenadine, astemizole, cisapride, quinidine or ergot alkaloid derivatives), the patient should be monitored for undesirable effects described in the product information of the orally administered CYP3A4 substrate.

1.8 Immunosuppression and Therapeutic Drug Monitoring

Immunosuppression involves an act that reduces the activation or activity of the immune system. Immunosuppressants are used to control severe manifestations of allergic, autoimmune and transplant-related diseases. Some drugs have a diffuse effect on the immune system while others have specific targets. Generally, immunosuppression is induced to prevent the rejection of a transplanted organ or for the treatment of autoimmune diseases such as rheumatoid arthritis or Crohn's disease. Immunosuppressive agents are required in solid organ transplantation for induction of immunosuppression in the early phase, maintenance of immunosuppression in the late phase or for the treatment of organ rejection (Moini et al., 2015).

Immunosuppressive regimens include calcineurin inhibitors, anti-metabolites, mTOR inhibitors, steroids and antibody-based therapies. These agents target different sites in the T cell activation cascade, usually by inhibiting T cell activation or proliferation or via T cell depletion. The selection of agents is based on an individual's medical history as well as on institution experience and preference. Most immunosuppressive regimens combine drugs with different sites of action of T cell response, allowing for dosage adjustments to minimize side effects and toxicities. Currently, the mainstay of maintenance immunosuppressive regimens are calcineurin inhibitors (CNIs), used in greater than 95% of transplant centers upon discharge, although there is a known increased risk of renal impairment, metabolic derangements, neurotoxicity and de novo malignancieswith the long-term use of these medications (Pillai & Levitsky, 2009).

Individualizing patient's drug therapy to optimize balance between therapeutic efficacy and the occurrence of adverse events is the main goal for physicians. This concept applies to all

drugs but is of particular importance for narrow therapeutic index (NTI) agents, such as the immunosuppressants. For these drugs the difference in the concentrations exerting therapeutic benefit and those causing adverse events is small and further complicated by a considerable between-subject pharmacokinetic variability, and a poor relationship between drug concentration and pharmacological response. This approach offers the opportunity to reduce the pharmacokinetic component of the variability by controlling drug therapy based on concentrations achieved in the body rather than by the dose alone. The advent of the genomic era has brought several new fields of study, including pharmacogenomics, which seek to link drug treatment with the individual's genetic makeup. Pharmacogenomics holds many promises for improved treatment of a large variety of medical conditions, including immunosuppression for organ transplantation. Those involved in TDM are now realizing the potential role of pharmacogenomics in influencing individual patient's exposure to immunosuppressive agents and concomitant therapy. As rapid techniques for assessing genetic polymorphisms become available, they are likely to play a significant part in planning the initial doses of immunosuppressive drugs and tailor maintenance therapy (Cattaneo D., 2005).

1.8.1 Intracellular TDM

Peripheral blood mononuclear cells (PBMCs), like many other biological matrices, are an important subject of investigation for TDM analysis. In fact, more and more studies show them to be a good matrix for therapeutic drug monitoring in several settings.

First, these cells are the best choice in case of therapies in which the drug target is the PBMC itself (Lemaitre, Antignac, Verdier, Bellissant, & Fernandez, 2013). Several studies indeed show significant correlations between concentrations and clinical outcomes (Capron et al., 2012), confirming the importance of this matrix for monitoring. Secondly, for many drugs, plasma or whole blood concentration are considered the best surrogate for levels inside the active side, but PBMC could be a better choice.

Challenges of intracellular dosing

While for many biological matrices, the extraction process is well-established and follows precise guidelines, for PBMC there are many discrepancies between the various studies produced to date that highlight the need for standardization (Bazzoli et al., 2010).

There are many aspects to be taken into account, but the pre-analytical ones are the most relevant:

1. PBMCs extraction: samples must be immediately available in the laboratory and there must be maximum an hour between sampling, isolation and extraction stages (Becher et al., 2002). The washing step is crucial and must be at 4^{o}C. Also, Yeo et al show that using Lymphoprep® instead of Ficoll-Paque gives some advantage in terms of repeatability and ease of use for the operators.
2. Cell count: In most studies cells are determined on a small aliquot with a Coulter Counter, or using a Malassez cell and a microscope. However this last procedure may suffer from insufficient accuracy and precision. The concentration is therefore expressed as amounts

per 10^6 cells and can be converted in amount per volume on the approximation that the PBMC volume would be 0.4 pL in order to compare intracellular and plasma concentrations (Gao, Cara, Gallo, & Lori, 1993). The accuracy of this volume may be questionable as it varies according to the state of the cells (quiescent or stimulated) or to the nature of the cells (cell volume of human lymphoblast: 2.1 pL) (Traut, 1994). This highlights the pitfalls of the conversion, and that's why measuring the mean corpuscular volume (MCV) for each patient/sample, during PBMC separation could compensate for inter-individual variability of corpuscular volume evaluation (D'Avolio et al., 2011).

3. Loss of drug during extraction: before discussing this aspect, it is important to remember that every drug has different characteristics that lead the molecules to be linked to the membrane or in the cytoplasm in free form or linked to proteins. Whatever the location, these molecules could be lost during cell preparation and extraction, so it could be very relevant and useful to quantify the washing solution after extraction in order to have a more precise and less unbiased data.

In conclusion, the standardization of extraction processes and cell counts, the expression of results in concentration and not mass / number of cells and finally the use of real MCV for normalization of the results could help in making these procedures more precise and repeatable.

1.9 Complications of prolonged immunosuppression after transplant

1.9.1 Infections

The most obvious consequence of immunosuppression is the increased risk of infection. Infections are in fact the most common causes of morbidity and mortality after transplantation (Girlanda, 2013). Improved immunosuppressive regimens while reducing the incidence of allograft rejection, have increased the susceptibility to opportunistic infections. The most common viral infections, bacterial infection and fungal infections are assumed in Table 7.

Adenovirus
Cytomegalovirus (CMV)
Epstein-Barr virus (EBV
Herpes simplex (HSV)
Influenza-Parainfluenza
Polyoma (BK)
Rotavirus
Varicella-zoster virus

Table 7: Post-transplant viral infections (Girlanda, 2013).

Infectious complications in children differ to some extent from those in adults and depend on the completeness and seroconversion achieved before transplantation for vaccine-preventable disease. Overall infections after pediatric liver transplantation and an analysis of risk factor have been reported by Shepherd et al. (Shepherd et al., 2008). The risk of death from infections was much higher in infants, whose rate of bacterial and fungal infections was 3 times greater than that of adolescents. Infections were the most common causes of death: the risk of death from an infection was 10 times greater than the risk of death from rejection. (Dhawan, 2011).

1.9.2 Growth Failure

Although poor growth has been found 6-12 months after liver transplant, most children display accelerated growth in 1- 4 years of transplantation. Their final height remains lower than that of adults not underwent transplantation. Growth may also be impaired by the use of high doses of immunosuppression (Dhawan, 2011).

1.9.3 Lymphoproliferative Disease

Lymphoproliferative disease occurs in 5-10% of children and is mainly caused by abnormal proliferation of B lymphocytes driven by Epstein-Barr virus (EBV). Other risk factors are the prolonged use of high dose immunosuppression and the use of anti-lymphocyte antibodies. Diagnosis of this condition is important because early interventions (immunosuppression reduction, immunotherapy, or both) can improve the outcome significantly (Dhawan, 2011).

1.9.4 Allergies

The pathogenesis of Transplant-acquired allergy (TAA) is not still completely understood. Most of the studies support the concept that the functioning liver itself, and not only tacrolimus immunosuppression, is one of the main contributors to TAA. Immature immunosystem play an important role in their predisposition to allergic disease (explained by their limited exposure to dietary antigens). An increased prevalence of food allergy noted in children under immunosuppression supports the hypothesis that selective suppression of Th1 lymphocytes by the interleukine (IL)-2 inhibitor promotes Th2 lymphocytes and an allergic immune response. TAC is also known to increase intestinal permeability, which may lead to increased exposure to allergenic proteins and a further shift toward Th2 cytokines and IgE production against these proteins (Ozdemir, 2013).

1.10 Pharmacogenetics: overview on TAC

To reach the intracellular target, the most of immunosuppressive drugs pass through the cell membrane by passive diffusion (as free fraction), since most of them are apolar and non-ionized, but partly by active transport. Once in the cytoplasm, part of the drug may be rejected out of the cells by efflux transport proteins, resulting in variable amount of intracellular drugs depending on the efflux activity. As active process, this efflux is subject of competition, saturation, inhibition, induction phenomena but may also depend on genetic polymorphism of the transport proteins expressed in cell membrane (Capron, Haufroid, & Wallemacq, 2016).

1.10.1 P-glycoprotein (P-gp)

Most IS drugs are substrates of the efflux pumps P-gp, which, for example, affect intracellular TAC and inter individual variability of its activity may influence the immunosuppressive effect of TAC (Vafadari et al., 2013). P-gp, the product of the ABCB1 gene, is of particular interest because it was found in membranes of lymphocytes (Callaghan, Crowley, Potter, & Kerr, 2008; Cascorbi, 2006; Coon et al., 1991; Leschziner, Andrew, Pirmohamed, & Johnson, 2007; Meaden, Hoggard, Khoo, & Back, 2002), their main therapeutic target, where it removes CNIs from the intracellular compartment of lymphocytes. In the liver, this efflux enhances the biliary excretion of drugs. Several SNPs have been reported for ABCB1 (Kim et al., 2001) three of them (1236 C>T, 2677 G>T/A and 3435 C>T) are in strong linkage disequilibrium. The SNP in exon 26 (for example 3435 C>T) has been associated with reduced mRNA expression, stability and changes in substrate specify (Elens et al., 2007). The ABCB1 3435CC genotype is associated with a higher ABCB1 function function compared with the 3435 CT and 3435 TT genotypes (Kimchi-Sarfaty et al., 2007). Vadhari et al. in 2013 showed that in TAC treated renal transplant patients with 3435 CC genotype, after treatment with a blocker of ABCB1, the TAC effects are enhanced. In contrast, this not influence

patients with TT genotype (Vafadari et al., 2013). This could be explained by the fact that in CC genotype patients, TAC is more effectively pumped out of the cells, which leads to lower TAC concentration at its target, so, these patients need higher TAC dose than those with the TT genotype. These results were in line with others of Hoffmeyer (Hoffmeyer et al., 2000). Although a few reports found that high intestinal levels of P-gp were associated with TAC disposition after liver transplantation (Perez-Tomas, 2006), most studies did not find any influence of ABCB1 genotypes on TAC pharmacokinetics (Chen et al., 2014; Summers, Moore, & McAuley, 2004), especially in pediatric recipients (Kock et al., 2007; Vafadari et al., 2013).

1.10.2 Cytochromes P450 (CYP450)

Immunosuppressors are also substrate of CYP450 enzymes, in particular of CYP3A4 and CYP3A5. Cytochromes oxidative activity is described within the lymphocyte. CYP3A5 is the major enzyme responsible for the TAC metabolism of TAC and is found in the liver. A polymorphism in the CYP3A5 gene (A>G) was found to be strongly associated with CYP3A5 protein expression. At least one CYP3A5*1 allele was found to express large amount of CYP3A5 protein, whereas homozygous for the CYP3A5*3 allele did not express significant quantities of CYP3A5protein, which causes severe decrease of functional CYP3A5. It was demonstrated that CYP3A5*1/*1 or *1/*3 (“expressors”) are significantly associated with lower dose adjusted TAC exposure and increased TAC dose requierements, in order to achieve target blood concentrations compared with CYP3A5*3/*3 (“non-expressors”). However, the impact of the CYP3A5 genotype of both recipient and donor (graft liver) should be taken into account when evaluating TAC pharmacokinetics (Chen et al., 2014). On the first day after transplantation, Calvo et al. observed statistically significant differences were observed between expressors and non-expressors. They observed also that at

the 1st day after transplant, the recipient combined genotype not seems to play an important role in TAC disposition in pediatric population, instead of the donor genotype: statistically significant differences in TAC concentration were observed between CYP3A5*1/*1 - *1/*3 and CYP3A5 *3/*3 groups (Calvo et al., 2017).

CYP3A4 is found in the liver, intestine and pancreas. Until today were identified 42 SNPs, of which CYP3A4*22B (392 A>G), CYP3A4*2 (222 Ser>Pro), CYP3A4*3 (445 Met>Thr) (140) are the mains. The most studied variant is CYP3A4*22B, characterized by a C>T transition in the six intron of the gene: this variant determine a lower enzymatic activity with an higher drug concentration (Gervasini, Vizcaino, Gasiba, Carrillo, & Benitez, 2005; Lamba et al., 2002; Sinues et al., 2007). Unfortunately the functional meaning of that genetic variant is controversial and studies have no demonstrated a real correlation between TAC pharmacokinetic and CYP3A4 (Cho et al., 2012; Hesselink et al., 2008; Roy, Barama, Poirier, Vinet, & Roger, 2006).

1.10.3 Immunosuppressors and intra-PBMC TDM

Therefore whole blood TDM contributed to improve efficacy and reduce toxicity of immunosuppressors, the relationship between blood concentration and acute cellular rejection (ACR) remains unclear. In fact, ACR remain an issue even though blood levels are within the therapeutic ranges, suggesting that immunosuppressants blood concentration do not totally reflect their pharmacological effect. The site of action of CNIs, mTORs and MPA is inside lymphocyte. If seems therefore reasonable to assume that drug concentration at the target site (intra lymphocytes) are more relevant than whole blood concentrations, in predicting treatment efficacy (Capron et al., 2016). Consequently, immunosuppressors concentration in peripheral blood mononuclear cells (PBMCs), a blood compartment enriched with lymphocytes, could represent a more reliable measure of immunosuppressive activity (Capron et al., 2012).

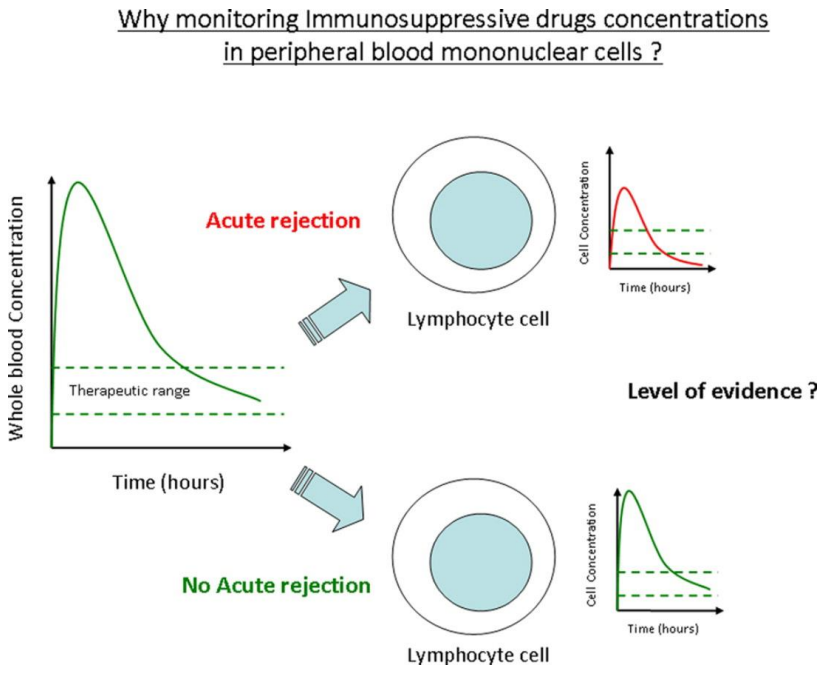


Figure 9: intra-PBMC monitoring importance

(Lemaitre, Antignac, & Fernandez, 2013)

Intra-PBMC TAC and EVE TDM

Measurement of TAC and EVE at their target site (tissue or lymphocyte) has been proposed as possible approach to monitor the risk of organ rejection (Lemaitre, Antignac, Verdier, et al., 2013).

Capron et al. highlighted that TAC concentration in PBMC significantly correlated with both the development and the severity of rejection: lower intra-PBMCs TAC levels were associated to istological rejection and, unlike whole blood levels, these levels were significantly correlated to the onset of rejection episodes at one week after transplantation (Capron et al., 2012).

Several HPLC, LC-MS and LC-MS/MS methods were reported for TAC quantification, but most of these evaluated TAC in whole blood (Kirchner, Meier-Wiedenbach, & Manns, 2004; Streit, Armstrong, & Oellerich, 2002; Tszyrsznic et al., 2013; Volosov, Napoli, & Soldin, 2001). Capron et al. published a method to measure TAC concentrations in PBMCs but its quantification was performed by LC-MS/MS only after a cumbersome and time-expensive extraction procedure (Capron et al., 2009).

There are limited data on intra PBMC EVE concentration; only two works were published with contrasting results on the correlation between intra-PBMC and whole blood EVE concentration.

Roullet-Renoleau et al. have shown a weak correlation between EVE whole blood and PBMC concentration. This results suggest that EVE concentration in PBMC do not reflect EVE whole blood concentration (Roullet-Renoleau et al., 2012). However Robertsen et al. shown a significant association between EVE whole blood and PBMC concentration (Robertsen et al., 2015).

Intra tissue TDM

Tissue analysis is not new. As early as 1950's researchers had performed tissue analysis to study drugs distribution into tissues for understanding their pharmacokinetic (PK) and pharmacodynamic (PD) profile (Hu, Lai, So, Chen, & Yao, 2012). Intra-tissue drug concentrations are fundamental to evaluate drug efficacy and toxicity at the site of action, as well as for monitoring drug interactions and inter-subject variability in drug response. Blood or plasma drug concentrations are typically used as surrogate for the ones at the site of action. However, plasma PK profiles do not necessary underline target drug penetration/accumulation. In fact, plasma exposure data do not take into account of uptake, efflux, metabolism and interstitial fluid flow. Although, the assumption that unbound drug concentrations in the systemic circulation mirror intracellular unbound drug concentrations at the site of action is widely acknowledge. Therefore, this assumption is based on the free-drug hypothesis that unbound drug concentrations on either side of a membrane are in thermodynamic equilibrium. Unfortunately, this is not valid for poorly permeable (charged or polar compounds), actively transported and highly protein-bound drugs: the majority of the drugs (Chu et al., 2013). For this reason, efficiently quantifying drugs at the real sites of action could be important. The sites of action of the most of the drugs, excluding only those acting on blood cell (erythrocytes, lymphocytes, leucocytes, monocytes) or on macrophages, are solid tissues. Method already published measure drug concentrations in homogenized tissue sample: the result is therefore an average of the extracellular and intracellular concentrations. However, because the cells make up about 70% of the volume of most tissue samples, the intracellular concentration has a dominant influence, so it is a good indicator of intracellular drug concentration (Greenwood, 2012). For anti-infective drugs, the infection site is typically located outside plasma, therefore, quantifying drug exposure in tissues represents

an opportunity to relate the pharmacologically active concentrations to an observed pharmacodynamic parameter, such as the MIC (Gonzalez, Schmidt, & Derendorf, 2013).

Thus bioanalysis of drugs and then metabolites in tissues can play an important role in understanding the pharmacological and toxicological properties of new drug candidates(Xue et al., 2012). To yield better drug candidates, we need to know where the drugs are distributed in the body, whether the drug accumulated in the tissue and reached a level that would lead to organ damage (Ho, 2012). Most drugs bind to plasma and tissue proteins, resulting in a decrease in free, pharmacologically active concentrations. Current methods for drugs intra-tissue quantification express results as "ng/mg of tissue", making these not comparable with plasma or blood concentrations, as volume ratio. The TAC blood concentration has contributed to improve efficacy and reduce the toxicity but the relationship between these concentration and rejection is still unclear, therefore seems to be necessary understand if these concentration mirror those at the graft site of action.

1.11 H/Ultra Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is is a technique in analytic chemistry used to separate the components in a mixture, to identify and/or quantify each component.

It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material.

Each component in the sample interacts slightly differently with the adsorbent material, causing different retention for the different components and leading to the separation of the components as they flow out the column.

During the run, the mobile phase can always be identical or can change over time, subjecting the molecules to a gradient of solvents: when the mobile phase affinity to the stationary phase overcomes the molecules one, the molecule detaches from the matrix and elutes.

The use of autosamplers allowed to apply such techniques for the analysis of large batchs of samples, containing the analytical variability and making possible the use of calibration curves, running simultaneously with the samples.

The HPLC system has been revisioned and updated, leading to new systems, called UPLC (Ultra Performance Liquid Chromatography), which work to a higher pressure, reducing the duration of analysis and improving the chromatographic separation.

The UPLC is based on the use of stationary phase consisting of particles less than 2 μm (while HPLC columns are typically filled with particles of 3 to 5 μm).

The underlying principles of this evolution are governed by the van Deemter equation, which is an empirical formula that describes the relationship between linear velocity (flow rate) and the Height Equivalent to a Theoretical Plate (HETP or H):

$$H = A + B/\mu + C^* \mu$$

Analyzing it in detail:

- A is dependent on the stationary phase characteristics and is defined as $A=2\lambda d_p$ were λ is the particle shape and d_p is particle diameter.

- B is a measure of the mobile phase interaction over the stationary phase and is defined as $B=2G*Dm$, were G is a constant and D_m is its diffusion coefficient (depending on viscosity and temperature).

- C is the mass transfer coefficient and is defined as $C=\omega*d_c^2/Dm + R*d_f^2/D_s$, were ω and R are constants, d_c is the capillary diameter, D_m is the mobile phase diffusion coefficient d_f is the film thickness and D_s in the diffusion coefficient of the stationary phase (depending on the chemical structure stationary phase and on temperature).

- μ is the linear velocity of the mobile phase flow (flow rate/column section).

From this equation is evident that the decrease in particle diameter decreases at the same time the H. So being **H=L**(*column length*)/**N**(*number of theoretical plates or column efficiency*), N results much higher in columns with a lower particle size. In general a three-fold reduction in particle size results in a threefold increase in N and, being the chromatographic resolution (R) equal to the square root of N, it increases of nearly 1.7.

The advent of UPLC has demanded the development of a new instrumental system for liquid chromatography, which can take advantage of the separation performance (by reducing dead volumes) and consistentwith the pressures (about 8000 to 15,000 PSI, compared with 2500 to 5000 PSI in HPLC). Efficiency is proportional to column length and inversely proportional to the particle size. Therefore, the column can be shortened by the same factor as the particle size without loss of resolution. The application of UPLC resulted in the detection of additional drug metabolites, superior separation and improved spectral quality.

1.11.1 UPLC system

Like HPLC systems, also UPLC systems consists of a pumping unit , an autosampler, a column heater and, finally, a detector. The pumping unit delivers mobile phases flow (from 1 to 4 phases) with a limit pressure of 15,000-psi (about 1000 bar), to take full advantage of the sub-2µm particle size in the stationary phase.

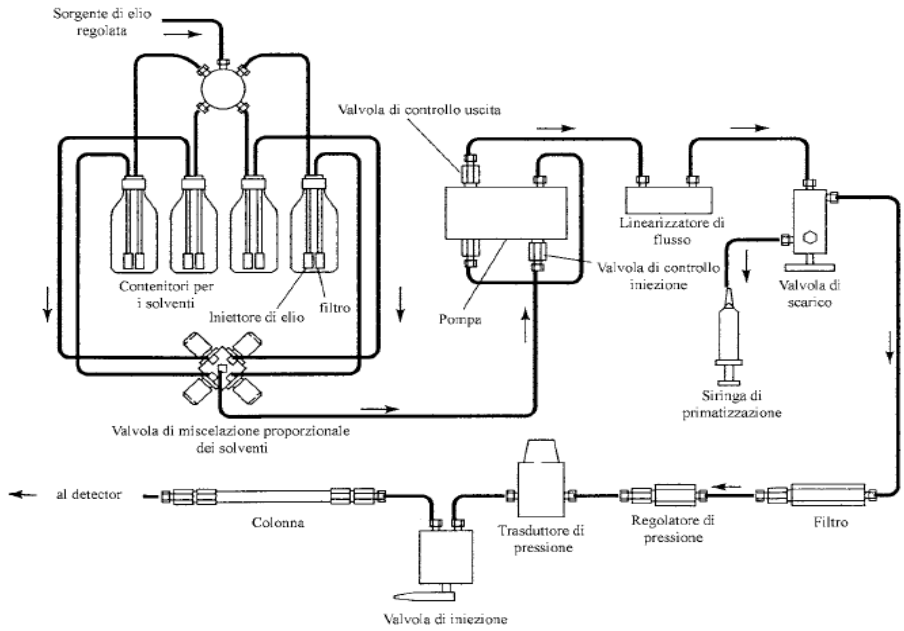


Figure 10: UPLC system overview.

Figure 11: Waters Acquity ® UPLC System with Triple Quadrupole Detector (TQD) with on-line SPE manager OSM (http://www.waters.com). 1.OSM; 2. Binary Solvent Manager (BSM); 3. Column Manager (CM); 4. Sample Manager (autosampler, SM); 5. TQD.

1.11.2 DETECTOR – Triple Quadrupole Mass Spectrometer

A chromatographic detector has to be capable of establishing both the identity and concentration of eluting components in the mobile phase stream. A broad range of detectors is available to meet different sample requirements: in particular triple-quadrupole mass spectrometer, also known as TQD, has recently become a standard for TDM purposes, because of its great sensibility and specificity.Basicly, in a tandem MS detectorthe firstand thirdquadrupoles act as mass filters and the second causes fragmentation of the analyte through interaction with a collision gas.The first quadrupole (Q1) selects the precursor ion of interest through a magnetic deflection of all other ions, which are so discarded, on the basis of its mass/charge ratio (m/z). The precursor ion is then transmitted to the collision cell (also considered a second quadrupole, Q2), where it is fragmented, usually by energetic collisions with a inhert gas (i.e. Argon), thenall the fragmented ions are collimated and passed into the third quadrupole (Q3). Just like Q1, Q3 performs a mass discriminationon the product ions (daughter scan) that compose the tandem mass spectra and are very specific for themolecular structure. So, the whole chromatographic and tandem MS process results in a sequential increase in specificity: chromatographydiscriminates temporally (retention times) the target molecules on the basis of their chemical properties, then, at the corresponding retention times, the Q1 selects the molecules with the right m/z ratio (mother ions) and Q2/Q3 further identifiy the target molecules on the basis of their specific molecular structure, which determines the resulting daughter ions. The resulting very low background noise allows the instrument to greatly increase the sensibility of the assay respect to previous detectors (single MS, PDA, fluorescence and UV).

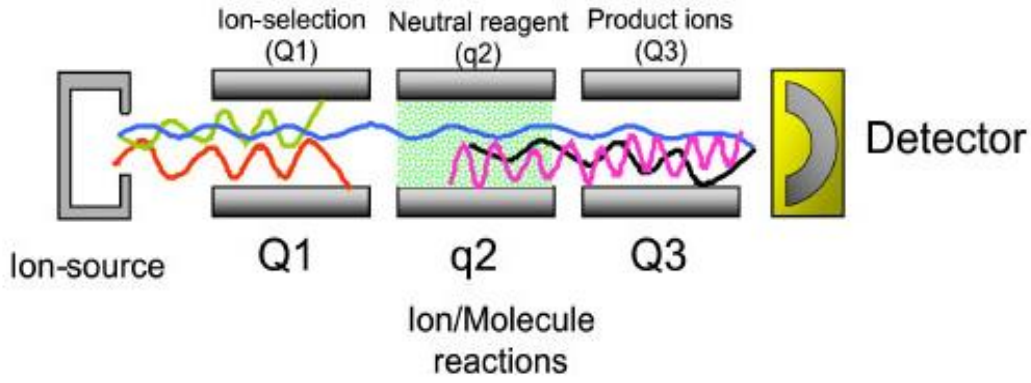


Figure 12*:* Triple Quadrupole detector overview. After ionization the target molecules, in the first quadrupole (Q1) are collimated through a magnetic field towards the collision cell (Q2), where the collision with inhert gas breaks down the mother ion in fragments; these fragments are selected and collimated through the third quadrupole (Q3) and finally detected.

1.12 Validation Of Bioanalytical Methods

The development of methods for the quantitative determination of drugs requires adequate validation. In order to harmonize the specific requirements for bioanalytical methods to be considered legit, international guidelines are regularly updated by the relevant organizations (eg FDA). The validation of HPLC methods is carried out in different ways, according to the situations:

- Full Validation: During development and implementation of a novel bioanalytical method. For analysis of a new drug entity. For revisions to an existing method that add metabolite quantification.
- Partial Validation: Partial validations evaluate modifications of already validated bioanalytical methods. It can range from as little as one intra-assay accuracy and precision determination to a nearly full validation.
- Cross Validation: comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies. An example of cross-validation would be a situation in which an original validated bioanalytical method serves as the reference, and the revised bioanalytical method is the comparator. The comparisons should be done both ways.

The full validation requires the repetition of chromatographic runs, containing a calibration curve and at least 3 quality controls at a known concentration, analyzed in duplicate. Once completed validation sessions, the following parameters are obtained:

• Specificity/selctivity: the ability of the bioanalytical method to measure and differentiate the analytes in the presence of components that may be expected to be present. These could

include metabolites, impurities, degradation products, concomitant drugs or matrix components.

• Sensitivity: is defined as the lowest analyte concentration that can be measured with acceptable accuracy and precision (i.e., LLOQ).

• LLOQ (Lowest Limit Of Quantification): the lowest value that can be quantified with precision and accuracy, with a coefficient of variation less than 20% (for calibrators above the LOQ, CV% should be less than 15%).

• LLOD (Lowest Limit Of Detection): the lowest concentration of an analyte that the bioanalytical procedure can reliably differentiate from background noise. By definition is the value in which the ratio between the signal of the analyte and that of the noise is greater than 2: 1.

• Recovery: The extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method. It must be repeatable and reproducible over time, but not necessary near to 100%.

• Stability: The chemical stability of an analyte in a given matrix under specific conditions for given time intervals.

2. Aims:

The main aims of the PhD project were the development and validation of a method for the quantification of TAC in its target (PBMCs) and, after the increase of enrolled patients in co-therapy with EVE or in EVE monotherapy, of another method for the quantification both of EVE and TAC in PBMC. The development of these methods was necessary for the evaluation of the presence or not of correlation between intracellular and whole blood data, which are those used in the clinical TDM routine of immunosuppressors. The obtained data would be used for studying, in a prospective study, the possible relationship between TAC concentrations data and advent of allergies, lymphoproliferative disorders and rejects; and also to evaluate possible cut off for TAC intra-PBMC and ratio concentrations.

A secondary aim, in collaboration with the Unit of Pediatric Gastroenterology and Hepatology of the University of Turin, was the correlation between genetic and adverse events and intra-PBMC concentrations obtained.

The third, and the last aim, was to develop a method for the quantification of immunosuppressants into tissue (firstly only on TAC) for obtaining results in a measure unit comparable with that of the data obtained with intra-PBMC and whole blood methods..

3. Materials and Methods

This section describe materials and methods used for the development of all of 3 methods:

1) **intra-PBMC TAC quantification**

2) **intra-PBMC TAC and EVE quantification**

3) **intra-tissue TAC quantification**

3.1 Patients and inclusion criteria

Pediatric patients (aged <18 years at transplant), managed on oral tacrolimus and/or everolimus after a liver transplant performed at A.O.U. Città della Salute e della Scienza, Torino, Italy, were enrolled into the study. DNA samples from the recipients and their donors were provided by the Regional Transplantation Centre (CRT Piemonte e Valle d'Aosta, Italy). Approval of the local ethic committee (protocol identification number 15386/28.3) was obtained, as was a written informed consent from the patients parents or guardians. TAC and EVE were orally administrated based on patient weight (0.015 mg/kg BID and 2/3 mg/kg OD). After an initial determination of optimal dose with hematic TDM of TAC concentration (first 15 days), as indicated by the treatment guidelines, the selected dose was maintained for 6 months. Blood samples were collected into CPT® tubes: 16 mL for 10-18 years old patients, 8 mL for 5-10 years old patients and 4 mL for < 5 years old patients. After separation, PBMCs samples were stored at -80°C until analysis. After each analysis, drug amounts quantified in PBMC samples were normalized by cell number and MCV in each single sample, as described by Simiele, according to the formula: $[DRUG]_{PBMC}=DRUG_{AMOUNT}/(Cell\ N° \times MCV)$ (D'Avolio, Pensi, Baietto, & Di Perri, 2014; FDA, 2013b; Pensi et al., 2015; Simiele et al., 2011; Vogeser & Seger, 2010). Intra-PBMC concentrations have been compared with whole blood concentrations obtained through UHPLC-MS/MS using MassTrak Immunosuppressants XE kit (CE-IVD marked; Waters, Milan, Italy). At this stage, no biopsy from real patients were already tested.

3.2 Chemicals, Reagents and Blank Matrices

TAC, EVE, 6,7-Dimethyl-2,3-di(2-pyridyl)quinoxaline, formic acid and ammonium acetate were purchased from Sigma-Aldrich Chemical Company (St. Luis, MO, USA). Ascomycin was obtained from Adipogen (Liestal, Switzerland). HPLC grade acetonitrile and methanol were purchased from J.T.Baker (Deventer, Holland). Ultrapure water was produced with the Synergy 185 water purification system from Millipore (Milan, Italy). Buffy coats from healthy volunteers were kindly provided from the blood bank of "Maria Vittoria" Hospital (Turin, Italy).

3.2.1 Blank PBMCs and Blank Tissue samples isolation procedure

Both blank PBMCs, used for the preparation of standards (STDs) and quality controls (QCs), and real PBMC samples were isolated following the same protocol, from buffy-coats of healthy volunteers and from patients blood samples, respectively. Briefly, isolation was performed with CPT tubes®, centrifuged at room temperature for 15 minutes (1600*g* for 15 min at 25°C with a 4227 R centrifuge [ALC, Milan, Italy]). The obtained PBMCs layer was collected into a falcon and washed twice in 40 mL ice-cold sodium chloride 0.9% solution, to prevent drug loss, and centrifuged at 2200 *g* for 6 min at 4°C with a Jouan model BR4i centrifuge (Saint-Herblain, France). To obtain red blood cells lysis, before the second wash, pellet was treated with 2 mL of ammonium salt solution (130 mM ammonium chloride + 7.5 mM ammonium carbonate) for 1 minute. After adjusting the volume again to 40 mL with sodium chloride 0.9% solution, 500 µL of cell suspension were diluted with 19.5 mL of Isoton in two beakers and used for cell count and determination of Mean Cell Volume (MCV) through an automated Beckman Coulter Z2 (Instrumentation Laboratory, Milan, Italy). Four counts for each sample (two for each beaker) were performed. Data were processed by Z2

AccuComp software (version 3.01). To obtain blank PBMC aliquots, the resulting PBMC pellet was dissolved with an extraction solution (methanol:water, 70:30 [vol:vol]) to cell concentration of $12x10^6$ cell/mL. The resulting cell lysates were divided in aliquots (500 µL), and then stored at −80°C. The same isolation procedure was used to obtain PBMCs samples from patients blood, which was directly collected into CPTs. The maximum cell concentration for each aliquot was $12x10^6$ cell/mL. The whole procedure took less than 1h.

Tissues used for the preparation of standards (STDs) and quality controls (QCs), derived from mice, cow and swine, after transplant, were rapidly washed two times with physiological salt solution (PBS), for eliminating, as much is possible, all blood contamination, and immediately stocked at -80°C. Before the analysis, frozen tissue was rapidly divided in portion of about 10-20 mg, each weighted with an analytical balance.

3.2.2 Preparation of standard and quality control sample

The stock solutions of TAC and EVE were prepared by dissolving powders into methanol and water (95:5 [vol:vol]), obtaining a concentration of 1mg/mL, and were stored at -80 °C. The two different internal standard (6,7-Dimethyl-2,3-di(2-pyridyl)quinoxaline and ascomycin) stock solutions used were prepared at 1 mg/mL in the same way: diluting the powders in pure ethanol. The resulting two internal standard (IS) working solutions (5 ng/mL[µmol/L]) were prepared by diluting stock solutions with methanol:water (50:50 [vol:vol]) at every analytical session. The calibrating solutions (containing only TAC or both TAC and EVE), used to spike standard samples (STDs 0-8) and quality controls (QCs), were prepared in water:methanol (50:50 [vol:vol]) by scalar dilutions from the stock solutions to achieve concentrations ranging from 50 ng/mL to 0.391ng/mL for calibrators, and of 40, 10 and 1 ng/mL for the preparation of high, medium and low QCs, respectively. These solutions were used to spike (100 µL) blank PBMCs samples with 6x106 cell/mL at each session.

3.2.3 PBMC isolation

PBMC from patients were extracted from blood (about 12 mL) through a separation on density gradient with two CPT tubes. After the separation (15 min, 1600 × g, 20 °C) the PBMC layer was transferred in a new falcon tube of 50 mL and washed with 40 mL of NaCl 0.9%. After this washing step, supernatant was discarded and pellet was added with 2 mL of ammonium salts solution(ammonium chloride and ammonium carbonate 7 and 0.072 g/L, respectively) and incubated for 1 min at room temperature, in order to eliminate eventually remaining erythrocytes. Then, NaCl 0.9% was added to reach a final volume of 40 mL and 500 µL of the resulting cell suspension was transferred in a beaker and put in a Bekman Coulter Z2(Instrumentation Laboratory, Milan, Italy), managed by Z2 AccuComp Software (Version 3.01) for the cell count. This counting method is eligible for the correct determination of the number and volume of PBMCs, evidencing moreover the absence of erythrocytes. After a new centrifugation, supernatant was discarded and cell pellet was suspended in 1 mL of H2O/methanol (30:70 v/v), divided in two 500 µL aliquots and stored at −80 °C. Blank PBMC from healthy donors were extracted from buffy-coat with the same procedure and then stored in aliquots of around 4 × 106 cells suspended in 500 µL of H2O/methanol (30:70 v/v) acidified with 0.5% formic acid.

3.3 Stability

The stability of stock solutions was evaluated for 6 months at -80 °C. The stability of TAC and EVE's calibrating solutions was evaluated for 3 months at -80 °C. TAC and EVE are known to be stable in these conditions (Capron et al., 2009; Roullet-Renoleau et al., 2012).

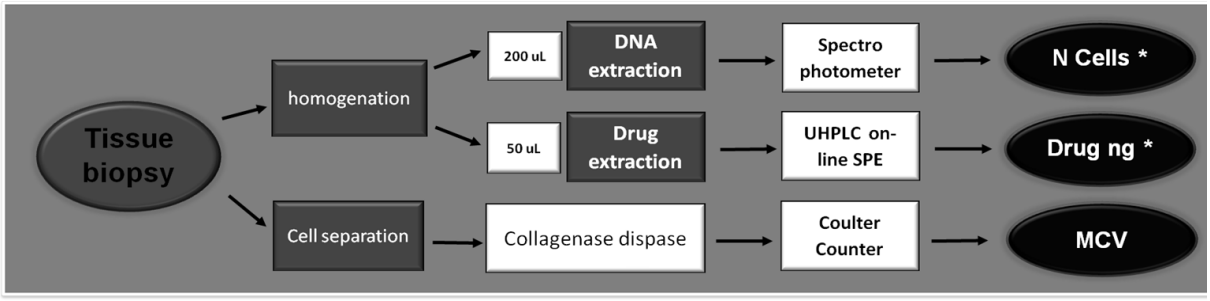
3.4 Sample preparation

3.4.1 Intra-PBMC quantification

40µL of internal standard solution were added to 500 µL of PBMC samples. Blank PBMCs were spiked with 100 µL of calibrating solutions, obtaining STDs 0-8 and QCs; 100 µL of a solution of methanol:water, 50:50 [vol/vol],without drug, were added to patients samples to reach the same volume. Each sample was vortex-mixed for 10 s, centrifuged at 21000 *g* at 4°C for 10 min and supernatant was transferred into total recovery vials. 100µL of methanol 100% were added to the residual pellet and, after vortex-mixing and another centrifugation step (21000*g* at 4°C for 10 minutes), 80 µL of the new obtained supernatant were transferred in the corresponding vials. 50 µL of the obtained extract was injected into the OSM®-UPLC MS/MS system.

3.4.2 Intra-tissue quantification

The entire methodological procedure is summarized into the following schema:



Homogenization:

10 uL of an IS, 10 uL of calibrant solution or H2O:MeOH[50:50] (for blank sample) and 200uL of Lysis Buffer Solution (Roche Diagnostic, Milan, Italy) each 10 mg of tissue were added. The homogenation was done using a MagNA Lyser Instrument (Roche Diagnostic,

Milan, Italy) for 40 sec at 4500 rpm. The supernatant obtained by homogenization was used both for DNA quantification and drug quantification.

Extraction procedure

35uL of the supernatant obtained by the homogenization are dissolved with 465 uL of extraction solution (H2O:MeOH [30:70]) and used for drug quantification. Each sample was vortex-mixed for 10 s and centrifuged at 21000g at 4°C for 10 min and supernatant was transferred into total recovery vials. 100µL of Methanol 100% were added to the residual pellet and, after vortex-mixing and another centrifugation step (21000g at 4°C for 10 minutes), 80 µL of the new obtained supernatant were transferred in the corresponding vials. 50 µL of the obtained extract was injected into the OSM®-UPLC MS/MS system.

DNA quantification and Spectrophotometric Analysis

PBMCs dsDNA calibration curve was obtained diluting a CAL 5 of 4.750.000 cell/mL to a CAL 1 of 296.875 cell/mL. The DNA was extracted using a MagNA Pure Compact Nucleic Acid Isolation Kit (Roche Diagnostic, Milan, Italy). After each DNA extraction were obtained 200 uL at which 10 uL of RNase were added. The corrispective dsDNA (ug/mL) was quantified using the spectrophotometer (Biophotometer, Eppendorf). The curve was used for evaluating the cell number of the tissue biopsy. In fact, the same DNA extraction procedure was made to 200 uL of the supernatant obtained by the sample homogenation: cell number was quantified by evaluating dsDNA absorbance into the calibration curve level.

MCV evaluation of tissue cells

From each biopsy, a part of tissue after collagenase-dispase treatment (Roche) was used to evaluate tissue cells Mean Cellular Volume (MCV) using a Beckman Coulter Counter.

3.5 Chromatographic system

The chromatographic system was an Acquity® UPLC (Waters, Milan, Italy), consisting in a binary pump, a refrigerated sample manager and a triple quadrupole detector (TQD) coupled with the new automated on-line solid phase extraction system (OSM®, Waters, Milan, Italy). Chromatographic separation was performed on an Acquity UPLC® BEH C18 1,7 µm (2,1 x 50 mm) column (Waters, Milan, Italy), protected by a pre-column frit (0.2 µm × 2.1 mm), heated at 45°C using a column thermostat. For the quantification of TAC and quinoxaline, the mass spectrometer was settled in the positive ion mode (ESI+), with a capillary voltage of 3kV, a source temperature of 150°C and a desolvation temperature of 500°C. While, for the quantification of TAC, EVE and ascomycin, the mass spectrometer was settled in the positive ion mode (ESI+), with a capillary voltage of 1kV, a source temperature of 150°C and a desolvation temperature of 400°C. In both methods developed, the nitrogen gas flow was 800 L/h and 50 L/h for desolvation and cone, respectively. For the method used only for the quantification of TAC and quinoxaline, the cone voltages and collision energies were, respectively, 58 V and 40 eV for quinoxaline , 30 V and 20 eV for TAC. In the second method the cone voltages and collision energies were, respectively, 17 V and 17 eV for IS, 25 V and 15 eV for EVE and 30 V and 20 eV for TAC. The monitored mass transitions were 821.53>768.3 for TAC, 975.5>908.5 for EVE, 809.39>756.45 for ascomycin and and 313.09>246.46 for quinoxaline, respectively. The chromatographic runs maximum duration was 6 minutes, with a flow rate of 0.4 mL/min for the method 6 minutes long, that quantify the 3 analytes, and of 0.5 mL/min for that that quantify TAC and quinoxaline in a run long just 5 min. Mobile phases, A and B, were water and methanol, respectively, both with 2 mM ammonium acetate and 0.1% formic acid (Table 1). Before chromatographic separation, the samples underwent an on-line solid phase extraction (conditions described in next subsection) on a dedicated single-use XBridge® C8 10 µm, 1x10mm OSM cartridge.

3.5.1 SPE method

On-line solid phase extraction were done with two different mobile phases. Phase C was water, with 2 mM ammonium acetate and 0.1% formic acid, and phase D is ACN. Before sample loading, each cartridge has been conditioned with 0.4 mL (flow 2mL/min) of phase D, and then equilibrated with 0.4 mL (flow 2mL/min) of a mixture of C and D (85:15 [vol:vol]). Sample loading was performed mixing 50µL of sample with 0.15 mL (flow 0.15 mL/min) of C and D (90:10 [vol:vol]), followed by a washing step with 0.10 mL (flow 0.1 mL/min) of the same mixture. Then, cartridges moved in elution position (on-line with the mobile phases flow to the column), where the gradient of A and B solvents allowed the elution (cartridges were held in the elution position for the whole run time). The whole process, except for elution step, was performed during the previous chromatographic run, maintaining the overall run-time of 5 or 6 min, depending on the quantification method used long.

3.6 Method validation

The assay for the intra-PBMC quantification was fully validated according to the FDA guidelines (revised 2013)(FDA, 2013b). The intra-tissue method received only a partial validation because no real patients sample were already enrolled for the study.

3.6.1 SPE-online method development

To develop the online-SPE method, analytes breakthrough, recovery and adsorption were evaluated using a dedicated function of the OSM® platform (advanced method development). Breakthrough is the fraction of analytes that is lost during of loading and washing steps. Conversely, adsorption is the fraction of analytes that are adsorbed to the fluidic system and not eluted from the cartridges, contributing to carry over and reducing recovery.

3.6.2 Specificity, selectivity and linearity

Interference from endogenous compounds was investigated by the analysis of five different blank PBMCs samples. Interference between compounds was also evaluated to avoid any cross-talk (interfering signal from other target analytes) (Vogeser & Seger, 2010). Linearity was assessed considering a minimum coefficient of determination (r^2) of 0.996 during the five validation sessions, considering a linear model forced through the origin of the axes. Processing was performed based on peak area.

3.6.3 Stability

Stock solutions and TAC and EVE's calibrating solutions were found to be stable at -80 °C for 6 and 3 months, respectively. TAC and EVE are known to be stable in these conditions (Capron et al., 2009; Roullet-Renoleau et al., 2012).

3.6.4 Accuracy, precision, and limits of quantification and detection

Intra-day and inter-day accuracy and precision were determined by performing the analysis of QC samples in multiple replicate (n=5 during each validation session). Inaccuracy was calculated as the mean percent deviation from the nominal concentrations. Mean value and relative standard deviation percent (RSD %) were calculated and used to express the intra- and inter-day imprecision. The lower limit of detection (LLOD) was considered as the concentration that yields a signal-to-noise ratio of 3. Lower limit of quantification (LLOQ) was considered as the concentration that yields a signal-to-noise of at least 5 and the level that showed a maximum inaccuracy and imprecision of 20%, as requested by FDA guidelines (FDA, 2013b; Pensi et al., 2015). Upper limit of quantification (ULOQ) was considered as the highest point of the calibration curve (STD 8).

3.6.5 Recovery

Recovery was considered as the relative difference between instrumental response obtained from the injection of extracts from QC samples and that obtained from injection of blank extracts added with the same concentrations of drugs (corresponding to 100% recovery) after the extraction (post-extraction addition protocol).

3.6.6 Matrix effect

Matrix effect was investigated by comparing peak areas corresponding to the injection of chemical mixes at QCs levels with those from the injection of blank PBMCs extracts spiked with the same amounts of drugs after sample cleanup (post-extraction addition protocol), as described by Taylor (Taylor, 2005). Matrix effect was evaluated at different cell concentrations ($3x10^6$, $6x10^6$, $12x10^6$, $24x10^6$ cell/mL). After the decision, in the second work published, of using another IS, more specific for the quantification of immunosuppressors and not just one that all laboratories could have (like quinoxaline, that is easy to find and cheap), the potential correcting power of IS was also investigated. This was done evaluating the "IS-normalized" matrix effect (nEM), calculated through the formula: $nEM = \{[(PA_{A\text{-}matrix}/PA_{IS\text{-}matrix})/(PA_{A\text{-}neat}/PA_{IS\text{-}matrix})\text{-}1]*100\}$. The RSD% value (measure of reproducibility of nEM and real indicator of the theoretical error) was calculated on the mean value of the $"(PA_{A\text{-}matrix}/PA_{IS\text{-}matrix})/(PA_{A\text{-}neat}/PA_{IS\text{-}matrix})"$ ratio.

Normalization of Results of tissue analysis

The results were normalize as ng/mL using the following formula:

$$\text{Concentration (ng/mL)} = \frac{\text{Drug ng}_{\text{in}}\ \text{N cells}}{\text{MCV}_{\text{x}}\ \text{N cells}}$$

The **N cells** is the number of cells into the biopsy. It is obtained through the quantification of dsDNA absorption of the cells derived by the homogenized sample.

The **Drug ng** is the amount of ng obtained from the homogenized sample through the UHPLC-MS/MS developed method.

The **MCV** is the Mean Cellular Volume of the tissue biopsy cells. It is obtained after a collagenase-dispase sample treatment and Coulter cell quantification.

3.7 Pharmacogenetic Evaluation

Blood aliquots were stored at -80°C before DNA extraction and genotyping. Genomic DNA extraction has been performed by using "FlexiGene DNA Kit" extraction kits (Qiagen, Valencia, CA).

Donor and recipient DNA samples were genotyped for CYP3A4*22, CYP3A5*3 and ABCB1 3435 C>T using the allelic discrimination reaction performed using TaqMan (Thermo Fisher Scientific, Waltham, MA, USA), same method used also in the article of Calvo et al. (Calvo et al., 2017). The CYP3A4*22 assay was validated by sequencing homozygous wild-type (*1/*1), heterozygous (*1/*22) and homozygous mutant (*22/*22) samples. Patients were classified as rapid metabolizers (RM) if the donor/recipient pair carried all, or at least five, CYP3A4/5*1 alleles; as extensive CYP3A4/5 metabolizers (EM), if they carried four CYP3A4/5*1 alleles; and as intermediate CYP3A4/5 metabolizers (IM), if they carried none, or up to three CYP3A4/5*1 alleles in their recipient and donor DNA.

3.7.1 Analysis of complication (EBV)

EBV infections are defined based on the hematic replication levels obtained with a PCR analysis : >1200 viral copies/mL, which is the cut-off used Virology Laboratory of Città della Salute e della Scienza Hospital (Turin, Italy).

3.8 Statistical Analysis

All the statistical analyses were performed through the SPSS ® software (IBM). The association between categorical variables was tested through Chi-square test. The correlation between continuous variables was tested through Pearson correlation test. Since the considered variables were not normally distributed, differences in continuous variables between groups were tested through non-parametric Mann-Whitney or Kruskall-Wallis tests, for two or more groups, respectively. Differences between two mutually dependent groups were tested through Wilcoxon test for paired groups. Variables predictivity for continuous or dichotomous variables were tested though linear or logistic regression analysis, respectively. Only evidences with a P-value lower than 0.05 were considered as statistically significant.

4. RESULTS

Two methods for quantification of TAC and TAC and EVE were developed, validated and published.

4.2 Paper 1

An UPLC-MS/MS method coupled with automated on-line SPE for quantification of tacrolimus in peripheral blood mononuclear cells.

A fully validated method for quantification of TAC intra-PBMC was developed and then applied to 100 PBMCs samples from 37 pediatric patients.

The main results reported into the article attached are:

"A good, but not excellent, correlation (r2=0.244, P= 0.002) between the intra-PBMC concentration and the blood one was observed. In particular, some outliers could be seen: these showed a high intra-PBMC TAC concentration but not also a high whole blood TAC concentration."

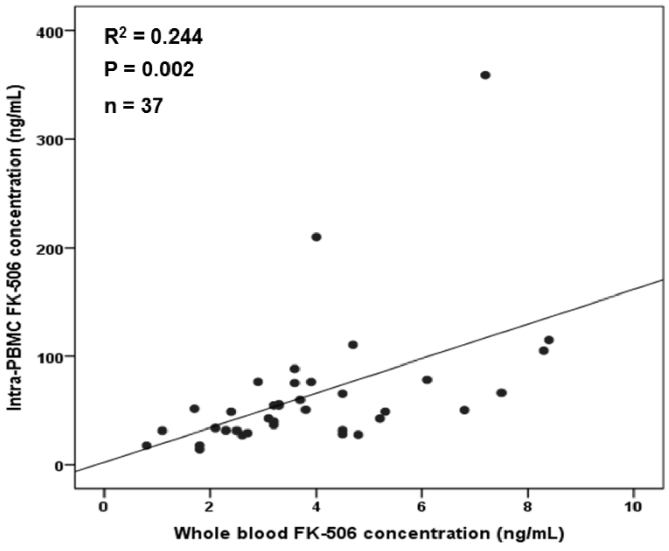


Figure 13: Correlation between the intra-PBMC and whole blood TAC (FK-506) concentrations from 37 patients.

“The observed intra-PBMCs concentrations resulted meanly 12.7 times higher than the blood one, highlighting a strong intracellular compartmentalization. Resulting very simple to use, fast and reliable, this method could be used in the future in the clinical routine for TAC TDM in PBMCs samples, giving to clinicians more reliable information about TAC exposure at the active site.”

Journal of Pharmaceutical and Biomedical Analysis 107 (2015) 512–517

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

An UPLC–MS/MS method coupled with automated on-line SPE for quantification of tacrolimus in peripheral blood mononuclear cells

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ARTICLE INFO

Article history:
Received 7 January 2015
Received in revised form 27 January 2015
Accepted 29 January 2015
Available online 7 February 2015

Keywords:
Tandem-mass
PBMC
Intracellular
FK-506
OSM

ABSTRACT

Background: Tacrolimus is an immunosuppressor used to treat patients undergoing liver transplantation. TDM of hematic tacrolimus by liquid chromatography became standard practice, but it does not necessarily reflect its concentration at its active site. Our aim was to validate a new method for tacrolimus quantification into target cells (peripheral blood mononuclear cells, PBMCs) and testing it on 100 real samples from 37 pediatric patients.

Methods: PBMCs were collected using cell-preparation-tubes; cells number and MCV were evaluated. Tacrolimus was quantified using UPLC–MS/MS coupled with a new automated on-line SPE platform. Chromatographic run was performed on an Acquity UPLC® BEH C18 1.7 μm (2.1 mm × 50 mm) column for 5 min, with a gradient of water and methanol (both with 2 mM/L ammonium acetate and 1 ml/L formic acid). XBridge® C8 10 μm (1 mm × 10 mm) SPE cartridges were used. The internal standard was 6,7-dimethyl-2,3-di(2-pyridyl)quinoxaline.

Results: Full validation following FDA guidelines was performed: the method showed high sensitivity and specificity (LLOQ of 0.010 ng; LLOD of 0.005 ng). Intra- and inter-day imprecision and inaccuracy were <15%. A positive and stable matrix effect was observed, with a good recovery for tacrolimus. All drug amounts in real samples resulted within the calibration range and calibration curves were linear ($r^2 = 0.998$). Concentrations from each patient were standardized using their evaluated MCV: intra-PBMCs concentration was meanly 12.7 times higher than the hematic one.

Conclusion: This method might be eligible and useful for a clinical routine use, giving more reliable data on drug concentration at the active site.

1. Introduction

Tacrolimus (TAC), a macrolide lactone, is a potent immunosuppressant drug used for prophylaxis of liver transplant rejection (primary immunosuppression and maintenance therapy) in adults and children [1]. Its use is characterized by considerable inter-individual variability in pharmacokinetics, making therapeutic drug monitoring (TDM) a standard of care to ensure appropriate drug exposure and reduce side effects [2]. Elevated TAC concentrations can cause serious toxicity and long-term morbidity, while under-dosing could lead to rejection. The individualization of TAC therapy through measured concentrations aims to achieve maximum therapeutic response with minimal adverse effects [3]. In routine clinical settings, TAC concentrations are measured in whole blood. In blood, TAC is mainly associated with erythrocytes (83.2 ± 4.0%), followed by plasma (16.1 ± 3.9%) and leucocytes

Abbreviations: TAC, tacrolimus; TDM, therapeutic drug monitoring; MS, mass; MS/MS, tandem mass; UPLC, ultra performance liquid chromatography; HPLC, high performance liquid chromatography; LC, liquid; ESI+, positive electrospray ionization; PBMC, peripheral blood mononuclear cell; CPT, cell preparation tube; MCV, mean cellular volume; SPE, solid phase extraction; OSM, on-line SPE manager; STDs, standards; QCs, quality controls; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification; LLOD, lower limit of detection; RSD, relative standard deviation.

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http://dx.doi.org/10.1016/j.jpba.2015.01.054
0731-7085/

(0.61 ± 0.4%), with a significant variability in the percentage of TAC associated with leucocytes [4]. Erythrocytes are the main reservoir for TAC in the blood of transplant recipients, but the percentage of the drug associated with these cells varied widely, supporting a concentration-dependent nonlinear binding of TAC to erythrocytes [5–7]. This variability in TAC blood seems to be due to inter-patient differences in hematocrit (range, 31–49%), concentration dependent distribution on the drug between blood and plasma and the drug binding capacity of erythrocytes [8]. The immunosuppressive effect of TAC is mediated through the inhibition of calcineurin in the cytoplasm of lymphocytes; therefore, a closer link to drug efficacy could be expected from direct quantification within this compartment, as compared with whole blood or even tissue concentrations [2].

The inhibition of calcineurin induces a lower activation of T-cells [9]. Measurement of TAC concentration at the target site (allograft tissue or lymphocytes) has been proposed as a possible approach to monitor the risk of organ rejection [10]. The percentage of TAC associated with leukocytes was significantly different during episodes of rejection than during episodes of stable graft function: the percentage of TAC associated with leukocytes and the unbound concentration of TAC were observed to be significantly lower during episodes of rejection than during periods of stable graft function [4]. Capron et al. highlighted that TAC concentrations in peripheral mononuclear blood cells (PBMCs) significantly correlate with both the development and the severity of rejection: lower intra-PCMCs TAC levels were significantly associated to histological rejection and, unlike whole-blood levels, these levels were significantly correlated to the onset of rejection episodes 1 week after transplantation [2]. Several HPLC, LC–MS and LC–MS/MS methods were reported for TAC quantification, but most of these evaluated TAC in whole blood [11–14]. Capron et al. published a method to measure TAC concentrations in PBMCs: quantification of TAC was performed by LC–MS/MS after a cumbersome and time-expensive extraction procedure [15]. Other methods were then published for TAC quantification in PBMCs, without resolving the issue of the low capability in clinical routine and without following a full validation [16–18]. The aim of this study is to develop and validate a rapid, sensible, specific and reliable method for TAC quantification in human PBMCs, and to verify the capability of this method to quantify TAC in "real-life" samples from pediatric patients undergoing liver transplantation.

2. Material and methods

2.1. Chemicals, reagents and PBMCs from healthy volunteers

TAC, formic acid, ammonium acetate and the internal standard (6,7-dimethyl-2,3-di(2-pyridyl)quinoxaline) (IS) were obtained from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). HPLC grade acetonitrile and methanol were purchased from J.T. Baker (Deventer, Holland). Ultrapure water was obtained with the Synergy 185 water purification system from Millipore (Milan, Italy). Buffy coats from healthy volunteers were kindly provided from the blood bank of Maria Victoria Hospital (Turin, Italy).

2.2. PBMCs isolation procedure

Both blank PBMCs used for the preparation of standards (STDs) and quality controls (QCs) and real PBMC samples were isolated following the same protocol. Blank PBMCs were isolated from buffy coats from healthy volunteers. Briefly, the cells were separated with CPT tubes®, which were centrifuged at room temperature for 15 min (1600 × g for 15 min at 25 °C with a 4227 R centrifuge [ALC, Milan, Italy]). The obtained PBMCs layer was collected into a falcon and washed twice in 40 mL ice-cold sodium chloride 0.9% solution, to prevent drug loss, and centrifuged at 2200 × g for 6 min at 4 °C with a Jouan model BR4i centrifuge (Saint-Herblain, France). To obtain red blood cells lysis, before the second wash, pellet was treated with 2 mL of ammonium salt solution (130 mM ammonium chloride + 7.5 mM ammonium carbonate). After adjusting again the volume to 40 mL with sodium chloride 0.9% solution, 500 μL of cell suspension diluted with 19.5 mL of Isoton in a beaker and used for cell count and determination of mean cell volume (MCV) with an automated Beckman Coulter Z2 (Instrumentation Laboratory, Milan, Italy). Four counts for each sample (two for each beaker) were performed. Data were managed by Z2 AccuComp software (version 3.01). To obtain blank PBMC aliquots, the resulting PBMC pellet was dissolved with extraction solution (methanol:water, 70:30 [vol:vol]) to obtain a concentration of 6×10^6 cell/mL, divided in aliquots (500 μL), and then stored at −80 °C. The same isolation procedure was used to obtain PBMC samples from patients blood, which was directly collected into CPTs, with the only difference that PBMC pellets from patients were always suspended in 1 mL of extraction solution: eventual dilution of samples with more than 6×10^6 cells have been performed immediately before the analysis. The whole procedure took less than 1 h.

2.3. Preparation of standard and quality control sample

The stock solution of TAC was prepared dissolving TAC powder into methanol and water (95:5 [vol:vol]), obtaining a 1 mg/mL solution, which was stored at −80 °C. The stability of this solution was evaluated for 6 months. Internal standard stock solution was prepared at 1 mg/mL in pure methanol, while the internal standard (IS) working solution (5 ng/mL [μmol/L]) was prepared by diluting stock solution with methanol:water (50:50 [vol:vol]) at every analytical session.

TAC calibrating solutions, used for the preparation of standard samples (STDs 0–9) and quality controls (QCs), were prepared into water:methanol (50:50 [vol:vol]) by scalar dilutions from TAC stock solution to achieve concentrations ranging from 100 ng/mL [μmol/L] to 0.391 ng/mL [μmol/L] for calibrators, and of 40, 10 and 1 ng/mL [μmol/L] for the preparation of high, medium and low QCs, respectively. These solutions were used to spike (100 μL) blank PBMC samples with 6×10^6 cell/mL at each session. TAC calibrating solutions were stored at −80 °C for less than 6 months. TAC is known to be stable in these conditions [15].

2.4. Sample preparation

Patients PBMC samples with a cell concentration higher than 6×10^6 cells were diluted to achieve the final concentration of 6×10^6 cell/mL. 40 μL of internal standard solution were added to 500 μL of PBMC samples. Blank PBMCs were spiked with 100 μL of calibrating solutions, obtaining STDs 0–9 and QCs; 100 μL of a solution of methanol:water 50:50 [vol:vol], without drug, were added to patients samples to reach the same volume. Each sample was vortex-mixed for 10 s and centrifuged at 21,000 × g at 4 °C for 10 min and supernatant was transferred into total recovery vials. 100 μL of methanol 100% were added to the residual pellet and, after vortex-mixing and another centrifugation step (21,000 × g at 4 °C for 10 min), 80 μL of the new obtained supernatant were transferred in the corresponding vials. 50 μL of the obtained extract was injected into the OSM®-UPLC MS/MS system.

2.5. Chromatographic system

The chromatographic system was an Acquity® UPLC (Waters, Milan, Italy), consisting in a binary pump, a refrigerated sample

Table 1
Chromatographic gradient: mobile phases, A and B, were H_2O and methanol, both with 2 mM ammonium acetate and 0.1% formic acid.

Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.500	65	35
0.10	0.500	65	35
1.00	0.500	52	48
3.70	0.500	30	70
3.75	0.500	0	100
4.3	0.500	0	100
4.35	0.500	65	35
5.00	0.500	65	35

manager and a triple quadrupole detector (TQD) coupled with the new automated on-line solid phase extraction system (OSM®, Waters, Milan, Italy). Chromatographic separation was performed on an Acquity UPLC® BEH C18 1,7 μm (2.1 × 50 mm) column (Waters, Milan, Italy), protected by a pre-column frit (0.2 μm × 2.1 mm), heated at 45 °C using a column thermostat. For the quantification of TAC and IS, the mass spectrometer was settled in the positive ion mode (ESI+), with a capillary voltage of 3 kV, a source temperature of 150 °C and a desolvation temperature of 500 °C. The nitrogen gas flow was 800 L/h and 50 L/h for desolvation and cone, respectively. The cone voltages and collision energies were, respectively, 58 V and 40 eV for IS, 30 V and 20 eV for TAC. The monitored mass transitions were 821.53 > 768.3 and 313.09 > 246.46 for TAC and IS, respectively. The chromatographic run duration was 5 min, with a flow rate of 0.5 mL/min. Mobile phases, A and B, were water and methanol, both with 2 mM ammonium acetate and 0.1% formic acid (Table 1). Before chromatographic separation, the samples underwent an on-line solid phase extraction (conditions described in next subsection) on a dedicated, single-use XBridge® C8 10 μm, 1 × 10 mm OSM cartridge.

2.5.1. SPE method

Before sample loading, each cartridge has been conditioned with 0.4 mL (flow 2 mL/min) of pure acetonitrile, and then equilibrated with 0.4 mL (flow 2 mL/min) of a mixture of water and acetonitrile (85:15 [vol:vol]). Sample loading was performed mixing 50 μL of sample with 0.15 mL (flow 0.15 mL/min) of water and acetonitrile (90:10 [vol:vol]), followed by a washing step with 0.10 mL (flow 0.1 mL/min) of the same mixture. Then, cartridges moved in elution position (on-line with the mobile phases flow to the column), where the gradient of A and B solvents allowed the elution. The whole process, except for elution step, was performed during the previous chromatographic run, maintaining the overall run-time of 5 min.

2.6. Method validation

The assay was fully validated according to the FDA guidelines (revised 2013) [19].

2.6.1. SPE-online method development

To develop the online-SPE method, analytes breakthrough, recovery and adsorption were evaluated using a dedicated function of the OSM® platform (advanced method development). Breakthrough is the fraction of analytes that is lost during of loading and washing steps. Conversely, adsorption is the fraction of analytes that are adsorbed to the fluidic system, contributing to carry over and reducing recovery.

2.6.2. Specificity, selectivity and linearity

Interference from endogenous compounds was investigated by the analysis of five different blank PBMC samples. Linearity was assessed considering a minimum coefficient of determination (r^2) of 0.996 during the five validation sessions, considering a linear model forced through the origin of the axes. Processing was performed based on peak area.

2.6.3. Accuracy, precision, and limits of quantification and detection

Intra-day and inter-day accuracy and precision were determined by performing the analysis of QC samples in multiple replicate ($n = 5$ during each validation session). Accuracy was calculated as the mean percent deviation from the nominal concentrations. Mean value and relative standard deviation percent (RSD %) were calculated and used to express the intra- and inter-day imprecision. The lower limit of detection (LLOD) was considered as the concentration that yields a signal-to-noise of 3. Lower limit of quantification was considered as the concentration that yields a signal-to-noise of at least 5 and that showed a maximum inaccuracy and imprecision of 20%, as requested by FDA guidelines [19]. Upper limit of quantification (ULOQ) was considered as the highest point of the calibration curve (STD 9).

2.6.4. Recovery

Recovery was considered as the relative difference between instrumental responses obtained from the injection of extracts from samples containing known amounts of drug (QC H [4 ng] and QC L [0.1 ng]) and that obtained from direct injection of a chemical mix with the same amount of drug (corresponding to 100% recovery). As additional experiment, recovery was evaluated at different cell concentrations (3×10^6, 6×10^6, 12×10^6, 20×10^6, 24×10^6 cell/mL).

2.6.5. Matrix effect

Matrix effect was investigated by comparing peak heights corresponding to chemical mix at QC H (4 ng) and QC L (0.1 ng) levels with those from the injection of blank PBMC extracts spiked with the same amounts of drugs after sample cleanup, as described by Taylor [20]. Matrix effect was evaluated at different cell concentrations (3×10^6, 6×10^6, 12×10^6, 20×10^6, 24×10^6 cell/mL).

2.7. Clinical application

Samples were obtained from 37 pediatric patients, in TAC treatment after liver transplant (Città della Salute e della Scienza Hospital, Turin, Italy).

Written informed consent has been obtained for each patient, according to the local review board indications.

TAC was orally administrated based on patients' weight (0.015 mg/kg BID). After an initial determination of optimal dose with hematic TDM of TAC concentration (first 15 days), the selected dose was maintained for 6 months. Blood samples were collected into CPT® tubes: 16 mL for 10–18 years old patients, 8 mL for 5–10 years old patients and 4 mL for <5 years old patients. After separation, PBMC samples were stored at −80 °C until analysis. After each analysis, drug amounts quantified in PBMC samples were normalized by cell number and MCV in each single sample, as described by Simiele, according to the formula: $[TAC]_{PBMC} = TAC_{AMOUNT}/(\text{Cell N}^\circ \times \text{MCV})$ [21–28].

Intra-PBMC concentrations have been compared with whole blood concentrations obtained through UPLC–MS/MS using Masstrak Immunosuppressants XE kit (CE-IVD marked; "Città della Salute e della Scienza" Hospital, Turin, Italy).

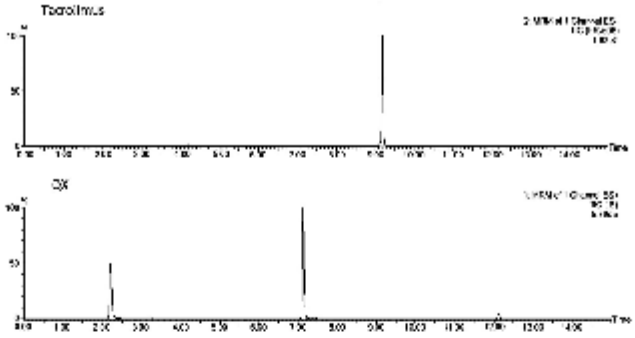

Fig. 1. Tacrolimus and IS chromatograms in advanced method development mode. Time between 0 and 5 min corresponds to breakthrough, 5–10 min to the SPE recovery and 10–15 min to the adsorption of the analytes to the fluidic system.

3. Results

3.1. Method validation

Method was validated on samples of 6×10^6 cell/mL (after demonstration of a good recovery and positive and stabilized matrix effect and an high recovery at this cellular concentration, see Section 3.1.4), according to the FDA guidelines [19].

3.1.1. SPE-online method validation

Breakthrough, recovery and adsorption of SPE were evaluated through a dedicated function of the instrument ("advanced method development"). Using C8 cartridges, TAC and IS recoveries, during on-line SPE, were 98.4 ± 1.1% and 64.3 ± 3.3% respectively (Fig. 1).

3.1.2. Precision, accuracy and limits of quantification and detection

A summary of precision (coefficient of variation) and accuracy for low, medium and high QC samples of TAC is reported in Table 2. The mean (intra- and inter-day) percent inaccuracy and imprecision at QCs levels were always lower than 15%, as required by the FDA guidelines [19]. LLOQ, LLOD, ULOQ were 0.010 ng, 0.005 ng and 10 ng, respectively (Table 2).

3.1.3. Specificity, sensibility and linearity

The calibration curve was linear within the concentration range (0.039–10 ng), with a mean determination coefficient (r^2) higher than 0.998. Typical TAC and IS chromatograms, compared with the chromatogram of a blank extract are shown in Fig. 2. No interfering peaks were observed at TAC and IS retention times of 4.19 ± 0.07 and 2.26 ± 0.07 min, respectively (Fig. 2).

3.1.4. Recovery and matrix effect

Recovery and matrix effect were evaluated at different cell concentrations (3×10^6, 6×10^6, 12×10^6, 20×10^6, 24×10^6 cell/mL). An impact of cell number on recovery and on matrix effect was observed. For samples with less than 6×10^6 cell/mL the determined recovery at the two QCs amounts (QCH and QCL) was +17 ± 6% for TAC and −50 ± 14% for IS. Was also observed at this cell concentration a positive and stable TAC matrix effect (+12 ± 10%). The matrix effect of IS was stable (−47 ± 1%). Conversely, cell concentrations higher than 6×10^6 cell/mL were associated with lower TAC recovery (63 ± 14%) and a negative matrix effect (−32 ± 20%). Therefore, all method was validated with samples of 6×10^6 cell/mL and all patients samples were diluted to obtain samples of this number of cells (Table 3).

3.2. Clinical application

The method was used for quantification of TAC in 37 patients in treatment after liver transplant. Every sample was analyzed in double replicate, to further evaluate the precision of the assay, confirming the validation precision data (inter-day RSD 13.5%). All patients TAC concentrations were in the calibration curve values range. Concentrations from each patient were standardized using the personalized MCV [28]. It was observed that intra-PBMCs concentration was meanly 12.7 times higher than the hematic one. A good, but not excellent, correlation ($r^2 = 0.244$, $P = 0.002$) between the intra-PBMC concentration and the blood one was observed (Fig. 3). In particular, some outliers could be seen: these showed a high intra-PBMC TAC concentration but not also a high whole blood TAC concentration.

4. Discussion and conclusions

The clinical use of TAC is complicated by a narrow therapeutic index and a significant inter-patients and intra-patient pharmacokinetic variability; hence TDM is highly recommended [15]. Nowadays the TDM of TAC is routinely performed only in whole blood [29]. Despite its simplicity to perform, TDM in this matrix often fails to give useful information to the clinicians. Probably this issue is because the real target of TAC is lymphocytes and

Table 2
Validation of the method: intra/inter-day precision and accuracy (n = 5) at 3 QCs concentrations, LLOQ, LLOD and ULOQ.

	Inter-day accuracy	Intra-day accuracy	Inter-day precision	Intra-day precision
QC H	−10.76%	−3.72%	3.2%	14.8%
QC M	−10.72%	3.66%	10.0%	13.2%
QC L	0.08%	2.68%	8.4%	14.9%
LLOQ		0.010 ng (signal-to-noise ratio < 5; RSD < 20%)		
LLOD		0.005 ng (signal-to-noise ratio < 3)		
ULOQ		10 ng		

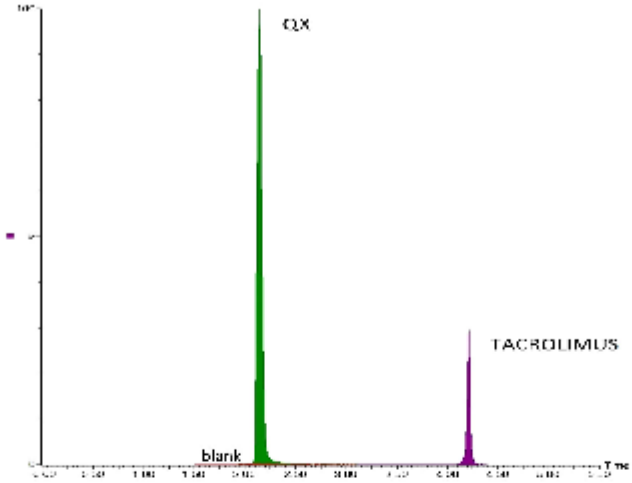

Fig. 2. Tacrolimus and IS chromatograms at LLOQ level, compared with the chromatogram of a blank extract. No interfering peaks were observed at TAC and IS retention times of 4.19 ± 0.07 and 2.26 ± 0.07 min, respectively.

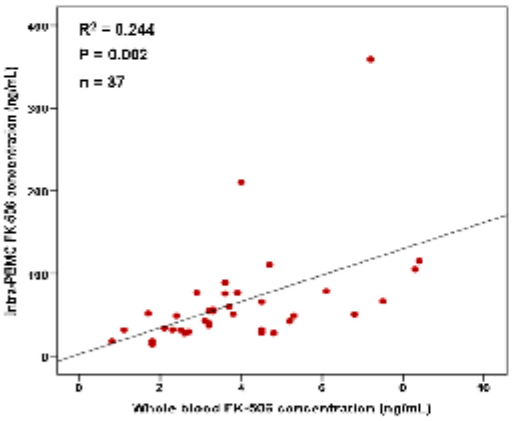

Fig. 3. Correlation between the intra-PBMC and whole blood TAC (FK-506) concentrations from 37 patients.

monocytes (PBMCs), so the need of a method capable of quantifying TAC into these cells has become mandatory. Moreover, the high variability in blood composition in terms of hematocrit (TAC penetrates into erythrocytes) and plasma proteins (TAC is highly bound by plasma proteins), makes the blood concentrations a poorly reliable marker of real exposure to TAC. Capron et al. demonstrated the usefulness of TAC PBMCs level as a marker to predict efficacy early after liver transplantation [2]. The same group developed method to quantify TAC in PBMCs but it resulted too laborious and time-consuming (5–6 h for PBMC isolation and extraction for only 10 samples) [15], and so not eligible for a use in the clinical routine. Others were then developed for TAC or everolimus quantification in PBMCs [16–18]. However, these methods had some critical points which made them not eligible for the use in the clinical routine: among these, the need of drying samples, which make the extraction cumbersome and time consuming, the expression of results in "ng/10^6 cells", which does not allow its correct standardization and comparison with whole blood concentration. One of these methods tried to fix this problem by considering a "standard" mean cellular volume of 200 fl, which, as stated by the authors themselves, "imprecisely reflects the exact volume of the different population cells" [17]. In this work, we developed one of the first methods using the new UPLC system coupled with OSM® technology, which allowed us to quantify TAC in PBMCs in an easy and rapid way, compatible with clinical routine. According to FDA

Table 3
Recovery and matrix effect in different cell numbers aliquots: higher recovery and less presence of matrix effect in population around 6×10^6 cell/mL.

	Cell/mL	TAC recovery mean (%)	TAC matrix effect mean (%)	IS recovery mean (%)	IS matrix effect mean (%)
QC H	3,000,000	21	11	−49	−41
	6,000,000	15	12	−50	−56
	12,000,000	−33	−28	17	17
	20,000,000	−42	−43	1	−16
	24,000,000	−57	−48	−3	−4
QC L	3,000,000	12	19	−54	−38
	6,000,000	17	8	−48	−55
	12,000,000	−18	−15	20	34
	20,000,000	−32	−18	10	31
	24,000,000	−36	−43	10	18

guidelines, the developed method resulted specific, sensitive, precise and accurate. The sensitivity, with a LLOQ of 0.010 ng and a LLOD of 0.005 ng, was high enough to allow a reliable quantification of all real samples. Intra- and inter-day imprecision and inaccuracy (RSD) are lower than 15%. Moreover, a contained and stable matrix effect was observed, with a stable recovery for both TAC and IS. The chromatographic run was only 5 min long, comprehensive of on-line SPE, thus resulting in a low "time for sample". This fast method was applied to 100 PBMC samples from 37 pediatric patients. All TAC amounts resulted within the calibration range (0.039–10 ng, mean r^2 = 0.998). Measured amounts of TAC from each sample were standardized and converted in concentrations using their sample-specific MCV [28–30], obtained for each sample through the automated cell counter. This represents a strong novelty point respect to the previous methods [15–18,31], because the standardization of analytical results on the basis of the real cell volume in the single sample removes an important source of bias. Moreover, this allowed a correct comparison and correlation between intra-PBMC and whole-blood concentrations of TAC. According to our results, the observed intra-PBMC concentrations in real samples resulted meanly 12.7 times higher than the hematic one, highlighting a strong intra-PBMC compartmentalization.

Resulting very simple to use, fast, accurate and precise, this method could be used in the future in the clinical routine for TAC TDM in PBMC samples, giving to clinicians more reliable information about TAC exposure at the active site.

Funding

Internal funding supported this study. The authors declare no competing financial interest.

Conflict of interest

The authors disclose no conflict of interest.

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4.3 Paper 2

First UHPLC-MS/MS method coupled with automated online SPE for quantification both of tacrolimus and everolimus in peripheral blood mononuclear cells and its application on samples from co-treated pediatric patients.

A fully validated method for quantification of TAC and EVE intra-PBMC was developed and then applied rug amounts in 15 "real" PBMCs samples from 5 pediatric patients in co-treatment.

The main results reported into the article attached are: "The observed intra-PBMCs concentrations resulted meanly 19.23 and 218.61 times higher than the blood one, for TAC and EVE, respectively, highlighting a strong intra-PBMC compartmentalization." "A significant, but not strictly tight correlation (r^2=0.773, P= 0.05) between the intra-PBMC and blood TAC concentrations was observed before changes of the EVE dosage (Figure 5). In particular, one outlier could be seen: this showed a high whole blood TAC concentration but not also a high intra-PBMC TAC concentration. A good correlation (r^2=0.971, P= 0.002) was observed between the intra-PBMC and blood EVE concentrations before dose adjustments. After an increment of EVE doses, this correlation was lost (r^2=0.734, P= 0.064)."

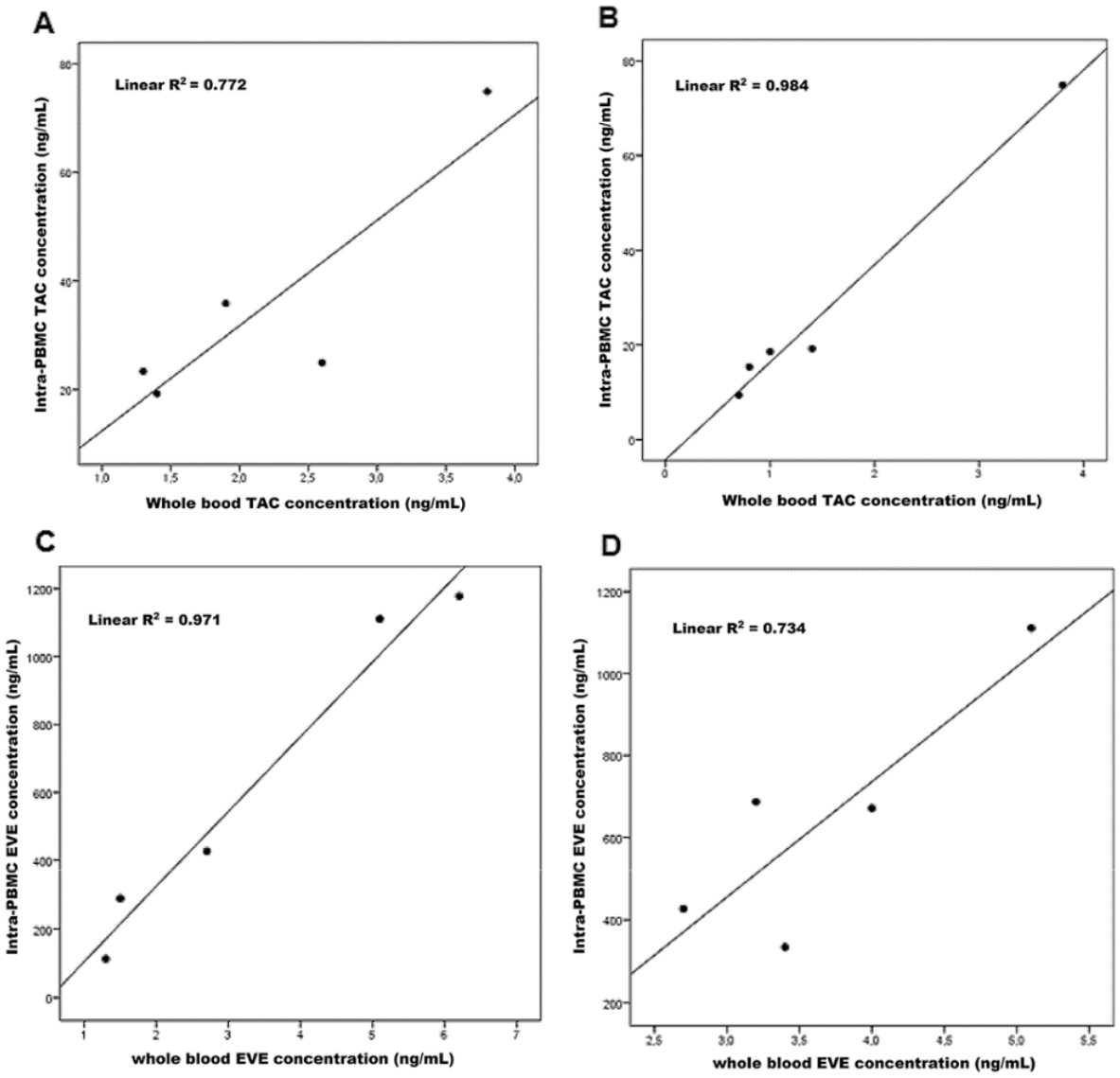

Figure 14: At the top, correlation between everolimus (EVE) concentrations in whole blood and peripheral blood mononuclear cells (PBMCs) before (A, $r^2 = 0.773$, P = 0.05) and after (B, $r^2 = 0.773$, P = 0.001) EVE dose adjustment. At the bottom, correlation between tacrolimus (TAC) concentrations in whole blood and PBMCs, before (C, $r^2 = 0.971$, P = 0.002) and after (D, $r^2 = 0.734$, P = 0.064) EVE dose adjustment

Featured article

Journal of MASS SPECTROMETRY

Received: 7 October 2016 Revised: 20 December 2016 Accepted: 12 January 2017 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/jms.3909

First UHPLC–MS/MS method coupled with automated online SPE for quantification both of tacrolimus and everolimus in peripheral blood mononuclear cells and its application on samples from co-treated pediatric patients.

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Tacrolimus (TAC, FK-506) and everolimus (EVE, RAD001) are immunosuppressors used to treat pediatric patients undergoing liver transplantation. Their hematic TDM by liquid chromatography became standard practice. However, it does not always reflect concentrations at their active site. Our aim was to develop and validate a new method for the simultaneous TAC and EVE quantification into target cells: peripheral blood mononuclear cells (PBMCs).

Peripheral blood mononuclear cells were collected using cell preparation tubes; cells number and mean cell volume were evaluated by an automatic cell counter. TAC and EVE were quantified using UHPLC–MS/MS coupled with an automated online solid-phase extraction platform. Chromatographic run was performed on an Acquity UPLC® BEH C18 1.7 μm (2.1 × 50 mm) column at 45 °C, for 6 min at 0.5 ml/min. Mobile phases were water and methanol, both with 2 mM ammonium acetate and 1 ml/l formic acid). XBridge® C8 10 μm (1 × 10 mm) SPE cartridges were used, and the internal standard was ascomycin.

Following Food and Drug Administration guidelines, method validation resulted in high sensitivity and specificity. Calibration curves were linear (r^2 = 0.998) and intra-day and inter-day impredsion and inaccuracy were <15%. A reproducible matrix effect was observed, with a good recovery for all compounds. Drug amounts in 15 'real' PBMCs samples from five pediatric patients in co-treatment resulted within the calibration range (0.039–5 ng). Concentrations from each patient were standardized using their evaluated mean cell volume: intra-PBMCs concentration was mean ly 19.23 and 218.61 times higher than the hematic one for TAC and EVE, respectively.

This method might be useful in clinical routine, giving reliable data on drugs concentration at the active site. Copyright © 2017 John Wiley & Sons, Ltd.

Keywords: tandem mass; PBMCs; intracellular; everolimus; tacrolimus; OSM

Introduction

Immunosuppressant drugs used in solid organ transplant include tacrolimus (TAC, FK506) and everolimus (EVE, RAD001), characterized by a narrow therapeutic index, potential drug interactions and significant inter-patient and intra-patient variability.[1–5] The incidence and severity of their side effects correlate with high exposure, while under dosed patients show a greater risk for allograft rejection.[1] After the approval of new immunosuppressant drugs (like EVE), the necessity to

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Abbreviations: ATDTAC (FK-506), tacrolimus; EVE (RAD001), everolimus; TDM, therapeutic drug monitoring; MS, mass spectrometry; MS/MS, tandem mass; UPLC, ultra performance liquid chromatography; HPLC, high performance liquid chromatography; LC, liquid chromatography; ESI+, positive electrospray ionization; PBMCs, peripheral blood mononuclear cells; CPT, cell preparation tube; MCV, mean cellular volume; SPE, solid phase extraction; OSM, online SPE manager; STDs, standards; QCs, quality controls; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification; LLOD, lower limit of detection; RSD, relative standard deviation; ME, matrix effect

understand how to manage the switch from a drug to the new one in difficult to treat patients became mandatory. Moreover, according to recent studies, also co-therapy approaches seem to be a good choice in some cases.[6–8] Nevertheless, in several studies, the combination therapy of TAC–EVE was prone to a higher onset of adverse events.[9] The majority of the currently available studies just analyze the co-therapy in new transplanted patients, in which only TAC dosage modification were studied.[7] Therapeutic drug monitoring (TDM) is used to optimize the immunosuppressive treatment, preventing rejection and avoiding toxicities. Although whole-blood TDM reduced acute rejection rate, this remains high. Monitoring immunosuppressant drugs in peripheral blood mononuclear cells (PBMCs) could represent a substantial improvement.[10] In fact, TAC is mainly associated with erythrocytes (83.2 ± 4.0%), followed by plasma proteins (16.1 ± 3.9%) and leukocytes (0.61 ± 0.4%): this variability in the percentage of TAC associated with leukocytes[11] can be clinically relevant.[12–14] The variability in whole-blood TAC concentrations seems to be due to inter-patient differences in hematocrit (range, 31–49%), to the concentration dependent drug distribution between blood and plasma and, finally, to the drug binding capability of erythrocytes.[15] Even EVE shows the same behavior.[16] Moreover, approximately 74% of EVE is bound by plasma protein.[17] P-glycoprotein plays an important role in EVE efflux from lymphocytes, and its expression can influence intracellular drug concentration and drug efficacy.[16,18,19]

Tacrolimus and EVE inhibit, respectively, calcineurin, inducing a lower activation of T cells,[20] and mTOR signaling pathways.[21] Whereas both targets are in the cytoplasm of lymphocytes, a closer link to drug efficacy could be expected from direct quantification within this compartment, as compared with whole blood or even tissue concentrations.[16,22,23] Measurement of TAC and EVE concentrations at the target sites has been proposed as a possible approach to avoid organ rejection.[1,10,24] In fact, both the fraction of TAC associated with leukocytes and its unbound concentration were observed to be significantly lower during episodes of rejection, rather than during periods of stable graft function.[11] Capron *et al.* highlighted that TAC concentrations in PBMCs were significantly associated with the onset, severity and timing of rejection: lower intra-PCMCs TAC levels were significantly associated to histological rejection and, unlike whole-blood levels, to the onset of rejection episodes as early as 1 week after transplantation.[22] Several chromatographic methods were reported for TAC quantification in whole blood,[25–28] meanwhile few methods were developed to measure TAC concentrations in PBMCs by LC–MS/MS.[29,30] Nowadays, only two artides are published on EVE monitoring in PBMCs, both using LC–MS/MS, with debatable results about the correlation between intra-PBMCs and whole blood concentrations.[16,19] Furthermore, no published works investigated this topic in patients undergoing TAC and EVE combination treatment and their potential pharmacokinetic interactions. The aim of this study was to develop and validate a rapid, sensitive, specific and reliable method for the simultaneous TAC and EVE quantification in human PBMCs, following a rapid and cheap extraction procedure, for the use on 'real-life' samples from pediatric patients undergoing liver transplantation. A secondary aim was to investigate on the presence of a possible correlation between whole blood and intra-PBMCs concentrations and to evaluate a possible pharmacokinetic interference between the two drugs.

Material and methods

Chemicals, reagents and PBMCs from healthy volunteers

Tacrolimus, EVE, formic acid and ammonium acetate were purchased from Sigma-Aldrich Chemical Company (St. Luis, MO, USA). The internal standard (IS; ascomycin) was obtained from Adipogen (Liestal, Switzerland). HPLC grade acetonitrile and methanol were purchased from J.T.Baker (Deventer, Holland). Ultrapure water was produced with the Synergy 185 water purification system from Millipore (Milan, Italy). Buffy coats from healthy volunteers were kindly provided from the blood bank of 'Maria Vittoria' Hospital (Turin, Italy).

PBMCs isolation procedure

Both blank PBMCs, used for the preparation of standards (STDs) and quality controls (QCs), and real PBMCs samples were isolated following the same protocol, from buffy coats of healthy volunteers and from patients' blood samples, respectively. Briefly, isolation was performed with cell preparation tube (CPT®), centrifuged at room temperature for 15 min at 1600 × *g* for 15 min at 25 °C. The obtained PBMCs layer was collected into a falcon and washed twice in 40 ml ice-cold sodium chloride 0.9% solution, to prevent drug loss, and centrifuged at 2200 × *g* for 6 min at 4 °C. To obtain red blood cells lysis, before the second wash, pellet was treated with 2 ml of ammonium salt solution (130 mM ammonium chloride + 7.5 mM ammonium carbonate) for 1 min. After adjusting the volume again to 40 ml with sodium chloride 0.9% solution, 500 µl of cell suspension were diluted with 19.5 ml of Isoton in two beakers and used for cell count and determination of mean cell volume (MCV) through an automated Beckman Coulter Z2 (Instrumentation Laboratory, Milan, Italy). Four counts for each sample (two for each beaker) were performed. Data were processed by Z2 AccuComp software (version 3.01). To obtain blank PBMCs aliquots, the resulting PBMCs pellet was dissolved with an extraction solution [methanol : water, 70 : 30 (vol : vol)] to cell concentration of 12 × 10^6 cell/ml. The resulting cell lysates were divided in aliquots (500 µl) and then stored at −80 °C. The same isolation procedure was used to obtain PBMCs samples from patients' blood, which was directly collected into CPTs. The maximum cell concentration for each aliquot was 12 × 10^6 cell/ml. The whole procedure took less than 1 h.

Preparation of standard and quality control sample

The stock solutions of TAC and EVE were prepared by dissolving powders into methanol and water [95 : 5 (vol : vol)], obtaining a concentration of 1 mg/ml, and were stored at −80 °C. IS stock solution was prepared at 1 mg/ml in pure ethanol, while the IS working solution (2500 ng/ml) was prepared by diluting the stock solution with methanol : water [50 : 50 (vol : vol)] at every analytical session.

Tacrolimus and EVE calibrating solutions, used to spike standard samples (STDs 0–8) and QCs, were prepared in water : methanol [50 : 50 (vol : vol)] by scalar dilutions from TAC and EVE stock solution to achieve concentrations ranging from 50 to 0.391 ng/ml for calibrators, and of 40, 10 and 1 ng/ml for the preparation of high, medium and low QCs, respectively (Table 3). These solutions were used to spike (100 µl) blank PBMCs samples with 6 × 10^6 cell/ml at each session.

Sample preparation

A 40 µl of internal standard working solution were added to 500 µl of PBMCs samples. Blank PBMCs were spiked with 100 µl of calibrating solutions, obtaining STDs 0–8 and QCs; 100 µl of a solution of methanol : water, 50 : 50 (vol/vol), without drug, were added to patients samples to reach the same volume. Each sample was vortex-mixed for 10 s and centrifuged at 21 000 × *g* at 4 °C for 10 min, and supernatant was transferred into total recovery vials. A 100 µl of methanol 100% were added to the residual pellet and, after vortex-mixing and another centrifugation step (21 000 × *g* at 4 °C for 10 min), 80 µl of the new obtained supernatant were transferred in the corresponding vials. A 50 µl of the obtained extract was injected into the OSM-UPLC MS/MS system.

Chromatographic system

The chromatographic system was an Acquity® UPLC (Waters, Milan, Italy), consisting in a binary pump, a refrigerated sample manager and a triple quadrupole detector coupled with the new automated online solid-phase extraction (SPE) system (OSM®, Waters, Milan, Italy). Chromatographic separation was performed on an Acquity UPLC® BEH C18 1.7 µm (2.1 × 50 mm) column (Waters, Milan, Italy), protected by a pre-column frit (0.2 µm × 2.1 mm), heated at 45 °C using a column thermostat. For the quantification of TAC, EVE and IS, the mass spectrometer was settled in the positive ion mode (positive electrospray ionization), with a capillary voltage of 1 kV, a source temperature of 150 °C and a desolvation temperature of 400 °C. The nitrogen gas flow was 800 l/h and 50 l/h for desolvation and cone, respectively. The cone voltages and collision energies were, respectively, 17 V and 17 eV for IS, 25 V and 15 eV for EVE and 30 V and 20 eV for TAC. The monitored mass transitions were 821.53 > 768.3 m/z for TAC, 975.5 > 908.5 m/z for EVE and 809.39 > 756.45 m/z for IS, respectively. The chromatographic run duration was 6 min, with a flow rate of 0.4 ml/min. Mobile phases, A and B, were water and methanol, respectively, both with 2 mM ammonium acetate and 0.1% formic acid (Table 1). Before chromatographic separation, the samples underwent an SPE (conditions described in next subsection) on dedicated single-use XBridge® C8 10 µm, 1 × 10 mm OSM cartridge.

SPE method

Online solid-phase extraction were performed with two different mobile phases. Phase C was water, with 2 mM ammonium acetate and 0.1% formic acid, and phase D is ACN. Before sample loading, each cartridge has been conditioned with 0.4 ml (flow 2 ml/min) of phase D and then equilibrated with 0.4 ml (flow 2 ml/min) of a mixture of C and D [85 : 15 (vol : vol)]. Sample loading was performed mixing 50 µl of sample with 0.10 ml (flow 0.15 ml/min) of C and D [90 : 10 (vol : vol)], followed by a washing step with 0.20 ml (flow 0.15 ml/min) of the same mixture. Then, cartridges moved in elution position (online with the mobile phases flow to the column), where the gradient of A and B solvents allowed the elution (cartridges were held in the elution position for the whole run time). The whole process, except for elution step, was performed during the previous chromatographic run, maintaining the overall run time of 6 min.

Table 1. Chromatographic gradient: mobile phases, A and B, were H_2O and methanol, both with 2 mM ammonium acetate and 0.1% formic acid

Time (min)	Flow (ml/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.400	60	40
2.00	0.400	60	40
4.00	0.400	1	99
5.00	0.400	1	99
5.05	0.400	60	40
6.00	0.400	60	40

Method validation

The assay was fully validated according to the Food and Drug Administration (FDA) (revised 2013) and EMA guidelines.[31–33]

Online SPE method development

Together with recovery, the fraction of analytes that was lost during loading and washing steps (breakthrough) and the fraction that was adsorbed to the fluidic system and not eluted from the cartridges (adsorption) were evaluated using a dedicated function of the OSM® platform (advanced method development).[30,34,35]

Specificity, selectivity and linearity

Interference from endogenous compounds was investigated by the analysis of six different blank PBMCs samples. Interference between target analytes (cross-talk) was also evaluated, by analyzing PBMCs samples spiked with single drugs at high QC concentration.[36] Linearity was assessed during the six validation sessions, considering a linear model forced through the origin of the axes. Processing was performed based on peak area.

Stability

The stability of TAC and EVE was evaluated in stock solutions, calibrating solutions and PBMCs samples at −80 °C up to 6 months. The bench-top and autosampler stabilities were evaluated up to 3 h and overnight, respectively.

Accuracy, precision and limits of quantification and detection

Intra-day and inter-day accuracy and precision were determined by performing the analysis of QC samples in multiple replicate ($n = 5$ during each validation session). Inaccuracy was calculated as the mean percent deviation from the nominal concentrations. Mean value and relative standard deviation percent (RSD%) were calculated and used to express the intra-day and inter-day imprecision. The lower limit of detection (LLOD) was considered as the concentration that yields a signal-to-noise ratio of 3. Lower limit of quantification (LLOQ) was considered as the concentration that yields a signal-to-noise of at least 5 and the level that showed a maximum inaccuracy and imprecision of 20%, as requested by FDA guidelines.[30,33] Upper limit of quantification was considered as the highest point of the calibration curve (STD 8).

Recovery

Recovery of SPE was evaluated through the dedicated 'advanced method development' function of the OSM, while the recovery of the pre-SPE steps was evaluated by comparing post-extraction spiked with pre-extraction spiked samples.

Matrix effect

Matrix effect (ME) was investigated by comparing peak areas corresponding to the injection of chemical mixes at QCs levels with

those from the injection of blank PBMCs extracts spiked with the same amounts of drugs after sample cleanup (post-extraction addition protocol), as described by Taylor.[37] ME was evaluated at different cell concentrations (3×10^6, 6×10^6, 12×10^6 and 24×10^6 cell/ml).

Moreover, the potential correcting power of IS was also investigated by evaluating the 'IS-normalized' ME (IS-nME), calculated through the equation: $IS\text{-}nME = [(PA_{A\text{-}matrix}/PA_{IS\text{-}matrix})/(PA_{A\text{-}neat}/PA_{IS\text{-}neat}) - 1] * 100$.

The RSD% value (measure of reproducibility of IS-nME and real indicator of the theoretical error in quantification associate with ME) was calculated on the mean value of the '$(PA_{A\text{-}matrix}/PA_{IS\text{-}matrix})/(PA_{A\text{-}neat}/PA_{IS\text{-}neat})$' ratio.

Clinical application and incurred sample reanalysis

Samples were obtained from five pediatric patients, in co-treatment with TAC and EVE after liver transplant (Città della Salute e della Scienza Hospital, Turin, Italy).

Written informed consent has been obtained for each patient, according to the local review board indications.

TAC and EVE were orally administered based on patients' weight (0.015 mg/kg BID and 2/3 mg/kg OD). After an initial determination of optimal dose with hematic TDM of TAC concentration (first 15 days), as indicated by the treatment guidelines, the selected dose was maintained for 6 months. The co-therapy with EVE started after determination of the TAC optimal dose. Blood samples were collected into CPTs: 16 ml for 10–18 years old patients, 8 ml for 5–10 years old patients and 4 ml for <5 years old patients. After separation, PBMCs samples were stored at −80 °C until analysis. After each analysis, drug amounts quantified in PBMCs samples were normalized by cell number and MCV in each single sample, as described by Simiele, according to the formula: $[DRUG]_{PBMCs} = DRUG_{AMOUNT}/(\text{cell } n \times MCV)$.[30,33–36,38–41]

Intra-PBMCs concentrations have been compared with whole blood concentrations obtained through UHPLC–MS/MS using MassTrak Immunosuppressants XE kit (CE-IVD marked; Waters, Milan, Italy).

All the tested samples were analyzed in two different sessions, in order to evaluate the incurred sample reanalysis precision, as suggested by EMA guidelines.[32]

Results

Method validation

Method was validated on samples of 6×10^6 cell/ml (after demonstration of a good recovery and positive and reproducible ME, see the section on Recovery and ME).[33]

Online SPE method validation

Breakthrough, recovery and adsorption were evaluated. Using C8 cartridges, TAC, EVE and IS recoveries, during online SPE, were >98%.

Precision, accuracy and limits of quantification and detection

A summary of precision (coefficient of variation) and accuracy for low, medium and high QC samples of TAC and EVE is reported in Table 2. The mean percent inaccuracy and imprecision at QCs levels were always lower than 15%, as required by the FDA guidelines.[33] LLOQ, LLOD and upper limit of quantification were 0.0195, 0.00975 and 5 ng for TAC and 0.039, 0.0195 and 5 ng for EVE, respectively (Table 3).

Table 2. Validation of the method: intra/inter-day precision and accuracy ($n = 5$) at three QCs concentrations

Tacrolimus	Accuracy	Precision	
		Intra-day (RSD%)	Inter-day (RSD%)
QC H	95.8	4.9	4.3
QC M	102.6	5.3	5.8
QC L	102.7	6.3	10
Everolimus	**Accuracy**	**Precision**	
		Intra-day (RSD%)	Inter-day (RSD%)
QC H	93.8	5.5	6
QC M	96.1	4.1	7.2
QC L	101.1	9.2	10.7

QC, quality control; RSD, relative standard deviation.

Table 3. Overview of STDs and QCs levels

	Tacrolimus		Everolimus	
	'Calibrating' solution (ng/ml)	Drug amount in STD and QC samples (ng)	'Calibrating' solution (ng/ml)	Drug amount in STD and QC samples (ng)
LOD	0.0975	0.00975	0.195	0.0195
LLOQ	0.195	0.0195	0.391	0.0391
STD1	0.391	0.0391	0.391	0.0391
STD8 (ULOQ)	50	5	50	5
QC H	40	4	40	4
QC M	10	1	10	1
QC L	1	0.1	1	0.1

The data processing was performed on the basis of drugs amount in samples; the calibrating solutions were dilutions of the corresponding stock solutions [in water : methanol (50 : 50)], used to spike PBMC aliquots, thus obtaining STD and QC samples.
LLOQ, lower limit of quantification; LOD, limit of detection; QC, quality control; STD, standard; ULOQ, upper limit of quantification.

Specificity, sensitivity and linearity

The calibration curves were both found to be linear within the drugs amount range between 0.039 and 5 ng, with a mean determination coefficient (r^2) higher than 0.998. No interfering peaks were observed at TAC, EVE and IS retention times of 4.15 ± 0.07, 4.25 ± 0.07 and 4.12 ± 0.07 min, respectively (Figs 1, 2). Typical TAC and EVE chromatograms at STD 1, LLOQ and LLOD levels, compared with the chromatogram of a blank extract are shown in Fig. 2. Absence of cross-talk was also evaluated for each compound (Fig. 3).

Recovery and ME

Mean recoveries for TAC, EVE and IS were 95.7%, 91.3% and 95.3%, respectively, stable for all compounds (RSD <4.70%). ME was evaluated at different numbers of cells (3×10^6, 6×10^6, 12×10^6 and 24×10^6 cell/ml) and at different drug concentrations (high and low QC levels), resulting in mean values of: −32% (RSD 15.8%), −28.5% (RSD 15.1%) and −32% (RSD 14.24%) for TAC, EVE and IS,

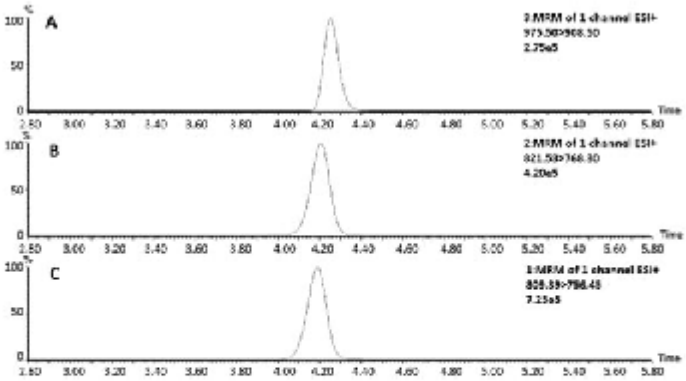

Figure 1. Typical chromatogram from the analysis of a high-quality control everolimus (A), tacrolimus (B) and internal standard (C) have retention times of 4.25 ± 0.07 min, 4.15 ± 0.07 min and 4.12 ± 0.07 min, respectively. ESI+, positive electrospray ionization; MRM, multiple reaction monitoring.

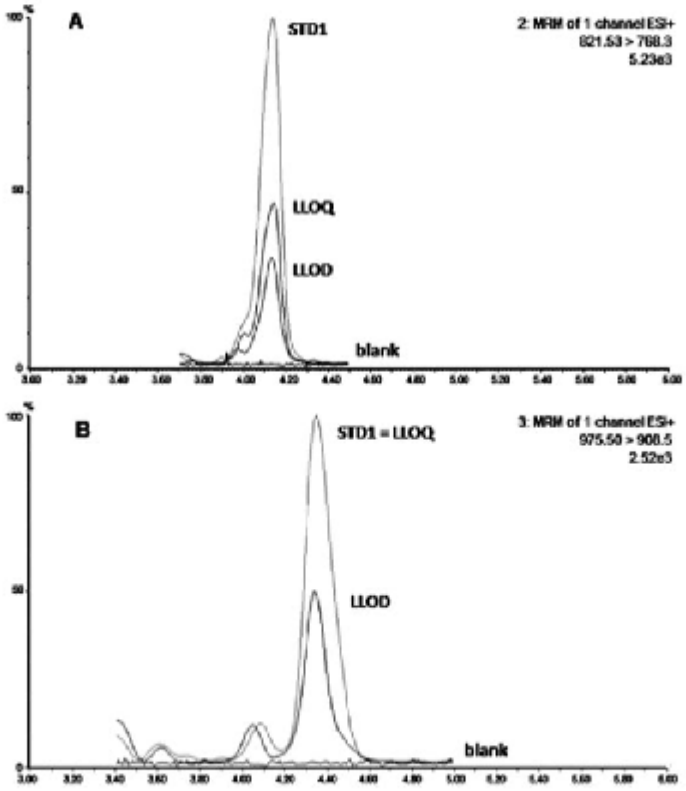

Figure 2. Tacrolimus (A) and everolimus (B) chromatograms at STD1, lower limit of quantification (LLOQ) and lower limit of detection (LLOD) levels compared with the chromatogram of a blank extract. No interfering peaks were observed at tacrolimus and everolimus retention times. ESI+, positive electrospray ionization; MRM, multiple reaction monitoring.

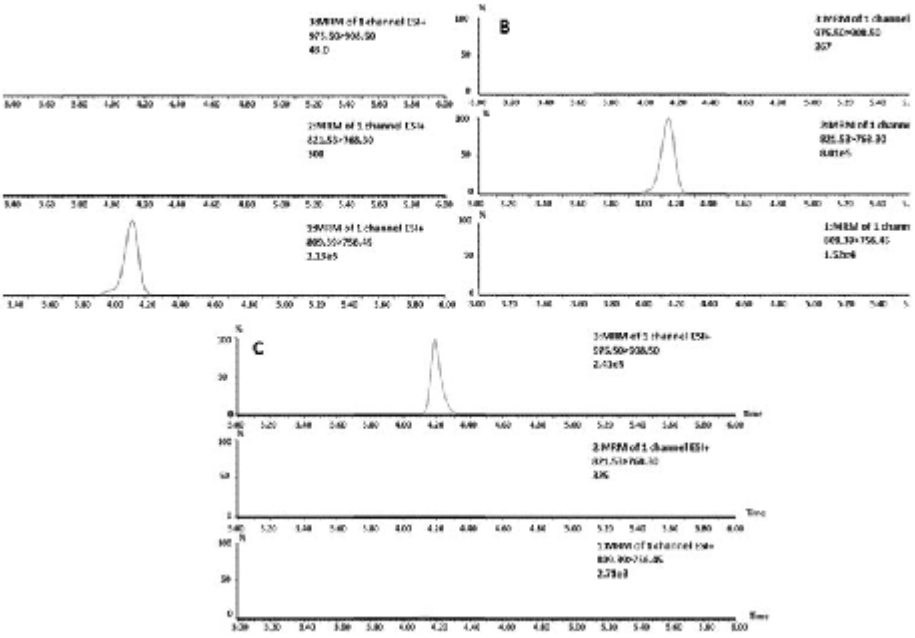

Figure 3. Chromatograms from injection of three chemical mixes, each one containing only one analyte: no cross-talk peaks have been observed. In the order: (A) injection of a mix containing only internal standard, (B) injection of a mix containing only tacrolimus and (C) injection of a mix containing only everolimus. ESI+, positive electrospray ionization; MRM, multiple reaction monitoring.

respectively. Because the ME resulted not reproducible for samples with 24×10^6 cells/ml, we decided to dilute all samples to 12×10^6 cell/ml or less.

Reevaluating ME in these conditions, it resulted to be −29.48% (RSD 3.35%), −14.87% (RSD 1.67%) and −31.71% (RSD 8.00%), fitting the minimum requested reproducibility for ME. Considering all cells number (including 24×10^6 cell/ml), the mean IS-nME resulted −0.1% (RSD 6.0%) and +5.0% (RSD 9.8%) for TAC and EVE, respectively. Excluding samples with 24×10^6 cells/ml, the mean IS-nME was +2.5% (RSD 5.0%) and +7.9 (RSD 8.8%), further enhancing the reproducibility (Fig. 4).

Stability

Tacrolimus and EVE resulted stable stored at −80 °C up to 6 months in stock solutions (−1.22% and −2.42%, respectively), in the calibrating solutions (−1.62% and 3.20%, respectively) and in PBMCs samples (−6.60% and −9.03%, respectively). The analytes were

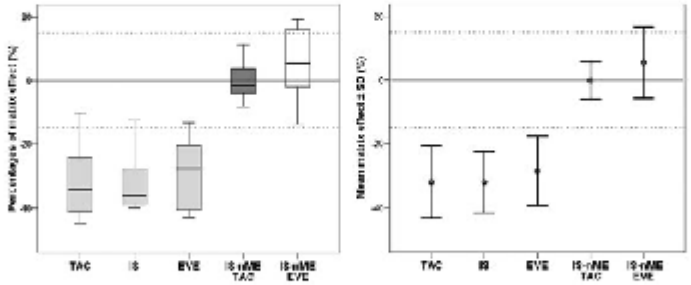

Figure 4. Graphical representation of the observed percentages of matrix effect both in the standard form and normalized based on the internal standard (IS), considering all cell concentrations (3×10^6, 6×10^6, 12×10^6 and 24×10^6 cell/ml). Despite the fact that IS is capable of reducing the mean value of matrix effect Q7 (ME) for both drugs, it performs better for tacrolimus (TAC) rather than everolimus (EVE), in terms of reproducibility. IS-nME, internal standard-normalized matrix effect.

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stable bench-top (−2.12% and −2.64%, respectively) and in the autosampler (−1.32% and −3.57%, respectively). Stability data in stock solution and calibrating solutions confirmed the ones already reported in literature.[16,29]

Clinical application

The method was used for quantification of TAC and EVE in samples from five patients in co-treatment after liver transplant, before and after a change of EVE dosage. Every sample was reanalyzed in two different sessions, in order evaluate the precision of the assay, confirming the acceptable reproducibility (inter-day RSD 13.5%). TAC and EVE amounts of all patients' samples were in the calibration curve range. Concentrations from each patient were calculated using the personalized MCV.[30,38–40] It was observed that intra-PBMCs concentration was meanly 19.23 and 218.61 times higher than the hematic one for TAC and EVE, respectively. A significant, but not strictly tight correlation ($r^2 = 0.773$, $P = 0.05$) between the intra-PBMCs and blood TAC concentrations was observed before changes of the EVE dosage (Fig. 5). In particular, one outlier could be seen: this showed a high whole blood TAC concentration but not also a high intra-PBMCs TAC concentration. A good correlation ($r^2 = 0.971$, $P = 0.002$) was observed between the intra-PBMCs and blood EVE concentrations before dose adjustments (Fig. 5). After an increment of EVE doses, this correlation was lost ($r^2 = 0.734$, $P = 0.064$).

Discussion

Tacrolimus and EVE have a narrow therapeutic index; therefore, TDM is highly recommended.[1–5,18,29] Nowadays, their TDM is routinely performed only in whole blood.[1,9,42] Despite to its simplicity to perform, TDM in this matrix often fails to give useful clinical information: In fact, whole-blood concentrations are influenced by an high variability in blood composition, and the real therapeutic targets are lymphocytes and, in a minor measure, monocytes. Thus, the necessity of a method capable of quantifying these two drugs within these cells became mandatory. Despite PBMCs consist not only in lymphocytes (the main site of action of these drugs), these could consider an optimal compromise: In fact, currently available methods for cell sorting take too much time, allowing drug exit from the cells. To date, no previous published methods are able to simultaneously quantifying TAC and EVE in PBMCs. Furthermore,

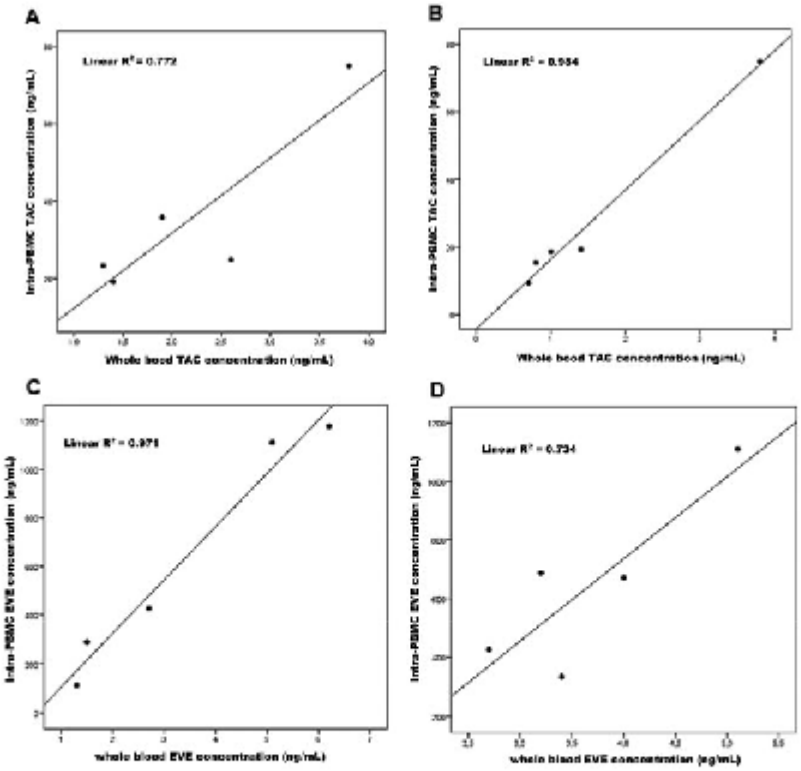

Figure 5. At the top, correlation between everolimus (EVE) concentrations in whole blood and peripheral blood mononuclear cells (PBMCs) before (A, $r^2 = 0.773$, $P = 0.05$) and after (B, $r^2 = 0.773$, $P = 0.001$) EVE dose adjustment. At the bottom, correlation between tacrolimus (TAC) concentrations in whole blood and PBMCs, before (C, $r^2 = 0.971$, $P = 0.002$) and after (D, $r^2 = 0.734$, $P = 0.064$) EVE dose adjustment.

the majority of current methods to singularly quantify TAC or EVE within this matrix are not eligible for a routine use. In a previous work, a method for only TAC quantification in PBMCs was described using OSM® technology. In this work, we updated and modified that method in order to simultaneously quantify TAC and EVE in PBMCs in an easy and rapid way, avoiding a long sample preparation procedure. This protocol resulted simpler and faster than previous published methods for EVE quantification.[14,19] According to FDA guidelines, it resulted specific, sensitive, precise and accurate. The sensitivity (with LLOQ and LLOD of 0.0975 and 0.0195 ng for TAC and 0.0195 and 0.039 ng for EVE, respectively) was high enough to allow a reliable quantification of both drugs in all real samples. As compared with our previous method for TAC quantification, this new method used ascomycin as IS instead of 6,7-dimethyl-2,3-di(2-pyridyl)quinoxaline, resulting a little more expensive but much more robust, because ascomycin has similar chemical properties and retention time to those of TAC and EVE: Among the several advantages, this allowed to used a stronger washing condition in the online SPE extraction, enhanding method performance.

Recovery resulted consistent and reproducible for all the analytes. On the other hand, considering the wide variability in the number of cells when approaching intracellular quantification, we tested the ME at four different cell concentrations. These tests highlighted a consistent and variable ME, directly proportional to the number of cells in the sample: In order to make this effect reproducible, we excluded from the test samples with 24×10^6 cells/ml, obtaining RSD values lower than 10%, thus resulting reproducible.

For this reason, all STDs, QCs and patients' samples were prepared at a cell concentration equal or lower than 12×10^6 cells/ml. The problem of ME on samples analysis was previously discussed as the main critical issue of mass spectrometry.[37] In order to evaluate the corrective impact of IS on analytes in this work, we proposed an equation for the calculation of 'IS-nEM', in further addition and support of FDA and EMA guidelines.[31–33] This equation considers the effect of matrix effect in each sample for both IS and target analyte and represents the capability of the IS of successfully counterbalancing the variability in analytical performance due to matrix effect. In this case, being the calibration curve prepared in matrix, the bias due to ME is mainly dependent on its variability (different samples and different blank PBMCs from healthy donors), expressed as the RSD%.

The low mean percentage of IS-nME and its very low RSD, confirmed ascomycin as an ideal 'non-deuterated' IS for TAC,[10,29,30] while it showed its slightly lower performance for EVE, as already described in literature.[43]

However, the real impact of ME on analytical results depends mainly on its variability among different samples: This variability in IS-nME (RSD lower than 15%) resulted contained for both analytes, also if samples with cell concentrations higher than 24×10^6 cells/ml were considered. This phenomenon was due to the same trend of ion inhibition for TAC, IS and EVE in different PBMCs lots and cell numbers (when analytes signal decreased, IS signal decreased as well). These results were again in accordance with both FDA and EMA guidelines.[17,33,44]

By a practical point of view, the chromatographic run was only 6 min long, comprehensive of online SPE, hence resulting in an acceptable 'time for sample' required. This fast method was applied to 20 PBMCs samples from five pediatric patients in co-treatment with TAC and EVE. Concentrations from each patient were standardized using their real cell number and MCV.[10,18,39] This represents an important innovation compared with previous methods, because the standardization of analytical results on the basis of the real cell volume in each single sample removes an important source of bias.[16,19,30,39,45]

The observed intra-PBMCs concentrations resulted meanly 19.23 and 218.61 times higher than the blood one, for TAC and EVE, respectively, highlighting a strong intra-PBMCs compartmentalization. Although the number could seem low, these correspond to a critical subpopulation of pediatric patients enrolled in a single center: Considering this, these patients represent a considerable fraction of treated patients. Moreover, these cases are among the most difficult to manage for clinicians and, probably, those which would have the strongest benefit from this new monitoring strategy. Anyway, this method can be applied on samples from adult patients with comparable performance. Nevertheless, a future study on larger cohorts of patients treated with this combination therapy will be warranted. In conclusion, this method results eligible for a future use in the clinical routine for TAC and EVE TDM in PBMCs samples, giving to clinicians more reliable information about these two drugs exposure at the active site.

Conflicts of interest

The authors disclose no conflicts.

Funding

This study was supported by internal funding.

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4.4 Other Results Not Already Published:

4.4.1 Genetic and adverse event analysis

In collaboration with the Gastroenterology Unit, 24 patients of the 37 considered into the first published work on the intra-PBMC TAC quantification were genotyped for the SNPs of CYP3A4, CYP3A5 and ABCB1 and was considered the presence or not of EBV infection, and also its negativization or persistent viremia, as an adverse event.

Also in this reduced group the correlation between the whole-blood and intra-PBMC concentrations was maintained (r^2=0.232, P=0.0017) (Figure 15).

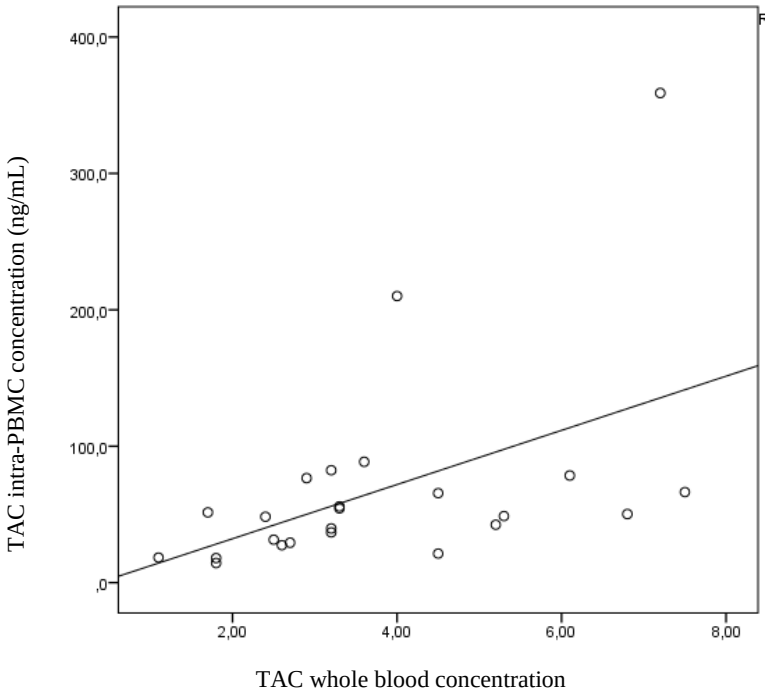

Figure 15: Correlation between the intra-PBMC and whole blood TAC (FK-506) concentrations from 24 patients r2=0.232, P=0.0017

P3A5*3 genotyping:

CYP3A5*1/*3 recipient genotype patients samples have a ratio (intra-PBMC TAC concentration /whole-blood TAC concentration) of 28.02 [13.95-44.96] instead of CYP3A5*3/*3 recipient genotype patients that have a ratio of 12.50 [8.94-16.87].

No significant difference between the two population was observed (P=0.09) but, as reported in figure 16, there is an evident difference also if it is not significant. This difference was higher considering only the TAC intra-PBMC concentrations and lower if is considered the whole-blood one.

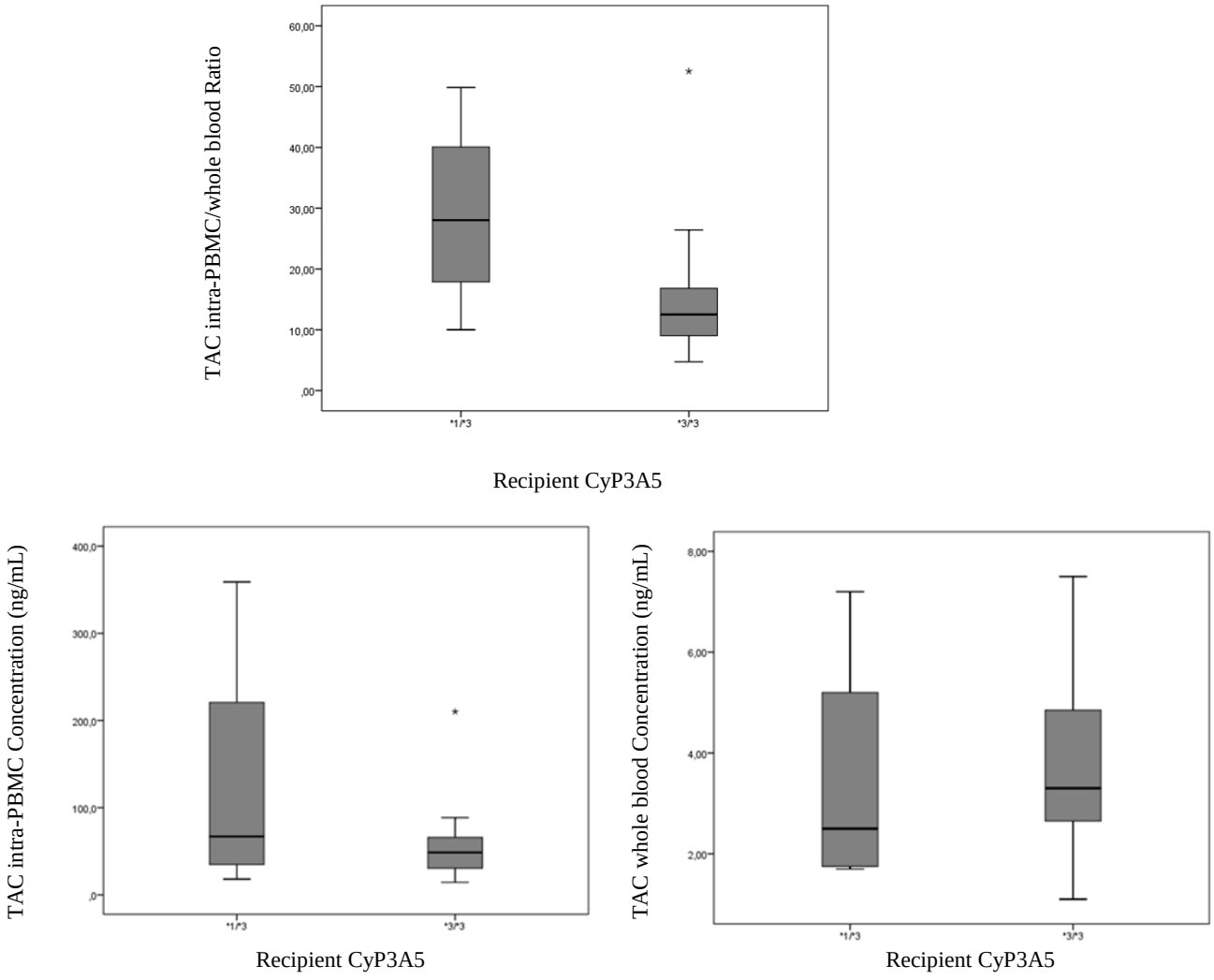

Figure 16: Box plots representing differences between TAC intra-PBMC, whole blood and Ratio in different recipient CyP3A5 population (*1/*3 vs *3/*3).

		TAC Ratio	*p value*
CYP3A5*1/*3	25°	13,95	0,09
	50°	**28,02**	
	75°	44,96	
CYP3A5*3/*3	25°	8,94	
	50°	**12,50**	
	75°	16,87	

Table 8: Recipients with CYP3A5*3/*3 have lower ratio values than those with CYP3A5*1/*3

ABCB1 genotyping

Considering two 3435 C>T and 1199 G>A P-glycoprotein polymorphisms, the TAC intra-PBMC/ whole blood concentrations ratio did not have significative differences between patients with C/C, C/T and T/T or with G/A and G/G (Figure 17).

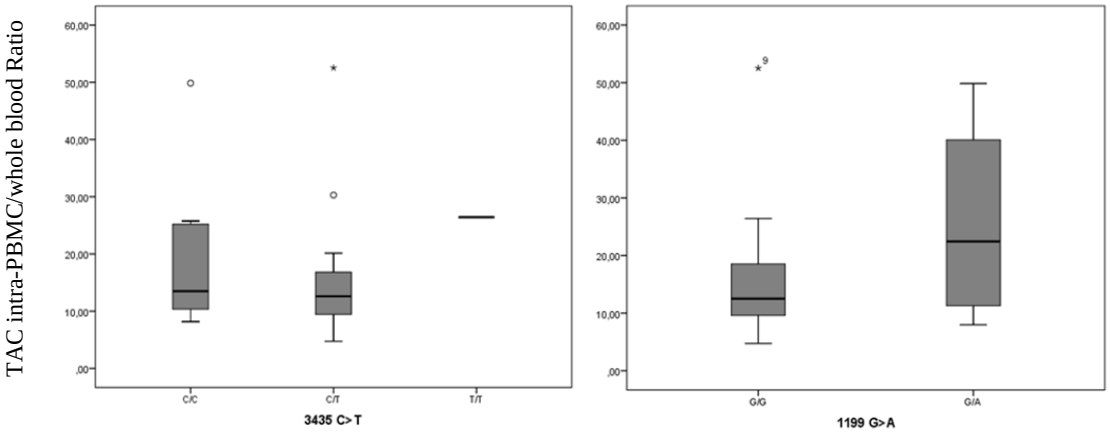

Figure 17: Box plots representing differences betweenTAC Ratio for 3435 C>T and for 1199 G>A.

EBV infection (adverse event):

Dividing patients based on the presence of infections or not , and on negativization or persistent viremia, was seen a significant difference between both the two groups considered (Figure 18).

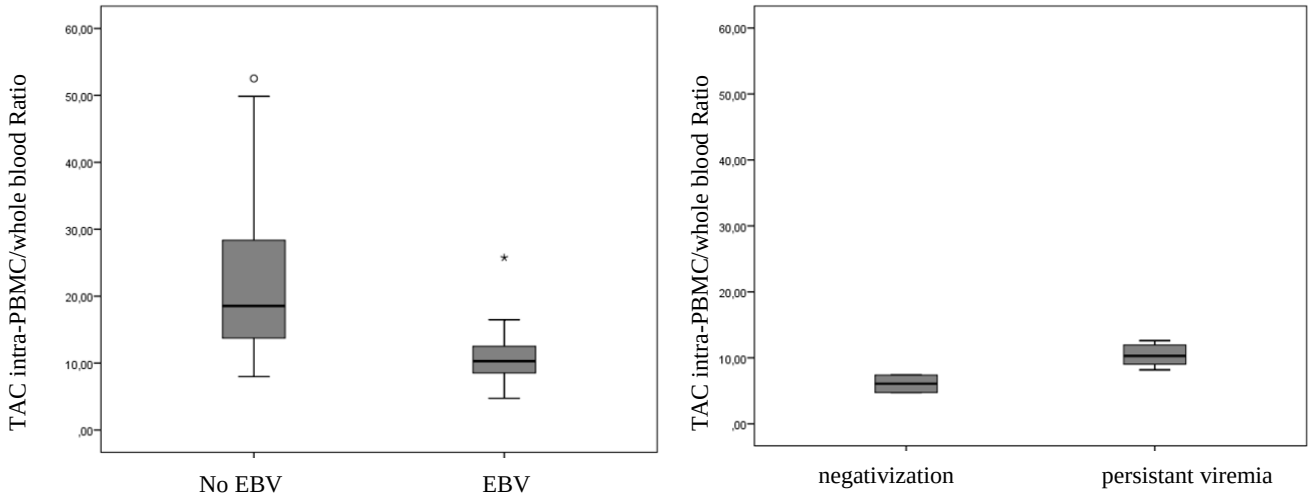

Figure 18: Difference in terms of TAC Ratio between patients with EBV and those without infection (p=0.022) and between with EBV negativization and those with persistant viremia (p=0.04)

		TAC Ratio	*p value*
No EBV	25°	13,30	0,02*
	50°	**18,53**	
	75°	29,33	
EBV	25°	8,34	
	50°	**10,28**	
	75°	12,55	

Table 9: Significant difference between TAC ratio of patients with EBV infection and of those without (p=0.02)

4.4.2 TAC Retrospective study

Allergies, lymphoproliferative disorders, rejects

For 27 paediatric patients were analysed the parameters reported in the Table 10:

	VALORI
Sex	15 M - 12 F
Mean Age	4,95 (IQR 2,46 - 7,1)
TAC dose/Kg	0,1 (IQR 0,08 – 0,17)
Mean Whole blood TAC concentration (ng/mL)	4,50 (IQR 3,2 - 7,5)
Mean Intra-PBMC TAC concentration (ng/mL)	77,98 (IQR 48,78 - 210,11)
Mean Intra-PBMC/Whole blood TAC ratio	20,63 (IQR 12,87 - 36,65)

TAC and EVE whole blood and intra-PBMC concentration were quantified two times for each patients (without change of dosage). Clinic history of the patients was evaluated: all the adverse events that occurred to paediatric patients undergone liver transplant and immunotherapy were considered and their appearance frequency in this population was evaluated (Table 11).

	No	**FREQ %**	**Yes**	**FREQ %**	**No info**	**FREQ %**	**N tot**	**FREQ %**
Rejects	20	74,1	7	25,9	-	-	27	100
Allergies	17	62,9	7	25,9	3	11,2	27	100
Lymphoproliferiatives disorders	24	88,9	3	11,1	-	-	27	100

Allergies

Of 24 patients analyzed, 7 manifested allergic adverse events. Those had also significant higher levels of intra-PBMC TAC than those without allergies (p=0.01), but this difference could not be visible considering hematic TAC levels (p=0.184).

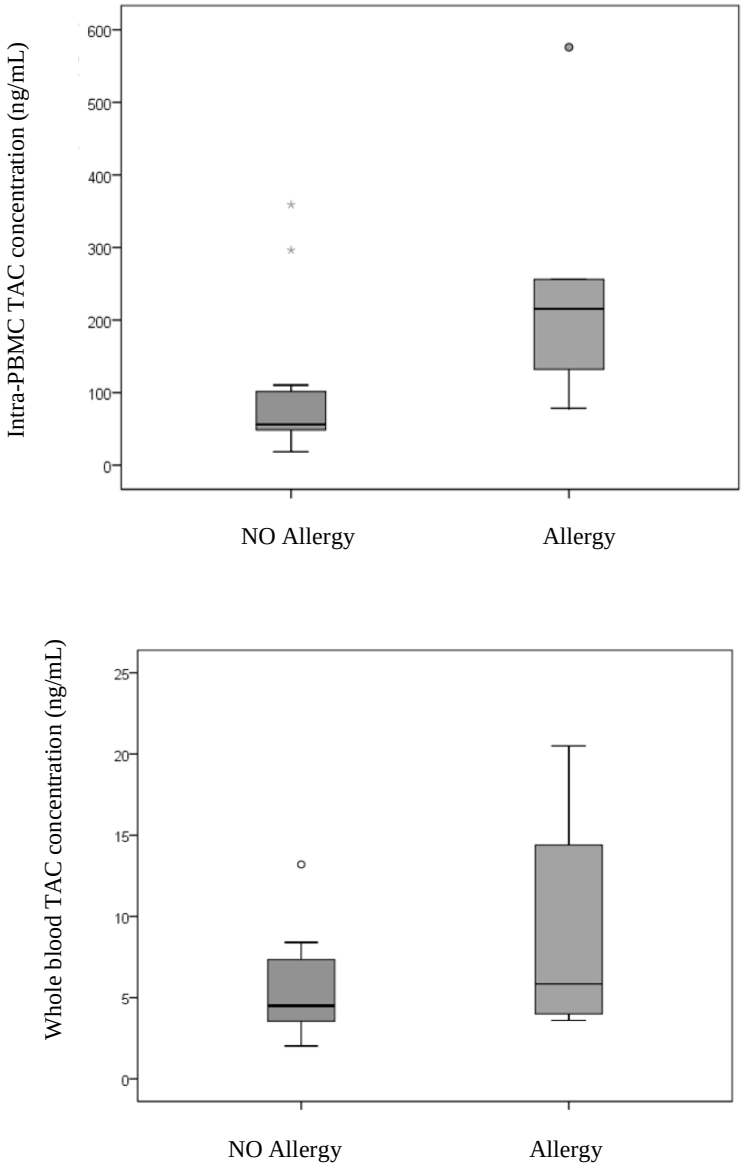

Figure 19: Box plots representing differences between TAC concentrations (whole blood and intra-PBMC) of patients that developed allergy and those without.

Lymphoproliferatives diseases

24 patients were analysed: hematic concentrations resulted the same in patients that developed PTLD and others (p = 0.924). Therefore, also if is not significant (p = 0.232), higher intra-PBMC TAC levels could be seen in patients with PTLD instead of those that not develop the PTLD. The ratio shows a correlation with PTLD near to the limit of significance (p = 0.094).

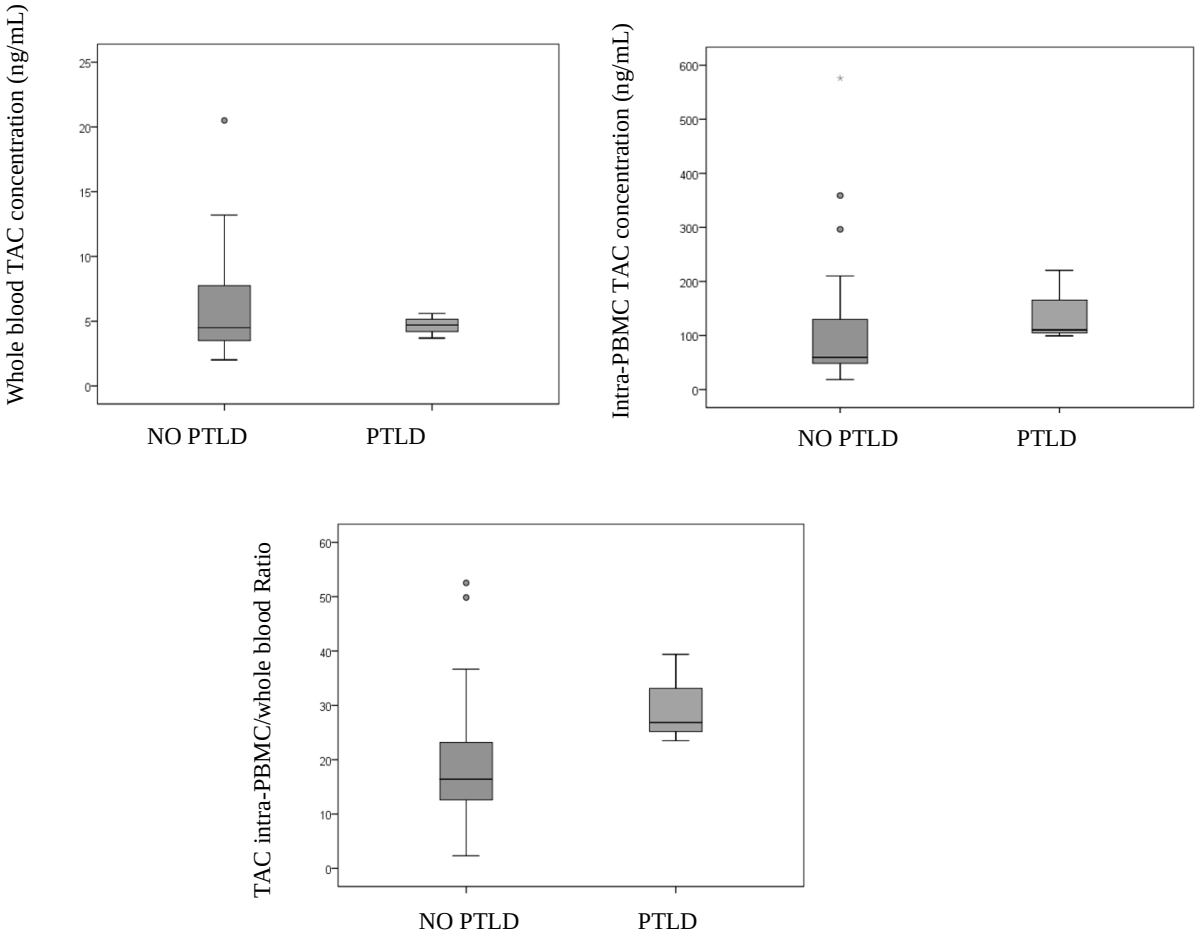

Figure 20: Box plots representing differences between TAC Ratio, intra-PBMC and whole blood concentrations in patients that developed PTLD and those that not developed lymphoproliferatives diseases.

Rejections

27 patients were considered: 7 have had acute or chronic reject phenomena. No correlation between whole blood TAC concentration and rejections (p = 0.602) instead of intra-PBMC TAC concentration (p = 0.053). Therefore, the ratio resulted strongly significant correlated to reject events (p = 0.021).

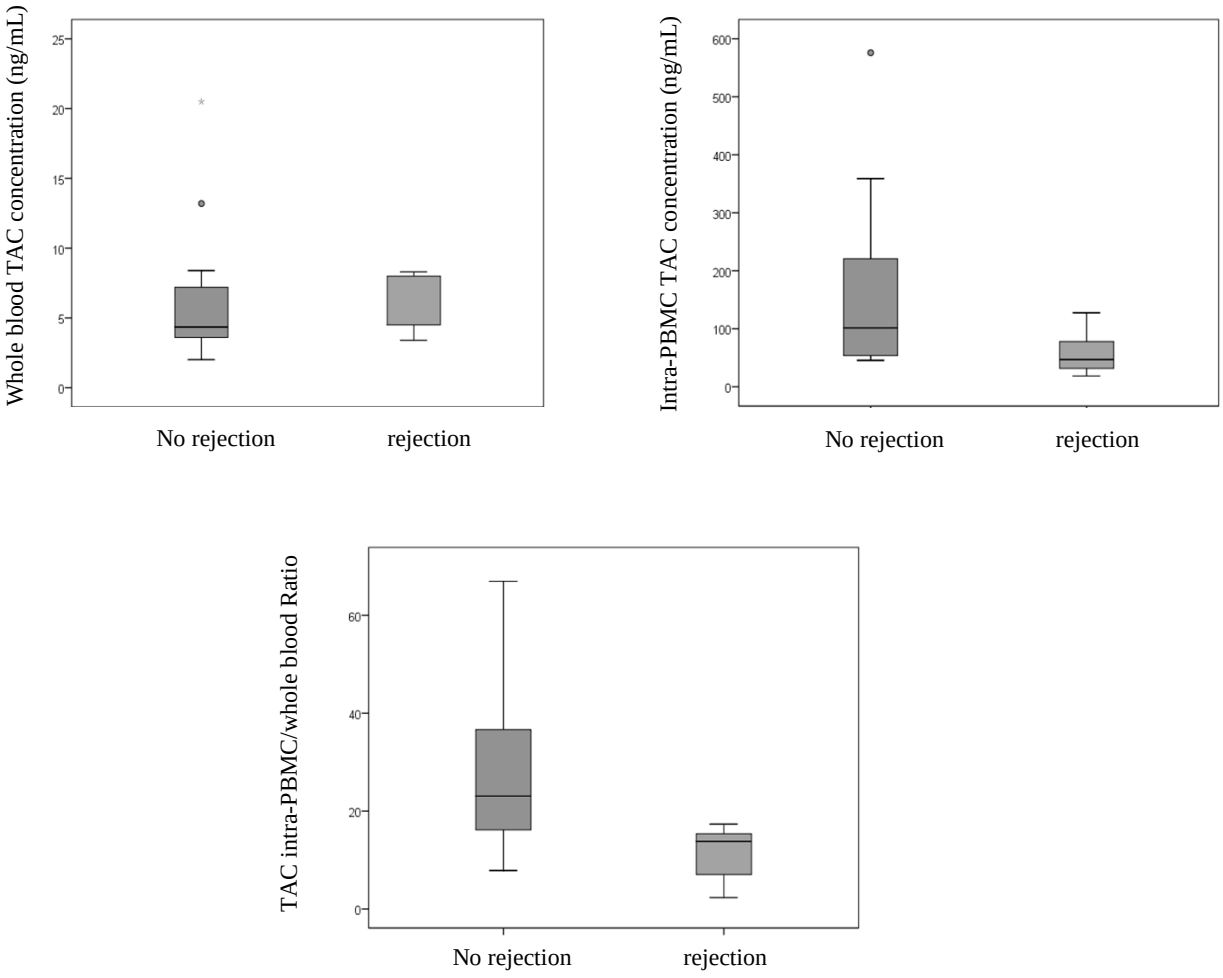

Figure 21: Box plots representing differences between TAC Ratio, intra-PBMC and whole blood concentrations in patients that had a reject phenomena and those that not.

Intracellular and ratio cut-off levels

The ROC (Receiver Operating Characteristic) curve were studied for the evaluations of possible cut off for TAC intra-PBMC and ratio concentrations: values of cut-off were expressed in terms of sensibility and specificity. For allergies was considered as cut-off a TAC intra-PBMC concentration of **121.19** ng/mL, for rejection were chosen two different TAC ratio values: **7.45** with a sensibility of 100% and **18.98** with a specificity of 100%.

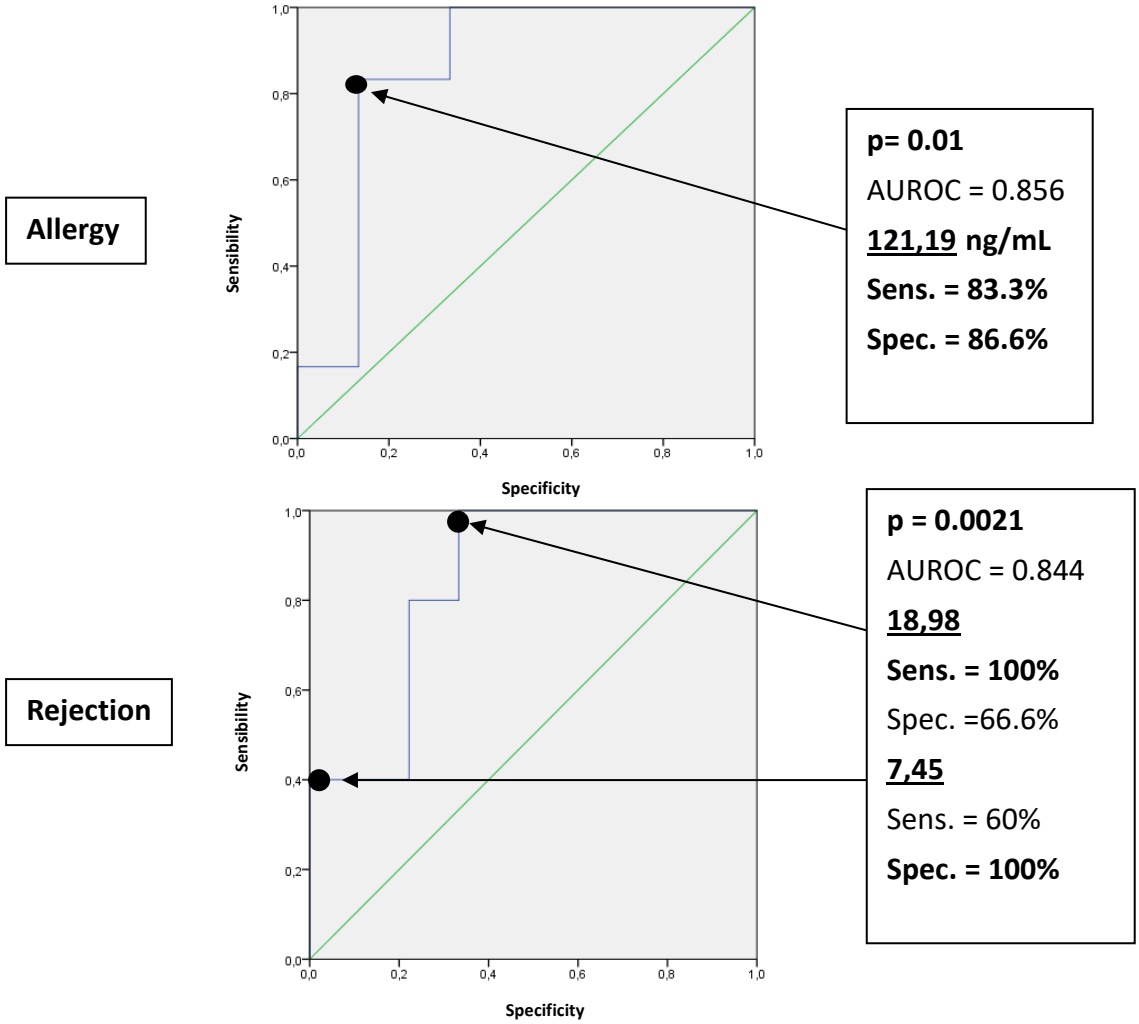

Figure 22: Roc curves for rejection and allergy determined on value of intra-PBM TAC concentrations and TAC ratio, cut off values are expressed in terms of sensibility and specificity.

4.4.3 Enrolments of more patients in the study:

The method published for the quantification of TAC and EVE was used for the quantification of a total of 61 patients treated with TAC and EVE. 117 samples were obtained from patients in TAC therapy and 31 with EVE therapy: some of the patients were switched from TAC monotherapy to a TAC and EVE co-therapy; others were switched to a co-therapy and then to an EVE monotherapy; others tried a period of co-therapy and then went back to a mono-therapy TAC regimen. Only one patient started with EVE monotherapy. Samples were collected in all these phases, mono (of either TAC or EVE) and co-therapy.

The correlations between whole-blood and intra- PBMC TAC concentrations were evaluated in 3 different groups of patients: (1) patients treated only with TAC; (2) patients treated with TAC (both in mono-therapy and co -therapy with EVE) and (3) patients in co-therapy with TAC and EVE.

(1) patients treated only with TAC: 101 samples of patients under TAC monotherapy were analysed: mean 21.96 [14.34-313.07]. A good correlation (r^2=0.496, P= 0.001) between the intra-PBMC concentration and the blood one was observed.

(1) patients treated with TAC (both in mono-therapy and co -therapy with EVE) : 117 samples of patients under TAC or TAC/EVE therapy were analysed: mean 25.62 [12.05-26.67]. A good correlation, but little worse compared with that obtained from samples in monotherapy, (r^2=0.476, P= 0.001) between the intra-PBMC concentration and the blood one was observed. A high variability of ratio was observed: a mean value of 237, but a median of 25.866

(2) <u>patients in co-therapy with TAC and EVE</u>: in this small subgroup of samples (n=16, mean= 48,68 [17.09-72.28]) from patients in TAC and EVE co-therapy the correlation between the intra-PBMC concentration and the blood one was lost (r^2=0.295, P= 0.268).

For studying better this difference observed between TAC whole-blood concentration, intra-PBMC concentration and ratio (intra PBMC/ whole blood concentrations) of patients in TAC mono-therapy and those of patients in co-therapy, a Mann-Whitney test analysis was done: only significant difference between the two hematic concentrations (P=0.002) was seen (Figure 22).

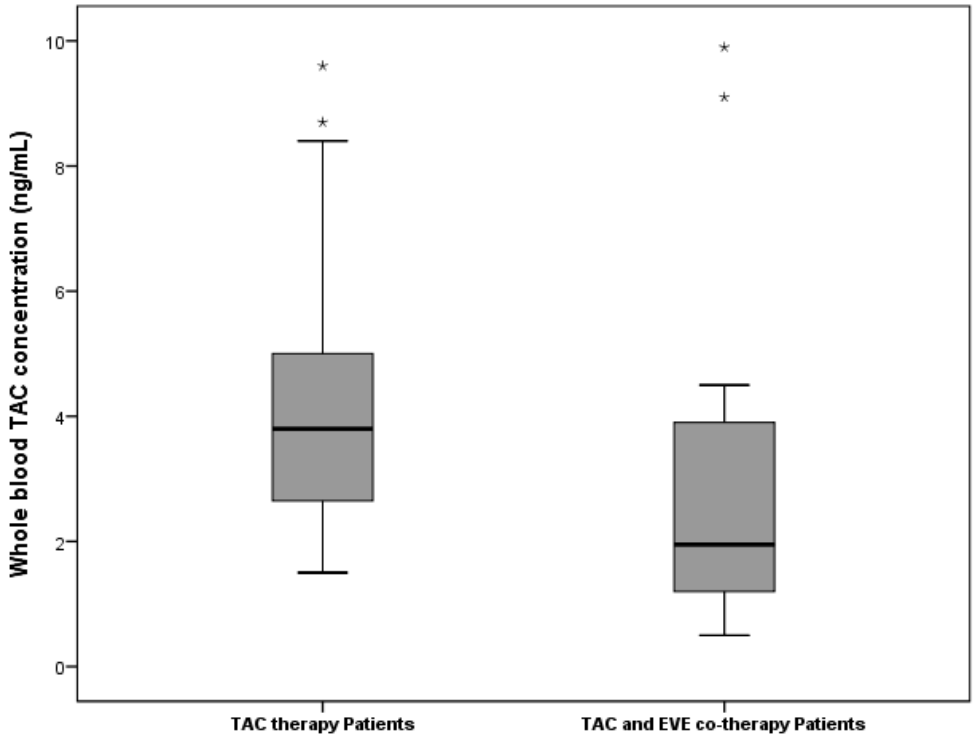

Figure 23: Box plots representing differences between TAC whole blood concentration in patients in TAC monotherapy and in TAC and EVE co-therapy.

The correlations between whole-blood and intra- PBMC EVE concentrations were evaluated in 3 different groups of patients: (1) patients treated only with EVE; (2) patients treated with EVE (both in mono-therapy and co -therapy with TAC) and (3) patients in co-therapy with EVE and TAC.

(2) patients treated only with EVE: 11 samples of patients under EVE monotherapy were analysed: mean 17.40 [11.35-21.58]. A correlation (r^2=0.496, P= 0.001) between the intra-PBMC EVE concentration and the blood one was observed but this not resulted significant.

(3) patients treated with EVE (both in mono-therapy and co -therapy with TAC) : 31 samples of patients under EVE or TAC/EVE therapy were analysed. No correlation was seen (r^2=0,182, P= 0.328) between the intra-PBMC EVE concentration and the blood one was observed. High variability of ratio results was observed: mean 237 [14.34-313.07].

(4) patients in co-therapy with EVE and TAC: in this subgroup of samples (n=14, mean = 477.41 [27.51-575.03]) from patients in EVE and TAC co-therapy the no significant correlation between the intra-PBMC EVE concentration and the blood one was observed (r^2=0.471, P= 0.089). A high variability of ratio results was observed also in this group of patients.

No significant difference between the data of whole-blood concentration, intra- PBMC concentration and ratio (intra PBMC/ whole blood concentrations) obtained from patients in EVE mono-therapy or in co-therapy was observed.

4.4.4 TAC intra-tissue method development

The method used for the quantification of TAC in tissue was the same of that developed for its quantification into-PBMC: it was adapted for the quantification in tissue. Tissues (liver and kidney) have different compositions of cells and extra-cellular components than those of PBMCs, therefore, for applying the method for TAC quantification on these matrix all the validations steps were reconsidered.

SPE-online method validation

Breakthrough, recovery and adsorption were evaluated. TAC and QX recoveries, during on-line SPE, were 98.4 ± 1.1 % and 80.3 ± 3.3 % respectively (Figure 24).

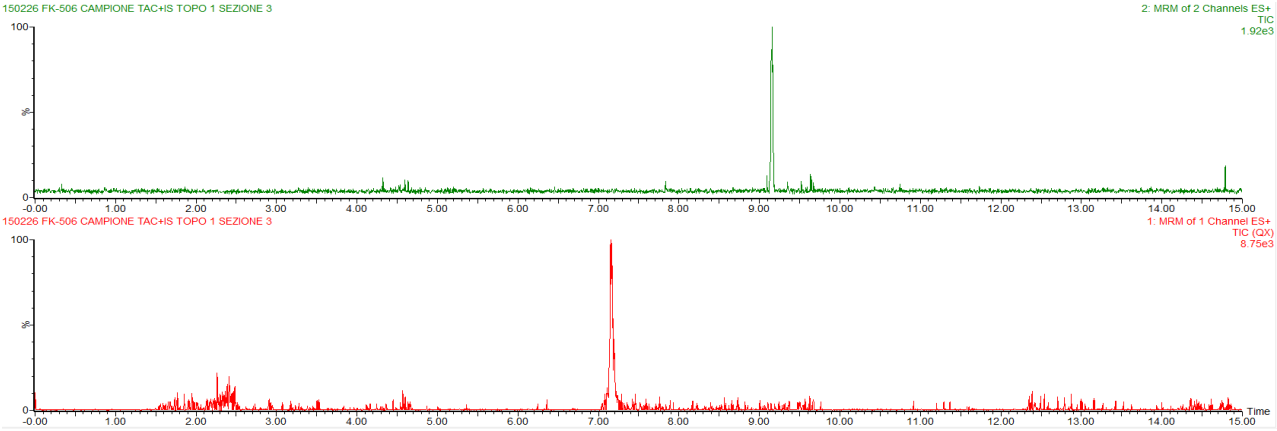

Figure 24: Tacrolimus and IS chromatograms in advanced method development mode. Time between 0 and 5 minutes corresponds to breakthrough, 5 to 10 minutes to the SPE recovery and 10 to 15 minutes to the adsorption of the analytes to the fluidic system.

Precision, accuracy and limits of quantification and detection

A summary of precision (coefficient of variation) and accuracy for low, medium and high QC samples of TAC is reported in Table 2. The mean (intra- and inter-day) percent inaccuracy and imprecision at QCs levels were always lower than 15%, as required by the FDA guidelines (FDA, 2013a). LLOQ, LLOD, ULOQ were 0.010 ng, 0.005 ng and 10 ng, respectively.

TAB 2		Accuracy	Inter-day precision	Intra-day precision
Liver	QC H	104.8%	84.6%	86.2%
	QC M	102.3%	85.6%	84.6%
	QC L	102.5%	81.4%	80.2%
Kidney	QC H	80.49%	93.1%	97.0%
	QC M	94.4%	94.7%	95.3%
	QC L	106.25%	90.1%	97.2%

Table 12: Validation of the method: intra/inter-day precision and accuracy (n=5) at QCH, QC M and QCL concentrations.

Specificity, sensibility and linearity

The calibration curve was found to be linear within the concentration range (0,039-10 ng), with a mean determination coefficient (r^2) higher than 0.998. Typical TAC and IS chromatograms, compared with the chromatogram of a blank extract are shown in Figure 25.

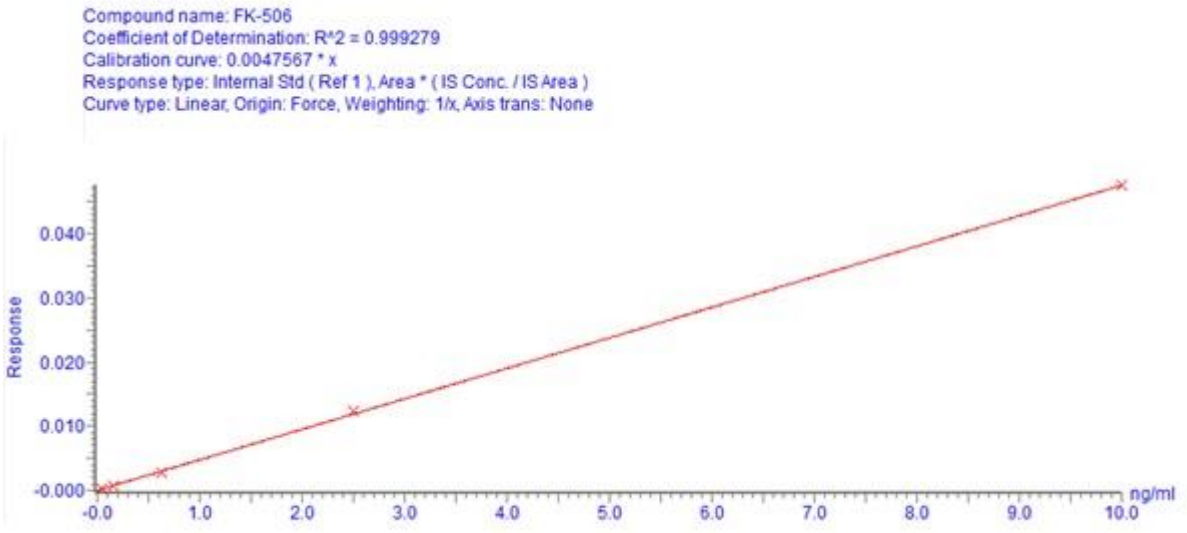

Figure 25 : Calibration curve of TAC in a range of 0.39-100 ng/mL, with a mean r^2 higher than 0.998

Also the calibration curve obtained by the quantification of the DNA extracted from scalar dilution of known number of PBMCs was found to be linear (r^2=0.998) (Figure 26)

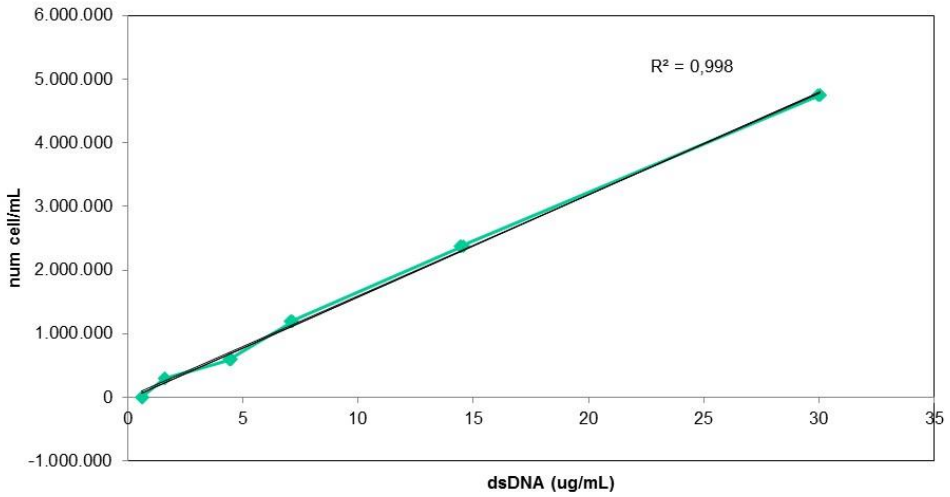

Figure 26: Calibration curve of PBMCs in range of 4.750.000-296.875 cell/mL, with a mean r^2>0.998.

No interfering peaks were observed at TAC and IS retention times of 4.17 ± 0.07 and 2.13 ± 0.07 min, respectively (Figure 27 and 28)

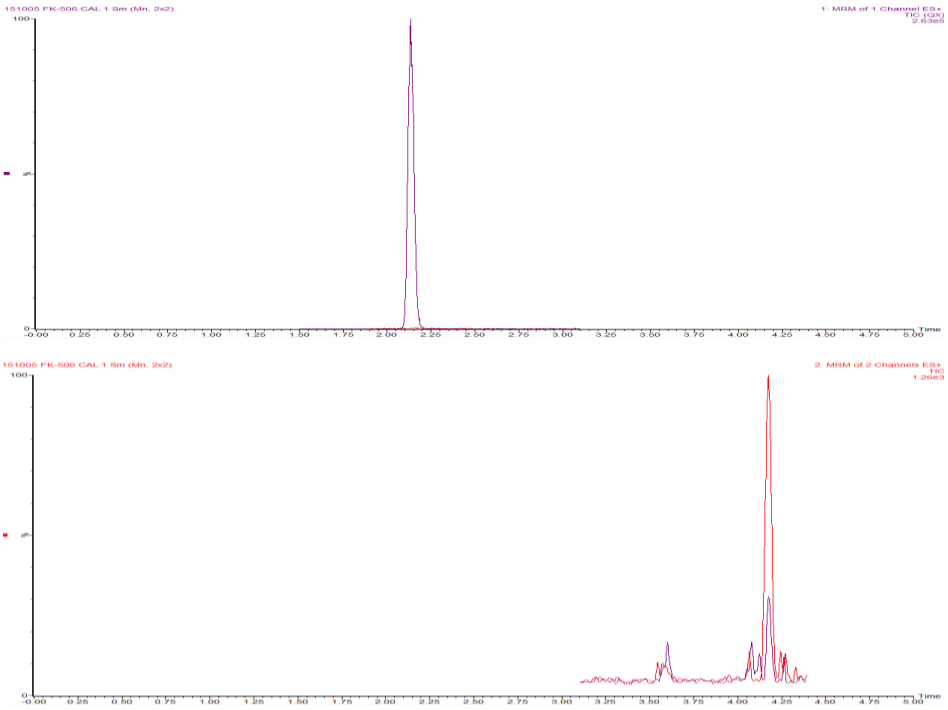

Figure 27 *:* Typical tacrolimus (LLOQ 0.39 ng/mL) and IS chromatograms, compared with the chromatogram of a blank extract. No interfering peaks were observed

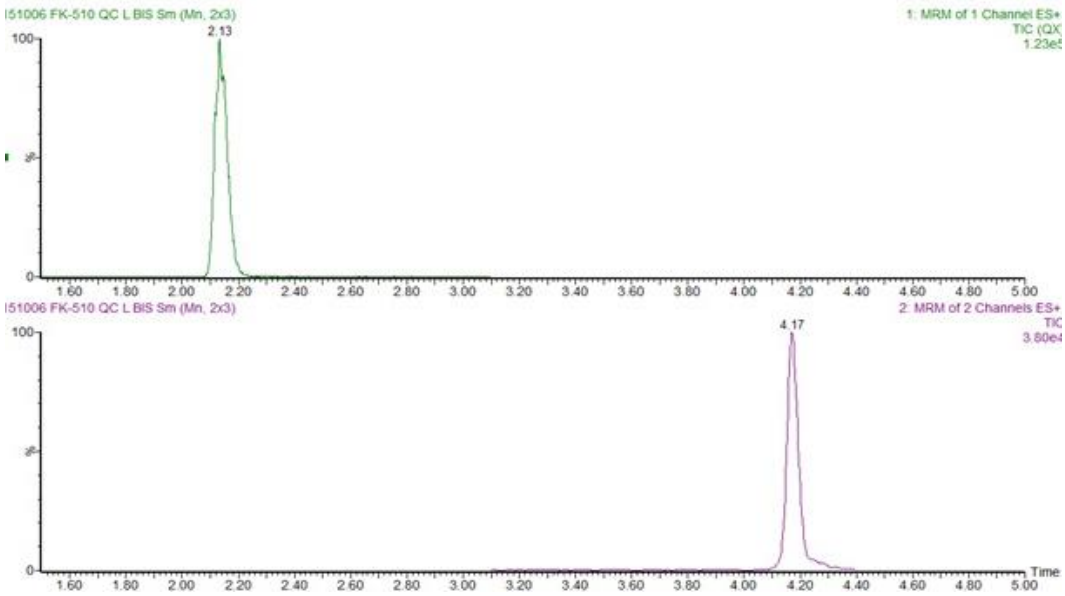

Figure 28: Typical tacrolimus QC L and IS chromatograms: TAC and IS retention times are 4.17 ± 0.07 and 2.13 ± 0.07 min, respectively.

Recovery and Matrix Effect

Recovery and matrix effect were evaluated in different tissues (kidney and liver). An impact of different type of tissue on recovery and on matrix effect was observed. The TAC recovery was higher on samples derived by kidney than liver for both of the two QCs amounts (QCL and QCH), respectively: 90,4% (RSD 0,1%) and 78,5 % (RSD 1,3%). A mean stable TAC matrix effect was observed for kidney tissue (+38,11±8,75%). The liver recovery was lower but also in this case the preliminary data confirm a stable and reproducible matrix effect: (+43,91±12,59%).

		Matrix Effect	Mean Tissue ME (RSD%)	Recovery	Mean Tissue REC (RSD%)
Liver	QC L	47.8%	43.91% (12.59%)	68.4%	57.92% (21.23%)*
	QC H	40.0%		47.5%	
Kidney	QC L	40.5%	38,11% (8,75%)	90.4%	90.48% (0.74%)
	QC H	35.75%		78.5%	

Table 13: Validation of the method: preliminary data of Matrix Effect and Recovery at QCH (40 ng/mL) and QCL (0.1 ng/mL) concentrations (* Higher RSD maybe due to the higher different tissue composition of the liver vs kidney).

No difference in stability and reproducibility between sample of PBMCs than tissue cells was underline in terms of matrix effect and recovery.

Test on Drugged Tissues

The entire procedure was tested on tissues, here are reported two example of the evaluation of the number of cells. The spectrophotometric analysis (Table 4) revealed a population of 1.392.135,67 and 1.405.505,33 cells into samples diluted 32 times for filling into the calibration curve made by the known PBMCs populations. Therefore, into each 200 uL derived from the two example slices of tissue, there were 44.548.341,33 and 44.976.170,67 cells, respectively. The portion of tissue used for these two examples were of 20 mg both, consequently, the corresponding numbers of cells were: 89.096.682,67 and 89.952.341,33.

	dsDNA (ug/mL)	num cell/mL	
cal 5	29,95	4750000	
cal 5	30,05	4750000	
cal 5	30,05	4750000	
cal 4	14,45	2375000	
cal 4	14,5	2375000	
cal 4	14,55	2375000	
CAL 3	7,05	1187500	
CAL 3	7,1	1187500	
CAL 3	7,15	1187500	
CAL 2	4,4	593750	
CAL 2	4,45	593750	
CAL 2	4,5	593750	
CAL 1	1,6	296875	
CAL 1	1,6	296875	
CAL 1	1,6	296875	
CAL 0	0,6	0	
CAL 0	0,65	0	
CAL 0	0,65	0	mean
ex 1	8,8	1.386.787,80	
ex 1	8,85	1.394.809,60	1.392.135,67
ex 1	8,85	1.394.809,60	
ex 2	8,9	1.402.831,40	
ex 2	8,9	1.402.831,40	1.405.505,33
ex 2	8,95	1.410.853,20	

Table 14: Examples of application of the spectophotometric dsDNA quantification.

5. Discussion and Conclusion

TAC and EVE have a narrow therapeutic index and significant inter-patients and intra-patient pharmacokinetic variability. For these reason their TDM is strongly recommended. Nowadays their TDM is routinely performed in whole blood. Despite the fact that it is very easy to perform, TDM in this kind of matrix do not give very useful information to the clinicians. Immunosuppressors whole blood concentrations are in fact influenced by high variability in blood composition, in terms of haematocrit, and plasma, considering in particular their affinity for the plasma protein and erythrocytes. Furthermore, their real target are lymphocytes and, in minor measure, monocytes. Thus, the develop of a reliable method for the quantification of TAC and then, after the advent of EVE regimen, also in co-therapy with TAC, of another one capable of quantifying both TAC and EVE in a routine clinical laboratory, became important. Despite PBMCs consist not only in lymphocytes, these could be consider an optimal compromise. Other methods, capable to sort cell types, take too much time and for this reason they could not be applied in a routine and, in some case, could allow drug exit from the cells. Capron et al. demonstrated the usefulness of TAC PBMCs levels as a marker to predict efficacy early after liver transplantation. The same group develop a method to quantify TAC in PBMCs but it resulted too laborious and time consuming, therefore not eligible for a clinical routine. Other methods were published both for quantification of TAC and EVE, but singularly and are not eligible for a clinical routine use for some critical points like: the need of drying samples (extraction became cumbersome and time consuming), expression of results like "ng/10^6 cells" (no comparison with whole blood results). The two methods developed in this thesis allowed to quantify TAC or TAC and EVE in an easy and rapid way, using the last generation UPLC system coupled with OSM® technology, the protocols applied resulted easy avoiding all long sample preparation procedure. According

with the FDA guidelines, both methods developed resulted specific, sensitive, precise and accurate, with a sensibility capable of quantifying the analytes in all patients sample. For the first method developed the internal standard was quinoxaline, which is a cheap and easy to find in clinical laboratories internal standard. In the second method, quinoxaline was substituted by ascomycin, resulting a little bit more expensive but more robust, because of its similar chemical properties and retention time to those of TAC and EVE. In this second work was also evaluated the effect of internal standard on matrix effect. In both validation was seen a matrix effect directly proportional to the number of cells: to make this effect reproducible, we excluded samples with 24 x 10^6 cells/mL. In order to evaluate the corrective impact of IS on analytes in the second work, was used for the first time the equation of "IS-nME", in addition to FDA and EMA guidelines. This equation consider the effect in each samples of both IS and target considering the real capability of IS of successful counterbalancing the variability caused by matrix effect. The low mean percentage of IS-nME confirmed, as reported in literature, of ascomycin is an ideal "non-deuterated" for TAC while a slight lower good internal standard for EVE.

The two developed methods were used for quantify a total of 37 patients and 100 samples. In the first published method TAC was quantified in 37 patients. In the second works were considered only 5 patients in TAC and EVE co-therapy. Up today, the number of patients enrolled increased: 61 patients treated with TAC and EVE. 117 samples were obtained from patients in TAC therapy and 31 with EVE therapy. Analyzing a major number of patients and samples the results obtained in the two previous published work were confirmed: there is a good correlation between intracellular and whole-blood data (r^2=0.496, P= 0.001), but just in TAC monotherapy. During co-therapy this good correlation is lost (r^2=0.295, P= 0.268).

The main data that we could report about intra-PBMC TAC concentration is that there is no significant difference between data observed in mono-therapy and in TAC/EVE co-therapy.

Otherwise, a significant difference (P=0.002) was seen between the whole-blood concentrations of mono-therapy and those of TAC/EVE co-therapy patients. These results could be explained by an influence of EVE on TAC whole-blood concentration, therefore, if these results will be confirmed, for clinicians changing of dosage only base on whole-blood TAC concentration in case of co-therapy could be reductive and no very reliable. Based on similar results an hypothesis of no difference of TAC diffusion in co-therapy, but only a difference on its adsorption and metabolism.

Conversely, no significant difference of concentrations (intra-PBMC, whole blood) and ratio between the patients group in EVE mono-therapy and other in co-therapy was seen. However an high variability of EVE ratio during co-administration with TAC was underlined. An absence of significant correlation between EVE whole blood and intracellular concentrations was seen, as reported with the first collected data in the second published work, in particular after a higher dosage of EVE during the co-therapy. Instead of the good correlation (r^2=0.496, P= 0.001) between the two EVE concentrations during the mono-therapy shown. It could be supposed a different EVE capability of entrance into cells during co-therapy with TAC. However, the number of sample and patients is very different between the two groups (mono and co-therapy with TAC), therefore the results must be confirmed before any hypothesis of modifying dosage based only on EVE intracellular concentration.

The data of TAC concentrations obtained were used for a retrospective study on the difference between these concentrations in patients that developed of not allergies, PTLD or had or not rejects. A strong statistical correlation was seen between high TAC intracellular concentration and alimentary allergies, in accord of the data present in literature, that shown a prevalence of food allergy in children under immunosuppression (Ozdemir., 2013). The ratio of TAC concentrations seems to have an influence, near to the significance (p=0.094), on the development of PTLD: the intracellular concentration is higher in those patients that

developed lymphoproliferative disease. In this study were also analyzed patients that had a reject: the intracellular data seems to be near to be significant ($p = 0.053$) for predicting the risk of rejection, and the ratio resulted significant ($p= 0.021$), instead of the hematic data that resulted not significant at all. This lack of correlation could be explained by the fact that the hematic TDM is used for adjusting the dosage of immunosuppressor, maintaining the whole blood TAC concentration inside of pre-defined range, therefore this could explain the rising of rejection also when TAC whole blood concentration resulted in this range, and in this case the data observed not correlate well with the capability of TAC of penetrate into lymphocytes.

After these evaluation, in this PhD thesis project seemed to be necessary to try to calculate a possible cut off for the rise of allergies and rejection. The one for the rising of allergies was calculated considering the intracellular TAC concentration of 121.19 ng/mL, which is the value at which there are both good sensitivity and good specificity. For the rejection were considered two value of TAC concentrations ratio: 7.45 with the highest specificity and 18.98 with the 100% of sensitivity.

In collaboration with the Unit of Pediatric Gastroenterology and Hepatology of the University of Turin, was study the correlation between genetic and adverse events and intra-PBMC concentrations. The genetic analysis were performed on three different genes: ABCB1, CYP3A5 and CYP3A4. TAC in fact is a substrate of p-glycoprotein and Cytochromes 3A4 and 3A5. An evident, also if not significant, difference between CYP3A5*1/*3 and CYP3A5*3/*3 recipient genotypes in terms of TAC ratio was observed. This difference was higher considering only the TAC intra-PBMC concentrations and lower if is considered the whole-blood one. Considering two 3435 C>T and 1199 G>A P-glycoprotein polymorphisms, the TAC intra-PBMC/ whole blood concentrations ratio did not have significative differences between patients with C/C, C/T and T/T or with G/A and G/G. Also if it was not significant, maybe for the small number of patients enrolled right now for the study, the recipient that

have 1199 G>A had higher intra-PBMC TAC concentration levels than those that not expressed p-glicoprotein, which is involved in the exit of the drug from the cells. This data was in accord with the literature (Capron, 2010; Elens, 2007).

The correlation of particular level of TAC with the presence or not of EBV infection, and also its negativization or persistent viremia, was study as an example of possible correlation of TAC concentration and the grow of averse event: differences in terms of TAC Ratio between patients with EBV and those without infection ($p=0.022$) and between with EBV negativization and those with persistent viremia ($p=0.04$) were seen. These data confirm that the status of opportunistic infections like EBV correlate well with the TAC concentration, in fact higher levels of TAC ratio correspond with positivization or reactivation of EBV infection.

The method for the quantification of TAC was applied and validated also for the quantification of TAC into tissues. Was developed a protocol capable to quantify drugs in tissue as ng/mL, a measure unit that let a comparison between intra-biopsy drug concentration data and others obtained by non-invasive methods (like PBMCs, plasma, urine, blood quantification methods). This protocol is easy to use and to apply to the routine laboratory in particular case of non-therapy adherence. It is characterized by three easy steps: (1) MCV evaluation, (2) quantification of the number of cell of the biopsy and (3) UHPLC-MS/MS on-line SPE drug quantification. MCV is evaluated from a part of the tissue, after a desegregation cells step, with an automated cell counter. The number of cells of the biopsy is obtained by the quantification of the dsDNA extracted from the tissue with a simple spectrophotometric absorption. The drug quantification is made using a simple, specific, sensitive, precise, rapid and accurate new method for TAC quantification. After a fully validation following the FDA guidelines, this protocol could be tested on real biopsy samples of patient under TAC treatment. The method proposed is an easy method for obtaining concentration as ng/mL from

a tissue, and for example using TAC as drug, no difference in stability and reproducibility between sample of PBMC than tissue cells was underline in terms of matrix effect and recovery. Since this protocol allows to correctly normalize intracellular analytical results, it could be useful in the near future to verify the correlation between plasma/blood/PBMC and tissue concentrations for many drugs, as TAC, to help clinicians to improve therapy and clinical outcome, and/or to evaluate their potential intracellular accumulations on the basis of data obtained from the target cells for research and clinic purposes.

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