Fernanda Ortiz, MD

Detection and progression of chronic allograft nephropathy

A study based on protocol biopsies
To my lovely daughters Guadalupe and Micaela,
my husband Marcus,
and my parents Margarita and Fernando
Contents

I. List of original publications .......................................................... 7
II. Abbreviations .............................................................................. 8
III. Abstract ..................................................................................... 9

1. Introduction ................................................................................ 11

Review of the literature ................................................................. 13
2. Pathophysiology of chronic allograft nephropathy (CAN):
   Immune-dependent factors ............................................................ 14
   2.1. HLA antigens ......................................................................... 14
   2.2. Sensitization .......................................................................... 15
   2.3. Acute rejection ....................................................................... 15
   2.4. Sub-clinical acute rejection ....................................................... 17

3. Pathophysiology of CAN: Non-immune-dependent factors ...... 18
   3.1. Donor factors .......................................................................... 18
   3.2. Donor brain death ................................................................... 18
   3.3. Both ischemia and reperfusion ................................................ 19
   3.4. Delayed graft function ............................................................. 19
   3.5. Sub-optimal immunosuppression .............................................. 19
   3.6. Hypertension .......................................................................... 20
   3.7. Dyslipidemia .......................................................................... 22
   3.8. Post-transplant diabetes mellitus (PTDM) ................................ 24

4. Other causes of kidney allograft damage .................................... 26
   4.1. Calcineurin inhibitors-related toxicity .................................... 26
   4.2. Recurrent and de novo glomerular disease ............................. 30
   4.3. Viral Infections ...................................................................... 30
5. **Diagnosis of kidney allograft damage** ................................. 33
   5.1. Classifications in kidney transplant pathology:
       Chronic allograft damage index .................................. 33
   5.2. Classifications in kidney transplant pathology: Banff:
       terminology and its evolution over time ......................... 35
   5.3. Protocol biopsies ..................................................... 37
   5.4. Markers of kidney graft dysfunction ............................. 38
   5.5. Immunohistochemical techniques: TGF-β ......................... 41
   5.6. Microarrays .......................................................... 43

6. **Aims of the study** ......................................................... 44

7. **Material and methods** .................................................. 45
   7.1. Patients ................................................................. 45
   7.2. Protocol biopsies ...................................................... 45
   7.3. Biopsy technique ...................................................... 46
   7.4. Histological analysis .................................................. 47
   7.5. Clinical variables ...................................................... 49
   7.6. Biochemical variables ................................................ 49
   7.7. Statistical analysis .................................................... 50

8. **Results** ........................................................................ 51
   8.1. Predictors of renal allograft histologic
       damage progression (I) .................................................. 51
   8.2. Timing and value of protocol biopsies (II) ....................... 52
   8.3. Sensitivity and specificity of several estimates of
       GFR to predict CADI (III) .............................................. 54
   8.4. Expression of TGF-β₁ in protocol biopsies (IV) ............... 55
       8.4.1. TGF-β₁ expression in protocol biopsies and its
               relationship with CADI and exposure to CsA .......... 55
8.4.2. TGF-β₁ expression in protocol biopsies and kidney allograft function

8.5. Summary of the histological results

8.5.1. Summary of CADI in protocol biopsies

8.5.2. Summary of AR, SAR and immunosupression

8.5.3. CsA toxicity in protocol biopsies

8.6. Summary of the progression of histological damage (ΔCADI)

8.7. Summary of the risk factors involved in the progression of CADI

8.8. CADI and ΔCADI as predictors of long-term kidney allograft function

8.9. Impact of SAR and borderline AR on long-term allograft function

9. Discussion

9.1. Pathophysiology of CAN

9.2. Tools for clinical surveillance of kidney allograft status

10. Conclusions

11. Finnish summary (Yhteenveto)

12. Acknowledgments

13. References
I. List of original publications

This thesis is based on the following publications referred to in the text by their Roman numerals:


IV Fernanda Ortiz, Ilkka Helanterä, Anne Räisänen-Sokolowski, Eero Honkanen and Petri Koskinen. *Association between cyclosporin A C2 levels with TGF-β1 expression in protocol kidney allograft biopsies*. Submitted to Transplant International

The original publications are reproduced with permission of the copyright holders.
ii. Abbreviations

ACEi: angiotensin converting enzyme inhibitor
AR: acute rejection
ARB: angiotensin II receptor blocker
AUC: area under the time concentration curve
CADI: chronic allograft damage index
CAN: chronic allograft nephropathy
CIT: cold ischemia time
CKD: chronic kidney disease
CMV: cytomegalovirus
CNI: calcineurin inhibitor
CRAD: chronic renal allograft dysfunction
CsA: cyclosporine A
CyC: cystatin C
C0: CsA blood concentration prior to the morning dose
C2: CsA blood concentration 2 h after the morning dose
DGF: delayed graft function
e-GFR: estimated glomerular filtration rate
GFR: glomerular filtration rate
HLA: human leukocyte antigen
HT: hypertension
IFTA: interstitial fibrosis and tubular atrophy
IGT: impaired glucose tolerance
KTX: kidney transplantation
MMF: mycophenolate mofetil
m-Tor: mammalian target of rapamycin
PAS: periodic acid Shiff
PTDM: post-transplant diabetes mellitus
RAAS: renin angiotensin aldosterone system
ROC: receiving operator characteristics
SAR: sub-clinical acute rejection
TGF-β: transforming growth factor β
iii. Abstract

Background. Kidney transplantation (KTX) is considered to be the best treatment of terminal uremia. Despite improvements in short-term graft survival, a considerable number of kidney allografts are lost due to the premature death of patients with a functional kidney and to chronic allograft nephropathy (CAN).

Aim. To investigate the risk factors involved in the progression of CAN and to analyze diagnostic methods for this entity.

Materials and methods. Altogether, 153 implant and 364 protocol biopsies obtained between June 1996 and April 2008 were analyzed. The biopsies were classified according to Banff ’97 and chronic allograft damage index (CADI). Immunohistochemistry for TGF-β₁ was performed in 49 biopsies. Kidney function was evaluated by creatinine and/or cystatin C measurement and by various estimates of glomerular filtration rate (GFR). Demographic data of the donors and recipients were recorded after 2 years’ follow-up.

Results. Most of the 3-month biopsies (73%) were nearly normal. The mean CADI score in the 6-month biopsies decreased significantly after 2001. Diastolic hypertension correlated with ΔCADI. Serum creatinine concentration at hospital discharge and glomerulosclerosis were risk factors for ΔCADI. High total and LDL cholesterol, low HDL and hypertension correlated with chronic histological changes. The mean age of the donors increased from 41–52 years. Older donors were more often women who had died from an underlying disease. The prevalence of delayed graft function increased over the years, while acute rejections (AR) decreased significantly over the years. Sub-clinical AR was observed in 4% and it did not affect long-term allograft function or CADI. Recipients’ drug treatment was modified along the Studies, being mycophenolate mophetil, tacrolimus, statins and blockers of the renine-angiotensin-system more frequently prescribed after 2001.

Patients with a higher ΔCADI had lower GFR during follow-up. CADI over 2 was best predicted by creatinine, although with modest sensitivity and specificity. Neither cystatin C nor other estimates of GFR were superior to creatinine for CADI prediction. Cyclosporine A toxicity was seldom seen. Low cyclosporin A concentration after 2 h correlated with TGF-β₁ expression in interstitial inflammatory cells, and this predicted worse graft function.

Conclusions. The progression of CAN has been affected by two major factors: the donors’ characteristics and the recipients’ hypertension. The
increased prevalence of DGF might be a consequence of the acceptance of older donors who had died from an underlying disease. Implant biopsies proved to be of prognostic value, and they are essential for comparison with subsequent biopsies. The progression of histological damage was associated with hypertension and dyslipidemia. The augmented expression of TGF-β1 in inflammatory cells is unclear, but it may be related to low immunosuppression. Serum creatinine is the most suitable tool for monitoring kidney allograft function on every-day basis. However, protocol biopsies at 6 and 12 months predicted late kidney allograft dysfunction and affected the clinical management of the patients. Protocol biopsies are thus a suitable surrogate to be used in clinical trials and for monitoring kidney allografts.
1. Introduction

Since the first successful kidney transplantation (KTX) in 1954 between identical twins, a new modality to treat patients with terminal kidney insufficiency was born. Although the results in the first decades were modest, continuous improvement has characterized this fascinating field. A major breakthrough was the introduction of the new immunosuppressant cyclosporine A (CsA) in the early 1980s. The fundament of its success was the ability to improve kidney graft survival considerably over the first year, and CsA is the cornerstone of immunosuppression even in the present decade.

KTX is considered the most suitable method for treating terminal uremia, taking into consideration patient’s outcome, quality of life and cost of treatment. The number of patients receiving a kidney transplant has increased during the past years. We are witnessing a remarkable growth in the number of patients entering dialysis, and unfortunately this is not paralleled by an equal increase in the kidney donor pool. The obvious consequence is that patients remain longer on dialysis while waiting for a kidney transplant. Several attempts to rectify this imbalance have been made, such as augmenting the donor pool by expanding the criteria for donation, e.g. by accepting cardiac death donors, and promoting living donation. The situation remains critical despite these efforts, and the increasing number of patients dying while waiting for a transplant is alarming. According to the newsletter of the Council of Europe, by December 2007, a total of 50,223 European dialysis patients were on the waiting list, and in the same year, 938 patients died while waiting for a kidney transplant.

The improvement of graft survival during the first years after transplantation has been largely achieved through a reduction in the rate of acute rejection (AR) episodes. In the Finnish population the AR rate fell from 37% in the mid-1980s to 20% at the beginning of this century (Salmela, 2004), and even lower rates, around 10%, have been reported in the past few years (Kyllönen, personal communication). This improvement was reflected in the longer kidney graft survival, which increased at five years from 73% to 94%. Despite the fact that short-term graft survival has improved considerably over the past 10 years, a similar success has not yet been achieved in the long run. The loss of functional kidneys due to the premature death of transplanted patients is of great concern. This represents the most common cause of graft loss, in addition to chronic allograft nephropathy (CAN). It is thus of vital importance to understand the mechanisms involved in the relentless damage of a kidney transplant.
aim is to prolong the survival of kidney allografts in order to overcome the shortage of donors.

The studies described in the following were conducted with the intention to elucidate some of the complex mechanisms behind the pathogenesis and prognosis of CAN, and to search for accurate methods for diagnosing it.
Review of the literature
2. Pathophysiology of chronic allograft nephropathy (CAN): Immune-dependent factors

The term CAN was proposed in 1991, and it replaced the previously used term “chronic rejection”. The intention was to unify chronic histological changes seen under light microscopy, such as interstitial fibrosis, tubular atrophy, transplant glomerulopathy and vasculopathy. The pathophysiology behind each of these features may nevertheless be different. The processes involved are approached by dividing them roughly into immunological and non-immunological factors, although they may be interrelated.

2.1. HLA antigens present in the donor but absent in the recipient are counted as HLA-mismatches. HLA-A, -B and -DR mismatches have been associated with poor graft survival. Although organ allocation relies on several non-immunological and logistic factors, a close HLA match is desirable. A report on the effect of HLA-mismatch on cadaver kidney allograft survival in 1999 was based on data reported by 301 transplantation centers. It was clearly demonstrated that the greater the number of mismatches, the poorer the survival at 5 years of follow-up. (Opelz, 1999) In 2004 data from cadaver kidney transplants performed during 1994 and 1998 were collected from the United Network of Organ Sharing (UNOS) and the United States Renal Data System (URSD). Altogether 33,433 recipients were followed up for 2.2 years and, in addition to HLA-mismatch, the effect of non-immunological factors such as cold ischemia time, donor age, gender and cause of death, donor and recipient body size were also analyzed. The authors concluded that the significance of HLA-matching is diminishing in the era of new immunosuppressive drugs, but that the impact of non-immunological factors on survival had remained unaltered. (Su, 2004) More efficacious immunosuppressive regimens (i.e. tacrolimus, mycophenolate mofetil (MMF), induction therapies) have gained popularity after the year 2000. The importance of HLA-mismatch in this era of modern immunosuppression was re-evaluated in the largest European study, where the results of two eras: 1985–1994 and 1995–2004, were compared. A total of 135,970 cadaver kidney transplants were followed up for 5 years. Although the survival rates improved over the years, HLA-mismatches still had a clear impact. A multiregression analysis of factors contributing to graft survival revealed that the impact of HLA-mismatches on graft survival was equally strong in the two decades compared. (Opelz, 2007)
2.2. Sensitization to foreign HLA is observed in patients on the transplant waiting list. The main routes of sensitization are blood transfusion, pregnancy and transplantation. The increased use of erythropoietin lessened the need for blood transfusions. Although leukodepleted transfusions are routinely used, they can still stimulate the production of HLA antibodies. (van de Watering, 2003) Antibodies can be identified in several ways, but the most commonly used is the complement-dependent toxicity test. The patient’s serum is tested against a panel of lymphocytes from HLA-typed individuals. If the serum contains antibodies, the addition of complement results in cytolysis that is visualized by staining, followed by microscopic evaluation. The frequency of positive reactions (i.e. % of panel-reacting antibodies) gives an indication of potential local organ donors with whom the patient would cross-match positively. Modern tests, such as flow cytometry and Luminex®, are able to measure these antibodies with increased sensitivity. Transplantation in the presence of donor-specific antibodies is associated with a high risk for acute rejection. This risk depends also on the titer of these antibodies. Lower titers might only be detected by the more sensitive techniques. (Fuggle, 2008) Thus, the clinical practice is to transplant patients with a negative cross-match, but in certain cases donor-specific HLA antibodies can be generated also after transplantation. There is robust evidence linking these donor-specific antibodies to the pathogenesis of chronic rejection. The histological hallmark is the detection of the diffuse deposition of a fragment of complement C4d in peritubular capillaries, in addition to the classical features of chronic rejection. (Racusen, 2006) Evidence was first presented in 2001 when biopsies from 38 patients with features of chronic rejection showed a positive staining for C4d, and 88% of them had donor-specific antibodies in their serum. (Mauiyyedi, 2001)

2.3. Acute rejection (AR) is a major determinant of both short-term and long-term graft survival. Already in 1994 it was pointed out that the graft half-life was significantly diminished if AR occurred later than one year after KTX, but even when it occurred within the first year, AR had also a negative effect. (Matas, 1994) Later, the impact of AR on CAN was compared in two different periods: 1984–1987 and 1991–1994. A reduction in the number of AR cases was interpreted as a decrease in chronic rejection from 31 to 14%, and the half-life of cadaver allograft kidneys was prolonged from 9 to 11 years. (Matas, 1999) Repeated AR was also demonstrated to affect graft survival negatively, and to be a risk factor for CAN. The half-life of grafts was reported to be shortened to 3.9 years if multiple AR occurred, compared to 16.9 years with no AR. (Ferguson 1994)

In a more recent study, the graft survival at 5 years had fallen to 52% if multiple episodes of AR occurred, vs 85% if there was only one episode of AR. Risk factors for multiple episodes of AR were early AR, severe AR, and
either delayed graft function (DGF) or slow graft function. (Humar, 2000)

The effect of the timing of AR on graft survival was re-evaluated in the present era of immunosuppressants. In a study published in 2003 on 654 KTX patients who were followed up for 10 years, early AR had a less negative impact than late AR. 85% of grafts with early AR (i.e. within the first 3 mo) survived, compared to 45% of those with late AR (over 3 mo). The subset of grafts without AR had the best outcome: 94% at 10 years when deaths were censored. (Sijpkens, 2003) (Figure 1)

![Figure 1. Kaplan-Meier graft survival, censored for causes other than chronic rejection for transplants without AR (solid line), with AR within 3 mo (dashed line) and with AR after 3 mo (dot line). (Sijpkens, 2003)](image_url)

There is, however, some discrepancy in the literature on the negative impact of early AR on death-censored graft failure or CAN, especially when more recent studies are included. (He, 2004) In the era of modern immunosuppression, not only the number but also the severity of AR has been successfully reduced. These improvements have been linked with the use of tacrolimus, MMF and induction therapies. (Pallardo Mateu, 2004) However, sensitization, HLA mismatches, prolonged cold ischemia times, and kidneys from older donors still represent risks for AR and subsequent chronic renal allograft dysfunction (CRAD).
2.4. **Sub-clinical acute rejection** (SAR). It has been known for a long time that biochemical monitoring of kidney function poorly reflects histological findings. The necessity to understand the mechanism behind CAN was the rational for obtaining biopsies from well functioning kidney allografts, hereafter called protocol biopsies. In a series of 70 protocol biopsies from 31 patients with stable kidney function obtained at one, two and three months post transplantation AR was diagnosed in 30% of the patients, the condition being named SAR. (Rush, 1994) SAR may represent either the evolving or the resolving phase of an alloimmune response. Under-diagnosed AR might be a risk factor for CAN, and this hypothesis was tested in a randomized control trial. Patients were biopsied by protocol during the first year after transplantation. SARs were treated with steroid pulses in the treatment arm, whereas the other arm was simply observed. After two years’ follow-up the treated group developed less AR, had lower chronic tubulointerstitial scores at 6 months, and lower levels of serum creatinine. (Rush, 1998) Thus far these results have not been reproduced by any other group in a larger scale.

The reduction in the AR rate with MMF, prednisone and CsA microemulsion was not associated with a decreased incidence of SAR, although the great majority of the cases were mild. (Nickerson, 1999) The prevalence of SAR decreases over time from transplantation onwards, varying from 25 to 60% at one month, 10 to 45% at three months and 8 to 18% at one year. (Kee, 2006) The great differences in the rate of SAR between institutions are explained by different immunosuppressive regimens, and by and the variable immunological risk of the population considered. (Nankivell, 2006) The early risk of SAR is influenced by HLA mismatches and prior AR. (Choi, 2005) The protocol biopsy policy aimed at detecting SAR has been questioned in a series of mainly living donor transplants treated with antilymphocyte induction, tacrolimus and MMF, in which the rate of SAR was only 2.6%. (Gloor, 2002) It thus appears that SAR implies a certain degree of immunoactivation, and if no therapeutic options are considered, SAR may lead to CRAD, CAN and finally reduced graft survival. Although there is no consensus on the treatment of SAR with steroid boluses, some authors have suggested that the level of immunosuppression be raised by replacing CsA with tacrolimus, or azathioprine with MMF, (Shishido, 2003) or by increasing the doses of the maintenance therapy. (Kee, 2006)
3. Pathophysiology of CAN: Non-immune-dependent factors

3.1. Donor factors are associated with graft outcome, in both living and cadaver donation. In a recent report on 248 living KTX, the donors’ age, systolic blood pressure, high cholesterol and decreased glomerular filtration rate (GFR) were associated with a lower recipients’ estimated GFR two years after transplantation. (Issa, 2007) Kidney volume, evaluated in living donor candidates by three-dimensional helical computed tomography, correlated with donor gender and body size, and was associated with kidney function at two years. Interestingly, smaller donor kidneys were prone to episodes of AR. (Poggio, 2006) The latter finding was also described in the case of cadaver donors, suggesting that low “nephron mass” affects the susceptibility to immuno-mediated injury. (Sanchez-Fructuoso, 2001) Female sex of the donor has been associated with poorer graft outcome irrespective of the recipient’s sex, reinforcing the concept of an imbalance between nephron supply and the recipient’s metabolic demands. (Zeier, 2002) Also a high donor/recipient age ratio was associated with graft loss in an analysis of 20,309 cadaver kidney transplants in the USA. (Swanson, 2002) The donor’s cause of death was also considered to influence early graft function, and it seemed to be associated with the early up-regulation of the immune system. (Nyhof, 2005) A review of the cause of death among donors in 2006 kidney transplants in Finland revealed that accidental death is decreasing, and there is a rising trend to accept cadaver kidneys from donors after surgical procedures, cardiopulmonary resuscitation or coronary disease. (Kyllönen, 2005) These factors, along with the higher age of the donors, are believed to adversely affect the prognosis of kidney allograft in the long run.

3.2. Donor brain death triggers an autonomic storm and the release of inflammation mediators. A vast majority of the data in this area comes from animal experiments. A study in humans was recently conducted to evaluate the cytokine gene expression in kidney biopsies obtained at organ procurement, after cold ischemia and after 30 min of reperfusion. The results were compared with biopsies obtained at the same time points from living donors. Tubular and glomerular expression of interferon-γ, TGF-β, PDGF-β and interleukin-2, -6 and -10 were all significantly increased in biopsies from cadaver donors compared to living donors. The early cytokine stimulation was linked to a further stimulation of the immune response and long-term kidney survival. (Kaminska, 2007)
3.3. Both ischemia and reperfusion induce profound changes in the graft. After ischemia, tissues suffer from a lack of oxygen and nutrients, and subsequently metabolic waste products accumulate. Reperfusion, on the other hand, causes sudden oxygenation of the tissue, leading to the formation of free oxygen radicals. This may overwhelm the capacity of scavengers to deal with this excessive offer. Reperfusion implies that leukocytes invade the graft, releasing chemokines and adhesion molecules to recruit more inflammatory cells that consequently propagate the damage. (Perico, 2004) A prolonged time of cold ischemia increases the risk for delayed graft function, with a direct adverse impact on graft survival. (Salahudeen, 2004)

3.4. Delayed graft function (DGF) is the term used to describe kidney allograft non-function immediately after transplantation. This lack of function is a consequence of ischemia-reperfusion injury and/or immunological injury. It is of vital importance to differentiate DGF from other causes of lack of function, such as volume depletion, AR, disease recurrence, CNI-toxicity and vascular or urinary tract complications. Risk factors for delayed graft function are cold-storage preservation, non-heart-beating donors; inotropic support, donor age over 55 years, marginal kidneys from diabetic or hypertensive donors, and the recipient's hypovolemia. (Perico, 2004, Peeters, 2008) The incidence on DGF varies between 4 and 50%. Unfortunately, in the literature there are many different definitions of DGF that it difficult comparisons between studies. (Yarlagadda, 2008) Patients with DGF are at increased mortality risk and hospitalization is prolonged as a consequence of their dependence on dialysis. DGF predisposes to both AR and CAN. It has been hypothesized that during DGF hidden foreign antigens are exposed to the immune system of the recipient. The addition of both DGF and AR has been suggested to predispose to CAN, affecting thereby graft survival. (Peeters, 2008) There are reports, however, on the negative impact of DGF on graft survival independent of AR. (Shoskes, 1998) A reduction in the nephron mass after the recovery from DGF has been suggested to be one of the most important variables for the development of CAN. Despite the efforts, there has not been a reduction of the incidence of DGF in the recent years. The deterioration in the donors’ quality is a possible explanation for this observation.

3.5. Sub-optimal immunosuppression could be the result of patient non-compliance with the treatment or a desired maneuver. The patients are more susceptible to infections and malignancies as a consequence of the use of immunosuppressants. Transplantation across HLA barriers requires more potent immunosuppression, increasing the occurrence of the above-mentioned complications even further. Whenever potentially life threatening conditions are recognized, a change in immunosuppression regimen is mandatory to save the patient. The cessation of immunosuppression carries
an inevitably high risk of graft loss due to acute and/or chronic rejection.

Overcoming non-compliance is also a great challenge for physicians. Numerous causes hide behind it, such as level of education, patient/physician relationship, side effects of the drugs, cost of the drugs, reimbursement policy, and inconsistency in the frequency of drug intake. A graft loss rate secondary to non-compliance are seen in all solid organ transplantations. A study on cadaver as well as living KTX revealed that 8% of the grafts were lost due to the patients’ non-compliance with the treatment. (Matas, 2002) Risk factors for non-compliance were young age, non-Caucasian race, unmarried status and emotional instability. (Jindal, 2003)

3.6. Hypertension (HT) is a well known contributor to chronic kidney disease (CKD) in native kidneys and in kidney transplants as well. A number of studies including different immunosuppression regimens and ethnologies have shown a negative impact of HT on graft survival. HT is particularly common among African Americans, in whom the prevalence of HT was 90% and it was usually poorly controlled. (Cosio, 1995) The majority of CKD patients suffer from HT prior to receiving a transplant, and in many of them it is cause of uremia. The number and dosage of hypertensive drugs frequently increases after transplantation. Pre-transplant HT is a strong risk factor for HT also after transplantation, and it may be generated by native kidneys. Other common causes of HT in KTX are the use of calcineurin inhibitors, particularly CsA, corticosteroids, transplant artery stenosis, and chronic renal allograft dysfunction (CRAD). (El-Amm, 2006) The prevalence of HT is already high when graft function is normal, but it increases significantly if CRAD develops. Over 90% of the patients are hypertensive when their creatinine level is above 260μmol/L. (Budde, 1997) Corticosteroids have been considered to be a risk factor for HT, but at present they are not believed to be a major contributor to HT due to the rapid tapering of the doses. (Midtvedt, 2002) Outstanding evidence of the deleterious effect of HT on graft survival came from the Collaborative Transplant Study, in which the systolic blood pressure of 55% of the patients was over 140 mm Hg one year after the transplant. Both systolic and diastolic blood pressures were identified as subsequent predictors of graft failure. (Opelz, 1998) For each 10 mm Hg increase in systolic, diastolic and mean blood pressure at one year after transplant, graft survival was shortened by 15%, 27% and 30%, respectively. (Mange, 2000) An association between HT and AR has also been described. In a review of 1505 charts from first cadaver KTX patients with an immunosuppression regimen based on CsA, there was no correlation between HT and 2-year graft survival in the absence of AR. On the contrary, the additive effects of AR and HT correlated negatively with graft survival. (Cosio, 1999) It is difficult to determine whether HT enhances the risk of AR, or whether it is a consequence of AR. The hemodynamic effects of HT, including glomerular hypertension, plus the up-regulation
of TGF-β and major histocompatibility complex I and II, might explain its contribution to the pathogenesis of CAN. (Sanders, 1995)

It is well established that HT increases the risk for cardiovascular disease and death in non-kidney diseases. HT is also a cardiovascular risk in KTX. It is unlikely for obvious ethical reasons that prospective trials were to be conducted in which patients would be randomized into optimal or suboptimal HT control groups. Despite the efforts to control HT, the task remains challenging. This was evidenced in an observational trial in which two groups of patients with blood pressure over or under 160/90 mm Hg were followed up for three years. Although the targets that these authors considered satisfactory is far above the recommendations of the Joint National Committee 7, they were able to recognize an increase in creatinine level and more cardiovascular events in the subgroup with the higher blood pressure. (Raiss Jalali, 2007) Data from The Finnish Registry of Renal Diseases reported that during the period 2004–2006, a great majority (86%) of the KTX patients was on hypertensive drugs, but only 22% had a blood pressure under the 130/85 mm Hg threshold.

It is evident that graft and patient survivals are enhanced if the patients’ blood pressure is controlled successfully. However, it is still unclear whether there are differences in the efficacy of the drugs prescribed to reach this goal. Calcium channel blockers are often considered the first choice because they reverse the vasoconstriction caused by CsA, and ameliorate its nephrotoxicity. (Zhang, 2003) Some authors claim that these drugs can also reduce the risk for AR, (Cosio, 1999) although the results of a meta-analysis were conflicting. (Ladefoged, 1994) The drugs most commonly used to treat chronic nephropathies are angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II receptor blockers (ARB). In KTX patients, the renin-angiotensin-aldosterone system (RAAS) is stimulated by CsA, among others. The widespread use of RAAS blockade has been hampered by its side effects, such as hyperkalemia, anemia and decreased glomerular filtration rate (GFR). The benefits might nevertheless be overwhelming, such as a reduction of glomerular hypertension, a decrease of proteinuria and a lowered production of TGF-β. In experimental models, both ACEi and ARB slowed the progression of CAN when compared to calcium channel blockers. (Cruzado, 2008) In addition to the nephroprotection that these drugs provide, they are also drugs of choice when left ventricular hypertrophy is present. They may also improve arterial stiffness, thus improving the cardiovascular risk factor profile. (Agarwal 2007) There are not, however, up-to-date sufficiently powered randomized trials to address an improvement in graft and patient survival provided by ACEi or ARB.

3.7. Dyslipidemia is a common finding in the kidney transplant population, its prevalence being 30 to 80%. The reported variability relies on differences among patient populations in regard to genetics, obesity, immunosuppressive regimen, age, post-transplant diabetes mellitus (PTDM),
CRAD, and the presence of proteinuria. (El-Amm, 2006) Data from The Finnish Registry of Kidney Diseases on the incidence of dyslipidemia in KTX patients during the period 2004–2006 indicates that 75% of them had triglycerides under 2 mmol/L, 90% had HDL cholesterol over 1 mmol/L, 81% had LDL cholesterol below 3 mmol/L and 64% had total cholesterol below 5mmol/L (www.musili.fi/smtr).

The fact that kidney grafts are mainly lost to CAN and the patient's death due to cardiovascular diseases, three key questions arise: Does dyslipidemia contribute to the progression of CAN? Is dyslipidemia involved in the cardiovascular deaths of transplanted patients to the same degree as in the normal population? Does treatment of dyslipidemia lower the risk of graft loss? To address the first question, there is evidence that hypercholesterolemia is indeed a risk factor for CAN, particularly when linked to AR. (Isoniemi, 1994) The lipid alterations typically present in CKD are increased concentrations of triglycerides, LDL, lipoprotein (a), apo C III and oxidized LDL, and decreased concentrations of HDL and apo A-1. (Crook, 2003) Studies in animals suggest that dyslipidemia might affect the kidney via inflammatory pathways, involving the recruitment of macrophages and monocytes. Macrophage infiltration is usually accompanied by the activation of pro-sclerotic growth responses by mesangial cells, including mesangial fibronectin, platelet-derived growth factor, and TGF-β. (Pawluczyn, 2000) Although animal experiments have provided abundant evidence on the association between dyslipidemia and CKD, such evidence is limited in humans. The potential role of lipoproteins in glomerulosclerosis and other glomerular diseases is suggested by the accumulation of cholesterol, LDL and apo B in the glomeruli. (Samuelsson, 1994) There is increased mesangial lipid deposition and enhanced expression of LDL receptors on mesangial and epithelial cells in patients with chronic glomerular diseases. (Takemura, 1993) The challenge in demonstrating that dyslipidemia is a primary risk factor for CKD is that it is usually linked to other cardiovascular risk factors, such as HT, diabetes mellitus or metabolic syndrome. A valuable contribution to elucidate this came up in the Helsinki Heart Study, in which patients with normal kidney function at the beginning of the investigation developed an increase in creatinine levels if they had low serum HDL level and an LDL/HDL ratio under 3.2 after 5 years of follow-up. (Manttari, 1995) A similar effect was noted even in patients with established CKD, when the progression of kidney dysfunction was faster if HDL was low. (Hunsicker, 1997) In KTX, hypertriglyceridemia, high concentrations of VLDL and lipoprotein (a) were independent risk factors for CRAD. (Castello 2002)

Dyslipidemia-related nephrotoxicity prompted investigators to study whether drug treatment could slow down or abolish the progression of CKD. In a trial published in 2003, patients already on RAAS blockers were randomized to receive additionally atorvastatin or placebo. Proteinuria
was significantly decreased and CKD did not progress in the atorvastatin group, which were not achieved in the placebo group. (Bianchi, 2003) The addition of pravastatin in KTX patients under CsA, azathioprine and methylprednisolone based immunosuppression seemed to reduce the probability of AR. (Katznelson, 1996) This beneficial effect was masked if azathioprine was replaced by MMF. (Kasiske, 2001) Positive results were also found in another randomized study in patients on CsA-based immunosuppression, comparing the effect of fluvastatin on AR. (Holdaas, 2001)

It is not clear whether the risk factors for cardiovascular disease in KTX are the same as those in the general population. KTX patients often have a decreased GFR; they are therefore CKD patients. An increase in creatinine of 80–100 μmol/L was found to double the risk for cardiac death, non-cardiovascular death, and overall mortality in KTX patients. (Soveri, 2006) Uremia may modify the characteristics of lipid moieties, turning them into a more atherogenic variant. (Kasiske, 2000) The largest multicenter randomized double-blind placebo-controlled study was the Alert trial. (Holdaas, 2003) Its goal was to assess the efficacy of fluvastatin in reducing cardiovascular events. After 5 years of follow-up there was a 17% reduction in the occurrence of major events (cardiac death, non-fatal myocardial infarction or a coronary intervention procedure), although it did not reach statistical significance. Cerebrovascular events and a composite endpoint of graft loss or doubling of creatinine, occurred at the same rate in both the treatment and placebo arms. One of the limitations attributed to this study was the fact that one inclusion criteria was patients who still had a functioning graft six months or more after transplantation, thus including also very old grafts. It is therefore possible that it was too late to reverse the trend of cardiovascular mortality in many of the patients. There was also a 14% crossover to fluvastatin in the placebo group, which limited the evaluation of the benefits of fluvastatin in intention-to-treat analysis. All in all, despite the fact that there is not irrefutable evidence provided by human trials favoring the use of statins to diminish CAN and cardiovascular events in KTX patients, there is no reason to believe that the risk factors of these patients are less harmful than those of the general population. On the contrary, KTX population is a selection of patients who have been exposed for various periods of time to the metabolic disturbances involved in uremia. Furthermore, many of them have a sub-optimal allograft function, in addition to the side effects of immunosuppressive drugs. A post-hoc analysis by the Alert Study Group showed that timing of the initiation of the lipid-lowering therapy had an impact on the frequency of cardiac deaths and non-fatal myocardial infarction. The major benefit was observed when the drug treatment was started 0 to 2 years after transplantation. (Holdaas, 2005)

There are some benefits beyond lipid modification, i.e. so-called “pleiotropic effects” which have been attributed to statins. Statins have been shown to improve endothelial function by reducing oxidative stress, thus
lowering cardiovascular risk. (Sorrentino, 2005) Statins may also lower the level of inflammatory markers, such as C-reactive protein. This effect is enhanced when another lipid-lowering agent, ezetimibe, is added to the treatment. The decrease in the concentration of C-reactive protein is directly linked to the decrease in LDL cholesterol levels. In the light of present research, it is difficult to elucidate the individual contribution of each factor to the cardiovascular risk. (Davidson, 2005)

A relative drawback in the use of statins in KTX is the pharmacokinetic interaction between CsA and certain statins because they share the metabolic pathway (CYP3A4). The result of this interaction is a potential increase in the plasma levels of the statin, possibly leading to rhabdomyolysis. (Ballantyne, 2003) This interaction may subdue the use of statins. The Alert study, however, reported a safety profile of fluvastatin, because it does not share the same metabolic pathway with CsA. (Holdaas, 2003)

3.8. Post-transplant diabetes mellitus (PTDM) In KTX the onset of PTDM represents a threat to both the patient and to graft survival (Jindal, 2000) as it augments their already increased cardiovascular risk. (Bostom, 2002) PTDM implies exposure to micro- and macrovascular complications, and raises medical costs. An analysis issued by the United Renal Data System disclosed a cumulative incidence of PTDM of 9.1% at three months, 16% at 12 months and 24% at three years. Risk factors for PTDM included black or Hispanic ethnicity, male donor, increasing HLA mismatches, hepatitis C, body mass index (BMI) over 30 kg/m² and use of tacrolimus. The relative risk for graft failure was 1.63 and for death-censored graft failure 1.46. The overall relative risk of death was 1.87. (Kasiske, 2003) A German study on 253 KTX patients with a mean follow-up of 3.3 years reported a prevalence of PTDM of 17%. Recipient age was significantly associated with PTDM, and there was a trend toward PTDM as BMI increased. In this study, neither an immunosuppressive regimen nor HLA mismatches increased the risk. (Schiel, 2005) Some authors have stated that tacrolimus is not associated with a higher risk of PTDM in a corticosteroid-free treatment regimen. (van Hooff, 2006) In the literature, the prevalence of PTDM has been reported to vary from 2 to 54%. The reasons for this wide variation are: lack of consensus in the definition of PTDM, mixed results on both cadaver and living-donor transplants, the use and doses of corticosteroids, use of tacrolimus, an allocation policy regarding HLA mismatches, differences in the length of the follow-up, and the prevalence of hepatitis C. Also CMV infections have been associated with PTDM. (Hjelmesaeth, 2005)

Special attention should be paid to the definition of PTDM as reported in the literature: from insulin dependency one week after transplantation, to insulin use at one or three months. The definition of PTDM merely according to insulin requirements rules out patients who can be treated with oral antidiabetic agents and diet, and those who fulfill the criteria of
diabetes mellitus based on the oral glucose tolerance test. This means that the PTDM incidence reported in the literature might be underestimated. Although the above-mentioned risk factors help to estimate roughly the probability of PTDM, the fasting plasma glucose concentration on the day of transplantation, and the oral glucose tolerance test on day 5 has been proposed to identify those at risk of PTDM. (Kuypers, 2008) The prevalence of impaired glucose tolerance (IGT) in KTX patients is even more obscure. It is known that IGT is related to cardiovascular diseases (Heldgaard, 2004) In a recent study in the Chinese population, the prevalence of IGT 6 months after transplantation was 6.7%, and the risk factors for IGT were very similar to those described for PTDM. (Chan, 2008) Another observational study from Australia reported an IGT prevalence of 28.5% and a PTDM prevalence of 11% after 6.6 years of follow-up for patients who were not previously diabetic. (Armstrong, 2006) In a recent study of 347 Spanish KTX patients without diabetes before or after transplant, impaired glucose metabolism was detected in 31.8% of them. This included IGT (24.6%) and those with isolated impaired fasting glucose (7.2%). (Delgado, 2008) These data further support the view that alterations in glucose metabolism are very common in KTX patients. An interpolation of the relationship between IGT or impaired fasting glucose and CAN might be justified, but there is scanty data available to support this.
4. Other causes of kidney allograft damage

4.1. Calcineurin inhibitors-related toxicity

Kidney transplant is susceptible to nephrotoxic drugs in a similar fashion as native kidney. Some nephrotoxic drugs, such as CNI, are commonly used as immunosuppressive agents in transplantation. CsA is capable of dramatically reducing the incidence of AR, which is the most relevant risk factor for CAN. However, an observation made already in 1978 directed attention to its nephrotoxicity.(Calne, 1978) This was confirmed later by the finding that kidney function deteriorates in non-kidney-transplant patients subjected to a CsA regimen.(Bennett, 1996) CsA nephrotoxicity can be either acute or chronic (Figure 2).

Acute nephrotoxicity is functional, and results from reduced kidney blood flow, increased vascular resistance of the afferent preglomerular arterioles, and consequently reduced GFR. It is caused by an imbalance between vasoconstrictors and vasodilators, due to the increased activity of endothelin and angiotensin II accompanied by a reduction in the synthesis and secretion of nitric oxide from the endothelium.(Fellström, 2004)

A common histological pattern is isometric vacuolization in proximal tubular cells. (Mihatsch, 1986) These acute changes are reversible, however, if CsA is discontinued or reduced. Conversely, changes related to chronic CsA nephrotoxicity are not reversible. This condition is histologically characterized by striped interstitial fibrosis and arteriolar hyalinization (Figure 3). It is believed to be mediated by several factors, such as, TGF-β, platelet-derived growth factor, fibroblast growth factor and tumor necrosis factor-α. (Khanna, 2002) The real prevalence of CsA chronic nephrotoxicity is difficult to estimate because conventional tissue staining cannot differentiate the initial injury behind interstitial fibrosis. The term CAN therefore covers interstitial fibrosis that could be the result of immune mechanisms, but also the consequence of chronic CsA toxicity. (Grinyo, 2004) The paradox is that the demonstrated short-term benefit of CsA via the reduction in AR rates is counterbalanced by its nephrotoxicity. This in fact partially explains why improved long-term outcomes have been difficult to achieve.
The other widely used CNI, tacrolimus, has also proved to be nephrotoxic. (Pirsch, 1997) Several strategies to overcome this problem have been proposed, such as either reducing the CNI exposure or discontinuing its use. This requires the introduction of some other non-nephrotoxic immunosuppressant; such as the mammalian target of rapamycin (m-Tor) inhibitors, i.e. sirolimus and everolimus. Unfortunately, it was noted that the rate of AR increased when CsA was discontinued, although rejections were usually easy to treat. (Fellström, 2004) The experience gained from the increasing use of m-Tor inhibitors in the recent years has revealed that these compounds are not free of nephrotoxicity, particularly when combined with CsA. (Morath, 2007)

CsA is known as a drug with a narrow therapeutic window. Its administration therefore requires constant monitoring in order to assure adequate immunosuppression and to avoid toxicity. The dosage of CsA has been initially based on mg/kg body weight, but the high incidence of adverse events has made it necessary to monitor drug exposure. This has been done over the years by testing the patient’s CsA concentration in blood before giving the morning dose (C0). In the early 1990’s it was observed that the C0 is not reliable in predicting freedom from AR, and that the best pharmacokinetic measure of CsA was the area under the time concentration curve (AUC_{0–12h}). (Lindholm, 1993) Figure 4.

Figure 2. Classification of CsA nephrotoxicity from a clinico-pathological context. (Morozumi . 2004)
Figure 3. CsA-related nephrotoxicity. (A) acute CsA toxicity evidenced by isometric vacuolization in proximal tubular cells. Hematoxin-eosin. Magnification X 400. (B) chronic CsA toxicity evidenced by striped interstitial fibrosis. Magnification X 100. In (C) CsA-related arteriolar hyalinosis, evidenced by the deposition of red pale homogenous material under the intima. Magnification X 400. (B) and (C) stained with Masson trichrome.
Although they are accurate for evaluating CsA exposure, the AUC$_{0-12h}$ measurements are not feasible for clinical follow-up. A great proportion of the variability of AUC$_{0-12h}$ was detected during the first 4 h. The AUC$_{0-4}$ also demonstrated a good correlation with drug exposure and freedom from AR. (Mahalati, 2001) Naturally, determination of the AUC requires multiple blood samples; a single time point measurement (more representative than C0) was therefore sought. Several studies have shown that the CsA blood concentration in samples taken 2 h after the morning dose (C2) correlated best with AUC$_{0-4}$ (Mahalati, 2001; Jorga, 2004) and with the maximal immunosuppressive effect of CsA (measured by the inhibition of calcineurin in lymphocytes). (Halloran, 1999) The implementation of C2 monitoring in clinical practice was recommended in 2002, initially for de novo kidney transplants and for the maintenance phase thereafter. (Cole, 2002) Numerous studies have described the long-term benefits of switching from C0 to C2, such as better kidney function and reduced CsA doses. (Citterio, 2005; Dominguez, 2005; Thervet, 2003, Pallardo, 2007) It was suggested that C0 monitoring leads to the patients’ overexposure to CsA. (Di Paolo, 2004; Midtvedt, 2003) One of the limitations of C2 monitoring is the inability to detect toxicity in slow CsA absorbers, or to consider the increasing CsA absorption over time in the early post-transplant period. (Einecke, 2004)

Despite the pharmacokinetic advantages of C2, there is no consensus on its targets at different time points. The initially proposed C2 targets are...
continuously challenged downwards with the aim to avoid CsA nephrotoxicity and to slow the progression of CAN. (Carstens, 2008)

4.2. Recurrent and de novo glomerular disease

Chronic glomerulonephritis is the primary disease in 15 to 30% of KTX patients. As many of the primary diseases are systemic, they may recur in the graft. Data from the Finnish Registry for Kidney Diseases on the frequency of primary disease in KTX patients are given in Figure 5.

The reported incidence of recurrent glomerulonephritis is around 10%, but there is great variability across institutions due to the differences in their biopsy policy regarding old dysfunctional grafts. Some diseases may recur soon after transplantation, such as atypical hemolytic uremic syndrome or focal and segmental glomerulosclerosis (FSGS). Others, like IgA nephropathy, tend to recur late. The data of the British Register of Kidney Diseases indicate that IgA nephropathy is the most common recurrent disease (ca 30% of all cases), and FSGS ranks second (ca 10% of all cases). (Furness, 2002)

Membranoproliferative glomerulonephritis type 1 tends to recur in 25–50% of the patients, type 2 being much more frequent, with a rate exceeding 80%. Graft loss due to recurrence occurs in 15–30% of the cases at 5 years. Idiopathic membranous glomerulopathy also tends to recur in 10% of the patients, but it should be differentiated from secondary causes (viral infections and malignancy) and from de novo disease. (Choy, 2006)

As kidney graft survival has improved, also diabetic nephropathy can develop in the allograft in the long term if the patient’s glucose control is not satisfactory.

4.3. Viral Infections

The most common infection affecting the post-transplantation period is by far caused by cytomegalovirus, and in the past decade BK polyoma virus (BKV) infection have been more frequently diagnosed. Transmission of BKV occurs during childhood via oral or respiratory tracts. Over 80% of adult population is seropositive, but the virus remains latent in the renourinary tract. BKV replicates if the patient receives excessive immunosuppression. (Egli, 2007) BKV associated nephropaty is histologically characterized by an exacerbated inflammatory response of monocytes, polymorphonuclear and plasmacytoid cells, with a cytopathic effect and severe tubular injury, often mistaken for AR. Viral DNA may be detected in blood by polymerase chain reaction (PCR), but when there is suspicion of pathology, the diagnosis should be confirmed by the presence of BK vi-
Virus in the graft, revealed by immunohistochemistry. Polyoma nephropathy has an ominous prognosis, and decreasing immunosuppression is an essential part of the treatment. (Blanckaert, 2006)

Virus-specific serology is used to stratify the risk for CMV-developing. The CMV seronegative recipients receiving a KTX from a positive donor are at the highest risk for CMV replication and disease. The risk also increases in seropositive CMV recipients with T-cell and/or B-cell depleting antibody treatment for induction or rejection. (Egli, 2007) The patients at higher risk for developing CMV disease receive either prophylactic or preemptive treatment. This policy reduced significantly CMV disease. However, when prophylaxis ends there is still a risk for virus replication. CMV identification in blood does not mean tissue invasion. Antiviral therapy is aim at eliminating viral replication to prevent CMV-disease. During the acute phase of cytomegalovirus infection, the kidney function may be affected, but persistent infection is associated with poor graft and patient survival. (Fishman, 1998) Persistent CMV infection in the graft stimulates inflammatory cells, which augment the production of several growth factors and cytokines. (Helanterä, 2006) The relation between cytomegalovirus and CAN has been the object of numerous studies. The results of a study on protocol biopsies suggested that a history of both previous AR and cytomegalovirus infection was associated with increased graft vasculopathy. (Helanterä, 2005)

A summary of the pathophysiological factors involved in CAN are depicted in Figure 6.
Figure 6. Pathophysiological factors involved in CAN. Factors whose pathogenic role in CAN are controversial or are lacking of robust evidence are displayed with *
5. Diagnosis of kidney allograft damage

5.1. Classifications in kidney transplant pathology: Chronic allograft damage index

The term chronic allograft damage index (CADI) was first proposed in 1992 (Isoniemi, 1992). It emerged as a necessity to discriminate histological findings two years after transplantation between four groups of patients undergoing different immunosuppressive regimens. The utility of this classification to predict chronic allograft rejection was reaffirmed in 1994 by the same authors (Isoniemi, 1994). The CADI score is a number generated by the sum of six individual scores, each graded from 0–3. These individual parameters are inflammation, interstitial fibrosis, tubular atrophy, mesangial matrix increase, intimal proliferation of the vessels and glomerulosclerosis. The CADI score ranges from 0 to a maximum of 18. Details on individual scoring are depicted in Table 1 and Figure 7.

Later on in a multicentre trial, CADI was proposed as a suitable surrogate marker of long-term graft survival (Yilmaz, 2003). In that study 621 protocol biopsies were obtained at baseline and at one and two years of follow-up, the CADI score progressed from the baseline to the end of follow-up, demonstrating a deterioration in histology. Patients with a CADI score under 4 had normal creatinine levels, and after a 3-year follow-up only 4.6% of the grafts were lost. Conversely, a CADI score above 4 was linked to an increased creatinine level, and 16.7% of the grafts were lost in this period.
Table 1. Individual scoring of CADI. (Isoniemi, 1994)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inflammation (focal/diffuse)</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>no or trivial inflammation</td>
</tr>
<tr>
<td>1</td>
<td>up to 25% of parenchyma inflamed</td>
</tr>
<tr>
<td>2</td>
<td>26–50% of parenchyma inflamed</td>
</tr>
<tr>
<td>3</td>
<td>&gt;50% of parenchyma inflamed</td>
</tr>
<tr>
<td><strong>Interstitial fibrosis (focal/diffuse/subcapsular)</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>no fibrosis</td>
</tr>
<tr>
<td>1</td>
<td>up to 25% of the interstitium affected</td>
</tr>
<tr>
<td>2</td>
<td>26–50% of the interstitium affected</td>
</tr>
<tr>
<td>3</td>
<td>&gt;50% of the interstitium affected</td>
</tr>
<tr>
<td><strong>Tubular atrophy</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>no tubular atrophy</td>
</tr>
<tr>
<td>1</td>
<td>tubular atrophy up to 15% of proximal tubules</td>
</tr>
<tr>
<td>2</td>
<td>tubular atrophy in 16–30% of proximal tubules</td>
</tr>
<tr>
<td>3</td>
<td>tubular atrophy in more than 31% of proximal tubules</td>
</tr>
<tr>
<td><strong>Mesangial matrix increase</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>no mesangial matrix increase</td>
</tr>
<tr>
<td>1</td>
<td>up to 25% of non-sclerotic glomeruli affected (at least moderate)</td>
</tr>
<tr>
<td>2</td>
<td>26–50% of non-sclerotic glomeruli affected (at least moderate)</td>
</tr>
<tr>
<td>3</td>
<td>&gt;50% of non-sclerotic glomeruli affected (at least moderate)</td>
</tr>
<tr>
<td><strong>Intimal proliferation (changes seen in at least 1 artery or 3 arterioles)</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>no intimal thickness</td>
</tr>
<tr>
<td>1</td>
<td>intimal thickness up to 25% of the remaining lumen</td>
</tr>
<tr>
<td>2</td>
<td>intimal thickness 26–50% of the remaining lumen</td>
</tr>
<tr>
<td>3</td>
<td>intimal thickness &gt;50% of the remaining lumen</td>
</tr>
<tr>
<td><strong>Sclerosis (increase in extracellular matrix, sclerotic areas positive stained with PAS)</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>no changes</td>
</tr>
<tr>
<td>1</td>
<td>&lt;15% of glomeruli affected</td>
</tr>
<tr>
<td>2</td>
<td>16–50% of glomeruli affected</td>
</tr>
<tr>
<td>3</td>
<td>&gt;50% of glomeruli affected</td>
</tr>
</tbody>
</table>

The CADI score has also been used to classify implant biopsies (Lehtonen, 2001). In this cohort the mean CADI score increased from 0.7 in implant biopsies to 2.9 after one year. It was also shown that patients with normal serum creatinine at one year nevertheless had an increased CADI score (2.5 ±1.5), reaffirming that histological changes might take place even in well functioning grafts.
Figure 1. Photomicrographs of four kidney biopsies showing a rising CADI score. (A) A 6-month protocol biopsy with normal histology, score: 0. (B) A 6-month protocol biopsy, score: 2, comprised of chronic vasculopathy (cv 1, not shown in the photomicrograph) and glomerulosclerosis (gs 1). (C) A 6-month protocol biopsy, score: 4, comprised of interstitial fibrosis (ci 1), tubular atrophy (ct 1), mesangial matrix increase (mm 1) and glomerulosclerosis (gs 1). (D) A 12-month protocol biopsy, score: 8, comprised of interstitial fibrosis (ci 1), tubular atrophy (ct 1), inflammation (i 1), glomerulosclerosis (gs 2) and severe chronic vasculopathy (cv 3). Masson's trichrome. Magnification X 100.

5.2. Classifications in kidney transplant pathology: Banff: terminology and its evolution over time

The Banff classification was created in a meeting held in Banff, Canada, in August 1991 and published in 1993. The Banff Working Classification of Renal Allograft Pathology is a schema developed to standardize the interpretation of kidney allograft pathology across frontiers. Banff ’97 is the most commonly used classification system in the literature on KTX. Details of the Banff ’97 classification are shown in Table 2.
Table 2. Banff ’97 diagnostic categories for renal allograft biopsies. (Racusen, 1999)

1. Normal

2. Antibody-mediated rejection, demonstrated to be due, at least in part, to antidonor antibody.
   - A. Immediate (hyperacute)
   - B. Delayed (accelerated acute)

3. Borderline changes suspicious for acute rejection. This category is used when no intimal arteritis is present, but there are foci of mild tubulitis (1 to 4 mononuclear cells/tubular cross section) and at least 11

4. Acute/active rejection

<table>
<thead>
<tr>
<th>Type (grade)</th>
<th>Histopathological findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>I A</td>
<td>Cases with significant interstitial infiltration (&gt;25% of parenchyma affected) and foci of moderate tubulitis (&gt;4 mononuclear cells/tubular cross-section or group of 10 tubular cells)</td>
</tr>
<tr>
<td>I B</td>
<td>Cases with significant interstitial infiltration (&gt;25% of parenchyma affected) and foci of severe tubulitis (&gt;10 mononuclear cells/tubular cross-section or group of 10 tubular cells)</td>
</tr>
<tr>
<td>II A</td>
<td>Cases with mild to moderate intimal arteritis (v1)</td>
</tr>
<tr>
<td>II B</td>
<td>Cases with severe intimal arteritis comprising &gt;25% of the luminal area (v2)</td>
</tr>
<tr>
<td>III</td>
<td>Cases with transmural arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells (v3 with accompanying lymphocytic inflammation)</td>
</tr>
</tbody>
</table>

5. Chronic/sclerosing allograft nephropathy

<table>
<thead>
<tr>
<th>Grade</th>
<th>Histopathological findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (mild)</td>
<td>Mild interstitial fibrosis and tubular atrophy without (a) or with (b) specific changes suggesting chronic rejection</td>
</tr>
<tr>
<td>II (moderate)</td>
<td>Moderate interstitial fibrosis and tubular atrophy (a) or (b)</td>
</tr>
<tr>
<td>III (severe)</td>
<td>Severe interstitial fibrosis and tubular atrophy and tubular loss (a) or (b)</td>
</tr>
</tbody>
</table>

6. Other
   - Changes considered not to be due to rejection.

Subsequent meetings have been held every other year to refine the classification. The modifications to the Banff schema made in 1997 synthesized two different classification systems: Banff ’95 and the classification developed by CCTT (Collaborative Clinical Trials in Transplantation) (Racusen,1999). The earliest versions of the Banff classification emphasized the diagnosis of AR, mainly because this was the most common problem in those days. CAN was graded into three categories – mild, moderate or severe – without an attempt to determine the pathophysiological mechanisms involved in this entity. The improvements in immunosuppressive regimens dramatically reduced the incidence of acute rejection, exposing
the problem of chronic allograft damage. The elucidation of the role of donor-specific antibodies, suggested by C4d staining (see below) caused a revolutionary modification of the Banff classification, and led to the omission of the general term “chronic allograft nephropathy”. (Solez, 2007) In an attempt to discriminate the underlying processes, the present recommendation distinguishes acute and chronic antibody-mediated rejection from acute and chronic T-cell mediated rejection. Unspecific interstitial fibrosis and tubular atrophy (IFTA) are now a separate category, graded from mild to moderate and severe.

5.3. Protocol biopsies

Protocol biopsies are the standard of care in many transplant centers around the world, but the majority of the units avoid taking a biopsy from a well functioning graft. There is no consensus regarding the possible benefits that might justify the risks related to this practice. Numerous reports have shown that the risks for major complications, such as bleeding, macroscopic hematuria with ureteric obstruction, peritonitis or graft loss, are only approximately 1%. The rate of minor complications resolving without intervention, such as asymptomatic arteriovenous fistula, gross hematuria, perirenal hematoma or vasovagal reactions, has been reported to vary between 0.5 and 7.3%. (Furness, 2003; Schwarz, 2005) The overall graft loss rate in five studies reporting complications after protocol biopsies was only 0.03% and patient survival was 100%. (Furness, 2003; Schwarz, 2005)

It is safer to take a biopsy by protocol than obtaining a biopsy of an inflamed graft. As bleeding is the major concern connected with this procedure, needles of different thicknesses used for the biopsies were compared. (Schwarz, 2005, Nicholson, 2000) It was found that 14-gauge needles were associated with more pain, but the samples were more representative, compared to samples taken with 16- or 18-gauge needles. The authors agreed that the 16-gauge needle was the best choice for the procedure, due to the low complication risk and adequate size of the sample.

A group of experts met in Boston in 2007 to discuss their experiences on protocol biopsies. (Mengel, 2007) They concluded that there is sufficient evidence from clinical studies confirming that protocol biopsies taken during the first six months after transplantation provide information that cannot be obtained in any other way. The morphological and ultrastructural changes correlated with later graft outcome. The best argument favoring protocol biopsy is that it is in fact the only tool for diagnosing SARs. The occurrence of SAR is high during the first year, decreasing notably afterwards. The policy of taking protocol biopsies with the intention of diagnosing SAR, and possibly altering the treatment is therefore justified during the first six months. Protocol biopsies taken at one year from transplanta-
tion reveal more chronic changes, although SAR might also be present, being a marker of a poor prognosis. (Nankivell, 2004)

Variations in histological grading of kidney biopsies due to the high interobserver bias, have aroused concern in the transplant community. (Furness, 2001) Unfortunately, if histological classification is understood in different ways by different pathologists, there is a great risk for heterogeneity in decision making. (Furness, 2003) The area of the cortex affected by interstitial fibrosis can be scored with a computer-based image. This method has been proposed to facilitate scoring and to lower the great interobserver variation. (Servais, 2007) Even so, the reproducibility of these techniques can be hampered by differences in tissue fixation, section thickness, levels of microscope light, and arbitrarily set thresholds. (Mengel, 2007)

Protocol biopsies are regarded fully justified under certain circumstances, such as delayed graft function, high-risk cross-match-positive patients, ABO-incompatible transplants, and as a surrogate of late graft dysfunction in clinical trials. (Nankivell, 2006) This group of patients is at increased risk for SAR, but other entities could also be diagnosed, such as CsA toxicity, polyoma nephropathy and recurrence of the original kidney disease. If such a strict protocol biopsy policy were to be applied, only a subset of patients would have a chance to be diagnosed accurately, and the SAR rate in the non-high-risk population would be uncertain. Protocol biopsies thus offer the possibility for a myriad of diagnoses, and help in understanding the physiopathological mechanisms involved in the early phases of pathological processes. These findings are time-dependent: early biopsies reveal more inflammation, while later protocol biopsies reveal more CAN as well as many other pathological conditions.

5.4. Markers of kidney graft dysfunction

The degree of kidney dysfunction is the scale used to categorize patients with CKD into five different stages. (National Kidney Foundation 2002) The scale is based on GFR, usually estimated by formulas based on the serum creatinine level. KTX patients are considered by definition as carriers of CKD, irrespectively of their GFR. (Levey, 2005) The equations used in non-KTX patients to calculate GFR do not necessarily perform equally well in KTX patients. (Gaspari, 2004) Despite the limitations of GFR estimates in KTX, they still remain the most simple and practical ways of monitoring graft status.

There are different methods of measuring GFR by using either exogenous or endogenous markers. Among the exogenous markers are inulin clearance, radioisotope clearance (i.e. $^{99m}$Tc-labeled DTPA, $^{51}$Cr-labeled EDTA or $^{125}$I-labeled iothalamate) and nonradioactive contrast agent clearance (i.e. cold iothalamate) (Poggio, 2007). The most commonly used endo-
genous markers for predicting GFR are serum creatinine concentration and creatinine clearance. Although the methods based on exogenous markers are unquestionably more precise, they are laborious and expensive. Creatinine concentration is therefore the most commonly used marker of kidney function due to its simplicity, wide availability and low cost. However, the assay used for measuring creatinine concentration should be calibrated to avoid bias. (Stevens, 2007) The experience on non-transplant patients has shown that weight, muscle mass and extremes of age affect creatinine concentration independently of the GFR. Numerous formulas for predicting GFR that take in consideration anthropometric factors are thus proposed in the literature. The most used formulas are Cockcroft and Gault, (Cockcroft, 1976) and four-variable MDRD, (Levey, 2006) and Nankivell’s formula has gained credibility in the transplant population. (Nankivell, 1995) All three formulas were compared with 125I-labeled iothalamate GFR in kidney transplant recipients. The four-variable MDRD equation performed the best, due to its lowest bias and highest precision. (Poggio, 2006) Nevertheless, an exogenous substance clearance should be used when a rigorous GFR measurement is needed, as for example in the assessment of renal function in living kidney donation. (Gaspari, 2004) Numerous studies have demonstrated that creatinine concentration does not predict pathological findings in kidney biopsies. (Isoniemi, 1992; Nankivell, 2003) A significant inverse correlation has been found between 99mTc-labeled DTPA and histological changes, but 99mTc-labeled DTPA nevertheless underestimated the degree of histological damage. (Nankivell, 2003)

The poor sensitivity to detect slight variations in GFR, together with several factors is influencing its concentration, make serum creatinine measurement for kidney function monitoring imprecise. Therefore, the proposal in 1985 of another endogenous substance, cystatin C (CyC), to predict kidney function seemed promising. CyC is a low-molecular-weight protein produced at a constant rate by all nucleated cells. It is freely filtered through the glomerular membrane and completely catabolized in the proximal tubules. (Simonsen, 1985) However, a drawback was encountered later: also factors other than GFR influence CyC concentration, such as thyroid function, (Fricker, 2003) cancer (Kos, 1998) and glucocorticoid therapy. (Risch, 2001) An increased extrarenal excretion of CyC has been described when GFR decreases. (Sjöstrom, 2005) Others have suggested that it can also be affected by age, sex, body size, cigarette smoking, and C-reactive protein, independently of GFR. (Knight, 2004)

The first extensive study of CyC performance in a cohort of 206 patients with various renal diseases concluded that CyC was more sensitive than creatinine. (Newman, 1995) Afterwards, two other large studies were carried out in a pediatric population. The authors presented a formula to estimate GFR from CyC, known as Bökenkamp and Filler formulas. (Bökenkamp, 1998; Filler, 2002) A comparison of the sensitivity of CyC with creatinine
was extensively reviewed in a meta-analysis published in 2007. The authors pooled over 2000 patients from 24 studies with very diverse pathologies and a wide range of ages. Using GFR under 60ml/min as the definition of CKD, the analysis of accuracy favored CyC. Cut-off values ranging from 0.9 to 1.4 mg/L ruled out CKD. (Roos, 2007) A literature review published at the same time concluded that a large number of studies favored CyC over creatinine for the estimation of GFR, while many others had found no superiority of one over the other. (Zahran, 2007) One of the reasons proposed for the reported discrepancy in the accuracy of CyC to detect CKD was the differences in the clinical presentations. In 2005 a study of 460 individuals, including healthy controls, CKD patients and transplant recipients, showed that in transplant patients CyC was 19% higher at the same GFR level measured by $^{125}$I-labeled iothalamate clearance. A strong association between CyC and GFR was found in native CKD patients, and the authors proposed a new formula for GFR estimation, based on CyC. (Rule, 2006) A cohort of CKD patients was selected in another study in which the gold standard was $^{51}$Cr-EDTA clearance. The study patients had CKD stages 2 to 3 (GFR 30–89 ml/min/1.73m$^2$). The authors concluded that CyC was a reliable marker of GFR in female patients with mild to moderate CKD. (Hojis, 2006)

The impact of steroids on plasma CyC concentration is of great importance due to their wide use in transplantation. This was first reported in an in vitro study using HeLa cells exposed to dexamethasone, revealing a dose-dependent increase in CyC concentration. (Bjarnadottir, 1995) The focus was therefore on the accuracy of CyC to predict GFR variations in the various circumstances that KTX patients go through. A prospective study on a case-control cohort undergoing different immunosuppressive regimens, including patients requiring steroid boluses to treat AR, reached the same conclusion as the in vitro studies: glucocorticoids affected CyC concentration in a dose-dependent fashion. (Risch, 2001) CyC may thus be useless for monitoring GFR when the steroid dose varies, as it does in the treatment of AR.

Results from pediatric KTX have encouraged physicians to switch from creatinine to CyC in the monitoring of CKD. (Filler, 2008) In adults, however, the opinions are divided. A Canadian study of 198 patients classified by $^{99m}$Tc-labeled DTPA into the five CKD categories revealed that the Filler equation correctly classified 76% of the patients, compared to 65% and 69% for the Cockcroft & Gault and MDRD equations, respectively. However, in stage 4 CKD the Filler equation diagnosed correctly only 60% and the MDRD 93% of the cases. (White, 2007) Nine CyC-based equations and 14 creatinine-based equations were compared in a study in which GFR measured by inulin was analyzed in 103 adult kidney transplants. The conclusion was that CyC did not perform better than creatinine. Therefore the authors do not justify the measurement of CyC for monitoring kidney graft function. (Zahran, 2007)
Overall, there are no studies demonstrating that creatinine is superior to CyC in predicting GFR. On the other hand, several studies point to a higher accuracy of CyC in estimating GFR. At least in the adult population, this supposed superiority of CyC seems to depend on the etiology of the kidney disease, the degree of impaired kidney function, and the use of steroids, among other factors.

5.5. Immunohistochemical techniques: TGF-β

Although not generally used in the routine analysis of kidney allografts, immunohistochemistry opens a possibility for detecting precursors of structural changes, revealed later by light microscopy. Several injuries to the kidney are ultimately manifested as interstitial fibrosis, tubular atrophy, glomerular sclerosis and vasculopahty. The overexpression of transforming growth factor β (TGF-β) plays an exclusive role in the final pathway of interstitial fibrosis.

TGF-β is synthesized by all cells in the body as a large precursor that is cleaved intracellularly and secreted. It is stored in the extracellular matrix as a complex of TGF-β-propeptide and the TGF-β-binding protein. (Blobe, 2000) Several factors can release TGF-β from this complex: alterations in pH, trombospondin 1, plasmin, and many other proteolytic enzymes. Three different isoforms of TGF-β have been identified in mammals: β₁, β₂ and β₃. They act as pleiotropic regulators of cell growth and differentiation, angiogenesis and arteriosclerosis, immunosuppression, inflammation, carcinogenenesis and tissue repair. TGF-β₁ acts as a major regulator of extracellular matrix production and degradation. (Pribylova-Hribova, 2006) TGF-β₁ can both stimulate and inhibit cell proliferation, playing an essential role in wound healing, as it restores the balance between inflammation and matrix cell deposition following an injury. (Jain, 2000) It has also immunosuppressive effects: TGF-β₁ promotes the differentiation of leukocytes and inhibits their proliferation and activation. (Letterio, 1998)

Drugs can alter the production of TGF-β. Some drugs are of major importance in kidney diseases, e.g., the calcineurin inhibitors CsA and tacrolimus are of particular interest due their wide use in KTX. They are the most potent and well known inhibitors of calcineurin, a phosphatase enzyme involved in lymphocyte activation. CsA binds in the cytoplasm of T-cells to cyclophilin, whereas tacrolimus binds to FK-binding protein. This complex activates calcineurin, which thereafter binds to calmodulin in the presence of calcium. (Gooch, 2007)

Calcineurin inhibition not only reduces the production of interleukin 2, but also affects other crucial molecules, such as other interleukins, nitric oxide synthetase, TGF-β, endothelin 1 and proteins involved in cellular protection against apoptosis. Particularly the increase in TGF-β production
is believed to be associated with CsA nephrotoxicity, although it may not be the only mechanism involved. (Cattaneo, 2004) The potential sources of TGF-β include macrophages, fibroblasts and tubular epithelial cells. It has been demonstrated that TGF-β expression is increased in kidney allografts compared to normal kidneys. (Lantz, 1996) In patients undergoing CsA treatment, the increased TGF-β expression in the biopsies was associated with a more rapid decline in kidney function. (Cuhaci, 1999) Lowering of the CsA dosage decreased circulating TGF-β along with further improvement in kidney function. (Hueso, 1998) CsA toxicity was found to be associated with a high intratubular expression of TGF-β. (Pankewycz, 1996) TGF-β showed a sustained increasing expression in sequential biopsies taken at implantation, one week after, and 6 months after transplantation. The authors noted that a history of delayed graft function was related to TGF-β expression, and that both calcineurin inhibitors, i.e. CsA and tacrolimus, exerted a similar influence on it. (Jain, 2002) TGF-β was found to be increased in rats lacking the α-isoform of calcineurin, and this was proposed to be the cause of increased fibronectin. It was thus suggested that the fibrosis and altered TGF-β expression associated with calcineurin inhibitors resulted from the inhibition of α-isoform of calcineurin activity. If so, targeted inhibition of the β-isoform of calcineurin may induce immunosuppression without nephrotoxicity. (Gooch, 2007)

Other widely prescribed drug families are the ACEi and ARB. A cross-talk between the renin-angiotensin-aldosterone system (RAAS) and TGF-β has been highlighted recently. (Wolf, 2006) Angiotensin II stimulates TGF-β expression in the kidneys and also upregulates its receptors. Also angiotensin III and aldosterone can activate TGF-β. A key issue regarding nephroprotection is the blockade of RAAS with either ACEi or ARB. ACEi-use has been linked to reduced serum TGF-β levels, and via this mechanism, among others, to protect the kidneys. (Campistol, 1999) A pilot study demonstrated a decrease in TGF-β expression in kidney grafts after 12 weeks of treatment with losartan. (Mas, 2004) There is no evidence, however, that ACEi or ARB can prolong graft survival.

The search for molecular predictors of kidney graft status in blood, urine or tissue has increased exponentially in recent years. Special attention was focused on AR, in the hope of finding a non-invasive method to diagnose this condition. This was achieved with quantitative PCR, detecting increased mRNA for perforin and granzyme in the urine of patients with AR. (Li, 2001) Increased urinary excretion of the adhesion molecule sICAM-1 was observed in patients a few days before AR. (Teppo, 2001) In the light of these promising results, attention was subsequently directed at the possibility of diagnosing CAN by the detection of adhesion molecular excretion in urine. For example, the cleaved amino-terminal peptide from type III collagen (PIIINP) is secreted into the surroundings in the initial phases of interstitial fibrosis. A higher urinary PIIINP/creatinine ratio was
measured from kidneys with fibrosis, along with elevated concentrations of urinary TGF-β. (Teppo, 2003) These findings were associated with a poor kidney allograft outcome. (Teppo, 2004)

Molecular techniques help to detect alterations at the molecular level, and their utility is not restricted to diagnostics, but they also have potential in the development of possible therapeutic targets. Although promising, these techniques cannot differentiate e.g. the type of rejection, or the cause of fibrosis. It is therefore unlikely that in the near future they can replace techniques based on histological morphology.

5.6. Microarrays

Genomic research has advanced remarkably after the development of microarray technology. The aim is to analyze simultaneous expression of tens of thousands of genes. Previously, the PCR technique made it possible to isolate individual genes. Microarrays open the possibility to identify simultaneous changes on multiple genes expression in different physiopathological processes. (Weintraub, 2006)

Regarding AR, transcriptional RNA-microarray brought to light distinct molecular patterns that ultimately correlated with a response to treatment and to graft outcome. The overexpression of genes involved in lymphocyte infiltration and activation is linked to a worse prognosis compared to the up-regulation of genes involved in cell-cycling and proliferation. (Sarwal, 2003) The gene expression of perforin, granzyme and FAS-ligand molecules differentiated biopsies, showing AR in control biopsies. (Scherer, 2003) In the case of CAN, there is wide up-regulation of genes involved in fibrosis, immune response and growth factors. However, these are hallmarks of late non-specific injury. (Hotchkiss, 2006) It is therefore challenging to stratify patients based on the mechanisms underlying the primary injury.

This new tool has several limitations. (Weintraub, 2006) Different patterns of gene expression from similar phenotypes have been found in individual studies. Gene expression differs in the cortex and medulla, despite the same underlying process. Thus the size and representativeness of the sample is critical in the interpretation of the results. Microarray analysis does not provide information on proteins and metabolic processes, as post-translational modifications can occur. In addition, the use of different tools for data processing and statistical analysis may lead to different conclusions between studies. Overall, the microarray techniques look promising, but they are not widely available, and they require validation in large-scale randomized controlled trials.
6. Aims of the study

The principal aim of this study was to find risk factors involved in the progression of CAN and to analyze the accuracy of diagnostic methods for this entity.

To achieve this, the following secondary aims were set:

- to analyze the risk factors implicated in the progression of chronic histological kidney allograft damage evolved from implant biopsies to six-month protocol biopsies (I);
- to find histological markers able to predict kidney allograft dysfunction during the first two years after transplantation (I and II);
- to compare the prognostic value of two different protocol biopsy policies (II);
- to compare the feasibility of the most commonly used estimates of kidney function, creatinine and cystatin C, for predicting CAN (III);
- to analyze the contribution of cyclosporin A concentration to CAN at the molecular level through the expression of a pro-fibrotic cytokine, transforming growth factor β1 (IV)
7. Material and methods

7.1. Patients

Altogether 553 patients living in the Helsinki University Central Hospital district received a kidney allograft between June 1994 and April 2008. Adult KTX patients were followed up at the same hospital. Living KTX accounted for 2% of the total kidney transplants.

The Immunosuppressive regimen was modified during the years, but the standard immunosuppression in this Institution is constituted by a calcineurin inhibitor, an antimetabolite and methylprednisolone. The most commonly used calcineurin inhibitor was CsA. CsA dosing was based on blood concentration of the drug before the morning dose (C0). The target level at 1 month was 150–180 μg/L; at 3 months 120–150 μg/L; at 6 months 100–140 μg/L and at 12 months and thereafter 80–120 μg/L. If the case was a re-transplantation, with previous sensitization or a poor HLA match, CsA was replaced by tacrolimus. Also tacrolimus was the CNI of choice in case of rapid steroids-tapering or steroid-free regimen to be adopted. An early discontinuation of corticosteroids was done if the patient developed severe complications related to its use, such as avascular necrosis of the femoral head or post-transplant diabetes mellitus. After 2003, corticosteroids were discontinued after the first year of transplantation. Azathioprine was the antimetabolite preferred up until to 2003, when it was replaced by MMF. Prior to 2003, MMF was however administered in selected cases by the physician’s discretion. The use of other medications believed to possibly affect the interpretation of the results, such as statins (Studies I and II) and antihypertensive drugs (Studies I, II and IV) was also recorded.

7.2. Protocol biopsies

A protocol biopsy from the functioning kidney allografts was obtained, with the patients’ full consent, if there was no medical contraindication for the procedure. Combined transplants (i.e. kidney-liver/ lung / heart / pancreas islets) were not considered for protocol biopsies. Before 2004, the policy at the Renal Division of the Helsinki University Central Hospital was to obtain a protocol biopsy at 6 months after transplantation. From 2004 to September 2007 the policy changed to protocol biopsies obtained
at two time points: 3 and 12 months. Between June 1994 and June 2000, 169 protocol biopsies were obtained at 6 months. Of those patients with representative material suitable for histological grading, 83 had an implant kidney biopsy available for comparison in Study I. Between December 2001 and October 2005, 45 6-month protocol biopsies and 41 3–12-month protocol biopsies were obtained, constituting the material for Study II. For the purpose of this study, implant biopsies from 34 and 36 patients, respectively, were available for comparison.

A plasma sample obtained concomitantly with the protocol biopsy was preserved at -20 °C. A representative protocol biopsy and frozen plasma samples were available from 105 cases in 1996–2002. This constitutes the material for Study III. From October 2007 onwards a single protocol biopsy has been taken at 6 months. Among the 112 out of 145 patients from whom protocol biopsies were obtained between 1 Jan. 2004 and 30 April 2008, 49 had a successful measurement of CsA concentration taken 2 h (± 15 min) after the morning dose. These 49 patients constitute the material for Study IV. (Figure 8)

7.3. Biopsy technique

Protocol biopsies were performed under ultrasonic guidance with either a Bard Magnum® or a Bard Biopty® automatic gun. An 18 gauge needle was used until 2007, and thereafter 16 gauge needles were applied. According to the policy of this Hospital, two core samples were taken. One of them was embedded in either plastic or Historesin® (Leica Instruments GmbH, Heidelberg, Germany), while the other was sectioned for immunofluorescence. The remaining tissue was frozen at -80 °C. The patients stayed in the Renal Ward for a mandatory 6 h of follow-up after the procedure, and before dismissal, an ultrasonic control was performed to screen for early complications.

Implant biopsies were obtained either after revascularization during implantation or during organ harvesting. The device used was a Bard Magnum® automatic gun and 18 gauge needles. In this case the whole core sample obtained was embedded in paraffin.
7.4. Histological analysis

Implant biopsies embedded in paraffin were cut into several serial tissue sections and stained with hematoxylin/eosin, periodic Acid Shiff (PAS) and Masson’s trichrome. Protocol biopsies embedded in either plastic blocks (until 2001) or Historesin and 3 μm thick serial tissue sections were stained with hematoxin/eosin, periodic Acid Shiff (PAS), silver methenamine (I–IV) and May-Grünwald-Giemsa (I) or toluidine (III) or Masson’s trichrome (II and IV). C4d staining was routinely performed since 2004 with immunoperoxidase, and since the beginning of 2008 with immunofluorescence.

Both implant and protocol biopsies were scored by two observers according to the CADI and Banff’97 classifications, described in detail elsewhere. (Isoniemi, 1994; Racusen, 1999) Briefly, interstitial inflammation, interstitial fibrosis, mesangial matrix increase, tubular atrophy, vascular intimal proliferation, and arteriolar hyalinosis were scored semi-quantitatively from 0–3 (0: none; 1: mild; 2: moderate; 3: severe) in both classification systems. In CADI also the percentage of sclerotic glomeruli was scored from 0–3 (0: none; 1: <15% of glomeruli are sclerotic; 2: 16–50% of glomeruli are sclerotic, 3: >50% glomerulosclerosis). Banff offers a categorical classification of CAN grades 1 to 3, whereas CADI scoring is based on the sum of the scores in six individual compartments (i.e., inter-
stitial inflammation, tubular atrophy, interstitial fibrosis, vascular intimal proliferation and percentage of sclerotic glomeruli) from a minimum of 0 to a maximum of 18. The definition of AR was taken from Banff ’97. Sub-clinical AR was defined as histological findings compatible with AR or borderline AR in a protocol biopsy from a patient whose graft function was unaltered. Details of CADI and Banf’97 classifications are depicted in tables 1 and 2.

The Banff ’97 requires a minimum of 10 glomeruli and 2 arteries. The threshold for a minimal sample was defined as 7 glomeruli and 1 artery. In Study I the biopsies included for the analyses had a minimum of 5 glomeruli and three arterioli per slice. The mean number of glomeruli in protocol biopsies was 9.5, being the reason why in 2007 the needle used for the procedure was changed to 16 gauge. Thereafter, the adequacy of the biopsy was upgraded to 7 glomeruli.

The Banff classification has evolved in the past decade, as described in 5.2. The term CAN has now been replaced by chronic rejection or IFTA, when the chronic rejection criteria are not fulfilled. The contemporary version of the Banff classification was adopted in this Institution after 2008. At the time when the histological material for this thesis was collected, the Banff ’97 version was used by renal pathologists. The term CAN is therefore used here in describing the results. CADI scoring system was extensively used in this thesis because its numerical stratification allows a follow-up of the histological changes semi-quantitatively. The numerical difference in the CADI score obtained from sequential biopsies at different time points (i.e. from implant to 3, 6 or 12 months) was assigned as delta CADI (ΔCADI) and used as a measure of progressing chronic histological damage in Studies I and II.

The pathologists’ interpretation was passed on to the clinicians with the purpose of adjusting the medication and for assessing prognosis. The remaining histological material was preserved in the laboratory for further investigations. In Study IV, 49 preserved paraffin blocks were cut into 3 μm thick slices for immunohistochemical analysis. They were stained with rabbit polyclonal antibody against a peptide mapping at the C-terminus of TGF-β1 of human origin (Santa Cruz Biotechnology Inc., CA, USA, TGF-β1(V): sc-146). TGF-β1 expression was scored semi-quantitatively from 0 to 3 in tubules, glomeruli, interstitial inflammatory cells and vessels by a single observer who was unaware of the medication or clinical status of the patients. The sum of the TFG-β1 in these compartments was acknowledged as TGF-β1 total score.
7.5. Clinical variables

Clinical data were obtained retrospectively from the patients’ hospital charts. Except for Study III, the data recorded were donor age and gender, donor type, donor’s cause of death (only in Study I), cause of end stage renal disease, dialysis modality (only in Study I), time on dialysis, number of HLA-AB and DR mismatches, percentage of panel-reactive antibodies (only in Study I), cold ischemia time (CIT), recipient’s race, gender and body mass index at the time of transplantation. During follow-up, the following data were recorded: occurrence of delayed graft function (DGF, defined as the need for dialysis after transplantation), AR events, weight, blood pressure, infections requiring hospital admission (only in Study I) and particularly CMV infections. The rate of complications after protocol biopsies was specifically recorded in Study II.

7.6. Biochemical variables

Kidney allograft function was estimated with serum (I) or plasma creatinine (II–IV), 24-h creatinine clearance (I), serum cystatin C (III), estimated glomerular filtration rate (GFR) based on creatinine (II and III) or CyC (III). For estimating GFR, creatinine expressed as μmol/L was converted to mg/dl by dividing by 88.8. CyC was measured from the samples taken at the time of protocol biopsy, and preserved at -20° C. The method used for CyC determination was the particle-enhanced turbidimetric immunoassay (Dako Cytomation, Glostrup, Denmark). GFR was estimated using CyC, creatinine and their reciprocals, CyC-estimated GFR (Larsson equation: estimated GFR= 77.24 x CyC(-1.2623)) (Larsson, 2004), Cockcroft & Gault (C&G= [(140-age) x weight] / (S-crea x 72) x 0.85 (if female)) (Cockcroft, 1976) and abbreviated Modification in Diet in Renal Disease (MDRD=175 x S-crea(-1.154) x age(-0.203) x (0.742 if female) x (1.21 if black)) formulas (Morath, 2003). Glycosylated hemoglobin, total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides were recorded (I and II). In addition, C-reactive protein, plasma albumin, 24-h proteinuria, parathyroid hormone, phosphorus and total calcium were collected (II). CsA concentration was obtained before the morning dose in Studies I, II and IV, and at 2 h after the morning dose in Study IV. CsA was measured with the enzymatic method (DiaSorin®). Also for the purpose of Study IV, mycophenolic acid concentration was measured at the time of the protocol biopsies (Cedia®).
7.7. Statistical analysis

Data are expressed as mean ± 1 SD, unless otherwise indicated. The Mann-Whitney U test was used to assess differences in the rank distributions of continuous variables between two groups. Fisher’s exact test served to evaluate the relation between two binary variables. Correlations between histological classifications, individual scorings and other continuous variables were calculated with the non-parametrical Kendall’s tau (I,III and IV) or Spearman’s test. Fisher’s exact test was used to correlate two binary variables (II). Differences between three groups were analyzed with the Kruskal-Wallis test, and the Dunn post-test was applied when considered convenient. Non-parametrical tests were used because of the relative small sample size and/or the lack of normal distribution of the variables (I, II, IV). CADI scores were transformed into binary variables using the mean value as cut-off point for logistic regression analysis, and the odds ratios were calculated (I). After testing for normality with the Kolmogorov-Smirnoff test, the t-test was applied to determine differences in GFR estimates in patients with either low or high CADI scores (III). Recipient-operator-characteristics (ROC) curves were used to compare the sensitivity and specificity for predicting CAN with several creatinine- and CyC-based GFR estimates (III). Positive and negative predictive values were obtained for different GFR estimates (III). Two-tailed P values lower than 0.05 were considered significant.

StatsDirect® statistical software (version 2.2.4) was used in Study I. SPSS® statistical software (versions 12.0, 13.0 and 16.0 for Windows, SPSS Inc., Chicago, IL, USA) and Excel 2003 (Microsoft Corporation) were used for the calculations in Studies II, III and IV. ROC curves were calculated with Prism® statistical software (version 4.0 Graph Pad Software Inc., San Diego, CA, USA).
8. Results

A total of 153 implant biopsies and 364 protocol biopsies were analyzed in this thesis. Because of the overlap in the time frame of individual studies, some of the biopsies were included in more than one investigation (Figure 8). The percentage of living-related transplantation remained low across time (2%). The most common causes of end stage renal disease were diabetic nephropathy, chronic glomerulonephritis and polycystic kidney disease. The percentage of female donors was 45.5%, and female recipients constituted 36.5% of the patient population. The mean donor age was 45 years (SD ±14) and mean recipient age 48 ±11 years. The mean total HLA mismatches varied slightly in these studies from 2.07–2.3 (P=ns) and the mean number of mismatches was 2.2 ±0.9. Despite the HLA-matching allocation policy, CIT was relatively short and remained unchanged across the study periods (mean 20 ±4h). The percentage of patients with DGF increased considerably from 23% (I) to 37% (II and IV). (P=0.03). The age of the recipients increased over the years, from 48 to 52 years (P=ns). Recipients’ male sex has remained predominant, but its prevalence rose to 73% of the recipients in the latest study (IV). No major complications occurred after protocol biopsies, and both patients and graft survival was 100% after the procedure.

8.1. Predictors of renal allograft histologic damage progression (I)

Biopsies at implantation retrieved from older donors and those died from a non traumatic cause had a higher CADI score. Predictors of a CADI score over 3 at 6 months were a CADI score over 1 at implantation, older age of the donor and higher number of HLA-AB mismatches. Patients who displayed moderate to marked progression of chronic histological changes in the 6 months following transplantation (ΔCADI) had a higher creatinine concentration at the time of hospital discharge. During the follow up, these patients had more elevate diastolic blood pressure than those who did not displayed deterioration on CADI score. A diastolic blood pressure over a cutoff point of 85mmHg also predicted a higher serum creatinine during 2 years of follow up. The total cholesterol and LDL cholesterol concentration at 6 months positively correlated with mesangial matrix increase. The HDL cholesterol concentration negatively correlated with vascular intima increase.
After testing CADI score components with logistic regression, using HLA-AB and HLA-DR mismatches, CIT, DGF, PRA, AR and serum creatinine at hospital discharge as independent variables, the risk for a ΔCADI over 1 was significantly increased by the degree of glomerulosclerosis in donor biopsy and the high serum creatinine concentration at hospital discharge. The odds ratios for having a ΔCADI over 1 are displayed in table 3.

### Table 3. Odds ratio for high CADI score in implant biopsies and in 6 month biopsies and odds for a ΔCADI over 1

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Odds for high CADI at transplantation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>donor age</td>
<td>1.07</td>
<td>1.01–1.14</td>
</tr>
<tr>
<td>non-traumatic donor cause of death</td>
<td>3.89</td>
<td>1.13–13.33</td>
</tr>
<tr>
<td>Odds for high CADI at 6 mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CADI in donor biopsies</td>
<td>3.82</td>
<td>1.19–12.21</td>
</tr>
<tr>
<td>Donor age, for 1-yr age increase</td>
<td>1.05</td>
<td>1.01–1.10</td>
</tr>
<tr>
<td>HLA-mismatches, for 1-unit increase</td>
<td>2.36</td>
<td>1.09–5.10</td>
</tr>
<tr>
<td>Odds for a ΔCADI &gt;1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% gs at transplantation, for 1%-gs increase</td>
<td>1.10</td>
<td>1.02–1.19</td>
</tr>
<tr>
<td>serum creatinine at hospital discharge, for 1μmol/L increase</td>
<td>1.01</td>
<td>1.00–1.02</td>
</tr>
</tbody>
</table>

### 8.2. Timing and value of protocol biopsies (II)

Twelve percent of 3 month protocol biopsies had chronic histological changes compatible with CAN. This percentage increased to 50% at 6 months but was only seen in 34% of the biopsies at 12 months. Among the 41 protocol biopsies diagnosed with CAN, only 4 of them were classified as II b (2 at 6 months and 2 at 12 months), being the rest of the cases graded as I a. The changes in CADI scores between the donor and protocol biopsies and between protocol biopsies at different time points are depicted in Figure 9. ΔCADI was negative in 19% of the biopsies between implantation and 6 months, 20% between implantation and 3 months, and 20% between implantation and 12 months.
Figure 9. Changes in CADI score between implantation, 3-, 6-, and 12 months biopsies

Borderline AR or subclinical AR were diagnosed in 7 biopsies. The consequences of these findings on patients’ management are depicted in table 4. Patients with borderline or subclinical AR did not show higher CADI score at 12 months compared with those without these features. The estimated GFR at 18 months was not significantly different among those with or without borderline or subclinical AR. CADI score at implantation, at 3-, 6-, and 12 months negatively correlated with estimated GFR at 18 months, but ΔCADI did not.

Steroids were withdrawn at one year after transplantation from 29 out of 45 patients in the subgroup of patients with 6 month biopsies and from 22 out of 41 in the subgroup of patients with sequential 3 and 12 months biopsies. A single episode of late AR occurred in the first subgroup, which was successfully treated with steroid boluses and conversion from CsA to tacrolimus.
Table 4. Consequences of protocol biopsies diagnosis on patients’ management

<table>
<thead>
<tr>
<th>Patient no. and time of biopsy</th>
<th>Finding in protocol biopsy</th>
<th>Consequence of the biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. (3 months)</td>
<td>Subclinical acute rejection Ia</td>
<td>Control biopsy at 5 mo. with normal histology</td>
</tr>
<tr>
<td>2. (3 months)</td>
<td>Subclinical borderline rejection</td>
<td>Control biopsy at 6 mo. with normal histology</td>
</tr>
<tr>
<td>3. (3 months)</td>
<td>Subclinical borderline rejection</td>
<td>Control biopsy at 6 mo. with normal histology</td>
</tr>
<tr>
<td>4. (3 months)</td>
<td>Tubular vacuolization</td>
<td>Tacrolimus dose reduction</td>
</tr>
<tr>
<td>5. (6 months)</td>
<td>Subclinical borderline rejection Ib</td>
<td>Intensification of immunosuppression</td>
</tr>
<tr>
<td>6. (6 months)</td>
<td>Subclinical borderline rejection</td>
<td>Conversion from cyclosporin to tacrolimus</td>
</tr>
<tr>
<td>7. (6 months)</td>
<td>CAN</td>
<td>Conversion from azathioprine to mycophenolate</td>
</tr>
<tr>
<td>8. (6 months)</td>
<td>CAN</td>
<td>Conversion from azathioprine to mycophenolate</td>
</tr>
<tr>
<td>9. (6 months)</td>
<td>CAN</td>
<td>Angiotensin-converting-enzyme inhibitor therapy (ACEi)</td>
</tr>
<tr>
<td>10. (6 months)</td>
<td>CAN and interstitial inflammatory infiltrate</td>
<td>Control biopsy at 12 mo. with no inflammation</td>
</tr>
<tr>
<td>11. (12 months)</td>
<td>Subclinical acute rejection Ia</td>
<td>Conversion from cyclosporin to tacrolimus and re-biopsy 1 mo later with normal histology</td>
</tr>
<tr>
<td>12. (12 months)</td>
<td>Subclinical borderline rejection</td>
<td>Control biopsy 1 mo later with normal histology</td>
</tr>
<tr>
<td>13. (12 months)</td>
<td>CAN</td>
<td>ACEi therapy, cyclosporin reduction</td>
</tr>
<tr>
<td>14. (12 months)</td>
<td>CAN</td>
<td>ACEi therapy, cyclosporin reduction</td>
</tr>
<tr>
<td>15. (12 months)</td>
<td>CAN, suspicion of calcineurin inhibitor toxicity</td>
<td>Cyclosporin dose reduction</td>
</tr>
<tr>
<td>16. (12 months)</td>
<td>Inflammatory infiltrates</td>
<td>Intensified clinical follow-up</td>
</tr>
</tbody>
</table>

8.3. Sensitivity and specificity of several estimates of GFR to predict CADI (III)

Creatinine, CyC and several formulas for estimating GFR that are based on these biochemical molecules were compared with kidney allograft histology. CADI, interstitial fibrosis, chronic vasculopathy and tubular atrophy correlated significantly with creatinine, 1/creatinine, MDRD, CyC, 1/CyC and the Larsson equation. Only creatinine and 1/creatinine correlated additionally with CAN and chronic glomerulopathy.
A subdivision of the biopsies using a CADI cutoff point of 2 demonstrated that mild histological changes were best revealed by creatinine, although with modest sensitivity/specificity. A plasma creatinine threshold of 111 μmol/L distinguished 74% of the patients with CADI>2 and excluded this condition in 66%. In the case of CyC, a plasma concentration threshold of 1.12 mg/dl had a positive predictive value of 69% and a negative predictive value of 60%.

The ROC area under the curve for creatinine was 0.72 (p<0.001). Creatinine and its reverse correlated best with CADI, chronic allograft nephropathy, chronic inflammation, tubular atrophy, vascular changes and glomerulopathy. Neither the C&G nor MDRD formulas to estimate GFR improved the performance of creatinine alone. CyC and Larsson’s formula performed similarly (for both, the ROC area under the curve was 0.67).

(Figure 10)

Figure 10. Comparison of ROC curves using a CADI cutoff point of 2. Crea: plasma creatinine; C&G: Cockcroft and Gault, MDRD: Modification of Diet in Renal Disease; CyC: cystatin C; Larsson: Larsson’s formula.

8.4. Expression of TGF-β₁ in protocol biopsies (IV)

8.4.1. TGF-β₁ expression in protocol biopsies and its relationship with CADI and exposure to CsA

Neither TGF-β₁ expression in different compartments nor total TGF-β₁ differed significantly among 3-, 6- and 12-month biopsies. Details of the
distribution of TGF-β1 in individual compartments at different time points are depicted in Figure 11. The expression of TGF-β1 in interstitial inflammatory cells correlated positively with CADI specifically at 6 months (tau 0.65 p=0.02). The only case of suspected acute CsA toxicity was diagnosed in a 6-month protocol biopsy, which displayed a considerable increase in TGF-β1 expression in all compartments. In this case C0 was, however, on target and C2 was 602 μg/L. Neither C0 nor C2 correlated with IFTA at any time point. Two cases of SAR or borderline AR were found, and they showed a remarkable increase in total TGF-β1 expression. (Figure 12)

The daily dose of CsA correlated negatively with total TGF-β1 expression in 12-month biopsies. A negative correlation was also found between the daily dose of CsA and TGF-β1 expression in interstitial inflammatory cells in 6-month biopsies (tau -0.66 P<0.01) and with TGF-β1 expression in glomeruli at 12 months (tau -0.42 P<0.01). The CsA dose in mg/kg correlated negatively at 6 months, both with TGF-β1 expression in interstitial inflammatory cells (tau -0.75 P=0.004) and in tubules (tau -0.52 P=0.04).

C0 did not correlate with the expression of TGF-β1 at any time point. On the contrary, C2 correlated negatively with TGF-β1 expression in interstitial inflammatory cells at 12 months (tau -0.38 P=0.02). Patients whose C2 levels were below 600 μg/L at 12 months expressed more TGF-β1 in interstitial inflammatory cells, but the significance was borderline (P= 0.058). When the C2 concentration was below 450 μgl/L at 12 months, more expression of TGF-β1 was detected in vessels (P= 0.043) and the total

Figure 11. Mean TGF-β1 expression in 3-, 6- and 12-month protocol kidney biopsies in tubules (TGF-b T), glomerulus (TGF-b G), endothelium (TGF-b V), inflammatory cells (TGF-b Infl) and the sum of individual scores (TGF-b total)
score was elevated (P = 0.043). The expression was slightly elevated also in
 tubules (P = 0.08) and glomeruli (P = 0.059).
 Altogether 8% of the patients were treated with ACEi or ARB at 3
 months and 52% at 12 months. A separate analysis of all patients on ACEi
 /ARB compared with those not using these drugs did not reveal differences
 in TGF-β₁ expression.

8.4.2. TGF-β₁ expression in protocol biopsies and
 kidney allograft function

We observed that patients with any expression of TGF-β₁ in interstitial in-
flammatory cells had a higher creatinine level at all time points compared to
those without any expression of the growth factor. The differences reached
statistical significance only at 6 months (creatinine 110 vs 128 µmol/L,
p<0.03). In other data points the significance was borderline (at 1 month
mean creatinine 124 vs 153 µmol/L, p = 0.057; at 3 months creatinine 112 vs
128 µmol/L, p = 0.08; at 12 months creatinine 115 vs 133 µmol/L, p = 0.07
and at 2 years 122 vs 153 µmol/L, p = 0.051).

Figure 12. Photomicrograph of a 6-month protocol biopsy displaying features of SAR
demonstrating TGF-β₁ expression in tubules (arrowhead), glomerulus (thin arrow),
inflammatory cells (open arrow) and vascular wall (thick arrow). The total TGF-β₁
score was 9, composed of the sum of T3, G2, Infl2 and V2. Indirect immunoperoxi-
dase. Original magnification X 200.
8.5. Summary of the histological results

8.5.1. Summary of CADI in protocol biopsies.

The mean CADI score in implant biopsies ranged from 0.6–1.35 (Studies I and II, respectively, P<.001). Absolute normal histopathology was found in 33% of the implant biopsies in Study I. The most common findings in these biopsies were glomerulosclerosis (mean 3%), chronic vasculopathy (mean score 0.24), tubular atrophy (mean 0.25) and interstitial fibrosis (mean 0.51). Any degree of glomerulosclerosis ranged from 27–43% of the implant biopsies, and the mean percentage varied from 3–9% (Studies I and II, respectively). Chronic vasculopathy (i.e. arterial fibrointimal thickening) was found in 16% of the donor biopsies, on average. (Studies I and II). Arteriolar hyalinosis was a common finding at implantation (mean 0.36, Study I).

The mean CADI score in 3-month biopsies was on average 1.25. Absolutely normal pathology was found in 31% of these biopsies, and CADI ≤1 was observed in 73% of these biopsies. The most common finding was inflammation (mean score 0.5), followed by interstitial fibrosis (mean 0.2) and tubular atrophy (mean 0.2). Less common were chronic vasculopathy (mean 0.18) and glomerulosclerosis (mean 0.1). Mean arteriolar hyalinosis was 0.15 (IV). Tubulitis was seen in three cases in Study II (two borderline AR and one SAR) and in one case of SAR in Study IV. On average, SAR and borderline AR were diagnosed in 5.5% of these biopsies.

The mean CADI score in 6-month biopsies fell from 3.27 in Study I to 2.2 in II and 1.55 in IV. (P=0.02) The lower CADI scores in Studies II and IV can be attributed to the lower scores of interstitial fibrosis (mean 0.27–0.74), tubular atrophy (mean 0.27–0.74) and chronic vasculopathy (mean 0.33–0.60). Inflammation was less common in the biopsies included in Study IV (mean 0.18) than in those included in Study II (mean 0.50). Mesangial matrix proliferation and chronic glomerulopathy were seldom observed. The mean arteriolar hyalinosis scores varied from 0.18–0.57 (IV and I, respectively). Tubulitis was seen in two cases in Study II and in one in Study IV. All studies taken together, SAR and borderline AR were detected, on average, in 3.3% of the 6-month protocol biopsies.

The mean CADI score in 12-month biopsies varied slightly from 1.9 (Study II) to 2.2 (Study IV). (P=ns) The most common findings were inflammation (mean 0.49), interstitial fibrosis (mean 0.44) and tubular atrophy (mean 0.37). Mean chronic vasculopathy varied from 0.1 (II) to 0.56 (IV). Mesangial matrix increase or chronic glomerulopathy were seldom seen. The mean arteriolar hyalinosis score was 0.2. Mild tubulitis compatible with borderline AR was observed in one case, in inflammatory cells and more intense tubulitis compatible with SAR was observed in another case, constituting altogether an incidence of 3%.
8.5.2. Summary of AR, SAR and immunosupression

AR. In the population included in Study I the cumulative prevalence of AR was 36%. In Study II the incidence of AR was 8.5% and in Study 4 it was 14%. More specifically, in Study IV the incidence of AR was 23% at 3 months, 18% at 6 months and 8% at 12 months. Late AR (i.e. after 12 months) was diagnosed in two patients in Study I, in one patient in Study II, and in three patients in Study IV. SAR was found in 3 out of 83 (3.6%) 6-month protocol biopsies in Study I. In Study II, SAR or borderline AR was detected in 3 out of 41 (7.3%) 3-month protocol biopsies, in 2 out of 45 (4.4%) 6-month protocol biopsies, and in 2 out of 41 (4.9%) 12-month protocol biopsies. In Study IV, one case of SAR at 3 months and one borderline AR at 6 months were observed out of 49 protocol biopsies (4%). The treatment of SAR or borderline AR was under physician discretion. Two patients in Study I were prescribed treatment for AR. Three patients in Study II and two patients in Study IV with SAR or borderline AR were switched from azathioprine to MMF or from CsA to tacrolimus. Four patients in Study II in whom immunosuppressive treatment was unchanged were later re-biopsied, and histology was normal in all of them.

Patients’ immunosuppressive regimen varied across the years. The proportion of patients on tacrolimus rose from 2.4% in Study I, to 10% in II and 23% in IV (P<0.0001). The use of MMF increased from 14.5% (I) to 97.5% (II and IV), respectively (P<0.0001). Methylprednisolone was routinely used during the first year after transplantation in 94% (I) to 100% of the patients (IV). An inhibitor of mammalian target of rapamycin was used in only one patient (IV) after discontinuing CsA due to drug-related neurotoxicity.

8.5.3. CsA toxicity in protocol biopsies

In Studies I and III, no cases of either acute or irrefutable chronic histological CsA toxicity were identified. In Study II, isometric vacuolization of proximal tubular cells compatible with calcineurin inhibitor toxicity was found in one 3-month protocol biopsy and one 12-month protocol biopsy. In Study IV, isometric vacuolization in proximal tubule cells and arteriolar hyalinization compatible with acute CsA nephrotoxicity was seen in one protocol biopsy. All in all, CsA nephrotoxicity was seldom seen in this series of protocol biopsies (0–2%).

8.6. Summary of the progression of histological damage (ΔCADI)

Progression of CADI from implant to 3, 6 and 12 months. There was an increase of 0.7–1.2 (P=ns) in mean CADI from the time of implant to the 3-month biopsies. This difference was caused by an increase in inflamma-
tion (mean increase from 0 to 0.5) and interstitial fibrosis (mean increase from 0 to 0.2). A higher ΔCADI was observed when the time period was longer: from implant up to 6 months the mean CADI rose from 1.35 to 3.27 in Study I (P<0.0001) and from 0.6 to 2.2 in Study II (P=ns). In these cases the difference was mainly due to interstitial fibrosis (mean increase from 0.5 to 0.74 in Study I and from 0 to 0.6 in Study II), tubular atrophy (mean increase from 0.25 to 0.74 in I and from 0.1 to 0.6 in II), increase in mesangial matrix (mean increase from 0.23 to 0.56 in I and from 0 to 0.3 in II) and inflammation (mean increase from 0 to 0.5 in II). A statistically significant increase was observed in interstitial fibrosis (P=0.01), tubular atrophy (P<0.0001), mesangial matrix (P<0.0001), transplant glomerulopathy (P=0.01) and CADI (P<0.0001) in Study I. Neither glomerulosclerosis nor chronic vasculopathy increased significantly from implantation up to 3 or to 6 months.

The mean CADI score rose from 0.7 in implant biopsies to 1.9 in 12-month biopsies. The difference was mainly due to interstitial fibrosis, tubular atrophy and inflammation, although it did not reach statistical significance. There was no substantial increment in chronic vasculopathy or glomerulosclerosis from implant to the 12-months biopsies.

Progression of CADI from three to six and twelve months. Study II included sequential biopsies taken at 3 and 12 months. Also in Study IV sequential biopsies at these time points in a subgroup of 7 patients was included in the analysis. There was a slight increase in CADI from 1.2 to 1.9 (P=ns). The mean CADI increased from 1.31 at 3 months to 1.55 at 6 months, and to 2.2 at 12 months (P=ns). This difference was mainly due to an increase in interstitial fibrosis (mean increase from 0.23 to 0.48), tubular atrophy (mean increase from 0.23 to 0.44) and chronic vasculopathy (mean increase from 0.15 to 0.56). The differences in ΔCADI were not statistically significant. Inflammation was present to a varying extent at all time points, and therefore did not affect ΔCADI.

8.7. Summary of the risk factors involved in the progression of CADI

The risk factors for a high CADI in implant biopsies were donor age (odds ratio 1.07) and non-traumatic cause of donor death (odds ratio 3.89). The risk factors for a high CADI at 6 months were CADI in implant biopsies (odds ratio 3.82), donor age (odds ratio 1.05) and HLA-AB mismatches (odds ratio 2.36). There were differences in the donor-related characteristics included in Studies I, II and IV. The mean age of the donors rose from 41 years in Study I, to 48 years in Study II and 52 years in Study IV (P=0.023). The cause of death of the older donors was more likely to be
of non-traumatic origin (e.g. a cerebrovascular accident), whereas a traumatic cause of death was more common among younger donors. Male sex of donors was prevalent in Study I, but the opposite was in the study involving more recent transplants (IV) (P=0.023).

The risk for a ΔCADI higher than 1 was elevated when glomerulosclerosis was found in the implant biopsies, and also if serum creatinine concentration remained elevated at the patient’s discharge from hospital after transplant surgery. Either a 1% increase in glomerulosclerosis in donor biopsies or a 10 μmol/L increase in serum creatinine at hospital discharge after transplantation increased the risk of having a ΔCADI over 1 by 10%.

Blood pressure and lipid profile correlated with individual components of the CADI score. Total cholesterol and LDL cholesterol correlated positively with the increase in mesangial matrix. HDL cholesterol correlated negatively with the increase in chronic vascular changes. Triglycerides did not have an impact on the development of chronic histological lesions from implant to 6-month biopsies. Both systolic and diastolic blood pressure correlated with tubular atrophy. Particularly diastolic blood pressure correlated with ΔCADI. Both systolic and diastolic blood pressure slightly fell after transplantation, in line with the increased number of patients under hypertensive treatment. Regarding dyslipidemia, clearly higher LDL cholesterol was observed in the patients in Study I (mean 3.6 mmol/L at 6 months) compared with Study II (mean 2.45 mmol/L) (P=0.001). Total cholesterol rose during the first year after transplantation, decreasing afterwards. Triglycerides, on the contrary, decreased after transplantation, and HDL cholesterol increased slightly during the two years of follow-up (I). Over the years we observed a marked increase in the use of statins. In Study I only 8% were on drug treatment for dyslipidemia, and the percentage rose to 59% of the patients included in Study II. (P<0.0001) A similar trend was observed with antihypertensive drugs, particularly with ACEi and ARB, which were seldom used in Study I, rising to 14% at 6 months post-transplantation of the patients in Study II, and to 33% of the patients in Study IV. (P<0.0001)

In Study I the mean BMI was 24 kg/m² and it did not increase in this population during two years of follow-up. Histological damage was more likely to progress in diabetic patients, but the difference did not reach statistical significance (p=0.06).

Neither AR nor CMV infections were associated with ΔCADI. The effect of SAR or borderline AR detected at 3 months on the histology at one year was analyzed in Study II. There was no difference in CADI score among those with or without SAR or borderline AR. Neither was a history of AR episodes associated with CADI at any time point.
8.8. CADI and ΔCADI as predictors of long-term kidney allograft function

CADI in implant biopsies, CADI at 6 months, and the difference between these two values (ΔCADI 0–6) correlated positively with serum creatinine at hospital discharge, 6 months, 12 months and two years after transplantation (Study I). Overall, CADI and ΔCADI 0–6 correlated positively with kidney allograft function at all time points, with the exception of CADI in implant biopsies and 12-month creatinine. Logistic regression analysis showed that the risk for having an abnormal serum creatinine concentration at two years from transplantation (i.e. > 115 μmol/L) was increased by 29% for each degree of increase in ΔCADI 0–6. The biopsies were subdivided into those with ΔCADI 0–6 > 1 and ≤1 and compared to kidney allograft function during two years of follow-up. The results of this analysis are shown in Figure 13.

Figure 13. Mean serum creatinine (with 95% confidence intervals) in patients with ΔCADI > 1 and patients with ΔCADI ≤ 1 at hospital discharge, biopsy time, one year and two years.

In Study II, kidney allograft function was estimated with the Cockcroft & Gault formula (e-GFR). CADI in all biopsies correlated negatively with eGFR at 18 months. A similar observation was made for ΔCADI 0–6 mo and ΔCADI 0–12 mo. In contrast, ΔCADI 3–12 mo did not correlate significantly with eGFR. The presence of CAN at 3 months did not have any
impact on eGFR at 18 months. However, IFTA (interstitial fibrosis plus tubular atrophy) at 3 months correlated negatively with eGFR (R = -0.47; P=0.004). Both CAN and IFTA at 6 or 12 months were inversely related to lower e-GFR at 18 months. Lower creatinine concentrations at 24 months were observed in the all-study comparison, and the difference between Studies III and IV was statistically significant (P=0.021) and also between Studies II and III (P=0.020).

8.9. Impact of SAR and borderline AR on long-term allograft function

The impact of AR and SAR on long-term allograft function was not analyzed separately in Studies I and IV due to the small number of such cases (3%). In Study II, patients with SAR and borderline AR were pooled, and their eGFR was compared to that of patients without these histological features. Kidney allograft function was found to be similar in both groups.
9. Discussion

This Study included protocol biopsies obtained in a 14 year period of time. The results of this thesis can be divided into two eras: the periods before and after the year 2001. Over these years, the prevalence of CAN changed and the patients’ treatment as well. The quality of the donors has worsened but immunosuppression and treatment of HT and metabolic disturbances were intensified along the years in this study population. The individual effect of these aspects on the pathophysiology of CAN is discussed below.

9.1. Pathophysiology of CAN

Before 2001 more kidneys from male donors after traumatic (i.e. accidental) death were transplanted, directly influencing the mean donor age in this period. After 2001 the trend was reverted: there was a significant increase in donor age, and the donors were mainly women whose death often resulted from an underlying disease. A similar observation was made in another study on the Finnish transplant population in 2005 (Kyllönen, 2005) and in a study based on the United Network of Organ Share (UNOS) data. (Su, 2004) This shows that the shortage of organs has prompted transplant physicians to accept kidneys from donors who in the past were considered marginal. The increased probability of accepting kidneys with some degree of damage is, however, overwhelmed by the longer survival and quality of life of the transplanted patients. The present study showed that older donor age and non-traumatic cause of death increase the probability of donor kidney damage, such as glomerulosclerosis, interstitial fibrosis and vasculopathy.

A proposed approach to the problem of marginal donors is to evaluate implant biopsies from older donors before accepting their kidneys for transplantation. In a study conducted in 2006, a score grading of implant biopsies was proposed as a tool to decide whether the kidney was suitable for transplantation. After 23 months of follow-up, histologically evaluated kidneys from donors older than 60 years had similar graft survival as kidneys from younger donors. The stratification of the kidneys from donors over 60 years of age, based of histological grading, resulted in a lowered prevalence of DGF, when compared to kidneys transplanted without this histological evaluation. (Remuzzi, 2006) In our Hospital a histopathological evaluation of implant biopsies of marginal donors before proceeding to implantation has not been done. In this Study absolute normal histology was found in one third of implant biopsies. The most common finding in
those with a CADI score ≥1 was glomerulosclerosis, and this finding was shown to be of prognostic value. A 1% increase of glomerulosclerosis in implant biopsies increased the risk for histologic damage progression and it was also associated with worse KTX function. It can be hypothesized that a thorough pathological evaluation before acceptance might prevent the progression of CAN in two thirds of the KTX.

In the studies included in this thesis, only data on CIT were collected, as information on the length of time between brain death and kidney harvesting was not available. Considering that free-radical-mediated injury starts before brain death and continues during organ preservation, shortening the CIT may reduce this type of injury. Brain death is associated with an autonomic storm that leads to tissue hypoperfusion and thus accumulation of free radicals. In addition, a systemic inflammatory response takes place, manifested by the upregulation of proinflammatory cytokines. Interstitial leukocytes and E-selectin adhesion molecules were detected in kidneys from cadaver donors when compared to living donors. TGF-β seemed to have a role in this process. (Nijboer, 2004) In a study from the year 2003, the oxidative stress in the kidneys from brain death donors was evaluated. A high concentration of malondialdehyde and low total antioxidant status were associated with poor short-term as well as long-term graft survival. (Kosieradzki, 2003) Therapies in which the cadaver donor is treated with antioxidant agents before kidney harvesting seem promising, but they require further investigation in large clinical trials.

The negative change in the quality of donors witnessed over the years may explain the considerable increase in the incidence of DGF from 23 to 37% before and after the year 2001, respectively. A report from UNOS stated that the prevalence of DGF was 22% in 1991 and 25% in 2003. (Cecka, 2003) DGF is directly influenced by donor age, CIT and hypovolemia. (Nyhof, 2005) Biopsies from patients with DGF may reveal acute tubular necrosis, AR or CNI toxicity. The policy in our Hospital consists in a protocol biopsy evaluation in patients with DGF. Although DGF did not prove to be a risk factor for CAN progression in the present Study, an elevated level of serum creatinine at hospital discharge was. This may have been the consequence of either DGF or grafts starting to function slowly. In a retrospective study on 611 KTX patients, the presence of concomitant AR and DGF shortened graft survival time independently of the duration of CIT. Prolonged CIT nevertheless seemed to predispose to AR, explaining why a stronger immunosuppressive regimen was proposed in this situation. (Mikhalski, 2008) In the population investigated in this thesis, the stronger factor influencing the increased prevalence of DGF was the older age of the donors, given the low prevalence of severe AR, rare CNI toxicity, and unchanged CIT.

The mean HLA mismatches remained nearly unchanged over the time, and CIT seldom exceeded 24 h in the present study. Lately, the kidney al-
lograft allocation based on HLA matching has been criticized in the USA because it delays the whole allocation process, consequently prolonging CIT. (Salahudeen, 2004) Also the impact of HLA mismatch on graft survival in the modern era of more potent immunosuppressive agents has been questioned. (Su, 2004) However, a large European study concluded that HLA matching still influences graft outcome positively. (Opelz, 2007) The kidney allocation policy in Finland is based on HLA matching and has led to excellent long-term results without significantly affecting CIT. (Salmela, 2004)

The nephrotoxicity of CNI is a side effect which comes along with its immunosuppressive effect. Thus, the options are to either avoid CNI or to use lower doses. The definition of a low dose is, however, difficult to determine because of the great variation in the risk profiles of different patients. There is evidence that minimizing or avoiding the use of CNI is safe and efficient, when established at least three months after transplantation. (Haller, 2009) In the Elite-Symphony study, the standard CsA through level ranged from 100–200 μg/L three months after transplantation, and the low-CsA target was 50–100 μg/L. The low-dose CsA concentrations mentioned above are actually standard target concentrations for the Finnish population, and might explain the low prevalence of CsA toxicity reported in the present study. The great variability in the absorption profiles of patients led to the development of a more predictable formulation of CsA, i.e. the microemulsion. (International Neoral Renal Transplantation Study Group, 2002) The patients included in the present study were all on CsA-microemulsion therapy. Due to the fact that optimal CsA exposure is critical in preventing both nephrotoxicity and under-immunosuppression, it is of utmost importance to evaluate the tools available for accomplishing it. In this study CsA exposure was monitored with through concentrations, C0. In accordance to the recommendations presented in the literature in recent years (Citterio, 2005; Dominguez, 2005; Pallardo, 2007), C2 was also measured concomitantly with protocol biopsies. The first limitation for the analysis was that the measurement of the CsA concentration within a time frame of 2 h ± 15 min succeeded in only 44% of the CsA users. The benefits of C2 monitoring have been described in many randomized controlled trials. The rate of successful C2 measurement in regular clinical practice, beyond rigorously controlled trials, has not been reported, however. Among the factors that may have affected the low percentage of successful C2 measurements in the present study are: the place where the samples were obtained (a busy laboratory of a general hospital), and insufficient information given to the patients and to the staff stressing the importance of the timing related to the procedure.

The hallmark of CsA nephrotoxicity is striped interstitial fibrosis. (Cattaneo, Zenoni et al. 2005) The present study was unable to show an association between either C0 or C2 and IFTA in protocol biopsies. A possible
explanation for this may be that IFTA is the result of many factors in addition to CsA. Kidneys biopsies from patients on CsA had enhanced the expression of TGF-β, and it has been suggested that CsA nephrotoxicity occurs via a TGF-β dependent pathway, although the exact mechanisms underlying this relationship are not completely understood. (Jain, 2002) In accordance with the above results, the decrease in CsA dose directly lowered the serum concentration of TGF-β and improved kidney function. (Hueso, 1998) The present study is the first one to report an association between C2 and TGF-β1 expression in protocol biopsies. The TGF-β1 staining of implant biopsies was not possible, but the expression of TGF-β1 did not increase from 3 to 12 months. An increasing expression of TGF-β1 was reported in sequential protocol biopsies in the literature. (Jain, 2002) This discrepancy may be explained by the low CsA targets in the population of the present study, which is in accordance with the low incidence of CsA toxicity. The significance of the expression of TGF-β1 in inflammatory cells is not known, but could be the consequence of under-exposure to CsA, leading to immune activation. An interesting observation in this study was the negative correlation between C2 and TGF-β1 expression in inflammatory cells. Recently, more attention has been paid to the subcapsular and perivascular inflammatory infiltrates detected in kidney biopsies, previously not believed to be of prognostic value. The last report on the Banff classification recommends scoring of the total inflammation and revision of this concept in future meetings. (Solez, 2008) In the light of the present study, the staining for TGF-β1 may bring valuable information about the pathophysiology of CAN. C2 below 450 μg/L at one year post-transplantation was associated with an increased total expression of TGF-β1, and particularly in the vessels. The increase in the expression of TGF-β1 in the vessels may be paralleled by the increase in chronic vasculopathy revealed by light microscopy. It can be hypothesized that TGF-β1 contributed to the development of allograft vasculopathy. An association between TGF-β1 expression and kidney function in the 6-month biopsies may add prognostic value to this finding. According to the results of the present study, a C2 target < 600 μg/L at one year might be discouraged in this patient population.

A drawback of low-CNI regimens or discontinuation of CNI is the increased risk of AR. That is why these regimens are usually associated with the initiation of an m-Tor inhibitor. (Fellström, 2004) In the present study, replacing CNI by rapamycin was acknowledged in one case only. The cumulative incidence of AR in this study was dramatically reduced from 36 to 14% before and after the year 2001, despite the relatively low daily doses of CsA and through target concentrations. In the present study, AR was not a risk factor for progression of CADI. This may be due to the fact that the majority of the AR were mild, steroid-sensitive and occurred early, i.e. before the third month. In addition to this, the prevalence of SAR
was constantly low in this study. Before 2001, when the standard immunosuppression regimen consisted of CsA, azathioprine and steroids, the incidence of AR was 36% and that of SAR 3%. After 2001 tacrolimus was used more frequently and, as mentioned earlier, azathioprine was replaced by MMF in the treatment of new transplant patients. In this latter period, the incidence of AR fell to 14% and the prevalence of SAR remained unchanged. Even patients in whom SAR was detected did not have a more adverse outcome than those free of SAR. It is worth mentioning that in half of the SAR cases the immunosuppression regimen was modified after the protocol biopsy. At 3 months, 73% of the protocol biopsies displayed an almost normal histology, and SAR was found in only 7%. This subgroup of SAR was not treated, and the tubulitis had spontaneously resolved in a subsequent biopsy. On the contrary, protocol biopsies at 6 months revealed more commonly chronic damage, and this correlated with worse kidney allograft function at two years. In these biopsies the incidence of SAR was 4%. This subgroup of patients was treated by increased immunosuppression and stricter follow-up. Whenever CAN was present in 6- and 12-months protocol biopsies, ACEi or ARB therapy was considered. Unfortunately, a follow-up protocol biopsy was not available from all these patients for evaluating the efficacy of these measures.

In the present study, the CADI score in 6-month biopsies fell from 3.27 to 1.55. Although the causes of this remarkable improvement may be multifactorial, a crucial change in drug therapy took place in 2003, when azathioprine was substituted with MMF. The more active use of ACEi/ARB and statins may also have played a role in this improvement. An increase in mesangial matrix correlated with total cholesterol and LDL. High HDL correlated with a decrease in the progression of chronic vasculopathy. Statins were more frequently prescribed after 2001, and this might in part explain the better dyslipemia profile described in 8.7. Similarly, the number of patients under drug treatment for hypertension increased over the years, and ACEi/ARB were the most commonly used drugs. This study showed that tubular atrophy was associated with hypertension, and that the progression of CAN was directly related to high diastolic blood pressure. A randomized controlled trial involving 162 KTX patients compared the effect of losartan, captopril or amlodipin on plasma concentration of TGB-β₁ and histopathological changes in serial biopsies. The authors found that biopsies from patients treated with losartan showed less progression of the histological changes and reduced plasma TGB-β₁ (el-Agroudy, 2003). Randomized controlled trials demonstrating prolonged graft and or patient survival attributable to treatment with ACEi/ARB or statins are still lacking (Cruzado, 2008). However, the present results support the use of these drugs to slow the progression of CAN.

The most common etiologies found in this study – diabetic nephropathy, chronic glomerulonephritis, and polycystic kidney disease – are in
agreement with the epidemiological data from the Finnish Kidney Transplant Registry (www.musili.fi/smtr) Although the etiology of primary kidney disease does not seem to affect CAN, patient survival might be affected if the follow-up period is long enough. In the present Study follow-up was limited to 2 years, and de novo or recurrent disease was seldom seen. In a large retrospective study including over 105,000 patients from UNOS and the United States Renal Data System (USRD), one fifth of the recipients had a history of cardiovascular disease prior to KTX. The most common cardiovascular diseases were unstable angina pectoris, coronary artery disease, and congestive heart failure. After 8 years' follow-up, both graft and patient survival were diminished if cardiovascular disease had been present prior to transplantation. Particularly diabetic recipients had a significantly higher prevalence of cardiovascular disease than non-diabetics (46 vs. 15%, respectively). The diagnosis of congestive heart failure or arrhythmia in this population was associated with worst graft survival. (Petersen, 2007) An abnormal glucose metabolism has been associated with poor patient survival, mainly due to death secondary to cardiovascular events. (Petersen, 2007; Hjelmesaeth, 2006) Graft survival was reported to be shortened in patients with PTDM, (Kasiske, 2003) but a relationship between abnormal glucose metabolism and CAN is missing. In the present study, a trend towards progression of histological damage in diabetics was observed. The lack of statistical significance (P=0.06) may be related to the fact that diabetic nephropathy is a slowly progressing disease, and the follow-up in this study was limited to two years. The lack of systematic protocol biopsies beyond the first year is another limitation to evaluating the impact of PTDM or IGT on the progression of CAN.

9.2. Tools for clinical surveillance of kidney allograft status

It has been generally accepted that the “well being” of kidney allografts is reflected by normal kidney function. This view was proven incorrect over 15 years ago when biopsies obtained from normally functioning kidney allografts displayed chronic damage. (Isoniemi, 1992; Freese, 2001, Legendre, 1998) In contrast, biopsies from suboptimally functioning kidney allografts can appear normal. The latter situation evidences the limitations of making an inference from a biopsy sample to represent the whole kidney allograft, the so-called sample bias. Sample bias may explain the negative ΔCADI scores obtained in some cases in this study. Another possible explanation is that among the CADI score components, inflammation, unlike fibrosis or tubular atrophy, may resolve over the time.

Protocol biopsy has been postulated as an appropriate surrogate of late graft function in clinical trials. (Mengel, 2007) It is also the standard
practice of care in many transplantation centers. Protocol biopsies have become more popular lately, despite the lack of consensus regarding this policy. Transplantation centers with a great deal of experience on this procedure have not yet proposed any suggestion about the timing of protocol biopsies. There is agreement concerning the necessity of protocol biopsies to monitor kidney allografts in the case of DGF, high-risk cross-match-positive patients, and ABO incompatibility. (Nankivell, 2006) Two different timings for protocol biopsies were compared in the present Study. In this population, a protocol biopsy obtained at 3 months displayed substantial histological changes in only 27% of the cases and did not predict late histological damage. Biopsy findings correlated with estimated GFR at 18 months, but did not add crucial information to that obtained from implant biopsies. Protocol biopsies obtained at 6 and 12 months revealed a wide variety of chronic changes that were of prognostic value for late graft function. Furthermore, the recognition of histological changes, both acute and chronic, helped clinicians to make modifications in the drug treatment of the recipients.

Although the protocol biopsies provided information that cannot be obtained in any other way, the most trusted everyday tool for monitoring kidney allograft function is the measurement of serum creatinine concentration. Serum creatinine has a poor sensitivity in detecting small changes in GFR, (Hoek, 2003) a highly critical situation especially in the monitoring of early kidney transplants. The search for another clinically useful biochemical marker of GFR led to the thorough evaluation of CyC as a suitable candidate. A notable drawback of CyC is the influence of glucocorticosteroids on its concentration, independently of variations in GFR. (Risch, 2001) This is a definite disadvantage when high steroid doses are needed to treat AR, making CyC useless for monitoring a graft recovering from the insult. The present study assessed whether CyC was more sensitive than creatinine for predicting CAN. The results pointed to a better positive and a negative predictive value of creatinine compared to CyC. It was not possible to determine whether the small dose of methylprednisolone used by 94% of the patients could have exerted an influence on these results. Numerous studies favor CyC as a better estimator of GFR, particularly in children and in patients whose GFR is slightly reduced. Several formulas have been proposed for estimating GFR based on CyC. (Filler, 2008; White, 2007) The discrepancy in the literature may be due to the differences in the populations studied and the variability in CKD stage, and the gold standard method employed. (Zahran, 2007) Based on the results of the present study, measurement of CyC afforded no benefit in the case of adult kidney transplants.
10. Conclusions

The progression of CAN in the present study seems to be affected by two major factors: the quality of the donor kidney and suboptimal control of the recipient's blood pressure. The age of donors has been on the rise in recent years due to the shortage of organs. Cadaver donors are more often females and with an underlying disease. This may explain the increasing prevalence of DGF found in this study. Careful hemodynamic control of the cadaver donor and efforts to shorten CIT are mandatory. Evaluation of biopsies from marginal kidneys prior to implantation is a strategy that ought to be considered, as it might influence CAN progression.

Diastolic blood pressure was associated with progression of CAN. Not only hypertension, but also dyslipidemia was associated with mesangial matrix expansion. These changes progressed already in the first 6 months after transplantation. The results may encourage the use statins and ACEi/ARB more widely to slow down the progression of CAN.

Implant biopsies proved to be of prognostic value, and they are essential for comparison with subsequent biopsies. Compared to implant biopsies, protocol biopsies at 3 months did not afford substantially superior prognostic value, and were not followed by modifications in the treatment. Protocol biopsies at 6 and 12 months were suitable surrogates of late kidney allograft function, and impacted the clinical management of the patients. The effect of this policy on CAN progression could not be evaluated, because this is an observational study. Nevertheless, the modifications to the treatment based on protocol biopsies may have contributed to the decrease in CADI score during the course of the study.

The prevalence of SAR was low in the present study, and AR was significantly reduced, despite the modest immunosuppressive treatment. The relevance of the expression of TGF-β1 in inflammatory cells remains to be confirmed, but it may be related to low immunosuppression. Thus, a CsA level below 600 μg/L at one year might be discouraged. Biopsies from patients with even lower C2 targets showed increased expression of TGF-β1 in the vessels, an observation that was linked to the progression of chronic vasculopathy in the graft. The expression of TGF-β1 in inflammatory cells of protocol biopsies was related to weaker graft function during follow-up.

The factors involved in the progression of CAN and the proposed strategies to manage it are summarized in Figure 14.

In routine clinical follow-up, creatinine measurement continues to be the most feasible way to monitor the function of kidney allografts, even
though its sensitivity to detect CAN is low. Protocol biopsies at 6 mo thus constitute a suitable surrogate to be used in clinical trials, and an advisable policy for the monitoring of kidney allografts.
11. Finnish summary (Yhteenveto)

_Tausta._ Munuaissiirto on paras tapa hoitaa kroonista munuaisten vajaatoomintaa. Vaikka tulokset ovat paranneet viime vuosina, huomattava määrä munuaissiirteistä menetetään siirtopotilaan ennenaikaiseen kuolemaan ja pitkäaikaiseen siirrännäisen nefropatiaan (CAN).

_Tavoite._ Analysoida CAN:in riskitekijöitä ja eri diagnostisten menetelmien arvoa.


12. Acknowledgments

This work was carried out at the Helsinki University Hospital, Department of Medicine, Division of Nephrology.

I wish to express my gratitude to Professor Carola Grönhagen-Riska for providing me with the opportunity to initiate a career as a researcher and with the possibility to join the division of Nephrology. I am greatly indebted to my supervisors, Docent Eero Honkanen and Docent Petri Koskinen. I am grateful to Eero Honkanen for the enthusiasm, patience, support and friendly attitude through all these years that gave me the courage to overcome the difficulties. It is hard for me to find enough words to thank Petri Koskinen for introducing me to the amazing world of kidney transplantation, for the enthusiasm we share about our work, for your support. I am glad to have you as a friend.

The careful review and constructive criticism of the two reviewers, Docent Kaj Metsärinne and Professor Hannu Jalanko, had a great impact on the final version of the manuscript. Thank you for your valuable comments.

I wish to express my gratitude to my co-authors: Docent Tom Törnroth, for your friendly attitude towards me and the valuable knowledge you transmitted to all of us. Special thanks to the outstanding young investigator Ilkka Helanterä: thanks for your help in so many ways. To Anne Räsänen-Sokolowski for the constructive comments and for providing the photomicrographs included in this thesis. Thanks to Professor Timo Paavonen, for introducing me in the field of the kidney allograft pathology. To Docents, Kaija Salmela and Lauri Kyllönen, thanks for your help and comments. Thanks to Professor Patrik Finne for the biostatistical guiding. I extend my thanks to Aimo Harmoinen and Heikki Helin for their contribution to this work.

The histological analysis this thesis is about would not have been possible without the collaboration of Marjatta Hellman, Erika Wasenius, Jaana Komulainen and Eeva Rouvinen from the Transplantation Laboratory. Thanks to all of you for your skilful assistance. I extend my gratitude to the nurses and secretaries of the Nephrology Division for your assistance. Also and want to thank the coordinators of the Transplantation office, Eero Hartikka and Heikki Norio for their friendly attitude and their assistance.

I want to express my gratitude to my colleagues in the Division of Nephrology. Especially I warmly thank Anna Salmela, Virpi Rauta, Agneta Ekstrand, Leena Martola, Kati Kaartinen. Sari Aaltonen, Jari Hartman, Mikko Haapio, Kristiina Ylinen, Mika Huuskonen, Seija Peltonen, Anja Corner and Per-Henrik Groop.
I am indebted to Terttu Kaustia for revising the English language of the manuscript.

To all my friends here and overseas, thank you for tolerating my endless talk about my thesis during all these years. I appreciate the medicine you gave to my soul during the time we have spent together.

This book is dedicated to my family. Today achievements would not have been possible without the love and support I received from my parents, Margarita and Fernando. I want to express my love to my father, from whom I have learned the meaning of dignity of work, honesty and for the eternal support. To my mother, for being the best example of mother I could ever imagine. Your will be forever present in the smile of my girls.

Finally, I have to confess that I received an enormous hand from my husband Marcus. Thank you for standing my humour, for believing in me and for taking care of our home during my trips to scientific meetings. Nothing would mean a thing to me without my amazing daughters Lupe and Milu. You girls give me the strength to go on and you fulfil me with joy. Forgive me for the long hours this profession takes from me, but there is a statistical probability that one day you will be proud of your mother.

This work was financially supported by research funds of the Helsinki University Central Hospital.
13. References


88


