# Molecular and genetic markers for the prediction of kidney transplant outcome

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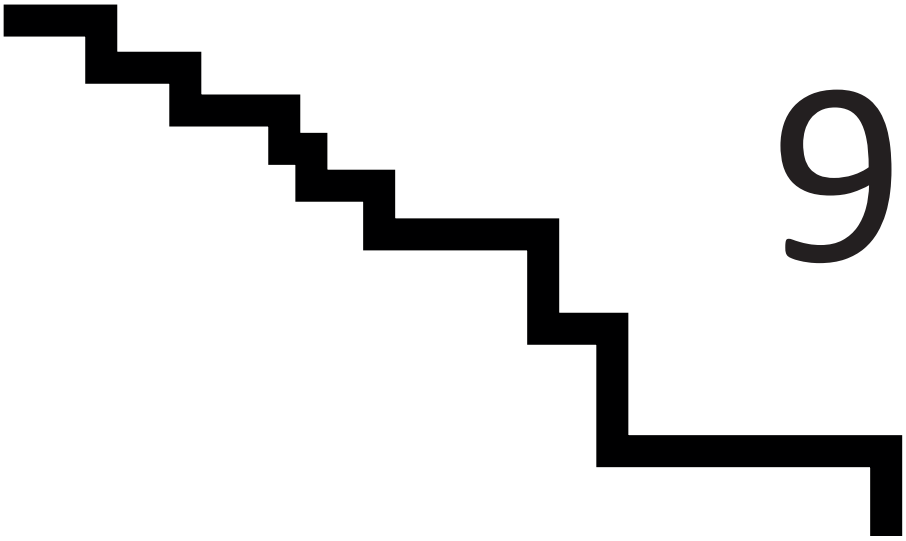
# Summary and general discussion



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**Summary and general discussion**

Kidney transplantation is the preferred treatment of patients with end stage renal disease, as it provides longer patient survival and better quality of life compared to dialysis (1). The allograft survival has been considerably improved over the past decades due to the advancement of surgical procedures, tissue typing, and immunosuppressive medication, especially the introduction of cyclosporine. Potential complications after kidney transplantation, such as delayed graft function and acute rejection (AR), remain risk factors for long-term graft outcome (2-6). Prediction of DGF, response to steroid resistant rejection and long-term graft outcome remain difficult when using merely clinical parameters. Numerous studies have reported on the predictive value of molecular markers for AR and worse graft outcome(7-13). However, the heterogeneity of AR and the variation among transplant centers leads to controversial results and preclude a more general clinical application. In the first part of this thesis, we aimed to investigate the molecular markers of steroid resistance and long-term graft survival on the basis of acute rejection biopsies. In the second part, we focused on genetic variants associated with acute rejection in kidney transplantation. In the final part we described the possible immune regulatory effect of S100 calcium binding proteins.

**Selection of SYBR green master mix**

Quantitative polymerase chain reaction (qPCR) is a sensitive and specific technique based on non-specific SYBR green chemistry to measure gene expression levels. The extraction and preservation protocols for obtaining high quality and quantity of RNA were optimized in order to increase the sensitivity of mRNA expression assessment (7). The PCR devices and master mixes also affect the accuracy and reliability of the gene expression assays. In chapter 2 we compared different commercial SYBR green PCR mixes using two different PCR machines with respect to the specificity and sensitivity of the qPCR assay.

Three commercial SYBR green PCR mixes: ABI, Bio Rad, and Roche, were tested for 79 immune-related transcripts targeted by specific primer pairs. We found that most primer sets (N=66, 94.3%) could generate a single sharp melting peak with all tested PCR mixes by strictly following the prescribed PCR protocol. However, 13 primer pairs (18.6%) produced suboptimal melting peak using Roche mixes. The use of ABI mixes often led to lower Cq values for cDNA and lower background levels for negative DNA samples compared to the other mixes. The PCR devices had a smaller influence on the results than the source of SYBR green mixes. Based on the data obtained in these studies, we decided to measure all molecular transcripts in biopsy samples using the ABI mix on Viia7 PCR equipment.

**Lack of association with DGF at the time of transplantation**

Delayed graft function, defined as requirement of dialysis within the first week after transplantation, is a risk factor for acute rejection (4, 5, 14). Ischemia reperfusion injury

(IRI) after transplantation resulting in acute tubular injury is considered as a main cause of DGF (15). Clinical parameters, such as donor creatinine level, prolonged ischemia time, and older recipient age, correlate with DGF (16-18). Accurate prediction of DGF using molecular profiles may allow the early intervention and prevention of further allograft injury. We tested the expression levels of Toll-like receptors (TLRs), complement and apoptosis related genes in pre-implementation biopsies and investigate the relationship with DGF in chapter 3.

In deceased donors none of the markers investigated in pre-implementation biopsies was predictive for the DGF, which is in contrast with a previous study showing that the BAX:BCL2 ratio was elevated in DGF group (19). We found that expression of C2 and C3, and BAX:BCL2 ratio were higher in deceased donors compared to living donors, indicative for a role of the complement and apoptosis pathways in ischemia reperfusion injury. This observation confirmed previous studies showing that complement components are significantly higher in deceased donors compared to living donors (20), and that apoptotic cell death is initiated as reflected by an increased BAX and decreased BCL2 during normothermic ischemia injury (21). Therefore, inhibition of complement and apoptosis pathway may act as therapeutic target to protect from the effects of IRI.

The complement regulators analyzed were not significantly different in their expression between living and deceased donors. TLR2, TLR4 or MyD88 deficient mice are protected from IR injury, and TLR4 expression was significantly higher in pre-implantation biopsies from deceased donors than that from living donors (22-24). However, we could not find any difference in the expression of TLRs between living and deceased donors. Our data suggest that the TLR pathway is not important for IRI and DGF after transplantation, and that even a low level of TLR expression is sufficient to initiate an immune response.



**Alteration of gene expression is the result of inflammatory cell infiltration**

Gene expression alterations between paired pre-implantation and acute rejection biopsies were analyzed in 75 patients. The majority of TLRs (TLR1-3 and TLR6-10), C2, and BAX:BCL2 mRNA levels were increased, whereas the expression levels of C4 and the complement regulators (CD46, CD55, and CD59) were decreased at the moment of AR compared to the situation before implantation. The changes in expression levels of TLR4, TLR5, C3, and CR1 varied among the patients with acute rejection. We speculate that the changes in mRNA expression are the result of infiltration of inflammatory cells. Therefore, the correlation between expression level of innate immunity genes and inflammatory markers (CD163, CD68, CD20, and CD3e) at time of AR was analyzed. The expression of TLR1, TLR4, TLR6-10, C2, C3, CR1, and BAX:BCL2 was positively correlated with one or more inflammatory markers, while the expression of CD46 and CD59 was negatively correlated with macrophage markers. In addition, immunohistochemical staining for TLR4, TLR9, and BCL2 confirmed that their expression was relative higher in acute rejection group than in patients with stable graft function. The influx of inflammatory cells can at least partly explain the altered

gene expression between implantation and AR biopsies. However, most of genes expressed during AR show no association with any of the Banff classification scores.

The TLR2 and TLR3 mRNA levels were minimally increased but did not correlate with any inflammatory makers, suggesting that these mRNA levels are dominantly expressed in renal parenchymal tissue and activated by inflammation, which is consistent with a previous study showing a similar TLR3 expression pattern (25). The expression of TLR4 was correlated with myeloid cell markers but the changes in expression varied among patients, suggesting that TLR4 is expressed in both myeloid and parenchymal cells. Indeed, immunohistochemical staining in our study and in other studies showed that TLR4 protein expression could be detected in both endothelial and tubular cells (24, 26-28). Thus, the altered TLR4 expression may depend on the extent of myeloid cell infiltration and kidney cell damage. Semi-quantitative immunohistochemical staining showed that patients with acute rejection have a significantly higher expression of TLR4 than patients with stable graft function. A possible explanation may be that inflammatory cells, especially myeloid cells, express high levels of protein but relative low levels of mRNA.

C3 and CR1 showed similar expression patterns as TLR4. The alteration of C3 and CR1 gene expression, which showed a wide range of expression in both pre-implantation and acute rejection biopsy samples, was further investigated with respect to its association with transplant outcome. Patients with increased and decreased gene expression did not differ with regard to the incidence of steroid resistant rejection and long-term graft outcome. The minor decrease in expression of C4 and CD55 lacked any correlation with inflammatory markers and may be the result of renal tissue damage during acute rejection.

**TCMR score provide new dimension for acute rejection assessment**

Histologic diagnosis of acute rejection according to Banff classification is poorly reproducible among pathologists and difficult to improve in accuracy (29, 30). This limitation may due to the fact that the principal lesions used for rejection diagnosis are also present in other inflammatory responses such as acute kidney injury (AKI), in combination with subjective interpretation. The recent Banff classification describes criteria of acute TCMR, ABMR, and mixed rejection, and it highlights molecular diagnostic techniques as new tools (31, 32). In chapter 4 we investigated the expression of TCMR related makers in an acute rejection cohort transplanted between 1995 and 2005.

TCMR related markers were mostly expressed in T cells, NK cells, and APCs, as was previously identified using microarray technique (33, 34). The T-score, the average z-score of all TCMR related transcripts, was significantly associated with the interstitial inflammation and tubulitis, but not with intimal arteritis. This is not surprising, since the T-score only reflects the degree of infiltration of inflammatory cells into the graft, and not the localization of the infiltrate. Intimal arteritis, characterized as inflammatory cell beneath the endothelium of arteries, could be induced by ABMR or AKI (35), which may explain

the lack of association with the T-score in the current study. Reeve et al also reported that the isolated v-lesions (intimal arteritis with insufficient infiltration of immune cells) had low TCMR scores (33). Therefore, patients with intimal arteritis with a relatively low T-score should not be categorized as TCMR and they would need to be carefully monitored.

Our studies showed that borderline rejection is reflected by a significantly lower T-score than tubulointerstitial rejection. However, in line with a previous study, several patients with borderline rejection had a relatively high T-score, which should be a reason to reclassify these patients as TCMR (33). Our findings give support to the notion that molecular assessment at time of acute rejection aids in predicting therapy sensitivity.

**Risk assessment of steroid resistance: E-score**

Steroid resistant rejection is associated with inferior long-term graft outcome (10, 36). Prediction of steroid resistance during acute rejection would open the possibility to treat patients immediately with the optimal immunosuppression and prevent unrepairable nephron damage during the period of steroid therapy. Sarwal et al showed that dense B cells infiltration is correlated with steroid resistant rejection and graft loss (10, 37). However, other studies failed to confirm the correlation between infiltration of B cells into the allograft and the response to steroid therapy (38, 39). In addition, the expression of other inflammatory markers in the graft tissue, including FasL (9), LAG-3, CD25:CD3e ratio (36), metallothioneins (8), granulysin (40), and CD68+ (macrophage) (41, 42), and FoxP3 expression in the urinary sediment (43) have been found to be associated with responsiveness to steroid therapy. In chapter 4 we found that an increased mRNA expression of endothelial-epithelial related genes at the moment of acute rejection predicts the responsiveness to steroid therapy.



Halloran's group established a molecular diagnosis system to identify TCMR and ABMR based on microarray data that was derived from over 700 biopsies (33, 34, 44-48). The transcripts used as ABMR classifiers are mainly expressed in endothelial cells, NK cells, and many of these are induced by INF-γ (47, 48). However, we could not identify any ABMR associated parameters in our studies and could not make any correlation with the E-score. Many patients with steroid-sensitive acute rejection showed comparable E-score as patients, who did not have acute rejection (protocol biopsy).

Endothelial cells line the interior surface of glomeruli and peritubular capillaries and they mediate crucial inflammatory processes. The endothelium-epithelium transcript profile, such as TM4SF18, PGM5, and CD34, which are involved in angiogenesis and biological adhesion, may reflect the integrity of nephron and ability of tissue repair after injury. This may explain why the decreased expression profile is associated with resistance of steroid treatment. In line with our finding, several studies showed that severe intimal arteritis and destruction of microvasculature predict steroid resistance of the rejection (49-51). Therefore, the endothelium-epithelium profiles may provide novel markers to predict steroid resistant rejection.

Univariate logistic regression analysis showed that the E-score is a predictor of steroid resistant rejection. Although the performance of the predictive model was modest (AUC=0.70), it might have a high specificity, representing patients with high expression of endothelium related transcripts who do respond to steroid treatment. In addition, the combination of the E-score and other inflammatory makers may generate a more powerful model for assessment of steroid resistance. This may tested further in future studies.

**Prediction of long-term graft survival**

Short-term graft survival has increased greatly over the last two decades. The half-life for deceased donors was 6.6 years in 1989 and increased to 8.8 years in 2005, which was driven by improvement of fist year attrition rates. However, the long-term attrition rates have hardly improved (52). Identification of molecular markers, which correlate with long-term graft survival, may allow clinicians to carefully monitor the renal function in high-risk patients and may open the way in the prevention of adverse graft outcome.

Steroid resistant rejection is a risk factor of long-term graft loss (10, 36). The effect of steroid resistant rejection on long-term graft survival was assessed in chapter 4. The patients with steroid resistance showed inferior long-term graft survival compared to patients who showed response to steroid therapy. Steroid resistant rejection is correlated with severe vascular rejection and low endothelium and epithelium expression profiles, suggesting that severe kidney injury during acute rejection results in chronic allograft damage.

In chapter 3 we showed that patients with high TLR4 expression during acute rejection have inferior graft survival compared to patients with low TLR4 expression. TLR4 in the allograft may bind to intracellular ligands released by dead cells, and provide additional proinflammatory signalling to enhance inflammation. Although the antirejection therapy successfully normalized kidney graft function, as reflected by decreased serum creatinine, the high expression of TLR4 may lead to production of higher levels of proinflammatory cytokines and chemokines that induce inflammatory cells infiltration into the allograft after the antirejection therapy and contribute to chronic allograft nephropathy.

The high BAX:BCL2 ratio reflects the high extent of apoptosis and it predicts inferior long-term graft survival in deceased donor groups. On the one hand, apoptosis of parenchymal cells directly leads to the loss of kidney function. However, apoptotic cells attract phagocytic cells into the graft and may be rapidly cleared (53, 54). The accumulated phagocytic cells can be triggered by a danger signal and mediate chronic allograft nephropathy (55). If the apoptotic cells in allograft are not rapidly cleared, they undergo necrosis and release damage-associated molecular patterns (DAMPs) that initiate immune responses (56). Thus, monitoring of the BAX:BCL2 ratio during AR may offer a predictive value with respect to long-term graft survival. Future studies should contain a more in-depth analysis of the presence and kinetics of dying cells in the graft, and their possible impact on outcome.

**Genetic risk factors in kidney transplant: lack of validation and small effect**

The role of HLA molecules in the transplantation field has been widely recognized: better matching between donor and recipient leads to better graft function. Numerous candidate single nucleotide polymorphisms (SNPs) have found to be associated with occurrence of acute rejection and with outcome, but most studies focused on immune response related genes (57-63). GWAS represent an unbiased approach to simultaneously analyse millions of SNPs and to identify novel makers involved in allograft rejection.

In chapter 5 we performed a GWAS of acute rejection in kidney transplantation. The significant candidate SNPs identified by current GWAS could not be verified in an independent cohort in another transplant center. In line with a previous study, we found that patients with acute rejection show a higher C allele frequency (rs1801274 in FCGR2) compared to the stable graft function group (64). Apart from this specific SNP, most of previously published SNPs could not be confirmed in the current GWAS. Consistent with a well powered GWAS in bone marrow transplantation, the previously reported genetic variants were most likely false positive findings (65). As discussed in chapter 7, the main limitation of GWAS is the requirement of stringent significance thresholds ($P<5\times10-8$). Only SNPs with a big effect on transplant outcome could be captured in our relatively small-sized GWAS. Thus, any false positive findings in our study may result from the small effect of individual genetic variants. Individual SNPs identified by GWAS usually have a small effect by themselves on a complex trait such as acute rejection, explaining only less than 10% of susceptibility to the disease even when all available genetic variants are combined (66). To identify true positive, single SNPs, which have a small effect, and to overcome the issue of validation, the only way is to increase sample size by international collaboration in the field of kidney transplantation.



The role of non-HLA antigens have increasingly been reported in kidney transplantation (67-69). As shown in chapter 6, we found no effect of genomic missense SNP mismatching on kidney transplant outcome. Besides, the mismatch load, reflecting the total amount of mismatching of SNPs in coding sequences between recipient and donor, does not have any effect on AR or long-term graft function. If the mismatch load had any effect, living related transplantations with lower mismatch load should have lower incidence of rejection and longer allograft survival compared to living unrelated transplantations that have higher mismatch load. However, a recent meta-analysis showed there was no difference between living related and unrelated kidney transplantations in acute rejection and graft survival rates (70). A reason for the negative finding may be that the effect of mismatching of missense SNPs is low, under the condition of HLA mismatching and efficient immunosuppressive therapy.

**The immune regulatory effect of calcium binding proteins**

High expression of S100A9 in kidney biopsies during AR is associated with a beneficial effect on long-term graft survival (71, 72). Most of S100A9+ cells are co-localized with CD68 and HLA-DR, and only one-third of them express CD163, suggesting a distinct macrophage population infiltrating the graft. We found in chapter 8 that S100A9 expression varies greatly among CD14 positive monocytes. Unfortunately we were not able to sort monocytes based on their expression level of S100A9 using SmartFlare RNA detection probes. Cytokine expression profiles between S100A9high and S100A9low subsets were not significantly different. We did find that overexpression of S100A8/A9 in monocyte-derived macrophages leads to increased reactive oxygen species (ROS) production, as well as increased IL-10 mRNA expression (Figure 1). The extracellular ROS may have a negative impact on T cell activation and their subsequent proliferation (73), which may dampen the immune response in the allograft. The consistent increase of IL-10 may represent another anti-inflammatory mediator in such immune response, even though the protein level of IL-10 could not be detected in the supernatant of the transfected cells. We hypothesize that the anti-inflammatory effect of S100A8/A9 proteins may explain their beneficial effect on kidney graft survival.

The THP1 macrophage cell line, which lacks co-stimulation molecules, is unable to stimulate allogeneic T cell activation. The cell viability and stimulation ability of monocyte-derived macrophage were negatively affected, probably as a result of the transfection of a large plasmid (~7000 base pairs), which prevented us from performing mixed lymphocyte cultures. To investigate the effect of S100A8/S100A9 on T cell activation and proliferation, downregulation of S100 proteins in macrophages by transfection of small siRNA constructs may be an alternative approach.

Numerous studies reported that the proinflammatory activity of S100 proteins can be used as biomarker of inflammation, infection, and autoimmune disease (74-80). In contrast, S100A9 was proposed as a novel marker of human monocytic MDSCs, which accumulate in kidney transplant recipients and are able to induce expansion of Treg cells in vitro (81, 82). We found that MDSCs in healthy PBMC express slightly higher levels of S100A9 compared to CD14+HLA-DR+ monocytes, but that S100A9 is not suitable as a specific marker of MDSC since all monocytes positively expressed S100A9. Since MDSCs are detected in higher quantities during inflammatory conditions, S100A9 expression should be tested in MDSCs obtained from kidney transplant recipients. Inhibition of S100A9 in such MDSCs should show whether S100A9 mediates the anti-inflammatory effect of MDSCs.

**Conclusions and future perspectives**

The results presented in this thesis demonstrate that several molecular and genetic markers are associated with kidney transplant outcome. We showed that a decreased expression profile of endothelial-epithelial cells during AR is associated with resistance to steroid

therapy, suggesting that endothelial cell integrity is involved in the efficacy of antirejection treatment. The elevated TLR4 expression and BAX:BCL2 ratio during AR independently predict inferior long-term graft survival. In addition, the genome-wide association study suggests that genetic risk factors in kidney transplantation confer a small effect and future efforts require large international collaborative studies. Furthermore, the overexpression of S100A8/A9 leads to increased ROS and IL-10 production by macrophages, which may explain the beneficial effect of these S100 molecules on kidney allograft survival.

Molecular assessment of renal biopsies has been established and may add a new dimension to histologic diagnosis. However, biopsy related complications such as bleeding, hematuria, and anuria are not completely eliminated. Assessment of noninvasive material, such as blood and urine, is a promising approach. Identification of rejection specific molecular markers in blood and urine may provide new tools to monitor the immune status of the allograft, and may allow for early detection of subclinical rejection and timely therapeutic intervention.

External validation of potential molecular predictors is necessary in biomarker discovery. The observed prognostic value of the E-score, TLR4, and BAX:BCL2 ratio need to be verified in an independent study cohort. Due to frequently reported false positive findings in genetic association studies, validation by international collaborative studies is highly recommended. In addition, a prospective cohort study is the golden standard to confirm the predictive value of candidate biomarkers before their clinical implication in kidney transplantation.

The potential immune regulatory effect of calcium binding proteins need to be further investigated. The knockdown of S100A8/A9 by transfecting siRNA into macrophages would be an alternative approach to investigate the macrophage stimulation ability by mixed lymphocytes cultures. For characterizing the cytokine expression profile, such as TNFα, IL6, IL10 and TGFβ, concentrated cell culture supernatant may provide valuable information. Thanks to the development of tissue imagine technique for simultaneous analysis of multiple markers by immunohistochemistry, the effect of inflammatory cell subsets infiltrating in the allograft and their possible relationship and interaction in space may be clarified in relation to the clinical outcome of kidney transplant patients.

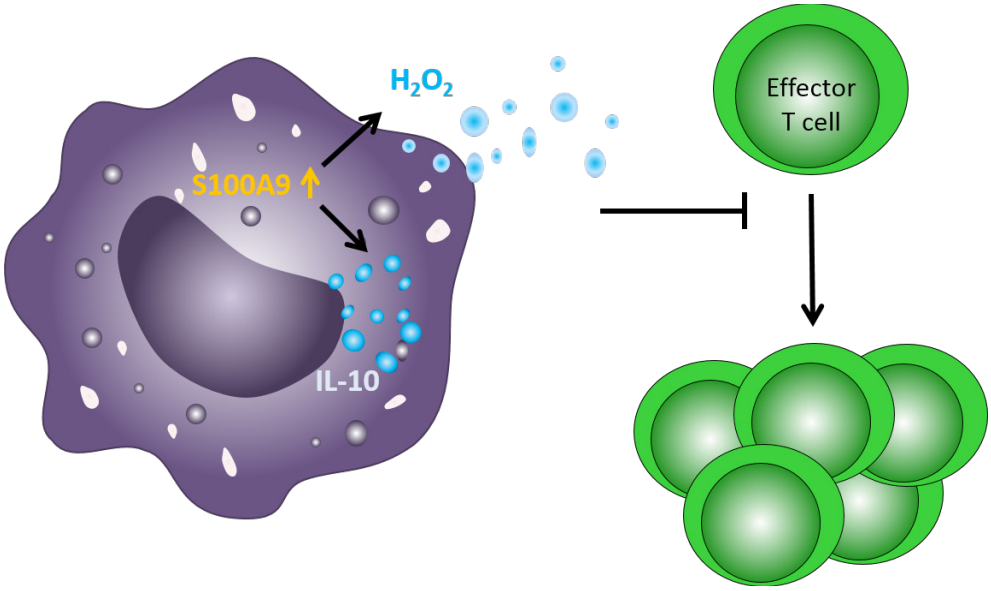


Figure 1. Increased expression of S100A9 in macrophage leads to increased ROS production and elevated IL-10 expression. ROS, reactive oxygen species.

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