Tacrolimus: The balance between good and evil

Pharmacogenetics in pediatric solid organ transplant recipients

Violette Gijsen

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Tacolimus: De balans tussen het goede en het kwade
Pharmacogenetica en orgaantransplantatie in kinderen

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The individualization of drug therapy

"Doctors are men who prescribe medicines of which they know little, to cure diseases of they know less, in human beings of whom they know nothing" Voltaire

In the ideal world, a doctor knows everything about his patient to treat him or her according to their specific needs. Unfortunately, this is not the case and may never be the case. That doesn't mean that for centuries we haven't tried to individualize our treatment to the patient's needs to the best of the knowledge known at the time. In ancient Egyptian times, attempts have been made to treat every patient according to their symptoms with hundreds of drugs being described in the literature. Similar practices can be found in ancient China in the 1st century BC, as well as ancient Greece in the 4th century BC. The struggle for the optimal therapy for every patient is clearly something that we have been dealing with for centuries and is still a cornerstone in clinical medicine.

Throughout my doctoral research project in Toronto, I was introduced to the field of clinical pharmacology and quickly fell in love. Dr. Saskia de Wildt, my co-supervisor, showed me all the possibilities of clinical pharmacology and how important it is in every day care of patients. She has been an absolute mentor to me, introducing me to the world of research, conferences and networking. Every conference I've been able to attend has been a new experience and made me more convinced that clinical pharmacology can have a major role in the care of patients. Saskia was key for my decision to keep being involved in clinical pharmacology.

The last couple of years have been an amazing and sometimes wild ride, but has resulted in this thesis. I hope you will read it with as much pleasure as I had in creating it.

Violette

General Introduction

General Introduction

Solid organ transplantation has dramatically increased the survival of both adult and children suffering from end-stage organ failure and improved the quality of life of these patients.[1,2,3,4] With the introduction of calcineurin-inhibitors (CNIs) the short-term survival of transplant recipients improved even further.[5] Presently, more than 90% of the immunosuppressive protocols in organ transplantation have CNIs incorporated as the primary immunosuppressant, resulting in CNIs being the mainstay of immunosuppressive therapy.[6,7]

In 1995, tacrolimus, a macrolide CNI originating from the fungus Streptomyces tsukubaensis, was introduced[8,9] and became part of clinical practice in 1997.[8] It is metabolized by the cytochrome P450 (CYP) enzymes CYP3A4 and CYP3A5, and is a substrate for the efflux pump ABCB1.[10] CYP3A4, CYP3A5 and ABCB1 are all expressed in the liver, intestine and kidney, and are therefore expected to influence the oral bioavailability as well as the systemic clearance of tacrolimus.

Tacrolimus has a narrow therapeutic window[11] and there is a clear relationship between the tacrolimus trough concentrations and the outcome of the patient. Subtherapeutic levels have been associated with rejection and supra-therapeutic levels with tacrolimus toxicity.[12]
Therefore, therapeutic drug monitoring (TDM) is recommended.[12,13,14,15] Yet, the monitoring of tacrolimus concentrations is relatively complicated due to the pharmacokinetic properties of tacrolimus; the effect of a dose adjustments may only be evident after a couple of days.[16] The predefined therapeutic range is dependent on the transplanted organ and the time after transplantation. Interestingly, only little evidence is available to support these therapeutic ranges.[17] Nevertheless, balancing between under-dosing and over-dosing is a critical aspect of the care of transplant patients, but is very complicated as many factors can influence tacrolimus trough concentrations.[18]

Despite the better short-term outcomes, the use of tacrolimus brings with it serious adverse effects, such as renal dysfunction, neurotoxicity, hypertension, glucose intolerance and liver function abnormalities.[19,20,21] Especially, tacrolimus-induced nephrotoxicity is of particular concern as it can result in a second transplantation or re-transplantation of the kidney.

Two forms of tacrolimus-induced nephrotoxicity have been described before. Acute nephrotoxicity is reversible and usually occurs within a couple of days after starting tacrolimus treatment. It may be counteracted with a dosage reduction or complete tacrolimus withdrawal. This acute form is characterized by a reduced glomerular filtration rate (GFR) due to a constriction of the afferent glomerular arteriole resulting in a decreased renal plasma flow. These changes seem to be due to an imbalance in the secretion and metabolism of nitric oxide, prostaglandines (both vasodilatory), thromboxane and endothelin (both vasoconstrictive) together with increased activity of the sympathetic nervous system. Tacrolimus may interfere with the normal tubular function and may cause mesangial cell contracting, thus altering the glomerular permeability.[22]

Chronic nephrotoxicity occurs with prolonged use of tacrolimus. The main clinical sign is a slow decline in renal function, which may result in end-stage renal failure. Most patients have hypertension as well, and the absence of proteinuria in these patients may help to distinguish tacrolimus-induced nephrotoxicity from other causes. However, this has not been proven yet. Chronic nephrotoxicity may not always resolve by dosage reductions or tacrolimus withdrawal. Initially, it has been reported that chronic nephrotoxicity is characterized by arteriolar hyalinosis, tubular atrophy, glomerular sclerosis, thickening of the Bowman's capsule and interstitial striped fibrosis.[22]

However, over the last couple of years a debate has developed on the existence of tacrolimus-induced nephrotoxicity. As chronic renal failure has multiple causes (i.e. CNI use, diabetes, HCV-associated glomerulonephritis, atherosclerosis), proving tacrolimus is the actual cause of the decline in renal function is challenging. Biopsies would be the preferred diagnostic tool; however, biopsies are not routinely taken. Additionally, the classical histological signs of tacrolimus-induced nephrotoxicity are currently under debate for its specificity.[23,24] For these reasons, we will use the term "renal failure" in the context of renal dysfunction in transplant recipients taking tacrolimus, as specific tacrolimus-induced nephrotoxicity cannot be proven at this point in time.

Renal failure is clinically diagnosed by an increase in the glomerular filtration rate (GFR), which is derived from the serum creatinine (SCr) levels. One of those formulas to estimate the GFR is the Schwartz formula. The Schwartz formula has been developed in 1987 especially for use in children and has been used ever since. It is especially adapted to take the gender and age of the children into account.[25] Additionally, the National Kidney Foundation clinical guidelines on chronic renal failure containing a classification system of the severity of renal failure (KDOQI).[26] This classification system can aid in the management of the patient with renal failure, but also to standardize research outcomes.
To identify a change in SCr concentrations and thus GFR, a decrease of 50% of renal function is needed and will not reflect accurate renal function unless steady state concentration have been reached.[34,35] Moreover, the SCr concentrations are influenced by factors such as muscle mass and meals.[34,35] There is a need for new acute kidney injury (AKI) biomarkers, since the use of SCr has several well-recognized drawbacks.

As renal failure is an important side effect of CNI therapy, in 2003 Ojo et al conducted a large landmark study looking into the prevalence of renal failure after non-renal transplantation. Overall, 11,426 (16.5%) of

the 69,321 non-renal solid organ transplant recipients developed chronic renal failure.[27] However, the majority of the patients were treated with cyclosporine (60%) rather than tacrolimus. No other study has been done since to determine the incidence of renal failure in patients treated with tacrolimus alone.

Tacrolimus disposition

Adults

A wide inter- and intra-variability in tacrolimus disposition is present among adult transplant recipients.[9] Several factors influencing this variation have been reported and include the patient's age, gender, ethnicity, concomitant medication, hematocrit and albumin levels.[12,19,28] In addition to these clinical factors, the polymorphically expressed *CYP3A5* gene, involved in the metabolism of tacrolimus genes has been reported to influence the tacrolimus disposition in adult transplant recipients.[19] In kidney, liver and heart transplant recipients it has been shown that *CYP3A5*-expressers (*CYP3A5*1/*1* or *CYP3A5*1/*3* carriers) had higher tacrolimus dosing requirements and lower tacrolimus dose adjusted trough concentrations compared to CYP3A5 non-expressers *(CYP3A5*3/*3* carriers). The influence of the efflux transporter *ABCB1* SNPs *3435C>T, 1236C>T* and *2677G>T/A* remains to be elucidated as results have been contradictory.[19]

As genetic variation in *CYP3A* is associated with the wide inter- and intra-variability in tacrolimus disposition, an impact of this variation on the risk of developing chronic renal failure may be present. In addition to polymorphisms in enzymes and transporters involved in tacrolimus disposition (i.e CYP3A and ABCB1), polymorphisms in genes involved in the pharmacodynamic effects (i.e. *ACE* gene, *TGF-ß*) of tacrolimus may play a role as well.

Children

Children have long been believed to be little version of adults and decisions on drug therapy have mainly been based on this hypothesis. Over the last decades, our knowledge on growth and development of children has largely expanded and we now recognize that the changes that children are undergoing will affect how the body handles drugs and their responses to these drugs.[29] It is therefore key to study children separately from adults, both for the disposition and effects of drugs.

Factors influencing tacrolimus disposition have not been as widely studied in children as in adults. A wide variability in tacrolimus disposition has been reported, yet, little is known about the impact of clinical factors (such as age, weight, hematocrit) or polymorphisms in metabolizing enzymes on tacrolimus disposition.

In pediatric liver and kidney transplant recipients it was shown that age, body weight, hematocrite and *CYP3A5* genotype are associated with altered tacrolimus disposition.[30] Children younger than 5 years of age needed higher tacrolimus dosages to achieve similar tacrolimus trough concentrations.[31,32] In addition, children with a hematocrite level of <33% had a higher tacrolimus clearance. This finding can be explained as tacrolimus is highly protein bound and accumulates in the erythrocytes. When patients experience low hematocrite and albumin concentrations this will results in a reduction of the whole-blood concentrations and higher tacrolimus clearance.[32] As reviewed by Quteineh et al. in pediatric liver and kidney recipients pediatric *CYP3A5*-expressers required higher tacrolimus dosages compared to *CYP3A5* non-expressers.[31,32] In pediatric liver transplant recipients, carriers of the *ABCB1 T-T-T* haplotype had higher tacrolimus dosing requirements (0.26 [0.15-0.32] versus 0.11 [0.01-0.25] mg/kg/12h) compared to non-carriers.[31]

To our knowledge, no studies have been done in pediatric heart, lung or small bowel transplant recipients.

The study by Ojo et al[27] reporting the prevalence of renal failure in non-renal solid organ transplant recipients mainly focused on adult patients. During childhood the kidney is still growing and developing, resulting in a low GFR at birth and exceeding adult values at 3 to 6 years of life.[33] For this reason, in addition to the age-related pharmacokinetics of tacrolimus, we cannot extrapolate the data from the study by Ojo et al.[27] to pediatric solid organ transplant recipients. Unfortunately, data in children on the prevalence of renal failure in pediatric solid organ transplant recipients treated with tacrolimus are scarce.

In conclusion, despite tacrolimus being part of most pediatric solid organ transplant protocols, our knowledge on the disposition and its adverse effects in children is still very limited. This leaves pediatric transplant recipients at an increased risk of therapy failure with subsequent graft loss but also of increased toxicity, with possible renal failure.
Therefore, the overall aim of this thesis was to identify clinical and genetic factors associated with tacrolimus pharmacokinetic and pharmacodynamic parameters in pediatric solid organ transplant recipients to ultimately optimize tacrolimus dosing.

In ***Chapter 2***; the available data on the prevalence of renal failure following solid organ transplantation in children is presented. In addition, in ***Chapter 3***, we reviewed the available literature on genes related to renal failure in both adult and pediatric solid organ transplant recipients.

In the second part of this thesis, we studied the impact of genetic polymorphisms in *CYP3A* and *POR* on the disposition and renal function of tacrolimus in pediatric solid organ recipients. In ***Chapter 4*** we determine the possible association between *CYP3A5* genotype and age on tacrolimus trough concentrations and dosages in pediatric heart transplant patients. With the discovery of the new SNP *CYP3A4*22* we reanalyzed our initial pediatric heart transplant cohort

and increased our sample size to determine if this new polymorphisms has an additional effect on tacrolimus disposition ***(Chapter 5)***. Since the variability in tacrolimus disposition can only partly be explained by differences in expression of *CYP3A4* and *CYP3A5*, polymorphisms in the gene encoding the P450 oxidoreductase (POR) protein that enables the activity of CYP enzymes, may be of interest. In ***Chapter 6*** we evaluated the possible relationship between *POR*28* genotype and tacrolimus disposition in pediatric kidney transplant recipients.

The third part of this thesis concerns the pharmacodynamic effects of tacrolimus, with a particular focus on renal failure in pediatric transplant recipients treated with tacrolimus. ***Chapters 7, 8 and 9*** are all part of a multicenter cohort study aimed to determine the relationship of genetic (96 SNPs) and non-genetic factors with the risk to develop renal failure in pediatric solid organ transplant recipients. ***Chapter 10*** of this thesis presents a pilot study on a potential novel AKI biomarker neutrophil gelatinase-associated protein (NGAL) in the first two weeks post-transplant. The final part summarizes the results of all studies and discusses the challenges encountered along with suggestions for future research topics ***(Chapters 11)***.

List of abbreviations

ABCB1	ATP-binding cassette sub-family B member 1
ACE	Angiotensin-converting enzyme
AKI	Acute Kidney Injury
CNI(s)	Calcineurin-inhibitor(s)
CYP	Cytochrome P450 enzyme
CYP3A	Cytochrome P450, family 3, subfamily A
CYP3A4	Cytochrome P450, family 3, subfamily A, polypeptide 4
CYP3A5	Cytochrome P450, family 3, subfamily A, polypeptide 5
GFR	Glomerular Filtration Rate
HCV	Hepatitis C Virus
KDOQI	The National Kidney Foundation Disease Outcomes Quality Initiative
NGAL	Neutrophil gelatinase-associated protein
POR	P450 oxidoreductase
SCr	Serum Creatinine
SNP(s)	Single Nucleotide Polymorphism(s)
TDM	Therapeutic Drug Monitoring
TGF-ß	Transforming Growth Factor beta

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Prevalence of renal dysfunction in tacrolimus-treated pediatric transplant recipients:

A systematic review

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Abstract

Background Renal dysfunction after non-renal transplantation in adult tacrolimus-treated transplant patients is well documented. Little is known about its prevalence in children. Age-related changes in both disposition and effect of tacrolimus as well as renal function may preclude extrapolation of adult data to children.

Objectives To systematically review the literature on renal dysfunction in non-renal pediatric transplant recipients treated with tacrolimus.

Methods PubMed/Medline, Embase and Google were searched from their inception till April 19th 2012 with the search terms "tacrolimus", "renal function", "transplantation" and "children".

Results Eighteen of 385 retrieved papers were considered relevant. Twelve dealt with liver, four with heart transplant, one with heart and lung transplant, and one with intestinal recipients. Reported prevalences of mild and severe chronic kidney disease ranged from 0%-39% and 0%-71.4%, respectively, for liver, and from 22.7%-40% and 6.8%-46%, respectively for heart and/or lung transplant recipients. Ranges remained wide after adjusting for follow-up time and disease severity. Possible explanations are inclusion bias and definitions used for renal dysfunction.

Conclusions A considerable proportion of pediatric non-renal transplant patients who receive tacrolimus-based immunosuppression, appear to suffer from chronic kidney disease. This conclusion warrants further research into the real risk, its risk factors and individualization of immunosuppressant therapy.

Introduction

Renal dysfunction is a serious complication of non-renal solid organ transplantation that may ultimately result in the need for dialysis or renal transplantation. In a large registry study by Ojo et al 11,426 (16.5%) of the total of 69,321 mainly adult, non-renal solid organ transplant recipients developed chronic kidney disease (defined as an estimated glomerular filtration rate [eGFR] ≤29 ml/min/1.73m^2) at a median of 35 months after transplantation (mean 46±38 months). In that study, the 5-year cumulative incidence of chronic kidney disease (CKD) ranged from 6.9% for heart and lung transplant recipients to 21.3% for intestinal transplant recipients. The majority of the patients included in this study used cyclosporine (60%) rather than tacrolimus as the primary immuno-suppressant.[1]

Data on the prevalence of CKD after non-renal solid organ transplantation in children are sparse. During childhood the kidney is still growing with nephron recruitment being completed at approximately 18 to 24 months. During this time the renal parenchyma increases with the patient's height and due to an increase of the filtration surface per body surface area.[2,3,4] Therefore, the GFR is low at birth, increases during the first year of life to exceed adult values at age 3 to 6 years.[5] As the kidney has not completely matured until after the age of 6 years, the impact of non-renal solid organ transplantation on kidney function may differ between children and adults. Furthermore, older age has been implicated as a risk factor for CKD, which could also result in a difference in the prevalence of CKD between adults and children.[1] Chronic kidney disease in children can have multiple causes. The chronic use of calcineurin-inhibitors, such as tacrolimus, might be more important in children as other possible causes (i.e. diabetes, HCV-associated glomerulonephritis, atherosclerosis) are less common in children compared to adults.

Tacrolimus, a macrolide calcineurin-inhibitor, was introduced in 1995 into clinical practice and was included in treatment protocols from 1997 onwards.[6] Originating from a fungus (Streptomyces tsukubaensis), the drug is an effective immunosuppressant agent. However, a range of serious adverse effects in addition to renal dysfunction, including neurotoxicity, glucose intolerance, liver function abnormalities and hypertension, complicates its use.[7,8] One of the most serious complications of chronic calcineurin inhibitor use is CKD.[9] Although its importance has recently been debated[10] there is compelling evidence that prolonged use of calcineurin inhibitors is indeed nephrotoxic.[11,12,13]

Although both cyclosporine and tacrolimus act by inhibiting calcineurin, the precise molecular mechanism causing CKD remains unclear and seems to differ between the two drugs.[9,14,15,16] Recently, Lamoureux et al. showed that exposure to tacrolimus and cyclosporine in a human kidney cell line resulted in different proteomic profiles, suggesting different molecular mechanisms of toxicity.[17] Similarly, in rats, proteins involved in the mechanism of kidney damage also showed different expression profiles for cyclosporine and tacrolimus.[18] These findings point at the necessity to separately study the adverse renal effects of cyclosporine and tacrolimus.

The prevalence of CKD in pediatric recipients of a non-renal organ treated with tacrolimus is unclear. Data from the adult population may not be extrapolated to children and the mechanisms underlying the nephrotoxicity of tacrolimus may be different from those of cyclosporine. Therefore, we aimed to determine the reported prevalences of CKD in children who had received a non-renal solid organ transplant and who received tacrolimus rather than cyclosporine as the primary immunosuppressant by systematically reviewing the literature.

Methods

Literature search

A literature search was conducted by reviewing Pubmed/Medline, Embase and Google from their inception till April 19th 2012 with the search terms "tacrolimus", "renal function", "transplantation" and "children". Subsequently, references of retrieved papers were screened for more references.

The inclusion criteria were: human studies, original research, tacrolimus, non-renal transplantation, renal function, and children. Only non-renal solid organ transplant papers were included as the cause for CKD in renal organ transplant recipients are multifactorial and therefore looking at non-renal solid organ transplantations might be a cleaner model. Case reports, case series and reviews were excluded. Conversion studies and studies with mixed patient groups (i.e. adults and children together, cyclosporine and tacrolimus together) were also excluded.

Outcome

The primary outcome of our review was to determine the prevalence of CKD post-transplantation in non-renal solid organ transplant recipients. The study author's definition of CKD was accepted. For a secondary analysis we defined CKD as mild (eGFR >60 and <90 ml/min/1.73m^2) and moderate-severe (eGFR <60 ml/min/1.73m^2) CKD. The prevalence of CKD for the secondary analysis was determined in those papers that reported prevalence numbers at least one year post-transplantation.

Quality assessment

To test the quality of the papers included, we used the checklist Strengthening the Reporting of Observational studies in Epidemiology.[19] We scored the items either by "yes", "partial" or "no" depending if the item in question could be found in the included paper. A partial response was given in case a question had multiple items and not every item was

reported. We would count the amounts of times we scored a "yes" or a "partial". The minimal score possible is 0 and the maximal score 34.

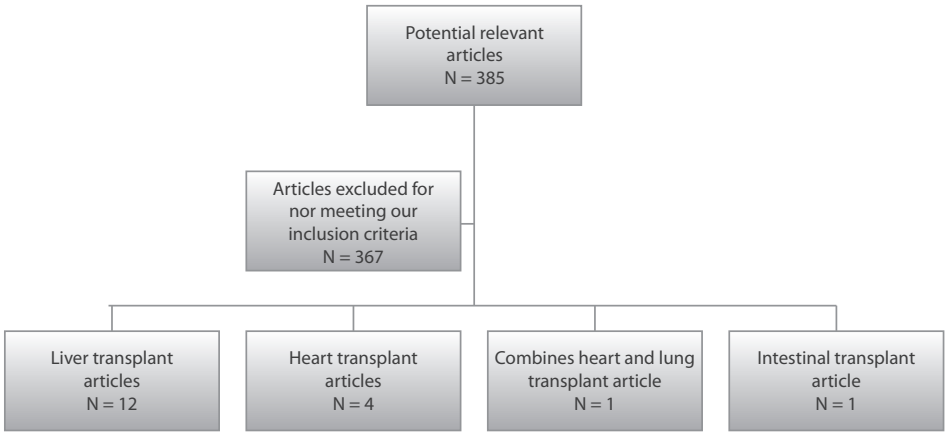
Data abstraction

Two researchers (VG, KC), not blinded to the papers' title or authors, reviewed titles and abstracts of the retrieved papers for eligibility. Disagreements about eligibility were resolved by discussion and consensus.

Results

The initial search retrieved 385 papers. After removing all duplicates and papers not meeting the inclusion criteria, eighteen papers were considered relevant. Twelve papers[20,21,22,23,24,25,26,27,28,29,30,31] reported on liver, one on combined heart and lung transplant,[32] four on heart transplant[33,34,35,36] and one on intestinal transplant recipients (Figure 1).[37]

Figure 1. Flow of process during the review process.



Liver transplant recipients

Twelve papers published between 1995 and 2008 reported the prevalence of CKD in liver transplant recipients (Table 1). Ages in these studies ranged from 3 months to 23.2 years; the follow-up time

ranged from one month to 14 years post-transplantation. Eleven studies[17,21,22,24,25,26,27,28,29,30,31] used clinical biomarkers, such as GFR or serum creatinine, to define CKD. One study did not mention the method used.[23] More specifically, two studies estimated GFR by measuring the inulin clearance[28] or the plasma disappearance of ^{99}mTc-DTPA. Others used serum creatinine and the Schwartz formula. All six studies using the Schwartz formula[21,24,25,28,29,31] employed an age and gender specific formula. Even though these six studies used the same measurement, a meta-analysis could not be done as the study designs were different and only two of these papers reported prevalence numbers. One study used the Counahan-Barratt formula.[26] Two studies[22,30] used serum creatinine.

Two studies used the KDOQI guidelines to grade the severity of chronic kidney disease and thus to determine the prevalence.[20,31] The other studies used different cut-offs for GFR or serum creatinine to identify patients with and without CKD.

Overall, only in 8 of the 12 papers a prevalence number could be extracted from the presented data; the reported prevalence of CKD ranged from 0%-88.5% in these papers. Four of the other studies reported a median decrease in CKD in the first year post-transplantation ranging from 8%-32%[25,26,28,29] with a slight improvement in renal function after the first year post-transplantation ranging from 1% till 29.6% compared to the first year post-transplantation.[25,28] However, renal function did not return to baseline values in any of these four cohorts. The fifth study showed a median decrease in serum creatinine from 0.87 ± 1.1 mg/dl (76.9 ± 97.2 umol/L) pre-transplant to 0.44 ± 0.16 mg/dl (38.9 ± 14.1 umol/L) on day 180 after transplantation.[30]
Further focusing on renal function one year post-transplantation and classifying CKD into mild (eGFR >60 and <90 ml/min/1.73m^2) and moderate-severe (eGFR <60 ml/min/1.73m^2), we identified six relevant studies: the reported prevalence ranged from 0%-39%[20,24,31] for mild

Table 1: Included papers on liver transplant recipients

Author	Year	Type of study	N on tacrolimus	Age	Follow-up time	Renal outcome measurement	Prevalence renal outcome	Exclusion criteria	Prevalence > 1 yr		STROBE (total: 34)
									Severe renal dysfunction(GFR < 60)	Mild renal dysfunction(GFR < 90)	
Bishop et al[20]	2008	Cohort study	60	73 (11–169) months	Up to 5 yrs	Stage 2 KDOQI: kidney damage with mild GFR reduction (60–90) Stage 3: kidney damage with moderate reduction (30–59) Stage 4: severe reduction in GFR (15–29) Stage 5: kidney failure (<15) mGFR measured by 99mTc-DTPA and expressed as mL/min/1.73 m2	13/60 (21.7%)	Patients who died within the rst year post-trans-plantation	1.67%	20.0%	Y: 18 P: 3
Loo et al[21]	2006	Case-control study	26	1.75 (0.72-19,24) years	Pre-transplant to 3 yrs post-transplant	cGFR (mL/min/1.73m2) by Schwartz: < 18 months k 0.45 18 months-puberty, and adolescent females: k 0.55 adolescent males after puberty k 0.70	cGFR<15: <12 months 88.5%, 13-24 months 13.3%, 25-36 months 0% cGFR 16-25: <12 months 0%, 13-24 months 66.7%, 25-36 months 54.5% cGFR >26: <12 months 11.5%, 13-24 months 20%, 25-36 months 45.5%	- Not on Tac or CsA - Not enough medical records data	24 months: 71.4% 36 months: 52.4%	No information available	Y: 16 P: 3
Staatz et al[22]	2004	Cohort study	35	5.7 +/- 4.5 (0.5-16.6) years	71% of samples from > 90 days post-op period	Significant rise in serum creatinine from its pre-transplant. Increase should be 0.04 mmol/L or more at a time when they were not taking concurrent medications gener-ally considered to be nephrotoxic.	8/35 (22.9%)	- Pre-existing history of renal failure before organ failure and transplanta-tion - Insufficient data	NA	NA	Y:22 P: 2
Colombani et al[23]	2000	Cohort study	30	28 months (3 months-7 yrs)	Up to 6 years post-transplant	not defined	6.67%	None men-tioned	Can't determine	Can't determine	Y: 10 P: 5
Wiesmayr et al[24]	2005	Cohort study	21	4 (0.3-18.1) years	1,3,6, and 12 and yearly thereafter months post-transplant	GFR between 50 and 80 ml/min/1.73m2 calculated by the Schwartz formula	0%	None men-tioned	0%	0%	Y: 12 P: 7
Tumgor et al[25]	2006	Cohort study	48	7.8 (0.5-17) years	Pre-transplant up to 4 years	Renal dysfunction was defined as serum creatinine (mg/dl) higher than normal ac-cording to age and sex at least two times with a one month interval GFR (ml/min/1.73m 2) was calculated using the Schwartz formula	GFR: 0 months: 111.1 ± 68.2 1 year: 99.5 ± 23.8 2 years: 107.8 ± 42.4 4 years: 129/1 ± 38/6 No mentioning of the amount of pa-tients experiencing renal dysfunction.	- Transplant-ed within the last 3 months - Patients who died within 6 months after transplanta-tion	Can't determine	Can't determine	Y: 15 P: 2

Table 1 continued: Included papers on liver transplant recipients

Author	Year	Type of study	N on tacrolimus	Age	Follow-up time	Renal outcome measurement	Prevalence renal outcome	Exclusion criteria	Prevalence > 1 yr		STROBE (total: 34)
									Severe renal dysfunction(GFR < 60)	Mild renal dysfunction(GFR < 90)	
Arora-Gupta et al[26]	2004	Cohort study	20	26 (3-177) months	Pre-transplantation up to 5 years	cGFR (ml/min/1.73m 2) was calculated using the modified Counahan-Barratt formula	Pre-transplant: 136 (44-196) 3 months: 32% fall 1 year: 30% fall	- Multiple liver transplants - Multi-visceral transplants - Renal dysfunction (cGFR < 35 ml/min/1.73 m2) pretransplant	Can't determine	Can't determine	Y: 19
Calvo-Garcia et al[27]	2008	Cohort study	146	4.1 +/- 4.9 years	Pre-transplantation up to 14 years	GFR was measured by a single injection of 99mTc-DTPA and expressed as ml/min/1.73m 2 Stage 2 CKD (KDOQI): GFR <90 ml/min/1.73m 2 Stage 3 CKD: GFR <60 ml/min/1.73m 2	Earliest mGFR: 115.7 ± 43.2 Latest mGFR: 127.0 ± 42.0 Stage 2: 39% Stage 3: 17%	-Patients who died within the 1st year of transplantation - Risk factors for renal cystic disease	17%	39%	Y: 20 P: 3
Berg et al[28]	2001	Cohort study	21	4.7 (0.7-23.2) years	Pre-transplantation up to 6 years	GFR was measured by inulin clearance as well as calculated using the Schwartz formula	Pre-transplantation: 123 ± 6 1 year: 95 ±7 4 years: 96 ± 9	None mentioned	Can't determine	Can't determine	Y: 8 P: 10
Heffron et al[29]	2003	Prospective clinical trial with control group	20	5.3 +/- 6.6 years	Pre-transplantation up to 180 days	Creatinine clearance was calculated using the Schwartz formula	Pre-transplant: Infant: 116 ± 40 Child: 133 ± 19 Adolescent: 140 ± 54 Day 180: Infant: 86 ± 39 Child: 123 ± 4.9 Adolescent: 161 ± 103	None mentioned	NA	NA	Y: 11 P: 7
Cacciarelli et al[30]	1996	Cohort study	19	1.8 (0.4-16.2) years	Pre-transplantation up to 180 days	Serum creatinin (mg/dl)	Pre-transplantation: 0.87 ± 1.1 Day 180: 0.44 ± 0.16	None mentioned	NA	NA	Y: 8 P: 8
Anastaze Stelle et al[31]	2012	Cohort study	24	4 +/- 0.985 years	Pre-transplant up to 3 years	Glomerular function was assed using creatinine clearance (CrCl). Abnormal < 90 ml/min/1.73m2	Pre-transplantation: 39% One month: 58.33% Three years: 20%	Deceased, Cyclosporine given, difficult follow-up, combined liver-kidney transplant, encephalitis.	0%	20%	Y:16 P: 4

Y = the amount of items scored with a "yes"
P = the amount of items scored with a "partial"

and 0%-71.4%[20,21,24,27,31] for moderate-severe CKD at least one year post-transplantation. However, one study reporting no CKD did not define renal dysfunction at all. Renal function was not one of their main outcome measures and their sample size was quite small.[24] Hence, the above findings should be interpreted with care. The study that reported the highest prevalence had a different design than the other studies. By categorizing the patients into three groups with either a GFR <15 or GFR 16-25 or GFR >26 ml/min/1.73m^2 it seems that only very sick patients were included.[21] Therefore, to correct for possible bias, we also analyzed the reported prevalence after excluding these two papers. This resulted in prevalences of 20%-39% and 1.67%-17% for mild and moderate-severe CKD, respectively.

Heart and lung transplant recipients

We identified five papers[32,33,34,35,36] reporting renal function after transplantation in either heart and lung transplant recipients[32] or heart transplant recipients[33,34,35,36] published between 1999 and 2011 (Table 2). Ages ranged from 7 hours until 18 years. Four papers[32,33,34,36] used the age and gender-adjusted Schwartz formula to calculate the GFR; one of these used a cut-off criterion to determine CKD.[32] The prevalence of CKD in these papers ranged from 6.8% - 46% with a follow-up time ranging from 14 days post-transplantation till 7 years post-transplantation. One paper[35] used serum creatinine as a biomarker to determine CKD, but did not mention a cut-off criterion to define CKD. Serum creatinine levels at 3 months post-transplantation were slightly higher compared to pre-transplant serum creatinine levels (0.9±0.1 ng/dl versus 0.7±0.1 ng/dl).[35] The prevalence of mild CKD at least one year post-transplantation ranged from 22.7%-40%; that of severe CKD ranged from 6.8% to 46%.[32,33,34]

Table 2: Included papers about heart and lung transplant recipients

Author	Year	Type of study	N on tac	Age	Type of transplant	Follow-up time	Renal outcome measurement	Incidence renal outcome	Exclusion criteria	Incidence > 1 year		STROBE (total:34)
										Severe renal dysfunction (GFR<60)	Mild renal dysfunction (GFR <90)	
Benden[32]	2008	Cohort study	29	14.3 (4.5-17.3) years	Heart and Lung	Pre-transplant 3 months, 12 months and last follow-up (+/- 23 months)	eGFR (ml/min/1.73m2) based on the Schwartz formula Stage III-IV CKD is eGFR < 60 ml/min/1.73m^2 Mild renal impairment is eGFR 60-80 ml/min/1.73m^2	Pre-transplant eGFR: 116 (35) 3 months: 72 (25), 33% decline 12 months: 8% decline 23 months: 62 (21), 46% stage III-IV CKD 38% mild renal impairment	None mentioned	46%	38%	Y: 18 P: 1
Simmonds[33]	2008	Cohort study	42	10.2 (0.3-17.3) years	Heart	Pre-transplant, 6 months and latest follow-up (1.2 years	eGFR (ml/min/1.73m2) was calculated using the Schwartz formula. Proportion or relative renal impairment was determined using the National Kidney Foundation Guidelines.	Immediate post-transplant eGFR: 66.7 6 months: 84.6 6 months - 1.2 yrs: 84.6-89.6 Latest follow-up: Mild renal impairment: 40% (17/42) Moderate renal impairment; 14% (6s/42)	Follow-up less than 1 year	14%	40%	Y: 12 P: 8
Di Filippo[34]	2005	Cohort study	88	7.6 +/- 6.5 years	Heart	Pre-transplant to 7 years post-transplantation	Creatinine clearance was calculated using the Schwartz formula. Renal dysfunction was defined as a persistent creatinine clearance < 80 ml/min/1.73m^2 two consecutive times during follow-up Severe renal dysfunction is defined as a CrCl < 50 ml/min/1.73m^2	Pre-transplant CrCl: 119.5 ± 53.5 1 year: CrCl 96.6 ± 37.9 2 years: CrCl 103.8 ± 33.7 5 years: CrCl 102.4 ± 30.2 7 years: 101.3 ± 38.1 Renal dysfunction: 21% Severe renal dysfunction: 6.8%	Patients who died within the first 6 months post-transplantation	Latest follow-up: 6.8%	Latest follow-up: 22.7%	Y: 17 P: 6
Armitage[35]	1993	Cohort study	26	7 hours – 18 yrs	Heart	Pre-transplantation up to 3 months	Serum creatinine	Pre-transplantation: 0.7 (0.1) ng/dl 3 months: 0.9 (0.1) ng/dl	None mentioned	NA	NA	Y: 8 P: 2
Gijsen[33]	2011	Cohort study	39	6 (IQR: 13.75) years	Heart	First 14 days post-transplantation	Creatinine Clearance was calculated using the Schwartz formula	Last available serum creatinine level: eGFR = 130.29 (IQR: 66.27) ml/min/1.73m^2	None mentioned	NA	NA	Y: 16 P: 3

Y = the amount of items scored with a "yes"; P = the amount of items scored with a "partial"

Intestinal transplant recipients

We identified one paper discussing the renal function after intestinal transplantation (Table 3).[37] The age of the children ranged from 0.6 till 15.6 years at the time of transplantation. The follow-up time ranged from pre-transplantation up to 9.8 years post-transplantation. Chronic kidney disease was defined as an eGFR <90 ml/min/1.73m^2 calculated by using the Schwartz formula. The pre-transplant eGFR was reported to be 138 ± 42 ml/min/1.73m^2 and decreased to 102 ± 35 ml/min/1.73m^2 at two years post-transplantation. We were not able to extract a prevalence number of mild or moderate-severe CKD, as the numbers of patients experiencing CKD were not mentioned in the paper.

Quality assessment

The quality of these studies varied between the papers, with scores ranging from 10 to 24. Separate scores can be found in Table 1. The study by Staatz et al[8] had the highest reporting quality with a score of 24. All 18 papers provided an informative and balanced summary in the abstract, explained the scientific background and rationale for the investigation, gave characteristics of the study participants and summarized the key results in the discussion. The worst reported items all concerned the statistical methods and included an explanation how the missing data was addressed (none of the papers), a description of any sensitivity analysis (1 paper), an explanation how the loss to follow-up was addressed (2 papers) and the description of any methods used to examine subgroups and interactions (4 papers).

Table 3: Articles included about intestinal transplantation

Author	Year	Type of study	N on tac	Age	Follow-up time	Renal outcome measurement	Incidence renal outcome	Exclusion criteria	Incidence > 1 year		STROBE (total:34)
									Severe renal dysfunction (GFR<60)	Mild renal dysfunction (GFR <90)	
Ueno[34]	2008	Cohort study	29	14.3 (4.5-17.3) years	Pre-transplant 3 months, 12 months and last follow-up (+/- 23 months)	eGFR (ml/min/1.73m2) based on the Schwartz formula Stage III-IV CKD is eGFR < 60 ml/min/1.73m2 Mild renal impairment is eGFR 60-80 ml/min/1.73m2	Pre-transplant eGFR: 116 (35) 3 months: 72 (25), 33% decline 12 months: 8% decline 23 months: 62 (21), 46% stage III-IV CKD 38% mild renal impairment	None mentioned	46%	38%	Y: 18 P: 1

Y = the amount of items scored with a "yes"; P = the amount of items scored with a "partial"

Discussion

Chronic kidney disease (CKD) is a potential serious complication of non-renal solid organ transplantation in children and needs to be studied in detail to optimize the management and protect the kidney while ensuring the transplanted organ is optimally functioning. This review aimed to determine the reported prevalence of CKD in pediatric, non-renal, solid organ transplant recipients treated with tacrolimus and the methods used to quantify this adverse event. We found an extremely wide prevalence range from 0% to almost 90%. The range remained wide after narrowing down the scope to CKD at least one year post-transplantation: a prevalence of mild CKD ranging from 0% till 40%; that of severe CKD ranging from 0%-71.4%. After excluding a study with possible selection bias and one in which the criterion of CKD was not defined, the mean prevalence of mild CKD ranged from 20%-40% and severe CKD from 1.67%-46%.

It would have been ideal to do a meta-analysis to have stronger evidence and a more definite answer to the question what the prevalence of CKD is after non-renal solid organ transplant recipients. Regrettably, we could not synthesize the studies into a meta-analysis in view of the widely ranging definitions and measurements. Additionally, the incidence of chronic kidney disease pre-transplantation, peri-operative and post-transplantation would be of more interest as it will reflect the new cases of CKD instead of an overall occurence. Yet, due to the cross-sectional nature of the available literature we were limited in reporting the prevalence instead.

The variability in the prevalence of chronic kidney disease may be explained by by inter-study variation in the factors cumulatively affecting renal function such as gender, ethnicity, intraoperative factors, such as hypotension and need for dialysis, postoperative factors (i.e. acute renal failure) and long-term exposure to different calcineurin inhibitors.[38,39]

Importantly, differences in the methods used to diagnose CKD may have

significantly contributed to inter-study variation. Renal function was determined in many different ways. However, every method has its own inaccuracies. Even though inuline clearance is considered the gold standard for measuring GFR in children, it could overestimate GFR if steady state has not been achieved.[40] The use of radioisotopes may introduce inaccuracies due to the different commercial products that are being used.[41] The use of estimated GFR values to determine if a patient experiences CKD also has its challenges. Although most of the included papers used the Schwartz formula,[42] the precision of the creatinine assay importantly determines its results.[43] This is of particular concern with low serum creatinine levels in which enzymatic creatinine values can run lower compared to those determined by the Jaffe method.[42,44] This will result in an overestimation of the GFR if the same "k" is used, Therefore, the constant in the Schwartz formula may have to be validated for each method used in all different centers.
As can be seen in the included papers, many different ways of determining the GFR have been used, each with their own limitation. However, to date no standardized method for determining the GFR exists. Furthermore, the use of cut-off values to determine (the severity of) chronic kidney disease in the included papers was variable. Two papers used the KDOQI cut-offs and others used non-defined/arbitrarily cut-offs. All of these reasons could contribute to the differences found in the prevalence of CKD.

Our findings suggest that the prevalence of CKD post-transplantation in children is greater than that in adults, although the results may be biased to a certain extent by the limitations above. Younger children (<6 yrs of age) will need on average higher tacrolimus doses to achieve similar target levels[36] and may consequently be exposed to higher levels of possibly nephrotoxic tacrolimus metabolites. Filler et al have shown that children metabolize sirolimus differently than adults, resulting in different metabolites.[45] As tacrolimus and sirolimus are both metabolized by CYP3A and share their FK-binding protein 12 pathway,

children might metabolize tacrolimus differently, as well. We speculate that an age-related difference in tacrolimus disposition, with possibly different metabolite formation as well, may be involved in a different effect of tacrolimus on the developing kidney. The developing kidney in itself may also be more susceptible to the nephrotoxic effects of tacrolimus.

Other methods to determine chronic kidney disease - Cystatin C, the CKiD formula and two different Schwartz formulas - were recently compared in pediatric heart transplant recipients. The conventional Schwartz formula tended to overestimate the GFR compared to Cystatin C, the CKid formula and one of the Schwartz formulas.[46] Additionally, the four methods yielded different classifications of patients according to the National Kidney Foundation's KDOQI classification methods. The conventional Schwartz formula classified 78% of the patients as having a normal GFR; the other methods arrived at no more than 31%-51%.[46] These results show that these newer methods might be a good alternative or addition to the methods used to date.

We were limited in that we could not always extract prevalence numbers. In seven studies[25,26,28,29,30,35,37] either no cut-off for defining CKD was given or the proportion of patients experiencing defined renal dysfunction was not specified. A further limitation is the wide range of the follow-up periods. This was a limitation when studies did not report the prevalence per time interval, distinguishing acute renal dysfunction from chronic kidney disease. Five of the included studies did not report the exact follow-up time, so it was not clear when the renal dysfunction occurred after transplantation.

The relatively small sample sizes in the included studies limit the generalizability of the data. Overall a mean of 43 transplant recipients were included per study; the mean number was 39 for the studies in

liver transplant recipients and 46 for those in heart and lung transplant recipients. And then, selection bias may have contributed to the large variation in reported prevalence. Four studies excluded patients who did not survive the first year post-transplantation.[20,26,27,33] Others excluded patients with a previous history of renal dysfunction[22] or patients transplanted within the last 3 months or having died within the first 6 months post-transplantation.[25,34] This may mean a predominance of patients with less severe disease.

Chronic kidney disease in children after solid organ transplantation can have multiple causes. The chronic use of calcineurin-inhibitors, such as tacrolimus, might be more important in children as other possible causes (i.e. diabetes, HCV-associated glomerulonephritis, atherosclerosis) are less common in children compared to adults. Nonetheless, proving that tacrolimus is the actual cause is difficult as renal biopsies are not regularly done in non-renal transplant recipients and there is no consensus on specific histological signs of renal failure caused by calcineurin-inhibitors. The classical histopathological signs of calcineurin-inhibitor nephrotoxicity (i.e. tubular vacuolizations, (striped) interstitial fibrosis, and arteriolar hyalinosis) are under debate for their reliability to determine tacrolimus-induced nephrotoxicity.[9,47] Yet, other causes than tacrolimus use can still be responsible for the post-tranpslant renal dysfunction (i.e. rejection) and need to be considered in differential diagnosis as a possible cause for the CKD.

Finally, reporting quality of the studies considered was quite variable, which may have contributed to the wide prevalence range. The two studies[22,47] with the best reporting quality are probably the most useful in estimating the real prevalence of CKD. They reported comparable prevalences of CKD in liver transplant recipients, i.e. 21.7% and 22.9%. However, only one study enabled to distinguish between mild and severe CKD; the respective prevalences were 21.7% and 1.67%.[20]

Consensus should be reached on the preferred standard methods to determine CKD and identify patients. Measuring GFR and analysis of biopsies at routine intervals will help to accurately identify CKD and its severity as well as the underlying histological changes. This may enable an accurate estimation of the severity of the problem and identify timely interventions in patients at risk. Using a checklist such as the STROBE statement[19] could improve reporting of studies and help to better understand the results.

Conclusion

We aimed to gather evidence on the prevalence of CKD in tacrolimus-treated, non-renal, pediatric solid organ transplant recipients. In the 18 relevant papers the reported prevalence ranged from 0%-88.5%.
Ranges remained wide after adjusting for follow-up time and severity of disease; 20%-40% for mild CKD and 1.67%-46% for severe CKD. Although the limitations of these studies demand cautious interpretation, it seems that CKD in transplant patients receiving tacrolimus is more frequent in children than in adults. This conclusion warrants further research into risk factors and individualization of therapy, taking the continuum of development into account.

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Tacrolimus-induced nephrotoxicity and genetic variability:

A review

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Summary

Background Calcineurin inhibition (CNI) is the mainstay of immunosuppressant therapy for most solid organ transplant patients. High tacrolimus levels are related with acute nephrotoxicity, but the relationship with chronic toxicity is less clear. Variation in disposition of tacrolimus is associated with genetic variation in *CYP3A5*. Hence, could genetic variation in *CYP3A5* or other genes involved in tacrolimus disposition and effect be associated with a risk for tacrolimus-induced nephrotoxicity? To perform a review of the literature and to identify if genetic variation in *CYP3A5* or other genes involved in tacrolimus disposition or effect may be associated with tacrolimus-induced nephrotoxicity and/or renal dysfunction in solid organ transplant recipients.

Material/Methods Pubmed/Medline, Embase and Google were searched from their inception till November 8th 2010 with the search terms 'tacrolimus', 'genetics', and 'nephrotoxicity'or 'renal dysfunction'. References of relevant articles were screened as well.

Results We identified 13 relevant papers. In kidney recipients, associations between donor *ABCB1*, recipient *CCR5* genotype and tacrolimus-induced nephrotoxicity were found. *CYP3A5* genotype studies in kidney recipients yielded contradictory results. In liver recipients, a possible association between recipient *ACE*, *CYP3A5*, *ABCB1* and *CYP2C8* genetic polymorphisms and tacrolimus-induced nephrotoxicity was suggested. In heart recipients, *TGF-ß* genetic polymorphisms were associated with tacrolimus-induced nephrotoxicity. The quality of the studies varied considerably.

Conclusions Limited evidence suggests that variation in genes involved in pharmacokinetics (*ABCB1* and *CYP3A5*) and pharmacodynamics (*TGF-ß*, *CYP2C8*, *ACE*, *CCR5*) of tacrolimus may impact a transplant recipients' risk to develop tacrolimus-induced nephrotoxicity across different transplant organ groups.

Background

Calcineurin inhibition (CNI) is the mainstay of immunosuppressant therapy for most solid organ transplant patients. Having been introduced in 1995[1,2] the macrolide calcineurin inhibitor drug tacrolimus was included in many treatment protocols from 1997 onwards[1]. Tacrolimus originates from a fungus (Streptomyces tsukubaensis) and it is an effective immunosuppressant agent. But its use brings with it serious adverse effects such as renal dysfunction, neurotoxicity, glucose intolerance, liver function abnormalities and hypertension.[3,4,5,6]

Tacrolimus has a narrow therapeutic window and as a serum concentration-response relationship exists between tacrolimus and acute nephrotoxicity, therapeutic drug monitoring is recommended to minimize tacrolimus-related acute renal dysfunction[7]. Tacrolimus-related acute and chronic nephrotoxicity is a well-recognized adverse effect[7,8] and a serious concern, often leading to permanent renal damage or even kidney loss. In the 2003 landmark study by Ojo et al[8], the five year cumulative incidence of chronic renal failure for a cohort of 70,000 adult non-renal transplant patients was 16.5% and end-stage renal disease (ESRD) developed in 28.9% of these patients. In that cohort, chronic renal failure was associated with an increased risk of death (relative risk: 4.55; $CI_{95\%}$ 4.38–4.74). However, most of the patients in this study received cyclosporine. Meanwhile, tacrolimus has become the primary calcineurin inhibitor for immunosuppressant treatment in solid organ transplant recipients.[9] Both cyclosporine and tacrolimus are associated with chronic nephrotoxicity, but the incidence and underlying mechanisms seem to differ.[10,11,12]

Furthermore, the adverse event profiles of the two drugs are different.[11,12,13,14,15] Both cyclosporine and tacrolimus are mainly metabolized by CYP3A4, but there is a bigger role for CYP3A5 in the metabolism of tacrolimus.[16] Although the risk of chronic renal failure in tacrolimus treated transplant patients is increased, not all patients develop renal failure. To identify patients at risk, more in depth knowledge of genetic and non-genetic risk factors is needed.

The genetically polymorphic cytochrome P450 3A5 (CYP3A5) is a phase I metabolizing enzyme involved in the metabolism of tacrolimus.[17] Carrying the *CYP3A5*3* allele results in a premature stop codon and the absence of the CYP3A5 protein (non-expressors).[18] Genetic variation in the drug-metabolizing enzyme cytochrome P450 3A5 (CYP3A5) is associated with interindividual differences in tacrolimus clearance in kidney, lung and heart transplant patients, not only in adults, but also in children.[19,20] *CYP3A5* expressors need higher doses of tacrolimus to reach the same therapeutic levels. And as high tacrolimus levels are associated with acute renal failure, genetic variation in *CYP3A5* may also impact the risk to develop chronic renal failure. The ABCB1 transporter actively transports substrates out of the cell.[21,22] As tacrolimus is a substrate for ABCB1, variation in ABCB1 expression rate is thought to influence the plasma and/orintracellular concentration of tacrolimus.[23]

The effect of tacrolimus on the kidney appears to be multi-factorial. Renal failure may occur due to an imbalance in the secretion and metabolism of nitric oxide, prostaglandines (both vasodilatory), thromboxane and endothelin (both vasoconstrictive) together with increased activity of the sympathetic nervous system. Consequently, variation in genes affecting these processes, such as *ACE*, *TGF-ß* and *CYP2C8* may also impact on the risk to develop renal failure while using tacrolimus. Transforming growth factor-*ß* (TGF-*ß*) seems to play a role in the development of fibrosis in the kidney, which is a typical histological feature of tacrolimus-induced nephrotoxicity[24,25] CYP2C8 is thought to counter the vasoconstrictive effect of tacrolimus, through a reduction of epoxyeicosatrienoic acids (EETs) formation by CYP2C8.[26,27]

The objective of the present study was to systematically review the current evidence for genetic associations with tacrolimus-induced nephrotoxicity and/or renal dysfunction in adult and pediatric renal and non-renal organ transplant recipients.

Material and Methods

Literature search

The Pubmed/Medline, Embase and Google databases were searched from their inceptions till November 8, 2010. The search terms were: 'tacrolimus', 'genetics', 'nephrotoxicity' and 'renal dysfunction'. Additionally, reference lists of articles were screened.

We reviewed the titles and abstracts of the retrieved papers for eligibility. The inclusion criteria were: human studies, original genetic research, tacrolimus. For renal transplant patients, we only included papers that used biopsy-proven nephrotoxicity defined as such by the authors of the papers. For the non-renal transplant patients we only included papers that used renal function, as defined by the authors.

Quality assessment

Study quality, in terms of internal and external validity and power, was assessed with the Downs- Black scale (Supplementary Figure 1).[28] To our knowledge quality assessment scales for genomic association studies are lacking. We looked for other scales that could be used alternatively. The Downs Black scale has been identified as a valuable tool to assess methodological quality of non-randomized studies. [29]
Two reviewers (VG, PM) applied the Downs-Black scale without blinding of authorship. Discrepancies were resolved by discussion and if necessary by a third reviewer (SW). The scale rated items with '1' if the item was reported, '0' if the item was not reported or 'U' if the reviewers were unable to make a determination. An exception is item number 5 (the distribution of the principal confounders): scored with '2' if all the confounders were listed, '1' if it was only partially reported, '0' if not at all. Another exception is item number 27 (the power of the study): scored from 0–5. The maximum total score is 32.

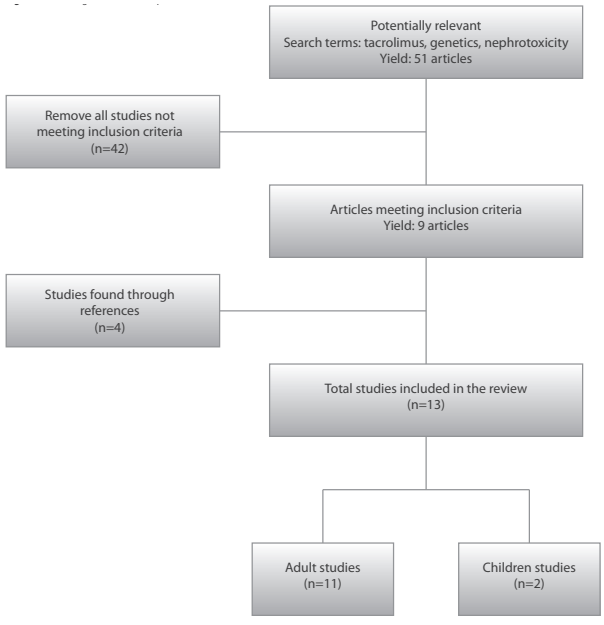
The reviewers used decision rules. For one, in the Downs-Black scale the term "intervention" was replaced with 'tacrolimus regimen' where applicable. As the scale can also be used for intervention studies, three items[14,23,24] pertaining to blinding and randomization were scored as

'U' and did not contribute to the total scores. The directions for use of the scale were otherwise followed. Study power was calculated using the data provided in the articles, if sufficient. We compared proportions yes/no renal dysfunction/nephrotoxicity for carriers with and without genetic polymorphism, using the power calculator for proportions from Sigmaplot (version 11.0, Systat software, 2008). We contacted authors of four papers to obtain more information needed to complete the power calculation. All studies were evaluated for a type-1 error rate with a=set to 0.05. Study power is scored as follows: A=<80% power → 0, B=80% power → 1, C=85% power → 2, D=90% power → 3, E=95% power → 4 and F=99% power → 5.

Results

Our initial search yielded 51 articles, of which 9 met the inclusion criteria. The reference lists yielded 4 more papers, resulting in a total of 13 papers (Figure 1). Table 1 shows detailed characteristics of the study populations, outcome measures and outcomes of all studies. Adult patients were studied in ten papers, pediatric patients in three papers.

Figure 1. Flow diagram of selection process.



Adults

Twenty-four polymorphisms in nine different genes (*CYP3A4*, *CYP3A5*, *ABCB1*, *CYP2C8*, *ACE*, *TGF-ß*, *CYP2J2*, *AGT1*, *AT1*) were investigated in adults. *YP3A* Seven articles studied the association between *CYP3A* polymorphisms and renal toxicity of tacrolimus.[30, 31, 32, 33, 34, 35, 36]

CYP3A

Kidney transplant

Kuypers et al.[31] reported a higher incidence of biopsy-proven CNI-nephrotoxicity in renal transplant recipients (n=95) carrying the *CYP3A4*1/CYP3A5*1* or *CYP3A4*1B/CYP3A5*1* expressor genotype compared to non-carriers of the alleles (37.5% vs. 11.2%, P=0.03 and 42.8% vs. 11.2%, P=0.02). With a larger cohort (n=304), three years later, the same group confirmed previous results that carrying the *CYP3A5*1* genotype increases the risk for biopsy-proven tacrolimusinduced nephrotoxicty (HR: 2.38 (1.15–4.92), P=0.01) at 3 months post-transplant.[36]

In this second study, de novo arteriolar hyalinization was used as of the histological definition of calcineurin- inhibitor toxicity (CNIT). In contrast to the two previous studies, a third study in 67 kidney recipients from a Chinese population showed a higher incidence of nephrotoxicity in the *CYP3A5*3/*3* genotype group, using protocol biopsies at one month post-transplant.[32] CNIT was graded based on histological changes from 0 to 3, with 3 being most severe. In patients with the *CYP3A5*3/*3* genotype, interstitial fibrosis (1.04±0.51 vs. 0.53±0.61, $p<0.01$) and vacuolization (0.89±0.63 vs. 0.40±0.63, $p<0.05$) were more severe than in patients with the *CYP3A5*1/*3* genotype. Similarly, 136 renal transplant recipients with the *CYP3A5*3/3* genotype presented with a non-significant trend towards a higher incidence of developing biopsy proven nephrotoxicity compared to *CYP3A5*1/*3* and *CYP3A5*1/*1* genotypes (33% vs. 9% and 10%, P=0.1).[33] Finally, Naesens et al.[35] did not find an association between donor and recipient CYP3A5 genotype and histological signs of nephrotoxicity in 252 renal transplant recipients.

Table 1: Included studies in the review

Author	Organ	Total n	N on TAC	Age (years)	Renal outcome measurement	DNA origin	Genes of interest	Outcomes	Total score
van de Wetering et al[38]	Heart	402	21	49 (15-64)	ESRF defined as need to start renal replacement	Recipient	*TGF-ß*	TGF-β Pro10 carriers: RR 2.9 for CNIT TGF-β Pro25 carriers: RR 2.6 for CNIT Data only analyzed pooled with cyclosporine.	22
Klauke et al[34]	Heart	53	21	50.2 ± 14.6	Serum creatinine ≥ 1.8 mg/dl at 3 ≥ occasions median 20 months post-transplant	Recipient	*CYP3A5, ABCB1, TGF-ß*	No relationship found between any of the genotypes and the renal outcome. Data only analyzed pooled with cyclosporine.	16
Fukudo et al[30]	Liver	60	60	55 (29-70)	Initial serum creatinine increase of > 0.5 mg/dl above pre-transplant baseline at 3,6,9,12 months post-transplant	Recipient Donor	*CYP3A5*	Recipient CYP3A5 expressors vs non-expressors CNIT 17% vs 46%. Donor CYP3A5 no association was found with CNIT.	20
Smith et al[39]	Liver	163	41	48 ± 10	Serum creatinine ≥1.6 mg/dl at 3 yrs post-transplant	Recipient	*CYP2C8, CYP2J2*	CYP2C8*3 OR 16.67 for CNIT. No relationship was found for the CYP2J2 genotype and CNIT.	21
Gallon et al[40]	Liver	143	100	55 ± 14	Serum creatinine ≥1.5 mg/dl at most recent follow-up (median 60 months post-transplant)	Recipient	*ACE, AGT1, AT1*	ACE D/D carriers: 57% nephrotoxicity vs 20% non nephrotoxicity (RR 4.3). No relationship was found for the AGT1, AT1 genotype and CNIT. Data only analyzed pooled with cyclosporine.	25
Hebert et al[37]	Liver	120	31	48 ± 12	Serum creatinine ≥1.6 mg/dl 3 years after transplantation or subjects who required hemodialysis or a kidney transplantation before 3 years post-transplantation	Recipient	*ABCB1*	ABCB1 2677 GT, 3435 CT 50%; 2677 GG, 3435 CC 31%; 2677 TT, 3435 TT 19% incidence of CNIT. Data only analyzed pooled with cyclosporine.	19
Hawwa et al[42]	Liver	51	51	2 (0.6-16)	30% reduction in GFR (Counahan-Barrat formula taken at 3,6,12 months) compared with pre-transplant baseline.	Recipient	*ABCB1*	CNIT at 6 months post-transplantation (1236T allele: frequency = 63.3% in nephrotoxic patients vs 37.5% in controls, P = 0.019; 2677T allele: frequency = 63.3% vs 35.9%, P = 0.012; 3435T allele: frequency = 60% vs 39.1%, P = 0.057). T-T-T haplotype 52.9% incidence vs 29.4% in controls, P = 0.029. None of ABCB1 alleles or haplotypes were associated with renal toxicity 1 year post-transplantation.	17
Kuypers et al[31]	Kidney	95	95	51.3 ± 14.1	Biopsies taken when clinically indicated. Biopsy proven using Banff 2001 any time up to 5 years post-transplant.	Recipient	*CYP3A5, ABCB1*	Both CYP3A4*1/CYP3A5*1 and CYP3A4*1B/CYP3A5*1 were associated with a higher incidence of CNIT compared to non-carriers (37.5% vs 11.2%, P = 0.03 and 42.8% vs 11.2%, P = 0.02 or combined CYP3A4*1/CYP3A5*1 and CYP3A4*1B/CYP3A5*1: 40% vs 11.2%, P = 0.005. No significance association found for the ABCB1 genotype and CNIT.	19

Table 1 Continued: Included studies in the review

Author	Organ	Total n	N on TAC	Age (years)	Renal outcome measurement	DNA origin	Genes of interest	Outcomes	Total score
Chen et al[32]	Kidney	67	67	39.28 ± 12.46	Protocol biopsy at 1 month, Banff 1997 criteria. Serum creatinine at 1 wk, 1,3,6,12 months post-transplant.	Recipient	*CYP3A5*	CYP3A5*3/*3 higher scores on all the histopathological changes for nephrotoxicity. No difference in serum creatinine between genotypes.	11
Quteineh et al[33]	Kidney	136	136	45 ± 11	Biopsy was obtained when possible and biopsy-proven nephrotoxicity defined according to Banff 2005 criteria.	Recipient	*CYP3A5, ABCB1*	CYP3A5*3/*3 trend for higher risk compared to patients with CYP3A5*1/*3 and CYP3A5*1/*1 (33% vs 9% and 10%, P=0.1). No significant association was found for the ABCB1 genotype and CNIT.	19
Kuypers et al[36]	Kidney	304	304	52.9 ± 14.1	Biopsy-proven nephrotoxicity based on the presence of de novo afferent arteriolar hyaline thickening at 3 months and every 12 months post-transplant.	Recipient	*CYP3A4, CYP3A5, ABCB1*	Carrying the CYP3A5*1 genotype results in a HR of 2.38 (1.15-4.92, P=0.01) No relationship was found for the ABCB1 and CYP3A4 genotypes and CNIT.	24
Naesens et al[35]	Kidney	252	252	54.5 ± 13.9	Histological signs of nephrotoxicty (IF/TA and arteriolar hyalinosis) at 3 years post-transplant	Recipient Donor	*ABCB1, CYP3A4, CYP3A5*	A significant association was found for carriers of the ABCB1 3435 TT genotype for recipients (OR: 1.8 (1.11-2.93), P=0.0175) and donors (OR: 1.76 (1.07-2.89), P=0.0263). The OR for a higher IF/TA grade is 3.9 (2.0-7.6, P<0.001) if both donor and recipient are homozygous for ABCB1 3435TT genotype vs no homozygosity for C3435T polymorphisms. OR is 3.7 (1.8-7.7, P<0.001), if both donor and recipient are homozygous for T variant of 3435 vs mixed combinations. No relationship was found between ABCB1 3435 genotypes and arteriolar hyalinosis or ABCB1 2677 genotypes and any signs of nephrotoxicity. No relationship was found for the CYP3A4 and CYP3A5 genotypes and CNIT.	23
Grenda et al[41]	Kidney	207	61	11 ± 5	Renal biopsy, observed consistent deterioration of renal function, accompanied with hyperuricaemia and/or tubuluar acidosis.	Recipient	*ABCB1, CYP3A5, IL10, IL6, CCR5, TNF-alpha, IL1B, IL1RN, MCP-1, VEGF, TGF- ß*	100% of the Tac patients with nephrotoxicity carried the CCR5Δ32 polymorphism vs 21% controls. No relationship was found for the other genotypes and CNIT, Data only analyzed pooled with cyclosporine.	11

Liver transplant

In 60 liver transplant recipients, *CYP3A5* nonexpressors had a higher risk to develop dysfunction HR 3.16 ($CI_{95\%}$ 1.01-6.16, $p<0.05$).[30]

Heart transplant

In contrast, Klauke et al.[34] found no association between the *CYP3A5* genotype and an increase in serum creatinine (SCr ≥1.8 mg/dl) in 53 heart transplant patients.

ABCB1

Kidney transplant

In 252 renal transplant recipients, a significant association was found between *ABCB1* genotype and histological signs of nephrotoxicity, as defined by interstitial fibrosis/tubular atrophy.[35] When both donor and recipient are homozygous for the T variant of *ABCB1 3435* there was an odds ratio of 3.9 ($CI_{95\%}$ 2.0–7.6, $P<0.001$) for higher Interstitial Fibrosis/ Tubular Atrophy (IF/TA) grades compared to no homozygosity for the *C3435T* polymorphism.

Liver transplant

In liver transplant patients, Hebert et al.[37] showed that 50% of patients with the *ABCB1 11/22* haplotype (*2677G,3435C/2677T,3435T*) experienced renal dysfunction (serum creatinine ≥1.6 mg/dl) compared to 31% of patients with the *ABCB1 11/11* haplotype (*2677G,3435C/2677G,3435C*) and 11.2% of patients with the *ABCB1 22/22* haplotype (*2677T,3435T/2677T,3435T*).
Three other studies did not identify a relation with ABCB1 genotype and nephrotoxicity.[31,34,36] For both cohorts, it was not possible to determine the association for the tacrolimus patients separately, as this was not reported in the papers.

CYP2C8 and CYP2J2.

Liver transplant

In 41 liver transplant recipients, carrying the *CYP2C8*3* polymorphism was associated with a higher risk OR 16.67 ($CI_{95\%}$ 2.8–99.6) to develop renal

dysfunction.[39] In the same cohort, no association was found with *CYP2J2* genotype and renal dysfunction.

ACE, AGT1 and AT1

Liver transplant

In 143 liver transplant recipients, (100 on tacrolimus), the *ACE D/D* genotype was significantly associated with a higher risk of renal dysfunction RR 4.3 ($CI_{95\%}$1.9–9.7, P=0.0001).[40] In the same cohort, no correlation was found between *AGT1* or *AT1* genetic variation and renal dysfunction.

Children

We identified only two studies in children. Grenda et al., studied twenty-four polymorphisms in ten different genes (*CYP3A5, ABCB1, TGF-ß, IL10, IL6, CCR5, TNF-alpha, IL2B, IL1RN, MCP-1, VEGF*) in 207 (61 on tacrolimus) pediatric kidney transplant recipients.[41] The rationale for studying these genotypes was not given by the authors of the paper. Except from *CCR5*, no relation between any of these genotypes and nephrotoxicity, defined by clinically indicated biopsy or deterioration of renal function, was found. We identified one other study in pediatric liver transplant recipients.[42]

ABCB1

Kidney transplant

In contrast to adults, in 207 (61 on tacrolimus) pediatric kidney transplant recipients no association between *ABCB1* genotype and nephrotoxicity, as defined by biopsy or reduced renal function was found.[41]

Liver transplant

In 51 paediatric liver recipients, *T-T-T* haplotype (*C1236T, G2677T, C3435T*) was associated with an increased risk of a reduction in creatinine clearance at 6 months post-transplant.[42] At 6 months post-transplant, 52.9% of the patients with renal dysfunction were carriers of the *T-T-T* haplotype compared to 29.4% in the patients without renal dysfunction (P=0.029).

CCR5

Kidney transplant

In the Grenda et al study, all transplant recipients with nephrotoxicity (n=18) carried *CCR5* wildtype compared to 79% of the no-nephrotoxicity group (n=28, P=0.041).[41]

Quality assessment

Overall, the quality of the studies was moderate: the mean score on the Downs-Black scale [28] was 17.8±4.1 (range 11–25). Separate scores are given in the Table 1. Appropriateness of the statistical tests used, the compliance of the intervention and whether patients were recruited from the same population were the best-reported items. These aspects were reported in all 13 studies. The worst reported items were on determining if the outcomes were blinded to genotype of the patients (only 3 papers) and the population pool subjects were recruited from (5 papers) and whether losses to follow-up were taken into account (4 papers).

Statistical power

Four papers[32,33,39,42] lacked sufficient data to recalculate the power. We were able to contact the authors of two of these papers[39,42] however only one[42] provided additional data. For those without sufficient data, the item was scored with a 'U' as in 'unable to determine'. Only three studies[36,38,40] had sufficient power, rated with 4 or 5 points. All studies considered a $p<0.05$ as statistically significant, and none made adjustment for multiple testing of genetic variants.

Discussion

This literature review shows that specific polymorphisms in six different genes have been implicated to be associated with the variation in the incidence of renal dysfunction in adults or children who have received tacrolimus for immunosuppression after solid organ transplant. The identified genes are *CYP3A5, ABCB1, CYP2C8, ACE, TGF-ß* and CCR5.

Five studies suggest a role for the *CYP3A5* gene (four kidney[31,32,33,36] one liver[30]), whereas three studies (two kidney,[35,41] one heart[34]) could not confirm such an association. For two papers, this lack of an association may be due to the small number of patients studied (n=61 and n=21, respectively).

In two studies researching kidney recipients, patients who are *CYP3A5* expressors had a higher risk of nephrotoxicity while the opposite was found in two other studies. The discrepancy between these studies may be explained by the outcome measure used. In the largest study to date, calcineurin inhibitor induced nephrotoxicity was defined as de novo arteriolar hyalinosis at 3 months after transplant and yearly thereafter. Hence, patients who received a donor kidney with arteriolar hyalinosis at transplant, would not be scored as CNIT, while this may have been the case in the other studies where any arteriolar hyalinosis was scored or other histological signs, according to Banff 2001, such as interstitial fibrosis/ tubular atrophy. The disparate results may further be explained by the timing of biopsies, e.g. early (at 1 month) versus late (over 3 months after transplant). When taking these differences and inherent limitations of the different studies into account, the strongest evidence points towards a positive association between *CYP3A5* expressors and a higher risk for calcineurin induced nephrotoxicity in adult renal transplant patients. For liver transplant patients only one paper (n=60) was identified, with the opposite finding for recipient *CYP3A5*. This may be explained by the effect of genetic differences in renal CYP3A5 activity, versus the genetic differences in hepatic *CYP3A5* in the renal transplant patients.

There is also a discrepancy with regard to *ABCB1*. Only 3 (one in kidney[35] and two in liver[37,42]) of the 8 papers on the association of *ABCB1* polymorphisms and tacrolimus-induced nephrotoxicity found a positive association. Again, similar reasons as for the *CYP3A5* associations for these discrepancies apply: small patient populations, mixed tacrolimus/cyclosporine cohorts, follow-up time, different organs and different *ABCB1* haplotypes. Taking these differences/ limitations into account, no conclusion can be drawn regarding the association between *ABCB1* genotype and renal dysfunction in adult heart or liver transplant recipients. For pediatric liver transplant patients, an association for early (less than 1 year after transplant) renal dysfunction and *ABCB1* genotype in patients on tacrolimus may be present. In adult and renal transplant patients, no association between recipient *ABCB1* genotype and CNIT seems to be evident, although this is dependent on the definition. If IF/TA are also considered due to tacrolimus, such an association may exist.

The discrepancies in the findings regarding *CYP3A5* and *ABCB1* could also be related to one another. A possible interaction between the CYP3A and ABCB1 expression has been proposed in the past, showing higher hepatic and intestinal CYP3A4 expression in *ABCB1 2677TT* carriers than homozygotes for *ABCB1 2677G*.[43] A gender effect on this interaction has been suggested as well, with women carrying the *ABCB1 2677TT-3435TT* haplotype showing significantly higher CYP3A4 mRNA expression levels in the native intestine than *ABCB1 2677GG-3435CC* carriers. This was not found in men.[44]

Smith et al.[39] reported a higher risk of developing renal dysfunction in adult liver recipients carrying the *CYP2C8*3* polymorphism. The patient group was a mixed one, but the patients receiving tacrolimus were also analyzed separately. The odds ratio for these patients was much higher at 16.67 ($CI_{95\%}$ 2.8–99.6). In spite of the very wide confidence interval, the risk is considerate since the lower limit is almost.[3] The results of this study strongly suggest a role for CYP2C8 in the risk of tacrolimus-related renal dysfunction in adult liver recipients. The ACE study[40] was assigned the

highest overall quality score of 25 and the highest power (99%). The liver transplant recipients group was mixed (tacrolimus or cyclosporine), but tacrolimus treatment was predominant (100 of 142 patients). The results suggest an association between *ACE* and renal dysfunction in adult liver recipients who receive tacrolimus.

The results for *TGF-ß* in adult heart transplant patients are interesting with an association between this genotype (*Pro10* and *Pro25* carriers) and an increased risk for end-stage renal failure. Only a small minority received tacrolimus (21 of 402), but considering the proposed mechanism of action in the kidney of both cyclosporine and tacrolimus, it is reasonable to assume that results may be similar if only patients on tacrolimus are studied.

For the other genes not mentioned in detail that were studied by different groups, no correlation between genotype and renal dysfunction and/or nephrotoxicity in solid organ transplant recipients was reported. Considering the limitations of the differentstudies discussed above, it can, however not be ruled out that these genes do not play a role in the risk to develop renal dysfunction after tacrolimus exposure.

However, the candidate-gene approach used in all of the included papers has a limitation in its design, as it only targets the genes thought to be important to the researcher are included in the design of the study. A Genome-Wide Association Study (GWAS) or a proteomic study might give a better understanding of the mechanisms involved in the pathophysiology of transplant-related diseases.

The first limitation of this review resides in the multifactorial background of renal dys-function after transplantation. Besides the use of calcineurin-inhibitors, such as tacrolimus, other possible causes (i.e. diabetes, atherosclerosis, HCV associated glomerulonephritis) might be the reason for the renal dysfunction experienced after transplantation.[45] Furthermore, renal biopsies are not regularly done creating difficulties tracking the progress of the renal dysfunction. Yet, even if they were, controversy exists

on the specific histological signs for calcineurin-inhibitor induced nephrotoxicity.[10,46] Secondly, the study design and reporting of the some of the studies that we identified are suboptimal. Reporting can be improved by using a recently published guideline on the reporting in genetic association studies (STREGA).[47] As we have discovered in our review, reporting of the methods in particular is sub standard. The laboratory methods, the genotyping methods as well as the error rates and call rates should be reported. The laboratory where genotyping was done should be identified as well. Additionally, it should be specified whether genotypes were assigned using all of the data from the study simultaneously or in smaller batches, and whether or how the Hardy- Weinberg equilibrium was considered. Although the papers in this review lacked much of this information and often results were not adjusted for multiple testing, they did report on other aspects mentioned in the guideline, especially in the results section. For example, the proportions of successfully genotyped patients were reported in 9 of the 13 papers.

Conclusions

Despite the fact that the factors discussed may limit overall conclusions on pharmacogenomic variation in tacrolimus-induced nephrotoxicity, we believe that interesting candidate genes have been identified.

The pharmacokinetic genes (e.g. *CYP3A5, ABCB1*) and some pharmacodynamic genes (e.g. *CYP2C8, TGF-ß*) deserve further investigation – also in children. Critically, it will be important to include all the target genes in a single predictive analysis, and not isolated ones. An important finding of the present analysis is that most studies had little statistical power to detect any genotype-phenotype associations. Although we realize that obtaining a large enough sample size is difficult regarding (pediatric) transplant recipients, we feel that future studies should try and increase the number of subjects studied, by genetic meta-analysis and/or consortia formation.

Conflict of interests

The authors declared no conflict of interest.

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Supplementary figure 1: Downs-Black scale[28]

Reporting

1. **Is the hypothesis/aim/objective of the study clearly described?**
   Yes 1 No 0

2. **Are the main outcomes to be measured clearly described in the Introduction or Methods Section?**
   If the main outcomes are first mentioned in the Results section, the questions should be answered no.
   Yes 1 No 0

3. **Are the characteristics of the patients included in the study clearly described?**
   In cohort studies and trials, inclusion and/or exclusion criteria should be given.
   In case-control studies, a case-definition and the source for controls should be given
   Yes 1 No 0

4. **Are the tacrolimus regimens clearly described?**
   Treatments and placebo (where relevant) that are to be compared should be clearly described.
   Yes 1 No 0

5. **Are the distributions of principal confounders in each group of subjects to be compared clearly described?**
   A list of potential confounders is provided.
   Yes 2 Partially 1 No 0

6. **Are the main findings of the study clearly described?**
   Simple outcome data (including denominators and numerators) should be reported for all major findings so that the reader can check the major analyses and conclusions. (This question does not cover statistical tests which are considered below)
   Yes 1 No 0

7. **Does the study provide estimates of the random variability in the data for the main outcomes?**
   In non normally distributed data the inter-quartile range of results should be reported. In normally distributed data the standard error, standard deviation or confidence intervals should be reported. If the distribution of the data is not described, it must be assumed that the estimates used were ap propriate and the question should be answered yes.
   Yes 1 No 0

8. **Have all important adverse events that may be a consequence of the tacrolimus regimen been reported?** This should be answered yes if the study demonstrates that there was a comprehensive attempt to measure adverse events. (A list of possible adverse events is provided.)
   Yes 1 No 0

9. **Have the characteristics of patients lost to follow-up been described?**
   This should be answered yes where there were no losses to follow-up were so small that findings would be unaffected by their inclusion. This should be answered no where a study does not report the number of patients lost to follow-up.
   Yes 1 No 0

10. **Have actual probability values been reported (e.g. 0.035 rather than <0.05) for the main outcomes except where the probability value is less than 0.001?**
    Yes 1 No 0

External validity

All the following criteria attempt to address the representativeness of the findings of the study and whether they may be generalised to the population from which the study subjects were derived.

11. **Were the subjects asked to participate in the study representative of the entire population from which they were recruited?** The study must identify the source population for patients and describe how the patients were selected. Patients would be representative if they comprised the entire source population, an unselected sample of consecutive patients, or a random sample. Random sampling is only feasible where a list of all members of the relevant population exists.
Where a study does not report the proportion of the source population from which the patients are derived, the question should be answered as unable to determine.
Yes 1 No 0 Unable to determine 0

12. **Were those subjects who were prepared to participate representative of the entire population from which they were recruited?** The proportion of those asked who agreed should be stated. Validation that the sample were representative would include demonstrating that the distribution of the main confounding factors was the same in the study sample and the source population.
Yes 1 No 0 Unable to determine 0

13. **Were the staff, places, and facilities where the patients were treated, representative of the treatment the majority of patients receive?** For the question to be answered yes, the study should demonstrate that the intervention was representative of that in use in the source population. The question should be answered no if, for example, the intervention was undertaken in a specialist centre unrepresentative of the hospitals most of the source population would attend.
Yes 1 No 0 Unable to determine 0

Internal validity - bias

14. **Was an attempt made to blind study subjects to the tacrolimus regimen they have received?**
For studies where the patients would have no way of knowing which intervention they received, this should be answered yes.
Yes 1 No 0 Unable to determine 0

15. **Was an attempt made to blind those measuring the main outcome of the tacrolimus regimen?**
Yes 1 No 0 Unable to determine 0

16. **If any of the results of the study were based on "data dredging", was this made clear?**
Any analyses that had not been planned at the outset of the study should be clearly indicated. If no retrospective unplanned subgroup analyses were reported, then answer yes.
Yes 1 No 0 Unable to determine 0

17. **In trials and cohorts studies, do the analyses adjust for different lengths of follow-up of patients, or in case-control studies, is the time period between the tacrolimus regiment and outcome the same for cases and controls?**
Yes 1 No 0 Unable to determine 0

18. **Where the statistical tests used to assess the main outcomes appropriate?**
The statistical techniques used must be appropriate to the data. For example non-parametric methods should be used for small samples sizes. Where little statistical analysis has been undertaken but where there is no evidence of bias, the question should be answered yes. If the distribution of the data (normal or not) is not described it must be assumed that the estimates used were appropriate and the question should be answered yes.
Yes 1 No 0 Unable to determine 0

19. **Was compliance with the tacrolimus regimen reliable?**
Where there was non compliance with the allocated treatment or where there was contamination of one group, the question should be answered no. For studies where the effect of any mis-classification was likely to bias any association to the null, the question should be answered yes.
Yes 1 No 0 Unable to determine 0

20. **Where the main outcome measures used accurate (valid and reliable)?**
For studies where the outcome measures are clearly described, the question should be answered yes. For studies which refer to other work or that demonstrates the outcome measures are accurate, the question should be answered yes.
Yes 1 No 0 Unable to determine 0

Internal validity – confounding (selection bias)

21. **Were the cases and controls (case-control studies) recruited from the same population?**
For example, patients for all comparison groups should be selected from the same hospital. The question should be answered unable to determine for cohort and case-control studies where there is no information concerning the source of patients included in the study.
Yes 1 No 0 Unable to determine

22. **Were the cases and controls (case-control studies) recruited over the same time period?**
For a study which does not specify the time period over which patients were recruited, the question should be answered unable to determine.
Yes 1 No 0 Unable to determine 0

23. **Were study subjects randomised to intervention groups?**
Studies which state that subjects were randomised should be answered yes except where the method of randomisation would not ensure random allocation. For example alternate allocation would score no because it is predictable.
Yes 1 No 0 Unable to determine 0

24. **Was the randomised intervention assignment concealed from both patients and health care staff until recruitment was complete and irrevocable?** All non-randomised studies should be answered no. If assignment was concealed from patients but not from staff, it should be answered no.
Yes 1 No 0 Unable to determine 0

25. **Was there adequate adjustment for confounding in the analyses from which the main findings were drawn?** This question should be answered no for trials if: the main conclusions of the study were based on analyses of treatment rather than intention to treat; the distribution of known confounders in the different treatment groups was not described; or the distribution of known confounders differed between treatment groups but was not taken into account in the analyses. In non randomised studies if the effect of the main confounders was not investigated or confounding was demonstrated but no adjustment was made in the final analyses the question should be answered as no.

Yes 1 No 0 Unable to determine 0

26. **Were losses of patients to follow-up taken into account?**

If the numbers of patients lost to follow-up are not reported, the question should be answered as unable to determine. If the proportion lost to follow-up was too small to affect the main findings, the question should be answered yes.

Yes 1 No 0 Unable to determine 0

Power

27. **Did the study have sufficient power to detect a clinically important effect where the probability value for a difference being due to chance is less than 5%?**

Sample sizes have been calculated to detect a difference or x% and y%.

	Size of smallest group	
A	$<n_1$	0
B	n_1- n_2	1
C	n_3- n_4	2
D	n_5- n_6	3
E	n_7-n_8	4
F	n_8^+	5

Age and *CYP3A5* genotype affect tacrolimus dosing requirements after transplant in pediatric heart recipients

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Summary

Background Tacrolimus is one of the commonly used immunosuppressive drugs for pediatric heart transplants. Large variation exists in pharmacokinetics during the direct post-transplant period, resulting in an increased risk of adverse events. Limited data are available on the interaction of age, *CYP3A5* and *ABCB1* genotype, and disease severity on the variation in disposition and outcome in pediatric heart transplant recipients.

Method We studied the relationship between age and *CYP3A5* and *ABCB1* genotype and the Pediatric Risk of Mortality (PRISM) score on tacrolimus dose (mg/kg), steady-state trough concentrations, and concentration/dose ratio, as well as rejection and renal function for 14 days after heart transplant in children.

Results Tacrolimus was administered to 39 children (median age, 6.0 years) after transplant. A correlation was found between the age at the time of transplant and the tacrolimus dosing requirements (r_s = –0.447, p = 0.004) and the concentration/dose ratio (r_s = 0.351, p = 0.029). *CYP3A5* expressors required median (interquartile range) higher doses of tacrolimus (0.14 [0.09] vs 0.06 [0.04] mg/kg/12 hours, p 0.001), and had lower concentration/dose ratios (45.34 [44.54] vs 177.78 [145.38] ng/ml per mg/kg/12 hours, p = 0.0001). This relationship was not seen with the *ABCB1* genotype. Age and *CYP3A5* genotype predicted the tacrolimus dosing requirements as well as the concentration/dose ratio (R^2 = 0.351, p = 0.001 and R^2 = 0.521, p = 0.001). No relationship was found between any of the *CYP3A5* or *ABCB1* genotypes and the estimated glomerular filtration rate.

Conclusion Younger age and *CYP3A5* expressor genotype were independently associated with higher dosing requirements and lower tacrolimus concentration/dose ratios.

Introduction

After its introduction into clinical use in 1997, tacrolimus became one of the most commonly used drugs for immunosuppressive treatment of solid-organ transplant recipients. In heart transplant recipients, it is often preferred to cyclosporin[1] for its lower incidence of hypertension, dyslipidemia, and fewer cosmetic adverse effects, such as hirsutism and gingival hypertrophy.[1,2,3] A narrow therapeutic window complicates tacrolimus dosing, however.

The first weeks after transplantation are generally marked by the highest risk for organ rejection. During this period, considerable variability in drug concentrations and pharmacokinetics can contribute to rejection risk with underdosing and drug toxicity (eg, nephrotoxicity, neurotoxicity) with overdosing. Sub-therapeutic tacrolimus concentrations confer a risk for biopsy-proven rejection in adult and pediatric heart transplant recipients.[4,5,6] Furthermore, in 112 adult cardiac transplant recipients, early renal insufficiency, defined as 10% rise in serum creatinine and a serum creatinine above 1.5 mg/dl on Day 3 after transplantation, was associated with tacrolimus levels.[7]

The pharmacokinetics of tacrolimus have been extensively studied in adults.[8,9] However, limited data exist on the sources of large interindividual and intraindividual variability of tacrolimus pharmacokinetics in children. Faster tacrolimus clearance rate in children aged younger than 6 years and higher tacrolimus doses per kilogram of body weight to achieve the target concentrations in this age group have been reported in liver, renal, and hematopoietic stem cell transplant recipients.[10,11,12,13] The causes for these differences are presently unknown but may be due to age-related changes in CYP3A activity and to the large size of the liver allograft relative to body size in children aged younger than 6 years.[14]

The relationship between the *CYP3A5* genotype and higher tacrolimus clearance has been well established in adult cardiac transplant patients[9,15] but only limited data are available in pediatric cardiac transplant recipients. A recent study[16] reported a relationship in 65 pediatric

patients between *CYP3A5* genotype and tacrolimus clearance at 3, 6 and 12 months after transplantation. This study suggested that *CYP3A5* expressors (*CYP3A5*1/*3*) need higher drug doses to maintain the same blood concentration at 3, 6 and 12 months after transplant. However, the effects of mutations in *ABCB1* genotypes on tacrolimus disposition in liver and kidney transplant recipients were inconsistent, with reports both supporting[17,18,19,20] or refuting such an association.[17,20,21] The study in pediatric heart transplant recipients demonstrated an association between *ABCB1 C3435T* and *G2677T/A* and tacrolimus dosing requirements at 6 and 12 months after transplantation.[16]

These studies did not evaluate the influence of genotype in relation to other clinical factors, such as age and comorbidity, on dose requirements in the early period after transplantation. A large variation in tacrolimus pharmacokinetics may occur in the early post-transplantation period due to critical illness-related factors, such as mechanical ventilation, altered cardiac output and consequent altered liver and kidney blood flow, body fluid, and plasma protein changes. Hence, we speculated that in patients with a higher severity of illness, as defined by the risk of mortality at intensive care unit admission by the Pediatric Risk of Mortality (PRISM) score, lower tacrolimus requirements would be observed. Our objective was to determine the effects of age, recipient *CYP3A5* and *ABCB1* genotypes, and PRISM score on tacrolimus disposition in the first 14 days after transplant in pediatric heart allograft recipients. In addition, we wanted to investigate the association between recipient *CYP3A5* and *ABCB1* genotypes and tacrolimus levels on transplant outcomes such as rejection and renal function.

Patients and methods

This study was approved by the Institutional Research Ethics Board, and informed consent was obtained from parents and/or children during enrollment. Pediatric heart transplant recipients (aged 18 years at the time of transplant) who received oral tacrolimus during the first 14 days after transplant between 1995 and 2008 at the Hospital for Sick Children,

Toronto, Ontario, were eligible for study entry.
DNA samples were derived from a cohort of patients prospectively enrolled in the Sickkids Heart Centre Biobank.

Immunosuppressive protocol
Induction therapy with anti-thymocyte globulin was started peri-operatively and given up to 2 to 5 days after transplantation in all patients. Tacrolimus was started at Day 2 to 3 after transplantation, with a starting dose of 0.2 mg/kg/day orally, divided twice daily. The treating physician used therapeutic drug monitoring to adjust the tacrolimus dose to a target level of 10 to 12 ng/ml. Additional immunosuppressive therapy consisted of a maintenance dose of mycophenolate mofetil and steroids.

Pharmacokinetic and pharmacodynamics outcome measures
As dependent variables, we collected tacrolimus dose (mg/kg/12 hours) and tacrolimus trough concentrations from patient health records. Dose-corrected tacrolimus concentrations were calculated by dividing the tacrolimus trough concentration by the weight-adjusted dose. Data were collected on the occurrence of rejection and renal function in our population. Rejection was graded according to the International Society of Heart and Lung Transplantation's (ISHLT) grading system.[22] Rejection was defined as an ISHLT grade 2R or higher. Creatinine clearance was estimated with the Schwartz formula, using the last available serum creatinine level during the study period.

Covariates
Patient sex, age, weight, the comedication received, *CYP3A5* and *ABCB1* genotype, and PRISM score were collected as independent variables.

Tacrolimus concentrations
Tacrolimus blood trough concentrations were determined in ethylenediaminetetraacetic acid (EDTA)-treated whole blood (0.25 ml) on the day of sampling, using liquid chromatography tandem mass spectrometry (LC-MS-MS) as previously described, as part of clinical care.[23]

Genotyping
Blood for genotyping was collected in EDTA-containing tubes, and DNA was extracted using a Magna-Pure LC (Roche Diangostics GmbH, Mannheim, Germany). Polymerase chain reaction (PCR)–restriction fragment length polymorphism for *CYP3A5*3* and *ABCB1 C3435T*, *G2677T*, and *C1236T* were performed as described previously.[24,25,26] Patients not carrying the *CYP3A5*3* allele were assigned the *CYP3A5*1/*1* genotype by default.

Statistical analyses
Data are presented as mean ± standard deviation or median and interquartile range (IQR) when the data were skewed. Differences were compared using the Mann-Whitney test or the Kruskal-Wallis test. The Spearman test was used to test possible correlations. A linear multivariate analysis was performed to test the influence of thepredictors on all dependent variables. All data analyses were performed using SPSS 16.0 software (SPSS, Chicago, IL). The Hardy-Weinberg equilibrium was calculated by using the method from Rodriguez et al.[27]

Results

Patient characteristics

The study comprised 39 eligible pediatric heart transplant recipients (25 boys, 14 girls) who were a median age of 6.0 (IQR: 13.75) years and a median weight of 13.1 (IQR: 25.5) kg. A detailed list of the patients' demographics can be found in Table 1.

Table 1: Demographics of the population

Variable	All patients (n=39)
Age in years < 1yrs	6.0 (13.75) 15/39 (38.5%)
Gender	
Female	14
Male	25
Weight in kg	**13.1 (25.5)**
Ethnicity	
Caucasian	28
African-American	2
Asian	4
Unkown	5
Diagnosis	
Dilated cardiomyopathy	22
Congenital heart disease	15
Unknown	2
PRISM score†	9.50 (10)
Need for pre-transplant mechanical ventilation	17.9% (7/39)
Need for post-transplant mechanical ventilation Days on mechanical ventilation	74.4 % (29/39) 2.56 (3.6)*
Tacrolimus oral dose (mg/kg/12h)	0.06 (0.06)
Tacrolimus trough level (ng/ml)	9.6 (2.08)
Concentration/Dose ratio (ng/ml per mg/kg/12h)	150.79 (173.3)

Table 1: Results are reported in median (IQR) unless noted otherwise. * mean ± sd The tacrolimus dose, tacrolimus trough level and concentration/dose ratio are averages of all values obtained during the 14 day post-transplant period. † = The PRISM score is based on variables collected during the first 24 hours of ICU admission after transplantation.

Tacrolimus disposition

During the 2-week post-transplant period, 258 tacrolimus concentrations were available for analysis. A median of 6 concentration measurements were available for each patient during the study period. The median tacrolimus trough concentration was 9.6 (IQR: 2.08) ng/ml, and the median dose requirement was 0.06 (IQR: 0.06) mg/kg/12 hours. The median concentration/weight-adjusted dose ratio (as a surrogate for estimated clearance) was 150.79 (IQR: 173.3) ng/ml/dose. Of all analyzed concentrations, 32.0% were above the target range and 53.7% were below the target range (Figure 1). On Day 7, 28% were above the target range and 51.3% were below the target range.

Figure 1: Histogram of tacrolimus concentrations

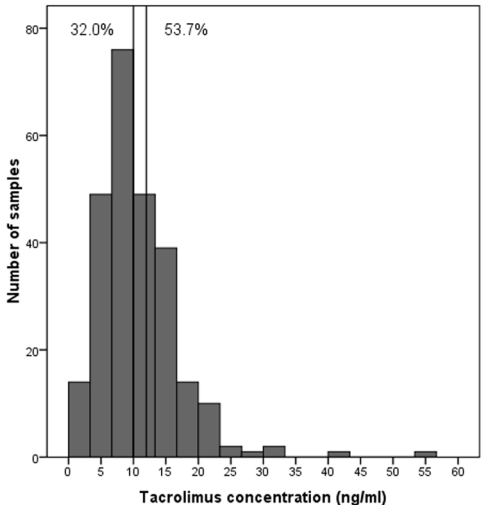

Figure 1: Vertical lines denote therapeutic range in first two weeks after heart transplant. % = percentage of samples outside of therapeutic window

Outcome

None of the patients were diagnosed with a grade 2R or higher of rejection in the first 14 days after transplantation. The median estimated glomerular filtration rate (eGFR) at the last available creatinine level was 130.29 (IQR: 66.27) ml/min/1.73 m^2.

Relationship with genotype

DNA for *CYP3A5* genotyping was available for 37 of the 39 patients. Only 1 patient carried the *CYP3A5*1/*1* genotype, 7 carried the *CYP3A5*1/*3* genotype, and 29 carried the *CYP3A5*3/*3* genotype. *CYP3A5* genotypes did not deviate from the Hardy-Weinberg equilibrium (X^2 0.49, p=0.5). *CYP3A5* expressors (*CYP3A5*1/*1* and *CYP3A5*1/*3*) required significantly higher doses of tacrolimus than the nonexpressors, at 0.14 (IQR, 0.09) vs 0.06 (IQR: 0.04) mg/kg/12 hour (p=0.001; Figure 2).

Figure 2: Relationship between CYP3A5 genotype and tacrolimus dosing requirements

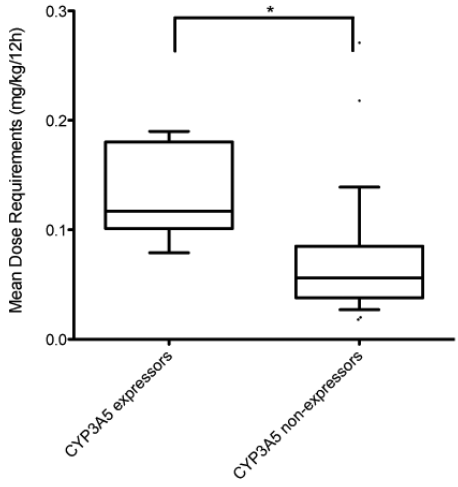

Figure 2: * = p<0.05 expressors vs. non-expressors, • = Outliers

Expressors also had significantly lower tacrolimus trough concentrations, at 7.7 (IQR: 5.85) vs 9.8 (IQR: 3.05) ng/ml (p=0.032), and lower concentration/ dose ratios of 45.34 (IQR: 44.54) vs 177.78 (IQR: 145.38) ng/ml per mg/kg/12 hours (p= 0.0001; Table 2).

Table 2: Relationship of CYP3A5 genotype with tacrolimus disposition

	Tacrolimus trough levels (ng/ml)			Tacrolimus dosing requirements (mg/kg/12h)			Concentration/dose ratio (ng/ml per mg/kg/12h)		
	N	Median (IQR)	P-value	N	Median (IQR)	P-value	N	Median (IQR)	P-value
CYP3A5	37		0.032*	37		0.002*	37		<0.0001*
Expressors	8	7.70 (5.85)		8	0.139 (0.09)		8	45.34 (44.54)	
Non-expressors	29	9.80 (3.05)		29	0.055 (0.04)		29	177.78 (145.38)	

* = p<0.05 expressors vs non-expressors

Table 3: ABCB1 genotype frequencies

Genotype	Frequency (percentage)	Hardy-Weinberg equilibrium	
		Chi-square	*p*-value
ABCB1		χ^2=1.29	P=0.2
3435 CC	12 (32%)		
3435 CT	15 (41%)		
3435 TT	10 (27%)		
ABCB1		χ^2=0.02	P=0.9
2677 GG	11 (30%)		
2677 GA	1 (3%)		
2677 GT	17 (46%)		
2677 TT	8 (21%)		
ABCB1		χ^2= 0.29	P=0.5
1236 CC	10 (27%)		
1236 CT	20 (54%)		
1236 TT	7 (19%)		

DNA for *ABCB1 C3435T*, *ABCB1 G2677T/A*, and *ABCB1 C1236T* genotyping was available for 37 of the 39 patients. The frequencies of each of the genotypes are described in Table 3. None of the *ABCB1* genotypes deviated from the Hardy-Weinberg equilibrium. No relationship was found between tacrolimus dosing requirements, tacrolimus trough concentrations, or concentration/dose ratio and *ABCB1 3435*, *2677*, and *1236* genotypes (Table 4).

Relationship with age and PRISM scores

Tacrolimus dosing requirements were higher in younger than older children (r_s=–0.447, p=0.004; Figure 3). Concentration/dose ratios were lower in younger children (r_s=0.351, p=0.029); however, the tacrolimus trough concentrations were not significantly correlated with age (r_s=0.052, p=0.752). No significant correlation was found between the PRISM score and tacrolimus dosing requirements (r_s=–0.29, p=0.09), concentration/dose ratios (r_s=0.20, p=0.25), or tacrolimus trough concentrations (r_s =–0.150, p=0.38).

Table 4: Relationship of ABCB1 genotype with tacrolimus disposition

	Tacrolimus trough level (ng/ml)			Tacrolimus dosing requirements (mg/kg/12h)			Concentration/Dose Ratio (ng/ml per mg/kg/12h)		
	N	Median (IQR)	P-value	N	Median (IQR)	P-value	N	Median (IQR)	P-value
ABCB1 3435	37		0.466	37		0.899	37		0.823
CC	12	9.85 (5.07)		12	0.062 (0.11)		12	150.16 (301.16)	
CT	15	9.50 (2.75)		15	0.055 (0.08)		15	150.79 (175.33)	
TT	10	10.03 (1.97)		10	0.073 (0.06)		10	145.10 (156.82)	
ABCB1 2677	37		0.318	37		0.963	37		0.531
GG	11	9.80 (5.70)		11	0.067 (0.09)		11	154.55 (331.46)	
GA/GT	18	9.50 (3.68)		18	0.073 (0.08)		18	139.54 (151.79)	
TT	8	10.17 (1.88)		8	0.072 (0.05)		8	174.12 (138.62)	
ABCB1 1236	37		0.413	37		0.835	37		0.776
CC	10	10.20 (5.40)		10	0.061 (0.06)		10	179.01 (283.16)	
CT	20	9.50 (2.88)		20	0.074 (0.08)		20	139.54 (178.71)	
TT	7	10.20 (2.30)		7	0.081 (0.04)		7	170.46 (144.19)	

Figure 3: The relationship between mean tacrolimus dosing requirements and patient's age at the time of transplant.

Interplay of age and *CYP3A5* genotype

Age and *CYP3A5* genotype both appeared to be associated with tacrolimus dosing requirements or the concentration/ dose ratio. The contribution of both parameters was assessed with multivariate linear regression. Age and *CYP3A5* genotype were independently associated with the tacrolimus dosing requirements (R^2=0.351, p=0.001) and with the concentration/dose ratio (R^2=0.521, p=0.001). This was reflected by the observation that in *CYP3A5* expressors younger than 6 years, the dosing requirements were more than 1.5 times higher than in *CYP3A5* expressors older than 6 years (0.15 [IQR: 0.08] vs 0.09 [IQR: 0.04] mg/kg/12 hours). *CYP3A5* non-expressors younger than 6 years also needed 1.5 times higher doses than *CYP3A5* non-expressors older than 6 years (0.07 [IQR: 0.18] vs 0.047 [IQR: 0.25] mg/kg/12 hours). In addition, the dosing requirements of *CYP3A5* expressors younger than 6 years were 3 times higher than *CYP3A5* non-expressors older than 6 years (0.15 [IQR: 0.08] vs 0.04 [IQR: 0.25] mg/kg/12 hours). When the analysis excluded 1 patient who received fluconazole and 2 patients who received amiodarone, which are *CYP3A* inhibitors, the results were similar for the relationship between *CYP3A5* and *ABCB1* genotype and age and tacrolimus disposition.

Relationship between genetic variation, tacrolimus levels, and outcomes

We did not find a relationship between eGFR at the last available creatinine level and median or highest tacrolimus trough level (r_s=0.128, p=0.439; r_s=–0.005, p=0.975). We also did not find a relationship between eGFR at the last available creatinine level and *CYP3A5* genotype for expressors (median eGFR, 125.37 [IQR, 56.77] ml/min/1.73m^2) vs non-expressors (130.43 [IQR: 72.65] ml/min/1.73m^2, p=0.941).

Discussion

Our data show that less than 15% of tacrolimus trough concentrations are within the (narrow) target range in the early post-transplant period in pediatric heart transplant recipients. Age and *CYP3A5* genotype, independently, both contribute to the variation in the tacrolimus dosing requirements in this cohort.

Limited data exist on pharmacogenetic influences in pediatric transplant recipients:

- Zheng et al[16] reported similar results at 3, 6, and 12 months after transplantation in 65 pediatric heart transplant recipients. These investigators showed a significant difference in the tacrolimus concentration/dose ratio between *CYP3A5* expressors and non-expressors, with the expressors requiring higher doses to maintain the same tacrolimus blood concentration.
- A lower tacrolimus oral clearance was reported by Zhao et al[28] for pediatric kidney transplant recipients with the *CYP3A5*3/*3* genotype compared with those with the *CYP3A5*1/*3* genotype less than 2 months after transplant.
- Two other studies of pediatric liver transplant recipients found no relationship between recipient *CYP3A5* genotype and tacrolimus disposition; in contrast, the liver donor's *CYP3A5* genotype was a significant predictor.[18,29]

Our study showed a *CYP3A5* recipient genotype-tacrolimus disposition relationship in the first 2 weeks after transplantation, arguably one of the most vulnerable periods. We did not find associations between *ABCB1* genotype and tacrolimus dosing requirements and disposition. This is consistent with a study in children done by Zheng et al,[16] which also failed to find an association at 3 months after pediatric heart transplant. In contrast, at 6 and 12 months after transplant, they found lower concentration/dose ratios in patients with the *GG* and *CC* haplotype (*ABCB1 G2677T/A* and *C3435T*, respectively). They explained this by higher cytokines concentrations in the early post-transplant period (ie, 3 months) that may have contributed to increased variability in P-glycoprotein

expression. Other studies also provide conflicting data about this association, with more studies showing positive associations late after transplant rather than the early period.[17,29]

The effect of age on tacrolimus pharmacokinetics has been reported.[30] Studies of pediatric renal[31] and liver[12,13,32] transplant recipients have shown that pre-pubertal children need 2 to 3 times higher doses than adults. In pediatric bone marrow transplantation, a higher clearance rate, compared with adults, was reported.[33] Within the pediatric population, age-related differences between younger and older children in tacrolimus pharmacokinetics have been reported as well. Przepiorka et al[10] documented a decreased tacrolimus clearance in the first 2 weeks after hematopoietic stem cell transplantation only for children aged older than 12 years. In addition, at steady state, the clearance rate was higher for those younger than 6 years than in older children. In pediatric renal transplant recipients, Kim et al[11] showed that the younger children (-5 years and 5–12 years) required 2.7 and 1.9 times higher dosages, respectively, than older children (12 years), and that a significant inverse correlation between dose/kg and age among all age groups was present. Naesens et al[34] reported that younger pediatric renal transplant recipients needed significantly higher doses to achieve comparable tacrolimus trough concentrations compared with older children. Our results show similar findings, with higher dose requirements and lower concentration/dose ratios (as surrogate marker for clearance) in younger pediatric heart transplant recipients.

Ontogeny in tacrolimus biotransformation may explain these findings. The hepatic metabolism of drugs is altered in younger children, with different ages for the different cytochromes to reach maturity, resulting in different metabolism rates[14] and consequent clearance rates. For many CYP3A4/5 substrates, it is widely established that clearance is increased in the age group between 6 months and 3 years. This has been attributed to higher CYP3A4/5 activity compared with adults, but others have suggested this is due to a larger liver/body size ratio in children than in adults.[14,35]

No patient experienced rejection within the 14 days after transplantation. Therefore, we were unable to test a relationship of genetic variation with outcomes. However, the potential influence of the pharmacokinetic variability in the first 14 days after transplantation on the long-term rejection risk still needs to be studied. In addition, we could not establish a relationship between genetic variability, tacrolimus levels, and renal function. Possible reasons could be the relatively small sample size as well as the limited 14-day interval. As an increase in serum creatinine is only apparent with a marked decrease in renal function, the 14-day interval may not have been big enough to see an effect of the high tacrolimus levels on causing a rise in serum creatinine. Factors other than tacrolimus-induced nephrotoxicity, such as comedication and altered hemodynamics, may affect renal function directly after transplant.

Importantly, our study shows that *CYP3A5* genotype and age are independently associated with tacrolimus disposition. As major changes occur in drug disposition during development, the effect of genetic variation in drug disposition should be studied in the context of this age-related variation. We observed that all *CYP3A5* expressors, independent of age, had higher tacrolimus dosing requirements than non-expressors. Taking age into account further amplified the genotype effect, with younger *CYP3A5* expressors needing, on average, tacrolimus doses that were 3 times higher than those needed by older *CYP3A5* non-expressors. One limitation of our study is the relatively small sample size, which affected the amount of confounders we could look at. In addition, the drug dosing data in our study were retrospectively collected, and this may have introduced unknown variation in the noted vs the actually administered dose, such as inaccuracies with drug dispensing and vomiting with repeated dosing. Although we only studied oral doses of tacrolimus, most of the children do receive tacrolimus orally. Some children, however, cannot tolerate oral tacrolimus and receive intravenous tacrolimus. These children need to be studied separately because differences exist between oral doses and intravenous doses.

The systemic exposure is different with intravenous tacrolimus because the first-pass metabolism (eg, metabolism by *CYP3A5* and *ABCB1* transport before absorption) is bypassed, although liver metabolism would still occur.

In conclusion, we showed that in the first 14 days after heart transplantation, younger age and *CYP3A5* expressor status were independently associated with higher tacrolimus dosing requirements and concentration/dose ratio (as surrogate marker for clearance). Drug dosing algorithms need to be developed to guide initial dosing that is individualized based on age and genotype, with the goal of optimizing the ability to safely and rapidly achieve therapeutic targets.

Disclosure statement

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The new CYP3A4 intron 6 C>T polymorphism (*CYP3A4*22*) and *CYP3A* combined genotypes both correlate with tacrolimus disposition in pediatric heart transplant recipients

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Abstract

Background Tacrolimus metabolism depends on CYP3A4 and CYP3A5. We aimed to determine the relationship between the *CYP3A4*22* polymorphism and combined CYP3A genotypes with tacrolimus disposition in pediatric heart transplant recipients.

Method Sixty pediatric heart transplant recipients were included. Tacrolimus doses and trough concentrations were collected in the first 14 days post-transplantation. CYP3A phenotypes were defined as extensive (*CYP3A5*1* carriers + *CYP3A4*1/*1*), intermediate (*CYP3A5*3/*3* + *CYP3A4*1/*1*) and poor (*CYP3A5*3/*3* + *CYP3A4*22* carriers) metabolizers.

Results *CYP3A4*22* carriers needed 30% less tacrolimus (p = 0.016) to reach similar target concentrations compared to *CYP3A4*1/*1* (n=56) carriers. Poor CYP3A metabolizers required 17% (p =0.023) less tacrolimus than intermediate and 48% less (p <0.0001) than extensive metabolizers. Poor metabolizers showed 18% higher dose-adjusted concentrations than intermediate (p=0.35) and 193% higher than extensive metabolizers (p<0.0001).

Conclusion Analysis of *CYP3A4*22*, either alone or in combination with *CYP3A5*3*, may help towards individualization tacrolimus therapy in pediatric heart transplant patients.

Introduction

Tacrolimus is the most commonly used drug for immunosuppressive treatment in heart transplant recipients.[1] Due to its narrow therapeutic window, accurate dosing and the attainment of target concentrations are challenging. This is true especially in the first weeks following transplantation where variability in drug concentrations are considerable and where information from therapeutic drug monitoring to guide therapy is limited. This early period is also marked by an increased risk for early organ rejection possibly due to under-dosing resulting in inadequate immunosuppression. Therefore, it is critical to study determinants of this variability in order to identify patients at risk.[2],[3]

Tacrolimus is metabolized by cytochrome P450 3A4 (CYP3A4) and 3A5 (CYP3A5). In adult heart transplant patients, the relationship between *CYP3A5* genotype and tacrolimus clearance rate has been well established.[4] [5] Two pediatric studies in heart transplant recipients have reported the relationship of *CYP3A5* genotype with tacrolimus pharmacokinetics.[6] [7] Our group has shown that *CYP3A5* expressers (carrying at least one *CYP3A5*1* allele) younger than 6 years of age needed 3 times higher doses and achieved lower tacrolimus trough concentrations than *CYP3A5* non-expressers older than 6 years of age in the first 14 days post-transplantation, consistent with an enhanced metabolism of tacrolimus in *CYP3A5* expressors.[7] These results were similar to Zheng et al showing higher dosing requirements for *CYP3A5* expressers compared to *CYP3A5* non-expressers at 3, 6 and 12 months post-transplantation.[6]
CYP3A4 is the major metabolizing enzyme of the CYP450 superfamily.[8] Up to 100-fold inter-individual variation in activity has been reported in the general population.[9][10][11][12] While numerous genetic polymorphisms have been described for *CYP3A4*, the frequency of these variants is very low (usually <1%), except for *CYP3A4*1B*. For this latter allele, however, the clinical effect is not very clear and has in fact been suggested to occur due to linkage to the CYP3A5*1 allele.[101] Therefore, to date a genetic explanation for the observed large variability in CYP3A4 activity is not readily available. Recently, a new single nucleotide polymorphism (SNP)

in intron 6 (rs35599367; 15389C>T; *CYP3A4*22*) was described showing decreased CYP3A4 mRNA hepatic expression and a lower microsomal CYP3A4 enzymatic activity for the T-variant allele.[13]

The effect of this newly described *CYP3A4* polymorphism, and the combined information of *CYP3A4* and *CYP3A5* genotypes on tacrolimus dosing requirements and on trough blood concentrations in stable adult renal transplant recipients has recently been described.[14] Carriers of the *CYP3A4*22* allele showed significantly higher dose-adjusted tacrolimus trough concentrations (179.4 [111.2-289.4] ng/ml per mg/kg/day) compared to *CYP3A4*1/*1* patients (88.9 [72.3-109.3] ng/ml per mg/kg/day; $p = 0.017$). These results were more pronounced when the *CYP3A4*22* and *CYP3A5*3* genotypes were combined, based on expected overall CYP3A activity: the predicted CYP3A poor metabolizers had significantly higher dose-adjusted trough concentrations (179.4 [111.2-289.4] ng/ml per mg/kg) compared to intermediate (110.1 [89.7-135.1] ng/ml per mg/kg/day) and extensive metabolizers (43.9 [31.7-60.8] ng/ml per mg/kg/day; $p < 0.001$).[14] In a second study, the same group reported in 185 de novo renal transplant recipients that the mean tacrolimus dose requirement when considering the first year post-transplantation was 33% lower for *CYP3A4*22* variant allele carriers compared to *CYP3A4*1/*1* patients. Moreover, on day 3 post-transplantation the risk of presenting a supra-therapeutic tacrolimus trough concentration was significantly higher for poor (OR: 8.3 $CI_{95\%}$ 1.3-57.0; $p = 0.027$) and intermediate CYP3A metabolizers (OR: 4.7 $CI_{95\%}$ 1.9-13.4; $p = 0.002$) compared to extensive CYP3A metabolizers.[15]

These results suggest a significant role for the newly described *CYP3A4* SNP in adult renal transplant recipients. Therefore, we aimed to determine the relationship of this newly described *CYP3A4* SNP and the combined CYP3A genotypes classification with tacrolimus disposition in the first 14 days post-transplantation in pediatric heart transplant recipients.

Methods

Pediatric heart transplant recipients transplanted between 1995 and 2008 at the Hospital for Sick Children, Toronto, Ontario, were eligible for this study if 1) the age at time of transplantation was younger than 21 years and 2) they had received oral tacrolimus during the first 14 days after transplantation. DNA samples were derived from the cohort of patients prospectively enrolled in the Sickkids Heart Centre Biobank. Informed consent was obtained from parents and/or children during enrollment. The study was approved by the Institutional Research Ethics Board.

The gender, age, weight of the patient, the co-medication received and *CYP3A4*, and *CYP3A5* genotypes, occurrence of rejection and renal function were recorded in the first two weeks post-transplantation. Rejection was graded according to the International Society of Heart and Lung Transplantation's (ISHLT) grading system.[16] Rejection was defined as grade 2R or higher. Renal function was estimated with the Schwartz formula, using the last available serum creatinine concentration during the study period.[17]

Immunosuppressive protocol

All patients received induction therapy consisting of anti-thymocyte globulin peri-operatively and up to 2 to 5 days after transplantation. Tacrolimus (tablets) was started at 0.2 mg/kg/day orally on day 2 or 3 post-transplantation. Therapeutic drug monitoring was used to adjust the tacrolimus dose to achieve a 12-hour post-dose trough concentration of 10-12 ng/ml. Additional immunosuppressive therapy consisted of a maintenance dose of mycophenolate mofetil (600 mg/m^2 twice daily) and a tapering steroid schedule. Tacrolimus dose (mg/kg/day) and tacrolimus trough concentrations were recalled from patient health records. Dose-adjusted tacrolimus concentrations were calculated by dividing the tacrolimus trough concentration by the weight-adjusted daily dose.

Tacrolimus trough concentrations

Tacrolimus blood trough concentrations were determined in ethylenediaminetetraacetic acid (EDTA)-treated whole blood (0.25 ml) on the day of sampling, using liquid chromatography tandem mass spectrometry (LC-MS-MS) as previously described, as part of routine clinical care.[18]

Genotyping

Blood for genotyping was collected in EDTA-containing tubes, and DNA was extracted using a Magna-Pure LC (Roche Diagnostics GmbH, Mannheim, Germany). Genotyping for *CYP3A4*22* and *CYP3A5*3* were performed as described previously.[14],[19],[20]

Patients were classified as a poor CYP3A metabolizer if they were a *CYP3A5* non-expresser and carried at least one *CYP3A4*22* allele, an intermediate CYP3A metabolizer was defined as either *CYP3A5* non-expresser carrying the *CYP3A4*1/*1* genotype or *CYP3A5* expressers carrying at least one *CYP3A4*22* allele and extensive metabolizers as *CYP3A5* expressers carrying the *CYP3A4*1/*1* genotype, as previously described (Table 1).[14]

Table 1: Genotype profiles

	CYP3A4*1/*22 OR CYP3A4*22/*22 *(CYP3A4*1/*22, n=4)*	***CYP3A4*1/*1*** (n=56)
CYP3A5 non-expressers *(CYP3A5*3/*3, n=49)*	Poor metabolizers (n= 4, 6.7%)	Intermediate metabolizers (n=45, 75%)
CYP3A5-expressers *(CYP3A5*1/*1, n=2, CYP3A5*1/*3, n=9)*	Intermediate metabolizers (n=0, 0%)	Extensive metabolizers (n=11, 18.3%)

Statistical analysis

Data are presented as mean ± standard deviation or median and inter-quartile range (IQR) when the data were skewed. The groups were compared using the Mann-Whitney test (two-tailed) or the Kruskal-Wallis test. A mixed-model analysis was used to compare the tacrolimus daily dose requirement, pre-dose concentrations and the dose-adjusted concentrations between different genotype groups. The mixed-model analysis was based on the maximum likelihood ratio, with patient *CYP3A4* genotype or *CYP3A4/5* phenotype status as the fixed factor and time following transplantation as the repeated measurement. A diagonal

covariance structure, which assumes heterogenous variances and zero correlation between elements, was imposed when considering between and within times of follow-up of the repeated tacrolimus measurements. Age, sex and ethnicity of the patients were introduced as random effects to adjust for these covariates. Percentage differences in geometric mean values of untransformed outcomes were determined by back-transforming coefficients estimated from mixed-model. A multivariate analysis was conducted to test the influence of age and genotypes on all dependent variables. The Hardy-Weinberg equilibrium was calculated by using the method of Rodriguez et al.[21] All data analyses were performed using Predictive Analytics Software (PASW) software, version 17.0 for Windows (IL, USA).

Results

This study is part of a larger pharmacokinetic-pharmacodynamic study; detailed information on the study participants can be found in a previous publication.[7] Sixty patients were included in the study with a median age of 4.0 (IQR: 12.0) years at the time of transplantation. Thirty-nine patients were Caucasian, 4 African-American, 3 Asians, 1 Native Canadian and for 13 patients ethnicity was unknown (Table 2).

DNA for *CYP3A4*22* and *CYP3A5*3* genotype information was available for all 60 patients (Table 1). The observed distribution of genotypes did not deviate from the Hardy-Weinberg Equilibrium (*CYP3A4:* $\chi^2 = 0.07$, $p > 0.05$; *CYP3A5:* $\chi^2 = 3.00$, $p > 0.05$). No linkage disequilibrium was observed between *CYP3A4*22* and *CYP3A5*3* ($\chi^2 = 0.62$, $p = 0.618$). No significant differences were found between both *CYP3A4* genotype groups concerning the patients' age ($p = 0.185$) and gender ($p = 0.875$) (Table 2).

Table 2: Patient demographics

Patient characteristics	All patients	CYP3A4*1/*1	CYP3A4*1/*22	p-value
Number	60	56	4	-
Gender (M/F)	33/27	31/25	2/2	0.875
Age (years)	4.00 (12.00)	3.00 (12.00)	8.50 (9.00)	0.185
Weight (kg)	13.30 (30.00)	12.60 (28.80)	14.15 (33.58)	0.898
Diagnosis				0.898
Dilated cardiomyopathy	34	31	2	
Congenital heart disease	26	24	2	
Unknown	1	1	-	
Ethnicity				0.808
Caucasian	39	36	3	
African-American	4	4	-	
Asian	3	3	-	
Native	1	1	-	
Unknown	13	12	1	
eGFR ml/min/1.73m2	128.70 (83.30)	128.70 (82.10)	154.85 (150.50)	0.611

All data are presented as median and interquartile range (IQR)

*CYP3A4*22 and tacrolimus disposition*

In a mixed model analysis, *CYP3A4*22* allele carriers needed 30% lower tacrolimus doses to reach target concentrations ($p = 0.016$) than *CYP3A4*1/*1* carriers when considering all follow-up time points. No significant difference in tacrolimus concentrations ($p = 0.953$) and the dose-adjusted concentration ($p = 0.211$) between *CYP3A4*22* carriers and non-carriers was observed.

Neither tacrolimus trough concentrations nor concentration/dose ratios per day were significantly different between *CYP3A4*1/*1* and *CYP3A4*22* allele carriers ($p > 0.05$, Table 3). Only on day 3 a significant association in tacrolimus dose requirements was found between *CYP3A4*1/*1* (0.10 [IQR: 0.11] mg/kg/day) and *CYP3A4*1/*22* carriers (0.02 [sd = 0.00]; $p = 0.002$).

Table 3: Relationship of *CYP3A4* genotypes with tacrolimus disposition

Time	Tac dose (mg/kg/day)			Tac level (ng/ml)			Concentration/Dose ratio		
	*CYP3A4*1/*1* carriers	*CYP3A4*1/*22* carriers	*p*-value	*CYP3A4*1/*1* carriers	*CYP3A4*1/*22* carriers	*p*-value	*CYP3A4*1/*1* carriers	*CYP3A4*1/*22* carriers	*p*-value
Day 2	0.10 (0.11)	-	-	3.30 (3.70)	-	-	81.07 (65.46)	-	-
Day 3	0.10 (0.11)	0.02 (0)†	0.002	6.10 (8.10)	14.40#	0.357	46.06 (75.12)	720.00#	0.074
Day 4	0.12 (0.08)	0.10 (0.11)*	0.230	10.00 (7.30)	8.15 (0.78)†	0.468	84.75 (83.42)	255.50 (359.00)†	0.317
Day 5	0.10 (0.10)	0.10 (0.18)*	0.641	11.80 (8.45)	-	-	91.42 (104.11)	-	-
Day 6	0.10 (0.09)	0.09 (0.14)*	0.775	10.90 (6.20)	8.85 (7.71)†	0.852	77.86 (81.21)	64.08 (14.83)†	0.619
Day 7	0.10 (0.08)	0.09 (0.11)	0.512	9.50 (7.60)	-	-	52.50 (58.71)	-	-
Day 8	0.10 (0.10)	0.09 (0.10)	0.457	9.40 (6.40)	8.55 (4.30)	0.852	85.83 (108.06)	94.63 (78.56)	0.640
Day 9	0.10 (0.09)	0.10 (0.09)	0.501	9.50 (7.75)	5.60#	0.462	86.36 (94.95)	140.00#	0.462
Day 10	0.12 (0.12)	0.10 (0.09)	0.447	8.90 (5.50)	9.90#	0.800	78.57 (75.00)	99.00#	0.800
Day 11	0.14 (0.11)	0.10 (0.09)	0.265	8.60 (4.10)	8.60#	1.000	61.43 (71.67)	53.75#	0.929
Day 12	0.14 (0.10)	0.10 (0.11)	0.228	9.35 (3.35)	-	-	55.00 (33.74)	-	-
Day 13	0.14 (0.12)	0.10 (0.12)	0.240	10.30 (5.10)	11.40 (7.49)†	0.686	85.83 (92.99)	118.00 (48.79)†	0.467
Day 14	0.15 (0.13)	0.10 (1.10)*	0.635	9.05 (5.43)	11.80 (16.90)*	.412	67.50 (76.26)	96.50 (30.41)†	0.442
Median Day 4-14	0.12 (0.10)	0.09 (0.10)	0.228	10.11 (3.27)	9.76 (4.59)	1.000	87.52 (75.10)	118.71 (86.77)	0.380

Only one sample available

† Only two sample available; mean and standard deviation are given instead

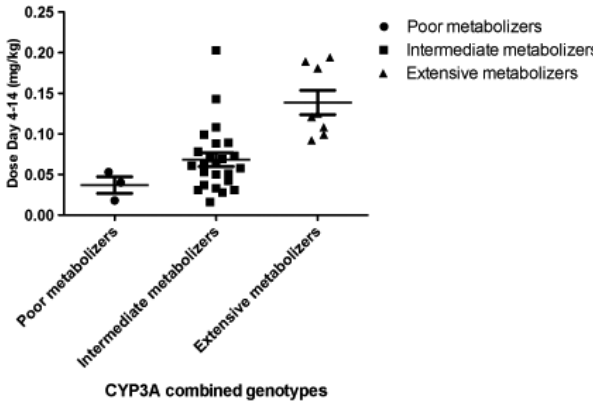
* Only three samples available; median and range given instead

Combined genotypes and tacrolimus disposition

In the mixed model, poor CYP3A metabolizers (*CYP3A4*1/*22* and *CYP3A5* non-expresser) required 17% less tacrolimus than intermediate (*CYP3A4*1/*1* and *CYP3A5* non-expresser, p =0.023) and 48% less than extensive CYP3A metabolizers (*CYP3A4*1/*1* and *CYP3A5*-expresser, p <0.0001), respectively, (Figure 1).

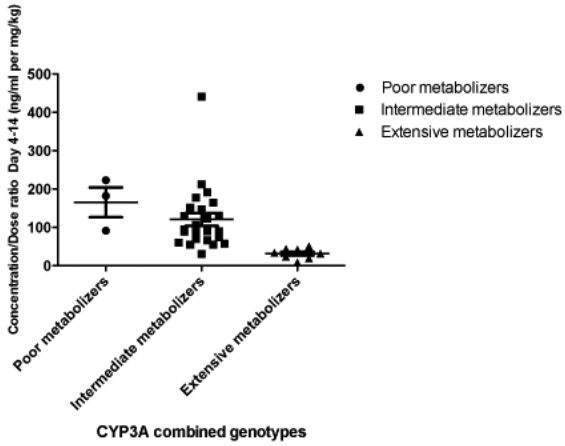
In addition, poor metabolizers showed 18% higher, albeit non-significant (p =0.35), dose-adjusted trough concentrations than intermediate metabolizers and 193% higher dose-adjusted concentrations (p <0.0001) than extensive metabolizers, respectively (Figure 2). No significant differences were found in tacrolimus trough concentrations between the combined CYP3A genotypes ($p = 0.832$ and $p = 0.200$).

Figure 1: Combined *CYP3A4/5* genotypes and tacrolimus dosing requirements



Tacrolimus dose requirements (mg/kg) from day 4 till 14 according to CYP3A activity. Individual values are reported.

Figure 2: Combined *CYP3A4/5* genotypes and tacrolimus concentration/dose ratio



Tacrolimus concentration/dose ratio (ng/ml per mg/kg) from day 4 till 14 according to CYP3A activity. Individual values are reported.

Starting from day 6, poor CYP3A metabolizers developed significantly lower median dosing requirements (Day 6: 0.09 [range: 0.14] mg/kg/day) compared to intermediate CYP3A metabolizers (0.10 [IQR: 0.06] mg/kg/day) and extensive CYP3A metabolizers (0.20 [IQR: 0.08] mg/kg/day; $p = 0.006$). The dosing requirements for all patients gradually increased throughout the first two weeks post-transplant. At day 14, poor CYP3A metabolizers needed 0.10 mg/kg/day (range: 0.10) compared to intermediate CYP3A metabolizers who needed 0.12 (IQR: 0.12) and extensive metabolizers, who needed 0.24 (IQR: 0.16) mg/kg/day ($p = 0.003$) (Table 4). Both age and the combined CYP3A genotype were independently associated with tacrolimus dose requirements considering the follow-up period from day 4 till 14 ($R^2 = 0.346$, $p < 0.0001$; $F_{age} = 8.59$ $p_{age} = 0.005$; $F_{cluster} = 22.78$ $p_{cluster} < 0.0001$) and the dose-adjusted concentrations day 4 till 14 ($R^2 = 0.273$, $p < 0.0001$; $F_{age} = 11.80$ $p_{age} = 0.001$; $F_{cluster} = 10.84$ $p_{cluster} = 0.002$).

Clinical outcome

Three patients experienced early graft rejection in the first 14 days post-transplantation. All patients experiencing rejection carried the *CYP3A4*1/*1* genotype. One patient was considered an extensive CYP3A metabolizer and the 2 others intermediate CYP3A metabolizers. No significant difference was found between the combined CYP3A genotypes and the occurrence of rejection ($\chi^2 = 0.627$, $p = 0.73$).

The median estimated glomerular filtration rate (eGFR) at the last available serum creatinine concentration was 128.7 (IQR: 83.3) ml/min/1.73m^2). eGFR at the last available serum creatinine concentration was not different between *CYP3A4*1/*1* homozygotes (128.7 [IQR: 82.1] ml/min/1.73m^2) and *CYP3A4*22* allele carriers (154.9 [IQR: 150.5] ml/min/1.73m^2; $p = 0.6$). Similarly, no significant difference in eGFR was found between poor CYP3A metabolizers (154.9 [IQR: 150.5] ml/min/1.73m^2), intermediate CYP3A metabolizers (125.2 [IQR: 79.2] ml/min/1.73m^2) and extensive CYP3A metabolizers (130.1 [IQR: 78.0] ml/min/1.73m^2, $p = 0.44$).

One patient had received fluconazole and two patients amiodarone, which are both CYP3A inhibitors. When these patients were excluded from the analysis, none of the results changed.

Table 4: Relationship of combined genotypes with tacrolimus disposition

Time	Tac dose (mg/kg/day)				Tac level (ng/ml)				Concentration/Dose ratio			
	Poor metabolizers	Intermediate metablizers	Extensive metabolizers	p-value	Poor metabolizers	Intermediate metablizers	Extensive metabolizers	p-value	Poor metabolizers	Intermediate metablizers	Extensive metabolizers	p-value
Day 2	-	0.10 (0.12)	0.10 (0.08)	0.309	-	3.30 (2.83)†	3.70 (2.83)†	1.000	-	81.07 (19.70)†	56.00 (55.15)†	0.683
Day 3	0.02 (0)†	0.10 (0.10)	0.10 (0.08)	0.114	14.40#	8.35 (7.85)	3.20 (4.50)	0.051	720.00#	67.50 (86.50)	42.86 (21.50)	0.121
Day 4	0.10 (0.09)*	0.10 (0.09)	0.13 (0.06)	0.447	8.15 (0.78)†	11.60 (7.70)	6.25 (5.93)	**0.016**	255.50 (253.85)†	104.25 (91.61)	45.73 (40.54)	0.012
Day 5	0.10 (0.18)*	0.10 (0.07)	0.17 (0.07)	0.098	-	13.10 (8.80)	7.40 (5.28)	**0.018**	-	127.17 (94.17)	45.47 (25.43)	0.001
Day 6	0.09 (0.14)*	0.10 (0.06)	0.20 (0.08)	**0.006**	8.85 (7.71)†	10.90 (6.30)	5.35 (7.65)	0.165	64.08 (10.49)†	88.57 (63.930	27.53 (30.81)	0.004
Day 7	0.09 (0.11)	0.10 (0.08)	0.20 (0.10)	**0.001**	-	9.60 (5.30)	7.30 (10.40)	0.650	-	65.00 (83.35)	47.33 (49.95)	0.079
Day 8	0.09 (0.10)	0.10 (0.08)	0.20 (0.14)	**<0.0001**	8.55 (4.30)	9.50 (6.05)	6.90 (6.00)	0.523	94.63 (78.56)	90.60 (101.43)	28.25 (45.53)	0.027
Day 9	0.10 (0.09)	0.10 (0.07)	0.22 (0.12)	**<0.0001**	5.60#	9.80 (7.98)	8.10 (5.70)	0.457	140.00#	96.50 (83.12)	27.50 (31.86)	0.010
Day 10	0.10 (0.09)	0.10 (0.07)	0.22 (0.09)	**<0.0001**	9.90#	8.45 (5.18)	11.40 (6.70)*	0.748	99.00#	86.94 (96.88)	43.85 (38.15)*	0.210
Day 11	0.10 (0.09)	0.12 (0.08)	0.24 (0.20)	**<0.0001**	8.60#	8.55 (2.97)	9.50 (5.90)	0.356	53.75#	80.50 (102.90)	39.90 (24.90)	0.141
Day 12	0.10 (0.11)	0.12 (0.10)	0.24 (0.20)	**<0.0001**	-	9.15 (3.12)	10.50 (4.25)	0.225	-	60.63 (41.81)	39.58 (20.95)	0.069
Day 13	0.10 (0.12)	0.12 (0.11)	0.24 (0.16)	**0.001**	11.40 (7.50)†	10.30 (5.77)	10.70#	0.833	118.00 (48.79)†	86.49 (93.40)	48.64#	0.524
Day 14	0.10 (0.10)*	0.12 (0.12)	0.24 (0.16)	**0.003**	11.80 (16.90)*	9.05 (3.40)	9.90 (11.70)	0.663	96.50 (30.41)†	77.14 (74.55)	31.03 (10.93)	**0.017**
Median day 4-14	0.09 (0.10)	0.12 (0.07)	0.20 (0.07)	**<0.0001**	9.76 (4.59)	10.45 (3.40)	9.29 (4.14)	0.198	118.71 (86.77)	91.62 (88.90)	43.63 (21.69)	**<0.0001**

only one sample available
† Only two sample available; mean and standard deviation are given instead
* only three samples available; median and range given instead

Discussion

In our study, we showed that this novel *CYP3A4*22* SNP is associated with changed tacrolimus dose requirement in pediatric heart transplant patients in the first 14 days after transplantation. This study is the first to show a relationship of this SNP in (pediatric) heart transplant recipients.

Patients carrying at least one *CYP3A4*22* allele needed 30% lower tacrolimus doses compared to *CYP3A4*1* carriers. Neither tacrolimus concentrations nor concentration/dose ratios differed between these two groups. This potential discrepancy may be explained as daily tacrolimus doses were available while concentrations (and consequently concentration/dose ratios) were not, as concentrations were not measured every day during the study period. This may have reduced the power in the mixed-model analyses for these two parameters.

In addition, *CYP3A* combined genotypes were strongly related with tacrolimus disposition in this population. Poor CYP3A metabolizers required almost 20% less tacrolimus than intermediate and approximately 50% less than extensive metabolizers, throughout the follow-up period post-transplantation.

The significant difference between poor and intermediate metabolizers in dosing requirements can only be contributed to the CYP3A4 genotype, as the only difference between poor CYP3A metabolizers and intermediate metabolizers in our study group is the *CYP3A4* genotype.

In addition, poor metabolizers had similar dose-adjusted trough concentrations as intermediate metabolizers, but significantly higher dose-adjusted concentrations than extensive metabolizers. The difference between poor and extensive metabolizers in dose requirements and dose-adjusted concentrations is likely a combined CYP3A5 and CYP3A4 effect.

From day 6 onwards, weight-normalized tacrolimus dosing requirements for extensive CYP3A metabolizers were the highest followed by intermediate CYP3A metabolizers and poor CYP3A metabolizers. The lack of significant differences in dosing requirement on day 2 and 3 between genotype groups could be explained by the fact that tacrolimus dosing is

usually adjusted based on tacrolimus concentrations starting from day 4 post-transplantation onwards and patients receive starting dosing in the first two days post-transplantation.

Our results are similar to what has previously been reported in adult renal transplant recipients, where patients carrying at least one *CYP3A4*22* allele had also 33% lower mean tacrolimus dose requirement considering the first year post-transplantation compared to *CYP3A4*1* homozygotes.[15] The association between *CYP3A4*22* and tacrolimus trough concentrations instead of dose requirement is, however, in our study less clear. We were not able to find such an association, which is similar to the study by Elens et al.[14] in 49 de novo adult renal transplant recipients. However, in their second study, an association at day 3 post-transplantation was found, showing higher tacrolimus trough concentrations for carriers of at least one *CYP3A4*22* allele (20.5 [15.2-27.7] ng/ml) compared to *CYP3A4*1/*1* homozygotes (14.9 [13.8-16.0] ng/ml, p = 0.05). However, this significant difference disappeared from day 10 post-transplantation onwards.[15] In contrast to our results, Elens et al. were not able to find a significant difference in the dose-adjusted tacrolimus trough concentration between the two *CYP3A4* genotype groups.[14],[15] Due to the limited amount of tacrolimus trough concentrations, the dose-adjusted tacrolimus trough concentrations are limited in number as well. This could explain why we have not been able to show an association between dose-adjusted tacrolimus trough concentrations and *CYP3A4* genotype.

As genotype does not change with age, it is conceivable that findings in adults would also be extrapolated to children, although this is not necessarily the case. Nonetheless, as the ontogeny of CYP3A enzymes is still a factor in children,[22],[23],[24] genotype might not match with the phenotype present at the age of the child as it does in adults.
We have shown that age and *CYP3A5* genotype appear to results in an additive effect on tacrolimus dosing. As *CYP3A5* genotype on average explains a two-fold variation in tacrolimus requirements, this variation becomes 4-fold when age is also taken into account. Younger *CYP3A5*-expressers needed 4 times higher tacrolimus doses than older *CYP3A5*

non-expressers.[7] This finding highlights the importance to study pharmacogenetics in children in the context of development.

As the first weeks after transplantation are characterized by an increased risk of early organ rejection,[2],[3] the importance of identifying key predictors becomes clinically valuable. As all three of our patient experiencing rejection where homozygous for *CYP3A4*1*, no analysis was done to test the possible influence of *CYP3A4* genotype on this outcome. We failed to show a possible relationship of the combined *CYP3A* genotypes and the occurrence of graft rejection.
As renal dysfunction is one of the major adverse events related to tacrolimus therapy, we a tested the relation between renal function as assessed by creatinine clearance, and the *CYP3A* genotypes classification. We could not find an association between the combined CYP3A genotypes and renal function defined by an eGFR of the last available serum creatinine concentration. Nevertheless, the time window of 14 days post-transplantation might be too short to accurately determine the relationship between genetic variability and renal function measured by serum creatinine. A decrease of about 50% of renal function is needed for serum creatinine concentrations to show changes.[25] Furthermore, serum creatinine concentration might not accurately reflect renal function until steady state concentrations of serum creatinine has been attained.[25]

This study has several limitations. The sample size is relatively small and only four carriers of the *CYP3A4*22* allele (also labelled as poor metabolizers) could be detected. Moreover, we did not have tacrolimus trough concentrations for every patient for every day. Due to the retrospective nature of this research we could not collect additional samples to enlarge our data set. The results per day have to be taken with caution as multiple testing was done with a limited sample size. Yet, it does provide a good overview of the tacrolimus disposition throughout the study period. Although the sample size was small, statistically significant relationships between the *CYP3A4*22* allele as well as between the combined *CYP3A* genotype classification and tacrolimus dose requirement were observed, suggesting a marked effect size. The use of dose-adjusted tacrolimus

concentrations to determine possible associations is but one way to determine the genetic influence on tacrolimus disposition. Unfortunately, area-under-the-curves or other pharmacokinetic parameters were not available for our cohort. Secondly, correcting our results for co-medication and altered hemodynamics could not be conducted, as our sample size was not large enough and would have increased the risk of spurious associations. Thirdly, the use of serum creatinine and the Schwartz formula for the estimation of renal function has its limits of its own. The Schwartz formula may provide falsely increased GFR values in patients experiencing chronic renal failure. Therefore, other methods for the assessment of renal function, such as neutrophil gelatinase-associated lipocalin (NGAL) and Cystatin C, which demonstrate promising results, may be used in the future to determine the association between genetic variability and drug-induced renal function.[26]

Conclusion

Despite the small sample size, our study shows an impact of the *CYP3A4*22* allele, as well as from the combined *CYP3A4* and *CYP3A5* genotypes on tacrolimus disposition in pediatric heart transplant recipients. To the best of our knowledge, this is the first study in heart transplant patients, and more specifically in children, that evaluates the association between the novel *CYP3A4*22* allele and tacrolimus disposition. However, our findings should be interpreted with caution as data on *CYP3A4*22* allele carriers were limited. Nonetheless, our findings are in line with previous reports in adults, suggesting pre-transplantation screening of patients for both the *CYP3A4*22* and *CYP3A5*3* polymorphism could potentially optimize tacrolimus therapy. By optimizing the individual therapy, we may minimize the risk for over- or under-dosing with the potential for preventing toxicity or organ rejection, although these results first need to be replicated in a larger, prospective study.

Executive summary

Introduction

- *CYP3A5* genotype has been associated with tacrolimus disposition in pediatric heart transplant patients
- Recently, a new polymorphism in *CYP3A4 (CYP3A4*22*) has been associated with altered tacrolimus disposition in adult renal transplant recipients

Results

- In the first 14 days post-transplantation *CYP3A4*22* carriers needed less tacrolimus compared to *CYP3A4*1* homozygotes
- Poor metabolizers of the combined *CYP3A4* and *CYP3A5* genotypes had lower tacrolimus dosing requirements and higher dose-adjusted tacrolimus trough concentrations.

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P450 oxidoreductase *28 *(POR*28)* and tacrolimus disposition in pediatric kidney transplant recipients:

a pilot study

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Submitted

Abstract

Background In a recent study, *POR*28* was associated with increased dosing requirements early after transplant in adult *CYP3A5*-expressing kidney transplant recipients. Both age and *CYP3A5* genotype are important determinants of tacrolimus disposition in pediatric kidney transplant recipients. The authors aimed to evaluate the additional contribution of *POR*28* to tacrolimus disposition in the first 14 days post-transplantation in pediatric kidney transplant recipients.

Method The authors studied data on tacrolimus dose and tacrolimus pre-dose serum concentrations in 43 pediatric kidney transplant recipients up to 14 days post-transplant. Recipient *POR*28* and *CYP3A5* genotype were determined.

Results *CYP3A5*-expressers carrying at least one *POR*28* allele had on average 18.3% lower tacrolimus pre-dose concentrations and 20.2% lower concentration/dose ratios compared to *CYP3A5*-expressers with *POR*1/*1* genotype ($p = 0.002$ and $p = 0.001$, respectively). No significant difference was found within the *CYP3A5* non-expressers between POR genotype and tacrolimus disposition.

Conclusion In this small cohort of pediatric kidney transplant recipients, *POR*28* genotype seems to explain part of the variability found in tacrolimus disposition, in addition to age and *CYP3A5* genotype. These results merit further study in a larger population to validate our findings and to evaluate the clinical impact of this genotype.

Introduction

Tacrolimus is a calcineurin inhibitor (CNI) with a narrow therapeutic window necessitating rigorous therapeutic drug monitoring. However, significant variability in tacrolimus pre-dose concentrations still remains an important clinical challenge.[1] While part of this variation can be explained by genetic variability in the drug metabolizing enzyme CYP3A5,[2,3] genetic polymorphisms in genes involved in the regulation of CYP3A4/5 enzymes have been explored.

P450 oxidoreductase (POR) is the protein that enables the activity of cytochrome P450 (CYP) enzymes by transferring electrons from NADPH to microsomal CYP enzymes.[4] To date, 41 haplotypes in the POR gene have been published by the Human Cytochrome P450 Allele Nomenclature Committee (http://www.cypalleles.ki.se/por.htm). Interestingly, the *POR*28* (rs1057868) SNP has been associated with isoform-specific effects on CYP activity. It was associated with 85% of wild-type in vitro activity of CYP1A2 but 113% activity of CYP2C19.[5] In vivo, the *POR*28* variant was found in 19.1% of the African-Americans, 26.4% of the Caucasians, 36.7% of the Chinese Americans and 31.0% of the Mexican Americans. This variant showed 56-67% of wild-type activity in the cytochrome c assays and 58-68% of wild-type activity in the P450c17 assays.[6]

Furthermore, homozygous carriers of the SNP *(POR*28)* T variant showed an increased in vivo CYP3A activity compared to CC carriers demonstrated by a 1.6-fold increase in the midazolam metabolic ratio in an adult cohort including heroin-dependent individuals treated with methadone as well as psychiatric patients receiving clozapine treatment.[7]

Recently, in 298 adults of whom 52 *CYP3A5*-expressing renal transplant recipients, *POR*28T* (also referred to as *POR*28*) allele carriers had lower tacrolimus pre-dose concentrations in the first days post-transplantation and reached the target levels much later compared to *POR*28CC* (also referred to as *POR*1/*1*) carriers. Additionally, in the first year post-transplantation *POR*28T* allele carriers had significantly higher tacrolimus

dosing requirements compared to *POR*28CC* homozygous patients. In *CYP3A5* non-expressers the pharmacokinetics of tacrolimus was not different between *POR*1* and *POR*28* carriers.[8]

Previously, we have shown that dosing requirements of pediatric kidney transplant recipients are not only associated with *CYP3A5* genotype, but also with age.[9] Dosing requirements of *CYP3A5*-expressers younger than 6 years of age were approximately four times higher than those in *CYP3A5* non-expressers older than 6 years. These results emphasize the need to consider maturation before extrapolating adult pharmacogenetic results to children.[10,11,12]

The objective of the present study was to determine the potential effect of the *POR*28* genetic polymorphism on tacrolimus disposition in the first 14 days post-transplantation in our previously studied cohort of pediatric kidney transplant recipients.

Methods

This was a retrospective cohort study in pediatric renal transplant recipients covering the first 14 days post-transplantation. Pediatric kidney transplant recipients transplanted between 2000 and 2008 were eligible if they were < 18 years of age at the time of transplantation and received tacrolimus in the first 14 days post-transplantation. All children were transplanted at the Hospital for Sick Children, Toronto, Ontario, Canada. This was the same cohort in which we have previously shown the relationship between age and *CYP3A5* genotype in tacrolimus disposition.[9]

Immunosuppressive protocol

Tacrolimus was started at 0.1 mg/kg twice daily orally. Therapeutic drug monitoring was used to adjust the tacrolimus dose to achieve a target level of 10-15 ng/ml. Additional immunosuppressive therapy consisted of a maintenance doses of mycophenolate mofetil and steroids. At the time of graft reperfusion, methylprednisolone (10 mg/kg) was administered intravenously and gradually tapered in the weeks after transplantation.

Outcome data

We collected data on tacrolimus dose and tacrolimus pre-dose levels in pediatric kidney transplant recipients up to 14 days post-transplant. Concentration/dose ratios (C/D, ng/ml per mg/kg/day) were calculated using tacrolimus pre-dose concentrations (ng/ml) divided by the tacrolimus dosing requirements (mg/kg/day), as a surrogate marker for tacrolimus clearance. Delayed graft function was defined as the need for dialysis treatment within the first week post-transplantation.[13]

Co-variates

The following patient characteristics were collected from SickKids electronic patient databases: transplant type, age at transplant, gender, and weight.

Tacrolimus pre-dose concentrations

Tacrolimus blood pre-dose concentrations were determined in ethylenediaminetetraacetic acid (EDTA)-treated whole blood (0.25 ml) on the day of sampling, using liquid chromatography-mass spectrometry.[14]

Genotyping

For DNA analysis, blood for DNA (0.5 ml) was collected during regular blood work at the Transplant Outpatient Clinic. In cases where DNA collection from blood was not possible, saliva was collected using the Oragene TM DNA elf-collection kit following the manufacturer's instructions (DNA Genotek, Kanata, ON). Blood and saliva were stored at -80°C until analysis. DNA was extracted using a Manga-Pure LC (Roche Diagnostics GmbH, Mannheim, Germany). Polymerase chain reactions (PCR)-restriction fragment length polymorphism for *CYP3A5*3* were performed as described previously.[15,16] Patients not carrying the *CYP3A5*3* allele were assigned the *CYP3A5*1/1* genotype by default. *POR*28* (rs1057868) genotype determination was done on an ABI PRISM 7500® Fast real-time PCR Systems (Applied Biosystems, CA, USA) using 20 ng genomic DNA, according to the manufacturer instructions. The assay was validated by direct sequencing of wild type and variant samples.

Statistical analysis

Data are presented as mean ± standard deviation or median and interquartile range (IQR) when the data were skewed. The groups were compared using the Mann-Whitney test or the Kruskal-Wallis test. A mixed-model analysis was used to compare the tacrolimus dosing requirements, tacrolimus pre-dose concentrations and the concentration/dose ratio between the different *POR* genotype groups. Data were analysed separately for *CYP3A5* expressors and non-expressors. As patient age was not statistically different between *POR* genotype groups, age was not added to the model as co-variate. The mixed-model analysis was based on the maximum likelihood ratio, with patient *POR* genotype as the fixed factor and time following transplantation as the repeated measurement. A diagonal covariance structure, which assumes heterogenous variances and zero correlation between elements, was imposed when considering between and within times of follow-up of the repeated tacrolimus measurements. Percentage differences in geometric mean values of untransformed outcomes were determined by back-transforming coefficients estimated from the mixed-model. The Hardy-Weinberg equilibrium was calculated by using the method from Rodriguez et al.[17] All data analyses were performed using Predictive Analytics Software (PASW) software, version 17.0 for Windows (IL, USA).

Results

Study population

A total of 43 renal transplant patients (28 male, 15 female) with a median age of 140.7 (IQR: 88.3) months and weight of 34.2 (IQR: 29.3) kilograms were included (Table 1). Two of the patients received thymoglobulin during the first 14 days post-transplantation. No differences were found between the *CYP3A5*-expresser group and the *CYP3A5* non-expresser group in demographic variables. Sixteen patients were *CYP3A5*-expressers and 27 *CYP3A5*-nonexpressers.

Within the *CYP3A5*-expressers group, 9 patients were homozygous for the *POR*1* allele, 4 patients were *POR*1/*28* carriers and 3 patients homozygous for *POR*28*. No difference in age was found between the

Table 1: demographics of the patients

	CYP3A5 expressers		CYP3A5 non-expressers		P-value
	POR*1/*1 (n=9)	POR*28 (n=7)	POR*1/*1 (n=15)	POR*28 (n=12)	
Age at transplant (months)	132.57 (83.46)	136.53 (155.47)	171.19 (95.80)	132.19 (101.98)	0.851
Gender (M/F)	5/4	7/0	10/5	6/6	0.127
ICU weight (kg)	29.40 (23.13)	33.20 (41.90)	42.70 (24.50)	29.75 (39.13)	0.775

POR genotype groups. Fifteen patients within the *CYP3A5*-nonexpresser group were *POR*1/*1* carriers, 7 *POR*1/*28* carriers and 5 *POR*28/*28* carriers. For this group also, no difference in age was found between the POR genotype groups. Overall allele frequencies were *CYP3A5*1* allele: 20.5% and **3* allele: 79.5%; *POR*1* allele: 69.3% and *28 allele: 30.7%, which are similar to what has previously been reported in Caucasian subjects.[6,7,16,] The observed distribution of genotypes did not deviate from the Hardy-Weinberg equilibrium ($\chi^2 = 2.91$, $p = 0.088$).

Genotype and tacrolimus pre-dose concentrations

In the mixed model, *CYP3A5*-expressers carrying at least one *POR*28* allele had on average 18.3% lower tacrolimus pre-dose concentrations compared to *CYP3A5*-expressers with the *POR*1/*1* genotype ($p = 0.002$). Within the *CYP3A5* non-expressers, no significant difference in tacrolimus

Figure 1: tacrolimus levels during the first two weeks post- transplantation

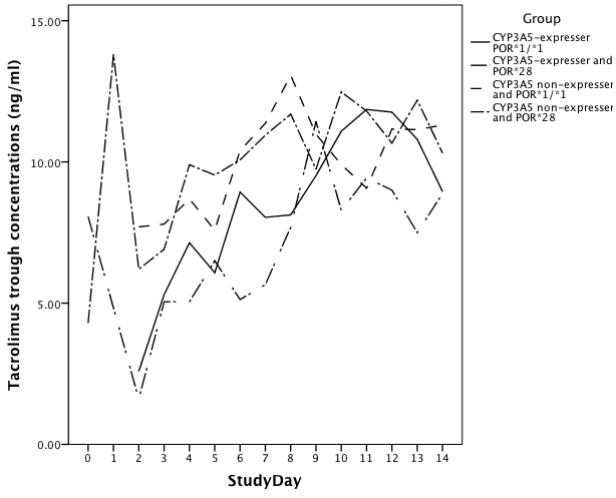

Table 2: Tacrolimus levels (ng/ml) in the first 14 days post-transplantation

Day	CYP3A5 expressers		P-value	CYP3A5 non-expressers		P-value
	POR*1/*1 (n=9)	POR*28 (n=7)		POR*1/*1 (n=15)	POR*28 (n=12)	
0	-	-	-	-	-	-
1	-	-	-	-	13.80#	-
2	2.50 (0.48)	1.60#	0.286	7.95 (5.85)	5.30 (6.45)	0.610
3	4.50 (2.60)*	5.20 (2.90)	1.000	3.95 (11.33)	6.95 (4.50)	0.562
4	6.15 (5.40)	4.35 (2.75)	0.461	6.40 (6.30)	8.05 (7.83)	0.408
5	6.90 (4.30)	6.40 (3.85)	0.699	7.40 (3.80)	8.00 (6.30)	0.438
6	8.50 (2.65)	4.50 (2.50)	0.005	10.90 (3.30)	8.40 (4.20)	0.557
7	8.60 (2.47)	5.00 (0.40)	0.072	10.25 (5.80)	11.85 (3.33)	0.821
8	7.75 (5.35)	7.80 (4.0)	0.779	12.20 (6.50)	10.50 (3.40)	0.365
9	8.50 (4.00)	8.60 (5.10)	1.000	11.05 (5.20)	10.90 (3.95)	0.643
10	11.90 (7.00)	8.30 (3.30)	0.259	10.00 (4.25)	13.90 (3.90)	0.025
11	10.90 (3.80)	9.55 (4.18)	0.295	8.80 (3.30)	12.00 (4.35)	0.030
12	12.70 (5.68)	10.30 (3.60)	0.072	11.40 (4.60)	12.50 (8.60)	0.941
13	9.30 (5.85)	6.10 (5.80)	0.132	10.50 (2.93)	11.80 (8.10)	0.477
14	8.95 (5.90)*	8.00 (4.53)	1.000	11.10 (1.20)	10.45 (7.08)	0.628

* With only 3 patients in this group a range was provided as an IQR could not be calculated
# Only 1 sample available

pre-dose concentrations between the *POR* genotype groups was found (p = 0.763)

When analyzed per day, *POR*28* allele carriers in the *CYP3A5*-expressor groups had significantly lower tacrolimus pre-dose concentrations on day 6 (4.50 [IQR: 2.50] ng/ml versus 8.50 [IQR: 2.65] ng/ml, p=0.005) post-transplantation compared to *POR*1/*1* carriers, but not on any other day.

Within the *CYP3A5* non-expressors, *POR*28* allele carriers had significantly higher tacrolimus pre-dose concentrations on day 10 (13.90 [IQR: 3.90] ng/ml versus 10.00 [IQR: 4.25] ng/ml, p=0.025) and day 11 (12.00 [IQR: 4.35] ng/ml versus 8.80 [IQR: 3.30] ng/ml, p=0.030) post-transplantation compared to *POR*1/*1* carriers (Table 2).

Genotype and tacrolimus concentration/dose ratio

In the mixed model, *POR*28* allele carriers within the *CYP3A5*-expressers had on average 20.3% lower concentration/dose ratios compared to the *POR*1/*1* genotype carriers (p = 0.001).

Figure 2: Concentration Dose ratio during the first two weeks post-transplantation

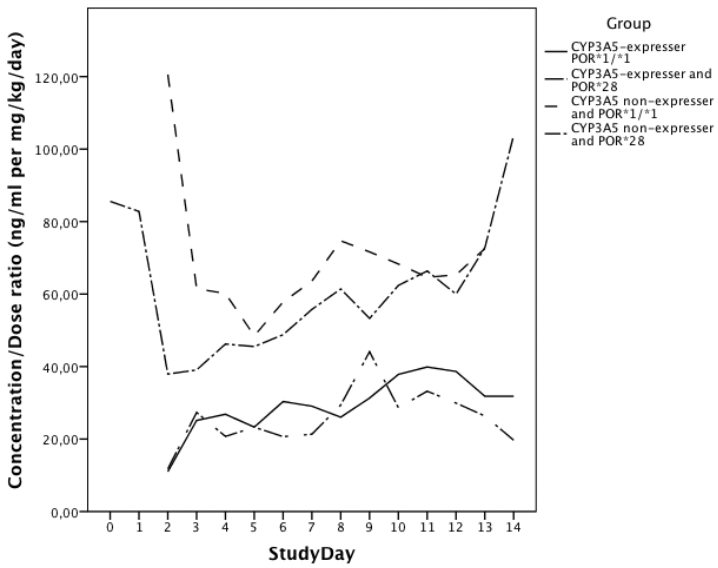

Table 3: Tacrolimus Concentration/Dose ratio (ng/ml per mg/kg/day) in the first 14 days post-transplantation

Day	CYP3A5 expressers		P-value	CYP3A5 non-expressers		P-value
	POR*1/*1 (n=9)	POR*28 (n=7)		POR*1/*1 (n=15)	POR*28 (n=12)	
0	-	-	-	-	-	-
1	-	-	-	-	82.80#	-
2	11.12 (5.84)	11.76#	0.857	68.08 (105.53)	27.50 (42.20)	0.257
3	19.0 (19.55)*	27.54 (20.90)	0.857	17.90 (91.39)	38.42 (21.58)	0.313
4	22.52 (26.43)	17.73 (18.83)	0.368	32.67 (42.63)	47.65 (24.04)	0.743
5	22.43 (18.10)	21.32 (12.33)	0.898	35.66 (24.57)	34.64 (32.28)	0.863
6	29.36 (11.37)	20.74 (10.38)	0.005	57.27 (24.48)	45.90 (27.17)	0.251
7	26.50 (11.90)	20.81 (15.88)	0.189	55.67 (31.22)	52.07 (29.93)	0.381
8	19.83 (16.58)	25.56 (20.19)	0.232	68.97 (33.95)	54.40 (31.91)	0.217
9	27.36 (13.12)	27.15 (35.09)	0.805	59.21 (47.77)	55.64 (39.52)	0.439
10	29.93 (24.76)	29.05 (22.54)	0.209	51.00 (38.75)	60.71 (65.72)	1.000
11	34.70 (32.86)	28.33 (33.77)	0.295	56.61 (41.19)	55.91 (64.12)	0.927
12	39.20 (25.24)	21.12 (35.05)	0.336	53.83 (38.75)	44.44 (65.22)	0.656
13	30.62 (20.03)	20.46 (25.05)	0.180	59.60 (48.09)	58.25 (89.75)	0.916
14	31.82 (3.63)*	19.69#	0.667	-	73.31 (100.89)*	-

* With only 3 patients in this group a range was provided as an IQR could not be calculated
# Only 1 sample available

In contrast, no significant difference between the *POR* genotypes within the *CYP3A5* non-expressers was observed (p = 0.055).
When analyzed per day, on day 6 *CYP3A5*-expressers carrying the *POR*28* allele had a significantly higher concentration/dose ratio compared to *CYP3A5*-expressers homozygous for *POR*1* (20.74 [IQR: 10.38] ng/ml per mg/kg/day per versus 29.36 [IQR: 11.37] ng/ml mg/kg/day, P=0.005). No significant differences were found in the *CYP3A5* non-expresser group between *POR*1/*1* carriers and *POR*28* allele carriers (Table 3).

Figure 3: Tacrolimus dosing requirements during the first two weeks post-transplantation

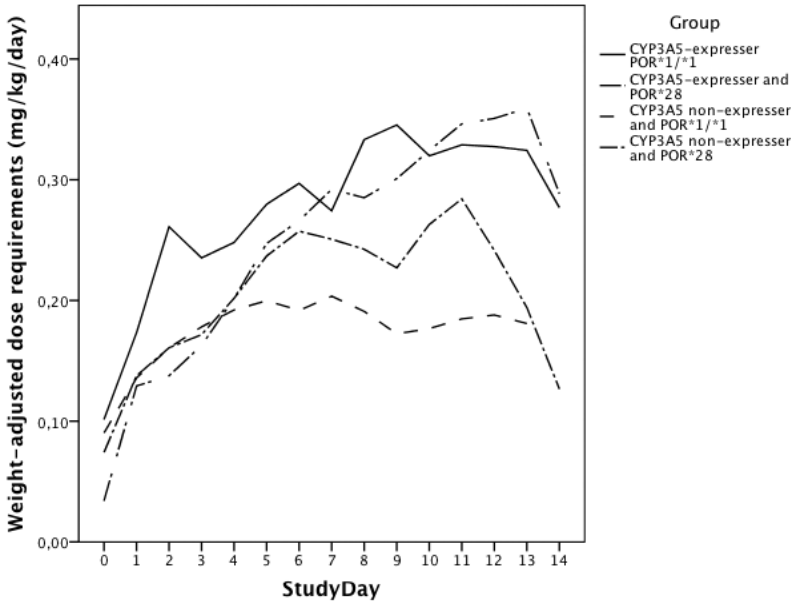

Table 4: Tacrolimus dosing requirements (mg/kg) in the first 14 days post-transplantation

Day	CYP3A5 expressers		P-value	CYP3A5 non-expressers		P-value
	POR*1/*1 (n=9)	POR*28 (n=7)		POR*1/*1 (n=15)	POR*28 (n=12)	
0	0.10 (0.02)	0.10#	0.229	0.09 (0.02)*	0.09 (0.05)	0.921
1	0.19 (0.10)	0.15 (0.12)	0.171	0.13 (0.09)	0.16 (0.11)	0.897
2	0.22 (0.05)	0.15 (0.08)	0.003	0.19 (0.08)	0.16 (0.07)	0.898
3	0.24 (0.08)	0.17 (0.07)	0.050	0.19 (0.07)	0.17 (0.08)	0.928
4	0.26 (0.08)	0.21 (0.19)	0.351	0.19 (0.07)	0.18 (0.07)	0.786
5	0.28 (0.11)	0.24 (0.19)	0.351	0.19 (0.06)	0.18 (0.09)	0.910
6	0.31 (0.10)	0.27 (0.20)	0.408	0.19 (0.07)	0.20 (0.08)	0.267
7	0.31 (0.17)	0.27 (0.21)	1.000	0.20 (0.08)	0.21 (0.10)	0.536
8	0.37 (0.17)	0.24 (0.23)	0.536	0.20 (0.11)	0.19 (0.11)	0.687
9	0.38 (0.15)	0.30 (0.21)	0.463	0.19 (0.12)	0.18 (0.15)	0.719
10	0.31 (0.15)	0.34 (0.27)	0.955	0.20 (0.13)	0.18 (0.14)	0.474
11	0.35 (0.15)	0.34 (0.28)	0.867	0.18 (0.13)	0.18 (0.14)	0.540
12	0.33 (0.14)	0.34 (0.30)	1.000	0.18 (0.11)	0.18 (0.14)	0.474
13	0.33 (0.13)	0.34 (0.30)	0.867	0.18 (0.12)	0.17 (0.14)	0.734
14	0.28 (0.15)*	0.28 (0.30)*	1.000	-	0.14 (0.11)*	-

* With only 3 patients in this group a range was provided as an IQR could not be calculated

Tacrolimus dosing requirements

In the mixed model no significant differences were found in the tacrolimus dosing requirements between the *POR* genotype groups within the *CYP3A5*-expressers (p = 0.16) or within the *CYP3A5* non-expressers (p = 0.25).

The daily tacrolimus dosing requirements (mg/kg) within the first two weeks after transplantation are shown in Table 4. In the *CYP3A5*-expressers group a significant difference was found only on day 2 (0.15 [IQR: 0.08] mg/kg versus 0.22 [IQR: 0.05] mg/kg, p=0.003) post-transplantation between *POR*28* allele carriers and *POR*1/*1* carriers (Table 4).

Outcome

None of our patients experienced delayed graft function and therefore a possible relationship with *POR*28* could not be tested. No significant difference was found in the last available serum creatinine (SCr) levels from the second week post-transplantation between *CYP3A5*-expressers (54.00 [IQR: 45.75] umol/L) and *CYP3A5* non-expressers (80.00 [IQR: 50.00] umol/L, p=0.33). Similarly, no significant difference in the last available SCr level from the second week post-transplantation was found within the *CYP3A5*-expresser group between at least one *POR*28* allele carriers (44.00 [IQR: 66.00] umol/L) and *POR*1/*1* carriers (59.00 [IQR: 21.50] umol/L, p=1.00) or within the *CYP3A5* non-expresser group between *POR*28* allele carriers (59.00 [IQR: 53.00] umol/L) and *POR*1* homozygotes (82.00 [IQR: 45.00] umol/L, p=0.42).

Discussion

To our knowledge, this is the first study in children exploring the possible association between *POR*28* and tacrolimus disposition. We were able to show that *CYP3A5*-expressers carrying at least one *POR*28* allele had 18.3% lower tacrolimus pre-dose concentrations and 20.2% lower concentration/dose ratios compared to *CYP3A5*-expressers carrying *POR*1/*1* in the first 14 days post-transplantation using mixed-model analysis. This is similar to what was found in adult de novo renal

transplant recipients showing lower tacrolimus pre-dose concentrations in the first few days post-transplantation for *CYP3A5*-expressers carrying the *POR*28* allele.[8]

We were not able to find a significant association between *POR* genotype and tacrolimus dosing requirements. This is different from the study by de Jonge et al. where *CYP3A5*-expressers carrying at least one *POR*28* allele had significantly higher dosing requirements than *CYP3A5*-expressers homozygous for *POR*1*.[8] Even though the tacrolimus pre-dose concentrations and concentration/dose ratios were significantly lower in our *CYP3A5*-expressers carrying at least one *POR*28* allele, the tacrolimus dosing requirements did not differ. A possible explanation could be that the tacrolimus pre-dose concentrations were still within the therapeutic window for most of the patients and therefore the tacrolimus doses were probably not adjusted.

The interplay of POR with CYP expression is not completely understood. The relationship with the *POR*28* genotype and CYP expression seems to be isoform-specific. The activity of specific POR mutants assayed by one P450 enzyme cannot be extrapolated to other P450 enzymes. This is due to the alterations in the conformation of the POR/P450 contact sites, caused by POR missense mutations, impairing the activity to different degrees, depending on the electron recipients used in the assay.[5,6] In vitro data show a decrease in CYP3A4 activity by using testosterone and midazolam as a substrate (61-77% lower wild-type activity), but no difference in activity when erythromycin and quinidine were used as a substrate (97% and 89% of wild-type activity).[18] The activity differed slightly between these CYP3A substrates used, suggesting that the ability of the *POR*28* sequence variant to affect the catalytic activity of CYP3A4/5 varied with the substrate used.[18,19]

In vivo, in 251 adults treated with either methadone or clozapine, the *POR*28* variant has been associated with increased CYP3A activity. The metabolic ratio of midazolam (1-OH-Midazolam/midazolam), as

surrogate marker of combined in vivo CYP3A4/5 activity, was 1.6-fold higher in *POR*28* homozygotes compared to *POR*1* allele carriers in both patient groups.[7] The reason why in vitro data show a decrease in CYP3A4/5 activity and in vivo data an increase in CYP3A4/5 is not completely known. A possible reason could be that patients in the study done by Oneda et al.[7] the patients also received other medications that could possibly induce CYP3A4/5 activity, resulting in difficulties comparing the data.
Despite the conflicting in vitro and in vivo data investigating the relationship of POR with individual CYP3A isoforms, our data and those from de Jonge suggest that this relationship is more prominent with CYP3A5 than with CYP3A4, as the in vivo relationship with POR and tacrolimus disposition was only found in the *CYP3A5* expressers.[8] This suggests a relationship between POR and CYP3A5, which may be different than for CYP3A4.

Although, age was not significantly different between our patient groups and we therefore did not correct for it in our analyses, it is a very important factor. The effects of *CYP3A5* genotype on tacrolimus disposition have been reported in both adults and children.[2,9,20] As genotype does not change with age, it is conceivable that findings in adults would also be replicated in children. Nonetheless, as the ontogeny of CYP3A enzymes is still a factor in children,[11,21,22] genotype might not match with the phenotype present at the age of the child being researched. Therefore, studies need to be done in children to elucidate the effect of age during childhood.

As acute nephrotoxicity is an important concentration-dependent adverse event of tacrolimus, genetic differences in its disposition and/or metabolite formation may explain inter-individual variation in renal function.[23] We were not able to find a significant association between POR genotype and the last available serum creatinine levels in *CYP3A5*-expressers and *CYP3A5* non-expressers in the first 14 days post-transplantation. We were not able to test the relationship with rejection as none of our patients experience rejection during the study period.

To our knowledge, this is the first study reporting on the possible association between *POR*28* genotype and the clinical outcome of the patient. However, our sample size is small and therefore these results need to be interpreted with caution. Nonetheless, the association between *CYP3A5* genotype and kidney function has been studied before. Yet, the results are conflicting showing an increased risk for both *CYP3A5*-expressers and *CYP3A5* non-expressers, demonstrating the need for studies with bigger sample sizes and kidney function as the primary objective.[24]
Our study has several limitations. First, a major limitation is that the sample size of our study is fairly small. This may explain why we only found a relationship between *POR* genotype and tacrolimus disposition using a mixed model, but not when data were analyzed by post-transplantation day. Nonetheless, we were able to replicate results similar to the adult study by de Jonge et al., even when correcting for *CYP3A5* genotype and age.[8] In addition, due to the retrospective design of the study, unexplained variation in dosing requirements and C/D may have been introduced by inaccuracies in the reporting of tacrolimus dosing, which are frequent in daily clinical care (e.g. mode of administration oral versus gastric tube, missed doses, repeated doses after vomiting).

Conclusion

In pediatric kidney transplant recipients, *POR*28* genotype explains part of the variability in tacrolimus disposition, in addition to age and *CYP3A5* genotype. These results merit further study in a larger population to validate our findings and to evaluate the clinical impact of this genotype.

Conflict of interest

The authors declare no contest of interest.

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Recipient genetic variation does not influence renal function in pediatric kidney transplantation patients receiving tacrolimus.

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Abstract

Background Post-transplant renal function decline is a major complication in pediatric renal transplant recipients. Potential genetic risk factors have recently been identified in adults. We determined the incidence as well as genetic risk factors of renal function decline after pediatric renal transplantation.

Methods In this multi-center retrospective cohort study, clinical data from the day of transplantation up to 10 years post-transplantation were analyzed. In multivariate analysis, the impact of genetic variation (77 SNPs) on renal function was studied using eGFR and age- and gender-normalized eGFR (z-scores).

Results Data of 199 renal transplant recipients were analyzed. Their median age was 13.0 (IQR: 7.0) years; the median follow-up was 0.72 years (range: 7 days–10 years). Chronic kidney disease (CKD, from 3 months post-transplantation onwards) stages 3-5 developed in 81 patients (40.7%): stage 3 in 55 (26.7 %); stage 4 in 17 (8.5%) and stage 5 in 9 (4.5%). None of the genetic polymorphisms studied was significantly associated with renal function.

Conclusion Renal function decline post-renal transplantation occurred in 40% of our pediatric renal transplant recipient cohort. Other than adult studies, this study failed to show a relationship of *CYP3A5*3* or other variant alleles tested with renal function.

Introduction

In kidney transplant recipients, more effective immunosuppressive therapy in the form of the calcineurin inhibitors tacrolimus and cyclosporine has greatly reduced the incidence of acute graft rejection.[1] Nonetheless, renal allograft long-term survival has barely changed in last few decades.[2] Renal function decline can ultimately necessitate re-transplantation and it is associated with a higher risk of premature death if persisting for at least three months post-transplantation.[3] Studies in adult renal transplant recipients have reported stage 2 chronic kidney disease (CKD) in 19%-46% of the patients for; stage 3 in 48%-61%; and stage 4 in 1%-19%.[4,5,6] Similar pediatric studies are scarce. One study in 23 pediatric kidney recipients reported CKD stage 2 in 35% and stage 3 in 43% of children at 3.4 ± 2.8 years post-transplantation.[7] Two other studies reported CKD stages 3-5 in 62% of 45, and stages 3-4 in 66% of 129 pediatric renal transplant recipients, respectively, at least one year post-transplantation. [8,9]

The reported causes for CKD following renal transplantation are largely similar between children and adults, and include ischemia-reperfusion injury, acute rejection, chronic allograft nephropathy and recurrent renal disease.[10,11] Additionally, urinary tract infections or obstruction damaging the graft, chronic CNI use, hypertension, diabetes mellitus and hepatitis C may increase the risk for CKD post-renal transplantation.[11,12,13] However, children more often than adults require renal transplantation for reason of congenital dysplasia, obstructive uropathy and focal segmental glomerulosclerosis.[11,14]

Apart from clinical risk factors, several SNPs have been studied in relation to renal function decline post-transplantation in adults.[15] Findings regarding *CYP3A5* genotype in relation to the risk of tacrolimus-induced nephrotoxicity were inconsistent.[16,17,18,19,20] Possible explanations are the use of different biopsy outcome measures to define tacrolimus-induced

nephrotoxicity and the relatively small patient cohorts in some studies.
Data from pediatric studies are also inconclusive so far. In 50 *de novo* pediatric renal transplant recipients on tacrolimus, a non-significant trend towards higher creatinin clearance was found in patients carrying at least one *CYP3A5*1* allele compared to those with the *CYP3A5*3/*3* genotype.[21] Pediatric kidney recipients with at least one *ACE D* allele showed a significantly steeper decline in GFR compared with homozygous carriers of the *ACE I* allele.[22] Others have confirmed this association,[11,23] but could not find associations of *AGT Met235 →Thr* and *AT1R A1166→C* polymorphisms with renal function decline in pediatric kidney recipients.[11]
In 207 pediatric kidney recipients, the presence of the *CCR5 wt/*Δ32 genotype was associated with significantly better graft function at 1 year post-transplant (GFR 115.10 ± 28.40 versus 86.43 ± 29.96 ml/min/1.73m^2; p=0.022).[24]
Most of these studies, however, concerned patients receiving cyclosporine or a mixed population of tacrolimus and cyclosporine treated patients, which leaves unresolved the true contribution of tacrolimus to renal failure following pediatric kidney transplantation.

Similarly, CKD prevalence following pediatric kidney transplantation has mainly been established in mixed populations. The calcineurin-inhibitors cyclosporine and tacrolimus are both associated with renal dysfunction, however, the responsible molecular mechanisms seem to differ between the two drugs, suggesting different mechanisms of toxicity.[25,26,27,28,29,30]
We therefore performed a study to determine the prevalence of CKD following kidney transplantation in children treated with tacrolimus. In addition, we studied possible associations between genetic variation of the recipient and renal function.

Methods

This is a retrospective cohort study of pediatric renal transplant recipients transplanted between January 1994 (date of introduction of tacrolimus in pediatric protocols) and 2011 at the Hospital for Sick Children, Toronto, Ontario or at Erasmus MC Sophia Children's Hospital, Rotterdam, the Netherlands. Patients were eligible for this study if 1) the age at time of transplantation was younger than 18 years; 2) they had received oral tacrolimus after their transplantation. Informed consent was obtained from parents and/or children during enrolment at both institutions. The study was approved by the Institutional Research Ethics Board of the Hospital for Sick Children. In Rotterdam, a waiver for REB approval was obtained, as only retrospective data and left over blood or saliva were used.

Data collection

Clinical data were obtained through electronic patient records and paper chart review. Data were collected from the day of transplantation, at 1 week, 1, 3, 6, 9 and 12 months and yearly thereafter until December 2009 or until data was available.

Endpoints

The primary end-point of this study was the cumulative incidence of chronic kidney disease following pediatric kidney transplantation by using the estimated glomerular filtration rate (eGFR), calculated according to the Schwartz formula.[31] Patients were next categorized for the severity of CKD according to the National Kidney Foundation Kidney Disease Outcome Quality Initiative (KDOQI) guidelines for chronic kidney disease.[32] Our secondary endpoints were absolute eGFRs and z-scores of eGFR to correct for age-related changes in eGFR. To calculate the z-scores, reference values for the mean and standard deviation from a healthy, mainly Caucasian, pediatric population were used.[33]

Other clinical data

Data were collected regarding patients' characteristics, tacrolimus concentrations, hematology and chemistry lab, concomitant medications, primary diagnosis, transplant information and the patient's outcome. Concomitant medication information was collected for: cyclosporine, gentamicin, cotrimoxazole, tobramycin, amphotericin B, valganciclovir, ganciclovir, spironolactone and vancomycin for their known influence on kidney function. If a patient received more than one renal transplant, the first transplantation was used in the analysis.

Immunosuppressive protocol

Tacrolimus was started according to the immunosuppression protocol from the Hospital for Sick Children or the Erasmus MC Sophia Children's Hospital. In both hospitals tacrolimus was started on day 1 post-transplantation. Tacrolimus starting dose was 0.2-0.3 mg/kg/day divided in two doses. Therapeutic drug monitoring was used to adjust the tacrolimus dose to achieve a target level of 10-15 ng/ml in the first weeks post-transplantation, 6-10 ng/ml from 6 weeks post-transplantation and 4-8 ng/ml after three months post-transplantation. Additional immunosuppressive therapy consisted of a maintenance dose of mycophenolate mofetil (600-1000mg twice daily) and a tapering steroids schedule.

Tacrolimus trough concentrations

Tacrolimus blood trough concentrations were determined in ethylenediaminetetraacetic acid (EDTA)-treated whole blood (0.25 ml) on the day of sampling, using liquid chromatography tandem mass spectrometry (LC-MS-MS) as previously described, as part of routine clinical care at the Hospital for Sick Children.[34,35] In Rotterdam, tacrolimus whole blood trough concentrations were determined ethylenediaminetetraacetic acid (EDTA)-treated whole blood (0.50 ml) on the day of sampling, using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) as previously described, as part of routine care.[36,37,38,39]

Genotyping

Blood or saliva samples were collected from patients at the first visit after informed consent was received or, in Toronto only, by access to DNA samples from either the Hospital for Sick Children's Biobank or the University Health Network HLA laboratory. Blood samples were collected in EDTA-containing tubes. Saliva samples were collected by using Oragene DNA OG-250 collection kits (DNA genotek, Ottawa, Ontario, Canada). Samples from both hospitals were stored at -80°C before purification of DNA using the QiaSymphony system (Qiagen, USA). Ninety-six SNPs were selected based on previous published associations, SNPs related to tacrolimus pharmacokinetic pathways and SNPs associated with renal failure (Supplemental table 1). DNA samples were genotyped for this custom set of 96 variants selected using BeadXpress genotyping platform using the manufacturer's protocol (Illumina, CA, USA) at the University of British Colombia, Vancouver, BC, Canada.[40]

Statistical analysis

Descriptive data are presented as mean ± standard deviation or median (IQR) for continuous variables and as percentage for categorical variables. CKD stage was designated as an eGFR below the cut-off values according to KDOQI CKD stages on two consecutive visits from 3 months post-transplantation onwards. Kaplan-Meier curves were plotted for the time course of CKD incidence. Follow-up visits were cyclosporine was used or tacrolimus was discontinued, temporarily or permanently, were not included in the analysis.

The Hardy-Weinberg equilibrium was tested for each polymorphism using the method by Guo et al.[41] Previously published and found to be significant genetic associations with eGFR were tested at a significance level of 0.05. All other genetic associations were tested at a significance threshold of 8.7×10^{-4} determined using a Bonferroni correction based on the effective number of independent tests (M_{eff}).[42] A univariate general linear mixed model was performed for the following covariates:

gender, age at time of transplantation, investigation site, tacrolimus levels, concomitant medication, year of transplantation, deceased donor, albumin and CRP levels, hematocrit, conversion from cyclosporin and the patient's weight and height. Covariates with a p-value < 0.05 were considered for retention in the regression model. A mixed model for the continuous endpoints was used for the genetic additive effect of each SNP by adjusting for the covariates retained in the stepwise selection. All statistical analysis for the genetic variables were done using SAS 9.3. All other statistical analyses, including figures were done using SPSS version 20.0 (IBM SPSS Statistics, Armonk, NY, USA).

Results

Patient population

Informed consent was received for 199 of the 205 eligible children and their clinical data were analyzed. There was a male predominance (58.8%) and median age at time of transplantation was 13.0 years (IQR: 7.0) (Table 1). The median follow-up was 0.72 years (range: 7 days – 10 years). The most prevalent pre-transplant diagnosis was renal dysplasia, (n=45; 22.6%) (Table 1). Ten patients (5.0%) underwent a secondary transplantation and one patient (0.5%) a third. Seven patients (3.5%) received dialysis post-transplantation, one of whom after both the first and second transplantation. Graft failure at any moment during the entire study period had been documented for 27 patients (13.6%); acute rejection post-transplantation for 63 (31.7%). Converted from cyclosporin to tacrolimus had been documented for 48 (24.0%) children. One patient (0.5%) had passed away (Table 1). DNA was available for 102 children (51.3%).

Renal failure following renal transplantation

Figure 1A and 1B depict the eGFR values and eGFR z-scores during the study period. The median eGFR at one year post-transplantation was 96.3 (IQR: 42.0) ml/min/1.73m^2 and the median eGFR z-score -1.4 (IQR: 2.0) (Table 1). The prevalence of each KDOQI CKD stage at various time points is presented in Figure 2. The cumulative incidence of CKD stage 3-5 was 40% (81/199) (Figure 3A). The majority of these 81 patients developed CKD stage 3 (55/199; 27.6%). Seventeen patients (17/199; 8.5%) developed CKD stage 4 and 9 patients (9/199; 4.5%) developed CKD stage 5 (Figure 3B). At one year post-transplantation, 7 renal transplant recipients (7/104; 6.7%) had moderate renal failure (Stage 3) and 2 patients (2/104; 1.9%) CKD stage 4. At 5 years post-transplantation, 2 patients (2/31; 6.5%) experienced CKD stage 3, 1 patient (1/31; 3.2%) CKD stage 4 and 2 patients CKD stage 5 (2/31; 6.5%) (Figure 2).

Figure 1A and 1B: eGFR over time and eGFR z-scores over time

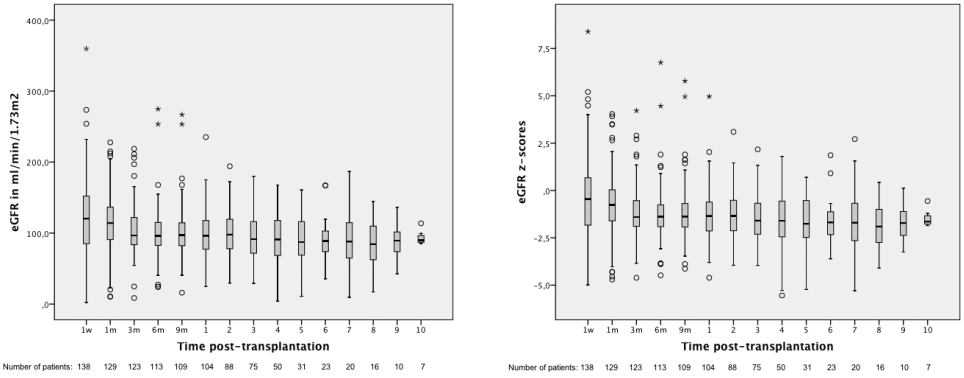


Table 1: Demographics of the kidney population

Variable	Kidney (all)	Kidney (DNA)
Number	199	102
Age at time of transplant (years) Mean ± SD Median (IQR)	 11.9 ± 5.2 13.0 (7.0)	 11 ± 5.7 11.0 (8.0)
Year of transplant Mean ± SD Median (IQR) Range	 2004.9 ± 3.3 2005 (6) 1994-2011	 2005.8 ± 3.5 2007 (4) 1994-2011
Follow-up time (years) Mean ± SD Median (IQR)	 1.54 ± 2.11 0.72 (1.93)	 1.86 ± 2.38 0.75 (2.75)
Gender (M/F)	M: 117 (58.8%) F: 82 (41.2%)	M: 59 (57.8%) F: 43 (42.2%)
Weight (kg) Mean ± SD Median (IQR)	 35.7 ± 17.3 34.3 (28)	 31.9 ± 18.6 25.5 (26.9)
Height (cm) Mean ± SD Median (IQR)	 142.8 ± 27.2 151 (35)	 138.5 ± 26.9 145.7 (42.8)
Transplant (n,%) 1st 2nd 3rd	 188 (94.5%) 10 (5%) 1 (0.5%)	 95 (93.1%) 6 (5.9%) 1 (1%)
Need for dialysis before transplant (n,%)	44 (22.1%)	32 (31.4%)
Acute rejection (n,%)	63 (31.7%)	26 (25.5%)
eGFR at 1 year post-transplantation Mean ± SD Median (IQR)	 99.62 ± 32.25 96.30 (42.0)	 104.31 ± 32.77 96.33 (36.27)
eGFR z-score at 1 year post-transplantation Mean ± SD Median (IQR)	 -1.25 ± 1.33 -1.35 (2.0)	 -1.05 ± 1.38 -1.36 (1.54)
Converted from CsA (n,%)	48 (24.1%)	34 (33.3%)
Mortality rate (n,%)	1 (0.5%)	1 (1.0%)
Donor age Mean ± SD Median (IQR)	 32.4 ± 10.3 37.0 (10.0)	 30.5 ± 12.8 31.5 (18.5)
Donor gender (M/F)	M:35, F:42	M:14, F:18
Deceased or living-related donor (n,%)	Deceased: 44 (22.1%), Living: 130 (65.3%) Unknown: 25 (126%)	Deceased: 27 (26.5%), Living: 48 (47.1%) Unknown:27 (26.5%)
Cold ischemia time (min) Mean ± SD Median (IQR)	 664.4 ± 554.9 685.5 (862.5)	 675.3 ± 497.3 722.5 (765)
Warm ischemia time (min) Mean ± SD Median (IQR)	 56.6 ± 121.9 32 (12.5)	 70.3 ± 151.8 33.5 (12)
Investigation site (n,%) Rotterdam Toronto	 49 (24.6%) 150 (75.4%)	 41 (40.2%) 61 (59.8%)
Primary diagnosis Dysplasia Cystic kidneys Glomerular nefritis/FSGS Hemolytic-Uremic Syndrome (HUS) Chronic renal failure Obstructive and reflux uropathy Other	 45 (22.6%) 14 (7%) 3(6.5%) 6 (3%) 17 (8.5%) 36 (18.1%) 68 (34.2%)	 26 (25.5%) 9 (8.8%) 4(3.9%) 2 (2%) 6 (5.9%) 13 (12.7%) 42 (41.2%)

Risk factors of renal failure

Four SNPs in 4 genes were not in Hardy-Weinberg equilibrium; *IL10 -1082G>A* (rs1800896), *TLR1 239G>C* (rs5743611), *SLCO1B1 521T>C* (rs4149056) and *APOL1 G1* (rs73885319) (Supplementary Table 2). More detailed information can be found in the supplementary material.

In the univariate analysis the year of transplantation, albumin levels, conversion from cyclosporine and body weight were associated with renal function (Table 2). In addition to these covariates, sex, age at time of transplantation, the investigation site and the tacrolimus levels were also added as covariates for their potential confounding relationship with renal function.

One SNP was significantly associated at the significance level of $p<0.05$ with eGFR in the genetic model; *VKORC1 1173C>T*. Homozygote carriers *VKORC1 1173TT* had lower eGFR values compared to heterozygotes and wild type carriers (Table 3). Nonetheless, none of the polymorphism reached the set significance level of 8.7×10^{-4} (Supplementary Table 3).

Figure 2: Prevalence of CKD at each time point

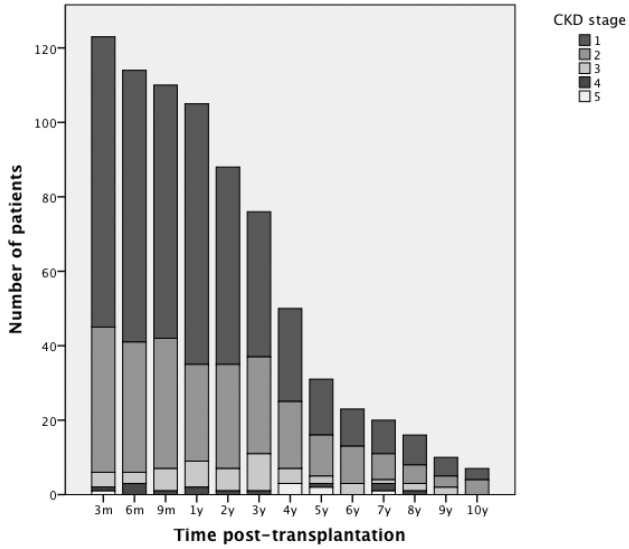
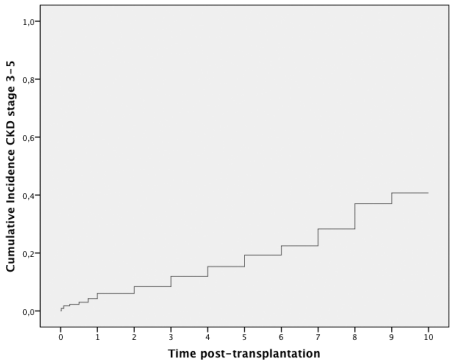
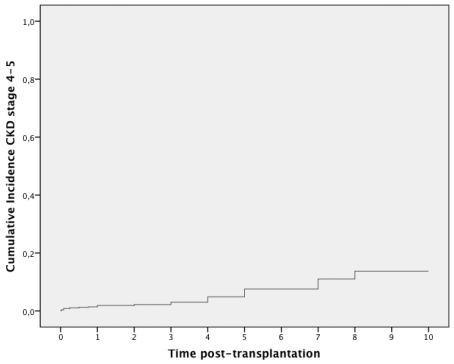
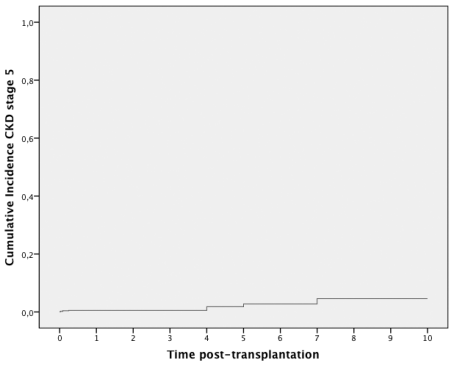


Figure 3: Kaplan-Meier curves of the cumulative incidence of CKD in 199 pediatric kidney transplant recipients







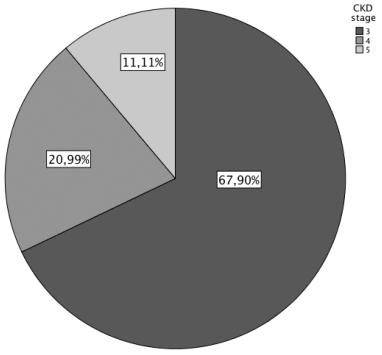


Table 2: Univariate analysis of covariates

Variable	N	P-value
Gender	459	0.56
Age at time of transplantaiton	459	0.11
Investigation site	459	0.45
Tacrolimus levels	459	0.98
Concomitant medication	459	0.11
Year of transplantation	459	0.01
Deceased donor	190	0.89
Albumin levels	369	0.03
Hematocrite levels	356	0.46
CRP levels	22	0.19
Converted to CsA	459	0.04
Weight	89	0.03
Height	406	0.11

Table 3: Genetic associations renal transplant recipients

Chr	Position	Gene	SNP	Genotype counts			N obs	N obs Estimate	SE	P-value of additive genetic effect*
				Minor homozygous	Heterozygous	Major Homozygous				
7	81700000	ABCB1 -1236	rs1128503	24	51	27	369	4.58	4.49	0.31
7	81701000	ABCB1 -2677	rs2032582	24	48	30	369	1.51	4.45	0.74
7	81702000	ABCB1 -3435	rs1045642	18	56	28	369	-0.78	4.65	0.87
7	99270539	CYP3A5	rs776746	3	30	69	369	3.76	5.83	0.52
16	31104878	VKORC1*	rs9934438	16	50	36	369	10.26	4.61	0.03

Positions based on Genome Build Version 37.1.
* Significance threshold = 0.05

Discussion

Post-transplant renal function decline is a major complication in pediatric renal transplant recipients. In this study, the cumulative incidence of CKD stages 3-5 was 40.7%, with 17 patients (8.5%) experiencing CKD stage 4 and 9 patients (4.5%) CKD stage 5. These incidences are lower than those reported in other pediatric studies using eGFR; i.e. 43-66%.[7,9] Previous studies used the last available follow-up, yet due to the small sample sizes the follow-up range was very wide. Therefore the time of onset of CKD post-transplantation is uncertain. The mixed population of cyclosporin and tacrolimus-treated pediatric kidney transplant recipients in one of the studies may also explain the higher prevalence.[7]

To our knowledge, we are the first to report kidney function and prevalence of CKD over time in addition to a cross-sectional design at one or five years post-transplantation. Kidney function, based on eGFR, slightly decreased in the first year post-transplantation and then stabilized. Four of 123 patients (3.3%) experienced CKD stage 3 at three months post-transplantation; 11 of 76 (14.5%) at 3 years post-transplantation (Figure 2). Prevalences of CKD stages 4 and 5 increased from three years post-transplantation and onwards an increase in the can be seen. This suggests that although the eGFR of the overall cohort seems stable, several patients experienced renal problems early on and then developed more serious renal failure.

The true prevalence of CKD may be higher as the use of eGFR underestimates the GFR measured by inulin clearance.[6] This is partially due to the serum creatinine assays used or to reduced muscle mass in some children.[43] One of the previous studies used mGFR, determined by ^{99}Tc DTPA, and found that 62% of 51 patients experienced CKD stages 3-5 at least one year post-transplantation. However, the sample size was small and patients also received cyclosporin.[8] Unfortunately, mGFRs were not routinely done during our study period and could therefore not be used

in our analysis. Measuring serum cystatin C concentrations is a promising new method to establish CKD. It better reflects developmental changes in children[44] and formulas incorporating cystatin C predict GFR better than does the Schwartz formula.[45] Measuring serum cystatin C levels is therefore recommended in the new Kidney Disease Improving Global Outcome (KDIGO) guidelines.[46]

We also looked at genetic variation, but could not identify any SNP predictive of a decline in renal function. One SNP, *VKORC1 1173C>T*, was significant at the 5% level but failed to reach the set significance threshold. In contrast to previous studies in adults and/or children, we did not find significant associations of *CYP3A4, CYP3A5, ABCB1, CCR5* genotypes with renal function.[15] Nevertheless, more recent studies, too, failed to show an association of *CYP3A5* genotype with graft outcome[15,17,20,24,47,48]
One study in 61 pediatric renal transplant recipients failed to show associations of *CYP3A5* and *ABCB1* genotypes with renal function decline as well as 8 other polymorphisms involved in inflammatory pathways.[24]
One explanation for these discrepant results on the effect of genetic variation in renal function post kidney transplant may be the use of biopsy data versus renal function as markers of tacrolimus-induced renal function decline.

Several limitations of this study should be addressed. In spite of the large sample size the number of SNPs limits the power of the genetic model. Furthermore, the candidate gene approach limited the chances of identifying genetic risk factors. In Genome Wide Association Studies hits may become apparent in genes we did not consider in our study. Moreover, we were unable to include donor DNA in the analysis. Inclusion of donor DNA might have had additive value as the donor kidney is an important contributor to renal function. A recent study showed that donor genotype determined tacrolimus metabolite disposition in the kidney, which may be of importance as tacrolimus

metabolites have been suggested to be involved in the pathogenesis of tacrolimus-related renal dysfunction.[49]

The use of kidney biopsies could have aided in differentiating between several causes of renal failure post-transplantation. Yet, mGFRs and kidney biopsies were not routinely done during the study period. Additionally, the retrospective nature of our study results in a dependency on the information available in the medical charts and the impossibility to complete the dataset

Conclusion

The fact that renal failure occurred in many pediatric renal transplant recipients points at the necessity of close monitoring. In contrast to previous reports, we did not find a significant association between the recipient's genetic variation and renal function. Further research could focus on the early detection of patients at risk for renal failure, e.g. with newer biomarkers such as NGAL or cystatin C. Moreover, closer monitoring of renal function with routinely measured GFRs or routine kidney biopsies may help optimize treatment.

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Supplementary Table 1: All SNPs analyzed

Chr	Position	Gene	SNP
1	60392494	C1orf87/CYP2J2	rs890293
1	162030688	OLFML2B/NOS1AP	rs10918594
1	184911126	PTGS2	rs4648279
1	186643541	PTGS2	rs3218625
1	186643836	PTGS2	rs5272
1	186643837	PTGS2	rs5273
1	186650751	PTGS2/PLA2G4A	rs689466
1	186646004	PTGS2	rs3218622
1	206946407	IL19/IL10	rs1800872
1	206946634	IL19/IL10	rs1800871
1	206946897	IL19/IL10	rs1800896
1	223285200	TLR5	rs5744168
2	228052600	COL4A3	rs2204862
2	234669144	UGT1A1	rs4148323
2	241542703	CAPN10	rs5030952
3	52261031	TWF2/TLR9	rs187084
3	119500035	NR1I2	rs3814055
3	119533733	NR1I2	rs6785049
3	119534153	NR1I2	rs2276707
3	119537254	NR1I2	rs3814057
3	121838319	CD86	rs1129055
4	38799710	TLR1	rs4833095
4	38800214	TLR1	rs5743611
4	38830350	TLR6	rs5743810
4	89052323	ABCG2	rs2231142
4	123377980	IL21/IL2	rs2069762
4	154626317	TLR2	rs5743708
4	187004074	TLR3	rs3775291
6	31241109	HLA-C	rs13191343
6	31543031	TNF/LTA	rs1800629
6	32809848	PSMB8	rs9357155
6	32811629	PSMB8	rs2071543
6	43736389	MRPS18A/VEGFA	rs699947
6	43737830	VEGF	rs1570360
6	90032942	UBE2J1/GABRR2	rs2064831
7	22766645	IL6/LOC541472	rs1800795
7	75615006	POR	rs1057868
7	81700000	ABCB1	rs1128503
7	81701000	ABCB1	rs2032582
7	81702000	ABCB1	rs1045642
7	87133470	ABCB1	rs17064
7	87230193	ABCB1	rs3213619

Chr	Position	Gene	SNP
7	99102509	CYP3A5	rs55965422
7	99194259	CYP3A4	rs17161886
7	99245013	ZNF498/CYP3A5	rs4646458
7	99245080	ZNF498/CYP3A5	rs4646457
7	99245914	CYP3A5	rs15524
7	99247772	CYP3A5	rs41279854
7	99250393	CYP3A5	rs41303343
7	99258139	CYP3A5	rs28383479
7	99262835	CYP3A5	rs10264272
7	99270539	CYP3A5	rs776746
7	99273821	CYP3A5	rs55817950
7	99325882	CYP3A7	rs2687136
7	99354114	CYP3A4/CYP3A7	rs12333983
7	99356324	CYP3A4	rs17161886
7	99358524	CYP3A4	rs4986910
7	99360870	CYP3A4	rs4646440
7	99365451	CYP3A4	rs2687117
7	99365983	CYP3A4	rs55785340
7	99366316	CYP3A4	rs35599367
7	99382096	CYP3A4/CYP3A43	rs2740574
7	99388017	CYP3A4/CYP3A43	rs2687102
8	139746208	COL22A1	rs4588898
9	120475302	TLR4	rs4986790
9	120475602	TLR4	rs4986791
9	125133507	PTGS1	rs3842787
9	125140241	PTGS1	rs3842789
9	125143707	PTGS1	rs3842792
9	125143973	PTGS1	rs5789
9	125148791	PTGS1	rs5791
9	125152507	PTGS1	rs5792
9	125152579	PTGS1	rs5793
10	90749963	FAS/ACTA2	rs1800682
10	96798749	CYP2C8	rs10509681
10	96818119	CYP2C8	rs1058930
10	101542578	ABCC2	rs717620
10	101611294	ABCC2	rs8187710
11	2857194	KCNQ1	rs2237895
11	2870108	KCNQ1	rs8234
11	17460712	ABCC8	rs2237982
11	112034988	TEX12/IL18	rs187238
11	112035458	IL18/TEX12	rs1946518
12	21329738	SLCO1B1	rs2306283

Chr	Position	Gene	SNP
12	21331549	SLCO1B1	rs4149056
12	68552522	IFNG	rs2430561
12	79481371	SYT1	rs12300068
16	31104878	VKORC1	rs9934438
17	32579788	ACCN1/CCL2	rs1024611
19	41858921	TGF-beta	rs1800470
21	28240574	ADAMTS5/AD-AMTS1	rs229109
22	35775889	HO-1	rs3761439
22	35776672	HMOX1/TOM1	rs2071746
22	36661906	APOL1	rs73885319
22	36662046	APOL1	rs71785313
22	36751101	MYH9	rs11089788
22	36774812	MYH9	rs5756168

A total of 19 SNPs had to be removed from the analysis; 16 SNPs had a allele frequency of 0, two SNPs had more than 5% missing genotypes and one SNP completely failed. A total of 77 SNPs were included in the analysis.

Supplementary Table 2: Hardy-Weinberg equilibrium

Chr	Position	Gene	SNP	Minor allele	Major allele	MAF frequency	H-W p-value	H-W Exact p-value
1	60392494	C1orf87/CYP2J2	rs890293	C	A	0.082802548	0.422814461	1
1	162030688	OLFML2B/NOS1AP	rs10918594	G	C	0.385350318	0.9276378	1
1	186643541	PTGS2	rs3218625	G	A	0.001592357		
1	186650751	PTGS2/PLA2G4A	rs689466	A	G	0.178343949	0.812656135	1
1	206946407	IL19/IL10	rs1800872	C	A	0.305111821	0.565836515	0.6167
1	206946634	IL19/IL10	rs1800871	G	A	0.283333333	0.714570206	0.7858
1	206946897	IL19/IL10	rs1800896	A	G	0.396496815	0.042740917	0.048
1	223285200	TLR5	rs5744168	G	A	0.046178344	0.108840512	0.2074
2	228052600	COL4A3	rs2204862	A	G	0.097133758	0.847978072	1
2	234669144	UGT1A1	rs4148323	G	A	0.009708738	0.960122881	1
2	241542703	CAPN10	rs5030952	G	A	0.012738854	0.920348217	1
3	52261031	TWF2/TLR9	rs187084	A	G	0.370607029	0.088927432	0.1149
3	119500035	NR1I2	rs3814055	G	A	0.404458599	0.125716199	0.1506
3	119533733	NR1I2	rs6785049	A	G	0.439490446	0.22287996	0.2282
3	119534153	NR1I2	rs2276707	G	A	0.208598726	0.743580897	0.7811
3	119537254	NR1I2	rs3814057	A	C	0.224522293	0.846002646	0.7832
3	121838319	CD86	rs1129055	G	A	0.265923567	0.565836515	0.6064
4	38799710	TLR1	rs4833095	A	G	0.356687898	0.269403832	0.2933
4	38800214	TLR1	rs5743611	G	C	0.071656051	0.006629165	0.0271
4	38830350	TLR6	rs5743810	G	A	0.294585987	0.393132894	0.4721
4	89052323	ABCG2	rs2231142	C	A	0.106687898	0.491830156	1
4	123377980	IL21/IL2	rs2069762	A	C	0.341853035	0.871239769	1
4	154626317	TLR2	rs5743708	G	A	0.02388535	0.759569923	1
4	187004074	TLR3	rs3775291	G	A	0.277070064	0.768342843	0.8019
6	31543031	TNF/LTA	rs1800629	G	A	0.157643312	0.7136281	0.7081
6	32809848	PSMB8	rs9357155	G	A	0.117834395	0.486261877	0.6087
6	32811629	PSMB8	rs2071543	C	A	0.133757962	0.666422804	0.6497
6	43736389	MRPS18A/VEGFA	rs699947	C	A	0.472929936	0.843021976	0.8446
6	90032942	UBE2J1/GABRR2	rs2064831	A	G	0.173566879	0.552452905	0.7367
7	22766645	IL6/LOC541472	rs1800795	C	G	0.305732484	0.664357678	0.6212
7	81700000	ABCB1	ABCB1-1236CT-r1128503	G	A	0.461783439	0.993023259	1
7	81701000	ABCB1	ABCB1-2677GT-r2032582	C	A	0.453674121	0.574740542	0.5576
7	81702000	ABCB1	ABCB1-3435CT-r1045642	A	G	0.498407643	0.27230394	0.3177
7	87133470	ABCB1	rs17064	A	T	0.068471338	0.892577745	1
7	87230193	ABCB1	rs3213619	A	G	0.039808917	0.247176386	0.2933
7	99245013	ZNF498/CYP3A5	rs4646458	A	C	0.100638978	0.129791211	0.1362
7	99245080	ZNF498/CYP3A5	rs4646457	A	C	0.159235669	0.812656135	1

Chr	Position	Gene	SNP	Minor allele	Major allele	MAF frequency	H-W p-value	H-W Exact p-value
7	99245914	CYP3A5	rs15524	A	G	0.151273885	0.90429921	1
7	99262835	CYP3A5	rs10264272	G	A	0.00477707	0.960320611	1
7	99270539	CYP3A5	rs776746	G	A	0.153354633	0.90429921	1
7	99325882	CYP3A7	rs2687136	A	G	0.165605096	0.780523249	0.7677
7	99354114	CYP3A4/CYP3A7	rs12333983	A	T	0.178913738	0.24080289	0.2574
7	99356324	CYP3A4	rs17161886	A	C	0.02388535	0.960320611	1
7	99358524	CYP3A4	rs4986910	A	G	0.00477707	0.960320611	1
7	99360870	CYP3A4	rs4646440	G	A	0.052547771	0.06007715	0.1065
7	99365451	CYP3A4	rs2687117	G	A	0.01433121	0.880181702	1
7	99366316	CYP3A4	rs35599367	G	A	0.042993631	0.759569923	1
7	99382096	CYP3A4/CYP3A43	rs2740574	A	G	0.060509554	0.6026503	1
7	99388017	CYP3A4/CYP3A43	rs2687102	A	G	0.012738854	0.920348217	1
8	139746208	COL22A1	rs4588898	G	A	0.294585987	0.876051221	0.8085
9	120475302	TLR4	rs4986790	A	G	0.076433121	0.609808154	0.4734
9	120475602	TLR4	rs4986791	G	A	0.070063694	0.609808154	0.474
9	125133507	PTGS1	rs3842787	G	A	0.046178344	0.527897302	1
9	125143707	PTGS1	rs3842792	A	C	0.001592357		
9	125143973	PTGS1	rs5789	C	A	0.02388535	0.759569923	1
10	90749963	FAS/ACTA2	rs1800682	A	G	0.48566879	0.846002646	0.8371
10	96798749	CYP2C8	rs10509681	A	G	0.087579618	0.422814461	1
10	96818119	CYP2C8	rs1058930	C	G	0.049363057	0.6026503	1
10	101542578	ABCC2	rs717620	G	A	0.204472843	0.662753305	1
10	101611294	ABCC2	rs8187710	G	A	0.071656051	0.25358574	0.2424
11	2857194	KCNQ1	rs2237895	A	C	0.420382166	0.206260662	0.224
11	2870108	KCNQ1	rs8234	A	G	0.357371795	0.093965944	0.1012
11	17460712	ABCC8	rs2237982	G	A	0.406050955	0.385473444	0.4118
11	112034988	TEX12/IL18	rs187238	C	G	0.246815287	0.732953406	1
11	112035458	IL18/TEX12	rs1946518	C	A	0.396496815	0.121764525	0.1519
12	21329738	SLCO1B1	rs2306283	A	G	0.447452229	0.388524151	0.4185
12	21331549	SLCO1B1	rs4149056	A	G	0.136942675	0.027409285	0.0379
12	68552522	IFNG	rs2430561	A	T	0.440514469	0.714876182	0.6991
12	79481371	SYT1	rs12300068	G	A	0.135350318	0.299621014	0.6081
16	31104878	VKORC1	rs9934438	G	A	0.415335463	0.843145878	1
17	32579788	ACCN1/CCL2	rs1024611	A	G	0.340764331	0.883629595	1
21	28240574	ADAMTS5/ADAMTS1	rs229109	G	A	0.304140127	0.768768069	0.817
22	35776672	HMOX1/TOM1	rs2071746	T	A	0.444089457	0.214387683	0.3229
22	36661906	APOL1	rs73885319	A	G	0.01433121	2.34488E-11	0.0157
22	36662046	APOL1	rs71785313	A	T	0.00477707		
22	36751101	MYH9	rs11089788	C	A	0.445859873	0.079535725	0.0783
22	36774812	MYH9	rs5756168	A	G	0.114649682	0.624316933	1

Table 3: Genetic associations renal transplant recipients

Chr	Position	Gene	SNP	Genotype counts			N obs	Estimate	SE	P-value of additive genetic effect*
				Minor homozygous	Heterozygous	Major Homozygous				
1	60392494	C1orf87/CYP2J2	rs890293		15	87	369	1.06	9.10	0.91
1	162030688	OLFML2B/NOS1AP	rs10918594	14	47	41	369	-3.20	4.61	0.49
1	186643541	PTGS2	rs3218625			102	369	0.00		
1	186650751	PTGS2/PLA2G4A	rs689466	3	31	68	369	3.01	6.74	0.66
1	206946407	IL19/IL10	rs1800872	8	37	57	369	-2.21	5.11	0.67
1	206946634	IL19/IL10	rs1800871	7	36	56	357	-1.90	5.38	0.72
1	206946897	IL19/IL10	rs1800896	24	40	38	369	2.68	4.22	0.53
1	223285200	TLR5	rs5744168	1	8	93	369	0.39	9.27	0.97
2	228052600	COL4A3	rs2204862	1	20	81	369	8.85	7.28	0.23
2	234669144	UGT1A1	rs4148323		1	100	365	-6.56	28.40	0.82
2	241542703	CAPN10	rs5030952		2	100	369	-11.57	22.06	0.60
3	52261031	TWF2/TLR9	rs187084	16	38	47	367	4.02	4.52	0.38
3	119500035	NR1I2	rs3814055	12	56	34	369	-8.06	5.32	0.13
3	119533733	NR1I2	rs6785049	22	44	36	369	-2.19	4.38	0.62
3	119534153	NR1I2	rs2276707	6	35	61	369	-7.85	5.11	0.13
3	119537254	NR1I2	rs3814057	6	36	60	369	-8.06	5.09	0.12
3	121838319	CD86	rs1129055	8	37	57	369	-6.71	4.97	0.18
4	38799710	TLR1	rs4833095	16	42	44	369	2.95	4.48	0.51
4	38800214	TLR1	rs5743611	3	12	87	369	0.96	8.22	0.91
4	38830350	TLR6	rs5743810	10	38	54	369	-0.38	5.02	0.94
4	89052323	ABCG2	rs2231142		13	89	369	11.30	9.62	0.24
4	123377980	IL21/IL2	rs2069762	10	45	47	369	-0.63	5.18	0.90
4	154626317	TLR2	rs5743708		6	96	369	-1.70	13.68	0.90
4	187004074	TLR3	rs3775291	8	39	55	369	-0.66	5.23	0.90
6	31543031	TNF/LTA	rs1800629	3	26	73	369	-4.13	6.01	0.49
6	32809848	PSMB8	rs9357155	2	19	81	369	1.39	6.74	0.84
6	32811629	PSMB8	rs2071543	2	21	79	369	-2.34	6.54	0.72
6	43736389	MRPS18A/VEGFA	rs699947	26	50	26	369	7.54	4.47	0.10
6	90032942	UBE2J1/GABRR2	rs2064831	2	30	70	369	2.49	6.49	0.70
7	22766645	IL6/LOC541472	rs1800795	8	38	56	369	-5.66	4.88	0.25
7	81700000	ABCB1	ABCB1-1236CT-r1128503	24	51	27	369	4.58	4.49	0.31
7	81701000	ABCB1	ABCB1-2677GT-r2032582	24	48	30	369	1.51	4.45	0.74
7	81702000	ABCB1	ABCB1-3435CT-r1045642	18	56	28	369	-0.78	4.65	0.87
7	87133470	ABCB1	rs17064	1	17	84	369	-7.18	9.47	0.45
7	87230193	ABCB1	rs3213619	1	10	91	369	-15.73	9.14	0.09
7	99245013	ZNF498/CYP3A5	rs4646458	3	18	81	369	6.87	6.55	0.30
7	99245080	ZNF498/CYP3A5	rs4646457	3	31	68	369	2.47	5.80	0.67

Chr	Position	Gene	SNP	Genotype counts			N obs	Estimate	SE	P-value of additive genetic effect*
				Minor homozygous	Heterozygous	Major Homozygous				
7	99245914	CYP3A5	rs15524	3	30	69	369	1.97	5.88	0.74
7	99262835	CYP3A5	rs10264272		1	101	369	0.00		
7	99270539	CYP3A5	rs776746	3	30	69	369	3.76	5.83	0.52
7	99325882	CYP3A7	rs2687136	5	33	64	369	1.82	5.36	0.74
7	99354114	CYP3A4/CYP3A7	rs12333983	7	31	64	369	0.65	4.98	0.90
7	99356324	CYP3A4	rs17161886		1	101	369	-6.79	27.96	0.81
7	99358524	CYP3A4	rs4986910		1	101	369	7.22	29.08	0.80
7	99360870	CYP3A4	rs4646440	2	12	88	369	0.70	7.25	0.92
7	99365451	CYP3A4	rs2687117		3	99	369	-12.99	22.81	0.57
7	99366316	CYP3A4	rs35599367		6	96	369	5.51	15.76	0.73
7	99382096	CYP3A4/CYP3A43	rs2740574		10	92	369	7.01	10.92	0.52
7	99388017	CYP3A4/CYP3A43	rs2687102		2	100	369	0.00		
8	139746208	COL22A1	rs4588898	8	40	54	369	0.94	5.26	0.86
9	120475302	TLR4	rs4986790	1	14	87	369	5.37	8.04	0.51
9	120475602	TLR4	rs4986791	1	14	87	369	4.15	7.92	0.60
9	125133507	PTGS1	rs3842787		12	90	369	14.37	9.46	0.13
9	125143707	PTGS1	rs3842792			102	369	0.00		
9	125143973	PTGS1	rs5789		6	96	369	-23.94	15.44	0.13
10	90749963	FAS/ACTA2	rs1800682	27	50	25	369	-1.81	4.38	0.68
10	96798749	CYP2C8	rs10509681		15	87	369	4.87	9.69	0.62
10	96818119	CYP2C8	rs1058930		10	92	369	1.51	11.22	0.89
10	101542578	ABCC2	rs717620	4	36	62	369	-3.79	5.87	0.52
10	101611294	ABCC2	rs8187710	2	16	84	369	1.93	7.49	0.80
11	2857194	KCNQ1	rs2237895	23	44	35	369	1.48	4.35	0.73
11	2870108	KCNQ1	rs8234	13	36	53	369	4.48	4.72	0.35
11	17460712	ABCC8	rs2237982	19	45	38	369	-6.88	4.39	0.12
11	112034988	TEX12/IL18	rs187238	7	42	53	369	-7.99	5.56	0.15
11	112035458	IL18/TEX12	rs1946518	16	58	28	369	-4.84	4.99	0.33
12	21329738	SLCO1B1	rs2306283	22	46	34	369	-1.09	4.49	0.81
12	21331549	SLCO1B1	rs4149056	5	20	77	369	0.19	6.90	0.98
12	68552522	IFNG	rs2430561	23	48	29	356	-2.30	4.63	0.62
12	79481371	SYT1	rs12300068		19	83	369	-0.51	8.30	0.95
16	31104878	VKORC1*	rs9934438	16	50	36	369	10.26	4.61	0.03
17	32579788	ACCN1/CCL2	rs1024611	12	45	45	369	1.14	5.07	0.82
21	28240574	ADAMTS5/ADAMTS1	rs229109	11	43	48	369	-1.59	5.26	0.76
22	35776672	HMOX1/TOM1	rs2071746	19	57	26	369	4.91	5.26	0.35
22	36661906	APOL1	rs73885319	1	1	100	369	-5.91	13.25	0.66
22	36662046	APOL1	rs71785313			102	369	0.00		
22	36751101	MYH9	rs11089788	27	42	33	369	0.92	4.19	0.83
22	36774812	MYH9	rs5756168	1	23	78	369	2.15	8.29	0.80

Genetic variation in relation to renal function among pediatric liver transplant recipients receiving tacrolimus.

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Abstract

Background Deterioration in renal function is a major complication in pediatric liver transplant recipients, which has been partially attributed to the nephrotoxic effects of the immunosuppressant tacrolimus. We aimed to determine the prevalence as well as genetic risk factors for renal function decline after pediatric liver transplantation.

Methods This retrospective cohort study considered clinical data, including renal function data, from the day of transplantation up to 10 years post-transplantation. In multivariate analysis, the impact of genetic variation (77 SNPs) on renal function was studied using eGFR and age-normalized eGFR (z-scores).

Results 135 liver transplant recipients treated with tacrolimus were included; their median age was 2.5 years (IQR: 8.2) and the median follow-up was 0.77 years (range: 7 days-10 years). Chronic kidney disease (CKD stages 3-5, at least 3 months post-transplant) had developed in 32 patients (24%). None of the polymorphisms studied were significantly associated with decrease in renal function.

Conclusion Renal function decline, a major complication in pediatric liver transplant recipients, occurred in 24% of the patients. In contrast to studies in adults, we were not able to show an effect of *CYP3A5*3* or other variant alleles on renal function.

Introduction

The reported prevalence of chronic renal failure following liver transplantation in adults ranges from 10% to 93%[1,2,3] and in children from 0%-88.5%.[4,5,6,7,8,9,10] This wide range may be explained by patient selection bias, differences in definitions of renal dysfunction, and differences in follow-up time.[1,10] The Studies in Pediatric Liver Transplantation (SPLIT) registry recently published a new report on the outcome of children receiving liver transplant in the United States and Canada. At 5-years post-transplantation, 45 children (13%) had an eGFR <90 ml/min/1.73m^2; at 10 years post-transplantation this was the case for 11 children (9%).[11,12] In a recent review of the pediatric literature we found reported prevalences of CKD stages 3-5), defined as an eGFR <60 ml/min/1.73m^2, ranging between 1.67% and 17%.[10] Hence, renal function decline after liver-transplantation is often seen.[1,13] Ultimately, decline can lead to end-stage renal disease and is associated with an elevated risk of death if persisting for at least three months post-transplantation.[1,14]

Clinical factors associated with renal dysfunction after liver transplantation can be categorized into pre-, peri- and post-operative factors. In addition to sex and race, pre-existing renal injury of the recipient, underlying metabolic disease, prior exposure to nephrotoxic medications and cirrhosis have been associated with post-transplant renal dysfunction.[14] Peri-operative hypotension and blood loss resulting in hemodynamic instability may result in renal function decline due to a decrease in renal blood flow and GFR.[14] Lastly, the chronic use of calcineurin inhibitors and hypertension may be considered post-operative factors.[14]

All such risk factors for pediatric liver transplant recipients have recently been reviewed by Matloff et al.[15] Some factors, however, may be less common in children, notably hepatitis C infection (HCV) and HIV.[15,16] Additionally, some metabolic diseases have been associated with post-transplantation renal function decline in children.[15,17]

To improve the long-term outcome of pediatric liver transplantation we need to further individualize current patient management. The fast developing field of pharmacogenetics may be of help here as it has the potential to "identify the right drug and the right dose for the individual patient".[23]

In adult liver transplant recipients, associations between the recipient *CYP3A5, ABCB1, CYP2C8*, and *ACE* genotypes and tacrolimus-induced nephrotoxicity have been reported.[24,25,26,27,28] These genotypes are of interest, as tacrolimus is a substrate for CYP3A5 as well as ABCB1, and CYP2C8 and ACE are involved in renal hemeostasis.[26,27,29] However, sample sizes in these studies were relatively small and definitions of renal failure varied, thereby limiting interpretation of these results. In 51 pediatric liver transplant recipients, an association was reported between *ABCB1* and tacrolimus-induced nephrotoxicity.[30] For in those who were *ABCB1 T-T-T* haplotype carriers, tacrolimus-induced nephrotoxicity was more often diagnosed than in the control group at 6 months post-transplantation. However, at one year post-transplantation this difference was no longer significant.[24,30]

Outcomes and genetic predictors of renal function after liver transplant may be affected by the calcineurin inhibitor administered. In adult liver transplant patients, administration of tacrolimus was associated with better renal function than administration of cyclosporine.[22] The molecular mechanisms by which these drugs may cause chronic kidney disease (CKD) are not fully understood but seem to differ between the two drugs.[18,19,20,21] As cyclosporine-treated patients are overrepresented in most adult, but also pediatric, studies, the results of these studies may not necessarily reflect outcome and risk factors in tacrolimus-treated pediatric liver transplant recipients.

In this study we aimed to identify CKD prevalence and genetic risk factors for post-transplantation renal function decline in pediatric liver recipients receiving tacrolimus.

Methods

We performed a retrospective cohort study of pediatric liver transplant recipients transplanted between January 1998 and June 2009 at the Hospital for Sick Children (Toronto, Canada). Patients were eligible for this study if 1) they were younger than 18 years at the time of transplantation and 2) they received oral tacrolimus after their transplantation. DNA samples were obtained if consent was provided. Informed consent was obtained from parents and/or children during enrolment. The study was approved by the Institutional Research Ethics Board of the Hospital for Sick Children.

Data collection

Clinical data were retrieved from electronic patient records and paper charts pertaining to the day of transplantation, 1 week, 1, 3, 6, 9 and 12 months and yearly thereafter until December 2009 or until available.

End-points

The primary end-point was the prevalence of CKD was established by the estimated glomerular filtration rate (eGFR) calculated according to the Schwartz formula.[31] Patients were categorized by CKD severity according to the National Kidney Foundation Kidney Disease Outcome Quality Initiative (KDOQI) guidelines, which distinguish 5 stages. The first is kidney damage with normal or elevated GFR (> 90 ml/min/1.73m^2); the second is kidney damage with mildly decreased GFR (60-89 ml/min/1.73 m^2); the third is moderate renal failure (GFR 30-59 ml/min/1.73m^2), the fourth is severe renal failure (15-29 ml/min/1.73m^2); and the fifth is complete renal failure (GFR < 15 ml/min/1.73m^2 or dialysis).[32]

The secondary end-points were the absolute eGFR and z-scores of eGFRs. The z-scores served to correct for age-related changes in eGFR and were based on reference values for the mean and standard deviation from a healthy pediatric, mainly Caucasian, population.[33]

Other clinical data

Other data collected concerned patients' demographic characteristics, tacrolimus levels, hematology and chemistry laboratory results, concomitant medications, primary diagnosis, transplant information and outcome. Information on concomitant medication known for nephrotoxic potential was collected: i.e. cyclosporin, gentamicin, cotrimoxazole, tobramycin, amphotericin B, valganciclovir, ganciclovir, spironolactone and vancomycin. Follow-up visits where cyclosporine was used or tacrolimus was discontinued, temporarily or permanently, were not included in the analysis. If a patient received more than one liver transplant, the most recent transplantation was used in the analysis.

Immunosuppressive protocol

Tacrolimus was started according to the immunosuppression protocol of the Hospital for Sick Children at the day of transplantation at 0.2 mg/kg/day twice daily. Therapeutic drug monitoring was used to adjust the tacrolimus dose to achieve a target level of 12-15 ng/ml in the first month post-transplantation, 10-12 ng/ml in the second and third months post-transplantation and a target level of 5-15 ng/ml afterwards. Additional immunosuppressive therapy consisted of a tapering steroid schedule.

Tacrolimus analysis

Tacrolimus serum concentrations were determined in ethylenediaminetetraacetic acid (EDTA)-treated whole blood (0.25 ml) on the day of sampling, using liquid chromatography tandem mass spectrometry (LC-MS-MS) as previously described, as part of routine clinical care.[34,35]

Genotyping

Blood or saliva samples were collected from patients at the first visit after informed consent was received or by access to DNA samples from either the Hospital for Sick Children's Biobank or the University Health Network HLA laboratory. Blood samples were collected in EDTA-containing tubes.

Saliva samples were collected using Oragene DNA OG-250 collection kits (DNA genotek, Ottawa, Ontario, Canada). Samples were stored at -80°C before purification of DNA using the QiaSymphony system (Qiagen, USA). Ninety-six SNPs were selected based on previous published associations, SNPs related to tacrolimus pharmacokinetic pathways and SNPs associated with renal function (Supplemental table 1). DNA samples were genotyped for this custom set of 96 variants using BeadXpress genotyping platform using the manufacturer's protocol (Illumina, CA, USA) at the University of British Columbia, Vancouver, BC, Canada.[36]

Statistical analysis

Descriptive data are presented as mean ± standard deviation or median (IQR) for continuous variables and as percentage for categorical variables. Kaplan-Meier curves were plotted for the time-course of CKD incidence. CKD stage was designated as an eGFR below the cut-off values according to CKD stages on two consecutive visits from 3 months post-transplantation onwards. The patient's sex, age at time of transplantation, investigation site, tacrolimus levels, concomitant medication, year of transplantation, albumin and CRP levels, hematocrit, conversion from cyclosporine, and weight and height were tested in a univariate general linear mixed model. Covariates with a p-value < 0.05 were considered for retention in the regression model. The Hardy-Weinberg equilibrium was tested for each polymorphism using the method by Guo et al.[37] Previously published[24] significant genetic associations with eGFR were tested at a significance level of 0.05. All other genetic associations were tested at a significance threshold of 8.7×10^{-4} determined by a Bonferroni correction based on the effective number of independent tests (M_{eff}).[38] A mixed model for the continuous endpoints served to analyze the genetic additive effect of each SNP by adjusting for the covariates retained in the stepwise selection. All statistical analyses for the genetic variables were done using SAS 9.3 (SAS institute Inc, Cary, NC, USA). All other statistical analyses, including figures were done using SPSS version 20.0 (IBM SPSS Statistics, Armonk, NY, USA).

Results

Patient population

One hundred and fifty-two patients were eligible for inclusion. Informed consent was received for 135 patients and they were included in the study. The median age was 2.5 years (IQR: 8.2); the median weight was 20.8 kg (IQR: 24.0) and 51.1% were females (Table 1). The median follow-up time was 0.77 years (range: 7 days – 10 years). The most common primary diagnosis for liver transplantation was biliary atresia (40.8%) (Table 1). Eleven patients (8.1%) were converted from cyclosporine to tacrolimus during the follow-up interval and 14 patients (10.3%) passed away (Table 1). Seven patients (5.2%) underwent re-transplantation. Recipient DNA was available for 78 patients.

Figure 1A and 1B: eGFR over time and eGFR z-scores over time

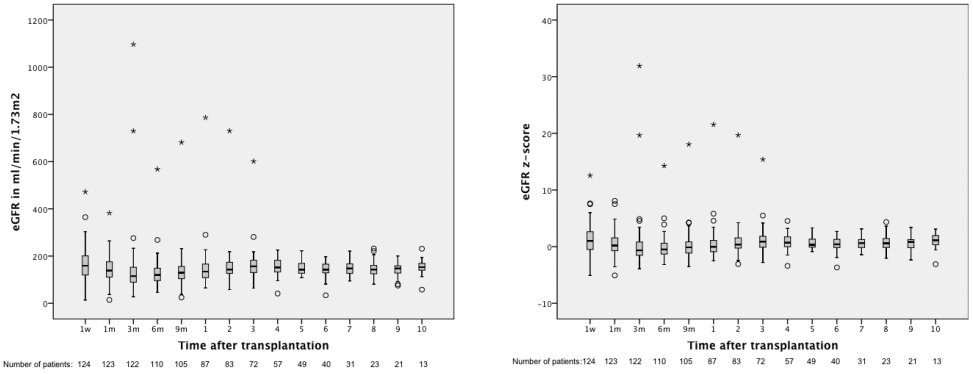


Table 1: Demographics of the patient population (continued)

Variable	Liver (All)	Liver (DNA)
Number	135	78
Age at time of transplant (years) Mean ± SD Median (IQR)	 5.2 ± 5.2 2.5 (8.2)	 4.4 ± 4.5 2.5 (4.8)
Year of transplant Median (Q1,Q3) Range	 2005 (2001, 2007) (1995, 2009)	 2004 (2001, 2007) (1995, 2009)
Follow up (year) Mean ± SD Median (IQR)	 1.96 ± 2.51 0.74 (2.77)	 2.2 ± 2.69 0.76 (3.67)
Gender	M: 69 (48.9%) F: 66 (51.1%)	M: 42 (53.8%) F: 36 (46.2%)
Weight (kg) Mean ± SD Median (IQR)	 26.8 ± 18.1 20.8 (24.0)	 25.2 ± 16.8 20.0 (18.1)
Height (cm) Mean ± SD Median (IQR)	 113.8 ± 34.1 113.0 (58.0)	 110.5 ± 32.2 109.7 (48.4)
Transplant (n, %) 1st 2nd	 128 (94.8%) 7 (5.2%)	 71 (91.0%) 7(9.0%)
eGFR at 1 year post-transplantation Mean ± SD Median (IQR)	 146.8 ± 80.5 134.4 (58.0)	 148.1 ± 38.0 140.0 (50.0)
Need for dialysis before transplant (n,%)	0 (0%)	0 (0%)
Converted from CsA (n,%)	11 (8.1%)	6 (7.7%)
Mortality rate (n,%)	14 (10.3%)	9 (11.5%)
Donor age Mean ± SD Median (IQR)	 30.70 ± 17.8 33.0 (31.0)	 27.95 ± 18.7 28.0 (34.0)
Donor gender	M: 26 (39%) F: 40 (61%)	M: 14 (40%) F: 21 (60%)
Deceased or living-related donor (n,%)	Deceased: 22 (16.3%) Living: 113 (83.7%)	Deceased: 7 (9.0%) Living: 71 (91.0%)
Cold ischemia time (min) Mean ± SD Median (IQR)	 466.8 ± 227.8 500.0 (346.0)	 488.8 ± 239.6 503.0 (378.3)
Warm ischemia time (min) Mean ± SD Median (IQR)	 60.1 ± 19.9 59.0 (22.0)	 64.2 ± 21.4 61.0 (20.0)
Primary diagnosis Biliary atresia Auto-immune Hepatoblastoma Fulminant liver failure Chronic liver failure Tyrosinemia Congenital Cholangitis Other	 60 (40.8%) 2 (1.4%) 12 (8.2%) 15 (10.2%) 13 (8.8%) 2 (1.4%) 4 (2.7%) 9 (6.1%) 30 (20.4%)	 38 (48.7%) 0 (0%) 7 (8.9%) 6 (7.7%) 8 (10.3%) 2 (2.6%) 2 (2.6%) 4 (5.1%) 11 (14.1%)

Renal failure following liver transplantation

None of the patients required dialysis before or after transplantation. The median eGFR for the 87 patients with data available at one year post-transplantation was 134.4 (IQR: 58.0) ml/min/1.73m^2 (Table 1) (Figures 1A and 1B). The incidences of the KDOQI CKD stages at various time points are presented in Figure 2. The cumulative incidence of CKD stages 3-5 is shown in Figure 3A. Thirty-two patients developed CKD stages 3-5. The majority of these 32 patients experienced CKD stage 3 (22.2%); and 2 patients developed stage 4 (1.5%) (Figure 3B). None of the patients experienced CKD stage 5. At 1 year post-transplantation only 5 patients (5.7%) patients experienced CKD stage 2, and none CKD stages 3-5. At 5 years post-transplantation none of the patients experienced CKD stages 2-5.

Figure 2: Incidence of CKD at each time point

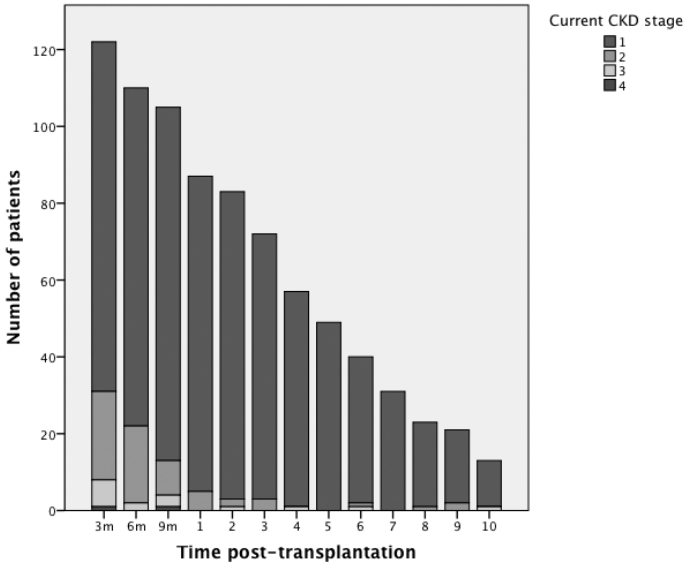
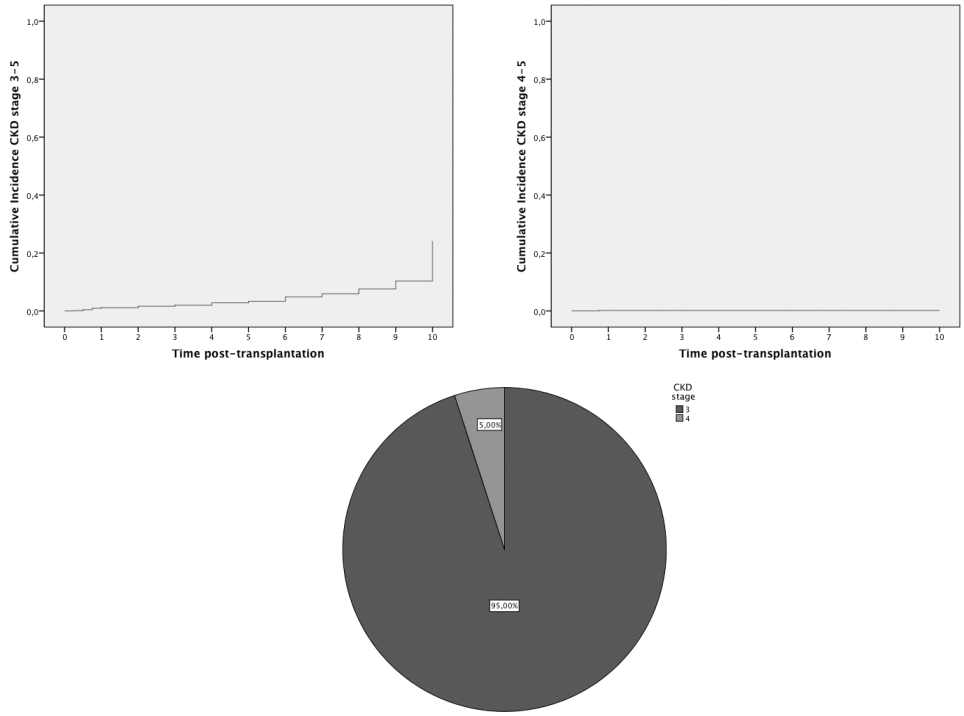


Figure 3: Kaplan-Meier curves of the cumulative incidence of CKD in 135 pediatric liver transplant recipients



Predictors of renal failure

Four SNPs in genes were not in Hardy-Weinberg equilibrium in our cohort: *UGT1A1*6* (rs4148323), *CYP3A4*3* (rs4986910), *CYP3A4*22* (rs35599367) and *CYP3A4* rs2687102 (Supplementary Table 2). In univariate analysis, tacrolimus trough concentrations, albumin levels, hematocrit and height and weight were significantly associated with eGFR (Table 2). In addition, sex and age at time of transplantation were entered into the regression model for their known influence of renal function. Two SNPs in two genes were significantly below the significance level of 0.05 in the genetic model; *TLR9 -1486C/T (rs187084)* and *IFN-gamma 874A/T (rs2430561)*. *IFN*-gamma *874* and *TLR9 -1486* variant carriers had lower eGFR values compared to their respective wild-type carriers (Table 3). Nonetheless, none of the polymorphisms tested reached the set significance level for multiple testing of 8.7×10^{-4} (Supplementary table 3).

Table 2: Univariate analysis of the covariates

Variable	N	P-value
Gender	466	0.47
Age at time of transplantation	466	0.77
Tacrolimus levels	466	<0.001
Concomitant medication	466	0.11
Year of transplantation	466	0.46
Albumin levels	405	0.002
Hematocrit levels	321	<0.001
Converted to CsA	466	0.38
Weight	140	0.001
Height	466	<0.001
Converted to CsA	459	0.04
Weight	89	0.03
Height	406	0.11

Table 3: Genetic association results

Chr	Position	Gene	SNP	Genotype counts			N obs	N obs Estimate	SE	P-value of additive genetic effect*
				Minor homozygous	Heterozygous	Major Homozygous				
3	52261031	TWF2/ TLR9*	rs187084	11	36	31	288	12.73	5.86	0.03
3	121838319	CD86	rs1129055	8	28	42	288	-11.01	5.83	0.06
6	31543031	TNF/LTA	rs1800629	2	17	59	288	13.79	8.10	0.09
7	81700000	ABCB1 -1236CT	rs1128503	16	42	20	288	-5.11	6.18	0.41
7	81701000	ABCB1 -2677GT	rs2032582	17	39	22	288	1.93	6.23	0.76
7	81702000	ABCB1 -3435CT	rs1045642	18	45	15	288	0.84	6.88	0.90
7	99270539	CYP3A5	rs776746	1	26	50	285	-0.83	7.97	0.92
10	90749963	FAS/ ACTA2	rs1800682	20	37	21	288	10.43	5.75	0.07
12	68552522	IFNG*	rs2430561	15	32	30	286	13.61	5.38	0.01
17	32579788	ACCN1/ CCL2	rs1024611	11	34	33	288	10.33	5.76	0.08

Adjusted for gender, age at time of transplantation, tacrolimus level, albumin level, hematocrite level and patient's height. Positions based on Genome Build Version 37.1.
* Significant at the level of 0.05

Discussion

The prevalence of CKD in in this pediatric cohort was quite high: 32 of the 135 patients (23.7%) experienced CKD stage 3-5, 2 (1.5%) experienced CKD stage 4, and none stage 5. Still, the cumulative incidence of CKD stages 4-5 was much lower than that reported by Ojo et al. (27%) in adult transplant recipients.[1]

Our results are similar to previous studies, which reported prevalences of moderate-severe CKD, defined as eGFR <60 ml/min/1.73m^2, ranged between 1.67% and 17%.[10] However, these prevalences were established at the time of the last available follow-up, which widely varied between patients and papers. Hence, it is hardly possible to identify a time window in which post-transplantation renal failure is of particular concern.

The prevalences of mild renal failure (CKD stage 2) at one year and 5 years post-transplantation in the present study were 5.7% and 0%, respectively. The SPLIT study reported a 13% prevalence of CKD stage 2 at 5 years post-transplantation and a 9% prevalence at 10 years.[11,12] The discrepancy between studies for the 5-years prevalence could be due to the fact that our sample at that time was relatively small compared to the SPLIT study (49 versus 352 patients).[11]

In contrast to the previous studies, we established the cumulative incidence as well as the prevalence of renal failure at several time points in the same cohort. The mean eGFR proved stable throughout the study period and the eGFR z-scores deviated little from those of healthy children. CKD stages 2 and 3 were more frequently seen in the first year post-transplantation than in later years. In the later years a smaller proportion of patients had renal failure. This approach represents the post-transplant outcome of liver transplant recipients more accurately than that in which patients with different follow-up periods are grouped together.

Two SNPs in the genetic model were significant at the 5% level for renal damage: *IFN-γ 874A/T* and *TLR9 -1486C/T*. Yet these are probably chance findings as the significance threshold for multiple testing was not reached. The results did not show a significant association between recipient *CYP3A5*, *CYP2C8*, *ACE* and also the *ABCB1* genotype, as has been suggested in studies in adults and, for *ABCB1*, in one previous pediatric study.[24] Two of the adult studies, however, unselectively pooled data from both cyclosporine and tacrolimus-treated patients, which brings into question the association between *ABCB1* and *ACE* genotypes with tacrolimus-related nephrotoxicity. Additionally, the definitions of renal failure were largely based on serum creatinine cut-offs.[24] The previous pediatric study only showed an association between *ABCB1* genotype and tacrolimus-induced nephrotoxicity at 6 months post-transplantation, which disappeared at one year post-transplantation. Hence, the genetic association of *ABCB1* genotype may not be relevant over the longer term post-transplantation.[30]

Several limitations of our study should be acknowledged. The limited sample size may have resulted in missing more subtle recipient genetic effects on renal function. Because we did not include donor DNA in our analysis, the donor contribution could not be established. The candidate gene approach of our study limits the detection of genetic risk factors to the genes included in the assay. A Genome Wide Association Study could perhaps reveal other candidates not yet thought of. The retrospective nature of the study limited us to the information available in database and medical charts, which did not include drug dosing, liver size and pre-transplant information. This was a single center study, and it could well be that similar studies in other transplant centers would yield different results. On the other hand, this study is the first of its kind determined genetic factors in relation to renal function for a prolonged period of time post-transplantation. Moreover, using eGFR and not CKD stage to identify association between genetic variation with renal dysfunction, increased

the sensitivity of our analyses. Nevertheless, the use of eGFR may not reflect the true prevalence of CKD in liver transplant recipients as this measure tends to overestimate the true glomerular filtration rate.[15] The "gold standard" of measured GFR (mGFR) would be preferable as it has been shown to result in a higher rate of CKD.[15]

In conclusion, this is the first pediatric liver transplant study reporting on the prevalence of CKD as well as genetic risk factors associated with decline in renal function. The higher CKD stages (4 or 5) were rare; however 24% of patients still experienced CKD stage 3. None of the SNPs studied were significantly associated with decline in renal function.

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Supplementary Table 1: All SNPs analyzed

Chr	Position	Gene	SNP
1	60392494	C1orf87/CYP2J2	rs890293
1	162030688	OLFML2B/NOS1AP	rs10918594
1	184911126	PTGS2	rs4648279
1	186643541	PTGS2	rs3218625
1	186643836	PTGS2	rs5272
1	186643837	PTGS2	rs5273
1	186650751	PTGS2/PLA2G4A	rs689466
1	186646004	PTGS2	rs3218622
1	206946407	IL19/IL10	rs1800872
1	206946634	IL19/IL10	rs1800871
1	206946897	IL19/IL10	rs1800896
1	223285200	TLR5	rs5744168
2	228052600	COL4A3	rs2204862
2	234669144	UGT1A1	rs4148323
2	241542703	CAPN10	rs5030952
3	52261031	TWF2/TLR9	rs187084
3	119500035	NR1I2	rs3814055
3	119533733	NR1I2	rs6785049
3	119534153	NR1I2	rs2276707
3	119537254	NR1I2	rs3814057
3	121838319	CD86	rs1129055
4	38799710	TLR1	rs4833095
4	38800214	TLR1	rs5743611
4	38830350	TLR6	rs5743810
4	89052323	ABCG2	rs2231142
4	123377980	IL21/IL2	rs2069762
4	154626317	TLR2	rs5743708
4	187004074	TLR3	rs3775291
6	31241109	HLA-C	rs13191343
6	31543031	TNF/LTA	rs1800629
6	32809848	PSMB8	rs9357155
6	32811629	PSMB8	rs2071543
6	43736389	MRPS18A/VEGFA	rs699947
6	43737830	VEGF	rs1570360
6	90032942	UBE2J1/GABRR2	rs2064831
7	22766645	IL6/LOC541472	rs1800795
7	75615006	POR	rs1057868
7	81700000	ABCB1	rs1128503
7	81701000	ABCB1	rs2032582
7	81702000	ABCB1	rs1045642
7	87133470	ABCB1	rs17064
7	87230193	ABCB1	rs3213619

Chr	Position	Gene	SNP
7	99102509	CYP3A5	rs55965422
7	99194259	CYP3A4	rs17161886
7	99245013	ZNF498/CYP3A5	rs4646458
7	99245080	ZNF498/CYP3A5	rs4646457
7	99245914	CYP3A5	rs15524
7	99247772	CYP3A5	rs41279854
7	99250393	CYP3A5	rs41303343
7	99258139	CYP3A5	rs28383479
7	99262835	CYP3A5	rs10264272
7	99270539	CYP3A5	rs776746
7	99273821	CYP3A5	rs55817950
7	99325882	CYP3A7	rs2687136
7	99354114	CYP3A4/CYP3A7	rs12333983
7	99356324	CYP3A4	rs17161886
7	99358524	CYP3A4	rs4986910
7	99360870	CYP3A4	rs4646440
7	99365451	CYP3A4	rs2687117
7	99365983	CYP3A4	rs55785340
7	99366316	CYP3A4	rs35599367
7	99382096	CYP3A4/CYP3A43	rs2740574
7	99388017	CYP3A4/CYP3A43	rs2687102
8	139746208	COL22A1	rs4588898
9	120475302	TLR4	rs4986790
9	120475602	TLR4	rs4986791
9	125133507	PTGS1	rs3842787
9	125140241	PTGS1	rs3842789
9	125143707	PTGS1	rs3842792
9	125143973	PTGS1	rs5789
9	125148791	PTGS1	rs5791
9	125152507	PTGS1	rs5792
9	125152579	PTGS1	rs5793
10	90749963	FAS/ACTA2	rs1800682
10	96798749	CYP2C8	rs10509681
10	96818119	CYP2C8	rs1058930
10	101542578	ABCC2	rs717620
10	101611294	ABCC2	rs8187710
11	2857194	KCNQ1	rs2237895
11	2870108	KCNQ1	rs8234
11	17460712	ABCC8	rs2237982
11	112034988	TEX12/IL18	rs187238
11	112035458	IL18/TEX12	rs1946518
12	21329738	SLCO1B1	rs2306283

Chr	Position	Gene	SNP
12	21331549	SLCO1B1	rs4149056
12	68552522	IFNG	rs2430561
12	79481371	SYT1	rs12300068
16	31104878	VKORC1	rs9934438
17	32579788	ACCN1/CCL2	rs1024611
19	41858921	TGF-beta	rs1800470
21	28240574	ADAMTS5/AD-AMTS1	rs229109
22	35775889	HO-1	rs3761439
22	35776672	HMOX1/TOM1	rs2071746
22	36661906	APOL1	rs73885319
22	36662046	APOL1	rs71785313
22	36751101	MYH9	rs11089788
22	36774812	MYH9	rs5756168
22	36751101	MYH9	rs11089788
22	36774812	MYH9	rs5756168

A total of 19 SNPs had to be removed from the analysis; 16 SNPs had a allele frequency of 0, two SNPs had more than 5% missing genotypes and one SNP completely failed. A total of 77 SNPs were included in the analysis.

Supplementary Table 2: Hardy-Weinberg equilibrium

Chr	Position	Gene	SNP	Minor allele	Major allele	MAF frequency	H-W p-value	H-W Exact p-value
1	60392494	C1orf87/CYP2J2	rs890293	C	A	0.082802548	0.588700555	1
1	162030688	OLFML2B/NOS1AP	rs10918594	G	C	0.385350318	0.337620766	0.3326
1	186643541	PTGS2	rs3218625	G	A	0.001592357	0.954561842	1
1	186650751	PTGS2/PLA2G4A	rs689466	A	G	0.178343949	0.69337799	1
1	206946407	IL19/IL10	rs1800872	C	A	0.305111821	0.48443888	0.477
1	206946634	IL19/IL10	rs1800871	G	A	0.283333333	0.648531759	0.6269
1	206946897	IL19/IL10	rs1800896	A	G	0.396496815	0.979868635	1
1	223285200	TLR5	rs5744168	G	A	0.046178344	0.816215886	1
2	228052600	COL4A3	rs2204862	A	G	0.097133758	0.290239465	0.3133
2	234669144	UGT1A1	rs4148323	G	A	0.009708738	2.22436E-05	0.0421
2	241542703	CAPN10	rs5030952	G	A	0.012738854	0.862516403	1
3	52261031	TWF2/TLR9	rs187084	A	G	0.370607029	0.915853751	1
3	119500035	NR1I2	rs3814055	G	A	0.404458599	0.200626485	0.2444
3	119533733	NR1I2	rs6785049	A	G	0.439490446	0.733967333	0.8194
3	119534153	NR1I2	rs2276707	G	A	0.208598726	0.416170618	0.465
3	119537254	NR1I2	rs3814057	A	C	0.224522293	0.238968827	0.2268
3	121838319	CD86	rs1129055	G	A	0.265923567	0.315567024	0.3974
4	38799710	TLR1	rs4833095	A	G	0.356687898	0.496905848	0.4923
4	38800214	TLR1	rs5743611	G	C	0.071656051	0.014156525	0.058
4	38830350	TLR6	rs5743810	G	A	0.294585987	0.442440668	0.7195
4	89052323	ABCG2	rs2231142	C	A	0.106687898	0.175696998	0.1702
4	123377980	IL21/IL2	rs2069762	A	C	0.341853035	0.865134745	1
4	154626317	TLR2	rs5743708	G	A	0.02388535	0.862516403	1
4	187004074	TLR3	rs3775291	G	A	0.277070064	0.341201578	0.5601
6	31543031	TNF/LTA	rs1800629	G	A	0.157643312	0.568614962	0.6143
6	32809848	PSMB8	rs9357155	G	A	0.117834395	0.674910112	0.6373
6	32811629	PSMB8	rs2071543	C	A	0.133757962	0.997848331	1
6	43736389	MRPS18A/VEGFA	rs699947	C	A	0.472929936	0.587040519	0.6306
6	90032942	UBE2J1/GABRR2	rs2064831	A	G	0.173566879	0.055846195	0.0564
7	22766645	IL6/LOC541472	rs1800795	C	G	0.305732484	0.786758383	1
7	81700000	ABCB1 -1236CT	rS1128503	G	A	0.461783439	0.48115569	0.6428
7	81701000	ABCB1 -2677GT	rs2032582	C	A	0.453674121	0.970930985	1
7	81702000	ABCB1 -3435CT	rs1045642	A	G	0.498407643	0.16949463	0.2538
7	87133470	ABCB1	rs17064	A	T	0.068471338	0.199695327	0.2568
7	87230193	ABCB1	rs3213619	A	G	0.039808917	0.678204173	1
7	99245013	ZNF498/CYP3A5	rs4646458	A	C	0.100638978	0.867957935	1
7	99245080	ZNF498/CYP3A5	rs4646457	A	C	0.159235669	0.16949463	0.2799
7	99245914	CYP3A5	rs15524	A	G	0.151273885	0.204608211	0.458

Chr	Position	Gene	SNP	Minor allele	Major allele	MAF frequency	H-W p-value	H-W Exact p-value
7	99262835	CYP3A5	rs10264272	G	A	0.00477707		
7	99270539	CYP3A5	rs776746	G	A	0.153354633	0.236443241	0.4366
7	99325882	CYP3A7	rs2687136	A	G	0.165605096	0.639487504	1
7	99354114	CYP3A4/CYP3A7	rs12333983	A	T	0.178913738	0.442232307	0.748
7	99356324	CYP3A4	rs17161886	A	C	0.02388535	0.816215886	1
7	99358524	CYP3A4	rs4986910	A	G	0.00477707	1.03041E-18	0.0067
7	99360870	CYP3A4	rs4646440	G	A	0.052547771	0.390537276	0.3644
7	99365451	CYP3A4	rs2687117	G	A	0.01433121	0.908684346	1
7	99366316	CYP3A4	rs35599367	G	A	0.042993631	1.7105E-05	0.0426
7	99382096	CYP3A4/CYP3A43	rs2740574	A	G	0.060509554	0.390537276	0.3783
7	99388017	CYP3A4/CYP3A43	rs2687102	A	G	0.012738854	1.03041E-18	0.0067
8	139746208	COL22A1	rs4588898	G	A	0.294585987	0.301771484	0.2938
9	120475302	TLR4	rs4986790	A	G	0.076433121	0.502860562	1
9	120475602	TLR4	rs4986791	G	A	0.070063694	0.588700555	1
9	125133507	PTGS1	rs3842787	G	A	0.046178344	0.954561842	1
9	125143707	PTGS1	rs3842792	A	C	0.001592357	0.954561842	1
9	125143973	PTGS1	rs5789	C	A	0.02388535	0.816215886	1
10	90749963	FAS/ACTA2	rs1800682	A	G	0.48566879	0.651605163	0.6539
10	96798749	CYP2C8	rs10509681	A	G	0.087579618	0.633082669	1
10	96818119	CYP2C8	rs1058930	C	G	0.049363057	0.678204173	1
10	101542578	ABCC2	rs717620	G	A	0.204472843	0.585992222	1
10	101611294	ABCC2	rs8187710	G	A	0.071656051	0.588700555	1
11	2857194	KCNQ1	rs2237895	A	C	0.420382166	0.609665876	0.6424
11	2870108	KCNQ1	rs8234	A	G	0.357371795	0.187623417	0.2336
11	17460712	ABCC8	rs2237982	G	A	0.406050955	0.609665876	0.6257
11	112034988	TEX12/IL18	rs187238	C	G	0.246815287	0.389689196	0.4982
11	112035458	IL18/TEX12	rs1946518	C	A	0.396496815	0.604954536	0.7997
12	21329738	SLCO1B1	rs2306283	A	G	0.447452229	0.528145106	0.5049
12	21331549	SLCO1B1	rs4149056	A	G	0.136942675	0.147061143	0.3436
12	68552522	IFNG	rs2430561	A	T	0.440514469	0.232560522	0.2356
12	79481371	SYT1	rs12300068	G	A	0.135350318	0.239968341	0.3572
16	31104878	VKORC1	rs9934438	G	A	0.415335463	0.64827917	0.6507
17	32579788	ACCN1/CCL2	rs1024611	A	G	0.340764331	0.640627488	0.6254
21	28240574	ADAMTS5/ADAMTS1	rs229109	G	A	0.304140127	0.315567024	0.4008
22	35776672	HMOX1/TOM1	rs2071746	T	A	0.444089457	0.472641902	0.6404
22	36661906	APOL1	rs73885319	A	G	0.01433121	0.954561842	1
22	36662046	APOL1	rs71785313	A	T	0.00477707		
22	36751101	MYH9	rs11089788	C	A	0.445859873	0.934129709	1
22	36774812	MYH9	rs5756168	A	G	0.114649682	0.825252819	0.5694

Table 3: Genetic associations renal transplant recipients

Chr	Position	Gene	SNP	Genotype counts			N obs	Estimate	SE	P-value of additive genetic effect*
				Minor homozygous	Heterozygous	Major Homozygous				
1	60392494	C1orf87/CYP2J2	rs890293		9	69	288	0.22	13.95	0.99
1	162030688	OLFML2B/NOS1AP	rs10918594	12	32	34	288	2.92	5.90	0.62
1	186643541	PTGS2	rs3218625		1	77	288	31.07	32.04	0.34
1	186650751	PTGS2/PLA2G4A	rs689466	2	24	52	288	4.20	7.50	0.58
1	206946407	IL19/IL10	rs1800872	13	34	31	288	-6.35	5.49	0.25
1	206946634	IL19/IL10	rs1800871	11	33	31	280	-7.75	5.63	0.17
1	206946897	IL19/IL10	rs1800896	10	36	32	288	5.18	6.01	0.39
1	223285200	TLR5	rs5744168		4	74	288	2.31	17.18	0.89
2	228052600	COL4A3	rs2204862	1	9	68	288	-10.40	10.70	0.33
2	234669144	UGT1A1	rs4148323	1	2	73	281	15.66	14.40	0.28
2	241542703	CAPN10	rs5030952		3	75	288	25.38	24.07	0.30
3	52261031	TWF2/TLR9*	rs187084	11	36	31	288	12.73	5.86	0.03
3	119500035	NR1I2	rs3814055	10	43	25	288	-7.75	6.66	0.25
3	119533733	NR1I2	rs6785049	16	37	25	288	-3.67	5.55	0.51
3	119534153	NR1I2	rs2276707	4	22	52	288	-4.89	6.84	0.48
3	119537254	NR1I2	rs3814057	6	24	48	288	-3.04	6.33	0.63
3	121838319	CD86	rs1129055	8	28	42	288	-11.01	5.83	0.06
4	38799710	TLR1	rs4833095	15	35	28	288	-6.14	5.64	0.28
4	38800214	TLR1	rs5743611	2	8	68	288	6.80	10.13	0.50
4	38830350	TLR6	rs5743810	2	27	49	288	2.79	7.97	0.73
4	89052323	ABCG2	rs2231142	3	16	59	288	4.99	7.67	0.52
4	123377980	IL21/IL2	rs2069762	9	34	35	288	9.48	6.01	0.12
4	154626317	TLR2	rs5743708		3	75	288	-43.46	29.99	0.15
4	187004074	TLR3	rs3775291	4	34	40	288	-2.58	6.88	0.71
6	31543031	TNF/LTA	rs1800629	2	17	59	288	13.79	8.10	0.09
6	32809848	PSMB8	rs9357155	2	18	58	288	-5.34	8.56	0.53
6	32811629	PSMB8	rs2071543	2	21	55	288	-4.18	8.25	0.61
6	43736389	MRPS18A/VEGFA	rs699947	16	36	26	288	1.93	5.65	0.73
6	90032942	UBE2J1/GABRR2	rs2064831	5	18	55	288	4.65	6.80	0.50
7	22766645	IL6/LOC541472	rs1800795	6	33	39	288	1.68	6.70	0.80
7	81700000	ABCB1 -1236CT	rs1128503	16	42	20	288	-5.11	6.18	0.41
7	81701000	ABCB1 -2677GT	rs2032582	17	39	22	288	1.93	6.23	0.76
7	81702000	ABCB1 -3435CT	rs1045642	18	45	15	288	0.84	6.88	0.90
7	87133470	ABCB1	rs17064	1	8	69	288	14.05	10.92	0.20
7	87230193	ABCB1	rs3213619		7	71	288	-3.50	14.80	0.81
7	99245013	ZNF498/CYP3A5	rs4646458	1	17	60	288	2.35	8.80	0.79
7	99245080	ZNF498/CYP3A5	rs4646457	1	28	49	288	0.94	7.94	0.91
7	99245914	CYP3A5	rs15524	1	27	50	288	-1.72	8.01	0.83
7	99262835	CYP3A5	rs10264272			78	288	0		

Chr	Position	Gene	SNP	Genotype counts			N obs	Estimate	SE	P-value of additive genetic effect*
				Minor homozygous	Heterozygous	Major Homozygous				
7	99270539	CYP3A5	rs776746	1	26	50	285	-0.83	7.97	0.92
7	99325882	CYP3A7	rs2687136	3	28	47	288	-4.70	7.17	0.51
7	99354114	CYP3A4/CYP3A7	rs12333983	3	30	44	283	0.90	7.19	0.90
7	99356324	CYP3A4	rs17161886		4	74	288	27.32	17.72	0.13
7	99358524	CYP3A4	rs4986910	1		77	288	-18.22	16.70	0.28
7	99360870	CYP3A4	rs4646440	1	10	67	288	17.15	10.24	0.10
7	99365451	CYP3A4	rs2687117		2	76	288	20.89	27.65	0.45
7	99366316	CYP3A4	rs35599367	1	2	75	288	-23.90	15.20	0.12
7	99382096	CYP3A4/CYP3A43	rs2740574	1	10	67	288	7.91	10.03	0.43
7	99388017	CYP3A4/CYP3A43	rs2687102	1		77	288	7.33	16.72	0.66
8	139746208	COL22A1	rs4588898	9	29	40	288	7.12	5.95	0.24
9	120475302	TLR4	rs4986790		11	67	288	-15.91	12.04	0.19
9	120475602	TLR4	rs4986791		9	69	288	-16.99	13.53	0.21
9	125133507	PTGS1	rs3842787		1	77	288	23.42	42.52	0.58
9	125143707	PTGS1	rs3842792		1	77	288	14.66	33.45	0.66
9	125143973	PTGS1	rs5789		4	74	288	16.24	21.49	0.45
10	90749963	FAS/ACTA2	rs1800682	20	37	21	288	10.43	5.75	0.07
10	96798749	CYP2C8	rs10509681		8	70	288	-2.37	13.78	0.86
10	96818119	CYP2C8	rs1058930		7	71	288	-22.91	14.52	0.12
10	101542578	ABCC2	rs717620	2	25	50	287	9.78	8.07	0.23
10	101611294	ABCC2	rs8187710		9	69	288	-17.26	12.90	0.19
11	2857194	KCNQ1	rs2237895	13	35	30	288	-4.63	6.08	0.45
11	2870108	KCNQ1	rs8234	15	31	30	283	-4.33	5.86	0.46
11	17460712	ABCC8	rs2237982	13	35	30	288	-0.38	6.14	0.95
11	112034988	TEX12/IL18	rs187238	5	24	49	288	10.34	6.98	0.14
11	112035458	IL18/TEX12	rs1946518	9	38	31	288	4.10	6.30	0.52
12	21329738	SLCO1B1	rs2306283	18	36	24	288	-1.64	5.90	0.78
12	21331549	SLCO1B1	rs4149056		22	56	288	0.21	9.11	0.98
12	68552522	IFNG*	rs2430561	15	32	30	286	13.61	5.38	0.01
12	79481371	SYT1	rs12300068	3	17	58	288	4.91	7.68	0.52
16	31104878	VKORC1	rs9934438	16	36	25	278	6.43	5.77	0.27
17	32579788	ACCN1/CCL2	rs1024611	11	34	33	288	10.33	5.76	0.08
21	28240574	ADAMTS5/ADAMTS1	rs229109	8	28	42	288	9.93	6.02	0.10
22	35776672	HMOX1/TOM1	rs2071746	12	41	25	288	9.75	6.26	0.12
22	36661906	APOL1	rs73885319		1	77	288	14.66	33.45	0.66
22	36662046	APOL1	rs71785313			78	288	0		
22	36751101	MYH9	rs11089788	15	38	25	288	6.91	6.11	0.26
22	36774812	MYH9	rs5756168	1	14	63	288	-11.41	9.32	0.23

Investigating recipient's genetic variability in predicting deterioration of renal function among pediatric heart transplant recipients receiving tacrolimus.

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Abstract

Background Pediatric heart transplant recipients receiving tacrolimus are at risk of renal failure. We determined the prevalence as well as genetic risk factors of renal function decline in pediatric heart transplant recipients.

Methods In this multi-center retrospective cohort study, clinical data from medical records from the day of transplantation up to 10 years post-transplantation were collected. In multivariate analysis, the impact of genetic variation on renal function was studied using age-normalized eGFR and the examination of 77 SNPs relevant to drug biotransformation or toxicity.

Results We analyzed data on 161 heart transplant recipients with a median age of 2.31 years (IQR: 9.89) and a median follow-up time of 0.78 years (range: 7 days – 10 years). Chronic kidney disease (CKD: eGFR<60 ml/min/1/73m^2 at two consecutive visits at least 3 months post-transplantation) developed in 55 patients (34.2%), 7 of whom (4.3%) developed stage 4 and one (0.6%) stage 5. None of the genetic polymorphisms studied were significantly associated with renal function.

Conclusion Chronic kidney disease occurred in 34.2% of pediatric heart transplant recipients in this study. Genetic risk factors were not identified.

Introduction

Renal failure is a serious complication following adult cardiac transplantation[1], as shown from the 6.9% five-year cumulative incidence reported in the landmark study by Ojo et al.[2] The International Society for Heart and Lung Transplantation reported that 6% of pediatric heart transplant recipients experienced renal failure at 1 year post-transplantation and 10% at both 5 and 10 years post-transplantation.[3,4] Reported prevalences in other pediatric studies range from 6.8%-46%.[5,6,7,8] Selection bias as well as different definitions for renal failure and different follow-up times may contribute to this wide variation.[8]

In pediatric cardiac transplant recipients, pre-transplant dialysis, hypertrophic cardiomyopathy, race, diabetes, pre-transplant extracorporeal membrane oxygenation treatment and previous transplant experience may be associated with risk of chronic kidney disease (CKD).[9]
These risk factors largely parallel risk factors reported in adult patients. Additional risk factors in adults are calcineurin inhibitor use and nephrotoxic co-medication.[2,10,11]

In addition to clinical risk factors, there may be a role for genetic variation.[12] In adult heart transplant recipients, *CYP3A5*1* carriers had higher estimated glomerular filtration rates (eGFR) compared to *CYP3A5*3* carriers up to 5 years post-transplantation.[13] Additionally, Pro-carriers of *TGF-β codons 10* and *25* had an increased risk for end-stage renal disease.[14] Nonetheless, others failed to find associations of *CYP3A5, ABCB1* and *TGF-β* genotypes with renal function in adult heart transplant recipients.[15]

Interestingly, pediatric heart transplant recipients identified as TGF-β high producers (*TGF-β codons 10* and *25; TC/GG, TT/GG*) had significantly lower creatinine clearance rates up to 6 years post-transplantation compared to intermediate/low TGF-β producers. *CYP3A5* and *ABCB1* genotypes have been associated with altered tacrolimus disposition in pediatric heart

transplant recipients,[16,17] but a recent study found no associations of 19 SNPs, including *CYP3A5*, *ABCB1* and *TGF-β* genotypes, with eGFR after adjusting for age at time of transplantation, race and sex in 302 pediatric heart transplant recipients.[18] In this study, however, the majority of patients received cyclosporine.

Both cyclosporine and tacrolimus are believed to contribute to the development of CKD following heart transplantation.[2,10] These drugs exert similar effects on the immune system, but seem to differ in the molecular mechanisms causing CKD.[19,20,21,22] Moreover, tacrolimus was associated with lower risk of CKD in adult liver recipients in comparison with cyclosporine.[23] So far, however, most of the relevant studies concerned patients receiving cyclosporine, even though tacrolimus currently is the calcineurin inhibitor of choice in pediatric heart transplant recipients. Hence, we aimed to determine the prevalence as well as genetic risk factors of renal function decline in pediatric heart transplant recipients.

Methods

This was a retrospective cohort study of pediatric heart transplant recipients transplanted between January 1998 and June 2009 at the Hospital for Sick Children, Toronto, Ontario, Canada or at the Erasmus MC - Sophia Children's Hospital, Rotterdam, the Netherlands. Patients were eligible for this study if 1) they were under 18 years of age at time of transplantation and 2) they had received oral tacrolimus for immunosuppression. At both institutions informed consent was obtained from parents and/or children at enrolment. The study was approved by the Institutional Research Ethics Board of the Hospital Sick Children. In Rotterdam, a waiver for REB approval was obtained, as only retrospective data and left-over blood or saliva samples were used.

Clinical variables

Clinical data were retrieved from electronic patient records and paper charts. Data were collected at the day of transplantation; at 1 week, 1, 3, 6, 9 and 12 months and yearly thereafter until December 2009 or until unavailable.

End-points

The primary end-point was renal dysfunction defined by using the estimated glomerular filtration rate (eGFR), calculated according to the Schwartz formula.[24] The severity of CKD was rated based on to the National Kidney Foundation Kidney Disease Outcome Quality Initiative (KDOQI) guidelines for chronic kidney disease. The KDOQI guidelines have categorized the renal function into 5 stages of severity. The first stage is kidney damage with a normal or elevated GFR (>90 ml/min/1.73m^2). The second stage, are patients with kidney damage with a mild decreased GFR (60-89 ml/min/1.73m^2); the third stage is moderate renal failure (GFR 30-59 ml/min/1.73m^2), the fourth stage severe renal failure (15-29 ml/min/1.73m^2) and the fifth stage is complete renal failure (GFR <15 ml/min/1.73m^2 or dialysis).[25] Our secondary end-points were the absolute eGFR and eGFR z-scores. Data were also analyzed using z-scores of eGFR to correct for age-related changes in eGFR. To calculate the z-scores, reference values from a healthy, mainly Caucasian, pediatric population were used.[26]

Other clinical data

Other data collected concerned patients' characteristics, tacrolimus dose schedule and serum concentrations during routine therapeutic drug monitoring, hematology and chemistry laboratory results, concomitant medications, primary diagnosis, transplant information and outcome Concomitant medication information was collected for cyclosporine, gentamicin, cotrimoxazole, tobramycin, amphotericin B, valganciclovir, ganciclovir, spironolactone and vancomycin as they are all potential

nephrotoxins as individual drugs or in conjunction with other nephrotoxic agents. If a patient received more than one heart transplant, the first transplantation was used in the analysis.

Immunosuppressive protocol

Immunosuppression was started according to either hospitals' protocols. Tacrolimus was started at 0.1-0.3 mg/kg/day divided into two doses. Therapeutic drug monitoring was used to achieve a target level of 12-15 ng/ml in the immediate post-transplant period and a target level of 9-12 ng/ml from 3-6 months post-transplantation. After six months post-transplantation, tacrolimus target concentrations were 6-8 ng/ml. Additional immunosuppressive therapy consisted of a maintenance dose of mycophenolate mofetil (600 mg/m^2 twice daily), anti-thymocite globulin (0.5 ml/kg/day up to 7 days) and a tapering steroid schedule.

Tacrolimus analysis

Tacrolimus serum trough concentrations were determined in ethylenediaminetetraacetic acid (EDTA)-treated whole blood (0.25 ml) on the day of sampling, using liquid chromatography tandem mass spectrometry (LC-MS-MS) as previously described, as part of routine clinical care.[27,28] In Rotterdam, tacrolimus whole blood trough concentrations were determined in ethylenediaminetetraacetic acid (EDTA)-treated whole blood (0.50 ml) on the day of sampling, using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), as part of routine care.[29,30,31,32]

Genotyping

Blood or saliva samples were collected from patients at the first visit after informed consent was received or, in Toronto only, by access to DNA samples from either the Hospital for Sick Children's Biobank or the University Health Network HLA laboratory. Blood samples were collected in EDTA-containing tubes. Saliva samples were collected by using Oragene

DNA OG-250 collection kits (DNA genotek, Ottawa, Ontario, Canada). Samples were stored at -80°C before purification of DNA using the QiaSymphony system (Qiagen, USA). Ninety-six SNPs were selected based on previous published associations, SNPs related to tacrolimus pharmacokinetic pathways and SNPs associated with renal failure (Supplemental table 1).DNA samples were genotyped for this custom set of 96 variants using BeadXpress genotyping platform using the manufacturer's protocol (Illumina, CA, USA) at the University of British Columbia, Vancouver, BC, Canada.[33]

Statistical analysis

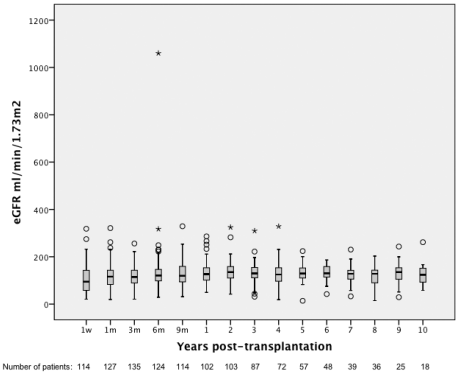
Descriptive data are presented as mean ± standard deviation or median (IQR) for continuous variables and as percentage for categorical variables. Kaplan-Meier curves were plotted for the time course of the CKD incidence. CKD stage was designated as an eGFR below the cut-offs values according to CKD stages on two consecutive visits. A univariate general linear mixed model was performed for the following covariates: sex, age at time of transplantation, investigation site, tacrolimus levels, concomitant medication, year of transplantation, albumin and CRP levels, hematocrit, conversion from cyclosporine and weight and height. Covariates with a p-value < 0.05 were considered for retention in the regression model. The Hardy-Weinberg equilibrium was tested for each polymorphism using the method by Guo et al.[34] Previously identified significant genetic associations with eGFR were tested at a significance level of 0.05. All other genetic associations were tested at a significance threshold of 8.7×10^{-4} determined using a Bonferroni correction based on the effective number of independent tests (M_{eff}).[35] A mixed model for the continuous endpoints was used to test the genetic additive effect of each SNP by adjusting for the covariates retained in the stepwise regression. All statistical analyses for the genetic variables were done using SAS 9.3 (SAS institute Inc, Cary, NC, USA). All other statistical analyses, including figures were done using SPSS version 20.0 (IBM SPSS Statistics, Armonk, NY, USA).

Results

Patient population (Table 1)

One hundred and seventy-four pediatric heart transplant recipients were eligible for inclusion. Informed consent was received for 161 patients and these were included in the study. The median age was 2.3 years (IQR: 9.9) with a median weight of 18.0 kg (IQR: 24.0) (Table 1). The median follow-up time was 0.78 years (range: 7 days – 10 years). The most common primary diagnosis was dilated cardiomyopathy (48.2%). Eleven children (6.8%) had undergone a second transplantation. Twenty-one (13%) patients had passed away (Table 1). Recipient DNA was available for 117 heart transplant recipients.

Figure 1A and 1B: eGFR over time and eGFR z-scores over time



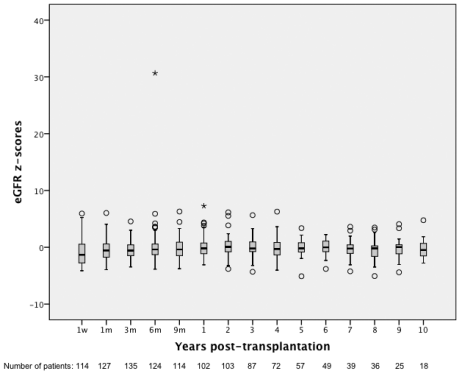


Table 1: Demographics of the patient population

Variable	Heart (All)	Heart (DNA)
Number	161	117
Age at time of transplant (years) Mean ± SD Median (IQR)	 5.2 ± 5.5 2.3 (9.9)	 4.8 ± 5.3 1.9 (8.5)
Year of transplant Median (Q1,Q3) Range	 2005 (2001, 2007) (1994, 2010)	 2004 (2001, 2007) (1995, 2009)
Follow up (year) Mean ± SD Median (IQR)	 2.15 ± 2.61 0.78 (2.88)	 2.16 ± 2.58 0.77 (2.81)
Gender	M: 86 (53.4%) F: 75 (46.6%)	M: 60 (51.3%) F: 57 (48.7%)
Weight (kg) Mean ± SD Median (IQR)	 21.4 ± 2.0 13.7 (25.7)	 19.4 ± 2.1 12.3 (23.3)
Height (cm) Mean ± SD Median (IQR)	 100.9 ± 33.3 90.5 (73.7)	 98.7 ± 38.5 88.0 (69.9)
Transplant (n, %) 1st 2nd	 150 (93.2%) 11 (6.8%)	 112 (95.7%) 5 (4.3%)
eGFR at 1 year post-transplantation Mean ± SD Median (IQR)	 130.9 ± 43.2 126.5 (53.0)	 132.8 ± 44.5 127.9 (53.0)
eGFR z-score at 1 year post-transplantation Mean ± SD Median (IQR)	 -0.3 ± 1.68 -0.19 (2.0)	 0.05 ± 1.73 -0.19 (1.93)
Need for dialysis before transplant (n,%)	0 (0%)	0 (0%)
Converted from CsA (n,%)	49 (30.4%)	32 (27.4%)
Mortality rate (n,%)	21 (13.0%)	15 (12.8%)
Donor age Mean ± SD Median (IQR)	 63.3 ± 5.9 60.5 (30.0)	 65.9 ± 8.2 61.0 (32)
Donor gender	M: 62 (53.0%) F: 54 (47.0%)	M: 45 (52.3%) F: 41 (47.7%)
Deceased (D) or living-related donor (L) (n,%)	D: 22 (13.7%) L: 139 (86.3%)	D: 14 (12.0 %) L: 103 (88.0%)
Cold ischemia time (min) Mean ± SD Median (IQR)	 220.9 ± 118.9 197.0 (176.0)	 234.9 ± 121.9 226.5 (209.8)
Warm ischemia time (min) Mean ± SD Median (IQR)	 65.0 ± 33.4 59.0 (36.0)	 59.3 ± 29.6 57.0 (37.0)
Primary diagnosis Dilated cardiomyopathy Congenital heart disease VSD Congestive heart failure Other	 76 (47.2%) 58 (36.0%) 5 (3.1%) 1 (0.6%) 21 (13.0%)	 58 (49.6%) 42 (35.9%) 3 (2.6%) 1 (0.9%) 13 (11.1%)

Renal failure following cardiac transplantation

None of the patients received dialysis either pre-or post-transplantation. At one year post-transplantation (n=102), the median eGFR was 126.5 (IQR: 53.0) ml/min/1.73m^2 and the median eGFR z-score -0.19 (IQR: 2.0) (Figures 1A and 1B). The incidences of the five CKD stages at the various time points are shown in Figure 2. Fifty-five patients (34.2%) developed CKD stages 3-5 (Figure 3A), i.e. 47 stage 3 (47/161; 29.2%); 7 stage 4 (7/161; 4.3%) and 1 stage 5 (1/161; 0.6%) (Figure 3B). At one year post-transplantation, the prevalence of CKD stage 2 was 8.8% (n=9); that of CKD stage 3 was 2.9% (n=3) (Table 2). At 5 years post-transplantation, 4 patients (7.0%) experienced CKD stage 2 and 1 patient (1.8%) CKD stage 5 (Figure 2).

Figure 2: Incidence of CKD at each time point

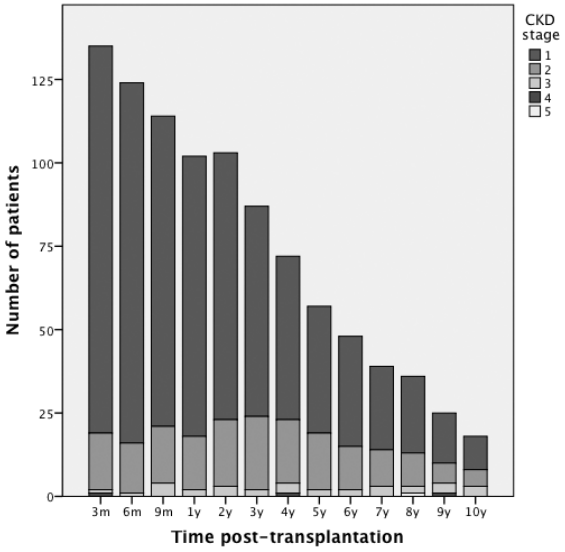
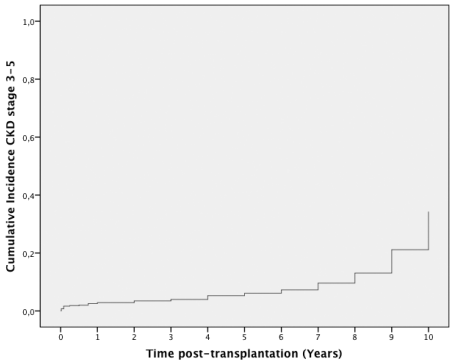
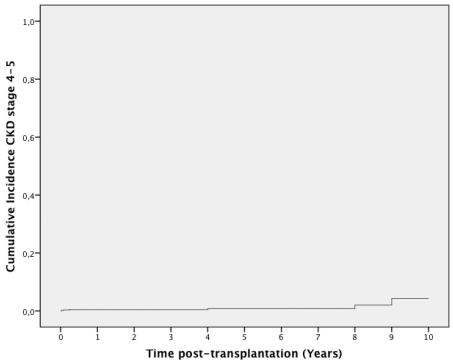
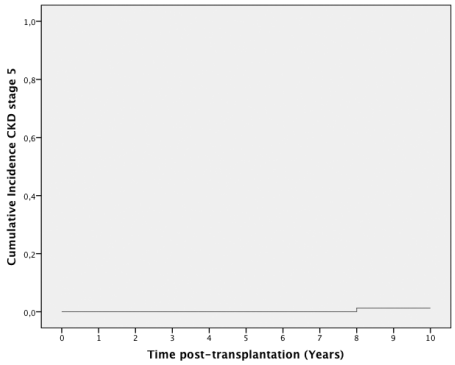
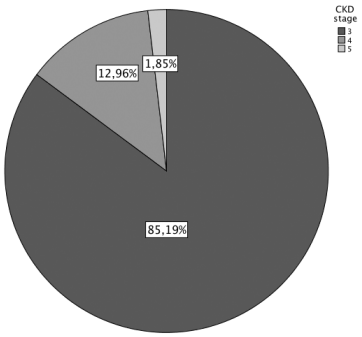


Figure 3: Kaplan-Meier curves of the cumulative incidence of CKD in 161 pediatric heart transplant recipients









Risk factors of renal failure

None of the SNPs deviated from the Hardy-Weinberg equilibrium (Supplementary Table 2). In the univariate analysis, age at the time of transplantation, height and weight, tacrolimus levels, year of transplantation, albumin levels and hematocrit were associated with renal function. As weight and height highly correlated, height only was entered into the multivariate analysis (Table 2). The number of determinations of albumin level was too small to incorporate in the multivariate analysis. In addition to the above-mentioned covariates, sex and investigation site were included in the multivariate analysis.

One polymorphism met the corrected significance level: *APOL1 G1* (rs73885319) showing a positive association (β=52.32; p=0.0001) with eGFR (Table 3). However, only one patient was homozygous for the variant and two patients were heterozygotes. The eGFR of the homozygous patient was significantly higher at all time points compared to the heterozygote and wild-type homozygous patients (Table 4). Nonetheless, at various time points no eGFR values were available for *APOL G1* allele carriers. In addition, the eGFRs of heterozygotes were very similar to wild-type carriers, suggesting the association is most likely a type I error.

Three other SNPs were significant at the significance level of 0.05: *TLR2 R753Q* (rs5743708), *KCNQ1 A/C* (rs2237895) and *ABCB1 G1199T/A* (rs229109). Carriers of the *ABCB1 1199* variant showed higher eGFR levels compared to wild-type carriers (β=10.64; p=0.04). Variant carriers of *TLR2* (β=-54.72; p=0.001) or *KCNQ1* (β=-11.06; p=0.01) had lower eGFR values compared to their wild-type carriers (Table 3). None of these SNPs reached the pre-defined significance threshold of 8.7×10^{-4} (Supplementary table 3)

Table 2: Univariate analysis of the covariates

Variable	N	P-value
Gender	534	0.57
Age at time of transplantation	534	<0.001
Investigation site	534	0.73
Tacrolimus levels	534	<0.001
Concomitant medication	534	0.60
Year of transplantation	534	0.06
Albumin levels	73	<0.001
Hematocrite levels	461	<0.001
CRP levels	25	0.06
Converted to CsA	534	0.52
Weight	196	<0.001
Height	502	<0.001
Height	406	0.11

Table 3: Genetic association results

Chr	Position	Gene	SNP	Genotype counts			N obs	Estimate	SE	P-value of additive genetic effect*
				Minor homozygous	Heterozygous	Major Homozygous				
4	89052323	ABCG2	rs2231142	4	22	91	432	11.43	6.60	0.09
4	154626317	TLR2*	rs5743708	0	6	111	432	-54.72	16.34	0.001
7	81700000	ABCB1 -1236	rs1128503	23	57	37	432	-2.65	4.91	0.59
7	81701000	ABCB1 -2677	rs2032582	24	53	39	431	-3.24	4.75	0.50
7	81702000	ABCB1 -3435	rs1045642	36	49	32	432	5.74	4.50	0.21
7	99270539	CYP3A5	rs776746	6	16	95	432	-0.23	6.46	0.97
10	101611294	ABCC2	rs8187710	0	13	104	432	-12.65	10.66	0.24
11	2857194	KCNQ1*	rs2237895	22	51	44	432	-11.06	4.44	0.014
21	28240574	ABCB1* -1199	rs229109	11	51	55	432	10.64	5.12	0.040
22	36661906	APOL1**	rs73885319	1	2	111	432	52.32	13.13	0.0001

Adjusted for gender, age at transplant, site, tacrolimus level, hematocrit level, and height. Positions based on Genome Build Version 37.1.

* Significance threshold = 0.05

**Significance threshold = 0.00087;

Table 4: eGFR values for each visit by genotype

SNP	Visit	N	eGFR		
			Wildtype	Heterozygotes	Homozygotes
APOL1 G1 (rs73885319)	1 week	54	102.7±47..3		318.2*
	1 month	68	113.0±43.8	141.6±33.8	321.1*
	3 months	51	124.9±47.7	126.3*	
	6 months	28	131.5±47.4		
	9 months	29	113.8±40.3		328.8*
	1 year	73	128.8±45.8		266.2*
	2 years	57	139.4±42.0	138.2*	278.7*
	3 years	50	137.8±27.3	114.6*	309.5*
	4 years	35	133.9±33.8		258.0*
	5 years	25	134.1±28.7		
	6 years	19	132.7±31.6		
	7 years	17	132.5±19.1		
	8 years	15	135.7±36.9		
	9 years	10	139.8±17.3		
	10 years	3	128.9±9.5		

* Only one patient available

Discussion

We evaluated the prevalence of renal failure and potential genetic risk factors for renal function decline in pediatric heart transplant population and found a 34.2% cumulative incidence of CKD stages 3-5, with 7 patients (4.3%) experiencing CKD stage 4 and one patient (0.6%) CKD stage 5. In our recent review of the pediatric literature the prevalence of moderate-severe renal failure (CKD stages 3-5) ranged from 6.8% to 46% and that of mild renal failure (CKD stages 2-5) from 22.7% to 40%.[8] The cumulative incidence of CKD stages 4-5 was much lower than the 18% reported by Ojo et al in adult heart transplant patients.[2]

The prevalence of CKD stages 3-5 at one year post-transplantation was 2.9% and the 1.7% prevalence of CKD stages 3-5 at five years were both lower compared to previous reports.[8] The prevalence of renal dysfunction reported in the Registry Reports of the International Society for Heart and Lung Transplantation from 2006 and 2010 was 10% at 5 and 10 years post-transplantation.[3,4] The discrepancy with our findings may be due to

a higher proportion of patients receiving cyclosporine than tacrolimus in the Registry Reports. Other than previous reports, we established the cumulative incidence of renal failure as well as the prevalence of renal failure at several time points. Throughout the study period, the eGFR and eGFR z-scores were stable. However, several patients had already developed mild CKD (stage 2) early after transplantation, which persisted throughout the study period. Only few patients experienced CKD stage 4 or 5 throughout the study period. We conclude that although the cumulative incidence was high, most patients experienced mild CKD.

One polymorphism met our significance threshold corrected for multiple testing; *APOL1 G1* (rs73885319). Our results showed a positive association of *APOL1 G1* with eGFR. Yet, only one patient was homozygous for the *APOL1 G1* allele and two patients were heterozygous. Therefore, the effect of *APOL1 G1* may be considered to be a patient-related factor rather than a genetic-related factor. Future studies with a higher allele frequency for *APOL* G1 may elucidate this finding. Three other SNPs (*TLR2 R753Q, KCNQ1* rs2237895 and *ABCB1 G1199T/A*) were associated with eGFR at the significance level of 0.05. These findings were probably due to chance as the significance threshold for multiple testing was not reached.
In contrast to results in adult heart transplant recipients, we failed to show an association between *CYP3A5* genotype and renal function.[13] This may be explained by differences in population characteristics and immunosuppressive regimens. Nonetheless, the association reported by De Denus et al[13] is surprising as the majority of the patients received cyclosporine as immunosuppressive therapy and CYP3A5 does not appear to play a major role in the metabolism of cyclosporine. The lack of associations in our study are similar to those reported in both adults and children,[15,18] but these studies pooled patients treated with tacrolimus together with those treated with cyclosporine. We are the first to include a large number of potential relevant SNPs in the analysis.

Our study has several limitations, of which the limited with sample size is the most important as it may obscure subtle genetic effects on renal function. The study design was retrospective and included a candidate gene approach, limiting the data set to the available information in medical records and the identification of genetic risk factors to those genes the investigators thought important. A Genome Wide Association Study could reveal different candidates never before associated with renal failure.
The use of eGFRs may underestimate the extent of renal failure following pediatric heart transplant recipients, as the Schwartz formula is known to overestimate GFR by approximately 20%.[36] Indeed, in 91 pediatric heart transplant recipients the Schwartz formula overestimated the measured GFR (99mTc DTPA method) by 33 ± 26 ml/min/1.73m^2.[37] This difference is approximately equal to one severity group higher on the CKD KDOQI classification. Therefore, measured GFR values are preferable, although this is an invasive procedure requiring a full day at the hospital. As this is quite burdensome it is not routinely done in clinical practice.

In conclusion, we showed that one third of the pediatric heart transplant patients experienced CKD stages 3-5, at least 3 months after transplant at two consecutive time points, which may present an important clinical problem. However, only few experienced CKD stage 4 or 5. None of the SNPs studied were significantly associated with renal function.

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Supplementary Table 1: All SNPs analyzed

Chr	Position	Gene	SNP
1	60392494	C1orf87/CYP2J2	rs890293
1	162030688	OLFML2B/NOS1AP	rs10918594
1	184911126	PTGS2	rs4648279
1	186643541	PTGS2	rs3218625
1	186643836	PTGS2	rs5272
1	186643837	PTGS2	rs5273
1	186650751	PTGS2/PLA2G4A	rs689466
1	186646004	PTGS2	rs3218622
1	206946407	IL19/IL10	rs1800872
1	206946634	IL19/IL10	rs1800871
1	206946897	IL19/IL10	rs1800896
1	223285200	TLR5	rs5744168
2	228052600	COL4A3	rs2204862
2	234669144	UGT1A1	rs4148323
2	241542703	CAPN10	rs5030952
3	52261031	TWF2/TLR9	rs187084
3	119500035	NR1I2	rs3814055
3	119533733	NR1I2	rs6785049
3	119534153	NR1I2	rs2276707
3	119537254	NR1I2	rs3814057
3	121838319	CD86	rs1129055
4	38799710	TLR1	rs4833095
4	38800214	TLR1	rs5743611
4	38830350	TLR6	rs5743810
4	89052323	ABCG2	rs2231142
4	123377980	IL21/IL2	rs2069762
4	154626317	TLR2	rs5743708
4	187004074	TLR3	rs3775291
6	31241109	HLA-C	rs13191343
6	31543031	TNF/LTA	rs1800629
6	32809848	PSMB8	rs9357155
6	32811629	PSMB8	rs2071543
6	43736389	MRPS18A/VEGFA	rs699947
6	43737830	VEGF	rs1570360
6	90032942	UBE2J1/GABRR2	rs2064831
7	22766645	IL6/LOC541472	rs1800795
7	75615006	POR	rs1057868
7	81700000	ABCB1	rs1128503
7	81701000	ABCB1	rs2032582
7	81702000	ABCB1	rs1045642
7	87133470	ABCB1	rs17064
7	87230193	ABCB1	rs3213619
7	99102509	CYP3A5	rs55965422
7	99194259	CYP3A4	rs17161886
7	99245013	ZNF498/CYP3A5	rs4646458
7	99245080	ZNF498/CYP3A5	rs4646457
7	99245914	CYP3A5	rs15524
7	99247772	CYP3A5	rs41279854
7	99250393	CYP3A5	rs41303343
7	99258139	CYP3A5	rs28383479
7	99262835	CYP3A5	rs10264272
7	99270539	CYP3A5	rs776746
7	99273821	CYP3A5	rs55817950
7	99325882	CYP3A7	rs2687136
7	99354114	CYP3A4/CYP3A7	rs12333983
7	99356324	CYP3A4	rs17161886
7	99358524	CYP3A4	rs4986910
7	99360870	CYP3A4	rs4646440
7	99365451	CYP3A4	rs2687117
7	99365983	CYP3A4	rs55785340
7	99366316	CYP3A4	rs35599367
7	99382096	CYP3A4/CYP3A43	rs2740574
7	99388017	CYP3A4/CYP3A43	rs2687102
8	139746208	COL22A1	rs4588898
9	120475302	TLR4	rs4986790
9	120475602	TLR4	rs4986791
9	125133507	PTGS1	rs3842787
9	125140241	PTGS1	rs3842789
9	125143707	PTGS1	rs3842792
9	125143973	PTGS1	rs5789
9	125148791	PTGS1	rs5791
9	125152507	PTGS1	rs5792
9	125152579	PTGS1	rs5793
10	90749963	FAS/ACTA2	rs1800682
10	96798749	CYP2C8	rs10509681
10	96818119	CYP2C8	rs1058930
10	101542578	ABCC2	rs717620
10	101611294	ABCC2	rs8187710
11	2857194	KCNQ1	rs2237895
11	2870108	KCNQ1	rs8234
11	17460712	ABCC8	rs2237982
11	112034988	TEX12/IL18	rs187238
11	112035458	IL18/TEX12	rs1946518
12	21329738	SLCO1B1	rs2306283
12	21331549	SLCO1B1	rs4149056
12	68552522	IFNG	rs2430561
12	79481371	SYT1	rs12300068
16	31104878	VKORC1	rs9934438
17	32579788	ACCN1/CCL2	rs1024611
19	41858921	TGF-beta	rs1800470
21	28240574	ADAMTS5/ADAMTS1	rs229109
22	35775889	HO-1	rs3761439
22	35776672	HMOX1/TOM1	rs2071746
22	36661906	APOL1	rs73885319
22	36662046	APOL1	rs71785313
22	36751101	MYH9	rs11089788
22	36774812	MYH9	rs5756168

A total of 19 SNPs had to be removed from the analysis; 16 SNPs had a allele frequency of 0, two SNPs had more than 5% missing genotypes and one SNP completely failed. A total of 77 SNPs were included in the analysis.

Supplementary Table 2: Hardy-Weinberg equilibrium

Chr	Position	Gene	SNP	Minor allele	Major allele	MAF frequency	H-W p-value	H-W Exact p-value
1	60392494	C1orf87/CYP2J2	rs890293	C	A	0.0828	0.294514598	0.2523
1	162030688	OLFML2B/NOS1AP	rs10918594	G	C	0.3854	0.85275999	1
1	186643541	PTGS2	rs3218625	G	A	0.0016		
1	186650751	PTGS2/PLA2G4A	rs689466	A	G	0.1783	0.266723957	0.3508
1	206946407	IL19/IL10	rs1800872	C	A	0.3051	0.892703129	1
1	206946634	IL19/IL10	rs1800871	G	A	0.2833	0.224341594	0.3039
1	206946897	IL19/IL10	rs1800896	A	G	0.3965	0.469494315	0.5694
1	223285200	TLR5	rs5744168	G	A	0.0462	0.49124341	1
2	228052600	COL4A3	rs2204862	A	G	0.0971	0.677391602	1
2	234669144	UGT1A1	rs4148323	G	A	0.0097	0.96264964	1
2	241542703	CAPN10	rs5030952	G	A	0.0127	0.925707324	1
3	52261031	TWF2/TLR9	rs187084	A	G	0.3706	0.976596504	1
3	119500035	NR1I2	rs3814055	G	A	0.4045	0.968185753	1
3	119533733	NR1I2	rs6785049	A	G	0.4395	0.505925593	0.5728
3	119534153	NR1I2	rs2276707	G	A	0.2086	0.041724997	0.0729
3	119537254	NR1I2	rs3814057	A	C	0.2245	0.006540498	0.0086
3	121838319	CD86	rs1129055	G	A	0.2659	0.072277541	0.0959
4	38799710	TLR1	rs4833095	A	G	0.3567	0.391351606	0.5236
4	38800214	TLR1	rs5743611	G	C	0.0717	0.524598008	1
4	38830350	TLR6	rs5743810	G	A	0.2946	0.797272822	0.84
4	89052323	ABCG2	rs2231142	C	A	0.1067	0.085807717	0.0888
4	123377980	IL21/IL2	rs2069762	A	C	0.3419	0.375008162	0.4317
4	154626317	TLR2	rs5743708	G	A	0.0239	0.775913232	1
4	187004074	TLR3	rs3775291	G	A	0.2771	0.888283895	1
6	31543031	TNF/LTA	rs1800629	G	A	0.1576	0.953675674	1
6	32809848	PSMB8	rs9357155	G	A	0.1178	0.603041666	0.6329
6	32811629	PSMB8	rs2071543	C	A	0.1338	0.446390914	0.4219
6	43736389	MRPS18A/VEGFA	rs699947	C	A	0.4729	0.641539871	0.7136
6	90032942	UBE2J1/GABRR2	rs2064831	A	G	0.1736	0.629176447	1
7	22766645	IL6/LOC541472	rs1800795	C	G	0.3057	0.336271939	0.4201
7	81700000	ABCB1	ABCB1-1236CT-r1128503	G	A	0.4618	0.90111287	1
7	81701000	ABCB1	ABCB1-2677GT-r2032582	C	A	0.4537	0.446590836	0.4563
7	81702000	ABCB1	ABCB1-3435CT-r1045642	A	G	0.4984	0.080819886	0.0928
7	87133470	ABCB1	rs17064	A	T	0.0685	0.49124341	1
7	87230193	ABCB1	rs3213619	A	G	0.0398	0.775913232	1
7	99245013	ZNF498/CYP3A5	rs4646458	A	C	0.1006	0.599685701	0.4655
7	99245080	ZNF498/CYP3A5	rs4646457	A	C	0.1592	0.000147298	0.0013

Chr	Position	Gene	SNP	Minor allele	Major allele	MAF frequency	H-W p-value	H-W Exact p-value
7	99245914	CYP3A5	rs15524	A	G	0.1513	0.106817362	0.1181
7	99262835	CYP3A5	rs10264272	G	A	0.0048	0.925707324	1
7	99270539	CYP3A5	rs776746	G	A	0.1534	0.000147298	0.0016
7	99325882	CYP3A7	rs2687136	A	G	0.1656	0.364329751	0.2901
7	99354114	CYP3A4/CYP3A7	rs12333983	A	T	0.1789	0.026959652	0.0431
7	99356324	CYP3A4	rs17161886	A	C	0.0239	1.58455E-12	0
7	99358524	CYP3A4	rs4986910	A	G	0.0048		
7	99360870	CYP3A4	rs4646440	G	A	0.0525	0.850785939	1
7	99365451	CYP3A4	rs2687117	G	A	0.0143	0.850785939	1
7	99366316	CYP3A4	rs35599367	G	A	0.0430	0.49124341	1
7	99382096	CYP3A4/CYP3A43	rs2740574	A	G	0.0605	0.000370758	0.0099
7	99388017	CYP3A4/CYP3A43	rs2687102	A	G	0.0127	0.888283895	1
8	139746208	COL22A1	rs4588898	G	A	0.2946	0.399086195	0.4975
9	120475302	TLR4	rs4986790	A	G	0.0764	0.312063228	1
9	120475602	TLR4	rs4986791	G	A	0.0701	0.367382156	1
9	125133507	PTGS1	rs3842787	G	A	0.0462	0.510901745	0.4255
9	125143707	PTGS1	rs3842792	A	C	0.0016		
9	125143973	PTGS1	rs5789	C	A	0.0239	0.850785939	1
10	90749963	FAS/ACTA2	rs1800682	A	G	0.4857	0.316701146	0.3491
10	96798749	CYP2C8	rs10509681	A	G	0.0876	0.613470663	1
10	96818119	CYP2C8	rs1058930	C	G	0.0494	0.003880649	0.0343
10	101542578	ABCC2	rs717620	G	A	0.2045	0.041724997	0.073
10	101611294	ABCC2	rs8187710	G	A	0.0717	0.524598008	1
11	2857194	KCNQ1	rs2237895	A	C	0.4204	0.297820652	0.339
11	2870108	KCNQ1	rs8234	A	G	0.3574	0.469593802	0.5597
11	17460712	ABCC8	rs2237982	G	A	0.4061	0.033352558	0.038
11	112034988	TEX12/IL18	rs187238	C	G	0.2468	0.329778441	0.4502
11	112035458	IL18/TEX12	rs1946518	C	A	0.3965	0.786839966	0.8397
12	21329738	SLCO1B1	rs2306283	A	G	0.4475	0.227416861	0.2526
12	21331549	SLCO1B1	rs4149056	A	G	0.1369	0.30566877	0.3786
12	68552522	IFNG	rs2430561	A	T	0.4405	0.801878879	0.8566
12	79481371	SYT1	rs12300068	G	A	0.1354	0.455403676	0.4824
16	31104878	VKORC1	rs9934438	G	A	0.4153	0.617298743	0.7017
17	32579788	ACCN1/CCL2	rs1024611	A	G	0.3408	0.605177623	0.6956
21	28240574	ADAMTS5/ADAMTS1	rs229109	G	A	0.3041	0.867700558	1
22	35776672	HMOX1/TOM1	rs2071746	T	A	0.4441	0.453426626	0.5772
22	36661906	APOL1	rs73885319	A	G	0.0143	1.07086E-07	0.0263
22	36662046	APOL1	rs71785313	A	T	0.0048	0.925707324	1
22	36751101	MYH9	rs11089788	C	A	0.4459	0.617478246	0.705
22	36774812	MYH9	rs5756168	A	G	0.1146	0.603041666	0.6396

Table 3: Genetic associations renal transplant recipients

Chr	Position	Gene	SNP	Genotype counts			N obs	Estimate	SE	P-value of additive genetic effect*
				Minor homozygous	Heterozygous	Major Homozygous				
1	60392494	C1orf87/CYP2J2	rs890293	2	18	97	432	-2.17	7.74	0.78
1	162030688	OLFML2B/NOS1AP	rs10918594	18	57	42	432	4.12	4.91	0.40
1	186643541	PTGS2	rs3218625	0	0	117	432	0		
1	186650751	PTGS2/PLA2G4A	rs689466	2	38	77	432	-6.70	6.49	0.30
1	206946407	IL19/IL10	rs1800872	8	46	62	431	-4.24	5.46	0.44
1	206946634	IL19/IL10	rs1800871	4	45	62	407	-5.95	6.33	0.35
1	206946897	IL19/IL10	rs1800896	17	60	40	432	7.19	4.94	0.15
1	223285200	TLR5	rs5744168	0	14	103	432	9.34	10.69	0.38
2	228052600	COL4A3	rs2204862	1	24	92	432	-6.41	7.57	0.40
2	234669144	UGT1A1	rs4148323	0	1	114	428	-35.51	34.40	0.30
2	241542703	CAPN10	rs5030952	0	2	115	432	-2.83	24.59	0.91
3	52261031	TWF2/TLR9	rs187084	17	55	45	432	1.46	5.00	0.77
3	119500035	NR1I2	rs3814055	20	57	40	432	6.10	4.92	0.22
3	119533733	NR1I2	rs6785049	24	54	39	432	5.25	4.97	0.29
3	119534153	NR1I2	rs2276707	8	30	79	432	7.31	5.80	0.21
3	119537254	NR1I2	rs3814057	10	29	78	432	5.95	5.51	0.28
3	121838319	CD86	rs1129055	12	38	67	432	4.48	4.85	0.36
4	38799710	TLR1	rs4833095	10	55	52	432	-1.07	5.30	0.84
4	38800214	TLR1	rs5743611	0	13	104	432	-6.61	11.07	0.55
4	38830350	TLR6	rs5743810	15	52	50	432	-4.47	4.99	0.37
4	89052323	ABCG2	rs2231142	4	22	91	432	11.43	6.60	0.09
4	123377980	IL21/IL2	rs2069762	13	58	45	428	-1.51	5.37	0.78
4	154626317	TLR2*	rs5743708	0	6	111	432	-54.72	16.34	0.0011
4	187004074	TLR3	rs3775291	9	48	60	432	5.37	5.52	0.33
6	31543031	TNF/LTA	rs1800629	3	32	82	432	6.00	6.76	0.38
6	32809848	PSMB8	rs9357155	2	22	93	432	-1.57	7.42	0.83
6	32811629	PSMB8	rs2071543	3	25	89	432	-2.65	6.76	0.70
6	43736389	MRPS18A/VEGFA	rs699947	27	61	29	432	-3.26	4.95	0.51
6	90032942	UBE2J1/GABRR2	rs2064831	3	36	78	432	-7.18	6.30	0.26
7	22766645	IL6/LOC541472	rs1800795	12	58	47	432	1.38	5.33	0.80
7	81700000	ABCB1	ABCB1-1236CT-r1128503	23	57	37	432	-2.65	4.91	0.59
7	81701000	ABCB1	ABCB1-2677GT-r2032582	24	53	39	431	-3.24	4.75	0.50
7	81702000	ABCB1	ABCB1-3435CT-r1045642	36	49	32	432	5.74	4.50	0.21
7	87133470	ABCB1	rs17064	0	14	103	432	13.90	10.82	0.20
7	87230193	ABCB1	rs3213619	0	6	111	432	4.33	16.11	0.79
7	99245013	ZNF498/CYP3A5	rs4646458	1	15	101	432	1.06	8.82	0.90
7	99245080	ZNF498/CYP3A5	rs4646457	6	16	95	432	-0.23	6.46	0.97

Chr	Position	Gene	SNP	Genotype counts			N obs	Estimate	SE	P-value of additive genetic effect*
				Minor homozygous	Heterozygous	Major Homozygous				
7	99245914	CYP3A5	rs15524	3	19	95	432	-4.65	7.47	0.53
7	99262835	CYP3A5	rs10264272	0	2	115	432	4.94	42.91	0.91
7	99270539	CYP3A5	rs776746	6	16	95	432	-0.23	6.46	0.97
7	99325882	CYP3A7	rs2687136	2	19	96	432	-3.17	8.08	0.70
7	99354114	CYP3A4/CYP3A7	rs12333983	4	19	94	432	-0.47	6.73	0.94
7	99356324	CYP3A4	rs17161886	3	3	111	432	13.71	9.47	0.15
7	99358524	CYP3A4	rs4986910	0	0	117	432	0		
7	99360870	CYP3A4	rs4646440	0	4	113	432	-23.04	18.09	0.21
7	99365451	CYP3A4	rs2687117	0	4	113	432	0.90	21.34	0.97
7	99366316	CYP3A4	rs35599367	0	14	103	432	-5.69	11.39	0.62
7	99382096	CYP3A4/CYP3A43	rs2740574	3	10	104	432	-12.41	8.95	0.17
7	99388017	CYP3A4/CYP3A43	rs2687102	0	3	114	432	-12.38	24.14	0.61
8	139746208	COL22A1	rs4588898	8	52	57	432	5.75	5.47	0.30
9	120475302	TLR4	rs4986790	0	20	97	432	9.34	8.79	0.29
9	120475602	TLR4	rs4986791	0	18	99	432	5.12	9.04	0.57
9	125133507	PTGS1	rs3842787	1	14	102	432	-2.70	8.76	0.76
9	125143707	PTGS1	rs3842792	0	0	117	432	0		
9	125143973	PTGS1	rs5789	0	4	113	432	5.49	17.75	0.76
10	90749963	FAS/ACTA2	rs1800682	20	63	34	432	6.19	5.00	0.22
10	96798749	CYP2C8	rs10509681	1	25	91	432	2.26	7.50	0.76
10	96818119	CYP2C8	rs1058930	2	9	106	432	-0.88	9.30	0.92
10	101542578	ABCC2	rs717620	8	30	79	432	-1.64	5.44	0.76
10	101611294	ABCC2	rs8187710	0	13	104	432	-12.65	10.66	0.24
11	2857194	KCNQ1*	rs2237895	22	51	44	432	-11.06	4.44	0.014
11	2870108	KCNQ1	rs8234	18	51	48	432	1.33	4.89	0.79
11	17460712	ABCC8	rs2237982	27	46	44	432	-0.16	4.46	0.97
11	112034988	TEX12/IL18	rs187238	5	47	65	432	4.21	5.97	0.48
11	112035458	IL18/TEX12	rs1946518	18	54	45	432	0.10	4.93	0.98
12	21329738	SLCO1B1	rs2306283	25	51	41	432	5.95	4.65	0.20
12	21331549	SLCO1B1	rs4149056	3	23	91	432	-3.45	6.70	0.61
12	68552522	IFNG	rs2430561	22	59	36	432	3.09	5.07	0.54
12	79481371	SYT1	rs12300068	4	29	84	432	-4.21	6.33	0.51
16	31104878	VKORC1	rs9934438	21	54	42	432	-5.08	4.69	0.28
17	32579788	ACCN1/CCL2	rs1024611	16	51	50	432	-0.21	5.03	0.97
21	28240574	ADAMTS5/ADAMTS1*	rs229109	11	51	55	432	10.64	5.12	0.040
22	35776672	HMOX1/TOM1	rs2071746	20	61	35	431	2.39	4.95	0.63
22	36661906	APOL1**	rs73885319	1	2	111	432	52.32	13.13	0.0001
22	36662046	APOL1	rs71785313	0	2	115	432	-27.18	26.84	0.31
22	36751101	MYH9	rs11089788	24	55	38	432	1.58	4.71	0.74
22	36774812	MYH9	rs5756168	2	22	93	432	2.08	7.57	0.78

Urinary NGAL levels early after pediatric kidney and liver transplantation: a pilot study

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Submitted

Abstract

Background Urinary neutrophil gelatinase-associated lipocalin (uNGAL) is a promising biomarker to monitor acute kidney injury (AKI). However, the pattern of uNGAL levels in the first 14 days after pediatric transplantation is not known. We aimed to describe the pattern of uNGAL levels in this population.

Method In this pilot study, we evaluated daily uNGAL, lnNGAL and uNGAL/Cr levels up to 14 days in 10 kidney (KT) and 12 liver transplant recipients (LT) receiving tacrolimus. uNGAL cut-offs of 2.2 and 135 ng/ml were used, as previously found in healthy children (<2.2 ng/ml) and children at risk for AKI (>135 ng/ml).

Results In KT recipients, the median uNGAL level was 14.9 (IQR: 29.6) ng/ml and the median uNGAL/Cr level 161.3 (IQR: 294.1) ng/mg. Sixty-two of the uGNAL measurements (92.5%) were above the 2.2 ng/ml and three (4.5%) in two KT patients were above 135 ng/ml. All KT patients had an uNGAL >2.2 ng/ml at least once during the first 14 days after transplantation. In LT recipients, the median uNGAL level was 13.6 (IQR: 21.9) ng/ml and the median uNGAL/Cr level 43.6 (IQR: 96.8) ng/mg. Ninety percent of all uNGAL measurements were above the 2.2 ng/ml. Seven uNGAL measurements (7%) in two LT patients were above the uNGAL 135 ng/ml.

Conclusion This is the first study in pediatric solid organ transplant recipients reporting uNGAL levels up to 14 days post-transplantation. Most liver and kidney recipients have higher than normal uNGAL levels, but infrequently uNGAL levels that were previously associated with AKI.

Introduction

Neutrophil gelatinase-associated lipocalin (NGAL) is a 25-kDa protein and member of the lipocalin superfamily. It is bound to gelatinase from neutrophils and expressed at low levels in the human kidney, lung and gastro-intestinal tract.[1,2] Serum NGAL is scarcely excreted in the urine as it is filtered by the glomerulus but then captured by the proximal tubule. Urinary NGAL is produced in the thick limbs of Henle and the collecting ducts and exclusively excreted in the urine.[3,4,5] The synthesis of NGAL is markedly induced in injured proximal tubular epithelial cells.[3,6]

Urinary NGAL can be detected early after transplant surgery. In adult kidney transplant (KT) recipients, uNGAL appears to be a good predictor of delayed graft function (OR = 5.1)[7] or acute rejection (ROC_{AUC} = 0.98).[8] In adult liver transplant (LT) recipients the uNGAL/urinary creatinine ratios appears to correlate with the occurrence of Acute Kidney Injury (AKI) within a few hours post-transplantation.[9,10] This mechanism suggests a role for NGAL following AKI, where the initial damage is targeted to the renal tubules. A previous animal study has shown that NGAL can be detected in the urine within 2 hours following ischemic kidney injury.[3]

In children undergoing cardiopulmonary bypass surgery for congenital heart disease, uNGAL at 2 hours post surgery was an independent predictor for AKI.[11,12,13,14] Additionally, the increase in uNGAL was more pronounced at 4 hours (25-fold) and 6 hours (26-fold) after surgery in patients who developed AKI.[12] The odds ratio (OR) for AKI increased by 32% for every 10 ng/ml increase in 2-hour uNGAL (OR: 1.32).[13] Furthermore, pediatric septic patients had a higher mean and peak uNGAL concentration than patients without sepsis.[14]

With regard to pediatric transplant recipients even fewer data are available. In pediatric, deceased donor, KT recipients (8-19 yrs) NGAL staining of the kidney transplant appears to be an early predictor for kidney failure.[15] NGAL staining intensity was significantly associated with both peak post-transplant SCr levels and dialysis requirements 2-3 days post-transplantation.[15] Furthermore, every 100 ng/mg increase in uNGAL/Cr in

the first 24 hours after KT was associated with a 20% increase in the odds of delayed graft function in a mixed population of adults and children.[16] In 79 pediatric heart transplant recipients at least 3 months post-transplantation with chronic kidney disease, 87% had an uNGAL level above 2.2 ng/ml[17] a level considered as the cut-off value in healthy children.[11]

Hence, a possible relationship between early uNGAL levels and the renal outcome of the patients has been reported. However, no study looking into patterns and reference levels of uNGAL has been done in pediatric liver or kidney transplant recipients up to 14 days post-transplantation. Therefore, the aim of this pilot study was to determine the pattern of uNGAL concentrations in this population.

Methods

Patients and setting

This was a prospective cohort study approved by the Institutional Research Ethics Board of the Hospital for Sick Children, Toronto, Ontario, Canada. This study was conducted in accordance with the ethical standards of the Helsinki Declaration. Patients were eligible for participation if they were 0-18 years of age at the time of their liver or kidney transplantation and if they received tacrolimus during the first 14 days following transplantation. This study was done in the Intensive Care Unit of the Hospital for Sick Children, Toronto, Ontario, Canada. Informed consent was obtained from all parents/legal guardians and/or children.

Immunosuppressive protocol

The transplant physician started all patients on tacrolimus, methylprednisolone and mycophenolate mofetil (MMF). The tacrolimus starting dose was 0.1 mg/kg orally twice daily for all patients. Therapeutic drug monitoring was used to adjust the tacrolimus dose to a target level of 10-15 ng/ml for both liver and kidney transplant recipients within the first two weeks after transplantation. Methylprednisolone (10 mg/kg) was intravenously administered at the time of graft reperfusion and gradually tapered after transplant. The use of induction therapy was left to the discretion of the attending physician.

Clinical data collection

All data were collected up to 14 days post-transplantation or for the duration of hospital stay in case a patient was discharged home before week 2. Patient's age, sex, weight, transplant type, tacrolimus dose and co-medication as well as SCr and tacrolimus concentrations were retrieved from the electronic patient chart. Urine aliquots (10ml) were taken daily (when practically feasible) from 24-hour urine collections for urinary NGAL (ng/ml) and urinary creatinine (mmol/L) for up to 14 days post-transplantation.

Laboratory measurements

Urine was stored at -80°C until urinary NGAL levels were determined using the NGAL kit from Abbott on an ARCHITECT® analyzer (Abbott Diagnostics, USA) at the Department of Clinical Chemistry, Erasmus MC, Rotterdam, the Netherlands. Tacrolimus blood concentrations were determined in ethylenediaminetetraacetic acid (EDTA)-treated whole blood (0.25 ml) on the day of sampling, using liquid chromatography tandem mass spectrometry (LC-MS-MS) as previously described.[18] Serum and urinary creatinine concentrations were measured by the hospital clinical laboratory.

Statistical analyses

Data are presented as mean plus standard deviation in case of a Normal distribution or as medians plus interquartile range (IQR) when data were not normally distributed. We determined the proportion of samples and patients with an uNGAL level above 2.2 ng/ml, which was reported to be the upper level of the healthy pediatric population.[17] A cut-off of >135.0 ng/ml was used to determine the proportion of patients with an increased risk of developing AKI.[19] Even though this cut-off value was established with an ELISA assay in children, no cut-off value for children is known for the ARCHITECT assay. To establish a cut-off for the ARCHITECT assay we have used the formula by Grenier et al which resulted in a cut-off >129.8 ng/ml.[20] Groups were compared by using the Chi-square test. All analyses were done using PASW 20.0 software (IBM, Chicago, IL, USA).

Results

Liver transplant recipients

Twelve LT recipients (5 male, 7 female) with a median age at the time of transplant of 5.5 (IQR: 14.8) years and a median weight of 20.4 (IQR: 43.1) kg were included in the study (Table 1). By day 14, ten patients had been discharged. Three patients received basiliximab and one patient received thymoglobulin.

Table 1: Demographics of the patients

Variable	**All** (n=22)	**Liver** (n=12)	**Kidney** (n=10)
Gender (M/F)	13/9	5/7	8/2
Age at time of transplant (years)	15.3 (IQR: 15.6)	5.5 (IQR: 14.8)	17.1 (IQR: 8.9)
Weight (kg)	28.0 (IQR: 44.1)	20.4 (IQR: 43.1)	43.0 (IQR: 45.3)
Days in ICU (days)	3.0 (IQR: 4.5)	5.0 (IQR: 8.3)	2.5 (IQR: 2.5)
Primary diagnosis			
Biliary atresia	5 (22.7%)	5 (41.7%)	-
Genetic	3 (25.0%)	2 (16.7%)	1 (10.0%)
Auto-immune	3 (25.0%)	2 (16.7%)	1 (10.0%)
Hepatoblastoma	1 (4.5%)	1 (8.3%)	-
Congenital	2 (9.0%)	-	2 (20.0%)
Glomerulonephritis	1 (4.5%)	-	1 (10.0%)
Unknown	1 (4.5%)	-	1 (10.0%)
Nephropathy	4 (18.2%)	-	4 (40.0%)
Donor			
Deceased	15 (68.2%)	12 (100.0%)	3 (30.0%)
Living-related	3 (13.6%)	0 (0.0%)	3 (30.0%)
Unknown	4 (18.2%)		4 (40.0%)
Retransplantation (yes/no)	1/22	0/12	1/10

All data are presented as median and interquartile range (IQR), unless noted otherwise.

The median uNGAL level was 13.6 (IQR: 21.9) ng/ml (Figure 1A), the median uNGAL/Cr level 43.6 (IQR: 96.8) ng/mg up to 14 days after transplantation. The daily NGAL levels can be found in Table 2a and Figure 1. On day 2, 3 and 6, NGAL levels were available for 9 out of the 12 patients. On day 2, 3 and 6; the median uNGAL levels were 12.1 (IQR: 16.2) ng/ml, 9.8 (IQR: 22.8) ng/ml and 13.8 (41.0) ng/ml, respectively.

Figure legends

Figure 1: Time profile of A) uNGAL levels (ng/ml) B) uNGAL/Cr levels (ng/mg) on a logarithmic scale and C) lnNGAL levels (ng/ml) in the first 14 days post-transplantation in pediatric liver transplant recipients.

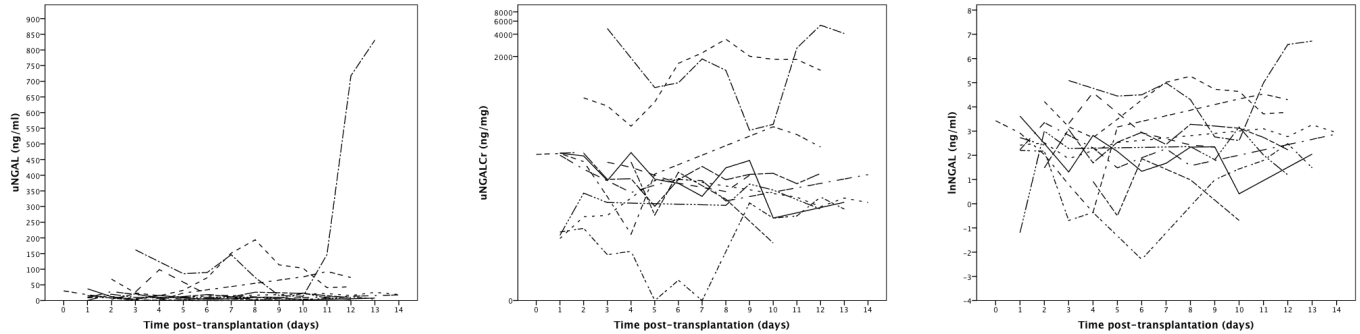


Table 2a: uNGAL levels liver transplant group

Study day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
N	1	7	9	9	8	8	9	5	8	7	8	7	7	4	2
uNGAL(ng/ml)	30.80*	11.3 (9.5)	12.1 (16.2)	9.8 (22.8)	7.8(15.3)	12.6 (25.0)	13.8 (41.0)	11.6 (141.4)	11.0 (55.5)	10.5 (18.6)	18.2 (60.6)	22.2 (85.3)	15.9 (64.1)	17.0 (624.9)	18.4± 0.7
uNGAL/Cr (ng/mg)	93.9*	91.4 (90.6)	74.7 (78.6)	40.9 (233.0)	52.9 (80.6)	41.8 (351.7)	42.3 (429.3)	64.1 (2007.0)	35.4 (967.1)	49.8 (163.0)	40.8 (224.1)	37.0 (1790.1)	51.4 (1279.0)	21.8 (3051.4)	34.8± 20.7
lnNGAL (ng/ml)	3.4*	2.4 (0.7)	2.45 (1.1)	2.3 (2.2)	2.0 (2.8)	2.5 (1.8)	2.6 (2.0)	2.5 (3.0)	2.4 (2.3)	2.4 (1.4)	2.9 (3.4)	3.1 (2.5)	2.8 (2.1)	2.7 (4.2)	2.9± 0.04

All data are presented as median and interquartile range (IQR), unless noted otherwise. In cases where only two samples are available, the mean and standard deviation are given. * n=1

Within the study period 90% of all uNGAL measurements (90/100) were above 2.2 ng/ml. All patients (100%) had an increased uNGAL (> 2.2 ng/ml) at least once during the first 14 days after transplantation.
No significant difference in having increased serum creatinine levels was found between the groups with or without uNGAL levels above 2.2 ng/ml ($X^2 = 0.34$, $P = 0.73$).

Table 3a: Cut-off levels and increased serum creatinine levels in the liver transplant recipients

	Increased Serum Creatinine	
uNGAL>2.2 ng/ml	**Yes**	**No**
Yes	3	87
No	0	10
uNGAL >135 ng/ml	Yes	No
Yes	0	7
No	3	90

Seven uNGAL measurements (7/100) in two patients were above the uNGAL cut-off of 135 ng/ml. Patients with or without an uNGAL above 135 ng/ml were not significantly different in having increased serum creatinine concentrations ($X^2 = 0.23$, $P = 0.80$) (Table 3a). As this cut-off suggest a high risk of kidney injury, we also looked at the serum creatinin levels of these patients. One of these patients experienced uNGAL levels above 135 ng/ml on day 7 and 8 with increased serum creatinine levels on day 9, 11 and 13. Yet, the second patient did not experience increased serum creatinine levels after having increased uNGAL (>135 ng/ml) on day 3, 7, 11, 12 and 13.
To correct for the use of a different assay, we also determined the proportion of patients with an uNGAL level >129.8 ng/ml. However, the results did not change with this new cut-off.

Kidney transplant recipients
We included ten KT recipients (8 male, 2 female) with a median age at time of transplantation of 17.1 (IQR: 8.9) years and a median weight of 43.0 (IQR: 45.3) kg (Table 1). Two patients were still admitted at day 14 post-transplantation, the other patients had been discharged home. Two patients received thymoglobulin.

The median uNGAL level was 14.9 (IQR: 29.6) ng/ml (Figure 2C), the median uNGAL/Cr level 161.3 (IQR: 294.1) ng/mg and the median lnNGAL level was 2.7 (IQR: 1.7) ng/ml for the first 14 days after transplantation. On day 4 and 5, NGAL levels for 8 out of the 10 kidney transplant patients were available. The daily levels for every day are found in Table 2b and Figure 2. On day 4 and 5, the median uNGAL levels were 10.0 (IQR: 22.1) ng/ml and 6.5 (IQR: 9.3) ng/ml, respectively.

Sixty-two of the uGNAL measurements (62/67; 92.5%) were above the 2.2 ng/ml. One hundred percent of the patients had an uNGAL >2.2 ng/ml at least once during the first 14 days after transplantation. Patients with an increased serum creatinine did not have an uNGAL above 2.2 ng/ml more frequently than patients without an increased serum creatinine ($X^2 = 2.30$, $P = 0.16$). Three uNGAL measurements (3/67; 4.5%) in two patients were above the 135 ng/ml (Table 3b).These results were also not significantly different ($X^2 = 1.34$, $P = 0.34$).

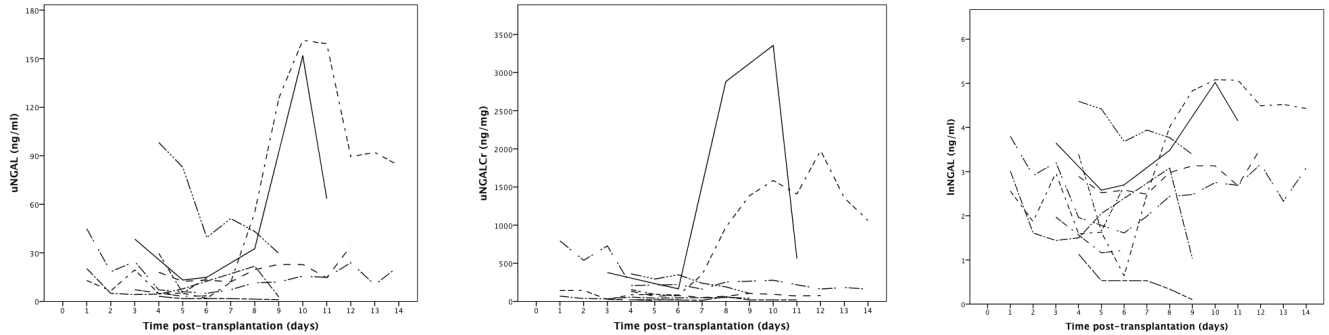
None of the two patients with an increased uNGAL levels (>135 ng/nl) showed an increase in serum creatinine levels thereafter. The results did not change when using the for assay-corrected uNGAL cut-off of 129.8 ng/ml.

Table 2b: uNGAL levels kidney transplant group

Study day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
N	0	3	3	5	8	8	7	5	7	6	4	4	3	2	2
uNGAL(ng/ml)		20.3 (32.0)#	6.5 (13.3) #	19.5 (25.9)	10.0 (22.1)	6.5 (9.3)	13.2 (11.5)	11.9 (27.2)	21.8 (31.7)	17.5 (51.3)	87.3 (141.5)	39.2 (120.6)	34.2 (65.6) #	51.20 ± 57.8	52.9 ± 44.0
uNGAL/Cr (ng/mg)		142.6 (724.8) #	143.7 (502.4) #	33.8 (526.3)	143.5 (259.3)	79.3 (171.1)	84.0 (149.3)	161.3 (261.5)	191.0 (910.5)	105.8 (509.8)	931.5 (2774.6)	389.7 (1087.6)	162.5 (1900.5) #	769.5 ± 830.8	610.7 ± 636.6
lnNGAL (ng/ml)		3.0 (1.2) #	1.9 (1.3) #	3.0 (1.7)	2.3 (1.7)	1.9 (1.3)	2.6 (1.5)	2.5 (2.0)	3.1 (1.3)	2.8 (3.0)	4.1 (2.2)	3.4 (2.2)	3.5 (1.3) #	3.4 ± 1.5	3.8 ± 1.0

All data are presented as median and interquartile range (IQR), unless noted otherwise. In cases where only two samples are available, the mean and standard deviation are given.
A range is given instead as n=3

Figure 2: Time profile of A) uNGAL levels (ng/ml) B) uNGAL/Cr levels (ng/mg) and C) lnNGAL levels (ng/ml) in the first 14 days post-transplantation in pediatric renal transplant recipients



Discussion

This is the first study reporting daily urinary NGAL levels up to 14 days post-transplantation in pediatric LT and KT recipients. We present uNGAL levels for every day in the first two weeks post-transplantation. In both the liver and kidney transplant patients a tentative pattern can be seen where the uNGAL levels initially decrease, only to start to increase again on day 10 in liver transplant recipients and day 8 in kidney transplant recipients. However, due to the limited sample size we have not been able to test this pattern statistically.

All patients had at least one uNGAL level >2.2 ng/ml during the first 14 days after transplantation, which is similar to the 87% reported by Abraham et al. at least 3 months post-transplantation in pediatric heart transplant recipients with chronic kidney disease.[17] These results show that the majority of our patients exhibited increased NGAL levels for the first 14 days post-transplantation compared to the healthy pediatric population.

These higher than normal uNGAL levels are not unexpected and may be due to pre-existing renal disease,[21] systemic or urinary tract infections or chronic kidney disease from the native kidneys.[22] Furthermore, a liver transplantation is a major surgical procedure and associated with hemodynamic changes possibly affecting the kidney that could account for the high uNGAL levels right after transplantation.[9]

Our results are in line with adult data showing similar uNGAL levels in the first week following transplantation.[7,8,9,10,16] However, most of the adult studies only present data within the first 24 hours, showing high uNGAL levels in the immediate post-transplant period. Unfortunately, we only had 1 patient with an uNGAL level at day 0 and therefore our results can not be compared with the literature.

As can be seen in figures 1 and 2 two liver and two kidney patients experienced high uNGAL levels (>135 ng/ml) in the last couple of days of the two week follow-up period. Interestingly, only one liver transplant

recipient experienced increased serum creatinine levels following those days. We speculate that either, the 135 ng/ml cut-off for AKI may be used directly (<6hrs) after transplant but is less useful thereafter, or, that the effect of kidney injury, as diagnosed by an increased NGAL level, on kidney function as diagnosed by an increase in serum creatinin further lags behind than one or three days.

This study has several limitations. The follow-up of our patients was only for 14 days limiting the possibility of determining the long-term outcome of the patients. Additionally, we were underpowered to conduct significant statistical analysis to better elucidate the predictive effect of uNGAL (i.e. ROC curves).
Even though the cut-off of uNGAL used in our population is determined by an ELISA assay in the original study,[17] we have tried to correct for this by converting the cut-off levels by using the formula reported by Grenier et al.[20] The results did not change by using this new cut-off. Nevertheless, the ELISA cut-off determined in the meta-analyses by Haase et al.[19] was based on studies reporting uNGAL levels within the first 6 hours post-surgery to determine the predictive value of uNGAL for renal function during follow-up time. Consequently, the predictive value of uNGAL levels in the first 14 days post-transplantation still needs to be established. Moreover, it would be favourable to have cut-off established by the ARCHITECT assay itself as this assay is also one of the assays used in clinical laboratories in contrast to the ELISA assays.

Conclusion

This is the first study in children reporting uNGAL levels in the first 14 days post-transplantation. This pilot study shows that a large proportion of pediatric LT and KT recipients have higher than normal uNGAL levels, but not necessarily above the increased uNGAL level (>135 ng/ml) in the first week after transplantation.

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General Discussion

Personalized tacrolimus therapy in pediatric solid organ transplant recipients: current state and future directions.

Solid organ transplantation

With the first successful kidney transplantation in 1954, a new treatment for end-stage organ failure was introduced. At this time, transplantation is the treatment of choice in adults and children for end-stage organ failure.[1]

The number of transplantations performed each year is still increasing, reaching about a thousand transplantations done in 2011 in Ontario, Canada (Trillium Gift of Life reports). At the Hospital for Sick Children in Toronto 71 children received a solid organ transplant, with the majority a liver or kidney transplant. In the same year in the Netherlands, also about a thousand transplantation were done, however, only a small portion was done in children (n=25) according to the reports by Eurotransplant (www.eurotransplant.org).

The introduction of immunosuppressive therapy (azathioprine, calcineurin inhibitors) has significantly improved the short-term outcome of transplant recipients.[1] Yet, the long-term outcome (patient and graft survival) of pediatric transplant recipients has remained stable.[2,3]

Need for research in children

Over the last few decades we have realized that children have different needs when administering drugs and they may react different to drugs compared to adults.[4] However, far less information is available for the use of drugs in children compared to the use adults. This lack of evidence based knowledge has been recognized internationally, resulting in a number of new legislations both in the USA (best pharmaceuticals for children act 2007) and in Europe (EMA 2007). Unfortunately, even today, off-label and unlicensed use of pediatric drugs is very common.[5,6]

Administration of appropriate dose schedules to children is challenging due to the many developmental changes. The different processes involved in the absorption of drugs (e.g. gastric pH, gastric emptying, intestinal transit time, drug transport and metabolism) have not been extensively studied in a systematic way, but seem to show different developmental patterns.[4,7] In addition, the body composition changes with age, with relatively higher water in neonates. Water soluble drugs, such as aminoglycosides, need to be dosed higher to reach similar concentrations.[4] The amount of circulating plasma protein levels in neonates and young infants is lower compared to adults, which could lead to a difference in the distribution of drugs that are highly protein bound.[4] The metabolism of drugs is done by Phase I and Phase II metabolizing enzymes. Both groups undergo maturation processes that appear to be isoform specific.[8,9,10] Due to these developmental changes drug metabolism mediated clearance may differ among children as well as the specific metabolites formation.[11] Drugs are eliminated either by renal or bile excretion. The clearance of renal cleared drugs is lower in neonates, as both GFR and also renal secretion are immature at birth.[4]

Prescribing and administrating drugs in children is even more complicated in patients with complex diseases. Renal failure, hepatic dysfunction and cardiac failure may all lead to altered drug clearance and are associated with critical illness.[12] The inflammatory processes underlying critical illness also contribute to the altered drug disposition due to a down regulation of major metabolizing enzymes.[13] However, data on changes required in the drug treatment of these children is lacking.[13]

In conclusion, simply extrapolating adult dosing guidelines to children may expose them to ineffective or drug dose schedules. Hence, it is key to study children in addition to adults for the disposition and the therapeutic/toxic effects of drugs.[14]

Tacrolimus in solid organ transplantation

Being introduced in 1995,[15] tacrolimus (a calcineurin inhibitor) has become the drug of choice in pediatric solid organ transplant recipients.[2,3] Although extensively studied in adults, data in children on the disposition and effect of tacrolimus in solid organ transplantation are scarce.

Tacrolimus is a highly protein bound drug with variable absorption rates.[15] It is metabolized by CYP3A4 and CYP3A5, and is a substrate for the efflux pump, ABCB1.[16] Developmental changes in absorption, distribution and metabolism could therefore affect tacrolimus disposition, however, data on this is currently lacking.

Developmental changes do not only impact the way the body handles the drug (pharmacokinetics), but also how the drug impacts the body (pharmacodynamics). The mechanism of action of tacrolimus involves the inhibition of T-cells by blocking the signalling pathway.[17] However, the immune system also undergoes developmental changes with high T-cell levels in the first year of life and decreases afterwards to reach adult levels in school children.[18] This immunological maturation may also impact tacrolimus efficacy. Interestingly, the therapeutic range for tacrolimus has never been determined in children of different ages.

Inter- and Intra-variability in tacrolimus disposition

Genetically driven inter-patient variability has been reported in tacrolimus disposition in children (***Chapter 4-6***).[19,20] Intra-patient variability further complicates the patient management and increases the risk of graft failure or toxicity.[17,21] Understanding factors that cause this variability will aid in a better management of the individual patient.

Tacrolimus disposition in children

Kidney transplant recipients

We previously showed that renal kidney transplant patients younger than 5 years of age need higher bodyweight-normalized tacrolimus levels than older children.[19] Also, supra-therapeutic tacrolimus levels have been reported more often in older children (<12 years)[22] and the tacrolimus area under the concentration-time curve was significantly larger in older children compared to children less than 6 years of age.[23] Body weight and hematocrit levels have also been related to the apparent oral clearance (CL/F) of tacrolimus.[24,25]

In addition to age, *CYP3A5* expressers (*CYP3A5*1* allele carriers) have higher weight normalized oral clearance, resulting in higher tacrolimus dose requirements to achieve similar tacrolimus trough concentrations,[24,19,26,27,28] and lower tacrolimus trough levels[26] compared to *CYP3A5* non-expressers (*CYP3A5*3/*3* carriers).[29] In contrast, no relationship has been found for *CYP3A4*1B, ABCB1* and *ABCC2* genotypes and tacrolimus dispositions in pediatric kidney recipients.[19,25,28]

The effect of age and *CYP3A5* genotype in children show an important aspect of the dangers in extrapolating adult pharmacogenetic data to children. In particular, we showed previously that *CYP3A5*-expressers younger than 5 years of age need four times higher tacrolimus doses compared to *CYP3A5* non-expressers older than 5 years of age, as for each of the factors alone (age or *CYP3A5* genotype), the effect is two-fold.[19]

The variability in tacrolimus disposition can only partly be explained by genetic variation in *CYP3A5*. Hence, polymorphisms in the gene encoding the P450 oxidoreductase (POR) protein that enables the activity of CYP enzymes may be of interest. We show (***Chapter 6***) that in addition to the *CYP3A5* genotype, the *POR*28* allele is also related to tacrolimus disposition. *CYP3A5*-expressers also carrying at least one *POR*28* allele had on average 18.3% lower tacrolimus trough concen-

trations and 20.3% lower concentration/dose ratios (C/D) compared to *CYP3A5*-expressers carrying the *POR*1/*1* genotype. However, this additional influence could not be found in *CYP3A5* non-expressers, suggesting that an interaction between POR and CYP3A5 is needed to show the additional effect of the *POR* genotype on tacrolimus disposition.

Heart transplant recipients

Similar to kidney transplant recipients, a significant negative correlation exists between age and weight-normalized tacrolimus dosing requirements (r^2 = -0.447, p = 0.004) and a positive correlation between age and the C/D ratio (r^2 = 0.351, p = 0.029) in the first 14 days after heart transplantation (***Chapter 4***). In addition to a confirmation of the influence of age, we have also been able to confirm previous findings by Zheng et al.[30] that *CYP3A5*-expressers needed higher tacrolimus doses (mg/kg) and had lower weight-normalized C/D ratios compared to *CYP3A5* non-expressers. An important new finding was the observation that age and *CYP3A5* genotype were independent factors related to tacrolimus disposition. No significant effect of the *ABCB1* genotype could be found (***Chapter 4***).[20] In 2010, a novel single nucleotide polymorphism (SNP) in *CYP3A4 (CYP3A4*22)* was discovered, associated with decreased CYP3A4 mRNA expression and activity.[31] We are the first to report that in addition to the *CYP3A5* genotype, this new SNP also influences tacrolimus dosing requirements in (pediatric) heart transplant recipients (***Chapter 4***). Moreover, when combining the *CYP3A4*22* genotype with the *CYP3A5* genotype the results were more pronounced with poor metabolizers (*CYP3A4*1/*22* and *CYP3A5* non-expresser) needing significantly less tacrolimus and showed higher C/D ratios compared to intermediate (*CYP3A4*1/*1* and *CYP3A5* non-expresser) and extensive metabolizers (*CYP3A4*1/*1* and *CYP3A5*-expresser) and had higher C/D ratios (***Chapter 5***). The more pronounced effect of the combined *CYP3A* genotypes suggests that patients should be screened for these genotypes a priori to establish a more individualized tacrolimus dosing regimen. The age of the

children should also be incorporated into the dosing adaptations to further optimize the immunosuppressive treatment of pediatric heart transplant recipients.

Liver transplant recipients

Similar to pediatric kidney and heart transplant recipients, higher body-weight corrected tacrolimus doses are needed in younger (< 5 years) pediatric liver transplant recipients. These children also show higher clearance rates compared to older children.[19,32,33] In addition, the apparent total clearance (CL/F) is higher in pediatric whole liver transplant recipients, but decreases with higher aspirate aminotransferase (AST) values, and increases with higher gamma-glutamyl transpeptide (GGT) values.[34] The volume of distribution (L) is negatively affected by the body-surface area and the bioavailability negatively affected by total bilirubin levels in pediatric liver transplant recipients.[32]

The association between the *ABCB1* genotype and tacrolimus disposition is unclear as the functionality of these variants is not completely known.[16,35] In addition, the mRNA expression of ABCB1 does not seem to be influenced by genetic variation in *ABCB1*.[36] Pediatric liver recipients with *CYP3A5*1* carrying donor livers had two-fold higher tacrolimus clearance than those with the *CYP3A5*3/*3* genotype.[36] Yet, no relationship has been found between the recipient's *CYP3A5* genotype and tacrolimus disposition.[19,36] The donor genetic make-up may be an important determinant of tacrolimus disposition in liver transplant recipients and it is therefore important to collect donor DNA to determine the influence of genetic variation of the donor on tacrolimus disposition.

For all organs studied, it is obvious that children 1-6 years of age need higher tacrolimus doses (per kg) than older children, which is a reflection of faster tacrolimus clearance rate. The underlying mechanisms for this observed faster clearance is unclear. It may be related to a relative large

liver:body size ratio and age-related changes in drug metabolism.[4,37] The larger liver:body size ratio results in an increased hepatic clearance of drugs. This ratio reaches its maximum around 2 to 3 years of age, partially explaining the high clearance of drugs in young children.[4]

Nevertheless, the purative between genotypes and tacrolimus have to be taken with caution. As we, and others, have shown, an additive effect of *CYP3A5* genotype and children's age in tacrolimus dosing requirements is present. However, the relative contribution of the genotype may be less prominent in children younger than one year of age as the ontogeny of CYP3A has not yet been completed.[24,38] Therefore, at this age the children may present as poor metabolizers, which is of particular concern in pediatric heart transplant recipients as a proportion of these patients are less than one year of age at the time of transplantation. In addition, the number of patients carrying *POR*28* or *CYP3A4*22* allele in our cohort was very small and consequently we could not analyse all genotype groups.

Post-transplantation decline in renal function

The definition problem

Even though renal dysfunction post-transplantation is a widely acknowledged adverse effect of tacrolimus, its relative contribution to renal dysfunction is still controversial. This debate has resulted in the questions:

- Does renal dysfunction caused by tacrolimus really exist?
- How to separate it from the adverse effect of low rate graft versus host disease (GVHD) and /or rejection of the kidney?

Several methods are currently being used to determine the kidney function, including histopathological changes and clinical markers (serum creatinine and GFR).

Histo-pathological changes

The Banff criteria for renal allograft pathology, are probably the most widely used guideline for defining pathological changes in kidney biopsies. Over the years, the criteria have version introduced several times with the latest change in 2007.[39] Poor inter-observer agreement and reproducibility have been major limitations of this staging tool.[40,41,42,43]

Four histological signs have been associated with CNI-toxicity; arteriolar hyalinosis, coarse vacuolization, interstitial fibrosis and tubular atrophy.[44,45,46,47,48,49,50] Recently, however, the specificity of these markers for CNI toxicity have been questioned.[51,45,52,53,54] Additionally, the use of biopsies for the diagnosis of CNI-induced nephrotoxicity has been a topic of discussion.[2,55]

All of the histo-pathological studies suffer from limitations. The diagnostic criteria and histological confirmations are very heterogenic, complicating comparison of the papers and results in variable evidence on the progressions of the classic histological signs and other non-specific factors.[56] Additionally, the majority of the articles include kidney transplant recipients and no other solid organ transplant recipients. In non-renal transplant recipients, renal failure thought to be cause due to CNI use may be the result of other factors, such as undiagnosed CKD pre-transplantation.[56] Therefore, the use of biopsies for the diagnosis of CNI-induced nephrotoxicity has currently no added benefit.

Considering the difficulty and controversy related to the diagnosis of CNI-induced toxicity, in our studies we have chosen to use the term "renal failure" or "renal dysfunction" in tacrolimus-treated patients instead.

Clinical markers of renal function

Despite the ongoing debate on the relative impact of tacrolimus on renal function in (pediatric) organ transplant recipients, it remains a

fact that solid organ transplant is associated with renal dysfunction and end-stage renal failure in a subset of patients. To adequately identify risk factors to further personalize tacrolimus therapy, different clinical markers of renal function have been used; serum creatinine and the glomerular filtration rate (GFR). The use of these different markers appears to result in potentially large variations in renal dysfunction prevalence (***Chapter 1***).

Serum creatinine

Serum creatinine has been the most widely used clinical tool to determine renal function. The extra-renal clearance of creatinine is relatively small in people with a normal kidney function, but about two-thirds of the total daily creatinine excretion can occur by extra-renal elimination in patients with CKD.[57] Serum creatinine has also been used to define renal function by setting a cut-off or following the change in serum creatinine. Although the most widely used marker of renal function, it is important to realize that serum creatinine levels can be misleading in several conditions, including in malnourishment and in liver or heart failure[58] and during renal replacement therapy such as CVVH.

Additionally, the use of serum creatinine in kidney patients is inaccurate due to contributing factors from the kidney (i.e. tubular or glomerular damage affecting filtration and secretion) as well as other factors.[59,60] Serum creatinine concentrations do not accurately reflect renal function unless steady state concentrations have been reached.[59,60] Moreover, the precision of the creatinine assay determines the results.[61] Especially with low serum creatinine levels, the creatinine values measured with the enzymatic method can run lower compared to those creatinine levels measured by the Jaffe methods.[62,63] Therefore, using serum creatinine as a tool for defining renal dysfunction is suboptimal, especially to detect fast changes in renal function and patients with multiple underlying conditions and/or nephrotoxic drugs.

Glomerular filtration rate

The glomerular filtration rate is currently considered the gold standard to reflect renal function. The GFR can be determined by direct measurement (mGFR) or by estimation using a formula (eGFR).

Measuring GFR

Over the years, many methods ways of measuring GFR have been discovered and used in clinical practice. An ideal marker for GFR is not protein bound and is freely filtered by the glomerulus. In addition, the markers should not be secreted, metabolized or reabsorbed by the renal tubules.[64]

The golden standard for measuring GFR is inulin clearance. However, the collection of timed urine specimens is very challenging in children who have not yet mastered toilet training or have conditions affecting the urine (i.e neurogenic bladder, dysfunctional voiding).[61] The inulin clearance can also be measured by a constant infusion technique estimating the plasma clearance from blood concentrations.[65] Yet, obtaining a constant inulin plasma or serum concentrations during intravenous infusion is difficult.[66] Steady-state concentrations are required, because unequilibrated samples will show lower or higher concentrations leading to an apparent over or underestimation of GFR.[66] Even though inulin clearance is probably the most accurate method for determining GFR, it is an invasive procedure and therefore not very frequently used in children.[67]

Another approach is the use of radioisotopes or other markers, such as ^{51}Cr EDTA clearance, ^{99}Tc DTPA clearance and ^{125}I iothalamate clearance. Nonetheless, the radioistopes are not available in every country[67] and there are some handling challenges.[67] The last method used to date is "cold" iothalamate and iohexol.[66] It has an advantage over the other methods since no radioactivity is involved and cold methods are equally accurate. A disadvantage for this method is the need to measure the compound in blood instead of a relatively simple radioactivity count.

The accuracy of all these GFR measurements methods is affected by the exact time and frequency of sampling. Poor standardization among centers and the inaccuracies introduced due to the different commercial products used are major hurldes.[68] To interpret the results, a single compartment model is used. However, the use of the single compartment model may result in overestimation of GFR.[66] Therefore, despite their technical advantages, each of these methods have several limitations.[67]

Estimating GFR

To estimate the GFR from serum creatinine levels, many formulas have been developed over the years with the Schwartz formula being especially designed for the use in children. To correct for age and gender, as a surrogate marker of the development change in muscle mass, an empirical constant "k" was added.[61] Logically, the same limitations pertaining the analytical method to measure serum creatinine and clinical factors impacting serum creatinine apply to estimated GFR.[69,70,71] The analytical limitations impacting serum creatinine concentrations, as described before, can lead to an overestimation of the eGFR.[61] Children with reduced muscle mass and CKD may also lead to an overestimation of the eGFR.[61]

Consensus

A consensus on the definition of renal dysfunction is highly desirable. There has been an ongoing discussion as described above. Several efforts have been made for standardization.

KDOQI

In 2002, the United States National Kidney Foundation published the Kidney Disease Outcome Quality Initiative (KDOQI) guidelines on the definition, classification and evaluation of CKD. Since its publication, many clinical practices and researcher have implemented this guideline. However, the staging of CKD is based on eGFR values, with all its

limitations. The validity of the KDOQI guidelines are in question if they are implemented in the current form, due to accuracy levels not being reached by several formulas used to date.[70] CKD stages 3-5 are of particular concern as with a GFR of 30-59 ml/min/1.73m^2 (CKD stage 3) the kidney function has deteriorated too much and an active management is started to treat complications. A decline in GFR to 15-29 ml/min/1.73m^2 (CKD stage 4) the patient needs to be prepared for renal replacement therapy and a further decline, GFR is <15 ml/min/1.73m^2 (CKD stage 5) the patient has complete renal failure and renal replacement is needed.[72]

KDIGO

The Kidney Disease: Improving Global Outcome (KDIGO) group is a global non-profit foundation committed to improving the care and outcomes of patients with kidney disease worldwide[73] Recently, they have published their guidelines for the evaluation and management of CKD, an update of the 2002 KDQOI guidelines. These new guidelines introduced a subgroup of CKD stage 3, separating patients with a GFR of 45-59 ml/min/1.73m^2 (CKD stage 3a) and those with a GFR of 30-44 ml/min/1.73m^2 (CKD stage 3b). The use of cystatin C has also been incorporated in these new guidelines as an additional tool for determining CKD. The use of these new guidelines in future research would aid in the possibilities of conducting meta-analyses to future understand the processes involved in CKD and improve the patient's outcome.

"Renal dysfunction" in tacrolimus-treated patients

Tacrolimus has been very successful in improving the outcome of pediatric transplant recipients. Nevertheless, it is associated with serious adverse effects including renal dysfunction.[52] This is important as the kidney is still developing during childhood as reflected in a change in glomerular filtration rates (GFRs) and renal excretion.[74]

Data on the prevalence of chronic kidney disease (CKD) after renal and non-renal transplantation in children are scarce and vary widely. We have shown in a systematic review of the literature an extremely wide prevalence of renal failure in pediatric non-renal transplant recipients (0%-90%). After adjusting for follow-up time (> one year) and severity of kidney disease, the reported prevalence narrowed to mild CKD ranging from 21.7% to 40% and severe CKD ranging from 1.67% to 46% (***Chapter 2***).[75] However, these studies were presented with several limitations, including patient selection biases, methodological differences in definition and tools used to measure renal function.

The majority of pediatric studies have examined a mixed populations of cyclosporine and tacrolimus treated children. In a recent Cochrane review, tacrolimus was found to be superior to cyclosporine with a lower risk of CKD in liver transplant recipients.[76] Therefore, studies in patients only treated with tacrolimus are needed to have a better understanding of CKD in this population.

In an effort to achieve some clarity on the extent of renal failure post-transplantation , we reported the prevalence of CKD in pediatric solid organ recipients treated with tacrolimus (***Chapters 7-9***). In a large, retrospective, multicenter, cohort study, we included 495 transplant recipients (135 liver, 161 heart and 199 kidney) with a follow-up time up to 10 years post-transplantation. The cumulative incidence of CKD stage 3-5 at least 3 months post-transplantation was 24% for liver, 34.2% for heart and 40% for renal transplant recipients. Although rare, CKD stage 4 and 5 are prevalent in pediatric solid organ transplant recipients. At one year post-transplantation, 6.7% of the pediatric kidney transplant recipients experienced CKD stage 3 and 1.9% CKD stage 4 and at five years post-transplantation 6.5% stage 3, 3.2% stage 4 and 6.5% stage 5. In liver transplant recipients none of the patients experienced CKD stage 3-5 at one or five years post-transplantation. The prevalence of CKD stage 3 was 2.9%

at one year post-transplantation and the prevalence of CKD stage 5 1.8% at five years post-transplantation in pediatric heart transplant recipients. However, our sample size at 10 years post-transplantation was limited as the majority of the children were transplanted in the recent years, resulting in a median time from transplantation of only 8 months! In addition, the median follow-up time was less than one year post-transplantation. Nonetheless, these numbers show that a significant proportion of pediatric solid organ transplant recipients will develop CKD post-transplantation. The prevalence of CKD may even be greater than we have reported as we used estimated GFR, which can overestimate the actual GFR measured by inulin clearance, as discussed above.[61,77,78] These findings illustrate the importance of monitoring the kidney function following pediatric solid organ transplantation. Although more invasive, routine mGFRs will better reflect the actual kidney function and should be considered in high risk patients.

Risk factors for renal failure

Causes of renal dysfunction

Despite the fact that tacrolimus therapy is associated with renal toxicity, it is not the only cause for kidney dysfunction in solid organ transplant recipients. It is probably multi-factorial as renal function can be cumulatively affected by intra-operative factors (hypotension and need for dialysis), post-operative factors (acute renal failure), concomitant disease, immunomodulation (e.g. rejection in kidney transplant and long term exposure to calcineurin inhibitors like tacrolimus.[58,79] Differentiating renal dysfunction caused by chronic use of tacrolimus from other causes is very challenging. In particular, in renal transplant recipients the underlying disease requiring transplantation and immune and non-immune related organ rejection can be major contributors.[58] In addition, liver transplant recipients tend to have pre-existing glomerular diseases as a result of inadequate clearance of immune complexes caused by liver disease.[58]

In pediatric solid organ transplant recipients, similar risk factors for developing renal failure post-transplantation as in adults have been reported.[78,80,81] Yet, we speculate that the chronic use of tacrolimus might be more important in children as the concomitant disease associated with renal dysfunction (e.g. diabetes, HCV-associated glomerulonephritis, atherosclerosis) are less common in children compared to adults. Non-adherence is of particular concern in adolescents and young adults[82,83] and may result in sub-therapeutic tacrolimus exposure and consequently increase the chances of renal dysfunction caused by rejection of the donated kidney. It may also lead to greater intra-patient variability in tacrolimus concentrations, possibly resulting in a higher risk for developing renal failure, in addition to the risk of transplant rejection.[17,21]

Pharmacogenetics and renal function

Genetic polymorphisms in metabolizing enzymes (CYP3A4, CYP3A5) and drug transporters (ABCB1) have been shown to influence tacrolimus disposition in both adults and children. Yet, the influence of genetic variation on the outcome of transplant patients is less well known. In ***Chapter 3***, we reviewed the available literature for promising gene candidates in relation to the risk of renal dysfunction after solid organ transplants, with tacrolimus therapy. Although both cyclosporine and tacrolimus are calcineurin inhibitors, they are different in physiological pathways which may explain their differential side effect profiles.[52,84,85,86,87,88] Hence, we tried to delineate the contribution of genetic variation, using a targeted gene approach, on renal function and an emphasis on tacrolimus.

In adult transplant recipients associations between *CYP3A5, ABCB1, TGF-β, CYP2C8* and *ACE* have been reported in liver, kidney and heart recipients. Results for *CYP3A5* and *ABCB1* were conflicting in kidney transplant recipients, associations between a higher risk of developing renal

dysfunction and *CYP3A5*-expressers has been reported[89,90] as well as with *CYP3A5* non-expressers.[91] In liver and heart transplant recipients, only one study has reported, showing an increased risk for *CYP3A5* non-expressers in liver transplant recipients[92] and no association in heart transplant recipients.[93] The *ABCB1* genotype also showed contradictory results with two studies (one kidney and one liver) showing that the T-variant in *ABCB1 3435* and *ABCB1 2677* is associated with an increased risk for renal dysfunction[54,94] while three other studies could not confirm such an association.[89,90,93] The associations found in the other genes, have only been reported in one study to date.

In pediatric kidney transplant recipients, carriers of the *CCR5Δ32* genotype were more frequently diagnosed with nephrotoxicity compared to those carrying the wildtype.[95] This study showed some methodological short-comings, which may reduce the certainty of the finding. At 6 months post-transplantation, pediatric liver recipients carrying the *ABCB1 T-T-T* haplotype were more frequently diagnosed with tacrolimus-induced nephrotoxicity. However, this significance disappeared at one year post-transplantation, suggesting that the protective effect of ABCB1 on the kidney may only be important in the first 6 months post-transplantation.[96] However, the possible protective effect of ABCB1 in the years following pediatric solid organ transplantation have not been studied yet.

Although, these results suggest promising genes for understanding the mechanisms behind renal dysfunction in transplant recipients, not all studies have shown consistency and methodological quality. The methods also differed considerably hampering meta-analysis. Many different definitions for renal function were used as well as varying follow-up times (***Chapter 3***).[97] As a next step, including all previously associated genes in one analysis in a large, preferably a multicentre, study would be interesting. This candidate gene approach may be more feasible compared to a Genome Wide Association Study (GWAS) as the pediatric transplant

population may not be large enough for a GWAS study design. We have been able to conduct a candidate gene approach study in pediatric liver, heart and kidney transplant recipients (***Chapters 7-9***).

We created an elaborate list of genetic polymorphisms, including all previously associated and published polymorphism as well as polymorphisms related to tacrolimus pharmacokinetic pathways and polymorphisms associated with renal function. For the first time, a total of 77 polymorphisms were analysed in relation to renal function (eGFR) in pediatric solid organ transplant recipients. Although, initial results were promising with several polymorphisms associated at the significance level of 0.05, only one polymorphism in pediatric heart transplant recipients was significantly associated at the set significance threshold corrected for multiple testing (***Chapters 7-9***). We used a targeted gene approach and could therefore have missed polymorphisms that have not been previously known to correlate with renal function. Genome-wide association studies or next generation sequencing are newer techniques in which hundreds of polymorphisms can be studied at once in large populations and potentially result in new disease or transplant related prognostic factors identifying patients at risk for developing renal failure.

Biomarkers

An optimal biomarker to monitor renal (dys)function is lacking. Recently, new potential biomarkers have been identified. The validation of these biomarkers is in different stages of development. At this time, the best validated marker is neutrophil gelatinase-associated protein (NGAL).
In adult kidney transplant recipients, uNGAL directly post-transplantation has been shown to be a good predictor for delayed graft failure (DGF), acute rejection and AKI.[98,99,100,101] In pediatric kidney transplant recipients, donor kidney NGAL staining has been shown to be an early predictor of kidney failure.[102] Additionally, an increase of 100 ng/mg in urinary creatinine-corrected uNGAL levels in the first 24 hours after kidney transplanta-

tion, has been associated with a 20% increase in the risk of developing DGF.[103] In pediatric heart transplant recipients experiencing CKD, uNGAL levels at least 3 months post-transplantation were above 2.2 ng/ml,[104] which is considered to be the cut-off in healthy children.[105]

Pediatric kidney and liver transplant recipients are at an increased risk for renal damage due to the surgery itself or the high tacrolimus concentrations in the first 14 days post-transplantation.[19] Hence, we aimed to explore the uNGAL levels in the immediate post-transplant period (***Chapter 10***). Although our study was a pilot study, in both the liver and kidney transplant recipients the majority of the patients exhibited uNGAL levels above 2.2 ng/ml, but not above 135 ng/ml, a cut-off associated with an increased risk of developing AKI in children.[106] This may suggest subclinical renal damage In contrast to previous reports, we failed to find an association between high tacrolimus concentrations and high uNGAL levels or between high uNGAL levels and AKI (***Chapter 10***). Our study has several limitations. Due to limited sample size, we were unable to establish ROC curves to determine the sensitivity of the early marker uNGAL. For future studies, it would be interesting to see if a rise in uNGAL can be detected when the tacrolimus concentrations rise in the absence of a rise in serum creatinine. This way, an early marker for acute tacrolimus nephrotoxicity could be established and possibly minimize the damage to the kidney. Nonetheless, knowledge about the natural evolution of uNGAL in several conditions is needed to distinguish between the natural pattern of uNGAL levels and other possible causes for the rise in the uNGAL levels. Yet, this natural pattern is currently not known.

THE FUTURE

The missing variables and tacrolimus pharmacokinetics

Clinical factors

We have demonstrated that children younger than 6 years of age needed higher tacrolimus doses compared to older children; hence adaptation of the starting dose of tacrolimus seems to be the next logical step. The effects of these dosing changes on the long-term outcome of children have not been studied. Moreover, as older children are at an increased risk for developing renal failure post-transplantation, a prospective study aimed to further delineate the reasons for this vulnerability is needed. Although many efforts are already being taken by the caregivers to increase the adherence to drugs in adolescents,[107] a focus group consisting of adolescent transplant recipients recipients to delineate their challenges to adhere to their medication, may reveal new reasons that can be incorporated into their care.

In the first two years of life, children eat differently compared to adults. The influences of breast milk, baby formula and non-solid foods on tacrolimus disposition are currently not known, but are important to take into account especially in pediatric heart transplant recipients, where a considerable proportion of the patients are less than one year of age. Additionally, children in the ICU often receive supplemental tube feeding, yet it is currently unknown if the composition of the enteral nutrition in these patients would affect tacrolimus absorption and therefore tacrolimus dosing requirements. The use of a buccal tube resulted in similar tacrolimus trough concentrations compared to the use of a nasogastric tube, suggesting the type of tube does not impact tacrolimus absorption.[108] An in vitro model to determine the influence of tube-feeding and different leading regimens on tacrolimus absorption may help us understand food related variation in tacrolimus disposition in children.

Some foods (grapefruit, pomelo, turmeric, ginger) are known to interact with these proteins and could therefore influence the tacrolimus disposition.[109,110,111,112,113,114,115,116,117] In animal studies, the influence of these types of food have been shown to increase the area-under-the-concentration-time-curve (AUC) of tacrolimus compared to those animals treated with water.[118] Yet, to our knowledge, none of these foods have been studied in humans in a systematic way and integrated in follow-up protocols to determine the influence on tacrolimus disposition and the frequency of the use of these foods in transplant recipients.

Inflammation has been associated with altered drug metabolism in both animal and human adult studies. C-reactive protein (CRP), a marker of inflammation, is associated with significantly lower CYP3A4 expression in adult liver tissue samples.[119] Similarly, in critically ill pediatric patients, the clearance of midazolam, a model drug for CYP3A4/5 activity, was significantly lower compared to children who underwent elective major craniofacial surgery or pediatric oncology patients.[120] In addition, higher degrees of organ dysfunction were associated with decreased metabolic clearance in pediatric intensive care patients.[121] Bilirubin, was also associated with lower CYP3A4 expression in liver tissue samples.[119] Although a relationship between bilirubin and tacrolimus disposition has been shown in adults,[122,123] contradictory results have been reported in pediatric transplant recipients.[33,124] A dosing algorithm incorporating all these factors amy be important.

Currently, tacrolimus is measured in whole blood samples. However, tacrolimus is highly protein and erythrocyte bound with a small unbound fraction.[125] Only this unbound fraction is responsible for the pharmacological action of tacrolimus. Hence, to really understand the exposure of pediatric transplant recipients to tacrolimus, it would be desirable to measure only the unbound fraction. However, technical difficulties and practical challenges (i.e. large sample volumes needed,

difficult assay) currently limit the ability to measure the unbound fraction. As mentioned, the metabolism of tacrolimus is age dependent. However, as shown with sirolimus[11] children may metabolize drugs differently compared to adults. This has not been studied yet for tacrolimus, but would be of interest as the possible different metabolites may be active as well and contribute to both effect and toxicity.

Concomitant medication

Several drugs have been reported to either inhibit or induce the activity of metabolizing enzymes.[126,127] Instead of excluding those patients receiving these medications from analysis, concomitant medications should be handled as covariates. The combination of the administered drugs as a covariate would be interesting as well, as transplant recipients generally receive multiple medications at the same time.

New insights in genetic polymorphisms

Various new insights in genetic polymorphisms and their use in research have further improved pharmacogenetic understanding of tacrolimus disposition. To date, mainly individual single nucleotide polymorphisms (SNPs) have been researched, but more data have become available on the use of haplotypes and linked polymorphism. A recent study by Wang et al has shown the importance of haplotypes, with *CYP3A5 A-T-T-T-G* haplotype carriers had significantly lower tacrolimus stable dose requirements ($p = 2.41 \times 10^{-5}$) compared to other haplotype carriers.[128] This emphasizes the importance of including haplotypes in pharmacogenetic analyses.

Genetic variation in the genes encoding the drug metabolizing enzymes does not explain all the variability reported in tacrolimus disposition in either adults or children. Therefore, other pathways may possibly influence tacrolimus disposition such as nuclear receptors (peroxisome proliferator-activated receptor-alpha, pregnane X-receptor, constitutive androstane receptor),[129,130,131,132,133] inflammatory genes

(interleukin-18, interleukin-10 and interferon-gamma)[123,134] and other drug transporters (multidrug resistance-associated protein 2).[135] We have not been able to show a relationship with some of these genes and renal function.

Donor's influence

So far, we have only focused on the recipient as the major factor. However, the donor may play a role as well. Especially in liver and kidney transplantation, the influence of the donor may be more important than the recipient's influence. This has been shown in both adult and pediatric patients.[136] A similar approach should be taken in kidney and small bowel transplantation as the donor DNA will determine renal and intestinal tacrolimus metabolism, respectively. In these solid organ transplant groups, it will be therefore key to include the donor's DNA or mRNA expressions of protein in futures studies.

Dosing algorithms

While the relationship between *CYP3A5* genotype and tacrolimus clearance has been well established in adults and children, the clinical impact of genotype-based dosing on tacrolimus therapy becomes more important. To study the potential impact of genotype-based dosing on the outcome of tacrolimus therapy, prospective studies will be needed. Studies in adults, using individualized dosing based on genotype have started, showing that patients treated with *CYP3A5* genotype-based tacrolimus doses had tacrolimus concentrations more often within the target range and achieved the target concentrations quicker than those patients with standard of care.[137]

Although the management of tacrolimus dosing requirements improved in the adapted group, the renal function at day 14 and 3 months post-transplantation was comparable between the two groups.[137] This finding raises the question if optimization of tacrolimus doses is actually the way to improve patient outcomes. However, the population studied was at a

low risk for acute rejection and hence the dosing algorithm needs to be validated in other patient groups where tacrolimus is started at the day of transplantation, as this is more commonly done in clinical practise.[138]
These results suggest the importance to incorporate clinically relevant endpoints in trials evaluating the effect of genotype-based dosing, but it also stresses the importance to prospectively study these patients, despite the significant costs and efforts needed to conduct such trials.

The missing variables and tacrolimus pharmacodynamics

Tacrolimus

With an ongoing discussion on the existence of tacrolimus-induced nephrotoxicty only the parent compound is taken into consideration as the possible cause for renal failure following transplantation. However, some authors have suggested that the metabolites of tacrolimus may contribute to nephrotoxicity. This hypothesis is supported by the renal expression of CYP3A5 and the renal clearance of tacrolimus being dependent of *CYP3A5* genotype.[139] Nonetheless, the nephrotoxic effects of tacrolimus metabolites have not been studied in either adults or children.[52]

Concomitant medication

Pharmacodynamic interactions have been reported with concomitant use of non-steroidal anti-inflammatory drugs (NSAIDs) and aminoglycosides. The concomitant use of these medications facilitates the progression of chronic changes in the kidney.[140,141] Due to these interactions, caution has to be taken to gather this information during research so the results can be adequately controlled for concomitant medication use. We have tried to control for the use by testing their use of concomitant medication against renal function (***Chapters 7-9***), but found no significant influence on renal function. However, we did not test combinations of medications, duration of the treatment and the dose of the concomitant medication. In future research,

the frequency and duration of these administered drugs need to be monitored and taken into consideration in the statistical analysis.

New insights in genetic polymorphisms

In a large genome wide association study (GWAS) potential pathways for chronic kidney disease were studied. Two SNPs were associated with serum creatinine levels: *SHROOM3* rs9992101 and *RAP2A* rs15358.[142] Shroom-related protein 3 is thought to be involved in epithelial cell shape regulation[143] and the *RAP2A* gene is thought to influence acetylation of NAT8. This is an interesting finding as NAT8 is strongly and almost exclusively expressed in the kidney.[142] Two SNPs (rs10206899 and *CEP89* rs4805834) have been associated with eGFR, cystatin C and CKD. Rs10206899 was in linkage disequilibrium with *RAP2A* rs15358. The last two SNPs associated with serum creatinine and eGFR were rs3127573 and *TBX2* rs8068318.[142] The *TBX2* gene encodes a member of the T-box family of transcription factors.[144] Unfortunately, the function of TBX2 is not yet known.[142]

In contrast, in another GWAS (n=4006) analysis containing 5 EUROSPAN studies and two replication cohorts, none of the studied SNPs met the multiple testing significance threshold of 1.55×10^{-7}.[145] The regions studied included those that were previously found to be significant. Instead, the authors focused on three promising regions for follow-up in the two replication cohorts: the collagen type XXII alpha 1(*COL22A1*) gene on chromosome 8, the synaptotagmin-1 (*SYT1*) gene on chromosome 12 as well as the gamma-aminobutyric acid receptor subunit tho-2 (*GABRR2*) and ubiquitin-conjugating enzyme E2 J1 (*UBE2J1*) genes on chromosome 6.[145] We have included their most significant SNPs into our custom assay, but we were unable to confirm a possible relationship between these SNPs and renal function in pediatric solid organ transplant recipients. Nevertheless, our sample size was significantly smaller than these GWAS studies and the candidate gene approach of our study may have limited our detection power.

Donor's influence

In addition to the differences in protein expression, the age of the donor has been reported to influence the graft outcome with kidney from older donors showing a higher susceptibility for renal graft failure.[146,147] An alteration of renal perfusion and a higher prevalence of pre-existing chronic vascular changes are thought to account for this increased susceptibility.[146,148]

To fully understand inter-individual variation in tacrolimus disposition and response, a systems biology approach may aid to individualize therapy and improve outcome. This approach may include pharmacokinetic and pharmacodyamic pathways. Prospective multi-centre trials may aid to collect samples large enough to be able to perform multiple testing and enable the use of replication cohorts. Next generation sequencing may be of help as it can reduce the costs of analysing hundred of thousands SNPs at the same time.

Other renal dysfunction biomarkers

Several new biomarkers have been studied for their potential to reflect renal function or renal damage.[149,150] The most promising new biomarker for renal function is serum cystatin C levels.[151,152,153,154,155] Formulas incorporating cystatin C show better accuracy in predicting the GFR compared to the conventional Schwartz formula used today in pediatric liver and kidney transplant recipients.[104,156,157] Kidney injury molecule 1 (KIM-1) and netrin-1 are not detectable in healthy kidney tissue,[158 159] and appear to be potential markers for kidney damage.[159,160] KIM-1 strongly correlates with serum creatinine levels and eGFR in adult renal allograft recipients[160,161] and has been identified as an independent risk factor for graft loss in adult renal transplant recipients.[161] Netrin-1 was highly expressed in the tubular epithelial cells of adult transplanted kidneys, also showing the potential as biomarker.[159]

Biomarkers of tacrolimus efficacy

The biomarkers we have discussed so far, dealt only with renal function. Yet, in addition to therapeutic drug monitoring, it would be ideal to be able to measure the effectiveness of tacrolimus treatment in patients. The mechanism of action of tacrolimus evolves around the inactivation of T-cells to prevent an immunological response of the host against the new graft. Therefore, the product of T-cell activation may be of interest for testing the effectiveness of tacrolimus. In a review by Oellerich et al. greater inhibition of interleukin-2 (IL-2), interleukin-4 (IL-4), IFN-γ and TNF-α was reported with increased cyclosporine A (CsA) concentrations.[162]

Other potential markers, such as the CD30, CD26 molecule or CD4 T-cells, have been proposed as a marker regarding acute rejection and renal allograft survival.[163,164,165] Unfortunately, most studies were done with CsA and the findings with CsA need to be replicated with tacrolimus due to the different mechanisms of action of both drugs.

At this time, the tacrolimus therapeutic window used by most pediatric transplant physicians is taken from adults. Importantly, the PK-PD relationship of tacrolimus in children has not been unequivocally shown. The developing child may respond differently to similar tacrolimus plasma levels than adults, even at different stages of development. This may be due to differences in tacrolimus metabolite formation and levels, but also differences in immunology and other organ functions (e.g. kidneys). Due to these differences, we wonder if the therapeutic range of tacrolimus may need to be adjusted for children. Traditionally, to measure tacrolimus pharmacodynamics, clinical endpoints such a rejection and adverse events have been used. The ideal study would collect data on tacrolimus (doses, tacrolimus and metabolite levels in blood and urine), chemistry and haematological laboratory results, concomitant medication, patient's characteristics (age, height, weight), genetic variation of the recipient and donor and transplant outcomes (rejection, renal failure, hypertension, neurotoxicity) to ultimately develop a model for treatment of the

individual patient. This would require a large sample size; yet with population PK-PD modelling it is possible to develop a PK-PD model with limited patient numbers as well as limited sample numbers. An approach found to be very helpful in small children in case of availability of limited samples.[166]

Determining the variables of importance for the PK-PD model is limited without a better understanding of the underlying mechanisms of renal failure in tacrolimus treated patients. A better understanding of the underlying mechanism may not only improve the PK-PD model, but may also identify possible therapy targets. It may be worthwhile to test drugs currently used to counteract the effect of other nephrotoxic drugs (ie. N-acetylcysteine for ifosfamide) in a tacrolimus-induced renal failure rat model.

Conclusions

In summary, in this thesis, I have shown the importance of *CYP3A5* and other novel polymorphisms (*POR*28, CYP3A4*22*) on tacrolimus disposition in pediatric transplant patients and the significant interplay with maturation. The novel approach of combining CYP3A genotypes, showed a significant improvement in explaining the variability in tacrolimus disposition. A large proportion of pediatric solid organ transplant recipients receiving tacrolimus develop CKD, warranting the need for future studies in which risk factors, clinical and genetic, are studied to ultimately develop a model for the identification of patient with an increased risk of developing renal failure post-transplantation. These data provide guidance for future personalized tacrolimus dosing in pediatric transplant recipients, with the ultimate goal to improve long-term outcome.

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English Summary

English Summary

The introduction of solid organ transplantation has dramatically increased survival rates of both adults and children with end-stage organ failure. The additional introduction of immunosuppressive therapy has further improved the short term survival yet, the long-term outcomes have barely improved.

Children have long been considered miniature adults. However, over the last decades we have come to better understand the developmental changes in children and realized that drug therapy needed to be adapted. In 1995, tacrolimus was introduced as a new immunosuppressive drug. It has since become the drug of choice in pediatric solid organ transplantation even though its pharmacokinetic and pharmacodynamic properties have hardly been studied in children.

Part I: Renal failure after solid organ transplantation
Renal failure is a serious complication of tacrolimus treatment in pediatric non-renal solid organ transplant recipients. In ***chapter 2***, we reviewed the current literature on the prevalence of this complication. We found a very wide range in reported prevalences, which we ascribed to the heterogeneity in methodology and renal failure definition in the different papers. Focusing on mild and severe renal failure at least one year post-transplantation, we narrowed the ranges to 0%-39% and 0%-71.4% for mild and severe failure, respectively, in liver transplant recipients; and 22.7%-50% and 6.8%-46%, respectively, in heart transplant recipients. These results show that a considerable proportion of pediatric liver and heart transplant recipients may experience renal failure post-transplantation.

In ***chapter 3*** we performed a systematic review of the literature to determine genetic risk factors for tacrolimus-induced nephrotoxicity in both adults and children. The limited evidence suggests that genetic variation

involved in pharmacokinetic (*ABCB1* and *CYP3A5*) and pharmacodynamic (*TGF-ß, CYP2C8, ACE, CCR5*) pathways of tacrolimus may impact the risk of developing tacrolimus-induced nephrotoxicity across different transplant organ groups. However, the quality of these studies varied considerably, limiting the conclusions that can be drawn.

Part II: Tacrolimus pharmacokinetics and genetic variability

Tacrolimus is metabolized by the phase I enzymes, CYP3A4 and CYP3A5. Its disposition can be affected by genetic variation in these enzymes, as has been shown in adults. Yet, data on the impact of these variations on tacrolimus disposition in children is scarce. ***Chapter 4*** presents the results of our study in pediatric heart transplant recipients, showing that younger children as well as *CYP3A5*-expressers required higher tacrolimus doses to reach similar tacrolimus trough concentrations as older children or *CYP3A5* non-expressers. The effects of age and *CYP3A5* genotype were cumulative because *CYP3A5*-expressers younger than six years of age required 1.5 times higher tacrolimus doses compared to *CYP3A5*-expressers older than six years of age and 6 times higher tacrolimus doses compared to *CYP3A5* non-expressers older than six years.

After the recent discovery of the new *CYP3A4*22* polymorphism, we reanalyzed our initial pediatric heart transplant data to determine if this new polymorphism has an additional effect to CYP3A5 on tacrolimus disposition. In ***chapter 5*** we show that despite the low allele frequency, *CYP3A4*22* carriers needed 30% less tacrolimus to reach similar target concentrations compared to *CYP3A4*1/*1* carriers. By combining the *CYP3A4* and *CYP3A5* genotypes, we were able to categorize the patients into CYP3A poor, intermediate or extensive metabolizers. The combined *CYP3A* genotypes were stronger associated with tacrolimus disposition as poor metabolizers needed significantly less tacrolimus and had higher concentration/dose (C/D) ratios compared to intermediate and extensive metabolizers. The new polymorphism *CYP3A4*22* appears to be the first functional

variation in CYP3A4. Combining the *CYP3A4* and *CYP3A5* genotypes into phenotypic categories may further reduce the intra-patient variability in tacrolimus disposition in the first 14 days post-transplantation.

Although very promising, the inter-patient variability cannot be completely explained by the *CYP3A4* and *CYP3A5* genotypes. Variants in the gene encoding P450 oxidoreductase (POR), the protein that enables the activity of cytochrome P450 (CYP) enzymes, may further explain the variability. *POR*28* has been associated with increased tacrolimus dosing requirements in adult kidney transplant recipients, but has not been studied in children. In ***chapter 6***, we report for the first time in pediatric kidney transplant recipients that the *POR*28* allele in *CYP3A5*-expressers explains some of the variability in tacrolimus disposition. *CYP3A5*-expressers carrying at least one *POR*28* allele had 18.3% lower tacrolimus trough concentrations as well as 20.2% lower tacrolimus C/D ratios compared to *CYP3A5*-expressers with the *POR*1/*1* genotype. This effect could not be shown in *CYP3A5* non-expressers, suggesting a more important role of POR in CYP3A5 activity than CYP3A4 in relation to tacrolimus disposition.

Part III: Renal failure after solid organ transplantation

The long-term outcome of pediatric solid organ transplant recipients can be further improved by individualization of the current patient management. Renal failure post-transplantation is a major complication and knowing the risk factors for renal failure may help optimize therapy. In addition to clinical factors, genetic variation may also explain renal function decline after pediatric solid organ transplantation. We developed a custom assay of 96 polymorphisms previously associated with tacrolimus-induced nephrotoxicity, tacrolimus disposition and renal function. In ***chapter 7***, we show a cumulative incidence of chronic kidney disease of 40% in 199 pediatric kidney transplant recipients. Renal function remained stable throughout the study period, yet a small number of patients developed chronic kidney disease already in the first months post-transplantation.

Chapter 8 presents the results for pediatric liver transplant recipients (n=135). The cumulative incidence of chronic kidney disease was 24% in this population. The prevalence of mild renal failure was 5.7% at one year post-transplantation and 0% at 5 years post-transplantation. In ***chapter 9*** we show that the cumulative incidence of chronic kidney disease in pediatric heart transplant recipients (n=161) was 34.2%. At one year post-transplantation the prevalence of moderate renal failure was 2.9% and at 5 years post-transplantation 1.7%. These results suggests that although the estimated glomerular filtration rate appears stable in all organ groups, a small group of patients experience renal problems early on and throughout the follow-up period develop more serious renal failure.
We also studied the impact of genetic variation on renal function. None of the genetic polymorphisms investigated was significantly associated with renal function decline in either of the three organ groups.

The use of serum creatinine and the estimated glomerular filtration rate for determining renal function has several limitations. One of the new potential biomarkers for renal failure is neutrophil gelatinase-associated protein or NGAL. Urinary NGAL levels have been associated with acute and chronic renal failure in adult transplant recipient, but have rarely been reported in pediatric kidney and liver transplant recipients. In ***chapter 10*** we show that uNGAL levels in pediatric liver and kidney transplant recipients were marginally increased, but seldom as high as previously associated with acute kidney injury. Although this was a pilot study, these results suggest subclinical renal damage in the majority of the children.

Part IV: Discussion and future directions

Chapter 11 discusses the results from the studies presented in this thesis, as well as challenges encountered. Furthermore, we present several gaps in knowledge as well as recommendations for future research. The main conclusions are the following:

- *CYP3A4/5* and *POR* genotypes as well as age explain a considerable proportion of the variability found in tacrolimus disposition in pediatric transplant recipients early after transplantation.
- A significant proportion of pediatric solid organ transplant recipients experience chronic kidney disease in the years following transplantation.
- Chronic kidney disease may already manifest in the first months post-transplantation and in a small proportion of pediatric transplant recipients renal function deteriorates over time.
- We were not able to identify genetic risk factors for CKD in pediatric transplant recipients.
- Urinary NGAL levels are a potential marker for subclinical renal damage in pediatric liver and kidney transplant recipients.

Nederlandse Samenvatting

Nederlandse Samenvatting

De introductie van orgaantransplantatie heeft de overlevingskans van volwassenen en kinderen met orgaan falen drastisch verbeterd. De bijkomende introductie van immuunsuppressiva (afweer onderdrukkende medicijnen) heeft de overleving op korte termijn verder verbeterd, maar dit heeft geen invloed gehad op de overleving op langere termijn.

Kinderen zijn lange tijd beschouwd als kleine volwassenen. Echter, in de laatste jaren hebben we meer inzicht gekregen in de relatie tussen de groei en ontwikkeling van het kind en de manier waarop het lichaam met geneesmiddelen omgaat. In 1995 is het afweer-onderdrukkende medicijn tacrolimus op de markt gekomen en dit is sindsdien de eerste keus bij kinderen die een geheel orgaan ontvangen. De farmacokinetische en farmacodynamische eigenschappen van tacrolimus zijn uitvoerig bestudeerd bij volwassenen, maar nauwelijks bij kinderen.

Deel I: Nierfalen na orgaan transplantatie
Nierfalen na een orgaantransplantatie is een ernstige complicatie bij kinderen die behandeld worden met tacrolimus. ***Hoofdstuk 2*** geeft een overzicht van de huidige literatuur over dit probleem. De gerapporteerde prevalenties (de mate waarin het voorkomt) verschillen sterk vanwege de verschillende onderzoeksmethodes en de diverse definities van nierfalen in de artikelen. We hebben de uitkomsten onderverdeeld in mild en ernstig nierfalen, en konden toen vaststellen dat bij kinderen na lever transplantatie de prevalentie van mild nierfalen varieert van 0%-39% en de prevalentie van ernstig nierfalen van 0%-71,4%. De respectivelijke percentages na hart transplantatie zijn 22,7%-50% en 6,8%-46%. Hieruit blijkt dat veel kinderen de kans lopen op nierfalen na lever- of niertransplantatie.

Voor ***hoofdstuk 3*** hebben we systematisch in kaart gebracht welke mogelijk erfelijke factoren voor tacrolimus-geïnduceerde nierschade in de huidige literatuur wroden beschreven, zowel voor volwassenen als kinderen. Er blijkt een vermoedelijke rol te zijn voor variaties in de genen die betrokken zijn bij de farmacokinetiek (*ABCB1* en *CYP3A5*) en farmacodynamiek (*TGF-β, CYP2C8, ACE, CCR5*) van tacrolimus. De kwaliteit van de studies was erg variabel, hetgeen het trekken van definitieve conclusies bemoeilijkt.

Deel II: De farmacokinetiek van tacrolimus en genetische variatie
Tacrolimus wordt omgezet in de darm, lever en nieren door de geneesmiddelmetaboliserende eiwitten CYP3A4 en CYP3A5. Bij volwassenen is aangetoond dat erfelijke variatie in deze genen deels verklaart hoe dit in zijn werk gaat (farmacokinetiek). Wat betreft kinderen is dit nog grotendeels een open boek. In ***hoofdstuk 4*** presenteren we de resultaten van onze studie naar hart transplantatie en het gebruik van tacrolimus. Het bleek dat jongere kinderen en kinderen bij wie CYP3A5 tot expressie komt hogere tacrolimus doseringen nodig hebben om dezelfde tacrolimus concentraties te bereiken als andere kinderen. Het effect wordt versterkt als jongere kinderen ook CYP3A5 tot expressie brengen, aangezien kinderen jonger dan 6 jaar gemiddeld 1.5 keer hogere doseringen nodig hadden dan oudere kinderen, en zelfs zes keer hogere doseringen dan oudere kinderen bij wie CYP3A5 niet tot expressie komt.

Na de recente ontdekking van een nieuwe erfelijke variatie in het CYP3A4 gen: *CYP3A4*22*, hebben we een nieuw onderzoek gedaan bij dezelfde kinderen om te bepalen of dit extra invloed heeft op de farmacokinetiek van tacrolimus na harttransplantatie. In ***hoofdstuk 5*** laten we voor de eerste keer zien dat kinderen met deze variant 30% minder tacrolimus nodig hebben om dezelfde tacrolimus concentraties te bereiken als *CYP3A4*1/*1* dragers. Door het combineren van de *CYP3A4* en *CYP3A5*

genotypes konden we de kinderen classificeren als langzame, gemiddelde en snelle omzetters. Het gecombineerde *CYP3A* genotype bleek sterker geassocieerd te zijn met de omzetting van tacrolimus: langzame omzetters hadden significant lagere tacrolimus doseringen en hogere concentratie/dosis (C/D) ratios vergeleken met gemiddelde en snelle omzetters. Deze nieuwe variant lijkt de eerste functionele variant in het *CYP3A4* gen te zijn die tot nu toe gevonden is. Het doseren van tacrolimus op basis van de combinatie van deze twee erfelijke varianten (*CYP3A5*3* en *CYP3A4*22*) kan de variatie in de tacrolimus spiegels bij kinderen na hart-transplantatie helpen verminderen. Alhoewel, niet alle variatie in tacrolimus spiegels tussen patiënten kan verklaard worden door alleen deze twee erfelijke varianten in de CYP3A genen.

Varianten in het gen coderend voor P450 oxidoreductase (POR), het eiwit dat de activiteit van cytochroom P450 enzymen bevordert, kunnen mogelijk deze variabiliteit verder verklaren. *POR*28* is gecorreleerd met hogere tacrolimus doseringen voor volwassen niertransplantatiepatiënten, maar dit is nog niet eerder onderzocht bij kinderen die een niertransplantatie ondergaan. Wij hebben dat wel gedaan en in ***hoofdstuk 6***, kunnen we concluderen dat bij kinderen bij wie CYP3A5 tot expressie komt, *POR*28* ook een deel van de variabiliteit in het omzetten van tacrolimus verklaart. Deze kinderen hadden 18% lagere tacrolimus concentraties en 20,2% lagere tacrolimus C/D ratios dan de kinderen met het *POR*1/*1* genotype. Aangezien dit effect niet werd aangetoond bij de kinderen bij wie CYP3A5 niet tot expressie komt, lijkt POR een grotere rol te spelen op de activiteit van CYP3A5 dan van CYP3A4.

Deel III: Nierfalen na orgaan transplantatie

Nierfalen na transplantatie is een ernstige complicatie. Als we weten welke factoren daarvoor verantwoordelijk zijn, kunnen we wellicht de kans daarop verkleinen. Dit zijn niet alleen klinische factoren, maar ook genetische factoren. Wij hebben daarom een assay ontwikkeld met 96

variaties in genen. Dit zijn variaties die anderen al eerder hebben geassocieerd met tacrolimus-geïnduceerde nierschade, tacrolimus dispositie en nierfalen. Uit ***hoofdstuk 7*** blijkt een cumulatieve incidentie van chronisch nierfalen van 40%; en dit betrof 199 kinderen die een niertransplantatie hadden ondergaan. Gedurende de gehele studie periode bleef de nierfunctie stabiel. Desalniettemin was bij een klein aantal kinderen ervoer chronisch nierfalen al in de eerste maanden na transplantatie zichtbaar.

In ***hoofdstuk 8*** laten we een cumulatieve incidentie van chronisch nierfalen van 24% zien in 135 kinderen die in levertransplantatie hebben ondergaan. Één jaar na transplantatie ondervond 5,7% van de levertransplantatie kinderen mild nierfalen en 5 jaar na transplantatie geen van de kinderen (0%). Tenslotte, de cumulatieve incidentie in 161 harttransplantatie kinderen was 34.2% (***hoofdstuk 9***). De prevalentie van nierfalen was 2,9% een jaar na transplantatie en 1,7% 5 jaar na harttransplantatie. Deze resultaten voor alle drie de vormen van transplantatie suggereren dat ondanks stabiele nierfuncties, een klein aantal nierschade ontwikkelt al vroeg na transplantatie en waarbij we ernstiger nierfalen zien op langere termijn.

Wat betreft het assay om de invloed van genetische factoren op nierfalen te bepalen: geen van de bestudeerde varianties was geassocieerd met een verslechterde nierfunctie, en dit gold voor alle drie vormen van transplantatie.

Iemands nierfunctie wordt vaak bepaald aan de hand van de creatinine concentratie in het serum en het schatten van de glomerulaire filtratie ratios. Deze methoden hebben diverse beperkingen. Een van de nieuwe veelbelovende biomarkers voor nierfalen is neutrofiel gelatinase-geassocieerd eiwit (NGAL). Bij volwassen transplantatie patienten zijn NGAL levels in de urine zijn geassocieerd met acuut en chronisch nierfalen, maar bij kinderen is dit nog weinig onderzocht. In ***hoofdstuk***

10 laten we zien dat kinderen na een lever of nier transplantatie licht verhoogde NGAL concentraties in de urine hebben, maar zelden in de mate die geassocieerd is met acuut nierfalen bij kinderen met andere aandoeningen. Deze pilotstudie getuigt van subklinische nierschade bij de meeste kinderen vroeg na lever- en nier transplantatie.

Deel IV: Discussie en toekomst perspectieven

In ***hoofdstuk 11*** bespreken we de resultaten van alle studies in dit proefschrift. Daarnaast presenteren we enkele kennishiaten en aanbevelingen voor de toekomst. De belangrijkste conclusies zijn:

- *CYP3A4/5* en *POR* genotypes evenals de leeftijd verklaren een groot deel van de variabiliteit in tacrolimus dispositie in de eerste twee weken na transplantatie bij kinderen na solide orgaan transplantatie.
- Een beduidend grote groep kinderen die een nier-, hart- of levertransplantatie ondergaan en tacrolimus toegediend krijgen lijden aan chronisch nierfalen in de jaren na transplantatie.
- Chronisch nierfalen treedt vaak al op vanaf 3 maanden na de transplantatie en bij een klein aantal patiënten verslechtert de nierfunctie in de latere jaren.
- Wij hebben geen genetische risico factoren voor chronisch nierfalen kunnen vaststellen bij kinderen die een nier-, hart- of levertransplantatie hebben ondergaan en tacrolimus toegediend krijgen.
- NGAL concentraties in de urine zijn een potentiële marker voor subklinische nierschade in kinderen vroeg na lever- en niertransplantatie.

Appendices

Curriculum Vitae

Violette Gijsen was born on the 12th of October 1985 as the youngest of four children. She finished Secondary School at the Valuascollege, Venlo, in 2003 and started studying Pharmacy at the University of Utrecht in 2003, but after 4 weeks she switched studies and began her Medical training at the Erasmus University Rotterdam. In 2007, she moved to Toronto, Canada, as part of her medical degree to study the effects of Reiki on post-operative pain in women who underwent a Caesarean section. After one year, she received her doctorate degree in Medical Science. She continued to stay in Toronto and started her PhD project. During her stay in Toronto she volunteered at a children's play, Tails, which was a weekly show especially for the sick children admitted to the Hospital for Sick Children. In September 2011, she moved back to Rotterdam to finish her PhD at the Erasmus MC Sophia Children's Hospital. She also started her clinical rotations for her Medical Training in March 2013.

In July 2009 she received the Bill Mahon Presentation Award for the best trainee presentation at the Canadian Society of Pharmacology and Therapeutics Conference in Saskatoon for her systematic review of the pharmacogenetics of tacrolimus-induced nephrotoxicity in adults. In June 2010 she was invited to present her project on the effect of age and genotype on the pharmacokinetics of tacrolimus in the first 14 days after transplantation at the Canadian Society of Pharmacology and Therapeutics Conference in Toronto as part of the trainee session.

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Erasmus MC department: Intensive Care and Pediatric Surgery
PhD Period: October 2008 - May 2013
Promotors: Prof. Dr. D. Tibboel, Prof. G. Koren
Supervisors: Dr. SN de Wildt, Dr. RHN van Schaik

	Year	Workload
(ECTS)		
General courses		
Research Design and Statistical Analysis	2009	4
Specific courses		
Clinical pharmacology	2008	4
Maternal-fetal Pharmacology	2010	2
Presentations		
ASCPT: oral (1x), poster (3x)	2012	1.0
ASCPT: poster	2011	0.3
CPNDS: oral	2011	0.4
CSPT: poster	2011	0.3
Pediatric Pharmacogenomics and Personalized Medicine: poster	2011	0.3
CSPT: oral	2010	0.4
ASCPT: poster	2010	0.3
CPNDS: oral	2010	0.4
CSPT: poster	2009	0.3
CPNDS: oral	2009	0.4

	Year	Workload
International conferences		
ASCPT, National Harbor, MD, USA	2012	1
ASCPT, Dallas, TX, USA	2011	1
CPNDS, Vancouver, Canada	2011	0.5
CSPT, Montreal, Canada	2011	1
Pediatric Pharmacogenomics and Personalized Medicine, Kansas City, MO, USA	2011	0.5
CSPT, Toronto, Canada	2010	1
ASCPT, Atlanta, GA, USA	2010	1
CPNDS:, Vancouver, Canada	2010	0.5
CSPT, Saskatoon, Canada	2009	0.5
CPNDS, Vancouver, Canada	2009	0.5
Workshops and Seminars		
Career Bootcamp	2012	0.1
Clinical Pharmacology Review	2011	0.1
Other		
Various research meetings at The Hospital for Sick Children, Toronto, Canada and Erasmus MC Sophia Children's Hospital, Rotterdam.	2008-2013	5
Teaching activities		
Pharmacokinetics and Dosing to first year medical students	2011	0.3
Clinical Pharmacology to first year medical students	2011	0.3

List of publications

1. **Gijsen VMGJ**, van Schaik RHN, Soldin OP, Soldin SJ, Nulman I, Koren G, de Wildt SN. *P450 oxidoreductase *28 (POR*28) and tacrolimus disposition in pediatric kidney transplant recipients – a pilot study.* Submitted.

2. **Gijsen VMGJ**, Zwiers AJM, van Schaik RHN, Soldin OP, Soldin SJ, Hesselink DA, Nulman I, Koren G, de Wildt SN. *Urinary NGAL levels early after pediatric kidney and liver transplantation: a pilot study.* Submitted.

3. **Gijsen VMGJ**, van Schaik RHN, Elens L, Soldin OP, Soldin SJ, Koren G, de Wildt SN. *The new CYP3A4 intron 6 C>T polymorphism (CYP3A4*22) and CYP3A combined genotypes both correlate with tacrolimus disposition in pediatric heart transplant recipients.* Accepted in Pharmacogenomics April 2013

4. **Gijsen VM**, Hesselink DA, Croes K, Koren G, de Wildt SN. *Prevalence of renal dysfunction in tacrolimus-treated pediatric transplant recipients: A systematic review.* Pediatr Transplant. 2013 Feb 28.

5. **Gijsen VM**, Madadi P, Dube MP, Hesselink DA, Koren G, de Wildt SN. *Tacrolimus-induced nephrotoxicity and genetic variability: a review.* Ann Transplant. 2012 Apr-Jun;17(2):111-21. Review.

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10. vanderVaart S, **Gijsen VM**, de Wildt SN, Koren G. *A systematic review of the therapeutic effects of Reiki.* J Altern Complement Med. 2009 Nov;15(11):1157-69.

11. **Gijsen VM**, de Wildt SN, Ito S. *Probability of rash related to gabapentin therapy in a child.* Ann Pharmacother. 2009 Feb;43(2):387-9.

12. **Gijsen V**, Fulga N, Garcia-Bournissen F, Koren G. *Does light drinking during pregnancy improve pregnancy outcome? A critical commentary.* Can J Clin Pharmacol. 2008 Fall;15(3):e782-6.

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