Novel diagnostics and therapeutics to prevent injury in native and transplanted kidneys

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Chapter 7

Summary and general discussion

Summary

Progression of kidney injury in native and transplanted kidneys has major implications for quality of life and patient survival. Chronic kidney disease led to 1.2 million deaths worldwide and was the 12th leading cause of death in 2017.[1] In addition, CKD led to 35.8 million disability-adjusted life years. Although kidney transplantation improves life quality and life expectancy in most patients, development of injury in kidney grafts leads to severe loss of quality of life in society.[2] Therefore, early recognition and prevention of kidney injury in both native and transplanted kidneys are of vital importance. The current strategy to recognize kidney injury is still dependent upon 'old' biomarkers, such as creatinine and proteinuria, which only recognizes advanced kidney injury. For prevention of kidney injury, physicians currently rely on regulation of blood pressure, minimizing proteinuria and promoting a healthy lifestyle.

Novel biomarkers are needed to recognize kidney injury in an early stage, when serum creatinine or proteinuria lack sensitivity. Therefore, two potential biomarkers, IGFBP7 and TIMP-2, were evaluated in **chapter 2** in the context of progressive kidney injury. These biomarkers proved their added value in the context of acute kidney injury,[3,4] but were not thoroughly investigated in chronic kidney injury. Chapter 2 describes higher circulating levels of both IGFBP7 and TIMP-2 in patients with diabetic nephropathy and to a lesser extend in diabetes patients with a preserved kidney function. IGFBP7 is mainly dependent upon kidney function, while TIMP-2 shows a different picture. As expected, type 1 diabetes patients, who received a simultaneous pancreas-kidney transplantation (SPKT) or kidney transplantation alone (KTA), had lower levels of IGFBP7 levels. However, TIMP-2 did not normalize and persisted to be higher, most likely due to other diabetes-related factors, such as systemic (micro)vascular damage. This finding is supported by a longitudinal study that followed type 1 diabetes patients the first year after SPKT. After one year, lower levels of circulating IGFBP7 persisted, while TIMP-2 levels at one year were comparable with pre-transplant levels. In short, TIMP-2 and IGFBP7 may offer interesting opportunities in monitoring early kidney injury.

Given the extensive amount of vascular injury in diabetes,[5] we next sought to investigate long noncoding RNAs (lncRNAs), since lncRNAs have recently been identified to be associated with vascular injury.[6,7] In **chapter 3**, nine lncRNAs were selected from a panel of 40,173 lncRNAs, in a pilot study of six healthy controls and six patients with diabetic nephropathy. These nine lncRNAs were studied further in the cohort described above. MALAT1, LIPCAR, and LNC-EPHA6 were present at higher circulating levels in patients with diabetic nephropathy. After SPKT MALAT1, LIPCAR, and LNC-EPHA6 normalized within one year. In addition, LIPCAR and LNC-EPHA6 correlated significantly with the vascular marker soluble thrombomodulin, while all three lncRNAs correlated with several vascular specific micro RNAs, supporting the association of these lncRNAs with vascular injury. Taken

together, although additional investigation is warranted, these LncRNAs may provide novel options to monitor vascular injury in diabetes patients.

If progression to end-stage renal disease occurs, kidney transplantation is the preferred treatment, concerning the quality of life and life expectancy.[8] Although transplantation offers several benefits, it comes with uncertainties for the patient. The risk for rejection is always present and injury, as a consequence of rejection, can be severe.[9,10] Since (micro)vascular injury is an important feature of acute rejection,[11] vascular lncRNAs, that we identified in chapter 3, were determined in a cohort of kidney recipients with acute rejection in **chapter 4**. Circulating LNC-EPHA6 appeared to be higher during a rejection episode, compared to healthy controls, and normalized one year after rejection to baseline levels. The correlation between LNC-EPHA6 and soluble thrombomodulin, already described in chapter 3, was confirmed in this cohort. This chapter pointed out the association of LNC-EPHA6 with vascular injury in the context of acute rejection in kidney recipients.

Especially acute antibody-mediated rejection (ABMR) can result in severe injury to the transplanted kidney.[12,13] ABMR is a rare condition and treatment options are only based on little evidence and expert opinion.[14] The primary aim is to avoid ABMR from developing. Risk assessment before transplantation is of vital importance. In **chapter 5**, the incidence and risk factors of ABMR are studied. The vast majority of kidney recipients from an unrelated living donor with ABMR in the first six months after transplantation consists of female recipients, who received a donor kidney from their male spouse. It is suggested that previous pregnancies caused an antibody response in the female recipient against the father of the child (and thus the donor of the kidney). Due to small numbers, a correlation between ABMR and pregnancies in this group was not observed. A retrospective, detailed risk assessment revealed pre-transplant donor specific antibodies (DSAs) in the majority of ABMR patients. The single antigen bead assay identified DSAs in 83% of female recipients of a male spouse, while the current detection strategy only identified 17%. Implementation of the single antigen bead assay as standard work-up in this group may prevent a proportion of ABMR to develop.

Chronic injury in the graft is characterized by the presence of fibrosis in the transplanted kidney and has multiple causes. A major cause of fibrosis formation is the use of calcineurin inhibitors (CNI), as part of the immunosuppressive regime.[15] Therefore, a cellular therapy with mesenchymal stromal cells (MSCs) is described in **chapter 6** with the aim to withdraw CNI at an early time point after renal transplantation. In this randomized controlled trial, MSCs were administered to kidney transplant recipients six and seven weeks after transplantation with subsequent withdrawal of the CNI (tacrolimus). Protocol renal biopsies 4 and 24 weeks after transplantation showed comparable fibrosis between treated patients and a control group with a standard immunosuppressive regime, including a CNI. Withdrawal of tacrolimus did not increase rejection rates significantly (3% in the MSC group)

and slightly less infection related adverse events were documented in the MSC group. Interestingly, regulatory T-cells were significantly higher in MSC patients 24 weeks after transplantation, compared with controls. Therefore, chapter 6 concludes that MSC therapy is a promising alternative for CNI in kidney transplantation, with comparable rejection rates.

General discussion

Early recognition and prevention of kidney injury remains a major challenge. In this thesis, biomarkers, such as IGFBP7, TIMP-2 and specific lncRNAs show their potential as novel means to identify kidney injury. However, before these biomarkers may be implemented in clinical practice, several steps have to be taken. Most importantly, the studies described here did not include patients having an early stage of kidney injury. In addition, prognostic value of these markers for decline in kidney function or kidney failure could not be determined, since this requires follow up of diabetes patients in a very early stage. Nonetheless, they offer the opportunity for further research into these novel biomarkers to identify patients at risk for developing end-stage renal disease in the earliest stage possible.

Chronic kidney injury

In the context of chronic kidney injury, novel biomarkers might be of added value to improve diagnostic approaches and potentially increase knowledge about the pathogenesis. Especially biomarkers in urine and blood can be important tools in the diagnostic process as an easy and cost-efficient way to improve knowledge about the amount of injury in the kidney. In chapter 2, we showed the value of circulating IGFBP7 and TIMP-2 in the discrimination between patients with or without kidney injury or systemic vascular injury, while these already showed their value as urinary biomarkers.[16] Urinary IGFBP7 and TIMP-2 are considered to be related to tubular injury,[17] while our study focusses on IGFBP7 and TIMP-2 in the circulation and their relation with endothelial injury as well. Since DM patients who received an SPKT had higher TIMP-2 levels, the amount of chronic systemic vascular injury, due to the long history of DM, may be associated with circulating TIMP-2 levels. This hypothesis is supported by the correlation with markers of vascular injury and higher levels of TIMP-2 in diabetes patients and chronic injury in kidney transplantation.[18] In chapter 3, we found a similar association between systemic vascular injury in DM patients and three lncRNAs (i.e. MALAT1, LIPCAR, and LNC-EPHA6). In addition, both LNC-EPHA6 and LIPCAR were correlated with the vascular marker soluble thrombomodulin and vascular-injury related micro RNAs. In order to study the early diabetic injury, studying a group of diabetes patients with an early stage of kidney injury, while still having a normal kidney function (eGFR >90ml/min/1.73m^2), would be very interesting. In these patients hyperfiltration occurs and vascular injury is already present. Studying these patients in time would also enable the prediction of development of more severe kidney injury with a decreased eGFR.

In studying noncoding RNAs, such as lncRNAs, it is important to note that noncoding RNAs not only play a role in transcription, splicing, and translation, but do also interact with other types of noncoding RNAs. E.g. Beermann et al. previously described that lncRNAs may function as a sponge for micro RNAs (and thereby alter their expression), next to transcription regulation and posttranscriptional control.[19] Therefore, studying noncoding RNAs should not be limited to one type of noncoding RNA, but should include other noncoding RNAs next to lncRNAs, such as micro RNAs, because of their presumed interactions. In addition, a more robust conclusion can be drawn, because specific micro RNAs are described in vascular injury as well. Most lncRNA levels are expressed at low levels[20], complicating their detection, especially compared to micro RNAs, and this may limit the implementation of lncRNAs in clinical practice. It would be beneficial if more sensitive detection methods would be developed for the clinical use of lncRNAs as biomarkers.

Although clear differences are observed in chapter 2 and 3, the studies described here are not suitable for analysis of the causative relationship between TIMP-2 and lncRNAs with systemic vascular injury. Previous research suggests an active role of both TIMP-2 and lncRNAs in the occurrence of vascular injury.[6,21] TIMP-2 is presumed to alter basement membrane degradation and rebuilding and MALAT1, as an example, is suggested to regulate hyperglycemia-induced endothelial inflammation. Further research is needed to clarify the relationship between these biomarkers and early diabetes related vascular injury. In addition, other factors, such as immunosuppressive drugs, may alter circulating levels of IGFBP7 and TIMP-2, as described before.[22] Nonetheless, we included DM patients with a kidney transplantation alone as a control group for DM patients with a simultaneous pancreas-kidney transplantation, that received largely similar immunosuppressive drugs.

Furthermore, we should be cautious yet in drawing strong conclusions from the studies performed in chapters 2 and 3, due to the limited group size. However, we show interesting changes in diabetes patients and pancreas-kidney recipients, that offer the opportunity to further investigate these groups of biomarkers for the detection and monitoring of chronic vascular injury. Additionally, this may also improve knowledge about the development of diabetes related injury.

Acute rejection

Impairment of kidney function due to vascular injury is not limited to native kidneys. After transplantation, vascular injury is an important cause of renal failure as well. Acute rejection is one of the major causes of vascular inflammation and subsequent injury and graft failure after transplantation. LNC-EPHA6 is described in chapter 3 to associate with vascular injury, due to diabetes and showed the same association with vascular injury due to T-cell mediated rejection in chapter 4. The same trend was observed in LIPCAR levels, without reaching statistical significance. Interestingly, we observed a correlation between LNC-EPHA6 and soluble thrombomodulin in both chapter 3 and 4. Given that sTM is a marker of

endothelial injury and dysfunction,[23] this also suggests a link of LNC-EPHA6 with endothelial cell injury. Previously, lncRNAs AF264622 and AB209021 were described as potential diagnostic biomarkers for acute rejection.[24] Since our study focused on previously selected vascular specific lncRNAs, we did not determine these lncRNAs.

Identification of biomarkers in the context of rejection can be complicated, since rejection frequently coincides with a diminished eGFR with subsequent changes in excretion of the biomarkers. Interestingly, in the longitudinal study, creatinine clearance did not change significantly from 1 month to 12 months after rejection, while we did find changes in vascular injury markers. This emphasizes the importance of sensitive biomarkers to detect vascular damage that doesn't translate into higher creatinine or increased proteinuria. Although speculative, the type of rejection treatment may also affect lncRNA levels. To rule out the influence of rejection treatment, LNC-EPHA6 should be determined in a larger cohort with a standardized rejection treatment. Since ABMR is characterized by even more vascular injury,[14] assessment of LNC-EPHA6 levels in a large ABMR cohort is very interesting. subsequently deteriorated graft function and even graft failure, as we found in chapter 5. A cohort, consisting of kidney recipients with unrelated living donors, was studied to assess the outcome of early acute ABMR in this population. One year graft survival was only 56% in patients with early acute ABMR, compared with 97% in the entire cohort. In the patients with a functioning allograft, kidney function was significantly worse in ABMR patients, compared with recipients with TCMR or no rejection. Interestingly, female recipients from a spousal donor kidney were at risk for ABMR. This is in accordance with previously described cases, where previous pregnancies in particular play a role in the development of preformed donor-specific antibodies and thereby increased risk for ABMR.[25,26] The main limitation in our analysis is that only a small proportion of the cohort developed ABMR. Although ABMR is a rare condition, the severe consequences make prevention of ABMR necessary. Stronger induction therapy (alemtuzumab) does not prevent ABMR in high risk patients. Therefore, more sensitive screening by the single antigen bead assay is needed in high risk populations to lower the initial risk of ABMR.

Chronic injury after kidney transplantation

Minimization of prescription of CNI is one of the strategies to decrease the amount of chronic injury after kidney transplantation.[15,27] However, complete avoidance of CNI leads to unacceptable rejection rates.[28] In chapter 6, a randomized, controlled trial is described, in which kidney recipients receive MSC therapy as a replacement for prescribed calcineurin inhibitors. As previously described, MSCs condition the immune system in different ways, resulting in Tregs that enable self-sustaining tolerogenic activity. In particular in the field of Hematology, MSCs proved their immunomodulatory capacities in graft-versus-host-disease.[29] Interestingly, we found higher numbers of Tregs in the MSC group, compared with the control group. This may have created a favorable immunological state in patients

to withdraw the CNI. After withdrawal of the CNI, also less infectious adverse events were reported in the MSC group.

In both the MSC group and the control group, rejection rates were low. Next to MSC therapy in the MSC group, alemtuzumab induction therapy may also play a role in the low incidence in the MSC patients without CNI. However, long term results (after the therapeutic window of alemtuzumab) show a low incidence of rejection as well. ABMR was not reported in both groups, but patients in the MSC group (and withdrawal of CNI) did have more de novo DSAs. Although these de novo DSAs did not lead to inferior graft survival or graft function, CNI was restarted in these patients.

Unfortunately, still a limited amount of randomized, controlled trials with MSC therapy has been performed worldwide. Further studies are required to increase knowledge about the clinical applications and their potential. We believe, that this study enables the next step to implementation of MSC therapy in the context of kidney transplantation in clinical practice.

In conclusion, we found specific tubular and vascular markers to be associated with the development of chronic kidney injury. Specific vascular lncRNAs increase in diabetic nephropathy and decrease after SPKT. Tubular markers IGFBP7 and TIMP-2 increase in diabetes. IGFBP7 decreases in case of an improved kidney function, while TIMP-2 remains high in patients who received an SPKT. Although these markers are not yet ready for implementation in the diagnostic process, they showed their potential as a biomarker and increase the knowledge about the pathophysiology of development of kidney injury. Secondly, we found the single antigen bead assay to be of added value in the screening process of female kidney recipients from a male spousal donor. This may prevent ABMR after kidney transplantation and therefore improve graft survival. Lastly, we demonstrated MSC therapy to be a feasible alternative for prolonged CNI use in kidney recipients. It was suggested that MSC therapy led to a regulatory response and fibrosis formation did not increase, compared with standard treatment.

References

1. Carney EF. The impact of chronic kidney disease on global health. Nat Rev Nephrol 2020; 16(5): 251.
2. Sussell J, Silverstein AR, Goutam P, et al. The economic burden of kidney graft failure in the United States. Am J Transplant 2020; 20(5): 1323-33.
3. Meersch M, Schmidt C, Van Aken H, et al. Urinary TIMP-2 and IGFBP7 as early biomarkers of acute kidney injury and renal recovery following cardiac surgery. PLoS One 2014; 9(3): e93460.
4. Dusse F, Edayadiyil-Dudásova M, Thielmann M, et al. Early prediction of acute kidney injury after transapical and transaortic aortic valve implantation with urinary G1 cell cycle arrest biomarkers. BMC Anesthesiol 2016; 16: 76.
5. WHO. Geneva, Switzerland. Global report on diabetes 2016. https://apps.who.int/iris/bitstream/handle/10665/204871/9789241565257_eng.pdf;jsessionid=4A26D369EAE6E7F61BFBFD8DFC58B7B7?sequence=1. 2018.

6. Lorenzen JM, Thum T. Long noncoding RNAs in kidney and cardiovascular diseases. Nat Rev Nephrol 2016; 12(6): 360-73.
7. Ignarski M, Islam R, Müller RU. Long Non-Coding RNAs in Kidney Disease. Int J Mol Sci 2019; 20(13).
8. Gill JS, Schaeffner E, Chadban S, et al. Quantification of the early risk of death in elderly kidney transplant recipients. Am J Transplant 2013; 13(2): 427-32.
9. El-Zoghby ZM, Stegall MD, Lager DJ, et al. Identifying specific causes of kidney allograft loss. Am J Transplant 2009; 9(3): 527-35.
10. Chand S, Atkinson D, Collins C, et al. The Spectrum of Renal Allograft Failure. PLoS One 2016; 11(9): e0162278.
11. Verma SK, Molitoris BA. Renal endothelial injury and microvascular dysfunction in acute kidney injury. Semin Nephrol 2015; 35(1): 96-107.
12. Solar-Cafaggi D, Marino L, Uribe-Uribe N, Morales-Buenrostro LE. Antibody-mediated rejection in the Banff classifications of 2007 and 2017: A comparison of renal graft loss prediction capability. Transpl Immunol 2018; 51: 40-4.
13. Sellarés J, de Freitas DG, Mengel M, et al. Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence. Am J Transplant 2012; 12(2): 388-99.
14. Loupy A, Lefaucheur C. Antibody-Mediated Rejection of Solid-Organ Allografts. N Engl J Med 2018; 379(12): 1150-60.
15. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Chapman JR, Allen RD. Calcineurin inhibitor nephrotoxicity: longitudinal assessment by protocol histology. Transplantation 2004; 78(4): 557-65.
16. Kashani K, Al-Khafaji A, Ardiles T, et al. Discovery and validation of cell cycle arrest biomarkers in human acute kidney injury. Crit Care 2013; 17(1): R25.
17. Mayer T, Bolliger D, Scholz M, et al. Urine Biomarkers of Tubular Renal Cell Damage for the Prediction of Acute Kidney Injury After Cardiac Surgery-A Pilot Study. J Cardiothorac Vasc Anesth 2017; 31(6): 2072-9.
18. Nicholson ML, Waller JR, Bicknell GR. Renal transplant fibrosis correlates with intragraft expression of tissue inhibitor of metalloproteinase messenger RNA. Br J Surg 2002; 89(7): 933-7.
19. Beermann J, Piccoli MT, Viereck J, Thum T. Non-coding RNAs in Development and Disease: Background, Mechanisms, and Therapeutic Approaches. Physiol Rev 2016; 96(4): 1297-325.
20. Boon RA, Jaé N, Holdt L, Dimmeler S. Long Noncoding RNAs: From Clinical Genetics to Therapeutic Targets? J Am Coll Cardiol 2016; 67(10): 1214-26.
21. Zaoui P, Cantin JF, Alimardani-Bessette M, et al. Role of metalloproteases and inhibitors in the occurrence and progression of diabetic renal lesions. Diabetes Metab 2000; 26 Suppl 4: 25-9.
22. Mazanowska O, Kamińska D, Krajewska M, et al. Increased plasma tissue inhibitors of metalloproteinase concentrations as negative predictors associated with deterioration of kidney allograft function upon long-term observation. Transplant Proc 2013; 45(4): 1458-61.
23. Martin FA, Murphy RP, Cummins PM. Thrombomodulin and the vascular endothelium: insights into functional, regulatory, and therapeutic aspects. Am J Physiol Heart Circ Physiol 2013; 304(12): H1585-97.
24. Ge YZ, Xu T, Cao WJ, et al. A Molecular Signature of Two Long Non-Coding RNAs in Peripheral Blood Predicts Acute Renal Allograft Rejection. Cell Physiol Biochem 2017; 44(3): 1213-23.
25. Pollack MS, Trimarchi HM, Riley DJ, Casperson PR, Manyari LE, Suki WN. Shared cadaver donor-husband HLA class I mismatches as a risk factor for renal graft rejection in previously pregnant women. Hum Immunol 1999; 60(11): 1150-5.
26. Sagasaki M, Nakada Y, Yamamoto I, et al. Antibody-mediated rejection due to anti-HLA-DQ antibody after pregnancy and delivery in a female kidney transplant recipient. Nephrology (Carlton) 2018; 23 Suppl 2: 81-4.
27. Nankivell BJ, Kuypers DR. Diagnosis and prevention of chronic kidney allograft loss. Lancet 2011; 378(9800): 1428-37.

28. Vincenti F, Ramos E, Brattstrom C, et al. Multicenter trial exploring calcineurin inhibitors avoidance in renal transplantation. Transplantation 2001; 71(9): 1282-7.
29. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005; 105(4): 1815-22.