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National Graduate School of Clinical Investigation  
Doctoral Programme in Clinical Research  
Children's Hospital  
University of Helsinki and  
Helsinki University Hospital  
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# **BIOMARKERS OF CHRONIC ALLOGRAFT INJURY IN CHILDREN AFTER RENAL TRANSPLANTATION**

JENNI MIETTINEN

ACADEMIC DISSERTATION

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## SUPERVISORS

*Professor Hannu Jalanko, MD, PhD*  
Children's Hospital  
University of Helsinki and  
Helsinki University Hospital  
Helsinki, Finland

*Docent Jouni Lauronen, MD, PhD*  
Finnish Red Cross Blood Service,  
Helsinki, Finland

## REVIEWERS

*Docent Ilkka Helanterä, MD, PhD*  
Abdominal Center  
Transplantation and Liver Surgery  
University of Helsinki and  
Helsinki University Hospital  
Helsinki, Finland

*Professor Matti Nuutinen, MD, PhD*  
Department of Pediatrics  
University of Oulu and  
Oulu University Hospital  
Oulu, Finland

## OPPONENT

*Professor Helena Isoniemi, MD, PhD*  
Abdominal Center  
Transplantation and Liver Surgery  
University of Helsinki and  
Helsinki University Hospital  
Helsinki, Finland

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## ABSTRACT

Renal transplantation (RTx) is a treatment of choice for children with end-stage renal disease. Excellent short-term survival is followed by moderate long-term results, and a considerable number of kidney grafts fail over the decades. Chronic allograft injury (CAI) is a multifactorial entity that manifests as a progressive deterioration of glomerular filtration rate (GFR), and histopathologically as interstitial fibrosis and tubular atrophy (IF/TA). CAI leads slowly to graft dysfunction and graft loss.

This thesis aimed to investigate potential biomarkers of CAI. Selected biomarkers reflect the consequences of post-transplant immune response or complications of immunosuppression. Post-transplant human leukocyte antigen (HLA) antibodies, immunohistochemical biomarkers, presence of anemia, low-grade inflammation and BK polyomavirus were analyzed to identify pediatric RTx recipients at risk for CAI. Understanding the effect of these post-transplant risk factors on allograft function is important for the adequate monitoring and early identification of graft failure.

The study cohort included 240 pediatric kidney transplant recipients who underwent RTx in Finland between 1988 and 2014. Data were retrospectively collected from patient records, and biomarker analyses were performed on stored serum samples or allograft biopsies. Luminex assay was used to detect donor-specific HLA antibodies (DSA) and immunoperoxidase staining to detect biomarker expression in biopsies. Finally, immunoassay method was used to detect inflammation and anemia related biomarkers and polyomavirus in blood samples.

HLA antibodies were detected in half of the routine follow-up samples of 123 pediatric RTx recipients. One-third of the patients had DSA, mostly against class II antigens. Donor-specificity, as such, was not predictive of subsequent deterioration of allograft function, questioning the need for modifications of immunosuppression in otherwise stable patients. Immunohistochemical staining of 165 biopsies from 56 patients revealed progressive IF/TA changes during the first 3 years post-RTx. Intense staining of collagen IV and vimentin associated with decreased GFR later on, although there was no additional prognostic value on graft function compared to routine IF/TA score. Post-transplant anemia and low-grade inflammation were common complications even years after RTx in 128 patients followed for a median of 10 years. Low Hb levels preceded IF/TA findings in protocol biopsies and associated with poor subsequent graft function. Anemia was not explained by low-grade inflammation or erythropoietin deficiency, and appeared early, rather than as a consequence of poor graft function. Inflammatory markers did not show a significant association with GFR at any time. BK viremia was detectable in nine patients with a tendency for decreased long-term graft function. Polyomavirus-associated nephropathy was detected in three patients.

The studied risk factors of post-transplant allograft nephropathy were rather common in this pediatric study population, but the clinical impact of a single biomarker on the long-term graft function was relatively minor. These findings support the follow-up of different pathophysiologic pathways in order to identify the high-risk recipients of CAI before the loss of graft function.

# TIIVISTELMÄ

Munuaisensiirto on käypä hoito munuaisten vajaatoiminnan loppuvaiheessa. Siirron jälkeinen ennuste on erinomainen, mutta pitkänajan ennustetta heikentää osalle potilaista kehittyvä munuaissiirteen krooninen vaurio ja siirteen vajaatoiminta, joka johtaa lopulta siirteen menetykseen vuosikymmenten kuluessa. Munuaissiirteen krooninen vaurio on monitekijäinen kokonaisuus, joka ilmenee munuaissiirteen toiminnan heikkenemisenä ja solutason muutoksina, kuten munuaiskudoksen arpeutumisenä ja munuaistiehyiden surkastumisena.

Tämän väitöskirjatyön tavoitteena oli tutkia munuaissiirteen kroonista vauriota kuvaavia biomerkkiaineita verestä sekä munuaissiirteestä otetuista koepaloista. Valitut merkkiaineet kuvastavat siirron jälkeisen immuunivasteen aktivoitumista ja seurauksia sekä hyljinnänestolääkitykseen liittyviä komplikaatioita. Tutkimme siirron jälkeisiä HLA (human leukocyte antigen) vasta-aineita ja immunohistokemiallisia kudokset merkkiaineita sekä matala-asteisen tulehduksen, anemian ja BK polyoomaviruksen (BKPyV) vaikutuksia munuaissiirteen toimintaan lapsipotilailla. On tärkeää selvittää siirron jälkeisten riskitekijöiden merkitys munuaissiirteen kroonisen vaurion kehittymisessä, jotta siirteen toimintahäiriölle alttiit riskipotilaat voidaan tunnistaa varhain.

Tutkimusaineistossa oli mukana 240 lapsipotilasta, joille on tehty munuaisensiirto Suomessa vuosina 1988–2014. Seeruminäytteistä ja munuaissiirteen kudokset koepaloista analysoitiin siirteen vauriota osoittavia merkkiaineita retrospektiivisesti. Verinäytteistä analysoitiin luovuttajaspesifisiä valkosoluvasta-aineita Luminex–menetelmällä ja anemiaa ja tulehdusmerkkiaineita immunologisella vasta-ainetunnistuksella. Immunoperoksidaasivärjäyksillä havainnollistettiin muutoksia munuaiskoepaloissa. Munuaistoiminnan mittarina ja pitkäaikaisseurannan vastemuuttujana käytettiin mitattua <sup>51</sup>Cr-EDTA-puhdistumaa.

HLA-vasta-aineita oli puolessa siirron jälkeisistä seurantanäytteistä. Kolmasosalla potilaista havaittiin luovuttajaspesifisiä HLA-vasta-aineita (DSA), jotka reagoivat pääosin HLA luokka II antigeenejä vastaan. DSA:n esiintyminen ei yksinään ennustanut tulevaa munuaistoiminnan heikkenemistä, mikä kyseenalaisti yksittäisen DSA löydöksen merkitystä ja tarvetta muuttaa hyljinnänestolääkitystä muutoin vakaavointisilla potilailla. Immunohistokemiallisista merkkiaineista kollageeni ja vimentiini olivat yhteydessä myöhempään munuaistoiminnan heikkenemiseen, mutta nämä merkkiaineet eivät tuoneet merkittävää lisää perinteisen Banff-luokituksen ennustearvoon. Anemia oli yleistä vielä vuosia siirron jälkeen. Varhaiset matalat hemoglobiini-arvot edelsivät munuaissiirteen kroonisen vaurion ilmaantumista ja heikkenevää munuaistoimintaa. Myös siirron jälkeisen BKPyV viremian havaittiin liittyvän munuaistoiminnan hiipumiseen jo ennen kudokset koepaloissa havaittavaa polyoomavirusinfektioon liittyvää munuaistautia.



Väitöskirjatutkimuksen tulokset lisäävät tietämystä kroonisen munuaisvaurion riskitekijöistä munuaisensiirtolapsilla. DSA-vasta-aineiden, anemian ja matalasteisen tulehduksen ilmaantuminen munuaissiirron jälkeen oli yleistä, mutta vain anemialla ja munuaiskoepalassa havaittavalla arpeutumisella todettiin selkeää ennustearvo munuaistoiminnan hiipumiseen. Yksittäisten merkkiaineiden kliininen hyöty munuaistoiminnan ennustajana on rajallinen, mutta löydökset tukevat eri patofysiologisten tekijöiden pitkäaikaisseuranta munuaissiirteen krooniselle vauriolle alttiiden riskipotilaiden varhaiseksi tunnistamiseksi.

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

- I Miettinen J\*, Peräsaari J\*, Lauronen J, Qvist E, Valta H, Pakarinen M, Merenmies J, Jalanko H. Donor-specific HLA antibodies and graft function in children after renal transplantation. *Pediatric Nephrology* 2012; 27:1011-1019. \* Authors contributed equally to this work
- II Miettinen J, Helin H, Pakarinen M, Jalanko H, Lauronen J. Histopathology and biomarkers in prediction of renal function in children after kidney transplantation. *Transplant Immunology* 2014; 31:105-111.
- III Miettinen J, Tainio J, Jahnukainen T, Pakarinen M, Lauronen J, Jalanko H. Anemia and low-grade inflammation in pediatric kidney transplant recipients. *Pediatric Nephrology*. DOI: 10.1007/s00467-016-3481-7. In press.
- IV Miettinen J, Lautenschlager I, Wernli M, Lauronen J, Hirsch H, Jalanko H. BK polyomavirus viremia in pediatric kidney transplant recipients. *Submitted*.

The publications are referred to in the text by their Roman numerals, and reprinted here with the permission of their copyright holders. Some previously unpublished data are also presented.

## ABBREVIATIONS

ABMR	antibody-mediated rejection
AR	acute rejection
AZA	azathioprine
BKPyV	BK polyomavirus
CAI	chronic allograft injury
CAN	chronic allograft nephropathy
CKD	chronic kidney disease
CAKUT	congenital anomalies of the kidney and urinary tract
CMV	cytomegalovirus
CNI	calcineurin inhibitor
<sup>51</sup> Cr-EDTA	Chromium-51 labeled ethylenediamine tetraacetic acid
CRP	C-reactive protein
CsA	cyclosporine A
CNF	congenital nephrotic syndrome of the Finnish type
DD	deceased donor
DSA	donor-specific HLA antibodies
<i>dn</i> DSA	<i>de novo</i> DSA
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-to-mesenchymal transition
EPO	erythropoietin
ESR	erythrocyte sedimentation rate
ESRD	end-stage renal disease
GFR	glomerular filtration rate
Hb	hemoglobin
HLA	human leukocyte antigen
hsCRP	high-sensitivity C-reactive protein
HUS	hemolytic uremic syndrome
IL-6	interleukin-6
IF/TA	interstitial fibrosis and tubular atrophy
JCPyV	JC polyomavirus
LD	living donor
MFI	mean fluorescence intensity
MMF	mycophenolate mofetil
pmarp	per million age-related population
PTLD	post-transplant lymphoproliferative disorder
PyVAN	polyomavirus-associated nephropathy
RRT	renal replacement therapy
RTx	renal transplantation
Tac	tacrolimus
TCR	T-cell receptor

# 1 INTRODUCTION

Renal transplantation is a treatment of choice for children with terminal renal failure. The goal of transplantation is to improve the health and quality of life of kidney graft recipients. In the past 50 years, transplantations have improved from the early experimental operations to an optimal mode of therapy in adults and children with end-stage renal disease (ESRD). Already in the 1960s, the short-term results of kidney transplantations were promising, but the risks of operations raised questions about rationality, especially among young pediatric patients. The lack of adequate immunosuppression hampered long-term results. Since the early 1980s, the use of Cyclosporine A (CsA) has reduced acute rejection rates and improved the long-term outcomes dramatically. Improvements in immunosuppressive therapy have allowed pediatric ESRD patients to grow and develop normally (Papalois, Najarian 2001, Sundaram et al. 2007)

In Finland, over 250 children and adolescents have undergone renal transplantation (RTx) at the Children's Hospital in Helsinki since 1986. Every year, on average 10 patients receive a kidney transplant, nearly half from a living donor (LD). The prevalence of pediatric patients on renal replacement therapy (RRT) in Finland is the highest (84.4 per million age-related population, pmarp) in the Europe. This is mainly due to the high incidence of the congenital nephrotic syndrome of the Finnish type (CNF); the first known, and the most common of the Finnish heritage diseases (Norio 2003).

To date, pediatric kidney recipients have the best long-term graft survival among recipients of all age groups. Short-term survival is excellent due to advances in immunosuppression, histocompatibility testing, surgery and perioperative management, whereas chronic allograft injury (CAI) and premature graft loss limit the long-term results (Dharnidharka et al. 2014). Importantly, improvements in immunosuppression also predispose patients to adverse effects, and patients are more vulnerable to infections and malignancy, which further challenge the post-transplant period.

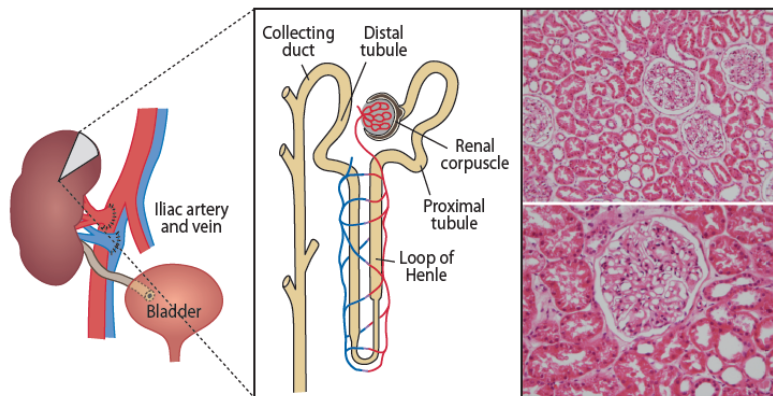
Clinical characteristics of CAI include slowly rising serum creatinine, proteinuria and hypertension (Pascual et al. 2002). Creatinine-based estimates of glomerular filtration rate (GFR) provide no insight into the underlying mechanisms of this clinic-pathological entity, and are insensitive to moderate changes in graft function, which may delay the diagnosis of the allograft nephropathy (de Souza et al. 2015). Current recommendations highlight the importance of early detection and follow-up with biopsies, although possible interventions may have only limited effect (Weir, Wali 2009). The main goal is to maintain long-term allograft function, especially among pediatric recipients with adult kidney grafts. This study aimed to identify the clinical relevance of early biomarkers of kidney allograft injury in pediatric RTx recipients.

## 2 REVIEW OF THE LITERATURE

### 2.1 Kidney

#### 2.1.1 Kidney anatomy and function

Kidneys are anatomically complex, bean-shaped organs in the retroperitoneal space. Microscopically, the structure relates closely to function (Figure 1). The main function of the kidneys is to maintain electrolyte and acid-base homeostasis and to remove excess water, salts and metabolic waste products from the blood to urine while restoring nutrients, glucose and amino acids back to blood (Moore, Dalley 2006). Adult kidneys filter about 180 liters of primary urine through glomeruli each day, but after reabsorption in tubules and collecting ducts, only about 1.5 liters of urine is excreted. In addition, kidneys produce essential hormones and enzymes. Interstitial fibroblasts produce erythropoietin, a hormone responsible for red blood cell production, and juxtaglomerular cells secrete renin, which regulates blood pressure.



**Figure 1** Schematic presentation of the kidney allograft in the right iliac fossa. In the renal cortex, each kidney contains roughly a million nephrons, which are responsible for ultrafiltration. Each nephron, the functional unit of the kidney, can be divided into glomerulus, proximal and distal tubule, loop of Henle and collecting duct. The hypertonic medulla contains the loops of Henle and collecting ducts, and is essential for reabsorption of water. Renal biopsy is required for the histological diagnosis of renal disease.

#### 2.1.2 Glomerular filtration rate

Renal clearance occurs in kidney glomeruli. GFR is a volume of filtrate formed in 1 minute corrected to the standard body surface area of  $1.73 \text{ m}^2$ . GFR is accurately measured by plasma clearance of chromium-51 labeled ethylenediamine tetraacetic

acid ( $^{51}\text{Cr-EDTA}$ ) (Fleming et al. 2004). The advantages of measured GFR are evident, especially among transplant recipients with multiple confounders (i.e., uremia, muscle wasting effect of corticosteroids, medications) that affect serum creatinine levels, making the interpretation of estimated GFR challenging (Stevens, Levey 2009).

Indirect assessment of GFR is more simple and common, and several algorithms exist based on the plasma creatinine value with specific modifications for children (Hoste et al. 2014, Hoste et al. 2015, Pottel et al. 2015, Vroiling et al. 2016). Estimated GFR is rather insensitive to moderate changes of accurate GFR, and only a substantial (50%) reduction in renal function increases the creatinine level above reference ranges (Piepsz et al. 2001, Stevens et al. 2006).

### 2.1.3 End-stage renal disease

ESRD is a rare but life-threatening condition in patients with various kidney diseases (Harambat et al. 2012). The stages of chronic kidney disease (CKD) represent the level of kidney function (Table 1). Dialysis is an alternative replacement of organ function, which is a unique opportunity compared to other solid organ transplantations. Most ESRD patients need dialysis before receiving a kidney transplant. Active maintenance dialysis before transplantation improves the metabolic and nutritional status, and allows children with ESRD to grow and maintain renal function (Warady et al. 2014).

**Table 1.** Stages of chronic kidney disease (CKD) (Karthikeyan et al. 2004)

CKD stage	GFR (mL/min/1.73 m <sup>2</sup> )	Description
1	90 +	Normal kidney function
2	60-89	Mild decrease in GFR
3	30-59	Moderate decrease in GFR
4	15-29	Severe decrease in GFR
5	< 15 or on dialysis	ESRD

CKD, chronic kidney disease; GFR, glomerular filtration rate; ESRD, end-stage renal disease.

The overall incidence of pediatric ESRD patients requiring RRT is approximately 5 cases per million of the age-related population (pmarp) in Europe (Chesnaye et al. 2014). In Finland, this number of new RRT patients per year is somewhat higher, around 9 pmarp (ESPN/ERA-EDTA registry report 2013), mainly due to the CNF, which accounts for the additional incidence to Finnish disease burden compared to the other European countries. These young patients, often under 2 years of age, require early dialysis at a median of 1.4 years before RTx (Laakkonen et al. 2008) to enhance growth, development and the quality of life (Jalanko et al. 2015).

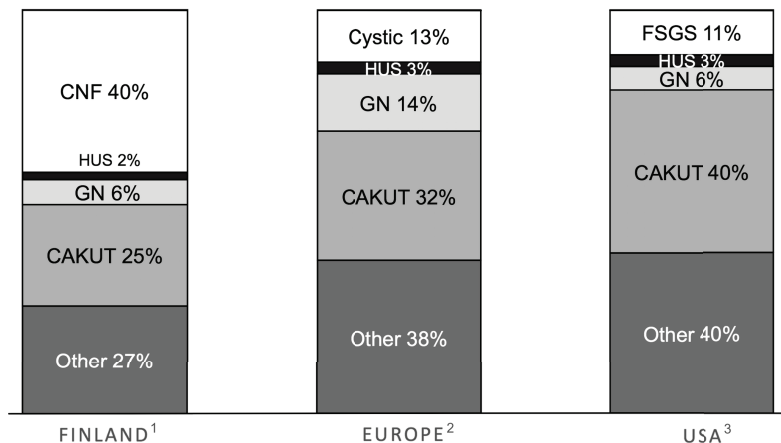
## 2.2 Renal transplantation in children

Kidney transplantation in children shares many aspects with transplantation in adults, i.e., surgery, medication, and acute rejection episodes (Dharnidharka et al. 2014). On the other hand, transplantations in children are unique and differ in terms of the primary kidney diseases (structural and congenital diseases in children), recipient's smaller size, and altered drug metabolism and pharmacokinetics. Also, primary viral infections, growth, and transition to adult care are specific topics among children (Dharnidharka et al. 2014).

### 2.2.1 Recipient

The major indications for RTx in children are different from those in adults (Figure 2). Most pediatric RTx recipients have congenital anomalies of the kidney and urinary tract (CAKUT) or inherited disorders, whereas diabetic nephropathy, hypertension and autosomal dominant polycystic kidney disease are the most common indications in adult RTx recipients (Holmberg, Jalanko 2015, Chesnaye et al. 2014, Dharnidharka et al. 2014).

Besides CAKUT, glomerulonephritis is the other common cause of renal failure in pediatric renal transplant recipients and affects more often older patients while younger ones are more likely to have hereditary or congenital disease. In Finland, RTx recipients have higher frequency (40%) of CNF (Figure 2), and patients require transplantation at a younger age compared to other Nordic countries (Jahnukainen et al. 2016).



**Figure 2** Etiology of end-stage renal disease (ESRD) in transplanted children in Finland, Europe and USA. CNF, congenital nephrotic syndrome of the Finnish type; HUS, hemolytic uremic syndrome; GN, glomerulonephritis; CAKUT, congenital anomalies of the kidney and urinary tract; FSGS, focal segmental glomerulosclerosis; Cystic, cystic kidney disease. <sup>1</sup> Children's Hospital, Helsinki; <sup>2</sup> ESPN/ERA-EDTA registry (Chesnaye et al. 2014); <sup>3</sup> NAPRTCS registry (NAPRTCS annual report 2014).

CNF is a Finnish heritage disease affecting 1 in 8000 live births (Norio 2003). In the Finnish patients, two main mutations (Fin-major, Fin-minor) appear in the NPHS1 gene, which encodes an essential component of the glomerular filtration barrier, a protein called nephrin. Mutation in NPHS1 results in massive proteinuria (Holmberg, Jalanko 2014). CNF patients with severe proteinuria require pre-transplant nephrectomy and initiation of dialysis at the age of 6-9 months, and thus undergo transplantation under the age of two years (Jalanko et al. 2015). Infants (0- to 24-month-old children) account for one-third of the pediatric RTx recipients in Finland, compared to 5.5% in the USA where most (47%) of the recipients are adolescent at the time of RTx (NAPRTCS Annual Report 2014).

### 2.2.2 Donor evaluation and surgery

Similarly to recipients, kidney donors require pretransplant evaluation. Preoperative assessment of a living donor follows generally accepted criteria in order to minimize risks for the donor. Physical and psychological health, renal vascular anatomy and kidney function are crucial for optimal and safe transplantation (Harmath et al. 2016). Moreover, careful selection of deceased donors ensures optimal graft quality and improves post-transplant survival (Israni et al. 2014). The demand for new kidney allografts exceeds deceased organ availability, resulting in an increase in the rate of living donors.

Pediatric patients receive mostly adult-sized kidneys from LD or deceased donors (DD). Living donors, in most cases recipients' parents, account for one-third of all donors for pediatric RTx recipients in Finland. In recent years, the number of LD kidneys has increased up to 50%, which is similar to numbers in North America (NAPRTCS Annual Report 2014). In the Nordic countries, LD kidneys are the most common (60%) graft type among pediatric recipients (Jahnukainen et al. 2016).

The benefits of LD kidneys comprise good donor quality, short cold-ischemia time and optimal timing of transplantation including pre-emptive transplantations (Jalanko et al. 2015). Additional advantages include elective surgery and alternative ABO incompatible (ABOi) transplantations. Pre-emptive RTx is a novel opportunity before initiating the dialysis if a living donor is available (Abramowicz et al. 2015). In Finland, the results of the first six pre-emptive transplantations have been promising.

Transplantation techniques in children are similar to adults, and the operations are highly successful. Generally, the recipients are over 10 kg and the graft is placed extraperitoneally. Smaller (6–10 kg) infants are successfully transplanted with intraperitoneal engraftment (Chavers et al. 2007, Jalanko et al. 2015). Extraperitoneal placement allows easy access to ultrasound investigations and kidney graft biopsies needed for rejection diagnostics (Neipp et al. 2002). Vascular anastomosis requires appropriate matching of blood vessel sizes to enable



adequate perfusion of the adult-sized donor graft (Chavers et al. 2007, Salvatierra et al. 2006) (Figure 1).

During transplantation, the kidney allograft may be exposed to several threats including prolonged cold-ischemia time, ischemia-reperfusion injury, and perfusion problems, all of which increase the risk for allograft injury (Galichon et al. 2013). Surgical complication occurs in 1 to 20% of the recipients (Rossi et al. 2016). The most common complication is lymphocele, which leads to perirenal fluid collection, and increases the risk for delayed graft function (Rossi et al. 2016, Ranghino et al. 2015). Vascular thrombosis is another important cause of graft loss secondary to technical problems (Salvatierra et al. 2006).

### 2.2.3 Histopathology

Percutaneous core needle biopsies are essential diagnostic tools in clinical nephrology (Fiorentino et al. 2016). Kidney graft biopsies enable a safe and established method for monitoring the post-transplant course (Galichon et al. 2013, Birk 2012). The main indication for kidney graft biopsy is graft dysfunction, including increasing serum creatinine, decreasing GFR, proteinuria and hypertension regardless of histopathology.

Histology reveals otherwise undetectable changes and is of invaluable importance when evaluating the degree and severity of the disorder, and to support diagnosis when the clinical manifestation is unclear. Vascular injury is characteristic of chronic changes that progress to fibrosis (Solez et al. 2007). Vasculopathy affects mainly the endothelium, which is the main target of immunological and non-immunological mechanisms of injury (Bruneau et al. 2012). Common histopathological features in biopsies include interstitial fibrosis and tubular atrophy (IF/TA), glomerular abnormalities, and arteriolar hyalinosis (Alexander et al. 2007).

CAI is a progressive process, which typically develops over years. The non-specific term '*chronic rejection*' (which originally implied immunological factors) was misleadingly used for late scarring of the graft, and in 1991 it was replaced by the more specific expression '*chronic allograft nephropathy*' (CAN). In turn, the term CAN became widely used as a non-specific description of fibrosis and graft dysfunction, despite the underlying disease processes. It was suggested that mixing many pathological processes with specific hallmark histopathology under the term CAN inhibited the accurate diagnosis and treatment of the real causes. Thus, CAN was replaced by a new definition, '*chronic allograft injury*', which is histologically defined by IF/TA with no evidence of any specific etiology (Solez et al. 2007).

The clinical manifestations of CAI include an increase in serum creatinine, proteinuria and hypertension, which appears as progressive renal dysfunction due to unrecognized causes. When specific causes are excluded, the non-specific term

CAI remains. In allograft biopsies, CAI is defined as IF/TA lesions, often in the presence of dynamic interplay between inflammation and fibrosis (Solez et al. 2007). Tissue injury activates infiltrated leukocytes and inflammatory cells to produce cytokines and proinflammatory molecules. Moreover, activated fibroblasts produce collagens, which induces renal fibrosis. In fibrogenesis, several profibrogenic cytokines, such as transforming growth factor- $\beta$  (TGF- $\beta$ ), bone morphogenetic protein (BMP), and hepatocyte growth factor (HGF) promote epithelial-to-mesenchymal transition (EMT), fibroblast activation and matrix deposition (Eddy 2014). IF/TA is defined as the excess accumulation of interstitial collagen and loss of normal tubular epithelial cell function in small, thin tubules (Zeisberg, Neilson 2010). Also, arterial lesions are prominent findings with intimal thickening and accumulation of foam cells, which leads to luminal narrowing. As larger arteries are generally affected while arterioles are spared, these chronic lesions may be undetectable on a kidney biopsy.

Fibrosis is the final manifestation of the CAI regardless of the underlying etiology. The combination of IF/TA is a common and unspecific finding in patients with chronic allograft dysfunction. IF/TA develops over months to years without addressing the underlying disease processes, and is followed by a decline in graft function (Haas 2014). As a response to prolonged graft injury, deregulated repair process may result in excess deposition of extracellular matrix (ECM), fibrous scars and loss of graft function. Visual assessment of IF relies mainly on standard trichrome-staining (Moreso et al. 2001), although computer-assisted morphometric analyses of trichrome, Sirius Red and collagen immunohistochemistry may increase the accuracy (Farris et al. 2011).

The etiology of CAI is multifactorial, and includes ischemia-reperfusion injury, acute rejection episodes, viral infections (cytomegalovirus, CMV; polyomavirus), chronic immunoactivity, and nephrotoxicity of calcineurin inhibitors (CNIs). Also, the diseases of the donor influence the progress of transplant destruction (Chapman et al. 2005), and lesions may have immunological or non-immunological origin (Table 2), although the roles of different mechanisms of injury are unclear. Thus, it is important to distinguish the underlying pathologic mechanisms in order to prescribe appropriate treatment (Halloran 2002, Galichon et al. 2013). Histopathologic lesions of CAI occur late and thus do not differentiate the underlying cause.

**Table 2.** Risk factors that influence kidney graft survival

<i>Non-immunological</i>	
Donor-related factors	
	Donor age and tissue quality
	Brain death-related stress
	Preservation and implantation injury
Recipient related-factors	
	Post-transplant stress factors, i.e., BKPyV and CMV infections
	Metabolic syndrome
	CNI toxicity
<i>Immune-mediated injury</i>	
Rejections	
TCMR	Severe rejections with endothelial injury
ABMR	Might occur late due to non-compliance
HLA mismatches	
Donor-specific HLA antibodies	
Under-immunosuppression/non-compliance	
Sensitization to foreign HLA	
Pre-RTx	Previous blood transfusions, pregnancies or transplantation
Post-RTx	DSA

BKPyV, BK polyomavirus; CMV, cytomegalovirus; CNI, calcineurin inhibitor; TCMR, T-cell mediated rejection; ABMR, antibody-mediated rejection; HLA, human leucocyte antigen; DSA, Donor-specific HLA antibodies; RTx, renal transplantation. *Adapted from (Halloran 2002, Galichon et al. 2013).*

The presence of abnormalities in implantation biopsies varies in up to 40%, depending on donor age and quality and kidney recovery after transplantation (Lehtonen et al. 2001). Irreversible allograft fibrosis is present in over half of the post-transplant biopsies by 10 years and associates with a decline in graft function (Nankivell et al. 2003). Observational studies have demonstrated that persistent inflammation, especially in the area of fibrosis, is harmful and associates with poor graft function (Heilman et al. 2010, Mengel et al. 2007, Cosio et al. 2005). However, the graft function is not a sensitive marker of the underlying severity of graft pathology (Legendre et al. 1998).

Semiquantitative histological evaluation of allograft biopsies follows standardized criteria. The Banff classification and its most recent revision, Banff'13 (Haas et al. 2014), are used worldwide for biopsy interpretation (Solez et al. 2007). Three main categories are divided into subcategories that comprise specific diagnostic criteria (Table 3). Other scoring systems, e.g. chronic allograft damage index (CADI) (Isoniemi et al. 1994) are also used to describe detailed histological findings and to further associate findings with graft outcomes (Yilmaz et al. 2003). Diagnosis of the graft biopsy is mainly based on light microscopy and immunohistochemistry.

**Table 3.** Banff classification

Diagnostic categories for kidney allograft biopsies according to Banff <sup>1</sup> classification	
1	Normal
2	Antibody-mediated rejection (ABMR) Acute/active ABMR Chronic/active ABMR C4d positivity without evidence of rejection
3	Borderline changes Mild tubulitis (t0-t1), mild interstitial inflammation
4	T-cell-mediated rejection (TCMR) Acute: IA, IB, IIA, IIB, III Chronic/active
5	Interstitial fibrosis and tubular atrophy Mild, moderate, severe
6	Other Changes not thought to be due to rejection i.e., CNI toxicity, chronic hypertension or obstruction, bacterial pyelonephritis, viral infection. May coincide with categories 2–5.

CNI, calcineurin inhibitor. <sup>1</sup> Banff '97 criteria, revised in '05 and '13. Modified from detailed Banff criteria (Racusen et al. 1999, Solez et al. 2007, Haas et al. 2014).

#### 2.2.4 Biomarkers of allograft injury

Biomarkers are objective indicators that can be classified as prognostic, predictive or surrogate end points (Lo et al. 2014). In kidney transplantation, many established biomarkers are in clinical use, i.e., serum creatinine and human leukocyte antigen (HLA) match between donor and recipient (Roedder et al. 2011). However, identification of novel biomarkers that could replace invasive biopsies and accurately reflect the allograft function is important.

Renal biopsy visualizes the current histopathology, but provides limited data on possible mechanisms of allograft injury (Henderson et al. 2011). Immunohistochemistry is a sensitive method used to detect structural mesenchymal markers, especially when biomarkers are expressed *de novo* (Galichon, Hertig 2011).

Serum biomarkers, which may improve the ability to diagnose specific pathologic mechanisms of CAI or predict outcomes, are presented in Table 4. Although the urinary biomarker candidates for acute kidney injury and acute rejection are numerous (Lo et al. 2014, Sigdel et al. 2016, Westhoff et al. 2016), none of the quantitative biomarkers of CAI has yet been validated for clinical use. Recently, varieties of non-invasive biomarkers have been studied as potential surrogates for CAI (Stegall et al. 2015, Stegall, Borrows 2015).

**Table 4.** Biomarkers involved in renal fibrogenesis or outcome

Indication/marker	Description of positivity/outcome	EMT	Ref.
<u>Markers of epithelial-to-mesenchymal transition (EMT)</u>			
<i>Cytoskeletal markers</i>			
Vimentin	Intermediate filament, mesenchymal cells	+	(Hertig et al. 2008)
$\alpha$ -SMA	Interstitial myofibroblasts	+	(Galichon, Hertig 2011)
$\beta$ -catenin	Translocation in the cytoplasm	+	(Hertig et al. 2006)
FSP1; S100A4	Tubular epithelial cell marker	+	(Vitalone et al. 2008)
Cytokeratin	Tubular epithelial cell marker	-	(Vongwiwatana et al. 2005)
<i>Extracellular matrix (ECM) proteins</i>			
Collagens	ECM and basement membrane constituents	+	(Rastaldi et al. 2002)
HSP47	Marker of collagen production	+	(Rastaldi et al. 2002)
<i>Cell-surface proteins</i>			
N-cadherin	Mesenchymal cells	+	(Zeisberg, Neilson 2009)
E-cadherin	Tubular epithelial cell marker	-	(Vitalone et al. 2008)
<i>Inhibitors of EMT</i>			
HGF	Anti-fibrotic molecule, counteracts TGF $\beta$	-	(Yang et al. 2005)
BMP-7	Anti-fibrotic molecule, counteracts TGF $\beta$	-	(Zeisberg et al. 2003)
<u>Markers of endothelial-to-mesenchymal transition</u>			
Fascin 1	Actin-bundling protein in endothelial cells		(Xu-Dubois et al. 2016)
<u>Markers associated with post-transplant outcome</u>			
KIM1	Marker of general renal injury		(Malyszko et al. 2010) (van Timmeren et al. 2007)
<i>Growth factors</i>			
TGF- $\beta$	Profibrotic factor, initiate/maintain EMT	+	(Zeisberg, Neilson 2009)
CTGF	Profibrotic molecule	+	(Xu-Dubois et al. 2013)
FGF-23			(Seifert et al. 2016)
<i>Proteases</i>			
MMPs			(Catania et al. 2007)
<i>Gene expression signatures (microRNA, microarray) in renal biopsies</i>			
	Correlated with AR, ABMR, IFTA, and GFR		(Baron et al. 2015) (Stegall, Borrows 2015) (Loupy et al. 2014) (Halloran et al. 2013) (Anglicheau et al. 2012)
<i>Immune monitoring</i>			
HLA antibodies	DSA with C1q and C3d binding		(Fichtner et al. 2016) (Cornoli et al. 2016) (Lefaucheur et al. 2016) (Loupy et al. 2013)
Non-HLA antibodies	MICA antibodies		(Opelz, Collaborative Transplant Study 2005)

EMT, epithelial-to-mesenchymal transition;  $\alpha$ -SMA, alpha-smooth muscle actin; FSP1, fibroblast-specific protein-1; ECM, extracellular matrix; HSP47, heat shock protein 47; HGF, hepatocyte growth factor; BMP-7, bone morphogenetic protein 7; KIM1, kidney injury molecule 1; TGF- $\beta$ , transforming growth factor beta; CTGF, connective tissue growth factor; FGF, fibroblast growth factor; MMP, matrix metalloproteinase; DSA, donor-specific HLA antibodies; MICA, major-histocompatibility-complex class I-related chain A.

The requirements for novel non-invasive, reliable and predictive biomarkers are high. The optimal time point for the measurement, appropriate thresholds and the features to reflect progression beyond other clinical conditions warrant additional clinical considerations. The development of novel candidate biomarkers, transcriptomics and proteomics, requires a variety of platforms (i.e., microarray, quantitative real-time PCR, and microRNAs) to assess their performance (Ying, Sarwal 2009, Anglicheau, Suthanthiran 2008). Validation of such markers would bring clear clinical value in the form of individualized therapy (Asadullah et al. 2015). This might decrease the rate of adverse effects, reduce costs and improve the long-term outcome (Ho et al. 2012).

## **2.3 Transplantation immunology and immunosuppression**

The immune system protects us from foreign pathogens and microbes. In the case of transplantation, the recipient immune system reacts against the foreign allograft and gradually rejects it, if the immune response cannot be circumvented with adequate immunosuppressive medication.

### **2.3.1 Human leukocyte antigen system**

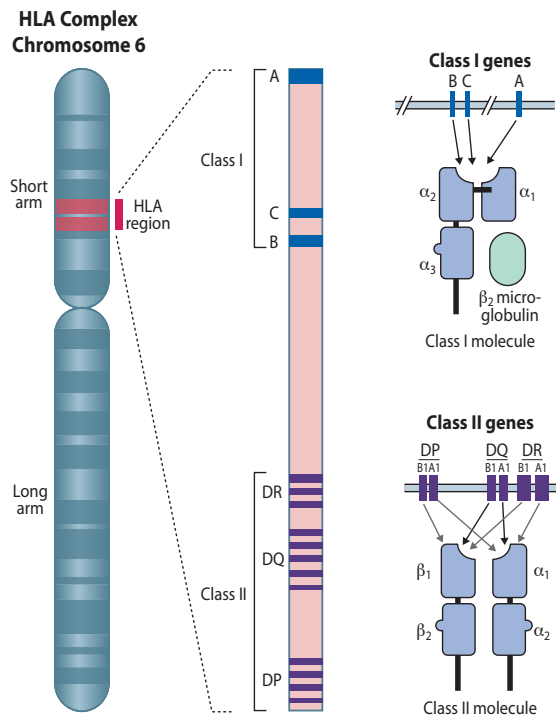
HLA molecules are polymorphic proteins and important targets for immune recognition in organ transplantation (Figure 3). To date, the known HLA polymorphism is extremely high and identifies over 10,000 allele sequences, double the number in the early 2010s (Thorsby 2009, Terasaki 2013). Polymorphism hampers HLA matching and predisposes to rejections, leading to a need for lifelong immunosuppression. In addition, alloreactivity increases with age, as circulating T- and B-cell subtypes and immunologic features develop from birth to adulthood, although children with ESRD may have suboptimal immune responses (Hartel et al. 2005, Dharnidharka et al. 2014).

HLA class I molecules are present in the cell surface of almost all nucleated cells, whereas HLA class II molecules are only found on the surface of antigen-presenting cells (APCs) such as dendritic cells, macrophages, B cells, monocytes and endothelial cells. APCs present HLA molecules with bound peptides for two types of cells. HLA class I molecule binds foreign proteins degraded inside the cell (endogenous pathway) and activates CD8 T cells (cytotoxic lymphocytes). Foreign protein degraded outside the cell (exogenous pathway) binds to HLA class II molecules, which in turn activates CD4 T lymphocytes (T helper cells) (Candon S, Marguiles DH 2004) (Figure 3).

### 2.3.2 Histocompatibility

Minimization of immunological differences between donor and recipient is crucial for successful kidney transplantation. Tissue typing prior to RTx includes four components: HLA and ABO matching, screening for HLA antibodies (HLAab), and cross matching.

ABO matching compares blood types of the kidney recipient and donor. Blood groups A, B and O have traditionally constructed the ABO barrier, which allows the transplantation of a kidney allograft with compatible antigens only to avoid rejection by anti-ABO antibodies. In ABO-incompatible (ABOi) transplantation, the recipient has antibodies against graft AB-antigens prior to preoperative management. In young children, the immature immune system allows organ transplantations across traditional ABO barriers. In the past 15 years, more than 3,000 ABOi living donor kidney transplantations have been reported worldwide. Long-term graft survival is comparable to ABO-matched LD kidneys (Zschiedrich et al. 2016), and ABOi transplantations are suggested to partly resolve the increasing need for new donors.



**Figure 3** In humans, the major histocompatibility complex (MHC) is termed the human leukocyte antigen (HLA) system. This highly polymorphic gene family is located on the short arm of chromosome 6, and divided into three main regions encoding different cell surface protein molecules, antigens. MHC class I genes encode HLA-A, HLA-B, HLA-C molecules while MHC class II genes encode HLA-DR, HLA-DQ, HLA-DP molecules. The MCH class III genes encode important molecules for inflammation, such as components of complement (Candon S, Marguiles DH 2004).

HLA typing compares tissue types of the recipient and donor. HLA-A, -B, and -DR antigens have an important role in transplantation, and are matched to reduce the risk of post-transplant rejection, although other antigens may also trigger rejection episodes. HLA-DR is the most immunogenic locus, followed by the HLA-B and HLA-A loci. This explains the importance of HLA-DR matching, although polymorphism complicates the matching of unrelated donors and kidney recipients (Candon S, Marguiles DH 2004).

The panel reactive antibody (PRA) test with cytotoxic panel identifies HLA reactivity before transplantation. It determines how likely the patient is to have antibodies against the donor organ, and is used as a risk assessment tool. Immunization against donor HLA antigens is possible prior to transplantation as a result of blood transfusions, pregnancy, or previous transplantation. Sensitization to HLAab often delays the finding of an acceptable donor, and sensitized recipients are at increased risk of severe rejection episodes if the antibody response is directed against donor-antigens or a cross-reactive polyclonal group.

Detection of HLAab is conventionally assessed by complement-dependent cytotoxic testing (CDC), although it lacks sensitivity in detecting clinically significant HLAabs. In turn, Luminex assay is a highly sensitive and specific method recently used in organ allocation and risk assessment by measuring pretransplant and posttransplant HLAab levels (Konvalinka, Tinckam 2015) (Table 5).

**Table 5.** Technological aspects of Luminex

	<b>Technological advantages</b>	<b>Technological limitations</b>
Qualitative	Precise identification of all antibody specificities	False positivity due to antibodies to denatured HLA
Comprehensive	Detection of all common alleles	Occasional background requires repeat testing and absorption protocols
Semiquantitative	Determination of high, intermediate, and low levels of ab	Variable HLA protein density in beads, risk for false-negative or misleading low assessments
Sensitive	Detects weak antibodies	
Rapid	Real-time ab monitoring, assists diagnosis of ABMR	Lot-specific variation
Non-HLA-specific antibodies	Detection of MICA	
Complement / non-complement fixing	Differentiation of C4d and C1q	Reagents not standardized

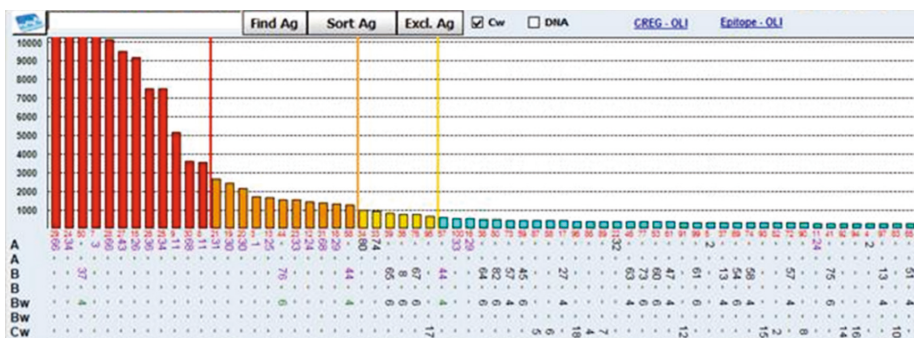
HLA, human leukocyte antigen; ABMR, antibody-mediated rejection; MICA, major-histocompatibility-complex class I-related chain A. Modified from (Tait et al. 2013).

Crossmatch testing is performed at the time of transplantation, and is designed to prevent hyperacute and acute rejections. Recipient serum is tested with lymphocytes from the donor. A positive crossmatch usually indicates the presence



of preformed donor-specific HLA antibodies (DSA), which results in rejection, and thus the transplantation is not performed.

Interpretation of solid-phase assays requires understanding of the challenges of the methodology to define the clinically relevant threshold for mean fluorescence intensity (MFI) positivity and to consider quantitative limitations (Figure 4). Serial testing of antibody levels over time is preferable to a single-antibody testing. The interpretation of results is affected by many variables, such as adherence to immunosuppression, latency period, and change in antibody levels due to memory B cell responses and dynamic changes in DSA MFI levels (Konvalinka, Tinckam 2015).



without memory. Acute infection and inflammatory cells increase cellular damage, which may provoke acute rejection early after transplantation. Innate immunity helps to activate the more specific adaptive immune response (Heeger 2003).

*In adaptive immune response*, recipient's T lymphocytes recognize foreign alloantigens, leading to alloantigen-specific immunity (Chalermkulrat et al. 2004). Adaptive immunity is slow, antigen-specific and poorly effective without innate immunity, but results in memory.

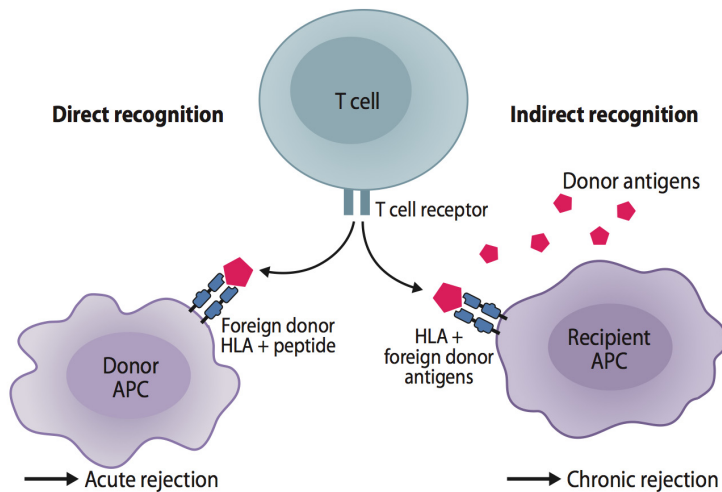
After transplantation, recipient's T-lymphocytes recognize allogeneic graft tissue as a foreign threat (T-cell allorecognition). HLA antigens may induce cellular (T cell-mediated) immunity or humoral (antibody-mediated) immunity. Cellular immunity to HLA antigens is further divided into direct and indirect allorecognition pathway.

*In direct recognition*, recipient's T cells interact with incompatible HLA antigens on the surface of donor APC cells. Self-restricted T cells are specialized to recognize these foreign allogenic HLA/peptide complexes (Heeger 2003). This leads to infiltration of recipient T-cell into the graft as a cellular immune response to initiate tissue injury. In general, HLA class I molecules activate CD8+ cytotoxic T cells, while HLA class II antigens activate CD4+ helper and effector T cells. Direct allorecognition early after RTx leads to severe immediate immune response, whereas the indirect pathway occurs over time (Figure 4).

*In indirect recognition* pathway, recipient's APCs take up donor-specific antigens and present these to the recipient's own T cells. Acute cellular rejection is an activation of alloreactive T cells and APCs. Different cell types are present, such as proinflammatory leukocytes, CD4+ helper cells, CD8+ cytotoxic T cells and antibody-forming B cells. The kidney graft is a continuous resource for donor antigens, which may lead to development of chronic allograft injury over time.

**In humoral immunity**, activated B cells mature and differentiate into alloantibody-producing plasma cells and memory B cells. Most alloantibodies react against class I and/or class II HLA antigens that are distinct from the recipient, but also non-HLA antibodies, such as anti-endothelial cell antibodies, have been regarded as an important risk factor for chronic rejection and graft failure (Delville et al. 2016). In fact, the rejection process involves the entire immunologic response system in addition to T cell and B cell cascades.

*Immunological tolerance* in transplantation biology would enable to silencing the immune response against the allogeneic graft tissue while immunity to infections and malignancy remained intact.



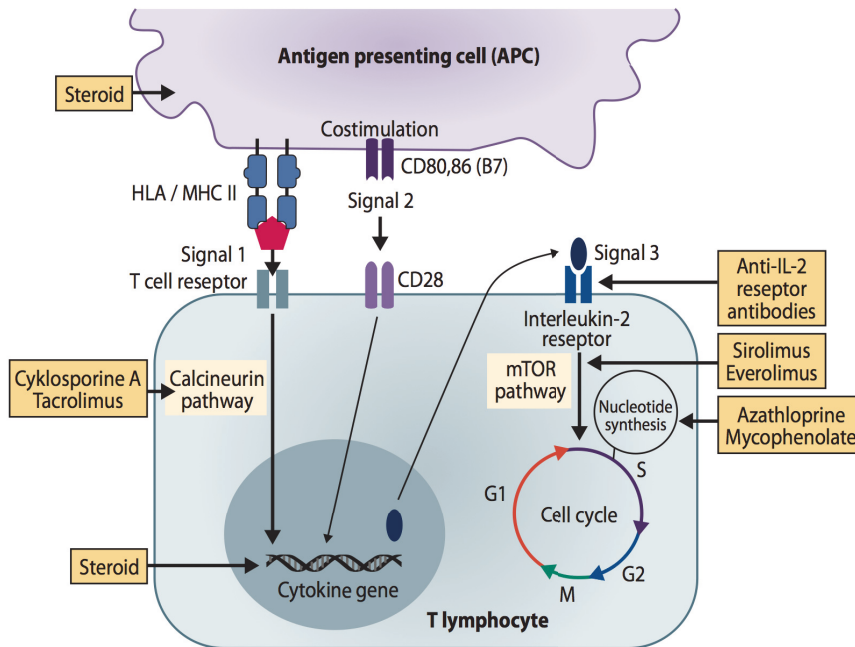
**Figure 5** Allorecognition, direct and indirect pathway. T-cell activation depends on the recognition of the HLA and foreign peptide complex. As the HLA/peptide complex engages the T-cell receptor (TCR), additional co-stimulatory and adhesion molecules are activated to provide the proper combination of signals. If T cells recognize this complex to self-antigens of the recipient, T cells remain inactive, whereas recognition to non-self antigens of the donor leads to T-cell activation, proliferation and rejection. Modified from (Fine et al. 2007).

### 2.3.4 Immunosuppression

Successful immunosuppression comprises a continuous balance between rejections and an acceptable number of side effects (Srinivas, Meier-Kriesche 2008). The early post-transplant period with intense immunosuppression is challenging, with individual variation in medications and differences across transplant programs (Axelrod et al. 2016).

The importance of immunosuppression is more pronounced early after RTx, as most acute rejections occur during the first 6 months post-Rtx (Nankivell et al. 2003). At the maintenance phase, graft adaptation reduces the need for rejection prophylaxis and doses are gradually reduced. Thus, the risk of rejection and the level of immunosuppression decrease with time.

Most immunosuppressive regimens act against the T lymphocytes. T cells require three signals to be activated, and all these are potential interaction sites for immunosuppressive drugs (Halloran 2004) (Figure 5). Medications interrupt lymphocyte proliferation, interfere with lymphocyte differentiation and cell co-stimulation, deplete cells and induce tolerance.



**Figure 6** Targets of immunosuppression. Modified from Halloran 2004.

Multidrug combination therapy provides synergistic efficacy and allows the use of reduced doses to avoid drug-related and dose-dependent side effects. Initial immunosuppression is tailored to the needs of the recipient in order to ensure efficacy and tolerability and to control the appearance of side effects (Augustine, Hricik 2007). The diversity of immunosuppressants has led to wide variation in protocols, and the medication used partly depends on center-level practice rather than on individual needs (Axelrod et al. 2016).

*Calcineurin inhibitors (CNIs)* have been the basis of modern immunosuppression over the past two decades. CNIs, cyclosporine A (CsA) and tacrolimus are effective immunosuppressants that induce good survival rates and graft function, although both are nephrotoxic (Srinivas, Meier-Kriesche 2008). They selectively suppress the activation and production of T cells and inhibit the release of IL-2 and other cytokines.

*Cyclosporine A* prevents transcription of the interleukin-2 gene, which, in turn affects cytotoxic T-cell precursors and inhibit the activation of T lymphocytes. As the therapeutic window between efficacy and toxicity is narrow, negative side effects are common and therapeutic drug monitoring mandatory (Tredger et al. 2006). CsA metabolism, via cytochrome P450 isoenzyme 3A4, causes individual variability in pharmacokinetics and significant drug interactions, and in general, children require higher doses of CsA than adults (Tredger et al. 2006).

*Tacrolimus* is the other main immunosuppressant, which has similar graft and patient survival outcomes as CsA. Tacrolimus might be more potent in rejection prophylaxis and thus be more favorable for renal function. Despite the reduction in acute rejection rate, the use of tacrolimus does not significantly improve the overall graft survival. Currently, no CNI is superior to another, and withdrawal or replacement of CNIs due to increased nephrotoxicity or early graft loss is no longer considered (Stegall et al. 2015).

*Antimetabolites* inhibit the synthesis of nucleic acids. Mycophenolates (mycophenolate mofetil, MMF and enterocoated mycophenolate sodium) inhibit the differentiation and proliferation of lymphocytes. Mycophenolates are selective for lymphocytes and provide specific immunosuppression with an acceptable side effect profile (gastrointestinal, CMV, bone marrow suppression). The use of MMF with prednisolone and CNI allows CNI sparing (Halloran 2004). MMF is commonly combined with tacrolimus. Most importantly, the combined use reduces the rejection rate, and MMF is not nephrotoxic and lacks increased cardiovascular risks (Srinivas et al. 2005).

*Azathioprine* is a purine analog, which unselectively inhibits DNA synthesis and the proliferation of lymphocytes (Tredger et al. 2006). Azathioprine was the first immunosuppressant used worldwide until the introduction of CsA. Currently, Aza has mostly been replaced by MMF in adults.

*Glucocorticoids* (corticosteroids) have remained a part of standard immunosuppressive therapy over the past 50 years of practice. Steroids bind to the glucocorticoid receptor and down-regulate inflammatory cytokines. This unspecific anti-inflammatory effect has many potential effects on the immune system, simultaneously with a large number of adverse effects. To reduce steroid-associated side effects, some centers use steroid withdrawal or steroid-free protocols. Steroid withdrawal is possible with careful planning, although the benefits are debated and depend on timing and co-medications (Grenda 2013, Webb et al. 2015).

*Inhibitors of the mammalian target of rapamycin (mTOR)*, sirolimus and everolimus, inhibit the proliferation of cytokine-dependent T lymphocytes, mesenchymal cells and tumor cells. As mTOR inhibitors have a highly synergistic effect with CNI, they allow a reduction in CNI levels without any impact on efficacy. Combination immunosuppression with sirolimus and MMF improves long-term graft function compared to the combination of CNI and MMF (Weir et al. 2016).

Induction therapy is prophylactic, perioperatively administered immunosuppression. It is effective in reducing initial rejections. Induction therapy includes polyclonal and monoclonal antibodies against T cells, such as anti-thymocyte globulin, which results in depletion of T cells. *Basiliximab* is a monoclonal interleukin-2 receptor antibody used in induction at the study center. Careful risk-benefit assessment is

necessary as induction therapy increases the risk for opportunistic infections and malignancy.

*Rituximab* is a monoclonal anti-CD20 antibody, which depletes peripheral B cells and is used in desensitization protocols, prophylaxis and treatment of humoral rejections, and in treatment of post-transplant lymphoproliferative disorder (PTLD). *Betacept*, in turn, blocks co-stimulation and is used as a part of CNI-free regimen. Desensitization protocols include the use of plasmapheresis, immunoabsorption, intravenous immunoglobulins (IVIG) and rituximab to remove circulating HLA antibodies.

*The side effects of immunosuppression* are in general non-specific, and comprising increased risk for malignancy and opportunistic infections. Infections are the main problem during the first year post-RTx, whereas malignancy is important in the long-term. Each immunosuppressant has additional, specific, often dose-dependent side effects, as listed in Table 6. CNIs are nephrotoxic, which may lead to functional or structural damage, which may be reversible or irreversible, respectively. Functional arteriolar vasoconstriction causes hypoperfusion of the kidney graft, which leads to a decreased GFR. This phenomenon is mostly reversible and dose-dependent, and induced by high CNI doses at the early post-RTx phase (Naesens et al. 2009). Striped fibrosis is a characteristic but non-specific histologic feature for chronic CNI toxicity. Vacuolar changes, hyaline deposits and thrombotic microangiopathy may indicate CNI nephrotoxicity, although histological characteristics are not specific for CNI toxicity (Mengel et al. 2011).

**Table 6.** Side effects of immunosuppressive medication

Side effects	CsA	Tacro	AZA	MMF	Steroids
Nephrotoxicity	+++	+++	-	-	-
Hypertension	+++	++	-	-	+++
Hypercholesterolemia	++	+	-	-	++
Diabetes	+	+++	-	-	++
Osteoporosis	+	+	-	-	+++
Neurotoxicity	++	+++	-	-	+
Myelosuppression	-	-	+	+	-
Gastrointestinal	+	+	+	+++	-
Hepatotoxicity	+	+	+	-	-
Cosmetic side effects	+	-	-	-	+
Risk of PyVAN	+	++	-	+	-

CsA, cyclosporine A; Tacro, tacrolimus; AZA, azathioprine; MMF, mycophenolate; PyVAN, polyomavirus-associated nephropathy (Webster et al. 2005, Halloran 2004).

## 2.4 Complications and long-term outcome

Post-transplant complications can be classified into graft-related (i.e., delayed graft function or acute tubular necrosis), technical (i.e., vascular thrombosis) or anatomical (i.e., obstruction). However, the immune response of the recipient causes the most problematic complications, such as rejections and secondary effects of immunosuppression, i.e., infections, malignancy and growth problems (Table 7).

**Table 7.** Post-transplant complications in relation to time after RTx in children

Time after RTx	Complication
Immediate, 0-1 week	<ul style="list-style-type: none"> <li>Delayed graft function (oliguria, need for dialysis)</li> <li>Acute tubular necrosis</li> <li>Vascular thrombosis</li> <li>Urologic complication (leak or obstruction)</li> </ul>
Early, 1-12 weeks	<ul style="list-style-type: none"> <li>Acute rejection</li> <li>CNI toxicity</li> <li>Urinary obstruction</li> <li>Hypovolemia</li> <li>Infections</li> <li>Recurrence of the primary disease</li> </ul>
Late chronic, years	<ul style="list-style-type: none"> <li>Cardiovascular and metabolic complications</li> <li>Hypertension</li> <li>Anemia</li> <li>Low-grade inflammation</li> <li>Growth and skeletal complications</li> <li>Psychosocial complications, i.e., non-compliance</li> <li>Malignancies (PTLD)</li> </ul>

RTx, renal transplantation; CNI, calcineurin inhibitor; PTLT, post-transplant lymphoproliferative disease.

### 2.4.1 Rejection-related graft injury

Most acute rejection episodes occur early during the first three months post-RTx, and are characterized by an increase in serum creatinine and specified findings on core needle biopsy (Nankivell et al. 2003). After that, late acute rejections frequently associate with under-immunosuppression or non-compliance. Subclinical rejection corresponds to histology of acute rejection without graft dysfunction. Acute rejection episodes are treated with high doses of intravenous methylprednisolone pulses. Conversion from CsA to tacrolimus, the use of antibody removal/neutralization by

plasmapheresis, anti B-cell therapies (Rituximab) or T-cell depleting agents (anti-lymphocyte globulin) may add efficacy. In the presence of CNI nephrotoxicity, reduction of CNI regimen may inhibit the histopathologic progression.

*In T cell-mediated rejection (TCMR)*, T lymphocytes infiltrate into the graft. Accumulation of T cells in the peritubular capillaries, tubule walls and interstitium is called tubulointerstitial rejection. This is the most common form of acute cellular rejection. Arterial cell-mediated rejection is characterized by accumulation of mononuclear leukocytes in the arterial walls, and these ominous lesions are less responsive to steroids. Acute transplant glomerulopathy is a rare and severe form of cellular rejection, detected as severe glomerular inflammation and cellular damage, which may exist without tubulo-interstitial rejection. No C4d positivity in tissue or DSA in blood is present. Recent studies suggest that TCMR shares common features with ABMR, and novel molecular methods are investigated to elucidate the more detailed pathogenesis of different types of graft injury (Reeve et al. 2016).

*Antibody-mediated rejection (ABMR)* response may appear as acute rejection within weeks to months or as chronic rejection within years. Variation in the time course of the antibody response is significant, making the screening/monitoring of antibodies demanding. Monitoring of HLA antibodies is necessary as circulating DSA may otherwise cause immediate hyperacute rejection and constitute a significant risk factor for transplant glomerulopathy and graft loss (Cardarelli et al. 2005). Biopsy is recommended in the presence of post-transplant DSA and the subsequent treatment is mainly based on the biopsy results (Tait et al. 2013).

Endothelial cells in the graft are exposed to recipient's serum and are a common site for antibody-initiated injury. In ABMR, donor-specific antibodies react against endothelial antigens. The key mechanism for the allograft injury and inflammation during ABMR includes the complement fixation by DSA and thus activation of the complement cascade (Thomas et al. 2015). It may appear as hyperacute, acute/active or chronic/active ABMR. Several subtypes of ABMR have recently been investigated (Halloran et al. 2016).

*In acute/active ABMR*, the main lesions are microvascular and may affect either, glomerular, arterial, or peritubular capillaries. Diagnosis is possible even in the absence of C4d complement component, as the most recent Banff classification (Banff 2013) includes C4d-negative ABMR (Haas et al. 2014). Previously, immunohistopathological evidence, i.e., C4d positivity, was required for the diagnosis of ABMR (Solez et al. 2007).

*Chronic/active ABMR* comprises chronic tissue injury manifesting as transplant glomerulopathy and arteriopathy, multilayering of peritubular capillaries, and evidence of microvascular inflammation and DSAs (Haas et al. 2014).



## 2.4.2 Infections

Post-transplant infections are a major post-transplant complication (Dharnidharka et al. 2014). Infections are the main cause of admission to the hospital after RTx in children, and account for 36% of the deaths in European pediatric RTx patients (Chesnaye et al. 2014), as viruses infect people early in life. Young pediatric recipients are at a higher risk of viral transmission from a seropositive adult donor organ with a latent virus, and thus at a higher risk of severe disease as compared to adults (Dharnidharka et al. 2014, Dharnidharka et al. 2009).

Early infections are usually caused by bacteria or yeast and related to invasive procedures affecting wounds and transplant (Table 8). The later period is characterized by the onset of opportunistic infections, which may be reactivated or transmitted from the donated organ due to intense immunosuppressive therapy. Epstein-Barr virus (EBV)-associated PTLD is a serious complication of immunosuppression, which accounts for about 95% of the malignancies in pediatric patients. Unlike in adults, non-PTLD malignancies are rare in pediatric RTx recipients.

**Table 8.** Infectious post-transplant complications among pediatric RTx recipients

Pathogen	Complication	Time of infection, months post-RTx	Frequency of post-RTx hospitalization*	
			At 1-5 mo	At 3 yrs
Total infections			51.4%	21.1%
Bacterial ( <i>Staph. Aureus/Epidermidis, E.Coli</i> )	UTI, septicemia	0-1	13.1%	5.3%
Viral		1-6	13.7%	4.7%
<i>Cytomegalovirus (CMV)</i>	CMV infection			
<i>Epstein-Barr virus (EBV)</i>	PTLD	> 6		
<i>Polyomaviruses, BK and JC</i>	PyVAN	> 6		
Fungal ( <i>Candida, Aspergillus</i> )	UTI, oral/skin	0-1	1.0%	0.2%

\*According to NAPRTCS 2014 Annual report, deceased donor recipients. UTI, urinary tract infection; PyVAN, polyomavirus-associated nephropathy, PTLD, post-transplant lymphoproliferative disorder.

Although CMV infections at 6 months post-RTx have increased from 10% to 30%, CMV rarely causes a life-threatening disease due to improved diagnostic tools, ganciclovir prophylaxis or pre-emptive treatment (Helanterä et al. 2010, Helanterä et al. 2014). Serial PCR monitoring for viral replication enhances early detection and interventions (Al Khasawneh et al. 2013). CMV prophylaxis is associated with preserved graft function (Hocker et al. 2016) and is recommended for a minimum of 3 months, or 6 months for high-risk (donor positive/recipient negative, D+/R-) recipients.

## **BK and JC polyomaviruses**

BK polyomavirus (BKPyV) has emerged as an important post-transplant pathogen in kidney transplant recipients. The increase in the incidence of BKPyV infections associates with the combined use of tacrolimus and mycophenolate (Hirsch et al. 2013). Among recipients using these agents, approximately 3–5% will develop BKPyV infection in the graft, and BKPyV is the main cause of polyomavirus-associated nephropathy (PyVAN).

The first sign of active virus replication is BK viremia, which precedes BK viremia and allows early prediction of PyVAN (Babel et al. 2009). Viral loads of > 10,000 copies/mL in blood and > 10 million copies/mL in urine are determined as clinically relevant and predictive for the pathology of PyVAN.

In RTx recipients, intense immunosuppression may induce reactivation of the latent BKPyV, which converts subclinical replication to viremia and viruria in 21–26% and 33–63% of the pediatric recipients, respectively (Haysom et al. 2004, Zarauza Santovena et al. 2015). BKPyVAN affects up to 10% of RTx patients and leads to allograft loss in 10–80% (Schaub et al. 2010). In pediatric patients, the corresponding prevalences are around 5% and 24% (Smith et al. 2007). Donor-derived *de novo* BKPyV infections associate with serostatus discordance (D+/R-) (Sood et al. 2013, Verghese et al. 2015, Schwarz et al. 2015).

PyVAN is diagnosed by histological evaluation of the kidney graft biopsy and immunohistochemistry using anti-simian virus 40 (SV40) antibodies. BKPyV replication causes viral cytopathy, increasing inflammatory infiltrates and IF/TA changes, which are difficult to distinguish from the morphology of acute rejection. Histological lesions are multifocal and randomly distributed; more than one-third of the results may thus be false negative due to uninvolved parenchyma at the biopsy sampling area. This hampers the differential diagnosis from acute cellular rejection, which has similar clinical symptoms but requires radically different therapy. In fact, studies of HLA-DR (MHC class II) expression on tubular epithelial cells and complement C4d deposits may provide help to distinguish BKPyVAN from rejection, as patients with BKPyVAN lack these features.

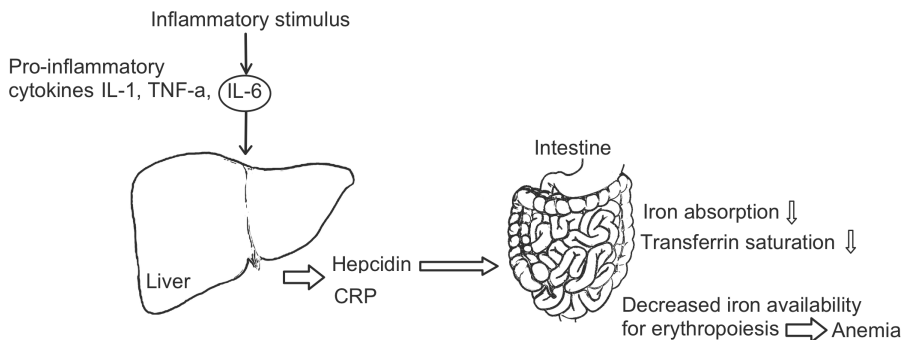
Treatment of BKPyVAN relies on reduction of immunosuppression, and the effectiveness of antiviral drug therapy is in doubt. Cidofovir and leflunomide have been used, although not validated, in adults or children (Dharnidharka et al. 2011). Virus-neutralizing antibodies show potency as a new anti-BKPyV therapy, although evidence is still lacking from *in vitro* studies on how these immune globulins would perform in clinical use (Randhawa et al. 2015, Pastrana et al. 2012).

### 2.4.3 Inflammation

Persistent inflammation is a common complication in patients with ESRD before renal RTx (Carrero, Stenvinkel 2009, Miyamoto et al. 2011). Transplantation diminishes inflammation and thus decreases the risk of cardiovascular complications and mortality. However, transplant recipients have an altered immune system, and low-grade inflammation increases the risk for graft-related complications and impaired long-term survival (Abedini et al. 2009, Cottone et al. 2007).

Inflammatory stimulus, such as kidney tissue injury, activates the rapid cascade of increasing cytokines interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and C-reactive protein (CRP). Upstream in the inflammatory cascade, IL-6 is the main inducer of the hepatic production of CRP (Gabay 2006) (Figure 7). IL-6 has an important role in the recruitment of monocytes and in activating the cellular immune response. In turn, any inflammatory state such as uremia or atherosclerosis may also hamper graft function (van Ree et al. 2007)

Post-transplant CRP levels were studied already a decade ago in the presence of allograft nephropathy (Sezer et al. 2004). Currently, the development of a sensitive CRP assay enables more precise measurements to detect the harmful effect of persistent low-grade inflammation on patient survival (Dahle et al. 2011). Inflammation markers IL-6 and high-sensitivity C-reactive protein (hsCRP) are independently predictive of cardiovascular events and mortality in renal transplant recipients (Abedini et al. 2009).



**Figure 7** Inflammatory cascade and the pattern of anemia of inflammation. IL-1, interleukin-1; TNF- $\alpha$ , tumor necrosis factor alpha; IL-6, interleukin-6; CRP, C-reactive protein.

#### 2.4.4 Anemia

Renal transplant recipients face the risk of developing hematologic abnormalities and post-transplant anemia (PTA), which increases the risk for impaired graft and patient survival after RTx. Multiple factors that contribute to late PTA include inadequate erythropoietin (EPO) levels, iron deficiency, chronic inflammation, transplant-associated medicines, and impaired allograft function.

The prevalence of PTA in pediatric RTx recipients varies between 26% and 83%, depending on the definition (hemoglobin, hematocrit) and time of assessment (Mitsnefes et al. 2005, Kausman et al. 2004, Kaidar et al. 2014). In adults, the reported rates of PTA are around 40% (Molnar et al. 2011a, Vanrenterghem et al. 2003, Shah et al. 2006). The biphasic pattern is characteristic for the prevalence of PTA. Early-onset anemia occurs within the first month after RTx and reaches a prevalence of 48–89% (Pascual et al. 2013, Fernandez Fresnedo et al. 2005, Jones et al. 2012, Imoagene-Oyedeji et al. 2006). This is partly explained by the demanding perioperative period, which is complicated by dilution, blood loss, frequent sampling, and poor early graft function (Poesen et al. 2011).

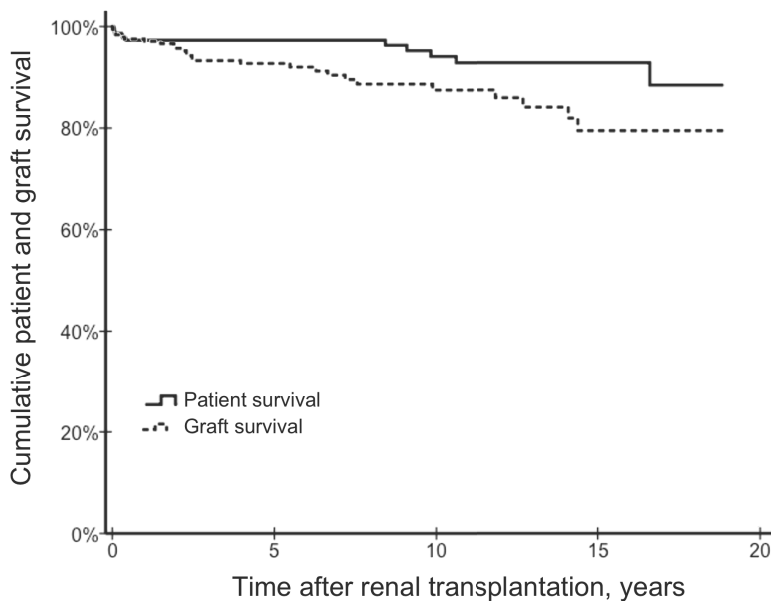
After successful transplantation, early erythropoietin deficiency recovers within the first year, resulting in a simultaneous Hb increase (Beshara et al. 1997, Sun et al. 1989). Usually, late PTA progresses over the following post-transplant years along with the deterioration of allograft function. Erythropoietin deficiency due to impaired allograft function is suggested as the main mechanism leading to anemia in renal transplant recipients (Yabu, Winkelmayr 2011). Other causes of PTA, such as iron deficiency, constant low-grade inflammation, and transplant-related medicines (immunosuppressants, antihypertensives, antimicrobials) induce the development of anemia by several mechanisms. These comprise decreased iron availability for erythropoiesis, increased production of erythropoiesis inhibiting inflammatory cytokines (Cooper et al. 2003, Molnar et al. 2011b, Molnar et al. 2011a) and myelosuppression by antiproliferative immunosuppressants AZA and MMF (Mitsnefes et al. 2005). The adverse effects of myelosuppressive drugs manifest in cytopenias, such as anemia, leucopenia and thrombocytopenia. Systemic inflammation and anemia underlie several chronic diseases and associate with impaired graft and patient survival.

Hepatic hepcidin-25, a biologically active 25-amino acid peptide, is the main regulator of iron homeostasis that binds to the cellular iron exporter ferroportin, limiting the availability of iron for erythropoiesis and circulation (Chan et al. 2013). Hepcidin-25 synthesis is induced by inflammation, especially pro-inflammatory cytokine IL-6 (Figure 6). Increased hepcidin-25 levels block the duodenal iron absorption and iron release from the reticuloendothelial system, which may lead to anemia in prolonged inflammatory states (Young, Zaritsky 2009).

### 2.4.5 Long-term outcomes

An important question is why long-term graft and patient survival of RTx patients remains unsatisfactory despite the excellent progression in short-term results, diagnostic methods and medications during the past decades (Van Arendonk et al. 2014, Meier-Kriesche et al. 2004). Toxicity of immunosuppressive medication, virus infections (BK polyomavirus), humoral rejections, and non-compliance - all these impair the survival rates. Although modern immunosuppressive therapy has improved survival rates and led to a reduction in acute rejection rates (Lamb et al. 2011, Dharnidharka et al. 2014), the long-term outcome of kidney transplant recipients continues to be impaired by simultaneous immunosuppression-related complications, such as CAI, infections, cardiovascular disease and malignancy continuously impair (Alangaden et al. 2006).

According to ANZDATA and NAPRTCS registries, pediatric kidney graft survival at 1 year exceeds 95% (Dharnidharka et al. 2014). The 5-year graft survival among European pediatric kidney transplant recipients is 84% (Lofaro et al. 2016). The best (90%) 5-year graft survivals was among pediatric RTx recipients with pre-emptive transplantation, and poorest (52%) among adolescents with long-term (> 2 years) dialysis (Lofaro et al. 2016). In Finland, the survival rates are excellent, with 5-year graft survival of 90.5% in adult RTx recipients (Peräsaari et al. 2015). In children, even the 10-year graft survival exceeds 80%, as shown in Figure 8.



**Figure 8** Long-term patient and graft survival in pediatric RTx recipients in Finland.

CAI is an important factor of graft failure, and of particular interest as there are distinct causes of injury at different time points and no effective therapy beyond early detection (Smith et al. 2013, Stegall et al. 2015). During the first few years, subclinical inflammation and recurrence of the primary disease play a major role, whereas alloantibodies and CNI-nephrotoxicity have only a minor role in early graft loss (< 5 years post-RTX) (Stegall et al. 2015).

In adults, 20-30% of the allografts will fail within the first 10 years (Stegall et al. 2015). The rate of premature graft loss is prominent among patients aged 13–25 years in the US and Canada (Foster 2015), and the risk for graft failure reaches the highest point between 17 and 24 years of age, independent of the age at the time of RTx (Foster et al. 2011). Risk of graft failure in adolescents and young adults increases at the time of transition from pediatric to adult care.

NAPRTCS registry data reports a 10-year patient survival of 91% (93% for a living-related donor and 88% for a deceased donor). Patient survival has improved significantly for living-donor patients, as the 5-year survival in the '87-95 era was 91%, compared to 96% for the '96-04 and '05-13 eras (NAPRTCS Annual Report 2014). In Finland, the patient survival rates are excellent as the 10-years patient survival exceeds 95% (Tainio et al. 2014).

Infections are an important (20%) cause of death among pediatric recipients, followed by other complications, such as cardiovascular disease and malignancies (Lofaro et al. 2016, NAPRTCS Annual Report 2014, Holmberg, Jalanko 2015). Increased cardiovascular morbidity and mortality are known side effects of immunosuppression and important factors for patient death with a functioning graft (Mitsnefes 2012, Smith et al. 2013).

*Adherence* to immunosuppressive therapy is demanding, especially for adolescents. The rate of reported nonadherence among pediatric recipients is around 30%, and among young adults even two thirds are classified as nonadherent (Massey et al. 2015, Dobbels et al. 2010, Shaw et al. 2003). Nonadherence increases the risk for acute rejection episodes and graft loss (Jarzembowski et al. 2004). Physical and cognitive development and the psychosocial functioning of pediatric patients are of highest importance. Real long-term outcomes in transplant recipients are measured with their ability to integrate into society, to study and work, and to raise a family. Improved outcomes turn the focus on complications and quality of life (Griva et al. 2013, Sundaram et al. 2007), and thus immunosuppressive therapy should aim to be less toxic and have fewer side effects to enable improvements in the quality of life among lifelong users.

### 3 AIMS OF THE STUDY

The aim of this thesis was to examine the biomarkers of chronic allograft injury in pediatric kidney transplant patients. Such markers would enable early identification of patients at risk of development of chronic allograft injury and prevent graft loss.

The specific aims of the study were:

1. To assess the incidence of HLA antibodies in pediatric RTx recipients, and to analyze the significance of DSA on kidney graft function.
2. To describe the expression of potential immunohistochemically detectable biomarkers in kidney graft biopsies, and to correlate these findings to graft histology and function.
3. To examine the association of post-transplant anemia and inflammation with graft function, and to evaluate the role of underlying anemia-related factors.
4. To assess the prevalence and impact of BKPyV infection on kidney transplant function.

## 4 PATIENTS AND METHODS

### 4.1 Patients

The patients included in this thesis underwent kidney transplantation at the Children's Hospital, Helsinki University Hospital, between 1988 and 2014. A total of 236 pediatric patients received a kidney transplant, and subgroups of 46 to 128 patients were included into studies I-IV. The patients were followed regularly at the transplantation center. Follow-up visits were performed at 3, 6, 12, 18, and 24 months post-RTx and annually thereafter until the transfer to adult care.

*Study I* (donor-specific HLA antibodies) included 123 patients who received kidney transplants between 1989 and 2004, and had stored plasma samples available for HLA analysis and adequate follow-up data for outcome measurements.

*Study II* (histopathology and biomarkers) included 56 patients transplanted between 1995 and 2004. All included patients had at least two biopsies available for Banff classification and immunohistochemical analyses.

*Study III* (anemia and inflammation) included 128 patients who were transplanted between 1988 and 2006 and had stored sera available for anemia and inflammation-related biomarker analyses.

*Study IV* (polyomavirus infections) comprised 46 patients who received a kidney transplant between 2009 and 2014 with adequate monitoring of BK viremia.

### 4.2 Methods

#### 4.2.1 Data collection

A retrospective collection of clinical data from the patient records included the following parameters: disease leading to RTx, date and age at RTx, donor type, HLA-A, -B, -DR mismatches, and cold ischemia time. Data on follow-up visits included laboratory tests (complete blood count, kidney function tests, and inflammation markers) and GFR measurements. GFR was the primary outcome measure in all studies (I-IV). Secondary outcomes were change in GFR over time and rate of biopsy-proven allograft injury.

*HLA typing* (HLA-A, -B or -DR) of each patient and kidney donor was determined using a serological assay and low-resolution sequence-specific primers (PCR-SSP; One Lambda, Inc., Canoga Park, CA) or additional sequence-specific



oligonucleotide probes (PCR-SSO; One Lambda, Inc., Canoga Park, CA). Also, ABO compatibility and negative complement-dependent lymphocytotoxicity crossmatch (CDC) against donor spleen lymphocytes were required. A mismatch of 2/1 (A and B loci/DR locus) or better was achieved in over 90% of the patients.

*Immunosuppression* was based on triple-drug immunosuppression including CNI (CsA or Tac), antimetabolite (AZA or MMF) and methylprednisolone. Combinations and dosing were individualized based on patient characteristics. Induction therapy with basiliximab was used since 2000.

*Acute rejections* were diagnosed by core needle and fine needle biopsies until the year 2000. Thereafter, only core needle biopsies were used for diagnosis. Episodes with clinical findings and biopsy verification were treated with intravenous methylprednisolone (10 mg/kg/day for 3-5 days).

*Kidney graft function* was followed by measured GFR using  $^{51}\text{Cr}$ -EDTA clearance with modified Brochner-Mortensen equation normalized to a standard body surface area of  $1.73 \text{ m}^2$  (Brochner-Mortensen et al. 1974, Haycock et al. 1978). The distribution values between 15% and 35% were acceptable. The annual GFR decline ( $\text{mL}/\text{min}/1.73 \text{ m}^2/\text{year}$ ) was assessed for every patient from 1.5 post-transplant years to the last follow-up visit. CKD stages were used as follows: GFR  $> 60 \text{ mL}/\text{min}/1.73 \text{ m}^2$  (stages 1-2), GFR  $30\text{--}59 \text{ mL}/\text{min}/1.73 \text{ m}^2$  (stage 3), GFR  $< 30 \text{ mL}/\text{min}/1.73 \text{ m}^2$  (stages 4–5). Graft failure was defined as a return to dialysis.

#### 4.2.2 Measurement of HLA antibodies using Luminex assay (I)

HLA class I (HLA-A, -B, -Cw) and class II (HLA-DR, -DQ, -DP) antibody screening was performed retrospectively, using LABScreen Mixed screening assay according to manufacturer's instructions (LABType, One Lambda, Canoga Park, CA). Positive samples required further analysis with LabScreen single antigen kits to specify antigen positivity and were assigned using HLA Visual software (One Lambda). Assigned antibodies were compared to donor HLA type to identify DSA. The strength of donor-specificity was determined using mean fluorescence intensity (MFI). MFI values  $> 500$  were considered positive.

#### 4.2.3 Histopathology (II, IV)

Percutaneous core needle biopsies were taken according to protocol at 3, 18 and 36 months or when indicated on clinical grounds, i.e., significantly decreased GFR, increased serum creatinine, or suspected acute rejection. Biopsy tissue samples were fixed in formaldehyde, embedded in paraffin and cut into thin 3–4  $\mu\text{m}$  sections for staining. Routine stains for light microscopy included hematoxylin and eosin, Masson's trichrome, periodic acid-Schiff (PAS), and methenamine silver PAS.

Routine histopathologic analyses were performed by a pathologist according to the Banff criteria. An additional scoring was blindly performed by one of the co-authors according to Banff05 criteria, and in selected cases, consensus-reading sessions were arranged with an experienced pathologist without knowledge of any clinical data (II). Combination of interstitial fibrosis (IF) and tubular atrophy (TA) scores was used to define chronic changes in allograft histology.

#### 4.2.4 Immunohistochemistry (II, IV)

Immunoperoxidase stainings were performed using routine methods according to manufacturer's instructions for vimentin, alpha-smooth muscle actin ( $\alpha$ -SMA), collagen IV, and P-selectin glycoprotein ligand-1 (PSGL-1) (Table 9). Biopsies from histologically normal kidneys that were removed due to other diseases than cancer were used as normal controls. Additional immunohistochemistry for C4d staining was performed routinely, and for polyomavirus (SV40T) when requested. Positivity was reported as present or absent. Positive nuclear immunoperoxidase staining for SV40 large T antigen (LTag) in a graft biopsy was diagnostic for PyVAN due to cross-reactivity among LTags of BKPyV, JCPyV and SV40 (IV).

**Table 9.** Antibodies used in immunohistochemical stainings in study II

Antibody	Manufacturer	Clone/Code	Dilution
Vimentin	DakoCytomation	3B4/M7020	1:100
$\alpha$ -SMA	DakoCytomation	1A4/M0851	1:400
Collagen IV	DakoCytomation	CIV 22/M0785	1:50
PSGL-1	Santa Cruz Biotech	Sc-13535	1:500

$\alpha$ -SMA, alpha-smooth muscle actin; PSGL-1, P-selectin glycoprotein ligand-1; DAB, 3,3'-diaminobenzidine.

*Microscopy and semiquantitative scoring* of immunopositivity (II) was performed using a standard Leica DM RX microscope and 20x magnification. Peritubular PSGL-1 infiltrates, vimentin expression in tubular epithelial cells, proportion of  $\alpha$ -SMA positive peritubular areas and collagen IV deposition in the interstitium were scored semiquantitatively from 0 to 3 (0, no staining; 1, comparable to normal controls < 10%; 2, mildly increased 10 to 24%; and 3, moderately to severely increased > 25% of renal cortex involved). In the statistical analyses, the scores 0–1 and 2–3 in the biomarker expression were defined as low and high, respectively.

#### 4.2.5 Enzyme-linked immunosorbent assay (III, IV)

Enzyme-linked immunosorbent assay (ELISA) was used to determine serum concentrations of the inflammation markers hsCRP and IL-6, and of the anemia markers EPO and hepcidin-25 from stored serum samples. Analyses were performed according to the manufacturer's protocol using the following kits:

Biovendor (Karasek, Czech Republic) for EPO, DRG (Marburg, Germany) for hepcidin-25, Circulex (Nagano, Japan) for hsCRP, and Diaclone (Besancon, France) for IL-6. Cut-off values for the inflammatory marker hsCRP were set at hsCRP < 1 mg/L, hsCRP 1–3 mg/L and hsCRP > 3 mg/L, as previously described (van Ree et al. 2007, Dahle et al. 2011).

#### 4.2.6 BkPyV monitoring by PCR (IV)

Patients were monitored for BK viremia every 3 to 6 months during the first 1.5 years and annually thereafter according to modified KDIGO guidelines (Kidney Disease: Improving Global Outcomes (KDIGO) Transplant Work Group 2009). Quantitative PCR was used to screen plasma BkPyV load in viral genome copies per milliliter as described previously (Dumoulin, Hirsch 2011). The lower limit for positivity was viral load of > 400 copies/mL and clinically significant limit of > 10,000 copies/mL in plasma (Hirsch et al. 2014).

#### 4.2.7 Statistical analyses

Results are expressed as means  $\pm$  standard deviations (SD) for continuous variables with normal distribution, and as medians and interquartile ranges (IQR) for parameters with skewed distribution. Statistical significance of differences between the groups was tested by t-test and ANOVA or Mann-Whitney U test, Kruskal-Wallis and chi-square tests, where appropriate. Univariate Pearson's correlation coefficient and Spearman rank test were used to assess the associations between variables. Abnormally distributed values were log-transformed before the linear regression analyses. Multivariable regression analyses were performed to identify independent predictors of continuous variables. Two-sided p-values < 0.05 were considered as statistically significant. All statistical analyses were performed using SPSS version 20.0 (IBM, Armonk, NY).

#### 4.2.8 Ethical considerations

The Ethics Committee for Pediatrics, Adolescent Medicine and Psychiatry of the Helsinki and Uusimaa Hospital District approved the study protocol (86/2007, 126/2007, 269/2009, 22/E7/2007).

## 5 RESULTS

The clinical characteristics of the pediatric RTx recipients included in studies I–IV are described in detail in Table 10.

**Table 10.** Characteristics of the pediatric RTx patients included into the studies I–IV

	I	II	III	IV
Study period, years	1989–2004	1995–2004	1988–2006	2009–2014
Number of patients	123	56	128	46
Gender, male, n (%)	80 (65)	38 (68)	84 (66)	28 (61)
Age at transplantation, yr	4.4 (1–18)	6.3 (1–15)	3.8 (1.7–9.3)	4.3 (1–18)
Cause of ESRD, n (%)				
CNF	47 (38)	19 (34)	61 (48)	20 (44)
CAKUT	31 (25)	21 (37)	28 (22)	7 (15)
Other hereditary	22 (18)	11 (20)	20 (16)	6 (13)
Inflammatory	11 (9)	0	8 (6)	4 (9)
Other	12 (10)	5 (9)	11 (9)	9 (20)
HLA-MM, mean±SD				
A/B	1.5 ± 0.8	1.4 ± 0.9	1.6 ± 0.9	1.9 ± 0.9
DR	0.7 ± 0.5	0.7 ± 0.5	0.7 ± 0.6	1.0 ± 0.5
LD, n (%)	32 (26)	14 (25)	34 (27)	21 (46)
Cold ischemic time, hours	18.8	15.9	18.4	9.7
CMV D+/R-, n (%)	51(41)	23 (44)	51 (40)	18 (39)
Graft outcome				
Follow-up, years	6.7 (4–20)	7.4 (1–10)	10 (7–14)	2.3 (1–6)
Graft loss, n	10	4	11	2

CNF, congenital nephrotic syndrome of the Finnish type; CAKUT, Congenital anomalies of the kidney and urinary tract; HLA-MM, HLA-mismatches; LD, living donor; CMV, cytomegalovirus; D+/R-, donor seropositive / recipient seronegative.

## 5.1 Donor-specific HLA antibodies (I)

### 5.1.1 HLA antibodies

Post-transplant HLAab findings were correlated to graft function and clinical outcome in 123 patients transplanted at the median age of 4.3 years and followed for over 10 years. HLAabs were measured retrospectively from samples (average of 2.4 samples per patient) that were routinely collected at different time points after RTx. Nearly two-third (64%) of the 123 patients had detectable HLAabs in half (140/294) of the samples. The proportions of HLAabs were comparable at different time points. Nearly half (44%) of the detectable HLAabs were donor-specific (DSA), whereas the other half were non-DSA (Table 11).

**Table 11.** HLA antibodies in pediatric renal transplant recipients

HLAab finding	Samples	Patients
Total	294	123
HLAab negative	154 (52%)	44 (36%)
HLAab positive	140 (48 %)	79 (64 %)
Non-DSA positive	78	37
DSA positive	62	42
Class I	15	12
Class II	40	23
Class I+II	7	7

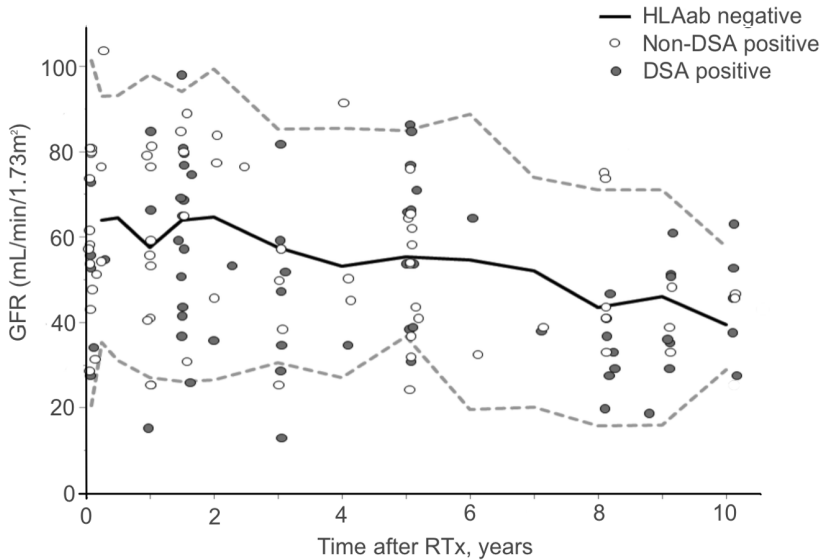
HLA, human leukocyte antigen; HLAab, HLA antibody; DSA, donor-specific HLA antibody.

### 5.1.2 Patients with DSA

DSA was detectable in 62 (21%) samples, and the proportion of DSA positive samples increased somewhat with time. Overall, DSA positivity was detectable in 42 (44%) patients. Most (55%) DSA antibodies reacted against class II antigens. DSA positivity fluctuated over time, but appeared always against the same antigen specificity in consecutive samples. The background data of the patients were comparable between DSA-positive and DSA-negative patients. However, early (< 3 months) immunoactivation was more common (71%) among patients with DSA compared to immunoactivation (46%) in those without any detectable HLA antibody ( $p = 0.035$ ). Five patients had detectable DSA (MFI levels 613, 1671, 1825, 2033, and 9064) before the late (> 3 months) acute rejection episode. Overall, late acute rejection episodes were treated in 21% (9/42) and 23% (10/44) of the patients with DSA and without HLA abs at any time, respectively.

### 5.1.3 DSA and graft function

The distribution of individual GFR values at the time of HLA antibody detection was similar in patients with and without HLAab or DSA, as shown in Figure 9. Even patients with high DSA antibody titers had variable GFR, which did not differ significantly from the other patient groups.



**Figure 9** Individual glomerular filtration rate (GFR) (mL/min/1.73 m<sup>2</sup>) values at the time of post-RTx HLA detection. Dashed lines,  $\pm 2$  SD for GFR of HLAab negative patients.

Detection of DSA did not associate with poor graft function in any time period of 0–2 years, 3–5 years or 6–10 years post-RTx (Table 12). Similarly, the annual GFR decline after HLAab detection was comparable between patient groups, and future graft function showed similar tendency regardless of the antibody finding (Table 12).

**Table 12.** Glomerular filtration rate (GFR) at the time of HLA antibody detection in time periods of 0–2, 3–5 and 6–10 years after RTx, and annual GFR decline ( $\Delta$ GFR)

	GFR (mL/min/1.73 m <sup>2</sup> )			
	0–2 yrs	3–5 yrs	6–10 yrs	$\Delta$ GFR /year
HLAab negative	63 $\pm$ 19 (78)	60 $\pm$ 19 (46)	39 $\pm$ 10 (22)	2.6 $\pm$ 3.7
Non-DSA positive	62 $\pm$ 20 (40)	50 $\pm$ 16 (22)	44 $\pm$ 15 (13)	2.8 $\pm$ 4.2
DSA positive	58 $\pm$ 25 (21)	55 $\pm$ 22 (18)	40 $\pm$ 13 (22)	1.3 $\pm$ 4.3

Values are presented as mean  $\pm$  SD (n). DSA, donor-specific HLA antibody;  $\Delta$ GFR, annual GFR decline (mL/min/1.73 m<sup>2</sup>/year).

### 5.1.4 DSA and biopsy findings

Post-RTx DSA positivity during the first three years was not significantly associated with chronic lesions (IF/TA) in protocol biopsies. Protocol core needle biopsy and simultaneous HLA ab measurement at 1.5 years and 3 years post-RTx were available from 64 and 75 patients, respectively. At 1.5 years post-RTx, chronic lesions were detected in 20 (31%) samples, associating with previous DSA positivity in five patients. At 3 years post-RTx, IF/TA lesions were observed in nearly half (47%) of the biopsies and were comparable between patients with and without DSA positivity (53% vs. 43 %,  $p = 0.450$ ).

An interesting question was what happens to graft function in patients with DSA positivity and chronic histological lesion during the first three years after RTx. The mean GFR in patients with DSA positivity and IF/TA lesions decreased 5–15 mL/min/1.73 m<sup>2</sup> compared to GFR in patients with normal biopsy findings, as shown in Table 13. However, the GFR was comparable to that of patients with chronic lesions without DSA, suggesting that chronic lesions, not antibody findings, correlated with the lower GFR values.

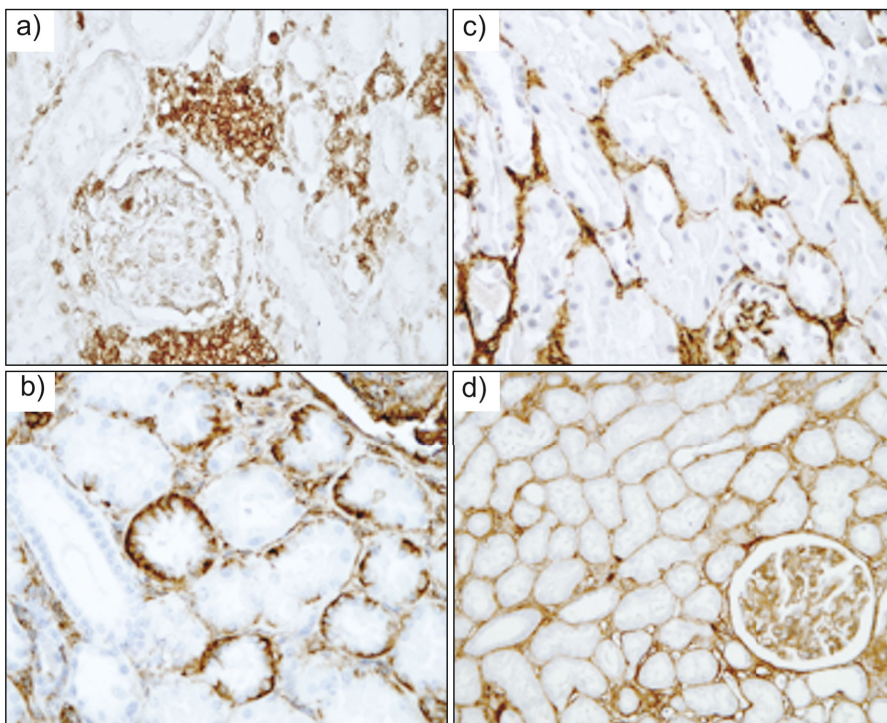
**Table 13.** Long-term graft function measured as glomerular filtration rate (GFR, mL/min/1.73 m<sup>2</sup>) in relation to post-transplant DSA finding at 0–3 years and biopsy finding at 1.5 or 3 years post-RTx

DSA, biopsy	GFR (mL/min/1.73 m <sup>2</sup> )			
	3 yrs (n=95)	5 yrs (n=84)	7 years (n=61)	10 years (n=32)
0–3 years				
DSA-, normal	64 ± 16 (35)	58 ± 15 (29)	51 ± 14 (22)	44 ± 10 (10)
DSA+, normal	56 ± 18 (11)*	54 ± 16 (11)	42 ± 15 (9)	40 ± 11 (6)
DSA-, IF/TA	52 ± 18 (36)	51 ± 16 (33)	47 ± 13 (23)	33 ± 14 (10)
DSA+, IF/TA	49 ± 18 (13)	49 ± 14 (11)	43 ± 14 (7)	34 ± 9 (6)

DSA, donor-specific HLA antibody; IF/TA, interstitial fibrosis and tubular atrophy. \*DSA+normal vs. DSA+ IF/TA,  $p = 0.040$ .

## 5.2 Histopathology and immunohistochemistry (II)

Histopathology and the expression of four biomarkers were studied in kidney graft biopsies to correlate biomarker findings to concomitant and subsequent graft function. A representative staining pattern of the biomarkers PSGL-1, vimentin,  $\alpha$ -SMA and collagen IV is shown in Figure 10.



**Figure 10** Immunohistochemical staining pattern of a) PSGL-1, b) vimentin, c)  $\alpha$ -SMA, and d) collagen IV in the kidney graft biopsy. Interstitial PSGL-1 expression in the areas of inflammation, vimentin expression in tubular cells, and  $\alpha$ -SMA and collagen IV expression in the interstitium.

### 5.2.1 Biomarker findings

Histopathology and biomarker scores were comparable in both protocol (75%, 123/165) and indication (25%, 42/165) biopsies. Only the correlation between biomarker scores and IF/TA decreased in indication biopsies. Overall, IF/TA lesions were common and increased with time. Mean Banff scores for inflammation, tubulitis, arterial hyalinosis and arterial sclerosis were  $0.53 \pm 0.75$ ,  $0.47 \pm 0.75$ ,  $0.09 \pm 0.28$ , and  $0.40 \pm 0.72$ , respectively.



**Table 14.** Banff scores in relation to biomarker expression in biopsies post-RTx

Banff score	Vimentin	$\alpha$ -SMA	Collagen IV	PSGL-1
Tubulitis (t)	0.170	0.118	-0.002	0.409 **
Interstitial inflammation (i)	0.153	0.138	0.134	0.415 **
Arteritis (v)	0.121	0.239 *	0.077	0.246 *
Glomerulitis (g)	-0.106	0.168	-	-
Interstitial fibrosis (ci)	0.245 *	0.370 **	0.416 **	0.303 *
Tubular atrophy (ct)	0.334 *	0.402 **	0.516 **	0.348 *
Glomerulopathy (cg)	0.101	0.045	0.119	-0.007
Mesangial matrix increase (mm)	0.179	0.233 *	0.166	0.247 *
Arterial sclerosis (cv)	0.156	0.251 *	0.126	0.197
Arterial hyalinosis (ah)	0.157	0.116	0.089	0.128

Spearman correlation, \*  $p < 0.05$ , \*\*  $p < 0.001$ .

Immunohistochemical staining for the interstitial biomarkers  $\alpha$ -SMA and collagen IV increased with time, similarly to IF/TA lesions. The expression of PSGL-1 and tubular vimentin was unrelated to time, although all four biomarkers correlated with the accumulation of fibrosis (Table 14). Overall, the biomarker scores were significantly higher in IF/TA positive biopsies compared to IF/TA negative biopsies (Table 15).

**Table 15.** Biomarker expression in biopsies with and without IF/TA lesions

Biomarkers	IF/TA negative	IF/TA positive	p
PSGL-1	1.0 $\pm$ 0.6 (63)	1.6 $\pm$ 0.7 (50)	< 0.001
Vimentin	0.8 $\pm$ 0.6 (69)	1.0 $\pm$ 0.6 (46)	0.011
$\alpha$ -SMA	1.4 $\pm$ 0.7 (61)	2.0 $\pm$ 0.9 (50)	< 0.001
Collagen IV	0.9 $\pm$ 0.8 (71)	1.7 $\pm$ 1.0 (51)	< 0.001

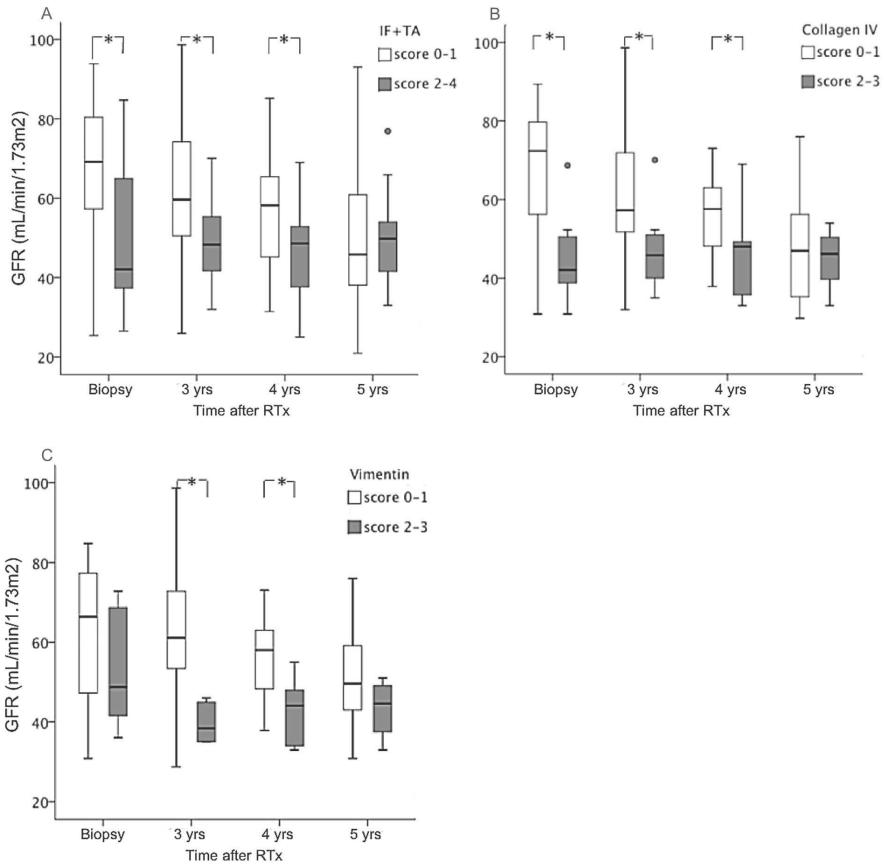
IF/TA, interstitial fibrosis/tubular atrophy; PSGL-1, P-selectin glycoprotein ligand-1;  $\alpha$ -SMA, alpha-smooth muscle actin.

### 5.2.2 Biopsies and graft function

Patients were divided into two groups according to biomarker scores (0–1 vs. 2–3) to evaluate the predictive value of each marker on GFR. At the time of the 1.5-years biopsy, patients with high (2–3) IF/TA or collagen IV scores showed decreased mean GFR (IF/TA: 50 vs. 68 mL/min/1.73 m<sup>2</sup>,  $p = 0.004$ ; Collagen IV: 45 vs. 65 mL/min/1.73 m<sup>2</sup>,  $p = 0.016$ ) compared to those with lower (0–1) scores. The other biomarkers showed similar, although statistically insignificant tendency.

Results

Early findings at 3-month biopsies were comparable between patients with low and high biomarker scores. However, the predictive value of 1.5-year biopsies was moderate as higher scores of IF/TA, collagen IV and vimentin associated with decreased GFR (Figure 11). In fact, the multivariable analysis revealed that none of the biomarkers showed superior predictive value for late graft function compared to routine IF/TA score according to Banff classification. Furthermore, the annual GFR decline was comparable between groups with low and high biomarker expression.



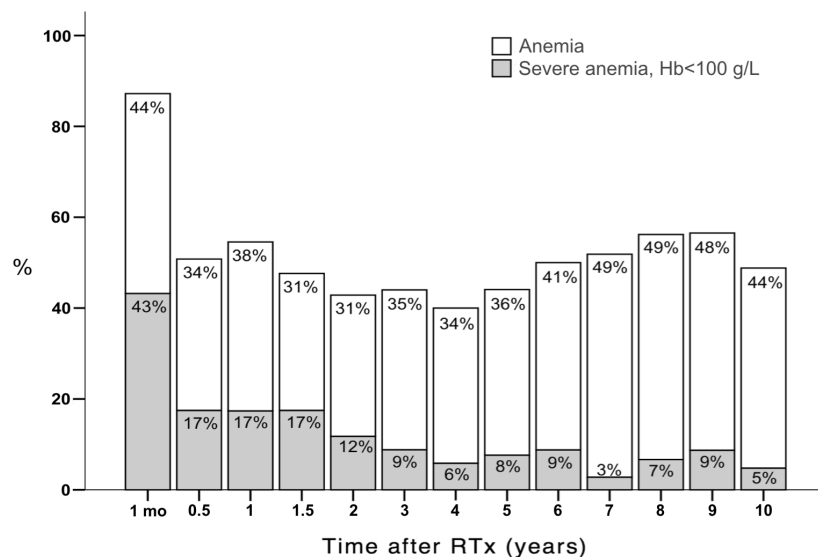
**Figure 11** Glomerular filtration rate (GFR, mL/min/1.73 m<sup>2</sup>) at the time of biopsy, 3, 4 and 5 years after RTx in patients with low and high IF/TA, collagen IV and vimentin scores in 1.5-year protocol biopsies.

### 5.3 Anemia and inflammation (III)

The association between post-transplant anemia, low-grade inflammation and allograft function of 128 pediatric RTx recipients transplanted between 1988 and 2006 was studied in a retrospective, observational analysis of a number of blood samples and their correlation with graft function.

#### 5.3.1 Prevalence of post-transplant anemia

The prevalence of anemia was 55% at 1 year and 49% at 10 years after RTx (Figure 12). The degree of anemia was mainly (73%) moderate (Hb > 100 g/L), and the prevalence of severely anemic patients decreased from 17% at 1 year to 5% at 10 years post-transplant.

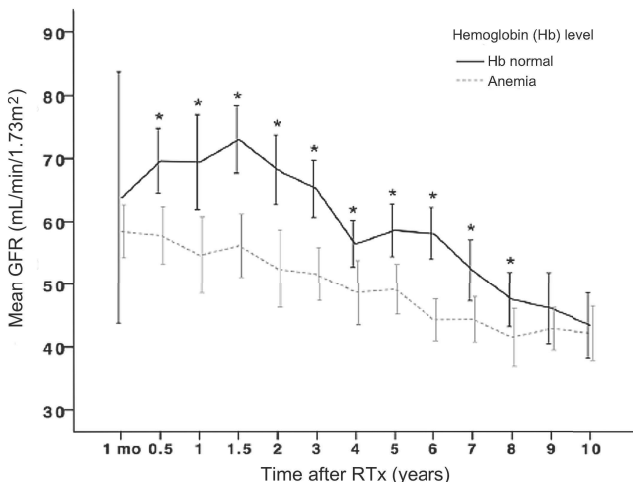


**Figure 12** The prevalence (%) of anemic samples after RTx. Anemia (white bars) and severe (Hb < 100 g/L) anemia (light grey bars).

#### 5.3.2 Biopsy findings and graft function

At the time of protocol biopsy at 1.5 and 3 years, anemia was detected in half (16/32) of the patients with fibrosis compared to 17% (3/18) in those without fibrosis ( $p = 0.033$ ). The mean levels of Hb were  $115 \pm 12$  g/L and  $121 \pm 13$  g/L, respectively ( $p = 0.034$ ). Low Hb levels even preceded graft fibrosis in patients who developed fibrosis after the first 3-month biopsy.

Compared with their non-anemic counterparts, the graft function of anemic patients was significantly lower from 6 months to 8 years post-RTx (Figure 13). The patients with good graft function ( $\text{GFR} > 60 \text{ mL/min/1.73 m}^2$ ) had a median Hb value of 119 g/L, whereas those with  $\text{GFR}$  of  $30\text{--}60 \text{ mL/min/1.73 m}^2$  and  $\text{GFR} < 30 \text{ mL/min/1.73 m}^2$ , had significantly lower Hb levels of 114 g/L and 112 g/L, respectively ( $p < 0.001$ ). In addition, the patients with anemia at 1.5 post-RTx years had significantly decreased subsequent GFR until 8 post-RTx years. Hemoglobin concentration was strongly related to concomitant and future GFR.



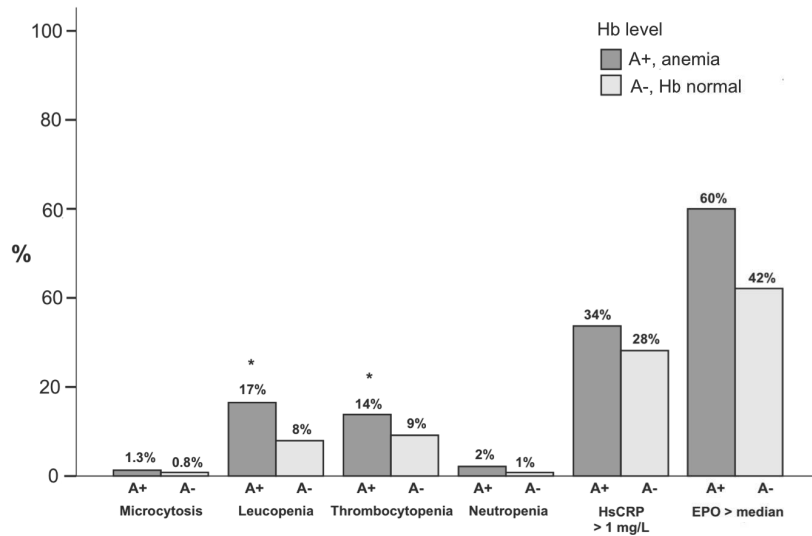
**Figure 13** Mean hemoglobin level in patients with and without anemia at the time of study point.

### 5.3.3 Anemia-related factors

Iron profile was assessed in 22% of the patients, and iron deficiency was observed in a total of 14%. Microcytosis was present in only 1% of the samples. Leucopenia and thrombocytopenia were more common (11%) and associated with simultaneous anemia ( $p < 0.01$ ) (Figure 14). Overall, pancytopenia was infrequent.

*Erythropoietin (EPO)* levels were measured in a sample collected at 1.5–4 years after RTx. Interestingly, the median EPO levels were higher in anemic samples compared to those with normal Hb (7.7 vs. 5.6 mIU/mL,  $p = 0.038$ ), and serum EPO level showed an inverse correlation with Hb concentration ( $r = -0.206$ ,  $p = 0.038$ ). EPO levels were unrelated to concomitant GFR ( $r = 0.025$ ,  $p = 0.818$ ) and comparable across CKD stages ( $p = 0.351$ ).

*Hepcidin-25* levels were also measured at the maintenance phase 1.5–4 years after RTx. Hepcidin-25 was unrelated to Hb level ( $r = -0.052$ ,  $p = 0.603$ ), but inversely correlated with concomitant GFR ( $r = -0.305$ ,  $p = 0.006$ ). Hepcidin-25 level at 1.5–2 years predicted GFR at 6 years ( $r = -0.336$ ,  $p = 0.001$ ).



**Figure 14** The prevalence of anemia-related factors in samples with anemia (A+) and with normal Hb (A-), \*  $p < 0.005$ .

#### 5.3.4 Low-grade inflammation

The inflammatory markers hsCRP and IL-6 were measured repeatedly to analyze the role of low-grade inflammation in allograft function. Inflammatory status was assessed in 731 samples drawn at various time points during the 10-year follow-up, with the median hsCRP value of 0.40 (IQR 0.2-1.5) mg/L, IL-6 value of 1.40 (IQR 0.2-5.0) and ESR of 19 (IQR 12-30) mm/hr. Elevated hsCRP levels tended to associate with decreased GFR, but only early after RTx. Similarly, ESR increased along the CKD stages and showed an inverse correlation with GFR, although in univariate analysis only. None of these markers of inflammation showed a clear significant correlation with concomitant or subsequent graft function.

The median hsCRP level was higher in anemic samples compared to those with normal Hb (0.43 vs. 0.39 mg/L,  $p = 0.019$ ). Accordingly, hsCRP and Hb showed a weak inverse correlation ( $r = -0.095$ ,  $p = 0.010$ ), which was prominent in the early post-transplant period (at 6 mo:  $r = -0.339$ ,  $p = 0.024$ ). Surprisingly, hsCRP also correlated with serum EPO level ( $r = 0.263$ ,  $p = 0.007$ ). IL-6 levels were unrelated to Hb level ( $r = -0.045$ ,  $p = 0.265$ ) and GFR ( $r = -0.041$ ,  $p = 0.694$ ), whereas ESR showed a strong inverse correlation with Hb level ( $r = -0.383$ ,  $p < 0.001$ ) and GFR ( $r = -0.276$ ,  $p = 0.005$ ) at 1.5–2 years after RTx.

## 5.4 BK polyomavirus (IV)

### 5.4.1 BK viremia

BK polyomavirus infection was studied in 46 kidney transplant recipients whose plasma was continuously monitored for BK viremia. During a median of 4 years of follow-up, 9 patients had a peak plasma BKPyV PCR >400 copies/mL in routine screening samples. BK viremia occurred at a median of 6 (range 3–22) months post-RTx with a median BKPyV load of 4,555 (IQR 1,350-17,300) copies/mL. All these patients had positive BKPyV PCR in at least two samples. Three patients had a high level (> 10,000 copies/mL) BK viremia, with a median peak BKPyV viral load of 61,500 copies/mL. High BK viremia occurred at a median of 6 (range 3–9) months post-RTx and lasted from 1 to 5 months. Background data and presumed risk factors of post-transplant BK viremia are presented in Table 16.

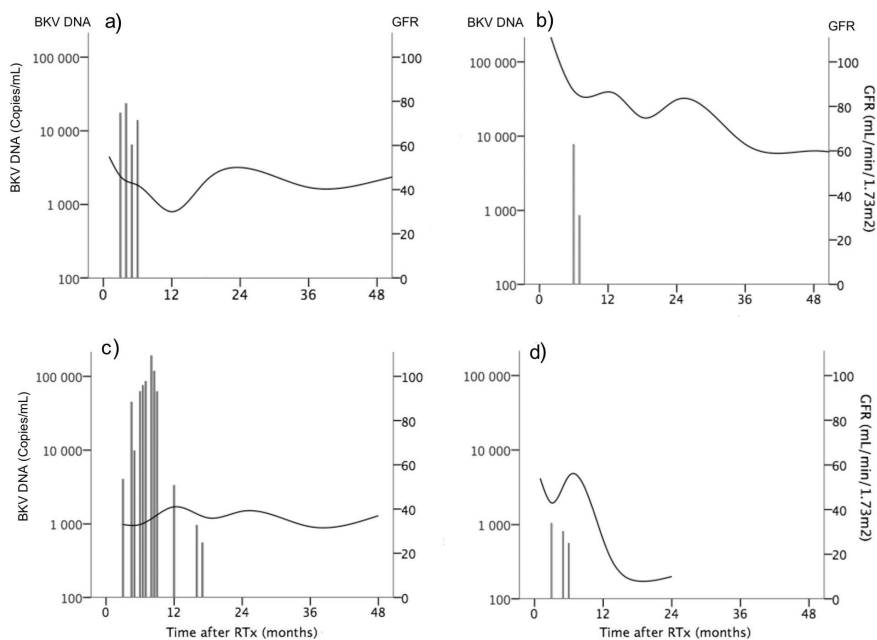
**Table 16.** Background data on patients with and without detectable BK viremia during the follow-up

	BK viremia (n=9)	No BK viremia (n=37)	p-value
Male gender, n	4 (44%)	24 (65%)	0.284
Age < 5 years at RTx, n	4 (44%)	20 (54%)	0.718
Living donor, n	4 (44%)	17 (47%)	0.881
Tacrolimus at discharge, n	3 (33%)	7 (19%)	0.384
Mycophenolate at discharge, n	2 (22%)	4 (11%)	0.581
Post-transplant DSA, n	3 (33%)	5 (14%)	0.176
Median plasma creatinine (umol/L)			
3 mo	75 (59-107)	49 (35-78)	0.121
6 mo	76 (54-92)	48 (34-83)	0.256
12 mo	85 (57-112)	47 (38-89)	0.128
24 mo	54 (50-77)	45 (36-75)	0.302
36 mo	55 (42-77)	52 (42-78)	0.765
Median GFR (mL/min/1.73 m <sup>2</sup> )			
3 mo	55 (41-61)	67 (57-82)	0.030
6 mo	58 (43-62)	60 (51-75)	0.288
12 mo	64 (36-77)	62 (53-74)	0.750
24 mo	60 (41-82)	68 (48-71)	0.874
36 mo	56 (41-63)	63 (58-76)	0.047

Values in table are presented as n (%) or median (interquartile range, IQR). DSA, donor-specific HLA antibodies; GFR, glomerular filtration rate.

### 5.4.2 BKPyV and graft function

Early GFR at 3 months post-RTx was available from 38 (83%) patients, of whom 6 with early BKPyV replication ( $> 400$  copies/mL) showed significantly decreased median GFR compared to 32 patients without BK replication (55 vs. 67 mL/min/1.73 m<sup>2</sup>,  $p = 0.030$ ) (Table 16). Similar, though statistically non-significant tendencies were seen in viremic patients at 6 months (58 vs. 60 mL/min/1.73 m<sup>2</sup>,  $p = 0.288$ ) and 12 months (64 vs. 62 mL/min/1.73 m<sup>2</sup>,  $p = 0.750$ ) post-RTx. At the time of high BKPyV load ( $> 10,000$  copies/mL, five samples), cystatin C level increased to a mean of  $2.1 \pm 0.4$  mg/L compared to  $1.4 \pm 0.3$  mg/L in 18 samples with a moderate (400–10,000 copies/mL) BKPyV load ( $p = 0.007$ ). The long-term graft function of four patients with BKPyV viremia is demonstrated in Figure 15.



**Figure 15** Long-term GFR (mL/min/1.73 m<sup>2</sup>) in four patients (a–d) with post-transplant BK viremia. Grey bars indicate the level of BKPyV viremia (copies/mL) and the black line demonstrates mean GFR (mL/min/1.73 m<sup>2</sup>) in relation to time (months) since RTx.

### 5.4.3 Polyomavirus-associated nephropathy

PyVAN developed in three patients during the first year post-RTx. One patient with PyVAN had simultaneous high BK viremia, whereas the other patient had high viral loads of JCPyV DNA in urine without detectable BKPyV replication in urine or plasma, as previously reported (Lautenschlager et al. 2014). In addition, one patient with BK viremia had BKPyV DNA extracted from biopsy, although the preceding SV40 staining remained negative. Two of these grafts were lost later due to ABMR.

## 6 DISCUSSION

This thesis evaluates noninvasive biomarkers of chronic allograft injury and long-term graft function in pediatric RTx recipients. The studies were retrospective, longitudinal and observational analyses of biomarkers that reflect the activation of immune response and the consequences of immunosuppression after kidney transplantation. The results showed that post-transplant HLA antibodies were common, but the detection of DSAs, as such, did not predict poor GFR at the time of sampling or later on. Fibrosis associated with decreased graft function and increased biomarker scores, but none of the biomarkers showed a superior predictive value for long-term graft function compared to routine IF/TA score. Anemia was related to poor graft function, which could not be explained by distinct low-grade inflammation or evident erythropoietin deficiency. In addition, BK polyomavirus infection associated with long-term complications, such as ABMR and allograft injury.

### 6.1 Can we predict graft function by detecting DSA? (I)

During the past decade, the significance of DSAs in chronic ABMR and poor graft survival has been well emphasized (Sellares et al. 2012, Hidalgo et al. 2009, Lee et al. 2009, Dunn et al. 2011). It is evident that sensitized recipients have a high risk for ABMR (Susal et al. 2009, Lucia et al. 2015), but also previously nonsensitized, low-risk recipients may develop post-transplant *dn*DSAs, which increases the risk for chronic ABMR and graft loss (Ginevri et al. 2012, Wiebe et al. 2012, Chaudhuri et al. 2013). In particular, pre-transplant DSAs increase the incidence of early ABMR from 1–6% to 21–55% (Amico et al. 2009, Lefaucheur et al. 2007, Kraus et al. 2009). Other independent predictors of early rejections are non-adherence to immunosuppression and HLA class II mismatches (Wiebe et al. 2012). Recommendations for post-transplant screening of *dn*DSA have suggested individual adaptation of immunosuppression in patients with DSA according to risk profile (Tait et al. 2013), but a recent cost-benefit analysis revealed only limited efficacy of screening stable recipients (Kiberd et al. 2016).

After publication of study I, the role of *dn*DSA on graft loss has been extensively studied in adult cohorts. We measured post-RTx HLAab in a pediatric cohort and correlated the detection of individual DSA with graft function. The clinical importance of post-transplant DSA on graft outcome is evident (Susal et al. 2015, Wiebe, Nickerson 2013, Loupy et al. 2012), but requires critical consideration together with other factors, such as age at RTx and nonadherence, when aimed to improve long-term survival (Sellares et al. 2012). Also, patients with combined TCMR and ABMR have unfavorable outcomes (Willicombe et al. 2014). Prospective multicenter studies of adult RTx recipients showed poor graft survival in patients



with *dn*DSA (Terasaki et al. 2007, Wiebe et al. 2012). The survival rate was 58% in patients with *dn*DSA compared to 81% in those without (Terasaki et al. 2007). However, no pretransplant DSA feature (class, number, MFI) reliably predicts graft outcome (Amico et al. 2009, Lefaucheur et al. 2010). And most importantly, many patients with DSA still have moderate outcome for years (Konvalinka, Tinckam 2015).

Interestingly, the optimal timing for post-transplant antibody testing, definitive treatment strategies, and the latency between the detection of DSA and clinical effect (i.e. graft dysfunction or histological injury) are still incompletely understood (Konvalinka, Tinckam 2015). We studied serum samples taken at various time points and detected a slight increase in the frequency of DSA over time. Detection of DSA was common in children, and was interestingly unrelated to a marked annual decline in GFR. Future determination of clinically feasible treatment strategies for stable patients with *dn*DSA requires a clinical trial (Archdeacon et al. 2011).

The cause of late allograft loss is often attributable to immune mediated chronic injury, detected as glomerulopathy, IF/TA with or without rejection, or polyomavirus nephropathy (El-Zoghby et al. 2009). The importance of alloimmunity is evident, although the specific mechanism of late ABMR, and especially the role of predominant class II DSA, remain somewhat unclear. Also, complement-fixing DSAs and C1q testing have been of special interest due to associations with poor outcomes (Loupy et al. 2013, Sutherland et al. 2012), and the IgG3-dominant (with the highest MFI level) DSA subclass associates with allograft failure (Lefaucheur et al. 2016). Focusing on these subtypes of DSA may be helpful to identify clinically relevant phenotypes of antibody-mediated injury. In our study, DSA reacted mostly against class II antigens and non-DSA against class I, as also reported in adults (Lachmann et al. 2009). When the results of adult studies are applied to pediatric cohorts it is important to note that B-cell subpopulations, which participate in the regulation of a variety of immune responses, mature with age (Smet et al. 2011).

To date, data on DSA in pediatric recipients are still scarce. According to two recent pediatric studies, one-third of the pediatric recipients have detectable *dn*DSA, which reacts mainly (70%) against class II antigens (Kim et al. 2014, Comoli et al. 2016). The rates of DSAs are similar to our finding (34%) with high prevalence of class II (65%) DSA. These rates are higher than reported (20%) in adult cohorts (Wiebe et al. 2012, Everly et al. 2013). This could be partly explained by noncompliance, stronger alloreactivity in children, or high sampling frequency (Comoli et al. 2016).

An important question is what to do with stable patients with detectable DSA after RTx. A recent study of complement fixing *dn*DSAs in a pediatric cohort suggests the removal or modulation of antibodies already at the time of the first confirmed positivity, since 60% of the patients with persistent DSA show ABMR (Comoli et al. 2016). However, the role of fluctuating DSA levels over time is incompletely

understood (Wiebe et al. 2012, Cooper et al. 2011). In our study, fluctuation was evident, although the limited number of sera per patient did not allow further analysis. Similarly, almost half of the DSAs became undetectable in another pediatric cohort (Kim et al. 2014). Also, Kimball et al. suggest longitudinal monitoring of DSA, as they showed favorable outcomes for patients who were able to eliminate DSA after RTx (Kimball et al. 2011).

The diagnostic criteria of AMBR were currently revised, and the C4d negative ABMR was included in the Banff'13 classification (Haas et al. 2014). Our results lacked the staining for C4d, which hampered the differentiation and classification of cellular and antibody-mediated episodes at that time. However, we found that IF/TA-findings in biopsies at 1.5 and 3 years post-transplantation correlated with poor outcome later on, but these lesions were not more prevalent in patients with DSA during the first three years. In accordance with this, the detection of DSA did not associate with marked chronic lesions in biopsies at 1.5 or 3 years or with a significant decrease in GFR at 3–10 years post-RTx. The wide distribution of GFR at the time of DSA detection was not different from that in patients without DSA.

Two pediatric studies reported increased plasma creatinine in DSA-positive recipients (Ginevri et al. 2012, Chaudhuri et al. 2013), and one showed poor graft function using Kaplan-Meier survival analysis (Kim et al. 2014). Interestingly, they reported that DSA-positive patients showed a more rapid decline in GFR, but other factors, such as microvascular injury, were independent risk factors for poor graft outcome in the multivariable analysis (Kim et al. 2014). The authors proposed a graded response to DSA MFI, in which the low MFI might resolve and the high MFI associate with poor GFR. Of note, no specific threshold for DSA positivity has yet been clearly established, but these results support the current concept of prospective HLAab monitoring also in pediatric patients. In our study cohort, one-third (23/62) of the DSA positive samples had MFI > 10,000, which was, however, unrelated to poor graft function later on. Moreover, the annual decline in GFR was comparable despite the antibody finding.

The adaptation of immunosuppression in the presence of high MFI levels might support the main aim to prevent the development of harmful microvascular injury. An ongoing clinical trial is performing a systemic evaluation of this concept (Dorling et al. 2014), and the interpretation of the results of a recent cost-benefit study (Kiberd et al. 2016) may specify the future focus of post-transplant monitoring of DSA. Our retrospective study design did not allow any adaptation to immunosuppression, and thus our findings reflect the natural course of clinical events and GFR, when no change was made due to DSA. The fact that no therapy is validated for patients with *dn*DSA puts the relevance of post-transplant monitoring of DSA in clinically stable patients into question (Tait et al. 2013, Roelen et al. 2012). The adaptation of immunosuppression has become a common policy in many transplant centers, although it partly hampers the goal to minimize immunosuppression.

## 6.2 Clinical value of immunohistochemical biomarkers (II)

CAI is a multifactorial process that develops over time and leads to excessive accumulation of extracellular matrix (ECM) and renal fibrosis. Early biomarkers are needed to detect the early molecular changes in cell structure, before the irreversible changes in visual histopathology (Birk 2012). The analysis of independent or supplement markers is essential to increase the diagnostic ability of biopsies to detect subclinical pathology.

To date, neither immunohistochemistry nor molecular techniques are able to differentiate reliably between IF/TA with different causes. The most accurate way is to combine clinical history, serology and histology to distinguish rejections, BKPyV-associated nephropathy, and recurrent or *de novo* diseases (Haas 2014). Protocol biopsies demonstrate the cumulative impact of donor factors on early IF/TA (Henderson et al. 2011). Early tubulointerstitial injury is detectable and precedes chronic IF/TA changes while the graft function is still normal. Thus, biomarkers that could visualize early injury would allow early interventions to slow down the development of fibrosis.

Epithelial-to-mesenchymal transition (EMT) is a well-known process in fetal development and pathologic cancer progression, but a debated phenomenon in human kidney fibrosis (Zeisberg, Duffield 2010). Most studies of EMT in renal tissue have been performed in animal models or adults. We used two mesenchymal markers, vimentin and  $\alpha$ -SMA, to study the prognostic value for early fibrogenesis. PSGL-1 was selected to detect interstitial inflammation, and collagen IV to visualize fibrosis. Neither immunohistological markers nor Banff chronic scores were found to be significant risk factors for poor graft function in our cohort. The results were similar with a single marker or when added up to a combined score.

Inflammation in biopsy indicates that the underlying disorder is actively progressing, and thus has adverse effects on graft survival (Sellares et al. 2011). Retrospective analysis of post-transplant DSA was available from fifty-two patients, one-fourth (13/52) of whom had detectable post-transplant DSA. These occasionally timed samples showed no significant association between DSA and simultaneous histopathology or biomarker scores (data not shown). Interstitial inflammation was studied with PSGL-1, a membrane-bound molecule and an important ligand for endothelial selectins on leucocytes enabling leucocyte recruitment (Ley 2003). We found a significant correlation between PSGL-1 expression and Banff inflammation (i) score, and IF/TA scoring. This demonstrated simultaneous processes of inflammation and fibrosis, a harmful combination for graft survival (Mannon et al. 2010, Mengel et al. 2009, Park et al. 2010, Sellares et al. 2011). Even low-grade inflammation alone shows adverse effects on graft function, especially when persistent (Mengel et al. 2007).

Early fibrogenesis is detectable as vimentin expression in tubular epithelial cells (de Matos et al. 2010, Kers et al. 2010, Xu-Dubois et al. 2013). The expression in glomeruli, arterioles and interstitial fibroblasts is normal, whereas in tubular epithelial cells (TECs) this intermediate filament protein is a marker of mesenchymal phenotype (Muramatsu et al. 2004, Hertig et al. 2008). While vimentin might be detectable already at the time of transplantation in TECs, the harmful *de novo* expression increases with time and associates with poor graft function (Kers et al. 2010, Hertig et al. 2008). We found the highest expression of tubular vimentin at 3 months post-RTx, but only a weak association with IF/TA score and no prognostic value for long-term GFR. A high vimentin score predicted poor GFR at 3–4 years post-RTx only at 1.5-year biopsies, but this was not confirmed by multivariable analysis. In fact, multiple confounders with different mechanisms and effects take part in the allograft injury (Alexander et al. 2007), which supports the idea of adding biomarkers to predictive panels.

The development of allograft injury may also be visualized by another mesenchymal marker,  $\alpha$ -SMA. It is strongly expressed in myofibroblasts (Roberts et al. 1997, Goumenos et al. 1994), and the interstitial expression of  $\alpha$ -SMA associates with collagen depositions in the interstitium and vimentin expression in TECs (de Matos et al. 2010, Kers et al. 2010, Vitalone et al. 2008). We found that  $\alpha$ -SMA expression associated with fibrosis, but not independently with long-term graft function. Also, increased collagen IV expression was demonstrated in the areas of fibrosis in which the extracellular matrix accumulates into the interstitium. Deposition of different type of collagens, including collagen type IV, is characteristic for these areas (Yan et al. 2010).

In theory, combining different immunohistochemical biomarkers, which demonstrate different mechanisms of allograft injury, could add the ability to predict poor graft function. However, we found that the most important predictor of graft function was the progression of fibrosis. Although biomarker expression might precede the appearance of visual IF/TA, the predictive value of molecular markers is always less prominent than the effect of structural change with a simultaneous loss of graft function. Due to the multifactorial process of chronic allograft injury, no panel of clinically relevant biomarkers is yet validated for routine use (Stegall, Borrows 2015).

### 6.3 Anemia and graft function: Cause or consequence? (III)

Inadequate management of post-transplant anemia may impair the long-term survival. Anemia is a risk factor for cardiovascular disease (CVD), which increases the risk for mortality among pediatric transplant recipients (Kaidar et al. 2014, Silverstein 2004). Similarly, adult renal transplant patients with anemia are shown to have an additional 25% risk for renal graft loss (Winkelmayer et al. 2006) and increased rate of mortality (Molnar et al. 2007, Chhabra et al. 2008). Interestingly, even partial correction of Hb levels might improve the health-related quality of life and physical functioning (Johansen et al. 2010) and prolong the long-term graft function and survival (Yabu, Winkelmayer 2011, Reindl-Schwaighofer, Oberbauer 2013).

The reported rates of PTA reach 31% in Australia (Chadban et al. 2007) and 42% in Europe (Molnar et al. 2011a). In our pediatric study cohort, the rates of PTA at 1 and 5 post-RTx years were 55% and 44%, respectively. Early PTA within the first post-RTx year involved up to 87% of patients, which is in accordance with a reported 91% prevalence of immediate (first 3 months) PTA in adults (Poesen et al. 2011). Our findings of decreasing frequency of anemia with time are in line with other reports suggesting that Hb levels increase with age and time since RTx (Krischock et al. 2016).

Moderate anemia was a common finding in patients at all ages in our study cohort. The study of Yorgin et al. reported a lower frequency of early anemia in patients less than 2 years at RTx compared to older children, and speculated that an adult size kidney graft possibly produces relatively more EPO and clears uremic toxins more effectively in a small recipients compared to older children (Yorgin et al. 2002a). However, we found the youngest children to be more anemic due to the high prevalence (76%) of young CNF patients. CNF patients in general had lower Hb levels already early after RTx, possibly due to nephrectomy prior to the operation. Severe anemia (Hb < 100 g/L), in contrast, was infrequent (range 3-9%) after the first few years. Similar frequencies (2–14%) of severe anemia were reported in both, adult (Vanrenterghem et al. 2003, Molnar et al. 2011a) and pediatric cohorts (Al-Khoury et al. 2006, Mitsnefes et al. 2005).

An unresolved question remains: what causes anemia in RTx patients? The hemoglobin levels of RTx patients are lower compared to general population, which highlights the role of transplant-associated factors (Chadban et al. 2007). Iron and EPO availability are the main regulators of erythropoiesis, and insufficient EPO production from renal peritubular fibroblasts leads to renal anemia. In normal kidneys, renal tissue hypoxia stimulates the production of EPO, but among CKD patients, pro-inflammatory cytokines have a negative effect on this regulation (Jelkmann 2011, Jelkmann 1998). Serum EPO levels were reported to be low in pediatric RTx recipients with anemia (Yorgin et al. 2002a), which is contrary to our findings of rather increased levels of EPO. However, the EPO values in our study

were close to the reported normal reference (6-32 mIU/mL) (Jelkmann 2011). EPO levels were inversely associated with Hb, but unrelated to GFR, similarly to the results reported by Sinnamon et al. (Sinnamon et al. 2007). A correlation between EPO and <sup>51</sup>Cr-EDTA clearance was not detected in CKD patients, either, regardless of anemia (Mercadal et al. 2012). These findings speak against the idea that impaired EPO production alone could explain the high frequency of post-transplant anemia after RTx.

Iron deficiency induces microcytic and hypochromic anemia. Iron deficiency has been reported in up to 33 % of pediatric RTx patients (Kausman et al. 2004, Yorgin et al. 2002a), when defined as ferritin levels of <100 µg/L (ng/mL) and TSAT of <20% (Galutira, Del Rio 2012). The use of serum iron, transferrin saturation, and ferritin is common, although problematic due to the large variety of cut-off levels and the acute-phase reactivity of the ferritin (Horl 2007, Lorenz et al. 2002). In our patient cohort, the overall frequency of microcytosis (1.3%) and hypochromia (9 %) was low. Hcpidin-25 levels were high in severely anemic samples, suggesting that inflammation and limited hepcidin-25 excretion due to reduced GFR were present rather than iron deficiency. These findings are consistent with a recent study of increased hepcidin-25 levels due to inflammation and poor GFR (Chan et al. 2013).

The myelosuppressive effect of the antimetabolites AZA and MMF is suggested to increase the risk of late PTA (Mitsnefes et al. 2005, Vanrenterghem et al. 2003, Yorgin et al. 2002b, Al-Uzri et al. 2003) although some studies lack clear evidence of a direct association (Pascual et al. 2013, Chhabra et al. 2008). In our study, patients transplanted in the 1990s received a constant dose of AZA (1 mg/kg/day), whereas MMF was used in only a minority of the patients. However, our data suggested that general myelosuppression was an infrequent cause of PTA.

In kidney graft biopsies, fibrosis and inflammation are characteristic findings before the loss of graft function. Inflammation, together with decreased oxygen delivery to the graft, may lead to progressive tubulointerstitial hypoxia and fibrosis (Fine, Norman 2008). On the other hand, fibrosis may impair the production of EPO by the peritubular cells. We found an association between Hb levels and IF/TA-findings in control biopsy samples. Interestingly, low Hb levels preceded the biopsy findings, suggesting that anemia may also partly worsen the graft histology.

The recent ESPN/ERA-EDTA registry data of over 3,600 pediatric RTx patients demonstrated that low Hb levels associated with worse eGFR and increased risk for graft failure (Krischock et al. 2016). Our longitudinal study showed a similar, up to 23% decrease in GFR among anemic recipients during the follow-up, verifying the previous cross-sectional observations. More importantly, the low Hb levels seemed to predict decreased subsequent GFR levels, which is in accordance with a recent report on adults showing that low Hb levels at one month were an independent prognostic factor for later graft loss (Pascual et al. 2013). The small number of graft losses did not allow us to analyze the effect of low Hb level on graft survival.

## 6.4 Low-grade inflammation and long-term prognosis (III)

Low-grade inflammation is a common complication of CKD and transplantation *per se* in RTx recipients. Inflammation is a pathogenic mechanism of CAI and a risk for graft-related complications. CAI induces the infiltration of inflammatory cells to the graft, and the sustained production of cytokines and pro-inflammatory molecules leads to chronic inflammation.

Chronic inflammation is known to induce anemia and atherosclerosis, and to impair graft function (Cottone et al. 2007). Pre-transplant CRP levels have been associated with biopsy-proven allograft nephropathy (Fink et al. 2002), and post-transplant hsCRP levels have been shown to predict cardiovascular events and mortality in RTx recipients (Abedini et al. 2009). Both IL-6 and hsCRP levels are important risk factors for long-term graft outcomes in adults (Dahle et al. 2011).

In our pediatric cohort, IL-6 and hsCRP levels were measured repeatedly (median 6 measurements/patient) over time to investigate the association between low-grade inflammation and graft function. Interestingly, inflammation markers fluctuated only slightly over time, demonstrating the peak levels of IL-6 at 1 month and hsCRP at 2 years post-RTx. In our cohort, serum hsCRP levels were low in most anemic patients and showed only a weak inverse correlation with the Hb levels. IL-6 levels correlated with hsCRP but not with hepcidin-25 or Hb levels. The longitudinal follow-up data on inflammatory markers did not favor the idea that low-grade inflammation would significantly induce the rate of anemia in pediatric RTx patients. Moreover, we found a positive correlation between hsCRP and EPO level, which is in agreement with the previous findings that inflammation may independently stimulate EPO production in CKD patients (Mercadal et al. 2012).

The markers of low-grade inflammation correlated poorly with either concomitant or subsequent graft function. A marker of increased red blood cell aggregation, ESR, increased significantly along CKD stages with decreasing GFR. The role of ESR in transplantation requires future studies to determine the ability of ESR to identify high-risk patients who would benefit from more cautious post-transplant monitoring. However, hsCRP and IL-6 showed no similar clear association with graft function, and the effect of low-grade inflammation on long-term graft function remained modest in multivariable analyses. This might reflect the high prevalence of low-grade inflammation among all RTx recipients and the multifactorial effect of inflammation on long-term prognosis.

## 6.5 Clinical characteristics of BKPyV infection (IV)

Polyomavirus infection is an important risk factor for allograft injury (Acott, Hirsch 2007). Over 80% of healthy children and adolescent are seropositive for BKPyV, and this reflects the high frequency of primary infections during childhood (Egli et al. 2009). BKPyV infection is asymptomatic and harmless for immunocompetent individuals, although the virus persists in the renoepithelial cells after the primary infection (Rinaldo et al. 2013).

One important question is how to identify patients at the highest risk for BKPyV in the early period. In pediatric studies, recipient seronegativity is a significant risk factor for BKPyVAN (Smith et al. 2004, Ginevri et al. 2003), although seropositivity does not protect the recipient from reactivation. We found BK viremia in 9 (20%) patients who showed a tendency for decreased GFR during the follow-up. Viral BKPyV replication in plasma occurred at a median of 6 months post-RTx.

High-risk patients would benefit from individualized selection and early adjustments of initial immunosuppression, although the continuous balancing between the risk for acute rejection and over-immunosuppression persists. Recent studies describe how the use of tacrolimus directly contributes to BKPyV replication, and thus increases the risk for BKPyV infection, whereas the use of CsA shows opposite effects (Hirsch et al. 2016, Hirsch et al. 2013).

Early BKPyV replication during the first weeks after transplantation might be donor-derived and increase further the risk for subsequent viremia and BKPyVAN (Saundh et al. 2013). The importance of donor-derived transmission is highlighted in a recent study of Schwarz et al. (Schwarz et al. 2015), where they demonstrate the dominance of the donor's BKPyV subtype, and suggest that pretransplant urinary BKPyV shedding is a major risk factor for posttransplant infection. However, neither recipient nor donor evaluation includes the routine serology for BKPyV. Instead, post-transplant BK viremia and viremia are often monitored according to protocols (Hirsch et al. 2014), as both BK viremia and viremia precede the progression of BKPyVAN. Overall, BKPyVAN affects 3–8% of the pediatric kidney recipients (Acott, Hirsch 2007). We found three patients with SV40 positivity in biopsies, a diagnostic sign of PyVAN. Two of these patients had BK viremia, and one demonstrated an uncommon case of JCPyV-associated nephropathy (JCVAN) (Lautenschlager et al. 2014). Two grafts were lost, both due to ABMR with DSAs in blood and clear C4d positivity in biopsies. Both patients were adolescents at the time of operation.



## 6.6 Methodology: advantages and pitfalls

Pediatric patients are a unique study population to evaluate the role of biomarkers in chronic allograft injury, as this patient cohort lacks many common confounders (e.g. hypertension and advanced age) seen in adult recipients. The strengths of our studies were the longitudinal study design, a relatively large study cohort of pediatric patients, and annual GFR assessment by  $^{51}\text{Cr}$ -EDTA clearance during up to 10 years of follow-up.

Limitations were identified due to the retrospective nature of the study. The lack of pre-transplant sera in studies I and IV limits the interpretation of the role of *dn*DSA. In addition, the solid-phase assay used is a qualitative detector of DSAs. Quantitative comparison of different DSA levels could be more specific (Reed et al. 2013). The other shortcomings of the Luminex technology are overdetection, unclear cut-off value for MFI to define DSA positivity, and the gradual decrease in antibody reactivity.

Immunohistochemistry was used to increase the diagnostic ability of invasive percutaneous core needle biopsies to characterize the allograft status. The biopsy procedure is safe, but sampling errors, inter-observer and interpretation variability challenge the accuracy of biopsy results. The major limitation at the time of study II was that we could not reliably associate biomarker findings with ABMR, as many of the biopsy samples were collected before the routine measurements of DSA and availability for the routine immunohistochemistry of C4d. Unfortunately, the staining of the paraffin-embedded samples for C4d afterwards was not reliable, as the grading of paraffin-embedded samples is unspecific and less sensitive than immunofluorescence for frozen sections (Seemayer et al. 2007). In addition, anemia-related factors, such as folate and vitamin B12 levels, were not available for analysis in study III. Also, the lack of iron status assessments limits the interpretation of the causality.

## 6.7 Future challenges and opportunities

Noninvasive biomarkers are studied to identify potential mechanisms and new diagnostic and therapeutic targets of chronic allograft injury. Early detection of clinical problems is important when the aim is to focus on therapeutic interventions in time. The use of biomarkers as surrogate end points decreases the time interval needed for sufficient outcome frequencies in long-term studies and enables the determination of efficacy. Recent approaches to promote long-term graft survival include individualized therapy and pre-emptive transplantations. The goal is to identify biomarkers that are sensitive to identify functioning grafts that will fail but could be rescued with early interventions. It is important to understand the mechanisms and features of chronic allograft injury and thus to develop and focus on early interventions for the right patients.

Recent studies with microarray analyses propose that the role of progressive CNI toxicity might be overestimated. Minimization strategies of immunosuppression may actually increase the risk for ABMR, and the role of ABMR in allograft injury might be underestimated (Halloran et al. 2014). Noninvasive microarray tests are powerful and diagnostic, but their incorporation into clinical transplantation requires distinct validation.

To date, renal function, DSAs, and graft histology are examples of biomarkers used as surrogate end points in clinical practice. Novel biomarkers are still often relatively non-specific and impractical, although gene expression methods enhance diagnostic accuracy and may add predictivity when combined with histology (Halloran et al. 2013). In the ongoing prospective IVIS trial, virus-specific T cells are successfully used as novel biomarkers of overimmunosuppression in pediatric RTx recipients (Ahlenstiel-Grunow et al. 2014). A combination of novel methods could be used as a molecular microscope to identify high-risk recipients before the early signs of graft failure (Loupy et al. 2014).

Future developments in the field of biomarkers in kidney transplantation include novel analytical methods for self-monitoring of individual drug levels. This would enable more customized therapies of immunosuppressants with a narrow therapeutic window and significant inter-individual variability. Although tools and technologies are already available to model disease pathophysiology, molecular signatures are far from the predictive biomarkers. Financial and collaborative efforts of different biosciences are needed to transfer the benefits of the complex technologies into clinical use.

## 7 CONCLUSIONS

The present studies focus on biomarkers that could identify patients at risk for chronic allograft injury.

*Study I* showed that circulating HLA antibodies were rather common in pediatric RTx patients, and DSA was detectable in one-third of the patients. DSA reacted mainly against class II antigens, but did not predict significantly worse graft function at the time of sampling or later on.

*Study II* demonstrated that biomarker expression associated with IF/TA lesions, which increased during the first three years post-RTx in protocol biopsies. Intense staining of collagen IV and vimentin associated with poor graft function, similarly to IFTA score. Histochemical biomarkers did not significantly add the ability to predict future graft function when compared to routine histopathology.

*Study III* described the correlation of anemia and inflammation with graft function over time. Both anemia and low-grade inflammation were common findings even years after RTx. Anemia associated with poor graft function, which was not explained by distinct inflammation or erythropoietin deficiency.

*Study IV* characterized the findings of BK viremia in pediatric RTX recipients. BK viremia and polyomavirus-associated nephropathy were rather uncommon, but patients with BK viremia showed a tendency for poor long-term graft function.

Development of chronic allograft injury is a multifactorial process with several immunological and non-immunological components that influence the allograft histopathology and function. Based on the results of this thesis, biomarkers of different pathophysiological mechanisms may identify patients at risk for poor kidney graft function. Patients with activated immune response or altered biochemical parameters require thorough evaluation, as no biomarker to date is well established to characterize the high-risk recipients of CAI.

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A handwritten signature in pink ink, appearing to read 'Jenni Miettinen', with a decorative flourish at the end.

Jenni Miettinen

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