8

General discussion and future perspectives

Prelude

An interaction between the kidney and the liver has mainly received attention in the context of acute liver failure, leading to renal failure in which kidneys fail subsequentially. A possible role of the liver in chronic kidney disease (CKD) has hardly been subject of investigation. There are, however, data that suggest that an interaction between the liver and the kidney, being the two major metabolic organs of the body, may be relevant to CKD as well. Therefore, the aim of the present thesis was to investigate aspects of liver function, in the non-failing liver, for their relevance in CKD and its complications.

Several aspects of liver function were studied. There are several traditional markers of liver function which might be interesting from the perspective of CKD. One of those is bilirubin, of which it has been suggested that it has a renoprotective effect. In this thesis we studied whether bilirubin protects against progression of CKD and whether the concentration of bilirubin can be elevated by lifestyle. Another aspect of liver function that we have studied is protein metabolism. The liver and the kidney are intrinsically linked in protein metabolism. One of the important roles of the liver in protein metabolism is breakdown of amino acids to urea. The kidney subsequently facilitates the excretion of this breakdown product in urine. The amount of urea excreted in urine provides an estimate of protein intake from food. We explored whether protein intake assessed from 24 hr urine excretion influences long-term outcomes after renal transplantation. Another aspect where liver function and kidney function come together is where they are both adversely influenced by obesity. In this field, it is important to realize that the measure of body mass index (BMI), which is of the used to define obesity is composed of both fat mass and other bodily masses, including muscle mass. Use of BMI as a marker of high fat mass therefore has its limitations. We sought to investigate the utility of a liver-derived marker of excess fat mass and a breakdown product of muscles which is excreted in urine as measures to better relate obesity to long-term outcome, including CKD.

Bilirubin and protection against chronic kidney disease

Diabetic nephropathy (DN) develops in approximately 20–40% of patients with diabetes and is the single leading cause of end-stage renal disease (ESRD) around the world (1). An important pathophysiological mechanism that has been identified in the development and progression of diabetic nephropathy is oxidative stress (2–4). Bilirubin is known to be a potent endogenous antioxidant (5) and accumulating evidence has shown that bilirubin has a protective effect against kidney disease (5–7).

Based on its anti-oxidant properties, we hypothesized that bilirubin is inversely associated with progression of diabetic nephropathy. To investigate this hypothesis, we tested the association of bilirubin with progression of diabetic nephropathy in a cohort of 1,498 patients with type 2 diabetes, hypertension, and nephropathy (**chapter 2**).

In this cohort, bilirubin was inversely and independently associated with progression of diabetic nephropathy. This supports a role for the anti-oxidant effect of bilirubin, exerting a mild protective effect on the kidney.

Besides in DN, oxidative stress has also been implicated in the progression of chronic allograft damage after renal transplantation (8–11). We therefore hypothesized that bilirubin protects against decline of renal function and late graft failure in renal transplant recipients (RTR) (**chapter 3**). To this end, we prospectively investigated 603 RTR with a functioning graft for more than one year. We found that the decline in renal function, determined by a decline in creatinine clearance, was least pronounced in RTR with highest bilirubin concentrations. We also found an independent inverse association of bilirubin with graft failure. Bilirubin was not associated with mortality.

Bilirubin: a causal association with chronic kidney disease?

Given the observational design of these studies, it remains unknown whether bilirubin truly protects against renal disease or whether bilirubin is merely an indicator of a favourable renal risk profile. Johansen et al. already pointed out that such a distinction is important: if bilirubin is a correlated biomarker, it might serve as a prognostic or diagnostic indicator for renal outcomes such as CKD, diabetic nephropathy or long-term graft failure (12). However, if bilirubin is causally related, it could serve as a potential therapeutic target against these diseases (12).

A method to estimate causal relationships is Mendelian randomization (13,14). Mendelian randomization is a statistical method that is based on the fact that genetic variability is less likely to be influenced by lifestyle or exposure to environmental influences compared to the phenotype (14). Mendelian randomization is increasingly used to limit biases such as (unmeasured) confounding and reverse causality that are often present in observational studies (14). Therefore, Mendelian randomization can be used as a tool to explore the possible causality of observed associations.

As much as 18% of the total variability in bilirubin is explained by the genetic variability in the gene UGT1A1. UGT1A1 encodes for the enzyme UDP-GT which is responsible for the regulation of bilirubin levels. Using Mendelian randomization, genetically elevated levels of bilirubin were associated with a lower incidence of type 2 diabetes in the general population (15). In contrast, Mendelian randomization studies rendered disappointing results for the association of bilirubin with cardiovascular disease. A genetically elevated level of bilirubin was neither associated with risk of ischemic heart disease in a prospective study of 10,264 individuals nor in a case control study of 5,133 cases and 5,133 healthy individuals (16). In line, a Mendelian randomization study of 868 healthy individuals found no association of genetically elevated levels of bilirubin with cardiovascular risk factors (17). These findings are in contrast to a number of observational studies which have demonstrated an inverse association

of circulating bilirubin with cardiovascular disease (18–20). To our knowledge, to date, no Mendelian randomization study has been performed to investigate the effect of bilirubin on renal outcomes.

Elevation of bilirubin

Assuming that variation in the circulating concentration of bilirubin is relevant to renal outcomes, we investigated the determinants of variability in the normal range (**chapter 4**), with emphasis on dietary factors and lifestyle factors such as drinking wine and smoking. To this end, a cross-sectional study was performed in 509 male subjects without major chronic diseases from the Zutphen elderly study aged 64–85 years. We investigated associations of bilirubin with individual food groups, a healthy dietary pattern, and lifestyle factors such as smoking. The dietary pattern was studied with the Mediterranean Diet Score (MDS). Both adherence to the MDS with more than 4 points and wine consumption were significantly associated with higher serum bilirubin concentrations in uni- and multivariable analyses. No data were available on oxidative stress in this population, it therefore remains therefore unknown whether this could have beneficial effects on oxidative stress.

Bilirubin: a correlated biomarker?

It is possible that there is no causal association of bilirubin with renal outcomes and that bilirubin is no more than a correlated biomarker. If so, the association of bilirubin with chronic kidney disease could be mediated by other factors, e.g. by metabolites that are generated during bilirubin production. These metabolites include carbon monoxide and biliverdin (5,21). Like bilirubin, carbon monoxide and biliverdin have been suggested to have a beneficial effect on the kidney. Administration of both carbon monoxide and biliverdin has been shown to attenuate ischemia reperfusion injury in rats (22). Administration of carbon monoxide alone has also been shown to inhibit chronic transplant dysfunction in rats (23) and protect against renal fibrosis in mice (24). Furthermore, administration of biliverdin has been shown to protect against diabetic nephropathy in mice (6).

A limitation of our studies is that only total bilirubin was measured. Total bilirubin is composed of two forms, conjugated (direct) bilirubin and unconjugated (indirect) bilirubin. It is important to distinguish between conjugated and unconjugated bilirubin. This is important because the conjugation of bilirubin may significantly alter its characteristics and thereby its influence on biological processes. It is also important because the amount of conjugated bilirubin may reflect the functionality of the liver to conjugate other metabolites. Such other metabolites include products from cellular catabolism, drugs, and vitamin D (25,26). Increased conjugation by the liver may cause unwanted excretion of substances with implied beneficial effects. In other words, if bilirubin

conjugation is impaired, the concentration of bilirubin may be higher than normal, but also the concentration of other metabolites may be higher. A logical next step, therefore, would be to investigate whether it is conjugated or unconjugated bilirubin that is associated with a more favorable renal risk profile.

Urea

The liver and the kidney are intrinsically linked in protein metabolism. Urea is synthesized by the liver from the breakdown of amino acids, as present in protein. Because the kidney facilitates the excretion of urea, circulating urea is used as a marker of kidney function. The urinary excretion of urea can be used a marker of protein intake.

Optimizing protein intake is an important component of dietary management of chronic kidney disease (CKD) patients. Because high protein intake is supposed to aggravate proteinuria (1–4), guidelines recommend daily allowances of 0.6 to 0.8 g protein/kg per day in patients with CKD stages 1 to 4 (3,5,6). Once patients are on dialysis, protein catabolism and protein losses must be compensated. Guidelines therefore recommend a minimal protein intake of 1.1 g/kg per day and preferably 1.2 to 1.3 g/kg per day in patients receiving dialysis (27–29). Unfortunately, advisable protein intake is unknown after transplantation. Therefore, we studied whether 24-h urinary urea excretion (UUE), as a marker of protein intake, was associated with graft failure and mortality in a cohort of 940 RTR with a functioning graft for more than one year **(chapter 5)**. We made use of the regular patient care program for outpatients. Data on repeated 24 hr urine collections was collected from our hospital laboratory system. In this cohort of RTR, optimal protein intake was higher than what is currently recommended in CKD patients (27,30,31), but comparable to recommended protein intake in patients treated with dialysis (≥ 1.1 g/kg/day) (27–29). UUE was not associated with graft failure in the overall population, but was inversely associated with graft failure in RTR with a BMI less than 25 kg/m^2, and in RTR with an eGFR of 45 mL per min per 1.73 m^2 or higher, independent of potential confounders. UUE was inversely associated with mortality, independent of BMI and eGFR. From these data, we conclude that a relatively high protein intake may be beneficial for long-term outcomes in RTR.

After renal transplantation, effects of the renal allograft, rejection episodes, and immunosuppressive drugs contribute to high protein and energy expenditure in RTR (32), possibly shifting optimal protein intake upward. Furthermore, a major side effect of chronic renal disease is anorexia with concordant low protein intake (33,34). When nutritional demands are not balanced by intake, protein and energy stores are utilized. In some patients, this utilization of energy stores can progress into the 'protein energy wasting syndrome'. The protein energy wasting syndrome is a generic term for malnutrition with inadequate protein intake, and is a risk factor for inflammation, oxidative stress, cardiovascular disease, and mortality (35–38). We hypothesized that, at some

point, the adverse effects of a high protein intake (i.e. renal function deterioration) may counterbalance the beneficial effects of high protein intake (i.e. tissue repair, more muscle mass, better nutritional status) in RTR.

Given the observational nature of our study, it remains to be established whether high consumption of protein indeed protects against graft failure and mortality in RTR. In addition, we did not discern between sources of protein (i.e. animal or plant protein), which may have opposite associations with outcome. Markers to determine the intake of meat protein are urinary carnosine, 1-methylhistidine and 3-methylhistidine (39). Therefore, it would be of interest to investigate associations of the source of protein (as measured by carnosine, 1-methylhistidine, and 3-methylhistidine) with renal outcomes in future studies.

Obesity, the liver, and the kidney

The kidney and the liver may also be parallel victims of adverse factors to which they are both exposed. Obesity is a common cause of both liver and kidney damage. However, the definition of obesity is a controversial issue. Several studies have surprisingly reported robust inverse associations of BMI with mortality in chronically ill individuals. This phenomenon has been referred to as the obesity paradox (40–44). Several explanations for this paradoxical association have been proposed. Survival bias has been suggested. Another potential explanation is that patients with a low BMI are at high risk for the protein energy wasting syndrome. As previously mentioned, the protein energy wasting syndrome is disadvantageous, and the long term effects of adiposity (i.e. cardiovascular morbidity and mortality) may not be relevant to patients with a short life expectancy. We propose that a high BMI can indeed reflect a high fat mass, but it can also reflect a high muscle mass, or a combination of both. It may be because of this notion that studies have reported inverse associations of BMI with mortality, and absent or even inverse associations of BMI with renal outcomes (40–44).

Another method to investigate the effects of a high fat mass is by studying hepatic fat. A healthy liver contains little or no fat, but approximately 70% of obese individuals have some degree of liver fat accumulation (NAFLD) (45–47). We hypothesized that alanine aminotransferase (ALT), as a marker of NAFLD might be a better predictor of progression of incident CKD than BMI. We tested this hypothesis in 1,187 patients with type 2 diabetes (**chapter 6**). ALT was elevated in few subjects. ALT was not significantly associated with incident CKD. To our knowledge, to date, the association of ALT with CKD has not yet been investigated, but it has been shown that the prevalence of CKD is significantly higher among patients with both diabetes and NAFLD compared to those without NAFLD (48,49). In those studies, the majority of patients had ALT levels within the normal range (48,49). Because NAFLD may be present even if ALT levels are normal, this may explain why we did not find an association of ALT with incident CKD.

Furthermore, ALT was inversely associated with all-cause mortality, particularly with non-cardiovascular mortality. These results are in line with several population-based studies that mainly included subjects with normal levels of ALT (50–55). It is tempting to speculate that both high and low levels of ALT are associated with mortality. Such a trend would be reflected in a U-shaped relationship or bimodal association. Indeed, other studies found bimodal associations of ALT with all-cause mortality (50,56). In line, the results of the present study also showed a significant bimodal association of ALT with all-cause mortality. A potential explanation for the association of ALT with noncardiovascular mortality is that lower levels of ALT reflect a low number of functional hepatocytes. This low amount of hepatocytes may reflect hepatic aging, which underlies extra-hepatic pathways that increase the risk of mortality (57,58). Because ALT is also produced in muscle cells (59), another explanation for this finding may be that low levels of ALT reflect a low muscle mass or frailty.

Another way to bring into view the harmful effects of a high fat mass is by taking both BMI and muscle into account. A recent meta-analysis by Nicoletto et al. concluded that obesity (i.e. body mass index [BMI] ≥ 30 kg/m^2) is a risk factor for neither graft failure nor mortality in renal transplant recipients (42). As mentioned, muscle mass is an important component of BMI. A high muscle mass is an established marker of better outcomes, both in RTR (60) and in other populations, including the general population (61,62). Accordingly, we investigated whether muscle mass, determined by 24 hr urinary creatinine excretion (UCE), confounds associations of BMI with graft failure and mortality in 916 RTR with a functioning graft for more than one year (**chapter 7**). In a univariable analysis, BMI was not associated with graft failure. The association of BMI with graft failure remained non-significant after adjustment for age and sex, but became uncovered by further adjustment for UCE. In a univariable analysis, BMI tended to be positively associated with mortality. This trend disappeared after adjustment for age and sex, but became uncovered by adjustment for UCE. In conclusion, adjustment for UCE seems to uncover an adverse association of high fat mass, for which BMI became a better measure after adjustment for UCE, with both graft failure and mortality. These findings provide an additional potential explanation for the absence of increased risk of BMI with graft failure and mortality, as previously observed in RTR (63,64). Based on these findings we infer that clinicians should not only monitor BMI, but also muscle mass, because preservation of muscle mass could improve outcome after transplantation.

Conclusion and future perspectives

Based on the findings of this thesis, we conclude that the non-failing liver may be involved in the pathophysiology of CKD. One aspect of liver function that we studied

was bilirubin. We found that bilirubin was significantly associated with CKD. However, because of the observational design of the studies, causality could not be ascertained. Causality of the association of bilirubin with CKD can be investigated using the Mendelian randomization approach in future studies. If bilirubin is no more than a correlated biomarker, it would be interesting to investigate the predictive capabilities of bilirubin and to compare its predictive capabilities to already existing biomarkers. It is also possible that bilirubin is a biomarker for a different causal association with CKD (e.g. for an association of carbon monoxide, or biliverdin with CKD). In that case, the association of bilirubin with CKD may disappear after controlling for the metabolites that are generated during the heme degradation cycle such as carbon monoxide and biliverdin. In addition, it is important that future studies distinguish between conjugated and unconjugated bilirubin, because, as pointed out earlier, the effects of conjugated and unconjugated bilirubin may be different.

Another aspect of liver function that we have studied is protein metabolism. From our research we learned that high protein intake may be beneficial for long-term outcomes in RTR. If these findings are confirmed by other studies, there is need for an intervention study that investigates the effect of high and low protein intake on outcomes after transplantation. It would also be interesting to determine the optimal range of protein intake for RTR. This information could aid clinicians in the treatment of RTR in the future. Furthermore, the most favorable source of protein intake (i.e. animal or plant protein) is unclear at present. It is also unknown why we found that the optimal protein intake of RTR was similar to the optimal protein intake of dialysis patients. We hypothesized that this was due to increased protein catabolism and/or the protein energy wasting syndrome in both RTR and dialysis patients, but we were not able to investigate this. A logical next question would be to determine whether a low protein intake is associated with increased markers of inflammation such as CRP, which are elevated as a consequence of the protein energy wasting syndrome. Furthermore, because a low protein intake could reflect an overall low dietary intake, it should be investigated whether the association of protein intake with outcomes in RTR is independent of caloric intake.

'Personalized medicine' is gaining increasing attention with medical decisions becoming tailored to a specific patient rather than a group of patients. Overall, renal transplantation induces protein losses that must be compensated for and muscle mass often decreases after transplantation. However, this may not be similar in every patient. The optimal moment at which action is warranted with respect to protein intake and muscle mass after renal transplantation may be an important area of investigation. Furthermore, it would be interesting to investigate whether preservation of muscle mass after transplantation is sufficient, or whether muscle mass should be increased before transplantation is initiated. Knowledge about this optimal moment of intervention would aid clinicians in adequate treatment of RTR (nutritional intervention and exercise), subsequently increasing the long-term prognosis after transplantation.

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