INDIVIDUALIZED IMMUNOSUPPRESSION AFTER RENAL TRANSPLANTATION IN CHILDREN

by

PAULA SEIKKU

ACADEMIC DISSERTATION

Supervisors

Professor Christer Holmberg
Pediatric Nephrology and Transplantation
Hospital for Children and Adolescents
Helsinki University Central Hospital
Helsinki, Finland

Docent Hannu Jalanko
Pediatric Nephrology and Transplantation
Hospital for Children and Adolescents
Helsinki University Central Hospital
Helsinki, Finland

Reviewers

Docent Helena Isoniemi
Transplantation and Liver Surgery Clinic
Helsinki University Central Hospital
Helsinki, Finland

Docent Timo Jahnukainen
Department of Paediatrics
Turku University Hospital
Turku, Finland

Official opponent

Professor Peter Hoyer
Department of Paediatric Nephrology
University of Essen
Essen, Germany

To Kai, Johannes, Markus, Henrik and Samuel

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1. ABSTRACT

Pediatric renal transplantation (TX) has evolved greatly during the past few decades, and today TX is considered the standard care for children with end-stage renal disease. In Finland, 191 children had received renal transplants by October 2007, and 42% of them have already reached adulthood. Improvements in treatment of end-stage renal disease, surgical techniques, intensive care medicine, and in immunosuppressive therapy have paved the way to the current highly successful outcomes of pediatric transplantation. In children, the transplanted graft should last for decades, and normal growth and development should be guaranteed. These objectives set considerable requirements in optimizing and fine-tuning the post-operative therapy. Careful optimization of immunosuppressive therapy is crucial in protecting the graft against rejection, but also in protecting the patient against adverse effects of the medication.

In the present study, the results of a retrospective investigation into individualized dosing of immunosuppresive medication, based on pharmacokinetic profiles, therapeutic drug monitoring, graft function and histology studies, and glucocorticoid biological activity determinations, are reported. Subgroups of a total of 178 patients, who received renal transplants in 1988–2006 were included in the study. The mean age at TX was 6.5 years, and 26% of the patients were <2 years of age. The most common diagnosis leading to renal TX was congenital nephrosis of the Finnish type (NPHS1).

Pediatric patients in Finland receive standard triple immunosuppression consisting of cyclosporine A (CsA), methylprednisolone (MP) and azathioprine (AZA) after renal TX. Optimal dosing of these agents is important to prevent rejections and preserve graft function in one hand, and to avoid the potentially serious adverse effects on the other hand. CsA has a narrow therapeutic window and individually variable pharmacokinetics. Therapeutic monitoring of CsA is, therefore, mandatory. Traditionally, CsA monitoring has been based on pre-dose trough levels (C0), but recent pharmacokinetic and clinical studies have revealed that the immunosuppressive effect may be related to diurnal CsA exposure and blood CsA concentration 0–4 hours after dosing. The two-hour post-dose concentration (C2) has proved a reliable surrogate marker of CsA exposure.

Individual starting doses of CsA were analyzed in 65 patients. A recommended dose based on a pre-TX pharmacokinetic study was calculated for each patient by the pre-TX protocol. The predicted dose was

clearly higher in the youngest children than in the older ones (22.9±10.4 and 10.5±5.1 mg/kg/d in patients <2 and >8 years of age, respectively). The actually administered oral doses of CsA were collected for three weeks after TX and compared to the pharmacokinetically predicted dose. After the TX, dosing of CsA was adjusted according to clinical parameters and blood CsA trough concentration. The pharmacokinetically predicted dose and patient age were the two significant parameters explaining post-TX doses of CsA. Accordingly, young children received significantly higher oral doses of CsA than the older ones. The correlation to the actually administered doses after TX was best in those patients, who had a predicted dose clearly higher or lower (> ±25%) than the average in their age-group. Due to the great individual variation in pharmacokinetics standardized dosing of CsA (based on body mass or surface area) may not be adequate. Pre-Tx profiles are helpful in determining suitable initial CsA doses.

CsA monitoring based on trough and C2 concentrations was analyzed in 47 patients, who received renal transplants in 2001–2006. C0, C2 and experienced acute rejections were collected during the post-TX hospitalization, and also three months after TX when the first protocol core biopsy was obtained. The patients who remained rejection free had slightly higher C2 concentrations, especially very early after TX. However, after the first two weeks also the trough level was higher in the rejection-free patients than in those with acute rejections. Three months after TX the trough level was higher in patients with normal histology than in those with rejection changes in the routine biopsy. Monitoring of both the trough level and C2 may thus be warranted to guarantee sufficient peak concentration and baseline immunosuppression on one hand and to avoid over-exposure on the other hand.

Controlling of rejection in the early months after transplantation is crucial as it may contribute to the development of long-term allograft nephropathy. Recently, it has become evident that immunoactivation fulfilling the histological criteria of acute rejection is possible in a well functioning graft with no clinical sings or laboratory perturbations. The influence of treatment of subclinical rejection, diagnosed in 3-month protocol biopsy, to graft function and histology 18 months after TX was analyzed in 22 patients and compared to 35 historical control patients. The incidence of subclinical rejection at three months was 43%, and the patients received a standard rejection treatment (a course of increased MP) and/or increased baseline immunosuppression, depending on the severity of rejection and graft function. Glomerular filtration rate (GFR) at 18 months was significantly better in the patients who were screened and treated for subclinical rejection in comparison to the historical patients (86.7±22.5 vs. 67.9±31.9 ml/min/1.73m^2, respectively). The improvement was most remarkable in the youngest (<2 years) age group (94.1±11.0 vs. 67.9±26.8 ml/min/1.73m^2). Histological findings of chronic allograft

nephropathy were also more common in the historical patients in the 18-month protocol biopsy.

All pediatric renal TX patients receive MP as a part of the baseline immunosuppression. Although the maintenance dose of MP is very low in the majority of the patients, the well-known steroid-related adverse affects are not uncommon. It has been shown in a previous study in Finnish pediatric TX patients that steroid exposure, measured as area under concentration-time curve (AUC), rather than the dose correlates with the adverse effects. In the present study, MP AUC was measured in sixteen stable maintenance patients, and a correlation with excess weight gain during 12 months after TX as well as with height deficit was found. A novel bioassay measuring the activation of glucocorticoid receptor – dependent transcription cascade was also employed to assess the biological effect of MP. Glucocorticoid bioactivity was found to be related to the adverse effects, although the relationship was not as apparent as that with serum MP concentration.

The findings in this study support individualized monitoring and adjustment of immunosuppression based on pharmacokinetics, graft function and histology. Pharmacokinetic profiles are helpful in estimating drug exposure and thus identifying the patients who might be at risk for excessive or insufficient immunosuppression. Individualized doses and monitoring of blood concentrations should definitely be employed with CsA, but possibly also with steroids. As an alternative to complete steroid withdrawal, individualized dosing based on drug exposure monitoring might help in avoiding the adverse effects. Early screening and treatment of subclinical immunoactivation is beneficial as it improves the prospects of good long-term graft function.

2. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles referred to in the text by Roman numerals I-IV:

I.   Seikku P, Hoppu K, Jalanko H, Holmberg C. Predictive value of pretransplantation cyclosporine pharmacokinetic studies on initial post-transplantation dosing in pediatric kidney allograft recipients. *Pediatric Transplantation 7: 102-10, 2003.*

II.   Seikku P, Krogerus L, Jalanko H, Holmberg C. Better renal function with enhanced immunosuppression and protocol biopsies after kidney transplantation in children. *Pediatric Transplantation 9: 754-62, 2005.*

III. Seikku P, Jalanko H, Holmberg C. Cyclosporine monitoring and clinical outcome in the early post-transplant period in children. *Submitted, 2007*

IV.   Seikku P, Raivio T, Jänne OA, Neuvonen PJ, Holmberg C. Methylprednisolone exposure in pediatric renal transplant patients. *American Journal of Transplantation 6: 1451-58, 2006.*

3. ABBREVIATIONS

APC   Antigen presenting cell
AR   Acute rejection
ATN   Acute tubular necrosis
AUC   Area under time – concentration curve
AZA   Azathioprine
B-CsA   Blood cyclosporine concentration
BID   Twice daily dosing
BKV   Polyoma virus, BK strain
C0   Blood cyclosporine through concentration
C2   Blood cyclosporine concentration two hours post-dose
CAD   Deceased donor
CAN   Chronic allograft nephropathy
CMV   Cytomegalo virus
CNF   Congenital nephrosis of the Finnish type
CNI   Calcineurin inhibitor
CRP   C-reactive protein
CsA   Cyclosporine A
CTL   Cytotoxic lymphocyte
EBV   Epstein-Barr virus
ESRD   End-stage renal disease
FNAB   Fine-needle aspiration biopsy
GBA   Glucocorticoid bioactivity
GC   Glucocorticoid
GFR   Glomerular filtration rate
GR   Glucocorticoid receptor
HLA   Human leucocyte antigen
LRD   Living related donor
MCH   Major histocompatibility complex
MMF   Mycophenolate mofetil
MP   Methylprednisolone
P-Crea Plasma creatinine concentration
PRA   Panel reactive antibodies
PTLD   Post-transplant lymphoproliferative disorder
Tac   Tacrolimus
TCR   T-cell receptor
TID   Three times daily dosing
TOR   Target of rapamycin
TX   Transplantation

4. INTRODUCTION

Organ transplantation (TX) has greatly changed the course of many diseases that previously lead to death after long, agonizing periods of treatments. During the past three decades kidney transplantation has become a highly successful treatment for children with end-stage renal disease (ESRD). Renal replacement therapy became applicable to children on routine basis in the late 1960s, but complications cast a shadow over the prospects of children with ESRD for years. Renal replacement therapy remained controversial in pediatric patients until the early 1980s, as expressed in a medical textbook on renal disease in 1979 "…we cannot escape the question whether children with end-stage renal failure should be treated or helped to die peacefully…", "…treating children under the age of 5…we do not always recommend…", and the prevailing treatment "…justified the hope that a substantial number of patients should survive 10–20 years and live a useful life" [1].

Kidney TX has proved a superior treatment for children with ESRD in comparison with long-term dialysis [2–6]. Today, more than 80% of children with renal transplants are expected to survive into adulthood [7–14]. Outcomes of organ TX have improved markedly over the past 20 years, mainly due to advances in surgical techniques, suitable choice of donors and recipients, better control of complications, and striking developments in immunosuppressive therapy. Most notable clinical advance in immunosuppression was the discovery of cyclosporine A [15] and its introduction in pediatric TX in the mid 1980s [16–18]. The primary aim of immunosuppression is to prevent acute and chronic rejection but it is essential that the aim is compatible with good quality of life. Organ transplant recipients are confronted with life-long immunosuppressive therapy with potentially serious adverse effects, e.g. nephrotoxicity, growth inhibition, increased risk for metabolic and cardiovascular disturbances, infections and malignancies. The pediatric patients continue to receive immunosuppressive medication for decades, which emphasizes the importance of optimal drug therapy.

Many immunosuppressive drugs in clinical TX share certain basic features, such as considerable interindividual variability in dosing requirements and relatively narrow therapeutic window, thus requiring therapeutic drug monitoring [19, 20]. Therapeutic protocols in children are often modified from those used in adults, although there are many fundamental differences in metabolism and pharmacokinetics in the growing and developing recipient [21]. Individualized immunosuppression with continuous monitoring and timely modification of therapy is imperative for successful long-term outcome in pediatric TX.

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5. REVIEW OF THE LITTERATURE

5.1 Renal transplantation in children

The incidence of ESRD varies between 5–10 children per million [22], and with improving survival and availability of treatment, the number of children receiving renal replacement therapy is increasing. Kidney TX is the optimal treatment for ESRD, leading to substantial improvement in quality of life. It is the consensus opinion that dialysis and/or renal TX should be considered for children when the glomerular filtration rate (GFR) falls below 15 ml/min/1.73m2 [23–25]. Etiology of ESRD in children differs from that in adults, so that congenital lesions such as obstructive uropathy and renal aplasia/dysplasia, together with focal segmental glomerulosclerosis (FSGS) account for nearly half of the transplants in the North America. The diagnostic categories listed for the indication for renal TX, according to the North American Pediatric Renal Transplant Cooperative Study (NAPRTCS), are displayed in Table 1. However, differences exist in the incidence of various diseases among countries. Chronic glomerulonephritis is a common cause (approximately 40%) for ESRD in developing countries. In Europe, hereditary familial nephropathies are reported three times more frequently than in the developing world. In Finland, congenital nephrotic syndrome of the Finnish type (CNF) accounts for nearly a half of renal TX in children, while in Sweden nephronophthisis is the most common singular cause, representing 20% of pediatric renal transplantation [26, 27].

Renal TX in children is often considered contraindicated in cases of severe neurological disease. In cases of concomitant infectious disease, active and rapidly progressed renal disease (e.g. hemolytic syndrome or crescentic glomerulonephritis) or malignancy, renal TX should be delayed until the underlying disease is controlled. Abnormalities in the urinary tract should be detected and corrected before TX. In some children with chronic kidney disease, it may be appropriate to perform TX before dialysis is needed (pre-emptive transplantation), thus improving the quality of life for these children, and perhaps improving the prognosis of the graft [28]. However, most children are on dialysis, either peritoneal or hemodialysis, prior to TX [29]. Pre-emptive TX is often performed from a living related donor (LRD), usually a parent [30]. Using a LRD transplant, timing of the operation can be decided in advance, thus shortening the waiting time on dialysis and limiting the related complications. The long-term results of

LRD kidney TX have been somewhat better than with a deceased donor (CAD). At the end of the last decade, the 1 – , 3 – , and 5 – year graft survival rates were 95, 90 and 83% using LRD, respectively, and 91, 82 and 75% in CAD transplantations [9]. However, the graft survival rates are improving for both donor types, and the difference between the two is narrowing [*30–32*]. The proportion of LRD in pediatric TX varies greatly from one country to another, from 86% in Scandinavia (excluding Finland) and 52% in USA, to less than 10% in France. Factors explaining the differences include activity of the cadaver transplant programs, the criteria for organ allocation to children, the way the parents are provided information about LRD and CAD transplantation, and cultural differences.

Apart from donor source, other risk factors for successful renal TX in children include recipient age, donor age, race (black vs. non-black), number of histocompatibility antigen mismatches, long cold-ischemia time (>24 hours), re-transplantations and prior blood transfusions, and the level of panel reactive antibodies (PRA). The number of pediatric deceased donors has decreased slowly over the decade. According to the US registry, 14% of kidney donors were under 18 years of age in 2004 [32]. Young deceased donor age (≤5 years) has been considered a risk factor for graft failure, although the results with grafts from very young donors are constantly improving [31]. Also, young recipient age (<24 months) may involve increased risk of graft failure because of greater immune reactivity and enhanced risk for graft thrombosis [*33*]. Prior transplantation, more than five life-time blood transfusions, black race and mismatches in the human leukocyte antigen (HLA) system all add to the risk of graft failure [30].

Table 1. Incidence of the most common diseases leading to renal transplantation in children. Data adapted from The North American Pediatric Renal Transplantation Cooperative Study [8].

Diagnosis	Incidence(%)
Aplastic/hypoplastic/dysplastic kidneys	16
Obstructive uropathy	16
Focal segmental glomerulosclerosis	12
Reflux nephropathy	5
Chronic glomerulonephritis	3
Polycystic kidney disease	3
Medullary cystic disease	3
Hemolytic uremic syndrome	3
Brune Belly	3
Congenital nephrotic syndrome	3
Cystinosis	2
Pyelo/interstitial nephritis	2
Membranoproliferative glomerulonephritis type I	2
Other or unknown	27

5.1.1 Pediatric renal transplantation in Finland

Pediatric renal TX program began in Finland in the mid 1980s, and by October 2007 191 children had received kidney transplants. One or more re-TX have been performed in 15 patients, and the number of kidney TX operations in children exceeds 200. Overall patient survival is 96%. The short-term patient and graft survival approaches 100%, and also the long-term outcome after renal TX in childhood is encouraging [*34, 35*]. Seventy-four (42%) patients who received a kidney transplant in childhood have reached adulthood.

The most common cause leading to ESRD in Finland is CNF, which results from mutations in *NPHS1* gene encoding nephrin, a transmembrane cell adhesion protein located in the podocyte slit diaphragm of kidney glomerulus [*36*]. Two mutations, Fin-major and Fin-minor, account for more than 90% of mutations in Finland [*37*], and are rare in non-Finnish patients [*38*]. Several other genes have also been implicated in nephrotic syndrome worldwide [*39*]. Mutations in *NPHS1* lead to massive proteinuria with secondary complications. In order to minimize the complications, these patients are bilaterally nephrectomized and dialyzed from an early age (<1 year). Continuous cycling peritoneal dialysis is used almost exclusively in the Finnish patients [*40*]. Seventy-two children in Finland have received kidney transplants because of CNF (38% of all pediatric renal TX patients), and 49 (68%) of them were under two years of age at the time of TX. Other causes leading to renal TX in Finland include urtheral valve (12%),

nephronophthisis (8%), polycystic kidneys (7%), glomerulonephritis (6%) and dysplastic kidneys (5%).

Young recipient age has been considered a risk factor for graft failure because of enhanced immunological reactivity and increased risk for graft thrombosis [41]. In addition to surgical complications acute tubular necrosis (ATN) is risk factor for arterial and venous thrombosis [42], and it is a more frequent complication in CAD than LRD TX [30]. Therefore, LRD TX has been advocated especially in young children. Because of the high incidence of CNF, a notable number of kidney TX patients are under two years of age in Finland. A third of pediatric recipients in Finland have received a LRD kidney, and 43% of them were ≤2 years of age. The incidence of rejection in the youngest patients in Finland does not appear to exceed that in the older patients [43], and graft thrombosis is an uncommon complication [44]. However, the long-term graft function in patients transplanted at an age less than two years, has not been as favorable as in the older children [45]. The reasons for the difference are not fully understood, but it has been postulated that the hemodynamic conditions in infants would not support sufficient arterial flow to an adult-sized graft [46]. Therefore, larger volumes of maintenance fluids have been suggested for the youngest patients to prevent chronic hypoperfusion of the graft [47].

An important aspect of care after transplantation is the child's growth. Improvement in patient care prior to TX has resulted in diminishing height deficit at the time of TX, which is an important determinant of final height. Long-term graft function as well as glucocorticoid therapy are other significant factors influencing growth [48]. In Finland, growth after renal TX in pediatric patients has been satisfactory, although not optimal [49, 44]. Declining graft function, especially in the youngest age group (<2 years) correlates with suboptimal catch-up growth, but glucocorticoid therapy inevitably affects growth as well. The use of glucocorticoids, although with tapering doses, is also related to excessive weight gain, which is a rather common problem after renal TX in Finland [44]. Disturbances in serum cholesterol and triglyceride concentrations are also rather common, although very mild in majority of the patients [50]. All pediatric renal TX patients in Finland receive triple immunosuppression consisting of cyclosporine A (CsA), azathioprine (AZA) and methylprednisolone (MP). Modifications of the standard protocol are made individually, when clinically required.

5.2 Transplant immunology

The success of organ TX is primarily limited by allograft rejection, an intrinsic part of the immune defense differentiating self from non-self, and protecting the organism from invaders. Only grafts between individuals

of the same genetic composition (syngeneic) are accepted, whereas grafts across these genetic barriers (allogeneic) are rejected.

Antigens encoded by the genes of the major histocompatibilty complex (MHC) on the short arm of chromosome 6, play a singular role in acting as major stimulants and targets of graft rejection. The MHC encodes cell surface protein molecules (MHC antigens), which in man are referred as the human leucocyte antigens (HLA). MHC is physically grouped into three regions – the class I and II regions (MHC-I and MHC-II) include the important histocompatibility loci, which encode the heavy chains of the HLA-A, -B and -C, and alpha and beta chains of the HLA-DR, -DP and -DQ molecules, respectively. The class III region encodes components of the complement pathway, among others.

Most nucleated cells express MHC-I antigens, which bind peptides generated through an endogenous pathway. A healthy cell supplies a sufficient representation of self-peptides displayed by the MHC-I molecule. A cell invaded by an intracellular pathogen produces MHC-I / foreign peptide complexes on its surface, signaling infection. The MHC-I / peptide complexes on the cell surface are then accessible for detection by T-cell or natural killer (NK) cell receptors. The MHC-II antigens, on the other hand, bind peptides generated through an exogenous pathway, and they are characteristic of B-lymphocytes, macrophages, dendritic cells, Langerhans cells, thymic epithelial cells and activated T-cells. These so-called "antigen-presenting cells" (APC) continually sample molecules from the extracellular space and introduce fragments of these on the cell surface in MHC-II / peptide complexes, where they are accessible for interaction with T-cell receptors (TCR) [51].

5.2.1 Allorecognition

Following transplantation of allogenic tissues, recognition by recipient T-lymphocytes of foreign proteins and peptides (T-cell allorecognition) initiates a cascade of immunological reactions resulting in rejection of the graft. This process is mediated via two distinct but non-exclusive mechanisms, the direct and indirect allorecognition pathway (Figure 1). The direct pathway represents a polyclonal T-cell response initiated via the presentation of allogenic MHC molecules by donor passenger leucocytes in the recipient's lymphoid organs. The multiplicity and high density of determinants created by the presence of allo-MHC on APCs results in enormous frequency of activated T-cells. Direct allorecognition is responsible for early sensitization of the host to donor antigens, leading to acute graft rejection. In contrast, self-MHC restricted indirect allorecognition (recipients own APC's) is oligoclonal and generally limited to few dominant allodeterminants, which, however may alter with time. The direct type response diminishes with time, whereas indirect alloresponses persist and seem to correlate with the chronic rejection process [52, 53].
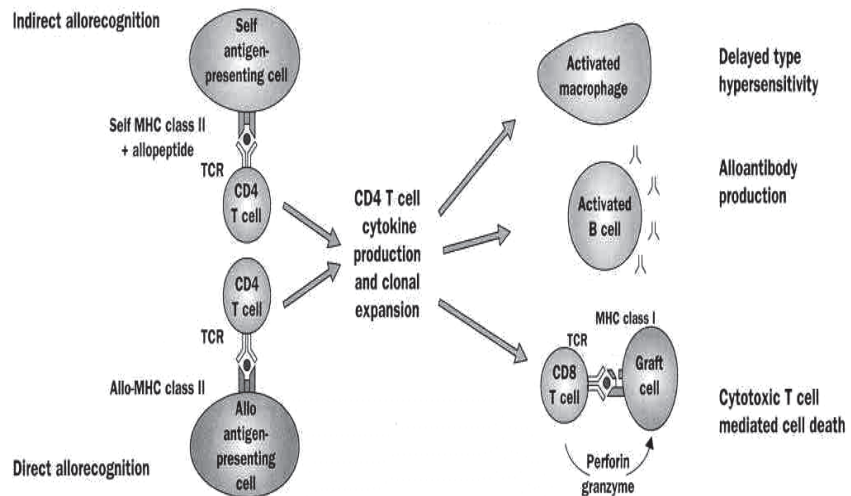
Figure 1. CD4+ T-cells recognize antigen through direct and indirect pathways, become activated, and undergo clonal proliferation. Activated CD4+ T-cells provide help for monocyte/macrophages, B-cells, and cytotoxic CD8+ T-cells by secreting cytokines and by cell-cell contact dependent mechanisms. Activated monocytes/macrophages release a range of noxious agents that mediate tissue injury. B-cell alloantibody production ultimately results in complement mediated tissue destruction. Activated CD8+ T cells kill graft cells in antigen-specific manner through induction of apoptosis and cell lysis. (Adapted from Denton et al [*54*]).

5.2.2 Mechanisms of allograft rejection

The anti-allograft response is contingent on the coordinated action of alloreactive T-cells and APCs, achieved through an elaborate network of cell surface receptor – ligand interactions. Naive lymphocytes are not programmed for a particular effector response nor do they recognize soluble forms of antigens. The initiation of rejection requires that foreign antigens are presented in association with MHC molecules on the surface of APCs, including macrophages, activated B-cells and the professional APCs, dendritic cells. Through the release of cytokines and cell-to-cell interactions, a diverse assembly of CD4+ helper T-cells, CD8+ cytotoxic T-cells, antibody-forming B-cells, and other proinflammatory leucocytes are recruited into the response. The repertoire of T-cells involved in allorecognition include CD4+ T-cells, which recognize donor MHC-II via the direct pathway, and those that are sensitized indirectly by donor peptides bound to self-MHC-II on recipient APCs. Some CD8+ T-cells directly recognize donor MHC-I peptides while another subset is cross-presented of processed antigens by recipient APCs in the context of MHC-I peptides [*55*]. Each T-lymphocyte clone has a unique TCR, which confers the cell the capability of binding to suitable ligand or antigen in

a MCH-specific manner. TCR is also bound to the CD3 surface protein, which initiates the signal transduction cascade after TCR-MHC peptide interaction [56]. The APC – T-cell interaction does not always result in T-cell activation, but costimulation by a class of cell surface molecules with no independent stimulatory capacity is required to allow full activation of naive lymphocytes [57, 58, 59]. The most significant costimulatory signals are the B7 – CD28 and CD40 – CD154 interactions [60].

Three potential effector mechanism have been implicated in allograft rejection: the production of cytokines and cytotoxic enzymes by CD4+ helper T-cells (Th) and CD8+ cytolytic T-cells (CTL), respectively, and promotion of production of alloreactive antibodies. CD4+ T-cells can contribute to rejection by providing signals (e.g. interleukin-2) that promote CTL activity of CD8+ T-cells, or by activating dendritic cells to promote CTL differentiation. They also provide signals that promote differentiation and activation of alloantibody-producing B-cells, or activation of antigen-independent effector leucocytes (delayed-type hypersensitivity reaction). Activated Th-cells can be segregated into Th1 and Th2 on the basis of their cytokine secretion. Interferon -γ (IFN-γ) and lymphotoxin are characteristic of Th1-cells, which enhance cell-mediated immunity, delayed-type hypersensitivity reactions, and auto-immune diseases. Typical cytokines of Th2-cells are interleukin (IL) -4, IL-10 and IL-13, which promote humoral and allergic responses. In an oversimplified view, Th1-cells are thought to be more responsible for allograft rejection, whereas Th2-cells may cause anergy and reduce the risk of rejection. However, Th2 cytokines are not essential for prolonged graft survival, and immunity driven either by Th1 or Th2 is damaging to the graft. Activated CD8+ T-cells damage grafts primarily by direct cytolysis of parenchymal or vascular cells bearing antigens that are recognized by the TCR of CTL's. Perforin and granzyme A and B represent molecular mediators of the lytic activity, while contact-dependent activation of the FasL pathway signals apoptotic death of the target cell [61, 62]. CD8+ T-cells express chemokine receptors as well as secrete a large number of chemokines, thus recruiting other effector cells. B-cells capture soluble antigens by surface immunoglobulins and process them into peptides to be presented within the surface MHC-II molecules. Primed Th-cells recognize the MHC-II / peptide complex expressed by B-cells and provide costimulatory signals, which enable B-cell activation, proliferation and differentation [63]. Alloantibodies produced by B-cells circulate freely and gain access to graft tissue, where antibody-coated cells can be killed by the activation of the complement cascade or NK-cell mediated cytotoxicity [64, 65].

The activation and proliferation of effector T-cells is regulated by a number of cell populations. Naturally occurring regulatory T-cells (Treg), which emerge from the thymus as a part of normal immunomaturation, constitute approximately 1–2% of the CD4+ cell population. Tregs

coexpressing CD4+CD25+ and a transcription factor FoxP3 play a crucial role in the prevention of organ-specific auto-immune disease [66]. Other regulatory cell types have also been identified, such as CD8+CD25+ and CD8+CD28- T-cells. Although the mechanisms of Tregs is only partly understood, it has become evident that these cells not only attenuate autoimmune phenomena and suppress tumor growth but also play a pivotal role in tolerance towards alloantigens [67, 68].

5.2.3 Consequences of allograft rejection

The terms acute and chronic rejection describe distinct clinical manifestations of the underlying rejection process. Anti-donor antibodies present at the time of TX may trigger immediate, hyper-acute rejection, which is a well-recognized, devastating antibody-mediated transplant injury. This form of graft failure can be largely avoided by pre-TX assessment of ABO blood group and anti-HLA antibodies and cross matching.

The immunopathologic injury in acute rejection (AR) is caused by T-cells (T-cell-mediated rejection) and antibodies (humoral rejection), either alone or together. Acute cellular rejection typically appears during the first 1–6 weeks after TX but may occur at any time, even after many years. T-cells infiltrate the tubulo-interstitium, glomeruli and arteries, separately or together. The most common form of cellular AR is tubulo-interstitial rejection, where T-lymphocytes accumulate in the peritubular capillaries and in the interstitium causing edema, and infiltrate the tubule walls (tubulitis). This results in epithelial cell damage and may disrupt the tubular walls. In cell-mediated arterial rejection T-lymphocytes accompanied by other leucocytes accumulate in arteries and arterioles undermining the endothelium. Arterial cell-mediated rejection may accompany tubulo-interstitial AR making the prognosis more ominous [69]. Glomerular inflammation and cellular damage caused by lymphocyte and monocyte infiltration (acute allograft glomerulopathy) is a very infrequent but severe form of cell-mediated rejection, which may be found in the absence of tubulo-interstitial AR. In acute humoral rejection antibodies are directed against endothelial cells of arteries or peritubular capillaries. In humoral arterial AR neutrophils, eosinophils and monocytes infiltrate the arterial wall causing inflammation and fibrin formation, hemorrhage and parenchymal infarction commonly ensue. This type of rejection is uncommon and associated with poor graft prognosis. Peritubular capillary form of humoral AR may coexist with tubulo-interstitial AR. The findings vary from peritubular capillary inflammation to acute tubular cell injury or necrosis. A stable breakdown product of complement component C4, C4d, binds to the site of rejection and is a characteristic finding in this type of rejection.

Chronic allograft nephropathy (CAN) is an insidious process, characterized morphologically by varying degrees of arterial and glomerular

lesions, and significant tubular atrophy with interstitial fibrosis. Arteries display intimal thickening with fibrosis, accumulation of macrophages and foam cells, and calcification. The glomerular changes are characterized by increase in mesangial matrix and cellularity, and double-contoured capillary walls. CAN gradually matures and may not dissipate over time, but results in deterioration of graft function over years, and responds poorly to non-specific immunosuppressive treatment. In addition to chronic rejection, other factors may compound the picture (e.g. viral infections, drug-induced injury) and all the insults collectively determine the onset and tempo of CAN.

5.2.4 Classification of renal allograft histopathology
From a practical point of view, standardization of allograft biopsy interpretation is necessary. The histologic criteria and grading of severity of acute and chronic rejection in renal biopsy specimen were defined as an international consensus statement in the Banff '97 classification [70], and updated thereafter [71, 72].

5.2.4.1 Banff classification of acute rejection
Tubilitis and vasculitis are the cardinal features of rejection. Grading of non-atrophic tubules according to number of cells per cross section ranges from t0 with no mononuclear cells in tubules to t3 with >10 cells per section. Inflammatory tubular injury and basement membrane destruction may be present in t3. Diagnosis of tubulitis requires it to be present in more than one focus in the biopsy, and that the most inflamed areas and tubules are sought. Likewise, in grading of arteritis, the focus should be in the most severely involved vessels. Grading ranges from v0 with no lymphocytic inflammation to v3 with transmural arteritis and/or fibrinoid change and smooth muscle necrosis, with accompanying inflammation in the vessel. Interstitial hemorrhage and/or infarction are marked with an asterisk added to the score. While not an independent criterion for rejection, a background interstitial inflammation is required for the diagnosis of tubulointerstitial rejection. Grading ranges from i0 with no inflammation to i3 with greater than 50% of the parenchyma infiltrated with T-lymphocytes and monocytes/macrophages. Remarkable numbers of other cell types are marked with an asterisk, and should evoke differential diagnoses. Glomerulitis is defined by mononuclear cell infiltrate and endothelial cell enlargement. Although not used as criterion for rejection, glomerulitis is graded from g0 with normal glomeruli to g3 with mostly global (>75%) glomerulitis. Types of acute rejection are categorized as Type I, tubulointerstitial rejection without arteritis, and Type II, intimal arteritis, and Type III, severe vascular rejection. Mild tubulitis with only mild focal interstitial inflammation is categorized as borderline rejection. The Banff '97 classification for acute rejection is summarized in Table 2.

5.2.4.2 Banff classification of chronic allograft nephropathy

Chronic changes in a renal allograft biopsy may be seen in glomeruli, interstitium, tubules and vessels. Interstitial fibrosis and tubular atrophy are non-specific findings, that are graded in the Banff '97 classification based on the percentage of parenchyma involved. Fibrosis is graded from ci0 with <5% to ci3 with >50% in cortical area, and tubular atrophy from ct0 with no findings to ct3 with atrophy in >50% of the area of cortical tubules. As a specific sign of transplant glomerulopathy, the presence of double contours in capillary loops, created by mesangial interposition, is graded from cg0 with <10% to cg3 with >50% of peripheral capillary loops affected. As a less specific finding, increase in mesangial matrix between adjacent glomerular capillaries is graded from mm0 with no matrix increase to mm3 with >50% of glomeruli affected. Vascular changes include disruptions of the elastica, inflammatory cells and proliferation of myofibroblasts in the intima, and formation of a second "neointima". These chronic changes are graded from cv0 with no findings to cv3 with >50% narrowing of the luminal area. Arteriolar hyaline thickening, indicative of calcineurin inhibitor toxicity, is graded separately from ah0 with no hyalinosis to ah3 with severe periodic-acid-Schiff –positive thickening in many arterioles. Tubular cell injury with isometric vacuolization may also be present in CNI toxicity. CAN is categorized as CI mild, CII moderate or CIII severe (Table 2).

Recently, accurate diagnosis of the underlying processes of chronic allograft dysfunction have been emphasized, and also the use of term CAN has bee questioned [72]. Chronic conditions such as hypertension, calcineurin inhibitor toxicicty, obstructive nephropathy, pyelonephritis or viral infections, diabetes and glomerular or vascular disease (recurrent or de novo) result in interstitial fibrosis and tubular atrophy, but also to recognizable morphological findings, and require specific therapies. CNI toxicity may occur acutely after TX and manifest in declining graft function. This is, however often reversible after modification of therapy. Chronic CNI toxicity may be more difficult to distinguish from other forms of chronic damage, and it may coexist with rejection and other chronic changes. It is also less responsive to dose reduction. Chronic alloimmune injury is an important cause of fibrosis and tubular atrophy in the graft. Recent data on circulating anti-donor antibodies and capillary-endothelial C4d deposits indicates a pathogenic role of humoral immunity in patients with chronic allograft dysfunction. The diagnostic criteria for identification of antibody-mediated rejection have been defined [71] and the diagnostic categories for renal allograft biopsies updated in the Banff '05 meeting report [72].

Table 2. The Banff '97 working classification of renal allograft pathology

Histopathological findings	Category
Acute rejection	
Suspicious for acute rejection: mild tubulitis and interstitial inflammation, no arteritis - t1 and at least i1	Borderline
Tubulointerstitial: significant interstitial infiltration and moderate tubulitis - t2 and at least i2	IA
Tubulointerstitial: significant interstitial infiltration and severe tubulitis - t3 and at least i2	IB
Vascular: mild to moderate intimal arteritis - v1	IIA
Vascular: severe intimal arteritis (>25% luminal area) - v2	IIB
Vascular: transmural arteritis and/or fibrinoid change and necrosis of medial smooth muscle cells - v3, lymphocytic inflammation	III
Chronic allograft nephropathy*	
Mild interstitial fibrosis and tubular atrophy - ci1 and ct1	I
Moderate interstitial fibrosis and tubular atrophy - ci2 and ct2	II
Severe interstitial fibrosis and tubular atrophy and tubular loss - ci3 and ct3	III

* Grading may be modified by "a" no changes suggestive of chronic rejection or "b" specific changes strongly suggestive of chronic rejection present.

5.2.5 Histocompatibility

HLA compatibility affects transplant immunity in several ways. Humoral immunity against HLA antigens is one major risk factor for chronic rejection [73, 74]. Direct recognition of HLA antigens on the surface of donor APCs results in strong T-cell response. This type of reactivity is extinguished with time, whereas indirect alloreactivity can be long-lasting due to the continuous supply of HLA antigens by the in situ transplant. The indirect mechanism may contribute to the development of chronic rejection [75]. Although HLA matching is beneficial in clinical TX, the enormous polymorphism of the HLA system makes it impossible to find a HLA identical unrelated donor. As the genes encoding for the HLA molecules are clustered and often inherited as a fixed haplotype, the chance to find a completely HLA-identical family donor is about 25%. However, it is clear that most patients will be transplanted a graft from a

HLA mismatched donor. To improve graft survival and enable tapering of immunosuppressive treatment, it is important to minimize the degree of HLA incompatibility.

Tissue typing for kidney TX includes HLA and ABO matching, serum screening for HLA antibodies and cross-matching with donor cells. HLA antigens coded by loci HLA-A, -B, and –DR are commonly considered in clinical matching protocols. In Finland, a maximum of three mismatches, with no more than two in HLA – A and – B, and no more than on in –DR loci is accepted. Transplants with zero ABDR mismatches have the best graft survival rates [76, 77]. However, many of these transplants fail and many ABDR mismatched transplants have good long-term function, reflecting the inadequacy of merely counting the mismatched HLA-A, -B, and –DR antigens. The immunogenity of HLA mismatches may differ, and certain "acceptable" mismatches are hardly recognized by the immune system of the recipient, while others are highly immunogenic in patients with some HLA phenotypes [78, 79]. Recent studies have revealed that anti-HLA antibodies participate in chronic rejection process and are an important risk-factor for long-term graft function [80, 81].

5.3 Immunosuppression

Prevention of acute rejection remained a major challenge for successful organ transplantation until the late 1970s and early 1980s when cyclosporine A (CsA) was introduced in clinical TX [82]. Today, the short- and medium-term results are impressive while the long-term graft survival remains a challenge, predominantly due to CAN. Currently a wide spectrum of different immunosuppressive drug schedules aimed at preventing or reversing rejection are available. The side-effects (e.g. nephrotoxicity, hypertension, hyperlipidemia) of some immunosuppressive agents have been implicated in the pathogenesis of CAN. Moreover, current immunosuppressive agents lack specificity, i.e. reduction in immune responsiveness to the allograft is reflected in reduced immunity to infection or malignant disease. In order to minimize the side effects of any single drug while maintaining adequate immunosuppression, combination therapy targeting at multiple steps of T-cell activation is essential (Figure 2). Immunosuppressive protocols consist of initial and maintenance therapies to prevent rejection, and short-course therapies to treat episodes of acute rejection. The maintenance immunosuppresive drugs may be categorized according to their mechanisms of action as 1) calcineurin inhibitors, 2) anti-proliferative agents, 3) glucocorticoids, and 4) TOR-inhibitors.
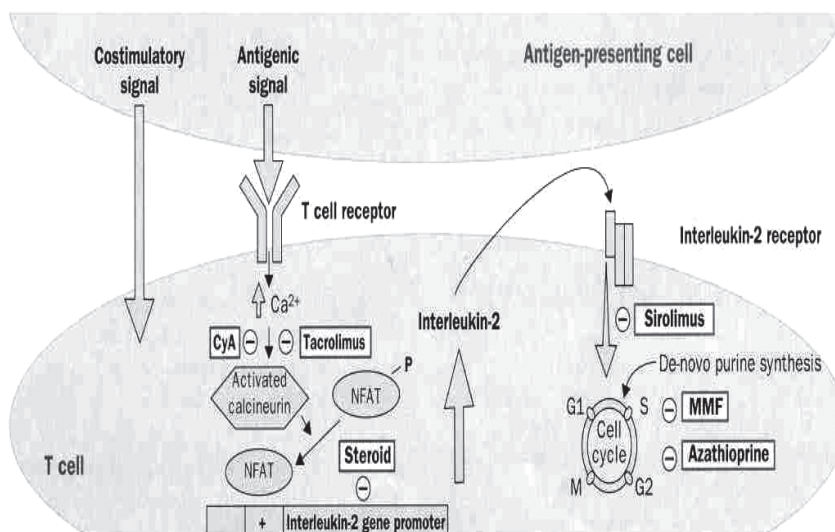
Figure 2. Multiple targets for immunosuppressive agents: Stimulation of T-cell receptor (TCR) results in calcineurin activation, a process inhibited by cyclosporine A (CyA) and tacrolimus. Calcineurin dephosphorylates NFAT (nuclear factor of activated T-cells) enabling it to enter nucleus and bind to interleukin (IL) -2 promotor. Corticosteroids inhibit cytokine gene transcription in lymphocytes and antigen-presenting cells by several mechanisms. Costimulatory signals are necessary to optimise T-cell IL-2 gene transcription, prevent T-cell anergy, and inhibit T-cell apoptosis. Experimental agents but not current immunosuppressive agents interrupt these intracellular signals. IL-2 receptor stimulation induces the cell to enter cell cycle and proliferate, a process that may be blocked by IL-2 receptor antibodies, or by sirolimus, which inhibits second messenger signals induced by IL-2 receptor ligation. Following progression into cell cycle, azathioprine and mycophenolate mofetil (MMF) interrupt DNA replication by inhibiting purine synthesis. (Adapted from Denton et al [54]).

5.3.1 Calcineurin inhibitors

CsA is the prime representative of agents inactivating intracellular calcineurin, a pivotal enzyme in T-cell receptor signaling. CsA binds to a cytoplasmic receptor, cyclophilin, and the complex inhibits the calcineurin-dependent IL-2 gene transcription during the early phase of T-cell activation, thereby inhibiting T-cell IL-2 production [83]. In the absence of IL-2, a powerful T-cell growth factor, the generation of cytotoxic T-cells is attenuated. The main target of CsA action is the Th-lymphocytes. Tacrolimus (Tac) has been developed as an alternative agent to CsA, and it has gained ground in clinical TX during the past few years [84]. Tac binds to a cytoplasmic receptor, FK-binding protein, and similarly to CsA, inactivates calcineurin. However, Tac is a more potent immunosuppressant than CsA, presumably due to a greater affinity to calcineurin [85].

Several important adverse effects are related to the therapeutic use of calcineurin inhibitors. Known adverse reactions are similar for both CsA and Tac, although the exact balance differs between the two [54, 86]. They

are roughly equivalent in nephrotoxicity, which may occur acutely after TX, or chronically over time. Acute nephrotoxic effects occur secondary to intrarenal vasoconstriction and may exacerbate ATN. The acute effects are reversible and respond to lowering of the dose. The permanent chronic nephrotoxic effects are propably a sequela of persistent vasoconstriction and ischemia as well as induction of fibrinogenic growth factors [87, 88]. These effects are characterized histologically by obliterative vasculopathy and interstitial fibrosis. Hypertension and hyperlipidemia are frequent findings in CsA treated patients, whereas diabetes mellitus and neurotoxic reactions are more common in patients receiving Tac. Hirsutism and gingival hyperplasia are usually related to CsA treatment [89].

5.3.1.1 Cyclosporine A pharmacokinetics

CsA is a drug of narrow therapeutic window with broad inter- and intra-individual pharmacokinetic variability [90]. Moreover, pharmacokinetics of CsA in children differ from that in adults, for example CsA metabolism is faster in young children [91]. CsA is metabolized via the 3A4 isoentzyme of cytochrome P450 (CYP) in the liver, and concomitant therapy with inducers or inhibitors of CYP3A4 may result in decreased or elevated blood CsA concentration [92]. The conventional formulation of CsA (Sandimmun®) exhibits considerable variability in bioavailability, whereas the more recent microemulsion formulation (Neoral®) shows more uniform intestinal absorption and greater bioavailability [93]. Albeit the microemulsion formulation has replaced the conventional formulation in clinical use, there remain considerable differences in bioavailability and clearance of CsA, especially in young children [94, 95]. Since serious clinical consequences may occur as a result of under- or overdosing of CsA, individualized dosing and therapeutic drug monitoring is necessary [96, 97].

Area under the concentration – time curve (AUC) reveals systemic exposure of a drug, but it is an inconvenient method for routine monitoring due to multiple sample collection requirements. CsA monitoring has been traditionally based on pre-dose (trough) blood concentrations, but poor correlation between blood CsA trough level (C0) and AUC [98, 99] has undermined the appropriateness of C0 monitoring in clinical practice. Pharmacokinetic studies have shown that the greatest intra- and inter-patient variability in CsA absorption occurs during the first 4 hours after dosing, and this absorption phase is crucial in determining the clinical outcome [99]. Moreover, the greatest calcineurin inhibition and the maximum inhibition of IL-2 production by CsA appear to occur during the first 1–2 hours after dosing [100]. Several limited sampling strategies have been proposed to predict the full-scale AUC of CsA, although none of them has gained popularity in clinical practice [101]. Instead, the two-hour post-dose concentration (C2) has become widely accepted as a single-point

estimate of CsA exposure. C2 correlates well with AUC0-4 hours [*102*], and adjustment of CsA dosing based on C2 monitoring appears clinically feasible [*103*]. In adults, adequate C2 levels are associated with reduced risk of AR [*104–106*], as well as reduced toxicity [*107*], and a target level has been defined at 1500 μg/L in the immediate post-TX period, tapering down to 800 μg/L after twelve months [*103, 108*]. C2 has proved a reliable surrogate marker for CsA AUC in children as well [*109–114*]. Similar concentrations to adults have been reported to relate to freedom from AR [*94, 113*], although conclusive target levels for C2 are yet to be defined in children [*115, 116*]. Furthermore, C2 monitoring may have some disadvantages. The steep slope of the concentration – time curve during the first four hours post-dose necessitates punctual sample collection in C2 monitoring with no more than 15 minutes error tolerance [*108*], which requires education of the patients and the medical staff. Secondly, C2 monitoring alone may not be sufficient to identify fast and slow absorbers and these patients may thus be predisposed to AR or toxicity. The individual variability in CsA AUC is schematically illustrated in Figure 3.

The individually variable pharmacokinetic parameters of CsA can be estimated by performing a pre-transplantation pharmacokinetic study for each patient [*91*]. The pharmacokinetic profile obtained in such a study may help to identify the patients who need very high or low doses of CsA, e.g. genetically fast or slow metabolizers, or poor absorbers. Also, young children may require two to three fold larger doses than school-aged or older children. The pharmacokinetic profile may be utilized to calculate individual dosing recommendations, aiming at a pre-defined target blood concentration. However, the profile is based on a single dose in the pre-transplantation state, and the calculated recommendations cannot be more than indicative of the individual doses needed after TX.
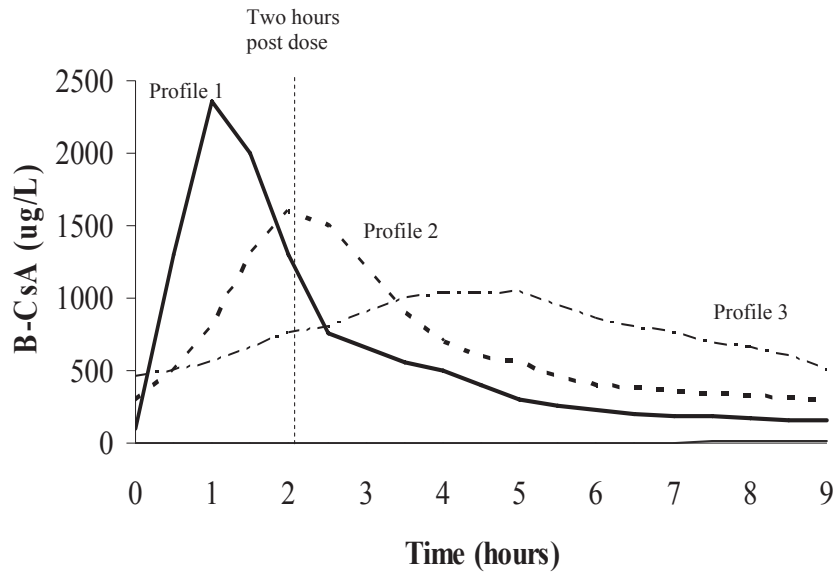
Figure 3. Examples of three potential CsA pharmacokinetic profiles after oral administration, illustrating very different C2, C0, and time of maximum concentration. Profile 2 is the most common, but other types of pharmacokinetic profiles are also encountered.

5.3.1.2 Tacrolimus

Tac shares with CsA the drawback of having a narrow therapeutic window. It also shows considerable intra- and inter-patient variability in its pharmacokinetics. As a substrate for the CYP3A enzymes and P-glycoprotein, tacrolimus metabolism may be influenced by concomitant use of inducers or inhibitors of the same mechanisms [117]. Several factors have been reported to influence the pharmacokinetics of tacrolimus, e.g. organ transplanted, hepatic function, patient age, ethnicity, time after TX, and corticosteroid dosage [118]. As a result, individualized dosing and drug level monitoring is required. Traditionally, Tac monitoring has been based on the trough level. In adults, the trough level has been shown to correlate with AUC as well as clinical outcome, although recent studies have questioned the reliance on trough monitoring [118].

Tac has become a potent alternative to CsA in pediatric recipients of liver or kidney transplants over the past decade [30, 119, 120]. Pharmacokinetic characteristics of Tac observed in adults may not be fully applicable to pediatric patients, and dosing requirements may thus be different. Young children clearly require higher doses than older children and adolescents [121–124]. In addition, large interindividual variability and poor correlation of drug exposure with trough levels has been observed in children receiving Tac [124].

5.3.2 Anti-proliferative agents

Antiproliferative agents prevent the expansion of alloactivated T- and B-cell clones. The prototype of antiproliferative agents is the purine analogue, azathioprine, which has been used as an immunosuppressive since 1960s'. AZA is converted in the liver into the active metabolite 6-mercaptopurine, and it inhibits DNA synthesis. The principal adverse effect is bone-marrow suppression, but AZA has been also implicated in liver damage and in the development of pancreatitis. However, AZA is usually well tolerated at the low doses (1–2 mg/kg/d) used in combined therapy. AZA is not effective in the treatment of an ongoing rejection. The efficacy of the drug correlates with the tissue concentration and monitoring of blood or serum levels is thus not useful for AZA.

Mycophenolic acid, which first became available as a prodrug formulation, mycophenolate mofetil (MMF), and later as an enteric-coated mycophenolate sodium formulation, is selective inhibitor of the de novo pathway of purine biosynthesis, thereby providing more specific and potent inhibition of T- and B-cell proliferation [125]. When used for maintenance immunosuppression in combination with calcineurin inhibitors, MMF appears more effective than AZA in preventing acute rejections [126]. Utilization of MMF in pediatric renal TX patients has increased markedly during the past decade [30]. The main side effects relating to MMF use are gastrointestinal disorders and to a lesser extent, myelotoxicity. In children, diarrhea, leukopenia, and anemia are the most common causes leading to dose reduction or interruption of MMF therapy [127]. There is considerable variability in the pharmacokinetics of MMF both within and between transplant patients [128]. In children, dosing is generally based on the body surface area (normally 1200 mg/m^2/d for MMF) and trough concentration monitoring is recommended, although the optimal drug monitoring strategy for MMF in children is unclear [127].

5.3.3 Glucocorticoids

Glucocorticoids (GCs) are non-specific anti-inflammatory agents. According to the classical model of GC action, the effects are mediated through glucocorticoid receptor (GR), a cytosolic ligand-activated transcription factor, belonging to the nuclear receptor superfamily [129]. The receptor-steroid complex translocates to the nucleus where it binds to steroid response elements in the promoters of a large number of genes, and either activates or represses gene expression [130]. GCs inhibit the production of several cytokines and growth factors by APCs, T-cells and macrophages, thereby disrupting antigen presentation, T-cell activation and macrophage-mediated tissue injury [131, 132]. They also inhibit vasodilatation and decrease vascular permeability. The actions of GCs at the cellular level are immensely complex, but in sum, the effects are highly immunosuppresive. GCs have numerous well recognized adverse effects including infection, adrenal suppression, hypertension, hyperlipidemia,

growth suppression, glucose intolerance and diabetes mellitus, osteoporosis, cataracts, acne, cushingoid appearance, weight gain and changes in mood or behavior [133].

High-dose course of GC treatment is used perioperatively in organ TX, and is the first-line therapy for AR [134]. In maintenance immunosuppression, GCs are commonly used in combination with antiproliferative agents and/or calcineurin inhibitors. According to the NAPRTCS database over 90% of children receive GC therapy after renal TX [30]. Prednisone, prednisolone and methylprednisolone are the most frequently used preparations in clinical transplantation. The anti-inflammatory potency of prednisone and MP is estimated to be 4 and 5 times greater than that of endogenous cortisol, respectively, with very little mineralocorticoid activity [133]. Both prednisone and MP have clinically significant growth-retarding effect, which is manifested in sub-optimal growth after TX [48]. Alternate day dosing of prednisone and MP may alleviate the adverse effects, particularly suppression of the hypothalamic-pituitary-adrenal axis [133]. Because of the far reaching adverse effects, steroid-free protocols in pediatric TX have been investigated. Although promising short-term results have been reported the long-term results remain unclear [135].

In immunosuppression protocols, dosing of GCs is often standardized, or based on weight, or less frequently on body surface area. GC pharmacokinetics, however, exhibit a broad range of interindividual variability, which may contribute to differences in immunosuppressive efficacy and occurrence of adverse effects [136, 137]. In pediatric TX patients MP exposure, instead of dose, has been shown to correlate with growth retardation and adrenal suppression [138]. Factors influencing GC pharmacokinetics include age, gender, obesity, and drug interactions. Considering the extensive use of this relatively old class of drugs, remarkably few studies on the pharmacokinetics of GCs in children have been conducted.

5.3.4 Target of rapamycin (TOR) -inhibitors

Rapamycin (sirolimus) was first investigated for antifungal properties, but was later (in 1988) discovered to possess immunosuppressive properties. Rapamycin inhibits TOR, a cytosolic enzyme that regulates differentiation and proliferation of lymphocytes. TOR-inhibitors may be important in long-term immunosuppression as they stimulate T-cell apoptosis and inhibit mesenchymal proliferation. TOR-inhibitors may be used in combination with other immunosuppressive drugs as the mechanism of action is different. The pharmacokinetics of rapamycin in children differs from that in adults [139], and blood level monitoring appears useful [140]. The major adverse effects of rapamycin are hyperlipidemia, thrombocytopenia and leucopenia [141]. Everolimus is a derivative of rapamycin and works similarly as an inhibitor of TOR.

5.3.5 Induction immunosuppression

The risk of graft rejection is highest in the immediate post-TX period. Immunosuppression is, therefore, initiated with higher doses of maintenance immunosuppresives, often accompanied with induction antibody therapy. Induction antibody therapy may involve a short course of potent anti-T-cell antibody preparation (anti-CD3 antibodies, anti-thymocyte and anti-lymphocyte globulins). In the early years, a polyclonal antibody prepared from horse serum was used. A monoclonal antibody, and standardized polyclonal antibodies were subsequently employed. In the recent years the use of antibodies against more specific targets, anti-IL-2 receptor antagonists (daclizumab, basiliximab) in particular, has increased [84]. While patients at increased risk for AR may benefit from antibody therapy, it may not be without the risk of serious adverse effects [*142–144*]. IL-2 receptor antibody induction therapy, however, appears effective and well-tolerated [*145*], also in children after renal TX [146]. According to the NAPRTCS database, 48% of pediatric patients received basiliximab or daclizumab induction therapy at renal TX [30]. These agents are generally administered for a limited period after the operation, but effective IL-2 receptor blockade is achieved for several weeks, thereby covering the critical period when AR is most common [*146, 147*].

5.3.6 Novel immunosuppressants

The introduction of monoclonal IL2-receptor antibodies in the 1990s marked the emergence of novel biologic agents in transplantation. A new generation of biologic agents is also being developed for maintenance immunosuppression with the purpose of replacing calcineurin inhibitors and GCs. While the costimulatory pathway in T-cell activation is an important therapeutic area, other potential targets include interleukins and adhesion molecules. Alemtuzumab is a potential T-cell depleting monoclonal antibody, targeting the CD25 antibody expressed on lymphocytes and monocytes. It has been used in induction therapy and maintenance immunosuppression after renal TX, but increase in rejection, changes in T-cell subpopulation and risk of malignancy and infections are disturbing drawbacks. Another potential target for inhibition is the IL-15 pathway. IL-15 is a cytokine promoting antiapoptosis signals, and elevated levels of IL-15 expression have been found in rejecting grafts. Interaction of CD28 and cytotoxic lymphocyte antigen-4 (CTLA-4) with their receptors CD80 and CD86 costimulates T-cell activation. Their blockade is the focus of new promising therapies [*148*]. Several small molecules other than antibodies with immunomodulatory and immunosuppressive properties have also been developed and investigated [*149*].

5.4 Rejection and pathology of the kidney allograft

Despite the improvements in the management of immunosuppressive regimens, rejection remains a serious concern as it may lead to graft loss and patient death. AR is an important risk factor for CAN leading to deteriorating graft function [*150–152*]. In recent years there has been a continuing trend of declining frequency of AR in pediatric TX patients, which may translate into less CAN [*30, 153*]. The 12-month probability of first rejection in LRD and CAD transplantations has decreased from 54% and 69% in 1987–90 to 13% and 16% in 2003–05, respectively [*154*]. However, the use of potent immunosuppressive therapy may increase the risk of post-TX infections and lymphoproliferative disorders [*30, 155–157*]. The management of pediatric TX patients requires continuous balancing between the risks of over-immunosuppression and the consequences of graft damage due to rejection. In the future, a better understanding of rejection mechanisms may hopefully allow for better adaptation of the immunosuppressive regimen to each patient.

5.4.1 Diagnosis of acute rejection

The classic signs of acute renal allograft rejection include tenderness and swelling of the graft, decreased urine outflow and fever. In the CsA era, the clinical signs are seldom seen and AR is often suspected on a rising serum creatinine concentration. Other causes of graft dysfunction cannot be distinguished with certainty from rejection without histologic examination. A renal core biopsy and grading of the findings according to the Banff criteria [*70, 72*] is the gold standard in diagnosing rejection. In this study, the Banff '97 classification has been used.

As a less traumatic method, that may be repeated frequently without general anesthesia in children, fine-needle aspiration biopsy (FNAB) allows diagnosis and follow-up of acute cellular rejection in pediatric patients [*43, 158*]. To describe the intensity of inflammation, a total corrected increment (TCI) [*159*] and the number of lymphoblasts per preparate (blast count) are recorded, and samples with a TCI value <3 and the blast count <3 are regarded normal. FNAB samples with a TCI score of 3–5 and the blast count up to five indicate mild immunoactivation, whereas a TCI score >5 and the blast count >5 yield the diagnosis of a cytological rejection [*160–162*]. A core needle biopsy for light microscopy and immunohistochemistry is necessary when vascular or steroid-resistant rejection is suspected, or a histological evaluation of the graft is needed. FNAB is suitable for routine screening of AR during the post-TX hospitalization, and it allows early detection of immunoactivation before major clinical signs appear, as well as differentiation of other causes for the clinical signs of rejection [*43*]. However, reliable FNAB diagnostics requires expertise in interpretation of the cytological findings.

5.4.2 Treatment of acute rejection

The most common treatment of AR in pediatric kidney allograft recipients is a course of intravenous GC, usually MP with doses upto 30 mg/kg/d for 3–5 days [163]. The therapy is often followed by increased doses of oral GC for several weeks [134]. Most acute rejection episodes are reversed with GC therapy, but some remain steroid-resistant and require other therapies, such as anti-thymocyte-globulin or anti-CD3-antibodies [164]. In patients with severe humoral rejection, non-standard treatment, including plasmaphresis, cyclophosphamide and immunoglobulin administration may be attempted [165].

The reasons why an AR episode occurred should be carefully analyzed. Occurrence of AR may be indicative of too weak baseline immunosuppression. Therefore, it is reasonable to consider changes in the baseline regimen after the AR has been reversed. In case of sub-therapeutic drug concentrations, adjustment of dosing and close drug concentration monitoring is warranted. A temporary increase in GC dosing may also be appropriate. On the other hand, if a rejection occurs despite adequate immunosuppression, conversion to other drugs might be indicated, e.g. CsA can be replaced with Tac [166], and/or AZA with MMF. Non-compliance is a common situation in patients with late AR (> 1 year after TX), most noticeably in the adolescent age-group [167–169].

5.4.3 Subclinical rejection

While the current immunosuppresive regimens often suppress the clinical signs of rejection, allograft immunoactivation cannot be excluded even in cases with no graft dysfunction. A subclinical form of allograft inflammation has been characterized, with the presence of histologic findings meeting the criteria for rejection in the absence of clinical manifestations or laboratory perturbations [170]. With protocol biopsies, the incidence of subclinical rejection has been reported at 30%–45% in adult renal TX patients [170–172]. Diagnosis and subsequent treatment of subclinical rejection has been shown to result in improved outcome in terms of lower serum creatinine and less chronic rejection two years after TX [173]. However, with the use of potent early immunosuppressive therapy (Tac, MMF, prednisone, induction therapy) the incidence of subclinical rejection 3 months after TX has been reported as low as 2.6% [174]. Furthermore, in some situations the natural course of subclinical inflammation may be benign [172, 175], although it has also been reported to be a risk factor for late graft damage and loss [176, 177]. In a recent study on Finnish pediatric renal TX patients, early treatment of AR (both clinical and subclinical) was found to be related to good long-term graft function [178].

The risk that AR will go unrecognized may be even greater in children than adults. The transplantation of an adult-sized kidney into a small child creates a disproportionately large renal mass related to body mass. It is

possible that this large renal mass conceals the clinical manifestations of AR, including rise in serum creatinine [179]. Similarly to adults, cellular rejection is detected in up to 50% of pediatric patients in protocol biopsies during twelve months after TX, and is associated with the progression of chronic allograft nephropathy [180, 181]. Furthermore, serum creatinine or calculated clearance may not accurately reflect the histologic severity of allograft nephropathy in children [180].

Currently, reliable non-invasive surrogate markers of rejection do not exist, and protocol biopsy is the method to allow early diagnosis of subclinical rejection [182]. The procedure of renal core biopsy is associated with a risk of complications, e.g. serious hemorrhage [172]. The risks must be balanced with the potential diagnostic benefits, although the actual frequency of significant complications is extremely low in pediatric patients [183].

5.4.4 Chronic allograft nephropathy

The past decades have witnessed dramatic improvements in reducing AR and early allograft failures after kidney TX, but whether there has been a substantial improvement in the rate of late allograft failure remains controversial [184, 185]. Chronic rejection, or CAN, is the most common cause for graft loss in pediatric renal TX recipients, accounting for one third of the reported graft losses [154]. CAN is used to denote the pathology of fibrosis and atrophy in a progressively failing renal allograft, and it remains a main clinical challenge. This time-dependent, variable, progressive allograft damage is mediated by a combination of alloimmune, ischemic and inflammatory stimuli [186]. Calcineurin inhibitors and steroids have been implicated in the increased production of TGF-β1, a cytokine initiating proinflammatory fibroproliferative cascades, which may have an important role in the development of CAN and nephrotoxicity [187]. Chronic CNI toxicity is characterized histologically by patchy tubular atrophy with striped interstitial fibrosis, and nodular hyalinosis of arteriole walls may be seen. Although not intrinsic to CNI toxicity, glomerulosclerosis may arise as a complication to long-standing toxicity. Methods advocated for distinguishing CNI toxicity from other chronic changes include analysis of tissue collagen type, assessment of tissue mRNA for laminin β2 and TGF-β. The histological features associated with CAN are schematically illustrated in Figure 4.

5.4.5 Viral infections

Immunosuppression is a major risk factor for infection following TX. All current regimens impair the host's ability to fight infection. Viral pathogens, especially those of herpesvirus family, are a major source of morbidity and mortality. The most common viral infection is by cytomegalovirus (CMV), and it can be caused by primary infection, reactivation of latent infection or superinfection with a different strain. Primary infection, typically acquired

from an organ donor, is associated with the greatest morbidity whereas reactivation tends to result in milder disease. The use of antiviral agents (e.g. ganciclovir) as prophylaxis and treatment has greatly decreased the rate and severity of CMV disease [188]. Epstein-Barr virus (EBV) infection is associated with post-transplant lymphoproliferative disorders (PTLD), especially in pediatric patients. Symptomatic EBV infection and PTLD in particular, is more common after primary infection (usually from an organ donor). Antiviral agents and intravenous immunoglobulins have been used as preventive strategies against PTLD [188]. Many micro-organisms have been described in transplanted kidneys but currently the most common and clinically important is polyoma virus, especially the BK strain (BKV). The major clinical manifestation of BKV infection is tubulointerstitial nephritis. Early identification (e.g. screening of urine or blood for the presence of BKV DNA) and due reduction of immunosuppression is recommended to prevent progression of BKV nephropathy to irreversible interstitial fibrosis and tubular atrophy [*188*].
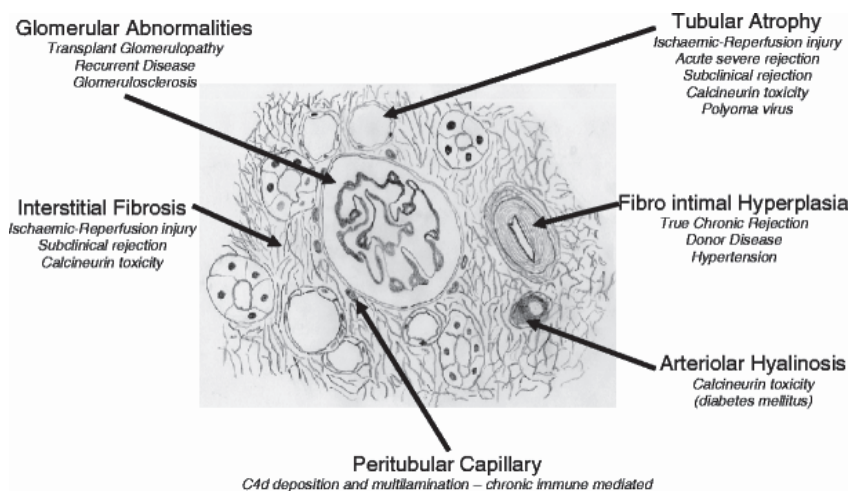
Figure 4. Schematic illustration of biopsy features of a renal allograft showing histopathological features characteristic of CAN. Italicized words indicate potential precipitating factors for CAN associated with the areas they specifically target (Adapted from Alexander et al [*189*]).

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The aggregate injury from immunologic and non-immunologic causes during the first six months after TX may be crucial in setting the fate of the kidney allograft [*190, 191*]. Surveillance biopsies obtained six months or later after TX may be very important in diagnosing CAN and predicting outcome. Due to the multiple pathophysiological causes of CAN (Figure 5), no single therapy will prevent or abrogate the injury. Ideally, individually tailored therapy should be initiated prior to, or during periods of active injury to prevent permanent nephron destruction. Recently it has become evident that the various causes leading to scarring and graft dysfunction should be categorized into more detail, particularly antibody-mediated chronic rejection should be classified as its own entity [*72*], allowing more specific therapies.
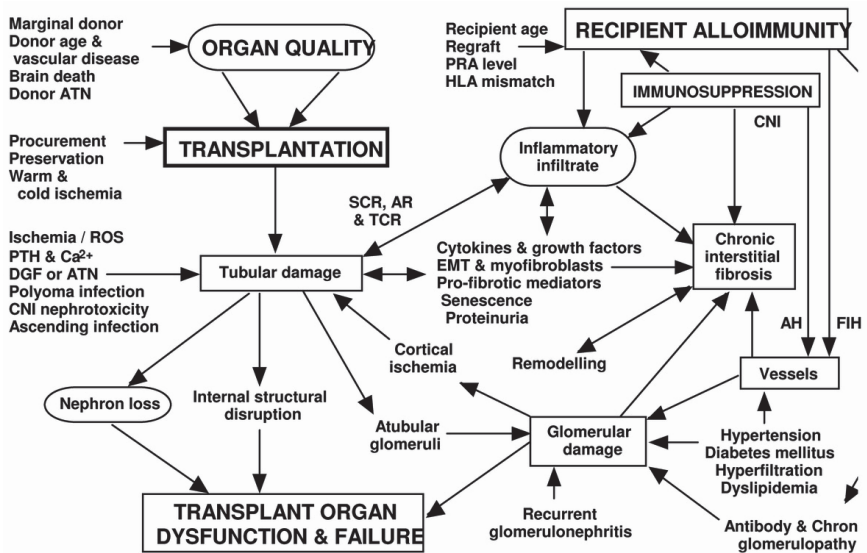
Figure 5. Pathophysiology of allograft damage. The interaction between donor quality, transplantation events and subsequent immune and nonimmune insults upon histological compartments leading to allograft damage and transplant failure. AH, arteriolar hyalinosis; AR, acute rejection; ATN, acute tubular necrosis; CNI, calcineurin inhibitors; DGF, delayed graft function; FIH, fibrointimal hyperplasia; HLA, human leukocyte antigen; PTH hyperparathyroidism, PRA, panel reactive antibodies; ROS, reactive oxygen species; SCR, subclinical rejection; TCR, true chronic rejection. (Adapted from Nankivell and Chapman [*186*]).

6. AIMS OF THE STUDY

Pediatric transplant recipients are faced with life-long, undisrupted use of immunosuppresive medication. Despite the rapid development of new immunosuppressive agents, the drugs currently in use influence the immune system in a non-specific manner, thus bearing the risks of significant adverse effects. In pediatric patients, the consequences of over- or under-immunosuppresion cast a shadow over the prospects of normal development and growth, and quality of life for decades. Many immunosuppresive protocols have been adopted from adults, although there are significant differences in the pharmacodynamics and pharmacokinetics in children. Moreover, it has become evident that an individualized approach in the care of transplant patients is a requisite for optimal outcome.

The aims of the study were to analyze whether individually tailored immunosuppression helps to reduce rejection and improve graft function, and to avoid steroid related adverse effects in children after kidney transplantation, with special emphasis on:

– Individualized cyclosporine A dosing based on pre-TX pharmacokinetic profiles.

– Diagnosis and treatment of subclinical rejection, based on early protocol biopsy and a frequent monitoring of graft function.

– Monitoring of cyclosporine A blood concentrations two hours after dose, in addition to pre-dose levels.

– Methylprednisolone exposure and its relation to the steroid-related weight-gain and growth retardation.

7. PATIENTS AND METHODS

7.1 Ethical considerations

The study design was approved by the Ethics Committee for pediatrics, adolescent medicine and psychiatry of the Hospital for Children and Adolescents, University of Helsinki. Informed consent was obtained from patients and/or parents prior to their inclusion in the study, when eligible.

7.2 Patients

All pediatric renal transplant patients were eligible for the study. A total of 178 children had received a kidney transplant by April 2006. Overlapping subgroups of these patients were included in studies I to IV. In all studies the most common single cause for renal TX was NPHS1, and the proportion of young (<2 years) recipients was approximately 30%. The average waiting time has slightly increased over the years, as has the number of CAD TX as well. Patient demographic data in the studies is presented in Table 3.

Study I (individual CsA dosing) included 65 patients, who had received their first renal transplants between 1988 and 1998. All patients had completed a pre-TX CsA pharmacokinetic study, and received CsA, MP and AZA after TX.

Study II (subclinical rejection) included 59 patients. Twenty-four patients, who received kidney transplants between 1999 and 2001, and were treated according to a revised protocol, were compared with 35 historical controls, who had received transplants between 1995 and 1999. The control group received CsA, MP and AZA after TX. The revised protocol included induction therapy with basiliximab, triple immunosuppression with CsA, MP and AZA, a renal core biopsy at 3 months, and adjustment of immunosuppression based on histology and graft function. Two patients in the revised protocol group died one and four months after TX.

Study III (C2 monitoring) included 47 patients, who received renal transplants between 2001 and 2006. The patients received induction therapy with basiliximab and triple immunosuppression with CsA, MP and AZA after TX. In addition to the conventional trough level, two-hour post-dose concentrations were utilized in CsA monitoring.

Study IV (MP exposure) included sixteen stable renal TX patients, who were scheduled for a regular follow-up visit to the hospital between September 2003 and July 2004. In compliance with the inclusion criteria for the study, all patients were over five years of age (12.9±2.8 years), at least six months time had elapsed after renal TX (7.0±3.1 years), the patients received alternate day doses of MP as a part of their maintenance immunosuppression, and they had normal adrenal function.

Table 3. Demographic data of patients included in studies I to IV

	Study I	Study II	Study III	Study IV
Number of patients (male / female)	65 (48/17)	59 (39/20)	47 (28/19)	16 (12/4)
Age at TX (mean±SD, years)	5.9±4.7	6.8±6.0	7.8±5.8	5.5±4.2
N:o (%) of patients <2 yrs at TX	20 (31)	16 (27)	12 (26)	4 (25)
Diagnosis NPHS1/other	30/35	17/42	14/30	7/9
Time on dialysis (mean±SD, years)	0.8±0.7	1.3±1.0	1.4±1.0	1.0±0.6
Donor source LRD / CAD	22/43	15/44	6/41	6/10
AB/DR mismatch (average)	n/a	1.7/0.6	1.6/0.7	1.6/0.6
Re-transplantations	0	2	1	1

7.3 METHODS

Children with a weight over 9 kg and without a major neurodevelopmental illness were accepted for transplantation. Patients with NPHS1 were nephrectomized bilaterally in order to normalize protein and coagulation status, nutrition, and sensitivity to infections prior to TX [*192*]. ABO compatibility and a negative T-cell crossmatch were prerequisites for TX. A maximum of three mismatches, with no more than two in HLA-A and -B and no more than one in –DR loci, were accepted. All LRD grafts were from a parent. The transplanted grafts were placed extraperitoneally in the right iliac fossa.

7.3.1 Immunosuppression protocol

Baseline protocol. All patients received triple immunosuppression consisting of MP, AZA and CsA. MP was started intra-operatively 100 mg i.v. divided in three doses, and continued post-operatively 1 mg/kg/day until 3 weeks when the dose was tapered down to 0.25 mg/kg/day. The dose was further reduced to 0.37 mg/kg every other day after three months. AZA was given intra-operatively 1.4 mg/kg i.v. divided in two doses, and continued post-operatively 2 mg/kg, which was reduced to 1 mg/kg/day after two weeks, and increased to 1.4 mg/kg/day after three months when the steroid was reduced. CsA was initiated pre-operatively with pharmacokinetically determined individual doses, aiming at a trough concentration 300 µg/L. Six months after TX the target concentration was reduced to 100 µg/L. CsA was administered in three doses in children younger than eight years, and in two doses in older children.

Revised protocol. The immunosuppression protocol was slightly revised in September 1999, when basiliximab induction therapy was introduced. Basiliximab was given in two doses, a bolus of 10 mg in children weighing <30 kg, and 20 mg in those weighing >30 kg, intra-operatively and on 4th day after TX. Triple immunosuppression was initiated according to the baseline protocol. After three months, immunosuppression was adjusted individually based on graft histology and function. MP dose was reduced (but not discontinued) in patients with well functioning grafts and normal histology. In cases of immunoactivation, MP was continued with daily doses. In some patients with immunoactivation (or drug related adverse effects in few patients) CsA was replaced with tacrolimus, and/or AZA with MMF.

7.3.2 Cyclosporine formulation, blood concentration and pretransplantation pharmacokinetic study

The conventional oil-based formulation of CsA (Sandimmun®) was used in standard immunosuppression until June 1995. After that oral CsA was administered as the microemulsion formulation (Sandimmun Neoral®). For historical reasons, two different analytical methods were used for determination of B-CsA. During the post-TX hospitalization, the daily B-CsA was analyzed using the FPIA technique (fluorescence polarization immunoassay, TDX, Abbott), and the results were available on the same day. In the pre-TX pharmacokinetic study, and at outpatient visits after TX, B-CsA was determined by the RIA method (specific monoclonal radioimmunnoassay, Cyclo-Trac SP Whole Blood, DiaSorin), and the results were available the next day. The hospital laboratory has investigated the differences in B-CsA levels in both adult and pediatric patients and reported 13–28% higher concentrations with the FPIA method. In pediatric kidney transplant recipients, a conversion factor 1.16 was suggested [193], and this has been used in the analyses to make the concentrations comparable. Therapeutic drug monitoring of CsA was based on trough (pre-dose) concentration solely until 2001 when two-hour post-dose concentration was included in routine monitoring. C2 was determined at 1–5 day intervals after the patient had been switched to oral administration of CsA, usually after the first 2–3 days. Even after introduction of C2 monitoring, the trough level remained as the primary monitoring parameter. C0 was targeted at 250–350 μg/L in the immediate post-TX period. After 6 months the target level for C0 was lowered to 100 μg/L. C2 concentrations 1500 -1800 μg/L early after TX, and 800–1000 μg/L after six months were considered appropriate, although these were not defined targets.

In the pre-TX pharmacokinetic study [194], CsA was given twice: as an intravenous 4-hour infusion of 3 mg/kg, and as a single oral dose of 10 mg/kg with a drug-free interval of at least 24 hours between the two administrations. The B-CsA was determined from samples taken before and at 0, 1, 2, 3, 4, 6, 9, 12, 16 and 24 hours after the oral dose, and before, in the middle, and at the end of the i.v. infusion, and 0.5, 1, 2, 3, 6, 9, 12, 16 and 24 hours after the i.v. infusion. The individual CsA dose to give target trough concentration on repetitive dosing was estimated as:

$$\text{Dose}_{i.v.} = \text{Dose}_{i.v.\ used} \times \left(\frac{C_t\ \text{target}}{C_t\ \text{observed}} \right) / f_{ss},$$

where $f_{ss} = AUC_{0-t} / AUC_{0-\infty}$ (fraction of the steady state concentration reached at time t), C_t target = target through concentration (300 μg/L), C_t observed = observed concentration, $\text{Dose}_{i.v.\ used}$ = the actual i.v. dose administered in the pre-TX study. The predicted oral doses were calculated using a similar formula, or from the i.v. doses with help of the

individual bioavailability F as: $Dose_{oral} = Dose_{i.v.} / F$. AUC was calculated using the trapezoidal method $AUC_{0-t} = \Sigma \ [(C_n+C_{n+1})^*(t_i - t_{i+1})]/2$, and $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$ (k_e = coefficient of elimination).

Three daily doses, instead of two, were recommended for individuals with fast elimination (e.g. children <8 years of age, enzyme-inducing co-medication). The trough level should equal in twice daily (BID) and three times daily (TID) dosing schemes but roughly 30% lower C2 should suffice in TID, if a uniform daily AUC exposure is the aim for both dosing schemes [195]. On the other hand, if sufficient peak concentration is the aim [100], similar C2 target concentrations may be applied in both schemes. Dose-interval AUC was approximated by two-point (C0 and C2) estimation in BID and TID patients using equations $AUC_{BID} = (9.50xC0)+(2.06xC2)+940.71$, and $AUC_{TID} = (10.80xC0)+(1.00xC2)+715.74$, respectively. In a previous study, these regression equations explained 77% and 82% of the AUC variation in Finnish pediatric renal TX patients on maintenance BID and TID dosing, respectively [195]. In this study, the diurnal AUC was estimated as AUC_{BID}^*2 and AUC_{TID}^*3 in BID and TID patients, respectively.

7.3.3 Acute rejection

Fine-needle aspiration biopsy was taken routinely on the 5th day after TX, and twice a week until the patient was discharged from the hospital. FNAB was also taken if a rejection was suspected on clinical grounds, i.e. fever, rise in serum creatinine and/or C-reactive protein concentration, tenderness of the graft, decreased urine output. In FNAB, a TCI higher than 5, and blast-cell count of at least 5 indicated AR [196] with or without fever and rise in serum creatinine concentration. In this study, AR was defined as an episode treated with MP (3 mg/kg/day) for five days or until the blast cell reaction subsided. If no response was seen after 5 days, a renal core biopsy was performed, and polyclonal antithymocyte globulin or anti-T-cell antibody was used if AR was still present.

Subclinical rejection was defined as histologic changes of the graft fulfilling the Banff '97 criteria of AR, grade IA or more, in the absence of clinical signs or laboratory perturbations. If subclinical rejection was detected in a biopsy, the patient received the standard treatment of AR, and the maintenance immunosuppression was continued with daily dosing of GCs. A follow-up biopsy was performed 1 month later. If the rejection had subsided GC was reduced to every other day dosing. In cases of severe rejection, unsatisfactory responsiveness to treatment, or declining graft function, GCs were continued with daily doses, CsA was replaced with tacrolimus and/or AZA with MMF. In case of borderline changes only, the maintenance immunosuppression was continued usually with daily administration of GCs. Borderline changes combined with declining graft function usually indicated modification of maintenance immunosuppression, and a follow-up biopsy within the next three

months. All modifications of maintenance immunosuppression were made individually based on graft histology, graft function, responsiveness to treatment and history of rejections.

7.3.4 Renal function

Serum creatinine concentration was monitored daily during post-operative hospitalization and at every control visit to the hospital after TX. Glomerular filtration rate (GFR) was measured by ^{51}Cr-EDTA clearance, corrected for a standard body surface area of 1.73m^2. GFR was routinely investigated before the patient was discharged from the hospital, and at 6 and 18 months after TX. From September 1999 onwards, GFR was investigated also at 3 and 12 months after TX.

7.3.5 Renal histopathology

Renal histology was routinely investigated 18 months after TX in all patients. From September 1999 onwards, histology was routinely investigated also at 3 months after TX. Additional biopsies were performed on clinical indications at any time. Percutaneous needle biopsies of the renal core were performed using an automated punch device. General anesthesia was used in young children, and when otherwise indicated. All the routine biopsies were examined by a pathologist on duty, and by pediatric nephrologists responsible for the treatment of the patients. In study II, the biopsies were also coded and examined by two investigators without the knowledge of kidney function or time after TX. The histologic findings were graded according to the Banff '97 criteria [70]. In addition, a more extensive scoring table was used [45] and the chronic allograft damage index (CADI) [197] was calculated.

7.3.6 Serum concentration of methylprednisolone and cortisol, and glucocorticoid bioactivity

The sixteen patients who participated in Study IV, received 0.3 mg/kg of MP orally (tablet Medrol, Pfizer, Ascoli Piceno, Italy) in the morning of the study day. MP or any other GC medication was not taken on the study day, or on the day before. All other prescribed medication was allowed to be taken normally, and food or liquid intake was not restricted. Blood samples for serum methylprednisolone (S-MP) and serum cortisol (S-cortisol) concentration, and serum glucocorticoid bioactivity (GBA) analyses were drawn on the day the patients would normally take their MP dose. Timed blood samples were drawn using an intravenous cannula before (0) and 1, 2, 3, 4, 6 and 8 hours after administration of MP. Serum was separated into two tubes, which were stored at – 70°C until analysis.

S-MP and S-cortisol were determined using ionspray-tendem mass spectrometry with the use of PE SCIEX API 300 LC/MS/MS system (Sciex Division of MDS Inc, Toronto, Canada) using dexamethasone as an

internal standard. The quantitation limit of the method was 2.5 ng/ml for both MP and cortisol.

GC effect at the target cell level (bioactivity) has been investigated by a recombinant cell bioassay, measuring GR-dependent reporter gene (luciferase) activity elicited by human serum. In the assay, mammalian cells (COS-1) were transfected with a mix of plasmids containing DNA of human GR, luciferase reporter and a steroid receptor coactivator (ARIP3). After transfection the cells were incubated with human serum, and the cell lysates were measured for ß-galactosidase and luciferase activities [198]. Glucocorticoid bioactivity (GBA) has been found to be increased in asthmatic children receiving inhaled GC therapy [198] as well as in cord plasma of preterm infants exposed to antenatal betamethasone regimen [199]. Suppression of GBA has been reported to relate to administration of mifepristone in women requesting emergency contraception [200]. Theoretically, measuring GBA may offer some advantages over the conventional methods for measuring serum steroids. For example, the different affinities of synthetic glucocorticoids to GR are revealed by GBA, and the bioassay is independent of the drug being used. In this study, serum GBA was determined using the bioassay, and the results were expressed in nmol/L (nM) cortisol equivalents.

S-MP was 0 ng/mL in all patients on the study day before administration of MP. Accordingly, GBA at time t =0 reflected the endogenous S-cortisol. After administration of MP, GBA levels accounted for both, endogenous S-cortisol and exogenous S-MP. Linear regression equation at t=0 was calculated for cortisol-induced GBA, and the equation was applied to estimate the cortisol-induced fraction of GBA (GBA$_{Cortisol}$) at t =1 – 8. GBA exceeding that caused by cortisol was calculated by subtracting GBA$_{Cortisol}$ from the measured total GBA. After ingestion of MP, most of the total GBA consisted of excess GBA, and the proportion of GBA$_{Cortisol}$ was marginal. In multiple regression analysis, S-MP was the only significant parameter explaining the total GBA. Consequently, total GBA was used in calculations, and "GBA" in text and tables refers to total GBA, unless otherwise stated.

7.3.7 Data collection
Medical records of the perioperational hospital stay (study I-IV), and of control visits thereafter (study II-IV) were reviewed. Data were collected and analyzed retrospectively in all studies. Information was collected concerning clinical and laboratory data, findings of FNAB and core needle biopsies, and medication, with special emphasis on immunosuppression.

7.3.8 Statistical analysis
The numerical results are generally expressed as mean±1 standard deviation (SD). Unpaired t-test was used for comparison of continuous parametric data. Analysis of variance (ANOVA) was used for comparison

of more than two groups. Pearson's correlation coefficient, and simple and multiple regressions were used for analysis of a linear relationship. Logistic regression was used for non-parametric data. A contingency table was used for comparison of nominal data, with either Fisher's exact test or the χ^2 (chi-square) test for more than two groups. A p-value of less than 0.05 was considered to imply statistical significance. All statistical analyses were performed using StatView by SAS Inc software.

8. RESULTS

Triple medication with CsA, AZA and MP has been the cornerstone of immunosuppression in Finnish pediatric renal TX patients for over two decades. Although the basic protocol is common for all patients, individualized adjustments are needed for optimal outcome. Individualized management of immunosuppression began in 1988 with calculation of recommended CsA doses for each patient already before TX. In order to prevent decline in GFR during the first years after TX, screening for subclinical rejection by early protocol biopsies was initiated in 1999. As it became evident that the CsA trough level monitoring may not be sufficient due to individually variable pharmacokinetics, the C2 monitoring was included in the routine management of CsA therapy in 2001. Although very useful in maintenance immunosuppression as well as treatment of AR steroids are notorious for numerous adverse effects. To explore steroid-related weight gain and growth retardation, a subgroup of pediatric renal TX patients was investigated for steroid exposure in 2004.

8.1. Individualized cyclosporine dosing (I)

Individualized CsA dosing during the post-TX hospitalization in 1988-1998 was analyzed in study I. Based on a pre-TX pharmacokinetic study, a recommended CsA dose was calculated for 65 patients (conventional formulation in 54 and microemulsion formulation in 11 patients), on an average 9.5 months before TX. In the pre-TX study, bioavailability was better in patients who received the microemulsion formulation than in those who received the conventional formulation (40 ± 13 vs. $24\pm10\%$). Accordingly, the calculated predicted dose was lower for the microemulsion than for the conventional formulation (11.8 ± 8.2 vs. 19.8 ± 10.3 mg/kg/d). The variability in the predicted doses was age-dependent, the dose recommendations being highest for the youngest patients (22.9 ± 10.4, 20.6 ± 9.6 and 10.5 ± 5.1 mg/kg/d in patients <2, 2–8 and >8 years of age, respectively; $p<0.05$).

Data on administered CsA doses was collected for three weeks after TX. The conventional formulation doses were higher than the microemulsion doses, and the youngest children received higher doses than the older ones, in concordance with the pre-TX pharmacokinetic studies. The administered doses were on the average very close to the predicted doses (Figure 6). Mean deviation from the predicted dose was 0.2, 0.2 and 1.5 mg/kg/d in patients <2, 2–8 and >8 years of age during three weeks after

TX, respectively. In multiple regression analysis the predicted dose and patient age were significant parameters explaining on the average 60% of the variability in the administered doses. The predicted dose explained the administered doses best in patients aged 2–8 years. Great variability was found in the predicted doses, especially in the youngest age group (Figure 6). The calculated predicted dose deviated more than 25% from the average in the age-group in 60% of the patients. When these patients were analyzed separately, the predicted dose explained on the average 66% of the variation in the administered doses (72% in patients <2 years of age).
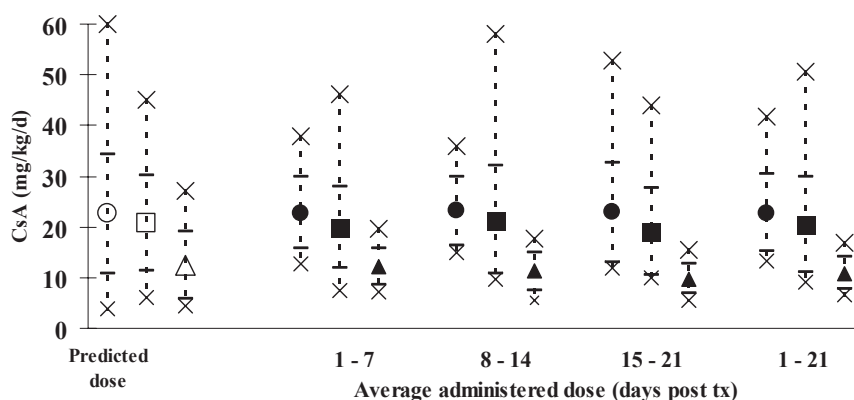
Figure 6. Predicted dose in the pre-TX pharmacokinetic study and actually administered CsA doses during 1st, 2nd, and 3rd week after renal TX in 65 pediatric patients in 1988–1999, divided into three groups according to age at TX:
< 2 years (n=20):   ○ = mean predicted dose, ● = mean administered dose.
2–8 years (n=24):   □ = mean predicted dose, ■ = mean administered dose.
>8 years (n=21):   △ = mean predicted dose, ▲ = mean administered dose.
— = ± 1 SD, X = minimum /maximum in all age-groups.

The targeted B-CsA trough levels were reached on the average after the first week post-TX. The average B-CsA was lower in patients <2 years of age than in the older ones. Also, patients who received the conventional formulation of CsA had lower B-CsA than those who received the microemulsion formulation (Table 4).

Table 4. B-CsA trough concentration§ in 65 pediatric patients three weeks after renal TX in 1988–1998.

| | Days post-TX | | | | | |
| | 1–7 | | 8–14 | | 15–21 | |
Age	Conv*	Microem†	Conv*	Microem†	Conv*	Microem†
<2	216±99	239±57	302±63	428±76	313±89	421±52
2–8	263±82	276±53	384±133	403±58	376±118	413±75
>8	282±97	372±127	375±84	419±73	336±108	386±46

§(mean± 1 SD; µg/L)
*Conventional and †Microemulsion formulation of CsA.

8.2 Subclinical rejection and graft function (II)

Graft function and histology in 24 patients treated according to the revised protocol (study group) in 1999–2001 were compared with 35 historical controls in 1995–1999. The study group and historical protocols are summarized in Table 5. One patient in the study group died one month after the TX in septic infection and multiple organ failure. Another patient in the study group died four months after TX as a result of recurrent nephrotic syndrome, severe rejection, surgical and infectious complications. None of the control patients died.

Table 5. Immunosuppression and follow-up protocol during 18 months after renal TX in study group (1999–2001) and in historical control patients (1995–1999).

	Study Group	Historical Controls
Initial immunosuppression	CsA, AZA, MP (daily) + Basiliximab induction	CsA, AZA, MP (daily)
GFR	At discharge, 3, 6, 12 and 18 months post-TX	At discharge, 6 and 18 months post-TX
Histology	3 and 18 months post-TX	18 months post-TX
Immunosuppression after 3 months	Individualized	MP (alternate day), tapering of CsA and AZA

The historical control patients experienced more early AR episodes than the patients treated according to the revised protocol (Table 6). One patient in the study group and three in the control group required dialysis early after TX. Three grafts were lost in the control group 4.5, 9 and 16 months after TX, and none in the study group (excluding the deceased patients). Twenty-three patients treated according to the revised protocol were studied for graft histology three months after TX. Signs of AR were found in 43% of the biopsies, and all but one of them were subclinical. The severity of immunoactivation was graded as borderline in three patients, and IA or more (according to Banff '97 classification) in the rest of the patients. Early AR did not correlate with the histological findings at three months.

Table 6. Early AR after renal TX in patients treated according to revised protocol (study group) in 1999–2001 and in historical control patients in 1995–1999.

	Study group (n=22)	Historical controls (n=35)
No of AR (per patient)	9 (0.38)	43 (1.23)
Number of patients with: 1 AR	7 (33%)	14 (40%)
2 or more AR	1 (4%)	12 (34%)
Average day (post-TX) of 1st AR	26*	14*
Average TCI in FNAB	1.8*	2.2*

*$p < 0.05$

Five patients with IA or more severe histological findings, and one patient with borderline changes and declining graft function three months after TX received AR treatment with MP, and continued daily dosing of maintenance MP. One patient with declining GFR was suspected for AR and received MP treatment, although graft histology proved normal. Six patients with borderline or IA changes (one patient with CI chronic changes) together with good graft function three months after TX did not receive treatment for AR but continued on daily dosing of maintenance MP. Based on the severity of the histological findings, history of AR, responsiveness to treatment, and graft function, the baseline immunosuppression was modified in six patients at three months – CsA was replaced with Tac in three, and AZA was replaced with MMF in three patients. Three more patients in the study group received other than the standard immunosuppression (e.g. cyclophosphamide, ATG). In the control group, four patients (11%) received other than the standard immunosuppression. AR was found only in a few patients 18 months after TX whereas chronic changes were more common. CAN was found more often in the control patients than in the study group at 18 months (Table 7).

Table 7. Histology according to Banff '97 classification in protocol biopsies 18 months after renal TX in study group (1999–2001) and control group (1995–1999) patients.

	Study group n =17	Control group n =30
Acute changes		
Normal	15 (88%)	26 (87%)
Borderline or more	2 (12%)	4 (13%)
Chronic changes		
Normal	12 (71%)	16 (53%)
C2	4 (24%)	12 (40%)
C2	1 (6%)	1 (3%)
C3	0	1 (3%)

Graft function was better in the study group than in the control patients. The difference increased with time and was most evident in the youngest patients (<2 years of age) (Figure 7). Serum creatinine concentrations were also significantly lower in the study than the control group (64±41 vs. 88±42 µmol/L in all patients, and 49±18 vs. 68±34 µmol/L in patients <2 years of age, respectively) 18 months after TX. However, this difference was evident already at discharge from the hospital, reflecting more delayed graft function in the control group. CAN was related to lower graft function 18 months after TX ($p=0.02$). Also, patients who experienced no rejections had better GFR at 18 months in comparison to those who were treated for rejection, although the difference was not significant.
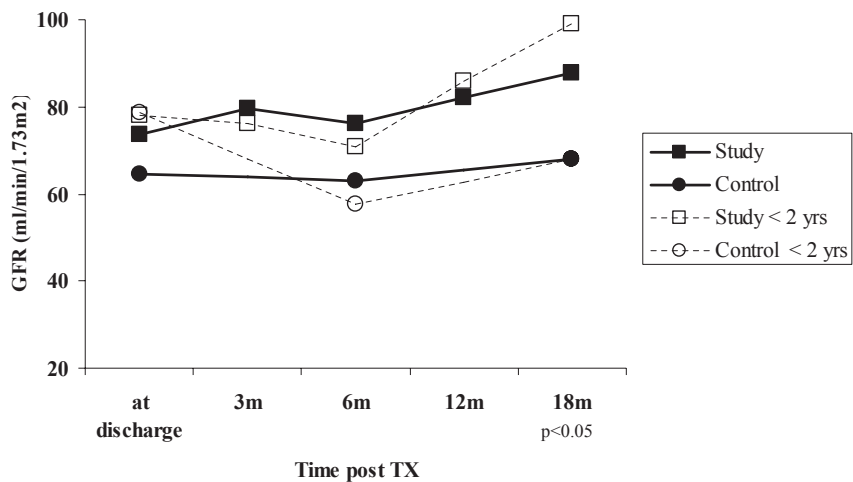
Figure 7. Glomerular filtration rate (GFR) during 18 months after renal TX in 22 study group patients in 1999–2001, and in 35 control group patients in 1995–1999. Patients under two years of age at TX are shown also separately for both groups.

8.3 Cyclosporine monitoring (III)

CsA two-hour post-dose monitoring was included in the routine protocol after renal TX in pediatric patients in 2001. Data on C2 and C0 concentrations during post-TX hospitalization were collected for 47 patients until 2006. C2 was determined on 1–5 day intervals after the patient had been switched to oral administration of CsA. CsA therapy was initiated intravenously in all patients but switched to oral administration after 2 days in 53% of the patients, and by the 5th postoperative day in 83% of the patients. Overall 547 and 916 values of C2 and C0, respectively, were available, concentrating on days 6 to 17 after TX.

Patient and graft survival was 100%, but two patients required dialysis early after TX due to delayed graft function. CsA was included in the initial immunosuppression in all patients but replaced with Tac in two patients three weeks after TX. These two patients were excluded from analyses. CsA was replaced with Tac in four more patients after discharge from hospital but before the three month control. These patients were included in the analyses covering the post-TX hospitalization period. CsA was started with a pharmacokinetically determined individual dose three times daily in all patients <8 years of age, and also in many of the older patients but switched to two daily doses before discharge from the hospital. Patients who received three or two daily doses of CsA invariably throughout their stay in hospital, were categorized as TID or BID patients, respectively. Those patients who initially received three oral doses daily, but were switched to two doses after no more than five days, were categorized as BID patients, and C2 data on the 1–5 days on TID was omitted from the analyses. Four patients were switched to BID after more than five days on TID, and were thus excluded from analyses covering the post-TX hospitalization period. Twenty-nine patients (71%) were categorized as TID and twelve patients (29%) as BID. Three months after TX, all patients ≥8 years of age were on BID, and the younger patients were on TID dosing.

Fourteen (29%) AR episodes were treated in 13 patients on the average 15 days after TX. The patients who experienced AR did not differ from those who remained rejection-free with respect to age, gender, pre-TX diagnoses or donor source (Table 8). Seven AR episodes occurred early (days 7–9) after TX and the remaining seven later (days 15–28). These AR episodes are referred as "early" and "late", respectively, in the following text and pictures. Nine AR episodes were diagnosed in TID patients, and five in BID patients (p=ns). FNAB was used routinely to diagnose AR, and a core needle biopsy was obtained in five patients (AR confirmed in 4 patients and 1 patient had normal histology). The patient with normal histology received treatment for vascular rejection, while all other AR episodes resolved after a standard treatment with MP.

Table 8. Patient characteristics in Study III shown separately for patients without acute rejection (AR) and with AR during hospitalization after renal TX.

	No AR n=34	AR n=13	p-value
Male/Female (n)	18/16	10/3	0.19
Pre-TX diagnosis (n)			0.16
NPHS1	9	5	
Polycystic kidneys	4	0	
Urethal valve	3	4	
Nephronphthisis	3	0	
Other	15	4	
Age at TX (years)	8.1±5.5	6.9±6.1	ns
< 2 years (n)	8	4	ns
CD/LRD (n)	31/3	10/3	0.33
Cold ischemia time (hours)	18.3±6.3	22.0±4.2	0.10
BID/TID (n)	7/20	5/9	ns
Re-TX (n)	1	0	-

The coefficient of variation in C2 during the post-TX hospitalization ranged from 0.17 to 0.59 from one patient to another, reflecting substantial day-to-day intra- and inter-patient volatility. No correlation between C2 and C0 was observed (R^2=0.03). C2 and C0 levels in patients who experienced AR were recorded for three days preceding the episode (pre-AR). The average pre-AR C2, C0 and diurnal abbreviated AUC was then compared with that in non-rejecting patients. Average C2, C0, and diurnal abbreviated AUC over days 5–9 and 13–17 after TX in the non-rejecting patients were used as reference levels for "early" and "late" AR, respectively. The C2 levels in the non-rejecting patients were slightly higher than the pre-AR levels in patients with "early" or "late" AR. C0 was significantly higher in rejection-free patients than the pre-AR C0 in patients with "late" AR. The pre-AR abbreviated AUC was slightly, although not significantly, lower in the "early" and "late" AR patients than in the rejection-free patients (Figure 8). In logistic regression analysis, the only significant parameter explaining AR was the pre-AR C0 level in patients with "late" AR.

8a.

8a.
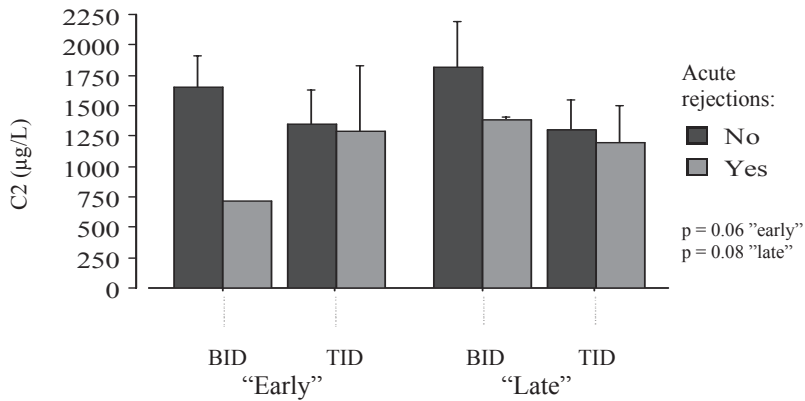
8b.
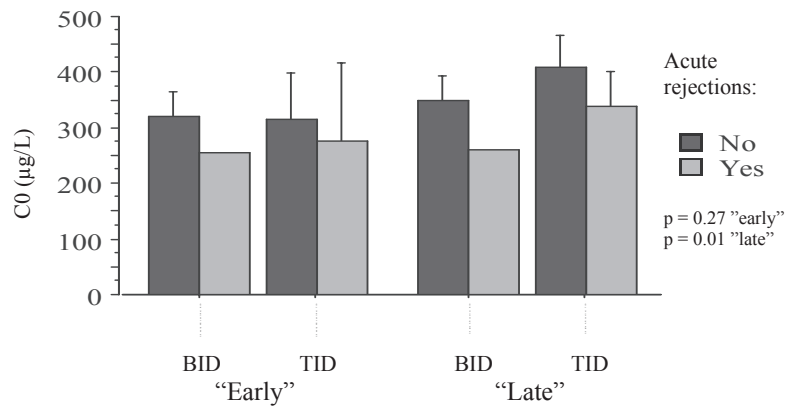
8c.
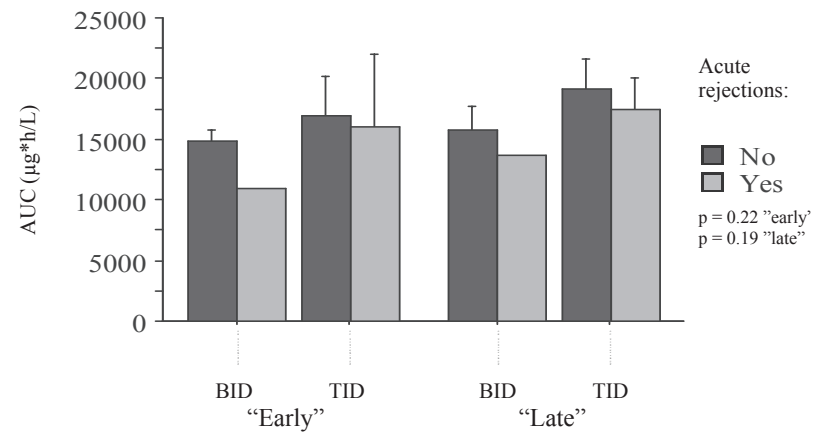
Figure 8. Average **a**. two-hour post-dose (C2), **b**. through (C0) concentration, and **c**. diurnal abbreviated AUC three days before diagnosis of "early" or "late" acute rejection (AR) compared to patients with no AR in days 5–9 and 13–17 after TX, respectively. The patients on two (BID) and three (TID) daily doses of CsA are shown separately. (The error bars denote ±1 SD)

The BID patients had higher C2 and lower C0 than the TID patients early (days 5–9) after TX (1558±385 vs. 1345±300 μg/L; p=0.09, and 313 ±47 vs. 310±91 μg/L; p=ns, respectively), and more significantly later (days 13–17) after TX, when a pharmacokinetic steady-state may be expected to be reached in most patients (1769±382 vs. 1284±250 μg/L; p<0.0001, and 340±50 vs. 396±62 μg/L; p=0.02, respectively). The diurnal abbreviated AUC was higher in TID than BID patients early (p=0.07), and more significantly later after TX (p<0.001).

C2 and C0 data was available in 40 patients three months after TX. Twenty-two (55%) of these patients had normal histology and 18 (45%) had acute rejection changes (Banff borderline or more) in the 3-month protocol biopsy. Rejection changes in the biopsy were not related to AR episodes during post-TX hospitalization. C2 levels three months after TX did not differ in patients with normal histology or rejection changes in the protocol biopsy. However, trough levels, and diurnal abbreviated AUC in patients with normal histology were slightly higher than in those with rejection (Figure 9). C2 or C0 levels during post-TX hospitalization were not related to graft histology three months after TX.

GFR at discharge from hospital was slightly but not significantly better in those patients, who did not experience acute rejections (76±34 vs. 66±23 ml/min/1.73m^2; p=0.34). In regression analysis, GFR at discharge was not related to C2 or C0 levels, or diurnal abbreviated AUC. Three months after TX, GFR was not significantly different in patients with normal histology than in those with rejection changes in protocol biopsy (66±25 vs. 64±17 ml/min/1.73m^2, respectively; p=ns). GFR was not correlated with C0 or C2 levels, or diurnal abbreviated AUC three months after TX.

9a.
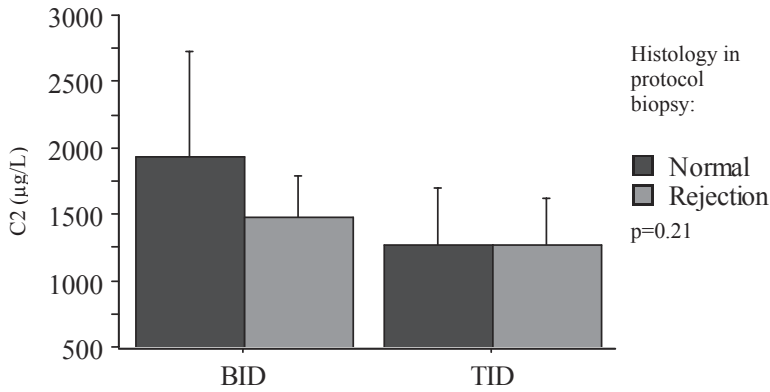
9b.
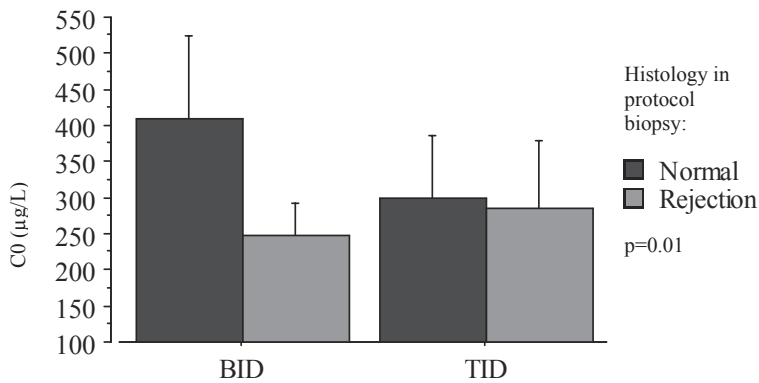
9c.
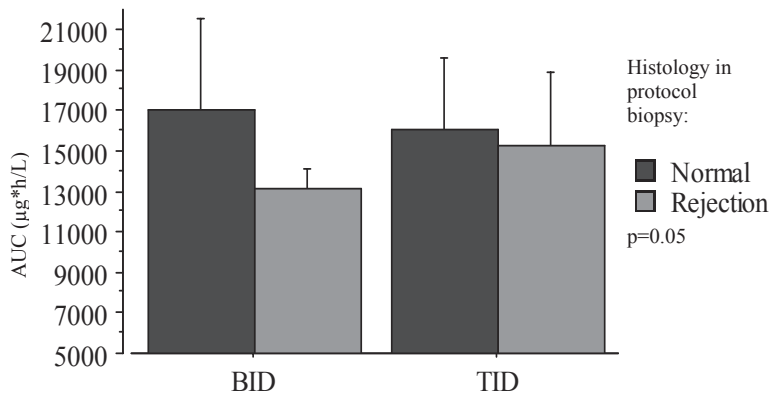
Figure 10. Cyclosporine **a**. two-hour post-dose (C2), and **b**. through (C0) concentration, and **c**. diurnal abbreviated AUC three months after TX in patients with normal histology or acute rejection changes (Banff borderline or more) in protocol biopsy. The patients on two (BID) and three (TID) daily doses of CsA are shown separately. (The error bars denote ±1 SD).

8.4 Methylprednisolone exposure and adverse effects (IV)

Eight-hour AUCs of MP, serum cortisol and serum GBA were analyzed in sixteen voluntary stable renal TX recipients, and great interindividual variability was observed (Figure 10). Suppression of endogenous cortisol secretion by administration of MP was seen as prompt decline in S-cortisol levels (Figure 10c). S-MP was linearly related with GBA (Figure 11), and the best correlation was found at six and eight hours post-dose ($R^2 = 0.73$, 79 respectively; $p < 0.001$).

10a.

10b.
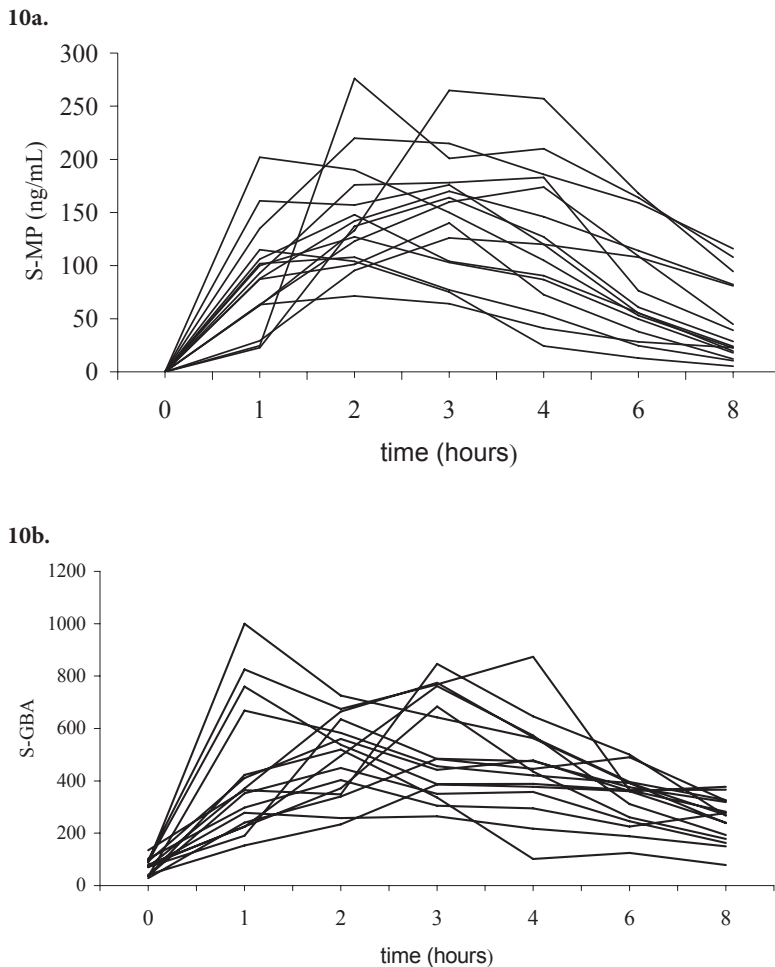
Figure 10.a. Serum methylprednisolone (S-MP), **b.** serum GBA, and **c**. serum cortisol concentration after oral dose of 0.3 mg/kg of MP in 16 stable renal TX patients.

10c.
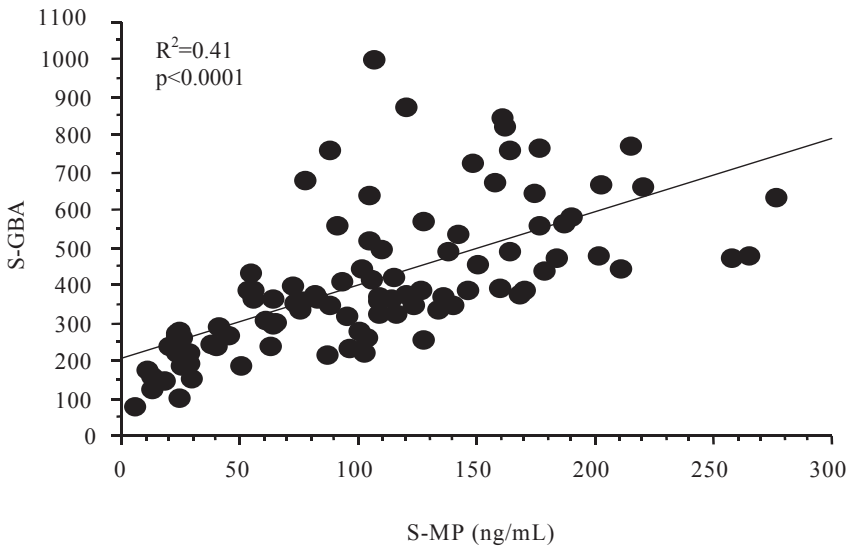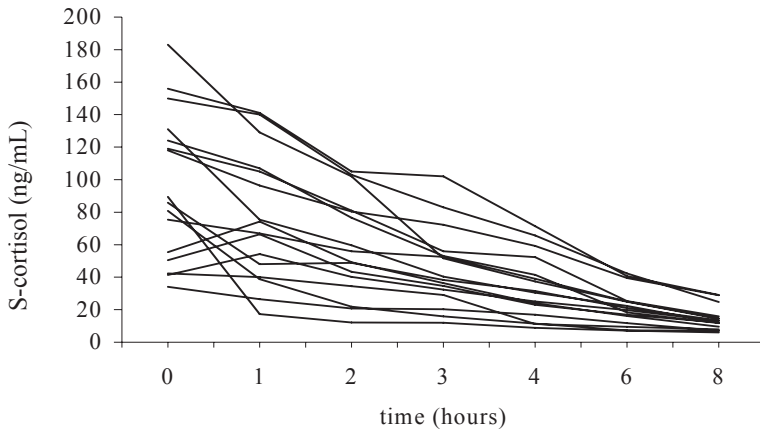
Figure 11. Linear regression of GBA and S-MP one to eight hours after oral administration of 0.3 mg/kg of MP in 16 stable renal TX patients.

At the time of the study (8.0±4.1 years after TX), all patients received low alternate day doses of maintenance MP (5.3±3.3 mg, which equals 0.13±0.04 mg/kg). The MP doses received earlier varied slightly depending on history of rejections. Twelve months after TX, the alternate day MP dose had been 0.29±0.08 mg/kg, and at discharge from hospital the daily dose had been 0.37±0.06 mg/kg. Six patients had gained excess weight (>10% increase in relative weight for height) within twelve months after TX, and three other patients later after TX. At the time of excess weight gain, all

58

patients had been >5 years of age. Average patient height at the time of the study was – 1.4±0.8 SDS, and change in height from TX to study date showed an average positive catch-up growth of 0.2±0.9 SDS.

Accelerated weight gain after TX correlated with MP-AUC (R^2 =0.63, p<0.001). GBA-AUC was not linearly related to weight gain, but GBA at t =6 hours was positively correlated with weight gain (R^2 =0.54, p=0.001). Since weight-related test-doses were used in this study, obese patients received larger doses in absolute terms than those with normal weight for height. However, the absolute MP dose was only weakly correlated with weight gain (R^2 =0.22, p=0.06), and MP dose was excluded by stepwise regression procedure in explaining obesity after TX. MP-AUC and patient age at TX remained the only significant variables (R^2 =0.84, p<0.0001). To further eliminate the potentially confounding effect of current obesity, patients were grouped according to absolute weight (in kilograms). MP-AUC and GBA-AUC were significantly higher in the obese than non-obese patients with similar weight (Figure 12). No correlation was found between early AR (when increased steroid doses were used) and weight gain after TX.

12a.
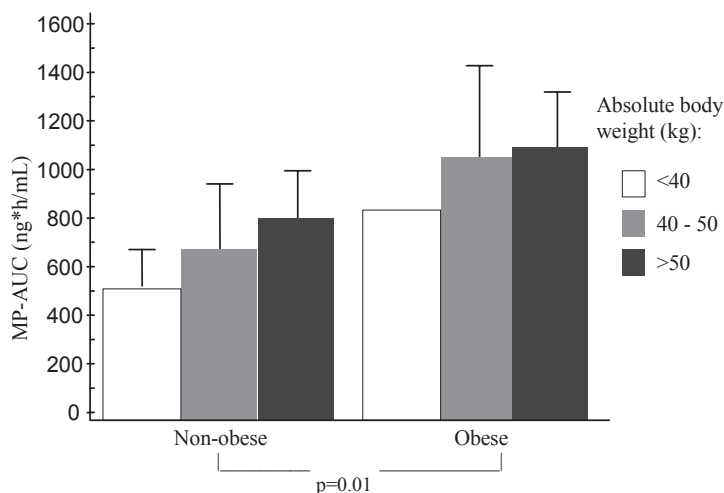
Figure 12. **a**. Methylprednisolone AUC (MP-AUC) and **b.** glucocorticoid bioactivity AUC (GBA-AUC) in the obese (weight for height >10%) and non-obese patients grouped according to absolute body mass at the time of the study.
The error bars denote ±1 SD.

12b.
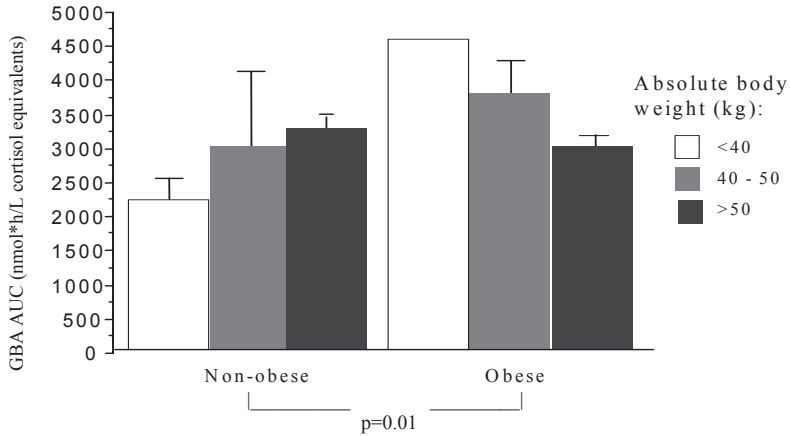
A linear relationship was observed between growth and MP-AUC (Figure 13). GBA-AUC was not linearly related to growth but GBA at $t = 6$ hours was negatively correlated with change in height ($R^2 = 0.38$, $p < 0.05$).

Blood glucose level, serum lipids, bone mineral density, serum creatinine or GFR were not linearly related to MP-AUC or GBA-AUC. Patients with normal graft histology in a recent biopsy had slightly but not significantly higher MP-AUC than those patients with rejection changes or CAN. Patients with suboptimal graft function (GFR <60 ml/min/1.73m², n =7) had higher GBA-AUC, most clearly at $t = 3–6$ hours, than those with good function ($p < 0.05$), with no difference in MP-AUC.
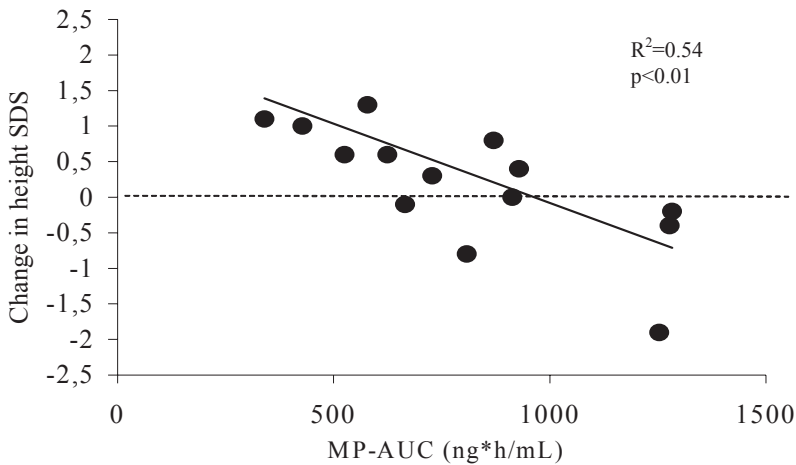
Figure 13. Linear regression of change in height (SDS) from TX to study date and methylprednisolone (MP) AUC in 14 stable renal TX patients.

9. DISCUSSION

A well-functioning kidney transplant may fully rehabilitate a patient with ESRD. However, a poorly functioning graft and intensive use of immunosuppressive medication are associated with serious morbidity and mortality. Improvements in post-TX care and the development of more potent immunosuppressive agents have led to a decrease in the incidence of AR and increase in short-term graft survival. As the potent immunosuppressive agents remain non-selective, the risk of post-TX infections and malignancies exist [155]. The goal of pediatric TX is good quality of life for decades, and therefore, careful judgment of adequate immunosuppression is required for each patient.

Etiology of ESRD in pediatric patients in Finland is heterogeneous, although the most common cause is NPHS1. Consequently, the proportion of very young children is relatively high among renal TX recipients, which may be considered a risk factor for post-TX complications and rejections. The age-dependent differences in pharmacokinetics and immune responsiveness should be incorporated in elaboration of immunosuppressive protocols. Added to that, great individual variability is inherent to many immunosuppressive agents. Triple medication with CsA, AZA and MP has remained the backbone of immunosuppression in the Finnish patients. To avoid the potential long-term adverse effects, the newest, rather potent immunosuppressive agents have not been included in the baseline protocol but used on individual basis. Pharmacokinetics, graft function, and histology are employed to assess the required level of immunosuppression in each patient.

9.1 Cyclosporine dosing and monitoring (I, III)

Even after introduction of the microemulsion formulation of CsA, great inter- and intraindividual variability is observed in CsA blood concentrations, or to put it the other way round, in dosing requirements to achieve a target concentration. Patients receiving the conventional oil-based formulation and patients receiving the microemulsion formulation of CsA were included in Study I. The numerical results must be interpreted separately for both groups, but the pharmacokinetic principles and analyses apply equally for both groups. In the pre-TX pharmacokinetic studies, the predicted doses in patients <2 years age were two-fold greater than in patients >8 years of age (Figure 6), in accordance with faster elimination of

CsA in young children [194]. The youngest patients, accordingly, received significantly higher doses of CsA after TX than the older children. Very large variability was observed in the predicted and administered doses. Patient age and the pharmacokinetically predicted dose were important determinants of the administered doses. The actually administered doses were best predicted by the recommended dose in those patients who required clearly lower or higher doses of CsA than the average patient of the same age group. Identification of these patients without a pre-TX pharmacokinetic study would be difficult. Using a standard starting dose in all patients may thus result in too low or high initial B-CsA concentration, and bear the risk of early acute rejection or CsA toxicity. However, calculation of the recommended doses is based on a trough level target, which may not be optimal in all patients. Trough level monitoring of CsA has proved insufficient in reflecting drug exposure and predicting outcome. Instead, the two-hour post-dose concentration has been shown to correlate with AUC. Currently, validation studies for C2 in children are lacking, and conclusive target levels are yet to be defined.

Abbreviated AUC based on C0 and C2, has been shown to correlate with CsA exposure [201, 202], and therefore, optimizing C0 and C2 may be expected to result in appropriate exposure. However, the true shape of an individual curve may still not be captured by the two-point estimation, thus bearing the risk of underestimation in slow absorbers and exaggeration in fast metabolizers, when aiming at uniform AUCs [203]. On the other hand, the maximum immunosuppressive effect occurring at peak concentrations, usually during the first two hours post-dose may be clinically more important than the complete exposure [100], thus emphasizing C2 as the prognostic parameter.

Young children metabolize CsA faster than school-aged and older children [91], and therefore, three daily doses instead of two, may be justified. Different dosing intervals may result in disproportionate AUC exposure with equal C2 targets for BID and TID patients. Approximately 30% lower C2-target in TID patients may be anticipated [204] when a uniform diurnal AUC is the objective. At the same time, this approach would compromise the peak concentrations in TID patients and may result in increased AR. In this study, C2 was clearly higher and C0 lower in BID than TID patients, but the diurnal abbreviated AUC was higher in TID patients. However, it may be possible that the true pharmacokinetic profiles in TID and BID patients differ in a non-linear fashion, resulting in lower than expected diurnal exposure in TID [205].

Whether sufficient peak concentration or adequate diurnal exposure is the optimal target in calcineurin inhibition –based immunosuppression is a fundamental question in designing dosing schemes. The findings in this study are suggestive that high C2 levels may be related to freedom of AR, especially during the early weeks after TX. However, the through level

appeared significant after the first two weeks, and also three months after TX. It may be hypothesized that during the early days after TX, when the supply of donor derived antigens is abundant, high peak concentrations are required to prevent AR driven by direct allorecognition mechanisms. Later after TX, when indirect mechanisms of allorecognition prevail, adequate trough level, and diurnal exposure may become more relevant to guarantee sufficient baseline immunosuppression.

CsA dosing and monitoring remains a challenge in children. The need of higher dosing in young children has become evident. In addition to patient age, the individually variable pharmacokinetic characteristics should be considered in optimizing CsA dosing. Much evidence support C2 as a good surrogate marker for CsA AUC, and sufficient C2 concentration appears to be related to less rejection. However, monitoring CsA therapy without knowledge of trough concentration may result in excessive or insufficient baseline immunosuppression [206], and potentially to consequent toxicity or rejection. The better understanding of CsA pharmacokinetics and mechanisms of action have provided guidelines for more accurate monitoring and dosing, but with some added complexity involved in the daily routines.

9.2 Subclinical rejection and graft function (II)

Graft function after renal TX in the Finnish patients has been relatively good. However, children transplanted at <2 years of age, were previously found to be at risk for insufficient increase in GFR to compensate for the growth of the child. In the youngest children the absolute GFR appeared to remain at the level reached 18 months after TX [35]. Since a significant proportion of the Finnish patients are transplanted at a very young age, there has been some reluctance to include the strongest immunosuppressants in the routine protocol in fear of the potential long-term risks of increased infections, malignancies and diabetes. In an attempt to prevent the observed reduction in graft function, the early immunosuppression was slightly enhanced and individualized in all patients 1999 onwards.

In study II the effect of the modification in protocol to AR and graft function was compared to historical control patients. The study and control groups in Study II were comparable with respect to patient age, gender, pre-TX diagnoses, cold-ischemia time, and AB/DR mismathches. However, some differences that may influence the outcome of TX were found between the groups, and could not be controlled. The study group included two re-TX patients and a greater number of patients < 2 years of age, both of which can be considered risk factors for succesfull TX. On the other hand, the number of LRD was higher in the study group, and the youngest patients received larger volumes of maintenance fluids after TX.

Although not similar, risk factors were found equally in both groups.

The introduction of basiliximab induction therapy resulted in lower FNAB TCI scores and fewer AR episodes soon after TX. The AR episodes were diagnosed on the average 12 days later than in the historical control patients. A probable cause for this delay was the effect of basiliximab, which increases the early post-TX immunosuppression enough to postpone the signs of immunoactivation. This bears the inherent risk of AR occurring after the patient has been discharged from hospital. The Finnish patients are typically discharged 3 to 4 weeks after TX, and hitherto all early AR episodes have been diagnosed during the hospitalization period.

Since 1999 a protocol biopsy has been obtained in all patients three months after TX. All protocol biopsy specimen obtained between 1999 and 2001 were systematically analyzed, and a subclinical rejection was diagnosed in 39% of patients. The subclinical rejection was not reliably predicted by the early FNAB TCI scores, and thus, not by early acute rejections. The observed frequency corresponds to the previously published incidence of silent immunoactivation of the graft [170, 171, 207]. Controversy exists weather increased baseline immunosuppression reduces the prevalence of subclinical rejection [174, 208]. In this study (II), most acute changes detected at three months subsided and little CAN developed. In a previous study in the Finnish renal TX patients, 30% of the grafts presented chronic rejection changes at 18 months [209]. In the present study, histologic changes consistent with slight CAN were present in 2 (9%) of the patients already three months after TX, and the number increased to 29% by the 18-month surveillance biopsy. The prevalence of chronic changes was higher (47%) in the historical control patients in the 18-month biopsy. Reduction in graft function 18 months after TX was related to graft histopathology both in the historical control patients and the patients treated according to the revised protocol.

Reduction in number of ARs, increased fluid volumes, controlling of subclinical immunoactivation and individualized dosing of immunosuppressive medication might all have limited the damaging immunologic or vascular processes, and thus contributed to the improved graft function in the patients treated according to the revised protocol. The improvement in GFR was most evident in patients ≤2 years of age at TX, which could be significant for the long-term prognosis as the potential to compensate for the growth of the child could be better preserved in these patients.

9.3 Methylprednisolone exposure (IV)

The individual pharmacokinetics may result in variable drug exposure when standardized or body weight related dosing schemes are followed

[210]. Pharmacokinetic parameters have been reported to relate to the immunosuppressive efficacy [136, 137] and to clinical side effects of GCs [138, 211], although with one contradiction [212]. To investigate the glucocorticoid milieu in Finnish pediatric TX patients receiving chronic GC therapy, two different analytical methods were used and the results were compared to GC-related adverse effects in a small cohort of volunteers. In previous studies, GBA has been found to be increased in asthmatic children receiving inhaled GC therapy [198] as well as in cord plasma of preterm infants exposed to antenatal betamethasone regimen [199]. Suppression of GBA has been reported to relate to administration of mifepristone in women requesting emergency contraception [200]. In this study (IV), GBA correlated moderately with serum MP concentrations. Best correlation between the data obtained using the two methods was observed in the serum samples taken six to eight hours post-dose, whereas during the earlier hours the values were more diverging. MP is rapidly metabolized with a mean half-life of 2–3 hours to inactive compounds and to a minor active compound, methylprednisone [213–216]. Renal excretion of unchanged MP is minimal. Accordingly, the MP AUC did not correlate with the GFR. However, the patients with decreased renal function had higher GBA values. It is possible, that in these patients some metabolites of MP may accumulate. The metabolites (or their deconjugation products formed during the assay) might possess GR activating capacity and be detected by the GBA assay, thus explaining the convergence of the linear relationship between MP concentration and GBA values towards 6 to 8 hours post-dose.

In a previous study, GC exposure, rather than dose, was related to adrenal suppression and growth retardation in Finnish pediatric TX patients [138]. In the present study (IV), MP AUC was related to growth inhibition and weight gain after renal TX. It is probable that these patients with marked GC side-effects have been exposed to higher plasma MP concentrations over the years after TX as compared to their counterparts with a similar weight-related dose but smaller AUC. Although body surface area related dosing of GCs appears to reduce interindividual variability in drug exposure [217], the findings in this study (IV) support monitoring of serum MP concentrations to avoid the long-term adverse effects.

All the patients in this study (IV), who had gained excessive weight after TX were over 5 years of age. This could be attributable to the fact that parents generally control the diets of young children, whereas school-aged children have more freedom in choosing their eating habits. Glucocorticoids are known to increase appetite and lipogenesis with a net increase in fat deposition [218, 219] and they may increase energy intake [220], although there are very few studies in children. Obesity after TX has been considered a risk factor for dyslipidaemia [221, 222]. In Finnish pediatric renal TX patients, mild hyperlipidaemia is a frequent finding (47–56%), and the

risk for dyslipidaemia increases with older age at TX [*50*]. Obesity is also associated with elevated blood pressure in children [*223–225*]. After renal TX, hypertension is common and associated with multifactorial etiology, including immunosuppressive drugs [*226*]. Taken together, school-aged TX patients are encountered with considerable risk for obesity. Thus, intensified efforts should be undertaken towards preventing weight gain in this age group of patients receiving GC treatment.

10. CONCLUSIONS

As the patient and graft survival after renal TX has improved remarkably during the past decades, it has become evident that fine-tuning and optimization of immunosuppression is imperative for good long-term graft function and quality of life. In this study, the individually variable pharmacokinetics of CsA and its clinical implications were analyzed. The influence of controlling subclinical rejection on subsequent graft function was investigated. Also, MP exposure and its association with steroid-related adverse effects after transplantation were analyzed. The main findings and conclusions of the study were:

1. CsA pharmacokinetics are highly variable depending on the patient age and other individual characteristics. Pre-TX pharmacokinetic studies of CsA are useful in predicting individually suitable starting doses after TX. The patients who need significantly higher or lower doses than the average of their age-group, appear to benefit most from a pre-TX pharmacokinetic study.

2. Trough level –based monitoring of CsA results in highly variable C2 levels, and TID patients have lower C2 and higher C0 than BID patients during the post-TX hospitalization. High C2 levels very early after TX may be protective against AR, but after the first few weeks sufficient trough concentration, or diurnal exposure, may be equally important. Three months after TX, sufficient trough level appears to be related to normal graft histology. Optimization of CsA therapy requires monitoring of both trough level and C2.

3. Histopathologic findings in asymptomatic patients with well-functioning grafts are common. Diagnosis and treatment of these subclinical rejections three months after TX results in improved graft function eighteen months after TX, most remarkably in the youngest patients.

4. Methylprednisolone exposure in pediatric patients is highly variable, and is associated with the steroid-related adverse effects of weight gain and growth retardation after TX. Individualized dosing in long-term steroid treatment might reduce the related adverse effects.

11. ACKNOWLEDGEMENTS

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