# IMPROVEMENT OF LONG-TERM OUTCOMES IN KIDNEY TRANSPLANTATION CLINICAL VALUE OF PROTOCOL BIOPSY

Ph.D. Thesis

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List of abbreviations

AHR – acute humoral rejection (antibody mediated rejection)
AR – acute rejection = acute T-cell mediated rejection
ATG – anti-thymocyte globuline
ATN – acute tubular necrosis
AUC – area under the curve
AZA – azathioprine
BPAR – biopsy proven acute rejection
Bx – biopsy
CAD – chronic allograft dysfunction
CAN – chronic allograft nephropathy
CNI – calcineurin inhibitor
CMV – cytomegalovirus
CR – chronic rejection
CsA – cyclosporine-A
CVD – cardiovascular disease
DM – diabetes mellitus
DWFG – death with functioning graft
eGFR – estimated glomerular filtration rate
GFR – glomerular filtration rate
HLA – human leukocyte antigen
IGT – impaired glucose tolerance
MMF – mycophenolate mofetil
NODM – new onset diabetes mellitus
PRA – panel reactive antibody
PTDM – post transplant diabetes mellitus
RAR – renal allograft rupture
SRL – sirolimus
Tac – tacrolimus
TDM – therapeutic drug monitoring
Tx - transplantation

# 1. INTRODUCTION

## 1.1. History of the kidney transplantation

The first successful experimental kidney transplantation was performed in 1902 by **Emerich Ullmann** (1861 – 1937) in Vienna. The kidney, autotransplanted in the neck of a dog, remained functional for five days. The next important step was the development of the technique of vascular sutures by **Alexis Carrel** (1873-1944), who was awarded, in 1912, by the Nobel Prize in Physiology or Medicine.
The first human kidney transplantation was performed by **Jean Hamburger** (1909-1992). Together with René Kuss, Hamburger defined the precise methods and rules for conducting renal transplantation surgery, and is attributed with founding the medical discipline of nephrology. The first successful human kidney transplantation was performed in Boston, in 23 December 1954, by **Joseph Murray** (1919- ), between the identical Herrick twins at the Peter Bent Brigham Hospital.

After the successful transplantations between identical twins, the obvious next step was to broaden the use of renal transplantation in humans. For this purpose, the development of immune biology and the immunosuppression has stepped into the focus of interest.

The history of the Hungarian transplantation started in Szeged, with the first kidney transplantation between siblings in 1962, performed by **András Németh** (1924 – 1999), urologist at the Medical University of Szeged. The kidney have functioned for 79 days, and then rejected due to the lack of immunosuppression. The kidney transplantation program started in 1973 in Budapest, leading by **Ferenc Perner**, who established later the Transplantation and Surgical Clinic at the Semmelweis University. This program was supported by the Ministry of Health, and had a legislation background. The next center in the Hungarian program was the pioneer university, Szeged, starting the kidney transplantation program in 1979. The director of the 1st Surgical Department of the Medical University was **Gábor Petri**, the same as in 1962. The two young doctors, **Ernő Csajbók**, surgeon and **Pál Szenohradszky**, urologist, have performed the first two deceased donor kidney transplantations, and established the Transplantation Unit inside the Surgical Clinic (further Szeged Transplant Center).

## 1.2. Improving results in kidney transplantation

### 1.2.1. Patient and graft survival after kidney transplantation

Results of the kidney transplantation are measured by the patient survival, and the graft survival rates. These two parameters were the same at the beginning, until the dialysis could give the full backup for the kidney transplantation. The care of renal transplant patients can be roughly divided into early and late post-transplant period. This division is justified by episodes of acute allograft rejection are most common in the first few months after transplantation when relatively large amounts of immunosuppressive medication, with their potential for complications, must be administered (1). Most statistical analyses use 12 months to define the onset of the late posttransplant period.

The incidence of acute rejection and early graft failure has declined dramatically as a result of new immunosuppressive medications. One-year graft survival is now close to 90% in most transplant centers (1).

The rate of late renal allograft failure is determined by both the rate of death and return to dialysis. There has been an increase in renal allograft half-life in the past several years. For patients receiving deceased donor renal transplants, the half-life had increased to 11.6 years. However, the half-life of two-haplotype-matched living-related kidney recipients over this same period was 22.8 years. This suggests that there remains a long way to go before the half-life for deceased donor renal transplants can be considered optimal (1, 2).

### 1.2.2. Causes of graft loss

Table 1. Causes of graft loss in the first three months after transplantation

| Non-immunological causes | Immunological causes |
|---|---|
| 1. Acute tubular necrosis<br>2. Vascular (obstruction or stenosis)<br>3. Urological<br>4. Infections<br>5. Thrombotic microangiopathy (TMA)<br>6. Nephrotoxicity (e.g. CNI toxicity) | 1. Antibody-mediated acute rejection<br>2. T-cell-mediated acute rejection |

The early posttransplant period refers to the first 3 posttransplant months. Generally, surgical issues tend to predominate in the first posttransplant days and medical and immunological issues tend to predominate thereafter. The causes of the graft dysfunction or even graft loss in this early posttransplant period are summarized in the Table 1.

The late posttransplant period is defined as more than 1 year after transplantation. Causes of graft loss after the first year are the death with functioning graft (DWFG) or the chronic graft dysfunction. The rates of different diagnoses are estimated by the US Renal Data Systems report of 2003 (Fig.1) and Szeged Transplant Center data (Fig.2).

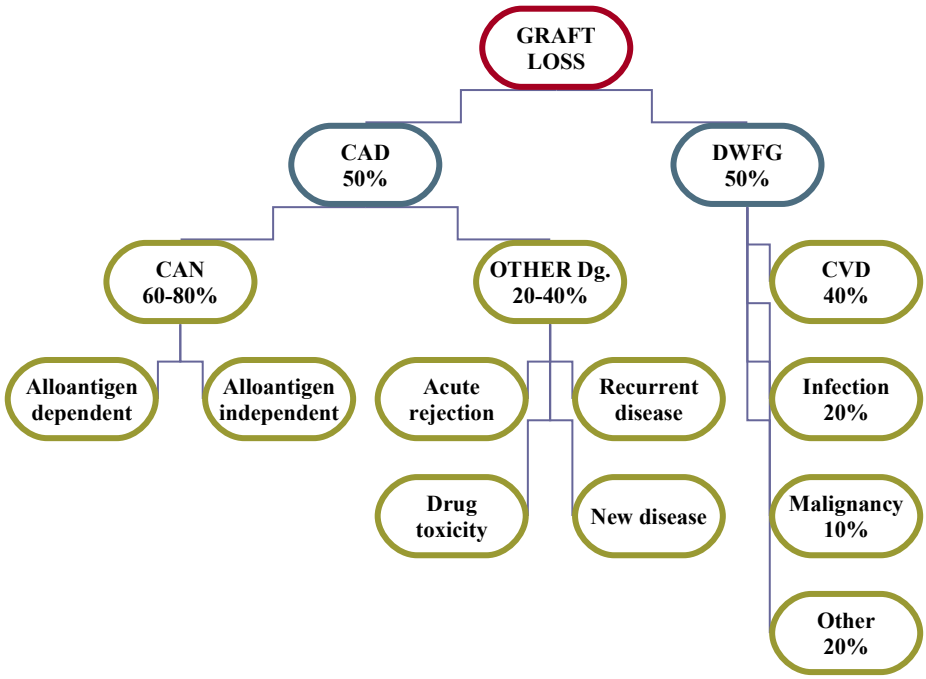



Figure 1. Kidney allograft loss after the first year. Data based on USRDS report of 2003.
CAD: chronic allograft dysfunction, DWFG: death with functioning graft, CAN: chronic allograft nephropathy, CVD: cardiovascular disease

The incidence of acute rejection (and the proportion of grafts lost during the first year after renal transplantation) has markedly decreased after the introduction of cyclosporine A. The reduction of the rate of graft loss after the first year, however, has been much less impressive.

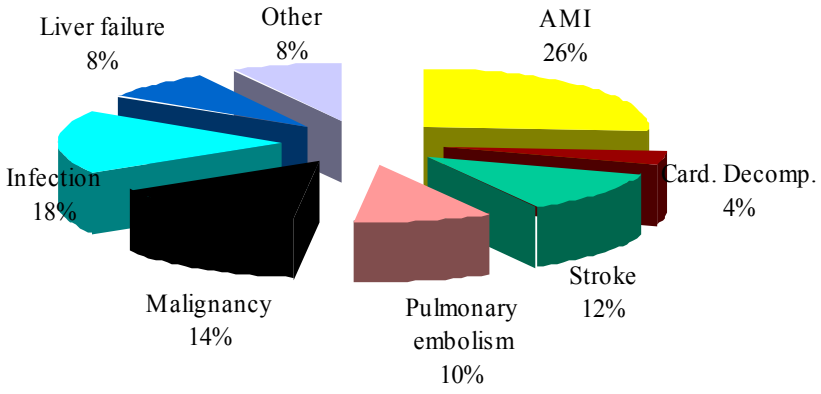



Figure 2. Causes of patients death. Szeged 1979-2003.

Chronic transplant nephropathy (CAN) have become the most common cause of late graft failure. CAN is defined by the histopathological features of interstitial fibrosis and tubular atrophy, but can also be associated with subclinical rejection, transplant glomerulopathy or transplant vasculopathy caused by smooth muscle cell proliferation. Even though the 8th Banff Conference on Allograft Pathology in 2005 eliminated the non-specific term "chronic allograft nephropathy" (CAN) from the Banff classification for kidney allograft pathology (3, 4), the term CAN is being employed quite widely to describe a clinical syndrome instead of defining the presence of interstitial fibrosis or tubular atrophy (5). CAN is characterized by slow deterioration of renal function, and it is strongly correlated with the number of AR episodes during the first year after renal transplantation. Later, subclinical AR episodes or chronic rejection may cause the graft damage.

The risk factors for these **immunological causes** of CAN are the suboptimal immunosuppression or medication non-compliance, besides the original immunological status, like prior sensitization, HLA mismatch or ongoing humoral injury.

**Non-immunological** risk factors for CAN include the donor-factors, type and age of the donor, ischemic injury (CIT), and infections (CMV, EBV), drug toxicity, hypertension, smoking or metabolic changes as DM or hyperlipidemia.

### 1.2.3. Development of immunosuppression

A short summary of the currently used immunosuppressive drugs is given in the Table 2, to show how progressive their development is, and to allow a quick overview.

Table 2. Summary of immunosuppressive drugs: mode of action and side effects

| Year of intro-duction | IS type | Drugs | Targets | Mode of action (inhibition) | Side effects |
|---|---|---|---|---|---|
| | Corticosteroids | prednisolon, methyl-prednisolon | T-cells<br>Macrophages<br>Neutrophils<br>Endothelial cells<br>Fibroblasts | Cytokine release (IL-1 and IL-2)<br>MHC-II expression<br>Adhesion<br>Expression of the adhesion molecules<br>Collagen synthesis | osteoporosis, diabetes, dyslipidaemia, hypertension, cataracta |
| | Anti-metabolites | Azathioprin | Purine-analog myelocytes | DNA synthesis<br>Promyelocytes proliferation | Neutropenia |
| 1984<br>1995<br>2000 | Calcineurin inhibitors (CNI) | Cyclosporin-A<br>Neoral<br>Tacrolimus | calcineurin phosphatase enzyme | IL-2 synthesis<br>T-cell activation | nephrotoxicity, TMA, HUS<br>hypertrichosis, gum hypertrophy<br>Hyperlipidaemia és hypertension, PTDM, tremor, neuro-toxicity |
| 1997<br><br>2004 | Purine synthesis inhibitors | Mycophenolate mofetil (MMF)<br>Mycophenolate sodium (MPS) | IMPDH (inosine mono-phosphate dehydrogenase) | *De novo* purine synthesis<br>B and T-cell proliferation | GI side-effects (diarrhea, abdominal pain)<br>Anemia, Leucopenia |
| 2002<br>2005 | mTOR-inhibitors | Sirolimus<br>Everolimus | T-cells<br>Endothelial cells | T-cell proliferation | hyperlipidaemia, anemia, Thrombocytopenia |
| 1992 | Polyclonal antibodies | Anti-thymocyte globuline (ATG) | T and/or B cells (activated)<br>Platelets | Blocking adhesion of lymphocytes and platelets to the endothelium | Cytokine release syndrome<br>Allergy, anaphylaxis,<br>Pan-cytopenia |
| 1998<br>2000 | Monoclonal antibodies | OKT3,<br>basiliximab,<br>daclizumab | CD3<br>CD25 | | |

Side effects of the immunosuppression, in general, are the infectious complications, and higher incidence of malignancies. These are not mentioned in the table as specific side effects.

## 2. AIMS

There has been a marked improvement in the results of kidney transplantation in Szeged, which is largely due to our continuous scientific-based efforts in the field. The present Ph.D. theses summarize our activities concerning diagnosis and effective treatment of early and late allograft dysfunction, including severe T-cell-mediated acute rejection episodes, introduction of new therapeutic regimens, and new diagnostic method, protocol biopsies in the Transplantation Unit of the Department of Clinical Surgery at the University of Szeged.

First of all, the major influencing factors of allograft function had to be determined, such as rejections as immunological, and the early and late non-immunological complications. In the first part of the present paper, the results of several retrospective analyses are presented (II, VI, XII).

Rejection episodes were confirmed by renal allograft biopsy. The early rejection rates were determined in different immunosuppressive regimes, which were changing in time, because of the introduction of newer and newer immunosuppressive drugs, such as microemulsion formulation of cyclosporine, MMF, basiliximab, daclizumab, tacrolimus and sirolimus.

Chronic rejection rate was also investigated after introduction of MMF, a promising drug to prevent long-term immunological reactions.

Non-immunological parameters, such drug toxicity or metabolic changes were also analyzed. The rate of posttransplant diabetes mellitus was determined, and compared in the two CNI-treated patient groups. The lipid profile was also compared in patients taking these two different immunosuppressive regimens.

In 2002, the Szeged Transplant Center, first in Hungary, introduced the protocol biopsy as a new diagnostic method. A prospective clinical study was designed to evaluate the benefit of this diagnostic tool. In the second part of the present paper, this new approach to the diagnosis of subclinical injuries is analyzed in several aspects. Safety, utility, time schedule and treatment were analyzed, and as the most important question, the impact on renal function also was investigated.

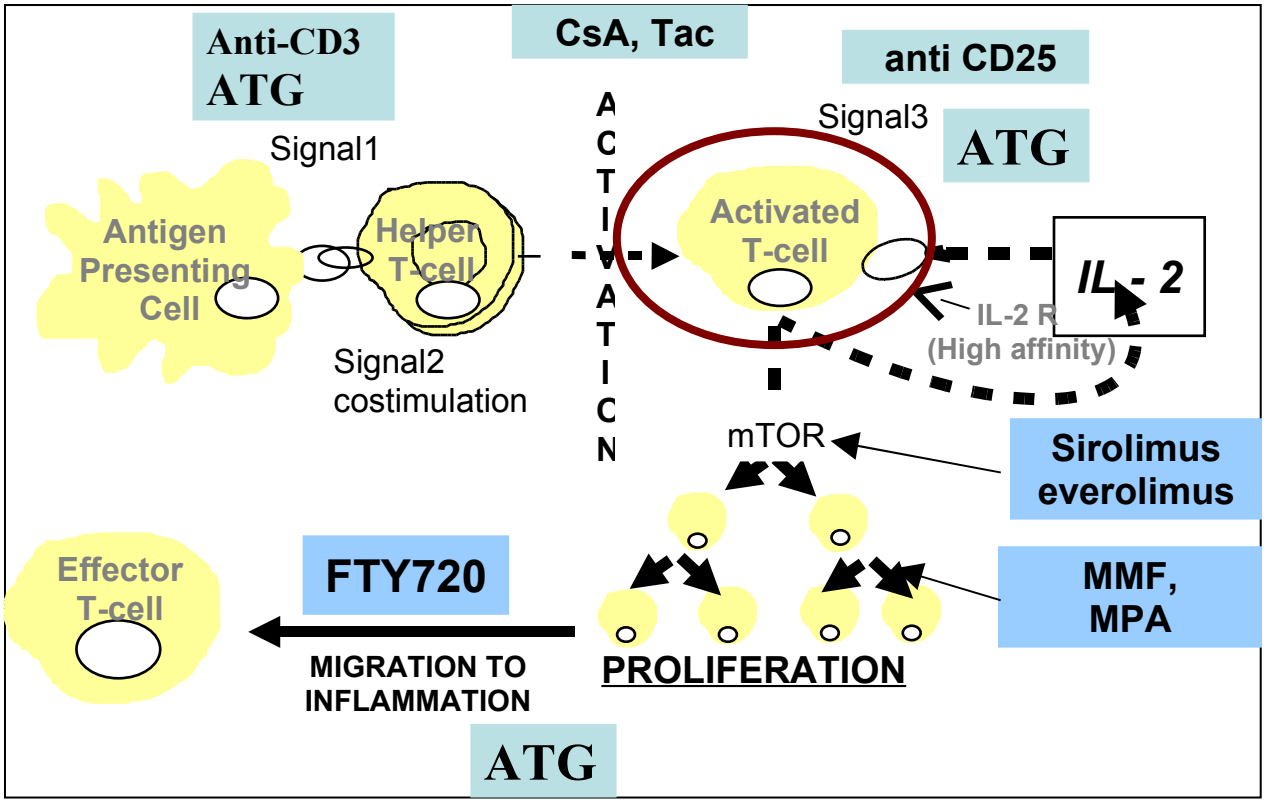
# 3. RETROSPECTIVE ANALYSIS OF CLINICAL DATA

## 3.1. Acute rejection

### 3.1.1. Immune response after transplantation

Immune response after kidney transplantation begins with antigen presentation (Fig.3). Interaction with helper T-cells together with costimulatory signals results in the generation of immune competent T-cells that start to proliferate, and finally to migrate to the transplanted organ tissues, where initiation of complement-dependent cellular toxicity largely mediated by cytotoxic T-lymphocytes occurs. This three-signal model of T-cell activation and subsequent cellular proliferation is a valuable tool for understanding the sites of action of the immunosuppressive agents.

Figure 3. Immune response after transplantation. Sites of action of immunosuppressive drugs




90% of acute rejections (AR) are T-cell mediated. However, 10% of the acute rejection episodes are still antibody mediated, so called acute antibody-mediated rejection (acute humoral rejection; AHR). AHR is clinically more dramatic, circulating donor specific cytotoxic antibodies can be detected by serology, and it is untreatable in most of the cases.

This simplification of the two types of acute rejection helps the clinician to treat their patients, but the two mechanisms are always mixing in different ratio in the acute rejection.

Clinically, the symptoms of AR are mainly inflammatory signs as fever, tenderness at the graft site and significant decrease of the urine output. Laboratory changes also prove the inflammatory response: increased leukocyte number and a clinically significant (>20%) increase of serum creatinine. However, in the modern immunosuppressive era, these clinical signs of the AR are not so severe, like they were earlier. Sometimes the only sign of AR is the graft dysfunction. That's why the allograft biopsy in the diagnosis of AR is mandatory, and only the biopsy proven acute rejection (BPAR) is considered as AR (6, 7).

### 3.1.2. The role of allograft biopsy after renal transplantation – allograft pathology

Standardization of renal allograft biopsy interpretation and reporting is necessary to guide therapy in transplant patients and to establish an objective end point for clinical trials of new antirejection agents. The Banff Working Classification of Renal Allograft Pathology is an international schema developed to fill this need. The classification, which originated in a meeting held in Banff, Canada on August 2 to 4, 1991, was published in 1993 (8), and is now widely used by center pathologists and in large international trials of immunosuppressive agents. Subsequent meetings have been held in every two years to refine the classification. The latest modification in 2007 was published in 2008 (3).

In our research, for evaluating transplanted kidney biopsies, we started to use the Banff 2003 classification (9), so remaining consistent, all the biopsies were evaluated according this scheme (Table 3).

Table 3. Banff 97 diagnostic categories for renal allograft biopsies – 2003 update (9)

| | |
|---|---|
| 1. | **Normal**, see Definitions |
| 2. | **Antibody-mediated rejection**<br>Rejection due, at least in part, to documented anti-donor antibody ('suspicious for' if antibody not demonstrated); may coincide with categories 3, 4 and 5<br>*Type (Grade)*<br>I. ATN-like – C4d +, minimal inflammation<br>II. Capillary- margination and/or thromboses, C4d +<br>III. Arterial – v3, C4d + |
| 3. | **Borderline changes**: 'Suspicious' for acute cellular rejection<br>This category is used when no intimal arteritis is present, but there are foci of mild tubulitis (1–4 mononuclear cells/tubular cross-section) and at least i1; may coincide with categories 2 and 5 |
| 4. | **Acute/active cellular rejection**<br>T-cell-mediated rejection; may coincide with categories 2 and 5<br>*Type (Grade) Histopathological findings*<br>**IA** - Cases with significant interstitial infiltration (>25% of parenchyma affected) and foci of moderate tubulitis (>4 mononuclear cells/tubular cross section or group of 10 tubular cells)<br>**IB** - Cases with significant interstitial infiltration (>25% of parenchyma affected) and foci of severe tubulitis (>10 mononuclear cells/tubular cross-section or group of 10 tubular cells)<br>**IIA** - Cases with mild to moderate intimal arteritis (v1)<br>**IIB** - Cases with severe intimal arteritis comprising >25% of the luminal area (v2)<br>**III** - Cases with 'transmural' arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells with accompanying lymphocytic inflammation (v3) |
| 5. | **Chronic/sclerosing allograft nephropathy**<br>Fibrosing changes in the allograft, with or without features of true alloimmune injury to the graft; may coincide with categories 2,3, and 4<br>*Grade Histopathological findings*<br>Grade I Mild interstitial fibrosis and tubular atrophy without (a) or with (b) specific changes suggesting chronic (mild) rejection<br>Grade II Moderate interstitial fibrosis and tubular atrophy (a) or (b) (moderate)<br>Grade III Severe interstitial fibrosis and tubular atrophy and tubular loss (a) or (b) (severe) |
| 6. | **Other**<br>Changes not considered to be due to rejection; may coincide with categories 2,3,4 and 5 |

### 3.1.3. Renal allograft rupture (II, VI)

*Introduction*

Renal allograft rupture (RAR) is a rare complication of kidney transplantation, mainly occurring within 2 weeks after transplantation. It is characterized by tears on the kidney surface, sudden onset of pain and tenderness at the graft site, perirenal hematoma, oliguria, and hypovolemic shock. Although conservative surgical management may be successful, most cases require immediate graftectomy. The pathogenesis of RAR is not known precisely. Major precipitating factors include acute rejection, ischemic acute tubular cell damage, renal vein thrombosis, mechanically damaged hilar lymphatics, and ureteral obstruction. The frequency of RAR in leading transplant centers has decreased to below 1%. The aim of our investigation was to gain an insight into the pathogenesis of kidney rupture and primarily to reveal and possibly eliminate some of its main causes and thus try to reduce the frequency of this complication in our unit.

*Patients*

Between 1979 and 1998, 628 renal allograft transplantations (mainly deceased donors) were performed in our center. Most of the grafts were perfused with Euro-Collins solution, in situ at 4°C. During the study period, the immunosuppressive regimen was changed. Until 1985, immunosuppression had been achieved by the administration of azathioprine (AZA) with prednisolon. In 1985, cyclosporine (CsA) was introduced, usually in combination with steroids. Since 1995, immunological high risk patients and those with a poor initial graft function have been treated with prophylactic polyclonal anti-lymphocyte or anti-thymocyte globulins (ALG or ATG). Two weeks after the transplantation, ATG was replaced with mycophenolate mofetil. Acute rejection episodes were treated with intravenous steroid bolus, and since 1992, steroid resistant cases has been treated with ALG or ATG.

*Study material and methods*

Graftectomy specimens due to rupture comprise the subject of our study. 37 nephrectomies due to renal allograft rupture were performed in 16 male (mean age 31 years; range 14-51) and 21 female (mean age 36 years; range 18-49) recipients.

Clinical and histopathological parameters were examined:

- cold ischemic time
- implantation time
- the change of size of the allograft (measured by ultrasound examination)
- allograft function (serum creatinine, and daily urine output)
- weight and size of the removed graft
- histology according to the Banff 97 classification (AR, intrarenal signs of obstruction, ATN, vascular thrombosis)

Controls: The weights of the ruptured kidneys were compared to 12 not implanted grafts and 18 no ruptured kidneys with acute rejection removed in the first 60 posttransplant days consecutively between 1990 and 1997.

*Results*

The median period between transplantation and graftectomy was 9 days (vs. 30 days in the controls). The total incidence of RAR leading to graftectomy was 6.3% (Table 4).

Table 4. Incidence of renal allograft rupture requiring graftectomy

| Time period | % | Graftectomy | Recipients | $\chi 2$ / $p$ value |
|---|---|---|---|---|
| 1979-1990 | 8.7 | 25 | 287 | - |
| 1991-1994 | 4.8 | 7 | 145 | 2.1179 / NS |
| 1995-1998 | 2.5 | 5 | 196 | 7.5857 / $p<0.001$ |

The initial graft function was poor in 26 patients. After graftectomy, all kidneys were grossly swollen, edematous, and their weight was significantly increased (306 *vs.* 177 g). No significant difference was demonstrated between the weights of rejecting grafts with rupture *vs.* no ruptured grafts with AR. Serial ultrasound examination revealed a gradual increase in the mean parenchymal width. The increase in the last measured volume compared to the baseline, was significant (increase in the mean parenchymal width 8.3 mm, $p<0.001$). The histological examination verified AR in 30 grafts (81%), 27 grafts also exhibited moderate or severe acute tubular cell injury. Venous thrombosis was observed in 15 grafts, and intrarenal signs of urinary tract obstruction on 11 occasions.

*Conclusions*

At rupture, all specimens were swollen, enlarged, and heavy. The histological evaluation verified that over 80% of RAR was secondary to acute rejection, a similar observation to others. In the present study, however, AR usually coexisted with other lesions, a fact not emphasized earlier. We observed AR and ATN in 75% of the specimens, accompanied with venous thrombi on 15 occasions and moderate or severe intrarenal signs of urinary tract obstruction on 11 occasions. AR, ATN, urinary tract obstruction and thrombi can all induce interstitial edema. Because edema is drained by the lymphatic vessels, the actual capacity of the renal lymphatic may be an important factor in the evolution of kidney swelling. In our data, there was a definitive decrease in the incidence of RAR. We believe that the intensification of anti-rejection prophylaxis and therapy with ATG is the factor that has positively influenced the frequency of the RAR since 1992.

### 3.1.4. Immunosuppression

The immune response can be suppressed at different points by the different immunosuppressive drugs, and it is reasonable to apply these drugs in combination. There were a lot of clinical trials to investigate the efficacy of the new immunosuppressive drugs or new combinations (12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, and 23).

Our center was also involved in several multicenter clinical trials (13, 14, 15, 16, 17, 18, 19, 20, 22), and based on their results; we introduced the newest drugs or combinations in the daily practice as soon as they were available (Table 5). Some new methods, applied in our transplantation practice, were analyzed regarding to the incidence of AR, as the main marker of the immunosuppressive effect. The aim of our studies was to determine, which immunosuppressive drug, or combination gives better results, measured as lower incidence of early AR.

In this chapter, an overview is given about the evolving immunosuppressive regimens in the Szeged Transplant Centre. The kidney transplantation program was started in 1979. The technical approach of the kidney transplantation was adopted properly. The graft survival was determined mostly by the immunological problems, namely the acute rejection. In this beginning era, the only immunosuppressants were the steroid and azathioprin combination, until 1984, when the cyclosporine-A was introduced as a “magic” immunosuppressive drug.

Then, the newer immunosuppressive drugs were introduced soon after they were available on the market. Development of our immunosuppressive protocols is summarized in the Table 5.

Table 5. Development of immunosuppression in Szeged Transplant Center

| Year of introduction | Immunosuppressive drug | Trade name |
|---|---|---|
| 1979 – 84. | Steroid + azathioprine | Prednisolon, Imuran |
| 1984. | Cyclosporine A | Sandimmun |
| 1992. | ALG, ATG | Pressimmun, ATG-Fresenius |
| 1995. | Cyclosporine microemulsion | Sandimmun-Neoral |
| 1997. | Mycophenolate mofetil | CellCept |
| 1999. | Introduction of C2 monitoring of cyclosporine | |
| 1998. | Basiliximab | Simulect |
| 2000. | Daclizumab | Zenapax |
| 2000. | Tacrolimus, FK-506 | Prograf |
| 2002. | Sirolimus, rapamycine | Rapamune |
| 2004. | Enteric coated mycophenolate sodium | Myfortic |
| 2005. | Everolimus | Certican |
| 2003. | *FTY 720* | *Study only* |
| 2006. | *LEA 29Y* | *Belatacept (study only)* |

### 3.1.4.1. Induction therapy – polyclonal antibodies, ATG

After kidney transplantation, the first contact between the recipients' immune system and the donor organ takes place after completion of the anastomoses during the reperfusion of the donor organ. From the immunological point of view, prevention of an immune response is better than interrupting or treating it after it has already started. The main rational of the "bolus", established within the past decade, is to interfere with the early recognition process in the cascade of immunological pathways. Antithymocyte globulin (ATG) infusion prior to reperfusion inhibits the adhesion of white blood cells including T-lymphocytes (24). This first step in recognition of the foreign organ happens in the post capillary system of the graft.

Conventional ATG-therapy starts on the day of transplantation - right after the operation is performed. A continuous infusion is applied at least 10 days. On the contrary, ATG-bolus induction is given prior to reperfusion, a high dose ATG in 90 min. lasting infusion. After this induction further lower ATG doses can be given for maximum 4 days, depending on the graft function – serum creatinine level.

The aim of the present study was to compare the two ATG protocols, related to the AR rate and the side effects.

*Patients*

In the Transplant Center of Szeged, 402 kidney transplantations were performed between 1995 and 2003. Polyclonal antibody prophylaxis was introduced to the therapy protocols in the year 1995. During this 9-year period, 76 patients were treated with ATG-Fresenius, 47 of them received the so called conventional therapy (group C), and 29 patients were given the ATG-bolus induction (group B). Indication of any kind of ATG therapy was the higher immunological risk (Table 6).

Table 6. Indication of ATG use (immunological risk)

| | Group C (n = 47) | Group B (n = 29) |
|---|---|---|
| Retransplantation | 16 (34%) | 9 (31%) |
| MM of DR 6 antigen | 6 (13%) | 3 (14%) |
| HLA MM > 3 | 17 (36%) | 11 (37%) |
| PRA > 15% | 8 (17%) | 6 (21%) |

Both of the two groups received steroid, cyclosporine prior to transplantation and mycophenolate mofetil (MMF) after the ATG treatment had been finished. Therapeutic protocols, including the dosage and timing are shown in Table 7. The control group was the 82 transplant patients, treated with CsA and steroid combination only.

Table 7. ATG therapeutic protocols

| | Conventional – group C<br>*n* = 47 | Bolus therapy – group B<br>*n* = 29 |
|---|---|---|
| Steroid dosage | 500 mg | 500 mg |
| timing | ***prior to reperfusion*** | pre-operatively |
| CsA dosage preop. | 12 mg/kg bw/day | 6 mg/kg bw/day |
| **ATG** D0 dose | 5 mg/kg bw/day | 9 mg/kg bw/day |
| Timing | Post transplant | ***Prior to reperfusion*** |
| Post TX dose | 5 mg/kg/day | 3 mg/kg bw/day |
| Duration of therapy | 10 days | Maximum 4 days |
| MMF | 2 g/day | 2 g/day |

Demographic data of our patients were similar in the two groups. Male and female ratio was 30:17 in group C, 15:14 in group B. The mean age of recipients were 41.0 years (range 18-59) in group C and 43.4 years (range 19-61) in group B. Donor age was also similar in the two groups: 47.1 years (14-68) and 41.2 years (18-59).

*Methods, data collection*

The primary end-points of our study were the rejection rate and graft function one month after transplantation. Rejection is defined as biopsy proven acute rejection (BPAR). Secondary end-points were hematological changes and complications, and of course patient and graft survival rates were compared in the two groups.

The early and late complications were observed continuously and detailed data were collected. The average follow-up time was 788 days (2.1 years, 1-9).

*Results*

The serum creatinine level had been decreased to normal level by the 14th day after transplantation in both groups. The serum creatinine level one month after transplantation was 144.5 (± 48.7) μmol/l in group B and 170 (± 83.73) μmol/l in group C ($p = 0.14$). At the end of the 1st month 20 patients (69%) had excellent graft function (under 200 μmol/l se creatinine) in group B, and 24 pts (51%) in group C ($\chi 2 = 2.3577$, $p > 0.10$).

Rejection rate in the first month was very low in both groups (11% in group C *vs* 15% in group B), but steroid resistant acute rejection occurred in 50 % of the BPAR cases. These AHR cases were also resistant to any other therapeutic interventions, so these grafts were lost in the first posttransplant month. AR rate, compared to the CsA + steroid treated control group, was significantly lower in the composite ATG-treated group: 11/76 (14%) *vs* 65/82 (79%); $\chi 2 = 66.33$, $p < 0.0001$ (Fig. 4).

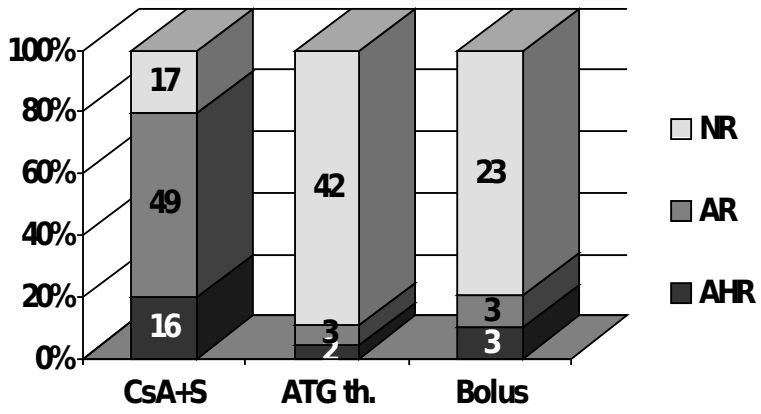



Figure 4. Acute rejection (AR) rate within 1 month after transplantation.
NR: non-rejection,

AR: acute cellular rejection, AHR: acute humoral rejection

In the infection rate, a significant difference was found between the two groups. In the B group there was no serious infection, 1 upper respiratory tract infection and 2 herpes zoster infections were observed, infection rate is 10 %. In the C group 16 pts had infection (34 %): 8 upper respiratory tract infections, 1 herpes zoster, 3 urinary tract infections, 1 pyelonephritis, 1 serious wound infection (MRSA), 1 pneumonia and 1 varicella. $\chi 2 = 5.3715$, $p < 0.05$.

Malignancies were developed only in the group C: one breast cc. 3 years after transplantation and one urinary bladder cc. 4 years after transplantation.

In the survival rates, there was no significant difference. One year patient survival was 94 % vs. 93 %, graft survival 85 vs. 83 % in group C and B. In the group C, 3 patients were lost with functioning graft, 2 of them because of septic complications and one because of cardiovascular death. In this group, 4 further grafts were lost: 3 due to AHR and one because of septic complication. From the bolus therapy group, 2 patients died with functioning graft, both due to a cardiovascular disease. Other 3 grafts were lost because of AHR.

The last evaluation of the patients was in February 2004. That time 23/29 pts (79 %) were alive with functioning kidney allograft in the bolus therapy group, and 35/47 (74 %) in the conventional ATG therapy group. The graft function was also similar in the two groups; serum creatinine is 136 vs. 143 μmol/l in group B and C.

*Conclusions*

Advantages of ATG prophylaxis in kidney transplantation are not questionable. As a result of it, there was a low rate of acute rejections (10-20 %), and a low rate of steroid resistant severe acute rejections, all together lead to better graft survival. As we published earlier (II), the rupture of the transplanted kidney almost disappeared thanks to using ATG prophylaxis and intensification of anti-rejection therapy with ATG.

ATG-bolus therapy, applying ATG prior to reperfusion, seemed to be more effective, as it extends action on the immune system before antigens can induce any response.

Compared the two different type of ATG-prophylactic protocol, it can be assumed immunological advantages are the same. What is more at “bolus-induction”, the lower rate of complications: the infection rate was significantly lower among bolus-treated group compared to the conventional therapy group (10 vs. 34 %). Malignancies occurred only after the long ATG treatment. Hematological changes were also better for patients treated with ATG-bolus.

In conclusion, polyclonal antibody prophylaxis (induction) in kidney transplantation, if it is necessary, is highly recommended with ATG-Fresenius bolus therapy, as it provides comparably good results in patient and graft survival rates, and causes less complication than other conventional therapies. Last, but not least, this type of ATG prophylaxis is much cheaper, than the other one, what might be important in the clinical practice.

#### 3.1.4.2. Mycophenolate mofetil (MMF)

MMF (CellCept) was introduced into clinical transplantation in 1995 after a series of clinical trials showed that it was more effective than azathioprine for the prevention of acute rejection in recipients of deceased donor kidney transplants when used in combination with CsA and prednisone. MMF is a prodrug, the active compound of which is mycophenolic acid (MPA), a fermentation product of several *Penicillium* species. The mofetil moiety serves to markedly improve its oral bioavailability. An enteric-coated form of MPA (ERL-080, Myfortic) became available in 2004. MPA is a reversible inhibitor of the enzyme inosine monophosphate dehydrogenase (IMPDH), a critical enzyme in the so-called *de novo* synthesis of purines and catalyses the formation of guanosine nucleotides from inosine. In principle, MMF is a more selective antimetabolite, which differs radically in its mode of action from the CNIs and SRL in that it does not affect cytokine production or the more proximal events following antigen recognition (1, 16, 17).

The anti-rejection effect may be the summary of the following effects:

- MMF blocks the proliferation of T and B cells
- Inhibits antibody formation
- Inhibits the generation of cytotoxic T cells.
- Downregulates the expression of adhesion molecules on lymphocytes, thereby impairing their binding to vascular endothelial cells.

- Inhibits the recruitment of mononuclear cells into rejection sites and the subsequent interaction of these cells with target cells.
- MMF has a preventive effect on the development and progression of proliferative arteriolopathy, a critical pathologic lesion in chronic rejection.

*Methods*

A retrospective analysis was performed to compare the rate of acute rejection in transplant patients treated with MMF-containing immunosuppression, and those receiving CNI and steroid without MMF. MMF was introduced into our practice in 1997.

Between 1997 and 1999, 75 transplant patients (50 male and 25 female) were treated with MMF without any induction therapy. MMF was added to the CsA and steroid combination. The historical control group was treated with CsA and steroid combination, 82 patients transplanted between 1995 and 1997. Early AR rate (within the first month) was investigated in these groups. The treatment failure and complication rate was also determined in the MMF group.

*Results*

There were no significant differences in the age, retransplant rate, HLA mismatch, PRA and the CIT between the two groups. AR rate was significantly lower in the MMF group (19/75; 25.3% *vs.* 65/82; 79%; $\chi 2 = 45.8$, $p < 0.001$). The steroid resistant rejection rate was also significantly lower in the MMF group: 6/75; 8% *vs.* 16/82; 20%; $\chi 2 = 4.31$, $p < 0.05$. (Fig. 5)

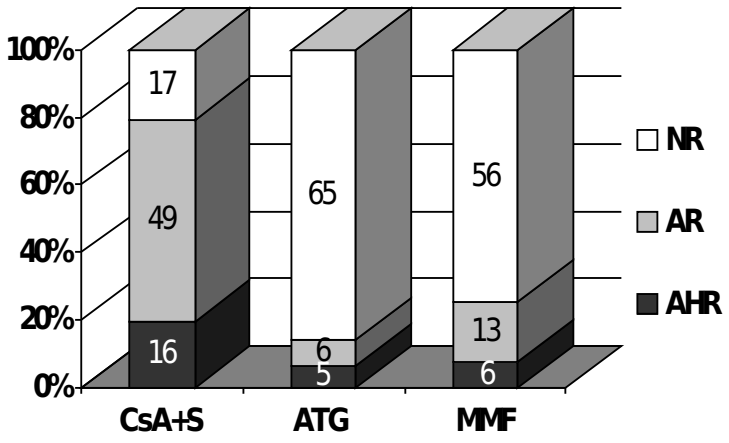



Figure 5. AR rate in the 1st month after transplantation, in different immunosuppressive treatment groups.
NR: non-rejection,
AR: acute cellular rejection
AHR: acute humoral rejection

At one year follow-up, 66 patients were still on the MMF therapy. 5 grafts were lost (4 death and 1 graftectomy due to AHR), and 4 patients did not tolerate the gastrointestinal side effects, such as the nausea and diarrhea. The GI side effects occurred in 19 cases (25.3%), but 15 of them were reversible after dose reduction. The infection rate was 11% (8 cases), 4 patients had CMV infection, 2 Herpes zoster infections and 2 Candida infections occurred.

*Conclusions*

In summary, the MMF/CsA/steroid regimen reduced the AR rate significantly, compared to the CsA/steroid/AZA regimen. However, the recommended therapeutic dose, 2 g/day was not tolerated by every patient, 30% of them needed to reduce the dose or even to withdraw the drug. The most common cause of intolerability of MMF is the gastrointestinal complications, like nausea and diarrhea. However, these symptoms might be the consequences of the infectious complications, like CMV gastroenteritis, or other viral or bacterial infections (25).

### 3.2. Chronic rejection

*Introduction*

The most important causes of late graft loss are the DWFG and the chronic allograft nephropathy (CAN) (Fig. 1.). In this category, it can be differentiated chronic rejection (CR), marked “b” by the Banff classification (9)**.** The chronic rejection is the most frequent reason for the late allograft dysfunction. Morphological signs of chronic rejection include arteriopathy, glomerulopathy, capillaropathy diagnosed with electron microscopy, and C4d positive peritubular capillaries by immune fluorescence microscopy.

In this study, we examined how the most recent immunosuppressive treatment protocols influence the prevalence of chronic graft rejection.

*Methods*

We studied 134 biopsy samples taken due to graft dysfunction from at least 1 year old transplanted kidneys using the combinations of light microscopy, immune fluorescence and electron microscopy. The diagnosis of chronic rejection was made by the verification of transplant arteriopathy, and/or transplant glomerulopathy, and/or transplant capillaropathy.

Patients were divided into two protocol treatment groups. In group one (n = 52), patients received CsA/steroid or CsA/steroid/AZA therapy. In group two (n = 82), patients received CsA/steroid/MMF or Tac/steroid therapy. The subgroup of 40 patients received CsA/steroid/ MMF, was also compared to the group one.

*Results*

There were no significant differences between the therapy groups in the serum creatinine level, in the time between the transplantation and the biopsy taking and in the number of HLA mismatch.

Chronic rejection was diagnosed significantly fewer among those patients who were treated with the new protocols' drug combination than were found in those treated with the earlier combination (49/82; 59.8% *vs.* 43/52; 82.7%; $p = 0.012$). (Fig. 6)

Examining the correlation between the CR and the use of MMF, a significantly lower rate of CR was in the MMF group (25/40; 62.5% *vs* 43/52; 82.7%; $p = 0.029$). There were no changes in the prevalence of diagnoses of AR, acute borderline rejection, de novo/recurred glomerulonephritis, and CNI toxicity.

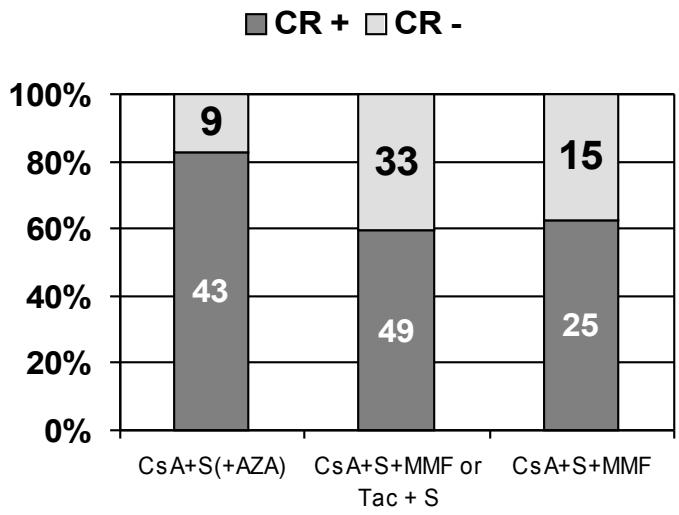



Figure 6. Incidence of chronic rejection (CR+) in different immunosuppressive protocols

*Conclusion*

The new treatment protocols reduced the frequency of chronic graft rejection, probably by the reduction of intensity of acute rejection episodes or a prevention of the sub-clinically progressing rejection.

### 3.3. Therapeutic drug monitoring
### – C2 monitoring in cyclosporine-treated patients

Therapeutic drug monitoring (TDM) is essential for drugs with narrow therapeutic range, where the effective and the toxic drug exposure are close together and there is a variation in interpatient and intrapatient metabolism. Cyclosporine was the first immunosuppressive drug, which improved significantly the results of kidney transplantation, although its nephrotoxic effect had been known at the moment of the introduction. The original formulation of cyclosporine has been replaced by the micro-emulsion formulation, Neoral. The new formulation has several advantages against the original formulation: the bioavailability is better, and there is less variability in cyclosporine pharmacokinetics. Peak concentrations (Cmax) of Neoral are higher and the trough level (Cmin) correlates better with the systemic exposure, as reflected by the area under the curve (AUC). The time of peak concentration after an oral dose also shows less variability, it is 2 hours in most of the cases (Fig. 7).

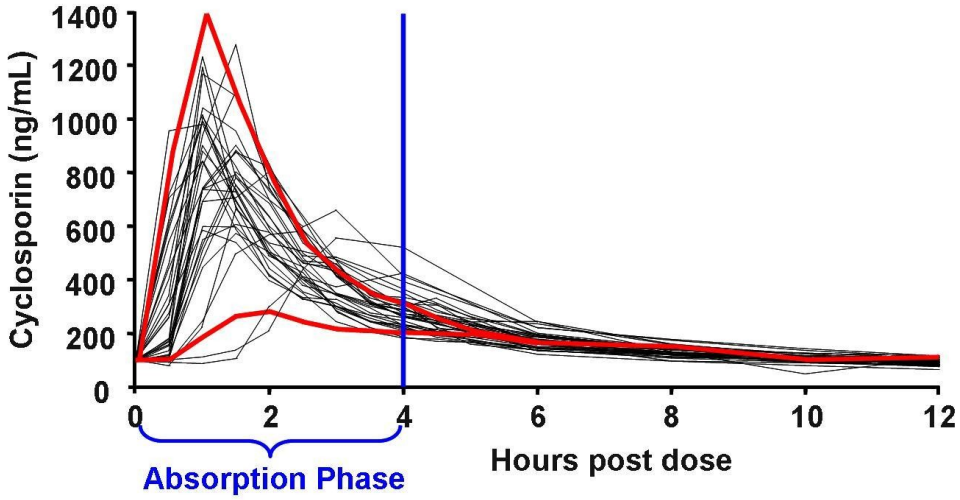



Figure 7. Pharmacokinetics of the cyclosporine after oral taking. The C2 value correlates with the AUC, more than the trough level.

Large multicenter study proved, that the AUC-controlled dosage is better, than the trough-level controlled one (14, 15). It is also proved, that the best correlation with the AUC is given by the Cmax, which is equal, in the case of Neoral, with the C2. Assuming these result, we decided to introduce the C2-monitoring in our practice.

*Patients and methods*

Between 1999 and 2002, 170 patients (109 male and 61 female), treated with cyclosporine (Neoral) were involved in the new TDM, and the Neoral dose was changed, if it had been necessary, based on the C2 monitoring results. The original trough levels were 250-400 ng/ml in the first posttransplant year and 150-350 ng/ml thereafter. At the time of introduction of the new monitoring method, both C0 and C2 measurements, and a two-sample AUC calculation were performed in every case. The target of the C2 was 1000-1300 ng/ml in the first year, correlating with the AUC target, and 800-1000 ng/ml thereafter. The dose change and the serum creatinine levels were analyzed.

*Results*

The Neoral dose had to be changed in 54 cases (32%), increased dose was introduced in 45 patients (27%), and the dose was decreased in 9 patients (5%). In the 54 cases, where the drug dose was changed, the graft function was analyzed 1 month later. The serum creatinine level decreased or remained stable in 43 cases (80%), and it increased in 11 patients (20%). Our results are correlated with the international studies, as the better method of TDM results an improved graft function in 25% of the CsA-treated patients.

*Conclusions*

The TDM is necessary for those, narrow therapeutic range drugs, like cyclosporine. The method of measurement is questionable even today. Recent data suggest that optimal cyclosporine (CsA) exposure early post-transplant significantly reduces the risk of acute graft rejection. They indicate that trough level monitoring is inadequate for precise concentration-controlled therapy, and suggest that absorption profiling may offer a superior approach for guiding clinical immunosuppression with Neoral.

A 2-h post-dose blood sample is the most consistent, accurate and robust single-point predictor of the absorption phase measured by AUC[0-4] and should replace trough level monitoring for accurate concentration-control of Neoral therapy in the clinical setting (14,15).

### 3.4. Metabolic changes due to immunosuppression

The incidence of cardiovascular disease (CVD) in the renal transplant population is more than four times higher than in the general population (26), leading to the main cause of mortality with a functioning graft is CVD. Risk factors associated with the post-transplant CVD include hyperlipidemia, hypertension and diabetes, plus other transplant-related factors, e.g. episodes of acute rejection, graft failure and immunosuppressive drugs. Immunosuppressants have differential effects on CVD risk factors. Numerous large, multi-center, randomized clinical trials demonstrated that new onset diabetes mellitus is significantly higher in Tac-treated patients (27, 28, 29), but switching from CsA to Tac resulted in improved lipid levels and blood pressure (30, 31). Clinical studies also have shown that Tac has a superior efficacy in preventing acute rejection, corticosteroid-resistant acute rejection and chronic rejection compared with CsA-based regimen, while maintaining a good safety profile.

The two CNIs, Tac and CsA are compared according their side effects, as CVD risk factors.

#### 3.4.1. Diabetes mellitus

One of the main risk factors for CVD is the DM, which often remains hidden in the ESRD population or after the renal transplantation. DM, developed after kidney transplantation is defined as “new onset DM = NODM” or “posttransplant DM = PTDM” with the earlier terminology. This NODM is not only a simple risk factor for CVD, but it is harmful for the kidney allograft itself, and decreases the long-term graft survival.

Aim of our investigation, performed first in Hungary, was to determine the incidence of NODM in our transplant patients, receiving CNI containing or CNI free immunosuppression. Based on the results of protocol biopsies, the graft morphology also was compared in the diabetic and non-diabetic patients.

*Patients and methods*

Kidney transplant recipients having acceptable renal function (serum creatinine < 250 µmol/l) in November 2005 were investigated. The 236 patients, who were non-diabetic before the transplantation, were involved into the retrospective analysis.

Patients were allocated into 3 groups, based on the definition of diabetes mellitus (32): non-diabetic (N), impaired glucose tolerance (IGT) and diabetic (NODM) group (Table 8).

Table 8. Demographic data and renal function in the patient groups based on the glucose metabolism

| | N<br>n = 167 | IGT<br>n = 46 | NODM<br>n = 23 | p |
|---|---|---|---|---|
| Age (years) | 52 ± 4 | 57 ± 7 | 50 ± 5 | N.S. |
| Gender (male/female) | 100/67 | 25/21 | 15/8 | N.S. |
| Body weight (kg) | 65 ± 8 | 75 ± 9 | 72 ± 6 | N.S. |
| Posttransplant time (years) | 4.8 ± 3 | 6.5 ± 2 | 4.8 ± 5 | N.S. |
| Serum creatinine (µmol/l) | 137 ± 48 | 138 ± 49 | 136 ± 48 | N.S. |
| $eGFR_{CG}$ (ml/min/1.73 m2) | 60.2 ± 11 | 62.3 ± 12 | 64.3 ± 11 | N.S. |

The renal function in these groups was measured by the serum creatinine and the eGFR calculated by Cockroft-Gault. One year protocol biopsy, when it was available, was evaluated by the Banff classification (Table 3), and the incidence of AR, CNI toxicity and CAN were compared in the 3 patient groups.

To compare the different immunosuppressive drugs, another allocation was applied. Patients were allocated, according to their immunosuppression, into a CNI-based group with the Tac and the CsA subgroups, and CNI free group (Table 9). In these patient groups, the incidence of NODM was analyzed.

Table 9. Demographic data of the patient groups based on the immunosuppression

| | CNI-based | | CNI free<br>n = 53 | p |
|---|---|---|---|---|
| | CsA n = 119 | Tac n = 64 | | |
| Age (years) | 49 ± 5 | 50 ± 3 | 46 ± 2 | N.S. |
| Gender (male/female) | 74/45 | 39/25 | 29/24 | N.S. |
| Body weight (kg) | 73 ± 8 | 71 ± 10 | 78 ± 7 | N.S. |
| Posttransplant time (years) | 5.3 ± 3 | 5.4 ± 5 | 5.4 ± 2 | N.S. |

*Results*

The incidence of NODM in our renal transplant patients were 9.7 % (23/236). The graft function was similar in all groups (Table 8). Analyzing the morphology in the one year protocol biopsies, available in 43 patients, AR rate and CAN seem to be remarkably more frequent in the diabetic group, however statistical significance cannot be count because of the small numbers (Fig. 8).

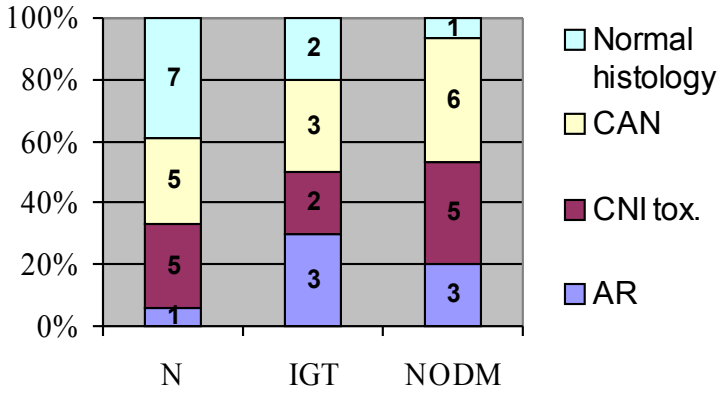
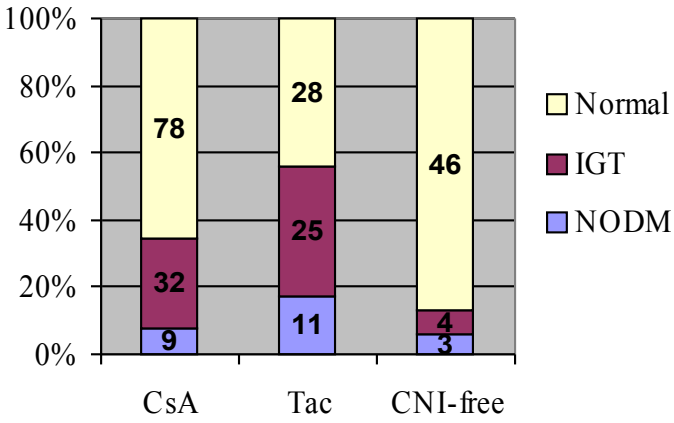



Figure 8. One year protocol biopsy in patient groups based on the glucose metabolism

Comparing the incidence of NODM in the patients receiving different immunosuppressive treatment, we found a significantly higher incidence of NODM in the Tac-based group than in the CsA (17% vs. 8%; $\chi^2 = 3.9602$, $p < 0.05$) or CNI free group (5%) (Fig. 9).

Figure 9. Incidence of diabetes mellitus in different immuno-suppressive protocols.




*Conclusions*

Cardiovascular risk of patients can be decreased by the appropriate diagnosis and control of the diabetes mellitus. The new onset diabetes mellitus after transplantation is more frequent in patients taking CNI, and significantly higher in the Tac-treated patient group. Our results

showed that the NODM may affect the graft survival, as the allograft damage is more frequent in the diabetic patients' 1-year protocol biopsy samples, a surrogate marker for the long-term graft survival. To achieve a better patient and graft survival, we suggest choosing immunosuppressive drugs with the lowest risk for the individual patient. Steroid is withdrawn early or Tac is avoided if patient is a high risk of DM. Regular control of the glucose metabolism is essential, by measurement not only of the fasting glucose, but the HbA1C, and glucose tolerance test should be performed if necessary. Other graft damaging factors also should be treated early, before affecting the graft function, so taking protocol biopsy is recommended. With this strategy, the risk of AR and the CV risk of immunosuppression (side effects of the drugs) taken together can be minimized.

### 3.4.2. Dyslipidemia, hyperlipidemia

*Patients*

In this open study, 21 adults free from diabetes were involved, at least 6 months after cadaver kidney transplantation. Another criterion of inclusion to the study was stable function of the transplanted organ. In these patients, CsA was changed to Tac. Cholesterol decreasing drugs had been stopped 6 weeks before the conversion of immunosuppressive treatment.
Between November 2001 and March 2004, 21 patients were converted from CsA to Tac because of cyclosporine side effects. Indications of conversion were: nephrotoxicity (8 cases), gingivitis and hirsutism (7 cases), hypertension (3 cases), cardio-toxicity (1 case), rescue therapy after rejection (1 case) and hyperlipidemia alone (1 case).
Before the conversion, further 16 patients have shown hypercholesterolemia.

*Methods*

CsA had been stopped in the evening and Tac was given from the next morning in a dose of 0.2 mg/kg body weight. Initial Tac trough level should have been 5-10 ng/ml. Average daily dose of Tac was 5,2 mg (range 2-12), average trough level has been achieved by the first month was 7,86 ng/ml.

Medical interview, physical examination and additional tests had been carried out before the beginning of the study, during and after study completion. The levels of total cholesterol,

HDL, LDL and se triglyceride have been compared before and after 12 months from the change of therapy. To establish the safety of this conversion, the frequency of complication of treatment and change in endogenous creatinine clearance has been assessed. The lipid metabolism was observed by the measurement of serum cholesterol, LDL, HDL and triglyceride levels before the conversion, 1, 2, 4 weeks after the conversion and further in each months. Apart from these values the blood pressure, kidney function, blood sugar level and clinical status of the patients were also measured and analyzed.

*Results*

16 patients completed the entire 12 months study period. Last patients' final visit was in March 2005. We have lost one patient because of cardiac death (with functioning graft) in the 6th month of the study. One patient lost graft at M11 due to chronic rejection, 3 more patients stopped taking Tac because of the following reasons: biopsy proven nephrotoxicity at M7, PTDM at M9, elevated blood sugar and patients wish at M10. The remaining 16 patients had no major complications, 3 temporary tremor occurred, which resolved by decreasing Tac doses.

According to the data analysis there was a significant improvement in the serum lipid profiles, such as in serum cholesterol levels that have decreased from 6.27 ± 1.22 mmol/l baseline value to 5.11 ± 0.82 mmol/l after 6 months ($p = 0.0002$) and to 5.09 ± 0.66 after 12 months ($p = 0.01$). Similarly, there was a beneficial improvement in the triglyceride levels from 2.84 ± 1.16 to 1.80 ± 0.61 mmol/l after 6 months ($p = 0.003$) and to 1.78 ± 0.59 mmol/l after 12 months treatment ($p = 0.004$). In the blood pressure values, no significant changes occurred. Results are summarized in the Table 10.

Table 10. Changes in the lipid profile and blood pressure after CsA – Tac conversion

| **Patient** | **se cholesterol (mmol/l)** | | | **se triglyceride (mmol/l)** | | | **RR (mean, Hgmm)** | | |
|---|---|---|---|---|---|---|---|---|---|
| | BL | after conversion | | BL | after conversion | | BL | after conversion | |
| | | 6 M | 12 M | | 6 M | 12 M | | 6 M | 12 M |
| **average** | 6.27 | 5.11 | 5.09 | 2.84 | 1.80 | 1.78 | 112 | 113 | 106 |
| **median** | 6.11 | 5.28 | 5.05 | 2.31 | 1.55 | 1.80 | 113 | 110 | 105 |
| **± SD** | 1.22 | 0.82 | 0.66 | 1.16 | 0.61 | 0.59 | 10 | 10 | 7 |
| ***p* value** compared to BL | | 0.0002 | 0.01 | | 0.0003 | 0.0038 | | 0.97 | 0.155 |
| ***p* value** compared to 6 M | | | 0.33 | | | 0.312 | | | |

BL: baseline, M: month

*Conclusions*

Introduction of tacrolimus in transplantation was important to have an alternative calcineurin inhibitor for patients, who have problems with side effects of cyclosporine. Even more, our study has shown that CsA-caused elevated serum lipids are not among the side effects of tacrolimus. According to the clinical results and our experiences, conversion of cyclosporine to tacrolimus is recommended when lipid levels are not in normal level, despite of lipid lowering therapy, or in the cases of cosmetic side effects of cyclosporine like hirsutism or gingivitis. Cyclosporine and tacrolimus has similar nephrotoxic effects, nevertheless tacrolimus therapy is a safe immunosuppression, with a beneficial effect on the serum lipids, resulting in the decreased risk of cardiovascular mortality and a better long-term patients survival (33, 34).

## 4. PROSPECTIVE CLINICAL STUDY WITH THE PROTOCOL BIOPSY

### 4.1. Introduction

Long-term kidney allograft survival is influenced by immunological and non-immunological factors such as acute rejection (AR), chronic rejection (CR), insults leading to interstitial fibrosis and tubular atrophy (‘chronic allograft nephropathy’ = CAN), infections, calcineurin inhibitor (CNI) toxicity and *de novo* or recurrent renal disease. A significant proportion of these conditions develop insidiously, without causing a measurable decrease in renal function, and are detected in biopsies performed at pre-specified post-transplant intervals, termed protocol biopsies (35, 36, 37, 38). The literature data indicate that the evaluation of protocol biopsies not only reveals the presence or absence of various subclinical injuries, but also facilitates validation of the efficacy of immunosuppressive regimens (39, 40). Although the potential risk of complications of the biopsy procedure is not negligible, studies on the complications of protocol biopsies have demonstrated that there are few major complications and those that do occur are of a benign nature (41, 42).

Despite the advantages of the early diagnosis and the relative safety of the procedure (43), protocol biopsies have not yet become standard practice in most transplant centers. This might be related to the lack of clear proof of the benefit of the early treatment of subclinical pathologies detected by protocol biopsy.

The question, whether early treatment of the pathologies had any impact on the long-term graft function, indicated the present study. The aim of the introduction of protocol biopsy program was to detect of subclinical AR and other graft-damaging disorders in the early and the late post-transplant period. Therefore, the incidences of subclinical AR, CR, CAN and CNI toxicity is analyzed by comparing the results of protocol biopsies taken at different posttransplant times. The complications of the biopsy procedure were reviewed concerning the safety of the procedure.

The main purpose of the clinical analysis was to compare the long-term (2 years and 3 years after transplantation) allograft function in those patients who were treated based on the protocol biopsy findings and those who had not undergone protocol biopsy taking with the consequence of being unable to be treated.

## 4.2. Material and Methods

### *4.2.1. Study plan*

The Szeged Protocol Biopsy Program, approved by the Regional Ethics Committee, was started in November 2002. Data collected between 2002 and 2006 were evaluated.

Patients, who participated in the Program on a voluntary basis, underwent protocol biopsy 3 months and/or 12 months after transplantation. In the morphological analysis we evaluated protocol biopsies also taken 3 years or later, 5 to 10 years after transplantation, however these late biopsies did not take part in the clinical study.

*Morphological analysis* – a retrospective analysis of all (229) protocol biopsy samples taken at different posttransplant times (3 months, 12 months, 36 months or later than 5 years). The incidence of AR, CNI toxicity, CR or CAN, and other pathologies like pyelonephritis, glomerulonephritis were determined. As the two CNIs were randomly used as maintenance immunosuppression, the two treatment groups are compared to each other.

*Clinical study* – a prospective, open, randomized study: to evaluate the impact of the early treatment of allograft pathologies detected by protocol biopsy on the graft function, the 3-

month and 12-month protocol biopsy patients were compared to the non-biopsy, control group (Fig. 11).

*4.2.2. Sampling*

Ultrasound-guided biopsy was performed with a 16G needle after the informed consent was signed. Two cores, if possible were taken in the 3rd month (79 pts), 12th month (86 pts), 3rd year (41 pts), and later (23 pts) after transplantation. Patients have been observed for 4-6 hours after biopsy taking for safety reasons. If any complication occurred, an overnight observation was applied.

*4.2.3. Biopsy evaluation*

The morphological examination included the standard light microscopic stainings (H&E, PAS, trichrome, methenamine silver), immunofluorescence analysis of the frozen sections with antibodies to HLA class II antigens, complement 4d (C4d), C3, IgG, IgA and IgM. Embedding for electron microscopy was carried out in all cases, and the ultrastructural evaluation was performed optionally. Renal lesions were graded and diagnosed according to the Banff 2003 schema (9) (Table 3).

*4.2.4. Patients*

*Morphological analysis*

Between November 2002 and December 2006, 229 protocol biopsies were performed in 175 patients (39 pts 2x, 7 pts 3x), 109 male and 66 female patients. The mean age was 46±10 years. All patients were clinically free of any symptoms, and had stable kidney functions (serum creatinine level 158 ± 43 μmol/l, eGFR 55,1 ± 15,8 ml/min). The induction therapy was ATG in 26 retransplanted patients and 10 highly sensitized patients (PRA≥50%), basiliximab in 82 patients and daclizumab in 48 patients. The subsequent immunosuppressive treatment was in all cases a combination of steroid, CNI (CsA, 84 pts or Tac, 91 pts) and mycophenolate mofetil (MMF).

*Clinical study*

Inclusion criteria 3 months after transplantation:

- adults (age > 18 years);

- a stable graft function (serum creatinine < 300 μmol/l);
- no clinical symptoms, no rejection episode within 1 month;
- taking CNI and MMF combination immunosuppressive therapy;
- a stable immunosuppressive drug trough level (tacrolimus (Tac) 5-15 ng/ml, cyclosporine (CsA) 100-250 ng/ml);
- good compliance;
- signed informed consent

164 patients, transplanted between November 2001 and February 2006, fulfilled the inclusion criteria 3 months after transplantation (baseline data). Randomly selected 113 patients participated in protocol biopsy taking, and 51 patients were selected to the control group (2:1 randomization). 66 patients had protocol biopsy at 3-month, and 47 patients were assigned to have only 12-month protocol biopsy. Three patients were lost to follow-up in the first posttransplant year (Fig. 10). 32 patients from the 3-month biopsy group underwent a second protocol biopsy taking 12 months after transplantation, while 33 of them did not. 3-year follow-up data were available on 142 patients (Fig. 10).

Baseline characteristics of the patient groups are demonstrated in Table 11. All patients received induction therapy, either ATG (highly sensitized and retransplanted patients) or anti IL-2 antibody induction, randomly chosen basiliximab or daclizumab. In the maintenance immunosuppressive combination, in all transplantation Tac or CsA was chosen by the 1:1 randomization.

Table 11. Demographic and baseline (3-month) data on protocol biopsy and non-biopsy patient groups (Patients with graft loss in the first year are excluded)

| Characteristics | Protocol biopsy ($n$ =112) | Non-biopsy ($n$ = 49) |
|---|---|---|
| Gender, M/F (%male) | 63/49 (56) | 29/20 (58) |
| Age (yr) | 44.1 ± 12 | 44.6 ± 12 |
| Donor age (yr) | 46.0 ± 10 | 44.5 ± 12 |
| Body weight (kg) | 69 ± 14 | 71 ± 14 |
| Systolic BP (Hgmm) | 132 ± 15 | 132 ± 13 |
| Diastolic BP (Hgmm) | 82 ± 8 | 82 ± 6 |
| HLA mismatches | 2.88 ± 0.84 | 2.94 ± 0.90 |
| Cold ischemic time (min) | 1115 ± 186 | 1095 ± 261 |

| | | |
|---|---|---|
| Retransplantation (%) | 18 (16) | 11 (23) |
| PRA≥50% (%) | 8 (7) | 3 (6) |
| Induction therapy: ATG/Sim/Zen | 26/53/33 | 14/22/13 |
| CNI use (CsA/Tac) | 53/59 | 24/25 |
| Se creatinine (µmol/l) | 160 ± 39 | 153 ± 39 |
| $eGFR_{CG}$ (ml/min) | 50.4 ± 13 | 53.2 ± 14 |
| $eGFR_{MDRD}$ (ml/min) | 44.2 ± 13 | 47.2 ± 14 |

pBx = protocol biopsy, Sim = Simulect (basiliximab), Zen = Zenapax (daclizumab)

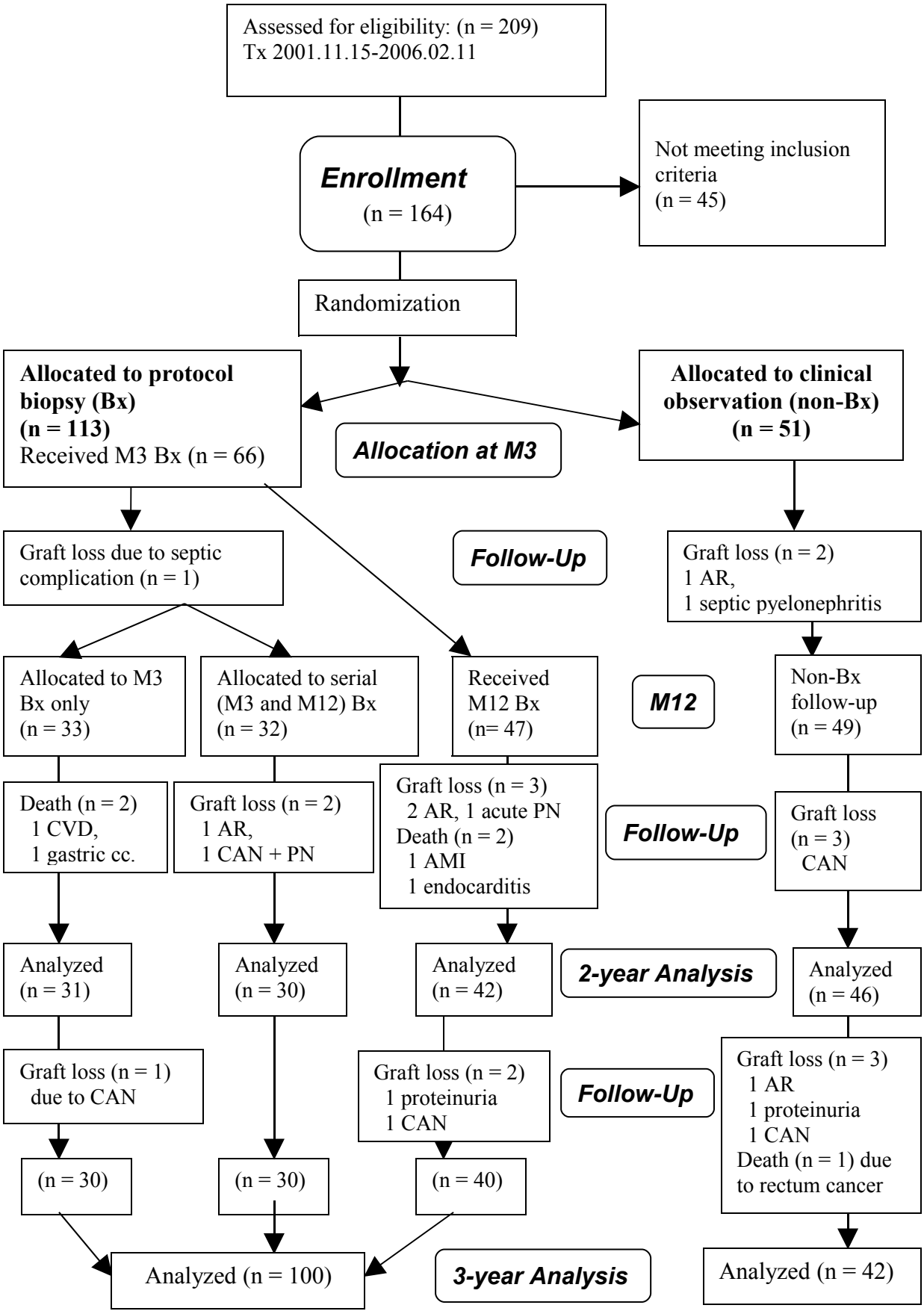



Figure 10. Protocol biopsy patient allocation flowchart

*4.2.5. Clinical follow-up and data collection*

All patients were regularly controlled at monthly intervals until 1 year post-transplantation, and then every 6-8 weeks. Physical examination, body weigh and blood pressure measurement were performed at each visit. Laboratory measurements included hematology biochemistry and immunosuppressive drug levels. Hypertension, anemia and the metabolic changes, diabetes or dyslipidemia, were treated appropriately. Clinically suspected rejections were investigated by biopsy, and the proven ones were treated by steroid bolus therapy as usual in all cases, regardless of the patient groups.

The treatment of pathologies detected by the protocol biopsy was introduced as follows. Cases of subclinical rejection, except borderline changes, were treated with steroid pulse therapy. Modification of the immunosuppression (dose reduction or drug change) was applied when CNI toxicity was diagnosed. Pyelonephritis was treated with antibiotics, and glomerulonephritis with steroid. CNI administration was stopped in 3 patients because of BK polyoma virus-induced nephropathy.

The graft function was measured via the serum creatinine and the glomerular filtration rate (GFR), estimated by the Cockroft-Gault method and by the MDRD formula (44, 45), the values being given as $eGFR_{CG}$ or $eGFR_{MDRD}$, at 3 months, 1 year, 2 years and 3 years after transplantation. All laboratory measurements were carried out by the accredited University Central Laboratory. Data for the study database were collected from the MedSolution patient record system.

*4.2.6. Statistical analysis*

Results are expressed as means ± standard deviation (SD) for continuous variables. Frequencies of categorical variables are given as counts and percentages. For continuous variables, such as the graft function, measured by the serum creatinine, eGFR, or the change in eGFR (ΔGFR), the two-sample t-test was used. The categorical variables were analyzed by the Fishers' exact and chi-square test. All p values were two-tailed and p values $<0.05$ were considered significant.

## 4.3. Results

### *4.3.1. Safety*

No major complications occurred. The minor complication rate was 5 % (9 pts), in 6 patients a transient hematuria was observed, which has been disappeared latest by the time of the third urination, so patients were able to be discharged after the 4 h observation time. In 3 patients a small arterio-venous fistula developed, which was detected later, by the control ultrasound examination.

### *4.3.2. Morphological findings*

The biopsy sample in 21 cases (9 %) was marginal, where the sample size was not sufficient for the pathologists' assessment according to the Banff criteria. In the early posttransplant period (3 months after transplantation) it was less (4/79; 5 %), while this rate was higher if patients were in the later posttransplant period (1 year: 9/86; 10%, 3 years 5/41; 12%, more then 5 years 3/23; 13%)

Biopsy evaluation (Table 12, Fig. 12.) showed that 71% of the samples had some significant pathological changes.

Table 12. Histology findings of protocol biopsies

| | N | Marginal | Acute rejection | | CNI tox. | CAN | | other | Norm. |
|---|---|---|---|---|---|---|---|---|---|
| | | Samples (%) | AR | BL | (%) | CR+ | CR- | | (%) |
| M3 | 79 | 4 (5) | 10 (13) | 19 (25) | 9 (12) * | 0 | 13 (17)** | 17 | 37 (49)* |
| 1 year | 86 | 9 (10) | 19 (24) | 23 (29) | 22 (28)* | 7 (9) | 37 (48)** | 17 | 17 (21)* |
| 3 years | 41 | 5 (12) | 7 (19) | 10 (27) | 18 (50)* | 8 (22) | 14 (38) | 5 | 5 (13) |
| Late | 23 | 3 (13) | 1 (5) | 6 (30) | 13 (56) | 5 (25) | 8 (40) | 3 | 2 (10) |
| **All** | **229** | **21 (9)** | **37 (18)** | **58 (28)** | **62 (30)** | **20 (10)** | **72 (35)** | **42** | **61 (29)** |

BL: borderline changes, * $p < 0.05$, ** $p < 0.01$

33 samples (16 %) showed normal histology and in further 28 cases (13 %) chronic allograft nephropathy (CAN) grade Ia, also considered as "normal". CNI toxicity was evident or suspected in 62 cases (30%), acute cellular rejection was observed in 37 cases (18%), borderline acute rejection occurred in 58 cases (28%), and chronic rejection was seen in 20 cases (10%). De novo glomerulonephritis was diagnosed in 11 biopsies (5%) and acute pyelonephritis in 14 biopsies (6%). In 37 samples (17%) chronic allograft nephropathy,

donor-related vascular disorder, nonspecific tubulointerstitial inflammation, or subcapsular scar was found. In three cases (1%), BK polyoma virus-induced nephropathy was diagnosed.

*4.3.3. Pathology in different posttransplant times*

Comparing the biopsy findings taken in different times after transplantation, no significant differences were found in the rate of subclinical acute rejection. However, acute cellular and borderline rejection taken together were significantly more frequent in 1 year protocol biopsy samples then in M3 biopsies ($\chi^2 = 3.8489$, $p < 0.05$)

In contrast, significant differences were found in the CNI toxicity rate and in the rate of chronic allograft nephropathy: CNI toxicity was diagnosed in 12% at 3 months, 28 % at 1 year and 50% at 3 year ($p < 0.05$), CAN was diagnosed in 17% of M3 biopsies, while in 57% of M12 samples ($\chi^2 = 25.69$, $p < 0.01$), but this rate has not increased after 1 year (Table 12., Fig. 11).

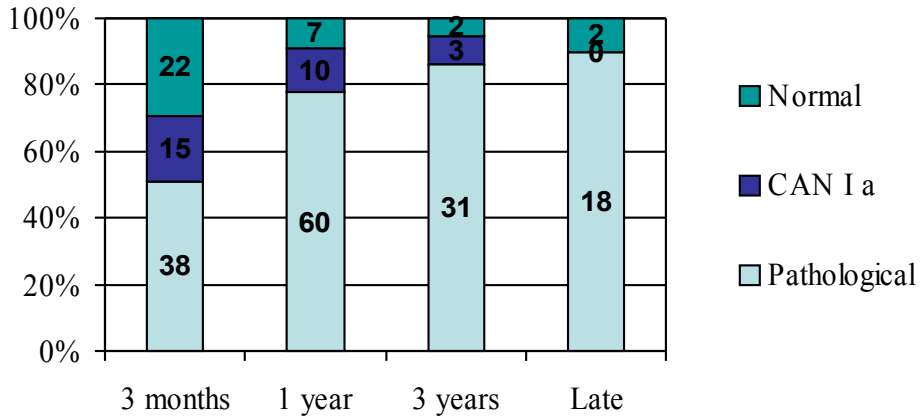



Figure 11. Protocol biopsy finding in different posttransplant times

*4.3.4. Comparison of different immunosuppressive protocols*

Comparing the Tac or CsA containing protocols, patients had protocol biopsy within 3 years after transplantation were examined. Significant differences were found between the patients receiving cyclosporine (CsA) and those taking tacrolimus (Tac). Although the all acute rejection rate was statistically not significant, higher grade acute rejections, Grade Ib and IIa were diagnosed more frequently in the CsA group then in the Tac group (14/84; 16% vs. 6/91; 7%, $\chi^2 = 4{,}38$, $p < 0{,}05$).

Analyzing the CNI toxicity rate, similar difference was observed, as it was 19% in the CsA group and 7% in the Tac group ($\chi^2$ = 4,93; p < 0,05). However, in the rate of mild or suspected toxicity was not significant difference between the two groups (Table 13).

Table 13. Different pathology in the protocol biopsy of patients taking CsA or Tac

| | N | AR (%) | AR > Ia (%) | CNI tox. (%) | Severe CNI tox. | CAN (%) | CR+ (%) |
|---|---|---|---|---|---|---|---|
| CsA | 84 | 23 | 16 | 27 | 19 | 52 | 15 |
| Tac | 91 | 13 | 7 | 24 | 8 | 35 | 4 |
| $\chi^2$ | | 2.67 | 4.38 | 2.35 | 4.93 | 6.12 | 7.65 |
| $p <$ | | NS | 0.05 | NS | 0.05 | 0.05 | 0.01 |

Occurrence of CAN and chronic rejection were also analyzed in these two groups. A significant advantage of the Tac group was observed: CAN occurred in 35% vs. 52% in the CsA group ($\chi^2$ = 6.12; $p$ < 0.05), similar rate was counted if only CsA/MMF and Tac/MMF combinations were compared, 33% vs. 51% ($\chi^2$= 4.19; $p$< 0.05). Chronic rejection (CAN CR+) was diagnosed also more frequently in the CsA group (15%), then in the Tac group (4%), and the difference was statistically significant ($\chi^2$ = 7.65; $p$< 0.01). Results are summarized in the Table 12 and Fig. 12.

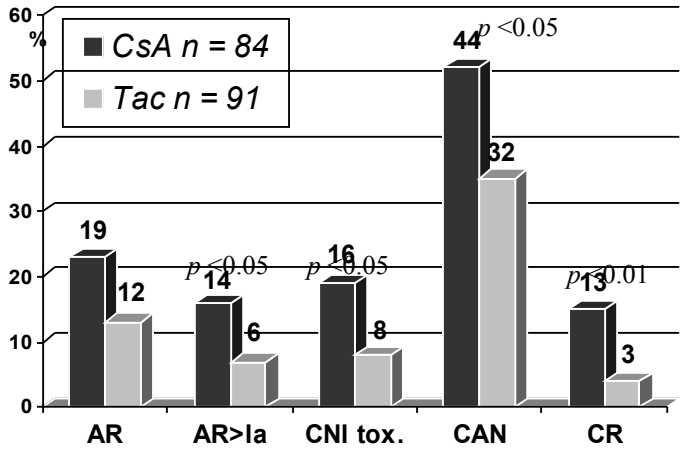



Figure 12. Comparison of CsA and Tac patients as concerns different pathologies revealed by protocol biopsy

*4.3.5. Kidney allograft function, graft survival*

There were no significant differences between the various patient groups, as concerns the demographic or baseline data (Table 1). The protocol biopsy group displayed a better graft

function 2 and 3 years after transplantation, relative to the patients who did not undergo biopsy. However, the differences at 2-year are not significant statistically (Table 14), a significantly better graft function was observed 3 years after transplantation in the protocol biopsy group.

Table 14. Graft function in different posttransplant times on protocol biopsy and non-biopsy groups

| Post TX time | Graft function | Protocol biopsy group ($n$ = 100) | Non-biopsy group ($n$ = 42) | $p$ value |
|---|---|---|---|---|
| 1-year | Creatinine (µmol/l) | 150 ± 33 | 157 ± 51 | 0.2 |
| | $eGFR_{CG}$ (ml/min/1.73m$^2$) | 54.3 ± 12.5 | 54.0 ± 16.2 | 0.4 |
| | $eGFR_{MDRD}$ (ml/min/1.73m$^2$) | 47.8 ± 11.8 | 47.4 ± 15.2 | 0.4 |
| 2-year | Creatinine (µmol/l) | 159 ± 45 | 187 ± 85 | 0.06 |
| | $eGFR_{CG}$ (ml/min/1.73m$^2$) | 52.5 ± 13.0 | 50.5 ± 18.0 | 0.3 |
| | $eGFR_{MDRD}$ (ml/min/1.73m$^2$) | 46.0 ± 12.7 | 44.3 ± 18.0 | 0.3 |
| 3-year | Creatinine (µmol/l) | 159 ± 45 | 217 ± 93 | **0.003** |
| | $eGFR_{CG}$ (ml/min/1.73m$^2$) | 51.1 ± 14.1 | 40.3 ± 15.5 | **0.002** |
| | $eGFR_{MDRD}$ (ml/min/1.73m$^2$) | 46.0 ± 13.8 | 35.4 ± 15.0 | **0.002** |

While the serum creatinine increased and the GFR decreased in the non-biopsy group, the renal function remained stable or was even better in the biopsy group ($\Delta eGFR_{MDRD}$ = 1.3 ± 7.8 vs. -9.2 ± 12.0 ml/min; $p$ = 0.002; $eGFR_{MDRD}$ = 35 ± 15 vs. 46.0 ± 13.8 ml/min; $p$ = 0.002; (Table 14, Fig. 13 and Fig. 14).

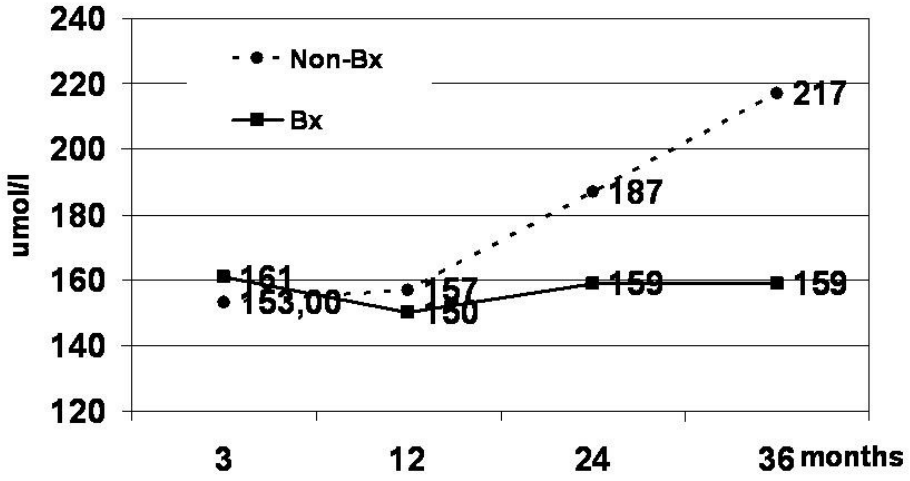



Figure 13. The 3-year serum creatinine is significantly higher in the non-biopsy group (p = 0.003)

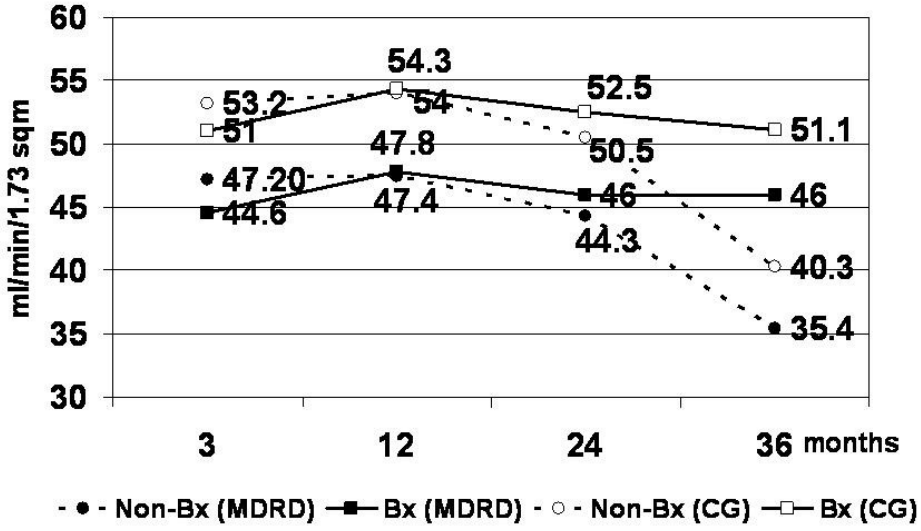



Figure 14. The estimated GFR values calculated both by Cockroft-Gault and MDRD formulas are significantly higher in the protocol biopsy patient group ($p = 0.002$).

Analyzing the rate of the graft function improvement, a significantly higher proportion of the patients had stable or better graft function in the biopsy group, than in the non-biopsy group: 59% *vs*. 33%, chi2 = 7.8002, $p < 0.01$ ( (Fig. 15).

4 years follow-up data are available from 97 patients by the end of year 2008, 65 from the biopsy group and 32 from the non-biopsy group. Graft loss occurred in 8 cases (2 DWFG and 6 returns to dialysis) from the biopsy group (4-year graft survival rate: 87.7%), whereas 13 (3

DWFG and 10 returns to dialysis) from the non-biopsy group (4-y graft survival: 59.4%), which is a highly significant difference ($\chi 2 = 10.1369$; $p < 0.001$).

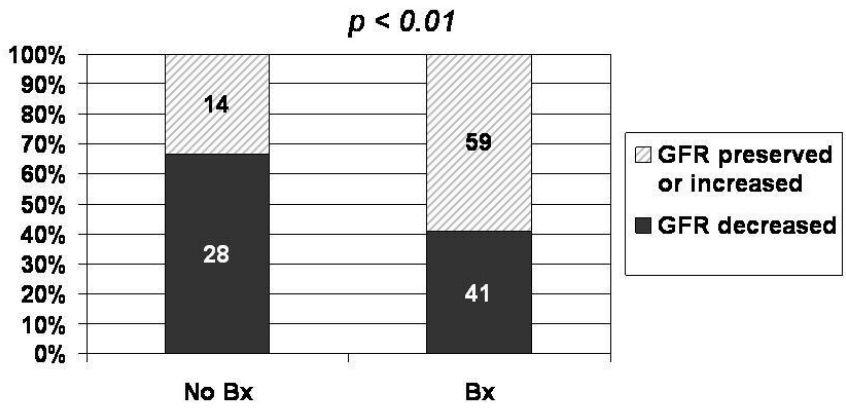



Figure 15. A significantly higher proportion of the patients have stable or better graft function ($\Delta GFR \geq 0$) in the protocol biopsy group (59% vs. 33%; $\chi^2 = 7.8002$; $p < 0.01$).

### 4.4. Conclusions

The present investigation was performed to answer several questions relating to protocol biopsies. The most important one was the question of the treatment of subclinical pathologies. We hypothesized that early detection and treatment should have beneficial effects on the long-term results such as the allograft function or even the graft survival. Other issues were the optimal time for the protocol biopsy, the effects of different immunosuppressive drugs, and the question of safety.

*What is the impact of protocol biopsy on the allograft function?*

The diagnosis of CAN may be useful to predict the quality of the graft function and the length of the graft survival (37), but the question remains of whether treat or not to treat subclinical pathologies.

The present clinical study has demonstrated that the dynamics of the renal function is better if patients participate in 1-year protocol biopsy taking after transplantation and any allograft pathology (not only subclinical AR, but other disorders too) is treated accordingly. Even after a 2-year follow-up period, the longest in similar previous studies (46, 47), a statistically significant change in the renal function has been found, but the importance of the difference

might be criticized by most clinicians, as a 3-5-ml/min change in GFR was not highly relevant. We considered that this tendency might predict a greater change later, and this proved to be correct 3 years after transplantation. The difference in the allograft function, of more than 10 ml/min for the biopsy group, is significant clinically and statistically.

Although the present study is a single-center trial, it has a great advantage that it involves an unselected patient population with standardized therapy of the detected subclinical AR, as advised by some experts (48), and also CNI toxicity or inflammatory diseases.

As the difference in the 4-year graft survival was statistically significant, we can conclude that early better graft function predicts a longer graft survival.

*What is the optimal time to perform the protocol biopsy?*

If the early detection and treatment of disorders can promote preservation of the allograft function, the question arises of the optimal time for protocol biopsy taking. Both morphological and clinical results must be considered here. The evidences from a study on a specific patient population recently led to the recommendation that the effects of both early (3-6-month) and 12-month protocol biopsies should be investigated (48).

In the present morphological study, a significantly lower rate of pathological disorders was found in the 3-month biopsies than in the 12-month biopsies. This correlated with the clinical finding that only the 1-year protocol biopsy patients demonstrated a significant difference in graft function from that in the non-biopsy group. A similar conclusion was drawn from an analysis (49) of the predictive value of protocol biopsies taken at different posttransplant times. Accordingly, the 1-year protocol biopsy seems to be more important than the 3-month biopsy. However, we suggest that early protocol biopsy taking should be performed not only in selected patient groups, such as immunological high-risk recipients (35, 50) or high CNI exposure patients (48), but in all transplanted patients, as 50% of the 3-month samples were found to reveal a possibly treatable pathology.

*Which calcineurin inhibitor should be used?*

CNIs have significantly reduced the incidence of AR after kidney transplantation. Nephrotoxicity, however, may contribute to a long-term allograft dysfunction (51). The

diagnosis of CNI nephrotoxicity is especially important, since this condition is reversible if immunosuppression is modified in time (52), though it is still not clear how long CNI should be continued to avoid CR (53).

The present comparison of the two CNIs demonstrated the advantages of Tac over CsA from several aspects. Although Tac and CsA have similar nephrotoxic effects (54), that of Tac seems to develop later: in this patient group, CNI toxicity was not found in the 3-month protocol biopsies, but only in 1-year biopsies; further, the prevalence of the CNI toxicity was significantly higher in the CsA group. Tac also offers more effective anti-rejection protection (55), confirming the findings (55, 56), of a better rejection rate and a better 3-year survival with Tac. We also observed a significantly lower rate of higher-grade (> Banff Grade Ia) AR in the Tac-treated group. We believe, that interstitial fibrosis and tubular atrophy might be consequences of earlier rejections (56) and/or drug toxicity, and hence can be reduced by using Tac instead of CsA. This hypothesis might be an explanation for the significant lower rate of CAN in the Tac-treated patients.

*Safety*

Finally, the clinical cost-benefit ratio was examined by analysis of the frequencies of different pathologies and complications. The safety of the protocol biopsy procedure was earlier tested on a high number of patients (41, 42), and it was concluded that the complication rate was low and utility rate was high. Although involving fewer patients, the present study indicated similar results: no major complication occurred and the minor complication rate (5%) was also very low. With the improved methodology, using a thicker (16-G) needle and taking a minimum of 2 cores, the utility rate reached 90%. Our morphological analysis result that a very high proportion of the allograft pathologies (71%) can be treated (66%) clearly demonstrates that the protocol biopsy should be an essential diagnostic tool in post-transplant patient care.

**In summary**, protocol biopsy taking is an excellent method for the early diagnosis of disorders developing in the transplanted kidney and for monitoring of the effects of immunosuppression. The protocol biopsy, followed by appropriate treatment, promotes preservation of the kidney allograft function, and therefore improves the long-term graft survival.

## 5. GENERAL CONCLUSIONS, NEW FINDINGS

In summary, the aim of my clinical research was to facilitate the improvement of long term kidney allograft function and survival, which was achieved by immediate introduction of the new immunosuppressive agents into the therapy, and by appropriate therapeutic response to the information based on the analysis of experiences and the consequent biopsies of the dysfunctional allografts. All these efforts resulted in nearly 90% of 1-year graft survival rate, and in longer term, 70% of 5-year graft survival after 2000, compared to the 55% between 1993 and 2000 (Fig.16).

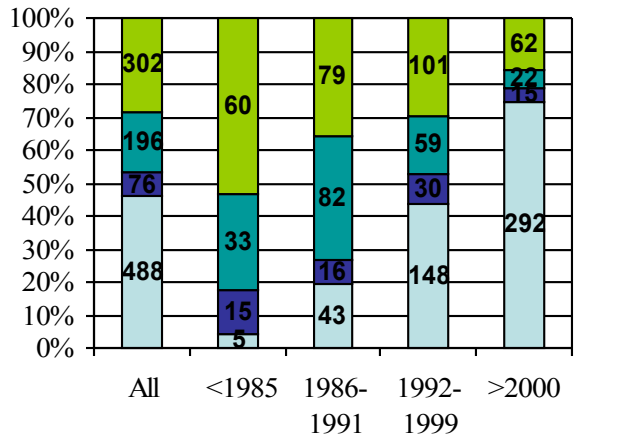



Figure 16. Graft loss in different era in the Szeged Transplant Center, 2008

I would like to highlight following new results:

1. It was pointed out first in Hungary that causality of the kidney allograft rupture is multifactor, and swelling of the renal parenchyma caused by T-cell mediated acute rejection is a major inducing process. Intensity of the T-cell mediated AR has been efficiently reduced by the introduction of anti-lymphocyte induction therapy as the part of immunosuppression, therefore threatening of the rupture of the transplanted kidney practically disappeared.

2. The ATG-bolus therapy has better clinical response and is more cost-effective, than the conventional, 10-day course.

3. The prevalence of the chronic rejection is significantly lower with mycophenolate mofetil, especially in combination with tacrolimus, as the lowest frequency of CR was found with Tac+steroid+MMF combination.

4. Our Center introduced the protocol biopsy as new diagnostic method into the clinical practice first in Hungary, and it is as far the only transplant center performing it. Treatment modifications based on the biopsy findings (subclinical AR, CNI toxicity, etc.) significantly improved the 4-year graft survival.

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# APPENDIX

# Publications

**Publications related to the subject of the thesis**

Full papers

I. Marofka F, Szenohradszky P, Csajbók E, **Szederkényi E**, Morvay Z, Iványi B. Ultrahangvizsgálat és biopszia szerepe és helye vesetranszplantáció után The role of sonography and biopsy after kidney transplantation. *Orv Hetil*. 1998; 139: 1843-1845

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**Publications, not related to the subject of the thesis**

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1. Márton János, Farkas Gyula, **Szederkényi Edit**, Avramov Katalin Az akut nekrotizáló pancreatitis szeptikus szövődményeinek kezeléséről Treatment of acute necrotizing pancreatitis and tis septic complications Orv. Hetil. 136: 893-895, 1995.
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