Chapter 9

Summary and general discussion
The overall aim of this thesis was to explore the potential relation of systemic markers of chronic low-grade inflammation with chronic transplant dysfunction (CTD) – leading to graft failure – and mortality after renal transplantation. A local inflammatory process in a tissue may lead to shedding of substances into the systemic circulation. The first part of the discussion of this thesis will be on markers of which variation in the systemic circulation may reflect variation in activity of ongoing local inflammatory processes in the transplanted kidney and future perspectives for research around these markers. A way to identify such markers is by investigating in a prospective study in renal transplant recipients whether they predict decline of renal function and/or occurrence of graft failure. If a marker would be predictive of mortality and not of decline of renal function or graft failure it may more reflect an ongoing systemic chronic inflammatory process, such as atherosclerosis, without particular involvement of the kidney. Such markers may be expected to predict (cardiovascular) mortality and/or cardiovascular events rather than decline of renal function and/or graft failure. The second part of the discussion of this thesis will be on such markers.

**SYSTEMIC MARKERS OF CHRONIC LOW-GRADE INFLAMMATION AND THE TRANSPLANTED KIDNEY**

The two most important causes of graft loss longer after renal transplantation are CTD and death with a functioning graft, the latter mainly due to atherosclerotic cardiovascular disease. Recent insights suggest that CTD and cardiovascular disease share chronic low-grade inflammation and accelerated atherogenesis in their pathogenesis. For CTD, this suggestion is supported by the histological features of CTD, in which equivalents of atherosclerosis, including glomerulosclerosis, hyalinosis, and perivascular inflammation are prominent. Equivalents of atherosclerosis are, however, not the only common manifestation of ongoing low-grade inflammation in transplanted kidneys: the interstitium can also be importantly involved. A recent revision of the original Banff scoring system for international standardization of histological findings in biopsies of transplanted kidneys importantly acknowledges this. During the 9th Banff conference held in 2007 a new lesion score, termed ‘ti’ (total interstitial inflammation), was added to the Banff schema. This lesion score uses quantitative criteria for mononuclear cell interstitial inflammation in total parenchyma (scarred and unscarred) scores.

Many centers are reluctant to perform surveillance biopsies, as this is an invasive procedure, with an ever existing risk of complications, including intractable bleeding necessitating removal of the graft. As a consequence, currently, noninvasively estimates of glomerular filtration rate (e.g., creatinine clearance or plasma creatinine) and proteinuria are used for the identification of renal transplant recipients at increased risk for CTD. However, once serum creatinine starts to rise or proteinuria develops, chronic structural
lesions are already present and it is usually too late for intervention. So, there is a great need for biomarkers that allow for earlier identification or prediction of CTD.

In search for new biomarkers it is important to choose an appropriate end-point for analyses. For a first exploration biomarkers for of CTD leading to graft failure the appropriate end-point would intuitively be graft failure, with censoring for death. If, however, the supposed disease process is importantly shared with the end-point of death due to cardiovascular disease, a biomarker for this shared process – like CRP for the process of atherosclerosis, with the process of atherosclerosis provoking both decline of function of the transplanted kidney and development of cardiovascular disease – might come out with huge underestimation of predictive performance if graft failure is used as end-point, because in particular those with declining renal function would tend to die from cardiovascular disease before they can reach the end-point of graft failure, particularly if death with a functioning graft would be more common than graft failure itself.

1. **C-reactive protein**

C-reactive protein (CRP) is considered a marker of the chronic low-grade inflammatory component of the process of atherosclerosis. So, C-reactive protein as a potential early marker for CTD could be particularly susceptible to this “confounding” by early death due to accelerated atherosclerosis. To overcome this theoretical problem for C-reactive protein we investigated whether C-reactive protein is a predictor of change of renal function until end of follow-up. If patients died, last creatinine clearance during follow-up was used for evaluation. We indeed found that elevated concentrations of hsCRP independently predict accelerated deterioration in renal function (Chapter 3). Our study is the first to show that hsCRP can be used for early identification of renal transplant recipients at risk for deterioration of graft function, independent of other clinically accepted predictors of graft failure such as proteinuria. At the time we performed the analyses for this study, too few renal transplant recipients (RTR) had developed graft failure defined by need for return to dialysis or retransplantation to allow for multivariate evaluation. At that time, rate of death was approximately two times higher than rate of graft failure. Later, this difference in rate appeared to persist. Interestingly, in a later study, in which we evaluated plasma procalcitonin (PCT) as a potential marker for CTD, it appeared that hsCRP is not predictive of death-censored graft failure, but strongly predictive of death (Chapter 4), which could be in agreement with our hypothesis that hsCRP is a marker for the inflammatory component of the process of atherosclerosis which also involves the transplanted kidney. It was, however, not possible to investigate whether hsCRP was still predictive of change in renal function, because at the time there were enough graft failures and deaths to allow for multivariate evaluation our laboratory
had shifted to another assay for plasma creatinine.

Like already outlined, the inflammatory process in transplanted kidneys frequently involves the interstitium in addition to vascular structures.\(^\text{10}\) Where it is currently even in biopsy studies still difficult – if not impossible – to determine whether the presence of inflammation is due to ongoing alloreactivity or not, this is even more difficult in epidemiological studies like the ones we perform. An important point that is not mentioned in reports on the Banff classification,\(^\text{10,19-21}\) is that presence of proteinuria in other proteinuric diseases like diabetic nephropathy, in which neither alloreactivity nor a primary inflammatory insult to the kidney are present, there is an interstitial inflammatory process characterized by the presence of macrophages, which strongly correlates with the magnitude of proteinuria/albuminuria.\(^\text{22-24}\) Thus, the presence of an interstitial inflammatory process in transplanted kidneys could well be aspecific and related to the extent to which filtered proteins induce inflammation in the interstitium of kidneys. Despite this potential non-specificity of the interstitial infiltrates, their presence offers opportunities for identification of new biomarkers for the ongoing inflammatory process that need not stringently be related to (peri)vascular inflammation like CRP supposedly is. Procalcitonin and hemopexin may be such biomarkers.

2. **Procalcitonin**

Procalcitonin (PCT) is best known as for being evaluated as a biomarker for bacterial infections and sepsis.\(^\text{25,26}\) Recently, however, it has been found that parenchymal cells stimulated by activated macrophages in infected tissues rather than peripheral blood mononuclear cells underlie very high concentrations of circulating PCT during sepsis.\(^\text{27,28}\) PCT might thus be a marker of non-infectious inflammation driven tissue damage. In **Chapter 4** we showed that PCT was independently associated with an increased risk for graft failure and, to a lesser degree, with mortality. Some of our findings in this study are of particular interest. First, the predictive performance for graft failure of PCT was similar to those of creatinine clearance and proteinuria, suggesting that measurement of PCT could be of additional value, next to measurement of creatinine clearance and proteinuria, for the identification of renal transplant recipients at risk for graft failure. Furthermore, the predictive performance of hsCRP, the prototypical acute phase reactant marker of inflammation, in predicting mortality was similar to that of PCT, while hsCRP was not a predictor for graft failure. This suggests that PCT is much more than hsCRP a specific marker for the ongoing inflammatory process local in the kidney. One of the possible underlying mechanisms linking increased PCT concentration to graft failure may lie in proteinuria. One of the hallmarks of proteinuric renal disease is infiltration of renal interstitial tissue by activated macrophages.\(^\text{22-24}\) Using adipocytes as a model for parenchymal cells of all kinds of tissues, it was recently demonstrated that parenchymal...
cells secrete PCT in response to stimulation by activated macrophages.\textsuperscript{27,28} Thus, our finding of high circulating PCT concentrations to predict graft failure may be a reflection of the release of PCT into the circulation by renal parenchymal cells in response to renal macrophage infiltration and activation in relation to proteinuria. Importantly, however, in our secondary analyses, we found PCT to be particularly predictive for development of graft failure in RTR without proteinuria. One possibility is that the cascade with macrophage activation and interstitial inflammation is already fully activated when the amount of protein in urine is still below the upper limit of the reabsorption capacity of the tubular epithelial cells. There is recent evidence that much more protein is filtered than has previously been thought and that active processing by tubular epithelial cells prevents it from appearing in urine.\textsuperscript{29-31} The concentration of PCT will then already be elevated in RTR when proteinuria is not yet detectable. This leads to the hypothesis that PCT is not only a biomarker for the existence of proteinuria, but could also be an early marker for a tendency for development of proteinuria. One way to substantiate this possibility would be by demonstration of a relationship between PCT and urinary albumin excretion in the general population, e.g. in the Groningen Prevention of End-stage Renal and Vascular Disease (PREVEND) study. Another possible mechanism linking PCT to development of graft failure may lie in alloreactivity. An ongoing chronic low-grade immunological response against non-self epitopes in the transplanted kidney may provoke an ongoing inflammatory process in the transplanted kidney which may lead to increased synthesis and release of PCT into the circulation from parenchymal renal cells and also from immune cells in the kidney. If proteinuria would be the trigger for PCT release from the kidney into the circulation, PCT should also be a marker of development of end-stage renal disease in non-inflammatory, non-transplant proteinuric diseases such as for instance diabetic nephropathy. If alloreactivity would be the trigger, PCT is more likely to not be a marker of development of end-stage renal disease in diabetic nephropathy. PCT might then also be promising as a marker of allograft dysfunction in other transplant populations such as, for example, lung, heart, liver transplant recipients.

It has been shown that immunoneutralization by an antiserum that is reactive to PCT ameliorates the symptomatology and also markedly improves survival of severely infected animals (i.e., hamsters and pigs).\textsuperscript{32-34} It may therefore also be interesting to intervene on PCT in RTR with high PCT concentrations and high risk for development of graft failure in the near future.

3. \textit{Hemopexin activity}

Hemopexin is mainly expressed in liver, and belongs to the acute phase reactants, the synthesis of which is induced after inflammation.\textsuperscript{35} Recent studies suggest a role for hemopexin, an acute phase protein, in inflammation and kidney damage. We
hypothesized that hemopexin might be involved in non-infectious inflammation driven deterioration of renal allograft function. In chapter 5 we showed that hemopexin activity late after transplantation is associated with a higher risk for graft failure in renal transplant recipients independent of possible confounders. There is accumulating evidence pointing to hemopexin as a key player in determining integrity of the glomerular filtration barrier. First, hemopexin infusion in rats induces reversible proteinuria. Second, in vitro data have indicated that hemopexin is involved in the cytoskeleton reorganisation of human podocytes, and is capable of increasing glomerular permeability by degrading the glycocalyx. In humans, the idea is supported by a study in children with minimal change nephrotic syndrome having higher levels of activated hemopexin. However in our study the predictive role of hemopexin activity for graft failure was independent of proteinuria, which suggests that proteinuria does not play a(n important) role in the mechanism underlying the association of hemopexin activity with increased graft failure. There are several putative underlying mechanisms explaining the association of hemopexin activity with increased risk for graft failure after renal transplantation. First, the association might be causal, which would mean that higher hemopexin activity itself leads to graft failure. Second, hemopexin activity may be a marker of an inflammatory process in the kidney. Inflammation in the kidney would then lead to local increased hemopexin activity, with hemopexin activity only acting as a marker for an increased tendency for development of graft failure after renal transplantation. More studies are needed to acquire further understanding of the role of hemopexin activity in development of graft failure. If hemopexin activity would be actively involved, it could lead to identification of new therapeutic agents and strategies.

**SYSTEMIC INFLAMMATORY PROCESSES AND MARKERS OF INFLAMMATION IN RENAL TRANSPLANT RECIPIENTS**

1. **Determinants of CRP in renal transplant recipients**

   In renal transplant recipients, slightly elevated levels of CRP have recently been demonstrated to be an independent predictor of coronary heart disease and total mortality. Importantly, a recent study has identified post-transplant CRP also as a predictor of chronic allograft nephropathy in a univariate analysis. However, in renal transplant patients it is not known what factors determine plasma CRP concentrations. In chapter 2 we have shown that waist circumference, as a measure of obesity, is the strongest, modifiable risk factor for a high hSCRP in renal transplant recipients. Adipose tissue is nowadays regarded as a very active endocrine organ, which secretes numerous hormones and pro-inflammatory cytokines, including tumor necrosis factor-α and IL-6 into the
Approximately 25% of basal circulating IL-6 originates in human adipose tissue, with production in intra-abdominal fat 3 times that of subcutaneous fat. These pro-inflammatory cytokines stimulate the liver in synthesis and secretion of CRP. The majority (60%) of renal transplant recipients in the United States are currently overweight or obese at the time of transplantation. Furthermore, many renal transplant recipients experience a 10% weight gain after transplantation, predominantly because of an increase in fat mass. Although there have been only few large, controlled studies that have rigorously assessed the effect of weight loss on CRP level, the studies that have been done suggest that weight loss may be an effective nonpharmacologic strategy for lowering CRP level. Whether this is also true in renal transplant recipients has to be shown in future studies.

2. Cytomegalovirus

Cytomegalovirus (CMV) is the most important pathogen after renal transplantation occurring in 20 to 60 percent of the renal transplant recipients. In part this reflects the ubiquitous nature of the virus as it is estimated that 60 to 70 percent of the general population are infected with CMV. However, renal transplant recipients are more susceptible for reactivation of latent CMV compared to the general population. This occurs in particular in the first months after transplantation as a consequence of a temporary disruption of an otherwise existing balance between immunological surveillance and viral replication by treatment with cytotoxic drugs and antilymphocyte antibody therapy and by systemic infection and inflammation. CMV reactivation from latency and primary infection are risk factors for both immunological rejection and mortality in the first year after transplantation. Relevance of CMV as an urgent medical problem slowly diminishes with time after transplantation in conjunction with return to latency. However, the virus may continuously smoulder under conditions of chronic immunosuppression, in particular in inflamed tissues. It has indeed been shown that latent CMV can be locally active in a transplanted organ with ongoing low-grade alloreactivity, without systemic signs of activity in the chronic phase after transplantation. In Chapter 8 it is described that both graft and recipient survival is significantly better in CMV seronegative renal transplant recipients compared to CMV seroconverted or seropositive renal transplant recipients when CMV serology is determined more than one year after transplantation. Furthermore, renal transplant recipients who are CMV IgG seropositive are at 2.7 times higher risk for graft failure than for death. These findings suggest that latent CMV is more active in a transplanted organ, potentially in association with chronic ongoing low-grade alloreactivity, or in kidneys in general. An important implication of this study would be that there should be more focus on matching for CMV status because, apparently, it is not only important to prevent CMV
disease, but to prevent occurrence of CMV infection at all. Current treatment guidelines recommend CMV prophylaxis for all recipients of solid organ transplants, who receive immunosuppression with antilymphocyte antibody products and for CMV negative recipients of CMV positive organs.\textsuperscript{70-72} However, it has been shown that up to 30% of CMV seronegative recipients receiving an organ from a CMV seropositive donor develop CMV disease after cessation of CMV prophylaxis (i.e., delayed-onset primary CMV disease).\textsuperscript{72,73} This suggests that the virus may continuously smoulder during prophylactic therapy and is reactivated after cessation of CMV prophylactic therapy. Another option to prevent CMV after renal transplantation is the development of a safe and effective vaccine. Several earlier trials tested a live, attenuated CMV vaccine in patients before renal transplantation but failed to show benefits in reducing the incidence of CMV disease. However, severe CMV disease was less frequent among the vaccinated CMV seronegative patients who received a kidney from a seropositive donor, and survival was significantly improved.\textsuperscript{74} Other recombinant vaccines from viral particles remain in preclinical or earlier clinical phases (I/II) of development.\textsuperscript{75-77}

3. \textit{Serum albumin as negative acute phase protein}  
Hypoalbuminemia is common after renal transplantation.\textsuperscript{78,79} Serum albumin is a negative acute phase protein, and hypoalbuminemia may therefore be reflecting ongoing chronic low-grade inflammation.\textsuperscript{80,81} Other potential explanations for hypoalbuminemia are poor nutritional status and loss of protein, in particular with proteinuria.\textsuperscript{80,81} Low serum albumin has been shown to be a predictor for both graft failure \textsuperscript{78,82} and mortality \textsuperscript{79,82-84} in renal transplant recipients. In the cross-sectional part of Chapter 6, we found independent inverse associations of serum albumin with hsCRP and urinary protein excretion. Prospectively, we found that RTR with low serum albumin concentrations at baseline are at increased risk for graft failure and mortality during follow-up, which is in line with existing literature.\textsuperscript{78,79,82-84} Despite the cross-sectional associations of serum albumin with hsCRP and urinary protein excretion at baseline, the association of serum albumin with graft failure was not explained by hsCRP, while the association of serum albumin with mortality was explained by hsCRP only to a very small extent (change in hazard ratio from 0.39 to 0.41). Importantly, we found that the association between serum albumin and graft failure was only present in RTR with proteinuria. This observation suggests that low albumin concentrations are not a risk factor for graft failure by themselves. Rather, it is strongly suggestive that proteinuria is involved. One reason may be that severity of proteinuria in some RTR is underestimated as a consequence of errors in collecting 24h urine samples. It is well-known that 24h urine collection is prone to collection errors.\textsuperscript{85-88} Another reason may be that low albumin concentrations in the presence of proteinuria are an indication of detrimental effects or urinary loss of peptides.
undetected by urinary protein assays as a consequence of tubular processing.\textsuperscript{29-31} For mortality, the impact of serum albumin was similar in RTR with and without proteinuria suggesting that malnutrition might be an important pathway linking serum albumin to mortality.

4. **Endothelial dysfunction as marker of the inflammatory process of the vascular wall**

Activation of the inflammatory cascade leads to endothelial dysfunction (ED) which sets the stage for both initiation and progression of atherosclerotic lesions.\textsuperscript{89} Besides inducing atherosclerosis, ED leads to increased vascular permeability for proteins resulting in, for example, proteinuria. Proteinuria is an established predictor of mortality, in particular cardiovascular mortality, in several populations, including renal transplant recipients.\textsuperscript{17,90-96} The association of proteinuria with ED is considered to be a mechanism underlying the elevated mortality in proteinuria.\textsuperscript{97-99} However, whether ED is involved in the prognostic impact of proteinuria in renal transplant recipients is unknown. In **Chapter 7** we show that urinary protein excretion and concentrations of sICAM-1 and sVCAM-1, as markers of ED, independently predict mortality late after transplantation. Furthermore, renal transplant recipients with proteinuria and high concentrations of sICAM-1 or sVCAM-1 appear to be at highest risk for death compared to renal transplant recipients without proteinuria and with low concentrations of sICAM-1 or sVCAM-1. These results suggest that ED plays a role in the association of proteinuria with mortality after renal transplantation.

**CONCLUSION**

In conclusion, we have investigated role of chronic low-grade inflammation in CTD and mortality after renal transplantation. One of the main findings is that hsCRP can be used as an early marker accelerated deterioration of graft function. We furthermore have shown that procalcitonin, to date only known to reflect microbial inflammation, is a strong predictor of graft failure, independent of creatinine clearance and proteinuria. The overall findings in this thesis support the growing notion that inflammation plays a role in the development of graft failure after renal transplantation.

Future studies are needed to investigate the mechanism underlying the role of chronic low-grade inflammation in the deterioration of renal function, the development of CTD, and mortality and whether treatment of chronic low-grade inflammation has a positive effect on graft and recipient survival after renal transplantation.
REFERENCES


42. van Ree RM, De Vries AP, Oterdoom LH et al. Abdominal obesity and smoking are important determinants of C-reactive protein in renal transplant recipients. Nephrol Dial Transplant 2005;20: 2524-2531.


