Summary and discussion
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This thesis is based on a case history of a young woman who lost her renal transplant from presumably chronic rejection (CR). She received a transplant from a cadaveric young male donor and was treated with maintenance prednisone and cyclosporine. She had 4% panel-reactive antibodies at the time of transplantation and there was a 1-2-0 HLA-A,-B,-DR mismatch. After an excellent first transplant year she participated in a randomized trial and was assigned for prednisone withdrawal. After a few weeks, an acute rejection episode (ARE) was diagnosed which responded on treatment with methylprednisolone, albeit without complete recovery of renal function. Gradually she developed proteinuria and a decline in renal function consistent with chronic transplant dysfunction (CTD). A biopsy demonstrated chronic allograft nephropathy (CAN) without signs of vasculopathy, glomerulopathy or cyclosporine toxicity. Renal dysfunction was progressive, despite treatment with a converting enzyme inhibitor; six years after transplantation she had to resume hemodialysis therapy.

What is CR? What are its clinical and histological manifestations? Did this patient loose her transplant from CR? What are the positive arguments to make the clinical diagnosis CR? To answer these questions we studied the epidemiological, clinical and histopathological features of CR in the Leiden cohort of renal transplants. The terms CR, CAN and CTD has often been used as synonym leading to ambiguity and even the proposal to abandon the term CR. We favour the following definitions (chapter 1). CR is an alloantigen-dependent process leading to CAN and CTD ultimately resulting in failure of the transplant. CAN is a descriptive term of the characteristic pathology consisting of fibrous intimal thickening of arteries, interstitial fibrosis and tubular atrophy. CTD is a clinical syndrome characterized by a slowly rising plasma creatinine concentration, increasing proteinuria and worsening hypertension. All definitions contain the adjective “chronic” that conveys different meanings. It describes late and lasting in time, i.e. beyond 3 months post-transplantation (CTD), persistent or recurrent injury (CR), and scarring and atrophy as pathological features (CAN). Because all these meanings apply in most patients undergoing CR, the term “chronic” is appropriate. In the Banff ‘97 classification of renal allograft nephropathy CAN has been graded by the severity of interstitial fibrosis and tubular atrophy. De novo vasculopathy or glomerulopathy supports the histological diagnosis of CR. CR should be differentiated from other causes of CTD, such as chronic calcineurin inhibitor (CNI) nephrotoxicity, recurrent or de novo glomerulonephritis, nephrosclerosis, transplant renal artery stenosis and BK virus nephropathy, entities which lead
to CTD and CAN and may occur alone or together. In the case report, the clinical diagnosis of CR could be made despite the lack of specific histological findings. The presence of immunological risk factors, a biopsy showing CAN, absence of other causes of CTD, and subsequent graft loss were considered as positive arguments supporting CR. This thesis focuses on the risk factors of graft loss from CR (chapter 2 and 3), prediction of graft failure (chapter 2, 4 and 5), cyclosporine nephrotoxicity (chapter 6), the different histological manifestations of CR (chapter 7 and 8), and one of the mechanisms of CR (chapter 8).

To evaluate the risk factors of CR, we studied all 654 cadaveric renal transplants performed in Leiden between 1983 and 1996 that had survived for more than six months (chapter 2). Biopsies obtained beyond 6 months were reviewed blinded for clinical information and scored according the Banff '97 classification. A total of 224 grafts were lost, mainly from patient death with a functioning transplant (53%). After exclusion of other causes, 82 transplants (36%) were lost from presumably CR and used as outcome variable (figure 1). In 62 out of these 82 cases graft histology was available showing CAN in absence of other causes of CTD. Recipient, donor, transplantation and clinical variables were collected and assessed as risk factors using uni- and multivariate Cox regression analysis. The impact of HLA matching was studied in detail at

![Figure 1](Causes of graft failure after 6 months, occurring in 224 out of 654 transplants between 1983 and 1996.)

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the level of broad and split antigens. Furthermore, MHC class I antigens were
assigned to one or more public epitopes, the so-called cross reactive groups
(CREG). Not only was the degree of mismatching between donor and recipient
studied but also the effect of sharing HLA-antigens. The term ‘shares’ was
used for the number of corresponding HLA antigens between donor and
recipient. HLA mismatches were not related with graft loss but in contrast,
sharing of HLA antigens, especially CREG, improved long-term graft survival.
The discrepancy between mismatches and shares could be explained by cross-
tabulation, demonstrating a non-reciprocal relationship. A higher percentage
of homozygosity of donors compared to recipients, as result of selection on
mismatches, appeared to be responsible for this finding. Hypothetically, more
shared antigens between donor and recipient may down regulate the response
to alloantigens. As known from the literature, ARE had a strong impact on
graft survival. We extended earlier data from our centre that acute vascular
rejection has also a detrimental effect on late outcome. Furthermore, the number
and timing of ARE were of importance. Young recipients were at risk likely as
result of an increased immune responsiveness and possibly non-compliance.
Old donor age influenced outcome, with increased immunogenicity, and
reduction in renal mass as possible mechanisms. Finally, smoking and
presumably the presence of atherosclerosis in recipients with nephrosclerosis
as baseline disease increased the risk of graft loss from CR. The reported
recipient was young, shared only one CREG with her donor and experienced a
late ARE, three factors explaining her risk to develop CR.

The late ARE was considered the most important risk factor of CR in this
patient. Between 1983 and 1996, ARE occurred frequently, allowing
epidemiological studies. In 384 of 654 transplant recipients (59%), one or more
treated ARE were documented. However, not all these ARE had an adverse
outcome. In most studies, early and late ARE are divided by the onset of the
first ARE. However, we observed more contrast in prognosis when the onset
of the last treated ARE was used as the time factor (chapter 2). The last ARE
occurred in 297 of 384 transplant recipients (77%) within 3 months and in 87
of 384 (23%) after 3 months (chapter 3). Ten-year graft survival rates censored
for causes of graft loss other than CR were 94%, 86%, and 45% for patients
without ARE, with early ARE, and with late ARE, respectively. In the latter
group, prognosis did not depend on the presence of previous early ARE.
Applying multivariate logistic regression analysis, the predictor variables of
the two groups were compared with transplants without ARE. Delayed graft
function, and HLA-DR mismatches were independent risk factors for ARE
within 3 months. In this immediate period after transplantation donor dendritic
cells are present in the graft. Allo-class II antigens expressed by these cells are
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recognized by T helper cells of the recipient in the so called direct pathway. The HLA-DR effect diminishes over time once the passenger cells disappear and donor-specific T helper cell hyporesponsiveness has been induced. In contrast to early ARE, young recipient age, old donor age, female donor gender, and CREG mismatches were associated with ARE beyond three months. These data strongly link late ARE with CR suggesting similarities in the pathogenesis with a role of the indirect pathway allorecognition. Hereby, recipient T cells recognize alloantigens that are shed from the graft, processed and presented by recipient antigen presenting cells. Interaction of helper T cells with B cells might be relevant for the induction of anti-donor antibodies possibly leading to subsequent chronic humoral rejection. Late rejection activity through the physiological indirect pathway might happen even under current immunosuppressive treatment. This explains the relatively minor change in long-term outcome despite significant reduction in the incidence of ARE and the increased impact of ARE on chronic allograft failure in recent time. Risk factors may operate differently in the process of graft attrition (chapter 4). They can influence the input and the pace of deterioration in function. Therefore, we compared the risk factors of a low intercept, defined as a creatinine clearance lower than 50 ml/min at 6 months, and of a negative slope of the reciprocal creatinine concentrations after 6 months. Two hundred of 654 grafts (31%) failed to reach optimal function because of old donor age, female gender of the donor, histoincompatibility, delayed graft function or ARE in the first 6 months. Forty-four percent (288/654) of all grafts displayed progressive deterioration of function over time, which was not related with donor factors and delayed graft function. However, the association with younger recipient age, sensitization, class I histoincompatibility, baseline immunosuppression and late ARE suggests an underlying immunological process. The influence of histoincompatibility and late ARE on both the intercept and the slope supports the link between late ARE and CR. The rate of decline is worse for grafts with a creatinine clearance of < 50 ml/min compared with grafts with a clearance of > 50 ml/min at 6 months posttransplantation. The negative impact of proteinuria and hypertension at six months on the slope is compatible with their role as progression factors. To improve the prediction of late graft failure we developed a model with time dependent renal function covariates (chapter 5). In contrast to time-fixed covariates, time-dependent covariates are measured repeatedly over time, where the number of observations and the time between the observations may vary between patients. All available serum creatinines taken beyond 6 months were used to obtain several time-dependent covariates. In a multivariate Cox proportional hazards model, cadaveric compared to living renal transplantation,
a lower reciprocal creatinine (RC) beyond 6 months and a lower ratio between RC and the RC at 6 months were independently associated with graft failure from CR or recurrent disease. This model was significantly better compared to a model with only time fixed parameters and therefore allows updates in prognosis during follow-up. In other words, renal dysfunction and a sharper decline in renal function beyond 6 months is more accurate predictor of subsequent graft failure compared to risk factors available at 6 months post-transplantation.

In our cohort, cyclosporine toxicity is the most important differential diagnosis of CR especially because of its therapeutic implications. In 1995-1996 we had to convert Sandimmune to Neoral, a micro-emulsion form of cyclosporine with a better bioavailability. Furthermore, a once-daily regimen with 24-hour target levels of 100 µg/l was changed to a twice-daily dosage aiming at a 12-hour target of 150 µg/l. Several patients developed a gradual decline in renal function and we decided to perform a retrospective cohort study to assess cyclosporine toxicity (chapter 6). Of 212 patients with a stable graft function pre-conversion clinical parameters at 1 and 12 months post-conversion were compared with those at time of conversion. The mean cyclosporine trough level rose from 87 µg/l at the time of conversion to 139 µg/l at 12 months post-conversion whereas the daily drug dose increased over the same period from 233 mg to 252 mg. Mean serum creatinine increased by 10% from 135 to 148 µmol/l. Cyclosporine nephrotoxicity was defined in 42 patients (20%) as a significant decline of the reciprocal of the serum creatinine concentration over time post-conversion in the absence of other obvious causes for declining graft function. Biopsies performed in 10/42 patients showing arteriolar hyalinosis and stabilisation of renal function after dose reduction or switch to mycophenolate mofetil supported the diagnosis. The increased exposure to cyclosporine allowed a risk assessment of nephrotoxicity. Cyclosporine dose and trough level did not predict nephrotoxicity but the use of beta blockers or calcium channel blockers reduced the risk of nephrotoxicity, independent from their effect on blood pressure. Both drugs may counteract cyclosporine induced vasoconstriction, which is mediated by sympathetic activation. As result of this study, we abandoned the target drug levels and reduced the cyclosporine dose on clinical grounds. Area under the curve (AUC) monitoring using 0, 2 and 3 hour blood samples is currently under evaluation to improve immunosuppressive drug dosing.

Chronic allograft nephropathy (CAN) is defined and graded in the Banff ’97 scheme by the severity of interstitial fibrosis and tubular atrophy. It has been denoted that the diagnosis of chronic rejection requires typical vascular lesions, consisting of fibrointimal thickening (chapter 1). As illustrated in the case
report we observed several patients who developed CAN without vascular changes or signs of cyclosporine toxicity and questioned the arguments for CR in this group of patients (chapter 7). Therefore, we categorized CAN according the Banff CV score in a group with and without transplant vasculopathy and assessed their risk factor profiles. Fifty-four transplants had CAN defined by a significant decline in renal function together with interstitial fibrosis and tubular atrophy without signs of cyclosporine nephrotoxicity or recurrent disease. Using the Banff CV score, 23 of 54 cases (43%) had a chronic vasculopathy score of 0 or 1 whereas 31 cases (57%) had a CV score of 2 or 3. Applying multivariate logistic regression, predictor variables of the two groups were compared with 231 transplants with a stable function for at least five years. Late ARE after three months post-transplantation was the strongest risk factor for both forms of CAN. CAN with vasculopathy was also associated with transplants performed in the 1980s, and with creatinine clearance at 6 months. In contrast, young recipient age and pre-sensitization were the other independent risk factors for CAN without vasculopathy suggesting an immune pathogenesis. Disruption of the tubular basement membrane, herniation of tubular cells with differentiation into myofibroblasts, as consequence of persistent tubulitis has been shown by others to link ARE with chronic interstitial rejection in the absence of arterial injury. As demonstrated in humoral ARE, pre-sensitization might also correlate with humoral CR, but the presence of donor specific antibodies and C4d deposits in PTC, an in-situ marker for humoral immunity, was not assessed in this study. However, in the transplant recipient reported at the start of this thesis, C4d staining was retrospectively performed and appeared to be negative, suggesting dominance of the cellular immune response. Compatible with the findings in this patient, we conclude that vascular lesions are not a condition sine qua non for the diagnosis of CR. Finally, we studied the epidemiology and (immuno-) histological features of 18 patients with chronic transplant glomerulopathy (CTG) (chapter 8). These patients had biopsies taken at 7.5 ± 3.5 years, on average 4 years later compared to 108 patients with CR without these glomerular lesions. Review of the histology revealed influx of mononuclear cells in the glomeruli in addition to the double contours of the glomerular basement membrane (GBM) together with a nonspecific immunofluorescence pattern. Multivariate analysis in comparison with 739 patients with a stable function revealed pre-transplant sensitization and ARE beyond 3 months as independent risk factors of CTG, similar to the risk profile of CR. Polyclonal anti-C4d antibody was used on paraffin sections for detection of C4d, a marker of humoral immunity. C4d stained positive in the glomeruli of 10/11 biopsies showing CTG in contrast to 1/14 positive biopsies in control patients with chronic rejection without CTG.
The risk factors and the presence of glomerular C4d deposits suggest a role of humoral immunity in the development of CTG. Additional results revealed that patients with glomerular C4d deposits had concomitant PTC deposits in 4/10 and donor specific anti HLA antibodies in 3/10 patients leaving the question open whether there are also tissue-specific antibodies involved. In a Fisher to Lewis rat model of CTG used in our centre IgG antibodies against the glomerular basement membrane (GBM) were found. Perlecan was identified as one of the antigens recognized. In clinical CTG, 9/13 sera contained antibodies directive to a non-Goodpasture antigen of the GBM. Preliminary results demonstrated that these antibodies might be reactive to agrin, a heparan sulphate proteoglycan with similar functions as perlecan. All C4d positive patients had anti-HLA and/or anti-GBM antibodies. Concerning the immunological risk factors, glomerular C4d deposits and presence of antibodies we concluded that CTG has to be considered a manifestation of humoral CR. In this thesis, we assessed the risk profiles of different manifestations of CR. Table 1 summarizes the most important predictors of CR, defined by a significant decline in renal function and histological features of CAN in the absence of chronic cyclosporine nephrotoxicity and glomerulonephritis. Independent of the presence of early ARE, recipients with ARE occurring beyond three months had the highest risk to develop one of the CR manifestations. The development of CAN could be explained by a fibrogenic response to intermittent or persistent tubulointerstitial injury. This unfavourable ARE correlated with CREG mismatches. Therefore, we conclude that histoincompatibility results in CR via late ARE. Pre-sensitization increased the risk of CR independent of late ARE. It correlated with chronic interstitial rejection and transplant glomerulopathy, perhaps mediated by circulating donor-specific antibodies post-transplantation. The glomerular deposits of C4d provided support of humoral CR. Tissue-specific antibodies might be involved in CR, explaining the differences in histological presentation. Fibrous intimal thickening was associated with older donor age, putting the specificity of chronic

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<th>Table 1. Independent risk factors of 54 cases with chronic rejection in comparison with 231 transplants with a stable function</th>
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<tr>
<td>Risk factor</td>
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<tr>
<td>Recipient age (per 10 years increase)</td>
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<tr>
<td>Smoking cigarettes</td>
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<tr>
<td>Panel reactive antibodies (per 10% increase)</td>
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<td>Last ARE &gt; 3 months versus no ARE</td>
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<td>Dipstick proteinuria &gt;1+ @ 6 months (%)</td>
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<td>ECC @ 6 months (10 ml/min increase)</td>
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Abbreviations: OR: odds ratio, CI: confidence interval, ARE: acute rejection episode, ECC: endogenous creatinine clearance
vascular lesions in doubt. On the other hand, early acute vascular rejection, in itself associated with HLA-DR mismatches, was also correlated with transplant vasculopathy suggesting a transition of the acute into the chronic form. The association of both class I and class II MHC mismatches and graft outcome warrants the current practice of HLA matching. Computer-based algorithms, such as HLAMatchmaker, that determine donor-recipient compatibility at the molecular level may better prevent CR despite a reduction in immunosuppressive drugs. Immune monitoring of both the cellular and humoral response post-transplantation may tailor treatment in the individual patient. We found that smoking cigarettes at time of transplantation increased the risk on CR by a factor 2. This habit should be stopped with the maximum aid of supportive care. Renal dysfunction, proteinuria and hypertension at 6 months post-transplantation and at time of the diagnostic biopsy were found as progression factors. Early and tight control of blood pressure and proteinuria, preferably with angiotensin converting enzyme and/or angiotensin II receptor blockers should prevent or postpone premature graft failure from CR.

This thesis concludes with the answers to the fundamental questions raised. What is CR? The CR concept includes a persistent or recurrent cellular and/or humoral alloimmune process resulting in a response to tissue injury. All anatomical compartments of the renal cortex can be affected by CR leading to transplant vasculopathy and/or glomerulopathy, and chronic interstitial rejection. Why does CR occur? CR may develop in patients with coexisting immunological risk factors and suboptimal immunosuppression. Young recipient age, histoincompatibility, sensitization, and especially late ARE explained the presence of CR in the case report. In patients with chronic transplant dysfunction, transplant physicians should take these risk factors into account as diagnostic tests influencing the chance on CR. Smoking, hypertension and proteinuria act as modifiable factors that accelerate progression. Multifactorial prevention and intervention strategies directed to these risk and progression factors are an achievable goal in our daily practice to prevent premature graft loss from CR.