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Edited by
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Contents

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Editorial

Recent Advances and Clinical Outcomes of Kidney Transplantation

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Abstract: Recent advances in surgical, immunosuppressive and monitoring protocols have led to the significant improvement of overall one-year kidney allograft outcomes. Nonetheless, there has not been a significant change in long-term kidney allograft outcomes. In fact, chronic and acute antibody-mediated rejection (ABMR) and non-immunological complications following kidney transplantation, including multiple incidences of primary kidney disease, as well as complications such as cardiovascular diseases, infections, and malignancy are the major factors that have contributed to the failure of kidney allografts. The use of molecular techniques to enhance histological diagnostics and noninvasive surveillance are what the latest studies in the field of clinical kidney transplant seem to mainly focus upon. Increasingly innovative approaches are being used to discover immunosuppressive methods to overcome critical sensitization, prevent the development of anti-human leukocyte antigen (HLA) antibodies, treat chronic active ABMR, and reduce non-immunological complications following kidney transplantation, such as the recurrence of primary kidney disease and other complications, such as cardiovascular diseases, infections, and malignancy. In the present era of utilizing electronic health records (EHRs), it is strongly believed that big data and artificial intelligence will reshape the research done on kidney transplantation in the near future. In addition, the utilization of telemedicine is increasing, providing benefits such as reaching out to kidney transplant patients in remote areas and helping to make scarce healthcare resources more accessible for kidney transplantation. In this article, we discuss the recent research developments in kidney transplants that may affect long-term allografts, as well as the survival of the patient. The latest developments in living kidney donation are also explored.

Keywords: kidney transplantation; renal transplantation; kidney transplant; renal transplant; transplant recipients; transplantation

1. Introduction

Kidney transplantation is the optimal treatment for improving survival and quality of life for patients with end-stage kidney disease (ESKD) [1]. Advances in surgical, immunosuppressive and monitoring protocols have led to a significant improvement in overall one-year kidney allograft survival of >95% [2]. Nonetheless, there has not been a significant change in long-term kidney allograft outcomes. In fact, chronic and acute antibody-mediated rejection (ABMR) has continued to cause kidney allograft failures [3]. In addition, non-immunological complications following kidney transplantation, such as the recurrence of primary kidney disease and other complications, such as cardiovascular diseases, infections, and malignancy also play important roles in poor long-term allografts and patient survival [4–6].

In their research into immunologic monitoring and diagnostics in kidney transplants [7–14], a number of groups have made attempts in the recent past towards determining the peripheral molecular fingerprints of ongoing rejection [7,8] and predicting acute rejection [7]. Contemporary researchers have measured the levels of donor-derived cell-free DNA (dd-cfDNA) and showed higher predictive abilities for acute rejection [9–12], especially antibody-mediated rejection (ABMR) diagnostics in cases with a combination of donor specific antibodies (DSA) and dd-cfDNA [13,14]. In addition, a molecular microscope diagnostic system for the evaluation of allograft biopsies has been recently introduced within transplant practice, particularly in complex cases. This has mainly been introduced for the purpose of enhancing histological diagnostics [15].

Recent studies have been conducted aimed at preventing or treating ABMR [16,17]. In 2017, imlifidase (IdeS), an endopeptidase derived from Streptococcus pyogenes, was utilized in a desensitization regimen in an open-label phase 1–2 trial [16]. An instant impact was observed by a significant decline in plasma IgG levels. Another single-center phase 2 study that focused mainly on the pharmacokinetics, effectiveness and safety of IdeS treatment was conducted and proved a reduction in anti-human leukocyte antigen (HLA) antibodies using a complement-dependent cytotoxicity test [17].

In recent years, there has been significant progress in research into kidney transplantation and kidney donation [18–84], including articles [20–60] published in our current Special Issue "Recent Advances and Clinical Outcomes of Kidney Transplantation" (https://www.mdpi.com/journal/jcm/special_issues/outcomes_kidney_transplantation).

In this article, we discuss the recent research developments in kidney transplantation that may impact long-term allografts and patient survival, as well as the latest developments in living kidney donation.

2. Non-HLA Antibodies in Transplantation

When it comes to solid organ transplantation, one major immunological obstacle is the detection the non-self structures that exist in the donor cells. Human leukocyte antigens (HLA) are considered the most important non-self allo-antigens in organ transplantation. In addition, patients can form antibodies against targets other than HLA [85]. Multiple targets for these non-HLA antibodies have been studied in kidney transplantation over the last decade (Figure 1). Recent studies have provided findings that suggest the an importance of non-HLA mismatches between donors and recipients in the development of acute rejection and long-term kidney allograft outcomes [68,78,86–92].

Types of Abs against Endothelial antigens in antibody-mediated immune responses	
Alloantibodies	ABO HLA MICA
Autoantibodies	AT1R ETAR Vimentin Perlecan Endoglin FLT3 ligand EDIL3 ICAM4 Fibronectin Collagen type 4

Figure 1. Post-transplant antibodies against human leukocyte antigen (HLA) and non-HLA antigens [68,78,86–92]. Abbreviations: human leukocyte antigen (HLA), major histocompatibility complex class I related chain A antigen (MICA); angiotensin type 1 receptor (AT1R); endothelin-1 type A receptor (Anti-ETAR); FMS-like tyrosine kinase 3 (FLT3); Epidermal growth factor-like repeats and discoidin I-like domain 3 (EDIL3); Intercellular adhesion molecule 4 (ICAM4).

3. Active AMR

Chronic active ABMR is one of the major causes of long-term allograft loss [93–95]. Tocilizumab, a humanized monoclonal antibody targeting the interleukin (IL)-6 receptor, has been assessed in patients with acute and chronic active ABMR [96–98], given that IL-6 mediates various inflammatory and immunomodulatory pathways, including the expansion and activation of T cells and B cells [98]. Furthermore, there is a genetically engineered humanized Immunoglobulin (Ig)G1 monoclonal antibody that binds to IL-6, inhibiting its interaction with IL-6R. Direct inactivation of IL-6 may limit a rebound induced by the accumulation of IL-6 [99,100]. Preliminary investigations from phase 1–2 trials demonstrated the efficacy of the C1q inhibitor for the prevention of a delayed graft function (DGF) and to lessen the occurrence of chronic active ABMR [101,102]. Although the inhibition of the first step in both the classical and lectin pathways of complement activation may serve as another tool to overcome critical sensitization, such data need to be validated in larger cohorts. Several trials are currently being conducted, and new developments will conceivably provide us with practical ways to counteract the deleterious consequences of ABMR [103].

4. Cardiovascular Diseases in Kidney Transplant Recipients

The burden of cardiovascular diseases on ESKD is improved after kidney transplantation [104]. However, it remains the leading cause of reduced early renal graft loss and mortality, as it is associated with significant morbidity and healthcare costs [104]. Major phenotypes of cardiovascular diseases among kidney transplant recipients include ischemic heart disease, congestive heart failure, valvular heart disease, arrhythmias and pulmonary hypertension (Figure 2).

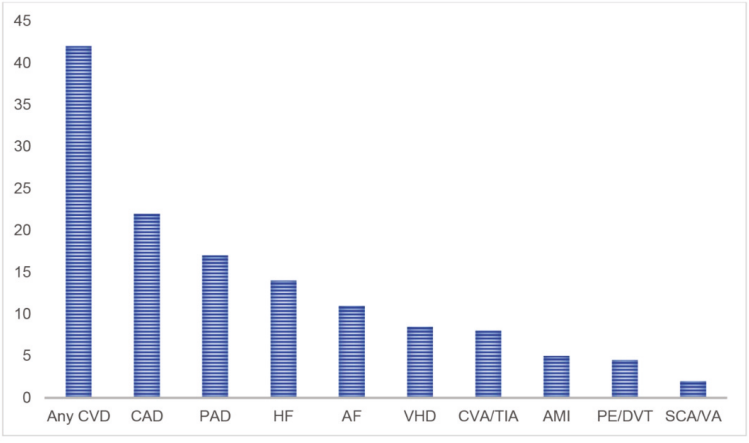

Figure 2. Incidence (%) of cardiovascular disease in kidney transplant recipients.

Reported risk factors for cardiovascular disease in kidney transplant recipients include inflammatory and immunosuppressive agents, episodes of allograft rejection, as well as traditional cardiovascular risk factors, such as hypertension, hyperlipidemia, smoking, obesity, chronic kidney disease, proteinuria, and diabetes mellitus, all of which add to a transplant recipient's cardiovascular risk profile [104]. Hypertension is common among kidney transplant recipients and uncontrolled hypertension in kidney transplant recipients is associated with increased cardiovascular mortality and morbidity, and reduced allograft survival [105]. Furthermore, weight gain is also a significant problem in post-kidney transplant patients. Weight gain after transplantation can unfavorably affect patient outcomes [106]. Identifying these risk factors and adopting strategies to abolish these risk factors may potentially prevent, and help manage, post-transplant obesity. The underlying mechanisms for the increased occurrence of dyslipidemia post-transplant are due to immunosuppressive medications, proteinuria, and post-transplant diabetes [107,108].

The medical management of risk factors includes strategies employed in the chronic kidney disease (CKD) population, with credence given to approaches specific for kidney transplant recipients, such as the choice of maintenance immunosuppression, steroid tapering or withdrawal, and particular anti-hypertensive regimens (Table 1). Overall, cardiovascular morbidity and mortality in kidney transplant recipients has decreased over the last few decades, likely due to improved detection and the timely management of risk factors. Recognition of these complications is important in assessing cardiovascular disease risk in kidney transplant recipients, and optimizing screening and therapeutic approaches. These include lifestyle and immunosuppressive regimen modification, as well as the best feasible regimen for glycemic and lipid controls according to an individual's metabolic profile and medical history.

Table 1. Cardiovascular risk factors among kidney transplant recipients and suggested management.

Cardiovascular Risk Factor	Suggested Management	Reference
Traditional risk factors		
Hypertension	• Monitor each visit • Target BP < 130/80 mmHg (ACC/AHA, 2017) • Initial treatment with CCB • ACEI/ARB if > 1 g/day proteinuria	[109–112]
Diabetes	• Monitor for post-transplant DM annually • Target HbA1c 7.0–7.5% (KDIGO, 2009) • Low-dose ASA in all atherosclerotic CVD	[109,113]
Cigarette smoking	• Screen annually • Offer intervention for smoke cessation	[109,114]
Dyslipidemia	• Monitor annually • Use of statins favored in all KTx (KDIGO, 2014)	[109,115]
Obesity	• Monitor BMI and weight circumference • Healthy diet and exercise • BMI target < 35 kg/m2	[109,116]
Non-traditional risk factors		
eGFR < 45 ml/min/1.73m2	• Increased use of living donor organs if possible • Check serum creatinine at least annually • Avoid nephrotoxic medications	[109,117]
Proteinuria	• ACEI/ARB if > 1 g/day proteinuria • Check urine analysis at least annually	[109,118,119]
Left ventricular hypertrophy	• Check ECG, echocardiography • Treat underlying hypertension	[109,120]
Anemia	• Treatment similar to CKD guidelines • Check CBC	[109,121,122]
Acute rejection episodes	• Treat rejections as per KDIGO, 2009	[109,123,124]

American College of Cardiology (ACC); angiotensin-converting enzyme inhibitor (ACEI); American Heart Association (AHA); angiotensin-II receptor blocker (ARB); aspirin (ASA); body mass index (BMI); blood pressure (BP); complete blood count (CBC); calcium-channel blockers (CCB); chronic kidney disease (CKD); cardiovascular disease (CVD); diabetes mellitus (DM); electrocardiography (ECG); estimated glomerular filtration rate (eGFR); Kidney Diseases Improving Global Outcomes (KDIGO); kidney transplant (KTx).

5. Preexisting Diabetes and Post-Transplantation Diabetes

Preexisting diabetes and post-transplantation diabetes confer reduced patient and graft survival in kidney transplant recipients [71,73,125]. Hyperglycemia is present in nearly 90% of kidney transplant recipients in the immediate postoperative period, but it is not sustained in the majority [126]. In addition to the general risk factors for diabetes, there are also certain transplantation-related factors (e.g., specific immunosuppressive agents, surgical stress and inflammation, nutritional interventions) placing kidney transplant recipients at elevated risk of hyperglycemia [126]. Some transplant immunosuppressive medications, including corticosteroids, calcineurin Inhibitors (CNIs), and mammalian target of rapamycin (mTOR) inhibitors, are associated with a higher incidence of metabolic complications such as post-transplantation diabetes. CNIs impair insulin secretion and sensitivity and directly damage pancreatic islet cells [127].

A robust evidence base guiding precise glycemic goals is currently lacking in kidney transplant recipients. Management is largely guided by evidence from the general diabetes population [71,73,125]. Hospital management of hyperglycemia is primarily achieved through an insulin regimen that takes into account rapid changes in glucocorticoid doses, nutritional modalities and renal function during the immediate post-transplantation period. There is an opportunity to use oral or non-insulin injectable agents in a considerable number of patients by the time they are discharged from the hospital, or in

the long run. The use of specific oral or non-insulin injectable agents is guided by patient specifics and the pharmacologic properties of medications. Although several studies have suggested the safe use of sodium glucose transport 2 (SGLT2) inhibitors in kidney transplant recipients [128], future studies assessing their efficacy and safety are needed, since SGLT2 inhibitor treatment also carries an increased risk of genital tract infections and, possibly, of urinary tract infections [129]; kidney transplant recipients are particularly susceptible to infections due to immunosuppressive regimens.

6. Posttransplant Malignancy

Cancer is one of the three major causes of death after kidney transplantation [130,131]. Posttransplant malignancy occurrence is widely recognized (Table 2). The effect of viral infections, induction and immunosuppressive maintenance regimens have been proposed as important risk factors for posttransplant malignancy. The increased risk of cancer may be due to viral reactivation induced by immunosuppressive agents or impaired immune surveillance leading to faster tumor growth [132]. A higher degree of immunosuppression is associated with an increased risk of malignancy, and calcineurin inhibitors can promote carcinogenesis [132].

Table 2. Standardized incidence ratio of cancers in kidney transplant recipients [133].

Cancer	Standardized Incidence Ratio (95% CI)
Lip cancer	29.45 (17.85–48.59)
Non-melanoma skin cancer	12.14 (6.37–23.13)
Renal cell carcinoma	10.77 (6.40–18.12)
Non-Hodgkin lymphoma	10.66 (8.54–13.31)
Thyroid cancer	5.04 (3.79–6.71)
Hodgkin lymphoma	4.90 (3.09–7.78)
Urinary bladder cancer	3.52 (1.48–8.37)
Melanoma	2.48 (1.08–5.67)
Hepatocellular carcinoma	2.45 (1.63–3.66)
Gastric cancer	1.93 (1.60–2.34)
Colon cancer	1.85 (1.53–2.23)
Lung cancer	1.68 (1.29–2.19)
Ovarian cancer	1.60 (1.23–2.07)
Pancreatic cancer	1.53 (1.23–1.91)
Breast cancer	1.11 (1.11–1.24)

Confidence Interval (CI).

7. Infection

Solid organ transplant recipients are at greater risk of infection than the non-immunosuppressed population (Table 3) [134]. Infections are the most common non-cardiovascular causes of mortality following kidney transplantation, accounting for 15%–20% of mortality [131,135]. The first six months post-transplant is the time of greatest infection risk. There are also times when patients encounter adverse reactions to immunosuppressive agents [136,137]. Among all infectious complications, viruses are considered to be the most common agents [138]. Herpes simplex virus, varicella zoster virus, BK polyomavirus, cytomegalovirus, Epstein–Barr virus, hepatitis B virus, and adenovirus are well-known etiologic agents of viral infections in kidney transplant patients worldwide [138]. In order to prevent opportunistic infections in kidney transplant recipients, antimicrobial prophylaxis is recommended after kidney transplantation. The recommended prophylactic method after transplant differs based on the organism, as well as individual patient characteristics.

Table 3. Infection post kidney transplantation.

<1 Month	1–6 Month	>6 Month
Bacterial infection * • UTI (mainly E Coli, Enterobacteriaceae, Pseudomonas, Enterococcus) • Respiratory • Catheter, drainage sites, wound, perinephric fluid collection, urinary stent infections • Bacteremia • C diff colitis	**Bacterial infection** • With prophylaxis ** -C diff colitis, Mycobacterium species • Without prophylaxis - Listeria, Nocardia	**Bacterial infection** • UTI • Pneumonia
Viral infection • HSV • Donor-derived—HIV, Hepatitis, CMV, BK, LCM virus, West Nile virus, Rabies	**Viral infection** • With prophylaxis—BK, Adenovirus, Influenza, EBV, HCV, Parvovirus • Without prophylaxis—HSV, CMV, VZV,	**Viral infection** • CMV (colitis or retinitis) • Hepatitis (B and C) • EBV, HSV, HHV-8, papillomavirus (associated with malignancy) • VZV, BK virus, parvovirus
Fungal infection • Candida (can be donor derived or pre—TX colonization)	**Fungal infection** • With prophylaxis—Aspergillus, Cryptococcus, Mucor • Without prophylaxis—Pneumocystis jiroveci	**Fungal infection** • Cryptococcus, Rhodococcus, Aspergillus, pneumocystis, Mucor
Parasitic infection • Donor-derived—Malaria, Babesia, Balamuthia, T. cruzi	**Parasitic infection** • Toxoplasma, Strongyloides, T. cruzi, Leishmaniasis	

* Center-dependent multidrug resistant bacteria like Methicillin-resistant Staphylococcus aureus (MRSA), Vancomycin-resistant Enterococcus (VRE), extended-spectrum beta-lactamases (ESBLs); ** With prophylaxis – with Bactrim and Gancyclovir/Valganciclovir; Abbreviations: cytomegalovirus (CMV), lymphocytic choriomeningitis virus (LCM), Epstein-Barr Virus (EBV), hepatitis B virus (HBV), hepatitis C virus (HCV), Trypanosoma cruzi (T. cruzi), Varicella Zoster virus (VZV), human herpes virus 8 (HHV-8).

8. Latest Developments in Living Kidney Donation

Living donor kidney transplants are the best option for many patients with ESKD for several reasons, including (1) better long-term graft survival, (2) no need to wait on the transplant waiting list for a kidney from a deceased donor, (3) transplant surgery can be planned and (4) lower risks of rejection and DGF [139]. Living donor kidney transplantation is the optimal treatment for patients with ESKD [139]. The expansion of living donor programs was made possible by new modes of living donation and by the extension of the living donor pool [139].

To expand the donor pool, a well-developed paired kidney donation program and the adequate reimbursement of costs associated with donation are fundamental elements [140]. Paired kidney donation provides living kidney donation for noncompatible donor/recipient pairs that otherwise would not be feasible or need desensitization [141]. Other possible approaches for increasing the donor pool include ABO-incompatible transplantation [142], the utilization of higher risk donors, advanced donation with a voucher system, and providing donors with financial incentives [141,143,144].

Over the past decade, the long-term risks of kidney donation have been described. Living donors seem to have a higher risk of ESKD, particularly in obese donors and also for African American donors with an apolipoprotein L1 (APOL1) high-risk genotype. In African American living kidney donors, those with the APOL1 high-risk genotype (prevalent in about 13% of African Americans in the United

States) had an almost three times more accelerated decline in estimated glomerular filtration rate (eGFR) after adjusting for pre-donation eGFR than those with a low-risk genotype [145].

9. Post-Transplant Hyperparathyroidism and Bone Disease

Successful renal transplantation results in a reduction in parathyroid hormone (PTH), especially during the first 3 months after transplantation [146]. However, elevated PTH levels can still be found in 30% to 60% of patients 1 year after transplantation. Persistent hyperparathyroidism following kidney transplantation can result in notable complications, such as fracture/bone diseases, cardiovascular disease, vascular calcification, and allograft dysfunction (Figure 3). Associated factors for persistent hyperparathyroidism are long dialysis duration, high PTH levels prior to transplantation, lower eGFR post-transplant, post-transplant hypercalcemia, and post-transplant high alkaline phosphatase.

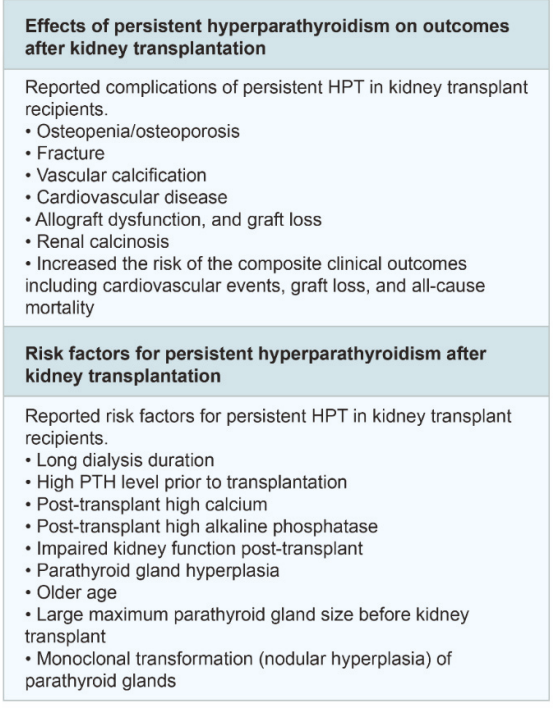

Figure 3. Effects and risk factors of post-transplant hyperparathyroidism.

10. Potential Directions and Future Scope

Researchers need to instantly shift their focus on the unaddressed concerns with respect to kidney transplants. Because of the limited supply of organs, numerous potential recipients still have to spend more time in dialysis, waiting for a transplant. Sensitization to HLA antigens inhibits the recipients' access to transplants, compromising the survival of the graft due to chronic and acute AMR. The publication of complete data from a multi-center second-phase test that explores how IdeS is useful in desensitization is underway (NCT02790437). The phase 3 trial, uncovering the impact of clazakizumab following transplantation, was launched recently, with the outcomes of the phase 2 trial to be released soon.

Moreover, the lack of experienced and skilled professionals could hinder the diagnostic correctness of complications following transplantation. Furthermore, medication non-adherence among patients

could increase the alloimune reaction. Notably, medical research on the costimulation blockade during kidney transplantation is underway. A randomized sixty-month multi-center study (CIRRUS, NCT03663335) in kidney transplant is also underway, with the aim of defining the range of dosage and assessing the tolerability, safety, and effectiveness of some newly developed anti-CD40 monoclonal antibodies in two distinct cohorts in comparison to a tacrolimus-based regimen. Recently, a phase 2a clinical trial, with the purpose of assessing how effective the dual costimulation blockade with anti-CD40 (VIB4920) is when combined with belatacept in kidney transplantation patients (NCT04046549), was registered.

Big data is increasingly being utilized, with the establishment of a large collection of cohorts and the usage of electronic health records (EHRs) in kidney transplantation and artificial intelligence, which might be useful in solving problems related to the survival analysis of patients who have gone through kidney transplantation [147–155]. In the present era, it is strongly believed that big data and artificial intelligence will greatly reshape the research done on kidney disease and, consequently, improve the general clinical practice of nephrology [156].

The benefits of telemedicine include reaching out to patients in remote areas and helping to make scarce healthcare resources more accessible. As telemedicine applications continue to proliferate, studies have demonstrated that telehealth for transplant care may be associated with a reduction in cost and time, and may also improve access to transplantation for ESKD patients [157,158].

11. Conclusions

The most recent endeavors in kidney transplantation tend to mainly focus on noninvasive monitoring, as well as the improvement of histological diagnostics with the aid of molecular techniques. Such studies offer creative means that can be used to find immunosuppressive agents, which can effectively overcome critical sensitization, prevent the creation of anti-HLA antibodies, treat chronic active ABMR, and reduce non-immunological complications following kidney transplantation, such as the recurrence of primary kidney disease and other complications, such as cardiovascular diseases, infections, and malignancy. In the present era of utilizing EHRs, it is strongly believed that big data and artificial intelligence will reshape the research done on kidney transplantation in the near future. In addition, the utilization of telemedicine is increasing, providing benefits such as reaching out to kidney transplant patients in remote areas and helping to make scarce healthcare resources more accessible for kidney transplantation.

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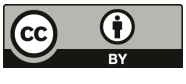

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High Plasma Branched-Chain Amino Acids Are Associated with Higher Risk of Post-Transplant Diabetes Mellitus in Renal Transplant Recipients

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Abstract: Post-transplant diabetes mellitus (PTDM) is a serious complication in renal transplant recipients. Branched-chain amino acids (BCAAs) are involved in the pathogenesis of insulin resistance. We determined the association of plasma BCAAs with PTDM and included adult renal transplant recipients (≥18 y) with a functioning graft for ≥1 year in this cross-sectional cohort study with prospective follow-up. Plasma BCAAs were measured in 518 subjects using nuclear magnetic resonance spectroscopy. We excluded subjects with a history of diabetes, leaving 368 non-diabetic renal transplant recipients eligible for analyses. Cox proportional hazards analyses were used to assess the association of BCAAs with the development of PTDM. Mean age was 51.1 ± 13.6 y (53.6% men) and plasma BCAA was 377.6 ± 82.5 μM. During median follow-up of 5.3 (IQR, 4.2–6.0) y, 38 (9.8%) patients developed PTDM. BCAAs were associated with a higher risk of developing PTDM (HR: 1.43, 95% CI 1.08–1.89) per SD change ($p = 0.01$), independent of age and sex. Adjustment for other potential confounders did not significantly change this association, although adjustment for HbA1c eliminated it. The association was mediated to a considerable extent (53%) by HbA1c. The association was also modified by HbA1c; BCAAs were only associated with renal transplant recipients without prediabetes (HbA1c < 5.7%). In conclusion, high concentrations of plasma BCAAs are associated with developing PTDM in renal transplant recipients. Alterations in BCAAs may represent an early predictive biomarker for PTDM.

Keywords: branched chain amino acids; post-transplant diabetes mellitus; biomarker; renal transplant recipients

1. Introduction

Post-transplant diabetes after transplantation (PTDM), often a result of insulin resistance and deficient insulin production [1], is a serious complication in renal transplant recipients [2]. PTDM develops in 10–20% of renal transplant recipients during the first year post-transplantation [3], although some studies reported incidences of up to 50% [4]. PTDM is an important risk factor for cardiovascular disease (CVD) and infections, contributing to impaired graft and patient survival [5–7]. Previous studies have shown that PTDM is an important risk factor for premature mortality in renal transplant recipients [8–10]. Since the main cause of death in these patients is cardiovascular-related [11–13]

and most of these patients die with a properly functioning graft [14], it is clinically relevant to identify patients that are at high risk of developing PTDM.

Non-modifiable risk factors for the development of PTDM are patient's age, race, genetic background, and family history of diabetes. On the other hand, modifiable risk factors include overweight and obesity, but also immunosuppressive medication, such as steroids and calcineurin inhibitors [5]. During the era of cyclosporine-based regimens, the largest number of incident cases of PTDM occurred beyond the first year after transplantation [2] and with current tacrolimus-based regimens, the number of incident cases of PTDM beyond the first year after transplantation is even higher [15]. Furthermore, it should be noted that if a renal transplant recipient is diagnosed with diabetes, it is considered PTDM, irrespective whether this occurs one-year post-transplantation or 10 years later [16].

Current research is focused on a better understanding of risk factors responsible for the development of PTDM. Branched chain amino acids (BCAAs), a group of three essential amino acids (i.e., valine, leucine, and isoleucine), can be obtained from diet and comprise about 15–25% of total protein intake [17]. BCAAs not only play an important role in protein metabolism, but also have metabolic functions [18]. They may stimulate protein synthesis and influence glucose homeostasis [19–21]. It is known that circulating concentrations of BCAAs are elevated in subjects with prediabetes, type 2 diabetes, metabolic syndrome, and obesity [20,22,23]. Previous studies have assessed the association of BCAAs with insulin resistance and development of type 2 diabetes [24,25]. Recently, we reported that high concentrations of BCAAs are associated with an increased risk of developing type 2 diabetes in a large prospective cohort study in the general population [26]. High concentrations of BCAAs might be the consequence of excess dietary consumption, dysbiosis of the gut microbiota, and reduced breakdown of BCAAs in skeletal muscle and adipose tissue [20,23,27,28]. It has been found that kidney transplant recipients suffer from dysbiosis of gut microbiota [29,30]. Chronic use of immunosuppressive medication, including glucocorticoids and calcineurin inhibitors, by kidney transplant recipients is moreover likely to influence skeletal muscle metabolism [31,32]. These factors could alter BCAAs in kidney transplant recipients compared to the general population and thereby contribute to development of diabetes.

Previous population-based studies have shown inverse associations between BCAAs and all-cause mortality [33–35]. Furthermore, it is known that disturbances in amino acid metabolism, particularly involving BCAAs, occur in patients with end-stage renal disease [36]. A previous study showed that levels of valine and leucine, but not isoleucine, were significantly lower in patients with stage I and II chronic kidney when compared to controls [37].

Whether BCAA plasma concentrations are associated with development of PTDM in renal transplant recipients has not yet been established. Therefore, we hypothesized that higher plasma BCAA concentrations are associated with a higher risk of developing PTDM in renal transplant recipients, as a primary endpoint. Furthermore, secondary endpoints of this study were all-cause mortality and death-censored graft failure, because these endpoints could potentially compete with development of PTDM as an endpoint.

2. Materials and Methods

2.1. Study Design and Population

In this large cross-sectional study with prospective follow-up, we included stable adult renal transplant recipients (≥18 y) with a functioning graft for at least one year after transplantation (i.e., on maintenance immunosuppression and with a stable renal function), therefore excluding patients with transient hyperglycemia post-transplantation. Between November 2008 and May 2011, patients who visited the outpatient clinic of the University Medical Center Groningen were invited to participate. Both subjects with known or apparent systemic diseases (i.e., malignancies, opportunistic infections) and subjects with a history of alcohol and/or drug addiction were excluded

from participation. Informed consent was given by 707 (86.5%) of 817 initially invited patients. We excluded patients with missing data on BCAA, resulting in 518 renal transplant recipients eligible for analyses. As previously described, we recorded age, sex, body composition and eGFR of the renal transplant recipients who did not consent [38]. Compared with participating renal transplant recipients, those who did not consent were slightly older (mean age ± SD, 58 ± 13 years versus 53 ± 13 years) and had lower eGFR (47 ± 19 ml/min per 1.73 m^2 versus 51 ± 20 ml/min per 1.73 m^2) [38]. For the analyses with PTDM, we also excluded patients with diabetes or a history of diabetes at baseline (n = 132). Of these 132 renal transplant recipients, 34 were diagnosed with diabetes before renal transplantation and 98 developed PTDM between time of transplantation and baseline, leaving 386 renal transplant recipients eligible for analyses (Supplementary Figure S1). The study protocol was approved by the institutional research board (METc 2008/186), which adheres to the Principles of the Declaration of Helsinki.

2.2. Data Collection

During a morning visit to the outpatient clinic, all baseline data were collected as described previously [39]. Body weight and height were measured. Body Mass Index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2). Systolic and diastolic blood pressure and heart rate were measured every minute for 15 minutes in a half-sitting position using a semi-automatic device (Dinamap®1846; Critikon, Tampa, FL, USA) to prevent white coat effects [40]. The average of the last three measurements was taken as blood pressure value. Information on medication was derived from patient records, whereas information on smoking behavior was obtained by questionnaire. Information on physical activity was obtained using the reliable and valid Short Questionnaire to Assess Health enhancing physical activity (SQUASH) score in time multiplied by intensity [41]. Alcohol consumption and total energy intake were measured using a reproducible, validated food frequency questionnaire (FFQ) [42], which consisted of 177 items and was updated several times. Blood samples were taken after an 8–12 h overnight fasting period in the morning after completion of 24 h urine collection. Renal transplant recipients were instructed to assure adequate urine collection. They were instructed to discard their first morning urine specimen and then collect their urine for the next 24 h, including the next morning's first specimen the day of their visit. Protein intake was measured using 24 h urinary urea excretion. Estimated Glomerular Filtration Rate (eGFR) was calculated using the serum creatinine-based Chronic Kidney Disease Epidemiology Collaboration equation [43].

2.3. Quantification of BCAAs

Plasma BCAA concentrations were measured at baseline using a Vantera Clinical Analyzer (LabCorp, Morrisville, NC, USA), a fully automated, high-throughput, 400 MHz proton (^{1}H) nuclear magnetic resonance spectroscopy (NMR) platform. Quantification of BCAAs by NMR was validated and had been previously described in detail elsewhere [25,44]. The within-laboratory (inter-assay) and within-run (intra-assay) imprecision for NMR-measured BCAAs are described in detail previously [44]. For total BCAAs, the coefficients of variation for inter-assay and intra-assay were 1.8–6.0% and 2.1–4.4%, respectively.

2.4. Clinical Endpoints

The primary outcome of this study was PTDM, which was defined as at least one of the following criteria: symptoms of diabetes (e.g., polyuria, polydipsia, unexplained weight loss) plus a non-fasting plasma glucose concentration of ≥200mg/dL (11.1 mmol/L); fasting plasma glucose concentration (FPG) ≥126 mg/dL (7.0 mmol/L); start of antidiabetic medication; or HbA1c ≥6.5% (48 mmol/L). This definition was according to the American Diabetes Association criteria for diabetes [45], including HbA1c levels as proposed by the International Expert Panel of the international consensus meeting on PTDM [46]. The secondary outcomes of this study were all-cause mortality and death-censored graft failure.

Death-censored graft failure was defined as return to hemodialysis treatment or retransplantation. All subjects received medical care at the University Medical Center Groningen alone or medical care shared with a secondary referral hospital. In accordance with the KDIGO guideline for renal transplant recipients, follow-up visits after the first year post transplantation were performed every 3 months [47]. Data on PTDM, all-cause mortality and death-censored graft failure were retrieved from patient files and verified with the corresponding nephrologist or the Municipal Personal Records Database in case of death. Endpoints were recorded until the end of September 2015. Since the outpatient program uses continuous surveillance systems, it guarantees correct and up-to-date information on patient status. No participants were lost to follow-up.

2.5. Statisical Analyses

Normal distributed data were presented as mean and standard deviation, whereas skewed distributed data were expressed as median and interquartile range. Categorical data were presented as number and percentage. Differences between diabetic and non-diabetic renal transplant recipients were compared using unpaired t-tests for normally distributed variables, Mann–Whitney U tests for skewed distributed variables and Chi-square tests for categorical variables. Differences between tertiles of total BCAA were compared using one-way ANOVA tests for normally distributed variables, Kruskal–Wallis tests for skewed distributed variables and Chi-square tests for categorical variables. Skewed distributed data were log-transformed when appropriate. Correlations between BCAAs and total energy intake, protein intake, physical activity, and HbA1c in non-diabetic renal transplant recipients were assessed using Pearson correlation coefficients.

Kaplan–Meier curves were plotted for the development of PTDM according to the highest tertile versus the two lowest tertiles of total BCAA. We performed crude and multivariable Cox proportional hazards regression analyses to assess the association of total BCAA with the development of PTDM. First, we performed crude analyses and analyses adjusted for age and sex (model 1). We further cumulatively adjusted for renal function parameters (eGFR, proteinuria, and time between transplantation and baseline) in model 2. To prevent overfitting by including too many covariates in relation to number of events [48], we adjusted for other potential confounders in additional models based on model 2. We additionally adjusted for total cholesterol and triglycerides in model 3; total energy intake, physical activity, and BMI in model 4; smoking status and alcohol consumption in model 5; prednisolone dose and trough levels of tacrolimus and cyclosporine in model 6. Total BCAA per 1 standard deviation (SD) was used as continuous variable, but also as categorical variable (highest tertile versus two lowest tertiles). Patients were censored at date of last follow-up or death. Hazards ratios and 95% CIs were given for the Cox proportional hazards analyses. Schoenfeld residuals of the BCAAs were checked and tested in STATA using the proportional hazard test by Grambsch and Therneau [49]. Furthermore, penalized splines analyses performed in R were used to visualize the association of total BCAA with the development of PTDM, adjusted for age and sex. Additionally, we evaluated potential effect modification by age, gender, BMI, eGFR and HbA1c by entering both main effects and the cross-product term in the crude model. When effect modification was observed, we proceeded with stratified analyses, with a HbA1c of 5.7–6.4% considered as prediabetic state [50,51].

In further analyses, we investigated whether plasma glucose and HbA1c could serve as mediator in the association of BCAAs and risk of PTDM. To investigate potential mediation, we performed mediation analyses using the mediation package of R [52], by which we tested significance and magnitude of mediation (see the Supplementary Materials for a detailed description). Competing risks occur when patients can experience or develop one or more events which compete with the outcome of interest [53]. To rule out competing risk of all-cause mortality with the development of PTDM, we performed competing risk analyses according to Fine and Gray [54]. We performed crude and multivariable Cox proportional hazards regression analyses to assess the association of total BCAA with the secondary outcomes all-cause mortality and death-censored graft failure. Total BCAA per 1 standard deviation (SD) was used as continuous variable, but also as categorical variable (lowest

tertile versus two highest tertiles). For the association of BCAAs with both all-cause mortality and death-censored graft failure, we evaluated the potential effect modification by diabetes.

A two-sided *p*-value of <0.05 was considered statistically significant. The main statistical analyses for the manuscript were performed using IBM Statistics SPSS version 23.0 (IBM Inc, Chicago, IL, USA). We used STATA version 11.0 (StataCorp LP, College Station, TX, USA) to check and test the Schoenfeld residuals by performing the proportional hazard test according to Grambsch and Therneau. We used R version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria) to perform penalized splines analyses and to perform mediation analyses. We used GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) to visualize the Kaplan–Meier curves for the development of PTDM.

3. Results

3.1. Patient Characteristics in Whole Cohort (n = 518)

Mean age of overall renal transplant recipients was 52.7 ± 13.0 y and 53.7% of the participants were men. Median time between baseline measurements and transplantation was 5.0 (IQR, 1.7–11.9) years. Diabetic renal transplant recipients had significantly higher plasma concentrations of total BCAA (424.6 ± 97.9 μM) when compared with non-diabetic renal transplant recipients (377.6 ± 82.5 μM). Baseline characteristics of the overall (*n* = 518), diabetic (*n* = 132) and non-diabetic (*n* = 386) population are shown in Table 1. Non-diabetic subjects were younger, had a lower weight and BMI, had a higher physical activity score and lower heart rate when compared with diabetic RTR. Furthermore, non-diabetic subjects had lower plasma glucose, HbA1c, and triglycerides, and higher HDL cholesterol concentrations. No differences were seen in medication, except for use of statins, which was more common in the diabetic renal transplant recipients than in the non-diabetic renal transplant recipients.

Table 1. Baseline characteristics in renal transplant recipients (n = 518).

	Total (n = 518)	Diabetic RTR (n = 132)	Non-Diabetic RTR (n = 386)	P-Value
General characteristics				
Age, years	52.7 ± 13.0	57.2 ± 10.1	51.1 ± 13.6	<0.001
Male sex, n (%)	278 (53.7)	71 (46.2)	207 (53.6)	0.97
Race (white), n (%)	515 (99.4)	132 (100.0)	383 (99.2)	0.31
BMI, kg/m^2	26.6 ± 4.8	28.7 ± 5.3	25.9 ± 4.5	<0.001
Physical activity score (time x intensity)	5075 (2190–8100)	3690 (1215–6563)	5590 (2810–8715)	<0.001
Smoking status, n (%)				0.16
Never	203 (39.2)	49 (37.1)	154 (39.9)	
Former	228 (44.0)	63 (47.7)	165 (42.7)	
Current	63 (12.2)	10 (7.6)	53 (13.7)	
Alcohol consumption, g/d	2.3 (0.0–11.1)	1.6 (0.0–7.4)	2.6 (0.0–11.7)	0.05
Total energy intake, kcal/d	2139 ± 634	2106 ± 599	2152 ± 647	0.49
Urea excretion, mmol/24h	389.4 ± 117.2	391.2 ± 124.3	388.8 ± 114.9	0.83
Circulation				
Heart rate, b.p.m.	68.6 ± 12.1	72.2 ± 11.3	67.3 ± 12.1	<0.001
SBP, mmHg	135.9 ± 17.7	138.2 ± 18.5	135.1 ± 17.4	0.07
DBP, mmHg	82.1 ± 11.0	82.0 ± 10.3	82.1 ± 11.3	0.93
Transplant characteristics				
Transplant vintage, years	5.0 (1.7–11.9)	6.2 (1.7–11.8)	4.9 (1.7–12.0)	0.59
Living donor, n (%)	182 (35.1)	37 (28.0)	145 (37.6)	0.05
Pre-emptive transplant, n (%)	91 (17.6)	14 (10.6)	77 (19.9)	0.02
Dialysis duration, months	42.0 (18.5–59.0)	36.0 (18.5–52.5)	44.0 (17.0–59.8)	0.58
Age donor, years	43.7 ± 15.2	41.9 ± 15.2	44.3 ± 15.2	0.12
Renal allograft function				
Serum creatinine, µmol/L	127.0 (101.0–167.3)	133.0 (102.3–166.0)	126.0 (101.0–168.0)	0.77
eGFR, ml/min per 1.73 m^2	50.6 ± 19.9	49.5 ± 20.8	51.0 ± 19.6	0.45
Proteinuria, n (%)	117 (22.6)	35 (26.5)	82 (21.2)	0.19
Glucose homeostasis				
Plasma glucose (mmol/L)	5.2 (4.8–6.0)	7.0 (5.4–8.1)	5.1 (4.7–5.5)	<0.001
HbA1c (%)	6.0 ± 0.8	6.9 ± 1.1	5.7 ± 0.4	<0.001
Lipids and lipoproteins				

Table 1. *Cont.*

	Total (n = 518)	Diabetic RTR (n = 132)	Non-Diabetic RTR (n = 386)	P-Value
Total cholesterol, mmol/L	5.2 ± 1.2	5.2 ± 1.2	5.1 ± 1.1	0.64
HDL-cholesterol, mmol/L	1.4 ± 0.5	1.3 ± 0.4	1.4 ± 0.5	0.03
LDL-cholesterol, mmol/L	3.0 ± 0.9	3.0 ± 1.0	3.0 ± 0.9	0.59
Triglycerides, mmol/L	1.7 (1.3–2.4)	1.9 (1.4–3.0)	1.7 (1.2–2.2)	<0.001
Medication				
Calcineurin inhibitor, n (%)				0.19
Cyclosporine	218 (42.1)	61 (46.2)	157 (40.7)	
Tacrolimus	93 (18.0)	27 (20.5)	66 (17.1)	
Trough level cyclosporine (µg/L)	108.0 (77.0–144.0)	102.5 (74.0–156.0)	111.0 (77.5–142.0)	0.78
Trough level tacrolimus (µg/L)	6.8 (5.0–9.0)	6.6 (5.4–9.9)	7.2 (4.9–9.0)	0.78
Proliferation inhibitor, n (%)				0.20
Azathioprine	100 (19.3)	22 (16.7)	78 (20.2)	
Mycofenol	333 (64.3)	82 (62.1)	251 (65.0)	
Prednisolone, n (%)	513 (99.0)	131 (99.2)	382 (99.0)	0.78
Prednisolone dose, mg/24h	10.0 (7.5–10.0)	10.0 (7.5–10.0)	10.0 (7.5–10.0)	0.71
Antihypertensive drugs, n (%)	462 (89.2)	121 (91.7)	341 (88.3)	0.29
Statins, n (%)	270 (52.1)	84 (63.6)	186 (48.2)	0.002
Amino acids				
Total BCAA, µM	389.6 ± 89.0	424.6 ± 97.9	377.6 ± 82.5	<0.001
Valine, µM	203.0 ± 44.7	217.0 ± 48.9	198.2 ± 42.2	<0.001
Leucine, µM	141.7 ± 37.3	157.1 ± 42.1	136.5 ± 34.0	<0.001
Isoleucine, µM	44.9 ± 19.1	51.8 ± 20.0	43.5 ± 17.8	<0.001

Data are represented as mean ± SD, median (interquartile range) or n (%). Differences were tested by unpaired T-test or Mann–Whitney U test for continuous variables and with χ^2-test for categorical variables. RTR, renal transplant recipients; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; LP-IR, lipoprotein insulin resistance index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BCAA, branched-chain amino acids.

3.2. Patient Characteristics in Subgroup of Non-Diabetic Renal Transplant Recipients (n = 386)

For the non-diabetic renal transplant recipients the baseline characteristics according to tertiles of total BCAAs are presented in Table 2. Subjects in the highest tertile of total BCAA were more often male, consumed more alcohol, had a lower heart rate, and a lower HDL cholesterol when compared with subjects in the lowest tertile. There were no differences in transplant characteristics, renal allograft function, and glucose homeostasis. Furthermore, we found that total BCAAs were positively correlated with protein intake (r = 0.25, p = <0.001) and HbA1c (r = 0.12, p = 0.02), but not with total energy intake (r = –0.01, p = 0.82) and physical activity (r = 0.10, p = 0.06). When we divided the non-diabetic renal transplant recipients in patients with prediabetes (HbA1c ≥ 5.7%) and without prediabetes (HbA1c < 5.7%), we found a positive correlation in the prediabetic renal transplant recipients (r = 0.23, p = 0.002), but not in renal transplant recipients without prediabetes (r = 0.003, p = 0.96).

Table 2. Baseline characteristics of non-diabetic renal transplant recipients according to tertiles of total BCAA (n = 386).

	Tertile 1 (n = 127)	Tertile 2 (n = 130)	Tertile 3 (n = 129)	P-Value
General characteristics				
Age, years	49.9 ± 13.3	52.8 ± 14.4	50.6 ± 12.9	0.19
Male sex, n (%)	81 (63.8)	73 (56.2)	88 (68.2)	<0.001
Race (white), n (%)	126 (99.2)	129 (99.2)	128 (99.2)	1.00
BMI, kg/m^2	25.4 ± 5.2	26.0 ± 4.2	26.3 ± 3.9	0.27
Physical activity score (time x intensity)	4930 (2100–7260)	5905 (3315–8625)	6100 (3120–9860)	0.05
Smoking status, n (%)				0.10
Never	54 (42.5)	58 (44.6)	42 (32.6)	
Former	47 (37.0)	51 (39.2)	67 (51.9)	
Current	21 (16.5)	17 (13.1)	15 (11.6)	
Alcohol consumption, g/d	1.6 (0.0–8.9)	2.9 (0.1–11.3)	4.3 (0.1–15.8)	0.03
Total energy intake, kcal/d	2178 ± 631	2184 ± 724	2096 ± 578	0.51
Urea excretion, mmol/24h	335.1 ± 92.5	406.6 ± 117.2	423.2 ± 114.2	<0.001
Circulation				
Heart rate, b.p.m.	70.3 ± 12.3	66.0 ± 12.5	65.8 ± 11.0	0.005
SBP, mmHg	134.9 ± 17.8	133.9 ± 18.2	136.4 ± 16.1	0.51
DBP, mmHg	81.7 ± 11.8	80.4 ± 11.4	84.1 ± 10.4	0.03
Transplant characteristics				
Transplant vintage, years	5.0 (1.8–14.9)	4.7 (1.7–12.0)	4.9 (1.3–10.8)	0.37
Living donor, n (%)	56 (44.1)	39 (30.0)	50 (38.8)	0.06
Pre-emptive transplant, n (%)	33 (26.0)	22 (16.9)	22 (17.1)	0.12
Dialysis duration, months	34.5 (11.0–63.0)	47.0 (14.0–60.5)	37.0 (22.5–58.5)	0.81
Age donor, years	42.7 ± 15.5	44.7 ± 15.6	45.5 ± 14.4	0.33
Renal allograft function				
Serum creatinine, µmol/L	124.0 (98.0–175.0)	123.0 (99.8–154.5)	134.0 (104.0–180.5)	0.23
eGFR, ml/min per 1.73 m^2	50.1 ± 21.1	52.8 ± 19.3	50.1 ± 18.2	0.44
Proteinuria, n (%)	28 (22.0)	28 (21.5)	26 (20.2)	0.92
Glucose homeostasis				
Plasma glucose (mmol/L)	5.1 (4.6–5.5)	5.1 (4.7–5.4)	5.0 (4.7–5.6)	1.00
HbA1c (%)	5.6 ± 0.3	5.6 ± 0.4	5.7 ± 0.4	0.10
Lipids and lipoproteins				

Table 2. *Cont.*

	Tertile 1 (n = 127)	Tertile 2 (n = 130)	Tertile 3 (n = 129)	P-Value
Total cholesterol, mmol/L	5.2 ± 1.0	5.1 ± 1.1	5.2 ± 1.3	0.62
HDL-cholesterol, mmol/L	1.5 ± 0.5	1.4 ± 0.4	1.3 ± 0.4	<0.001
LDL-cholesterol, mmol/L	3.0 ± 0.8	3.0 ± 1.0	3.1 ± 1.0	0.43
Triglycerides, mmol/L	1.6 (1.2–2.1)	1.6 (1.2–2.2)	1.7 (1.3–2.4)	0.19
Medication				
Calcineurin inhibitor, n (%)				0.57
Cyclosporine	53 (41.7)	54 (41.5)	50 (38.8)	
Tacrolimus	19 (15.0)	19 (14.6)	28 (21.7)	
Trough level cyclosporine (µg/L)	112.0 (78.3–143.3)	102.0 (74.8–141.5)	105.0 (74.5–156.5)	0.85
Trough level tacrolimus (µg/L)	5.5 (3.9–8.0)	7.7 (6.0–9.7)	7.4 (6.0–9.6)	0.08
Proliferation inhibitor, n (%)				0.04
Azathioprine	34 (26.8)	25 (19.2)	19 (14.7)	
Mycofenol	71 (55.9)	84 (64.6)	96 (74.4)	
Prednisolone, n (%)	127 (100.0)	128 (98.5)	127 (98.4)	0.37
Prednisolone dose, mg/24h	10.0 (7.5–10.0)	10.0 (7.5–10.0)	10.0 (7.5–10.0)	0.19
Antihypertensive drugs, n (%)	107 (84.3)	117 (90.0)	117 (90.7)	0.21
Statins, n (%)	53 (41.7)	66 (50.8)	67 (51.9)	0.20
Amino acids				
Total BCAA, µM	297.1 ± 33.7	366.6 ± 20.4	467.9 ± 64.7	<0.001
Valine, µM	159.1 ± 20.7	194.5 ± 18.4	240.5 ± 35.3	<0.001
Leucine, µM	107.6 ± 21.5	133.3 ± 17.3	168.2 ± 29.3	<0.001
Isoleucine, µM	31.6 ± 10.0	39.1 ± 11.2	59.1 ± 18.0	<0.001

Data are represented as mean ± SD, median (interquartile range) or n (%). Differences were tested by analysis of variance or Kruskal–Wallis for continuous variables and with χ^2-test for categorical variables. SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; LP-IR, lipoprotein insulin resistance index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BCAA, branched-chain amino acids.

3.3. BCAAs and Risk of Developing PTDM

In the subgroup of non-diabetic renal transplant recipients at baseline (n = 386) during a median follow-up of 5.3 (IQR, 4.2–6.0) y, 38 (9.8%) subjects developed PTDM. Of the renal transplant recipients in the highest tertile of total BCAA 17.3% developed PTDM versus 8.0% in the lowest two tertiles (p = 0.02). The Kaplan–Meier curves for the development of PTDM according to the highest tertile versus the two lowest tertiles of total BCAA is shown in Figure 1.

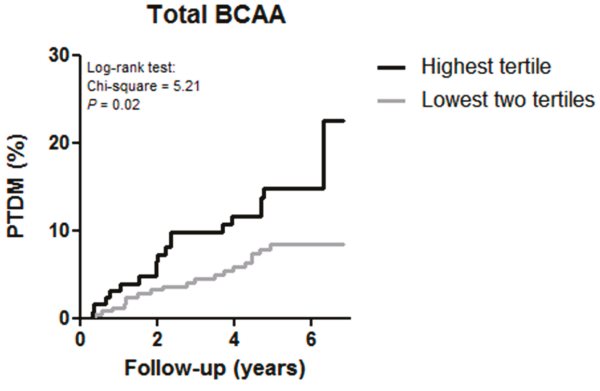

Figure 1. Kaplan–Meier curves for the development of post-transplant diabetes mellitus (PTDM) according to the highest tertile versus the two lowest tertile of total branched chain amino acids (BCAA) in renal transplant recipients.

Cox regression analyses with total BCAA per standard deviation (SD) as continuous variable showed that higher total BCAA was associated with a higher risk of developing PTDM (HR: 1.43, 95% CI 1.08–1.89, p = 0.01), independent of age and sex (Table 3, model 1). After adjustment for other potential confounders, including renal function parameters, lipids, dietary and lifestyle factors, and use of medication the association did not materially change (Table 3, model 2–6). In additional Cox regression analyses with total BCAA divided in the highest tertile versus the two lower tertiles, total BCAA was again significantly associated with development of PTDM, independent of age and sex (HR: 2.07; 95% CI 1.07–3.99, p = 0.03). Further adjustment for potential confounders did not change the association (Table 3, model 2–6). To illustrate the association of total BCAA with development of PTDM, an age and sex adjusted penalized spline is shown in Figure 2. We found no significant effect modification by age ($p_{interaction}$ = 0.75), gender ($p_{interaction}$ = 0.17), BMI ($p_{interaction}$ = 0.31), and eGFR ($p_{interaction}$ = 0.50) in the association of total BCAA per SD with PTDM, but we did for HbA1c ($p_{interaction}$ = 0.02). We continued with stratified analyses (Supplementary Figure S2). BCAAs were associated with PTDM in renal transplant recipients without prediabetes (HbA1c < 5.7%), but not in renal transplant recipients with prediabetes (HbA1c ≥ 5.7%), independent of age, sex, eGFR, proteinuria, and time since transplantation.

Table 3. Association of plasma BCAAs with post-transplant diabetes mellitus in renal transplant recipients (n = 386).

	Per SD as Continuous Variable (μmol/L)		Highest Tertile vs. Lower Two Tertiles		
BCAA					
No. of events	38		19	19	
	HR (95% CI)	*P*	Reference	HR (95% CI)	*P*
Crude	1.43 (1.09–1.88)	0.009	1.00	2.06 (1.09–3.90)	0.03
Model 1	1.43 (1.08–1.89)	0.01	1.00	2.07 (1.07–3.99)	0.03
Model 2	1.43 (1.07–1.90)	0.02	1.00	1.97 (1.02–3.82)	0.05
Model 3	1.37 (1.03–1.84)	0.03	1.00	1.82 (0.93–3.57)	0.08
Model 4	1.42 (1.06–1.90)	0.02	1.00	1.90 (0.95–3.80)	0.07
Model 5	1.47 (1.10–1.96)	0.009	1.00	2.09 (1.05–4.17)	0.04
Model 6	1.42 (1.08–1.85)	0.01	1.00	2.12 (1.09–4.12)	0.03

Cox proportional hazards regression analyses were performed to assess the association of BCAAs with PTDM. Model 1: adjustment for age and sex; model 2: model 1 + adjustment for eGFR, proteinuria, and time since transplantation; model 3: model 2 + adjustment for total cholesterol and triglycerides; model 4: model 2 + adjustment for total energy intake, physical activity, and BMI; model 5: model 2 + adjustment for smoking status and alcohol intake; model 6: model 2 + adjustment for prednisolone dose and trough levels of tacrolimus and cyclosporine;. BCAA, branched chain amino acids; PTDM, post-transplant diabetes mellitus; eGFR, estimated glomerular filtration rate.

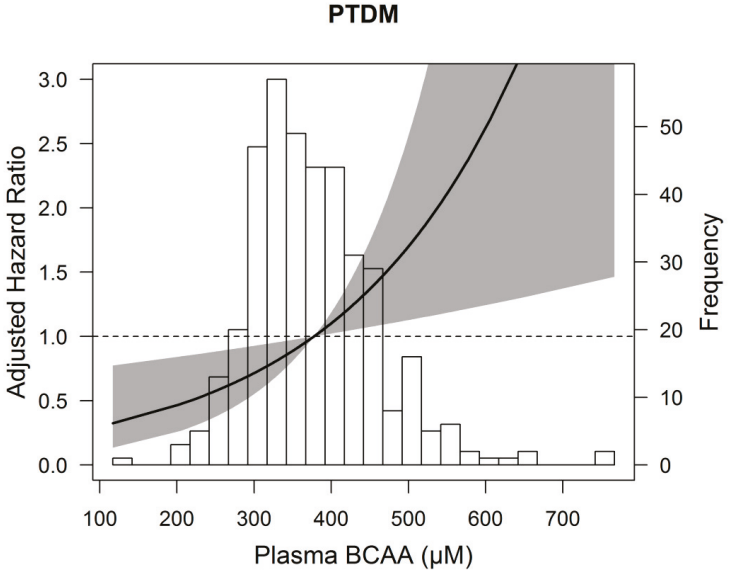

Figure 2. Association between plasma branched chain amino acids (BCAA) and post-transplant diabetes mellitus (PTDM) in 386 renal transplant recipients. Data were fit by a Cox regression model based on penalized splines and adjusted for age and sex. The gray area represents the 95% confidence interval.

3.4. Secondary Analyses

In mediation analyses, we found that HbA1c mediated 53% of the association between BCAAs and PTDM in renal transplant recipients, whereas plasma glucose was not a significant mediator in this association (Supplementary Table S1), after adjustment for age and sex. The results of competing risk analyses did not materially differ from those with Cox regression for the association of total BCAA per SD as continuous variable and development of PTDM (HR: 1.44, 95% CI 1.08–1.92, p = 0.01), adjusted for age and sex (Table 3, model 2 for comparison). Also, the analysis with total BCAA divided

in the highest tertile versus the two lower tertiles did not differ in the competing risk analysis (HR: 2.09, 95% CI 1.10–3.96, $p = 0.02$), adjusted for age and sex (Table 3, model 2 for comparison).

3.5. BCAAs and Risk of All-Cause Mortality and Death-Censored Graft Failure

In the total population of both diabetic and non-diabeticrenal transplant recipients at baseline ($n = 518$), 114 (22.0%) subjects died during a median follow-up of 5.4 (IQR, 4.7–6.2) y, whereas 65 (12.5%) subjects developed graft failure during a median follow-up of 5.3 (IQR, 4.5–6.0) y. There was no significant association between total BCAA and the individual BCAAs with all-cause mortality and death-censored graft failure (Supplementary Table S2). We found no effect modification by diabetes for the association of total BCAA with all-cause mortality ($p_{interaction} = 0.22$) and death-censored graft failure ($p_{interaction} = 0.41$).

4. Discussion

In this large cross-sectional study with prospective follow-up, higher concentrations of total BCAAs are associated with a higher risk of developing PTDM in renal transplant recipients. This association did not change after adjustment for relevant confounders, including age, sex, renal function parameters, lipids, dietary and lifestyle factors, and use of immunosuppressive medication. Subsequently, this association was modified by HbA1c; total BCAAs were significantly associated with PTDM in renal transplant recipients without prediabetes (HbA1c < 5.7%), but not in renal transplant recipients with prediabetes (HbA1c ≥ 5.7%). Furthermore, we show that the association between total BCAA and PTDM was mediated to a considerable extent (53%) by HbA1c. In addition, no association of total BCAAs with all-cause mortality and death-censored graft failure in renal transplant recipients was found.

It is known that BCAAs are elevated in subjects with prediabetes, type 2 diabetes, and obesity [22]. In this cohort, BCAA concentrations were elevated in diabetic renal transplant recipients when compared with non-diabetic renal transplant recipients (424.6 ± 97.9 μM vs. 377.6 ± 82.5 μM, respectively), as observed in previous studies in the general population [23,55,56]. The BCAA concentrations of the diabetic renal transplant recipients can be compared to the BCAA concentrations of 439 ± 95 μM in patients with type 2 diabetes mellitus in the general population [25]. The BCAA concentrations of the non-diabetic renal transplant recipients are comparable to the mean plasma levels of 370.3 ± 88.6 μM in a large prospective population-based cohort study [26].

BCAAs are a group of essential amino acids, comprising valine, leucine, and isoleucine, and can only be obtained from diet. They comprise about 15–25% of total protein intake [17]. Previous studies have shown that plasma BCAA levels are modifiable by a higher or lower consumption of protein. Prior work showed that higher consumption of BCAAs is significantly associated with higher plasma levels of BCAAs [57]. The correlation was moderate, but comparable to other diet-plasma biomarker correlations. It has been shown that dietary protein reduction lowers serum levels of BCAAs [58]. Recently, a randomized controlled crossover trial even showed that short term dietary reduction of BCAAs decreases postprandial insulin secretion [59]. It is known, that around 80% of dietary BCAAs reach the blood circulation [60], but circulating plasma levels of BCAAs can also be affected by their catabolism [61]. The initial site of the BCAA metabolism is skeletal muscle, because of the high branched-chain-amino-acid aminotransferase (BCAT) activity in the muscle [62]. This metabolism is sensitive to changes in the amount and composition of food. A high protein diet leads to higher concentrations of BCAAs, whereas a low protein diet lowers the plasma BCAA concentrations [62,63]. Indeed, in our study, subjects in the highest tertile of total BCAA had a higher 24 h urinary urea excretion, which is an objective measurement for total protein intake, when compared to subjects in the lowest tertile of total BCAA.

The results of the prospective analysis with PTDM are consistent with previous studies that reported the association of BCAAs with type 2 diabetes in the general population [21,22,64]. Recently, we showed in a prospective cohort study that high concentrations of BCAAs are associated with

increased risk of developing type 2 diabetes [26]. The fact that total BCAAs were significantly associated with PTDM in subjects without prediabetes, but not in prediabetic subjects suggest that alterations in total BCAAs might be an early signal of deterioration of glycemic control. A previous study has shown that elevated BCAAs levels may appear long before other markers of insulin resistance become abnormal [21]. Elevations in circulating BCAAs can occur before any alterations in insulin action are detectable. Moreover, it has been reported that plasma BCAAs might serve as a better indicator of impaired insulin resistance when compared to plasma glucose levels [65], since in patients without prediabetes the metabolic status is not deteriorated enough to alter plasma glucose levels.

The secondary outcomes, all-cause mortality and death-censored graft failure, were not associated with total BCAAs. This is in contrast to a previous study that showed an inverse association of total BCAAs and death in patients at risk for coronary artery disease [33], supporting the underlying malnutrition-inflammation syndrome hypothesis. Furthermore, the large Estonian biobank study also observed inverse associations between BCAAs and all-cause mortality [34]. Moreover, in the ADVANCE study including individuals with type 2 diabetes, low levels of leucine and valine were associated with increased all-cause mortality [35]. In our study 114/518 (22.0%) renal transplant recipients died during a median follow-up of 5.4 years, resulting in a death rate of 4.1% per year, which is slightly higher when compared to the FAVORIT trial, a large multi-center double-blind randomized controlled trial in 4110 stable renal transplant recipients (age 52 ± 9.4 years, 62.8% male at 5 years after transplantation) in which 493/4110 (12.0%) subjects died within 4.0 years of follow-up, resulting in a death rate of 3.0% per year [66]. Prior work showed that levels of valine and leucine, but not isoleucine, were significantly lower in patients with stage I and II chronic kidney disease, when compared with controls [37], suggesting potential use as a biomarker for renal dysfunction.

Currently, there are several potential mechanisms that could explain the contribution of BCAAs to the development of insulin resistance, type 2 diabetes, and PTDM, although these mechanisms are not completely understood. One mechanism proposes that BCAAs interfere with insulin signaling through activation of the mammalian target of rapamycin complex 1 (mTORC1) in skeletal muscle and serine phosphorylation of insulin receptor substrate 1 and 2, which promotes insulin resistance and can lead to the development of type 2 diabetes [20]. However, conflicting results regarding the role of BCAAs to elicit insulin resistance have been reported [67] and do question whether mTORC1 activation is sufficient or necessary in the development of insulin resistance. Others assume that BCAA dysmetabolism, especially in obesity, contributes to a rise in BCAAs, which results in accumulation of potential toxic BCAA metabolites, which could induce cellular damage [20]. These BCAA metabolites might lead to mitochondrial dysfunction and β-cell apoptosis, which is common in insulin resistance and type 2 diabetes [20]. Nevertheless, the association of total BCAAs and PTDM in our study was independent of BMI. Moreover, a previous study in the general population showed that the association of BCAAs with insulin resistance was independent of leptin and adiponectin, both valid biomarkers of adipose tissue dysfunction, when taking BMI into account [25], suggesting the association is presumably mainly driven by another mechanism.

To the best of our knowledge, this is the first study that studied the association of BCAAs with the development of PTDM in renal transplant recipients. Strengths of this study include the complete follow-up and use of clinical endpoints (PTDM, all-cause mortality, and death-censored graft failure), which are relevant in daily clinical practice. Furthermore, this study had a considerable follow-up period. Another strength is the use of stable patients who had a functioning graft for at least 1 year, which resulted in exclusion of patients with transient hyperglycemia post-transplantation, which occurs frequently and is evident in about 90% of renal transplant recipients in the first few weeks post-transplantation [68,69]. Hyperglycemia can also occur as a result of rejection therapy, infections, and other critical conditions [46]. Therefore, it is important to diagnose PTDM in stable patients (i.e., on maintenance immunosuppression, stable renal function and in absence of acute infections) [46]. This study also has several limitations. First, it is a single-center study, with a study population mainly consisting of Caucasians. As ethnicity is an independent risk factor for developing PTDM [5],

it is important to repeat this study in more diverse populations. Renal transplant recipients in the current study were included at a median of 5.0 years after transplantation. Therefore, extrapolating our results to patients in early stages after renal transplantation should be done with caution. The clinical significance or impact of the diagnosis of PTDM early or late after transplantation has yet to be determined [16]. In addition, age at time of transplantation in our cohort is lower when compared to other European cohorts [70]. Moreover, the prevalence of living donor grafts is higher in the Netherlands [71], which might also contribute to a lower age in our cohort, since younger subjects have a broader social network and therefore likely a higher chance of finding a compatible living donor at younger age. Furthermore, it is known that oral glucose tolerance tests (OGTTs) are the gold standard diagnostic tool to diagnose PTDM. Unfortunately, OGTTs were not performed, but recently it has been shown that the combined use of fasting plasma glucose and HbA1c criteria appears to be a diagnostic strategy for PTDM in stable renal transplant recipients [72]. Another limitation of this study is that only 38 subjects developed PTDM during follow-up, which led to a lack of power. Unfortunately, a comorbidity index was not available in our cohort and we do not have data on weight gain post-transplantation. It cannot be excluded that change of weight could serve as a source of bias and could spuriously strengthen or weaken the association of BCAAs with development of PTDM. Finally, longer-term intervention studies are required to determine whether BCAAs are causally related to the development of diabetes mellitus or merely act as markers of underlying pathophysiology.

5. Conclusions

In conclusion, this single-center cross-sectional assessment of BCAA in stable renal transplant recipients showed that high plasma concentrations of total BCAA are associated with a higher risk of developing PTDM during prospective follow-up. Alterations in BCAA levels might be an early signal of deterioration of glycemic control in renal transplant recipients. Further research is needed to investigate the possible mechanism/role of BCAAs in the development of post-transplant diabetes.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/2/511/s1, Figure S1: Flowchart of the study, Figure S2: Stratified analyses of the association of branched chain amino acids (BCAA) and post-transplant diabetes mellitus (PTDM) in both patients with normal glucose tolerance (HbA1c < 5.7%) and prediabetes (HbA1c ≥ 5.7%) adjusted for age, sex, eGFR, proteinuria, and time since transplantation, Figure S3: Mediation analysis on the association of branched chain amino acids (BCAA) on post-transplant diabetes mellitus (PTDM), Table S1: Mediating effect of HbA1c on the association of BCAA with PTDM, Table S2: Association of BCAAs with all-cause mortality and death-censored graft failure in renal transplant recipients (n = 518).

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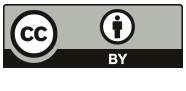

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The Effect of Proton Pump Inhibitor Use on Renal Function in Kidney Transplanted Patients

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Abstract: Recently, proton pump inhibitor (PPI) intake has been linked to acute kidney injury and chronic kidney disease. The objective of this study was to assess the effect of PPIs on renal function and rejection rate in kidney transplant patients. We performed a single center, retrospective analysis of 455 patients who received a kidney transplant between May 2010 and July 2015. Median follow-up time was 3.3 years. PPI prescription was assessed in half-year intervals. Primary outcome parameters were the estimated glomerular filtration rate (eGFR), change in the eGFR, and >30% and >50% eGFR decline for different time periods (up to four years post-transplantation). Our secondary outcome parameter was occurrence of biopsy proven acute rejection (BPAR) in the first two years after transplantation. Except for >30% eGFR decline from half a year to two years post-transplantation ($p = 0.044$) and change in the eGFR, >30% and >50% eGFR decline showed no association with PPI intake in our patient cohort ($p > 0.05$). Similarly, by analyzing 158 rejection episodes, BPAR showed no correspondence with mean daily PPI intake. We conclude that prolonged PPI intake has no relevant adverse effect on kidney transplant function or rejection rates. Polypharmacy, however, remains a problem in renal transplant recipients and it is thus advisable to question the necessity of PPI prescriptions when clear indications are missing.

Keywords: proton pump inhibitor; kidney transplantation; transplant rejection; GFR

1. Introduction

With only a single transplanted kidney and oftentimes reduced renal filtration rates, kidney transplant (KTx) recipients are particularly vulnerable to the nephrotoxic adverse effects of drugs. Care is taken to avoid such drugs that could further impair kidney function. For this reason, recent epidemiological studies that have observed a relationship between acute kidney injury (AKI), chronic kidney disease (CKD) and proton pump inhibitor (PPI) intake have been of special interest for practitioners involved in the care of KTx patients [1–5].

Furthermore, in the setting of KTx, two medication interactions of possible relevance are the interaction between PPIs and mycophenolate mofetil (MMF) and between PPIs and tacrolimus. The first may lead to decreased blood levels of the active metabolites of MMF [6–12], which may result in increased rejection rates [13–16]. Tacrolimus is known to be nephrotoxic and it is thought that interactions with PPIs may change its uptake and/or metabolism [17–19], potentially increasing tacrolimus blood concentration. This could be detrimental to kidney transplant function.

In the face of surgical stress and long-term polypharmacy, upper gastrointestinal symptoms are frequent in KTx patients [20]. Even in those patients receiving acid suppressive therapy, the risk of ulcer disease is still elevated [20]. Gastrointestinal complications have also been associated with

decreased graft survival [21]. As PPIs are a very effective form of acid suppression, they are frequently given as prophylaxes among transplant recipients [20,22,23]. In our center, they are the standard of care for KTx patients.

In light of the mentioned studies and the possibility of adverse drug interactions between PPIs and mycophenolate mofetil or tacrolimus, we carried out this study. Previous PPI studies in KTx patients have focused on the possible interaction of MMF with PPIs [22,24,25] or the occurrence of other PPI intake related adverse events [23]. We retrospectively evaluated if a relationship between PPI intake and renal function could be found. We analyzed follow-up data of up to four years after transplantation. To our knowledge, no study exists to date that has specifically analyzed the changes in renal function after KTx with regard to PPI intake over a comparable time frame. Additionally, we compared rejection rates, as these may be of relevance regarding MMF and PPI interactions.

2. Patients and Methods

2.1. Patients

Prior to analysis, the data of all patients was anonymized. The local ethics committee (Ethik Kommission der Ärztekammer Westfalen-Lippe und der Medizinischen Fakultät der Westfälischen Wilhelms-Universität, No. 2014-381-f-N) approved the study. Methods in this study were carried out in accordance with the current transplantation guidelines and the Declarations of Istanbul and Helsinki. Written informed consent was given by all participants at the time of transplantation for recording their clinical data.

We herein performed an explorative, retrospective, single-center cohort study. We enrolled all patients receiving a KTx between May 2010 and July 2015 at the University Hospital Muenster. Inclusion criteria were patient age ≥18 years and PPI therapy at primary hospital discharge post-transplantation (pTx). Patients receiving multiple organ transplants remained included. The recipient and donor data was collected from the patients' electronic files. The following data was collected and used: recipient and donor age and sex; recipient body mass index (BMI); prior renal transplants; transplant under European Senior Program; donor type (living or deceased); delayed graft function (DGF; dialysis within the first week pTx); cold ischemia time; pre-transplant time dialyzed; pre-transplant arterial hypertension; pre-transplant diabetes; presence of peripheral arterial occlusive disease; cerebral arterial occlusive disease or stroke; coronary heart disease or myocardial infarction; anticoagulant prescription; antiplatelet drug prescription (including acetylsalicylic acid (ASA)); statin prescription; MMF dosage at primary discharge and two years pTx; cortisone intake at primary discharge and one year pTx; tacrolimus dose and blood level three months pTx; prior smoking history; continuation of smoking after KTx; number of human leukocyte antigen (HLA) mismatches; ABO incompatibility of transplant; induction therapy; pre-transplant donor specific antibody (DSA) occurrence; panel reactive antibodies; transplant rejection occurrence and type according to Banff criteria; eGFR from half a year to four years pTx; and data on PPI prescription. At primary discharge, none of the patients had any non-steroidal anti-inflammatory drug prescriptions, except for ASA. To improve adjustment for confounding through comorbidities, additional data was collected to calculate the Charlson comorbidity index [26] at transplantation [27].

The induction therapy was chosen according to the immunological risks of the patients. One gram of mycophenolate mofetil was given twice a day; the dosage was reduced in case of adverse events. Prednisolone was started at 500 mg intravenously (i.v.) before KTx, followed by 100 mg for three days; then reduced by 20 mg/day. A dosage of 20 mg/day was maintained until day 30 and then slowly reduced to 5 mg/day. Immunosuppressive maintenance therapy usually consisted of a calcineurin inhibitor (tacrolimus or cyclosporine A), mycophenolate sodium or mycophenolate mofetil and prednisolone.

2.2. Proton Pump Inhibitors—Data Collection

Data on the prescription of PPIs (agent and dose prescribed) were collected in half year intervals for all patients starting at primary discharge. PPI intake was assumed according to this prescription until the next interval. If data was not present for a certain interval, the prescription from the preceding interval was assumed.

Due to the favorable drug interaction profile of pantoprazole in comparison to other PPIs [28], pantoprazole is used as the PPI of choice at our center. Therefore, it has also been used as the standard PPI in this study. In order to also use data from different PPIs, equivalent doses were calculated for the two other agents (omeprazole and esomeprazole) used. We used the ratio: 40 mg pantoprazole ≙ 20 mg omeprazole ≙ 20 mg esomeprazole [29,30].

At our center, patients are instructed to ingest tacrolimus and MMF on an empty stomach one hour before intake of PPIs. Both tacrolimus and MMF are usually taken twice daily, while PPIs are mostly taken once per day.

2.3. Group Formation

Based on PPI intake (n = 363) and non-intake (n = 82) at half a year pTx, two patient groups were formed. These were used for a direct comparison of GFR and change thereof.

For the outcome measures >30% and >50% eGFR decline and the number of rejections, the groups 0 mg, 1–20 mg, 21–40 mg and >40 mg mean daily PPI intake were compared. The standard dose at our center is 40 mg pantoprazole, 20 mg is the common reduced dose, and above >40 mg (often 80 mg) is an elevated dose (rationale for the group formation).

2.4. Outcome Measures

Primary outcome measures were: the eGFR (at six months, one year, two years, three years and four years), change in the eGFR (from six months pTx to one year, two years, three years and four years), eGFR decline >30% and eGFR decline >50% (from six months to two years and two years to four years). All eGFR-values were calculated using the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation [31].

Our secondary outcome was biopsy proven acute rejection (BPAR) in months one to six, seven to twelve and in the second year pTx. For each time period, every patient with a rejection was counted (not only a patient's first rejection). The usual indication for biopsy in our center is a rise in creatinine with no apparent cause.

2.5. Statistical Analysis

Statistical analysis was performed using IBM SPSS® Statistics 24 for Windows (IBM Corporation, Somers, NY, USA). Microsoft Excel was used for data collection, simple calculations, and graphing.

This is an explorative study and no adjustment was made for multiple testing. p-values ≤ 0.05 were regarded as statistically noticeable. Normally distributed continuous variables are displayed as mean ± standard deviation (SD), non-normally distributed as median and interquartile range (IQR), and categorial variables as frequencies and percentage of total. Pairwise comparisons of independent samples were performed using student's t-test or Welch's unequal variance t-test for normally distributed data and the Mann–Whitney U test was used for non-normally distributed data. For categorial variables, groups were compared using Fisher's exact test.

For the group comparison of the eGFR and change in the eGFR, the Mann–Whitney U test and multivariable linear regression were performed. Further information on model building and the included variables is found in Supplementary Materials 2A.

A possible relationship between >30% and >50% eGFR decline endpoints and mean daily PPI intake was investigated using Fischer's exact test and multivariable logistic regression analysis. Mean daily PPI intake was calculated by averaging the prescribed PPI dose at half year intervals up to half a

year before the final relevant eGFR value. Information on model building and the herein included variables can be found in Supplementary Materials 2B.

Correlation of BPAR in months one to six, seven to twelve and in the second year pTx with mean daily PPI intake, respectively, was investigated univariably using Fischer's exact test and multivariably using logistic regression analysis with forward selection of confounders (same procedure as for >30% and >50% eGFR decline endpoints). A list of the variables included and further information on the testing can be found in Supplementary Materials 2C.

3. Results

3.1. Patients

A total of 511 patients were transplanted in the study period. The following exclusions were made: 37 patients as they were <18 years of age, 13 as they did not receive PPIs at primary discharge, five due to non-onset of graft function after transplantation and one due to death before primary discharge. A final number of 455 patients were included; the median follow-up time was 3.3 years (IQR, 2.2–4.9). In our cohort, 12 patients died and 10 experienced graft loss within the first year; seven died and eight graft losses occurred within the second year pTx. Most patients (96%) initially received pantoprazole as their PPI agent. Patient and donor characteristics are displayed in Table 1 (and Supplementary Materials 1).

Table 1. Patient characteristics at primary hospital discharge post-transplantation (additional information included in Supplementary Materials 1).

Patient Characteristic	All Patients (n = 455)	PPI Group (n = 363)	No PPI Group (n = 82)	p-Value of Group Comparison
Recipient age, mean ± SD (years)	52.6 ± 14.2	53.1 ± 13.9	49.3 ± 14.5	0.026
Recipient male gender, n (%)	279 (61.3)	219 (60.3)	52 (63.4)	0.707
Recipient BMI, mean ± SD (kg/m^2)	25.9 ± 4.4	26.0 ± 4.3	24.9 ± 4.5	0.053
Prior renal transplantation, n (%)	64 (14.1)	45 (12.4)	16 (19.5)	0.109
Age of donor, mean ± SD (years)	53.1 ± 14.0	53.3 ± 14.4	51.4 ± 11.9	0.204
Living donor, n (%)	153 (33.6)	112 (30.9)	41 (50.0)	0.001
Male donor, n (%)	208 (45.7)	170 (46.8)	33 (40.2)	0.326
Delayed graft function, n (%)	79 (17.4)	59 (16.3)	11 (13.4)	0.616
European Senior Program, n (%)	76 (16.7)	62 (17.1)	10 (12.2)	0.322
Cold ischemia time (hours), median (IQR)	7.8 (2.5–11.6)	7.8 (2.7–11.7)	5.2 (2.3–11.1)	0.053
Pre-Tx time dialyzed (months), median (IQR)	45.3 (21.0–86.0)	48.2 (23.2–88.5)	32.4 (8.6–67.2)	0.002
Tacrolimus therapy at primary discharge, n (%)	432 (94.9)	347 (95.6)	76 (92.7)	0.265
Cyclosporin therapy at primary discharge, n (%)	23 (5.1)	16 (4.4)	6 (7.3)	0.265
MPS therapy at primary discharge, n (%)	76 (16.7)	57 (15.7)	18 (22.0)	0.191
MMF therapy at primary discharge, n (%)	341 (74.9)	278 (76.6)	57 (69.5)	0.200
MMF mean daily dosage (mg), median (IQR)	1000 (500–1000)	1000 (500–1000)	1000 (0–1063)	0.851
Cortisone intake at primary discharge, n (%)	444 (97.6)	353 (97.2)	81 (98.8)	0.698
CCI, median (IQR)	2 (2–4)	3 (2–4)	2 (2–3)	<0.001
HLA mismatch on A, B and DR, mean ± SD	2.9 ± 1.7	2.9 ± 1.7	2.9 ± 1.7	0.875
Basiliximab induction, n (%)	363 (79.8)	293 (80.7)	61 (74.4)	0.272
ATG induction, n (%)	14 (3.1)	13 (3.6)	1 (1.2)	0.482
ABO blood type incompatible transplant, n (%)	37 (8.1)	26 (7.2)	11 (13.4)	0.077
PRA >20%, n (%)	60 (13.2)	48 (13.2)	10 (12.2)	1.000

The two compared groups were formed based on PPI (proton pump inhibitor) intake (PPI Group) or non-intake (No PPI Group) at half a year post-transplantation. Results are presented as mean ± standard deviation (SD), median and interquartile range (IQR) or as absolute and relative frequencies. Abbreviations: BMI, body mass index; Tx, transplantation; MPS, enteric-coated mycophenolate sodium; MMF, mycophenolate mofetil; CCI, Charlson comorbidity index; HLA, human leukocyte antigen; ATG, Antithymocyte globulin; PRA, panel reactive antibodies. Along with HLA mismatch (n = 3) and Basiliximab induction (n = 7), four other variables have one patient with missing values.

Lost to follow-up rates in the PPI group and no PPI group were 16/363 (4.4%) and 2/82 (2.4%) at one year, 64/363 (17.6%) and 6/82 (7.3%) at two years, 152/363 (41.9%) and 28/82 (34.1%) at three years, 229/363 (63.1%) and 54/82 (65.9%) at four years, respectively.

3.2. Group Comparison

The two groups differed noticeably regarding recipient age, donor type (living or deceased), time dialyzed before transplantation, active smoking, smoking history, cerebral artery occlusive disease or stroke, coronary artery disease or myocardial infarction and Charlson index at the time of transplantation. These characteristics were favorable for the no PPI group. A noticeable difference was also found in the number of fast tacrolimus metabolizers three months pTx. (Table 1 and Supplementary Materials 1).

The PPI group showed significantly lower eGFR compared to the no PPI group at half a year, one year and two years pTx ($p < 0.05$) (multivariable). For the third and fourth year, the difference was not statistically significant (Supplementary Materials 3). The trend in the mean eGFR can be seen in Figure 1.

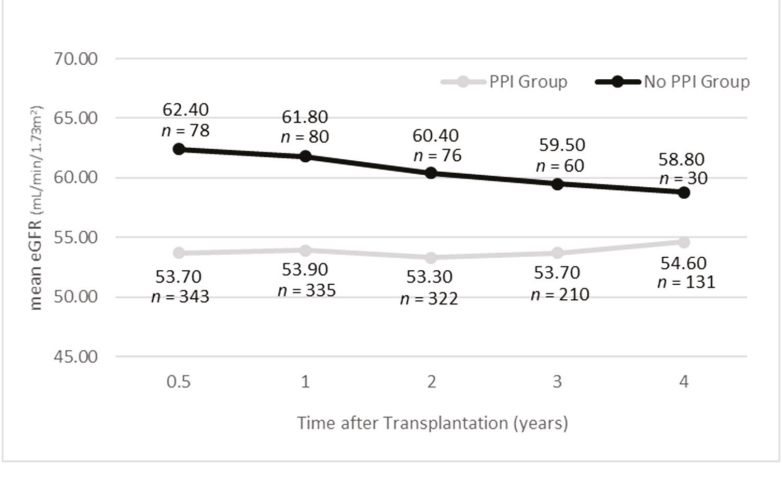


Figure 1. Trend in mean estimated glomerular filtration rate (eGFR). Mean eGFR is plotted against time in the two patient groups. Grouping is according to PPI intake or non-intake at half a year post-transplantation.

Regarding the change in the eGFR, both groups were similar over all time periods (Table 2).

Table 2. Results of the PPI group comparison of change in the eGFR.

Time Period of Analyzed eGFR Change	Groups 1 = PPI 0 = No PPI	*n*	Mean Change in the eGFR ± SD (mL/min/1.73 m²)	Median (IQR) Change in the eGFR	*p*-Value in Univariable Analysis	*p*-Value (CI) in Multivariable Linear Regression Model
0.5–1 year	1	323	−0.6 ± 12.0	1.0 (−6.0–6.5)	0.488	0.498 (−1.9–3.8)
	0	78	−0.5 ± 8.3	−0.5 (−5.6–4.4)		
0.5–2 years	1	310	−1.6 ± 14.2	0.2 (−9.0–7.5)	0.274	0.542 (−2.3–4.5)
	0	74	−2.4 ± 10.2	−1.5 (−9.2–5.2)		
0.5–3 years	1	202	−0.8 ± 15.1	0.35 (−8.0–8.4)	0.331	0.452 (−2.6–5.8)
	0	58	−2.5 ± 11.0	−1.3 (−7.0–5.4)		
0.5–4 years	1	125	−0.1 ± 14.5	−0.1 (−7.3–8.6)	0.101	0.228 (−2.2–9.1)
	0	28	−4.2 ± 9.1	−1.7 (−10.5–2.9)		

Results of the comparison of the change in the eGFR, values between groups. The eGFR value measured at half a year is always used as the reference value and was subtracted from that of the later date. Groups were formed based on PPI intake or non-intake at half a year pTx (post-transplantation). For the linear regression models, the patient number is slightly reduced (<3 patients difference per test) due to missing covariables in a few patients. Abbreviation: CI, confidence interval.

3.3. >30% and >50% eGFR Decline

Multivariable logistic regression was only carried out for the outcome >30% eGFR decline from half a year to two years pTx. In all others, only univariable analysis was performed (due to the low number of events). eGFR decline >30% from half a year to two years showed statistical correlation with higher PPI doses in multivariable logistic regression ($p = 0.044$). All other eGFR decline endpoints showed no relation to mean daily PPI intake. eGFR decline >50% from two to four years showed some tendency in the same direction ($p = 0.056$). However, here, only three events occurred. Tables with the results can be viewed in Supplementary Materials 4A–D.

3.4. Secondary Outcomes

BPAR occurred in 96 patients in months one to six, in 32 patients in months seven to twelve and in 36 patients in the second year pTx. For the rejection analysis, patients who did not complete a follow up of at least 5/6 of the analyzed time points were excluded from analysis. Those with death or transplant loss with prior rejection, however, were included. Twelve patients were excluded from multivariable analyses due to missing data. The results of the analysis are presented in Figure 2A–C. Regarding rejection types, mean daily PPI intake only showed a correlation with antibody mediated rejections (AMR) in the second year pTx in univariable analysis ($p = 0.027$); multivariable (logistic regression) analysis was not feasible in this case due to zero events in one group. All other tests did not show any association.

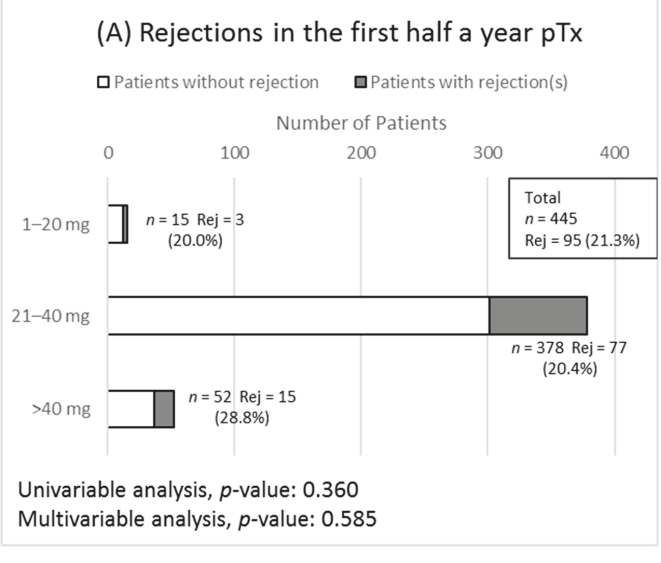
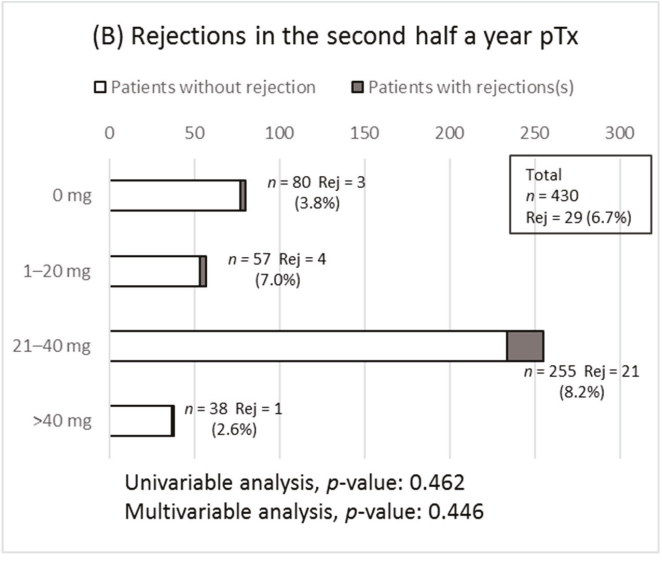


Figure 2. *Cont.*



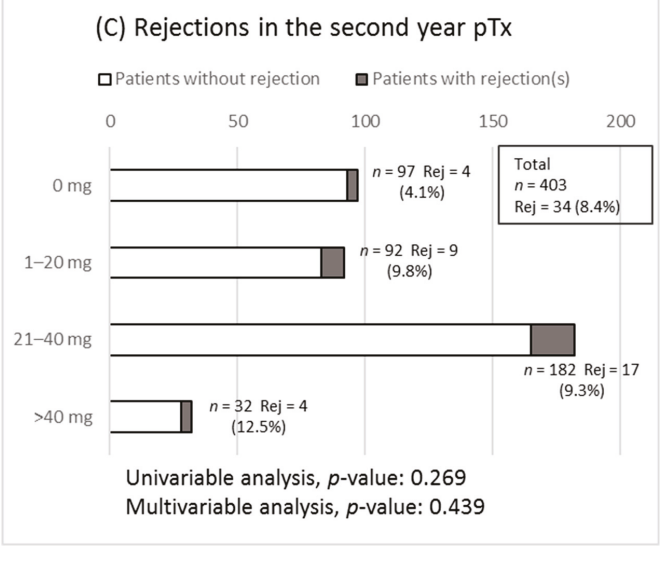


Figure 2. Results of the biopsy proven rejection analyses are shown. Grouping is according to PPI intake (pantoprazole equivalent) in the relevant time period. Rejection prevalence is shown as absolute number and as percentage of patients in the group. The dark part of each bar represents the number of patients with rejections. Abbreviations: *n*, total number of patients in the group; Rej, number of patients who experienced a graft rejection; pTx, post transplantation. (**A**) Analysis of rejections in months 1–6 pTx. (**B**) Analysis of rejections in months 7–12 pTx. (**C**) Analysis of rejections months 13–24 pTx.

As the possible drug interaction of MMF with PPIs was of special interest, all rejection analyses were repeated, analyzing the data of patients who had MMF at primary discharge only. None of these tests showed any significant association.

4. Discussion

Our results demonstrate that prolonged PPI intake after KTx does not lead to any meaningful decline in kidney function within the first four years after transplantation. Additionally, our analyses of rejection rates are in line with recent studies showing no relevant association between rejection rates and PPI-intake after KTx [22,24,25].

Several large epidemiological studies observed a relationship between AKI, CKD, and PPI intake [1–5]. From case studies, an association between PPI intake and interstitial nephritis was previously assumed [32]. Estimates of the impact of this finding have not been around as long [33–35]. However, the relevance and the stake of interstitial nephritis for AKI in PPI observational studies still remains unclear [35,36]. As AKI can lead to CKD, it was not surprising that an association between CKD and PPI intake was recently proposed [1]. Nevertheless, Xie and colleagues provided evidence that PPI-associated CKD even occurred in the absence of AKI [3]. The pathomechanism, however, remains unknown. Proposed mechanisms include elevation of plasma asymmetric dimethylarginine levels [37], microinflammation due to gut microbe dysbiosis [38], endothelial senescence [39] and PPI-induced hypomagnesemia [40]. In addition to hypomagnesemia, PPI intake was recently associated to be dose-dependently linked to iron deficiency and hypomagnesemia in a kidney transplant cohort from the Netherlands [41,42]. The authors speculated that the effects were associated with reduced intestinal absorption of both elements under PPI therapy. However, our center's policy is to monitor the iron status and to replace magnesium after KTx, because iron-deficiency is common and calcineurin inhibitors frequently lead to magnesium loss. Considering these findings, our results may relieve unwarranted fear when prescribing PPIs in KTx patients.

Following the Kidney Disease Improving Global Outcomes (KDIGO) guideline, a form of mycophenolate acid (MPA) together with the calcineurin inhibitor tacrolimus and low dose corticosteroid therapy is the preferred maintenance therapy after KTx at our center (Table 1 and Supplementary Materials 1) [43]. The tacrolimus target level in our center was 6–10 ng/mL from months one to three and 4–8 ng/mL for the following time.

Tacrolimus and PPI potentially interfere e.g., at the cytochrome P450 system (CYP3A) [17,18]. Another mechanism proposed is that PPI can increase the uptake of tacrolimus in the small intestine [19,44]. Both mechanisms could increase blood tacrolimus levels. Usually, these interactions are not clinically noticeable as several factors have a more profound effect on tacrolimus metabolism and exposure [45]. However, as tacrolimus can be nephrotoxic, slight increases in exposure may be relevant in the course of time [18,46]. Although analysis of tacrolimus blood levels was not the goal of this study, it is interesting to note that tacrolimus blood levels at three months pTx showed a tendency ($p = 0.07$) to be higher in the PPI group (Supplementary Materials 1). Furthermore, significantly more fast tacrolimus metabolizers were found in the no PPI group ($p = 0.035$). However, the previously mentioned Dutch KTx magnesium study found no relevant interaction between PPI and tacrolimus in their observational study [42]. Thus, these interesting findings may warrant further investigation.

It may also be worth mentioning that we did not investigate a possible effect of PPIs on the intrapatient variability of tacrolimus. It has been shown that the intrapatient variability of tacrolimus correlates with poor long-term outcomes in kidney transplant recipients [47]. It may be assumed that any drug with the possibility of interfering with tacrolimus pharmacokinetics may potentially change the intrapatient variability of tacrolimus [48]. To our knowledge, no study exists which has directly investigated a potential relationship between PPI intake and increased intrapatient variability in kidney transplant recipients. However, we suspect this effect to be minimal when adherence to our center's instruction of ingesting tacrolimus on an empty stomach is followed. As we did not see any differences regarding the eGFR changes between the groups, a clinically relevant effect of PPIs on transplant function does not seem to exist.

Mycophenolate mofetil is a prodrug that is hydrolyzed to the active metabolite MPA. It acts as a selective uncompetitive inhibitor of inosine monophosphate dehydrogenase (IMPDH) and thus inhibits de novo guanosine synthesis [6]. Pharmacokinetic studies have shown that PPIs may reduce

MPA exposure in patients receiving concomitant MMF and PPI treatment, whereas the alternative drug enteric-coated mycophenolate sodium is not affected [6–12]. Decreased MPA exposure can increase rejection rates [13–16]. However, in a recent pharmacokinetic blinded cross-over study [49], this mentioned interaction was not found. In line with this and with previously published observational studies, rejection rates in our cohort were comparable between groups [22,24,25].

Previous PPI studies in KTx patients relevant to our investigation have focused on the possible interaction of MMF with PPIs. Van Boeckel and colleagues compared 125 patients taking pantoprazole with 77 patients using ranitidine [22]. The primary outcome was BPAR and secondary outcomes were creatinine and the eGFR at three months pTx. No significant differences were found in any of the outcomes. Knorr and colleagues [24] came to similar results in their comparative study of 213 patients receiving PPIs, and 390 with ranitidine. The primary outcome was BPAR in the first year pTx. Both groups had comparable rejection rates and eGFRs. Notably, in the subgroup of African American recipients (predominantly fast tacrolimus metabolizers), PPI intake and rejection rates correlated. The recent study by Patel and colleagues [25] compared rejection rates in 183 patients taking PPIs and 339 using histamine-2 receptor antagonists. The primary outcome was the incidence of acute rejection within one year pTx, but eGFR values at one month and one year were also compared between the two groups. None of these parameters showed a significant difference.

Our study adds to the existing literature as we extended the analyses of the eGFR and changes thereof to a longer time period (half a year to four years) than previous studies (analyzing three to twelve months).

A simple comparison of the eGFR reflected differences in patient characteristics (Table 1 and Supplementary Materials 1). These differences were not a result of PPI therapy as is shown by comparable changes in the eGFR in both groups during follow-up (Table 2).

Moreover, similar rejection rates in the groups taking different doses of PPI, namely 0 mg, 1–20 mg, 21–40 mg and >40 mg pantoprazole equivalent, led us to conclude that the relevance of PPI intake for MMF efficacy is at best minimal. This is in line with the previously mentioned observational studies that used different methodologies. Correction for various potentially relevant confounders did not change the results with regards to kidney function or rejection rates. In line with our observations is a recently published meta-analysis that evaluated the data of 6786 KTx patients. The authors found that PPI use was linked to hypomagnesemia, but not associated with acute rejection, graft loss, or one-year mortality [40].

One limitation of our study is the retrospective study design analyzing a limited number of patients from one center. For part of the rejection analysis and >30% and >50% eGFR decline, the inclusion of covariables in the multivariable logistic regression analyses was limited due to the low number of events. Confounding by indication proved a serious difficulty in this study. Patients with longer, higher PPI intake showed a tendency to higher comorbidity and risk factors (Table 1 and Supplementary Material 1). To cope with this, we included information on the Charlson comorbidity index. PPI therapy post-transplant is the standard of care at our center. Discontinuance of PPI medication was assumedly due to clinical evaluation or patient choice, not by standard procedure.

For the statistically observed relation of second year AMRs and >30% eGFR decline (from half a year to two years) with PPI average intake, we presume residual confounding to be the reason because in a post-hoc comparison of the groups used for the >30% eGFR decline analysis, we found significant differences for the following patient characteristics: recipient age and BMI, pre-transplant dialysis time, prior renal transplant, donor type (living or deceased), pre-transplant diabetes, ABO transplant incompatibility, cerebral arterial occlusive disease or stroke, coronary heart disease or myocardial infarction, statins, prior smoking history, continuation of smoking after KTx, and Charlson comorbidity index. All of these, apart from prior renal transplant and ABO incompatibility, showed an unfavorable tendency with higher PPI intake.

5. Conclusions

We conclude from our data that prolonged PPI therapy is safe in regard to KTx function. However, further studies into a possible interaction between PPIs and tacrolimus may be of interest. In addition, our findings highlight the importance of examining changes in the eGFR rather than single eGFR measurements in similar studies. Polypharmacy is a relevant problem in the transplant population [50] and it is always advisable to question unnecessary medication [51]. This may include PPI therapy.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/1/258/s1. Supplementary Materials 1: Additional patient information; Supplementary Materials 2: Statistical analysis; Supplementary Materials 3: Results of the group comparison of the absolute eGFR; Supplementary Materials 4: Results of the >30% and >50% eGFR decline with mean daily PP-dose tests.

Author Contributions: D.J.G.F., K.S.-N., S.R. and conceived and designed the study, D.J.G.F. and K.S.-N. collected the data. D.J.G.F. and K.S.-N. analyzed the data. H.P. and B.S. supervised the project and discussed the data, D.J.G.F., K.S.-N. and S.R. wrote the main manuscript text. All authors have read and agreed to the published version of the manuscript.

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Circulating Arsenic is Associated with Long-Term Risk of Graft Failure in Kidney Transplant Recipients: A Prospective Cohort Study

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Abstract: Arsenic is toxic to many organ systems, the kidney being the most sensitive target organ. We aimed to investigate whether, in kidney transplant recipients (KTRs), the nephrotoxic exposure to arsenic could represent an overlooked hazard for graft survival. We performed a prospective cohort study of 665 KTRs with a functional graft ≥1 year, recruited in a university setting (2008-2011), in The Netherlands. Plasma arsenic was measured by ICP-MS, and dietary intake was comprehensively assessed using a validated 177-item food-frequency questionnaire. The endpoint graft failure was defined as restart of dialysis or re-transplantation. Median arsenic concentration was 1.26 (IQR, 1.04-2.04) μg/L. In backwards linear regression analyses we found that fish consumption (std $\beta = 0.26$; $p < 0.001$) was the major independent determinant of plasma arsenic. During 5 years of follow-up, 72 KTRs developed graft failure. In Cox proportional-hazards regression analyses, we found that arsenic was associated with increased risk of graft failure (HR 1.80; 95% CI 1.28–2.53; $p = 0.001$). This association remained materially unaltered after adjustment for donor and recipient characteristics, immunosuppressive therapy, eGFR, primary renal disease, and proteinuria. In conclusion, in KTRs, plasma arsenic is independently associated with increased risk of late graft failure.

Keywords: arsenic; diet; fish consumption; oxidative stress; kidney transplantation; graft failure

1. Introduction

Arsenic is toxic to many organ systems, the kidney being the most sensitive target organ [1,2]. Free radical mediated-oxidative damage is the cornerstone of arsenic-induced pathology [3]. Arsenic induces morphological alterations of mitochondria that lead to uncontrolled formation of free radicals [4], whilst it inhibits the production of glutathione that protects cells from oxidative damage, ultimately yielding irreversible cell damage [5,6]. The kidney being a major player in removal of arsenic from the system, it is also very much exposed to arsenic and therefore susceptible to arsenic-induced toxicity [7–10].

A large variety of arsenic compounds are known, divided into the elemental metal, inorganic, and organic compounds with a large variety of toxicity [1,2,11,12]. While an extraordinary cause for arsenic intake has been described as hydroarsenicism—contamination of drinking water with arsenic in the US, Chile, and Taiwan—arsenic in food is an increasingly recognized pathway of environmental exposure. Thus, upon background regional differences, arsenic exposure substantially derives from rice consumption, as well as vegetables, fruits, and herbal tea [13–19]. Of note, however, seafood is thought to be a major route for arsenic intake, followed by alcohol consumption, with the latter mainly due to contaminated wine, therewith representing an evident public health threat [20,21].

Basic and clinical evidence has linked arsenic exposure to nephrotoxicity, tubular necrosis, diffuse interstitial fibrosis, decline of kidney function, incident chronic kidney disease, and progress of native chronic kidney disease, among several other conditions such as hypercalciuria, albuminuria, and nephrocalcinosis [22–29]. Kidney transplant recipients (KTRs) are particularly vulnerability to the harmful effects of nephrotoxic agents. However, no study has been devoted to evaluating whether arsenic may be an otherwise overlooked modifiable risk factor in the post-kidney transplantation setting. The current study, therefore, aimed to identify independent environmental and system determinants of plasma arsenic levels and to evaluate the potential association of plasma arsenic levels with long-term risk of graft failure in a large cohort of well-characterized KTRs.

2. Methods

2.1. Design and Study Population

In this prospective cohort study, outpatient adult KTRs with a functioning graft ≥1 year, no alcohol or drug addiction, and without known systemic illnesses (i.e., malignancies, opportunistic infections) were invited to participate. The recruitment of patients took place at the University Medical Center Groningen between November 2008 and March 2011. In total, 817 KTRs were invited for the study, of whom 707 (87%) provided written informed consent to participate. All patients with missing plasma arsenic levels were excluded, resulting in 665 KTRs eligible for statistical analyses. Multiple imputations (n = 5) were used to account for missingness of data among variables other than data on circulating arsenic. The present study was approved by the Institutional Review Board (METc 2008/186) and was conducted in accordance with the Declaration of Helsinki.

The primary outcome of this study was death-censored graft failure, defined as end-stage kidney disease requiring dialysis or re-transplantation. The continuous surveillance system of the outpatient clinic of our university hospital, in which patients visit the outpatient clinic with declining frequency in accordance with the American Transplantation Society Guidelines, ensured updated information on patient status [30]. General practitioners or referring nephrologists were contacted in case the status of a patient was unknown. Endpoints were recorded until September 2015. No patients were lost to follow-up.

All KTRs were transplanted at the University Medical Center Groningen following the establishment of standard antihypertensive and immunosuppressive therapies. Relevant characteristics including recipient age, gender, cardiovascular history, and transplant-related information were extracted from patient records. Dietary intake, clinical parameters, and laboratory measurements were extensively assessed at baseline.

2.2. Assessment of Dietary Intake

Dietary intake was assessed using a validated semi-quantitative food frequency questionnaire (FFQ) developed and updated at Wageningen University [31]. The questionnaire consisted of 177 food items to record intake during the last month, taking seasonal variations into account. For each item, the frequency was expressed in times per day, week, or month. The number of servings was recorded in natural units (e.g., slice of bread or apple) or household measures (e.g., cup or spoon). The FFQ was self-administered and then checked by a trained researcher on the day of visit to the outpatient clinic. Inconsistent answers were verified with the patients. The results of the FFQ were converted into total energy and nutrient intake per day by using the Dutch Food Composition Table of 2006 [32].

2.3. Clinical Parameters and Definitions

All measurements were performed during a morning visit to the outpatient clinic. Blood pressure was determined with a semi-automatic device (Dinamap 1846, Critikon, Tampa, FL, USA), measuring every minute for 15 min. The last three measurements were averaged, following a strict protocol as described previously [33]. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2), and body surface area (BSA) was estimated in meters squared (m^2) by using the universally adopted formula of DuBois and DuBois [34]. Diabetes was defined as use of antidiabetic medication, fasting plasma glucose ≥ 7.0 mmol/L, and/or HbA_{1C} higher than 6.5% [35]. Kidney function was assessed by means of estimated glomerular filtration rate (eGFR) according to the Chronic Kidney Disease Epidemiology Collaboration equation [36].

2.4. Laboratory Methods and Arsenic Measurement

Blood was drawn after a fasting period of 8–12 h, which included no medication intake. Serum high-sensitivity C-reactive protein (hs-CRP), HbA_{1C}, triglycerides, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and total cholesterol were measured using routine laboratory methods. Serum creatinine was determined using a modified version of the Jaffé method (MEGA AU 510, Merck Diagnostica, Darmstadt, Germany). Serum cystatin C was determined using Gentian Cystatin C Immunoassay (Gentian AS, Moss, Norway) on a modular analyzer (Roche Diagnostics, Mannheim, Germany). Class I and class II human leukocyte antigens (HLA) antibodies were assessed by ELISA (LATM20×5, One Lambda, Canoga Park, CA, USA) as described elsewhere [37]. According to a strict protocol, all participants were instructed to collect a 24 h urine sample the day before to their visit to the outpatient clinic. Total urinary protein concentration was determined using the Biuret reaction (MEGA AU 150, Merck Diagnostica, Darmstadt, Germany).

Arsenic plasma concentrations were assessed from EDTA plasma samples that were stored frozen at −80 °C. Arsenic plasma concentrations were determined using inductively coupled plasma mass spectrometry (ICP-MS, Varian 820-MS; Varian, Palo Alto, CA, USA) with a modified method for the measurement of low concentrations of heavy metals in plasma using a standard addition method. Standards were made by addition to blanc plasma known amounts of arsenic to obtain added concentrations of 0.500, 1.00, 2.00, 3.00, 4.00, and 5.00 μg/L. Control samples were made by spiking blanc plasma with known amounts of arsenic to obtain added concentrations of, respectively, 0.75 (low), 2.5 (medium), and 4.5 μg/L (high). Sample preparation consisted of diluting 100 μL sample with 1.0 mL dilution reagent. The dilution reagent contained 0.005% Triton X100, 0.005% EDTA, and 0.1 mg/L Yttrium as internal standard. Characteristics of this method are summarized in Table 1.

Table 1. Bias and precision of arsenic measurements.

Cadmium Concentration	*n*	µg/L	Bias (%)	Inter-Assay Coefficient	
				SD (µg/L)	CV (%)
Low	36	0.75	−13	0.26	40
Medium	36	2.5	−9.2	0.38	17
High	37	4.5	−6	0.48	11

n, number of control samples; SD, standard differentiation; CV, coefficient of variation.

2.5. Follow-Up of Plasma Arsenic Levels in a Sample Population of the TransplantLines Cohort and Biobank Study

Additionally, to investigate plasma arsenic levels over time, we requested follow-up plasma samples (3 months, 6 months, 1 year, and 2 years post-kidney transplantation) from 46 consecutive KTRs enrolled between February 2016 and May 2017 in the ongoing TransplantLines Prospective Cohort and Biobank Study [38]. Arsenic plasma concentrations were determined using inductively coupled plasma mass spectrometry (ICP-MS, Varian 820-MS; Varian, Palo Alto, CA, USA) with a modified method for the measurement of low concentrations of heavy metals in plasma using a standard addition method, as described hereby in the preceding section.

2.6. Statistical Analyses

Data analyses were performed using SPSS version 23.0 software (SPSS, Inc., Chicago, IL, USA) and R version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria). Continuous variables were summarized using mean (SD) for normally distributed data, whereas skewed distributed variables are given as median (IQR). Categorical variables were summarized as numbers (percentage). In all analyses, a two-sided $p < 0.05$ was considered significant. Linear regression analyses were performed to evaluate the association of baseline characteristics with arsenic concentrations, adjusted for (i) age and sex, and additionally (ii) eGFR. The assumption of homoscedasticity and normality of residual variance were verified, and a natural log-transformation was applied when appropriate. Std. β coefficients represent the difference (in SD) in arsenic per 1-SD increment in continuous characteristics or for categorical characteristics the difference (in SD) in arsenic compared to the implied reference group. In order to study, in an integrated manner, which baseline characteristics were independently associated with and were determinants of plasma arsenic, we performed forward selection of baseline characteristics according to preceding multivariable linear regression analyses (p for inclusion < 0.2), followed by stepwise backwards multivariable linear regression analyses (p for exclusion 0.05). Finally, we also performed a stepwise backwards multivariable linear regression with exclusion of eGFR in the initial model in order to isolate environmental determinants of plasma arsenic levels.

The prospective association of plasma arsenic with risk of graft failure during follow-up was examined incorporating time to event and accounting for death-censoring, by means of univariable and multivariable Cox proportional-hazards regression analyses with time-dependent covariates to calculate hazard ratios (HR) and 95% confidence intervals (CI). Schoenfeld residuals were calculated to assess whether proportionality assumptions were satisfied. Associations are shown with plasma arsenic as a continuous variable and according to tertiles of the plasma arsenic distribution. Following univariable analyses (model 1), we first performed multivariable adjustment for the most important environmental determinants of arsenic levels according to the results of our backwards linear regression analyses (model 2). To avoid overfitting, further models were performed with additive adjustments to model 2, defined as the primary multivariable model [39]. Thus, we performed additional adjustments for intake of fruits, vegetables, potato, rice, bread, and total energy intake (model 3); transplant characteristics (donor and recipient age, donor type, HLA mismatches, circulating anti-HLA class I antibodies, circulating anti-HLA class II antibodies, transplant vintage, and immunosuppressive

therapy; model 4); risk factors of graft failure (eGFR, hs-CRP, systolic blood pressure, total cholesterol, and triglycerides concentration; model 5); and primary renal disease and proteinuria in model 6.

The intra-individual coefficient of variation (CV) for plasma arsenic levels in KTRs of the TransplantLines Cohort and Biobank Study was calculated using the formula CV = (SD/mean) × 100, in which SD is the standard deviation and mean is the mean value for plasma arsenic concentrations as measured in follow-up samples taken at 3 months, 6 months, 1 year, and 2 years post transplantation. Next, box plots were used to illustrate medians (interquartile range) of plasma arsenic levels during follow-up visits. Finally, significance of potential change during follow-up visits was tested using the Kruskal Wallis test.

3. Results

3.1. Baseline Characteristics and Cross-Sectional Analyses

Mean (SD) age of the 665 KTRs was 53 (13) years, of whom 383 (58%) were male. Median (IQR) plasma arsenic concentration was 1.26 (1.04–−2.04) µg/L. The baseline characteristics of the study participants along with the results of age- and sex- as well as eGFR-adjusted linear regression analyses are shown in Table 2. In stepwise backward multivariable linear regression analysis, fish consumption ($\beta = 0.26$; $p < 0.001$), eGFR ($\beta = -0.11$; $p = 0.02$), and proteinuria (std $\beta = 0.18$; $p < 0.001$) were identified as independent determinants of plasma arsenic concentrations (Table 2). If analyses were performed with eGFR excluded from the initial model, fish consumption ($\beta = 0.27$; $p < 0.001$) was identified as the only independent determinant of arsenic (Table 2).

3.2. Prospective Analyses

During a follow-up of 5 years, 72 (11%) patients developed graft failure. Chronic allograft dysfunction was the major cause of graft failure accountable for 50 (69%) of all graft failures. Other causes for graft failure included return of primary kidney disease (11%), infection (4%), acute rejection (4%), BK nephropathy (4%), vascular complications (3%), and others (4%). From low to high tertiles of the plasma arsenic distribution, 18, 25, and 29 patients developed graft failure, respectively. Prospective analyses of the association of plasma arsenic with death-censored graft failure are shown in Table 3. Multivariable-adjusted Cox proportional hazards models showed that plasma arsenic was directly associated with graft failure (HR 1.80; 95% CI 1.28–2.53, $p = 0.001$), independent of major environmental determinants of arsenic concentration, i.e., alcohol and fish consumption. In analyses with further adjustment for potential confounders, the association remained materially unchanged (Table 3). We did not find signs of a non-linear association between plasma arsenic levels and risk of death-censored graft failure (Supplementary Materials Table S1). Figure 1 illustrates the association between plasma arsenic concentration and risk of death-censored graft failure using Cox regression analyses with mean concentration of plasma arsenic as reference, adjusted for age, sex, fish intake and alcohol consumption, and in relation to the histogram of plasma arsenic distribution.

Table 2. Baseline characteristics of 665 kidney transplant recipients (KTRs) and their association with plasma arsenic.

Baseline Characteristics	Overall KTRs $n = 665$	† Plasma Arsenic (ln), µg/L	‡ Plasma Arsenic (ln), µg/L	Backwards Linear Regression	§ Backwards Linear Regression
		Std. β	Std. β	Std. β	Std. β
Plasma arsenic, µg/L, median (IQR)	1.26 (1.04–2.04)	–	–	–	–
Demographics and body composition					
Age, years, mean (SD)	53 (13)	–	–		
Sex (male), *n* (%)	383 (58)	–	–		
Diabetes mellitus, *n* (%)	160 (24)	−0.07 *	−0.07 *	~	~
Body surface area, m^2, mean (SD)	1.94 (0.22)	−0.02	−0.05		
Body mass index, kg/m^2, median (IQR)	26.0 (23.3–29.4)	−0.003	−0.02		
Waist circumference, cm, mean (SD)	99 (14)	0.003	−0.02		
Cardiovascular history and lifestyle					
History of cardiovascular disease, *n* (%)	325 (49)	−0.01	−0.01		
Heart rate, beats per minute, mean (SD)	69 (12)	0.01	0.02		
Systolic blood pressure, mmHg, mean (SD)	136 (17)	−0.04	−0.06 *	~	~
Use of antihypertensives, *n* (%)	586 (88)	0.001	−0.04		
Current or former smoker, *n* (%)	382 (57)	0.04	0.03		
Alcohol consumption > 10 g/d, *n* (%)	169 (25)	0.14 ***	0.14 ***	~	
Dietary intake					
Bread, g/day, mean (SD)	133 (59)	−0.09 **	−0.08 *	~	~
Vegetables, g/day, median (IQR)	90 (50–118)	−0.03	−0.03		
Fruit, g/day, median (IQR)	123 (61–232)	−0.04	−0.04	~	~
Potato, g/day, median (IQR)	119 (72–161)	−0.11 ***	−0.11 **	~	~
Rice, g/day, median (IQR)	15 (4–32)	0.07 *	0.06 *	~	~
Fish, g/day, median (IQR)	11 (4–21)	0.32 ***	0.31 ***	0.26 ***	0.27 ***
Coffee, mg/day, median (IQR)	500 (250–625)	−0.001	0.01		
Tea, mg/day, median (IQR)	250 (54–375)	0.03	0.01 *	~	~
Laboratory measurements					
Albumin, g/L, mean (SD)	43 (3)	−0.05	−0.03		
Calcium, mmol/L, mean (SD)	2.40 (0.15)	−0.06 *	−0.04		
Phosphate, mmol/L, mean (SD)	0.97 (0.21)	0.09 **	0.03		
eGFR, mL/min/1.73 m^2, mean (SD)	53 (20)	−0.18 ***	–	−0.11 **	–
Proteinuria, *n* (%)	150 (23)	0.12 ***	0.09 **	0.18 ***	
Alkaline phosphatase, U/L, median (IQR)	67 (54–84)	0.02	0.02		
ASAT, U/L, median (IQR)	22 (18–27)	0.06 *	0.07 *	~	~

Table 2. *Cont.*

Baseline Characteristics	Overall KTRs $n = 665$	† Plasma Arsenic (ln), µg/L	‡ Plasma Arsenic (ln), µg/L	Backwards Linear Regression	§ Backwards Linear Regression
		Std. β	Std. β	Std. β	Std. β
ALAT, U/L, median (IQR)	19 (14–25)	0.01	0.04		
Gamma-GT, U/L, median (IQR)	26 (18–41)	0.05 *	0.05		
Lipids					
Total cholesterol, mmol/L, mean (SD)	5.1 (1.1)	0.03	0.02		
HDL cholesterol, mmol/L, median (IQR)	1.3 (1.1–1.6)	0.04	0.08 *	~	~
LDL cholesterol, mmol/L, mean (SD)	3.0 (0.9)	0.02	0.01		
Triglycerides, mmol/L, median (IQR)	1.7 (1.2–2.3)	−0.01	−0.04		
Inflammation and oxidative stress					
Leukocyte count, per 10^9/L, mean (SD)	8.1 (2.6)	0.01	0.01		
hs-CRP, mg/L, median (IQR)	1.6 (0.7–4.5)	−0.01	−0.02		
Malondialdehyde, µmol/L, median (IQR)	2.5 (1.9–3.7)	−0.02	−0.01		
Primary kidney disease and kidney transplantation					
Primary kidney disease					
Glomerulosclerosis, *n* (%)	190 (29)	0.02	0.01		
Glomerulonephritis, *n* (%)	51 (8)	0.01	−0.01		
Tubulointerstitial nephritis, *n* (%)	76 (11)	0.05	0.06		
Polycystic kidney disease, *n* (%)	136 (21)	−0.09	−0.07		
Kidney hypo/dysplasia, *n* (%)	29 (4)	0.02	0.02		
Renovascular disease, *n* (%)	38 (6)	−0.05	−0.04		
Diabetes, *n* (%)	32 (5)	0.04	0.04		
Other/miscellaneous, *n* (%)	113 (17)	0.02	0.02		
Donor type, living *n* (%)	229 (34)	−0.05	−0.04		
Donor age, years, median (IQR)	46 (31–54)	−0.01	−0.06 *	~	~
Transplant vintage, years, median (IQR)	5.5 (2.0–11.9)	−0.03	−0.01		
Immunosuppressive therapy					
Prednisolone dose, grams, median (IQR)	10.0 (7.5–10.0)	0.01	0.02		
Use of calcineurin inhibitor, *n* (%)	381 (57)	0.05	0.003		
Use of proliferation inhibitor, *n* (%)	553 (83)	−0.001	0.02		
Acute rejection treatment, *n* (%)	176 (26)	0.04	0.03		

* $p < 0.2$; ** $p < 0.05$; *** $p < 0.01$. † Linear regression analysis; adjusted for age, sex, ‡ and eGFR. Std β coefficients represent the difference (in SD) in arsenic per SD increment in continuous characteristics or for categorical characteristics the difference (in SD) in arsenic compared to the implied reference group. For inclusion and exclusion in stepwise backwards linear regression analyses *p* values were set at 0.2 and 0.05, respectively. § eGFR was removed from the initial model. ~ Excluded from the final models. ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; HLA, human leukocyte antigens; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein.

Table 3. Prospective analyses of the association of plasma arsenic with death-censored graft failure in 665 kidney transplant recipients.

	Plasma Arsenic				
	Tertile 1	Tertile 2	Tertile 3	Continuous (ln)	
	Ref.	HR (95% CI)	HR (95% CI)	HR (95% CI)	*p*
n_{events}	18	25	29	72	
Model 1	1.00	1.41 (0.77–2.59)	1.69 (0.94–3.04)	1.47 (1.08–2.01)	0.02
Model 2	1.00	1.58 (0.86–2.92)	2.12 (1.14–3.95)	1.80 (1.28–2.53)	0.001
Model 3	1.00	1.55 (0.84–2.87)	2.05 (1.10–3.82)	1.74 (1.24–2.45)	0.001
Model 4	1.00	1.40 (0.75–2.61)	2.00 (1.06–3.77)	1.90 (1.32–2.73)	0.001
Model 5	1.00	1.32 (0.71–2.45)	1.76 (0.93–3.32)	1.56 (1.10–2.23)	0.01
Model 6	1.00	1.29 (0.70–2.40)	1.84 (0.99–3.42)	1.53 (1.09–2.14)	0.01

Cox proportional-hazards regression analyses were performed to assess the association of plasma arsenic with risk of death-censored graft failure (number of events = 72). Associations are shown with plasma arsenic concentration as a continuous variable and according to tertiles of the plasma arsenic distribution (tertile 1: ≤1.1 µg/L; tertile 2: 1.1-1.67 µg/L; tertile 3: ≥1.67 µg/L). Model 1 is univariable. Multivariable model 2 was adjusted for fish intake and alcohol consumption. Subsequently, additive adjustment was performed for intake of fruits, vegetables, potato, rice, bread, and total energy intake (model 3); donor and recipient age, donor type, human leukocyte antigen mismatches (HLA), circulating anti-HLA class I antibodies, circulating anti-HLA class II antibodies, transplant vintage, and immunosuppressive therapy (model 4); eGFR, high-sensitivity C-reactive protein, systolic blood pressure, total cholesterol, and triglyceride concentration (model 5); primary kidney disease and proteinuria (model 6).

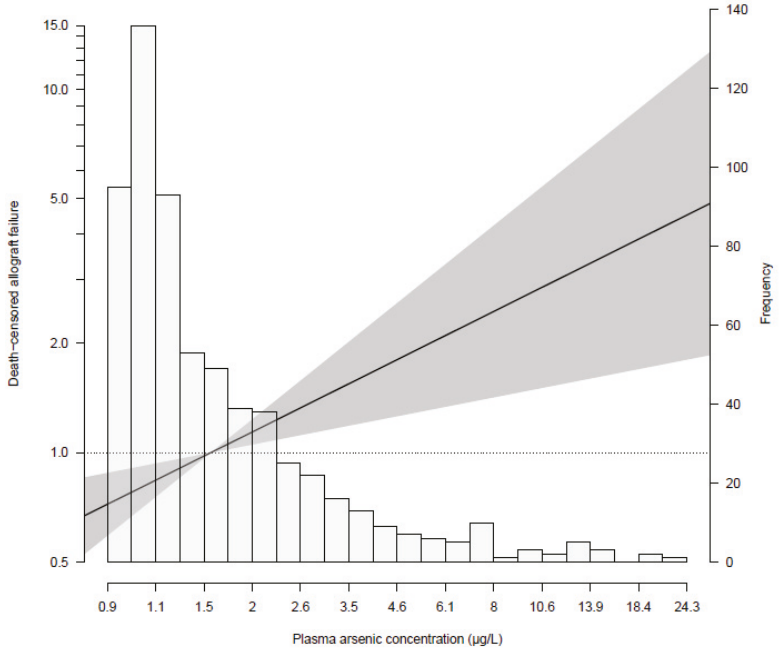

Figure 1. Association between plasma arsenic concentration and risk of death-censored graft failure using Cox regression analyses with mean concentration of plasma arsenic as reference, adjusted for age, sex, fish intake, and alcohol consumption, and in relation to the histogram of plasma arsenic distribution.

3.3. Follow-up of Plasma Arsenic Levels in a Sample Population of the TransplantLines Cohort and Biobank Study

In Supplementary Materials Figure S1 we show box plots with medians (IQR) of plasma arsenic concentration of 46 KTRs (mean age 52 ± 14 years-old, eGFR 43 ± 28 mL/min/1.72 m^2) from the

TransplantLines Prospective Cohort and Biobank Study, at different follow-up visits post-kidney transplantation. Median (interquartile range) plasma arsenic concentrations were 1.61 (1.51–1.99), 1.64 (1.52–2.05), 1.64 (1.43–1.94), and 1.59 (1.46–2.26) µg/L at 3 months, 6 months, 1 year, and 2 years post-kidney transplantation, respectively. Median (interquartile range) intra-individual coefficient of variation was 12.2% (6.7–28.7%), and we did not find signs of a significant change in plasma arsenic levels over time ($p = 0.64$).

4. Discussion

In these analyses of 665 well-characterized individuals from a Dutch cohort of KTRs, we identified fish consumption as the major environmental determinant of plasma arsenic levels. Prospective analyses showed that higher plasma arsenic levels are associated with increased long-term risk of graft failure, independent of donor and recipient characteristics, immunosuppressive therapy, eGFR, and proteinuria. These data pose arsenic as a potentially modifiable risk factor for late graft failure in KTRs, emphasizing the need for specific recommendations regarding arsenic exposure, as well as patient monitoring and management of arsenic-induced kidney injury, particularly in populations highly susceptible to nephrotoxic agents such as KTRs.

Being the major organ involved in arsenic clearance, the kidney is highly susceptible and the most sensitive target organ to arsenic exposure [1,2,9,10]. Arsenic-induced oxidative stress has been suggested to be the cornerstone of pathological mechanisms leading to kidney injury and development of chronic kidney disease [3,40]. On the one hand, decreased antioxidant capacity has been shown in individuals exposed to arsenic [41], wherein depletion of glutathione has been consistently described [5,42,43]. Of note, by protecting cells from oxidative damage, inhibition of glutathione production and subsequent glutathione depletion ultimately reverberates into increased vulnerability of cells to arsenic damage. On the other hand, it has been shown that arsenic induces morphological alterations of mitochondrial integrity that lead to uncontrolled free radical formation [4], which further feeds the circle of oxidative challenge and tissue injury. Indeed, basic and clinical evidence has linked arsenic exposure to nephrotoxicity, tubular necrosis, diffuse interstitial fibrosis, decline of kidney function, incident chronic kidney disease, and progress of native chronic kidney disease, amongst other conditions such as hypercalciuria, albuminuria, and nephrocalcinosis [22–29]. Subsequently, diminished kidney clearance of arsenic and enhanced production of reactive oxygen species longitudinally contribute to perpetuate tissue insult and progression of chronic kidney disease [22,23]. Previous studies have also shown an association between arsenic and hypertension and type 2 diabetes mellitus, both suggesting additional mechanisms for secondary kidney damage [44,45]. Ecological studies from the United States, Chile, and Taiwan have shown that arsenic exposure is associated with increased mortality from kidney disease [13–15,22,26,28,46–49]. KTRs are particularly vulnerable to harmful effects of nephrotoxic agents. End-stage kidney disease and maintenance immunosuppressive therapy are constant sources of oxidative challenge for the graft tissue, which shortens the capacity of oxidative stress defenses against additional environmental hazards. To our knowledge, the current study is the first to provide evidence of an independent prospective association between circulating arsenic levels and risk of late kidney graft failure.

Further supportive evidence for the key role of oxidative stress in arsenic-induced pathogenic mechanisms—and suggestive of potential management alternatives—was provided by the observation that co-administration of ascorbic acid and α-tocopherol to arsenic-exposed rats led to a reduction in the levels of lipid peroxidation, protein carbonyls, and hydrogen peroxide along with increased levels of reduced glutathione, ascorbic acid, and α-tocopherol. Investigation aimed to evaluating whether ascorbic acid and α-tocopherol supplementation may improve arsenic-induced altered microsomal functions in the kidney is warranted [50].

An increasing body of evidence supports that the kidney is a primary site of arsenic uptake and accumulation. Recently, X-ray fluorescence spectrometry allowed detection of arsenic accumulation, specifically at level of the kidney cortex [51]. X-ray fluorescence spectrometry may provide

comprehensive information of bioaccumulation for biomedical and toxicological research by allowing direct measurement of the distribution of arsenic at tissue, cellular, and subcellular level. Next, X-ray absorption spectroscopy has been shown to allow in vivo assessment of whole-body distribution, which is key information for the development of chelation therapies [52]. Future studies using these analytical methods may provide essential research data to understand the sequence of specific mechanisms of nephrotoxicity and deepen the understanding of the association between long-term arsenic exposure and kidney damage [51].

The current study is etiological in nature, which needs to be separated from prediction research [53]. Whereas the latter is a distinct field of epidemiologic research aimed at predicting the risk of an outcome according to a model of statistically significant predictors, which not necessarily represents causal associations, etiological studies aim to understand a certain pathway of a disease in an attempt to prevent its onset or progression [53]. Taken together, our findings and the aforementioned studies may support an etiological role of arsenic in pathways of disease that contribute to increased risk of death-censored graft failure.

Data on the average diet-derived arsenic exposure in The Netherlands are scarce. One study reported an estimated median (range) exposure of 37.8 (20.6–70.1) μg/day [54]. This was corroborated by a more recent study of Hoogenboom et al. stating that the average diet-derived arsenic exposure is <50 μg/day. In agreement with our findings, higher intake of arsenic most frequently originates from higher fish consumption [55]. A monitoring program from the Dutch Agriculture Advisory Committee (LAC), conducted in the 1980s, demonstrated that levels of arsenic in fish landed in The Netherlands varied between 0.8 and 6.8 mg/kg wet weight, showing a slight decreasing trend over time. Likewise, the arsenic levels in shrimps decreased from 4.3 to 1.3 mg/kg wet weight during that period (LAC program, 1991, in reference [41]). However, more recent data regarding arsenic-contaminated fish landed in The Netherlands are lacking and needed to evaluate strategies aiming to reduce the dietary consumption of arsenic by the population. Next, although in The Netherlands, naturally occurring arsenic concentrations in drinking water are usually below the concentrations required by the European drinking water standard (<10 μg/L in all countries, except Denmark, where it is <5 μg/L), health risks cannot be excluded at this level, and it has been recommended to optimize water supply to arsenic levels <1 μg/L [56,57].

The current study was performed in a large cohort of extensively phenotyped KTRs, allowing us to control our main findings for several potential confounders, including donor and recipient characteristics, immunosuppressive therapy, proteinuria, and eGFR. Moreover, patients were monitored for an extensive period and patient status was updated without losses to follow-up, allowing the study of the long-term association of arsenic with graft failure. Despite considerable improvement of short-term graft survival during last decades, improvement of long-term outcomes continues to lag behind, emphasizing that future advances in the field of kidney transplantation are expected from the amelioration of long-term graft attrition [58]. Systematic description of modifiable risk factors is key to promote preventive strategies particularly addressed for this population of solid organ patients.

Our study derived from a single university center from the northern part of The Netherlands, which calls for prudence to extrapolate our results to different populations regarding potential environmental arsenic contamination and exposure. Additionally, the observational design of the current study does not allow hard conclusions on causality, nor could the potentiality of reversed causation or residual confounding be eliminated, despite the substantial number of potential confounders for which we adjusted. Furthermore, the technique used in the current study does not allow different species of arsenic to be distinguished, while arsenic species have major varieties in toxicity [1–4,11,12]. Elemental arsenic is nontoxic as the metal is insoluble in bodily fluids, and inorganic species of arsenic, e.g., arsenite and arsenate, are especially toxic to humans. Organic species vary in toxicity; the most common species, monomethylarsonic acid and dimethylarsinic acid, are less toxic compared to inorganic species, and arsenobetaine and arsenosugars have a very low toxicity [1,5,9,11,59–61]. Further studies utilizing techniques with the ability to distinguish between the different species of arsenic,

e.g., high-performance liquid chromatography–inductively coupled plasma-mass spectrometry, could provide more information on the impact of the different species on graft failure in KTRs. A further limitation is that adjustment for immunological factors as potential confounders of the association was limited to adjustment for HLA matching, circulating anti-HLA class I antibodies, and circulating anti-HLA class II antibodies, since we had no data on donor-specific anti-HLA antibodies and biopsy findings. Finally, it should be acknowledged that graft failure can be the consequence of multiple, heterogenous causes. Unfortunately, in our study the numbers of cause-specific cases of death-censored graft failure was too small to allow for meaningful separate analyses [62]. Larger studies are warranted to comprehensively evaluate the association of plasma arsenic with different causes of death-censored graft failure. It should be noticed, however, that this study is the first to indicate a prospective association of arsenic with the hard endpoint graft failure, thus holding a plea for future studies which to only investigate arsenic plasma concentrations, but also take into account concentrations of arsenic in drinking water, and not only in KTRs to investigate associations with death-censored graft failure, but also in other populations, such as patients with diabetes and the general population.

5. Conclusions

In conclusion, the current study shows for the first time that circulating arsenic levels are independently associated with higher risk of late kidney graft failure, emphasizing the need for specific recommendations regarding arsenic exposure, as well as patient monitoring and management of chronic arsenic-induced kidney damage. Our findings point towards arsenic as an otherwise overlooked modifiable risk factor for adverse long-term kidney outcomes, especially in populations of vulnerability to oxidative stress challenge, *e.g.*, KTRs. Further studies are warranted to confirm our results and investigate the longitudinal association between arsenic exposure and graft failure in KTRs from populations with different dietary and environmental exposure.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/2/417/s1, Figure S1: Plasma arsenic concentration of 46 kidney transplant recipients from the TransplantLines Prospective Cohort and Biobank Study [38], at different follow-up visits after transplantation, Table S1: Verification of linearity of the association between plasma arsenic and risk of death-censored graft failure.

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Physical Activity and the Development of Post-Transplant Diabetes Mellitus, and Cardiovascular- and All-Cause Mortality in Renal Transplant Recipients

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Abstract: (1) Background: Little is currently known about the health impacts of daily-life moderate-to-vigorous physical activity (MVPA) in relation to the development of post-transplant diabetes mellitus (PTDM) and the long-term survival of renal transplant recipients (RTRs). (2) Methods: We analyzed self-reported data on MVPA within non-occupational and occupational domains, estimated with the SQUASH questionnaire, from a prospective cohort study of RTRs (n = 650) with a functioning graft exceeding 1 year. PTDM diagnoses were based on plasma glucose levels (≥126 mg/dL), HbA1c (≥6.5%), and the use of antidiabetic medication. Mortality data were retrieved from patient files up to the end of September 2015. (3) Results: During a median follow-up period of 5.3 years, 50 patients (10%) developed PTDM and 129 (19.8%) died. Of these deaths, 53 (8.9%) were caused by cardiovascular disease. Cox regression analyses showed that higher MVPA levels among patients were associated with a lower risk of PTDM (hazard ratio (HR); 95% confidence interval (95%CI) = 0.49; 0.25–0.96, p = 0.04), cardiovascular- (0.34; 0.15–0.77, p = 0.01), and all-cause mortality (0.37; 0.24–0.58, p < 0.001) compared with No-MVPA patients, independently of age, sex, and kidney function parameters. Associations of MVPA with cardiovascular and all-cause mortality remained significant and materially unchanged following further adjustments made for transplant characteristics, lifestyle factors, metabolic parameters, medication use, and creatinine excretion (muscle mass). However, the association between MVPA and PTDM was no longer significant after we adjusted for metabolic confounders and glucose levels. (4) Conclusion: Higher MVPA levels are associated with long-term health outcomes in RTRs.

Keywords: physical activity; renal transplant recipients; transplantation; post-transplant diabetes mellitus; cardiovascular mortality; mortality

1. Introduction

Renal transplantation is a more effective treatment strategy than chronic dialysis in patients with end-stage renal disease [1]. However, post-transplant patients are at an increased risk of developing cardiometabolic diseases that lead to high morbidity and mortality among renal transplant recipients (RTRs) [2,3]. The cardiovascular mortality rate is estimated to be 10 times higher for RTRs compared

with the general population [2]. Moreover, approximately 20% of RTRs develop post-transplant diabetes mellitus (PTDM) [3]. This situation necessitates an investigation aimed at developing strategies for improving the management of long-term health outcomes in RTRs.

Studies have consistently found that physical activity (PA) is a modifiable factor that contributes to reducing the risk of cardiometabolic diseases and premature mortality within the general population [4,5]. However, there is limited data on the impacts of PA on RTRs [6–10]. Studies conducted on the benefits of PA have mostly focused on the intermediate outcomes of clinical trials entailing exercise training programmes [6–8]. A few studies found that a low PA is significantly associated with substantial weight gain and with risks of cardiovascular and all-cause mortality in RTRs [11–14]. Moreover, it remains unclear whether the benefits of increased PA in relation to patients' long-term outcomes are independent of their health and transplant characteristics (i.e., kidney function and duration of pre-transplant dialysis), lifestyle factors, and use of medication [11–13].

Clinical guidelines for the general population recommend the performance of at least 150 min of moderate-to-vigorous physical activity (MVPA) per week [15]. There are no specific clinical guidelines or recommendations for RTRs. A recent position statement on exercise for solid organ transplant recipients released in 2019 recommending that it is a key step toward raising awareness of the importance of exercise training in the patients among transplant professionals [16]. The available data show that the level of daily-life PA is lower for RTRs compared with individuals within the general population [17–19]. It is unclear whether individuals within the general population as well as RTRs can attain the recommended MVPA level by engaging in different domains of daily-life activities, such as non-occupational and occupational activities. Results showed that occupational MVPA should not be included within assessments of healthy daily-life PA and should not be deemed a substitute for leisure time MVPA. Specifically, it is not known whether both non-occupational and occupational PA can contribute to the improved health of RTRs [20–22].

Therefore, we aimed to investigate the association between daily-life MVPA and the risk of developing long-term health outcomes, such as PTDM as well as cardiovascular and all-cause mortality in RTRs sampled from a large prospective study. We also examined whether these associations were independent of several variables, including age, sex, kidney function, transplant characteristics, lifestyle factors, medication use, metabolic parameters, and anthropometric measures. Moreover, we assessed the benefits of non-occupational MVPA as well as total daily-life MVPA, including occupational PA.

2. Methods

2.1. Study Population

This study was conducted in a large single-center prospective cohort of stable outpatient RTR [23,24]. A total of 817 adult RTRs who met the study's eligibility criteria, namely having a functioning graft for at least 1 year and no history of alcohol and/or drug addiction, were invited to participate in the study. Further exclusions were apparent systemic diseases, such as malignancies or active infections. In total, 707 (86.5%) RTRs signed written informed consent. Baseline data were collected between November 2008 and May 2011. We excluded 57 RTRs, whose PA questionnaires were incomplete, from the analysis, leaving a total of 650 RTRs. Subsequently, 148 and 61 RTRs, respectively, with a history of diabetes and cardiovascular diseases (CVDs) prior to undergoing transplants, were excluded from the analyses of PA and the development of PTDM and cardiovascular mortality. The study was conducted according to the Helsinki Declaration and was approved by the UMCG's review board (METc 2008/186).

2.2. Measurements at Baseline

All baseline measurements have been previously described in greater detail elsewhere [25]. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). A semiautomatic device (Dinamap®1846; Critikon, Tampa, FL, USA) was used to measure blood pressure in a half-sitting

position and the average of the final three readings of blood pressure was used. Information on medication was derived from patient records. Daily caloric intake and alcohol consumption were calculated from a validated Food Frequency questionnaire. Information on smoking was obtained by a questionnaire. The serum creatinine-based Chronic Kidney Disease Epidemiology Collaboration equation was used to calculate the estimated glomerular filtration rate (eGFR) [26]. Creatinine excretion—a marker of muscle mass—was calculated from the 24-h urine collection as described earlier [27].

2.3. Assessment of Physical Activity

The SQUASH is a validated questionnaire used to estimate habitual physical activities performed during a normal week [28]. The SQUASH is pre-structured into four domains: commuting, leisure time and sports, household, and occupational activities. Questions consisted of three main queries: days per week, average time per day, and intensity. In this study, we used activities at the moderate (4.0–6.5 MET) to vigorous (≥6.5 MET) level. Metabolic equivalent (MET) values were assigned to activities according to Ainsworth's Compendium of Physical Activities [29]. We used the combination of leisure-time and commuting (non-occupational) moderate-to-vigorous physical activity (MVPA) minutes per week (min/week) as a measure of PA in this study, since active commuting of high intensity and longer duration is often replacing sports activities, like cycling. We did not include occupational MVPA in the main analysis because of its health benefit is not clear in the general population [20–22,30]. In an additional analysis, we investigated the association between total MVPA, including occupational MVPA, with clinical endpoints. Participants were subdivided into three categories based on their levels of non-occupational MVPA. RTRs who did not engage in PA at a moderate-to-vigorous level were deemed 'inactive' (no-MVPA), and the remaining participants (MVPA > 0) were divided into two groups based on median values of non-occupational MVPA (less active, MVPA-1 and active, MVPA-2). The MVPA min/week (median, interquartile range (IQR)) was used to define the MVPA groups (MVPA-1 and MVPA-2): 5-197 (120, 60–150) and 200–1680 (360, 260–540).

2.4. Endpoints of the Study

Endpoints of interest in this study were post-transplant diabetes mellitus (PTDM), cardiovascular and all-cause mortality. PTDM was defined according to the presence of at least one of the following criteria: diabetes symptoms (e.g., polyuria, polydipsia, or unexplained weight loss) along with a non-fasting plasma glucose concentration of ≥200 mg/dL (11.1 mmol/L); fasting plasma glucose concentration (FPG) ≥ 126 mg/dL (7.0 mmol/L); start of antidiabetic medication; or HbA1c ≥ 6.5% (48 mmol/L). This definition matched the diagnostic criteria for diabetes applied by the American Diabetes Association, including HbA1c levels, as proposed by the expert panel constituted at the international consensus meeting on PTDM [31,32]. Cardiovascular mortality and all-cause mortality were monitored through continuous surveillance conducted within the outpatient program and retrieved from patients' files up to the end of September 2015. No participants were lost to follow-up.

2.5. Statistical Analysis

The patient characteristics are expressed as means with a standard deviation for normally distributed variables or as medians with interquartile range (25th to 75th percentile) for non-normally distributed variables and numbers with percentages in case of categorical data. The differences between groups were tested using 1-way analysis of variance tests or Kruskal–Wallis tests for normally and non-normally distributed continuous variables, respectively. The frequency distributions of categorical variables were analyzed using the Pearson Chi-Square test.

We adopted MVPA as a continuous and categorical variable in a Cox regression analysis. First, we tested associations of non-occupational MVPA, considered as a continuous variable, on clinical endpoints. In this analysis, MVPA (measured in min/week) was log-transformed to obtain a normal distribution. Thereafter, multivariate Cox regression analyses were performed to examine whether

higher non-occupational MVPA is associated with lower risks of PTDM, cardiovascular mortality, and all-cause mortality independently of potential confounders which are clinically known confounders in the relating associations of physical activity with long-term outcomes [6,7,12–14,33]. In these analyses, we first adjusted for age and sex (model 1) as well as kidney function parameters, including eGFR, proteinuria, the time lapse between transplantation and the baseline measures, and primary renal disease (model 2). We further adjusted model 1 for transplant characteristics (acute rejection, preemptive transplantation, and living donor status) in model 3. Similarly, we adjusted model 1 for lifestyle factors, such as smoking, alcohol consumption, and daily caloric intake (model 4); calcineurin inhibitors and prednisolone used as immunosuppressive medication (model 5); systolic blood pressure, use of antihypertensive drugs, high-density lipoprotein cholesterol (HDL), and triglycerides (model 6), BMI and waist circumference (model 7), and 24-h creatinine excretion (model 8). With regard to potential collinearity (model 7), we tested the correlation between BMI and waist circumference ($r = 0.84$, $p < 0.001$ for men and $r = 0.81$, $p < 0.001$ for women). Then we performed Cox-regression analyses using separate models adjusted for BMI and waist circumference separately (Supplementary materials, Table S1. We found no differences relating to the confounding effects of total fat and fat distribution. Finally, we included those variables in the same model. In addition, we adjusted model 1 for baseline hemoglobin A1C and fasting plasma glucose (model 9) relating to the association between MVPA and PTDM. We also adjusted model 1 relating to the association between MVPA and PTDM for diet quality (Model 10). We furthermore investigated whether diet quality might modify the association of MVPA with development of PTDM by additional inclusion of a product-term of the continuous variables of diet quality and MVPA in the concerned model, to assess potential interaction between the two. Mediterranean diet score was used as diet quality and assessed with a 177-item validated food frequency questionnaire which is described in greater detail elsewhere [33]. All models (1-10) include up to 6 variables to fulfil the rule of thumb which allows 1 variable per 7–10 events. This is now fulfilled for all analyses [34,35]. Hazard ratios were reported with 95% confidence intervals. Proportional hazard assumptions were tested using the Schoenfield residuals method developed by Grambsch and Therneau [36]. Penalized splines were constructed to visualize the association of non-occupational MVPA with PTDM as well as cardiovascular and all-cause mortality independently of age and sex.

We performed additional analyses to explore the role of work within this population by investigating the associations between total MVPA, including occupational MVPA, and non-occupational MVPA with clinical endpoints for the RTRs who worked ($n = 322$, 49.5%). Occupational status was defined using the answers for the questions related to occupational PA. If responders answered as not applicable, we considered them as unemployed. Another subgroup analysis was performed to address changes in these associations across age categories. The population was categorized as being over or under 55 years of age, based on the WHO guideline on the prevention of CVD [37]. Finally, to rule out competing mortality risks associated with the occurrence of PTDM, we conducted competing risk analyses following the procedures outlined by Fine and Gray [38].

A two-sided statistical significance was set at $p < 0.05$ for all tests. All statistical analyses were performed using SPSS software V.22 (IBM Inc., Chicago, IL, USA,) R software V.3.2.2 (R Foundation for Statistical Computing, Vienna, Austria), STATA version 13.0 (StataCorp LP, College Station, TX, USA) and Graph Pad Prism 7 (Graph Pad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Baseline Characteristics

A total of 650 RTRs (men: 56.3%, mean age: 51.8 ± 13.2 years old) were examined in this study. Baseline measurements were taken 5.7 years (median value; interquartile range (IQR): 1.9–12.1 years) post-transplantation. Of the total sample of RTRs, 37.8% ($n = 246$) did not perform daily MPVA at all within any domain. The other RTRs spent a median of 200 min (IQR = 120–360 min per week) engaged in non-occupational MVPA. Table 1 shows the baseline characteristics of RTRs according to their

non-occupational MVPA levels. RTRs in the active groups (MVPA > 0) had lower values for BMI, waist circumference, and systolic blood pressure and higher creatinine excretion values compared with the values of the inactive group (no-MVPA). Moreover, higher alcohol consumption, lower concentrations of triglycerides and HDL-C, haemoglobin A1C, and less proteinuria and diabetes at the baseline level along with more 'living donors' were observed for the 'active' groups compared with the 'inactive' group. Table S2 presents the baseline characteristics of the RTRs according to the presence of clinical endpoints. Figure S1 further shows levels of daily MVPA according to the participants' ages and work status. As expected, total MVPA values, including those for occupational MVPA, were significantly higher in RTRs who were working (n = 322). However, when working status or age was considered, the levels of non-occupational MVPA did not differ significantly.

Table 1. Characteristics of the study population, according to MVPA level.

Variable	Total (n = 650)	No-MVPA (n = 246)	MVPA-1 (n = 201)	MVPA-2 (n = 203)	p-Value
Age (years)	52.6 ± 12.8	54.1 ± 11.8	51.9 ± 13.4	51.6 ± 13.3	0.08
Male gender (%, n)	56.3 (366)	56.1 (138)	55.7 (112)	57.1 (116)	0.96
Current smoking (%, n)	12.3 (80)	15.3 (36)	12.4 (25)	9.5 (19)	0.20
Occupational status: Employed * (%, n)	49.5 (322)	44.7 (110)	51.2 (103)	53.7 (109)	0.14
Alcohol use (g/day)	2.61 (0.1–11.1)	1.45 (0.1–9.7)	2.51 (0.1–9.7)	3.95 (0.1–14.1)	0.01
Total energy intake (kcal/d)	2174.9 ± 640.7	2114.7± 720.4	2247.5 ± 598.7	2173.2 ± 573.9	0.11
Non-occupational MVPA (min/week)	90 (0–240)	0	120 (60–150)	360 (260–540)	–
Anthropometric measures					
Body mass index (kg/m^2)	26.7 ± 4.84	27.9 ± 5.49	25.7 ± 4.26	26.1 ± 4.23	0.001
Waist circumference, men (cm)	101.1 ± 13.4	104.0 ± 13.7	98.9 ± 13.5	99.7 ± 12.4	0.01
Waist circumference, women (cm)	95.0 ± 15.8	99.7 ± 12.4	91.5 ± 14.6	93.1 ± 14.1	0.01
Creatinine excretion (mmol/24h)	11.7 ± 3.49	11.2 ± 3.80	11.8 ± 3.29	12.2 ± 3.23	0.01
Lipids and blood pressure					
Total cholesterol (mmol/L)	5.14 ± 1.11	5.18 ± 1.18	5.17 ± 1.10	5.07 ± 1.02	0.48
Triglyceride (mmol/L)	1.68 (1.2–2.33)	1.85 (1.3–2.6)	1.67 (1.2–2.4)	1.59 (1.2–2.05)	0.001
HDL-C in men (mmol/L)	1.27 ± 0.41	1.23 ± 0.41	1.27 ± 0.36	1.32 ± 0.45	0.21
HDL-C in women (mmol/L)	1.56 ± 0.51	1.39 ± 0.43	1.62 ± 0.54	1.70 ± 0.53	0.001
Systolic blood pressure (mm Hg)	136.2 ± 17.3	138.3 ± 18.5	135.6 ± 16.7	134.2 ± 16.3	0.04
Diastolic blood pressure (mm Hg)	82.8 ± 10.9	83.1 ± 11.2	82.5 ± 11.3	82.6 ± 10.3	0.77
Cardiovascular medication use					
Antihypertensive (%, n)	88 (572)	92.7 (228)	81.1 (163)	89.2 (181)	0.001
A2 antagonist (%, n)	14.8 (96)	15.4 (38)	12.9 (26)	15.8 (32)	0.68
ACE inhibitor (%, n)	32.2 (209)	32.1 (79)	30.3 (61)	34.0 (69)	0.74
RAAS blockers (%, n)	47.8 (311)	49.2 (121)	44.8 (90)	49.3 (100)	0.58
Beta-blockers (%, n)	63.2 (411)	63.4 (156)	62.2 (125)	64.0 (130)	0.93
Calcium channel blockers (%, n)	24.5 (159)	26.0 (64)	20.4 (41)	26.6 (54)	0.27
Diuretics (%, n)	40.0 (260)	52.0 (128)	27.4 (50)	37.9 (77)	0.001
Vitamin K antagonist (%, n)	11.4 (74)	13 (32)	10.4 (21)	10.3 (21)	0.60
mTOR inhibitor (%, n)	1.8 (12)	3.3 (8)	1 (2)	1 (2)	0.60
Anti-diabetic drugs (%, n)	14.8 (96)	18.7 (46)	14.4 (29)	10.3 (21)	0.045
Statin (%, n)	51.8 (337)	54.9 (135)	52.7 (106)	47.3 (96)	0.27
Glucose metabolism					
Fasting plasma glucose (mmol/L)	5.67 ± 1.82	5.78 ± 192	5.76 ± 2.13	5.46 ± 1.28	0.13
Heamoglobin A1C (%)	5.94 ± 0.78	6.03 ± 0.77	5.94 ± 0.90	5.83 ± 0.65	0.021
Kidney function					
eGFR (mL/min/1.73m^2)	52.0 ± 20.2	49.9 ± 22.1	53.8 ± 18.7	52.9 ± 18.8	0.09
Albumin excretion (mg/24h)	267.3 ± 734.6	307.2 ± 777.5	175.1 ± 378.5	308.7 ± 917.5	0.11
Proteinuria (%, n)	21.5 (140)	28.0 (69)	16.9 (34)	18.2 (37)	0.01

Table 1. *Cont.*

Variable	Total (n = 650)	No-MVPA (n = 246)	MVPA-1 (n = 201)	MVPA-2 (n = 203)	p-Value
Primary renal disease (%, n)					0.01
Glomerulosclerosis	28.8 (187)	30.1 (74)	28.4 (57)	27.6 (56)	
Glomerulonephritis	7.7 (50)	5.7 (14)	8.0 (16)	9.9 (20)	
Tubulointerstitial nephritis	11.8 (77)	9.8 (24)	12.9 (26)	13.3 (27)	
Polycystic kidney disease	20.9 (136)	20.7 (51)	19.9 (40)	22.2 (45)	
Renal hypodysplasia	3.5 (23)	4.1 (10)	3.0 (6)	3.4 (7)	
Renavascular diseases	5.7 (37)	7.7 (19)	4.0 (8)	4.9 (10)	
Diabetes mellitus	4.6 (30)	6.5 (16)	5.5 (11)	1.5 (3)	
Others	16.9 (110)	15.4 (38)	18.4 (37)	17.2 (35)	
Duration of dialysis before the transplantation (months)	25 (8–48)	29 (11–51)	19 (4–49)	25 (9–43)	0.51
Transplant characteristics					
Transplant vintage (months)	14.0 (2.0–39.5)	17.0 (2.0–41.0)	12.0 (2.0–44.8)	16.0 (0.5–41.0)	0.49
Cold ischemia time (h)	15.2 (2.8–21.1)	16.4 (3.6–22.0)	15.1 (2.6–21.3)	13.6 (2.5–20.5)	0.10
Living donor (%, n)	34.8 (226)	26.4 (65)	37.3 (75)	42.4 (86)	0.001
Pre-emptive transplant (%, n)	16.6 (108)	13.4 (33)	20.9 (42)	16.3 (33)	0.11
Acute rejection	27.2 (177)	27.2 (67)	27.9 (56)	26.6 (54)	0.96
Immunosuppressive medication					
Calcineurin inhibitor (%, n)	58.3 (379)	59.8 (147)	59.2 (119)	55.7 (113)	0.52
Proliferation inhibitor (%, n)	82.6 (537)	80.1 (197)	84.1 (169)	84.2 (171)	0.62
Prednisolone dose (mg)	10.0 (7.5–10.0)	10.0 (7.5–10.0)	10.0 (7.5–10.0)	10.0 (7.5–10.0)	0.48

Data are presented as mean ± SD or median (interquartile range) and percentage (%, number). MVPA = moderate-to-vigorous physical activity, HDL-C = high-density lipoprotein cholesterol, eGFR = estimated glomerular filtration rate, A2 = angiotensin 2, ACE = angiotensin-converting-enzyme, RAAS = renin–angiotensin–aldosterone system, mTOR = mammalian target of rapamycin. * the number of patients that do have employment.

3.2. Post-Transplant Diabetes Mellitus

A total of 50 RTRs (10%) had developed PTDM after a median follow-up period of 5.3 years (4.1–6.0 years). The multivariable Cox proportional hazard models showed that the group with the highest level of non-occupational MVPA was associated with a lower risk of PTDM (hazard ratio (HR); 95% CI = 0.49; 0.25–0.96, p = 0.04) compared with the no-MVPA group, independently of age, sex, and kidney function parameters (model 1, Table 2). This association remained significant after we made further adjustments for kidney function parameters, transplant characteristics, lifestyle factors, Mediterranean diet score and 24-h creatinine excretion quantities (considered as a marker of muscle mass) (models 2–4, 8, and 10). Following adjustments made for immunosuppressive medication (model 5), metabolic parameters (model 6), anthropometric measures (model 7), and baseline glucose levels (model 9), the highest level of MVPA was no longer associated with PTDM. However, when MVPA was applied as a continuous variable in the Cox regression analysis, as opposed to using groups of MVPA levels, a higher non-occupational MVPA was associated with a lower risk of PTDM independent of all of the above-mentioned confounders apart from the adjustment of metabolic parameters and glucose level.

Table 2. Association of non-occupational MVPA with long-term health outcomes.

Physical Activity	MVPA (cont.)		No-MVPA (Ref)	MVPA-1		MVPA-2	
	HR (95% CI)	*p*		HR (95% CI)	*p*	HR (95% CI)	*p*
Post-transplant DM							
No. of events	50/502		23	14		13	
Model 1	0.88 (0.79–0.97)	0.01	1.00	0.57 (0.29–1.10)	0.09	0.49 (0.25–0.96)	0.04
Model 2	0.88 (0.79–0.98)	0.02	1.00	0.61 (0.31–1.20)	0.15	0.49 (0.25–0.96)	0.04
Model 3	0.88 (0.79–0.98)	0.02	1.00	0.55 (0.28–1.07)	0.08	0.48 (0.24–0.95)	0.04
Model 4	0.87 (0.79–0.97)	0.01	1.00	0.57 (0.29–1.12)	0.10	0.46 (0.22–0.94)	0.03
Model 5	0.89 (0.80–0.99)	0.03	1.00	0.59 (0.30–1.26)	0.11	0.52 (0.26–1.03)	0.06
Model 6	0.91 (0.82–1.01)	0.09	1.00	0.70 (0.36–1.40)	0.31	0.60 (0.29–1.22)	0.16
Model 7	0.88 (0.79–0.99)	0.03	1.00	0.63 (0.31–1.25)	0.19	0.50 (0.25–1.03)	0.06
Model 8	0.87 (0.79–0.97)	0.01	1.00	0.55 (0.29–1.08)	0.08	0.47 (0.24–0.93)	0.03
Model 9	0.91 (0.82–1.01)	0.12	1.00	0.72 (0.36–1.41)	0.34	0.59 (0.30–1.19)	0.14
Model 10	0.87 (0.78–0.97)	0.01	1.00	0.58 (0.32–1.25)	0.11	0.44 (0.21–0.92)	0.03
Cardiovascular mortality							
No. of events	53/589		26	14		13	
Model 1	0.84 (0.74–0.94)	0.01	1.00	0.45 (0.22–0.94)	0.03	0.34 (0.15–0.77)	0.01
Model 2	0.84 (0.75–0.95)	0.01	1.00	0.49 (0.23–1.02)	0.06	0.35 (0.16–0.80)	0.01
Model 3	0.86 (0.76–0.96)	0.01	1.00	0.51 (0.25–1.05)	0.07	0.40 (0.18–0.91)	0.03
Model 4	0.87 (0.77–0.98)	0.02	1.00	0.56 (0.26–1.21)	0.14	0.43 (0.19–0.94)	0.046
Model 5	0.84 (0.74–0.94)	0.001	1.00	0.45 (0.21–0.93)	0.03	0.36 (0.16–0.81)	0.01
Model 6	0.85 (0.76–0.96)	0.001	1.00	0.49 (0.23–1.02)	0.06	0.38 (0.17–0.86)	0.02
Model 7	0.85 (0.75–0.96)	0.01	1.00	0.51 (0.23–1.11)	0.09	0.40 (0.17–0.92)	0.03
Model 8	0.87 (0.77–0.98)	0.02	1.00	0.55 (0.26–1.16)	0.12	0.44 (0.19–0.99)	0.051
All-cause mortality							
No. of events	129/650		76	27		26	
Model 1	0.84 (0.78–0.89)	<0.001	1.00	0.39 (0.25–0.61)	<0.001	0.37 (0.24–0.58)	<0.001
Model 2	0.85 (0.79–0.91)	<0.001	1.00	0.43 (0.27–0.67)	<0.001	0.40 (0.26–0.63)	<0.001
Model 3	0.85 (0.79–0.91)	<0.001	1.00	0.41 (0.27–0.64)	<0.001	0.41 (0.26–0.64)	<0.001
Model 4	0.83 (0.77–0.89)	<0.001	1.00	0.41 (0.21–0.64)	<0.001	0.35 (0.22–0.58)	<0.001
Model 5	0.83 (0.78–0.89)	<0.001	1.00	0.39 (0.25–0.61)	<0.001	0.37 (0.23–0.58)	<0.001
Model 6	0.85 (0.79–0.91)	<0.001	1.00	0.42 (0.27–0.66)	<0.001	0.41 (0.26–0.65)	<0.001
Model 7	0.84 (0.78–0.89)	<0.001	1.00	0.40 (0.25–0.63)	<0.001	0.37 (0.23–0.59)	<0.001
Model 8	0.86 (0.80–0.92)	<0.001	1.00	0.45 (0.29–0.70)	<0.001	0.44 (0.28–0.69)	<0.001

DM = Diabetes mellitus, MVPA = moderate-to-vigorous physical activity. Model 1: adjusted for age and sex. Model 2: model 1 + adjustment for kidney function (eGFR, urinary protein excretion, time between transplantation and baseline, and primary renal disease). Model 3: model 1 + adjustment for transplant characteristics (acute rejection, pre-emptive transplantation, donor type). Model 4: model 1 + adjustment for lifestyle factors (smoking, alcohol consumption, daily caloric intake). Model 5: model 1 + adjustment for immunosuppressive medication (calcineurin inhibitors, prednisolonee). Model 6: model 1 + adjustment for lipids and blood pressure (systolic blood pressure, use of antihypertensive drugs, triglycerides, HDL-C). Model 7: model 1 + adjustment for BMI and waist circumference. Model 8: model 1 + adjustment for 24-h creatinine excretion. Model 9: model 1 + adjustment for fasting plasma glucose and HbA1c. Model 10: model 1 + adjustment for Mediterranean diet score.

3.3. Cardiovascular and All-Cause Mortality

During the follow-up period, 129 (19.8%) patients died. Of these deaths, 53 (8.9%) were caused by cardiovascular disease (CVD). In the multivariable Cox proportional hazard models, the highest level of non-occupational MVPA was associated with a lower risk of cardiovascular mortality (HR; 95% CI = 0.34; 0.15–0.77, $p = 0.01$) compared with the no-MVPA group, independently of age, sex, and kidney function parameters (model 1–2, Table 2). This association remained significant after further adjustments were made for transplant characteristics, immunosuppressive medication, metabolic parameters, and anthropometric measures (models 3, 5–7). However, the association was no longer

significant after adjusting for lifestyle factors (model 4) and creatinine excretion (model 8). Moreover, the association of MVPA with cardiovascular mortality was sustained independently of all of the potential confounders when non-occupational MVPA was applied as a continuous variable in the Cox regression (models 1–9, Table 2).

With regard to all-cause mortality, the group with the highest level of non-occupational MVPA was associated with a lower risk of all-cause mortality (HR; 95% CI = 0.37; 0.24–0.58, $p < 0.001$) compared with the no-MVPA group (model 1, Table 2). This association remained significant after we adjusted for potential confounders (models 2–8). However, the association weakened after we adjusted for transplant characteristics (model 3), metabolic parameters (model 6), and creatinine excretion (model 8). When log-transformed non-occupational MVPA was applied as a continuous variable in the Cox regression analysis, the association was independent of all of the above-mentioned confounders, apart from creatinine excretion (model 8), remaining materially unchanged.

To illustrate these associations further, age-and sex-adjusted penalized splines and the Kaplan-Meier survival curves are shown in Figures 1 and 2.

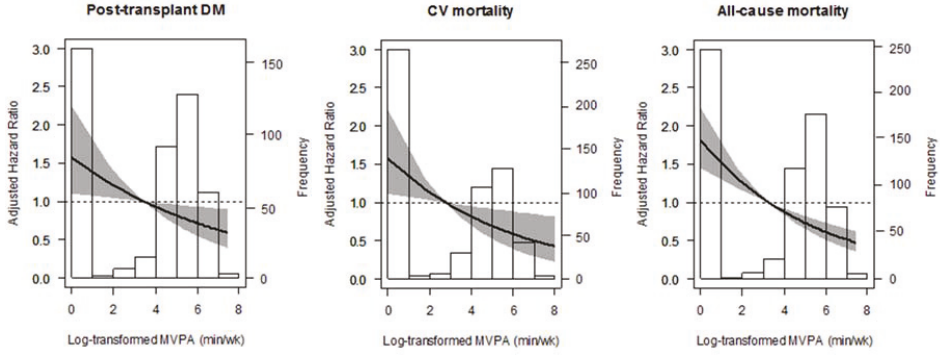

Figure 1. Association between non-occupational MVPA and post-transplant diabetes mellitus (PTDM), Cardiovascular (CV)mortality, and all-cause mortality in renal transplant recipients (RTRs).

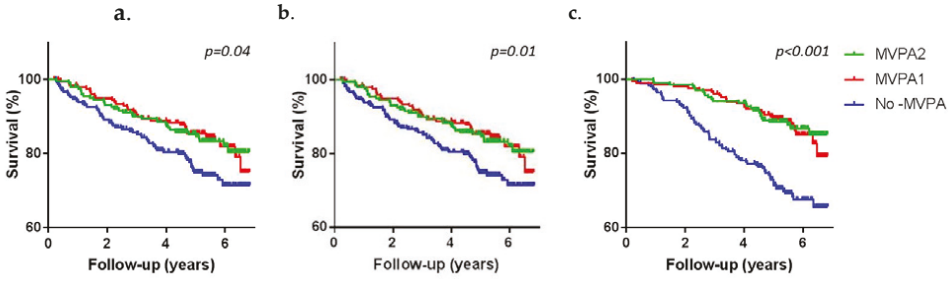

Figure 2. Probability of survival for PTDM (**a**), cardiovascular mortality (**b**), and all-cause mortality (**c**) according to non-occupational MVPA level.

3.4. Additional Analyses

Additional analyses of the subgroup of working RTRs revealed that the inclusion of occupational PA in the estimate of MVPA resulted in the attenuation of the HRs of all of the significant and non-significant associations (Table 3). The association of non-occupational MVPA with cardiovascular and all-cause mortality was stronger compared with that of total MVPA, which includes occupational MVPA.

Table 3. Additional analysis on the associations of MVPA with long-term health outcomes in RTRs who are working (n = 322).

Physical Activity	MVPA (cont.)		No-MVPA		MVPA > 0		
	HR^ (95% CI)	*p*-value	N *	Reference	N *	HR^^ (95% CI)	*p*-Value
Post-transplant DM							
Non-occupational PA	0.87 (0.74–1.03)	0.113	10	1.00	10	0.46 (0.18–1.13)	0.076
Total PA	0.91 (0.78–1.06)	0.212	8	1.00	12	0.48 (0.20–1.20)	0.056
Cardiovascular mortality							
Non-occupational PA	0.63 (0.48–0.83)	0.001	12	1.00	3	0.11 (0.11–0.42)	0.001
Total PA	0.75 (0.63–0.91)	0.003	9	1.00	6	0.23 (0.10–0.58)	0.051
All-cause mortality							
Non-occupational PA	0.76 (0.66–0.87)	<0.01	25	1.00	11	0.21 (0.14–0.51)	<0.01
Total PA	0.82 (0.74–0.92)	0.001	19	1.00	17	0.30 (0.18–0.58)	<0.01

Total PA was the sum of non-occupational and occupational MVPA in min/week. DM = diabetes mellitus, MVPA = moderate-to-vigorous physical activity, HR=hazard ratio, CVD = cardiovascular disease, N * = number of events. ^ Analyses were adjusted for age, gender, and kidney function parameters. ^^ Analyses were adjusted for age and gender (kidney function parameters excluded in this analysis due to fulfill the rule of thumb).

Age-stratified analyses revealed that associations of MVPA with long-term health outcomes were stronger in older adults (Figure 3).

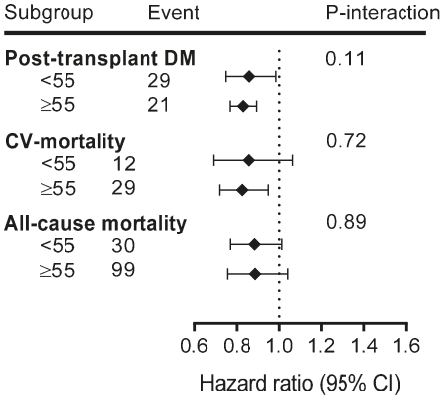

Figure 3. Subgroup analysis for the associations of MVPA with long-term health outcomes over age categories.

The results of the competing risk analyses showed that there was no strong influence of a competing risk of all-cause mortality on the association of MVPA with PTDM. For instance, the competing HR was 0.51 (0.30–0.92, p = 0.04) for the highest MVPA with PTDM after adjusting for age and sex. By comparison, the HR was 0.49 (0.25–0.96, p = 0.04, model 1, Table 2) when competing risks were discounted.

4. Discussion

We found that increased daily-life MVPA is associated with a reduced risk of PTDM, cardiovascular mortality, and all-cause mortality in RTRs independently of age, sex, baseline kidney function parameters, transplant characteristics, and other lifestyle habits. The association of MVPA with PTDM was affected by the adjustments we made for baseline glucose levels and metabolic parameters,

but it did not seem to be affected by other potential confounders, notably anthropometric and immunosuppressive medication. The associations of MVPA with cardiovascular and all-cause mortality were not substantially affected by adjustments made for the above-mentioned confounders. These results confirm the importance of PA in the long-term healthcare management of RTRs.

Previous studies have found that PTDM is highly prevalent in RTRs [3,39]. However, data on lifestyle interventions for improving glucose tolerance or observational data on the association of increased PA with incidences of PTDM are lacking [13,40]. An intervention study showed that lifestyle modifications, including the incorporation of exercise training, improved 2-h postprandial glucose levels in RTRs who were glucose intolerant [40]. One observational study found that higher levels of PA are associated with a lower risk of glucose intolerance in RTRs [13]. However, this study entailed a cross-sectional design and did not test whether this association of PA is independent of other potential confounders. In our longitudinal study, the association of MVPA with PTDM was found to be independent of age, sex, baseline kidney function parameters, transplant characteristics, and other lifestyle factors, such as smoking, alcohol use, and diet (daily caloric intake and Mediterranean diet score). However, the association was affected by adjustments made for immunosuppressive medication, anthropometric measures (BMI and waist circumference), baseline glucose levels, and metabolic parameters. It is widely accepted that obesity is associated with the development of diabetes within the general population [41]. The use of immunosuppressive medications play a role in the development of PTDM through a pathway of stimulation of gluconeogenesis affecting increased blood glucose which can leads to insulin resistance in combination with other mechanisms [39]. However, when we applied log-transformed continuous MVPA, significant associations were observed after we adjusted for immunosuppressive medication and anthropometric measures, indicating that statistical power issues may also play a role. Thus, further large-scale studies of a longer duration should be conducted to explore whether or not MVPA is associated with PTDM independently of immunosuppressive medication and obesity. Furthermore, diet is an important factor in the development of diabetes. A previous analysis by Osté et al. for our study population showed that Mediterranean style diet predicts the development of PTDM [33]. We found that the association between MVPA and PTDM became slightly stronger when adjusted for Mediterranean diet score indicating the importance of diet, but there was no effect modification by diet quality (P-interaction = 0.147).

A previous study, investigating another sample of RTRs, found that a lower PA is strongly associated with an increased risk of cardiovascular and all-cause mortality [14]. In their Cox regression analyses, these authors found that the association was independent of potential confounders, including the history of CVD, muscle mass, and Framingham CVD risk score factors. However, they did not adjust for some clinical variables, such as kidney function and transplant characteristics (e.g., transplant vintage and donor type). Our study supports an independent association of PA with the risk of cardiovascular and all-cause mortality. Many studies have pointed to the benefit of PA within the general population in preventing premature mortality [4,5]. One of the mechanisms proposed to explain the effects of increased PA entails the improvement of all organ systems, especially the cardiovascular system. Specifically in RTRs, improved cardiovascular function is associated with improvements in kidney function. Increased physical activity can support perfusion and oxygen delivery in the kidneys. Studies have shown that higher levels of daily-life PA are associated with a lower risk of renal function decline within the general population and in patients with chronic kidney disease [42–44]. Consequently, increased PA, by improving kidney function, may be of benefit for long-term graft survival. This effect may also be due to improvements in metabolic dysfunctions, such as insulin resistance, impaired glucose tolerance, dyslipidemia, and hypertension, all of which are related to (central) adiposity [45–47]. Furthermore, a number of studies on diet analysed in-depth the effect of dietary factors on the same outcomes such as PTDM, renal function decline and mortality [23–25,33,48]. They suggest that lifestyle is very important for RTR, however, it should be noted that MVPA in daily life has not gotten that much attention. Taken together, these findings suggest that the improvement of daily-life MVPA needs to be evaluated as a therapy for improving patients' long-term survival.

Within this RTR population, MVPA levels were lower than those within the general population. In our study, 38% of RTRs were inactive (no-MVPA), whereas in the Lifelines cohort, a population-based study for which the same questionnaire (SQUASH) and comparable data processing methods were used, the prevalence of inactivity (no-MVPA) was 10% (n = 125,402, 40.5% males, median age of 45) [30,49]. Even in different age groups and gender, it was lower, ranging between 7.5% (n = 42,661, 40% of males, median age of 40) and 12.5% (n = 34,506, 45.6% of males, median age of 56) in the Lifelines. Lower PA levels among RTRs may be attributed to lower muscle mass (a structural abnormality) and muscle weakness (a functional abnormality) [6]. Our descriptive analysis indicated that inactive RTRs had a lower 24-h creatinine excretion value (a marker of muscle mass) compared with that of active RTRs. We also found that the duration of pre-transplantation dialysis was longer in inactive RTRs, although not significantly so. Studies concluded that low muscle mass can be caused by low PA levels [6,7,9]. This conclusion is in line with our findings, indicating that the association was slightly attenuated after we adjusted for renal factors and muscle mass but that the effect of PA remained evident. Actually a shorter time on dialysis would thus also help post-transplant health because studies showed that the level of PA declines in patients with end-stage kidney diseases and it increases after transplantation [7]. Finally, both recovery of activity after transplantation, as well as prevention of inactivity and loss of muscle mass in people with longstanding kidney disease is important for long-term health after transplantation.

A growing body of evidence is showing that occupational MVPA may have no clear benefit on health in the general population [20–22,30,49]. This was tested in our study including a specific patient population, the RTR. Even in the case of RTR, where being at work may be indicative of relatively good health, individuals who were much more active in terms of their occupational MVPA may not obtain any additional benefits for health. A clear mechanism that prevents occupational PA from generating health benefits is missing. There is always the possibility of residual confounding by factors such as sex, socioeconomic status, work-related stress, and body weight in the association between occupational PA and health outcomes [20,22,30,49]. Studies attempted to explore the possibility of residual confounding, but also found no clear association of occupational MVPA and health outcomes. Thus, we suggest that it is important to be aware that occupational MVPA should not be considered as a substitute for leisure time MVPA in RTR.

The potential benefit of PA seems to be more pronounced in older adults, a phenomenon that was described before in the general population [50]. In the general population, studies concluded that the benefit of PA can be gained more easily when there is more room for improvement, like as in older people. However, it might also be that its effects will be potentially outweighed by other, more important clinical factors (e.g., comorbidities and medication use). Therefore, we attempted to test the effect of physical activity in specific groups such as in RTR in two age groups. We found that a higher MVPA is strongly associated with the development of long-term outcomes such as PTDM and cardiovascular mortality in younger and older adults, but is especially stronger in older adults. Thus, older RTR who are able to remain active despite their longstanding condition are likely to remain relatively healthy.

The strengths of this study include its prospective design, long duration, and complete follow-up. Another strength is we included stable RTRs after transplantation and studied relevant clinical outcomes. Nevertheless, there are some limitations to our study. The observational nature of the study precludes us from drawing conclusions regarding causality. A limitation of this study was its use of self-reporting, which is subject to recall bias, for the PA assessment. However, the SQUASH questionnaire has been validated within general as well as specific populations, such as patients who have undergone total hip arthroplasty and those with ankylosing spondylitis [28,51,52]. Furthermore, PA was assessed at a single point in time. However, in RTR, after 1-year of transplantation, PA is increased by 30% and remained materially unchanged the next 5-years [18]. In this study, we included RTR > 1 year graft functioning with a median of 5.7 years post-transplantation. Another limitation is that we could not fully control for the history of all cardiometabolic diseases in the association of MVPA with all-cause

mortality. Patients with a history of diabetes or CVD before the transplantation were excluded from the analyses on the association between MVPA and PTDM or CV mortality. However, cardiometabolic diseases might be more prevalent in 'No-MVPA' group after transplantation as well. A limitation is that we could not have data on functional evaluations, like e.g., a 6 min walking test, which could have provided important information on cardiovascular efficiency. Finally, single-center nature of study, which mainly consisted of white people is unclear whether our findings can be extrapolated to other populations. It would be relevant to repeat our study in other patient populations.

5. Conclusions

Higher daily-life MVPA is associated with a reduced risk of PTDM as well as cardiovascular and all-cause mortality in RTRs, suggesting that PA has a positive influence on the long-term health management of RTRs. The associations of MVPA with cardiovascular and all-cause mortality were not substantially affected by adjustments made for potential confounders, such as age, sex, baseline kidney function parameters, transplant characteristics, lifestyle habits, metabolic parameters, anthropometric measures, and immunosuppressive medication. The association of MVPA with PTDM was affected by adjustments of metabolic parameters and glucose levels. The potentially beneficial effects of daily-life PA apply to non-occupational activities at the moderate-to-vigorous level (e.g., commuting, leisure activities, or sport). By contrast, a higher level of occupational MVPA is not directly associated with the development of long-term outcomes. The associations of non-occupational MVPA and the risk of PTDM and cardiovascular mortality were also stronger in older adults. Finally, we suggest that because of the long-term importance of PA, it should be embedded in the healthcare management of RTRs. Furthermore, large scale interventional studies are needed to test the ab initio effect of physical activity after transplantation on the development of post-transplant diabetes mellitus.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/2/415/s1, Figure S1: Level of daily-life PA according to occupational status (A) and age (B), Table S1: Model 7 and separate adjustments for BMI and waist circumference in the associations of MVPA of with long-term health outcomes in RTRs. Table S2: Baseline characteristics of RTRs according to the presence of long-term health outcomes.

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The Preliminary Results of Bortezomib Used as A Primary Treatment for An Early Acute Antibody-Mediated Rejection after Kidney Transplantation—A Single-Center Case Series

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Abstract: Proteasome inhibitor bortezomib has been used in the treatment of refractory cases of acute and chronic antibody-mediated rejection (AMR) in kidney transplant recipients. However, its efficacy and safety as a primary treatment for early AMR has been scarcely investigated. We herein present our preliminary experience with bortezomib- and plasmapheresis-based primary treatment for early AMR. Thirteen patients transplanted between October 2015 and September 2019 were treated (starting at median 19th post-transplant day) with bortezomib/plasmapheresis protocol for early biopsy-proven AMR. Twelve out of thirteen patients received 4 doses and one patient recieved 3 doses of bortezomib (1.3 mg/m^2 per dose). In 11/13 patients, 4–7 concomitant plasmapheresis sessions were performed, with or without intravenous immunoglobulin (IVIG). Of note, rituximab was not used in all study patients. The kidney graft and patient survival were 100%. The mean 3-month estimated glomerular filtration rate (eGFR) was 55.3 (95%CI: 44.9–65.8) mL/min/1.73m^2, 8/13 patients completed 12-month follow-up with mean eGFR 60.4 (45.4–75.4) mL/min/1.73m^2, and 6/13 patients completed a 24-month follow-up period with mean eGFR 73.9 (56.7–91.1) mL/min/1.73m^2. Neutropenia < 1 G/L was observed in one patient, third or fourth grade thrombocytopenia in two patients, and eleven patients needed a blood transfusion (median: 2 units/patient). The mid-term results of a primary bortezomib-based treatment for kidney AMR showed its non-inferiority as compared to preceding regimens and acceptable safety. However, our data should be validated in a multicenter randomized trial.

Keywords: acute humoral rejection; first-line therapy; outcomes; proteasome inhibitor

1. Introduction

Acute antibody-mediated rejection (AMR) accounts for 20–30% of all acute rejection episodes after kidney transplantation and is often associated with poor allograft survival [1]. It rarely occurs in unsensitized patients, but may occur in up to 50% of highly sensitized recipients [2,3]. Diagnosis of AMR is based on the histopathologic features of the graft biopsy (glomerulitis, arterial-transmural lesions, thrombotic microangiopathy, etc.) and the presence of donor-specific antibodies (DSAs), with or without positive C4d staining [4]. Nowadays, most frequently used treatment modalities for AMR include plasmaphereses (PF) or immunoadsorption (IA), intravenous immunoglobulin (IVIG), anti-T-cell therapy (antithymocyte globulin, ATG), and anti-B-cell therapy (rituximab). Unfortunately, long-term results of such therapy remain suboptimal [5,6]. The rationale for PF or IA is the removal of readily available antibodies and, therefore, limiting the acute tissue damage. Currently, PF is the standard-of-care for the treatment of AMR despite the evidence uncertainty [6].

As the plasma cells are the main source of antibody production, the proteasome inhibitor, bortezomib (Velcade®, Milennium Pharmaceuticals, Cambridge, MA, USA), had been introduced into the AMR treatment, mostly as a rescue therapy in refractory cases [7–10]. In general, kidney graft outcomes were significantly better in acute than chronic AMR [8,10–12]. In some reports, such an adjuvant therapy was effective in decreasing DSAs and stabilizing kidney graft function in mid-term observation [7,9]. Nonetheless, the rate of graft loss during longer follow-up periods was high and the excretory function of still-functioning transplanted organs was markedly decreased [7,9,13,14]. As early as 2010, Walsh et al. reported the first use of bortezomib along with a single dose of rituximab in two patients as a primary therapy in early post-transplant AMR, with a rapid DSAs elimination and excellent renal function at five and six months of observation [15]. Later on, Waiser et al. compared the effect of bortezomib- versus rituximab-based AMR therapies, but both study subgroups contained acute and chronic AMR cases with a substantial age difference between groups [16]. Since then, only a few case reports have been published [17,18]. The existing patophysiologic evidence of the potential mechanisms leading to the therapeutic effect of bortezomib indicates its ability for causing apoptosis of antibody-producing plasma cells, blocking the secretion of class IgG antibodies against human leukocyte antigens (anti-HLA) [19], and decreasing the number of plasma cells within the graft [20]. In order to maximize the bortezomib efficacy in AMR treatment, in all previously reported protocols, each dose was administered after plasmapheresis session, which aimed to decrease the amount of circulating antibodies. Therefore, we herein present the largest-to-date cohort of kidney transplant recipients with early AMR diagnosed in graft biopsy and primarily treated with bortezomib-based therapy without concomitant rituximab administration.

2. Methods

2.1. Study Group

We present a retrospective observational study, including all consecutive kidney graft recipients (KTRs) from our center, transplanted between October 2015 and September 2019 and diagnosed with early biopsy-proven AMR. All patients received their organs from deceased donors and all had negative complement-dependent cytotoxicity (CDC) crossmatch performed immediately prior to transplantation. Flow cytometric crossmatches were not performed. In our country, only patients with negative CDC crossmatch are listed for the transplant centre in order to choose the recipients of organs procured from the donor. Based on the routine pre-transplant screening and the last Luminex results, the presence of DSA with mean fluorescence intensity (MFI) $\geq$ 5000 eliminates the potential kidney transplant candidate from the ongoing procedure. A routine kidney graft early protocol biopsy was introduced at our center in the second half of 2015. Since than, all patients who were diagnosed with AMR were then assigned to primary therapy, which included bortezomib. The study was conducted in accordance with the Declaration of Helsinki. As this drug is not registered for the AMR therapy, the Bioethic Committee of the Medical University of Silesia was consulted and all patients gave their informed consent for the off-label use of bortezomib in their therapy. Notably, the guidelines for immunosuppressive therapy after kidney transplantation, issued by the Polish Transplant Society, have allowed the use of bortezomib in KTRs with AMR since 2012 [21].

2.2. Immunosuppressive Protocol

The standard immunosuppressive protocol included tacrolimus 2 × 0.1 mg/kg twice daily (with target through level 7–12 ng/mL) and mycophenolate mofetil 750 mg twice daily, both started immediately prior to operating procedure, and steroids, starting with the dose of 500 mg of methylprednisolone intravenously (i.v.) during the operation. Induction therapy was based on the rabbit antithymocyte globulin (rATG) (Thymoglobuline®, Genzyme Europe B.V., Amsterdam, Holland) in immunologically high-risk recipients (maximum panel reactive antibodies (PRA) titer > 25% and/or the presence of pre-transplant DSAs) or the anti-interleukin 2 receptor blocker basiliximab (Simulect®,

Novartis Europharm Europe, Dublin, Ireland). Pre-transplant anti-HLA antibodies were evaluated by solid-phase assays using bead arrays and a Luminex platform. Pre- and post-transplant DSAs were determined using a single-antigen bead assay and results were expressed as mean fluorescence intensity (MFI). Among 4 patients who did not receive rATG induction, in one of those patients, the pre-transplant DSA were undetermined and IL-2RB was given, one patient did not received rATG or IL-2RB induction due to the lack of information concerning the presence of DSA at the time of transplantation, and 2 others had their DSA only in HLA class I and in a relatively low titers (i.e., 1992 and 541), so IL-2RB was used. Each administration of rATG was preceded by metyloprednisolone 125 mg i.v., paracetamol 1.0 g i.v., and an antihistaminic drug, and the first dose was started preoperatively and then continued with intermittent dosing based on lymphocyte count. Additionally, routine fluconazole (100 mg), valgancyclovir (labeled dose adjusted to the kidney graft function), and sulfamethoxazole-trimethoprim (2 × 480 mg) prophylaxis was given in rATG-treated patients.

2.3. Primary Treatment Protocol of AMR

After diagnosis, bortezomib (four doses, each 1.3 mg/m^2) was administered subcutaneously the day after the PF session. Concomitantly, 4–8 PF sessions were performed every second/third day, with a plasma exchange rate of 2.0–2.5× patient's plasma volume. In patients who did not receive rATG induction, the routine valgancyclovir and sulfamethoxazole-trimethoprim prophylaxis was started prior to bortezomib therapy. Additionally, antibiotic prophylaxis with piperacillin/tazobactam was started and continued during the bortezomib/plasmapheresis therapy. Immediately before every subcutaneous administration of bortezomib, above i.v. metyloprednisolone, paracetamol, and an antihistaminic drug were given as a premedication.

2.4. Kidney Graft Function and Protocol Biopsies

Kidney graft estimated glomerular filtration rate (eGFR) was calculated based on MDRD (Modification of Diet in Renal Disease) formulation at the 3rd, 12th, and 24th post-transplant month.

Kidney graft protocol biopsies were performed usually at the 8th–11th post-transplant day. All biopsies were evaluated by one experienced pathologist according to the revised Banff classification [4,22–24]. Each kidney biopsy specimen was routinely stained for hematoxyllin and eosin, PAS, Masson trichrome, and silver methenamine. Additionally, SV40 antigen staining specific for polyoma BK virus infection and von Kossa staining for the presence of calcium-phosphate deposits within the tubular lumen or interstitium were performed. We also analyzed histologic signs of potential calcineurin inhibitor nephrotoxicity. Immunohistochemistry was routinely performed (CD4, CD8, CD20, CD68, and C4d) and described semi-quantitatively based on the grade of infiltration (as scattered cells, foci, clusters, groups, or diffused infiltration). AMR was diagnosed based on the following criteria: (1) the presence of histologic signs of microvascular injury (glomerulitis, peritubular capillaritis (PTC-itis), arteriitis, acute tubular injury/necrosis, (2) positive C4d staining, and (3) presence of DSAs. During the follow-up period, control protocol biopsies were performed and analyzed in 9 patients.

2.5. Statistics

Statistical analysis was performed using the Statistica software (StatSoft Polska, Cracow, Poland). Values were presented as means with 95% confidence interval (CI) or medians with Q25–Q75 quartile values. The comparison of kidney graft function before and after bortezomib treatment was performed using the Student's *t*-test. *p*-values below 0.05 were considered as statistically significant.

3. Results

3.1. Baseline Characteristics

Thirteen KTRs (7 males and 6 females) with early acute AMR treated with bortezomib-based primary therapy were analyzed. Their demographic and clinical characteristics are presented in

Table 1. The mean recipient age was 53 years (minimum 30, maximum 68), mean body mass index (BMI) was 26.4 (95%CI: 24.0–28.7), and the median dialysis vintage before transplantation was 43 (IQR: 27–64) months. The history of previous blood transfusions was positive in nine, negative in one, and unknown in two patients. Out of six females, four reported past pregnancies. Only three patients had historical and two patients had the last pre-transplant panel-reactive antibodies (PRA) ≥ 25%, whereas nine patients presented positive results of virtual PRA, calculated based on the Eurotransplant Reference Laboratory HLA database version 2.0. Pre-transplant DSAs were present in twelve patients, with median MFI 10,706 (IQR: 2741–11,415) (Table 2). Induction therapy was used in twelve patients, including rATG in nine and basiliximab in three patients, respectively.

3.2. AMR Diagnosis and Treatment

AMR was diagnosed based on the first protocol biopsy, performed at a median 10 (IQR: 9–10) post-transplant day (Table 2). Due to technical constraints, the histopatologic biopsy results were available after 2–5 working days. In the majority of patients, the CD4, CD8, and CD68 infiltration was predominantly seen, whereas CD20 staining revealed only single cells or scattered foci, except in three patients, in whom, CD20 clusters and/or groups were described (Figure 1). Hence, a primary AMR treatment started at median 19th post-transplant day. In eight patients, all three Banff criteria of AMR were fulfilled. In the next four patients, the suspected diagnosis was C4d-negative AMR. In one patient, results of pre-transplant single-antigen bead assay were not available, but the screening test for class II anti-HLA antibodies was positive (Table 2). Immediately after AMR diagnosis, the primary treatment was started as described above. One patient received only 3 out of 4 planned doses of bortezomib due to the observed gastrointestinal side effects. In the first three patients, PF treatment with fresh frozen plasma (FFP) was completed. In the next eight patients, FFP together with 5% human albumin was used, in the 1:1 volume proportion. The last two patients were treated with a modified protocol (bortezomib, ATG with total dose 5 mg/kg and IVIG single dose 1g/kg) as they initially received basiliximab induction, whereas we were not able to plan and perform PF sessions at that time. Besides, dialysis therapy was required in five patients before and partially also during the AMR treatment.

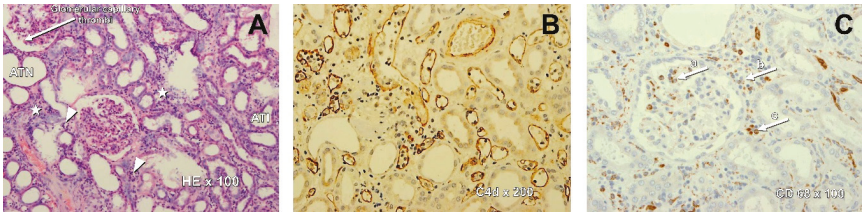

Figure 1. Microphotograph presenting typical histologic findings in kidney transplant recipients with early antibody-mediated rejection. (**A**) Hematoxylin and eosin, magnification 100×. Interstitial edema (tubules are not back to back) with inflammatory infiltrates—asterisks, acute tubular injury (ATI) with the flattening of epithelium cells with the absence of brush border, acute tubular necrosis (ATN) with the tubular basement membranes denuded of epithelial cells, peritubular capillaritis (PTC-itis)—arrowheads, and hypoperfused glomeruli with microthrombi—arrow. (**B**) A diffused C4d staining pattern around peritubular capillaries. Magnification 200×. (**C**) Immunostaining demonstrating CD68-positive macrophages within glomeruli (a), interstitial space (b), and peritubular capillaries (c). Magnification 100×.

Table 1. Baseline demographic and clinical characteristics of kidney transplant recipients with bortezomib-treated antibody-mediated rejection (AMR).

Patient	Gender	Age (years)	Cause of ESRD	KTx No	PRA (%)	PRA max (%)	vPRA Class I	vPRA Class II	vPRA Class I+II	Pre-Treatment DSA (MFI)	Induction	Early Graft Function
1	M	30	GNC	2	50	87	98.108	0	98.108	A2 (3489)	rATG	SGF
2	M	41	Unknown	1	0	15	0	0	0	A2 (15,101)	rATG	SGF
3	M	61	ADPKD	2	0	0	31.732	0	31.732	A1 (1968)	rATG	SGF
4	F	61	PNC	1	0	15	46.798	99.476	99.607	DR 4 (10,284)	rATG	IGF
5	M	68	PNC	1	0	0	n/a	n/a	n/a	n/a	IL-2RB	SGF
6	F	52	GNC	1	0	0	30.335 *	80.568	85.604	DR 13 (1500)	rATG	SGF
7	M	61	GNC	2	0	0	0	41.179	41.179	DR10 (11,509)	None	DGF
8	F	63	GNC	1	25	40	68.006	0	68.006	B18 (11,321)	rATG	SGF
9	F	56	Unknown	1	0	0	12.620	93.770	94.541	A11 (1798)	rATG	DGF
10	M	47	GNC	3	5	70	96.361	42.897	97.977	A68 (13,936)	rATG	DGF
11	F	51	ADPKD	1	0	0	75.197	48.049	86.827	B35 (4899)	rATG	DGF
12	M	47	ADPKD	1	0	0	0	0	0	A2 (1992)	IL-2RB	DGF
13	F	60	DM	1	0	0	0	0	0	A3 (541)	IL-2RB	DGF

* Only Cw antigens. AMR, acute antibody-mediated rejection; ESRD, end-stage renal disease; KTx, kidney transplantation; PRA, panel-reactive antibodies; vPRA, virtual PRA; DSA, donor-specific antibodies; MFI, mean fluorescence intensity; GNC, glomerulonephritis; PNC, pyelonephritis; ADPKD, autosomal dominant polycystic kidney disease; DM, diabetes mellitus; rATG, rabbit antithymocyte globulin; IL-2RB, interleukin-2 receptor blocker; SGF, slow graft function; IGF, immediate graft function; DGF, delayed graft function; n/a, non available.

Table 2. Protocol biopsy findings and serum creatinine concentration before primary AMR treatment and during the follow-up period.

Patient	Bx POD	Banff Biopsy Scoring										C4d	Other Diagnosis	DSA	Diagnosis	Serum Creatinine Concentration (mg/dL)				
		(g)	(t)	(i)	(v)	(ah)	(ptc)	(cg)	(ct)	(ci)	(cv)					Pre-Treatment	Post-Treatment	3 Month	12 Month	24 Month
1	10	2	0	0	0	0	2	0	0	0	0	1		1	AMR	5.7	2.2	1.1	1.1	1.1
2	8	2	0	0	0	1	2	0	0	0	0	1		1	AMR	2.5	1.8	1.2	1.3	1.3
3	9	2	0	1	1	0	1	0	1	0	0	1		1	AMR	1.8	0.8	1.1	0.9	0.8
4	10	1	0	0	0	0	1	0	0	0	0	1		1	AMR	0.9	0.7	0.8	1.1	0.8
5	9	2	0	0	0	0	1	0	0	0	0	1		0	AMR susp	1.6	1.3	1.2	1.4	1.3
6	18	0	0	2	0	0	2	0	0	0	0	1		1	AMR	6.2	1.5	0.9	0.9	0.9
7	9	3	0	3	1	0	2	0	0	0	0	0	TCMR	1	AMR susp	12.7	1.5	1.3	1.5	
8	10	0	0	0	1	1	0	0	1	0	0	0	TCMR IIA	1	AMR susp	2.7	1.7	1.5	1.6	
9	10	0	0	0	0	2	0	0	0	1	0	1		1	AMR	5.9	1.7	1.7		
10	10	2	0	0	1	0	2	2	0	0	0	1		1	AMR	8.5	3.0	1.4		
11	8	0	0	0	3	0	2	0	0	0	2	1		1	AMR	3.9	1.9	1.7		
12	11	1	1	2	0	2	2	0	0	0	1	0		1	AMR susp	4.0	1.2	1.9		
13	9	2	0	1	1	0	2	0	1	1	1	0	CNI	1	AMR susp	3.1	1.4	1.4		

AMR, acute antibody-mediated rejection; AMR susp, the suspected AMR; Bx, protocol biopsy; POD, post-operative day; DSA, donor-specific antibodies; TCMR, T-cell mediated rejection; CNI, calcineurin inhibitor toxicity.

3.3. Kidney Graft Function and Survival

After bortezomib-based primary AMR therapy, kidney graft function improved in all patients (serum creatinine concentration decreased from mean 4.6 (2.6–6.6) mg/dL before to 1.6 (1.3–1.9) mg/dL after treatment; $p < 0.001$). Mean 3-month serum creatinine concentration (S_{Cr}) was 1.35 ± 0.3 mg/dL (eGFR 55.3, 95%CI: 44.9–65.8 mL/min/1.73m^2), 8/13 patients completed 12-month follow-up with mean S_{Cr} 1.2±0.3 mg/dL (eGFR 60.4, 95%CI: 45.4–75.4 mL/min/1.73m^2), and 6/13 patients completed a 24-month follow-up period with mean S_{Cr} 1.0 ± 0.2 mg/dL (eGFR 73.9, 95%CI: 56.7–91.1 mL/min/1.73m^2) (Table 2). In post-treatment control protocol biopsies (Figure 2), four patients presented normal histology, one patient showed the partial resolution of microvascular inflammation, one patient presented mild signs of acute tubular necrosis, and one specimen was inadequate for histopatologic diagnosis. The signs of acute humoral rejection (C4d-) were still present in one patient. One patient was transferred to other transplant center and the results of her control biopsy are unknown.

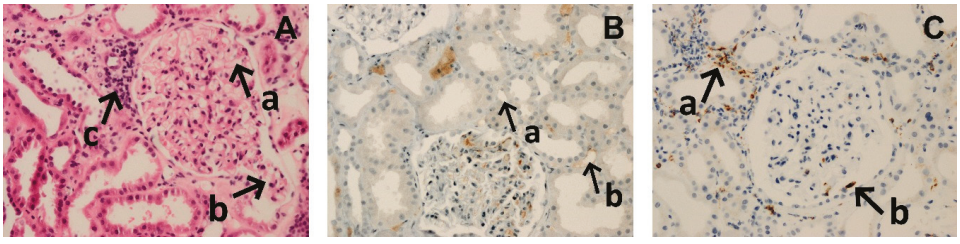

Figure 2. Microphotograph presenting histological findings after bortezomib-based therapy of acute antibody-mediated rejection. (**A**) Hematoxylin and eosin, magnification 200×. Patent capillary lamina without signs of microangiopathy or glomerulitis (a). Prolapse of capillary tuft into the lumen of proximal tubule (b). Small foci of interstitial inflammatory infiltrates (c). (**B**) Negative (a) and nonspecific (b) peritubular capillary C4d staining. Magnification 200×. (**C**) Small interstitial foci of CD68+ cells (a). Single CD68+ cells within glomeruli (b). Magnification 100×.

In the follow-up period of median 21 (IQR: 6–30) months, both patient and kidney graft survival was 100%. Post treatment DSAs were determined in nine patients and were absent in three of them. Median MFI was significantly lower (1373 (IQR: 0–3046)) than prior to treatment.

3.4. Treatment Safety

Despite the preceded induction therapy with rATG in nine patients treated with bortezomib/plasmapheresis AMR protocol approximately 2–3 weeks later, the treatment was generally well tolerated. In one patient, the last dose of bortezomib was cancelled due to gastrointestinal toxicity. Additionally, in one patient, due to the serious hemorrhage after each of the first two PF sessions and the need of reoperation due to large hematoma, the next six PF had to be performed using citrate to avoid heparin administration. In one patient, we observed ascites of unkonwn reason, which resolved thereafter, and in another, the nasal ulceration was noticed. One patient developed a urinary tract infection during AMR treatment, and another patient presented a reccurrent upper respiratory tract infection within a few post-transplant months, which finally resolved thereafter. Based on the laboratory parameters, neutropenia < 1000 cells/μL was observed in one patient, third or fourth grade thrombocytopenia (<50,000 cells/μL) was observed in two patients, and ten patients needed a blood transfusion (median: 2 units/patient). Finally, in seven patients, the transient mild elevation of liver function tests was noted. No other serious adverse events, including neurotoxicity, opportunistic infections, or malignancy, were observed during the follow-up period.

4. Discussion

In this study, we presented our results of the first-line treatment of early post-kidney transplantation AMR based on the administration of bortezomib accompanied by plasma exchange and/or rATG and IVIG. Those early and mid-term outcomes, quantified by kidney graft function, seem to be adequate, being significantly better than previously reported outcomes in KTRs with the diagnosis of early AMR, in whom the first-line treatment was based on ATG, plasmaphereses, IVIG, and/or rituximab, and then the rescue treatment with bortezomib was applied. Additionally, the overall safety profile during and after the AMR treatment was acceptable.

The use of proteasome inhibitor in the treatment of early or late AMR was postulated because it targets antibody-producing plasma cells [25]. Notably, the vast majority of AMR episodes are diagnosed during the later post-transplant period, as pre-transplant DSA titers are increasing or de novo DSAs are produced as a consequence of substantial HLA mismatch, immunosuppressive regimen minimization, non-adherence which is increasing over time, and other relevant factors [26,27]. Hence, until now, the main evidence regarding the effectiveness of multimodal AMR treatment in KTRs is based on late AMR episodes, where its success rate is only moderate. Moreover, bortezomib was usually used as a second-line therapy, after the failure of the initial treatment. Regarding its potential utility as a first-line medication, the literature evidence is scarce. To date, after the first report of two cases [15], the same group published the more comprehensive study, including ten patients who received bortezomib-based primary AMR treatment [11]. However, in all these patients, this primary treatment consisted of bortezomib, plasmapheresis, and a single dose of rituximab. The only previous evidence of the sole effect of bortezomib/plasmapheresis as a primary AMR treatment is the comparison of ten patients treated with bortezomib-based regimen with the historic control group of nine patients who received the rituximab-based AMR regimen [16]. The 18-month graft survival was 6/10 in the bortezomib group, much worse than in our present report. However, they diagnosed AMR episodes based on the indication, not protocol biopsies, which may suggest later diagnosis and treatment as compared to our study. Secondly, there were acute and chronic AMR cases mixed together in both groups and the exact post-transplant timing of AMR therapy diagnosis and treatment initiation was not given. Additionally, all PF sessions were performed using only 4% albumin, whereas in our cohort, a fresh frozen plasma constituted approximately 50% of total PF exchange volume. These particular differences, especially those involving the timing of bortezomib therapy initiation, may partly explain the considerable difference in outcomes of kidney grafts after an AMR episode.

We decided to introduce the protocol based on the four labeled doses of bortezomib associated with the concomitant plasmapheresis sessions. In two patients without plasmaphereses, rATG and IVIG were administered instead. Of note, rixutimab was not used in study patients. At the time, as well as the negative literature concerning the efficacy of rituximab in AMR treatment, we also kept in mind the relatively high cost of our previously used rituximab protocol (approximately $1352 USD/dose in patients with 1.8 m^2 of body surface). Instead, the cost of bortezomib is negligible (~$44 USD/dose in a given patient). Our present case series results suggest an adequate effectiveness of such protocol. In fact, the mean serum creatinine concentration after 12 and 24 months was just about optimal. Of note, the previously reported kidney graft outcomes after the refractory AMR treatment were noticeably worse [7,9,13,14], with several graft losses and suboptimal kidney graft excretory function. We can only hypothesize that some specific properties of bortezomib may condition its effect in the early AMR. As was shown by Perry et al. [19], bortezomib, but not rATG or rituximab, completely abolished anti-HLA antibody production against all HLA specifities. Besides, it also induced the significant increase of apoptotic plasma cells' percentage in vitro [19]. It is possible that the removal of circulating antibodies by PF results in a rebound of their production, thereby enhancing the sensitivity to proteasome [28]. It could partly explain the efficacy of our primary approach to early post-transplant AMR, with bortezomib, but not rituximab, given immediately as a first-line agent accompanied by PF. As the 12-month serum creatinine concentration was shown to be a good predictor of the long-term

kidney graft survival [29], we may expect that the routine primary AMR therapy including bortezomib could also optimize the long-term results of kidney transplantations in this group of patients.

When analyzing the safety features, it is worth to notice that the spectrum of potentially bortezomib-related hematologic and gastrointestinal disturbances is generally similar to those observed after rATG induction, which was received by the majority of patients presented in this study. Thus, we may assume that the use of bortezomib did not increase the risk of adverse events in subjects who received both medications. Only one patient with leukopenia after initial rATG induction later presented aggravated leukopenia and neutropenia during the bortezomib/plasmapheresis treatment. Besides, in four patients who were not treated with rATG, but received bortezomib, we observed only mild leukopenia in two, thrombocytopenia in two, and slight elevation of liver enzymes activities in seven. Overall, the observed adverse events spectrum in our cohort was similar to the previously reported abnormalities obtained during the treatment of refractory AMR [7,9]. In patients with multiple myeloma, neurotoxicity often limits the use of bortezomib [30]. In this case series, however, we did not observe neuropathy associated with bortezomib use.

We are aware about the study limitations, namely its retrospective character and the low number of patients. However, we present the largest case series of KTRs with early diagnosed AMR, primarily treated with bortezomib. Heterogeneity of AMR treatment regimen is the another limitation, with 2/13 patients treated without plasmaphereses (but with rATG and IVIG instead), and with plasmaphereses performed in the first three patients only with sole albumin supplementation. Nevertheless, rituximab was not used in these patients, whereas the labeled dose of bortezomib was the core of the primary AMR therapy. Also, the last few patients had a shorter follow-up period and a lack of post-treatment biopsies and assessment of DSAs. Nevertheless, 6/13 patients completed the 24-month and 8/13 completed the 12-month observation period. Finally, the authors considered the lack of control group as a serious limitation.

In conclusion, the mid-term observation of primary bortezomib-based treatment of early post-kidney transplant AMR showed its non-inferiority as compared to previously proposed regimens and acceptable safety profile. It could encourage clinicians to perform early protocol biopsies, especially in patients with high immunological risk, and initiate such a therapeutic protocol as a first-line treatment. However, our data should be confirmed in a larger, multicenter randomized trial.

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Altered Gut Microbial Fermentation and Colonization with *Methanobrevibacter smithii* in Renal Transplant Recipients

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Abstract: Renal transplant recipients (RTRs) often suffer from posttransplant diarrhea. The observed dysbiosis in RTR may influence the fermentation processes in the gut. In this study, we aimed to investigate whether fermentation differs between RTRs and healthy controls (HCs), by measuring breath H_2 and CH_4 concentrations. Additionally, we determined the fecal presence of the methanogen *Methanobrevibacter smithii* (*M. smithii*), which plays a main role in the process of methanogenesis. Data from the TransplantLines Biobank and Cohort Study (NCT03272841) was used. A total of 142 RTRs and 77 HCs were included. Breath H_2 concentrations in RTRs were not significantly different from HCs. Breath CH_4 concentrations in RTRs were significantly lower compared with HCs (median [interquartile range (IQR)] 7.5 [3.9–10.6] ppm vs. 16.0 [8.0–45.5] ppm, $p < 0.001$). *M. smithii* was less frequently present in the feces of RTRs compared to HCs (28.6% vs. 86.4% resp., $p < 0.001$). Our findings regarding the altered methanogenesis in the gut of RTRs show similarities with previous results in inflammatory bowel disease patients. These findings provide novel insight into the alterations of fermentation after renal transplantation, which may contribute to understanding the occurrence of posttransplant diarrhea.

Keywords: posttransplant diarrhea; methanogenesis; *Methanosphaera stadtmanae*; mucins; sulfate-reducing bacteria

1. Introduction

Renal transplantation is the preferred treatment for patients with end-stage renal disease [1–3]. Part of its success has been made possible by improved therapeutic options, such as ameliorations in surgical techniques and perioperative care [4]. Despite the success of transplantation, the burden of morbidity in renal transplant recipients (RTRs) remains high [5].

Patients often experience gastrointestinal complaints such as diarrhea, which is associated with premature kidney allograft failure and mortality, and which affects quality of life [5,6]. This posttransplant diarrhea is believed to be non-infectious and induced by the use of medication [5,7]. Recently, a study in RTRs showed that dysbiosis in the gut might cause or contribute to this posttransplant diarrhea [7]. Lee et al. demonstrated in this study that the gut microbiota diversity of RTRs with diarrhea was significantly lower than in RTRs without diarrhea. In addition, RTRs with diarrhea had a lower diversity of commensal bacterial taxa in the gut, creating a dysfunctional metabolic state. These commensal bacterial taxa are important for the degradation of complex molecules such as complex carbohydrates. During this degradation, among many other molecules, short-chain fatty acids are produced, which contributes to overall gut health [8,9]. It has been proposed that posttransplant diarrhea might be the consequence of a diminished ability to digest complex sugars [7]. A proportion of complex polymers such as fibers escape digestion and absorption in the small bowel. These complex polymers are then fermented to short-chain fatty acids (acetate, butyrate and propionate) and gases (hydrogen (H_2) and carbon dioxide (CO_2)) [10].

In order to maintain fermentation, it is essential that H_2 concentration is reduced by H_2-consuming microorganisms [11]. H_2 can be used as an electron donor in sulfate respiration, methanogenesis or acetogenesis to produce hydrogen sulfide (H_2S), methane (CH_4) and acetate, respectively [12]. Production of H_2S is most favorable, followed by the production of CH_4 and acetate, respectively. However, for the production of H_2S, the presence of sulfate is necessary [13]. The production of CH_4 is performed by archaea. *Methanobrevibacter smithii* (*M. smithii*) and *Methanosphaera stadtmanae (M. stadtmanae)* are the two methanogens usually detected in the human gut. *M. smithii* is the predominant methanogen in the human colon [14]. Next to H_2, formate can be used for the methanogenesis as well [15]. The produced CH_4 and the remaining H_2 are excreted in breath and flatus. Therefore, both gases can be measured in exhaled breath [13]. Measuring breath CH_4 concentrations is a simple way to investigate the metabolism of intestinal methanogens, since no significant catabolism elsewhere in the human body has been observed [12]. The presence of *M. smithii* can be measured in the feces, as has previously been performed in studies investigating patients suffering from inflammatory bowel disease (IBD) [12,16].

The dysbiosis in RTRs may influence the fermentation in the gut and the processes following fermentation, possibly leading to or contributing to posttransplant diarrhea. To gain more insight into pathogenesis of this diarrhea, we aimed to investigate the fermentation and methanogenesis in the gut in RTRs. Firstly, we aimed to investigate whether breath H_2 and CH_4 concentrations differ between RTRs and HCs. Secondly, we aimed to investigate whether the presence of *M. smithii* in feces differs between RTRs and HCs, and finally we aimed to identify the determinants of CH_4 production.

2. Methods

2.1. Study Population

For this study we used data from the TransplantLines Biobank and Cohort Study (ClinicalTrials.gov identifier: NCT03272841). A detailed description of the study design, inclusion and exclusion criteria has been described previously [17]. In addition to the standard protocol, we measured breath H_2 and CH_4 concentrations and analyzed the presence of *M. smithii* in feces for the current study. (Potential) living organ donors were used as a healthy control group for comparison. Our inclusion period was between February and December 2017. The study protocol has been approved by the Institutional Review Board (METc 2014/077) (METc UMCG), adheres to the UMCG Biobank Regulation, and is in accordance with the WMA Declaration of Helsinki and the Declaration of Istanbul [17].

2.2. Patient Comorbidities

Diabetes mellitus was defined according to the guidelines of the American Diabetes Association [18]. The estimated glomerular filtration rate (eGFR) was calculated using the serum creatinine-based chronic

kidney disease epidemiology collaboration (CKD-EPI) formula. Data regarding the history of allograft rejection and primary renal disease before transplantation were retrieved from patients' medical files.

2.3. Breath H_2 and CH_4 Concentration Measurement

For H_2 and CH_4 measurements, breath samples were collected using a 50 cc syringe with an opening of 6 mm in diameter at approximately 40 cc with a 3-way-stopcock. Subjects were instructed to inhale normally and exhale fully in the syringe, with the plunger set at 50 cc and the 3-way stopcock open. After full expiration, the opening was immediately closed by the subject's finger, the plunger was set to 30 cc and the 3-way stopcock was closed. This resulted in breath samples that were not diluted by environmental air. Two breath samples were taken subsequently per study subject. Breath samples were analyzed within 12 h after sample collection. H_2, CH_4 and CO_2 measurements were performed using a solid-state gas-chromatography device (Breathtracker SC, QuinTron Instrument Company, Inc., Milwaukee, WI, USA). The device separates the components by the basic principle of gas chromatography, using room air as the carrier gas, which is pumped through the system by an internal circulating pump. H_2 and CH_4 are separated from all other reducing gases and from each other, and are carried past a solid-state sensor [19]. The sensors are reported to be affected only by reducing gases, so it is unaffected by other gases in the sample; it can also employ a CO_2 correction factor [19]. The analytical sensitivity is 1 ppm for H_2 and CH_4 and 0.1% for CO_2. The Breathtracker has a linear analytical range of 2–150 ppm for H_2, 2–75 ppm for CH_4 and 1000–70,000 ppm for CO_2. To ensure reliable breath measurements, study subjects were not allowed to smoke for at least one hour before the sample collection [20].

2.4. M. Smithii Measurement in Feces

Fecal samples were collected the day prior to the TransplantLines visit, using a FecesCatcher (TAG Hemi VOF, Zeijen, The Netherlands) and were immediately frozen after collection. The feces samples were transported in cold storage to the TransplantLines visit, and immediately stored at −80 °C (−112 °F) [17]. After thawing, DNA was extracted with the RBB and Qiagen method, as performed by Yu et al. with modifications described by de Goffau et al. [21,22]. To measure the quantity of *M. smithii*, real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) (7500 real time PCR system, applied Biosystems, Thermo Fisher Scientific, Waltham, USA) was performed. Primers were taken as described by Johnston et al., and differentiation between *M. smithii* and other organisms in the sample was assessed using *nifH* genes [23]. The number of *nifH* genes are equal to the number of *M. smithii*, since only one gene of *nifH* is present in each *M. smithii* [24]. Analyses were performed using the Taqman machine and processed using SDSShell (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA). The quantifiable presence of *M. smithii* was determined using a cycle threshold value. Values < 40 cycles were regarded as positive, and values ≥40 were regarded as negative. For analyses, CT-values ≥40 were regarded as negative and concentrations of *M. smithii* in these patients were regarded as 0 *M. smithii*/gram feces. A detailed method description is attached in Supplementary File 1.

2.5. Statistical Analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 23.0 (IBM corp.; Armonk, NY, USA). In all analyses, $p < 0.05$ was regarded as statistically significant. Categorical variables are presented as n (%), normally distributed variables as mean ± standard deviation (SD) and non-normally distributed variables as median [interquartile range]. Normality was assessed using Q–Q plots. Differences between groups with normally distributed variables were assessed using independent T-tests. Non-normally distributed data were compared using the Mann–Whitney U test. Comparison of categorical variables was performed using a chi-square test for groups with $n \geq 5$ and a Fisher's exact test for groups with $n < 5$. For all other tests and visualizations, the mean of the duplicate measurements of the breath H_2 and CH_4 concentration in breath was used. To correct for environmental CH_4, 2 ppm was subtracted from each breath

CH_4-measurement [25,26]. Possible determinants of breath CH_4 were identified using univariable linear regression. All variables with a p-value <0.2 were included in a multivariable linear regression model run backward to identify the determinants of breath CH_4 production. Because H_2 is used by *M. smithii* for the conversion to CH_4, an interaction term of H_2 and *M. smithii* was added in the analysis. Log_{10} transformations were performed if necessary to reach conditions in all performed analyses.

3. Results

We included 219 study subjects, of whom 142 (64.8%) were RTRs and 77 (35.2%) were HCs. Among RTRs, 91 (64.1%) were male, and the mean age was 56.3 ± 13.7 years. Among HCs, 39 (50.6%) were male, and the mean age was 56.4 ± 10.6 years. Baseline characteristics are shown in Table 1. A Consort Flow diagram is presented to provide an overview of subgroups that were used in different analyses (Figure 1). Breath H_2 concentrations of the RTRs were not significantly different compared with HCs (Table 1). The RTRs had, however, lower breath CH_4 concentrations compared to the HCs (7.5 [3.9–10.6] ppm vs. 16.0 [8.0–45.5] ppm, $p < 0.001$). Data distributions of breath H_2 and CH_4 concentrations are shown in Supplementary File 2. Raw data are shown in the Supplementary data.

Table 1. Baseline characteristics.

Characteristics	Renal Transplant Recipients	Healthy Controls	*p*-Value
Number of subjects, *n* (%)	142 (64.8)	77 (35.2)	*n*/a
Fermentation parameters			
Breath H_2 concentration, ppm	11.3 [4.0–30.0]	10.5 [4.5–28.3]	0.9
Breath CH_4 concentration, ppm	7.5 [3.9–10.6]	16.0 [8.0–45.5]	<0.001
Quantifiable abundance of *M. smithii* in feces, *n* (valid %)	22 (28.6)	38 (86.4)	<0.001
Abundance of *M. smithii* in feces samples, *M. smithii*/gram	0.0 [0.0–4.0 × 10^5]	5.9 × 10^7 [1.2 × 10^6–8.9 × 10^8]	<0.001
Demographics			
Age, y	56.3 ± 13.7	56.4 ± 10.6	0.6
Number of males, *n* (%)	91 (64.1)	39 (50.6)	0.05
BMI, kg/m^2	28.0 ± 5.2	26.4 ± 3.8	0.01
Time since transplantation, y	1.0 [0.5–8.0]	-	n/a
Lifestyle parameters			
Current smokers, *n* (valid %)	23 (16.7)	14 (18.9)	0.4
Alcohol intake per day, units	0.0 [0.0–0.2]	0.25 [0.0-0.5]	0.003
Laboratory parameters			
Hemoglobin, g/dL	13.8 ± 1.9	14.4 ± 1.3	0.008
Hematocrit, L/L	0.42 ± 0.06	0.43 ± 0.04	0.2
Leukocytes, 10^9/L	7.4 ± 2.5	6.5 ± 1.9	<0.003
Platelets, 10^9/L	250.6 ± 78.4	261.2 ± 56.6	0.3
C-reactive protein, mg/L	2.3 [1.1–5.0]	1.2 [0.8–4.0]	0.035
Albumin, g/L	44.2 ± 3.1	45.4 ± 2.5	0.003
Glucose, mmol/L	6.0 ± 1.7	5.5 ± 0.7	0.005
HbA1c, mmol/mol	42.3 ± 7.8	36.9 ± 3.5	<0.001
eGFR, ml/min/1.73 m^2	49.8 ± 16.5	69.3 ± 18.7	<0.001
Creatinine, µmol/L	130 [103.0–156.8]	92.0 [81.0–106.0]	<0.001
Urea, mmol/L	9.4 ± 4.4	5.8 ± 1.6	<0.001
Medication use			
Antibiotics, *n* (%)	37 (16.9)	0 (0.0)	<0.001
Immunosuppressants, *n* (%)			
Prednisolone, *n* (%)	140 (98.6)	-	*n*/a
Mycophenolate mofetil, *n* (%)	112 (78.9)	-	*n*/a
Tacrolimus, *n* (%)	102 (71.8)	-	*n*/a

Table 1. *Cont.*

Characteristics	Renal Transplant Recipients	Healthy Controls	*p*-Value
Cyclosporine, *n* (%)	14 (9.9)	-	*n*/a
Everolimus, *n* (%)	7 (4.9)	-	*n*/a
Azathioprine, *n* (%)	10 (7.0)	-	*n*/a
Statins, *n* (%)	70 (49.3)	8 (10.4)	<0.001
Proton pump inhibitors, *n* (%)	108 (76.1)	0 (0.0)	<0.001
Insulin, *n* (%)	11 (7.8)	0 (0.0)	0.009
Biguanides, *n* (%)	7 (4.9)	0 (0.0)	0.09
Macrogol, *n* (%)	8 (5.6)	1 (1.3)	0.200
Lactulose, *n* (%)	2 (1.4)	0 (0.0)	0.500
Loperamide, *n* (%)	1 (0.7)	0 (0.0)	1.000
Antidepressants, *n* (%)	16 (7.3)	4 (5.2)	0.4
Primary renal disease before transplantation			
Unknown, *n* (%)	23 (16.2)	-	*n*/a
Inflammatory disease	55 (38.7)		
Congenital and hereditary kidney disease, *n* (%)	41 (28.9)	-	*n*/a
Renal vascular disease, excluding vasculitis, *n* (%)	13 (9.2)	-	*n*/a
Diabetic nephropathy, *n* (%)	10 (7.0)	-	*n*/a
Others			
Diabetes mellitus, *n* (%)	27 (19.0)	1 (1.3)	<0.001
History of allograft rejection, *n* (%)	14 (9.9)	-	*n*/a

Data are presented as mean ± standard deviation (SD), median with interquartile ranges (IQRs) or number with percentages (%). Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rate; CH_4, methane; H_2, hydrogen; HbA1c, hemoglobin A1c; *M. smithii*, *Methanobrevibacter smithii*.

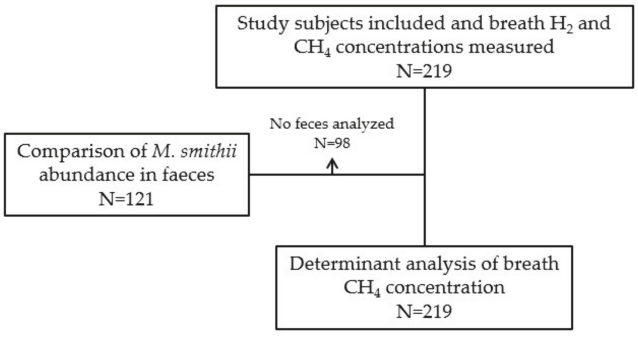

Figure 1. Consort flow diagram. Abbreviations: CH_4, methane; H_2, hydrogen; *M. smithii*, *Methanobrevibacter* smithii.

3.1. M. Smithii in Feces

The feces of 98 study subjects was not available for analysis. *M. smithii* abundance was analyzed in the feces samples of 77 RTRs and 44 HCs (i.e., 121 of 219 study subjects, see Figure 1). Among the RTRs, 22 (28.6%) had quantifiable concentrations of *M. smithii* in their stool samples. Among HCs, 38 (86.4%) had quantifiable concentrations of *M. smithii* in their feces samples. The median abundance of *M. smithii* in the feces of those study subjects was 5.9×10^7 [1.2×10^6–8.9×10^8] per gram feces. A quantifiable concentration of *M. smithii* was significantly less frequently observed in RTRs compared to HCs ((22 (28.6%) vs. 38 (86.4%) resp.; $p < 0.001$) (Table 1). In addition, the abundance of *M. smithii* was positively correlated with breath CH_4 concentrations ($r = 0.69$, $p < 0.001$).

3.2. Determinants of Breath CH_4

Determinants of breath CH_4 were analyzed using linear regression analysis in all 219 study subjects, and these results are presented in Table 2. Breath H_2 and the presence of a quantifiable abundance of *M. smithii* in feces were associated with higher breath CH_4 concentrations (standardized beta (st. β) 0.57, $p < 0.001$ and st. β 0.94, $p < 0.001$ resp.). A negative interaction was found between both determinants on breath CH_4 (st. β −0.51, $p = 0.001$), indicating that in the presence of *M. smithii* the magnitude of the correlation between H_2 and CH_4 in breath decreases from overt to virtually absent ($r = 0.88$, $p < 0.001$ vs. $r = 0.09$, $p = 0.5$ resp., Figure 2). In addition, the use of mycophenolate mofetil was associated with a lower breath CH_4 concentration (st. β −0.18, $p = 0.014$). The described determinants explained 55.0% of the total variation in breath CH_4 concentrations.

Table 2. Linear regression analysis of $\log_{10}$ breath CH_4 concentration.

	Univariable Linear Regression Analysis		Multivariable Linear Regression Analysis *	
	St. β	***p*-Value**	**St. β**	***p*-Value**
A medical history of renal transplantation (yes vs. no)	−0.42	<0.001		
Fermentation parameters				
Log_{10} Breath H_2, ppm	0.32	<0.001	0.54	<0.001
Quantifiable abundance of *M. smithii* in feces (yes vs. no)	0.55	<0.001	0.95	<0.001
Interaction between $\log_{10}$ breath H_2 and *M. smithii* in feces	0.48	<0.001	−0.51	0.001
Demographics				
Age, y	0.07	0.3		
Gender (yes vs. no)	−0.02	0.7		
BMI, kg/m^2	−0.18	0.012		
eGFR, mL/min/1.73 m^2	0.25	<0.001		
Intoxications				
Smoking (yes vs. no)	−0.10	0.1		
Alcohol (units per day)	−0.01	0.9		
Medication use (yes vs. no)				
Antibiotics	−0.12	0.1		
Immunosuppressive medication (yes vs. no)				
Prednisolone	−0.40	<0.001		
Mycophenolate mofetil	−0.36	<0.001	−0.18	0.014
Tacrolimus	−0.27	<0.001		
Cyclosporine	−0.06	0.4		
Azathioprine	0.00	1.0	−0.10	0.1
Everolimus	0.04	0.5		
Statins	−0.15	0.024		
Proton pump inhibitors	−0.26	<0.001		
Macrogol	0.06	0.4		
Lactulose	0.10	0.1		
Loperamide	0.02	0.8		
Biguanide drugs	−0.04	0.6		
Insulin	−0.05	0.5		
Antidepressants	−0.14	0.044		
Primary renal disease of RTR (yes vs. no)				
Unknown	−0.04	0.6		
Inflammatory disease	0.02	0.8		
Congenital and hereditary kidney disease	0.00	1.0		
Renal vascular disease, excluding vasculitis	0.09	0.3		
Diabetes Mellitus	−0.08	0.3		
Others (yes vs. no)				
Suffering from Diabetes Mellitus	−0.06	0.4		
History of allograft rejection	−0.02	0.8		

Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rate; CH_4, methane; H_2, hydrogen; *M. smithii*, *Methanobrevibacter smithii*; St. β, standardized beta. $R^2 = 0.550$. * Run backwards.

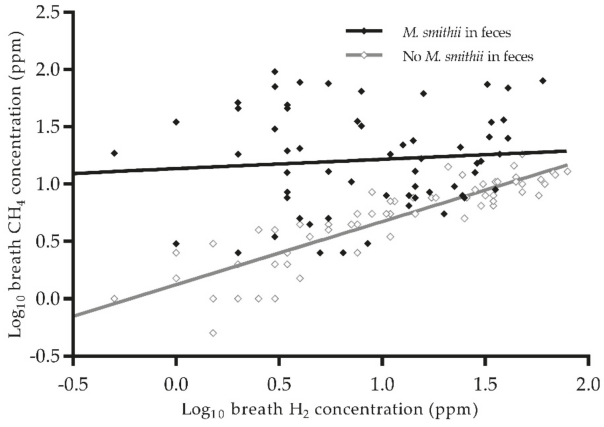

Figure 2. Scatterplot of $\log_{10}$ breath H_2 and CH_4 concentration by presence of *M. smithii* in feces. There is a difference between the relation between H_2 and CH_4 in subjects with and without *M. smithii*. Abbreviations: CH_4, methane; H_2, hydrogen; *M. smithii*, *Methanobrevibacter smithii*. $N = 121$. Pearson correlation in the absence of *M. smithii*, $r = 0.88$, $p < 0.001$. Pearson correlation in the presence of *M. smithii*, $r = 0.09$, $p = 0.5$.

4. Discussion

We have shown that although no significant difference in breath H_2 concentration was found between RTRs and HCs, breath CH_4 concentrations were significantly lower in the RTRs compared with the HCs. In addition, we found a significantly lower presence of *M. smithii* in the feces of RTRs compared with HCs. Breath H_2 and the presence of *M. smithii* in feces were associated with higher breath CH_4 concentrations. Moreover, the association between breath H_2 and CH_4 concentrations disappeared in presence of *M. smithii* in feces. Finally, mycophenolate mofetil was associated with a lower breath CH_4 concentration.

The reduced breath CH_4 concentration in RTRs compared to HCs which we observed might be explained by the reduced presence of *M. smithii* in the feces of RTRs, since *M. smithii* is the most abundant methanogen in the human gut [12]. One reason for the lower prevalence of *M. smithii* in the feces of RTRs may be the result of an increased presence or activity of sulfate-reducing bacteria (SRB). It has been described that a high concentration of either methanogens or sulfate-reducing bacteria is present in the feces of healthy individuals. These two groups of microorganisms appear to be competing for H_2, with the prevailing group becoming the predominant organism [27,28]. However, since no mechanism of direct competition between SRB, methanogens and acetogens has been observed, at this point it is impossible to predict any dominance of one of these hydrogenotrophs [29]. If the gut in RTRs is more colonized with SRB, or if these SRB are more active, more hydrogen sulfide (H_2S) is produced. H_2S is highly toxic to the colonocytes and impairs their metabolic function, especially the butyrate oxidation [30]. Butyrate has a known anti-inflammatory effect and several other health-promoting functions [31]. The presence of butyrate in the lumen and the oxidation by colonocytes are both involved in the regulation of water and sodium absorption from the colon [30]. SRB and the consequent disturbance of butyrate oxidation is believed to play a key role in the pathogenesis of IBD [12]. If SRB are indeed more present in RTRs, this might also be an explanatory factor for the occurrence of any of the gastrointestinal complaints of RTRs [16], especially since the butyrate concentration in RTRs seems to be lower due to the reduced prevalence of bacteria taxa that produce butyrate [7].

In addition, more colonization with SRB, and consequently more H_2S, may diminish the positive effects of the butyrate in the gut of RTRs. However, the presence of SRB was not measured in this study. Although our results regarding the decreased presence of *M. smithii* do support this hypothesis, future studies will have to further test this hypothesis.

Another reason for the lower prevalence of *M. smithii* in feces might be a lower presence of mucins in the gut of RTRs. We observed no significant correlation between breath H_2 and CH_4 concentrations in the presence of *M. smithii*. Therefore, the produced CH_4 by *M. smithii* may be derived from endogenous substrates such as mucins, formate or other unknown substrates [12,32]. Importantly, for mucins it has not yet been settled whether they contribute to methane production or rather inhibit it, or under which circumstances stimulation may shift towards inhibition [32,33]. Mucins cover the epithelium and form a protective layer in the gut, thereby providing a protective layer against pathogenic organisms [34]. Deficiencies of mucin in the intestinal barrier are associated with an abnormal mucosal inflammatory response, which is present in IBD [34]. The role of mucins in the fermentation processes in RTRs has, to our best knowledge, never been investigated.

We also observed a strong correlation between breath H_2 and CH_4 concentrations in the absence of *M. smithii* in feces (Figure 2). Possibly, other methanogens that flourish in the absence of *M. smithii* are more dependent upon H_2 concentrations. One of these methanogens might be *M. stadtmanae*, an archaeon that is the second most common archaeon in the healthy gut after *M. smithii* [35]. It is known that the CH_4 production by *M. stadtmanae* is highly dependent upon the presence of H_2 and methanol [36,37]. An increased prevalence of *M. stadtmanae* has been observed previously in IBD patients in a study by Lecours et al. [38]. Interestingly, it has been reported that *M. stadtmanae* can induce an inflammatory cytokine response from monocyte-derived dendritic cells, which may contribute to pathological conditions in the gut [39]. In order to gain more insight into gut health in RTRs, the prevalence of *M. stadtmanae* needs to be further investigated [12,39,40].

In addition, our study shows that patients using mycophenolate mofetil exhale lower concentrations of CH_4. Previous studies have shown that mycophenolate mofetil is associated with gastrointestinal injury and diarrhea, although any underlying mechanisms are incompletely understood [6]. Lower CH_4 levels are also associated with diarrhea [12]. Future studies may investigate whether methanogenesis plays a role in the association between mycophenolate mofetil and diarrhea.

Our observations are in line with previous studies in IBD patients [12,41]. This is interesting, since RTRs and IBD patients have similarities: both groups suffer from intestinal dysbiosis, often have diarrhea and often need to take immunosuppressive medication [38,42–44]. Scanlan et al. observed a significantly lower presence of methanogen-positive feces samples in patients suffering from ulcerative colitis compared to healthy controls (24% versus 48%). In addition, a lower presence of methanogens in patients suffering from Crohn's disease was observed (30% versus 48%), although this association was not statistically significant [41]. In another study by Ghavami et al., significantly higher amounts of *M. smithii* were found in the feces samples of HCs compared to IBD patients [16]. Our results suggest that the reduced colonization with *M. smithii*, and possibly the methanogenesis of IBD patients, might be comparable to RTRs.

It is known that CH_4 reduces inflammation, oxidative stress and apoptosis in the human body [45]. Our findings show significantly lower breath CH_4 concentrations in RTRs compared to HCs, while the protective properties of CH_4 appear especially important in RTRs in the context of (prevention of) renal rejection, inflammation and high levels of oxidative stress [46]. Future studies may further investigate the associations of (breath) CH_4 concentrations with patient outcomes, such as renal rejection. If the hypotheses regarding the protective properties of CH_4 are confirmed, the relatively low CH_4 levels in RTR may be a therapeutic target, since CH_4 concentrations in the body can be increased iatrogenically by inhalation or injection [47,48].

No difference in breath H_2 concentration was found in the current study. This is in line with other studies: the matter of hydrogenotrophics in the gut is highly complex, and is dependent upon many variables [49].

It is a limitation of our study that we did not measure mucin concentrations or potential colonization by SRB or *M. stadtmanae*. In addition, we did not measure H_2 and CH_4 concentrations in flatus, although it has been found that the concentration of both gases are higher in flatus than in breath when the concentrations are high [50]. No data regarding menopausal status was available in our study,

although it is known that menopausal status does affect the gut microbiome [51]. Additionally, we did not measure breath H_2 and CH_4 concentrations and the abundance of *M. smithii* in the feces of RTRs before transplantation. Further limitations of our study are that it was performed in a single center, and that our RTRs were included at different time points after transplantation. Another limitation of this study is that for logistical reasons we were unable to analyze *M. smithii* in the feces of all our study subjects. In addition, although we measured the breath H_2 concentrations in the morning, shortly after breakfast, it was in a non-fasting state. Finally, the current study uses cross-sectional data, and therefore no conclusions regarding causal relationships can be drawn.

5. Conclusions

To the best of our knowledge, this is the first study to investigate both breath and feces samples in RTRs. The study shows that breath CH_4 concentration and the prevalence of *M. smithii* in feces are significantly lower in RTRs compared to HCs. Our findings regarding the altered methanogenesis in the gut of RTRs show significant similarities with previous results in IBD patients. We observed that in the absence of *M. smithii*, breath CH_4 production is highly dependent on H_2 concentration, while this is not the case in the presence of *M. smithii*. Apparently, methanogenesis differs significantly depending on presence of *M. smithii*. Finally, the use of mycophenolate mofetil was associated with methanogenesis. These findings provide novel insight into the alterations of fermentation after renal transplantation, which may contribute to the occurrence of posttransplant diarrhea. In addition, this study has raised important hypotheses regarding the potential role of SRB and *M. stadtmanae* in post-transplant diarrhea. Future studies are needed to investigate the role of SRB and *M. stadtmanae*. Additionally, future research may study whether altered methanogenesis is associated with clinical outcomes, such as posttransplant diarrhea.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/2/518/s1, Supplementary File 1: extended description of the methods used to quantify the number of M. smithii in fecal samples, Supplementary File 2: data distributions of breath H_2 and CH_4 concentrations, Supplementary data: raw data.

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C3d-Positive Preformed DSAs Tend to Persist and Result in a Higher Risk of AMR after Kidney Transplants

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Abstract: C3d-binding assays have been introduced as methods for the prediction of the presence of complement-binding functional antibodies; however, the prognostic value of C3d-positive preformed donor-specific antibodies (pDSAs) has not been fully evaluated. In this study, we performed a retrospective investigation of the association of pDSAs and their C3d-binding capacity with one-year clinical outcomes. pDSAs were defined as donor-specific antibodies (DSAs) that were produced before kidney transplants (KTs) (pre-pDSAs) or within the first four weeks after KTs, owing to rebound immune response (post-pDSAs). Of 455 adult KT recipients, pre-pDSAs and post-pDSAs were found in 56 (12.3%) and 56 (12.3%) recipients, respectively, and C3d-positive post-pDSAs were found in 13 recipients (2.9%) in total. Approximately half of the C3d-negative pre-pDSAs (37/73, 50.7%) disappeared after transplantation; however, all C3d-positive pre-pDSAs (8/8, 100%) persisted after transplantation despite desensitization ($p = 0.008$). C3d-positive pDSAs were significantly associated with a higher incidence and risk of AMR ($p < 0.001$, OR 94.467–188.934). Identification of the C3d-binding activity of pDSAs before and early after KT is important for predicting the persistence of pDSAs and the risk of AMR induced by the presence of pDSAs.

Keywords: kidney transplant (KT); donor-specific antibodies (DSA); C3d-binding assay; antibody-mediated rejection (AMR)

1. Introduction

Donor-specific human leukocyte antigen antibodies (DSAs) are a critical factor in kidney transplantation (KT), as antibody-mediated rejection (AMR) induced by the binding of DSAs to the allograft represents a major post-transplant complication. If DSAs are detected before transplantation, desensitization procedures, such as the administration of rituximab or intravenous immunoglobulin (IVIG), or plasmapheresis, may be performed to reduce the DSA titer and lower the risk of AMR [1].

Single antigen bead-based antibody identification (SAB) assays are generally used as the standard for DSA monitoring. However, the clinical relevance of all the detected DSAs remains unclear because

the presence of DSAs does not always correlate with complement-mediated cytotoxicity crossmatching and may not induce AMR. Recently, C1q- and C3d-binding assays were introduced as methods for predicting the presence of complement-binding functional antibodies; however, the prognostic value of these tests remains controversial [2–8]. In particular, the clinical significance of preformed DSAs (pDSAs) with complement-binding activities has been not fully evaluated.

Human leukocyte antigen (HLA) antibodies at a titer below the SAB assay cut-off level or diluted across multiple beads that share target epitopes may not be appropriately detected in SAB assays. The titer of those cryptic antibodies can be elevated by immunological memory response shortly after KT [9]. Wiebe et al. suggested that false-negative pDSAs and their increased titer after transplantation, due to memory B cell activation, may create the false impression of de novo DSA (dnDSA) early post-transplantation. They suggested that no dnDSA was detected prior to 6 months when using two strict definitions: (1) all historic and current samples were DSA-negative, with an MFI cut-off of 300 and special attention to grouped epitopes; (2) no AMR in protocol biopsies at 6 months after transplantation [10]. Therefore, we thought that DSAs that were found only before KT should not be defined as pDSAs. In this study, we considered all of the DSAs produced within one month following KT as pDSAs and then investigated the production of pDSAs in pre- and post-transplantation and evaluated their effect on the occurrence of acute rejection and clinical outcome associated with their C3d-binding activity.

2. Materials and Methods

2.1. Study Population

Of the 560 adult recipients who underwent KT between January 2013 and July 2017 at the Samsung Medical Centre, Seoul, Korea, 455 patients (279 men and 176 women) were included in this study. Multi-organ, ABO-incompatible, or combined kidney and bone marrow transplantation cases were excluded (Figure 1). Recipients who underwent desensitization owing to a high level of panel reactive antibodies (PRA) (over 50%) without DSAs were excluded. All cases were negative for complement-dependent cytotoxicity (CDC), crossmatched on the day of transplantation. DSA status was monitored pre-transplantation (within 1 month before KT) and post-transplantation (at 1 and 4 weeks after KT). The recipients were divided into four groups according to the presence of DSAs before (pre-pDSA) and after transplantation (post-pDSA): Group 1, the pDSA-negative group (recipients without pre- and post-pDSAs); Group 2, the cryptic pDSA rebound group (comprising recipients with post-pDSA only); Group 3, the pDSA reversed group (recipients with pre-pDSA only); and Group 4, the pDSA persistent group (recipients with both pre- and post-pDSAs). To determine the effect of C3d-binding capacity on clinical outcomes, the post-pDSA positive groups (Groups 2 and 4) were subdivided according to their C3d-binding capacity. A schematic of the study is shown in Figure 2. This study was approved by the Institutional Review Board of Samsung Medical Centre, Seoul, Korea (SMC-2016-07-140-003), and the requirement for the subjects' informed consent was waived.

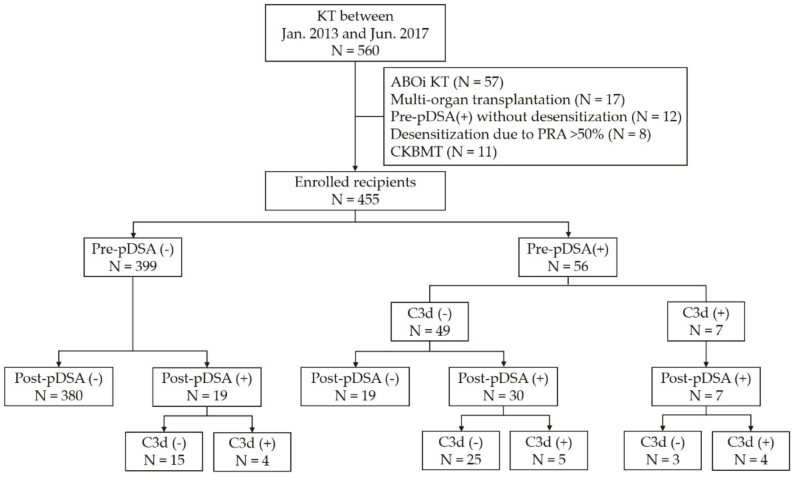

Figure 1. Study population and recipient groups according to donor-specific antibody (DSA) presence and C3d-binding capacity. Pre-pDSA, DSA confirmed before transplantation; Post-pDSA, DSA confirmed within 1 month of KT; KT, kidney transplantation; ABOi, ABO-incompatible; pDSA, preformed donor-specific HLA antibody; PRA, panel-reactive antibody; CKBMT, combined kidney and bone marrow transplantation.

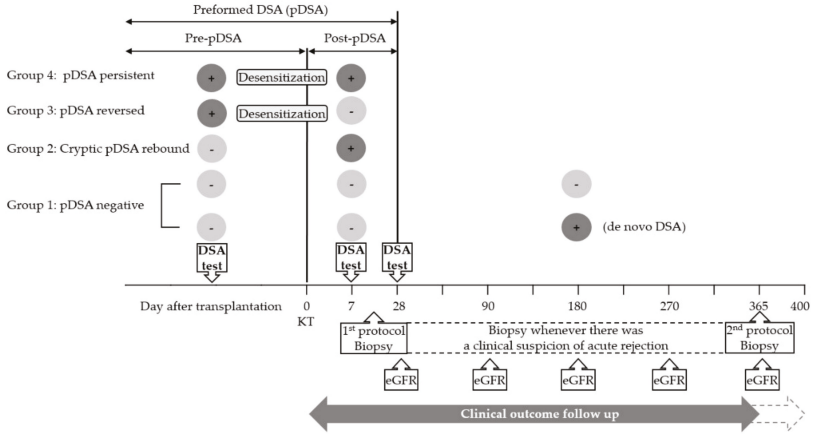

Figure 2. The study scheme showing definitions of recipient groups and a summary of clinical outcome follow up. All DSAs presented within 1 month before KT and produced within 1 month after KT were considered as preformed DSAs (pDSAs). Pre-pDSA, DSAs confirmed before transplantation; Post-pDSA, DSAs confirmed at 1 week and/or 4 weeks after KT; KT, kidney transplantation; pDSA, preformed donor-specific HLA antibody; SAB, single antigen bead-based antibody identification assay; eGFR, estimated glomerular filtration rate.

2.2. Desensitization and Immunosuppression

The desensitization protocol consisted of the administration of rituximab (Genentech Inc., San Francisco, CA, USA), treatment with intravenous immunoglobulin (IVIG) (Green Cross, Seoul, Korea), and plasmapheresis by using a COBE Spectra (Gambro BCR, Lakewood, CO, USA) before transplantation. In recipients with a pre-pDSA mean fluorescence intensity (MFI) of ≥2,500 by SAB assay, all three desensitization protocols were performed; in recipients with a low pre-pDSA MFI of

<2,500 by SAB assay, only rituximab was administered. For the induction of immunosuppression, recombinant anti-thymocyte globulin (rATG) (Genzyme, Cambridge, MA, USA) was administered on Day 0; it was administered post-transplantation on Days 1 and 2. Basiliximab (Simulect, Novartis, Basel, Switzerland) was used to induce immunosuppression in recipients without pre-pDSA. Desensitization and immunosuppression protocol details have been described elsewhere [11].

2.3. Immunologic Assays

HLA-A, -B, -C, -DRB1, and -DQB1 loci HLA typing for donors and recipients was performed by using polymerase chain reaction with sequence-specific primer (PCR-SSP) (One Lambda, Canoga Park, CA, USA) or reverse sequence-specific oligonucleotide probes (rSSOP) (Immucor, Peachtree Corners, GA, USA).

Anti-HLA antibody measurements were performed at 1 and 4 weeks after KT. Anti-HLA class I and II IgG antibodies were tested by using a Luminex bead-based detection assay. The LABScreen Mixed kit (One Lambda, Waltham, MA, USA) was used to screen for class I and II antibodies in conjunction with the HLA Fusion software v3.0 (One Lambda, Canoga Park, CA, USA). Sera that were positive in the screening test were subsequently tested for HLA antibody specificities and the presence of DSA using the LIFECODES LSA Class I and Class II SAB kit (Immucor, Stamford, CT, USA) in accordance with the manufacturer's recommendations; the results were analyzed by using Match-It software v1.2 (Immucor, Norcross, GA, USA). All sera were subjected to SAB treatment with 50 mM dithiothreitol (DTT) for 30 min. Antibody-positive results were assigned when more than two criteria were calculated from background MFI, control MFI was calculated, and the normalization factors recommended by the manufacturer were met.

The complement-binding capacity of DSAs in sera stored at −70 °C was measured using the LIFECODES C3d assay kit (Immucor, Stamford, CT, USA) in accordance with the manufacturer's instructions.

2.4. Data Collection and Statistical Methods

Data describing patient characteristics and their clinical outcomes were obtained from medical records. Categorical variables were summarized by number and percentage (%) and compared among groups by using Fisher's exact test or the chi-square test as appropriate. For continuous variables, the Shapiro–Wilks test was performed beforehand to examine the normality of distributions, and then summarized with mean (SD, standard deviation) or median (IQR, interquartile range) and compared among groups using one-way ANOVA or the Kruskal–Wallis test according to the normality of their distribution.

2.4.1. Recipient Characteristics

Data on demography, underlying diseases, transplantation conditions, conditioning, and immunosuppression regimens were included in the analysis.

2.4.2. HLA Antibody Characterization

Preformed DSAs (pDSAs) were defined as DSAs that were produced before KT (pre-pDSAs), including Groups 3 and 4, or within the first 4 weeks after KT due to rebound immune response (post-pDSAs), such as Group 2. One of our hypotheses was that the cryptic pDSA rebound group (Group 2) would be useful for the assessment of the effects of pDSAs that were undetectable before transplantation using current antibody tests, and patients were therefore not subjected to the pre-transplant desensitization, in contrast to the sensitization in the pre-pDSA positive groups (Groups 3 and 4). Receiver operating curves (ROC) were plotted to assess SAB MFI performance in an effort to predict the C3d-binding activities of HLA antibodies. Optimal cut-offs exhibiting maximal sensitivity and specificity (Youden index) were obtained for risk assessment of the presence of DSAs.

2.4.3. Clinical Outcome

To assess clinical outcomes, graft rejection rate, rejection-free survival, and graft function were evaluated. Graft biopsy was performed on Day 14 and 1 year post-KT, or whenever there was clinical suspicion of acute rejection. Biopsy results up to 400 days after KT were included in the analysis because the protocol-mandated biopsy schedule was delayed for some patients due to hospital or patient circumstances. Acute cellular rejection (ACR) and AMR were diagnosed in accordance with the Banff Criteria 2013 [12]. The rates of ACR and AMR occurrence were compared among recipient groups using Fisher's exact test or the chi-square test, as appropriate. The univariable logistic regression was repeatedly used for the four-group comparison (Groups 1, 2, 3, and 4) and also for two- or four-subgroup comparisons: Group 1, Group 4 C3d (-) subgroup, and Group 4 C3d (+) subgroup. Wald's chi-square test was used for pairwise comparison with Bonferroni's correction. Rejection-free survival rates were estimated by using the Kaplan–Meier method, and the four groups were compared via the stratified log-rank test. Graft function was evaluated using the estimated glomerular filtration rate (eGFR) at 1, 3, 6, 9, and 12 months post-KT. eGFR was calculated using the modification of diet in renal disease (MDRD) study equation. Generalized estimating equation (GEE) analyses were applied to repeated measurements of eGFR. $p < 0.05$ was considered statistically significant.

2.4.4. Statistical Software

Statistical analyses were computed by using SAS v9.4 (SAS Institute, Cary, NC, USA) and SPSS v22.0 (IBM, Armonk, NY, USA). Analyse-it v5.10 (Analyse-it Software, Leeds, UK) was used for graphical analyses.

3. Results

3.1. Recipient Characteristics

Fifty-six recipients (12.3%) had detectable DSAs prior to KT (re-pDSA; Figure 1). The patients were divided into four groups according to their pre- and post-KT DSA status: the pDSA negative group (Group 1; $n = 380$, 83.5%), the cryptic pDSA rebound group (Group 2; $n = 19$, 4.2%), a pDSA reversed group (Group 3; $n = 19$, 4.2%), and a pDSA persistent group (Group 4; $n = 37$, 8.1%). All recipients with pre-pDSA (Groups 3 and 4) underwent desensitization. The recipient characteristics for each group are summarized in Table 1.

In pre-transplantation, seven recipients (12.5%) had C3d-positive pDSAs; however, the number of recipients having C3d-positive pDSAs increased to 13 recipients (23.2%) within the first month after KT. Five recipients exhibited persistently C3d-positive pDSAs before and after transplantation. HLA classes of total and C3d (+) DSAs in Groups 2, 3, and 4 are summarized in Table 2. Class II DSAs were assessed with limited loci, as –DR and -DQB1. In contrast with the higher frequencies of class I HLAs in pre-transplantation, class II HLAs were more frequent in post-transplantation, not only among total DSAs but also among C3d (+) DSAs. However, there were no significant differences in the distribution of post-pDSA class and SAB MFI between Groups 2 and 4 ($p = 1.000$; $p = 0.327$ in class I and $p = 0.882$ in class II, respectively; Supplementary Table S1).

Table 1. Patient characteristics.

Characteristics	Total	Group 1: pDSA negative	Group 2: Cryptic pDSA rebound	Group 3: pDSA reversed	Group 4: pDSA persistent	*p*-Value *
Number	455	380	19	19	37	
Age, median (IQR)	52.0 (43.0–59.0)	52.0 (42.0–59.0)	49.0 (40.0–56.3)	51.0 (44.2–57.7)	52.0 (48.0–60.0)	0.635
Sex (male) (%)	279 (61.3)	259 (68.2)	7 (36.8)	1 (5.3)	12 (32.4)	<0.001
Dialysis duration, median (IQR)	810.0 (50.2–2173.7)	843.5 (51.4–2164.1)	260.0 (47.3–2107.2)	172.0 (1.2–1652.7)	1305.0 (82.7–2428.3)	0.333
Underlying diseases (%)						
DM	125 (27.5)	114 (37.1)	3 (15.8)	3 (15.8)	5 (13.5)	
GN (1 – 3)	71 (15.6)	61 (16.1)	1 (5.3)	1 (5.3)	8 (21.6)	0.034
IgA	62 (13.6)	49 (12.9)	4 (21.1)	6 (31.6)	3 (8.1)	
Other	176 (38.7)	141 (37.1)	10 (52.6)	5 (26.3)	20 (54.1)	
Re-transplantation (%)	46 (10.1)	28 (7.4)	4 (21.1)	0 (0.0)	14 (37.8)	<0.001
DDKT (%)	230 (50.5)	192 (50.5)	10 (52.6)	9 (47.4)	19 (51.4)	0.989
Desensitization						
RTX	46 (10.1)	0 (0.0)	0 (0.0)	13 (68.4)	33 (89.2)	NA
RTX + PP	10 (2.2)	0 (0.0)	0 (0.0)	6 (31.6)	4 (10.8)	
Induction therapy						
rATG	291 (64.0)	226 (59.5)	10 (52.6)	19 (100.0)	36 (97.3)	NA
Basiliximab	164 (36.0)	154 (40.5)	9 (47.4)	0 (0.0)	1 (2.7)	
Maintenance regimen						
CsA + MMF (PD)	6 (1.3)	6 (1.6)	0 (0.0)	0 (0.0)	0 (0.0)	
FK + MMF (PD)	446 (98.0)	372 (97.9)	18 (94.7)	19 (100)	37 (100)	NA
Sirolimus/Everolimus combination	3 (0.7)	2 (0.5)	1 (5.3)	0 (0.0)	0 (0.0)	
Pre-sensitization (PRA %)						
Class I	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–11.7)	37.0 (5.4–77.0)	54.0 (0.0–87.7)	<0.001
Class II	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–14.0)	0.0 (0.0–63.8)	36.0 (0.0–74.3)	<0.001
HLA mismatches, median (IQR)	3.0 (2.0–4.0)	3.0 (2.0–4.0)	4.0 (2.0–4.0)	3.0 (2.2–4.8)	3.0 (2.0–4.0)	0.642

* $p < 0.05$ was considered statistically significant. IQR, interquartile range; DM, diabetes mellitus; GN, glomerulonephritis; IgA, IgA nephropathy; DDKT, deceased donor kidney transplantation; pDSA, preformed donor-specific HLA antibody; NA, not applicable; MFI, mean fluorescence intensity; RTX, rituximab; PP, plasmapheresis; rATG, recombinant anti-thymocyte globulin; CsA, cyclosporine A; MMF, Mycophenolate mofetil; PD, prednisolone; FK, FK506; PRA, panel reactive antibody.

Table 2. HLA classes and complement binding activities of pre- and post-transplantation donor-specific HLA antibodies in recipient groups.

HLA Classes of pDSA	Group 2: Cryptic pDSA Rebound		Group 3: pDSA Reversed		Group 4: pDSA Persistent	
	Total pDSA	C3d (+) pDSA	Total pDSA	C3d (+) pDSA	Total pDSA	C3d (+) pDSA
Pre-transplantation			19	**0 (0.0)**	37	7 (18.9)
Class I only	NA	NA	13 (68.4)	0 (0.0)	13 (35.1)	0 (0.0)
Class II* only	NA	NA	5 (26.3)	0 (0.0)	14 (37.8)	7 (18.9)
Class I + II*	NA	NA	1 (0.53)	0 (0.0)	10 (27.0)	0 (0.0)
Post-transplantation	19	4 (21.1)			37	9 (24.3)
Class I only	6 (31.6)	1 (5.3)	NA	NA	11 (29.7)	0 (0.0)
Class II* only	11 (57.9)	3 (15.8)	NA	NA	21 (56.8)	9 (24.3)
Class I + II*	2 (10.5)	0 (0.0)	NA	NA	5 (13.5)	0 (0.0)

* Class II pDSAs were assessed limited loci; as –DR, and -DQB1. Bold letters indicate significant results. pDSA, preformed donor-specific HLA antibody; MFI, mean fluorescent intensity; NA, not applicable.

3.2. HLA Antibody Characteristics, Including Complement Binding Capacities

In total, 105 pDSAs derived from 75 recipients (Groups 2, 3, and 4) were identified; the median number per recipient was 1.4 (range: 1–6) (Supplementary Table S2). Of these pDSAs, 81 (77.1%) were persistent pre-pDSA, and 24 (22.9%) were rebound cryptic pDSAs that were newly produced within 4 weeks post-KT. C3d-binding capacities were observed in 9.9% (8/81) of pre-pDSA and 20.6% (14/68) of post-pDSA. Among the 73 C3d-negative pre-pDSAs derived from 49 recipients, 50.7% (37/73) became negative (pDSA reversed), 43.8% (32/73) persisted as C3d-negative, and 5.5% (4/73) became C3d-positive after transplantation. Pre-pDSA SAB MFIs were significantly higher in Group 4 than in Group 3 ($p < 0.001$). Importantly, all 8 C3d-positive pre-pDSAs identified in 7 recipients persisted after transplantation, although 25% (2/8) became C3d-negative, which was significant when compared with the C3d-negative pre-pDSAs ($p = 0.008$; Figure 3, red lines).

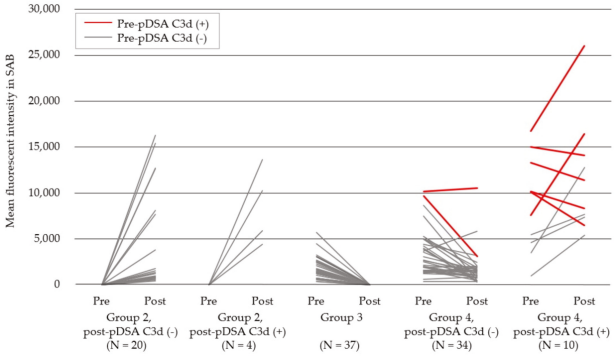

Figure 3. Production and MFI changes of pDSAs within 4 weeks of kidney transplantation in different patient groups. The red lines indicate cases of C3d-positive pDSAs and the grey lines highlight cases of C3d-negative pDSAs. pDSA, preformed donor-specific HLA antibody; MFI, mean fluorescence intensity.

To determine the SAB MFI cut-offs for HLA antibodies predicting C3d-binding activities, all 1515 HLA antibodies from 112 recipients were analyzed (Figure 4). The numbers of observed HLA antibodies to A, B, C, DR, DQB1, and DPB1 were 322, 526, 71, 370, 159, and 67, respectively. SAB MFIs of C3d-positive antibodies were significantly higher than those of C3d-negative antibodies in all loci. For class I antibodies, 15.1% (139/919) of the HLA antibodies were C3d-positive, and their median SAB MFI was 9429 (IQR: 5457–16,016), whereas that of the C3d-negative antibodies was 1988 (IQR: 1271–3190). The optimal cut-off value of the total class I loci for predicting C3d-binding activities was 7797, and the area under the curve (AUC) was 0.908. For class II antibodies, 42.6% (254/596) of the HLA antibodies were C3d-positive, and their median SAB MFI was 10,341 (IQR: 6693–14,207), whereas that of the C3d-negative antibodies was 1,711 (IQR: 1002–3401). The optimal cut-off value of total class II loci was 4460, and the AUC was 0.914 (Supplementary Table S3, Supplementary Figure S1).

3.3. Rejection Episodes and Graft Function

Overall, 177 recipients (38.9%) were diagnosed with rejection episodes, including ACR and AMR, with an incidence of 168 (36.9%) and 13 (2.9%), respectively; in addition, 4 (1.7%) recipients were diagnosed with both ACR and AMR (Table 3). The incidence of ACR among the four groups was not significantly different. In contrast, the incidence of AMR among the four groups was significantly different. In both Groups 2 and 4, recipients with C3d-positive post-pDSA exhibited a significantly higher incidence of AMR (2/4, 50.0% and 3/9, 33.3%, respectively) than recipients with C3d-negative post-pDSA (1/15, 6.7% and 4/28, 14.3%, respectively). The odds ratio (OR) of AMR risk was significantly increased in the Group 2 C3d (+) subgroup and both C3d (-) and (+) subgroups in Group 4 compared with Group 1 (Table 4). Although it had marginal statistical significance (adjusted $p = 0.0876$),

the C3d-positive subgroup, but not the C3d-negative subgroup in Group 2, exhibited quite different OR (OR = 0.056 and OR = 0.778, respectively) compared with Group 3.

The 1-year AMR-free survival was also significantly different among all groups (Figure 5a), and it was the lowest in the C3d-positive subgroup of Group 2, the cryptic pDSA rebound cases (Figure 5b).

During the maximum 400 day follow-up period (median 17.5, IQR 12.0–330.0), graft failure was not observed in any recipients, and the eGFR differences observed among the groups were not significantly different at any time point ($p = 0.575$; Supplementary Figure S2).

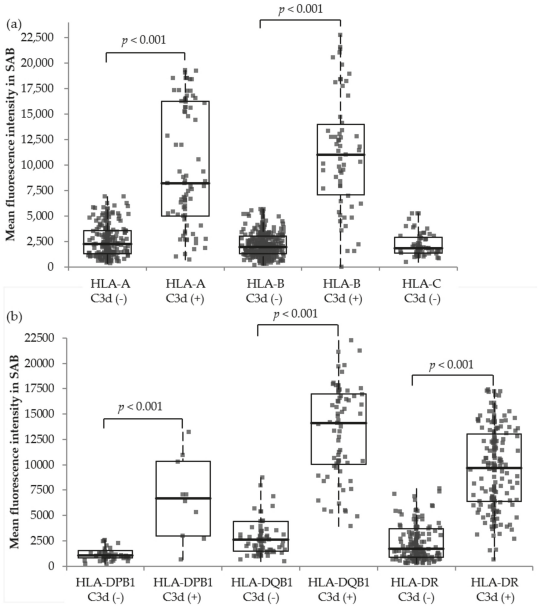

Figure 4. Mean fluorescence intensity distribution of 1522 Class I and II HLA antibodies according to their loci and C3d-binding capacities (**a**,**b**). Box plot, 1st to 3rd quartile range and whiskers extend to the furthest observation within 1.5× interquartile range from the quartiles. SAB, single antigen bead-based antibody identification assay.

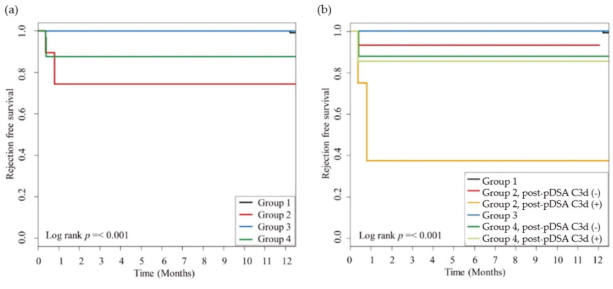

Figure 5. AMR-free survival according to the presence of pre-and post-pDSAs and C3d-binding capacities. The incidences of AMR were significantly different among the groups (**a**) and when considering the presence of post-pDSA C3d-binding capacities (**b**) ($p < 0.001$, respectively). The statistical difference between the Kaplan–Meier survival curves was evaluated by using the log-rank test, and p-values of <0.05 were considered statistically significant. AMR, antibody-mediated rejection; pDSA, preformed donor-specific HLA antibody.

4. Discussion

Both preformed DSAs present before (pre-pDSAs) and early after KT (post-pDSAs) were associated with the risk of AMR when they had C3d-binding activities. In particular, C3d-positive pre-pDSAs tended to persist after transplantation, despite the pretransplant desensitization.

4.1. Cryptic DSAs

pDSAs that were produced within 1 month of transplantation were considered as cryptic pDSAs with anamnestic reactions [10]. Using this study design, we compared the clinical effect of pDSAs with or without pre-transplantation desensitization. Our results indicated that the presence of rebound pDSA was primarily associated with AMR, followed by that of persistent pDSA, reversed pDSA, and negative pDSA.

The incidence of newly produced DSAs after transplantation, which usually occurs within 1 year of KT, with a variable median time between 6 months and 4.6 years, has been reported in 13% to 30% of pDSA-negative recipients before KT [10,13,14]. Several studies have analyzed the effect of early produced DSAs, which were defined as those produced within 1 year of transplantation [15,16], but it remained unclear whether this was dnDSA or pDSA [16]. King et al. reported that DSAs that were produced sooner than 1 month after transplantation exerted more pronounced effects on recipients and allografts than the effects of those produced later [16]. Cryptic DSAs may exist below the detection limit of the current antibody tests and can be induced rapidly by anamnestic reactions after re-stimulation by the donor graft. This is in contrast with dnDSAs, which develop gradually through the primary immune reaction associated with an indirect pathway after encountering new alloantigens [9,10]. Anamnestic reactions lead to the production of C3d-positive pDSAs, and this was related to the highest incidence of AMR in this study. Therefore, further studies regarding the management of cryptic pDSAs are necessary.

4.2. Persistent pDSAs

pDSA persistence is known to be associated with AMR. Kimball et al. reported a higher AMR incidence in persistent pDSA groups than in negatively converted groups (43% and 3%, respectively) [17]. In addition, Marfo et al. demonstrated that recipients with persistent pDSAs experienced more acute and chronic rejection ($p = 0.006$) than recipients with reversed pDSA [18]. The independent risk factor associated with persistence of pDSA was pre-transplant MFI, as shown by Redondo-Pachon et al., and class II DSAs persisted more frequently [19]. Similarly, we found that persistent pDSAs possessed higher MFIs. Although the risk of AMR was not statistically different between the persistent pDSA group and reversed pDSA group, it tended to be higher in the persistent pDSA group. The C3d-positivity (33.3%, 3/9) of persistent pDSA resulted in an increased risk of AMR (OR = 9.000, $p = 0.0781$) compared with C3d-negative group (OR = 3.000, $p = 0.3439$).

Table 3. Comparison of the occurrence of rejection in recipient groups.

	Total	Group 1: pDSA Negative	Group 2: Cryptic pDSA Rebound			Group 3: pDSA Reversed	Group 4: pDSA Persistent			*p*-Value*
			Post-pDSA C3d (-)	Post-pDSA C3d (+)	*p*-Value		Post-pDSA C3d (-)	Post-pDSA C3d (+)	*p*-Value	
Number	455	380	15	4		19	28	9		
ACR (%)	168 (36.9)	142 (37.4)	7 (46.7)	2 (50.0)	0.906	7 (36.8)	7 (25.0)	3 (33.3)	0.624	0.479
AMR (%)	13 (2.9)	2 (0.5)	1 (6.7)	2 (50.0)	**0.035**	1 (5.3)	4 (14.3)	3 (33.3)	0.204	< **0.001**

* *p*-values among the four groups without consideration of post-DSA C3d-binding activity. $p < 0.05$ was considered statistically significant. Bold letters indicate significant results. pDSA, preformed donor-specific HLA antibody; ACR, acute cellular rejection; AMR, antibody-mediated rejection.

Table 4. Estimated risk of antibody-mediated rejection between recipient groups according to C3d-binding activities of post-transplant pDSAs.

Comparison groups	Odds ratio (95% CI)	95% Confidence limits		*p*-Value*	Adjusted *p*-value*
Group 1 vs. Group 2	**35.425**	5.528	227.022	**< 0.001**	**0.001**
Group 1 vs. Group 2 C3d (-)	**13.495**	1.154	157.782	**0.038**	0.076
Group 1 vs. Group 2 C3d (+)	**188.934**	17.098	>999.999	**<0.001**	**<0.001**
Group 1 vs. Group 3	10.496	0.909	121.203	0.060	0.358
Group 1 vs. Group 4	**44.084**	8.771	221.57	**<0.001**	**<0.001**
Group 1 vs. Group 4 C3d (-)	**31.489**	5.491	180.593	**<0.001**	**<0.001**
Group 1 vs. Group 4 C3d (+)	**94.467**	13.275	672.252	**<0.001**	**<0.001**
Group 2 vs. Group 3	0.296	0.028	3.142	0.313	0.999
Group 2 C3d (-) vs. Group 3	0.778	0.045	13.559	0.863	0.999
Group 2 C3d (+) vs. Group 3	**0.056**	0.003	0.923	**0.044**	0.088
Group 2 vs. Group 4	1.244	0.283	5.48	0.773	0.999
Group 2 C3d (-) vs. Group 4 C3d (-)	2.333	0.237	22.999	0.468	0.999
Group 2 C3d (-) vs. Group 4 C3d (+)	7.000	0.6	81.674	0.121	0.965
Group 2 C3d (+) vs. Group 4 C3d (-)	0.167	0.018	1.546	0.115	0.919
Group 2 C3d (+) vs. Group 4 C3d (+)	0.500	0.045	5.514	0.571	0.999
Group 3 vs. Group 4	4.200	0.477	36.978	0.196	0.999
Group 3 vs. Group 4 C3d (-)	3.000	0.308	29.182	0.344	0.688
Group 3 vs. Group 4 C3d (+)	9.000	0.781	103.723	0.078	0.156

* *p*-values of < 0.05 were considered statistically significant; bold letters indicate significant results; adjusted *p*-values were computed with Bonferroni's correction for multiple comparisons. pDSA, preformed donor-specific HLA antibody; CI, confidence interval.

4.3. Complement-Binding Capacities and Clinical Outcome

SAB was designed to detect all IgG antibody isotypes, irrespective of their complement-binding capacity. IgG3 was the most potent complement binder among the subclasses of IgG and significantly affected the occurrence of rejection and graft loss after transplantation via IgG3-induced C1q-binding [20]. Honger et al. reported that IgG pre-pDSA was composed of 39% IgG1 and/or IgG3, 7% IgG2 and/or IgG4, and a 54% mixture of both complement-binding and weak/non-complement-binding subclasses [21]. These findings suggested that not all SAB-positive antibodies promoted complement activation, rejection, and graft loss. Therefore, two modified SAB assays targeting different complement derivatives were recently introduced (the C1q- and C3d-binding assays). dnDSAs harboring C1q-binding capacity were known to affect clinical outcomes such as graft survival, acute AMR, and transplant glomerulopathy [8,22–27], but the role of pDSA in AMR or poor graft survival prediction is controversial [28–31]. C3d-positive dnDSA increased the risk of graft loss, AMR, proteinuria, C4d histological staining, and rapid progression to graft dysfunction [3,6,32,33]; however, C3d-positive pre-pDSA was reported not to increase the risk of graft failure significantly [34]. In the present study, we performed a C3d-binding assay, rather than a C1q-binding assay, for two reasons. First, targeting C3d is more relevant because C3d is derived after the initiation of the complement activation cascade, so it may reflect more of the functional aspects of antibodies [7]. Second, less data are available on the significance of C3d-positive pDSAs than that of C1q-positive pDSAs. We also found that C3d-positive pDSAs tended to persist after KT, and were associated with higher AMR incidence, regardless of desensitization.

The threshold of SAB MFI, which was correlated with C1q- and C3d-binding capacities, has been reported [3,7], with the purpose of estimating the risk of identified antibodies in a timely manner before carrying out the subsequent complement binding assay. The suggested cut-off MFIs for predicting C3d-binding capacities in previous reports ranged from 4225–17,057 and 8356–15,027 for HLA class I and class II antibodies, respectively [6,32,35]. Class II HLA antibodies were reported to possess higher MFIs than those of class I in regard to C3d-binding capacity. In this study, the median MFI of C3d-positive antibodies also tended to be higher in class II than those of class I, but the optimal cut-off value of class I (MFI, 7797; sensitivity, 61.9%; specificity, 97.9%) was higher than that of class II (MFI, 4460; sensitivity, 88.9%; specificity, 83.9%). Such a discrepancy may be due to different sensitivities and specificities, as determined by different studies or the limited number of HLA antibodies analyzed in our study. Kamburova et al. reported that 95% of C3d-positive antibodies exhibit SAB MFI values of 4000 or more, but only 56% of antibodies exhibiting an MFI of 4000 or more possessed C3d-binding capacity. Based on this, they suggested that the C3d-binding capacity was correlated with the SAB MFI; however, positivity cannot be completely predicted based on SAB MFI [34].

This study has a few limitations. First, the definition of pDSAs, which included cryptic pDSAs, was not verified by using donor-specific mBCs, and the possibility of rapidly produced dnDSAs cannot be excluded. Second, the number of recipients who progressed to AMR was small, although the study was able to determine statistical significance from these data. Finally, the contribution of HLA-DP DSAs could not be estimated, as donor and recipient DP typing was not performed.

5. Conclusions

The monitoring of pDSA persistence, particularly that with C3d-binding capacities that occurred despite desensitization, and of DSA production immediately following KT likely reflects that elevated cryptic pDSAs, due to the anamnestic response, are critical for the prediction of AMR. This approach would aid the initiation of timely therapeutic intervention to reduce the risk of DSAs.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/2/375/s1, Table S1: Donor-specific antibody characteristics in Group 2 and 4 recipients, Table S2: MFI of 105 DSAs from 75 recipients, Table S3: Distribution of mean fluorescence intensity of 1,515 anti-HLA antibodies in a single antigen bead assay according to their C3d-binding capacity and optimal cut-offs to predict C3d-binding capacity Figure S1: Receiver operating curves analysis of the mean fluorescence intensity of single antigen bead-based antibody

identification assay performance in an effort to predict complement binding capability in class I (a) and class II (b); Figure S2: eGFR differences observed among the groups were not significantly different. eGFR was calculated by the modification of diet in renal disease (MDRD) study equation.

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Female Specific Association of Low Insulin-Like Growth Factor 1 (IGF1) Levels with Increased Risk of Premature Mortality in Renal Transplant Recipients

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Abstract: Associations between insulin-like growth factor 1 (IGF1) and mortality have been reported to be female specific in mice and in human nonagenarians. Intervention in the growth hormone (GH)-IGF1 axis may particularly benefit patients with high risk of losing muscle mass, including renal transplant recipients (RTR). We investigated whether a potential association of circulating IGF1 with all-cause mortality in stable RTR could be female specific and mediated by variation in muscle mass. To this end, plasma IGF1 levels were measured in 277 female and 343 male RTR by mass spectrometry, and their association with mortality was assessed by Cox regression. During a median follow-up time of 5.4 years, 56 female and 77 male RTR died. In females, IGF1 was inversely associated with risk (hazard ratio (HR) per 1-unit increment in log2-transformed (doubling of) IGF1 levels, 95% confidence interval (CI)) of mortality (0.40, 0.24–0.65; $p < 0.001$), independent of age and the estimated Glomerular filtration rate (eGFR). In equivalent analyses, no significant association was observed for males (0.85, 0.56–1.29; $p = 0.44$), for which it should be noted that in males, age was negatively and strongly associated with IGF1 levels. The association for females remained materially unchanged upon adjustment for potential confounders and was furthermore found to be mediated for 39% by 24 h urinary creatinine excretion. In conclusion, low IGF1 levels associate with an increased risk of all-cause mortality in female RTR, which may link to conditions of low muscle mass that are known to be associated with poor outcomes in transplantation patients. For males, the strongly negative association of age with IGF1 levels may explain why low IGF1 levels were not found to be associated with an increased risk of all-cause mortality.

Keywords: insulin-like growth factor 1; growth hormone; muscle mass; patient survival; physical activity; renal transplant recipients

1. Introduction

The peptide hormone insulin-like growth factor 1 (IGF1) is a key mediator of the biochemical/endocrine effects of growth hormone (GH) [1]. Synthesis of IGF1 is regulated by GH and mainly takes place in the liver after which IGF1 is secreted and transported to other tissues, where it acts as an endocrine hormone [2,3]. IGF1 provides a stable, integrated measure of the activity of the somatotropic axis thereby contrasting with GH secretion which is highly variable [3].

Reduced GH and IGF1 signaling extends lifespan in many laboratory models, including worms, yeast, and drosophila [4]. A specific role for IGF1 receptor signaling in mammalian longevity was first established in IGF1 receptor-(haplo) insufficient mice. These mice lived 33% longer than their wildtype littermates, yet this effect was restricted to females [5], which was subsequently confirmed in two follow-up studies in mice [6,7]. A similar link between IGF1 receptor-insufficiency and longevity has been proposed for humans following observations in several studies [8–10]. Moreover, IGF1 levels predict better survival in nonagenarians (i.e., people between the age of 90 and 99), and, notably, the corresponding association between IGF1 levels and longevity was found to be female specific [11]. It remains, however, unclear whether circulating levels of IGF1 are also associated with longevity in middle-aged subjects and whether such association is female specific.

Studying the association between IGF1 levels and longevity (survival) in specific patient groups appears to be interesting as well, for example, following the growing interest in ghrelin receptor agonists targeting the GH-IGF1 axis to potentially reverse the anorexia–cachexia syndrome in a variety of conditions, including renal insufficiency [12–15]. An important mechanism by which stimulation of the GH-IGF1 axis may improve long-term outcome is through stimulation of muscle mass accretion [15,16]. To this regard, a large and growing group of patients that might be worthwhile studying is that of renal transplant recipients (RTR), in which protein–energy wasting is always lurking [17–19]. In fact, it has been found that the risk of premature mortality in this population is 6–7 times higher compared to the general population [20], and this risk was particularly high in RTR with low muscle mass, as reflected by low 24 h urinary creatinine excretion [21,22]. Recent studies furthermore suggested that 24 h urinary creatinine excretion may be a noninvasive, easily accessible, inexpensive, and direct measurement of total body muscle mass [19], while this measure is often not included in clinical studies to complement the imaging technique armamentarium which is applied for evaluation of muscle mass in observational and clinical intervention studies [23–25].

In this study, we aimed to investigate (1) the nature of the association between circulating levels of IGF1 and mortality in RTR, (2) whether such (potential) association is female specific, and (3) furthermore whether such (potential) association could, in part or as a whole, be mediated by variation in muscle mass, as reflected by 24 h urinary creatinine excretion.

2. Experimental Section

2.1. Study Population

All RTR (aged ≥ 18 years) that were transplanted at the University Medical Center Groningen (UMCG) and that were one year or longer post-transplantation were approached for participation in this study during outpatient clinic visits between 2008 and 2010, as described previously [26]. The RTR included in this study had no known or apparent systemic diseases (e.g., malignancies, active infections) at inclusion. Written informed consent was obtained from 707 (87%) of the 817 RTR that were initially invited, and plasma IGF1 levels were measured in 620 RTR (76%). For this study, ethical approval has been granted by the UMCG's review board (METc 2008/186), and the study adheres to the Declaration of Helsinki. The study is registered as 'TransplantLines Food and Nutrition Biobank and Cohort Study (TxL-FN)' at ClinicalTrials.gov (NCT identifier 'NCT02811835').

2.2. Data and Sample Collection

Measurement of clinical parameters has been described in detail previously [26]. Physical activity was assessed with the Short QUestionnaire to ASsess Health-enhancing physical activity (SQUASH) as developed and validated by the Dutch National Institute of Public Health and Environment to assess daily life physical activity in the Dutch adult population [27]. Information on medical history and medication use was obtained from patient records. Diabetes was defined as the use of antidiabetic medication or fasting plasma glucose of at least 7.0 mmol/L. Twenty-four h urine was collected (per strict protocol) a day before the outpatient clinic visits while blood was drawn in the morning on the day of the outpatient clinic visit, yet after completion of the 24 h urine collection.

2.3. Laboratory Procedures

Blood and urine markers were measured by routine laboratory procedures with the exception of serum creatinine which was assessed using a modified version of the Jaffé method (MEGA AU 510; Merck Diagnostica, Darmstadt, Germany), and the urine total protein concentration which was obtained using the Biuret reaction (MEGA AU 510; Merck Diagnostica). Renal function was estimated with the 2012 Chronic Kidney Disease Epidemiology (CKD-EPI) Collaboration equation using both serum creatinine and cystatin C [28]. IGF1 was assessed in plasma samples (which had not undergone any previous freeze–thaw cycle) using a semi-automated mass spectrometric IGF1 assay [29] which was validated according to FDA guidelines [30]. The samples were analyzed in 13 analytical runs containing up to 81 clinical samples per run, as well as nine calibration samples and duplicate quality control (QC) samples at three concentrations (i.e., low, midrange, and high IGF1 levels). All runs met the acceptance criteria stipulated in the FDA guidelines thereby featuring 75% (though at least six) of the calibration samples with back-calculated levels within 15% (or 20% for the lowest level calibration sample) of their expected value, and at least 67% of the QC samples (though at least one replicate per QC level) yielding IGF1 levels within 15% of their respective nominal value (see Figures S1 and S2 in the Supplementary Material).

2.4. Outcome Ascertainment

All-cause mortality was the primary outcome of this study and was recorded until the end of September 2015. Up-to-date information on patient status was obtained on the basis of a continuous surveillance system of the outpatient program. In case the status of a patient was unknown, general practitioners or referring nephrologists were contacted. There was no loss to follow-up for the outcome. Specific causes of mortality were secondary outcomes of this study. This information was obtained by linking patient numbers to the database of the Dutch Central Bureau of Statistics (CBS) to retrieve causes of mortality reported by physicians. Infectious mortality was defined as mortality from infectious causes [31]. Cardiovascular mortality was defined as mortality caused by cardiovascular pathology, coded by ICD-10 codes I10-I52 [32]. Mortality due to malignancies was defined as mortality caused by malignant diseases. Miscellaneous causes of mortality were defined as other causes of death, not included in mortality from infectious causes, cardiovascular mortality, or mortality due to malignancies.

2.5. Statistical Analyses

Data analyses were performed using IBM SPSS Statistics for Windows (version 23.0.0.0; IBM Corp., Armonk, NY, USA) and STATA/SE (version 15.1; StataCorp, College Station, TX, USA). All p-values are two-tailed, and a p-value lower than 0.050 was considered statistically significant. Baseline characteristics are presented according to tertiles of plasma IGF1 levels for female and male RTR. Continuous data are presented as mean with SD for normally distributed variables and as median with interquartile range (IQR) for variables with skewed distributions, whereas categorical variables are presented as percentages. Differences in baseline characteristics across the tertiles were tested by one-way ANOVA, Kruskal–Wallis test, and linear-by-linear association χ^2 test for normally distributed

continuous, skewed continuous, and categorical variables, respectively. Multivariable linear regression was performed to assess associations between patients' characteristics and plasma IGF1 levels in female and male RTR. Models were included for analyses that were adjusted for age alone, for age and estimated glomerular filtration rate (eGFR), and for multiple variables selected following (automatic) stepwise backward elimination. The prospective associations of plasma IGF1 levels with all-cause mortality, as primary endpoint, and with cause-specific mortality, as secondary endpoint, were assessed by Cox proportional hazards regression. In order to verify the existence of effect modification by sex for the primary endpoint, we performed Cox regression analyses for the association of plasma IGF1 levels with all-cause mortality in which female and male RTR were grouped together, with additional inclusion of an interaction term of plasma IGF1 and sex in the Cox regression model. Hazard ratios (HR) and 95% confidence intervals (CIs) were calculated per 1 unit increment in log2-transformed IGF1 levels. Thereafter, we proceeded with sex-stratified prospective analyses for all-cause mortality as a primary endpoint. In addition to crude analyses, we performed analyses with adjustment for age and eGFR with and without additional physiological, lifestyle, routine clinical chemistry, transplantation, medication, and comorbidity related variables. Subsequently, we performed mediation analyses using the method as described by Preacher and Hayes, which allowed for testing the significance and magnitude of (potential) mediation [33]. In these analyses, mediation was assessed by computing bias-corrected confidence intervals upon running 2000 bootstrap samples. The proportion of mediation was obtained by dividing the indirect effect coefficient by the total effect coefficient, which were adjusted for age and eGFR. Mediation analyses were performed using IGF1 as a potential risk factor and 24 h urinary creatinine excretion, a marker of muscle mass, as potential mediator while also vice versa, because the observational nature of our study does not allow for drawing conclusions on cause–effect relationships. At last, we performed Cox-regression analyses for the association of plasma IGF1 levels with cause-specific mortality as secondary endpoints. Due to lower numbers of events, the exploratory nature of these analyses, and the generally accepted rule of thumb that allows for one variable to be included for each 7–10 events in Cox regression models [34], we performed sex-stratified crude analyses Cox regression analyses and sex-stratified age- and eGFR-adjusted analyses for the separate causes of mortality.

3. Results

3.1. RTR Characteristics

Baseline characteristics according to tertiles of plasma IGF1 levels for female and male RTR are shown in Table 1. At baseline, median IGF1 levels were 153 ng/mL (IQR: 118–196) in female and 168 ng/mL (IQR: 128–224) in male RTR (see Figure 1).

Female RTR who had higher IGF1 levels were more likely to have a larger waist circumference and a higher 24 h urinary creatinine excretion. In turn, the prevalence of diabetes mellitus as primary renal disease, the use of insulin therapy, the cumulative prednisolone dose, and the time between transplantation and baseline measurements were lower for these subjects.

For male RTR with higher levels of IGF1, subjects were more likely to be younger, to have a higher body weight and SQUASH score, and to have a lower waist circumference, prevalence of diabetes mellitus as primary renal disease, and cumulative prednisolone dose. Male RTR in the highest tertile of IGF1 levels were furthermore more likely to have received a graft from a living donor, to have undergone dialysis before transplantation, to have a shorter time between transplantation and baseline measurements, to use calcineurin inhibitors, whereas these subjects were less likely to use coumarin derivatives. Lastly, levels of serum creatinine, plasma albumin, plasma triglycerides, and 24 h urinary creatinine excretion were more likely to be higher whereas plasma aspartate transaminase (AST), gamma-glutamyltransferase (GGT), and high sensitivity C-reactive protein (hs-CRP) were more likely to be lower for these subjects.

Table 1. Baseline characteristics according to tertiles of plasma IGF1 levels in female and male RTR [1].

Variable	Tertiles of Plasma IGF1 Levels for 277 Female RTR				Tertiles of Plasma IGF1 Levels for 343 Male RTR			
	<131 ng/mL	131–181 ng/mL	>181 ng/mL	*p*-Value for Trend [2]	<141 ng/mL	141–202 ng/mL	>202 ng/mL	*p*-Value for Trend [2]
Age, y	56 (48–64)	54 (44–63)	54 (41–60)	0.42	59 (48–65)	55 (46–61)	49 (38–61)	<0.001
BMI, kg/m^2	25 (22–30)	26 (23–30)	27 (23–30)	0.31	25 (23–28)	26 (24–30)	26 (23–28)	0.07
Body weight, kg	67 (61–84)	73 (65–81)	74 (65–86)	0.20	80 (73–90)	86 (76–97)	84 (74–93)	0.03
Body length, cm	167 (161–171)	167 (162–173)	168 (163–171)	0.49	179 (174–183)	180 (174–184)	180 (174–185)	0.29
Waist circumference, cm	90 (78–104)	95 (87–106)	95 (88–105)	0.04	100 (91–108)	104 (94–112)	99 (89–107)	0.01
Blood pressure, systolic, mmHg	133 (121–147)	131 (120–142)	132 (124–145)	0.47	138 (127–151)	135 (124–145)	136 (125–145)	0.46
Blood pressure, diastolic, mmHg	80 ± 11	80 ± 12	82 ± 10	0.15	83 ± 10	84 ± 12	84 ± 10	0.27
Lifestyle:								
Smoking status, current, %	11.6	8.7	9.0	0.56	9.8	17.4	16.4	0.19
Alcohol consumption, yes, %	87.2	83.3	79.3	0.18	88.0	91.3	92.5	0.28
SQUASH score, ×1000	4.9 (2.6–7.6)	4.3 (1.7–7.1)	5.0 (2.0–6.7)	0.60	5.2 (1.8–7.3)	6.4 (3.0–10.7)	5.4 (2.5–9.6)	0.02
Primary renal disease:								
Primary glomerulosclerosis, %	23.9	20.4	27.2	0.60	28.9	39.5	32.2	0.61
Glomerulonephritis, %	10.9	8.6	4.3	0.10	9.6	4.4	8.7	0.79
Polycystic kidney disease, %	19.6	24.7	27.2	0.23	14.0	19.3	18.3	0.40
Renal hypoplasia/dysplasia, %	4.3	5.4	2.2	0.45	2.6	3.5	5.2	0.31
Diabetes mellitus, %	8.7	5.4	0.0	0.005	11.4	4.4	1.7	0.002
Other primary renal diseases, %	32.6	35.5	39.1	0.36	32.5	28.9	33.9	0.81
Kidney and transplantation related variables:								
eGFR, mL/min per 1.73 m^2	42 (27–57)	45 (32–57)	39 (25–51)	0.31	45 (32–61)	43 (29–55)	41 (31–55)	0.19
Serum creatinine, µmol/L	110 (87–148)	107 (87–140)	118 (96–162)	0.23	128 (102–167)	137 (115–171)	148 (116–175)	0.04
Living donor, %	28.3	40.2	39.1	0.13	26.3	31.9	42.6	0.009
Graft rejection, %	21.7	22.6	18.5	0.59	37.7	32.5	27.0	0.08
Dialysis before transplantation, %	87.0	75.3	78.3	0.14	91.2	89.5	79.1	0.007
Time between transplantation and baseline visit, y	7.5 (3.4–12.5)	5.0 (1.9–14.6)	3.7 (1.1–7.9)	0.003	7.5 (4.2–13.7)	7.0 (2.9–13.9)	2.6 (1.0–7.5)	<0.001

Table 1. *Cont.*

Variable	Tertiles of Plasma IGF1 Levels for 277 Female RTR				Tertiles of Plasma IGF1 Levels for 343 Male RTR			
	<131 ng/mL	131–181 ng/mL	>181 ng/mL	*p*-Value for Trend [2]	<141 ng/mL	141–202 ng/mL	>202 ng/mL	*p*-Value for Trend [2]
Blood markers:								
ALT, U/L	18 (13–25)	18 (13–22)	18 (13–23)	0.81	21 (16–29)	20 (16–27)	19 (14–27)	0.32
AST, U/L	23 (18–29)	22 (18–26)	20 (18–25)	0.08	24 (20–31)	22 (19–26)	21 (17–25)	<0.001
GGT, U/L	29 (19–49)	25 (16–37)	25 (18–34)	0.09	32 (21–48)	27 (19–43)	24 (18–33)	0.004
Albumin, g/L	42 (40–45)	42 (41–45)	43 (42–45)	0.10	42 (41–45)	43 (41–44)	44 (42–45)	0.003
Glucose, mmol/L	5.0 (4.6–6.2)	5.2 (4.7–5.9)	5.2 (4.7–5.6)	0.66	5.4 (4.9–6.2)	5.3 (4.9–6.2)	5.3 (5.0–5.9)	0.86
HbA1c, %	5.7 (5.4–6.0)	5.9 (5.5–6.3)	5.9 (5.5–6.3)	0.39	5.8 (5.5–6.2)	5.8 (5.5–6.3)	5.8 (5.5–6.2)	0.68
Triglycerides, mmol/L	1.7 (1.2–2.6)	1.7 (1.3–2.1)	1.7 (1.3–2.3)	0.97	1.6 (1.1–2.2)	1.7 (1.2–2.9)	1.8 (1.4–2.3)	0.03
Total cholesterol, mmol/L	5.5 (4.6–6.4)	5.1 (4.4–6.1)	5.2 (4.6–5.9)	0.32	5.1 (4.3–5.9)	4.8 (4.2–5.6)	4.8 (4.3–5.6)	0.29
HDL cholesterol, mmol/L	1.6 (1.1–1.9)	1.4 (1.2–1.8)	1.4 (1.2–1.8)	0.22	1.3 (1.1–1.6)	1.2 (0.9–1.4)	1.2 (1.0–1.4)	0.05
LDL cholesterol, mmol/L	3.0 (2.3–3.7)	3.0 (2.2–3.9)	3.0 (2.5–3.5)	0.94	2.9 (2.3–3.7)	2.8 (2.3–3.5)	2.8 (2.3–3.5)	0.43
hs-CRP, mg/L	1.8 (0.9–5.2)	2.0 (0.9–5.4)	1.7 (0.8–3.0)	0.23	1.8 (0.8–5.5)	1.9 (0.8–5.1)	1.3 (0.5–3.4)	0.03
Follicle-stimulating hormone, U/L	52 (7–90)	51 (5–81)	47 (5–78)	0.87	5.2 (3.0–11.0)	5.7 (3.8–10.7)	4.9 (2.9–8.3)	0.20
Follicle-stimulating hormone ≥ 34 U/L, yes, % [3]	64.0	65.2	63.6	0.95	3.8	5.8	1.8	0.43
Luteinizing hormone, U/L	32 (8–55)	28 (6–56)	32 (8–58)	0.95	5.0 (3.4–8.3)	5.1 (3.6–8.5)	5.1 (3.4–6.9)	0.52
Urine markers:								
Urinary creatinine excretion, mmol/24 h	8.6 (7.3–10.1)	9.6 (8.0–11.3)	10.1 (9.0–11.3)	<0.001	12.3 (10.3–14.5)	13.1 (11.0–15.4)	13.6 (11.4–15.7)	0.008
Urine total protein, g/24 h	0.14 (0.02–0.47)	0.15 (0.02–0.29)	0.15 (0.02–0.29)	0.67	0.24 (0.02–0.52)	0.25 (0.02–0.59)	0.21 (0.02–0.34)	0.29
Medication use:								
Proliferation inhibitors, yes, %	81.5	84.9	83.7	0.69	79.8	86.8	80.0	0.98
Coumarin derivatives, yes, %	14.1	9.7	7.6	0.15	16.7	11.4	7.8	0.04
Calcineurin inhibitors, yes, %	54.3	51.6	66.3	0.10	48.2	55.3	80.0	<0.001
Sirolimus, yes, %	1.2	4.5	0.0	0.55	4.6	0.9	1.0	0.07
Antihypertensive drugs, yes, %	87.0	81.7	89.1	0.67	88.6	92.1	91.3	0.48
Statins, yes, %	48.9	55.9	51.1	0.57	53.1	60.5	43.5	0.14
Diabetes, yes, %	28.3	23.7	26.1	0.74	27.2	22.8	20.0	0.20
Antidiabetics, yes, %	21.7	15.1	13.0	0.11	17.5	15.8	12.2	0.26
Metformin, yes, %	3.3	4.3	4.3	0.71	7.9	2.6	3.5	0.11
Insulin therapy, yes, %	17.4	10.8	7.6	0.04	10.5	7.9	6.1	0.22
Prednisolone, yes, %	97.8	98.9	98.9	0.54	100.0	97.4	100.0	1.00
Prednisolone, cumulative dose, g [4]	23 (10–37)	19 (6–40)	11 (4–25)	0.01	23 (14–42)	23 (10–46)	10 (4–28)	<0.001

[1] Continuous variables are reported as 'mean ± standard deviation' when normally distributed (Shapiro–Wilk test $p \geq 0.05$) or 'median (interquartile range)' when not normally distributed (Shapiro–Wilk test $p < 0.05$), and categorical variables are reported as percentage. [2] Determined by linear-by-linear association χ^2 test (categorical variables), Kruskal–Wallis test (continuous variables, not normally distributed), or one-way ANOVA (continuous variables, normally distributed). [3] A follicle-stimulating hormone cut-off level of ≥ 34 U/L was used to derive a surrogate marker of post-menopause [35]. Abbreviations: ALT: Alanine transaminase; AST: Aspartate transaminase; BMI: Body mass index; eGFR: Estimated glomerular filtration rate; GGT: Gamma-glutamyltransferase; HbA1c: Glycated hemoglobin; HDL: High-density lipoprotein; hs-CRP: High sensitivity C-reactive protein; LDL: Low-density lipoprotein; SQUASH: Short QUestionnaire to ASsess Health-enhancing physical activity [27]. [4] The cumulative dose of prednisolone was calculated as the sum of the maintenance dose of prednisolone until inclusion and the dose of prednisolone or methylprednisolone required for treatment of acute rejection (a conversion factor of 1.25 was used to convert methylprednisolone dose to its prednisolone dose equivalent).

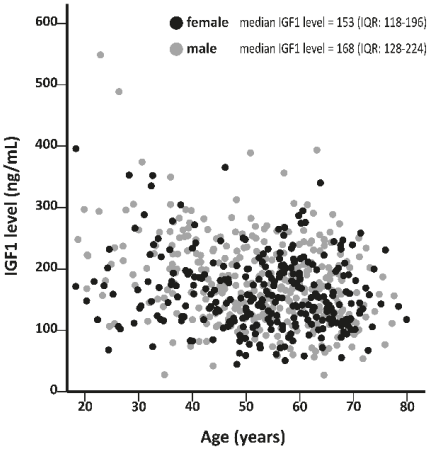

Figure 1. Association between insulin-like growth factor 1 (IGF1) levels and age for female and male renal transplant recipients (RTR).

3.2. Association of Plasma IGF1 with Selected Variables in RTR

Associations between plasma IGF1 levels and variables of interest adjusted for age alone, for age and eGFR, and for multiple variables which were selected following stepwise backward elimination are shown in Table 2.

For female RTR, the analyses adjusted for age featured positive and significant associations between plasma IGF1 and body weight, 24 h urinary creatinine excretion, and calcineurin inhibitor use. Significant inverse associations with plasma IGF1, independent of age, were observed for the prevalence of diabetes mellitus as primary renal disease, the time between transplantation and baseline measurements, the cumulative prednisolone dose, and GGT. After further adjustment for eGFR, the magnitude, direction, and significance of all associations generally remained the same. The final stepwise backward model featured an adjusted R^2 of 0.14 and revealed significant positive associations between plasma IGF1 and both 24 h urinary creatinine excretion and calcineurin inhibitor use, but also significant inverse associations with the prevalence of diabetes mellitus as primary renal disease, GGT, HDL cholesterol, and hs-CRP.

For male RTR, analyses adjusted for age showed significant positive associations between plasma IGF1 and both albumin and calcineurin inhibitor use. Significant inverse associations were observed between plasma IGF1 and eGFR, the prevalence of diabetes mellitus as primary renal disease, the cumulative prednisolone dose, AST, and GGT, and the time between transplantation and baseline measurements. Further adjustment for eGFR did not lead to major changes in the magnitude, direction, or significance of these associations. Lastly, an adjusted R^2 of 0.28 was obtained for the final stepwise backward model which featured significant positive associations between plasma IGF1 and both albumin and calcineurin inhibitor use. Significant inverse associations were furthermore revealed between plasma IGF1 and age, eGFR, the prevalence of diabetes mellitus as primary renal disease, and GGT.

Table 2. Multivariable linear regression analysis with plasma IGF1 as the dependent variable in female and male RTR [1].

	Female RTR (N = 277)						Male RTR (N = 343)					
	Age Adjusted		Age and eGFR Adjusted		Backward (adj. R^2 = 0.14)		Age Adjusted		Age and eGFR Adjusted		Backward (adj. R^2 = 0.28)	
Variable	Stand. β	*p*-Value	Stand. β	*p*-Value	Stand. β	*p*-Value	Stand. β	*p*-Value	Stand. β	*p*-Value	Stand. β	*p*-Value
Age, y	−0.16	0.007	−0.17	0.006			−0.32	<0.001	−0.35	<0.001	−0.27	<0.001
eGFR, mL/min per 1.73 m^2	−0.05	0.37	−0.05	0.37			−0.18	0.001	−0.18	0.001	−0.19	0.001
Body weight, kg	0.12	0.05	0.11	0.06			0.01	0.87	−0.01	0.88		
Body length, cm	0.09	0.14	0.09	0.14			0.00	0.97	−0.02	0.74		
SQUASH score	−0.05	0.43	−0.04	0.49	−0.11	0.09	0.02	0.64	0.05	0.38		
Diabetes mellitus, yes vs. no	−0.13	0.04	−0.13	0.03	−0.13	0.04	−0.12	0.02	−0.14	0.008	−0.13	0.009
Living donor, yes vs. no	0.02	0.71	0.03	0.63			0.02	0.66	0.04	0.44		
Graft rejection, yes vs. no	−0.05	0.37	−0.07	0.28			−0.10	0.06	−0.10	0.05	−0.09	0.06
Dialysis before transplantation, yes vs. no	0.07	0.28	0.07	0.23			0.05	0.39	0.06	0.26		
Time between transplantation and baseline visit, y	−0.14	0.02	−0.14	0.02			−0.15	0.003	−0.15	0.004		
AST, U/L	−0.09	0.16	−0.09	0.16			−0.21	<0.001	−0.15	0.005	−0.10	0.06
GGT, U/L	−0.12	0.04	−0.13	0.03	−0.14	0.02	−0.22	<0.001	−0.20	<0.001	−0.18	0.001
Albumin, g/L	0.09	0.15	0.10	0.11			0.12	0.03	0.17	0.003	0.15	0.01
Triglycerides, mmol/L	−0.06	0.36	−0.07	0.23	−0.11	0.10	0.09	0.08	0.06	0.26		
HDL cholesterol, mmol/L	−0.08	0.20	−0.07	0.27	−0.14	0.03	−0.09	0.08	−0.05	0.32		
hs-CRP, mg/L	−0.10	0.09	−0.10	0.09	−0.15	0.01	−0.04	0.49	−0.06	0.22		
Urinary creatinine excretion, mmol/24 h	0.24	<0.001	0.25	<0.001	0.25	<0.001	0.07	0.19	0.06	0.22		
Coumarin derivatives, yes vs. no	−0.09	0.13	−0.10	0.11			−0.02	0.67	−0.05	0.34		
Calcineurin inhibitors, yes vs. no	0.15	0.01	0.15	0.02	0.16	0.01	0.20	<0.001	0.16	0.003	0.18	0.001
Sirolimus, yes vs. no	−0.07	0.23	−0.07	0.23			−0.06	0.24	−0.05	0.30		
Insulin therapy, yes vs. no	−0.10	0.10	−0.11	0.08			−0.06	0.26	−0.07	0.19		
Prednisolone, cumulative dose, g [2]	−0.12	0.05	−0.12	0.05			−0.13	0.01	−0.13	0.01		

[1] Variables showing *p*-values below 0.10 for the trend of tertiles of IGF1 in at least one of the sexes (see Table 1), with the exception of highly correlated variables (e.g., BMI, waist circumference, serum creatinine), as well as body weight and body height were included for multivariable linear regression analysis. Abbreviations: AST: Aspartate transaminase; eGFR: Estimated glomerular filtration rate; GGT: Gamma-glutamyltransferase; HDL: High-density lipoprotein; hs-CRP: High sensitivity C-reactive protein; SQUASH: Short QUestionnaire to ASsess Health-enhancing physical activity [27]. [2] The cumulative dose of prednisolone was calculated as the sum of the maintenance dose of prednisolone until inclusion and the dose of prednisolone or methylprednisolone required for treatment of acute rejection (a conversion factor of 1.25 was used to convert the methylprednisolone dose to its prednisolone dose equivalent).

3.3. Association of Plasma IGF1 with All-Cause Mortality in RTR

Median follow-up was 5.4 years (IQR: 4.8–6.0 years) for female and 5.4 years (IQR: 4.8–6.3 years) for male RTR. During this prospective follow-up, 56 female and 77 male RTR died. We first investigated whether the association of plasma IGF1 levels with all-cause mortality was modified by sex. In these analyses, with data of female and male RTR combined, we found that higher plasma IGF1 levels were associated with a significantly decreased risk (HR per log2 increment of plasma IGF1, 95% CI) of all-cause mortality (0.61, 0.47–0.80; $p < 0.001$). Furthermore, inclusion of a product-term of (log2-transformed plasma) IGF1 levels and sex in the basic multivariable model (i.e., with adjustment for age and eGFR) revealed the existence of significant effect modification by sex (p for interaction = 0.02). After finding this significant interaction by sex, we proceeded with sex-stratified analyses of the association of (log2-transformed) plasma IGF1 levels with all-cause mortality. For female RTR, the crude analyses showed that higher plasma IGF1 levels were associated with a significantly decreased risk of all-cause mortality (0.42, 0.26–0.66; $p < 0.001$; see Figure 2 and Table 3), while a nonsignificant trend towards a decreased risk was observed for male RTR (0.74, 0.52–1.04; $p = 0.09$; see Table 3 and Figure 2).

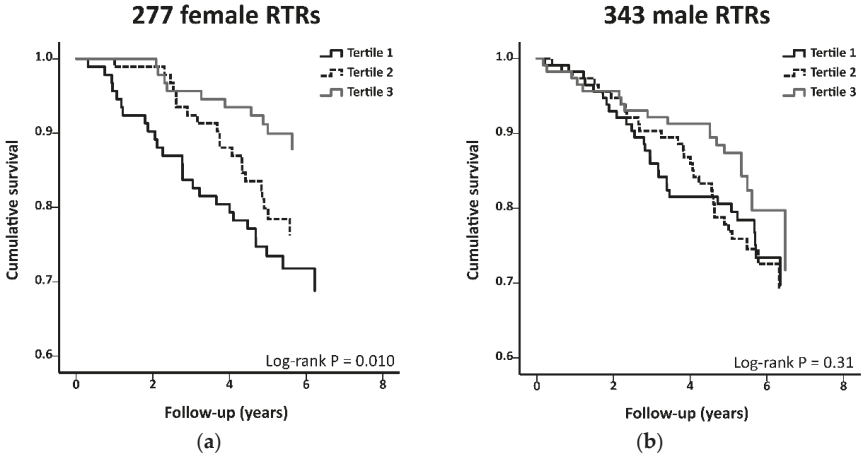

Figure 2. Kaplan–Meier curves for all-cause mortality according to tertiles of plasma insulin-like growth factor 1 (IGF1) in (**a**) female and (**b**) male renal transplant recipients (RTR). For female RTR, IGF1 levels of the tertiles 1, 2, and 3 are below 131 ng/mL, range between 131 and 181 ng/mL, and are above 181 ng/mL, respectively. For male RTR, IGF1 levels of the tertiles 1, 2, and 3 are below 141 ng/mL, range between 141 and 202 ng/mL, and are above 202 ng/mL, respectively.

In the model with adjustment for age and eGFR, the significant inverse association of IGF1 with all-cause mortality remained in female RTR (0.40, 0.24–0.65; $p < 0.001$) and the association in male RTR remained insignificant (0.85, 0.56–1.29; $p = 0.44$). Further adjustment for potential confounders, which was assessed based on seven different multivariable models, did not substantially affect the associations between plasma IGF1 and mortality for both female and male subjects (see Table 3). Lastly, mediation analysis (according to the procedures of Preacher and Hayes [33]) was carried out for the female subjects and revealed 24 h urinary creatinine excretion as significant mediator (p-value for indirect effect < 0.05) accounting for 39% on the association between plasma IGF1 and all-cause mortality (see Table 4). Since the observational nature of our study does not allow for drawing conclusions regarding cause–effect relationships, we also performed alternative mediation analyses with 24 h urinary creatinine excretion as potential risk factors and plasma IGF1 levels as potential mediators. In these analyses, we found that plasma IGF1 levels as significant mediators (p-value for indirect effect

< 0.05) accounted for 9% on the association between 24 h urinary creatinine excretion and all-cause mortality (see Supplemental Table S5).

Table 3. Association between log2-transformed plasma IGF1 levels and the risk of all-cause mortality in female and male RTR [1].

	277 Female RTR (56 Events)			343 Male RTR (77 Events)		
Variable	**HR (log2)**	**95% CI**	***p*-Value**	**HR (log2)**	**95% CI**	***p*-Value**
Crude model	0.42	0.26–0.66	<0.001	0.74	0.52–1.04	0.09
Model 1 [2]	0.40	0.24–0.65	<0.001	0.85	0.56–1.29	0.44
Model 2 [3]	0.47	0.27–0.81	0.006	0.88	0.58–1.34	0.55
Model 3 [4]	0.33	0.16–0.64	0.001	0.88	0.54–1.42	0.60
Model 4 [5]	0.38	0.23–0.63	<0.001	0.81	0.51–1.27	0.35
Model 5 [6]	0.39	0.24–0.65	<0.001	0.87	0.57–1.32	0.50
Model 6 [7]	0.34	0.20–0.57	<0.001	0.94	0.61–1.45	0.78
Model 7 [8]	0.36	0.21–0.61	<0.001	1.06	0.66–1.69	0.82
Model 8 [9]	0.41	0.24–0.69	0.001	0.85	0.55–1.29	0.44

[1] Hazard ratios (HR) per 1 unit increment in log2-transformed plasma IGF1 levels and corresponding 95% confidence intervals (CI) were derived from Cox proportional hazards models. [2] Multivariable model adjusted for age and estimated glomerular filtration rate (eGFR). [3] Multivariable model adjusted for age, eGFR, body length, body weight, waist circumference, systolic blood pressure, and diastolic blood pressure. [4] Multivariable model adjusted for age, eGFR, smoking status, alcohol consumption, and Short QUestionnaire to ASsess Health-enhancing physical activity (SQUASH) score [27]. [5] Multivariable model adjusted for age, eGFR, glucose, glycated hemoglobin (HbA1c), triglycerides, serum total cholesterol, high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol. [6] Multivariable model adjusted for age, eGFR, serum creatinine, and urine total protein. [7] Multivariable model adjusted for age, eGFR, primary renal disease, graft rejection, dialysis before transplantation, time between transplantation and baseline visit, and donor status. [8] Multivariable model adjusted for age, eGFR, aspartate transaminase (AST), gamma-glutamyltransferase (GGT), serum albumin, high sensitivity C-reactive protein (hs-CRP), follicle-stimulating hormone, and luteinizing hormone. [9] Multivariable model adjusted for age, eGFR, antidiabetics, antihypertensive drugs, coumarin derivatives, proliferation inhibitors, calcineurin inhibitors, insulin, and prednisolone.

Table 4. Mediation analysis of the relationship between plasma IGF1, 24 h urinary creatinine excretion, and all-cause mortality in female RTR.

		Multivariable Model [1]	
Potential Mediator	**Effect [2]**	**Coefficient (95% CI, *bc*) [3]**	**Proportion Mediated [4]**
24 h urinary creatinine excretion	indirect effect (*ab* path)	−0.11 (−0.18–−0.06)	39.3%
	direct effect (*c′* path)	−0.17 (−0.33–−0.02)	
	total effect (*ab* + *c′* path)	−0.28 (−0.44–−0.12)	

[1] Coefficients and corresponding 95% confidence intervals (CI) of the indirect and total effects are standardized for the standard deviations of the potential mediator, plasma IGF1, and all-cause mortality. [2] Coefficients are adjusted for age and estimated glomerular filtration rate (eGFR). [3] 95% CIs for the indirect and total effects are bias-corrected confidence intervals after running 2000 bootstrap samples. [4] The size of (statistically significant) mediated effects is calculated by dividing the standardized indirect effect by the standardized total effect followed by multiplication by 100.

3.4. Association of Plasma IGF1 with Cause-Specific Mortality in RTR

Next, we performed sex-stratified analyses of the association of log2-transformed plasma IGF1 levels with mortality from specific causes of death, namely death from infectious diseases, cardiovascular mortality, death from malignancies, and other, miscellaneous causes of death. In females, we found that higher plasma IGF1 levels were strongly associated with a significantly decreased risk of infectious disease-related mortality (0.17, 0.07–0.38; $p < 0.001$; see Supplemental Table S1, Model 1). In females, we also found a borderline significant association of higher plasma IGF1 levels with cardiovascular mortality (0.43, 0.18–1.00; $p = 0.05$; see Supplemental Table S2, Model 1), but neither a significant association with cancer-related mortality (1.50, 0.45–4.93; $p = 0.51$; see Supplemental Table S3, Model 1), nor with mortality from miscellaneous causes (0.43, 0.10–1.78; $p = 0.24$; see Supplemental Table

S4, Model 1). In males, no significant associations with cause-specific mortality were encountered (see respective Supplemental Tables S1–S4).

4. Discussion

This study showed that low plasma IGF1 levels were independently associated with an increased risk of all-cause mortality in female RTR. Such association was less pronounced and insignificant in male RTR, which should be seen in the context of IGF1 levels being negatively and strongly associated with age in males, which may explain why low plasma IGF1 levels were not associated with mortality in males. Adjustment for potential confounders did not alter the association observed in women, and 39% of this association was found to be mediated by 24 h urinary creatinine excretion, a marker of muscle mass. In alternative analyses, we found that 9% of the association of urinary creatinine excretion with mortality in women was mediated by plasma IGF1 levels. In secondary analyses, in which the association of plasma IGF1 with cause-specific mortality was assessed, we found a particularly strong association of low plasma IGF1 levels with increased risk of mortality due to infectious causes in females.

To our knowledge, this is the first study that investigated the association between IGF1 and long-term outcomes in RTR, hence we were limited in comparing our study with existing literature. Studies addressing associations between IGF1 and outcomes in other clinical settings are available, yet such studies are scarce and generally do not assess female and male subjects separately. When attempting to compare our results to studies on IGF1 in which both sexes were analyzed separately, we found inconsistent evidence. For example, in a cross-sectional study of 5388 US adults, the magnitude of the (positive) association between high IGF1 levels and the risk of chronic kidney disease was found to be stronger for males than for females [36]. In addition, a study of 183 healthy nonagenarians (i.e., people between the age of 90 and 99) reported a significant association between low IGF1 levels and longer survival in female subjects which was not observed for males [11]. Recently, a prospective population-based study on 1618 elderly adults reported that men featured greater decreases in IGF1 and its most important binding protein (i.e., IGF binding protein 3) with age as compared to females [37]. A recently described cross-sectional study on 200 elderly subjects furthermore reported a (negative) association between IGF1 levels and co-existent frailty and low muscle mass in female subjects whereas such association was not found for male subjects [38]. The difference between females and males as we observed in our study therefore links to previous data but also connects to why gender-specific reference ranges for IGF1 are being employed in routine clinical practice [39–41]. It should, however, be noted that all these results were obtained using different analytical methods, and it is known that different methods may yield different analyte levels, particularly in the case of IGF1 [42,43]. Moreover, IGF1 predominantly circulates being bound to IGF binding proteins [44], and the efficiency of dissociating such complexes may vary between (immuno)assays from different vendors and thereby lead to biased, or at least to incomparable results [45].

With respect to the observed association between IGF1 and mortality in female RTR, several other findings which were put forward in our study should be taken into consideration. Firstly, the identification of 24 h urinary creatinine excretion as a strong mediator in this association represents an interesting finding of our study. The fact that 24 h urinary creatinine is a widely available and accepted marker reflecting muscle mass [46–48] and the recognition of IGF1 as a growth hormone involved in muscle growth [49,50] support the biological plausibility of a link between IGF1 and physical fitness. Low physical activity is, in fact, known to be a risk factor for morbidity and mortality in RTR [51–53], hence further studies on IGF1 in this context are warranted. Secondly, the significant association between IGF1 and the use of calcineurin inhibitors should be viewed in this context as well. The target of these drugs, calcineurin, has been described as a regulator of muscle mass, although it should be noted that much is still unknown about the underlying mechanisms [54–56]. Thirdly, the observed strongly significant association of higher plasma IGF1 levels with lower risk for infectious disease-related mortality may be interesting as well in this regard. At last, it should be noted that

evidence for a potential link between IGF1 and physical fitness is currently still circumstantial and that further research is needed to verify and explore our findings.

Important limitations of this study include the facts that it represents a single-center study and that it addresses a population consisting mainly of Caucasian participants. It is unknown whether our findings can be extrapolated to other populations, and repeating this study in other populations is therefore desirable. Moreover, there may be untested or residual confounding relevant for the observed association, as is often true for observational studies. Moreover, laboratory markers were analyzed only once at baseline, hence corresponding changes over time could not be addressed in the present study. With respect to the IGF1 measurements, it should be noted that measurements were carried out using biobanked samples which had been stored for several years at −80 °C. Sample stability parameters (e.g., freeze-thaw stability, benchtop stability) were addressed during validation of our IGF1 method thereby following the US Food and Drug Administration (FDA) guidelines on bioanalytical method validation [30]. Nonetheless, storage conditions comparable to those applying to the long-term stored plasma samples could not possibly be addressed during validation, as is often the case when targeting biobanked samples. We could, however, monitor the extent of IGF1 oxidation which represents a prominent feature of our mass spectrometric IGF1 assay [29] considering that protein oxidation is a (unwanted) chemical modification occurring during storage of proteins [57]; yet, no abnormalities in IGF1 oxidation were observed. In order to reduce the (potential) impact of corresponding pre-analytical variability on the quality of our data, we only included samples which had not undergone any previous freeze–thaw cycle and we verified that the samples had not been exposed to deviating storage conditions, for example caused by power outages or freezer malfunctions.

Strengths of this study are its prospective design, the relatively large cohort of well-characterized, stable RTR, the complete follow-up for all-cause mortality, the availability of detailed data on potential confounders, and the use of a mass spectrometric IGF1 assay which allowed for highly selective IGF1 quantification.

In conclusion, low plasma IGF1 levels were found to be associated with an increased risk of all-cause mortality in female RTR, and this association was not found (to be significant) for male RTR. The association in females was mediated for a substantial proportion by 24 h urinary creatinine excretion which hints at a possible link with conditions of low muscle mass (e.g., poor physical fitness, poor nutritional state). Secondary analyses pointed towards a particularly strong association of low plasma IGF1 levels with mortality from infectious causes. Further research is, however, needed to explore the existence and/or relevance of such a link, and also to investigate whether IGF1 can be useful as a (predictive) marker of mortality in female RTR possibly by reflecting physical fitness in this population.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/2/293/s1. Figure S1: Overview of calibration data for the thirteen runs carried out for quantification of insulin-like growth factor 1 (IGF1) in the clinical samples. Figure S2: Overview of the quality control data obtained during the thirteen runs carried out for quantification of insulin-like growth factor 1 (IGF1) in the clinical samples. Table S1: Association between log2-transformed plasma IGF1 levels and the risk of infectious disease-related mortality in female and male RTR. Table S2: Association between log2-transformed plasma IGF1 levels and the risk of cardiovascular mortality in female and male RTR. Table S3: Association between log2-transformed plasma IGF1 levels and the risk of cancer-related mortality in female and male RTR. Table S4: Association between log2-transformed plasma IGF1 levels and the risk of miscellaneous-cause mortality in female and male RTR. Table S5: Mediation analysis of the relationship between 24 h urinary creatinine excretion, plasma IGF1 levels, and all-cause mortality in female RTR.

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Conflicts of Interest: The authors declare no conflict of interest.

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Review

Ischemia and Reperfusion Injury in Kidney Transplantation: Relevant Mechanisms in Injury and Repair

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Abstract: Ischemia and reperfusion injury (IRI) is a complex pathophysiological phenomenon, inevitable in kidney transplantation and one of the most important mechanisms for non- or delayed function immediately after transplantation. Long term, it is associated with acute rejection and chronic graft dysfunction due to interstitial fibrosis and tubular atrophy. Recently, more insight has been gained in the underlying molecular pathways and signalling cascades involved, which opens the door to new therapeutic opportunities aiming to reduce IRI and improve graft survival. This review systemically discusses the specific molecular pathways involved in the pathophysiology of IRI and highlights new therapeutic strategies targeting these pathways.

Keywords: ischemia reperfusion injury; kidney transplantation; delayed graft function; innate immune system; adaptive immune system; apoptosis; necrosis; hypoxic inducible factor; endothelial dysfunction

1. Introduction

To date, 10% of the worldwide population suffers from chronic kidney disease (CKD). The prevalence of the disease will most likely grow over the next decade due to the increase in the elderly population and the growing incidence of diabetes and hypertension. In 2015, CKD was ranked 12th in the global list of causes of death [1]. The population of patients needing renal replacement therapy (RRT) worldwide was estimated to be approximately 4.902 million (95% CI 4.438–5.431 million) in a conservative model and 9.701 million (95% CI 8.544–11.021 million) in a high estimate model, illustrating the magnitude of the disease burden of end stage renal disease (ESRD) [2].

For patients with ESRD, transplantation is still the optimal treatment. Long-term survival with kidney transplantation is dramatically better than dialysis and transplantation provides a sustainably higher quality of life. Unfortunately, there is a worldwide shortage of suitable donor organs for (kidney)

transplantation. The number of renal transplantations performed worldwide in 2018 was 75.664 [3]. Due to the persistent shortage of donor kidneys, many transplant centres have established large living donor programmes and transplant teams are also now accepting increasing numbers of older and higher risk organs, retrieved from deceased donors. The use of these extended criteria donors (ECD) has affected outcomes after transplantation due to an often-suboptimal quality of the donor organ [4,5]. As we will face more complex donors in the future with a reduced viability such as unstable donation after brain death (DBD) donors, donation after circulatory death (DCD) donors, and ECD, the challenge in transplantation is to be able to use these donor sources, however, without compromising successful immediate function and long-term graft survival after transplantation. It is therefore imperative that the condition of every graft-to-be is optimised prior to or at the time of transplantation and that additional injury is minimized in order to achieve the best possible post-transplant function and avoid primary non function (PNF), delayed graft function (DGF), and rejection with chronic graft failure.

Ischemia and reperfusion injury (IRI) is inevitable in (kidney) transplantation and one of the most important mechanisms for non- or delayed function immediately after transplantation [6–8]. It is accompanied by a proinflammatory response and is associated with acute rejection due to an increased immunogenicity favouring T-cell mediated rejection as well as anti-body mediated rejection (ABMR) [9,10]. In addition, it may result in progressive interstitial fibrosis and is associated with chronic graft dysfunction due to interstitial fibrosis and tubular atrophy (IFTA) [11]. In the past decade more insight has been gained in the complex molecular pathophysiology of IRI. This may open a door to new therapeutic targets aiming to reduce IRI. The aim of this review is to systematically highlight these molecular mechanisms and to discuss potential therapeutic strategies specifically targeting these molecular pathways.

2. Ischemia and Reperfusion Injury

IRI consists of a complex pathophysiology involving activation of cell death programs, endothelial dysfunction, transcriptional reprogramming and activation of the innate and adaptive immune system [8]. Numerous pathways and signalling cascades are implicated (Figure 1) and it is while worthy to dissect the distinct effects of ischemia and reperfusion (I/R).

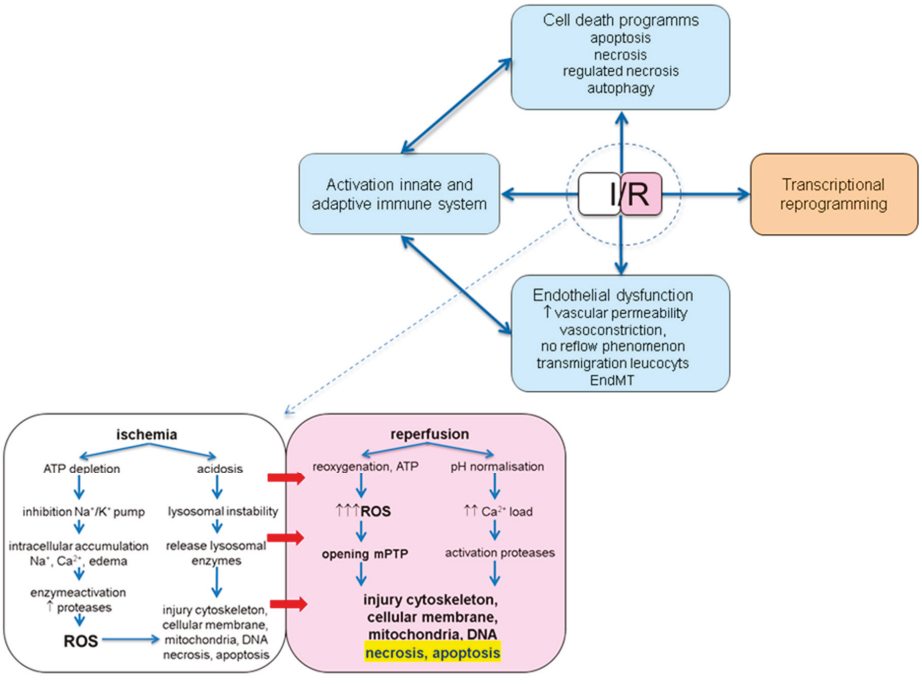

Figure 1. Schematic overview of the pathophysiological consequences of ischemia and reperfusion. I/R: ischemia/reperfusion; ATP: adenosine triphosphate; EndMT: endothelial to mesenchymal transition; ROS: reactive oxygen species; mPTP: mitochondrial permeability transition pore.

2.1. Ischemia

Due to a decrease in oxygen supply, cells will switch from an aerobic to an anaerobic metabolism, which results in a decrease in adenosine triphosphate (ATP) production and intracellular acidosis due to the formation of lactate. This causes destabilisation of lysosomal membranes with leakage of lysosomal enzymes, breakdown of the cytoskeleton and inhibition of membrane-bound Na^+/K^+ ATPase activity [12–14]. This last process gives rise to an intracellular accumulation of Na^+ ions and water with as a consequence cellular oedema. Due to declined Ca^{2+} excretion, there is an intracellular Ca^{2+} accumulation, which causes activation of Ca^{2+} dependant proteases like calpains. Due to the acidosis, these calpains stay inactive during the ischemic period but may damage the cell after normalisation of the pH during reperfusion. The remaining ATP is broken down to hypoxanthine, which will accumulate in the cell, since further metabolism into xanthine requires oxygen [15]. In the mitochondria, the Ca^{2+} overload is responsible for generation of reactive oxygen species (ROS) [8]. This will lead to opening of the mitochondrial permeability transition pores (mPTP) after reperfusion. During the ischemic period, only small amounts of ROS are produced compared to the entire I/R due to the reduction of cytochromes, nitric oxide synthases, xanthine oxidase and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation [16–19].

2.2. Reperfusion

During reperfusion, oxygen levels increase, and the pH normalises which is harmful for the previously ischemic cells. The intracellular Ca^{2+} level further increases, which activates the calpains causing injury to the cell structure and cell death [8]. Restoration of normoxemia leads to the production of large amounts of ROS, together with a reduction in the antioxidant capacity [20]. This burst of

ROS production was thought to be due to a generalised dysregulation of the electron transport chain with electrons leaking out at non-specific sites [21]. Recently, however, Chouchani et al. [22] showed that this superoxide production is generated by reverse action of complex I of the electron transport chain driven by a pool of succinate, a metabolite of the citric acid cycle, accumulated during ischemia. This massive amount of mitochondrially produced ROS is responsible for the activation of various injurious pathways through carbonylation of proteins or lipid peroxidation. This may contribute to injury of the cell membranes, the cytoskeleton and DNA and may lead to a disruption of ATP generation and induction of mPTP [20]. Additionally, the combination of ROS, dysfunctioning of the mitochondrial machinery and increase in mitochondrial Ca^{2+} load causes opening of the mPTP and release of substances like cytochrome C, succinate and mitochondrial DNA (mtDNA), which are able to induce cell death through apoptosis and necrosis and may act as danger/damage associated molecular patterns (DAMPs) entailing activation of the innate and subsequently the adaptive immune system [23–26].

Recent insights in the pathophysiological mitochondrial mechanisms and general understanding of the pivotal role of the mitochondria in IRI has led to various strategies targeting mitochondria with the aim to reduce IRI including limiting oxidative stress and mitochondrial ROS generation [20]. Both lipophilic cations and mitochondrial targeted proteins have been developed to deliver antioxidants to the mitochondria [27]. Triphenylphosphonium (TTP), a lipophilic cation, is rapidly taken up by mitochondria where it releases covalently bonded bioactive compounds. MitoQ, with its bioactive compound ubiqinone, is the most investigated of these molecules. In the mitochondria ubiqinone is reduced to ubiquinol, a powerful ROS scavenger. Administration of MitoQ in renal I/R models resulted in reduced markers of oxidative stress, reduced renal injury and improved function [28–30]. Regarding the mitochondrial targeted proteins, the Szeto-Schiller (SS) proteins are the best known. Exact mechanism of action is poorly understood but a possible explanation of action is through interaction with cardiolipin, an important component of the inner mitochondrial membrane. SS peptides have shown to reduce renal IRI in rodents [31], and its lead compound SS-31 (Elamipretide, Stealth BioTherapeutics-Alexion Pharmaceuticals) is currently being investigated in humans for its efficacy in reducing IRI post-angioplasty for renal artery stenosis. A pilot study administration of SS-31 before and during percutaneous transluminal renal angioplasty and stenting has shown to attenuate post-procedural hypoxia, increased renal blood flow and improved kidney function [32].

Another strategy to reduce ROS generation is reduction of succinate formation by inhibition of succinate dehydrogenase, preventing the accumulation of succinate, a driving force of reverse action of complex I. This has been shown to be effective in various in vivo models of IRI including the heart but has yet been unexplored in renal IRI [22,33].

3. Pathophysiological Consequences of IRI

3.1. Cell Death: Necrosis, Apoptosis, Regulated Necrosis and Autophagy

3.1.1. Necrosis

I/R leads to the activation of cell death programs. Of these programs, necrosis is the most uncontrolled form. It is due to swelling of the cell and subsequent rupture of the cellular membrane [34]. This will lead to an uncontrolled release of cellular fragments into the extracellular space. These fragments act as DAMPs and are able to activate the innate and adaptive immune system, entailing infiltration of inflammatory cells into the tissue and release of different cytokines.

3.1.2. Apoptosis

In contrast to the uncontrolled process of necrosis, apoptosis is a highly regulated and controlled process in which activation of the caspase signalling cascade results in a self-limiting programmed cell death. These caspases, a family of proteases, are essential in this process. There are two types of

caspases: initiator caspases (2,8,9,10) and effector caspases (3,6,7) [35,36]. The initiator caspases are activated by binding to a specific activator protein complex (death-inducing signalling complex (DISC), apoptosome) [37]. The formed complexes then activate the effector caspases through proteolytic cleavage upon which these proteolytically degenerate various intracellular proteins. Apoptosis gives rise to apoptotic bodies, containing these intracellular protein fragments, via the process of membrane blebbing. The apoptotic bodies will undergo phagocytosis before they can spill their content into the extracellular space and therefore will generate a less immune stimulating impulse compared to necrosis. Apoptosis can be initiated through the intrinsic pathway (mitochondrial dependent pathway) in which the initiating signal comes from within the cell (e.g., damaged DNA, hypoxia, metabolic stress) or the extrinsic pathway (cell death receptor pathway) due to signals from out of the cell (tumor necrosis factor-α (TNF-α), first apoptosis signal (Fas)-ligand, FasL) (Figure 2) [37].

A protein family playing an important role in the regulation of apoptosis is the B-cell lymphoma 2 (BcL-2) family [38]. Members of this family can act as protectors (BcL-2, BcL-xL) inhibiting apoptosis, sensors (BH3 only proteins, Bad, Bim, Bid) inhibiting the protectors, or effectors (Bax, Bad) initiating apoptosis by enhancing the permeability of the mitochondrial membrane [39]. In case of intrinsic signalling, intracellular signals of cell stress will lead to an increase in the BH3 only proteins resulting in inhibition of the protectors and activation of the effectors. These effectors increase the permeability of the mitochondrial membrane resulting in leakage of pro-apoptotic proteins upon which a caspase activator complex, the apoptosome, is formed in the intracellular space [40–43]. The apoptosome cleaves procaspase-9 to its active form of caspase-9, which in turn is able to activate the effector caspase-3. In case of the extrinsic signalling, binding of TNF-α (TNF path) or the FasL, expressed on cytotoxic T lymphocytes, (Fas path) to receptors of the TNF receptor (TNFR) family will lead to the formation of a complex called the death-inducing signalling complex (DISC) [44–46]. The DISC, amongst others, consisting of a death effector domain and three procaspase-8 or -10 molecules, cleaves and activates the procaspases [47]. Activation of the initiator caspase-8 by both paths directly activates other members of the caspase signalling cascade such as the effector caspase-3 but also can lead to an increase in BH3-only proteins (Bim, Bid) and trigger the intrinsic pathway (Figure 2) [48].

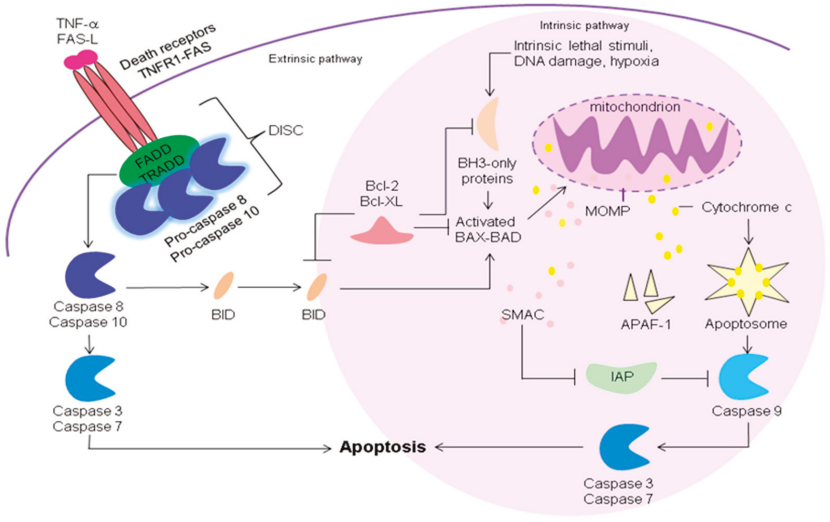

Figure 2. Extrinsic and intrinsic apoptotic pathway. The intrinsic pathway is mediated by intracellular signals of cell stress leading to an increase in the BH3 only proteins (members of the B-cell lymphoma 2 (Bcl-2) family) resulting in an inhibition of the protectors and activation of the effectors. The effectors Bax and Bad increase the permeability of the mitochondrial membrane (MOMP: mitochondrial outer membrane permeabilisation) resulting in leakage of apoptotic proteins. One of these proteins, known as second mitochondria-derived activator of caspases (SMAC), binds to proteins that inhibit apoptosis (IAPs, by suppression of the caspase proteins) causing an inactivation of these IAPs. Another protein released from the mitochondria is cytochrome c, which binds to Apoptotic protease activating factor-1 (Apaf-1) and ATP. This complex binds to procaspase-9 creating a complex, the apoptosome. The apoptosome cleaves procaspase-9 to its active form of caspase-9, which in turn is able to activate the effector caspase-3. The extrinsic pathway is mediated through receptors of the tumor necrosis factor (TNF) receptor (TNFR) family either via the TNF path or the Fas (first apoptosis signal) path. In the TNF path binding of TNF-α to a trimeric complex of TNFR1 molecules induces activation of the intracellular death domain and the formation of the receptor-bound complex 1 made up of TNF receptor-associated death domain (TRADD), receptor-interacting protein kinase 1 (RIPK1), two ubiquitin ligases (TNFR-associated factor (TRAF)-2 and cellular inhibitors of apoptosis (clAP)1/2) and the linear ubiquitin assembly complex (LUBAC). This complex 1 can lead to a pro-survival pathway or to apoptosis. In case of apoptosis the TRADD dependant complex IIa (consisting of TRADD, Fas-associated death domain protein (FADD) and caspase-8) or the RIPK-1 dependant complex IIb also known as the ripoptosome (consisting FADD, RIPK1, RIPK3 and caspase-8) is formed. In the Fas path, presence of the Fas ligand (FasL, expressed on cytotoxic T lymphocytes) causes three Fas receptors (CD95) to trimirize. This clustering and binding to the FasL initiates binding of FADD. Three procaspase-8 or -10 molecules can then interact with the complex by their own death effector domains. The complex formed is the death-inducing signalling complex (DISC) which cleaves and activates procaspase-8 and 10. Activation of the initiator caspase-8 by both paths directly activates other members of the caspase signalling cascade such as the effector caspase-3 but also can lead to an increase in BH3-only proteins (Bim, Bid) and trigger the intrinsic pathway).

3.1.3. Regulated Necrosis

Recently, new pathways of a more regulated form of necrosis have been described. These processes show features of apoptosis as well as necrosis. One of the best-known pathways of regulated necrosis is via TNFR-1 and is called necroptosis [46]. In the absence of active caspase-8, phosphorylation of receptor-interacting protein kinase 1 (RIPK1) and RIPK3 in complex IIb leads to formation of a

complex called the necrosome. The necrosome recruits Mixed Kinase Domain-Like protein (MLKL), which is then phosphorylated by RIPK3 [46]. MLKL activates the necrosis phenotype by entering the bilipid membranes of organelles and the cellular membrane. This causes formation of pores in these membranes and leads to release of cellular contents, functioning as DAMPs, into the extracellular space [49]. As in necrosis the DAMPs are able to activate both the innate and adaptive immune system promoting proinflammatory responses that activate rejection pathways [50,51]. A recent study in a kidney transplant mouse model showed that RIPK3-deficient kidneys had better function and longer rejection-free survival [52]. Therefore RIPK3-inhibiting drugs might be of interest in the reduction of IRI in organ transplantation. Next to TNFR-1, other death receptors and toll like receptors (TLR) have also shown to be able to induce necroptosis [46]. Other forms of regulated necrosis include mitochondrial permeability transition (MPT)-associated death (involving opening of mPTP leading to necrosis instead of apoptosis), ferroptosis (involving iron and gluthation metabolism), parthanatos (also known as PARP-1 (Poly(ADP-ribose) polymerase-1) dependent cell death, involving the accumulation of PAR (poly(ADP-ribose)) and the nuclear translocation of apoptosis-inducing factor (AIF) from mitochondria) and pyroptosis (involving caspase-1 and -11 in mice and caspase-4 and -5 in humans) [53]. The role of pyroptosis in IRI in the kidney, however, is unclear.

3.1.4. Autophagy

Cells can preserve their metabolic function and escape cellular death. This is due to autophagy of damaged cell parts. There are several pathways of autophagy, namely, macro-autophagy, micro-autophagy and chaperone-mediated autophagy—the last two are beyond the scope of this review. Macro-autophagy (hereafter called autophagy) involves formation of autophagosomes containing damaged cell parts or unused proteins. These double membrane autophagosomes travel through the cytoplasm to fuse with lysosomes (autolysosome) leading to degradation of the damaged cell parts. This process is continuously active at low basal levels, preserving cellular homeostasis, but stimulated upon stress through various signals like nutrient deprivation, ROS formation, hypoxia, free amino acids, etc. [54–56]. Cellular building blocks obtained from recycling of damaged cell parts by autophagy may serve as anti-stress responses and energy source promoting cell survival.

The first step in autophagy, the initiation, is regulated by two kinases: mammalian target of rapamycin complex 1 (mTOR, mTORC1) and adenosine monophosphate-activated protein kinase (AMPK) [54,57,58]. Together, they regulate the activity of the Unc-51 like autophagy activating kinase 1/2 (ULK1/2) complex [59,60]. Activation of mTOR leads to the phosphorylation of this complex and inhibition of autophagy (for instance, through the phosphatidylinositol 3-kinase (PI3K)/Protein kinase B (AKT) or the mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (Erk) 1/2 signalling pathway). On the other hand, activation of AMPK, upon intracellular AMP increase, activates autophagy [61]. This occurs by inhibition of the mTORC1 through dissociation of mTORC1 from ULK1/2 (indirect) or in a direct way by phosphorylation of ULK1/2 forming the ULK1/2-complex [62,63]. Next to the ULK1/2 complex, inducible beclin-1 complex (or class III PI3K complex) is involved in initiation of autophagy. This complex is activated by the ULK-1/2 complex and inhibited by Bcl-2 and Bcl-XL. The ULK1/2 and class III PI3K complexes join to form the phagopore and eventually the autophagosme which will fuse with a lysosome [64–69]. The content of this formed autolysosome is degenerated, and the components are released to be reused to synthesise new proteins or to function as an energy source for the cell (Figure 3) [70].

In renal IRI, autophagy is considered a doubled-edged sword. Upon I/R, it is mostly upregulated, but both protective and harmful effects are observed, proposing a dual role for autophagy in renal IRI [71,72]. Decuypere et al. [71] hypothesize that autophagy can switch roles depending on the severity of the ischemic injury. The exact mechanism behind this switch is unclear but may depend on the survival vs death properties of beclin1 and its interaction with the Bcl-2 family proteins [71,73]. Autophagy can be considered a protective mechanism in (oxidative) stress injured cells through restoring cellular homeostasis. Kidneys from older donors are at increased risk of DGF.

The age-dependent decline in autophagy activity and age-dependant autophagic dysfunction may be one of the underlying mechanisms of this phenomenon [74]. Extensive oxidative stress (amount or duration), however, may have detrimental effects which eventually could trigger the switch to aggravation of the injury through autophagy dependant cell death. Excessive or prolonged ROS exposure may lead to the oxidative modification of macromolecules making them only partially degradable by the autolysosome [75]. Furthermore, an energy dependent process of autophagy could deprive the cell of necessary energy. In this light, excessive autophagy seen after prolonged cold ischemia time in particular in DCD donors seems to be one of the underlying mechanisms behind augmentation of reperfusion injury seen in these circumstances, thereby increasing the risk of DGF [71,76]. Based on this dual role of autophagy in renal IRI and transplantation the goal would be to restrict autophagy levels within a protective window. Upon severe ischemia (prolonged cold ischemia time (CIT)) autophagy inhibitors most likely outweigh the activators [71]. Continuing efforts have to be made to elucidate the mechanism of autophagic transition from protective to harmful function.

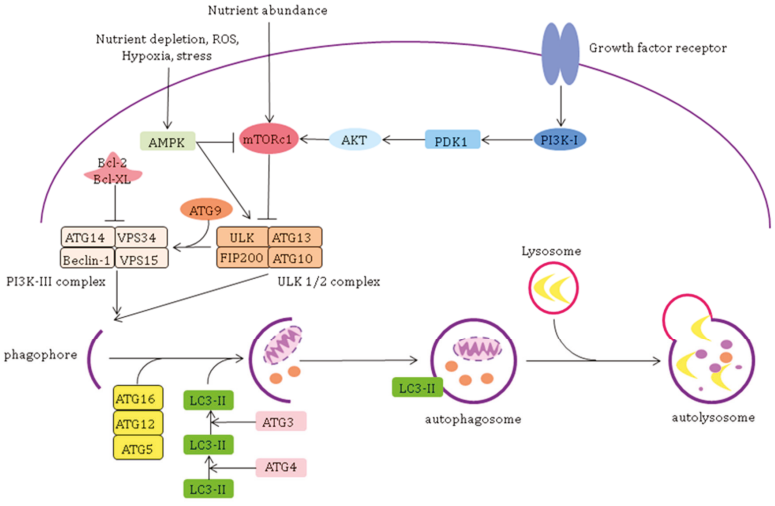

Figure 3. Pathways of macro-autophagy. Initiation of autophagy is regulated by mTORC1 (mammalian target of rapamycin complex 1) and AMPK (AMP-activated kinase). Together, they regulate the activity of the ULK1/2 complex consisting of ULK1/2 (Unc-51 like autophagy activating kinase), FIP200 (FAK family kinase interacting protein of 200 kDa) and the autophagy related proteins (ATG) ATG13 and ATG10. Activation of mTOR leads to the phosphorylation of this complex and inhibition of autophagy (for instance, through the phosphatidylinositol 3-kinase (PI3K)/ Protein kinase B (AKT) or the mitogen-activated protein kinase (MAPK)/ extracellular signal–regulated kinase (Erk) 1/2 signalling pathway) whereas activation of AMPK activates autophagy. AMPK, activated upon intracellular AMP increase, is able to activate autophagy by inhibition of the mTORC1 through dissociation of mTORC1 from ULK1/2 allowing ULK1/2 to be activated. AMPK, is also able to initiate autophagy in a direct way by phosphorylation of ULK1/2 forming the ULK1/2-complex.Another complex involved in the initiation is the autophagy inducible beclin-1 complex (or class III PI3K complex) which consists of Vps34 (phosphatidylinositol 3-kinase), beclin-1 (a BH3 only domain protein member of the Bcl-2 family), vps15 and ATG14. This complex is activated by the ULK-1 complex and inhibited by Bcl-2 and Bcl-XL. The ULK1/2 and class III PI3K complexes join to form the phagopore and eventually the autophagosme. This process is mediated by the ATG5-ATG12-ATG16 complex and the formation of phosphatidylethanolamine-conjugated Light Chain (LC) 3 (LC3-II) facilitating elongation of the bilipid membrane to form a closed autophagosme. The autophagosome fuses with a lysosome and the content of the autolysosome is degenerated and the components are released to be reused to synthesise new proteins or to function as an energy source for the cell. PDK-1: pyruvate dehydrogenase kinase-1.

The different cell death programs described above are induced in response to common stimuli. Several proteins in the autophagy and apoptosis pathway are shared resulting in an intimate crosstalk between apoptosis and autophagy. Regulation of these proteins determines cellular fate to cell survival or cell death. Caspase-mediated degradation of several autophagy regulation proteins limits autophagosome formation and therefore autophagy [77–79]. Apoptosis inhibitors Bcl-2 and Bcl-XL also inhibit autophagy by binding to Beclin-1 limiting its availability to form the classIII PI3K complex [80,81]. Inhibition of cisplatin induced autophagy enhanced caspase-3 activation and apoptosis in renal proximal tubular cells [82,83]. On the other hand, overexpression of ATG5 and beclin-1 prevented cisplatinum induced caspase activation and apoptosis [84]. Additionally, there is evidence that autophagy induction regulates necroptosis. Inhibition of autophagy has shown to prevent necroptosis and vice versa inhibition of necroptosis is able to supress autophagy [85,86].

3.1.5. Targeting Cell Death Programs

Targeting pathways of cell death programs to reduce IRI seems very attractive, since it directly preserves cellular function. Secondly, dead cells releasing DAMPs elicit a strong immune response not only in the organ exposed to I/R but also in other organs of the individual, so called remote organ injury. Therefore, interfering with this process might be immunosuppressive and organ protective. The relative contribution of each of the cell death programs to IRI and outcome in transplantation, however, has to be elucidated.

Nydam et al. [87] showed in a syngeneic mouse transplant model that administration of the pan-caspase inhibitor Q-VD-OPh during graft retrieval and cold preservation resulted in decreased caspase-3 expression and activity, reduced apoptosis in renal tubular cells and improved renal function post-transplantation. The pro-apoptotic gene p53 is activated upon hypoxia, oxidative stress and DNA damage and is able to induce cell cycle arrest, which enables DNA-repair proteins to repair the sustained injury. However, in case of severe DNA damage it induces apoptosis by initiating the intracellular pathway.

Inhibition of P53 in proximal tubular cells has been shown to decrease apoptotic cell death and provide protection against IRI [88,89]. QPI-1002 is a synthetic small interfering ribonucleic acid (siRNA) designed to reversibly and temporarily inhibit p53. In pre-clinical models it has been shown that QPI undergoes rapid glomerular filtration and uptake by proximal tubular epithelial cells [89]. Administration of QPI-1002 has shown to be safe in humans. Two phase I dose escalating safety and pharmacokinetics studies in patients undergoing major cardiovascular surgery (NCT00554359, NCT00683553) has been executed without dose-limiting toxicities or safety issues. A phase I/II study has been executed to evaluate QPI-1002 for the prevention of DGF in recipients of kidneys from deceased donors (NCT00802347) in which treatment with QPI-1002 resulted in lower incidence and severity of DGF [90]. Recently, a phase 3 randomized, double-blind, placebo-controlled study in recipients (n = 594) of (older) DBD donor kidneys (>45 years) has been completed (NCT02610296, ReGIFT-study). Results have not been reported yet.

Various pharmacological substances like necrostatins (RIPK1 inhibitors, necroptosis), ferrostatins (ferroptosis), sanglifehrin A (MPT-associated death) and olaparib (parthanatos) and many others have been developed to target specific key molecules of the different programs of regulated necrosis and are currently tested in various animal and disease models (Figure 4) [91,92]. The question remains how safe it will be to inhibit non-apoptocic cell death pathways in patients, since these pathways also function as a backup system when apoptosis fails or is inhibited for instance, by caspase inhibitor expressing viruses. Of these molecules, RIPK1 inhibitors have now entered clinical trials and their safety is being tested in healthy volunteers [93,94].

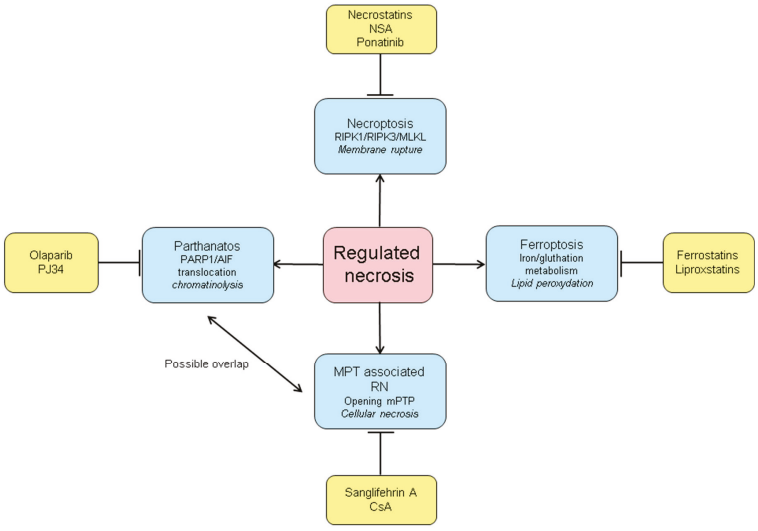

Figure 4. Programs of regulated necrosis and their inhibitors. RIPK1: receptor-interacting protein kinase 1; RIPK3: receptor-interacting protein kinase 3; MLKL: Mixed Kinase Domain-Like protein; MPT: mitochondrial permeability transition; mPTP: mitochondrial permeability transition pore; RN: regulated necrosis; CsA: cyclosporin A; PARP1: poly (ADP-ribose) polymerase-1; AIF: apoptosis-inducing factor.

3.2. Endothelial Dysfunction

At a vascular level, I/R leads to swelling of the endothelial cells (ECs), loss of the glycocalyx and degradation of the cytoskeleton. As a consequence, intercellular contact of endothelial cells is lost, increasing vascular permeability and fluid loss to the interstitial space [95]. Furthermore, the endothelium will produce vasoactive substances like platelet-derived growth factor (PDGF) and Endothelin-1 (ET-1), causing vasoconstriction [96]. This vasoconstriction can be enhanced by a reduced nitric oxide (NO) production during reperfusion due to decreased endothelial nitric oxide synthase (eNOS) expression and increased sensitivity of the arterioles for vasoactive substances like angiotensin II, thromboxane A2 and prostaglandin H2 [97–99]. Eventually this can lead to the so called no reflow phenomenon characterized by the absence of adequate perfusion on microcirculatory level despite reperfusion.

The regenerative capacity of ECs in peritubular capillaries is limited and injury to the microcirculation may lead to permanent peritubular capillary rarefaction [100,101]. Chronic hypoxia in these regions may induce transcription of fibrogenic genes like transforming growth factor-β (TGF-β) and connective tissue growth factor (CTGF) together with an accumulation of α-smooth muscle actin (α-SMA) [101]. In the end, this may lead to development of IFTA, a process which has mainly been attributed to resident fibroblasts. More recently, however, the role of endothelial-to-mesenchymal transition (EndMT) in this process has been described [102,103]. During EndMT, ECs lose their endothelial phenotype (such as expression of specific endothelial markers like Von Willebrand factor (VWF)) and acquire the phenotype of multipotent mesenchymal cells (MSC). These cells show an increased expression of α-SMA, neuronal (N)-cadherin, vimentin and fibroblast-specific protein-1 and exhibit enhanced migratory potential and increased extracellular matrix production [104–106]. In a porcine I/R model Curci et al. [102] showed that 20%–30% of the total α-SMA+ cells emerging after IRI were also CD31+ suggesting a different origin compared to resident activated fibroblasts. Man et al. [107] showed that in kidney transplant recipients experiencing IFTA and allograft dysfunction, progression of EndMT plays an important role. EndMT is controlled by complex signalling pathways

and networks. In their porcine I/R model, Curci et al. [102] showed a critical role of complement in this process. Kidneys of pigs treated with recombinant C1 inhibitor (C1-INH) showed preserved EC density, significant reduction of α-SMA expression and limited collagen deposition 24 h after I/R compared to untreated pigs. The ECs in the treated pigs showed preserved physiological conformation and position tight to the basal layer of the vessels. The number of transitioning ECs was significantly lower in the treated animals. In an additional in vitro experiment activating ECs with the anaphylatoxin C3a, they showed that C3a induced down regulation of the expression of VWF whilst upregulating α-SMA, by activating the Akt pathway. Activation of the ECs with C5a showed a similar response [102]. Targeting signalling pathways in EndMT in kidney transplantation could be of interest to reduce IFTA and enhance long-term graft survival. More insight however has to be gained to the exact role of EndMT in renal transplantation and what suitable targets to aim for. Furthermore, since EndMT gives rise to multipotent MSC this placidity could be of interest to push these MSCs in the direction of regeneration rather than fibrosis.

An important feature of IRI is the chemotaxis of leukocytes, endothelial adhesion and transmigration of these cells into the interstitial compartment [108]. This process is initiated by increased expression of P-selectin on the endothelial cells and interaction of P-selectin with P-selectin glycoprotein 1 (PSGL-) expressed on the leukocytes. This interaction results in rolling of the leukocytes on the endothelium. Subsequently, firm adherence of the leucocytes to the endothelium is achieved by the interaction of the β2-integrins lymphocyte function-associated antigen 1(LFA-1) and macrophage-1 antigen (MAC-1 or complement receptor 3, CR3) on the leukocyte and the intracellular adhesion molecule 1 (ICAM-1) on the endothelial cells. Platelet endothelial cell adhesion molecule 1 (PECAM-1) thereafter facilitates transmigration into the interstitial space. Once activated, these leukocytes will release several toxic substances like ROS, proteases, elastases and different cytokines in the interstitial compartment which will result in further injury like increased vascular permeability, oedema, thrombosis and parenchymal cell death (Figure 5) [109].

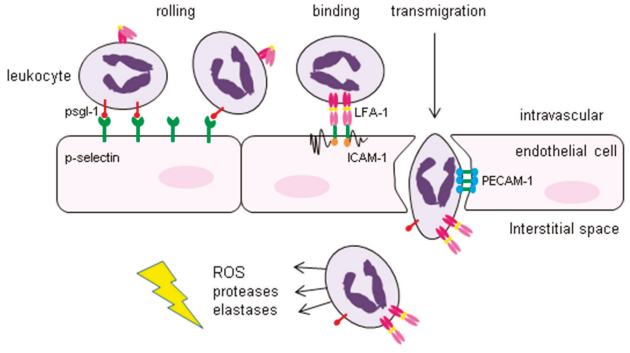

Figure 5. Interaction of leukocytes and endothelial cells in the process of transmigration of leukocytes. The increased expression of P-selectin on the endothelial cells upon I/R facilitates interaction with P-selectin glycoprotein 1 (PSGL-) expressed on the leukocytes. This results in rolling of the leukocytes on the endothelium. Subsequently, firm adherence of the leucocytes to the endothelium is achieved by interaction of lymphocyte function-associated antigen 1(LFA-1) and macrophage-1 antigen (MAC-1 or complement receptor 3, CR3) on the leukocyte and the intracellular adhesion molecule 1 (ICAM-1) on the endothelial cells. Finally, platelet endothelial cell adhesion molecule 1 (PECAM-1) facilitates transmigration of the leukocytes into the interstitial space. Once activated, these leukocytes will release several toxic substances like ROS, proteases, elastases and different cytokines in the interstitial compartment resulting in further injury like increased vascular permeability, oedema, thrombosis and parenchymal cell death.

3.3. Innate and Adaptive Immune Response

IRI is accompanied by sterile inflammation in which the innate as well as the adaptive immune system are involved.

3.3.1. Innate Immune Response

The innate, or non-specific, immune system is evolutionary the oldest part of the immune system. It acts on infection or injury with a fast, short-lasting and non-specific response in which different cells and systems are involved.

Toll-Like Receptor Signalling

In the innate immune response, the toll-like receptors (TLRs) play an important role [110]. TLRs are transmembrane proteins and members of the interleukin-1 receptor (IL-IR) superfamily. They function as pattern recognition receptors (PRR) and are present on the cellular membrane and in the cytosol of cells like leukocytes, endothelial cells and tubular cells [111]. The human TLR family contains 10 members, TLR1–TLR10 [112]—of which, TLR2 and TLR4 have shown to be upregulated in tubular epithelial cells upon ischemia [113–117]. Both are attributed an equal importance in initiating apoptosis in a genetic knock-out renal I/R mouse model [115]. TLR activation leads to the downstream recruitment of various adapter molecules (TNF receptor-associated factor 6 (TRAF6), Myeloid differentiation primary-response protein 88 (MyD88), toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM)) activating different kinases (IL-1 receptor-associated kinase (IRAK)-1 (IRAK-1), IRAK-4, inhibitor of nuclear factor-κB kinase (IKK), TANK-binding Kinase-1 (TBK1)), leading to activation of transcription factors (nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), IFN-regulatory factor 3 (IRF3) resulting in transcription of proinflammatory genes and the subsequent inflammatory response [8,112].

TLR2 and TLR4 have polyvalent ligand binding activity and can be activated by exogeneous (e.g., lipopolysaccharide, LPS) and endogenous ligands comprising DAMPs released upon I/R. These DAMPs vary depending on type of injury and tissue involved. High-mobility group box-1 (HMGB-1), an intracellular protein involved in the organisation of DNA and the regulation of gene transcription, is one of the DAMPs linked to the pathogenesis of IRI [118–120]. From the nucleus, HMGB-1 can be released into the cytosol or extracellular space by passive leakage from injured cells or through active secretion by immune cells [121,122].

In IRI in the kidney, TLR4 plays an important role. Bergler et al. [123] showed that TLR4 is highly upregulated after renal IRI, and that high TLR4 expression is strongly correlated with graft dysfunction in an allogenic renal transplant model in rats. Furthermore, TLR4-deficient mice are protected against renal IRI and kidneys from donors with a TLR4-loss of function allele show less pro inflammatory cytokines in the kidney after transplantation and a higher percentage of immediate graft function [118,124]. Activation of TLR4 in renal IRI has various consequences on the graft. First of all it promotes the release of different proinflammatory mediators like IL-6, IL-1β and TNF-α, accompanied by an increased expression of macrophage inflammatory protein-2 (MIP-2) and monocyte chemo attractant protein-1 (MCP-1) involved in the recruitment of neutrophils and macrophages [124]. Second, TLR-4 activation leads to increased expression of adhesion molecules ICAM-1, vascular cell adhesion molecule 1 (VCAM-1) and E-selectin facilitating leukocyte migration and infiltration into the interstitial space. TLR-4 signalling seems mandatory for this increased expression. Chen et al. [125] showed that increased expression of adhesion molecules after renal IRI was absent in TLR4 knockout mice in vivo and the addition of HMGB-1 to isolated endothelial cells increased adhesion molecule expression on cells from wild-type but not from TLR4 knockout mice. Thirdly, activation of TLR4 on circulating immune cells of the innate immune system leads to activation of these cells. Neutrophils and macrophages are involved in an early stage after reperfusion. Neutrophils are regarded as the primary

mediators of injury and their activation leads to ROS release, secretion of different proteases and renal tissue injury [126]. Upon activation, macrophages release proteolytic enzymes and proinflammatory cytokines like TNF-α, IL-1β and interferon-γ (IFN-γ) [127]. In TLR-4 knockout mice subjected to IRI, neutrophil and macrophage infiltration was reduced [124]. Finally, the TLR4-facilitated immune response is linked to renal fibrosis. The upregulation of TLR4 upon I/R induces a strong inflammatory response accompanied by tubular necrosis, loss of brush border, formation of casts and tubular dilatation [124]. Such a robust inflammation is known to potentiate interstitial fibrosis [128].

Proposed endogenous ligands for TLR-4 in renal IRI include HMGB-1, extracellular matrix (ECM) components like biglycan, heparin sulphate and soluble hyaluronan, and heat shock proteins (Hsps) [129–134]. Upon ligand binding, activation of TLR4 leads to downstream signalling via the MyD88-dependent and MyD88 independent pathway (Figure 6). The MyD88-dependent pathway in which MyD88 and TIRAP or MyD88 adapter-like (Mal) recruits and activates members of the IRAK family is considered to be the dominant pathway [124,135]. Wang et al. [136] demonstrated that MyD88- and TRIF-deficient mice showed a significant reduction in interstitial fibrosis reflected by α-SMA and collagen I and II accumulation Furthermore, Administration of the MyD88 specific inhibitor TJ-M2010-2, a small molecular compound, inhibiting the homodimerisation of MyD88, in a renal I/R model in mice has shown to prolong the survival rate, preserve renal function and attenuate the inflammatory responses and apoptosis in the kidney. In the long term, inhibition of the TLR/MyD88 signalling pathway with TJ-M2010-2 attenuated renal fibrosis via inhibition of TGF-β-induced epithelial to mesenchymal transition [137]. Liu et al. [138] showed that pre-treatment with the synthetic TLR4 inhibitor eritoran (Eisai co., Ltd, Tokyo, Japan) in an renal I/R rat model resulted in reduced expression of TNF-α, IL-1β and MCP-1, attenuated monocyte infiltration in the kidney and improved renal outcome Altogether in view of the pivotal role of TLR4 in renal IRI, inhibition of TLR4 or upstream or downstream mediators could be an interesting target in reducing IRI and optimising graft survival.

Next to TLR4, TLR 2 is markedly upregulated upon ischemic injury in the kidney and its upregulation is associated with the initiation of an inflammatory response [139]. Kidneys of TLR2-/- mice subjected to I/R showed less tubular damage compared to TLR2+/+ mice. Reduced levels of MIP-2, MCP-1, and IL-6 and reduced levels of infiltrating leucocytes were seen [140]. The role of TLR2 in the development or progression of renal fibrosis, however, is less clear. Leemans et al. [139] showed that in a mouse model of obstructive nephropathy TLR2 does not play a significant role in renal progressive injury and fibrosis. In addition to this de Groot et al. [141] showed in human allograft biopsies that TLR2 expression 6, 12 and 24 months after transplantation is associated with superior graft outcome in the long run Currently, the humanized immune globuline (Ig) G4 (IgG4) monoclonal antibody against TLR2 OPN-305 (Tomaralimab, Opsona Therapeutics Ltd, Dublin, Ireland) has entered phase 2 trials (NCT01794663) with the aim to reduce delayed graft function in recipients of post-mortal donor kidneys. In the first part (A) of this study a single dose of 0.5 mg/kg administered 1h before reperfusion was associated with full inhibition of TLR2 and an 80% reduction of IL-6 [142]. Subsequently, this dose has been used in part B of the study, which has been completed but results have not been reported yet.

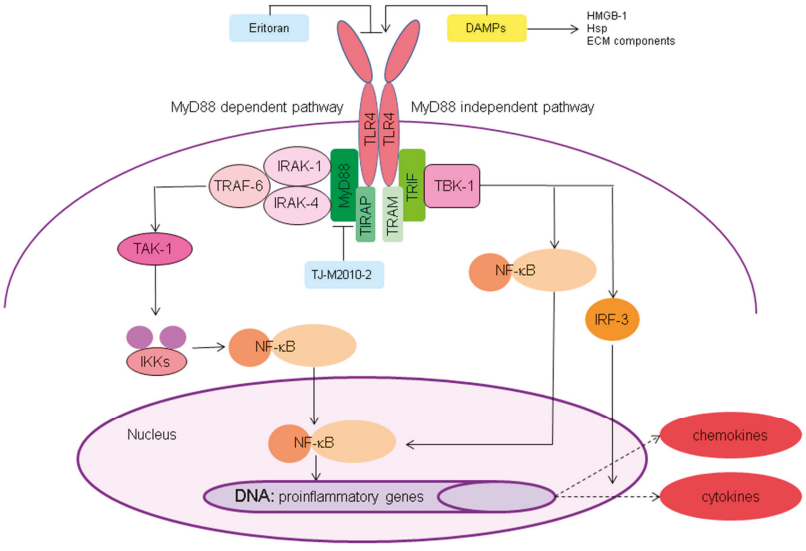

Figure 6. Toll-like receptor 4 signalling. Activation of toll-like receptor 4 (TLR4) by danger associated molecular patterns (DAMPs), like high mobility group box-1 (HMGB-1), heat shock proteins (hsp) and extracellular matrix (ECM) components, leads to downstream signalling via the MyD88 (Myeloid differentiation primary-response protein 88) dependent and MyD88 independent pathway. The MyD88-dependent pathway in which MyD88 and TIRAP (toll-interleukin 1 receptor (TIR) domain containing adaptor protein) or MyD88 adapter-like (Mal) recruits and activates members of the IL-1 receptor-associated kinase (IRAK) family is considered to be the dominant pathway. IRAK activation leads to recruitment of TRAF6 (TNF receptor-associated factor 6) and subsequently activation of transforming growth factor beta-activated kinase 1 (TAK1). Activation of TAK1 then leads to the activation of inhibitor of nuclear factor-κB kinase (IKK), which results in the release of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) from its inhibitor, promoting translocation to the nucleus. The MyD88 independent pathway is mediated by the adapter molecules TIR-domain-containing adapter-inducing interferon-β (TRIF)/TRIF-related adaptor molecule (TRAM) and downstream signalling leads to activation of 2 inhibitor of nuclear factor-κB kinase (IKK) homologs IKKε and TANK-binding Kinase-1 (TBK1), which possibly form a complex together and activate transcription factors NF-κB and IFN-regulatory factor 3 (IRF3). From here, proinflammatory gene transcription is initiated. TLR4 signalling is inhibited by Eritoran and TJ-M2010-2.

Complement System

The complement system is the second crucial player in the innate immune response in IRI. The system consists of soluble proteins, regulatory proteins and membrane-bound receptors and comprises three pathways. DAMPs released upon I/R are able to activate all three pathways via binding to C1q (classical pathway), C3 (alternative pathway) or PRRs of the lectin pathway (LP).

Recently, the LP has been pointed out as the primary route of renal complement activation after I/R [143]. Activation of the LP can take place through various PRRs like collectins (manose binding lectin (MBL) and collectin-11) [144] and ficolins (ficolin 1-3) [145]. Upon binding of the collectin–mannan-binding lectin serine protease (MASP) complex to carbohydrate-bearing ligands (for instance, mannose or fructose expressed on stressed cells) the MASPs are activated to cleave complement component (C) 4 (C4) and C2. LP activation is critically dependant on the action of MASP-2 [146,147]. In an isograft transplantation model in wild-type and MASP-2-deficient mice, Asgari et al. [147] showed that renal function was preserved with MASP-2 deficiency After complex-ligand interaction,

LP proceeds with cleavage of C4 and C2, mediated by MASP-2, leading to the synthesis of the classical pathway C3 convertase. Recently, a C4 independent bypass in the LP pathway was also demonstrated [122]. This could explain why C4-deficient mice are not protected against renal I/R and cellular mediated rejection [148,149]. One of the PRRs assigned an important role in the LP is collectin-11 (CL-11), a soluble C-type lectin containing a carbohydrate recognition domain and MASP binding domain [150]. In renal tissue, tubular cells are the main source of CL-11 and expression increases after IRI [151]. CL-11 has been appointed an important role in complement activation in the kidney. It has been shown that CL-11 engages L-fucose at sites of ischemic stress and inflammation initiating the LP [147]. In a renal I/R model, CL-11-deficient mice showed no post-ischemic and complement mediated injury supporting the importance of CL-11 in triggering renal complement activation.

All activating routes converge and lead to the formation of the C3 convertase (C4b2b, C3bBbP). C3 convertase cleaves and activates additional C3, creating C3a and C3b. C3b together with C4b2b forms the C5 convertase, which will cleave C5 into C5a and C5b. C5b together with C6–9 will then form the Membrane Attack Complex (MAC, C5b-9). The formed complement effectors will lead to opsonisation (C3b), chemotaxis of neutrophils and macrophages (C3a, C5a) [143]. The formed MAC inserted into the cellular membrane is associated with a proinflammatory response via noncanonical NF-KB signalling (Figure 7) [152,153].

Next to inducing inflammation and cell death, the complement system is able to modulate antigen presentation and T cell priming via C3a and C5a and is therefore playing a role in donor antigen sensitisation and rejection [154]. Antigen-presenting cells (APC) express C3 and C5 along with complement receptors C3aR and C5aR1. Upon complement activation in the extracellular space, C3a and C5a increase the presentation of alloantigens and expression of co-stimulatory molecules on the APC enhancing APC priming of T cells [143]. Furthermore, C3a and C5a promote T-cell differentiation of CD4+ and CD8+ T-cells. CD8+ cells mediate vascular and cellular T-cell mediated rejection. Upon activation, CD4+ T-cells can stimulate further CD8+ T-cell differentiation, they can proliferate and differentiate to memory and effector CD4+ cells which can activate macrophages, recruit leukocytes and stimulate inflammation and finally CD4+ cells stimulate B-cell differentiation and in the end antibody production [143]. The B-cells response can also be enhanced in a direct manner via C3b and C3d on the APC and the complement receptor 2 (CR2) on the B-cell. Activation of the B-cell by binding to the donor alloantigen induces class switching of the donor specific antibody from IgM to IgG. Subsequently, ABMR occurs when IgG donor specific antibodies (DSA) recognizes antigens in the kidney graft and engage with C1q, C1r and C1s to activate the classical pathway [143]. Under normal physiological circumstances, formation of the complement effectors is controlled by proteins (soluble or surface bound) that mediate break down of the C3 and C5 convertases. After I/R this balance shifts to uncontrolled complement activation predisposing the graft to complement mediated injury and rejection [155].

Many interventions on the level of C3, C5, and regulatory proteins in I/R injury and especially kidney transplantation have been evaluated in pre- and clinical studies [156]. Eculizumab (Soliris®, Alexion Pharmaceuticals, New Haven, CT, USA) is to date the best studied complement inhibitor in kidney transplantation. Therapeutic inhibition of C5 with the use of eculizumab, an anti-human C5 micro antibody, showed potential in the prevention and/or treatment in AMBR [157–159] and has been investigated as such in several phase 2/3 clinical trials (NCT01567085, NCT01106027, NCT01399593). All studies report a safety profile of the drug that is consistent with that reported for eculizumab's approved indications like atypical haemolytic uremic syndrome. Results of these trials suggest a potential role of eculizumab in the prevention and treatment of ABMR in patients with DSA [160,161]. Next to ABMR, eculizumab has been investigated for the prevention of DGF (NCT01919346, NCT02145182). Again, the safety profile was good but pre-treatment with eculizumab had no effect on the incidence of DGF. Groups in these studies, however, were rather small [162]. Another anti-C5 antibody Tesidolumab (LFG-316, MorphoSys, Novartis) has currently entered phase 1 studies (NCT02878616).

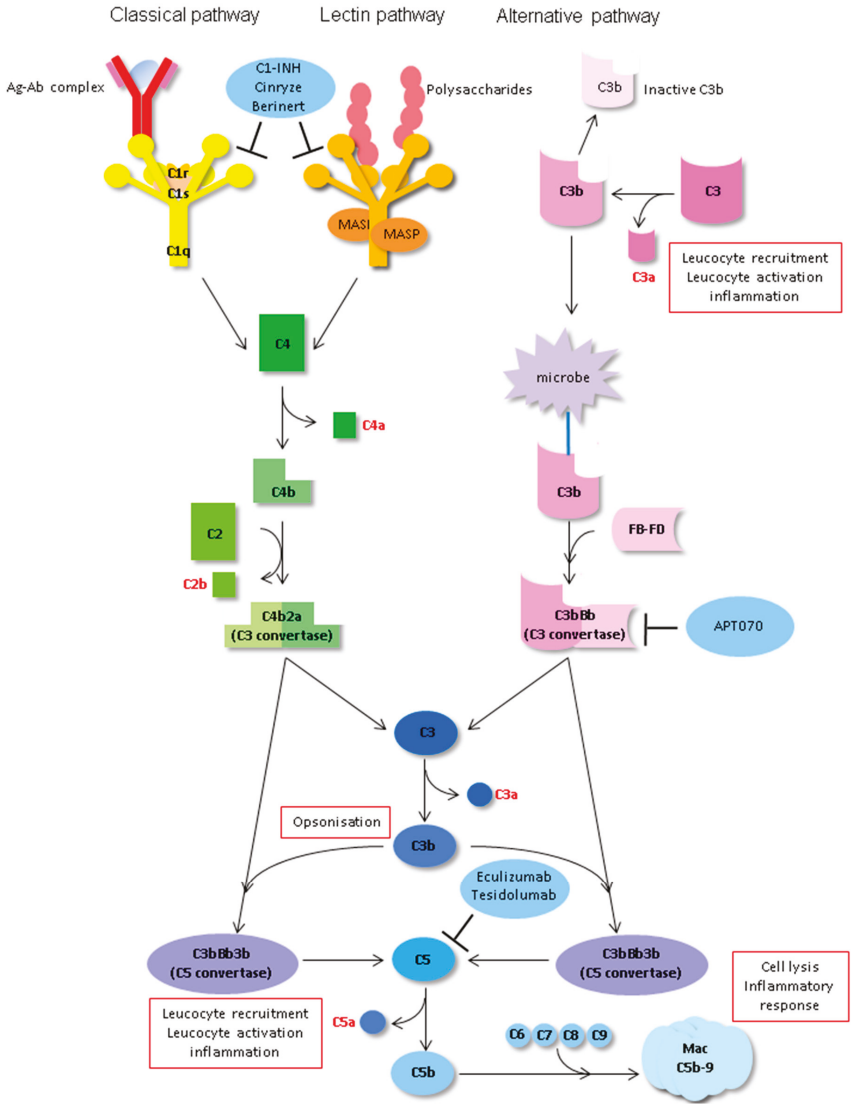

Figure 7. Routes of the complement system with its inhibitors currently studied in kidney transplantation. Damps released upon I/R are able to activate all three pathways via binding to C1q (classical pathway), C3 (alternative pathway) or pattern recognition receptors (PRRs) of the lectin path. All activating routes converge and lead to the formation of the complement component (C) 3 (C3) convertase (C4b2b, C3bBbP). C3 convertase cleaves and activates additional C3, creating C3a and C3b. C3b together with C4b2b forms the C5 convertase, which will cleave C5 into C5a and C5b. C5b together with C6–9 will then form the Membrane Attack Complex (MAC, C5b-9). The formed complement effectors will lead to opsonisation (C3b), chemotaxis of neutrophils and macrophages (C3a, C5a). The formed MAC inserted into the cellular membrane is associated with a proinflammatory response via noncanonical NF-KB signalling. C1-inhibitors (C1-INH), Cinryze®and Berinert®target complement initiation and APT070 complement amplification. Eculizumab and Tesidolumab inhibit complement activation at the level of C5.

In addition to targeting terminal complement pathways, therapeutics targeting complement initiation (C1) and amplification (C3, convertases) have been developed. C1 esterase inhibitors (C1-INH) should not be considered complement-specific inhibitors, since these broad protease inhibitors and their functions extend beyond the classical pathway and even beyond the complement system [163]. The C1INH Cinryze®(Shire US Inc., Lexington, MA, USA) is recently being evaluated for treatment of ABMR (NCT02547220). The study was terminated May 2019 following a pre-scheduled interim analysis, it was determined that the study met the pre-specified criteria for futility. Cinryze®is still listed to be tested as a pre-treatment to reduce IRI and DGF (NCT02435732). Another C1INH, Berinert®(CSL Behring, King of Prussia, PA, USA), has been evaluated in a phase 1/2, double-blind, placebo-controlled study assessing its safety and efficacy for prevention of delayed graft function in recipients of deceased donor kidneys [164]. Although the primary outcome measure (DGF) was not met, treatment with Berinert®was associated with significantly fewer dialysis sessions 2 to 4 weeks post-transplantation. In addition, a better renal function was seen at 1 year compared with the placebo treated group. No significant adverse events were noted in this study [164]. Finally, Mirococept (APT070) a membrane-localising C3 convertase inhibitor is currently being evaluated in a double-blind randomised controlled investigation its efficacy for preventing IRI deceased donor kidneys (EMPIRIKAL-trial, ISRCTN49958194) [165].

Translation to the Adaptive Immune System

The link between the innate and adaptive immune response is made by dendritic cells (DCs, Figure 8). DCs are APCs and play an essential role in the pathogenesis of IRI. Immature DCs can be activated by DAMPs via TLRs and the complement system. After maturation, they are able to activate the adaptive immune system in a direct manner by antigen presentation to B- and T-cells or indirectly via cytokine signalling [8,166]. This process can already start in the donor in which in case of a DBD donor, DCs are activated by oxidative stress or C5a and present donor antigens to T-cells of the recipient [167]. Furthermore, it is thought that DCs (subtype CDC11c+ and F4/80+) play an important role in the early pathophysiology of IRI by secretion of TNF-α, Chemokine (C-C motif) ligand 5 (CCL5), IL-6 and MCP-1within the first 24h after IRI [168]. Further, at a later stage, DCs contribute to allograft dysfunction. Batal et al. [169] looked at kidney transplant biopsies performed > 15 days after transplantation and found that a high DC density was independently associated with poor graft survival. Additionally, they found that high DC density was correlated with an increased T-cell proliferation and poor patient outcome in patients with high total inflammation scores of biopsies, including inflammation in areas of tubular atrophy. In these patients, DC density could predict allograft loss. When looking at the origin of the DCs they showed that initially donor DC predominated but found that in late biopsies the majority of DCs were of recipient origin. These data suggest a potential rationale to target DCs influx in the kidney to improve long-term allograft survival.

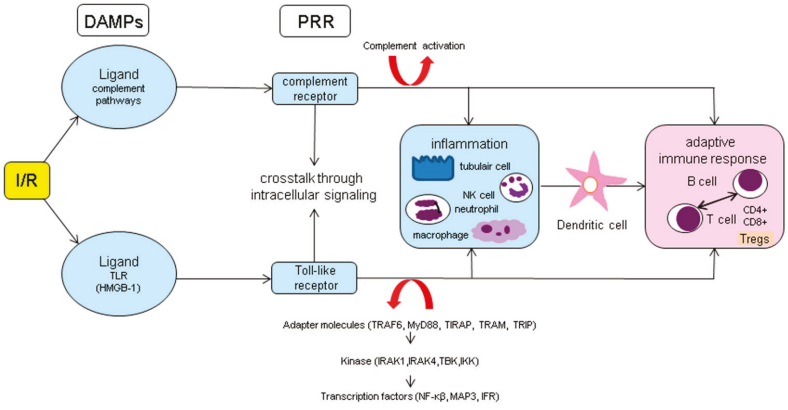

Figure 8. Interaction of the innate and adaptive immune system in the pathophysiology of ischemia and reperfusion injury. DAMPs released upon I/R are able to activate the innate immune system by binding to PRRs like complement receptors and TLRs. Activation of these receptors will lead to production of pro-inflammatory cytokines and chemokines and chemotaxis, opsonisation and activation of leucocytes like macrophages, neutrophils and natural killer (NK) cells. Additionally, immature dendritic cells can be activated, which, after maturation, are able to activate the adaptive immune system in a direct manner by antigen presentation to B- and T-cells or indirectly via cytokine signalling. Treg: regulatory T-cell.

3.3.2. Adaptive Immune Response

In contrast to the non-specific nature of the innate immune response, the role of the adaptive immune system is to recognize alloantigens and to react with an alloantigen-specific response, simultaneously generating immunological memory. Involved cells are B- and T-cells.

T-Cells

Activation of T-cells occurs through binding of the T-cell receptor (TCR) on the surface of the T-cell, to the major histocompatibility complex (MHC, in case of humans the human leucocyte antigen (HLA) system) on the APC. This can be in a direct way when the TCR binds to unprocessed allogenic MHC on the APC of the donor or in an indirect manner when MHC proteins of the donor have been taken up by APC of the recipient, processed and presented by the MHC of the recipient [170]. In case of IRI, CD4+ T helper (Th) cells as well as CD8+ cytotoxic T-cells are found in the kidney and are important mediators of IRI [171–174]. T-cell-deficient mice showed attenuated renal IRI and adoptive T-cell transfer experiments in athymic mice resulted in acute kidney injury (AKI) [175–177].

The TCR on CD4+ T-cells can only bind to MHC class 2 molecules (HLA DP, DQ, DR). Upon activation, these CD4+ T-cells become cytokine producing effector cells harming the graft through cytokine mediated inflammation [170]. The effector CD4+ Th cells can differentiate into three major subtypes Type 1 (Th1), Type 2 (Th2) and Th17 cells depending on the cytokines they produce and the transcription factors they express. This differentiation process, referred to as polarisation, starts with induction in lymphoid tissue. Cytokines produced by APCs (DCs and macrophages), NK cells, basophils and mast cells act on T-cells stimulated by the antigen and co-stimulators. This induces transcription of cytokine genes characteristic for the particular subset. Upon continued activation, genetic modifications occur, keeping the characteristic cytokine genes in a transcriptionally active state (commitment). The cytokines produced by the subset promote development of this subset and inhibit differentiation toward other subsets (amplification) [170]. The main effector cytokine of Th1 cells is IFN-γ and the key Th1 transcription factors are signal transducer and activator of transcription (STAT) 4 (STAT-4) and the T-box transcription factor T-bet. Main effector cells are macrophages, B-cells,

CD8+ T-cells and CD4+ T-cells (amplification). IFN-γ secreted by Th1 cells will activate macrophages leading to secretion of inflammatory cytokines (TNF, IL-1 and IL-2), an increased production of toxic substances like ROS, NO and lysosomal enzymes and finally stimulation of expression of costimulatory molecules enhancing the efficiency of the macrophage as APC [170]. The main effector cytokines of Th2 are IL-4, IL-5 and IL-13 and key transcription factors are GATA binding protein 3 (GATA-3) and STAT-6. IL-4 act on B-cells to stimulate production of IgE antibodies which can lead to mast cell degranulation upon binding of IgE with mast cells. IL-5 activates eosinophils, inducing defence against helminthic infections. IL-4 and IL-13 are involved in alternative macrophage activation promoting development of M2 macrophages which have anti-inflammatory effects and may promote tissue repair and fibrosis [170]. Signature cytokines of Th17 are IL-17 and IL-22. Differentiation into this subtype is mediated by IL-6 and TGF-β leading to activation of transcription factors STAT-3 and retinoic acid-related orphan receptor γ*t* (RORγ*t*) respectively. IL-17 act on leukocytes and tissue cells and stimulates production of several chemokines and cytokines (TNF-α, IL-1β, IL-6) that recruit neutrophils and to a lesser extend monocytes to generate an inflammatory response. IL-22 produced in epithelial cells is primarily involved in maintaining the barrier function of epithelia [170]. Th17 T-cell most likely play a significant role in IRI-induced inflammation. STAT-3 KO mice are protected from renal IRI via downregulation of Th17 activity [178]. The differentiated T-cells can convert from one subtype to another by changes in activation circumstances [179]. It is suggested that Th1/Th2 ratio plays an important role in the pathogenesis of IRI [180,181]. Yokota et al. [181] demonstrated that STAT-6-deficient mice with a defective Th2 phenotype have enhanced renal I/R injury whereas STAT-4-deficient mice have mild improved function In addition, Loverre et al. [182] showed that kidney transplant recipients experiencing DGF predominantly expressed Th1 phenotype within the graft In literature both Th1 and Th17 cells are associated with T-cell mediated rejection [183–188].

The TCR on CD8+ T-cells can only bind to MHC class 1 molecules (HLA A, B, C) presented on APCs. Upon activation in lymphoid tissue, they differentiate into cytotoxic T-cells (CTLs) or memory cells. This differentiation is facilitated by CD4+ Th1 cells by secreting cytokines that act directly on the CD8+ cells [170]. The main cytokines involved are IL-2 (proliferation, differentiation CTL/memory cell), IL-12/IFN (differentiation CTL), IL-15 (memory cell survival), IL-21 (memory cell induction). The CTLs are able to kill cells which present the allogenic class 1 MHC of the donor in the graft. This through binding on the target cell and release of granule content into the immune synapse. These granules contain perforin and granzymes. Perforin induces the uptake of granzymes into the target cell. These granzymes are capable of activating caspases and inducing apoptosis. The killing of the target cell can also be Fas/Fas-L mediated in which the CTL expose the Fas ligand on the membrane which will bind to the Fas receptor on the target cell inducing apoptosis. Only CTLs that are activated in the direct way (by donor MHC on donor APC) are able to kill graft cells [170]. Like CD4+ Th cells, CTL secrete inflammatory cytokines, (predominantly IFN-γ) that attribute to inflammation and injury of the graft. The role of CD8+ cells in early phase of renal IRI is unclear, in a mouse model CD4+ deficient mouse was protected from IRI but CD8+ deficient mouse was not [176].

Ko et al. [189] showed that already 6 h after renal IRI, transcriptional activity occurs in T-cells and that these gene expression changes persist up to 4 weeks after the event. Genes involved in immune cell trafficking and cellular movement were most upregulated in the early phase (6 h, 3 days). On day 10 this was shifted to genes related to cellular development products involved in immune responses and on day 28 to genes involved in cellular and humoral immune response involved in antigen presentation. In addition, they found that the CC motif chemokine receptor 5 (CCR5) was one of the most upregulated genes at all time points, which was confirmed at a protein level. Subsequently, the addition of CCR5 antibody attenuated IRI and led to decreased T-cell activation [189].

B-Cells

Next to alloreactive CD4+ and CD8+ T-cells, antibodies (immune globulins, Ig) against the graft contribute to rejection. Most of these Igs are produced by Th dependant alloreactive B-cells. The naive

B-cell recognizes allogenic MHC-molecules, processes these MHC-molecules and presents them to Th cells that were activated previously by the same alloantigen presented by APCs. The produced Igs (IgM/IgG) are then able to induce complement activation, and activation of neutrophils, NK cells and macrophages. The T-cells are responsible for T-cell mediated rejection and B-cells together with complement activation for ABMR [170].

Regulatory T-Cells

The T-cells which most likely play a protective role in renal IRI are regulatory T-cells (Tregs), a subset of CD4+ T-cells whose function is to supress the innate as well as the adaptive immune response and maintain self-tolerance. Tregs can be discriminated from other T-cells by expression of FoxP3 amongst other proteins like CD25. FoxP3 is probably the most important transcription factor for Treg differentiation. The mechanism of action of Tregs is production of immune suppressive cytokines IL-10 and TGF-β, reduction of APC is to stimulate T-cells (possibly by binding to B7 proteins on the APC) and finally consumption of IL-2, an important growth factor for other T-cells [170]. TGF-β inhibits various immune cells amongst which: proliferation and effector functions of T-cells, macrophages, neutrophils and endothelial cells. It regulates differentiation of FoxP3+ Tregs and promotes polarisation towards Th17 cells. Furthermore, TGF-β promotes tissue repair by the ability to stimulate collagen synthesis and matrix modifying enzyme by macrophages and fibroblasts. IL-10 inhibits the production of IL-12 by activated macrophages and DCs, therefore inhibiting these cells and their IFN-γ production. It also inhibits T-cell activation by inhibiting the expression of co-stimulators and MHC-II molecules on DCs and macrophages [170].

Tregs play a potentially promising role in the reduction of IRI and graft tolerance [190–193]. Currently, several clinical trials are running evaluating the safety and effeciacy of FoxP3 cellular therapy in kidney transplantation (NCT02091232, NCT03284242, NCT01446484) [194,195]. However, all that glitters is not gold, since recent studies have shown that human FoxP3+ T-cells show great variations in gene expression phenotype and function [196–199]. Furthermore, recently a subset of FoxP3+ Tregs mimicking Th cells was discovered that secreted pro-inflammatory cytokines [200]. Also, the effect of different immune suppressive agents on the Treg phenotype needs to be elucidated, since these drugs might influence Treg phenotype [200,201]. Altogether, more insight in function and biology is needed before this therapy finds its way to clinical settings.

3.4. Transcriptional Reprogramming

Finally, cells can protect themselves from hypoxia and ischemia and maintain homeostasis via an evolutionary conserved mechanism with the use of oxygen sensors and activation of specific transcription factors. These so called hypoxic inducible factors (HIFs) regulate various genes involved in the metabolic cell cycle, angiogenesis, erythropoiesis, energy conservation and cell survival and are therefore able to induce a protective cell response to hypoxia [202].

HIFs are heterodimeric transcription factors consisting of an α and β subunit. There are two types of α subunits, HIF-1α and HIF-2α, which have common, but also subunit-specific target genes. In the kidney, HIF-1α is predominantly localized in tubular and glomerular cells, whereas HIF- 2α can be found in glomerular cells, peritubular endothelial cells and fibroblasts [203–205]. In aerobic circumstances, HIFs are inactive. Oxygen-sensing prolylhydroxylase (PHD) hydroxylates the amino acid proline on the HIF-1α/HIF-2α subunit. This induces a conformational change enabling von Hippel–Lindau tumour suppressor protein (pVHL) to bind with the α-subunit, leading to degradation of the HIF-α subunit. Ischemia/hypoxia will lead to inhibition of the oxygen-dependent PHD, which enables nuclear translocation of the α subunit, binding of the α and β subunit and formation of HIF. In the nucleus HIF binds with the hypoxia response promotor element (HRE) leading to the transcription of various genes like glycolysis enzymes Glut-1 and aldolase (enabling ATP production under hypoxic circumstances), NF-κB, TLRs, adenosine receptors, vascular endothelial growth factor (VGEF), CD73 and erythropoietin. Activation of HIF can also occur in normoxemic circumstances, for instance, by ROS, LPS, various

cytokines and TCR-CD28 stimulation. Transcriptional reprogramming is a consequence of I/R that should be considered a protective mechanism (Figure 9) [206].

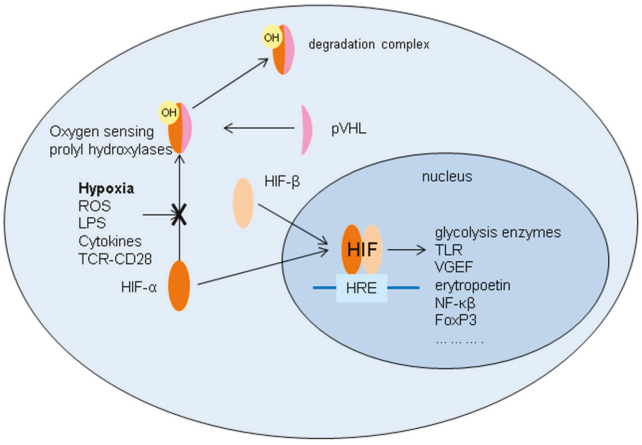

Figure 9. Intracellular stabilisation and activation of hypoxic inducible factor. Under normoxemic conditions, proline on the hypoxic inducible factor (HIF) α (HIFα) subunit is rapidly hydroxylated by oxygen-sensing prolyl hydroxylase (PHD). This induces a conformational change enabling von Hippel–Lindau tumour suppressor protein (pVHL) to bind with the α-subunit, leading to degradation of the HIF-α subunit. Ischemia (or other signals like lipopolysaccharide (LPS), various cytokines, etc.) will lead to inhibition of the oxygen-dependent PHD, enabling nuclear translocation of the α subunit, binding of the α and β subunit and formation of HIF. In the nucleus, HIF binds with the hypoxia response promotor element (HRE) leading to the transcription of various genes. VGEF: vascular endothelial growth factor.

Conde et al. [207] showed in various models and human post-transplantation biopsies that HIF-1α is induced in a biphasic manner namely during the hypoxic as well as the reperfusion phase. They pointed out the PI3K/Akt mTOR pathway to be responsible for this HIF-1α accumulation during the normoxemic reperfusion phase. In their study, this second increase (e.g., during reperfusion) seemed crucial for tubular cell survival and recovery. During the hypoxic phase, an increase in HIF-1 resulted predominantly in the upregulation of PHD3 and VGEF mRNA, which remained elevated during oxygenation. EPO mRNA was upregulated upon reperfusion. EPO and VGEF have been suggested to be involved in proximal tubular regeneration [208–210]. Their human post-transplantation biopsies revealed HIF-1α expression in proximal tubular cells without ischemic damage or features of regeneration suggesting a protective role for HIF-1α during I/R [207]. Oda et al. [211] had similar findings in patients receiving a DBD/DCD donor kidney. Their analysis of 46 post-transplant biopsies, gained 1h after reperfusion, showed that expression levels of PI3K, Akt, mTOR and HIF-1α were significantly higher in patients without DGF compared to patients experiencing DGF (76% of the patients). The expression levels of HIF-1α and donor type (DCD) were independently associated with DGF HIF-2α expression in renal endothelial cells is suggested in several studies to be protective against renal IRI via protection and preservation of the vasculature endothelium by upregulation of angiogenic factors like VGEF and their receptors Tie2 and VGEFreceptor-2 (FLK-1) [212–215]. Increased production of HIF in myeloid and lymphoid cells influences the innate and adaptive immune response. T-cell activation and proliferation is reduced under hypoxic conditions [216]. A study of Zhang et al. [217] revealed a hypoxia/HIF-2α/adenosine2A receptor axis to be responsible in reduction of NK T-cells activation and renal IRI upon I/R. HIF-1α induces a shift from Th1 to Th2 cells (decrease

Th1/Th2 ratio) accompanied by a decrease in excretion of inflammatory cytokines. Furthermore, HIF-1α promotes transcription of FoxP3 and therefore generation activation of Tregs.

Various PHD inhibitors have been developed and tested in animal I/R models. In a rat model, Wang et al. [218] showed that use of the PHD-1 inhibitor acetate prior to the ischemic event was able to stabilize HIF in a dose-dependent manner and was associated with improved renal outcome. In addition, in an allogenic renal transplant model in rats, the use of the PHD inhibitor FD-4497 pre-donation was associated with increased HIF expression and improved graft outcome and reduced mortality of recipients [219]. Hence, activation and/or upregulation of HIF could be an interesting approach to reduce renal IRI and improve renal transplant outcome. Several PHD inhibitors are currently being tested in clinical trials in order to treat anaemia in patients with chronic kidney disease but have not been tested in the field of transplantation yet.

4. Summary

The past decade's research in kidney transplant recipients has focussed on post-transplant patient management, with a predominant emphasis on immunosuppression. However, the biggest 'hit' to the donor organ is encountered during the process of donation and reperfusion at time of transplantation, i.e., ischemia and reperfusion injury. An important initiating step in IRI is the uncontrolled ROS formation during reperfusion and dysfunction of the mitochondrial machinery leading to the opening of mPTP and the release of DAMPs in the intra- and extracellular space. From here, several injury cascades are activated, including activation of cell death programs like apoptosis and (regulated) necrosis, endothelial dysfunction implicating increased vasoconstriction upon reperfusion, loss of specific phenotype of endothelial cells and transmigration of leucocytes into the interstitial space. Activation of the innate and subsequently the adaptive immune system will take place through binding of DAMPs to the toll-like receptors and activation of the complement system, leading to further injury of the graft, increased immunogenicity favouring T-cell and antibody mediated rejection and the initiation of fibrosis associated with chronic graft dysfunction. Currently, several novel agents targeting various pathways are tested and, although most are still in the preclinical phase, some have already entered clinical trials. Intervention early in this cascade of events (e.g., on a mitochondrial level), seems very attractive, since mitochondrial dysfunction plays a pivotal role in the initiation of IRI. Due to the complexity of the pathophysiological mechanisms, however, it may be predicted that a multiple treatment strategy using a combination of agents given at various time points during the donation, preservation and transplantation process will most likely be the best strategy to reduce IRI.

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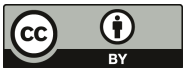

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Urinary Excretion of N^1-methyl-2-pyridone-5-carboxamide and N^1-methylnicotinamide in Renal Transplant Recipients and Donors

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Abstract: N^1-methylnicotinamide (N^1-MN) and N^1-methyl-2-pyridone-5-carboxamide (2Py) are successive end products of NAD^+ catabolism. N^1-MN excretion in 24-h urine is the established biomarker of niacin nutritional status, and recently shown to be reduced in renal transplant recipients (RTR). However, it is unclear whether 2Py excretion is increased in this population, and, if so, whether a shift in excretion of N^1-MN to 2Py can be attributed to kidney function. Hence, we assessed the 24-h urinary excretion of 2Py and N^1-MN in RTR and kidney donors before and after kidney donation, and investigated associations of the urinary ratio of 2Py to N^1-MN (2Py/N^1-MN) with kidney function, and independent determinants of urinary 2Py/N^1-MN in RTR. The urinary excretion of 2Py and N^1-MN was measured in a cross-sectional cohort of 660 RTR and 275 healthy kidney donors with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Linear regression analyses were used to investigate associations and determinants of urinary 2Py/N^1-MN. Median 2Py excretion was 178.1 (130.3–242.8) µmol/day in RTR, compared to 155.6 (119.6–217.6) µmol/day in kidney donors ($p < 0.001$). In kidney donors, urinary 2Py/N^1-MN increased significantly after kidney donation (4.0 ± 1.4 to 5.2 ± 1.5, respectively; $p < 0.001$). Smoking, alcohol consumption, diabetes, high-density lipoprotein (HDL), high-sensitivity C-reactive protein (hs-CRP) and estimated glomerular filtration rate (eGFR) were identified as independent determinants of urinary 2Py/N^1-MN in RTR. In conclusion, the 24-h urinary excretion of 2Py is higher in RTR than in kidney donors, and urinary 2Py/N^1-MN increases after kidney donation. As our data furthermore reveal strong associations of urinary 2Py/N^1-MN with kidney function, interpretation of both N^1-MN and 2Py excretion may be recommended for assessment of niacin nutritional status in conditions of impaired kidney function.

Keywords: N^1-methyl-2-pyridone-5-carboxamide; N^1-methylnicotinamide; urinary excretion; renal transplantation; kidney function; biomarker; niacin status; tryptophan; vitamin B_3

1. Introduction

Niacin, or vitamin B_3, is the precursor of the nicotinamide nucleotide coenzyme NAD^+. An adequate niacin status is vital to provide reducing equivalents for energy metabolism, and substrates of NAD^+ consuming enzymes, including adenosine diphosphate (ADP)-ribosyl transferases and deacetylases, that transfer ADP-ribose moieties from NAD^+ and $NADP^+$ [1,2].

Niacin nutritional status is most commonly assessed by the 24-h urinary excretion of N^1-methylnicotinamide (N^1-MN) as a breakdown product of NAD^+, and recommended as such by authorities, including the WHO and the European Food Safety Authority (EFSA) [3,4]. However, N^1-methyl-2-pyridone-5-carboxamide (2Py) is the end product of NAD^+ catabolism, after aldehyde oxidase (AOX1)-dependent oxidation of N^1-MN (Figure 1) [5,6]. Although the 24-h urinary excretion of N^1-MN has shown the most sensitive response to oral test doses of niacin equivalents [3,7], excretion of 2Py, whether or not combined with that of N^1-MN, has also been implicated for the assessment of niacin status [8–11].

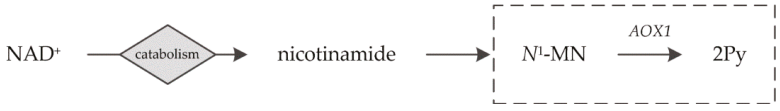

Figure 1. Schematic overview of NAD^+ catabolism. 2Py is the end product of NAD^+ catabolism after AOX1-dependent oxidation of N^1-MN, framed by the dotted line. AOX1, aldehyde oxidase; N^1-MN, N^1-methylnicotinamide; 2Py, N^1-methyl-2-pyridone-5-carboxamide.

In a recent study, we found that N^1-MN excretion is lower in renal transplant recipients (RTR) than in healthy controls [12]. As this discrepancy could not be explained by lower dietary intake of niacin equivalents, enhanced enzymatic conversion of N^1-MN to 2Py by AOX1 might be present in this population due to the suggested contribution of AOX1 to N^1-MN clearance with lower kidney function [13,14]. It is unclear whether 2Py excretion is increased in RTR, and if so, whether a shift in excretion of N^1-MN to 2Py can be attributed to kidney function.

Hence, to evaluate the applicability of N^1-MN excretion as a biomarker of niacin nutritional status in conditions of impaired kidney function, we measured the 24-h urinary excretion of 2Py and N^1-MN in RTR and kidney donors before and after kidney donation, allowing us to (1) compare the 24-h urinary excretion of 2Py in RTR and kidney donors, (2) investigate the effect of kidney donation on the excretion of 2Py and N^1-MN in kidney donors, (3) assess whether the urinary ratio of 2Py to N^1-MN (2Py/N^1-MN) is associated with kidney function, and (4) identify determinants of urinary 2Py/N^1-MN in RTR.

2. Materials and Methods

2.1. Study Population

This cross-sectional study was based on a well-characterized, single-center cohort of 707 RTR (aged ≥18 years) who visited the outpatient clinic of the University Medical Center Groningen, Groningen, the Netherlands, between 2008 and 2011, with a functioning graft for at least 1 year and no history of alcohol and/or drug abuse [15–17]. As a control group, 367 healthy kidney donors were included who participated in a screening program before kidney donation, and of whom biomaterial was collected before and, after declared eligible, 3 months after kidney donation. Exclusion of subjects with missing biomaterial or niacin supplementation use left 660 RTR and 275 kidney donors, of which 85 underwent donor nephrectomy during the inclusion period, eligible for statistical analyses. Signed informed consent was obtained from all participating subjects and the study protocol was approved by the institutional review board (METc 2008/186) adhering to the Declaration of Helsinki. This study included the same cohort of 660 RTR and 275 kidney donors for data collection as reported previously [12].

2.2. Data Collection and Measurements

Participants were instructed to collect a 24-h urine sample on the day before their morning visit to the outpatient clinic, and to fast overnight for 8 to 12 h. Urine samples were collected under oil, and chlorhexidine was added as an antiseptic agent. Fasting blood samples were drawn after completion of the urine collection. Laboratory measurements were performed directly with spectrophotometric-based routine clinical laboratory methods (Roche Diagnostics, Rotkreuz, Switzerland). Body composition and hemodynamic parameters were measured according to a previously described, strict protocol [15]. Diabetes was diagnosed if fasting plasma glucose was ≥7.0 mmol/L or antidiabetic medication was used. Proteinuria was diagnosed if total urinary protein excretion was ≥0.5 g/day as measured by a biuret reaction-based assay (MEGA AU510; Merck Diagnostica, Darmstadt, Germany).

Dietary intake was assessed with a validated semi-quantitative food frequency questionnaire (FFQ) [18,19]. The self-administered questionnaire was filled out at home and inquired about 177 food items over the last month. During the outpatient clinic visit, the FFQ was checked for completeness by a trained researcher and inconsistent answers were verified with the participant. The FFQ was validated for RTR as previously reported [16]. Dietary data were converted into daily nutrient intake using the Dutch Food Composition Table of 2006 [20]. Intake of niacin equivalents was calculated by adding up niacin and one-sixtieth of tryptophan intake. Subjects who were using niacin supplementation were excluded. Smoking behavior was assessed with a separate questionnaire [21]. Data on medication and vitamin supplements use, and medical history were obtained from medical records [21].

The estimated glomerular filtration rate (eGFR) was calculated by the combined creatinine and cystatin C-based Chronic Kidney Disease Epidemiology Collaboration equation [22], which has shown to be the most accurate equation in RTR [23]. The glomerular filtration rate (GFR) was measured by infusion of ^{125}I-Iothalamate as described previously [24].

2.3. Assessment of 2Py and N^1-MN Excretion

Measurement of 2Py and N^1-MN concentrations was performed with a validated liquid chromatography (Luna HILIC column; Phenomenex, Torrance, CA, USA) isotope dilution-tandem mass spectrometry (Quattro Premier; Waters, Milford, MA, USA) (LC-MS/MS) method, as described previously [25], with the addition of N^1-methyl-2-pyridone-5-carboxamide-d_3 in acetonitrile as an internal standard. The 24-h urinary excretion of 2Py and N^1-MN (μmol/day) was obtained by multiplying concentrations (μmol/L) by total urine volume calculated from weight (L/day).

2.4. Statistical Analysis

Data are presented as the mean ± SD, median (IQR) and absolute number (percentage) for normally distributed, skewed and nominal data, respectively. Assumptions for normality were checked by visual judgments of the corresponding frequency distribution and Q-Q plot.

Baseline characteristics of RTR and the total cohort of kidney donors were compared by means of *t*, Mann–Whitney, and Chi-Square tests, of which age, sex, body surface area, N^1-MN excretion and eGFR have been reported previously [12]. Crude associations of 2Py and N^1-MN excretion with intake of niacin equivalents were investigated with linear regression analyses. Characteristics of kidney donors before and after kidney donation were compared by means of paired samples *t* and Wilcoxon signed rank tests.

Linear regression analyses were used to investigate associations of urinary 2Py/N^1-MN with kidney function in RTR and kidney donors, with additional adjustments for age and sex. Effect modification between either age or sex and kidney function with urinary 2Py/N^1-MN was assessed by including the corresponding cross product term in the linear regression model.

Linear regression analyses were furthermore employed to investigate cross-sectional associations of urinary 2Py/N^1-MN with baseline variables in RTR. Variables were 2-base log-transformed when assumptions of normality and homogeneity of variance of the residuals, based on visual judgement of

P-P and scatter plots, respectively, were not met. Multivariable linear regression analyses were used to identify determinants of urinary 2Py/N^1-MN, by entering terms with p-value <0.1 in univariable analysis, and eliminating the least significant term stepwise until the remaining terms contributed significantly to the model.

For all statistical analyses, a two-sided p-value of less than 0.05 was considered to indicate statistical significance and SPSS Statistics version 23.0 (IBM, Armonk, NY, USA) was used as software.

3. Results

3.1. Excretion of 2Py in Kidney Donors and RTR

The total cohort consisted of 660 stable RTR (57% male; mean age 53.0 ± 12.7 years), included at a median time of 5.6 (2.0–12.0) years after transplantation, and 275 healthy kidney donors (41% male; mean age 53.3 ± 10.7 years) (Table 1). Differences in the 24-h urinary excretion of 2Py and N^1-MN and kidney function are shown in Table 1. 2Py excretion was higher in RTR than in kidney donors (178.1 (130.3–242.8) versus 155.6 (119.6–217.6) μmol/day, respectively; $p < 0.001$), while N^1-MN excretion was lower in RTR than in kidney donors (22.0 (15.8–31.8) versus 41.4 (31.6–57.2) μmol/day, respectively; $p < 0.001$). Kidney function was significantly lower in RTR than in kidney donors (eGFR: 45.8 ± 18.7 versus 91.0 ± 14.2 mL/min/1.73 m^2, respectively; $p < 0.001$ and GFR: 52.4 ± 17.4 versus 82.3 ± 29.7 mL/min/1.73 m^2, respectively; $p < 0.001$). Urinary 2Py/N^1-MN was significantly higher in RTR than in kidney donors (8.7 ± 3.8 versus 4.0 ± 1.4, respectively; $p < 0.001$), while the sum of 2Py and N^1-MN excretion was similar (198.3 (155.9–269.4) versus 203.7 (149.4–274.7) μmol/day, respectively; $p = 0.98$). The urinary fraction of 2Py was higher in RTR than in kidney donors (89.1% (86.4%–91.3%) versus 79.0% (75.6%–82.1%), respectively; $p < 0.001$), and that of N^1-MN was lower (10.9% (8.7%–13.6%) versus 21.0% (17.9%–24.4%), respectively; $p < 0.001$). The 24-h urinary excretion of 2Py, N^1-MN and the sum of 2Py and N^1-MN, but not urinary 2Py/N^1-MN, were directly associated with intake of niacin equivalents (Table S1).

3.2. Excretion of 2Py and N^1-MN before and after Kidney Donation in Kidney Donors

The 24-h urinary excretion of 2Py and N^1-MN and kidney function in 85 kidney donors before and after kidney donation are shown in Table 1 and Figure 2. At a median time of 1.64 (1.61–1.87) months after kidney donation, 2Py excretion did not change significantly (152.8 (124.4–215.1) to 161.7 (116.6–227.8) μmol/day, respectively; $p = 0.31$), while N^1-MN decreased (40.9 (31.0–58.2) to 32.5 (23.4–44.0) μmol/day, respectively; $p < 0.001$). Kidney function decreased significantly after kidney donation (eGFR: 92.8 ± 13.9 to 60.1 ± 12.1 mL/min/1.73 m^2, respectively; $p < 0.001$ and GFR: 103.7 ± 16.7 to 65.3 ± 10.4 mL/min/1.73 m^2, respectively; $p < 0.001$). Urinary 2Py/N^1-MN increased after kidney donation (4.0 ± 1.4 to 5.2 ± 1.5, respectively; $p < 0.001$), while the sum of 2Py and N^1-MN excretion did not change (198.3 (162.3–270.8) to 189.7 (141.9–271.6) μmol/day, respectively; $p = 0.90$). The urinary fraction of 2Py increased after kidney donation (78.3% (75.5%–81.8%) to 83.5% (80.0%–86.0%), respectively; $p < 0.001$), and that of N^1-MN decreased (21.7% (18.2%–24.5%) to 16.5% (14.0%–20.0%), respectively; $p < 0.001$).

3.3. Associations of Urinary 2Py/N^1-MN with Kidney Function

Urinary 2Py/N^1-MN was associated with kidney function in RTR (eGFR: $\beta = -0.40$; $p < 0.001$ and GFR: $\beta = -0.39$; $p < 0.001$) and the total cohort of kidney donors (eGFR: $\beta = -0.17$; $p = 0.03$ and GFR: $\beta = -0.20$; $p = 0.003$), but not in the pre- (eGFR: $\beta = -0.01$; $p = 0.94$ and GFR: $\beta = -0.02$; $p = 0.89$) and post-donation subgroups of kidney donors (eGFR: $\beta = -0.11$; $p = 0.42$ and GFR: $\beta = 0.15$; $p = 0.27$), with adjustment for age and sex (Table 2). No significant interaction between either age or sex with kidney function was found in the association with urinary 2Py/N^1-MN in RTR and kidney donors.

Table 1. Excretion of 2Py and N^1-MN and kidney function in RTR and kidney donors before and after kidney donation [1].

Variable	RTR $n = 660$	Kidney Donors $n = 275$	Population Difference	Kidney Donors $n = 85$		Paired Difference
	Total Cohort	Total Cohort	p-Value [2]	Pre-Donation	Post-Donation	p-Value [3]
Age, years	53.0 ± 12.7	53.3 ± 10.7	0.68	52.2 ± 10.5	52.6 ± 10.4	<0.001
Male, n (%)	379 (57)	112 (41)	0.001	43 (51)	43 (51)	-
Body surface area, m^2	1.9 ± 0.2	1.9 ± 0.2	0.90	2.0 ± 0.2	1.9 ± 0.2	0.01
BMI, kg/m^2	26.6 ± 4.8	25.9 ± 3.4	0.01	26.0 ± 3.4	25.7 ± 3.2	0.03
Urinary excretion						
2Py, µmol/day	178.1 (130.3–242.8)	155.6 (119.6–217.6)	0.001	152.8 (124.4–215.1)	161.7 (116.6–227.8)	0.31
N^1-MN, µmol/day	22.0 (15.8–31.8)	41.4 (31.6–57.2)	<0.001	40.9 (31.0–58.2)	32.5 (23.4–44.0)	<0.001
2Py/N^1-MN	8.7 ± 3.8	4.0 ± 1.4	<0.001	4.0 ± 1.4	5.2 ± 1.5	<0.001
Sum of 2Py and N^1-MN, µmol/day	198.3 (155.9–269.4)	203.7 (149.4–274.7)	0.98	198.3 (162.3–270.8)	189.7 (141.9–271.6)	0.90
2Py fraction, % [4]	89.1 (86.4–91.3)	79.0 (75.6–82.1)	<0.001	78.3 (75.5–81.8)	83.5 (80.0–86.0)	<0.001
N^1-MN fraction, % [4]	10.9 (8.7–13.6)	21.0 (17.9–24.4)	<0.001	21.7 (18.2–24.5)	16.5 (14.0–20.0)	<0.001
Kidney function						
eGFR, $mL/min/1.73\ m^2$	45.8 ± 18.7	91.0 ± 14.2	<0.001	92.8 ± 13.9	60.1 ± 12.1	<0.001
GFR, $mL/min/1.73\ m^2$	52.4 ± 17.4	82.3 ± 29.7	<0.001	103.7 ± 16.7	65.3 ± 10.4	<0.001

[1] Data are presented as mean ± SD, median (IQR) and absolute number (percentage) for normally distributed, skewed and nominal data, respectively. [2] p-value for difference between RTR and the total cohort of kidney donors was tested by t and Mann–Whitney tests for normally and skewed distributed continuous variables, respectively. [3] p-value for difference between kidney donors before and after kidney donation was tested by paired samples t and Wilcoxon signed rank tests for normally and skewed distributed continuous variables, respectively. [4] The urinary fraction of 2Py or N^1-MN (percentage) was calculated by dividing 2Py or N^1-MN excretion by the sum of 2Py and N^1-MN excretion, respectively, and multiplying by 100. BMI, body mass index; eGFR, estimated glomerular filtration rate; GFR, glomerular filtration rate; N^1-MN, N^1-methylnicotinamide; RTR, renal transplant recipients; 2Py, N^1-methyl-2-pyridone-5-carboxamide; 2Py/N^1-MN, ratio of 2Py to N^1-MN.

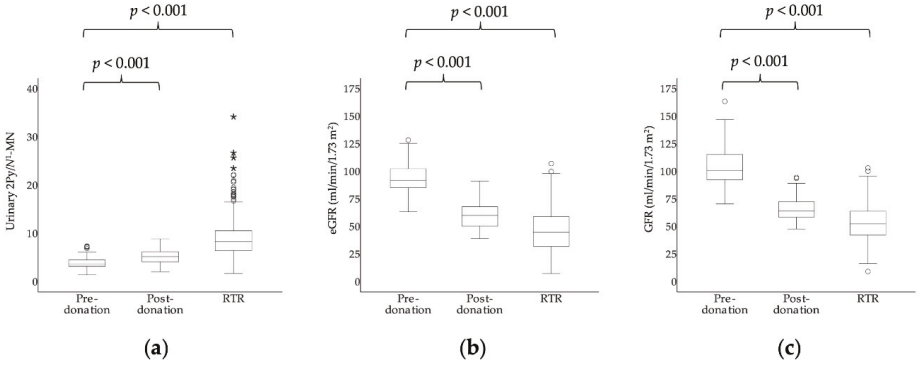

Figure 2. Box plots of (**a**) urinary 2Py/N^1-MN, (**b**) eGFR and (**c**) GFR in kidney donors before (n = 85) and after kidney donation (n = 85) and RTR (n = 660), respectively. Boxes, bars and whiskers represent IQRs, medians and values <1.5 × IQR, respectively, whereas outliers (1.5–3 × IQR) are indicated by circles and extreme outliers (>3 × IQR) by asterisks. p-value for difference between kidney donors before and after kidney donation was tested by paired samples t and Wilcoxon signed rank tests for normally and skewed distributed continuous variables, respectively. p-value for difference between RTR and kidney donors before donation was tested by t and Mann–Whitney tests for normally and skewed distributed continuous variables, respectively. eGFR, estimated glomerular filtration rate; GFR, glomerular filtration rate; N^1-MN, N^1-methylnicotinamide; RTR, renal transplant recipients; 2Py, N^1-methyl-2-pyridone-5-carboxamide; 2Py/N^1-MN, ratio of 2Py to N^1-MN.

Table 2. Associations of urinary 2Py/N^1-MN with kidney function in RTR and kidney donors before and after kidney donation [1].

Variable	RTR	Kidney Donors		
		Total Cohort	Pre-Donation	Post-Donation
	n = 660	n = 275	n = 85	n = 85
eGFR, mL/min/1.73 m^2				
Standardized β	−0.40	−0.17	−0.01	−0.11
p-value	<0.001	0.03	0.94	0.42
GFR, mL/min/1.73 m^2				
Standardized β	−0.39	−0.20	−0.02	0.15
p-value	<0.001	0.003	0.89	0.27

[1] Linear regression analyses were performed to investigate associations of urinary 2Py/N^1-MN with kidney function, with adjustment for age and sex. eGFR, estimated glomerular filtration rate; GFR, glomerular filtration rate; N^1-MN, N^1-methylnicotinamide; RTR, renal transplant recipients; 2Py, N^1-methyl-2-pyridone-5-carboxamide; 2Py/N^1-MN, ratio of 2Py to N^1-MN.

3.4. Characteristics and Associations with Urinary 2Py/N^1-MN in RTR

Characteristics of the RTR cohort are shown in Table 3. Urinary 2Py/N^1-MN was positively associated with body surface area, body mass index (BMI), glucose homeostasis parameters, triglycerides, mean arterial pressure, high-sensitivity C-reactive protein (hs-CRP), proteinuria, and use of antidiabetics, antihypertensives, acetylsalicylic acid, proton pump inhibitors and tacrolimus. Inverse associations were found between urinary 2Py/N^1-MN and smoking, alcohol consumption, energy intake, vitamin B_6 intake, high-density lipoprotein (HDL) and eGFR.

Table 3. Associations of urinary 2Py/N^1-MN with characteristics in 660 RTR [1,2].

Variable	Value	Standardized β	*p*-Value
Urinary 2Py/N^1-MN	8.7 ± 3.8	-	-
2Py excretion, µmol/day	178.1 (130.3–242.8)	-	-
N^1-MN excretion, µmol/day	22.0 (15.8–31.8)	-	-
Age, years	53.0 ± 12.7	0.03	0.09
Male, *n* (%)	379 (57)	−0.004	0.92
Body surface area, m^2	1.9 ± 0.22	0.11	0.006
BMI, kg/m^2	26.6 ± 4.8	0.17	<0.001
Creatinine excretion, mmol/day	11.7 ± 3.4	−0.05	0.22
Time since transplantation, years	5.6 (2.0–12.0)	−0.07	0.07
Lifestyle			
Current smoker, *n* (%)	78 (13)	−0.10	0.02
Alcohol consumption, g/day	3.1 (0.0–11.9)	−0.15	<0.001
Nutrition			
Energy intake, kcal/day	2182 ± 642	−0.09	0.03
Niacin equivalents intake, mg/day	35.6 ± 9.2	−0.06	0.14
Vitamin B_6 intake, mg/day	1.8 ± 0.5	−0.09	0.03
Glucose homeostasis			
Glucose, mmol/L	5.3 (4.8–6.0)	0.15	<0.001
HbA1c, %	5.8 (5.5–6.2)	0.12	0.002
Diabetes, *n* (%)	152 (23)	0.16	<0.001
Lipid homeostasis			
Total cholesterol, mmol/L	5.1 ± 1.1	0.03	0.38
LDL, mmol/L	3.0 ± 0.9	0.07	0.10
HDL, mmol/L	1.3 (1.1–1.7)	−0.20	<0.001
Triglycerides, mmol/L	1.7 (1.2–2.3)	0.17	<0.001
Hemodynamic			
Systolic blood pressure, mmHg	135.8 ± 17.3	0.07	0.08
Diastolic blood pressure, mmHg	82.5 ± 11.0	0.08	0.05
Mean arterial pressure, mmHg	107.0 ± 15.0	0.10	0.02
Inflammation			
Hs-CRP, mg/L	1.6 (0.7–4.6)	0.19	<0.001
Kidney function			
eGFR, $mL/min/1.73\ m^2$	45.8 ± 18.7	−0.40	<0.001
Proteinuria, *n* (%)	132 (20)	0.08	0.04
Nonimmunosuppressive medication			
Antidiabetic, *n* (%)	96 (15)	0.14	<0.001
Statin, *n* (%)	349 (53)	0.06	0.15
Antihypertensive, *n* (%)	581 (88)	0.09	0.02
Acetylsalicylic acid, *n* (%)	127 (19)	0.09	0.03
Proton pump inhibitor, *n* (%)	326 (49)	0.08	0.04
Immunosuppressive medication			
Prednisolon dose, mg/day	3.0 (2.0–3.0)	0.07	0.07
Proliferation inhibitor, *n* (%)	548 (83)	−0.02	0.71
Tacrolimus, *n* (%)	120 (18)	0.11	0.007
Cyclosporine, *n* (%)	253 (38)	−0.01	0.72

[1] Data are presented as mean ± SD, median (IQR) and absolute number (percentage) for normally distributed, skewed and nominal data, respectively. [2] Linear regression analyses were performed to investigate associations of urinary 2Py/N^1-MN with baseline variables, of which standardized β and *p*-value are presented. BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; N^1-MN, N^1-methylnicotinamide; RTR, renal transplant recipients; 2Py, N^1-methyl-2-pyridone-5-carboxamide; 2Py/N^1-MN, ratio of 2Py to N^1-MN.

3.5. Determinants of Urinary 2Py/N^1-MN in RTR

Stepwise multivariable linear regression analyses with backward elimination revealed smoking, alcohol consumption, diabetes, HDL, hs-CRP and eGFR as independent determinants of urinary 2Py/N^1-MN in RTR (Table 4). In the final model, urinary 2Py/N^1-MN was positively associated with diabetes (β = 0.10; $p = 0.01$) and hs-CRP (β = 0.10; $p = 0.009$), and inversely associated with smoking (β = −0.13; $p = 0.001$), alcohol consumption (β = −0.12; $p = 0.002$), HDL (β = −0.12; $p = 0.002$) and eGFR (β = −0.38; $p < 0.001$).

Table 4. Independent determinants of urinary 2Py/N^1-MN in RTR.

Variable	Univariable		Multivariable [1]	
	Standardized β	*p*-Value	Standardized β	*p*-Value
Age, years	0.03	0.09	-	-
Male, *n* (%)	−0.004	0.92	-	-
BMI, kg/m^2	0.17	<0.001	-	-
Time since transplantation, years	−0.07	0.07	-	-
Lifestyle				
Current smoker, *n* (%)	−0.10	0.02	−0.13	0.001
Alcohol consumption, g/day	−0.15	<0.001	−0.12	0.002
Nutrition				
Energy intake, kcal/day	−0.09	0.03	-	-
Niacin equivalents intake, mg/day	−0.06	0.14	-	-
Vitamin B_6 intake, mg/day	−0.09	0.03	-	-
Glucose homeostasis				
Diabetes, *n* (%)	0.16	<0.001	0.10	0.01
Lipid homeostasis				
LDL, mmol/L	0.07	0.10	-	-
HDL, mmol/L	−0.20	<0.001	−0.12	0.002
Hemodynamic				
Mean arterial pressure, mmHg	0.10	0.02	-	-
Inflammation				
Hs-CRP, mg/L	0.19	<0.001	0.10	0.009
Kidney function				
eGFR, mL/min/1.73 m^2	−0.40	<0.001	−0.38	<0.001
Proteinuria, *n* (%)	0.08	0.04	-	-
Nonimmunosuppressive medication				
Antihypertensive, *n* (%)	0.09	0.02	-	-
Acetylsalicylic acid, *n* (%)	0.09	0.03	-	-
Proton pump inhibitor, *n* (%)	0.08	0.04	-	-
Immunosuppressive medication				
Prednisolon dose, mg/day	0.07	0.07	-	-
Tacrolimus, *n* (%)	0.11	0.007	-	-
R^2	0.28		0.26	
Adjusted R^2	0.25		0.25	

[1] Stepwise multivariable linear regression with backward elimination was performed to identify determinants of urinary 2Py/N^1-MN, of which standardized β and *p*-value are presented. BMI, body mass index; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; N^1-MN, N^1-methylnicotinamide; RTR, renal transplant recipients; 2Py, N^1-methyl-2-pyridone-5-carboxamide; 2Py/N^1-MN, ratio of 2Py to N^1-MN.

4. Discussion

This study aimed to investigate the 24-h urinary excretion of both 2Py and N^1-MN as major catabolic products of NAD^+ with regard to kidney function. We assessed 2Py and N^1-MN excretion in RTR and healthy kidney donors as a model of renal disease, and in kidney donors before and after unilateral nephrectomy as a model of isolated renal function impairment. In RTR, 2Py excretion was significantly higher compared to that in kidney donors. Urinary 2Py/N^1-MN increased significantly in kidney donors after donation. In both RTR and kidney donors, urinary 2Py/N^1-MN was associated with kidney function. Kidney function was furthermore revealed as the strongest determinant of urinary 2Py/N^1-MN in RTR.

NAD^+ is formed either de novo from tryptophan via the kynurenine pathway, or via salvage pathways from preformed nicotinamide, nicotinic acid and nicotinamide riboside [26], commonly known as niacin, or vitamin B_3. NAD^+ catabolism proceeds via nicotinamide and its downstream metabolites N^1-MN and 2Py, respectively (Figure 1), and these products are found in both plasma and urine [27]. N^1-MN itself exhibits anti-inflammatory properties, and is produced by muscle in response to hypoxia and depletion of energy stores, besides its primary production in the liver [28]. Whereas nicotinamide is reabsorbed by renal tubules and only small amounts appear in urine, N^1-MN and 2Py account for 20%–35% and 45%–60%, respectively, of all urinary NAD^+ metabolites [29]. The WHO and the EFSA recommend the 24-h urinary excretion of N^1-MN for laboratory assessment of niacin nutritional status accordingly [3,4]. In a previous study, we found that N^1-MN excretion is clearly reduced in RTR compared to healthy kidney donors [12]. The fact that this is paralleled by a significant elevation of 2Py excretion in the present study, raises speculation that enhanced enzymatic conversion of N^1-MN to 2Py by AOX1 may be present in RTR. Furthermore, the opposing shifts of 2Py and N^1-MN excretion in kidney donors after donation, may imply a putative isolated effect of renal function impairment on urinary 2Py/N^1-MN.

Regarding kidney function, urinary 2Py/N^1-MN was positively associated with kidney function in both RTR and the total cohort of kidney donors. Renal clearance of N^1-MN is affected by lower kidney function [13,14], being freely filtered at the glomerulus and tubular secreted, with negligible and saturable tubular reabsorption [30,31]. 2Py has previously been classified as a uremic retention product by the European Uremic Toxin Working Group [32,33], though specific mechanisms of its renal clearance have yet not been characterized. Whereas plasma concentrations of 2Py are reported to increase progressively with chronic kidney disease stages [34], those of N^1-MN are suggested to be less sensitive to kidney function because of the contribution of AOX1 to N^1-MN clearance [13,14]. In view of this, we can speculate upon slower excretion of N^1-MN, hence prolonged exposure to 2Py-forming AOX1 that is related to kidney function, rather than retention of 2Py primarily. This speculation is supported by the fact that kidney function appeared to have only a minor effect on the daily excretion of the sum of 2Py and N^1-MN in all groups. The presence of a significant association of urinary 2Py/N^1-MN with kidney function in the total cohort of kidney donors, but not in the pre- and post-donation subgroups, is most likely due to smaller effect sizes in the latter subgroups of kidney donors being declared eligible after pre-donation screening.

The identification of eGFR as the strongest independent determinant of urinary 2Py/N^1-MN in RTR further supports the notion of an isolated effect of kidney function. Other identified determinant factors include those that are known to affect the enzymatic activity of the aforementioned 2Py-forming AOX1 and most likely contribute as such. In fact, urinary 2Py/N^1-MN has been used as an index to estimate in vivo AOX1 levels and activity [35], being regulated by a wide variety of endogenous and exogenous factors [36]. Smoking and alcohol consumption are well-known factors [37,38] that showed an inverse association with urinary 2Py/N^1-MN in RTR. Diabetes and inflammatory mediators [37], including hs-CRP [39,40], have also been implicated in AOX1 activity, as well as HDL-cholesterol-levels via interaction of AOX1 with the ATP-binding cassette transporter A1 (ABCA1) which is a regulator of HDL metabolism [41,42]. Surprisingly, medication use did not appear to affect urinary 2Py/N^1-MN in RTR, despite the significant function of AOX1 in metabolizing xenobiotics. Importantly, the fact that

urinary 2Py/N^1-MN has multiple determinants in addition to eGFR, precludes its use as a biomarker of kidney function.

Excessive poly (ADP-ribose) polymerase (PARP) activation induced by stressors such as inflammation, oxidative stress and DNA damage that are predominant in RTR [43,44], has also been implicated in higher production of 2Py from NAD^+ degradation [45,46]. One would, however, expect that this would be reflected by an overall increase of NAD^+ catabolites, which is opposed by the two-fold reduction of N^1-MN excretion in our RTR population.

In general, higher urinary output of NAD^+ metabolites indicates higher niacin nutritional status, being excreted after the pool of pyridine nucleotide coenzymes is filled [47]. Acute stress may alter this output, but not steady state conditions, in which elimination and production rates are equal [48]. However, the ratio of metabolites is subject to factors that affect not only the activity of 2Py-forming AOX1, but according to our data also kidney function. In a previous study, we found N^1-MN excretion to be lower in RTR independent of dietary intake of niacin equivalents, as well as to be positively associated with kidney function [12]. According to the present study, the latter association remains when taking into account 2Py excretion, by means of urinary 2Py/N^1-MN. Therefore, although urinary excretion of N^1-MN is the most common and recommended index [3,4], our findings suggest that this index might be of limited value in conditions of kidney function impairment and future studies may confirm whether 2Py excretion should at least be additionally interpreted for evaluation of niacin nutritional status.

The speculative presence of slower excretion, hence prolonged exposure of N^1-MN to 2Py-forming AOX1 with kidney function impairment has not been confirmed in previous studies. In fact, this speculation indicates straight substrate conversion kinetics, which is unlikely to fully account for the previously reported, increased serum concentrations of 2Py in patients with chronic renal failure [46]. More specifically, Rutkowski et al. suggested high serum concentrations of 2Py in chronic renal failure to be a result of kidney function impairment, based on the fact that serum concentrations of 2Py were approximately 20-fold higher in patients with advanced renal failure than in healthy subjects (15.5 ± 5.8 μmol/L versus 0.83 ± 0.18 μmol/L), with only a transient drop after dialysis, and a permanent reduction after kidney transplantation [46]. Accordingly, given its accumulation, along with a deterioration of kidney function, and its toxic properties due to significant inhibition of PARP activity, 2Py has been identified as a uremic toxin [32,45,46]. As we only measured urinary excretion of 2Py, it cannot be ruled out whether increased urinary excretion of 2Py is solely the consequence of increased serum concentrations of 2Py, due to decreased renal clearance, rather than conversion kinetics of N^1-MN to 2Py by AOX1, and future studies are warranted to address this matter.

Strengths of this study include the large sample size of a specific patient group and the availability of healthy kidney donors before donation as a control group, and after donation as a model of isolated renal function impairment. Moreover, the extensive characterization of RTR allowed us to control for other factors that could affect 2Py/N^1-MN in 24-h urine, and to comprehensively identify determinants of urinary 2Py/N^1-MN. The ratio of metabolites in 24-h urine provides a measure to demonstrate changes in metabolism related to renal function, while being the least sensitive to 24-h urine collection errors. Limitations of this study are its observational nature, which prohibits causal inferences, as well as final conclusions on underlying mechanisms of increased urinary 2Py/N^1-MN in RTR and kidney donors after kidney donation, and associations with kidney function. Therefore, it remains to be determined whether the association of urinary 2Py/N^1-MN with kidney function is a causal relation. The observational design of this study did neither allow us to rule out increased serum concentrations of 2Py due to decreased renal clearance, or higher production of 2Py from NAD^+ degradation due to PARP activation by means of an experimental design. Conclusions are yet additionally supported by the presence of direct associations of the 24-h urinary excretion of 2Py, N^1-MN, and the sum of 2Py and N^1-MN, but not urinary 2Py/N^1-MN, with niacin nutritional intake (Table S1). Future studies are strongly encouraged to elaborate on serum concentrations of 2Py and N^1-MN along with their urinary excretion. The present study is confined to the urinary excretion of the major NAD^+

metabolites, comprising the most common and recommended indices of niacin nutritional status according to existing literature and authorities, including the WHO and the EFSA [3,4], respectively. Other indices, including serum or erythrocyte concentrations of niacin and its metabolites [49], are considered inferior as urinary concentrations have shown the most sensitive response to oral test doses of niacin equivalents [3,7]. Given the aforementioned limitations, this study should be conceived as a descriptive report that precludes final conclusions on the applicability of N^1-MN excretion as a biomarker of niacin nutritional status in conditions of impaired kidney function. Finally, although niacin deficiency is considered to be uncommon in the developed world, it might be prevalent in subpopulations, including RTR [12]. Still, it should be emphasized that assessment of niacin nutritional status might not be feasible in the developing world given the costs.

5. Conclusions

The 24-h urinary excretion of 2Py is higher in RTR than in kidney donors, and urinary 2Py/N^1-MN clearly increases after kidney donation. Urinary 2Py/N^1-MN is associated with kidney function in both RTR and kidney donors, and kidney function is identified as the strongest determinant of urinary 2Py/N^1-MN in RTR. Therefore, interpretation of both N^1-MN and 2Py excretion, rather than N^1-MN alone, may be recommended for assessment of niacin nutritional status in conditions of impaired kidney function.

Supplementary Materials: The following is available online at http://www.mdpi.com/2077-0383/9/2/437/s1, Table S1: Associations of 2Py and N^1-MN excretion with niacin equivalents intake in RTR and kidney donors.

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Management of Immunosuppression in Kidney Transplant Recipients Who Develop Malignancy

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Abstract: The risk of cancer increases after transplantation. However, the consensus on immunosuppression (IS) adjustment after diagnosis of malignancy is lacking. Our study aims to assess the impact of IS adjustment on mortality of post-kidney transplant patients and allograft outcomes. We retrospectively reviewed the data in our center of 110 subjects. Our results showed IS dose adjustment was not statistically associated with mortality risk (HR 1.94, 95%CI 0.85–4.41, $p = 0.12$), and chemotherapy was the only factor that was significantly related to mortality (HR 2.3, 95%CI 1.21–4.35, $p = 0.01$). IS reduction was not statistically associated with worsening graft function (OR 3.8, 95%CI 0.77–18.71, $p = 0.10$), nor with graft survival (SHR 4.46, 95%CI 0.58–34.48, $p = 0.15$) after variables adjustment. Creatinine at cancer diagnosis and history of rejection were both negatively associated with graft survival (SHR 1.72, 95%CI 1.28–2.30, $p < 0.01$ and SHR 3.44, 95%CI 1.25–9.49, $p = 0.02$). Reduction of both mycophenolate and calcineurin inhibitors was associated with worsening graft function and lower graft survival in subgroup analysis (OR 6.14, 95%CI 1.14–33.15, $p = 0.04$; HR 17.97, 95%CI 1.81–178.78, $p = 0.01$). In summary, cancer causes high mortality and morbidity in kidney transplant recipients; the importance of cancer screening should be emphasized.

Keywords: malignancy; cancer; kidney transplant; immunosuppression; graft failure; survival

1. Introduction

The number of solid organ transplants has increased in the past decade, with 21,167 kidney transplants performed in the United States in 2018. Multiple studies have shown that there is an increased risk of malignancy in transplant recipients [1]. The overall cancer incidence rate is 90 per 1000 patients at 10 years after transplant, which is twice as high as in the general population, while the dialysis population has a 1.35 standardized cancer incident ratio compared to the general population [2]. Nonmelanoma skin cancer is even more frequent, with an incidence rate 14 times higher in transplant recipients compared to the general population.

The burden of malignancy in kidney transplant patients is very high, and the mortality risk in kidney transplant recipients diagnosed with cancer is also greater than nontransplant patients. The median survival of kidney transplant patients with cancer is significantly lower than kidney transplant patients without cancer (2.1 years vs. 8.3 years). Malignancy is currently the second most common cause of death in kidney transplant patients after cardiovascular disease [3].

Despite the surging incidence of cancer in kidney transplant recipients, there is very limited data of how immunosuppression (IS) should be managed after malignancy diagnosis. In current practice, the consensus is that IS dose should be decreased in renal transplant patients with newly diagnosed malignancy, since there is evidence supporting that IS is associated with an increased risk of malignancy and can promote tumor growth [4]. However, specific recommendations regarding how to adjust

IS after diagnosis of malignancy in kidney transplant patients are lacking, and management varies depending on institutions, and even by provider in the same practice. The regimen adjustment ranges from no dose reduction, dose reduction, or cessation of one or more immunosuppressive medications, to class switch. The aim of our study was to assess the impact of changes of IS on patient survival and graft function by retrospectively reviewing data on patients who were diagnosed with malignancy after kidney transplantation in our center.

2. Methods

This is a retrospective data analysis, in which we identified subjects by manual search of medical records of patients who had kidney transplantations and cancer diagnosis from January 1990 to December 2018 at Beth Israel Deaconess Medical Center, Boston, MA, USA. Data on immunosuppressive regimen, creatinine at cancer diagnosis and one year after diagnosis were extracted from medical records. Time from transplant to cancer diagnosis, patient and graft survival data were calculated from actual dates. Study data were collected and managed using REDCap (Research Electronic Data Capture) electronic data capture tools hosted at Harvard Catalyst—Beth Israel Deaconess Medical Center [5]. Data were collected by chart review following HIPAA guidelines. Institutional Review Board approval was obtained for data collection and analysis, with a waiver for individual consent.

We included all adult patients (18 years or older) who were diagnosed with malignancy after renal transplantation, as seen in Figure 1. Nonmelanoma skin cancer patients who did not require chemotherapy or radiation for cancer treatment were excluded from the analysis. The primary outcome in this study was patient survival. Secondary outcome included graft failure (defined as renal replacement therapy requirement) and worsening renal function (defined as glomerular filtration rate (GFR) reduction of more than 30% or developed graft failure at one year after cancer diagnosis). The mortality and graft function information were obtained from medical records. Our primary variable of interest was dose reduction defined by any types of IS dose reduction. Variables considered to have potential confounding effect were included in the multivariable models, specifically we included demographics of the subjects (i.e., age, race, gender), creatinine at cancer diagnosis, history of rejection, cancer type, donor type, history of chemotherapy, and history of radiation therapy. Races were divided into black and nonblack, which includes Asian, Hispanic, and others. Cancer types were differentiated as solid organ malignancy and hematologic malignancy. Missing data and loss to follow-up were excluded from the analysis. For survival analysis, loss to follow-up cases were censored.

We stratified the population based on whether individuals had IS dose reduction. Means and standard deviations were used to summarize continuous variables with normal distribution. Median (interquartile range) was used for skewed continuous variables. Categorical variables were summarized as percentage. We used t-test to assess the differences in continuous variables that were normally distributed. We tested the difference in categorical variables with Fisher's exact test. Wilcoxon rank sum test was used to test skewed continuous variables. We used logistic regression to assess variables for worsening graft function at one year after cancer diagnosis. For graft failure outcome, competing risk survival analysis (Fine and Gray model) was used to assess cumulative graft failure incidence, and the covariable effect on graft failure was reported as subdistribution hazard ratio. Death without graft failure was considered as a competing outcome. The Cox proportional hazards model was used to assess risk factors for mortality, and the data was censored by last follow-up date. Patient survival was analyzed using the Kaplan–Meier method with significance tested using the log-rank test.

For subgroup analysis, patients were divided into groups according to type of IS reduction (mycophenolate mofetil (MMF), calcineurin inhibitor (CNI), and reduction of both MMF and CNI). We compared each group to the group without any IS changes to assess the risk of worsening graft function and graft failure between these groups. Propensity score adjustment was utilized for subgroup analysis given the small number of subjects in each group. Propensity score of each subject was calculated based on significant factors derived from initial analysis of worsening graft function and graft failure.

Then, we performed regression analysis for worsening graft function outcome and Cox regression model for graft failure outcome. Propensity score was applied to the model for adjustment.

All multivariable models ware built based on clinical risk factors and statistically significant variables from univariable analyses. $p < 0.05$ was considered to be significant. The data collected were analyzed using the Stata software version 15.0 (Stata Corp., College Station, TX, USA).

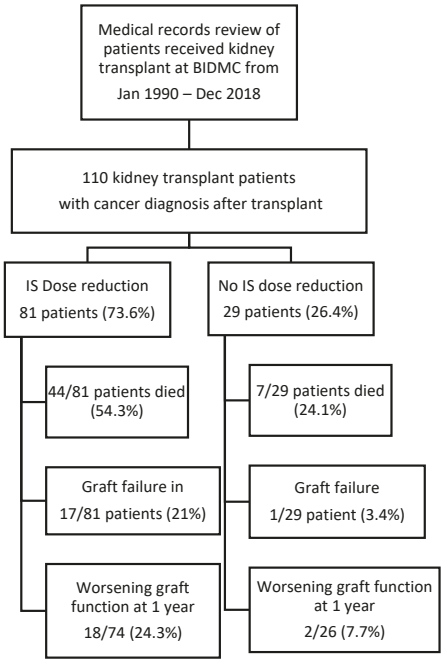

Figure 1. Summary of the study.

3. Results

3.1. Baseline Characteristics of the Study Population

One hundred and ten subjects who underwent kidney transplantation and developed malignancy were included in our analysis, as seen in Figure 1. Patients' demographics are shown in Table 1.

The mean age at cancer diagnosis was 60.2 years. Male gender contributed to 65.5% of subjects. The ethnicities of subjects were 77.3% non-Hispanic White, and 11.8% non-Hispanic Black. Our study population underwent transplantation during 1971–2018 (1971–1999 in 24 patients and 2000–2019 in 86 patients). Of the study population, 73.6% underwent IS regimen changes (dose reduction or class switch), 26.4% patients had no changes in their IS regimen. Among patients with IS reduction, 26 patients had reduction of both mycophenolate mofetil (MMF) and calcineurin inhibitor (CNI), 19 patients had reduction of CNI only, while 25 patients had reduction of MMF only.

The IS regimen of our patient population is presented in Figure 2. Degree of dose reduction for each IS was showed as median of percent reduced from precancer diagnosis dose in Table 2.

Table 1. Baseline characteristic of subjects (N = 110).

Characteristics	Dose Reduction (N = 81)	No Dose Reduction (N = 29)	*p* Value
Sex			
Male	55 (67.9%)	17 (58.6%)	0.37
Race			
White	61(75.3%)	24 (82.8%)	0.17
Black	8 (9.9%)	5 (17.2%)	
Asian	6 (7.4%)	0	
Hispanic	6 (7.4%)	0	
Age at cancer diagnosis	60.1 (11.2)	60.2 (9.1)	0.87
Primary disease			
Diabetes	28 (34.6%)	7 (24.1%)	0.33
Glomerulonephritis	15 (18.5%)	8 (27.6%)	
PKD	7 (8.6%)	3 (10.3%)	
Reflux	1 (1.2%)	2 (6.9%)	
Other	30 (37.0%)	9 (30.0%)	
Transplant type			
Deceased donor	41 (50.6%)	16 (55.2%)	0.77
Living unrelated donor	24 (29.6%)	9 (31.0%)	
Living related donor	16 (19.8%)	4 (13.8%)	
Mean creatinine at cancer diagnosis (mg/dL)	1.65 (1.44–1.87)	1.49 (1.27–1.70)	0.39
Type of cancer			
Hematological cancer	19 (23.5%)	4 (13.8%)	0.27
Solid organ cancer	62 (76.5%)	25 (86.2%)	
History of chemotherapy	48 (59.3%)	8 (27.6%)	<0.01
History of radiation	27 (33.3%)	11 (37.9%)	0.66
History of rejection	14 (17.3%)	5 (18.5%)	0.22

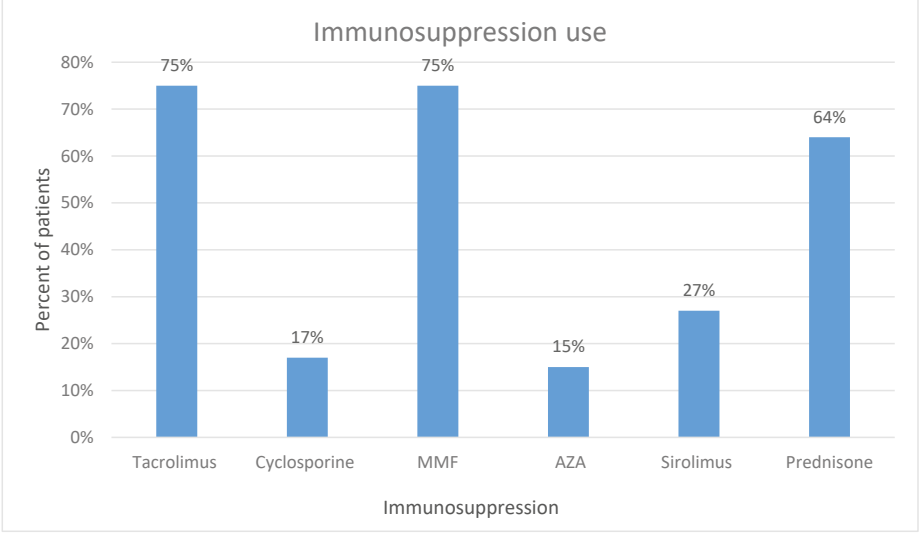

Figure 2. Types of immunosuppression (IS) used by subjects in the study. MMF = mycophenolate mofetil, AZA = azathioprine.

Table 2. Median percent dose reduction of each immunosuppression (100% = completely discontinuation of immunosuppression).

Immunosuppression	Median Percent Dose Reduction (IQR)
Tacrolimus	60% (29.17%–100%)
Cyclosporine	100% (100%–100%)
Mycophenolate mofetil (MMF) or mycophenolic acid	50% (50%–100%)

Medians of percent dose reduction were 60% for tacrolimus, 100% (completely discontinued) for cyclosporine, and MMF or mycophenolic acid. Solid organ malignancies represented 79.1% of the cases; the remainders were hematological cancers. Number of subjects for each type of malignancy are shown in Figure 3.

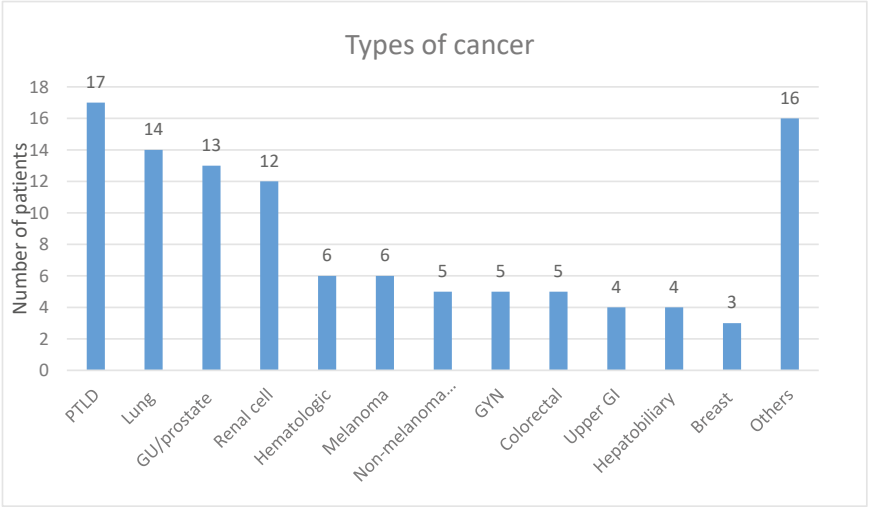

Figure 3. Number of patients in each type of cancer; PTLD = Post-transplant Lymphoproliferative Disorders, GU = Genitourinary, GYN = gynecology, GI = gastrointestinal. Other cancers are head/neck, Kaposi sarcoma, other sarcoma, brain, and unknown origin.

Deceased donor kidney transplant constituted 51.8% of the transplants, and the remainders were from living donors. Mean baseline creatinine at time of cancer diagnosis was 1.6 mg/dL (interquartile range 1.1–1.8 mg/dL). Median time of cancer diagnosis was 6.76 years after transplantation (interquartile range 2.7–11.7 years).

3.2. Mortality

The mortality rate was very high, at 46.4 % (51/110), with median survival time of 1.8 years after cancer diagnosis (interquartile range 0.7–5.6 years). Thirty patients died within one year of cancer diagnosis. Analysis of mortality in the transplantation eras before and after 2000 was performed by chi-square test, mortality rate between both eras was not statistically significant, $p = 0.65$. Of 51 patients who died, malignancy was the cause of death in 27 patients. Infection was the cause of death in four patients. Eighteen patients had no cause of death recorded. Other causes of death were cardiovascular disease and unknown cause. Kaplan–Meier curve and log-rank test revealed that IS dose reduction significantly increased mortality, $p = 0.01$, as seen in Figure 4.

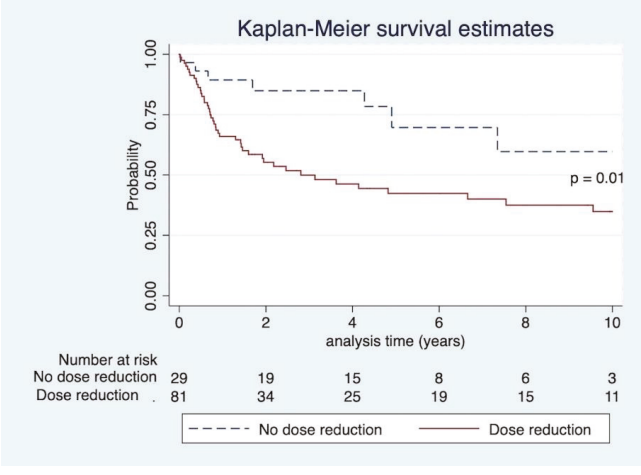

Figure 4. Kaplan–Meier curve and log-rank test of IS dose management and mortality risk.

We performed univariate Cox regression analysis to assess relationship of each variable to mortality, as shown in Table 3. According to our univariate regression analysis model, older age, male gender, IS dose reduction, and chemotherapy were associated with higher mortality. However, in the multivariate model, only chemotherapy remained significant (HR 2.3, 95%CI 1.21–4.35, $p = 0.01$). When we excluded patients who died within six months of cancer diagnosis, the results did not change.

Table 3. Effect of immunosuppression dose reduction on patients' mortality. Multivariable analysis was adjusted for age, IS dose reduction, chemotherapy history, and gender. Nonblack race = White, Asian, Hispanic, and other races. * = Statistically significant, $p < 0.05$.

Variables	Univariate Model		Multivariable Model	
	Hazard Ratio (95%CI)	*p*-Value	Hazard Ratio (95%CI)	*p*-Value
Age at cancer diagnosis	1.04 (1.02–1.07)	<0.01 *	1.02(0.99–1.05)	0.13
IS dose reduction	2.68 (1.21–6.00)	0.02 *	1.94 (0.85–4.41)	0.12
Chemotherapy	3.08 (1.69–5.61)	<0.01 *	2.30 (1.21–4.35)	0.01 *
Male	2.44 (1.24–4.77)	0.01 *	1.97 (0.98–3.99)	0.06
History of rejection	0.78 (0.37–1.67)	0.53		
Cr at cancer diagnosis	1.06 (0.80–1.42)	0.68		
Black Race +	0.36 (0.11–1.17)	0.09		
Solid organ cancer	1.24 (0.58–2.65)	0.57		
Radiation therapy	1.59 (0.91–2.79)	0.10		
Deceased donor	1.62 (0.92–2.84)	0.09		

We also checked the interaction between chemotherapy and dose reduction; the p value of 0.36, indicates no strong interaction between those two variables. The spearman correlation coefficient between chemotherapy and dose reduction was 0.28.

3.3. Worsening Graft Function

There were 100 patients who had post-cancer diagnosis creatinine at one year available. Twenty percent of patients (20/100) developed worsening graft function. In univariate logistic regression, creatinine at cancer diagnosis and female gender were associated with worsening renal function. Those variables remained significant in the multivariable analysis after adjusting for creatinine at cancer diagnosis, IS dose reduction, age, and gender. Interestingly, cancer type, chemotherapy, and donor type were not associated with worsening graft function at one year. The result is shown in Table 4.

Table 4. Impact of immunosuppression dose reduction on worsening GFR > 30% at one year after cancer diagnosis. Multivariable analysis was adjusted for age, creatinine at cancer diagnosis, IS dose reduction, and gender. Nonblack race = White, Asian, Hispanic, and other race. * = Statistically significant, $p < 0.05$.

Variables	Univariable Model		Multivariable Model	
	Odds Ratio (95%CI)	p-Value	Odds Ratio (95%CI)	p-Value
Age at cancer diagnosis	0.99 (0.94–1.03)	0.59	1.02 (0.97–1.08)	0.48
Cr at cancer diagnosis	2.37 (1.28–4.40)	<0.01 *	2.67 (1.35–5.28)	<0.01 *
IS dose reduction	3.86 (0.83–17.94)	0.09	3.80 (0.77–18.71)	0.10
Male	0.43 (0.16–1.16)	0.01 *	0.22 (0.06–0.77)	0.02 *
Black Race +	0.33 (0.04–2.72)	0.30		
Solid organ cancer	0.54 (0.18–1.63)	0.27		
Chemotherapy	2.05 (0.74–5.68)	0.17		
Radiation therapy	0.47 (0.13–1.36)	0.15		
Deceased donor	1.11 (0.41–2.96)	0.84		

It is important to note that the direction and magnitude of the estimates for IS dose reduction suggest a potentially strong effect on worsening graft function and mortality outcome, but our study did not have enough power to detect this, given the small number of patients.

3.4. Graft Failure

In our study, the graft failure rate was 16.4% (18/110). Median graft survival after cancer diagnosis in patients with graft failure was 2.97 years (interquartile range 0.56–4.22 years). Causes of graft failure were acute kidney injury in five patients, "chronic allograft nephropathy" in five patients, and acute rejection in five patients. BK nephropathy, multiple myeloma, and unknown cause contributed to the remaining patients.

As shown in Table 5, in competing risk survival model, creatinine at cancer diagnosis, history of rejection and hematologic cancer were associated with increased risk of graft failure in univariable analysis. After adjusting for age at cancer diagnosis, creatinine at cancer diagnosis, IS dose reduction, malignancy type, and history of rejection, our result showed that creatinine at cancer diagnosis and

history of rejection have remained statistically significant with SHR 1.72, 95% CI 1.28–2.30, $p < 0.01$ and SHR 3.44, 95% CI 1.25–9.49, $p = 0.02$, respectively.

Table 5. Impact of immunosuppression dose reduction on graft survival. Multivariable analysis was adjusted for age, creatinine at cancer diagnosis, history of rejection, IS dose reduction, and cancer type. Nonblack race = White, Asian, Hispanic, and other races. * = Statistically significant, $p < 0.05$.

Variables	Univariable Model		Multivariable Model	
	SHR (95%CI)	*p*-Value	SHR (95%CI)	*p*-Value
Age at cancer diagnosis	0.97 (0.93–1.01)	0.16	0.99 (0.94–1.03)	0.62
Cr at cancer diagnosis	1.83 (1.45–2.30)	<0.01 *	1.72 (1.28–2.30)	<0.01 *
History of rejection	3.63 (1.45–9.08)	0.01 *	3.44 (1.25–9.49)	0.02 *
IS dose reduction	6.19 (0.82–46.73)	0.08	4.46 (0.58–34.48)	0.15
Solid organ cancer	0.35 (0.13–0.95)	0.04 *	0.48 (0.16–1.42)	0.18
Black Race +	0.91 (0.23–3.61)	0.90		
Male	0.67 (0.27–1.66)	0.39		
Chemotherapy	1.39 (0.56–3.46)	0.48		
Radiation therapy	0.93 (0.36–2.44)	0.89		
Deceased donor	0.80 (0.32–1.98)	0.62		

IS was reduced in all the patients who had graft failure, except for one patient who did not have his IS adjusted, as he was only on low dose tacrolimus monotherapy due to BK viremia. PTLD diagnosis contributed to five out of 18 cases of graft failure.

3.5. Subgroup Analysis

3.5.1. Worsening Graft Function

We performed subgroup analysis in patients who had IS reduction, defined by reduction of CNI (19 patients), reduction of MMF (25 patients), and reduction of both (29 patients), compared to 29 patients who had no IS change at all to analyze their impact on worsening graft function at one year. After adjusting for gender, age at cancer diagnosis, creatinine at cancer diagnosis using propensity score, reduction of two types of IS was a significant factor for worsening graft function at one year in logistic regression, OR 6.14, 95% CI 1.14–33.15, $p = 0.04$, as seen in Table 6.

Table 6. Impact of each type of IS reduction compared to no dose reduction on worsening GFR > 30% at one year after cancer diagnosis Adjusted for gender, age at cancer diagnosis, and creatinine at cancer diagnosis. * = Statistically significant, $p < 0.05$.

Immunosuppression Reduction (N)	OR (95% CI)	*p*-Value
CNI Reduction (19/29)	1.31 (0.16–10.59)	0.80
MMF Reduction (25/29)	5.28 (0.86–32.55)	0.07
Reduction of all IS (26/29)	6.14 (1.14–33.15)	0.04 *

3.5.2. Graft Failure

Subgroup analysis was also performed to assess the impact of different IS reduction regimens on graft failure. The patient groups are the same as subgroup analysis in worsening graft function. In the Cox model adjusted for age at cancer diagnosis, creatinine at cancer diagnosis, history of rejection, and cancer type using propensity score, reduction of both CNI and MMF was associated with graft failure, HR 17.97, 95%CI 1.81–178.78, $p = 0.01$, as seen in Table 7.

Table 7. Impact of each type of IS reduction compared to no dose reduction on graft survival. Adjusted for age at cancer diagnosis, creatinine at cancer diagnosis, history of rejection and cancer type. * = Statistically significant, $p < 0.05$.

Immunosuppression Reduction	HR (95%CI)	*p*-Value
CNI Reduction (19/29)	6.52 (0.46–92.70)	0.17
MMF/myfortic Reduction (25/29)	0.66(0.04–11.14)	0.77
Reduction of all IS (26/29)	17.97 (1.81–178.78)	0.01 *

4. Discussion

Although there is increasing evidence of high morbidity and mortality of kidney transplant patients diagnosed with malignancy, specific recommendation on how to adjust IS is lacking. A randomized trial comparing low cyclosporine dose to regular dose found no difference in graft survival or function, although the low-dose regimen was associated with fewer malignant disorders and more frequent rejections [6]. Another randomized controlled trial in 489 kidney transplant patients with 20-year follow-up showed that azathioprine and cyclosporine-based regimens were associated with similar overall long-term cancer risks. In addition, gender, previous antithymocyte globulin (ATG) exposure, and graft failure showed no association with development of malignancy, excluding skin cell carcinoma [7]. One retrospective observational study in heart transplant patients showed that everolimus treatment was associated with lower malignancy risk than MMF [8]. Previous studies showed that sirolimus was associated with reduction in the risk of malignancy and nonmelanoma skin cancer in kidney transplant recipients; however, it was associated with increased mortality risk [9].

KDIGO guidelines published in 2010 recommend considering a reduction of IS for kidney transplant recipients with malignancy (2C recommendation). Important factors to consider (not graded) include the stage of cancer at diagnosis, malignancies which are likely to be exacerbated by IS, available therapies, and whether IS interferes with ability to administer standard chemotherapy [10]. The likelihood of cancer being exacerbated by IS can be assessed using standardized incidence ratio (SIR), which compares the malignancy risk in kidney transplant patients to that in the general population. Cancers with SIR > 3, such as Kaposi's sarcoma, PTLD, and ano-genital cancer, are mostly associated with viral infections, e.g., Human Herpesvirus 8 (HHV8), Epstein–Barr virus (EBV), human papillomavirus (HPV). It has been shown that the incidence of Kaposi's sarcoma, non-Hodgkin's lymphoma, HPV related ano-genital cancer, and melanoma were significantly elevated in patients with functioning transplant graft, but not after transplant failure, when patients were back on dialysis, suggesting that IS has significant effect on these types of cancer. As a consequence, IS adjustment should be strongly considered in these types of malignancy [11,12].

Our study showed that mortality rate in kidney transplant patients with diagnosis of malignancy was high (46.4%), with median survival time of 1.8 years after cancer diagnosis (interquartile range 0.7–5.6 years). Mortality rate was not significantly different between patients who had transplantation before and after year 2000. Interestingly, in our study, malignancy was the main cause of death in subjects whose cause of death was recorded, while the leading cause of death in kidney transplant recipients in general is cardiovascular disease. This data suggests that malignancy contributes to major of mortality in kidney transplant recipients with cancer diagnosis. In addition, more than half

of deceased subjects died within two years of their cancer diagnosis, possibly reflecting advanced cancer at presentation and/or aggressive disease in transplant patients. Our data emphasizes that the appropriate cancer screening could reduce mortality and its importance should be particularly stressed in transplant recipients.

The possible causes of increased mortality risk in this population have been attributed to reduction of immune surveillance in the setting of IS and limited use of certain chemotherapy regimens due to reduced renal function. Notably, kidney transplant recipients and patients with HIV share a similar pattern of increased risk of cancer. Consequently, the increased risk of malignancy after kidney transplantation is thought to be caused by viral infection along with chronic IS use [2].

The significant variable between dose reduction and no reduction groups was whether patients required chemotherapy, suggesting that physicians are more inclined to reduce IS when the cancer is more advanced. The type of cancer (hematologic or solid organ malignancy) did not appear to affect the decision of changing the IS. According to Kaplan–Meier analysis, mortality was significantly higher in the dose reduction group, which is likely confounded by the fact that patients with more advanced stage malignancy tended to have their IS adjusted. Our result is comparable to a previous study in a different center [13]. For multivariate analysis, our study demonstrated that chemotherapy is the only variable associated with mortality, which could be similarly explained by the severity of disease.

As expected, patients with baseline poor kidney function had higher risk of graft failure. The degree of IS dose reduction was significant in majority of patients (IS dose was reduced by at least 50% to completely stopped) putting patients at higher risk of acute allograft rejection. Interestingly, our data showed a novel and important factor in subgroup analysis, reduction of both CNI and MMF put patients at higher risk of graft failure. As a consequence, we recommend that providers should carefully weigh the risks and benefits before drastically changing IS in transplant recipients after cancer diagnosis. A multidisciplinary approach is necessary, focusing on the individual patient's wishes and goals in terms of survival, quality of life, and factor in the possibility of graft failure and return to dialysis. Patients with renal allograft failure returning to dialysis seem to have inferior quality of life and higher rate of depression compared to wait-listed transplant naive patients [14].

Based on our cohort, patients with PTLD had the highest mortality (seven out of 17 patients). Graft failure incidence in patients diagnosed with PTLD was also the highest compared to any other malignancy, as five out of 18 patients who had graft failure were diagnosed with PTLD.

Our study has many limitations. First, it is an uncontrolled retrospective study; therefore, the direct and independent effect of IS changes on mortality could not be clearly determined. Second, our database is from a single center, which has a relatively small number of subjects and heterogeneous cancer types, which might contribute a major confounder. Third, despite adjusting for chemotherapy and radiation therapy, cancer staging was not included in our analysis due to lack of record and heterogeneity of cancer diagnosis. While some chemotherapy regimens could have been a cause graft failure, we did not include this data in our analysis. Lastly, we disregarded the effect of sirolimus and steroid adjustment since both drugs are not part of the standard immunosuppressive regimen at our transplant center.

5. Conclusions

Our study shows no difference in mortality and graft survival outcomes between reduction and no reduction of IS in kidney transplant recipients diagnosed with cancer. However, it is important to note that the direction and magnitude of the estimates for IS dose reduction suggest a potentially strong effect on worsening graft function and mortality outcome, but a lacking power, caused by the small group of subjects, prevented us to detect the differences. The mortality rate in this population is high and malignancy is usually aggressive; therefore, kidney transplant patients would benefit from early detection of disease by routine cancer screening. The data from our study reveals a novel finding: the risk of graft failure appears remarkably higher after adjusting two immunosuppressive medications. Most importantly, providers should have an extensive discussion with patients regarding the risk

and benefit of IS adjustment, chances of prolonging survival from cancer treatment, and worsening quality of life in case patients develop kidney allograft failure requiring dialysis. As a future direction, a prospective study might be the key to define the temporal effect of IS adjustment on patient's survival, malignancy, and allograft outcomes in kidney transplant recipients.

Author Contributions: All authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Conceptualization, Supervision, F.C.; Methodology, Data Curation, Data Analysis, Draft Preparation, D.Y. and N.T. D.Y. and N.T. equally have contributed to this manuscript as first authors.

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Conflicts of Interest: F.C. has been part of the Natera scientific board. D.Y. and N.T. declare no conflict of interest.

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Beliefs of UK Transplant Recipients about Living Kidney Donation and Transplantation: Findings from a Multicentre Questionnaire-Based Case–Control Study

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Abstract: Differing beliefs about the acceptability of living-donor kidney transplants (LDKTs) have been proposed as explaining age, ethnic and socioeconomic disparities in their uptake. We investigated whether certain patient groups hold beliefs incompatible with LDKTs. This questionnaire-based case–control study was based at 14 hospitals in the United Kingdom. Participants were adults transplanted between 1 April 2013 and 31 March 2017. LDKT recipients were compared to deceased-donor kidney transplant (DDKT) recipients. Beliefs were determined by the direction and strength of agreement with ten statements. Multivariable logistic regression was used to investigate the association between beliefs and LDKT versus DDKT. Sex, age, ethnicity, religion, and education were investigated as predictors of beliefs. A total of 1240 questionnaires were returned (40% response). DDKT and LDKT recipients responded in the same direction for 9/10 statements. A greater strength of agreement with statements concerning the 'positive psychosocial effects' of living kidney donation predicted having an LDKT over a DDKT. Older age, Black, Asian and Minority Ethnic (BAME) group ethnicity, and having a religion other than Christianity were associated with greater degree of uncertainty regarding a number of statements, but there was no evidence that individuals in these groups hold strong beliefs against living kidney donation and transplantation. Interventions should address uncertainty, to increase LDKT activity in these groups.

Keywords: living kidney donation; living-donor kidney transplantation; beliefs; inequity

1. Introduction

Living-donor kidney transplantation offers the best treatment in terms of life-expectancy and quality of life [1–6] for most people with kidney failure. The healthcare costs associated with living-donor kidney transplants (LDKTs) are less than for dialysis or deceased-donor kidney transplants (DDKTs) [7,8]. The medium-term risks of donating a kidney are small [9–12] and the quality of life of donors returns to pre-donation levels after donation [13,14].

Only 20% of those listed on the UK national transplant waiting list receive an LDKT each year [15]. Certain individuals with renal disease appear to be disadvantaged: people from Black and Asian ethnic groups in the UK are less likely to receive an LDKT when compared to White people with kidney

disease [16,17]. Socioeconomic deprivation is also associated with reduced access to living-donor kidney transplantation [16,17]. Older people with kidney disease are less likely to receive an LDKT when compared to younger patients [17], and women are less likely to receive an LDKT when compared to men [18,19]. Ensuring equity in living-donor kidney transplantation has been highlighted as a UK and international research priority by patients and clinicians [20–22]. Differing beliefs in the acceptability of living kidney donation and transplantation have been proposed as a possible explanation for the observed differences in access [17,23,24].

In this questionnaire-based case–control study, we compared the beliefs of LDKT and DDKT recipients about the acceptability of living kidney donation and transplantation. We investigated whether beliefs about living-donor kidney transplantation were associated with an individual's sex, age, ethnicity, religion and education. We aimed to identify groups with beliefs against living-donor kidney transplantation, that may explain the observed disparities in the uptake of LDKTs.

2. Experimental Section

2.1. Participants

The study was based at 14 UK hospitals (listed in Supplementary Methods). We wanted to investigate beliefs about living-donor kidney transplantation specifically, and not kidney transplantation in general. Therefore, we did not invite people with Chronic Kidney Disease or those on dialysis to participate, as some of these individuals may have held beliefs against transplantation in general, as opposed to living-donor kidney transplantation specifically. We obtained from each site an anonymised list of all individuals who received kidney transplants between 1 April 2013 and 31 March 2017, stratified by LDKT and DDKT status. Individuals aged <18 years at the time of transplantation, and individuals lacking mental capacity according to the Mental Capacity Act 2005, were excluded. We performed stratified random sampling using Stata 15 [25] to select, on average, 110 LDKTs and 110 DDKTs from each site, weighted by the number of transplants performed annually at each study site. Sex and 5-year age group strata-matched sampling was used to ensure a similar sample distribution by age and sex. The case–control study was designed to detect a 7-point difference in a continuous measure of patient activation (analysis of this variable not presented here) between LDKT cases and DDKT controls with 90% power, assuming a 5% significance level. The calculation indicated that 170 patients would be needed, and that, therefore, a total of 944 would be needed to allow analyses stratified by Index of Multiple Deprivation rank quintile and allow for 10% missing data. This sample size allows for the detection of a far smaller difference (0.16 Standard Deviation) for a dichotomous exposure or between 6–8% for a categorical outcome [26].

2.2. Questionnaire Content and Survey Tools

Paper questionnaires were mailed by post to participants by research collaborators at the study sites. Questionnaires were accompanied by a patient information sheet, an invitation letter and a return postage paid envelope. A website-address was provided so that participants could complete the questionnaire online if preferred. Non-responders were sent a second questionnaire after 4–6 weeks. Anonymised data were extracted from returned paper questionnaires at the University of Bristol and uploaded onto a secure REDCap database [27].

Transplant beliefs were assessed using questions developed by Stothers et al. [28,29]. In development, the questions were reviewed by three expert focus groups, then evaluated in a pilot study to test content reliability and validity [28]. Test–retest analysis was reported as demonstrating excellent internal consistency, and there was no evidence of 'skew' or 'halo' effects (an overall perception/feeling of satisfaction that influences all responses rather than allowing a thoughtful consideration of each individual question) [28]. Participants were asked to read ten statements describing a belief regarding living-donor kidney transplantation (Box 1). These included statements regarding the acceptability of receiving a donated kidney, coercion or pressure on family to donate,

rewards for the donor, required closeness of relationship, the subsequent effect on relationship, beliefs about recipients asking family to donate, donation from offspring to parents, and the risks of donation. Participants were asked to tick one of the following options: (i) Strongly disagree, (ii) Disagree, (iii) Agree, (iv) Strongly agree, (v) Don't know.

Box 1. Belief statements.

1. It is morally acceptable to take a kidney from a healthy person.
2. Donors often agree to donate due to feelings of guilt or family pressure.
3. Donating a kidney is a rewarding experience for the live donors.
4. Donating a kidney to someone requires an extremely close personal relationship.
5. A living-donor kidney transplant may strengthen the relationship between the donor and recipient.
6. Approaching a potential donor who then says no will change the relationship between the two people.
7. Asking someone to donate makes the recipient seem selfish.
8. It is acceptable for a parent to receive a kidney from his/her child (over 18 years old).
9. Decisions about donation should be made by the donor alone. The recipient should not ask for a kidney.
10. Since the donor operation is not risk free, someone who needs a kidney transplant should wait for a kidney from someone who has died.

Questionnaires assessed participant demographics as indicated in Box 2.

Box 2. Participant demographic data collected.

- Sex
  - Male; Female
- 10-year age group
  - 10–19 years; 20–29 years; 30–39 years; 40–49 years; 50–59 years; 60–69 years; 70–79 years; 80–89 years
- Religion
  - No religion; Christian; Muslim; Jewish; Hindu; Sikh; Buddhism
- Socioeconomic position
  - No formal education; Primary school; Secondary school; Vocational/Technical; University—undergraduate; University—postgraduate; Other
- Ethnicity coded using the UK's Office for National Statistics 2011 census categories [30]
  - White;
  - Asian/Asian British;
  - Black/African/Caribbean/Black British;
  - Mixed/Multiple (White and Black Caribbean, White and Black African, Any other Mixed/Multiple ethnic background);
  - Other (Arab, Any other ethnic group)

2.3. Statistical Analysis

We compared demographic characteristics between DDKT and LDKT recipients using chi^2 tests. The proportion of DDKT and LDKT recipients selecting each level of agreement with a belief statement was calculated and initially compared using chi^2 tests. We used multivariable logistic regression to look at the association of transplant type (LDKT versus DDKT) with a recipient's agreement with a belief statement. For the multivariable logistic regression, the response options were coded 1–4 (1 = strongly disagree, 2 = disagree, 3 = agree, 4 = strongly agree) with 'Don't know' coded as missing.

For each belief statement we ran an unadjusted model and one adjusted for potential confounders. We specified, a priori, potential confounders including sex, age, education level, ethnicity and religion. We used robust standard errors to account for clustering within renal centres. Statistical analyses were performed in Stata 15 [25].

Basic descriptive statistical tests (chi^2 tests) then were performed to look for differences in response (agreement = strongly agreed and agreed; disagreement = strongly disagreed and disagreed; and don't know) across different patient demographic groups. For these analyses, age was dichotomised into age <60 years and age ≥60 years, ethnicity into White, Black, Asian and Minority Ethnic (BAME) groups, education into university education or no university education, and religion divided into three categories: no religion, Christianity, or other religion. Small numbers of respondents from certain ethnic groups and from religions other than Christianity or none limited subgroup analysis. Small numbers and single participant responders in some groups risked identification: we were therefore required to combine Islam, Hinduism, Judaism, Buddhism, and Sikhism as 'religions other than Christianity' for analysis.

2.4. Ethical Approval and Consent

We received NHS Research Ethics Committee (REC) (REC reference 17/LO/1602) and Health Research Authority (HRA) approval. A consent form formed the first page of the questionnaire. The study was funded by a Kidney Research UK Project Grant (RP_028_20170302). The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul as outlined in the 'Declaration of Istanbul on Organ Trafficking and Transplant Tourism'.

3. Results

A total of 1240 questionnaires were returned from 3103 patients (40% response). Participant characteristics are reported in Table 1.

LDKT recipients were more likely to respond than DDKT recipients (46% vs. 34%) and women were more likely to respond than men (43% vs. 37%) (Table S1). However, the study participants were a population representative sample (Table S2). Overall, the proportion of missing data was small (<3% for belief questions and <10% for all demographic variables) (Supplementary Missing data).

3.1. Comparison of LDKT and DDKT Recipients

DDKT recipients expressed greater uncertainty than LDKT recipients regarding all belief statements, with a greater proportion of DDKT than LDKT recipients selecting 'Don't know' for every question (Table 2).

The direction of belief for DDKT and LDKT recipients was the same for nine statements (Table 2). The majority of both DDKT and LDKT recipients agreed with the statements: (1) It is morally acceptable to take a kidney from a healthy person; (3) Donating a kidney is a rewarding experience for live donors; (5) A living-donor kidney transplant may strengthen the relationship between the donor and recipient; (8) It is acceptable for a parent to receive a kidney from his/her child (over 18 years old); (9) Decisions about donation should be made by the donor alone. The recipient should not ask for a kidney. The majority of both DDKT and LDKT recipients disagreed that: (4) Donating a kidney to someone requires an extremely close personal relationship; (10) Since the donor operation is not risk free, someone who needs a kidney transplant should wait for a kidney from someone who has died. For these seven statements, DDKT and LDKT recipients who indicated that they had a belief (rather than did not know) reported the same direction of belief but for all questions a greater proportion of LDKT recipients indicated a stronger belief than DDKTs.

No difference between DDKT and LDKT recipients was found with either direction or strength of belief with respect to Statement (3)—'Asking someone to donate makes the recipient seem selfish'. Statement (6)—'Approaching a potential donor who then says no will change the relationship between

the two people'—was associated with the greatest uncertainty for all participants; 36% of DDKT recipients and 34% of LDKT recipients selecting 'Don't know' for this question.

DDKT and LDKT recipients differed in the direction of their belief with respect to only one statement. For statement (2)—'Donors often agree to donate due to feelings of guilt or family pressure'—the majority of LDKT recipients disagreed whilst DDKT recipients were split between disagreement, agreement and not knowing (Table 2).

3.2. Predictors of Case–Control Status

The strength of agreement with seven belief statements predicted case–control status, even after adjustment for potential confounders (Table 3). A greater level of agreement with statements 1, 3, 5, and 8 predicted being an LDKT over a DDKT recipient. These statements concern the 'acceptability' of living donation and transplantation, and its 'positive effects' ('rewarding experience' and 'strengthening relationship'). A greater level of disagreement with statements 2, 6 and 10 predicted being an LDKT over a DDKT recipient. These statements relate to beliefs about individuals experiencing 'pressure to donate' and the 'risks/negative impacts of living donation'.

3.3. Participant Characteristics and Beliefs (Table S3a–e)

3.3.1. Sex

For only one of the ten statements, responses from women and men differed. The majority of women and men agreed with Statement 8—'It is acceptable for a parent to receive a kidney from his/her child (over 18 years old)'—but a greater proportion of women disagreed compared to men (14% versus 8%, chi^2 p-value < 0.001 across all categories of agreement).

3.3.2. Age

For four of the ten statements, older respondents indicated greater uncertainty by selecting 'Don't know' rather than indicating a direction of belief. Individuals aged ≥60 years were more likely than individuals aged <60 years to answer 'Don't know' for statement (2)—'Donors often agree to donate due to feelings of guilt or family pressure' (36% versus 24%, chi^2 p-value < 0.001 across all categories of agreement), statement (5)—'A living-donor kidney transplant may strengthen the relationship between the donor and recipient' (23% versus 16%, chi^2 p-value 0.02 across all categories of agreement), statement (6)—'Approaching a potential donor who then says no will change the relationships between the two people' (41% versus 31%, chi^2 p-value < 0.001 across all categories of agreement), and statement (7)—'Asking someone to donate makes the recipient seem selfish' (32% versus 18%, chi^2 p-value < 0.001 across all categories of agreement).

For one statement, statement (9)—'Decisions about donation should be made by the donor alone. The recipient should not ask for a kidney'—the direction of belief differed with age. People aged ≥60 years were much more likely to agree compared to people aged <60 years (73% versus 57%, chi^2 p-value < 0.001 across all categories of agreement).

Table 1. Participant characteristics by case–control status [a].

Characteristics		Cases [b] (LDKTs) $n = 672$	Controls [b] (DDKTs) $n = 565$	Chi^2 Comparing Cases and Controls
Sex	Male	382 (57)	322 (57)	$p = 0.95$
	Female	279 (42)	235 (42)	
	Missing	11 (2)	8 (1)	
Age (years)	20–29	47 (7)	27 (5)	$p = 0.39$
	30–39	80 (12)	57 (10)	
	40–49	106 (16)	102 (18)	
	50–59	178 (27)	153 (27)	
	60–69	167 (25)	132 (23)	
	>70	77 (12)	79 (14)	
	Missing	17 (3)	15 (3)	
Ethnicity	White	581 (87)	445 (79)	$p = 0.005$
	Asian	38 (6)	41 (7)	
	Black/African/Caribbean	19 (3)	39 (7)	
	Mixed/Multiple	5 (0.7)	5 (0.9)	
	Other	10 (2)	14 (3)	
	Missing	19 (3)	21 (4)	
Religion	No religion	191 (28)	144 (26)	$p = 0.01$
	Christian	402 (60)	315 (56)	
	Muslim	10 (2)	11 (2)	
	Other religions [c]	37 (6)	56 (10)	
	Missing	22 (3)	39 (7)	
Highest level of education	No formal education/Primary school	10 (2)	20 (4)	$p = 0.03$
	Secondary school	202 (30)	191 (34)	
	Vocational/Technical	171 (26)	143 (25)	
	University-undergraduate	145 (22)	98 (17)	
	University-postgraduate	73 (11)	46 (8)	
	Other	33 (5)	24 (4)	
	Missing	38 (6)	43 (8)	

[a] The three participants for whom transplant type/case–control status was missing are excluded from this table. [b] Percentages may not total 100% due to figures being presented to the nearest whole number. [c] Hindu, Jewish, Sikh, Buddhist and Other combined due to single participant responders in some groups risking identification.

Table 2. Beliefs about living donation and living-donor kidney transplantation.

Belief Statement	Transplant Type	Strongly Disagree *n* (%)	Disagreen *n* (%)	Agreen *n* (%)	Strongly Agree *n* (%)	Don't Know *n* (%)	Chi^2 *p*-Value
1. It is morally acceptable to take a kidney from a healthy person.	DDKT [a]	8 (2)	22 (4)	293 (53)	172 (31)	52 (10)	<0.001
	LDKT [a]	24 (4)	11 (2)	252 (39)	340 (52)	28 (4)	
2. Donors often agree to donate due to feelings of guilt or family pressure.	DDKT	63 (11)	172 (31)	117 (21)	22 (4)	177 (32)	<0.001
	LDKT	134 (20)	262 (40)	81 (12)	9 (1)	170 (26)	
3. Donating a kidney is a rewarding experience for the live donors.	DDKT	6 (1)	5 (0.9)	260 (47)	158 (29)	123 (22)	<0.001
	LDKT	11 (2)	4 (0.6)	269 (41)	314 (48)	60 (9)	
4. Donating a kidney to someone requires an extremely close personal relationship.	DDKT	78 (14)	286 (52)	79 (14)	47 (9)	62 (11)	0.004
	LDKT	121 (18)	331 (50)	110 (17)	59 (9)	38 (6)	
5. A living-donor kidney transplant may strengthen the relationship between the donor and recipient.	DDKT	8 (2)	55 (10)	254 (46)	77 (14)	158 (29)	<0.001
	LDKT	13 (2)	65 (10)	314 (48)	198 (30)	69 (11)	
6. Approaching a potential donor who then says no will change the relationship between the two people.	DDKT	47 (9)	185 (34)	85 (16)	33 (6)	200 (36)	0.001
	LDKT	91 (14)	235 (36)	96 (15)	14 (2)	222 (34)	
7. Asking someone to donate makes the recipient seem selfish.	DDKT	45 (8)	204 (37)	120 (22)	41 (8)	139 (25)	0.45
	LDKT	68 (10)	256 (39)	145 (22)	38 (6)	151 (23)	
8. It is acceptable for a parent to receive a kidney from his/her child (over 18 years old).	DDKT	23 (4)	45 (8)	292 (53)	106 (19)	86 (16)	0.002
	LDKT	17 (3)	40 (6)	365 (56)	169 (26)	68 (10)	
9. Decisions about donation should be made by the donor alone. The recipient should not ask for a kidney.	DDKT	19 (4)	112 (20)	203 (37)	127 (23)	90 (16)	<0.001
	LDKT	42 (6)	121 (18)	213 (32)	220 (33)	62 (9)	
10. Since the donor operation is not risk free, someone who needs a kidney transplant should wait for a kidney from someone who has died.	DDKT	87 (16)	311 (56)	52 (9)	10 (2)	92 (17)	<0.001
	LDKT	265 (40)	336 (51)	5 (0.8)	8 (1)	44 (7)	

[a] DDKT = deceased-donor kidney transplant; LDKT = living-donor kidney transplant.

Table 3. Strength of agreement and likelihood of being an LDKT recipient over a DDKT recipient.

Belief Statement	Association between Agreement with Statement and Likelihood of Being an LDKT Recipient over a DDKT Recipient		Interpretation
	Unadjusted OR (95% CI)	Adjusted OR [a] (95% CI)	
1. It is morally acceptable to take a kidney from a healthy person.	1.47 (1.26–1.71)	1.47 (1.29–1.68)	Agreement with statement predicts being an LDKT recipient
2. Donors often agree to donate due to feelings of guilt or family pressure.	0.56 (0.45–0.70)	0.57 (0.45–0.73)	Disagreement with statement predicts being an LDKT recipient
3. Donating a kidney is a rewarding experience for the live donors.	1.56 (1.24–1.94)	1.42 (1.13–1.78)	Agreement with statement predicts being an LDKT recipient
4. Donating a kidney to someone requires an extremely close personal relationship.	0.97 (0.84–1.13)	0.94 (0.79–1.12)	
5. A living-donor kidney transplant may strengthen the relationship between the donor and recipient.	1.42 (1.20–1.68)	1.45 (1.21–1.74)	Agreement with statement predicts being an LDKT recipient
6. Approaching a potential donor who then says no will change the relationship between the two people.	0.69 (0.62–0.78)	0.62 (0.55–0.71)	Disagreement with statement predicts being an LDKT recipient
7. Asking someone to donate makes the recipient seem selfish.	0.88 (0.75–1.02)	0.86 (0.71–1.04)	
8. It is acceptable for a parent to receive a kidney from his/her child (over 18 years old).	1.31 (1.10–1.56)	1.29 (1.04–1.60)	Agreement with statement predicts being an LDKT recipient
9. Decisions about donation should be made by the donor alone. The recipient should not ask for a kidney.	1.09 (0.98–1.21)	1.05 (0.95–1.19)	
10. Since the donor operation is not risk free, someone who needs a kidney transplant should wait for a kidney from someone who has died.	0.36 (0.27–0.47)	0.38 (0.27–0.54)	Disagreement with statement predicts being an LDKT recipient

[a] Adjusted for sex, 10-year age-group, ethnicity (White and Black, Asian and Minority Ethnic (BAME) groups), religion (No religion, Christian, Other), university education (university education or no university education.

3.3.3. Education

For two of the ten statements, a greater proportion of those who did not go to university disagreed with the statement compared to those who did: statement (5)—'A living-donor kidney transplant may strengthen the relationship between the donor and recipient' (13% vs. 7%, chi^2 $p = 0.008$), and statement (6)—'Approaching a potential donor who then says no will change the relationship between the two people' (49% versus 42%, chi^2 p-value 0.03). For statement (9)—'Decisions about donation should be made by the donor alone. The recipient should not ask for a kidney'—individuals without a university degree were more likely to agree than those with (66% versus 58%, chi^2 p-value 0.04).

Individuals without a university degree indicated greater uncertainty with respect to statement (7)—'Asking someone to donate makes the recipient seem selfish'—with a higher proportion selecting 'Don't know' compared to those with a university degree (26% versus 18%, chi^2 p-value 0.01).

3.3.4. Ethnicity

The majority of both white and non-white individuals agreed with statement (1) regarding the moral acceptability of taking a living-donor transplant (89% and 81%), but of the remainder, non-white individuals were more likely to select 'Don't know' than white individuals (13% versus 6%, chi^2 p value = 0.002). Statement (10)—'Since the donor operation is not risk free, someone who needs a kidney transplant should wait for a kidney from someone who has died'—generated the greatest ethnic difference in opinion: non-white individuals were less likely to say they disagreed with this statement (69% versus 85%) and more likely to indicate that they did not know (21% versus 9%, chi^2 $p < 0.001$).

3.3.5. Religion

For statement (1)—'It is morally acceptable to take a kidney from a healthy person'—a greater proportion of people from the 'Other religions' group selected 'Don't know' (13%) compared to those of no religion (5%) and Christians (7%) (Chi^2 $p = 0.01$). Similarly, for statement (3)—'Donating a kidney is a rewarding experience for the live donors'—individuals from the group comprising religions other than Christianity were less likely to agree, and more likely to select 'Don't know' (24%) compared to those of no religion (19%) and Christians (11%) (Chi^2 $p < 0.001$). For statement (10)—'Since the donor operation is not risk free, someone who needs a kidney transplant should wait for a kidney from someone who has died'—a smaller proportion of people in the 'Other religions' group said that they disagreed with this statement (65%) compared to people of no religion (89%) or Christians (89%), and a greater proportion selected 'Don't know' (24%) compared to Christians (10%) and people with no religion (8%) (chi^2 $p < 0.001$).

For statement (6)—'Approaching a potential donor who then says no will change the relationship between the two people'—a slightly greater proportion of Christians (49%) disagreed with the statement compared those of 'Other religions' (43%) or none (42%) (chi^2 $p = 0.008$).

4. Discussion

In this questionnaire-based case–control study, we compared the beliefs of LDKT and DDKT recipients about the acceptability of living kidney donation and transplantation. We found no evidence that DDKT recipients hold strong beliefs against living-donor kidney transplantation. Rather, DDKT recipients hold similar beliefs to LDKT recipients, but report less conviction and greater uncertainty. We did not investigate the source of beliefs in this questionnaire, but it would be interesting to investigate whether the greater uncertainty in the DDKT respondents influences or reflects the beliefs of family members and potential donors. Uncertainty may reflect differing or conflicting beliefs within a family regarding the acceptability of living-donor kidney transplantation.

We aimed to identify groups with beliefs against living-donor kidney transplantation that may explain observed sex, age, ethnic and socioeconomic disparities in the uptake of LDKTs. Overall,

we did not find any evidence of significant difference in the direction of belief with sex, age, ethnicity, religion or education. This suggests that inequality in LDKT uptake associated with sex, age, ethnic, or socioeconomic position is not explained by disproportionately high numbers of individuals in these groups holding beliefs that are incompatible with living-donor kidney transplantation.

BAME group ethnicity and having a religious affiliation other than Christianity were both associated with greater uncertainty regarding a number of belief statements. BAME individuals were particularly uncertain as to whether one should wait for a DDKT, given that living kidney donation is not risk free. Uncertainty regarding organ donation and transplantation has previously been reported in qualitative research amongst certain ethnic and religious groups, attributed specifically to uncertainty regarding religious edicts [31,32]. One qualitative study from the Netherlands identified a lack of awareness about the 'official' position of an individual's religion regarding living organ donation within communities, and confusion due to differing interpretations of religious texts [32]. Research from the USA has shown that, amongst church-attending African-American individuals without kidney disease, 37% disagreed with living donation [33], and members of the clergy were more likely to express reservations about living donation than deceased donation (33.3% versus 16.7%) [33]. These studies suggest that faith leaders might play an important educational role, that their opinion might be influential, and that clarity over the position of the religion on living-donation needs to be made explicit [32–34]. To this end, during the preparation of this manuscript, a new fatwa clarifying Islamic approval of living and deceased organ donation and transplantation was published in the UK [35].

Older people reported greater uncertainty in their beliefs about the impact of donation on the family, and whether asking is selfish on the recipient's part. Older people have been reported as being unhappy to accept an organ from a younger living donor [36,37], in part due to parents believing they should protect their children from harm [36,37]. This belief regarding the acceptability of living-donor kidney transplantation might be influenced by clinicians: research from the USA has suggested that eligible older people with kidney disease are less likely to be encouraged to seek a transplant by their nephrologists [38].

Our findings suggest that the majority of DDKT recipients believe living kidney donation and living-donor kidney transplantation are acceptable, appropriate and justifiable. The majority of demographic groups believe that there are benefits from LDKTs to both the donor and the recipient. Given these beliefs, it suggests that there is capacity to increase LDKT activity in the UK. There should be no assumption that people of certain groups (BAME or older people) have strong beliefs against an LDKT—but rather, any uncertainty should be taken as an opportunity to engage in discussion. Attitudes towards living kidney donation are often open to change and, accordingly, can be influenced [39]. Conversations with religious leaders may help to overcome specific uncertainties regarding a particular religion's position on living donation [34,35].

The belief statements in this study were first developed and used in a Canadian population [29]. LDKT recipients and wait-listed patients surveyed in Canada were found to have the same direction of response as LDKT recipients and DDKT recipients in the UK for all statements except for Statements (4) and (10). For Statement (4)—'Donating a kidney to someone requires an extremely close personal relationship'—69% Canadian LDKT recipients agreed or strongly agreed with this statement, compared to 26% of UK LDKT recipients. For statement (10)—'Since the donor operation is not risk free, someone who needs a kidney transplant should wait for a kidney from someone who has died'—a greater proportion of UK DDKT recipients disagreed with this statement when compared to Canadian wait-listed patients (72% versus 52%). These differences may reflect transplant practice and beliefs changing over time, since the Canadian study was undertaken over 15 years earlier. However, these differences may in part explain why the UK's LDKT activity is greater than Canada's [40], and this requires further investigation.

In our study, statement (10)—'Since the donor operation is not risk free, someone who needs a kidney transplant should wait for a kidney from someone who has died'—generated the

most difference in opinion; therefore, how beliefs will change with the UK's move to an opt-out deceased-donation law in 2020 will need to be investigated.

This was a large, multicentre study. To our knowledge, this is the first quantitative study to investigate beliefs about living-donor kidney transplantation amongst transplant recipients. The questionnaire was evaluated in cognitive interviews prior to use, validated and then piloted [26]. The proportion of missing data was small. However, the study has limitations: (i) Although our response rate was reasonable for an unincentivized postal survey, and compares to the response rate of other postal surveys in the UK [41,42] and that of previous a previous European transplant survey [43], there is a risk of self-selection bias. We have reported in our results that our population appeared population representative (Table S2). In addition, we compared our findings to those from the Access to Transplantation and Transplant Outcome Measures (ATTOM) study (which had 72% participation), and found the same effect sizes between socioeconomic position and likelihood of an LDKT (see Table S4) providing further evidence our sample is fairly representative of the total population of such patients. (ii) A total of 14% of participants were from BAME groups—this is not a surprising finding as in the UK between 2013 and 2017 BAME individuals comprised 17% of LDKT recipients and 27% of LDKT kidney transplant recipients [44], but this did prevent the analysis of individual ethnic groups (e.g., Asian, Black, Chinese).

The questionnaire was administered to LDKT and DDKT transplant recipients, both of whom have experienced transplantation; thus in the analyses examining the relationship between beliefs and transplant type, one might expect responses to be subject to a range of cognitive biases, including justifying their decision, and endowment effects. However, evidence against a significant endowment effect on the direction of belief includes the finding that the majority of DDKT recipients expressed positive beliefs about living donation and transplantation. Were there significant endowment effects, we would not have expected the majority of DDKT recipients to express positive beliefs about LDKTs. Cognitive biases do not explain the differences in beliefs between different demographic groups.

5. Conclusions

The majority of both DDKT and LDKT recipients across all demographic groups reported holding positive beliefs about living donation and transplantation. This encouraging finding suggests that, at least on the part of the transplant candidate, beliefs that are incompatible with LDKT are not a major barrier to living-donor transplantation in the UK, and that there is capacity to increase LDKT activity.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/1/31/s1, Supplementary Methods; Table S1. Responders and non-responders, Table S2. Responders compared to national denominator population, Supplementary Missing Data, Table S3a. Difference in beliefs with participant sex, Table S3b. Difference in beliefs with participant age, Table S3c. Difference in beliefs with participant education, Table S3d. Difference in beliefs with participant ethnicity, Table S3e. Difference in beliefs with participant religion, Table S4. Comparison with participants in ATTOM study.

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Intraoperative Fluid Restriction is Associated with Functional Delayed Graft Function in Living Donor Kidney Transplantation: A Retrospective Cohort Analysis

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Abstract: Background: In 2016 we observed a marked increase in functional delayed graft function (fDGF) in our living donor kidney transplantation (LDKT) recipients from 8.5% in 2014 and 8.8% in 2015 to 23.0% in 2016. This increase coincided with the introduction of a goal-directed fluid therapy (GDFT) protocol in our kidney transplant recipients. Hereupon, we changed our intraoperative fluid regimen to a fixed amount of 50 mL/kg body weight (BW) and questioned whether the intraoperative fluid regimen was related to this increase in fDGF. Methods: a retrospective cohort analysis of all donors and recipients in our LDKT program between January 2014–February 2017 (n = 275 pairs). Results: Univariate analysis detected various risk factors for fDGF. Dialysis dependent recipients were more likely to develop fDGF compared to pre-emptively transplanted patients ($p < 0.001$). Recipients developing fDGF received less intraoperative fluid (36 (25.9–50.0) mL/kg BW vs. 47 (37.3–55.6) mL/kg BW ($p = 0.007$)). The GDFT protocol resulted in a reduction of intraoperative fluid administration on average by 850 mL in total volume and 21% in mL/kg BW compared to our old protocol ($p < 0.001$). In the unadjusted analysis, a higher intraoperative fluid volume in mL/kg BW was associated with a lower risk for the developing fDGF (OR 0.967, CI (0.941–0.993)). After adjustment for the confounders, prior dialysis and the use of intraoperative noradrenaline, the relationship of fDGF with fluid volume was still apparent (OR 0.970, CI (0.943–0.998)). Conclusion: Implementation of a GDFT protocol led to reduced intraoperative fluid administration in the LDKT recipients. This intraoperative fluid restriction was associated with the development of fDGF.

Keywords: fluid management; kidney transplantation; delayed graft function; goal-directed fluid therapy

1. Introduction

During the procedure of organ donation and transplantation a number of potentially harmful processes will inevitably occur, affecting the viability of the kidney graft. Both donor and recipient are

subjected to anesthesia and surgery, which will produce a sequence of systemic and local changes, including a significant proinflammatory and procoagulatory response [1]. The donor organ is by definition, exposed to a number of phases of injury from the moment the donor suffers from cerebral injury (in case of brain death) until the kidney is reconnected to the circulation in the recipient. These phases include a profound systemic and local proinflammatory and procoagulatory response during donor management and retrieval, associated with hypoxia and ischemia of the kidney. In addition, prolonged warm ischemia in the deceased circulatory death (DCD) donor will affect the viability of the donor kidney. These combined effects on the graft-to-be result in a cascade of renal damage that will reveal itself at the time of transplantation, when the donor kidney is reperfused in the recipient and has been named an ischemia-reperfusion injury (IRI) [2]. Typically, IRI will clinically manifest as immediate nonfunction of the transplant with the need for dialysis treatment until the graft recovers from the insult and starts eventually to function. This 'secondary' recovery is called delayed graft function.

DGF, a form of acute kidney injury post-transplantation, is an uncommon complication after living donor kidney transplantation (LDKT), most likely due to very short ischemia times and healthy living donors. Incidences reported vary between 1%–8% [3,4]. In transplantation with kidneys from deceased brain death (DBD) donors, however, the incidence of DGF increases to 15%–25% and may rise up to 72% in transplantation with kidneys from deceased DCD donors [5,6]. DGF is a risk factor for acute rejection (AR) and the combination of DGF and AR reduces graft and patient survival [7–9]. Also in the absence of AR, DGF has been shown to be an independent risk factor for long term graft loss. Reported risk factors for DGF are: deceased donor, longer ischemia times, donor and recipient older age, female donor, male recipient, history of dialysis, higher body mass index (BMI), hypertension in the donor, diabetes in the recipient, retransplantation, higher panel-reactive antibody levels, and higher human leukocyte antigens (HLA) mismatch [3,5,7,10]. This variety of risk factors underscores the complex pathological mechanisms underlying DGF.

Regarding the intraoperative period, several studies suggest that an adequate/supranormal fluid state is associated with a reduced risk of DGF [5,7,11–14]. These studies, however, are mainly retrospective and often comprise a variety of donor types with variable incidences of DGF hampering an adequate analysis. Central venous pressure (CVP)-guided fluid therapy has been suggested until recently [11,12], but CVP does not correlate well with intravascular fluid state and its use to guide fluid therapy is currently discouraged [15]. Blood pressure and heart rate are also affected by several variables, unrelated to the circulatory state of the patient, like pain, temperature, anesthetics, and analgesics, making them less suitable as an indicator of the intravascular volume [16,17].

Recently, goal directed fluid therapy (GDFT) has been shown to improve patient outcomes after major (abdominal) surgery [18–20]. During 2015, our department implemented a GDFT approach in kidney transplant recipients to replace our standard intraoperative fluid regimen of four to five liters (L) of balanced crystalloids. In the first half year of 2016 a marked increase in DGF and functional (f)DGF in our LDKT population was noticed. During 2014 and 2015, respectively, 8.5% and 8.8% of the patients experienced fDGF. From January to June 2016 the incidence of functional delayed graft function (fDGF) rose to 23.0%, which was a significant increase compared to 2014 and 2015 ($p = 0.039$ and $p = 0.021$, respectively). Since the incidence of fDGF in this population has been stable over the past two decades and no protocol changes were implemented with the exception of the GDFT protocol, we questioned whether this increase in fDGF was due to the altered fluid regimen. To our surprise, a retrospective analysis revealed that the implementation of GDFT protocol had resulted in a reduced intraoperative fluid administration which seemed associated with the increase in fDGF. Based on these results, we promptly changed the intraoperative fluid protocol in September 2016 to a fixed amount of 50 mL/kg BW with a lower limit of 2500 mL and upper limit of 6000 mL (50 kg–120 kg), unless patients comorbidity determined otherwise. After six months the incidence of fDGF was back to baseline at 8.2%.

Since we were interested in whether the amount of fluid administered intraoperatively was indeed an independent factor predicting fDGF in this LDKT population, we performed a retrospective cohort analysis of all donors and recipients in our living donor program between January 2014–February 2017.

2. Materials and Methods

2.1. Study Design and Population

This retrospective cohort analysis comprised all consecutive donor and recipient pairs of the LDKT program of the University Medical Centre of Groningen (UMCG) between January 2014 and February 2017. The Institutional Review Board approved the study (METc 201600968), which was conducted in adherence to the Declaration of Helsinki. Due to the observational and retrospective character of the analysis, the requirement for informed consent was waived.

2.2. Definition of DGF

Twenty-two definitions of DGF were identified in literature based on dialysis, serum creatinine levels, urine output or a combination of these 3 [21], Most commonly used was dialysis requirement the first week after transplantation (also used in this analysis for DGF). This dialysis-based definition, however, is criticized for its subjectivity since there are center- or physician-specific thresholds for the use of dialysis after transplantation [22]. Furthermore, since approximately half of our LDKT population was transplanted preemptively, this dialysis-based definition was unsuitable for this analysis. Another definition, referred to as functional (f)DGF, is failure of serum creatinine level to decrease spontaneously by at least 10% daily on 3 consecutive days during the first postoperative week, discounting creatinine decreases due to dialysis. Moore and colleagues showed that fDGF is independently associated with reduced death-censored graft survival in contrast to DGF based on the dialysis definition and suggested a superiority of this definition over the dialysis-based definition [23]. To prevent misclassification in patients with excellent early graft function, failure of creatinine to decrease on postoperative day three was not classified as fDGF if optimal graft function had already been achieved by day 2. In this analysis, we compared patients undergoing LDKT with fDGF and without fDGF (nofDGF).

2.3. Intra- and Postoperative Management and Surgical Procedure

Anesthetic management was according to local protocol. Propofol was used for induction of anesthesia and either propofol or sevoflurane were used for maintenance of anesthesia. Sufentanil or remifentanil were used to control nociception and rocuronium or cis-atracurium for muscle relaxation. Until the implementation of the GDFT protocol, donors and recipients were given 4–5 L of balanced crystalloids throughout the procedure unless their comorbidity determined otherwise. During 2015, a GDFT protocol was gradually implemented in the recipients (not in the donors). For a detailed description of this protocol, see below. From September 2016 fluid protocol in recipients was changed to a fixed amount of 50 mL/kg BW intraoperatively. Timeline of fluid management in recipients is given in Figure 1. Fluid management in donors was not actively changed during our observation period. Regarding the type of fluid, predominantly Ringers' lactate (RL) was used. If hyponatremia occurred RL was replaced by 0.9% saline. Colloids were not given and administration of blood products was according to our local transfusion protocol with thresholds based upon patients comorbidity. Regarding hemodynamics, the goal was to keep the blood pressure within 80% range of the baseline blood pressure of the patient. As baseline, we used blood pressure measured at the preoperative visit. If hypotension occurred, the first step was to adjust depth of anesthesia or analgesia. If that was insufficient or not possible, patients received one or more doses of ephedrine or phenylephrine or a continuous infusion of noradrenaline was started. Kidney donation was performed using a hand-assisted laparoscopic approach. Thereafter the kidney was flushed and perfused with cold University of Wisconsin solution (ViaSpan®, BMS, Bruxelles, Belgium or CoStorSol®, Bridge to Life, Elkhorn, WI, USA) and stored on

ice. Transplantation was performed according to local, standardized protocol. Postoperative fluid management comprised 1 L NaCl 0.45%-Glucose 2.5% per 24 h, complemented with the volume of diuresis in the former hour.

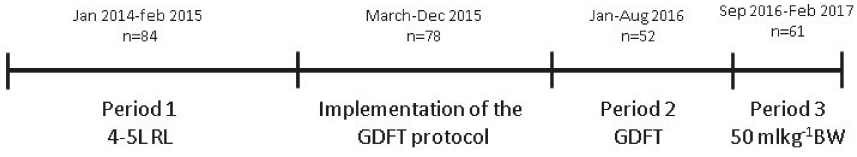

Figure 1. Timeline of various intraoperative fluid protocols in recipients. L: liters; RL: Ringers' lactate; GDFT: goal directed fluid therapy, BW: body weight.

2.4. Goal-Directed Fluid Therapy Protocol.

GDFT was performed with the use of the FloTrac®in combination with the EV1000®monitor (Edwards Lifesciences Corporation, Irvine, CA, USA). The system was used according to manufacturer's instructions. A standard institutional GDFT protocol was used with adjustment of the goal. Instead of a stroke volume variation (SVV) < 12%, commonly used in abdominal surgery, we aimed for a SVV < 10% throughout the procedure. When the SVV was >10% additional fluid was given until SVV was <10%. If SVV < 10%, fluid administration was left to the discretion of the attending anesthesiologist, however, when cardiac index (CI) was below age-adjusted normal values, a noradrenaline infusion was started. If measurement of the SVV was not possible (e.g., due to cardiac arrhythmias) a protocol based on stroke volume (SV) was used. In this case, if a fluid bolus of 250 mL resulted in an increase of the SV of 10%, additional fluid was given, if not, the trend of the SV was monitored and fluid administration was left to the discretion of the attending anesthesiologist. When SV decreased >10%, additional fluid was given. The FloTrac®was used with the EV1000 monitor, which does not communicate with our digital PDMS. Therefore SV, SVV, and CI values could not be retrieved for this analysis.

2.5. Patient Data

Demographic and postoperative data were obtained from digital patient medical records. The following variables were taken into account: age, gender, BMI, smoking, hypertension, use of antihypertensive drugs, measured glomerular filtration rate (mGFR) with use of iodine 125-iothalamate in the donor, blood pressure (measured the day of hospital admission), difference in blood pressure between donor and recipient measured by systolic/diastolic/mean of the recipient minus systolic/diastolic/mean of the donor, underlying kidney disease, number of HLA mismatches, history of dialysis, related or unrelated donor transplantation. For all recipients, the age-adjusted Charlson comorbidity index (CCI) [24] and length of hospital stay was calculated. Intraoperative data were retrieved from our digital patient data monitoring system (PDMS, CS-EZIS, Chipsoft B.V., Amsterdam, the Netherlands) and consisted of duration of surgery, intraoperative volume and type of fluid, cumulative hypotensive periods defined as a systolic blood pressure < 80 mmHg and MAP < 60 mmHg, intraoperative use of vasoactive substances, ischemia times, left/right kidney, side of implantation, number of arteries, sacrifice of an accessory artery, and urinary output the first 2 h postoperatively. Regarding the use of vasoactive substances, patients were scored on receiving one or more boluses of ephedrine and/or phenylephrine and whether or not noradrenaline was administered as a continuous infusion. Additionally, the maximum noradrenaline infusion rate during the procedure was noted. This was grouped into 3 categories: low infusion rate (0.02–0.10 mg/h), intermediate (0.10–0.20 mg/h), and high (>0.20 mg/h) infusion rate.

2.6. Statistics

For the statistical analysis SPSS version 23 (IBM Corp, Armonk, NY, USA) and GraphPad Prism version 7.02 (GraphPad Software Inc, La Jolla, CA, USA) were used. We performed univariate analyses

to identify factors associated with fDGF. Categorical data were analyzed by chi-square or Fisher's exact tests. Continuous data were analyzed with an unpaired t-test in the case of normally distributed values. If variables were not normally distributed Mann–Whitney test was applied. Multivariate analysis was performed by means of binary logistic regression. We adjusted the amount of fluid administered intraoperatively in recipients for potentially relevant confounders with high significance in the univariate analysis. Additionally, we were interested in the impact of implementation of our GDFT protocol on the incidence of fDGF and on the amount of fluid administered intraoperatively. We therefore analyzed these data between the different time periods 1–3 (described above) with the use of Fisher's exact test and Kruskal–Wallis test. Post-hoc analysis with Mann–Whitney was used. Values are given as number (%), mean ± standard deviation (SD) or median with interquartile range (IQR). All reported *p*-values are two-sided. A *p*-value of 0.05 or less was considered significant.

3. Results

3.1. Univariate Analysis

3.1.1. Patient Characteristics

Between January 2014 and February 2017, 275 living donor kidney transplant procedures were performed in our center. Of the 275 recipients, 31 patients experienced fDGF and 244 recipients did not (nofDGF). Donor and recipients characteristics of fDGF and nofDGF kidneys are listed in Table 1. There were no statistically significant differences in baseline characteristics and kidney function (mGFR) in donors of kidneys with our without fDGF. Recipients developing fDGF were more likely to be dialysis-dependent at the time of transplantation (25 (81%) vs. 105 (43%), $p < 0.001$). The composition of the group of dialysis dependent patients did not differ between nofDGF and fDGF recipients. In the nofDGF group 76 (72%) patients were on hemodialysis at the time of transplantation and 29 (28%) on peritoneal dialysis. In the fDGF group, this was the case for 19 (76%) and six (24%), respectively. All patients on hemodialysis were dialyzed the day before transplantation to 1 kg above dry weight.

Table 1. Donor and recipient demographics. Data given as number (%), mean (SD), or median (IQR).

	nofDGF	**fDGF**	***p***
Donor	***N = 244***	***N = 31***	
Age year	54 (11.6)	51 (12.4)	0.104
Gender male	117 (48%)	20 (65%)	0.089
BMI	26.1 (3.0)	25.1 (2.7)	0.075
Smoking	67 (27%)	13 (42%)	0.140
Blood pressure			
S-RR mmHg	136 (15.3)	136 (11.8)	0.848
D-RR mmHg	79 (73–84)	81 (73–86)	0.548
MAP mmHg	98 (9.4)	98 (6.7)	0.897
Hypertension	38 (16%)	2 (6%)	0.277
Anti-hypertensive drugs			
Diuretics	11	1	>0.999
B-blocker	13	1	>0.999
Ca antagonist	10	0	0.610
ACE-I	4	0	>0.999
AT-II-ant.	16	1	0.703
mGFR			
Non-stimulated mL/min	109 (97–23)	107 (95–128)	0.846
Stimulated mL/min	116 (103–133)	118 (100–140)	0.764
ΔGFR	7 (2–12)	7 (−1–12)	0.810

Table 1. *Cont.*

	nofDGF	fDGF	*p*
Recipient	***N = 244***	***N = 31***	
Age year	54 (41–61)	55 (43–62)	0.991
Gender male	138 (57%)	21 (68%)	0.254
BMI	25.6 (22.6–28.4)	25.8 (24.0–29.8)	0.267
Smoking	45 (18%)	7 (23%)	0.626
Blood pressure			
S-RR mmHg	143 (20.4)	138 (23.7)	0.196
D-RR mmHg	79 (73–84)	81 (73–86)	0.548
MAP mmHg	97 (9.4)	98 (6.6)	0.897
Δ blood pressure with donor			
Δ S-RR mmHg	7.1 (22.8)	2.5 (29.1)	0.308
Δ D-RR mmHg	3.1 (13.9)	1.0 (15.0)	0.336
Δ MAP mmHg	4 (−6–14)	8 (−10–12)	0.756
Hypertension	175 (72%)	21 (68%)	0.675
Antihypertensive drugs			
Diuretics	84 (34%)	8 (25%)	0.421
B-blocker	124 (51%)	10 (32%)	0.058
Ca antagonist	131 (54%)	15 (48%)	0.703
ACE-I.	46 (19%)	5 (16%)	0.811
AT-II-ant	55 (23%)	7 (23%)	>0.999
CCI	3 (2–4)	3 (2–6)	0.157
Underlying kidney disease			
DM	15 (6%)	5 (16%)	0.358
PKD	57 (23%)	5 (16%)	0.495
Systemic autoimmune diseases	25 (10%)	3 (10%)	>0.999
Glomerulonephritis	47 (19%)	4 (13%)	0.4713
Other	100 (41%)	14 (45%)	0.701
HLA mm < 3	55 (23%)	8 (25%)	0.655
Dialysis dependent	105 (43%)	25 (81%)	<0.001 *
LURD	164 (67%)	19 (61%)	0.547

fDGF: functional delayed graft function; BMI: body mass index; S-RR: systolic blood pressure; D-RR: diastolic blood pressure; MAP: mean arterial pressure; ACE-I: angiotensin-converting enzyme inhibitor; AT-II-ant: angiotensin II receptor antagonist; CCI: Charlson comorbidity index; mGFR: measured glomerular filtration rate measured with use of iodine 125-iothalamate; DM: diabetes mellitus; PKD: polycystic kidney disease; HLA: human leucocyte antigen; LURD: living unrelated donation; *: statistically significant.

3.1.2. Intra- and Postoperative Data

Intraoperative data of donors of fDGF and nofDGF kidneys showed no differences with exception of the total amount of fluid, in which donors of fDGF kidneys received less fluid intraoperatively, which was the case for total volume (3545 mL (778.2) vs. 3845 mL (799.1), $p = 0.050$) and mL/kg BW (45 mL/kg BW (10.3) vs. 49 mL/kg BW (11.4), $p = 0.053$).

Recipients who developed fDGF received significantly less intraoperative fluid, which was the case for the total amount of fluid (3000 mL (2250–3680) vs. 3500 mL (2900–4075), $p = 0.023$) and mL kg-1BW (36 mL/kg BW (25.9–50.0) vs. 47 mL/kg BW (37.3–55.6), $p = 0.007$). Predominantly RL was given, but in case of hyponatremia RL was partially replaced by saline. This was the case in 48 (20%) of the recipients without fDGF and in 8 (26%) of the patients with fDGF ($p = 0.477$). Median volume replaced by saline was 1000 mL (500–2000) in the nofDGF group and 800 mL (500–1075) in the fDGF group ($p = 0.865$). Blood loss was comparable between groups and transfusion of red blood cells was

applied in 10 (4.1%) of the patients in the noFDGF group and two (6.4%) of the fDGF group. Patients showed no difference in hypotensive periods, but recipients experiencing fDGF were treated more frequently with noradrenaline continuous infusion ($p = 0.034$), which was only the case for low dose infusion with a maximum of 0.1 mg/h. For noradrenaline administered at higher dosage (>0.1 mg/h), there was no difference between the two groups. fDGF was associated with a lower urine output during the first two hours after transplantation ($p = 0.005$ for the first hour and $p = 0.002$ for the second hour). Ten patients in the fDGF group were dialyzed after transplantation versus zero patients in the nofDGF group ($p < 0.001$). Eight of these kidneys gained function after a mean of 10.3 (3.1) days. Two kidneys suffered primary nonfunction due to a combination of ATN and mild antibody-mediated rejection (patient 114, transplanted June 2015) and non-HLA-mediated hyperacute rejection (patient 273, transplanted November 2016). Recipients experiencing fDGF showed a longer hospital stay (14 (10–20) vs. 9 (7–13) days $p < 0.001$) (Table 2).

Table 2. Intra- and postoperative donor and recipient data. Data given as number (%), mean (SD), or median (IQR).

	nofDGF	**fDGF**	***p***
Donor	***n = 244***	***n = 31***	
Duration min	227 (38.2)	216 (36.8)	0.134
Fluid			
Total mL	3845 (799.1)	3545 (778.2)	0.050*
mL/kg BW	49 (11.4)	45 (10.3)	0.053
Intraoperative blood pressure			
S-RR ≤ 80 mmHG	137 (56%)	21 (68%)	0.251
Cumulative duration (min)	10 (5–15)	10 (5–15)	0.772
Vasoactive substances			
Ephedrine	178 (73%)	25 (71%)	0.515
Phenylephrine	22 (9%)	4 (13%)	0.512
Noradrenaline	61 (25%)	11 (35%)	0.277
Recipient	***n = 244***	***n = 31***	
Duration min	212 (189–239)	224 (190–260)	0.390
Fluid			
Total mL	3500 (2900–4075)	3000 (2250–3680)	0.023*
mL/kg BW	47 (37.3–55.6)	36 (25.9–50.0)	0.007*
Intraoperative blood pressure			
S-RR < 80 mmHg	49 (20%)	6 (19%)	>0.999
Cumulative duration min	5 (5–10)	7.5 (4.5–11.2)	0.679
MAP < 60 mmHg	93 (38%)	11 (35%)	0.846
Cumulative duration min	10 (5–10)	5 (5–20)	0.759
Vasoactive substances			
Ephedrine	93 (38%)	16 (52%)	0.174
Phenylephrine	26 (11%)	3 (10%)	>0999
Noradrenaline	129 (53%)	23 (74%)	0.034*
0.02–0.10 mg h^{-1}	37 (15%)	10 (32%)	0.024*
0.10–0.20 mg h^{-1}	42 (17%)	7 (23%)	0.459
>0.20 mg h^{-1}	49 (20%)	6 (19%)	>0.999
Ischemia times (min)			
WIT	3 (3–4)	3 (3–4)	0.724
CIT	154 (140–173)	158 (141–178)	0.646
WIT2	39 (33–45)	38 (33–45)	0.982

Table 2. *Cont.*

	nofDGF	fDGF	*p*
Kidney left	177 (73%)	19 (61%)	0.209
Right fossa	203 (83%)	26 (84%)	>0.999
>1 artery	49 (20%)	8 (26%)	0.482
Artery sacrificed	11 (5%)	4 (13%)	0.074
Blood loss (mL)	250 (150–400)	250 (162.5–500)	0.499
Urineproduction	***n* = 230**	***n* = 30**	
1st h (mL)	405 (250–675)	255 (75–512)	0.005*
2nd h (mL)	350 (250–550)	183 (64–462)	0.002*
	n = 244	***n* = 31**	
Dialysis after transplantation	0 (0%)	10 (32%)	<0.001*
Length of hospital stay days	9 (7–13)	14 (10–20)	<0.001*

Min: minutes; BW: bodyweight; S-RR: systolic blood pressure; MAP: mean arterial pressure; WIT: warm ischemia time; CIT: cold ischemia time: WIT2: warm ischemia time 2; *: statistically significant

3.2. Multivariate Logistic Regression Analysis

In the unadjusted analysis, a higher intraoperative administered fluid volume was associated with 3% lower odds for the development of fDGF per mL/kg BW (OR 0.967, CI (0.941–0.993), model 1). We adjusted for potentially relevant confounders with high significance in the univariate analysis, i.e., a history of dialysis and the use of intraoperative noradrenaline, after which the relationship was still apparent (OR 0.970, CI (0.943–0.998), model 2). Since the intraoperative amount of fluid in the donors approached significance in the univariate analysis with lower volumes given in the fDGF group, we also adjusted for amount of fluid in the donor, after which the relationship was still apparent (OR 0.969, CI (0.941–0.997), model 3) (Table 3).

Table 3. Multivariate logistic regression on risk factors of functional delayed graft function (fDGF).

Model	Odds ratio (95% CI)	*p*
1. Unadjusted analysis, model 1		
• Amount of fluid administered intraoperatively, recipient, mL/kg BW	0.967 (0.941–0.993)	0.015
2. Adjusted analysis, model 2		
• Amount of fluid administered intraoperatively, recipient, mL/kg BW	0.970 (0.943–0.998)	0.036
• No dialysis dependence at time of transplantation	0.186 (0.073–0.475)	<0.001
• Use of noradrenaline continuous infusion yes/no	2.018 (0.834–4.878)	0.119
3. Adjusted analysis, model 3		
• Amount of fluid administered intraoperatively, recipient, mL/kg BW	0.969 (0.941–0.997)	0.029
• No dialysis dependence at time of transplantation	0.181 (0.071–0.464)	<0.001
• Amount of fluid administered intraoperatively, donor, mL/kg BW	0.978 (0.942–1.014)	0.231

3.3. Influence of the GDFT Protocol on the Intraoperative Fluid Volume.

Additionally, we were interested in the impact of implementation of our GDFT protocol on the incidence of fDGF and on the amount of fluid administered intraoperatively. The GDFT protocol was gradually implemented during 2015 and in 2016 (up to September) all recipients were treated following this protocol (Figure 1). Data of the EV1000 monitor were not recorded in our PDMS, therefore we were unable to see which patients in 2015 were treated according the GDFT protocol and disregarded this period (March 2015–December 2015) in this specific analysis. We compared patients transplanted between January 2014–February 2015 (period 1, $n = 84$, old protocol) to patients transplanted between January 2016–June 2016 (period 2, $n = 52$, GDFT protocol) and patients transplanted between September 2016–February 2017 (period 3, $n = 61$, new protocol).

Incidence of fDGF during the different periods are shown in Figure 2. Implementation of GDFT was accompanied by an increase in fDGF from 8.3% in period 1 to 23% in period 2. The implementation of the new protocol in period 3 resulted in a reduction of the incidence of fDGF back to baseline (8.2%, $p = 0.029$).

Total amount of intraoperative administered fluid and mL/kg BW in recipients in the different time periods are shown in Figure 3A,B, respectively. Total amount of fluid and mL/kg BW were significantly different between the three time periods ($p < 0.001$, $p < 0.001$). Implementation of the GDFT (period 2) resulted in a decrease of intraoperative fluid administration compared to our old protocol (period 1), which was the case for total volume (2775 mL (2313–3500) vs. 3625 mL (3213–4000), $p < 0.001$) and mL/kg BW (38 mL/kg BW (30.3–45.3) vs. 48 mL/kg BW (40–60), $p < 0.001$). The implementation of the new protocol (period 3) resulted in an increase in intraoperative fluid administration to 4150 mL (3475–4575) mL and 54 mL/kg BW (47.4–60.1) compared to the old (total volume $p = 0.037$, mL/kg BW $p = 0.053$) and GDFT (total volume $p < 0.001$, mL/kg BW $p < 0.001$).

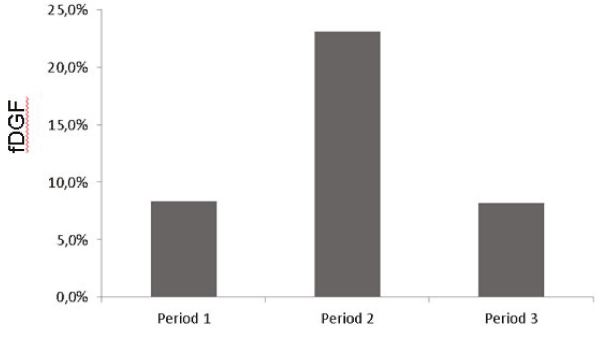

Figure 2. Incidence of fDGF in recipients during the different time periods. Period 1: January 2014–February 2015, old protocol, 4–5 L RL. Period 2: January–June 2016, GDFT protocol. Period 3: September 2016–February 2017, new protocol, 50 mL/kg BW. $p = 0.029$.

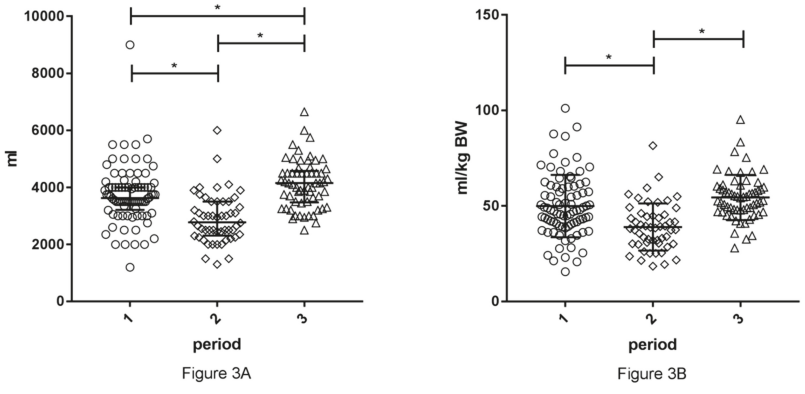

Figure 3. Volume of fluid administered intraoperatively in recipients during the different time periods. Period 1: January 2014–February 2015, old protocol, 4–5 L RL. Period 2: January–June 2016, GDFT protocol. Period 3: September 2016–February 2017, new protocol, 50 mL/kg BW. Volumes are given in mL (**A**) and mL/kg BW (**B**).

4. Discussion

This retrospective cohort analysis study shows that intraoperative fluid restriction in recipients is associated with fDGF in living donor kidney transplantation. Additionally, we showed that the implementation of a GDFT with a goal set at SVV < 10% led to a reduction of intraoperative fluid administration, on average by 850 mL in total and 21% in mL/kg BW, compared to our old protocol of 4–5 L of RL. In our opinion, this analysis provides valuable information for other centers when changes in intraoperative fluid management during kidney transplantation are considered.

Four to five liters of RL was the standard intraoperative fluid protocol in kidney transplantation in our center for over 15 years. This may seem rather liberal, but problems due to hypervolemia were rarely seen. However, following new trends on GDFT [24], a personalized intraoperative fluid approach seemed more appropriate in this group of patients presenting with a variety of fluid states at the time of surgery. Therefore, when in 2015 an intraoperative GDFT protocol was introduced in our center for several surgical procedures, we included the kidney transplant program in this implementation. Since there is no evidence in current literature on what goal to aim for, we adjusted the standard institutional GDFT protocol of SVV < 12%, commonly used in abdominal surgery, to a more generous goal in fluid administration of SVV < 10%. The implementation of this protocol resulted in a reduction in the amount of fluid administered intraoperatively in contrast to previous studies comparing GDFT to a "standard" protocol, which generally reported an increase of the amount of fluid. This could be due to the fact that most of these studies compare GDFT with a rather restrictive fluid protocol, which was general practice before GDFT was introduced. Kidney transplantation, however, has always been an exception on this restrictive trend and most centers use a rather liberal fluid protocol during this procedure. Another factor could be the performance of the FloTrac®-system in predicting fluid responsiveness in this specific patient category. GDFT and the performance of the FloTrac®-system has predominantly been validated in cardiac and abdominal surgery, liver transplantation, and septic patients. Patients with end-stage renal disease (ESRD) and especially patients on HD develop morphologic and functional cardiovascular changes. They often present with severe arterio- and atherosclerosis, inducing arterial stiffening and systolic or diastolic dysfunction. Since SVV is calculated as the percentage change of SV to the mean, derived from an arterial pulse contour analysis, it is conceivable that these cardiovascular changes influence the performance of the FloTrac®-system in predicting patients fluid state. Only one pilot study presents the effect of fluid loading on SVV measured with the use of the FloTrac®-system in patients with ESRD on HD. In this study, HD patients undergoing vascular surgery presented with a broad range of SVV (16.2 ± 6.0) after

induction of anesthesia. After a fluid bolus of only 500 mL of a colloid solution almost all patients showed a SVV < 10% (6.2 ± 2.8), the threshold in our protocol [25].

The debate on perioperative fluid management is still ongoing. Controversy exists regarding assessment of the intravascular volume state, which goals to aim for, how to measure these goals, and what type of fluid should be used. Hypovolemia leads to a decreased oxygen supply to organs and tissues and may cause hypoxia, which can lead to organ dysfunction. Hypervolemia, on the other hand, can damage the endothelial glycocalyx resulting in a fluid shift from the intravascular compartment to the interstitial space and tissue edema [26]. Shin and colleagues report in their large cohort analysis of 92.094 patients undergoing noncardiac surgery that both too little and too much intraoperative fluid is associated with increased morbidity, mortality, costs, and length of hospital stay [27]. Myles and colleagues randomLy assigned 3000 patients undergoing a major abdominal procedure to a restrictive or liberal fluid regimen. In their study, a restrictive regimen was associated with increased risk of acute kidney injury with a hazard ratio of 1.71 (95% CI 1.29–2.27) [28]. These studies, however, do not take kidney transplant recipients into account. In the normal kidney, blood flow is regulated by an autoregulatory mechanism, ensuring adequate perfusion in a broad blood pressure range by afferent and efferent arterioles. In the transplanted, denervated kidney, this haemodynamic autoregulation is impaired making the renal blood flow linearly dependent on the systemic blood flow [29–31]. Furthermore, reperfusion of the ischemic kidney can be followed by vasoconstriction in the afferent arterioles. This may result in a reduced GFR due to a decrease in glomerular transcapillary hydraulic pressure difference [7,32,33]. Ensuring an adequate volume state in this specific patient category, therefore, is essential to obtain an adequate circulation both on macro- and microcirculatory level. Recently, Cavalari and colleagues reported the results of their prospective observational study, in which they compared a prospectively observed cohort of 33 deceased donor kidney transplant recipients treated with a GDFT protocol to a historical cohort of 33 kidney transplant recipients treated with their conventional fluid therapy [34]. They observed a significant reduction of cardiovascular complications, DGF. and surgical complications in the GDFT group. Surprisingly, in this study both groups received the same amount of fluid throughout the transplant procedure. Studies including deceased donor kidneys, however, comprise a variety of donor types with variable incidences of DGF hampering an adequate analysis and conclusions.

The most important predictor of fDGF in our analysis was dialysis dependency at the time of transplantation. A history of dialysis and especially hemodialysis prior to transplantation is a known risk factor of DGF [5,7,35,36]. Hypovolemia at the time of transplantation is one of the proposed underlying mechanisms [37]. Our hypothesis before implementation of the GDFT protocol was that these hypovolemic dialysis patients would present with higher SVV at time of surgery, demanding more fluid intraoperatively, compared to the relatively normovolemic or slightly hypervolemic preemptively transplanted patients. Surprisingly, comparable amounts of fluids were given to the two groups.

In our GDFT protocol, noradrenaline was used when CI was below an age-adjusted value. Therefore, an increased use of noradrenaline was seen in period 2 compared to period 1 (71% vs. 41% $p = 0.001$) due to the implementation of the GDFT. In period 3, the use of noradrenaline decreased to 50% of the patients. In the univariate analysis, the use of noradrenaline was correlated with development of fDGF, but after multivariate logistic regression this was no longer the case. However, Morita and coworkers showed that in a rat model, transplanted kidneys responded to sympaticomimetics with a reduction in renal blood flow (RBF) in contrast to the increase in RBF seen in native rat kidneys [38].

There are some limitations of this analysis that have to be addressed: A major limitation is that we were unable to evaluate outcome directly according to the fluid protocol (4–5L RL vs. GDFT) and are unable to present information or draw any conclusions regarding actual SV, SVV, CO or CI values and their relation to the observed increase of fDGF. Other limitations are those of a retrospective observational trial. There is the potential of confounding by unmeasured factors. Regarding postoperative fluid volume, the exact amount of fluid given could not be retrieved in a reliable way from our PDMS and is therefore not implemented in this analysis. Postoperative fluid

management was according to a standardized protocol and comprised of 1 L NaCl 0.45%-Glucose 2.5% per 24 h, complemented with the volume of diuresis in the former hour. This means that when the kidney produces less urine the patient will be given less fluid postoperatively. Since fDGF was associated with a lower urinary output the first two hours, it is very likely that patients experiencing fDGF received less fluid postoperatively. Whether this contributed to development of fDGF or is more of a symptom remains unknown. Backpressure from congested tubules obstructed with cellular debris may contribute to a reduction in GFR [39,40]. A higher volume of urine in the first hours may have led to washout of this debris.

Finally, due to the fact that there are only 31 events there is always the possibility of overestimating the strength of associations using a multivariate analysis. A strong argument, however, is that no policy changes were implemented during the study period with the exception of the intraoperative fluid regimen. Furthermore the incidence of fDGF in our LDKT population has been stable over many years and after changing the fluid regimen back to a more liberal fixed amount of 50 mL/kg BW the incidence of fDGF instantly returned to baseline.

DGF after transplantation is a clinically relevant problem. It is associated with an increase in morbidity, patient anxiety, increased risk of acute rejection, and additional diagnostic procedures and costs. In our population the median hospital stay in patients experiencing fDGF was prolonged by five days. Furthermore, this study shows that strict protocols for perioperative fluid management are needed when studies in kidney transplantation are designed. Fluid restriction can be an important risk factor for DGF, a frequently used primary end point, even in the setting of LDKT.

5. Conclusions

Implementation of a goal-directed approach to fluid administration with a goal set at a SVV < 10% throughout the procedure led to reduced intraoperative fluid administration in the LDKT recipients in our center. This intraoperative fluid restriction was associated with the development of more fDGF. A thorough validation of GDFT protocols in patients with renal insufficiency is warranted before these are implemented in this population.

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Correction

Correction: Fast Tac Metabolizers at Risk—It is Time for a C/D Ratio Calculation. *J. Clin. Med.* 2019, *8*, 587

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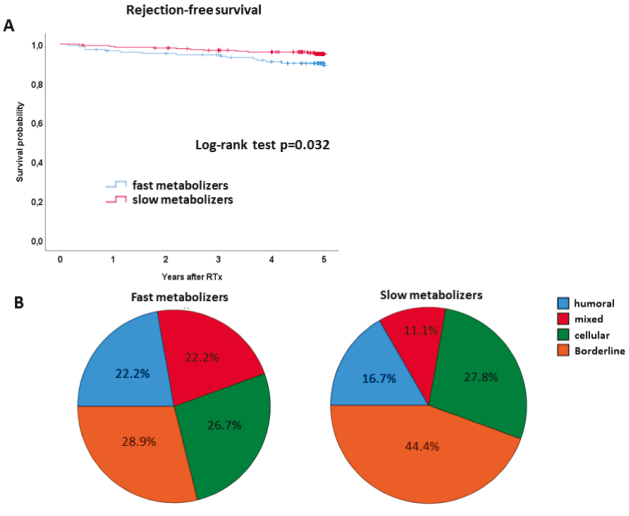
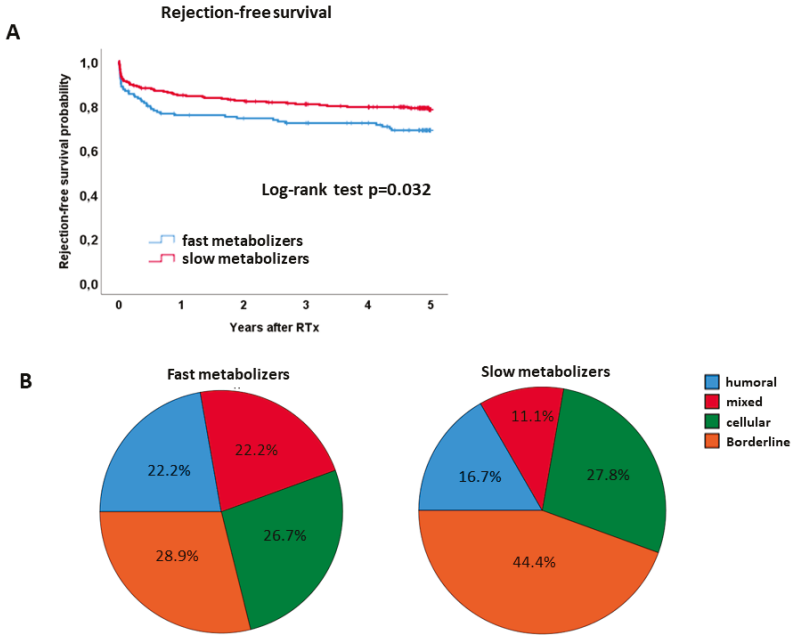
The authors wish to make the following corrections to this paper [1].

The authors made an error regarding the rejection-free survival curve in Figure 4A. Figure 4 needs to be corrected.



should be replaced with



The authors apologize to the readers for any inconvenience caused by these changes. It is important to state that this correction do not affect our study's results and involve no changes or modifications in the original data supporting our results. The original manuscript will remain online on the article webpage, with reference to this Correction.

Reference

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Article

Proton-Pump Inhibitors and Hypomagnesaemia in Kidney Transplant Recipients

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Abstract: Proton-pump inhibitors (PPIs) are commonly used after kidney transplantation and there is rarely an incentive to discontinue treatment. In the general population, PPI use has been associated with hypomagnesaemia. We aimed to investigate whether PPI use is associated with plasma magnesium, 24-h urinary magnesium excretion and hypomagnesaemia, in kidney transplant recipients (KTR). Plasma magnesium and 24-h urinary magnesium excretion were measured in 686 stable outpatient KTR with a functioning allograft for ≥1 year from the TransplantLines Food and Nutrition Biobank and Cohort-Study (NCT02811835). PPIs were used by 389 KTR (56.6%). In multivariable linear regression analyses, PPI use was associated with lower plasma magnesium (β: −0.02, $P = 0.02$) and lower 24-h urinary magnesium excretion (β: −0.82, $P < 0.001$). Moreover, PPI users had a higher risk of hypomagnesaemia (plasma magnesium <0.70 mmol/L), compared with non-users (Odds Ratio (OR): 2.12; 95% confidence interval (CI) 1.43–3.15, $P < 0.001$). This risk tended to be highest among KTR taking high PPI dosages (>20 mg omeprazole Eq/day) and was independent of adjustment for potential confounders (OR: 2.46; 95% CI 1.32–4.57, $P < 0.005$). No interaction was observed between PPI use and the use of loop diuretics, thiazide diuretics, tacrolimus, or diabetes ($P_{interaction} > 0.05$). These results demonstrate that PPI use is independently associated with lower magnesium status and hypomagnesaemia in KTR. The concomitant decrease in urinary magnesium excretion indicates that this likely is the consequence of reduced intestinal magnesium absorption. Based on these results, it might be of benefit to monitor magnesium status periodically in KTR on chronic PPI therapy.

Keywords: proton-pump inhibitors; magnesium; hypomagnesaemia; kidney transplantation

1. Introduction

Proton-pump inhibitors (PPIs) are frequently used after kidney transplantation for their gastro- protective properties in the setting of immunosuppressive therapy, which usually includes glucocorticoids. Since their first introduction in the late 1980s, numerous case reports and observational studies have been published that associate PPI use with unfavorable clinical outcomes, including an

increased risk of hypomagnesaemia [1–8]. Recently, this observation has been strengthened by a large population based cohort study which demonstrated a two times higher risk of hypomagnesaemia among subjects from the general populations on chronic PPI therapy compared to non-users [9].

Magnesium homeostasis depends mainly on the balance between intestinal Mg^{2+} uptake, storage and resorption from bones and urinary excretion of Mg^{2+} via the kidneys [10]. It is postulated that PPIs induce hypomagnesaemia through inhibition of pH-dependent active magnesium absorption via transient receptor potential melastatin (TRPM) 6 and 7 channels in the intestine [11,12]. Moreover, increased renal magnesium retention has been observed in magnesium depleted subjects using chronic PPI therapy, indicating a defect in intestinal magnesium absorption or increased losses into the gastrointestinal tract, rather than renal magnesium wasting [1,7,13].

Hypomagnesaemia is very common after kidney transplantation and it is generally thought to be a side effect of immunosuppressive therapy, especially of calcineurin inhibitors (CNI) which are known to induce renal magnesium wasting [14]. It has been shown that hypomagnesaemia is not only present in the immediate post transplantation period, but persists in about 20% of kidney transplant recipients (KTR) for many years after transplantation [15,16]. Importantly, hypomagnesaemia has been associated with onset of post-transplant diabetes mellitus (PTDM) in KTR [17,18] and has also been associated with increased risk of cardiovascular morbidity [19,20] and mortality [21] in the general population. Whether use of PPIs contributes to hypomagnesaemia in KTR has not been well established. To our knowledge only one cohort study investigated the association between PPI use and hypomagnesaemia in 512 KTR, with negative results [22]. Reasons for absence of an association were unclear, but may have included a low prevalence of PPI use of 20%, which could have led to low statistical power of the study. Thus, whether PPI use negatively affects magnesium status after transplantation remains to be determined. We aimed to investigate whether PPI use is associated with magnesium status and hypomagnesaemia in a large single center cohort of stable outpatient KTR, in which plasma magnesium measurements were not part of routine clinical care but were assessed from samples that had been stored in a biobank.

2. Methods

2.1. Study Design and Population

This is a cross-sectional analysis using data from a previously described prospective cohort study registered at clinicaltrials.gov as 'TransplantLines Food and Nutrition Biobank and Cohort-study', NCT02811835 [23]. In summary, all adult KTR with a functioning graft beyond the first year after transplantation and without known or apparent systemic illnesses (i.e., malignancies other than cured skin cancer, opportunistic infections, overt congestive heart failure) who visited the outpatient clinic of the University Medical Center Groningen (UMCG) between November 2008 and March 2011, were asked to participate. A total of 707 out of the initially 817 invited KTR signed informed consent. We excluded KTR with missing biomaterial (n = 8), missing data on PPI dosage (n = 1), with on-demand PPI use (n = 3) or using magnesium supplements (n = 6) from statistical analyses, leaving 689 cases eligible for analysis. Study measurements were performed during a single study visit at the outpatient clinic. All study procedures were conducted in adherence with the Declaration of Helsinki and Declaration of Istanbul. The institutional review board of the UMCG approved the study protocol (METC 2008/186, approved on 17 September 2008).

2.2. Exposure Definition

PPI type and daily dosage were obtained from electronic patient records and are demonstrated in Table S1. KTR using any PPI on a daily basis during a period of at least 3 months prior to the study visit were defined as chronic PPI users as described previously [24]. To investigate a potential dose–response relationship, KTR were divided into three groups based on daily PPI dose defined in

omeprazole equivalents: no PPI, low PPI dose (≤20 mg omeprazole equivalents/day (Eq/day)) and high PPI dose (>20 mg omeprazole Eq/day) [24,25].

2.3. Assessement of Plasma and Urinary Magnesium

Plasma magnesium was measured in samples containing lithium heparin, using a xylidyl blue method (Roche Modular analyzer, Roche Diagnostics, Mannheim, Germany). Urinary magnesium excretion was assessed in 24 h-urine samples and measured on a MEGA clinical chemistry analyzer (Merck, Darmstadt, Germany). Hypomagnesaemia was defined as plasma magnesium <0.70 mmol/L.

2.4. Assessment of Dietary Magnesium Intake

Dietary magnesium intake was calculated using a validated semi quantitative food frequency questionnaire (FFQ) developed and updated at the Wageningen University, which was filled out at home [26,27]. Dietary data were converted into daily nutrient intake using the Dutch Food Composition Table of 2006 [28].

2.5. Assessment of Covariates

Medical history was obtained from electronic patient records as described previously [23]. History of cardiovascular disease was classified according to the International Classification of Diseases, 10th revision (ICD-10) code Z86.7. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Blood pressure was measured as described in detail previously [29]. Information on alcohol use and smoking behavior was obtained using a questionnaire. Medication use, including the use of PPIs, H2-receptor antagonists, diuretics, prednisolone, mycophenolate mofetil (MMF), tacrolimus, cyclosporine, and sirolimus was recorded at baseline. Routine immunosuppressive therapy consisted of: A combination of azathioprine and prednisolone from 1968 to 1989; a combination of cyclosporine and prednisolone from 1989 to 1996. In 1997 mycophenolate motefil was added to the standard immunosuppressive regimen and cyclosporine was slowly withdrawn after the first year in KTR without complications. In 2012 cyclosporine was replaced by tacrolimus, and KTR received triple-immunosuppressive therapy with prednisolone, tacrolimus and mycophenolate mofetil. PPIs were routinely prescribed after kidney transplantation for their gastro-protective properties with concurrent use of prednisolone. Blood samples were collected after an 8–12 h fasting period. Serum creatinine was measured using an enzymatic, isotope dilution mass spectrometry-traceable assay (P-Modular automated analyzer, Roche Diagnostics, Mannheim, Germany). Estimated glomerular filtration rate (eGFR) was calculated using the serum creatinine based Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation. Serum potassium, calcium, parathyroid hormone (PTH), glucose and hemoglobin A1c (HbA1c), were determined using standard laboratory methods. Proteinuria was defined as urinary protein excretion ≥0.5 g/24 h.

2.6. Statistical Analyses

Statistical analyses were performed using SPSS, version 23.0 (IBM corp., Armonk, NY, USA). Data are presented as mean ± SD for normally distributed data, median with interquartile range (IQR) for skewed data and number with percentage for nominal data. Differences between PPI users versus PPI non-users were tested using independent sample *T*-tests, Mann–Whitney U-tests and Chi-square tests or Fishers exact tests when appropriate.

To study the effect of PPI use on plasma magnesium linear regression analyses were performed with adjustment for potential confounders of magnesium status including: age, sex, BMI, eGFR, proteinuria, time since transplantation, alcohol use, diabetes, history of cardiovascular disease, use of loop diuretics, thiazide diuretics, tacrolimus, cyclosporine, MMF, and dietary magnesium intake. To investigate the association between PPI use and hypomagnesaemia we performed logistic regression analyses with adjustment for the same potential confounders used in multivariable linear regression analyses. Effect modification by loop diuretics, thiazide diuretics, tacrolimus and diabetes was tested

by inclusion of interaction terms. To investigate a potential dose–response relationship we performed additional analyses in which KTR were divided into three groups based on daily PPI dose defined in omeprazole equivalents: No PPI, low PPI dose (≤20 mg omeprazole Eq/day) and high PPI dose (>20 mg omeprazole Eq/day) [24,25]. Tests of linear trend were conducted by assigning the median of daily PPI dose equivalents in subgroups treated as a continuous variable. We performed sensitivity analyses in which H2-receptor antagonist (H2RA) users (n = 18) were excluded to assess the robustness of the association between PPI use and hypomagnesaemia. Lastly, we investigated which KTR are at increased risk of developing hypomagnesaemia. A two-sided P-value < 0.05 was considered statistically significant in all analyses.

3. Results

3.1. Baseline Characteristics

Baseline characteristics are shown in Table 1. PPIs were used by a small majority of 389 (56.5%) KTR and omeprazole was the most often prescribed PPI (n = 340). Other PPIs used were esomeprazole (n = 30), pantoprazole (n = 16), and rabeprazole (n = 3). KTR who used PPIs were older than KTR who did not use PPIs, had a higher BMI and had shorter time between transplantation and baseline measurements. Diabetes was significantly more prevalent in PPI users compared with non-users (28.3% vs. 18.3%, P < 0.002). Plasma magnesium and 24-h urinary magnesium excretion were significantly lower in PPI users and 102 (26.2%) PPI users had hypomagnesaemia compared with 43 (14.3%) non-users (P < 0.001). Dietary magnesium intake was not significantly different between PPI users and non-users. Loop diuretics, cyclosporine and MMF, were more often used by PPI users compared with non-users. Triple immunosuppressive therapy consisting of MMF, cyclosporine and prednisolone, was more common in PPI users compared with non-users. Duo therapy consisting of MMF-prednisolone, MMF-cyclosporine, and cyclosporine-prednisolone was more common in PPI users compared with non-users.

Table 1. Baseline characteristics of 689 kidney transplant recipients.

Characteristics	Total Population	Non-PPI Users	PPI Users	*P*
Number of subjects, *n* (%)	689 (100)	300 (43.5)	389 (56.5)	n/a
Demographics				
Age, year	53 ± 13	51 ± 13	54 ± 12	0.001
Men, *n* (%)	395 (57.3)	177 (59.0)	218 (56.0)	0.4
BMI, kg/m^2	26.6 ± 4.8	25.9 ± 4.6	27.1 ± 4.8	0.002
Diabetes Mellitus, *n* (%)	165 (23.9)	55 (18.3)	110 (28.3)	0.002
History of CV disease, *n* (%)	274 (39.8)	92 (30.7)	182 (46.8)	<0.001
Time since transplantation, year	5.5 (1.9–12.1)	9.6 (4.1–15.0)	4.2 (1.1–8.7)	<0.001
Lifestyle parameters				
Current smoker, *n* (%)	84 (13.0)	35 (12.4)	49 (13.6)	0.7
Alcohol consumer, *n* (%)	436 (70.3)	198 (72.8)	238 (68.4)	0.2
Magnesium intake, mg/day	329.9 ± 88.7	333.0 ± 89.2	327.6 ± 88.4	0.5
Renal function parameters				
eGFR, mL/min/1.73 m^2	52.3 ± 20.2	55.1 ± 19.9	50.2 ± 20.1	0.002
Serum creatinine, µmol/L	124 (100–160)	119 (98–152)	128 (101–168)	0.03
Proteinuria (≥0.5 g/24 h), *n* (%)	157 (22.9)	71 (23.7)	86 (22.2)	0.7
Laboratory parameters				
Hypomagnesaemia, *n* (%)	145 (21.0)	43 (14.3)	102 (26.2)	<0.001
Plasma magnesium, mmol/L	0.77 ± 0.11	0.79 ± 0.09	0.76 ± 0.11	<0.001
24-h urinary magnesium excretion, mmol/24 h	3.3 (2.3–3.3)	3.8 (2.8–4.8)	3.1 (2.0–3.9)	<0.001
Serum potassium, mmol/L	3.98 ± 0.46	3.97 ± 0.47	3.99 ± 0.46	0.6
Serum calcium, mmol/L	2.40 ± 0.15	2.40 ± 0.15	2.40 ± 0.15	0.8
PTH, $_p$mol/L	9.0 (6.0–14.8)	8.7 (6.0–13.6)	9.2 (5.9–16.3)	0.2
Glucose, mmol/L	5.3 (4.8–6)	5.2 (4.7–5.8)	5.3 (4.8–6.2)	0.01
HbA1c, mmol/mol	40 (37–44)	39 (36–42)	41 (38–45)	<0.001

Table 1. *Cont.*

Characteristics	Total Population	Non-PPI Users	PPI Users	*P*
Medication use				
Mycophenolate mofetil, *n* (%)	452 (65.6)	178 (59.3)	274 (70.4)	0.002
Tacrolimus, *n* (%)	124 (18.0)	49 (16.3)	75 (19.3)	0.3
Cyclosporine, *n* (%)	272 (39.5)	97 (32.3)	175 (45.0)	0.001
Sirolimus, *n* (%)	13 (2.0)	8 (2.8)	5 (1.4)	0.3
Prednisolone, *n* (%)	682 (99.0)	298 (99.3)	384 (98.7)	0.7
Loop diuretics, *n* (%)	160 (23.2)	41 (13.7)	119 (30.6)	<0.001
Thiazide diuretics, *n* (%)	120 (17.4)	53 (17.7)	67 (17.4)	0.9
H2-receptor antagonists, *n* (%)	18 (2.6)	17 (5.7)	1 (0.3)	<0.001
Combination therapy				
MMF + Tac + pred, *n* (%)	78 (11.3)	32 (10.7)	46 (11.8)	0.6
MMF + Cyclo + pred, *n* (%)	175 (25.4)	51 (17.0)	124 (31.9)	<0.001
MMF + Tac, *n* (%)	81 (11.8)	33 (11.0)	48 (12.3)	0.6
MMF + Pred, *n* (%)	447 (64.9)	176 (58.7)	271 (69.7)	0.003
MMF + Cyclo, *n* (%)	177 (25.7)	52 (17.3)	125 (32.1)	<0.001
Cyclo + Pred, *n* (%)	269 (39.0)	96 (32.0)	173 (44.5)	0.001
Tac + Pred, *n* (%)	120 (17.4)	48 (16.0)	72 (18.5)	0.4

Data are presented as mean ± SD, median with interquartile ranges (IQR) or number with percentages (%). Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; PTH, Parathyroid hormone; MMF, mycophenolate mofetil; Tac, tacrolimus; Pred, prednisolone.

3.2. Association of PPI Use with Plasma Magnesium and 24-h urinary Magnesium Excretion

PPI use was significantly associated with lower plasma magnesium (β = −0.03; 95% CI −0.04; −0.01 mmol/L, $P = 0.001$) and lower urinary magnesium excretion (β = −0.86; 95% CI −1.10; −0.06 mmol/24 h, $P < 0.001$) as compared to non-users, Table 2. After adjustment for potential confounders, PPI use remained significantly associated with lower plasma magnesium levels (β = −0.02, 95% CI −0.04; −0.003, $P = 0.02$) and 24-h urinary magnesium excretion (β = −0.82, 95% CI −1.07; −0.57, $P < 0.001$).

Table 2. Association of proton-pump inhibitor (PPI) use with plasma magnesium and 24-h urinary magnesium excretion in 689 kidney transplant recipients.

	Plasma Magnesium, mmol/L			Urinary Magnesium Excretion, mmol/24 h		
	β	95% CI	*P*	β	95% CI	*P*
Crude	−0.03	−0.04; −0.01	0.001	−0.86	−1.10; −0.06	<0.001
Multivariable model	−0.02	−0.04; −0.003	0.02	−0.82	−1.07; −0.57	<0.001

Multivariable analyses were adjusted for age, sex, BMI, eGFR, proteinuria, time since transplantation, alcohol use, diabetes, history of CV disease, loop diuretics, thiazide diuretics, tacrolimus use, cyclosporine use, MMF use and dietary magnesium intake. Abbreviations: CI, confidence interval.

3.3. Association of PPI Use with Hypomagnesaemia

In crude logistic regression analysis, PPI use was associated with a more than two times higher risk of hypomagnesaemia compared with no use (OR: 2.12; 95% CI 1.43–3.15, $P < 0.001$), as shown in Table 3. The association remained independent of adjustment for potential confounders including age, sex, eGFR, proteinuria, time since transplantation, alcohol use, diabetes, history of cardiovascular disease, medication use (loop diuretics, thiazide diuretics, tacrolimus, cyclosporine and MMF) and dietary magnesium intake (OR: 2.00; 95% CI 1.21–3.31, $P = 0.007$). No significant interaction was observed between PPI use and the use of loop diuretics, thiazide diuretics, tacrolimus, or diabetes for the association with hypomagnesaemia ($P_{interaction} = 0.2$, $P_{interaction} = 0.7$, $P_{interaction} = 0.7$, $P_{interaction} = 0.9$, respectively).

Table 3. Logistic regression analyses investigating the association of PPI use with hypomagnesaemia in 689 kidney transplant recipients.

	Hypomagnesaemia		
***N* = 689**	**Odds Ratio**	**95% CI**	***P***
Crude	2.12	1.43–3.15	<0.001
Multivariable model	2.00	1.21–3.31	0.007

Multivariable analyses were adjusted for age, sex, BMI, eGFR, proteinuria, time since transplantation, alcohol use, diabetes, history of CV disease, loop diuretics, thiazide diuretics, tacrolimus use, cyclosporine use, MMF use and dietary magnesium intake. Abbreviations: CI, confidence interval.

3.4. Dose–Response Analyses

Based on daily dose equivalents of omeprazole, 251 KTR received a low PPI dose (≤20 mg omeprazole Eq/day) and 138 KTR received a high PPI dose (>20 mg omeprazole Eq/day). As shown in Table 4 and Figure 1, risk of hypomagnesaemia tended to be highest among KTR taking a high PPI dose (OR: 2.53; 95% CI 1.55–4.11, $P < 0.001$). The association remained materially unchanged after multivariable adjustment (OR: 2.46; 95% CI 1.32–4.57, $P < 0.005$), Table 4. Moreover, a significant trend between PPI dose and risk of hypomagnesaemia was observed ($P_{trend} = 0.004$).

Table 4. Subgroup analyses of the association of PPI use with hypomagnesaemia in 689 kidney transplant recipients.

	Categories of PPI Use						
	No PPI		**Low PPI Dose**		**High PPI Dose**		
Number of subjects	300		251		138		
	Odds ratio (95% CI)	*P*	Odds ratio (95% CI)	*P*	Odds ratio (95% CI)	*P*	*P*-Trend
Hypomagnesaemia							
Crude	1.00 (reference)	n/a	1.92 (1.25–2.96)	0.003	2.53 (1.55–4.11)	<0.001	<0.001
Multivariable model	1.00 (reference)	n/a	1.79 (1.04–3.08)	0.04	2.46 (1.32–4.57)	0.005	0.004

Multivariable analyses were adjusted for age, sex, BMI, eGFR, proteinuria, time since transplantation, alcohol use, diabetes, history of CV disease, loop diuretics, thiazide diuretics, tacrolimus use, cyclosporine use, MMF use, dietary magnesium intake. Low PPI dose (≤20 mg omeprazole Eq/day), High PPI dose (>20 mg omeprazole Eq/day). Abbreviations: CI, confidence interval.

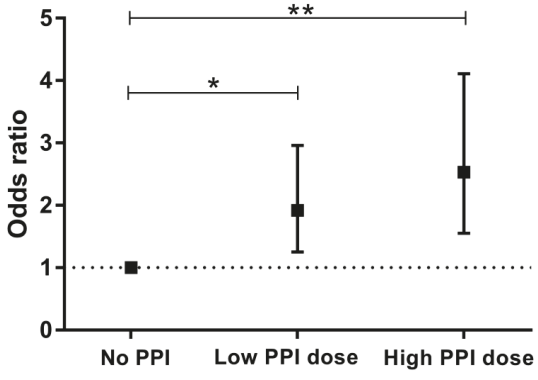

Figure 1. Crude association between PPI use and risk of hypomagnesaemia stratified by subgroups of PPI use. No PPI, Low PPI dose (≤20 mg omeprazole Eq/day), High PPI dose (>20 mg omeprazole Eq/day). Presented are odds ratio's with 95% confidence intervals. * $P = 0.004$; ** $P < 0.001$; $P_{trend} < 0.001$.

3.5. Sensitivity Analyses for Risk of Hypomagnesaemia

To account for the use of other important gastric acid reducing medication, we performed sensitivity analyses in which H2RA users (N = 18) were excluded form statistical analyses (Table S2). The association between PPI use and hypomagnesaemia remained materially unchanged when H2RA users were excluded (OR: 2.17, 95% CI 1.29–3.67, P = 0.004). We also performed analyses to investigate which KTR are at increased risk of developing hypomagnesaemia. These analyses are presented in Table S3. We found that patients with a history of cardiovascular disease, patients at shorter time after transplantation, not consuming alcohol, PPI users, thiazide diuretic users and patients using tacrolimus based immunosuppressive regimens were at increased risk of developing hypomagnesaemia. Moreover, KTR with hypomagnesaemia had higher fasting glucose levels, HbA1c and lower serum calcium levels compared with KTR without hypomagnesaemia.

4. Discussion

The present study is to our knowledge the largest cohort study to date exploring the association between PPI use and hypomagnesaemia in a cohort of KTR. Our results demonstrate a higher risk of hypomagnesaemia among KTR using PPIs, with subsequently lower plasma magnesium levels in combination with lower renal magnesium excretion. The association between PPI use and risk of hypomagnesaemia remained significant after adjustment for important potential confounders and tended to be highest among KTR taking high PPI dosages.

Our results confirm previous case-series and cohort studies investigating the association between PPI use and increased risk of hypomagnesaemia [1,2,7,9]. In a large cohort study (N = 9818) among subjects from the general population, it was shown that PPI users had significantly lower serum magnesium levels and had a two times higher risk of hypomagnesaemia compared with non-users [9]. Our results are in line with observations from this large cohort study and show a similar increased risk of hypomagnesaemia (OR 2.12).

So far, only one other study by van Ende et al. investigating the association between PPI use and magnesium status in KTR has been published [22]. Contrary to our findings, van Ende et al. found no association between PPI use and serum magnesium levels. Reasons for the lower proportion of PPI users in the study by van Ende et al. are unclear, though underreporting may have played a role, given that it was not specified how data regarding PPI use was obtained. It was also unclear whether the data of van Ende et al. were derived from routine outpatient assessment of plasma magnesium concentrations, which may have provided an incentive for stopping PPI use in KTR with low magnesium concentrations. This could have biased their results and could possibly also explain the large difference in PPI use between our study and their study, because in our center no plasma magnesium data were available at the time of the study. It was furthermore unclear whether it concerned on-demand or chronic PPI use. Furthermore, data regarding PPI dose, type and magnesium supplementation were not reported, which may have influenced the outcome.

In a recently published meta-analysis, a similar risk of hypomagnesaemia among KTR was demonstrated (pooled OR = 1.56, 95% CI 1.19–2.05) [30]. This meta-analysis by Boonpheng et al. was based on one published paper and seven abstracts presented at medical conferences. Our study adds that it investigated a dose–response relationship, and provides data on dietary magnesium intake and 24-h urinary magnesium excretion.

In the present study, both plasma magnesium and 24-h urinary magnesium excretion were lower in PPI users, suggesting that PPI induced hypomagnesaemia is caused by impaired gastrointestinal absorption rather than renal magnesium wasting. In general, hypomagnesaemia can be the consequence of either a decreased intestinal uptake, a decrease in dietary magnesium intake or an increase in renal magnesium excretion. It is postulated that PPIs inhibit the active magnesium absorption via the TRPM 6 and 7 channels in the intestine [11,12]. In KTR other contributing factors than PPI use may add to the risk of hypomagnesaemia. For example, decreased intestinal magnesium absorption can also be the consequence of chronic post-transplant diarrhea, which is highly prevalent and

often complicated by hypomagnesaemia [10,31]. Data regarding symptoms of severe diarrhea were unfortunately unavailable in this study, therefore we could not correct for this potential confounder. Likewise, hypomagnesaemia can be the result of insufficient intake of foods rich in magnesium. In our study, mean dietary magnesium intake was 329.9 ± 88.7 mg/day, which was slightly lower than the mean habitual intake of magnesium among the general Dutch population, as reported in the Dutch National Food Consumption Survey 2007–2010 [32]. A low dietary magnesium intake can also be a reflection of an overall poor diet. Nonetheless, when we adjusted for dietary magnesium intake in our logistic regression analyses, the relationship between PPI use and risk of hypomagnesaemia remained materially unchanged, indicating that the observed risk associated with PPI use was not confounded by dietary magnesium intake.

The main strength of this study is measurement of three important pillars of magnesium status: plasma magnesium, 24-h urinary magnesium excretion and dietary magnesium intake. Because of this, we were able to confirm that PPI use does not lead to increased renal magnesium wasting but very likely impairs intestinal magnesium absorption. Furthermore, we only included KTR who were using PPIs for at least 3 months before blood sampling. It is previously noted that hypomagnesaemia occurs mainly in patients on prolonged PPI therapy suggesting that it takes time before magnesium stores are meaningfully depleted [6,7,33]. Moreover, we excluded KTR using magnesium supplements and adjusted for potential confounders, including CNI use, which did not alter the association.

A limitation of our study is its cross-sectional design. Therefore, a causal relationship between PPI use and hypomagnesaemia remains to be determined and changes over time in magnesium status parameters were unknown. Furthermore, no information regarding compliance to PPI treatment was available, which may have led to underestimation of effect sizes. PPI users had a shorter time between transplantation and baseline measurements. However, adjustment for time since transplantation did not alter the association between PPI use and hypomagnesaemia. Lastly, the possibility of residual confounding or bias by indication remains, which may have led to overestimation of the role of PPIs since on average PPI users were less healthy than non-users. A strength of the current study is, that no routine outpatient monitoring of plasma magnesium was performed and that we measured plasma and urine magnesium in samples that had been stored in a biobank, which reduces the change of selection bias in our cohort.

Our findings may be of clinical importance. KTR with low magnesium levels seem to develop post-transplant diabetes mellitus (PTDM) more frequently [17]. In this study we also found that KTR with hypomagnesaemia had higher fasting glucose levels and HbA1c. Next to that, a higher degree of arterial stiffness, as assessed by a carotid-femoral pulse wave velocity (PWV) measurement, has been found in KTR with low magnesium levels [34]. This same PWV measurement was found to be an independent predictor of cardiovascular events in KTR [35]. Moreover, hypomagnesaemia has been associated with cardiovascular morbidity [19,20] and mortality [21] in the general population. However, whether this association is also present in KTR is currently unknown. Another clinical significance lies in the association with lower calcium levels, which potentially points to an increased risk of developing osteoporosis. Long-term PPI use has indeed been associated with decreased bone mineral density and increased risk of fractures [36]. Because many patients use PPIs without evidence based indication [37–39], we believe that reevaluation of treatment indication in KTR on chronic PPI therapy might be of benefit. In situations in which PPIs are clinically needed, it would be judicious to assess and follow-up magnesium levels periodically during treatment, as recommended by the US Food and Drug administration and stated in the summary of product characteristics of all PPIs.

5. Conclusions

This study demonstrates that PPI use is associated with lower magnesium status and hypomagnesaemia in KTR. Moreover, risk of hypomagnesaemia was higher among KTR taking a high PPI dosage. Healthcare professionals should be aware of this additional risk and should

consider regular monitoring of magnesium levels, especially in this patient population at high risk of hypomagnesaemia.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/8/12/2162/s1, Table S1: Types and daily dosages of proton-pump inhibitors used by 389 kidney transplant recipients. Table S2: Logistic regression analyses investigating the association of PPI use with hypomagnesaemia in 617 kidney transplant recipients (H2RA users excluded). Table S3: Baseline characteristics of 689 RTR with and without hypomagnesaemia.

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Urinary Biomarkers α-GST and π-GST for Evaluation and Monitoring in Living and Deceased Donor Kidney Grafts

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Abstract: The aim of this study was to analyze the value of urine α- and π-GST in monitoring and predicting kidney graft function following transplantation. In addition, urine samples from corresponding organ donors was analyzed and compared with graft function after organ donation from brain-dead and living donors. Urine samples from brain-dead ($n = 30$) and living related ($n = 50$) donors and their corresponding recipients were analyzed before and after kidney transplantation. Urine α- and π-GST values were measured. Kidney recipients were grouped into patients with acute graft rejection (AGR), calcineurin inhibitor toxicity (CNI), and delayed graft function (DGF), and compared to those with unimpaired graft function. Urinary π-GST revealed significant differences in deceased kidney donor recipients with episodes of AGR or DGF at day one after transplantation ($p = 0.0023$ and $p = 0.036$, respectively). High π-GST values at postoperative day 1 (cutoff: >21.4 ng/mg urine creatinine (uCrea) or >18.3 ng/mg uCrea for AGR or DGF, respectively) distinguished between rejection and no rejection (sensitivity, 100%; specificity, 66.6%) as well as between DGF and normal-functioning grafts (sensitivity, 100%; specificity, 62.6%). In living donor recipients, urine levels of α- and π-GST were about 10 times lower than in deceased donor recipients. In deceased donors with impaired graft function in corresponding recipients, urinary α- and π-GST were elevated. α-GST values >33.97 ng/mg uCrea were indicative of AGR with a sensitivity and specificity of 77.7% and 100%, respectively. In deceased donor kidney transplantation, evaluation of urinary α- and π-GST seems to predict different events that deteriorate graft function. To elucidate the potential advantages of such biomarkers, further analysis is warranted.

Keywords: kidney transplantation; urinary biomarkers; α-GST; π-GST; acute rejection; delayed graft function; nephrotoxicity

1. Introduction

Kidney transplantation is by far the best therapeutic option for patients with end-stage renal disease (ESRD). After transplantation, the main challenges, besides surgical complications, are acute graft rejection, delayed graft function, and adverse effects of immunosuppressants [1]. Acute graft

rejection still occurs in up to 25% of recipients and is a significant prognostic factor for long-term graft survival [2]. The improvements of immunosuppressive drugs have turned transplantation into a safe and widely predictable therapy; however, many of the agents used today still contribute to graft failure due to their nephrotoxic potential [3]. Delayed graft function, defined by the need for dialysis within the first week after transplantation and mainly caused by acute tubular necrosis, is mostly due to long ischemia times, advanced donor age, and comorbidities [4,5]. Recognizing the cause of graft dysfunction may be challenging, yet immediate diagnosis and therapy are essential for optimal graft survival.

The signs of graft dysfunction are decreased diuresis and impaired creatinine blood levels. Monitoring immunosuppressive drugs and their toxicity through serum levels is of limited value, since the difference between therapeutic and toxic levels is not fixed [6]. In order to define the pathomechanism of graft dysfunction, a graft biopsy is required in most cases. However, this is an invasive procedure and risks associated complications endangering the transplanted kidney [7].

α- and π-GST, which are specifically present in the kidney tubules, are two isotypes of the glutathione-S-transferases. Beyond their biochemical differences, they are also located in different parts of the tubule system [8]. α-GST is found in cells of the proximal tubules, which are predominantly affected by ischemia time and nephrotoxic substances. π-GST is located in distal tubules, which are damaged during acute graft rejection [9]. Their release into the urine as a result of cell damage gives an accurate prediction of the impaired part of the tubules system and therefore the underlying cause of graft dysfunction [10,11]. Analyses of α- and π-GST have been reported to be promising for discriminating between the different causes of graft dysfunction [11–13].

The aim of our study was to determine the value of measuring α- and π-GST concentrations in urine as biomarkers for monitoring graft function and predicting postoperative events in the first week after transplantation in living and deceased donor kidney transplantation.

2. Experimental Section

2.1. Samples and Data Collection

This study was approved by the Ethics Committee of Berlin's Charité University Hospital (EA2/137/10). We prospectively analyzed blood and urine samples as well as demographic data from 160 patients: 30 brain-dead donors and their 30 corresponding recipients; and 50 living kidney donors as well as their 50 corresponding living donor kidney recipients. All surgeries were carried out at the Department of General, Visceral and Transplantation surgery of Charité University Hospital, Berlin. Machine perfusion was not performed for any kidney allograft in this study. Except for brain-dead donors, all patients were followed during the first week after surgery. Blood and urine samples were collected at the following time points: day 0, day 1, day 3, day 5, and day 7. Samples from brain-dead organ donors were obtained on day 0, the day of the organ donation surgery. Urine samples were collected from recipients after transplantation through an externalized uretero-vesico-cutaneous stent and therefore exclusively reflected the α- and π-GST content of the transplanted grafts.

2.2. Immunosuppression Events and Subgroups

All recipients received a triple immunosuppressant consisting of prednisolone, mycophenolate mofetil (MMF), and a calcineurin inhibitor. All deceased donor kidney recipients received tacrolimus, whereas living donor kidney recipients were treated with either tacrolimus or cyclosporine. Recipients were divided into subgroups according to the events in the first postoperative week: acute graft rejection (AGR, G1), calcineurin-induced nephrotoxicity (CNI, G2), both acute kidney rejection and calcineurin-induced nephrotoxicity (AGR + CNI, G3), delayed graft function (DGF, G4), and event-free (healthy, G5) subgroup (Figure 1). An acute graft rejection was confirmed by graft biopsy and classified according to the BANFF criteria. Calcineurin-induced nephrotoxicity was defined by serum levels of agents (tacrolimus >15 ng/mL, cyclosporin >250 ng/mL). It is worth noting that delayed graft function,

characterized by the need for dialysis in the first week after transplantation, was not recorded in any of the living donor recipients. In case dialysis was required due to a known graft-damaging event such as AGR or CNI, those patients were enrolled in subgroups of primary cause and not defined as DGF. Recipients with no signs for any of the above events were considered healthy and were used as our control group.

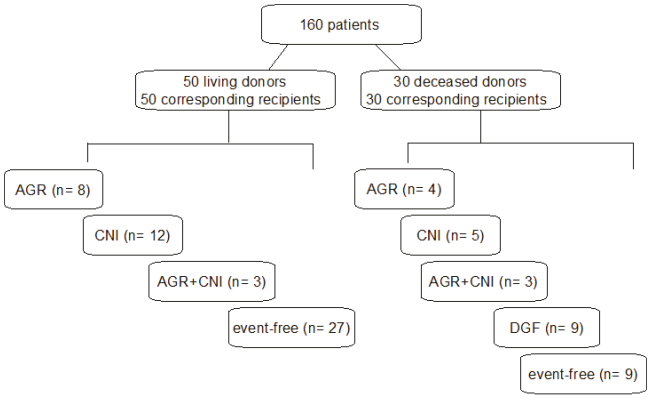

Figure 1. Patients, groups, and subgroups.

2.3. GST Analysis and Statistics

Urinary α- and π-GST values were measured using a commercially available ELISA test kit provided by Argutus Medical Ltd. (Dublin, Ireland). To consider the physiological differences in urine concentrations, α- and π-GST values were standardized to urine creatinine. The resulting unit for GST was ng/mg uCrea. Reference ranges of urinary α- and π-GST were determined as recommended from the measured GST values in the healthy population of this study group. For this purpose, the α- and π-GST values in the urine of healthy living donors were considered before donor nephrectomy. The reference interval for α-GST is 2.7–7.6 ng/mg uCrea and for π-GST 4.1–13 ng/mg uCrea in this study.

For statistical analysis, GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) was used. Quantitative data are given as the mean and standard deviation. To compare normally distributed variables, *t*-tests such as Mann-Whitney and Wilcoxon were performed. For the comparison of multiple variables, we used two-way ANOVA; here we applied Tukey and Holm-Sidak tests for post hoc analysis of the subgroups. The area under the curve was calculated in ROC analysis and a log-rank (Mantel-Cox) test was performed in survival analysis. A p-value less than 0.05 was defined as significant.

3. Results

3.1. Demographic Data

There were no differences regarding gender and BMI between the patient groups. The mean eGFR before graft recovery or transplantation (d0) did not differ between the groups. Recipients of deceased donor kidneys were significantly older than those of living donors (58 ± 13 and 48 ± 15 respectively, $p = 0.006$), whereas the donor's age was not different. Cold as well as warm ischemic time were significantly higher in the deceased donor group (both $p < 0.0001$). The demographic data of donors and recipients are given in Table 1. The increase in the eGFR of recipients after transplantation was, as expected, more noticeable in living donation scenarios (Figure 2).

Table 1. Demographic data of donors and recipients.

	Deceased Donor Grafts	Living Donor Grafts	*p* Value
Donor	30	50	
Age (years)	58 ± 15	53 ± 10	n.s.
Sex (male/female)	11/19	22/28	n.s.
BMI	27.3 ± 6.5	25.3 ± 3.2	n.s.
eGFR, d0 (mL/min)	96 ± 39	97 ± 18	n.s.
Diuresis in last hour (mL)	152 ± 81	-	
Recipient	30	50	
Age (years)	58 ± 12	47 ± 15	<0.05
Sex (male/female)	22/8	35/15	n.s.
BMI	26.4 ± 4	25.8 ± 4.9	n.s.
eGFR, d0 (mL/min)	9 ± 3	8 ± 4	n.s.
Primary disease			
Glomerulonephritis	9	24	n.s.
Hypertensive nephrosclerosis	7	9	n.s.
Polycystic kidney disease	6	2	n.s.
Autoimmune disease	2	5	n.s.
Diabetes	2	3	n.s.
Urologic disease	2	2	n.s.
Calcineurin-induced nephrotoxicity	0	2	n.s.
Others	2	3	n.s.
Dialysis			
Hemodialysis	29	28	n.s.
Peritoneal dialysis	1	3	n.s.
No dialysis	0	19	<0.05
Cold ischemia time (minutes)	613 ± 269	193 ± 62	<0.05
Warm ischemia time (minutes)	34 ± 11	23 ± 7	<0.05

BMI, body mass index; eGFR, estimated glomerular filtration rate; n.s., not significant.

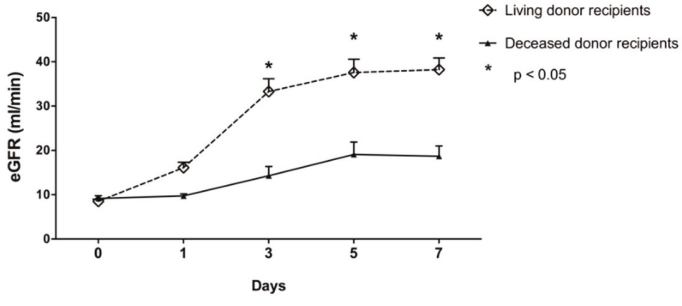

Figure 2. eGFR comparison between deceased and living donor recipients before and during the first week after transplantation.

3.2. α- and π-GST in Recipients

Neither α- nor π-GST correlated with age, BMI, and cold or warm ischemic time in any group. However, both GST isoenzymes correlated with renal function in living donors and in the healthy recipients subgroup. In deceased donor recipients and living donor recipients we observed acute rejection in four (13.4%) and eight patients (16%), calcineurin-induced nephrotoxicity in five (16.7%) and 12 (24%) patients, and both simultaneously in three (10%) and three (6%) patients, respectively. Delayed graft function occurred only with deceased donors (nine patients, 30%). Patients with an uneventful postoperative course in the deceased donor recipient group numbered nine (30%) and, in the living donor recipient group, 27 (54%). Both α- and π-GST were significantly elevated at 1st postoperative day (POD) in deceased donor recipients, with acute rejection when compared with the corresponding healthy subgroup (α-GST: Mean 473.5 ± 818 vs. 15.6 ± 21.2 ng/mg uCrea,

$p = 0.0094$; π-GST: mean 477.8 ± 804 vs. 8 ± 6.4 ng/mg uCrea, $p = 0.0023$). In living donor recipients, only π-GST showed an increase, reaching a peak at day 5 (mean 21.5 ± 28.2 ng/mg uCrea); however, there was no significant difference between this group and the healthy subgroup. In patients with CNI toxicity, α-GST performed better in both recipient groups; in deceased donor recipients, the mean was 316 ± 704.5 ng/mg uCrea at 1 POD and was easily discriminated from the uneventful subgroup ($p = 0.06$). Also, in the living donor group, a rise of α-GST to 68.9 ± 219 ng/mg uCrea was noted at day 5, when CNI toxicity occurred. When both acute rejection and CNI toxicity were recorded, neither α- nor π-GST was able to distinguish those patients; however, it is worth mentioning that the number of subjects in this subgroup was very low in our study. In the case of delayed graft function, present only in deceased donor recipients in our study, α- as well as π-GST were elevated in the urine, with means at 1st POD of 81.5 ± 201.3 and 151.6 ± 270.6 ng/mg uCrea, respectively. Only π-GST levels proved significant when compared to the control subgroup ($p = 0.036$) (Figure 3).

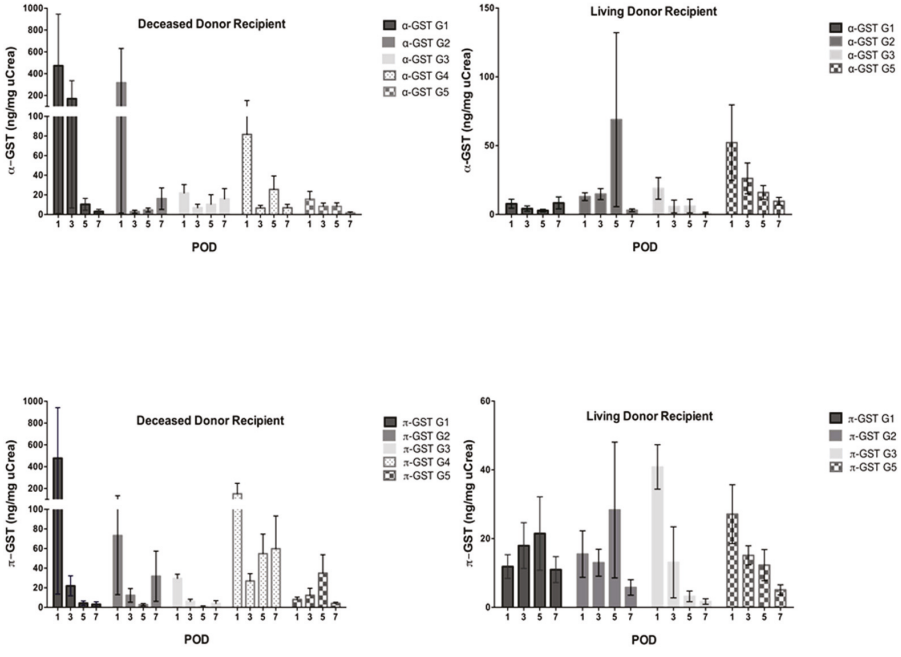

Figure 3. Urinary α- and π-GST levels in subgroups of deceased and living donor recipients during the first week after transplantation; G1: Acute graft rejection (AGR), G2: Calcineurin-induced nephrotoxicity (CNI), G3: Simultaneous acute graft rejection and calcineurin-induced nephrotoxicity (AGR + CNI), G4: Delayed graft function (DGF), G5: Event-free (control).

Furthermore, we performed ROC curve and survival analysis on the most outstanding biomarkers at a specific point in the study. π-GST showed the most promising results in deceased donor recipients with acute graft rejection and delayed graft function at day 1 after transplantation. Patients who developed a biopsy confirmed acute graft rejection within the first week after transplantation had significantly higher levels of urinary π-GST at POD 1. With an estimated cutoff of 21.4 ng/mg uCrea, π-GST was able to distinguish the occurrence of AGR from a rejection-free course with 100% and 66.6% sensitivity and specificity, respectively (Figure 4). Similar reliability for π-GST was observed in the DGF subgroup; however, the estimated cutoff at POD 1 was slightly lower at 18.3 ng/mg uCrea (sensitivity, 100%; specificity, 62.6%). Higher urinary π-GST levels could be seen in recipients with

delayed graft function; this was observed at POD1, POD 3 as well as POD 7. π-GST was not able to differentiate between the causes of graft dysfunction in the early postoperative period (Figure 5).

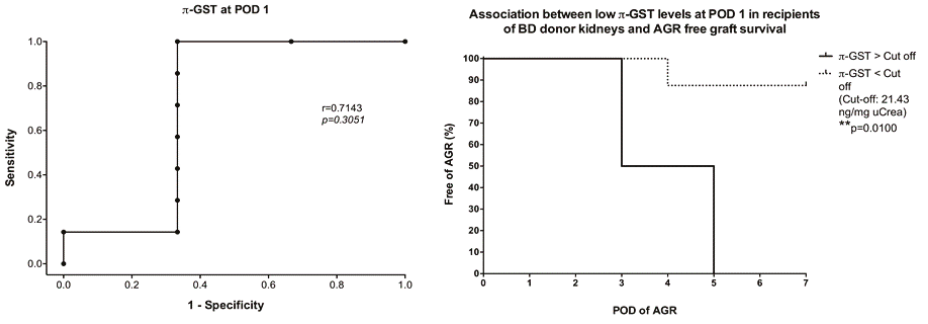

Figure 4. Correlation between urinary π-GST levels in recipients of brain-dead donors' grafts on POD 1, and probability of graft survival without acute graft rejection (AGR) during the first week after transplantation.

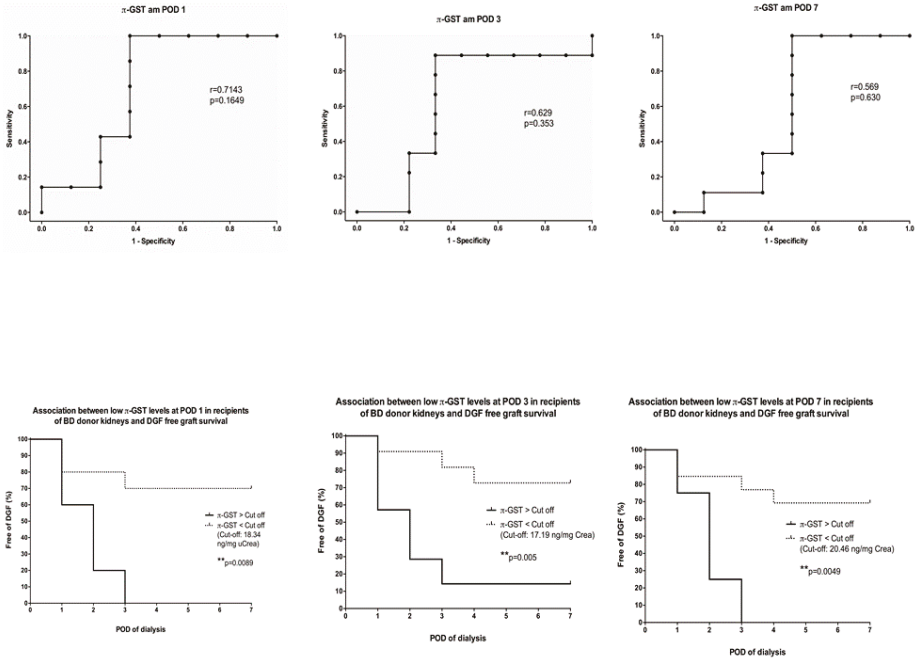

Figure 5. Correlation between urinary π-GST levels in recipients of brain-dead donors' grafts on POD 1, 3, and 7; and probability of graft survival without delayed graft function (DGF) during the first week after transplantation.

3.3. α- and π-GST in Donors

α- and π-GST showed interesting results when measured in deceased donor urine before organ harvesting as they were elevated in those with poorer graft function after transplantation, and seemed to predict a foreseeable event such as acute rejection or delayed graft function, which was 3- to 8-fold more likely to occur than in those recipients with an uneventful course of treatment. α-GST stood out in the subgroup with both acute rejection and CNI toxicity ($p = 0.02$), while α- and π-GST were

remarkably higher (but not significantly so) in the DGF subgroup compared to the control group (Figure 6). As for the predictive value of GST when measured in donor urine, α-GST stood out with significant results in the AGR subgroup when ROC curve analysis was performed, and a cutoff value of >33.97 ng/mg uCrea was calculated (AUC, 0.86; sensitivity, 77.7%; specificity, 100%). Based on the donor's urinary α-GST alone, all four renal grafts from deceased donors that showed acute rejection in recipients were distinguished from those who had an AGR-free course in the survival curve analysis ($p = 0.0109$) (Figure 7).

On the other hand, urinary GST in living donors showed no differences between subgroups and the corresponding control group, and therefore failed to predict future events in recipients.

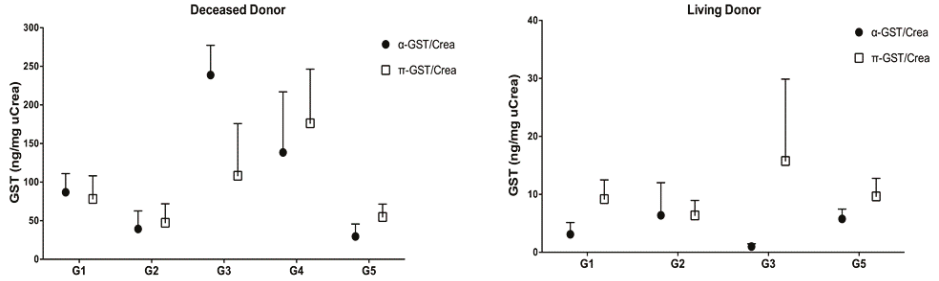

Figure 6. Urinary α- and π-GST levels in deceased donors before transplantation; G1: Acute graft rejection (AGR), G2: Calcineurin-induced nephrotoxicity (CNI), G3: Simultaneous acute graft rejection and calcineurin-induced nephrotoxicity (AGR+CNI), G4: Delayed graft function (DGF), G5: Event-free (control).

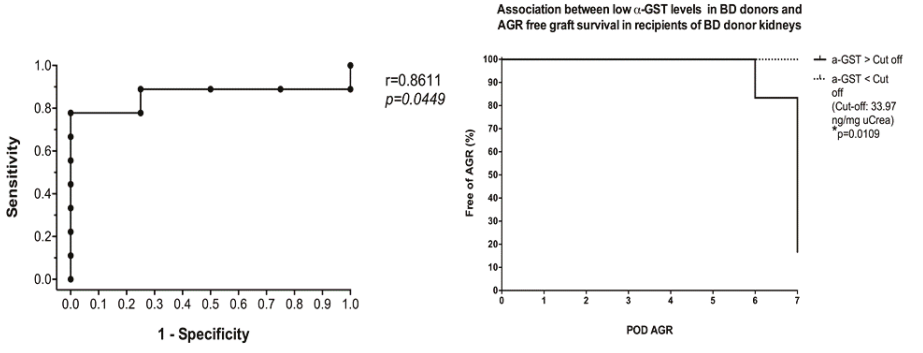

Figure 7. Correlation between urinary α-GST levels in brain-dead donors before transplantation and probability of graft survival without acute graft rejection in corresponding recipients during the first week after transplantation.

3.4. Six- and 12-Months Graft Survival

We followed up recipients of the subgroups G1 (AGR) and G4 (DGF) at six and 12 months after transplantation, as correlations of α- and π-GST in those cohorts showed the most promising results. However, in subgroup G1 only one patient out of four lost the graft due to recruiting nephritis; in subgroup G4 all three grafts were lost due to death not associated with graft function (cancer or cardiac arrest).

4. Discussion

The results of our prospective study, evaluating urinary α- and π-GST in deceased as well as living kidney donors and their corresponding recipients as biomarkers for graft quality and function,

suggest the potential value of these enzymes. Previous studies showed the ability of urinary α- and π-GST to predict acute renal damage in kidney graft recipients and demonstrated a release of these enzymes in malfunctioning grafts [10–15]. However, none of these groups has compared the course of α- and π-GST in donors as well as in corresponding recipients. In addition, we investigated the differences in the markers in two settings, brain-dead/deceased and living organ donation. Our analyses reveal that the determination of urinary π-GST concentration in deceased donor recipients, especially on the first day after graft transplantation, could be valuable and indicative of kidney allograft function and survival without AGR or DGF. Secondly, we found higher concentrations of α- and π-GST in the urine of deceased kidney donors, whose grafts performed poorly in the corresponding recipients; α-GST was able to predict AGR before transplantation. A determination between the causes of impaired allograft function could not be reached by assessing urinary α- and π-GST alone, though it is unlikely that in the complex setting of transplantation a single biomarker will reliably distinguish between the pathogenesis of multifactorial elements; therefore, the proposed markers should be seen as an useful tool in addition to established methods.

Research studies investigating α- and π-GST in living donation are extremely limited: in our review of the literature we only found one publication on this issue, and this concerned liver rather than kidney transplantation [16]. Our findings showed lower concentrations of α- and π-GST in the urine of living donor kidney recipients than in that of deceased donor kidney recipients. This was observed in almost all subgroups, and especially on POD 1. Except for a notable, yet insignificant, rise of π-GST in living donor recipients with AGR when compared to the control group, our results find no further significances of urinary α- and π-GST in living donor recipients when harmful events occurred. Considering the superior organ quality and logistics in living donation transplantation, as demonstrated by over half of living donor grafts surviving the first week after transplantation event-free compared to 30% of deceased donor grafts, lower urinary concentrations of α- nor π-GST in living donor transplantation are to be expected. Daemen et al. found a correlation between α-GST and warm ischemic time in grafts from donation after cardiac death [17]. In our study neither α- nor π-GST had a proven correlation with ischemic time, although it should be noted that a different type of graft was investigated in the work mentioned.

The toxic effect of CNI agents on renal grafts and its association with the excretion of α-GST has been described in the past [10,18]. Our results failed to indicate such a correlation in deceased or living donor recipients. This might be due to our definition of the toxic range of CNI serum levels. Therapeutic and toxic serum levels of several drugs and especially immunosuppressants have been known to be inconsistent and even overlapping [6,19]. In the 1990s, serum levels of tacrolimus in the early period after transplantation were suggested to be below 20 ng/mL in order to avoid side effects, whereas later on levels above 15 ng/mg were proven to be associated with a higher risk of toxicity [20,21]. On the other hand, the risk of acute rejection is significantly higher when there are low concentrations of the agent [22]. A helpful step would be to find the toxic serum level of immunosuppressants that is agreed upon by transplant communities; currently, despite all efforts, this varies significantly between transplant centers. Another concerning factor is the design of the study, which did not include daily and therefore more precise surveillance on that matter. In order to investigate the correlation between toxic exposure to immunosuppressants and the excretion of GST into the urine, a closer observation with more frequent sample collection is required.

DGF was observed only in deceased donor recipients in our study. It occurred with an incidence of 30%, which is similar to the findings of a recent work by Willicombe et al. [23]. Risk factors and characteristics of donors and recipients associated with DGF such as cold ischemic time and donor age have been established in previous publications [4,5,24]. However, taking these factors into consideration, it is to be expected that DGF is less common in living donation. It has been demonstrated that α-GST excretion would be increased in the case of DGF due to its location in the renal tubular system and the association between DGF and proximal tubular necrosis [8,25]. On the other hand, π-GST has been shown to be of predictive value in terms of the need for dialysis in a publication by

Seabra et al. including 245 patients with acute kidney injury [26]. Hall et al. investigated α- and π-GST in a perfusate solution during machine perfusion of kidney allografts from deceased donors, and suggested an independent association between π-GST and DGF [27]. Our findings demonstrated the consistent significance of urinary π-GST in differentiating between DGF and normally functioning grafts when measured in deceased donor recipients. This was observed at several time points of the study and had a strong power of sensitivity and specificity. Little is known about the behavior of the proteins under dialysis, so it is unclear whether α-GST is more dialyzable than π-GST or the other way round. The sample collection from patients undergoing dialysis in our study did not occur with respect to dialysis time, which should be seen as another possible disturbance factor.

Further limitations of this study are the small number of patients in certain subgroups and the overall high standard deviations. We distinguished well between the causes of impaired graft function and took into consideration simultaneous events. The time frame of our observation was limited in that it focused only on the first week after transplantation. We believe that multiple serial samples and an extended study design would be beneficial in future projects.

5. Conclusions

In summary, the elevation of urinary π-GST in deceased donor kidney recipients at day 1 after kidney transplantation could be a helpful monitoring parameter, in addition to urinary output and serum creatinine, to determine graft function in recipients. It might be indicative of acute rejection or a need for dialysis. The measuring of urinary α- and π-GST should also be considered in deceased donors as this seems to be of predictive value in terms of graft outcome and might help with assessing allograft quality. Our findings reveal an association between urinary α-GST in deceased donors and AGR in corresponding recipients. Thus, further investigation of α- and π-GST in a larger population and daily sample collection should be considered. Although urinary α- and π-GST in living kidney donation showed no relevant correlation with harmful events in our analyses, this is, to the best of our knowledge, the first study demonstrating differences in biomarkers between deceased and living kidney donation, so subsequent investigations will be needed in order to confirm or contradict our findings.

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Urinary Oxalate Excretion and Long-Term Outcomes in Kidney Transplant Recipients

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Abstract: Epidemiologic studies have linked urinary oxalate excretion to risk of chronic kidney disease (CKD) progression and end-stage renal disease. We aimed to investigate whether urinary oxalate, in stable kidney transplant recipients (KTR), is prospectively associated with risk of graft failure. In secondary analyses we evaluated the association with post-transplantation diabetes mellitus, all-cause mortality and specific causes of death. Oxalate excretion was measured in 24-h urine collection samples in a cohort of 683 KTR with a functioning allograft ≥1 year. Mean eGFR was 52 ± 20 mL/min/1.73 m^2. Median (interquartile range) urinary oxalate excretion was 505 (347–732) µmol/24-h in women and 519 (396–736) µmol/24-h in men ($p = 0.08$), with 302 patients (44% of the study population) above normal limits (hyperoxaluria). A consistent and independent inverse association was found with all-cause mortality (HR 0.77, 95% CI 0.63–0.94, $p = 0.01$). Cause-specific survival analyses showed that this association was mainly driven by an inverse association with mortality due to infection (HR 0.56, 95% CI 0.38–0.83, $p = 0.004$), which remained materially unchanged after performing sensitivity analyses. Twenty-four-hour urinary oxalate excretion did not associate with risk of graft failure, post-transplant diabetes mellitus, cardiovascular mortality, mortality due to malignancies or mortality due to miscellaneous causes. In conclusion, in KTR, 24-h urinary oxalate excretion is elevated in 44% of KTR and inversely associated with mortality due to infectious causes.

Keywords: oxalate; hyperoxaluria; kidney transplant recipients; graft failure; post-transplantation diabetes mellitus; all-cause mortality; cardiovascular mortality; infectious mortality

1. Introduction

Kidney transplantation is considered the gold standard treatment for end-stage renal disease (ESRD) [1,2]. Short-term survival of kidney transplant recipients (KTR) has improved markedly in the past decades [3,4]. Although a better understanding of modifiable risk factors has been achieved over the recent years [5,6], patients perceive the ever existing threat of premature graft failure (GF) as most compelling, and would like to know whether factors such as lifestyle and diet can contribute to prevention of it [7,8]. Another factor of interest influencing long-term KTR survival is

post-transplantation diabetes mellitus (PTDM), which has become increasingly common and may affect patient and graft survival [9]. Further, an increased risk of premature mortality, in particular, increased risk for premature death from cardiovascular and infectious causes remain significant problems in the post-transplantation setting. In KTR, conventional risk factors for cardiovascular mortality are abundantly present, such as hypertension, diabetes mellitus and dyslipidemia. On top of that, KTR had pre-existent renal diseases, which additionally increases the cardiovascular risk [10]. Mortality due to infection is significantly higher in KTR than in the general population due to multiple reasons, to which immunosuppressive therapy is a large contributing factor [11]. Furthermore, KTR are at a two to threefold higher risk of cancer-related mortality compared to the general population [12].

Although different mechanisms underlying these long-term complications of kidney transplantation have been found, substantial unknown mechanisms particular to the post-kidney transplantation setting remain to be identified in order to provide rationale for the markedly high risk of premature mortality in KTR [13]. A recent prospective cohort study in 3123 patients with chronic kidney disease (CKD) stages 2 to 4, found urinary oxalate as a potential risk factor for progression of CKD [14]. In the post-kidney transplantation setting, the study of oxalate remains overlooked. Whether urinary oxalate (reference value ≤455 µmol/24-h) [15] may be prospectively associated with adverse outcomes in KTR remains unknown.

The current study aims to assess the potential association of urinary oxalate excretion with adverse long-term outcomes in a large cohort of extensively phenotyped KTR with a functioning graft ≥1 year. For this purpose, the prospective associations of 24-h urinary oxalate excretion with GF, PTDM, and overall and cause-specific mortality were systematically investigated.

2. Experimental Section

2.1. Study Design and Population

This is a single-center prospective cohort study, initiated in 2008 on with follow-up of endpoints until 2015. KTR with a functioning allograft for at least one year or more who visited the outpatient clinic of the University Medical Center Groningen (Groningen, The Netherlands) between November 2008 and March 2011. Exclusion criteria were no known or apparent systemic illnesses, insufficient knowledge of the Dutch language and history of drug or alcohol addiction according to their patient files. KTR received anti-hypertensive and standard maintenance immunosuppressive therapy. Of the 817 invited KTR, 706 (87%) signed informed consent. Patients missing 24-h urinary oxalate excretion were excluded from the analyses, resulting in 683 KTR eligible for statistical analyses. The study was conducted in concordance with the guidelines formulated in the Declaration of Helsinki and Istanbul, and approved by the Institutional Review Board of the UMCG (METc 2008/186) [16]. The continuous surveillance system according to the American Society of Transplantation was followed for the correct collection of data [17]. When status of patients was unknown, the referring nephrologist or general practitioners were contacted in order to obtain the missing information. There was no loss due to follow-up.

2.2. Study Endpoints

The primary endpoint of this study is death-censored GF. Secondary endpoints are PTDM, all-cause mortality and cause-specific mortality. GF occurrence in this study is defined as ESRD requiring re-transplantation or return to dialysis. A subject was considered to have developed PTDM when the fasting plasma glucose exceeded 7 mmol/L, the HbA1c exceeded 6.5% or use of antidiabetics after transplantation as registered in the patient database [18,19]. Among specific causes of death, we studied cardiovascular mortality, death from infection, death from malignancies, and other causes of death (miscellaneous). Cardiovascular mortality is defined as mortality caused by cardiovascular pathophysiology, coded by ICD-10 codes I10-I52. This information was obtained from linking the patient number to the database of the Central Bureau of Statistics and then, by physicians, reported

mortality cause. Infectious mortality and mortality due to malignancies were defined as mortality caused by infectious causes or malignant causes. Miscellaneous causes of mortality have been defined as other causes of death besides the previously described outcomes.

2.3. Baseline Measurements and Definitions

At the outpatient clinic, baseline data was gathered according to a strict and detailed protocol described previously [20]. Anthropometrics were obtained without shoes and heavy garments. Systolic and diastolic blood pressures (SBP and DBP) were measured by means of an automatic device (Philips Suresign VS2+, Andover, MA, USA) according to a standard clinical protocol [16]. Mean arterial pressure (MAP) was automatically calculated by (SBP + DBP × 2)/3. History of cardiovascular disease was searched for in the patient files under ICD-10 code Z86.7.

Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2) [21]. Estimated glomerular filtration rate (eGFR) was calculated using the CKD Epidemiology Collaboration (CKD-EPI) creatinine equation as shown in Formula (S1) [22].

2.4. Assesments of Physical Activity and Dietary Intake

Physical activity was quantified using the Short Questionnaire to Assess Health-enhancing physical activity (SQUASH). Activity was expressed in intensity multiplied by the amount of hours [23]. Dietary intake was assessed using a semi-quantitative Food Frequency Questionnaire (FFQ) [24,25]. To obtain the energy of a certain product, the Dutch Food Composition Table of 2016 was used [26]. Micro and macronutrients were adjusted for total energy intake (kCal), because of the potential of correlation and confounding [27].

2.5. Laboratory Measurements

For the collection of 24-h urine samples, the patients were asked to start the collection the day prior to their visit to the outpatient clinic. Collection was done in concordance with a strict protocol, i.e., discarding the first morning urine, collecting the subsequent in 24 h including the next morning's urine [16]. Subsequently, urine samples for oxalate analysis were acidified and stored at −80 °C. Urine oxalate analysis was performed using a validated ion-exchange chromatography assay with conductivity detection (Metrohm, Herisau, Switzerland). Inter-assay precision was monitored using three urine pool samples. Inter-assay precision was 8.2% at 0.17 mmol/L, 7.0% at 0.38 mmol/L and 9.0% at 0.52 mmol/L. Comparison of this method with a routine laboratory GC-MS method showed no systemic difference and no proportional difference. Reverse-phase high performance liquid chromatography (HPLC) was used to measure urinary thiosulfate [28].

2.6. Statistical Analyses

Data was analyzed using IBM SPSS version 23.0 (SPSS Inc., Chicago, IL, USA); Stata version 14.0 (StataCorp., College Station, TX, USA), GraphPad Prism version 7.02 (GraphPad Software, La Jolla, CA, USA), and Rstudio version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria). A two-sided $p < 0.05$ was considered significant in all following analyses.

Normally distributed variables are expressed as mean ± standard deviation (SD), skewed data as medians (Interquartile range (IQR)), and categorical data as given number and percentage. Baseline characteristics were described for the overall population and by sex-stratified tertiles of 24-h urinary oxalate excretion. Data are presented in tertiles to allow for assessment of linearity of cross-sectional associations of 24-h urinary oxalate excretion with other variables. Sex-stratified tertiles were created by first separately distributing all female subjects according to tertiles and distributing all male subjects according to tertiles, and thereafter combining the tertiles of females and males. We generated sex-specific tertiles because of differences between women and men in oxalate excretion [29–32]. Analyses of difference in baseline characteristics across sex-stratified tertiles of 24-h urinary oxalate excretion were tested by ANOVA for normally distributed continuous variables, Kruskal-Wallis

for skewed continuous variables and $\chi 2$ test for categorical data. Sex-stratified tertiles of 24-h oxalate excretion were tested for associations with outcomes by Kaplan-Meier analysis, including the log-rank test.

Linear regression analyses were performed to investigate the association of baseline characteristics with 24-h urinary oxalate excretion. Normality was assessed by means of a *p–p* plot, and a natural log transformation was performed when appropriate. Homoscedasticity was controlled in a scatterplot.

Cox regression analyses were used to investigate the association of 24-h urinary oxalate with primary and secondary outcomes. Model 1 of the Cox proportional-hazards regression analysis was adjusted for demographics, i.e., sex and age. Model 2 was additionally adjusted for transplantation related variables, namely primary renal disease, BMI, donor age, time from transplantation to follow-up, eGFR and proteinuria. In the next models, baseline characteristics which were cross-sectionally associated with 24-h urinary oxalate excretion were subsequently included, and potential confounding of urinary thiosulfate was investigated due to its role in the anion transporters in the proximal renal tubuli (Model 3) [33]. In addition, we also looked for lactate dehydrogenase (LDH) because of its importance in the conversion of glyoxylate (Model 4) [34], for 24-h urinary pH because of its influence on the reaction of oxalate with calcium (Model 5) [35], for fibroblast growth factor 23 (FGF23) because of the relationship with gastrointestinal calcium absorption and oxalate bioavailability [36,37] (Model 6), and for fruits and vegetables as main dietary sources of oxalate [38–40] (Model 7). To allow for detection of a potential threshold effect, which was found in an earlier study on urinary oxalate excretion and CKD [14], Cox regression analyses were also performed according to sex-stratified tertiles with the first tertile as reference.

Spline regression were created to visualize the association of 24-h urinary oxalate excretion for outcomes, for which we consistently found significant associations. Nonlinearity was tested by using the likelihood ratio test, comparing models with linear or linear and cubic spline terms. Restricted cubic splines were knotted at the minimum, median and maximum. The splines were adjusted according to Model 6 of the primary prospective analyses.

Sensitivity Analyses

Several sensitivity analyses were performed to examine the robustness of the associations between 24-h urinary oxalate excretion and outcomes. For that purpose, we reanalyzed the data excluding subjects with potential inadequate 24-h urine collection (i.e., overcollection or undercollection), which was defined as the upper and lower 2.5% of the difference between the estimated and measured volume of a subject's 24-h urine sample. The following formula was used to calculate the estimated 24-h urine volume: $\frac{1}{4}$ ((urine creatinine) * (24-h urine volume)/(serum creatinine)), where creatinine clearance was estimated using the Cockcroft-Gault Formula [41,42]. These analyses were analogous to Model 6 of the primary prospective analyses.

Furthermore, we performed competing risk analyses of outcomes of interest with all-cause mortality as competing event according to Fine and Gray [43]. For that purpose, we performed multivariable Cox regression analyses analogously to Model 6 of the primary prospective analyses.

3. Results

3.1. Baseline Characteristics

In total 683 KTR were included in the analyses (mean age 53 ± 13, 43% female, 99.6% Caucasian ethnicity). Median urinary oxalate excretion was 505 (IQR, 347–732) µmol/24-h in women and 519 (IQR, 396–736) µmol/24-h in men ($p = 0.08$). Forty-four percent of the patients were above the range of clinical hyperoxaluria of ≤455 µmol/24-h. All 227 study subjects in tertile 3 were above the clinical cutoff point for hyperoxaluria, and all 227 subjects in tertile 1 were below the clinical cutoff point. Mean eGFR was 52 ± 20 mL/min/1.73 m^2. Additional baseline characteristics and analyses are shown overall and by sex-stratified tertiles of 24-h urinary oxalate excretion in Table 1.

Table 1. Baseline characteristics of the overall population, and by sex-stratified tertiles of 24-h urinary oxalate excretion. [a]

Baseline Characteristics	Overall KTR *n* = 683	Sex-Stratified Groups of 24-h Urinary Oxalate Excretion			*p*
		♀≤ 390; ♂≤ 431 µmol/24-h	♀391–633; ♂432–632 µmol/24-h	♀≥ 633; ♂≥ 632 µmol/24-h	
Oxalate					
Oxalate in 24-h urine, µmol [b]	514 (378–732)	339 (278–278)	514 (461–563)	882 (732–1137)	—
Demographics					
Age, years	53 ± 13	54 ± 13	53 ± 12	51 ± 13	0.04
Sex (female), *n* (%)	295 (43)	98 (43)	99 (43)	98 (43)	1.00
Ethnicity (Caucasian), *n* (%)	680 (99.6)	226 (99.6)	228 (99.6)	226 (99.6)	1.00
Body composition					
BSA, m^2	1.94 ± 0.22	1.92 ± 0.21	1.98 ± 0.21	1.94 ± 0.23	0.05
BMI, kg/m^2	26.6 ± 4.8	26.3 ± 4.8	27.0 ± 4.6	26.6 ± 4.8	0.34
Waist circumference, cm	98 (89–108)	97 (89–105)	100 (90–110)	96 (87–106)	0.02
Lifestyle					
Current smoker, *n* (%)	81 (12)	42 (19)	19 (9)	20 (9)	0.001
Alcohol consumption					0.30
None, *n* (%)	22 (3)	6 (3)	8 (4)	8 (4)	
0–10 g/day, *n* (%)	426 (62)	144 (64)	146 (64)	136 (60)	
10–30 g/day, *n* (%)	137 (20)	44 (19)	44 (19)	49 (22)	
>30 g/dag, *n* (%)	37 (5)	10 (4)	12 (5)	15 (7)	
SQUASH-score	5070 (2040–7800)	4440 (1680–7240)	5400 (2323–8475)	5580 (2280–7980)	0.10
Cardiovascular					
History of CV disease, *n* (%)	295 (50)	92 (41)	103 (45)	100 (44)	0.72
SBP, mmHg	136 ± 18	137 ± 17	136 ± 18	135 ± 18	0.42
MAP, mmHg (calculated)	100 ± 12	101 ± 11	100 ± 12	100 ± 13	0.73
LDL cholesterol, mmol/L	3.0 ± 0.9	3.1 ± 0.9	3.0 ± 0.9	2.9 ± 0.9	0.54
Triglycerides, mmol/L	1.7 (1.2–2.3)	1.7 (1.3–2.3)	1.6 (1.2–2.4)	1.7 (1.2–2.2)	0.69
Glucose homeostasis					
Diabetes mellitus, *n* (%)	162 (24)	52 (23)	58 (25)	53 (23)	0.78
Plasma glucose, mmol/L	5.3 (4.8–6.0)	5.3 (4.8–5.9)	5.2 (4.8–6.2)	5.3 (4.7–6.1)	0.85
Diet					
Av. energy intake, kCal/day	2092 (1720–2536)	2045 (1705–2479)	2104 (1735–2557)	2171 (1759–2589)	0.45
Av. daily fat intake, g/d [c]	84 (65–106)	80 (63–101)	85 (66–106)	86 (64–110)	0.31
Av. daily protein intake, g/d [c]	81 (67–95)	80 (65–95)	81 (66–95)	81 (68–95)	0.82
Glycine, mg/d [c]	3276 ± 806	3228 ± 805	3261 ± 817	3337 ± 794	0.38
Ascorbic acid, mg/d [c]	84 (60–118)	70 (53–101)	82 (60–114)	103 (73–138)	<0.001
Vegetables, g/d	93 ± 58	94 ± 53	90 ± 53	96 ± 66	0.54
Fruits, g/d	123 (65–232)	111 (50–226)	121 (64–228)	165 (81–247)	0.001

Table 1. *Cont.*

Baseline Characteristics	Overall KTR *n* = 683	Sex-Stratified Groups of 24-h Urinary Oxalate Excretion			*p*
		♀≤ 390; ♂≤ 431 µmol/24-h	♀391–633; ♂432–632 µmol/24-h	♀≥ 633; ♂≥ 632 µmol/24-h	
Transplantation characteristics					
Age donor, years	43 ± 15	43 ± 15	43 ± 16	43 ± 16	0.85
Sex donor (female), *n* (%)	322 (47)	108 (49)	99 (44)	15 (52)	0.24
Donor type (living), *n* (%)	231 (34)	67 (30)	81 (35)	83 (37)	0.24
Serum markers					
Venous pCO2, kPa	5.9 ± 0.8	5.9 ± 0.9	5.9 ± 0.8	5.8 ± 0.8	0.53
Leukocyte count, per 10^9/L	8.2 ± 2.7	8.3 ± 2.6	8.1 ± 2.8	8.1 ± 2.6	0.52
HsCRP, mg/L	1.6 (0.7–4.6)	1.6 (0.8–4.4)	2.0 (0.8–5.3)	1.4 (0.6–3.8)	0.04
Hemoglobin, mmol/L	8.2 ± 1.1	8.1 ± 1.1	8.2 ± 1.1	8.3 ± 1.1	0.11
FGF-23	61 (43–99)	63 (43–107)	61 (42–98)	61 (45–97)	0.66
LDH, U/L	198 (170–232)	195 (169–232)	203 (174–238)	196 (170–223)	0.35
Vitamin B6, nmol/L	29 (18–29)	27 (16–47)	26 (15–44)	36 (22–57)	<0.001
Renal allograft function					
Creatinine, µmol/L	125 (100–160)	126 (99–164)	126 (101–164)	122 (100–157)	0.66
eGFR, mL/min/1.73 m^2	52 ± 20	51 ± 20	52 ± 20	54 ± 20	0.26
Serum cystatin C, mg/L	1.7 (1.3–2.2)	1.7 (1.3–2.5)	1.7 (1.3–2.2)	1.6 (1.3–2.1)	0.25
Proteinuria ≥ 0.5 g/24-h, *n* (%)	152 (22)	49 (22)	49 (21)	54 (24)	0.59
24-h urine					
pH	6.0 ± 0.5	6.0 ± 0.5	6.0 ± 0.5	6.1 ± 0.5	0.48
UUN excretion, mmol	389 ± 114	349 ± 100	407 ± 111	412 ± 1	<0.001
Phosphate excretion, mmol	25 ± 9	22 ± 8	25 ± 8	27 ± 9	<0.001
Thiosulfate excretion, µmol	7.0 (3.9–12.0)	6.7 (3.7–11.0)	6.9 (4.2–12.5)	7.5 (3.8–12.6)	0.57
Protein excretion, mg	196 (15–367)	163 (15–281)	221 (15–380)	200 (15–417)	0.10

Abbreviations: ♀, female; ♂, male; KTR, kidney transplant recipients; *n*, number; β, standardized beta; BSA, body surface area; BMI, body mass index; SQUASH, Short Questionnaire to Assess Health-enhancing physical activity; SBP, systolic blood pressure; MAP, mean arterial pressure; LDL, low density lipoprotein; Av., average; hs-CRP, high sensitivity C-reactive protein; LDH, lactate dehydrogenase; eGFR, estimated glomerular filtration rate; UUN, urinary urea nitrogen; FGF-23, fibroblast growth factor 23. [a] Normally distributed variables are expressed as mean ± standard deviation (SD), skewed data as medians (25th–75th inter quartile range (IQR)), categorical data is given as number and percentage, *n*, (%). Analyses of difference in baseline characteristics across sex-stratified tertiles of 24-h urinary oxalate excretion were tested by ANOVA for normally distributed continuous variables; Kruskal-Wallis for skewed continuous variables; χ^2 test for categorical data. [b] To convert oxalate in µmol/24-h to mg/24-h, multiply by 0.088. [c] Adjusted for energy intake.

3.2. Cross-Sectional Analysis

We found that age ($p = 0.04$), current smoking status ($p = 0.01$), and cystatin C ($p = 0.03$) were inversely associated with 24-h urinary oxalate excretion, whereas plasma glucose ($p = 0.01$), ascorbic acid ($p < 0.001$), fruit consumption ($p < 0.001$), vitamin B6 ($p < 0.001$), urinary urea nitrogen excretion ($p < 0.001$), and phosphate excretion ($p < 0.001$) were positively associated with 24-h urinary oxalate excretion.

3.3. Prospective Analyses

GF and mortality were recorded during a follow-up of 5.3 years (IQR, 4.5–6.0). During follow-up, 83 (12%) patients developed GF, 55 (9%) patients developed PTDM and 149 (22%) patients died, of which 59 deaths (40%) were due to cardiovascular causes, 41 deaths (28%) due to infectious causes, 26 deaths (17%) due to malignancies and 23 deaths (15%) due to miscellaneous causes (Table 2).

Table 2. Association of baseline characteristics with 24-h urinary oxalate excretion. [a]

Baseline Characteristics	β	*p*
Demographics		
Age, years	−0.08	0.04
Lifestyle		
Current smoker	−0.11	0.01
Plasma glucose, mmol/L	0.10	0.01
Diet		
Ascorbic acid, mg/d [C]	0.24	<0.001
Fruits, g/d	0.16	<0.001
Blood markers		
Vitamin B6 in blood, nmol/L	0.20	<0.001
Renal allograft function		
Cystatin C, blood, mg/L	−0.16	0.03
24-h Urine		
UUN excretion, mmol	0.24	<0.001
Phosphate excretion, mmol	0.25	<0.001

[a] Multivariate linear regression, adjusted for age, sex and eGFR.

3.3.1. GF, PTDM, Cardiovascular Mortality, Mortality due to Malignancies, and Miscellaneous Mortality

A Kaplan-Meier curve for the association of tertiles of 24-h urinary oxalate excretion with GF is shown in Figure 1A ($p = 0.20$, p for trend 0.08). Results of multivariate Cox regression analyses did not show a consistent association of 24-h urinary oxalate excretion with GF (HR 0.71, 95% CI 0.53–0.98) (Table 3). Uni- and multivariate analyses of the associations of 24-h urinary oxalate excretion and potential confounders with GF are shown in Table S1.

A Kaplan-Meier curve for the association of tertiles of 24-h urinary oxalate excretion with PTDM is shown in Figure 1B ($p = 0.24$, p for trend 0.37). Results of multivariate Cox regression analyses showed no association of 24-h urinary oxalate excretion with PTDM (HR 0.95, 95% CI 0.71–1.27) (Table 3).

A Kaplan-Meier curve for the association of tertiles of 24-h urinary oxalate excretion with cardiovascular mortality is shown in Figure 1C ($p = 0.08$, p for trend 0.08). Results of multivariate Cox regression analyses showed cardiovascular mortality is not associated with 24-h urinary oxalate excretion (HR 0.78, 95% CI 0.56–1.10) (Table 4).

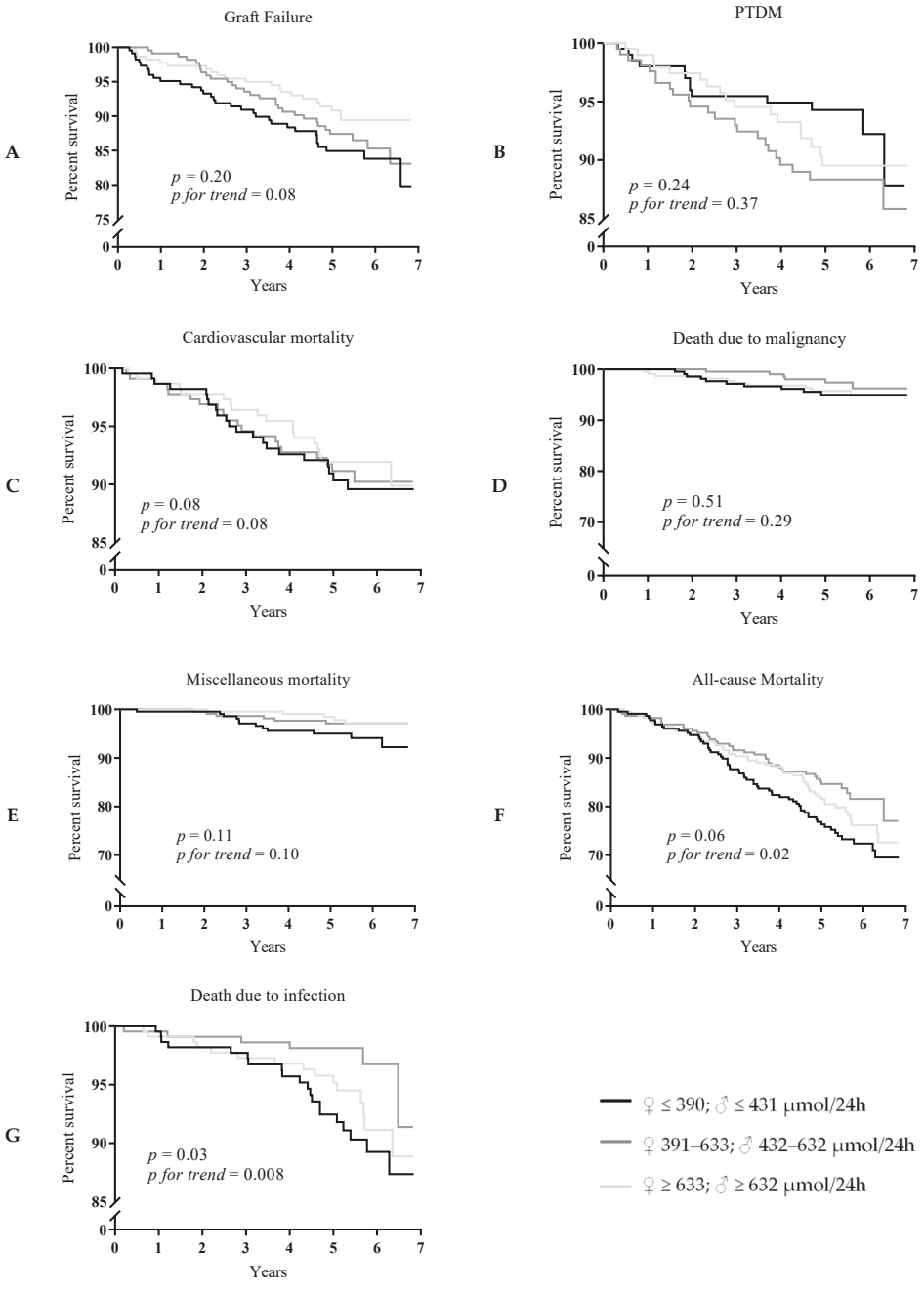

Figure 1. (**A**) Graft failure, (**B**) PTDM, (**C**) cardiovascular mortality, (**D**) death due to malignancy, (**E**) miscellaneous mortality (**F**) all-cause mortality, and (**G**) death due to infection according to sex-stratified tertiles of 24-hour urinary oxalate excretion over approximately 7 years of follow-up.

Table 3. Association of 24-h urine oxalate excretion with graft failure and PTDM.

	Continuous,		Tertiles				
	per 1–SD		Tertile 1	Tertile 2		Tertile 3	
	HR	95%CI	Ref	HR	95% CI	HR	95% CI
Graft Failure							
Model 1	0.80	0.64–1.00	1.00	0.82	0.50–1.36	0.58	0.33–1.00
Model 2	0.78	0.61–1.02	1.00	0.77	0.45–1.32	0.61	0.35–1.08
Model 3	0.72	0.54–0.94	1.00	0.68	0.39–1.17	0.48	0.26–0.86
Model 4	0.71	0.53–0.93	1.00	0.68	0.40–1.18	0.45	0.24–0.82
Model 5	0.71	0.53–0.93	1.00	0.66	0.38–1.15	0.43	0.23–0.80
Model 6	0.71	0.53–0.98	1.00	0.69	0.39–1.20	0.44	0.24–0.83
Model 7	0.75	0.56–1.00	1.00	0.70	0.40–1.25	0.48	0.19–0.77
PTDM							
Model 1	0.93	0.71–1.22	1.00	1.27	0.68–2.37	0.71	0.34–1.46
Model 2	0.91	0.69–1.23	1.00	1.23	0.66–2.32	0.68	0.33–1.41
Model 3	0.91	0.68–1.22	1.00	1.32	0.70–2.50	0.61	0.28–1.33
Model 4	0.94	0.70–1.25	1.00	1.39	0.73–2.68	0.66	0.30–1.44
Model 5	0.95	0.73–1.27	1.00	1.50	0.77–2.91	0.71	0.32–1.57
Model 6	0.95	0.71–1.27	1.00	1.50	0.77–2.91	0.76	0.34–1.73
Model 7	0.99	0.73–1.33	1.00	1.45	0.74–2.83	0.75	0.34–1.69

Multivariate Cox regression were performed for the association of 24-h urinary oxalate excretion with graft failure and PTDM. Model 1: age and sex adjusted. Model 2: Model 1 + adjustment for BMI, primary renal disease, donor age, transplant vintage, eGFR, and proteinuria. Model 3: Model 2 + adjustment for thiosulfate in 24-h urine. Model 4: Model 3 + adjustment for LDH in blood. Model 5: Model 4 + adjustment for pH of 24-h urine. Model 6: Model 5 + adjustment for FGF23. Model 7: Model 6 + adjustment for fruit and vegetables intake.

Table 4. Association of 24-h urine oxalate excretion with all-cause and cardiovascular mortality.

	Continuous,		Tertiles				
	per 1–SD		Tertile 1	Tertile 2		Tertile 3	
	HR	95% CI	Ref	HR	95% CI	HR	95% CI
All-cause mortality							
Model 1	0.83	0.70–0.98	1.00	0.86	0.59–1.25	0.72	0.48–1.74
Model 2	0.81	0.67–0.97	1.00	0.84	0.57–1.23	0.73	0.48–1.14
Model 3	0.76	0.62–0.93	1.00	0.85	0.58–1.25	0.56	0.35–0.88
Model 4	0.76	0.62–0.92	1.00	0.80	0.54–1.18	0.53	0.34–0.83
Model 5	0.77	0.63–0.94	1.00	0.79	0.53–1.17	0.54	0.34–0.86
Model 6	0.77	0.63–0.94	1.00	0.75	0.50–1.13	0.55	0.34–0.86
Model 7	0.83	0.68–1.03	1.00	0.74	0.48–1.15	0.67	0.41–1.11
Cardiovascular mortality							
Model 1	0.90	0.69–1.19	1.00	0.90	0.48–1.69	1.09	0.59–2.00
Model 2	0.87	0.65–1.17	1.00	0.84	0.44–1.62	1.11	0.59–2.08
Model 3	0.78	0.56–1.09	1.00	0.87	0.45–1.69	0.81	0.40–1.63
Model 4	0.77	0.56–1.08	1.00	0.81	0.42–1.57	0.75	0.37–1.53
Model 5	0.79	0.57–1.09	1.00	0.82	0.42–1.59	0.78	0.38–1.57
Model 6	0.78	0.56–1.10	1.00	0.82	0.41–1.62	0.77	0.37–1.59
Model 7	0.79	0.55–1.13	1.00	0.80	0.39–1.66	0.75	0.33–1.70

Multivariate Cox regression were performed for the association of 24-h urinary oxalate excretion with all-cause and cardiovascular mortality. Model 1: age and sex adjusted. Model 2: Model 1 + adjustment for BMI, primary renal disease, donor age, transplant vintage, eGFR, and proteinuria. Model 3: Model 2 + adjustment for thiosulfate in 24-h urine. Model 4: Model 3 + adjustment for LDH in blood. Model 5: Model 4 + adjustment for pH of 24-h urine. Model 6: Model 5 + adjustment for FGF23. Model 7: Model 6 + adjustment for fruit and vegetables intake.

A Kaplan-Meier curve for the association of tertiles of 24-h urinary oxalate excretion with death due to malignancy is shown in Figure 1D (p = 0.51, p for trend 0.29). Results of multivariate Cox

regression analyses showed mortality due to malignancies is not associated with 24-h urinary oxalate excretion (HR 1.14, 95% CI 0.73–1.77) (Table 5).

A Kaplan-Meier curve for the association of tertiles of 24-h urinary oxalate excretion miscellaneous mortality is shown in Figure 1E (p = 0.11, p for trend 0.10). Results of multivariate Cox regression analyses showed miscellaneous death causes are not associated with 24-h urinary oxalate excretion (HR 0.75, 95% CI 0.45–1.26) (Table 5).

Table 5. Association of 24-h urine oxalate excretion with death due to infection, malignancy and other causes.

	Continuous, per 1–SD		Tertiles				
			Tertile 1	Tertile 2		Tertile 3	
	HR	95% CI	Ref	HR	95% CI	HR	95% CI
Death due to infection							
Model 1	0.67	0.49–0.92	1.00	0.75	0.39–1.47	0.33	0.13–0.83
Model 2	0.58	0.40–0.83	1.00	0.75	0.38–1.49	0.31	0.12–0.79
Model 3	0.54	0.36–0.81	1.00	0.70	0.35–1.40	0.25	0.09–0.68
Model 4	0.56	0.38–0.82	1.00	0.65	0.32–1.30	0.23	0.09–0.63
Model 5	0.57	0.38–0.84	1.00	0.65	0.32–1.32	0.24	0.09–0.66
Model 6	0.56	0.38–0.83	1.00	0.62	0.31–1.26	0.25	0.09–0.67
Model 7	0.58	0.38–0.88	1.00	0.57	0.27–1.21	0.30	0.11–0.83
Death due to malignancy							
Model 1	1.01	0.69–1.50	1.00	1.31	0.54–3.17	0.78	0.28–2.20
Model 2	0.98	0.65–1.47	1.00	1.31	0.54–3.18	0.74	0.26–2.09
Model 3	1.02	0.68–1.53	1.00	1.50	0.60–3.77	0.84	0.29–2.45
Model 4	1.03	0.68–1.55	1.00	1.56	0.62–3.94	0.88	0.30–2.59
Model 5	1.08	0.71–1.62	1.00	1.44	0.56–3.71	0.95	0.32–2.81
Model 6	1.10	0.71–1.71	1.00	1.24	0.45–3.41	1.01	0.33–3.07
Model 7	1.14	0.73–1.77	1.00	1.08	0.36–3.8	1.18	0.37–3.71
Death due to other causes							
Model 1	0.76	0.48–1.21	1.00	0.63	0.23–1.71	070	26–1.89
Model 2	0.82	0.51–1.35	1.00	0.53	0.19–1.47	0.62	0.22–1.74
Model 3	0.77	0.45–1.29	1.00	0.59	0.21–1.65	0.43	0.13–1.41
Model 4	0.76	0.45–1.27	1.00	0.53	0.19–1.51	0.39	0.12–1.29
Model 5	0.76	0.46–1.28	1.00	0.53	0.19–1.51	0.39	0.12–1.29
Model 6	0.75	0.45–1.26	1.00	0.46	0.16–1.35	0.36	0.11–1.20
Model 7	0.96	0.56–1.63	1.00	0.64	0.19–2.19	0.75	0.20–2.74

Multivariate Cox regression were performed for the association of 24-h urinary oxalate excretion with death due to infection, malignancy and other causes. Model 1: age and sex adjusted. Model 2: Model 1 + adjustment for BMI, primary renal disease, donor age, transplant vintage, eGFR, and proteinuria. Model 3: Model 2 + adjustment for thiosulfate in 24-h urine. Model 4: Model 3 + adjustment for LDH in blood. Model 5: Model 4 + adjustment for pH of 24-h urine. Model 6: Model 5 + adjustment for FGF23. Model 7: Model 6 + adjustment for fruit and vegetables intake.

3.3.2. All-Cause and Infectious Mortality

A Kaplan-Meier curve for the association of tertiles of 24-h urinary oxalate excretion with all-cause mortality is shown in Figure 1F (p = 0.06, p for trend 0.02). Results of multivariate Cox regression analyses showed, however, that all-cause mortality is independently associated with 24-h urinary oxalate excretion (HR 0.77, 95% CI 0.63–0.94) (Table 4). Uni- and multivariate analyses of the associations of 24-h urinary oxalate excretion and potential confounders with all-cause mortality are shown in Table S2. The association of 24-h urinary oxalate excretion with all-cause mortality demonstrated a nonlinear relationship, as shown by a restricted cubic spline (Figure 2A).

A Kaplan-Meier curve for the association of tertiles of 24-h urinary oxalate excretion with infectious mortality is shown in Figure 1G (p = 0.03, p for trend 0.008). Results of multivariate Cox regression analyses showed infectious mortality was independently associated with 24-h urinary oxalate excretion

(HR 0.58, 95% CI 0.38–0.83) (Table 5). The association between 24-h urinary oxalate excretion and infectious mortality demonstrated a nonlinear relationship, as shown by a restricted cubic spline (Figure 2B).

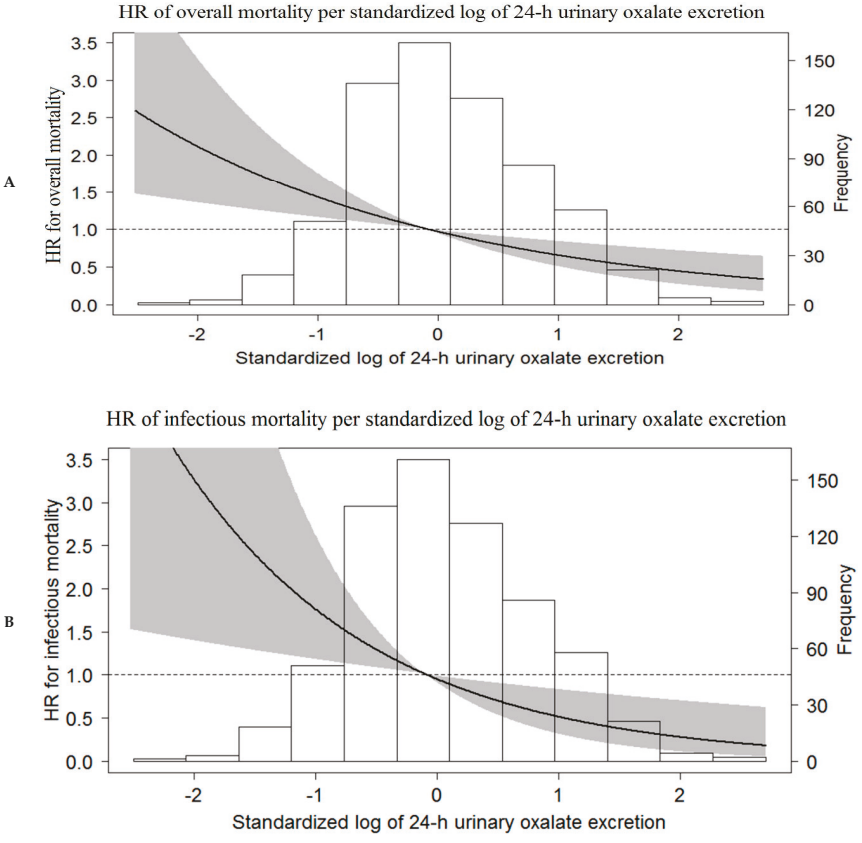

Figure 2. Adjusted association of standardized log 24–hour urinary oxalate excretion with (**A**) all-cause mortality, and (**B**) infectious mortality, based on restricted cubic spline regression, fitted with Model 6. The black line in the graph represents the HR, 95% CI is shown and the gray area.

3.4. Sensitivity Analyses

When we restricted the analyses to subjects with no potential over or undercollection of 24-h urine samples based on differences in expected and observed 24-h urinary creatinine excretions (n = 650), generally similar results were found for GF (HR 0.74, 95% CI 0.55–0.99), PTDM (HR 0.93, 95% CI 0.69–1.26), cardiovascular mortality (HR 0.68, 95% CI 0.47–0.98), mortality due to malignancies (HR 1.10, 95% CI 0.70–1.72), mortality due to miscellaneous causes (HR 0.71, 95% CI 0.41–1.24), all-cause mortality (HR 0.74; 95% CI 0.59–0.92), and infectious mortality (HR 0.58, 95% CI 0.37–0.89).

When competing risk analyses were performed, generally similar results were found for PTDM (HR 1.15, 95% CI 0.91–1.48), cardiovascular mortality (HR 0.82, 95% CI 0.55–1.23), mortality due to malignancies (HR 1.16, 95% CI 0.69–1.95), mortality due to miscellaneous causes (HR 0.84, 95% CI 0.54–1.31), and infectious mortality (HR 0.61, 95% CI 0.45–0.85). The risk of GF was not consistently significant (HR 1.14, 95% CI 0.64–2.26).

4. Discussion

In KTR, median excretion of 24-h oxalate was higher than the clinical cut-off point for hyperoxaluria. No association of 24-h urinary oxalate excretion was found with GF, PTDM, cardiovascular mortality, or mortality due to malignancy or miscellaneous causes, but an independent, inverse association with all-cause mortality and infectious mortality was found. There was respectively a 23% and 44% decrease in hazard ratio per standard deviation increase of 24-h urinary oxalate excretion. The associations remained materially unchanged after adjusting for potential confounders. The association with all-cause and infectious mortality remained materially unchanged after performing sensitivity analyses.

A single-centered prospective study had previously already found an elevated plasma oxalate level in KTR [44]. However, no previous study has provided data on oxalate excretion. The elevated urinary oxalate excretion reflects one of the major findings of this study, being that 44% of the stable KTR are within the clinical range of hyperoxaluria.

To the best of our knowledge, there have not been any previous studies investigating the association of urinary oxalate with GF, PTDM and (cause-specific) mortality in stable KTR. However, a recent study of Waikar et al. with CKD patients stage 2 to 4 found 24-h urinary oxalate excretion to be positively associated with all-cause mortality [14]. With regards to the study of Waikar et al., their first four quintiles can be considered to be below the range of hyperoxaluria of 455µmol/24-h, whereas in our population, only the first tertile can be considered normal with regard to urinary oxalate excretion. This, in part, might explain the difference in association of urinary oxalate excretion with the outcome variables.

The difference between Waikar et al.'s and our study cannot be explained by a higher BMI or diabetes contributing to hyperoxaluria through higher effective renal plasma flow and glomerular hyperfiltration in the Waikar et al. population (respectively, BMI of 32.1 ± 7.7 and 26.6 ± 4.8 and diabetes in 48.9% and 24% of the population) [45]. Low density lipoprotein (LDL) profile was not published in the Waikar et al. report, therefore, difference in oxalate excretion through dyslipidemia cannot be determined [46]. In both studies, the urinary samples were stored at −80 °C. Storage at this temperature can lead to underestimation of oxalate levels through calcium oxalate precipitation [14]. Since the difference of storage time of the samples until measurement is not known, we cannot exclude this as a potential clarification of the found difference. Additionally, spontaneous oxalate generation over the course of the storage might have increased the sample oxalate levels in either studies. Another hypothesis for the interesting difference in 24-h urinary oxalate excretion between the study of Waikar et al. might be found in the possible absence of *Oxalobacter formigenes* in the gut microbiome. KTR have been exposed to antimicrobial prophylactic therapies to lower the risk of opportunistic infections. This greatly affects the diversity of the human microbiome and can cause dysbiosis [47]. Dysbiosis in KTR could contribute to a decrease of *O. formigenes* and therefore, increased gastrointestinal absorption of oxalate, leading to an increased oxalate serum concentration and consequently, elevated urinary excretion.

We found no association with GF, PTDM, mortality due to malignancies, nor mortality due to miscellaneous causes. The results of the proportional hazards models show that the inverse overall association with mortality is mainly driven by infectious mortality. We hypothesized that because 24-h urinary oxalate excretion was positively associated with ascorbic acid, which is inversely associated with overall mortality in RTR through reducing inflammation, an increase in oxalate might contribute to a lower infectious mortality [48]. However, the exact mechanism behind the association of 24-h urinary oxalate excretion with infectious mortality remains to be further investigated, since to our knowledge, there are no studies available showing a potential theoretical explanation.

The strength of this study lays in its prospective design, with a large cohort of stable KTR who were closely monitored according to standardized protocols and continuous surveillance system according to the American Society of Transplantation without loss due to follow-up during a median follow-up of 5.4 years for (specific cause) mortality. The KTR were extensively phenotyped at baseline measurement, providing a broad array of potential confounders to adjust for. The inclusion of the

FFQ gives the possibility to assess the associations with dietary intake, rather than just the urinary excretion. Furthermore, urine was collected as 24-h collecting samples, according to a previously described strict protocol, which eliminates possible daily variances in fluid balance and excretion to give a more accurate excretion estimate. Additionally, potential over- or undercollection of the 24-h urine samples was accounted for by means of sensitivity analyses, which showed that the results remained materially unchanged after restricting the study population as described previously.

However, we also acknowledge limitations of the current study. First, we were unable to adjust our results for socioeconomic status at baseline. Next, although the FFQ and SQUASH are validated questionnaires, they are self-reported, which may lead to possible over or underreporting of dietary intake and physical activity. We also acknowledge that our population consists almost entirely of Caucasian ethnicity, therefore, our results call for caution to extrapolate our results to different populations with regard to ethnicity. Finally, data on nephrolithiasis was not documented; therefore, we were unable to assess the association of urinary oxalate with the outcome nephrolithiasis, which remains a rather overlooked topic in KTR. Nevertheless, our results show for the first time a high prevalence of hyperoxaluria in the post-kidney transplant setting, thus emphasizing the need for future studies in which such analyses are performed. Additionally, because the study of the microbiome was beyond the scope of the current study, the hypothesized mechanism of increased gastrointestinal absorption of oxalate to explain the observed levels of hyperoxaluria cannot be further confirmed.

In conclusion, in stable KTR, 24-h urinary oxalate excretion is quantitatively higher than in the general population. Forty-four percent of the current study population showed urinary oxalate levels above the range of clinical hyperoxaluria. This hyperoxaluria might suggest a role of dysbiosis by leading to diminished *O. formigenes* and therefore, higher oxalate absorption and excretion in the current study population. Twenty-four-hour urinary oxalate excretion was not associated with risk of graft failure, post-transplant diabetes mellitus, cardiovascular mortality, mortality due to malignancies, nor death from miscellaneous causes. However, a consistent and independent inverse association was found with infectious mortality. Our data encourages further studies to validate our findings on the associations of oxalate with long-term outcomes in KTR. Future studies are warranted to investigate specific causes of death and the effect of hyperoxaluria post-kidney transplantation.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/8/12/2104/s1, Formula (S1): CKD-EPI creatinine according to Levey et al., Supplementary Table S1: Uni- and multivariate analyses of the associations of 24-h urinary oxalate excretion and potential confounders with graft failure; Supplementary Table S2: Uni- and multivariate analyses of the associations of 24-h urinary oxalate excretion and potential confounders * with all-cause mortality.

Author Contributions: Conceptualization, S.J.L.B.; data curation, E.v.d.B.; formal analysis, A.T. and C.G.S.; funding acquisition, C.G.S. and S.J.L.B.; investigation, A.T., C.G.S. and M.Y.S.; methodology, A.T., C.G.S., I.M., T.F., M.H.d.B., M.Y.S., G.J.N. and S.J.L.B.; project administration, S.P.B. and S.J.L.B.; software, M.Y.S.; supervision, C.G.S.; visualization, A.T. and S.J.L.B.; writing—original draft, A.T.; writing—review & editing, C.G.S., A.P., M.Y.S., R.M.D., R.R. and S.J.L.B.

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Conflicts of Interest: The authors declare no conflict of interest.

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Plasmapheresis Reduces Mycophenolic Acid Concentration: A Study of Full $AUC_{0–12}$ in Kidney Transplant Recipients

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Abstract: Background: Mycophenolic acid (MPA), a crucial immunosuppressive drug, and plasmapheresis, an effective immunoreduction method, are simultaneously used for the management of various immune-related diseases, including kidney transplantation. While plasmapheresis has been proven efficient in removing many substances from the blood, its effect on MPA plasma levels remains unestablished. Objectives: To evaluate the full pharmacokinetics of MPA by measuring the area under the time–concentration curve ($AUC_{0–12}$), which is the best indicator for MPA treatment monitoring after each plasmapheresis session, and to compare the $AUC_{0–12}$ measurements on the day with and on the day without plasmapheresis. Methods: A cross-sectional study was conducted in kidney transplantation recipients who were taking a twice-daily oral dose of mycophenolate mofetil (MMF, Cellcept®) and undergoing plasmapheresis at King Chulalongkorn Memorial Hospital, Bangkok, Thailand, during January 2018 and January 2019. The MPA levels were measured by an enzymatic method (Roche diagnostic®) 0, 1/2, 1, 2, 3, 4, 6, 8, and 12 h after MMF administration, for $AUC_{0–12}$ calculation on the day with and on the day without plasmapheresis sessions. Plasmapheresis was started within 4 h after administering the oral morning dose of MMF. Our primary outcome was the difference of $AUC_{0–12}$ between the day with and the day without plasmapheresis. Results: Forty complete AUC measurements included 20 measurements on the plasmapheresis day and other 20 measurements on the day without plasmapheresis in six kidney transplant patients. The mean age of the patients was 56.2 ± 20.7 years. All patients had received 1000 mg/day of MMF for at least 72 h before undergoing 3.5 ± 1.2 plasmapheresis sessions. The mean AUC on the day with plasmapheresis was lower than that on the day without plasmapheresis (28.22 ± 8.21 vs. 36.79 ± 10.29 mg × h/L, $p = 0.001$), and the percentage of AUC reduction was 19.49 ± 24.83%. This was mainly the result of a

decrease in $AUC_{0–4}$ of MPA (23.96 ± 28.12% reduction). Conclusions: Plasmapheresis significantly reduces the level of full $AUC_{0–12}$ of MPA. The present study is the first to measure the full $AUC_{0–12}$ in MPA-treated patients undergoing plasmapheresis. Our study suggests that a supplementary dose of MPA is necessary for patients undergoing plasmapheresis.

Keywords: mycophenolic acid; immunosuppression; plasmapheresis; kidney transplantation

1. Introduction

Mycophenolic acid (MPA) is one of the main powerful immunosuppressive drugs widely used for many immunological diseases. There are two MPA compounds available, i.e., mycophenolate mofetil (MMF, Cellcept®) and enteric-coated mycophenolate sodium (EC-MPS, Myfortic®). Both MMF and EC-MPS are similar in terms of efficacy and safety. EC-MPS was developed to improve the side effects of upper gastrointestinal symptoms. The time to reach maximum plasma MPA concentration (t_{max}) of MMF is usually within 1–2 h after an oral dose, while EC-MPS reveals a median lag time from 0.25 to 1.25 h [1]. After absorption from the gastrointestinal tract, 97 to 99% of MPA, which is the active form, will bind to serum albumin. MPA is converted by uridine diphosphate-glucuronosyltransferase (UGT) into inactive mycophenolic acid glucuronide (MPAG), which is mainly excreted by the renal tubules. MPAG can also be excreted in the biliary tract by multidrug-resistant protein (MRP), which can lead to enterohepatic recycling. [1]

Plasmapheresis is one of the most effective methods utilized for rapid immunoglobulin removal in various immunological diseases. Many proteins and protein-bound substances, including medications, can also be removed during plasmapheresis sessions [2,3]. Substances which are likely to be removed during plasmapheresis have the following characters: (1) high blood concentration, (2) high protein bound, (3) low volume of distribution (Vd), and (4) undergoing high-dose/high-efficiency plasmapheresis [4].

Several immunologically mediated diseases can be treated by MPA together with plasmapheresis, i.e., systemic lupus erythematosus (SLE), lupus nephritis, myasthenia gravis, Guillain–Barré syndrome, psoriatic arthritis, relapsed/refractory thrombotic thrombocytopenic purpura (TTP), severe polymyositis/dermatomyositis, inflammatory bowel disease, pemphigus vulgaris, and kidney transplantation [5–7]. Unintentional removal of MPA may result in inadequate immunosuppression and unfavorable outcomes. Of interest, the effect of plasmapheresis on MPA concentration has been studied only in a case series of two patients, one kidney transplant recipient and one patient with myasthenia gravis [8]. MPA removal were measured by considering MPA levels at only two time points—before and after each plasmapheresis session. The MPA removal was calculated on the basis of MPA concentration in plasma effluent. The authors concluded that plasmapheresis of 3 L of plasma did not significantly alter post-plasmapheresis MPA concentration. Currently, there are no available data regarding the effect of plasmapheresis on the area under the concentration–time curve from 0 to 12 h ($AUC_{0–12}$) of MPA, which is the best indicator of MPA exposure of patients.

The present study was conducted in kidney transplant recipients who were taking stable doses of MMF and had indication for plasmapheresis to examine the effects of plasmapheresis on MPA exposure.

2. Methods

An observational study of patients who were taking MMF (Roche, Basel, Switzerland) in combination with plasmapheresis treatment was conducted in King Chulalongkorn Memorial Hospital, Bangkok, Thailand, during January 2018 and January 2019. The inclusion criteria were kidney transplant recipients older than 18 years, who were under an immunosuppressive regimen of tacrolimus, MMF, low-dose prednisolone and had an indication for plasmapheresis. The dosage of MMF had to be

500 mg orally every 12 h for at least one week. Exclusion criteria were patients with serum albumin concentration lower than 2 g/dL and patients who were coadministered a proton pump inhibitor.

Plasmapheresis sessions were initiated within 4 h after the morning dose of MMF. The plasmapheresis machine was Plasauto EZ®, and the dialyzer was Plasmaflo® with a maximum pore size of 0.3 μm. The total treatment volume was 1.5 plasma volume per session. The blood flow rate was 150 mL/h. The replacement fluid was 5% albumin in the same volume as the treatment volume. The number of sessions required was determined on the basis of the clinical judgment of the attending nephrologists.

Plasmapheresis was performed on an alternate day basis for patients who were prescribed more than one plasmapheresis session.

Patients had to strictly take a stable dose of MMF, i.e., 500 mg orally every 12 h for at least one week, before entering the study. MMF dosage adjustment was not allowed during the study period. Patients were not allowed to have a meal for one hour before and two hours after taking the MMF dose. MPA level was measured by an enzymatic immunoassay method (Roche-diagnostic®). The $AUC_{0–12}$ was calculated with the trapezoidal rule from the MPA levels at nine time points after the morning dose of MMF (C0, C0.5, C1, C2, C3, C4, C6, C8, and C12) (Figure 1). The full $AUC_{0–12}$ was measured on the day just before the day patients underwent plasmapheresis and compared with the $AUC_{0–12}$ of the following day, in which patients received the plasmapheresis treatment. Blood samples were taken via a heparin lock in the arm by using the double-syringe technique.

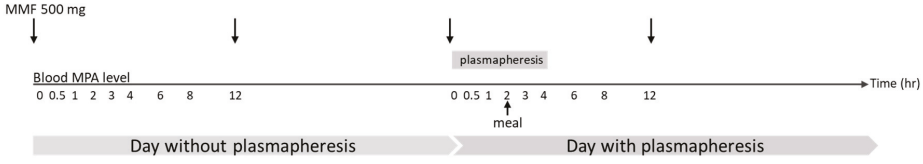


Figure 1. Timing of mofetil (MMF) dosage, plasmapheresis sessions, and meal on the day before and on the day with a plasmapheresis session. MPA: mycophenolic acid.

A complete clinical evaluation including vital signs and body weight was performed. The baseline characteristics including age, cause of end-stage renal disease, type of kidney transplantation, time after kidney transplantation, renal function, indications for plasmapheresis, session of plasmapheresis, and plasma volume per session were recorded.

Absolute and relative frequencies were used for qualitative data. Mean and standard deviation were utilized for numerical data. The chi-squared test was used for comparisons between categorical data. Paired-samples *t*-test was used to compare the $AUC_{0–12}$ of the day with plasmapheresis and the $AUC_{0–12}$ of the day without plasmapheresis. Data were analyzed using the SPSS statistic version 22 (IBM; New York, NY, USA).

This study was approved by The Research Ethics Review Committee for Research Involving Human Research Participants, Health Sciences Group, Chulalongkorn University (IRB No.CF 333/61). The study was registered with the Thai Clinical Trials Registry (TCTR20190211001).

3. Results

Six kidney transplant recipients were enrolled, with a total of 20 plasmapheresis sessions. There were 40 $AUC_{0–12}$ measurements (each AUC consisted of measurements of MPA levels at 9 time points), 20 of which were recorded on the day just before the day patients underwent plasmapheresis, and the other 20 were recorded on the following day, when patients underwent a plasmapheresis session. The mean (±SD) age of the patients was 56.2 ± 20.7 years, and five patients (83.3%) were men (Table 1). At baseline, the mean (±SD) estimated glomerular filtration rate (eGFR) was 49.7 ± 10.9 mL/min/1.73 m^2, serum albumin concentration was 3.8 ± 0.4 g/dL, and hemoglobin concentration was 10.3 ± 1.4 g/dL. Indication for plasmapheresis was antibody-mediated rejection (ABMR) for all

six patients, who were diagnosed by pathological presentation and donor-specific antibody (DSA) detection. The number of plasmapheresis sessions per patient was 3.5 ± 1.2 (range of 1–4 sessions).

Table 1. Baseline characteristics of the patients.

Characteristics	
Age, year (mean ± SD) (range)	56.2 ± 20.7 (25–80)
Male (*n*, %)	5/6, 83%
Cause of ESRD before kidney transplantation Unknown (*n*, %)	 6/6, 100%
Type of kidney transplantation Living donor kidney transplantation (*n*, %)	2/6, 33%
History of previous kidney transplantation (*n*, %)	1/6, 16.7%
HLA mismatch (*n*, %) 0 1–5 6	 0/6 6/6, 100% 0/6
Panel reactive antibody (*n*, %) 0% 1–80% More than 80%	 4/6, 66.7% 0/6 2/6, 33.3%
Induction immunosuppression (*n*, %) Anti-IL2 receptor antibody Anti-thymocyte globulin	 4/6, 66.7% 2/6, 33.3%
Time after transplantation, month (mean ± SD) (range)	97.1 ± 69.5 (1.97–196.52)
Body weight, kg (mean ± SD) (range)	62.2 ± 12.4 (42.7–79.3)
eGFR CKD-EPI, mL/min/1.73 m ^{2} (mean ± SD)	49.7 ± 10.9
Serum albumin, mg/dL (mean ± SD) (range)	3.8 ± 0.4 (3.0–4.2)
Hemoglobin, mg/dL (mean ± SD) (range)	10.3 ± 1.4 9.0–12.2
Liver enzyme, U/L (mean ± SD) SGOT (range) SGPT (range)	32 ± 42 (10–117) 33 ± 36 (10–104)
Type of plasmapheresis Conventional plasmapheresis (*n*, %)	 6/6, 100%
Indication for plasmapheresis (*n*, %) ABMR Acute ABMR Chronic active ABMR	 6/6, 100% 2/6, 33.3% 4/6, 66.7%
Plasma volume per session, mL (mean ± SD)	4,041 ± 749
Number of plasmapheresis session in each patient (mean ± SD)	3.5 ± 1.2

ESRD: end-stage renal disease, ABMR: antibody-mediated rejection; eGFR: estimated glomerular filtration rate; CKD-EPI: chronic kidney disease epidemiology collaboration; SGOT: serum glutamic-oxaloacetic transaminase; SGPT: serum glutamate-pyruvate transaminase.

The mean of MPA $AUC_{0–12}$ of the day with plasmapheresis was significantly lower than that of the day without plasmapheresis (28.22 ± 8.21 vs. 36.79 ± 10.29 mg × h/L, $p = 0.001$) (Figure 2). The

percentage reduction of $AUC_{0–12}$ was 19.49 ± 24.83% (Table 2). The early part of the AUC was affected by plasmapheresis sessions. The $AUC_{0–4}$ of the day with plasmapheresis was significantly lower than that of the day without plasmapheresis (15.79 ± 6.46 vs. 21.78 ± 5.66 mg × h/L, $p < 0.001$), while the $AUC_{4–12}$ was not significantly different between the day with and that without plasmapheresis (12.43 ± 5.02 vs. 15.00 ± 7.56 mg × h/L, $p = 0.125$).

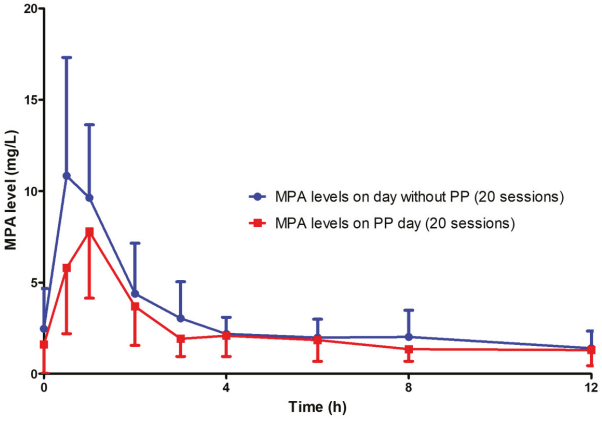


Figure 2. MPA levels on the day with plasmapheresis (20 sessions) compared with those on the day without plasmapheresis (20 sessions). PP: plasmapheresis.

Table 2. Comparison of MPA AUCs recorded on days with and without plasmapheresis, from 0 to 12 h, from 0 to 4 h, and from 4 to 12 h.

Parameters	Day without Plasmapheresis	Day with Plasmapheresis	*p*-Value
$AUC_{0–12}$ mg × h/L (mean ± SD)	36.79 ± 10.29	28.22 ± 8.21	$p = 0.001$
Percentage reduction of $AUC_{0–12}$ (%)	19.49 ± 24.83		-
$AUC_{0–4}$ mg × h/L (mean ± SD)	21.78 ± 5.66	15.79 ± 6.46	$p < 0.001$
Percentage reduction of $AUC_{0–4}$ (%)	23.96 ± 28.12		-
$AUC_{4–12}$ mg × h/L (mean ± SD)	15.00 ± 7.56	12.43 ± 5.02	$p = 0.125$
Percentage reduction of $AUC_{4–12}$ (%)	3.88 ± 42.89		-
$AUC_{0–12}$ of the first day with plasmapheresis session, mg × h/L (mean ± SD)	41.66 ± 10.66	32.26 ± 9.42	$p = 0.001$
Percentage reduction of $AUC_{0–12}$ of the first day with plasmapheresis session (%)	22.86 ± 6.99		-

(AUC; area under the time–concentration curve).

The reduction of MPA $AUC_{0–12}$ was detected as early as the first session of plasmapheresis. The MPA $AUC_{0–12}$ of the day before and of the day of the first session of plasmapheresis were 41.66 ± 10.66 and 32.26 ± 9.42mg × h/L, respectively ($p = 0.001$) (Table 2 and Figure 3). The percentage reduction of MPA $AUC_{0–12}$ of the first day of plasmapheresis session was 22.86 ± 6.99%. The $AUC_{0–12}$ of the day before the second to that of the day of the forth plasmapheresis sessions could be rebounded from the $AUC_{0–12}$ of the day with plasmapheresis. However, the rebounded $AUC_{0–12}$ gradually decreased with the number of sessions of plasmapheresis that the patients received (Figure 4). Given that the target therapeutic $AUC_{0–12}$ of MPA is 30 to 60 mg × h/L for kidney transplantation recipients [9], 17 out of 20 (85%) $AUC_{0–12}$ measured on the day without plasmapheresis achieved the target therapeutic range, compared with only 9 out of 20 (45%) $AUC_{0–12}$ measured on the day with plasmapheresis ($p = 0.008$) (Figure 5).

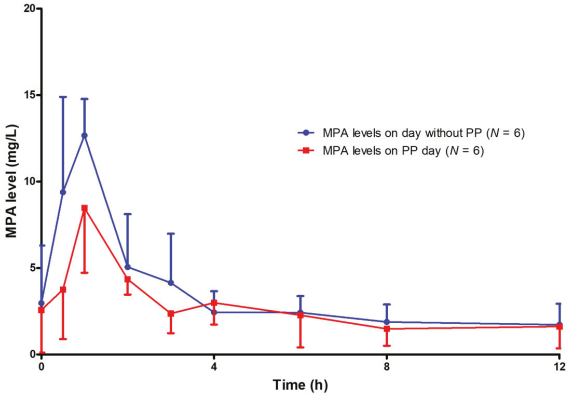
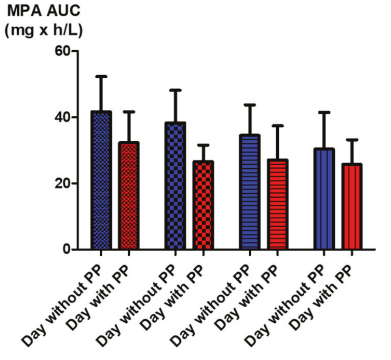


Figure 3. MPA levels on the day before the first plasmapheresis session ($N = 6$) compared with MPA levels on the day with the first plasmapheresis session ($N = 6$).



	AUC_{0-12} without PP	AUC_{0-12} with PP
1st session	41.66 ±10.66	32.26 ±9.42
2nd session	38.31 ±9.86	26.53 ±5.03
3rd session	34.54 ±9.24	27.07 ±10.33
4th session	30.36 ±11.10	25.73 ±7.47

Figure 4. Comparison of the mean MPA $AUC_{0–12}$ between the day with and that without plasmapheresis from the first plasmapheresis session to the fourth session.

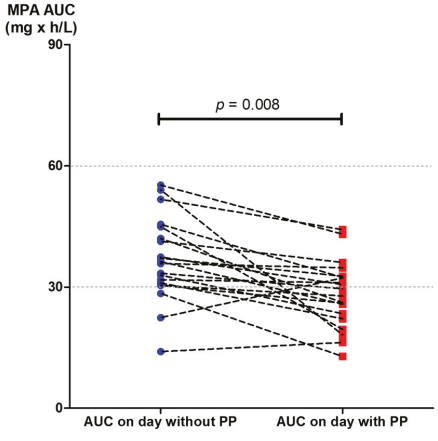


Figure 5. The MPA $AUC_{0–12}$ achieved the target level between the day just before a plasmapheresis session (20 measurements) and the following day, when plasmapheresis was administered (20 measurements).

4. Discussion

The present study is the first to demonstrate the effect of plasmapheresis on MPA exposure by using the full MPA $AUC_{0–12}$. The $AUC_{0–12}$ of MPA was significantly affected by plasmapheresis. This effect was found starting from the first session of plasmapheresis (Figures 2 and 3). One-fifth of the total $AUC_{0–12}$ was lowered by plasmapheresis. The component of AUC most affected by plasmapheresis was the early part ($AUC_{0–4}$). Undergoing plasmapheresis treatment immediately after an oral dose of MMF can lower the MPA peak level, leading to exposure to a subtherapeutic level of MPA. Consecutive sessions of plasmapheresis could increase the risk of underimmunosuppression by lowering the rebound of MPA $AUC_{0–12}$ (Figure 4).

MMF is one of the major immunosuppressive agents widely used to treat many immunological diseases. Since overimmunosuppression can lead to many side effects and underimmunosuppression can cause unfavorable treatment outcomes, MPA level monitoring has been recommended to maintain MPA concentration at the therapeutic level [9,10]. Plasmapheresis is one of the most effective methods for rapid immunoglobulin G (IgG) reduction [5]. Many high-molecular-weight substances can also be removed during a plasmapheresis session, especially proteins and albumin, which makes albumin replacement necessary. Since 97 to 99% of MPA is protein-bound, MPA should be theoretically removed from patients during plasmapheresis treatment.

The effect of plasmapheresis on MPA plasma level was reported in only two patients who were administered MMF in combination with plasmapheresis [8]. Plasmapheresis sessions were started 4 h after MMF administration, and MPA removal was assessed at only two time points (pre- and post-plasmapheresis) together with MPA concentration in plasma waste. The authors concluded that a plasmapheresis session starting later than 4 h after the administration of an oral MMF dose did not significantly alter MPA concentration. Since serum proteins can be trapped in the dialyzer and bloodline, monitoring of MPA removal by only measuring MPA in plasma waste may not reflect total MPA removal. Our study monitored MPA exposure by full $AUC_{0–12}$ measurement on the day with a plasmapheresis session as the study arm and on the day without plasmapheresis as the control arm. Alteration in $AUC_{0–12}$ between the day with and the day without plasmapheresis is the best indicator of the effect of plasmapheresis on MPA plasma levels. The early phase of the full MPA AUC (peak level, $AUC_{0–4}$) is the one mostly affecting MPA exposure and represents more than 50% of $AUC_{0–12}$. The plasmapheresis sessions designed in the present study started within one hour after oral administration of an MMF dose which is the most crucial period for determining the effects of plasmapheresis on MPA.

MPA together with plasmapheresis is mainly utilized for the treatment of many immunologic conditions and diseases which require potent immunosuppression, such as kidney transplant rejection, severe lupus nephritis, or relapsed/refractory thrombotic thrombocytopenic purpura. The patients enrolled in the present study were kidney transplant recipients who were taking MMF and experienced antibody-mediated rejection, which is indicated for plasmapheresis treatment. The present study reveals that MPA administration without dosage adjustment during consecutive sessions of plasmapheresis can lead to unexpected underimmunosuppression and may increase the failure rate of treatment. The present study demonstrated that MPA $AUC_{0–12}$ is reduced by 20% when a plasmapheresis session is started within 4 h after oral administration of MMF (Table 2, Figure 2). The higher the number of consecutive sessions of plasmapheresis performed, the higher the chance of MPA underexposure (Figure 4). We also further examined the role of MMF dose increments in two patients who underwent plasmapheresis and found that increasing the MMF dose from 1000 mg/day to 1250 mg/day can prevent subtherapeutic $AUC_{0–12}$ during plasmapheresis sessions (unpublished data). An MMF dosage increment of 20% may be required to maintain a therapeutic level of MPA on the day patients undergo plasmapheresis. A further comprehensive study of therapeutic drug monitoring in patients with increased dose of MPA before undergoing plasmapheresis is crucially required. Otherwise, a 4 h delay of the plasmapheresis session after administration of an MMF dose may reduce the effect of plasmapheresis on MPA exposure (Figure 6).

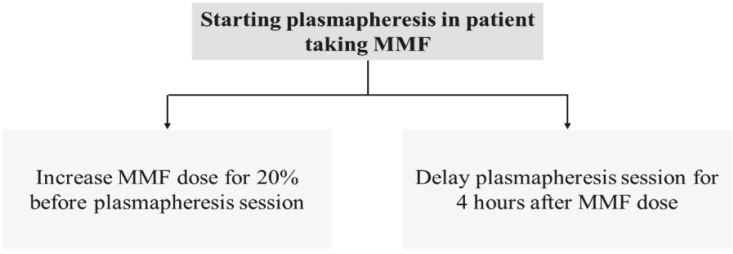


Figure 6. Recommendations for MMF dose or plasmapheresis adjustment in patient receiving concomitant MMF and plasmapheresis treatment.

The MMF dose used in the present study was relatively low. This is because the target population of patients enrolled in this study were kidney transplant recipients who were in the maintenance phase of immunosuppression. Moreover, a study on Asian patients showed that most of the patients achieved the target MPA level with an MMF dose of 1000 mg/day [11]. Besides conventional plasmapheresis, a study of the effects of others apheresis techniques such as double-filtration plasmapheresis and immunoadsorption, which have different kinetics of protein removal, should be carried out.

5. Conclusions

Plasmapheresis significantly reduces MPA plasma levels, particularly in the early phase after oral administration of an MPA dose. This effect should be addressed when combining MPA administration together with plasmapheresis in a treatment protocol.

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Plasma Vitamin C and Cancer Mortality in Kidney Transplant Recipients

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Abstract: There is a changing trend in mortality causes in kidney transplant recipients (KTR), with a decline in deaths due to cardiovascular causes along with a relative increase in cancer mortality rates. Vitamin C, a well-known antioxidant with anti-inflammatory and immune system enhancement properties, could offer protection against cancer. We aimed to investigate the association of plasma vitamin C with long-term cancer mortality in a cohort of stable outpatient KTR without history of malignancies other than cured skin cancer. Primary and secondary endpoints were cancer and cardiovascular mortality, respectively. We included 598 KTR (mean age 51 ± 12 years old, 55% male). Mean (SD) plasma vitamin C was 44 ± 20 μmol/L. At a median follow-up of 7.0 (IQR, 6.2–7.5) years, 131 patients died, of which 24% deaths were due to cancer. In Cox proportional hazards regression analyses, vitamin C was inversely associated with cancer mortality (HR 0.50; 95%CI 0.34–0.74; $p < 0.001$), independent of potential confounders, including age, smoking status and immunosuppressive therapy. In secondary analyses, vitamin C was not associated with cardiovascular mortality (HR 1.16; 95%CI 0.83–1.62; $p = 0.40$). In conclusion, plasma vitamin C is inversely associated with cancer mortality risk in KTR. These findings underscore that relatively low circulating plasma vitamin C may be a meaningful as yet overlooked modifiable risk factor of cancer mortality in KTR.

Keywords: Kidney transplant; vitamin C; cancer mortality; oxidative stress.

1. Introduction

Although kidney transplantation improves the prognosis of patients with end-stage renal disease (ESRD), kidney transplant recipients (KTR) remain at higher mortality risk compared to healthy individuals [1]. Since the beginning of kidney transplantation, the main cause of death has been cardiovascular [2–4]. In recent years, however, there has been a changing trend in mortality causes in KTR, with a decline in death due to cardiovascular causes along with a relative increase in cancer mortality [2,5–7]. Among non-cardiovascular deaths, malignancies lead the individual causes of death [8,9]. Noteworthy is that overall risk of death associated with cancer in KTR is ten-fold higher than in the general population [9]. Given this relative increase in cancer mortality in KTR, further studies to explore potential risk factors and underlying mechanisms are needed.

Post-transplantation immunosuppression as well as chronic uremic state have been recently proposed as risk factors, with oxidative stress as a potential underlying mechanism [2,10,11]. Vitamin C is a well-known radical scavenger and reducing agent [12], and due to its antioxidant, anti-inflammatory and immune system enhancement properties, it could offer protection against cancer incidence in KTR [13]. There is evidence supporting that low plasma vitamin C may lead to an increased risk of dying from cancer in the general male population [13], and is also inversely associated with gastric cancer risk in the general population [14].

Increased oxidative stress occurs when there is an imbalance between antioxidant and pro-oxidant species, leading to oxidative damage. Malondialdehyde (MDA), a decomposition product of peroxidized polyunsaturated fatty acids, is a widely used and sensitive biomarker of oxidative damage [15]. Gamma-glutamyl transpeptidase (GGT) is also currently used as an indicator of whole body oxidative stress [16,17]. Uric acid in plasma acts as antioxidant in presence of vitamin C [18]. Higher levels of free thiol groups have been proposed to be protective against oxidative damage, similarly to vitamin C [19]. Under the hypothesis that anti-carcinogenic properties of vitamin C are mainly driven by its antioxidant properties, the potential protective effect of vitamin C against cancer mortality would be expected to vary upon changes in oxidative stress biomarkers.

This evidence suggests that vitamin C could be a simple and widely available modifiable risk factor for cancer mortality in KTR. Nevertheless, studies focusing on the prospective association of vitamin C and long-term cancer mortality in this clinical setting are lacking. In this study, in primary analyses we aimed to investigate the association of circulating plasma vitamin C concentrations with long-term cancer mortality in a large cohort of KTR. As oxidative stress is considered a potential underlying mechanism, we aimed to assess whether the potential association of plasma vitamin C with cancer mortality would vary upon changes in oxidative stress biomarkers, i.e., uric acid, free thiol groups, MDA and GGT. In secondary analyses, we aimed to investigate the association of circulating plasma vitamin C concentrations with cardiovascular mortality.

2. Materials and Methods

2.1. Study Design and Patients

We performed a post hoc analysis in the TransplantLines Insulin Resistance and Inflammation Biobank and Cohort Study, number NCT03272854. Outpatient KTR (≥18 years old) with a functioning graft for at least 1 year were invited to participate between August 2001 and July 2003. Patients with overt congestive heart failure and patients diagnosed with cancer other than cured skin cancer (squamous cell or basal cell carcinoma successfully treated by a dermatologist) were not considered eligible for the study. The outpatient follow-up constitutes a continuous surveillance system in which patients visit the outpatient clinic with declining frequency, in accordance with the American Transplantation Society guidelines [20]. A total of 847 KTR were invited to be enrolled, of which 606 (72%) patients provided written informed consent to participate. Data were extensively collected at baseline. Patients with missing plasma vitamin C concentration (n = 8) were excluded for the statistical analysis, resulting in 598 KTR, of whom data are presented in the current study (Figure S1). The present study was approved by the Institutional Review Board (METc 2001/039), and was conducted in accordance with declarations of Helsinki and Istanbul.

2.2. Kidney Transplant Recipients Characteristics

Relevant characteristics including recipient age, gender, and transplant date were extracted from the Groningen Renal Transplant Database. This database contains detailed information on all kidney transplantations that have been performed at the University Medical Center Groningen since 1968. Details of the standard immunosuppressive treatment were described previously [21]. Smoking status was obtained using a self-report questionnaire at inclusion. Details about collection of dietary history have been described before [22]. In brief, a semi-quantitative food-frequency questionnaire was used

to assess fruit and vegetable intake. Fruit intake was assessed by asking participants 'How many servings of fruit do you eat per day on average?' Vegetable intake was assessed by asking participants 'How many tablespoons of vegetable do you eat per day on average?' Respondents were asked to choose among five possible frequency categories: 0, 1, 2, 3, ≥4 per day. Collection of data on use of vitamin C or multivitamin supplements containing vitamin C was systematically performed, by means of self-report, at baseline.

2.3. Laboratory Measurements

All measurements were performed during a morning visit to the outpatient clinic. Diabetes mellitus was defined according to the guidelines of the American Diabetes Association [23]. Proteinuria was defined as urinary protein excretion ≥0.5 g/24 h. Kidney function was assessed by estimated Glomerular Filtration Rate (eGFR) applying the Chronic Kidney Disease Epidemiology Collaboration equation [24].

Blood was drawn after a fasting period of 8–12 h, which included no medication intake. According to a strict protocol, patients were instructed to collect a 24-hour urine sample the day before their visit to the outpatient clinic. Total cholesterol, low-density lipoprotein cholesterol (LDL), plasma triglycerides, plasma glucose levels, plasma insulin concentration, and glycated hemoglobin (HbA_{1C}) were determined as described previously [25]. Plasma high sensitivity C-reactive protein (hs-CRP) was measured by enzyme-linked immunosorbent assay, as described previously [26]. MDA was measured fluorescently after binding to thiobarbituric acid as described before [27]. Ellman's reagent was used for the determination of free thiol groups in cell culture and a cell-free solution of L-cysteine as described previously [28]. Plasma creatinine concentration was determined using a modified version of the Jaffé method (MEGA AU510; Merck Diagnostica). Total urinary protein concentration was analyzed using the Biuret reaction (MEGA AU510; Merck Diagnostica).

2.4. Plasma Vitamin C Measurement

After phlebotomy, blood was directly transferred to the laboratory on ice, deproteinized and stored in the dark at −20°C until analysis. For quantitative measurement ascorbic acid is enzymatically transformed to dehydroascorbic acid, which in turn is derivatized to 3-(1,2-dihydroxyethyl) furo-[3,4-b] quinoxaline-1-one. Then, reversed phase liquid chromatography with fluorescence detection is applied (excitation 355 nm, emission 425 nm).

2.5. Cause-Specific Mortality and Graft Failure

The primary endpoint for analyses was mortality from cancer, defined according to a previously specified list of International Classification of Diseases, Ninth Revision (ICD-9) codes 140–239 [29]. Secondary endpoint was mortality from cardiovascular causes, defined as death due to cerebrovascular disease, ischemic heart disease, heart failure, or sudden cardiac death according to ICD-9 codes 410–447. Information on the cause of death was derived from the patients' medical records and was assessed by an adjudication committee. Information about death-related type of cancer was ascertained by contacting the general practitioners who were in charge of deceased cancer patients. Graft failure was defined as return to dialysis or need for a re-transplantation. The continuous surveillance system of the outpatient program ensures up-to-date information on patient status and cause of death. There was no loss to follow-up.

2.6. Statistical Analyses

Data analysis was performed using SPSS version 23.0 software (SPSS Inc., Chicago, IL, USA), STATA 14.1 (STATA Corp., College Station, TX, USA), and R version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria). In all analyses, a two-sided $p < 0.05$ was considered significant. Continuous variables were summarized using mean (standard deviation; SD) for normally distributed data, whereas skewed distributed variables are given as median (interquartile range; IQR). Categorical variables were summarized as numbers (percentage). Multiple imputation was performed to account

for missingness of data among variables other than data on plasma vitamin C [30]. The percentages of missing data were 0.2, 0.2, 0.2, 0.2, 0.3, 0.3, 0.3, 0.3, 0.5, 0.7, and 0.7% for waist circumference, HbA_{1C}, albumin, alkaline phosphatase, proteinuria, leukocyte concentration, MDA, cumulative dose of prednisolone, uric acid, GGT, and prior history of cardiovascular disease, respectively. The percentages of missing data were maximally 11, 21, and 33% for free thiol groups, free fatty acids, and fruit and vegetable intake, respectively.

Age- and sex-adjusted linear regression analyses were performed to evaluate the association of plasma vitamin C concentrations with baseline characteristics. Residuals were checked for normality and variables were natural log-transformed when appropriate. In order to study in an integrated manner which patient- and transplant-related variables of interest were independently associated with and were determinants of plasma vitamin C concentrations, we performed forward selection of baseline characteristics by including all the variables that were associated with plasma vitamin C with a $p < 0.1$ in the preceding age- and sex-adjusted linear regression analyses. Selected variables were then used to perform stepwise backwards multivariable linear regression analyses ($P_{out} > 0.05$). Standardized beta coefficients represent the difference (in standard deviations) in plasma vitamin C per 1 standard deviation increment in continuous baseline characteristics, or for categorical characteristics the difference (in standard deviations) in plasma vitamin C compared to the implied reference group.

To analyze whether plasma vitamin C was prospectively and independently associated with cancer mortality, we performed multivariable-adjusted Cox proportional hazards regression analyses. For these analyses plasma vitamin C concentrations were used as log-transformed values with a log2 base, in order to obtain the best fitting model. We tested proportionality assumptions of Cox proportional hazards regression analyses, and they were satisfied, indicating that the association of baseline vitamin C with outcome is constant over follow-up time of the current study. The selection of covariates was made a priori, considering their potential confounding effect based on previously described risk factors for all-cause mortality in KTR and generally accepted risk factors for cancer mortality in the general population and in KTR [9,10,13,31]. We adjusted for age, sex, and smoking status (Model 1); eGFR, dialysis vintage, time since transplantation and proteinuria (Model 2); and, fruit and vegetable intake (Model 3). To avoid overfitting and inclusion of too many variables for the number of events, further models were performed with additive adjustments to Model 3 [32]. We performed additional adjustments for diabetes mellitus, hs-CRP and prior history of cardiovascular disease (Model 4); immunosuppressive therapy (use of calcineurin inhibitors (CNI), use of antimetabolites, use of mammalian target of rapamycin (m-TOR) inhibitors, and cumulative dose of prednisolone, calculated as the sum of maintenance dose of prednisolone since kidney transplantation until inclusion in the study and the dose of prednisolone or methylprednisolone required for treatment of acute rejection (a conversion factor of 1.25 was used to convert methylprednisolone to prednisolone dose). For acute rejection, different amounts of prednisolone or methylprednisolone were administered, which was taken into account in the calculations. Rejection episodes after inclusion were not included [33]; Model 5); and transplantation era (Model 6). Transplantation eras, with corresponding immunosuppressing medications, have been previously well described [34]. In secondary analyses, the aforementioned Cox proportional hazards regression analyses were performed for cardiovascular mortality. The analyses for both cancer death and cardiovascular death were performed by fitting multivariable-adjusted proportional cause-specific hazard models. In each of these models, the competing events were treated as censored observations, causing the regression parameters to directly quantify the hazard ratio among those individuals who are actually at risk of developing the event of interest, i.e., cancer mortality or cardiovascular mortality [35]. Hazard ratios (HR) are reported with 95% confidence interval (CI). The HR of each model is given per doubling of vitamin C concentration.

To adhere to existing recommendations for good reporting on survival analyses [36,37], we tested for potential interaction of all potential confounders and the oxidative stress biomarkers with vitamin C, namely, uric acid, free thiol groups (corrected by total serum protein) [19], MDA, and GGT by fitting models containing both main effects and their cross product terms. For these analyses, $P_{\text{interaction}} < 0.05$

was considered to indicate significant interaction. We also performed subgroup analyses according to the aforementioned oxidative stress biomarkers, with adjustment for age, sex, smoking status, eGFR, dialysis vintage, time since transplantation, proteinuria, and fruit and vegetable intake. Cut-off points of originally continuous variables used in the stratified analyses were determined so they would allow for an as much as possible similar number of events in each subgroup, and thus allow for similar statistical power for the assessment of the primary association under study (plasma vitamin C and cancer mortality) in each subgroup after stratification of the overall population. Whenever and as much as possible, these criteria were matched with clinical cut-off points.

In sensitivity analyses, we performed graft failure-censored Cox proportional hazards regression analyses of the association of plasma vitamin C with cancer mortality and cardiovascular mortality. In addition, we performed Cox proportional hazards regression analyses of the association of plasma vitamin C with cancer mortality with adjustment for HbA1c instead of diabetes mellitus.

3. Results

3.1. Baseline Characteristics

A total of 598 patients (51 ± 12 years old, 55% male) were included at a median of 5.9 (IQR, 2.6–11.4) years after kidney transplantation. None of the patients used vitamin C supplements or multivitamin supplements containing vitamin C. Mean plasma vitamin C concentration was 44 ± 20 µmol/L, mean eGFR was 47 ± 16 mL/min/1.73 m^2. Patient-related variables of interest, including transplant-related characteristics and immunosuppressive therapy are summarized in Table 1. The results of the age- and sex-adjusted linear regression analyses are shown in Table 2. In stepwise backward multivariable linear regression analysis, fruit intake (std. $\beta = 0.22$; $p < 0.01$), dialysis vintage (std. $\beta = -0.09$; $p < 0.05$), proteinuria ≥0.5 g/24 h (std. $\beta = -0.11$; $p < 0.05$), HbA_{1C} (std. $\beta = -0.14$; $p < 0.01$), diastolic blood pressure (std. $\beta = -0.16$; $p < 0.01$), alkaline phosphatase (std. $\beta = -0.15$; $p < 0.01$), hs-CRP (std. $\beta = -0.17$; $p < 0.01$) and male sex (std. $\beta = -0.18$; $p < 0.01$) were identified as independent determinants of plasma vitamin C (Table 2). The overall R^2 of the final model was 0.21.

Table 1. Baseline characteristics of 598 kidney transplant recipients.

Baseline Characteristics	All Patients
Study subjects, *n* (%)	598 (100)
Plasma vitamin C, µmol/L, mean (SD)	44 (20)
Demographics	
Age, years, mean (SD)	51 (12)
Sex, male, *n* (%)	328 (55)
Caucasian ethnicity, *n* (%)	577 (97)
Body composition	
Body mass index, kg/m^2, mean (SD)	26.0 (4.3)
Body surface area, m^2, mean (SD)	1.9 (0.2)
Waist circumference, cm, mean (SD) [a]	97 (14)
Kidney allograft function	
estimated Glomerular Filtration Rate, mL/min/1.73 m^2, mean (SD)	47 (16)
Proteinuria ≥0.5 g/24 h, *n* (%) [b]	166 (28)
Tobacco use	
Never smoker, *n* (%)	214 (36)
Ex-smoker, *n* (%)	251 (42)
Current smoker, *n* (%)	131 (22)
Blood pressure	
Systolic blood pressure, mmHg, mean (SD)	153 (23)
Diastolic blood pressure, mmHg, mean (SD)	90 (10)
Prior history of cardiovascular disease	
History of myocardial infarction, *n* (%) [c]	48 (8)
History of cerebrovascular accident or transient ischemic attack, *n* (%) [c]	32 (5)

Table 1. *Cont.*

Baseline Characteristics	All Patients
Diet	
Fruit intake, servings/day, mean (SD) [d]	1.5 (1.0)
Vegetable intake, tablespoons/day, mean (SD) [d]	2.5 (0.8)
Diabetes and glucose homeostasis	
Diabetes, *n* (%)	105 (18)
HbA_{1C}, %, mean (SD) [a]	6.5 (1.1)
Insulin, µU/mL, median (IQR)	11.2 (8.0–16.3)
Glucose, mmol/L, median (IQR)	4.5 (4.1–5.0)
Laboratory measurements	
Leukocyte concentration, $\times 10^9$/L, mean (SD) [b]	8.6 (2.4)
hs-CRP, mg/L, median (IQR)	2.0 (0.8–4.8)
Albumin, g/L, mean (SD) [a]	41 (3)
Lipids	
Total cholesterol, mmol/L, mean (SD)	5.6 (1.1)
HDL cholesterol, mmol/L, mean (SD)	1.1 (0.3)
LDL cholesterol, mmol/L, mean (SD)	3.5 (1.0)
Free fatty acids, µmol/L, mean (SD) [e]	403 (180)
Triglycerides, mmol/L, median (IQR)	1.9 (1.4–2.6)
Oxidative stress	
Uric acid, mmol/L, mean (SD) [f]	0.45 (0.13)
Malondialdehyde, µmol/L, mean (SD) [b]	5.6 (1.8)
Gamma-glutamyl transpeptidase, U/L, median (IQR) [c]	24 (18–39)
Alkaline phosphatase, U/L, median (IQR) [a]	72 (57–94)
Kidney transplant and immunosuppressive therapy	
Dialysis vintage, months, median (IQR)	27 (13–48)
Time since transplantation, years, median (IQR)	6 (3–11)
Donor type (living), *n* (%)	83 (14)
Use of calcineurin inhibitor, *n* (%)	470 (79)
Cyclosporine, *n* (%)	386 (65)
Tacrolimus, *n* (%)	84 (14)
Use of antimetabolites, *n* (%)	441 (74)
Azathioprine, *n* (%)	194 (32)
Mycophenolate acid, *n* (%)	247 (41)
Use of mammalian target of rapamycin inhibitors, *n* (%)	10 (1.7)
Cumulative dose of prednisolone, g, median (IQR) [b]	21 (11–38)

Data available in: [a] 597, [b] 596, [c] 594, [d] 400, [e] 471, [f] 595. Abbreviations: hs-CRP, high-sensitive C reactive protein; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; HbA_{1C}, glycated hemoglobin; SD, standard deviation.

Table 2. Association of baseline characteristics with plasma vitamin C in 598 kidney transplant recipients.

Baseline Characteristics	Plasma Vitamin C (Log_2), µmol/L	
	Linear Regression [†]	Backwards Linear Regression [§]
	Std. β	Std. β
Study subjects, *n* (%)	—	—
Plasma vitamin C, µmol/L, mean (SD)	—	—
Demographics		
Age, years	−0.56	
Sex, male	−0.19 ***	−0.18 ***
Caucasian ethnicity	−0.21	
Body composition		
Body mass index, kg/m^2	−0.08 *	~
Body surface area, m^2	−0.06	
Waist circumference, cm	−0.15 ***	~

Table 2. *Cont.*

Baseline Characteristics	Plasma Vitamin C (Log_2), μmol/L	
	Linear Regression †	Backwards Linear Regression §
	Std. β	Std. β
Kidney allograft function		
estimated Glomerular Filtration Rate, mL/min/1.73 m^2	0.11 ***	~
Proteinuria ≥0.5 g/24 h	−0.11 ***	−0.11 **
Tobacco use		
Never smoker	0.03	
Ex-smoker	0.08 *	~
Current smoker	−0.11 ***	~
Blood pressure		
Systolic blood pressure, mmHg	−0.12 ***	~
Diastolic blood pressure, mm Hg	−0.1 **	−0.16 ***
Prior history of cardiovascular disease		
History of myocardial infarction	−0.01	
History of cerebrovascular accident or transient ischemic attack	−0.04	
Diet		
Fruit intake, servings/day	0.22 ***	0.22 ***
Vegetable intake, tablespoons/day	0.09*	~
Diabetes and glucose homeostasis		
Diabetes	−0.11 ***	~
HbA_{1C}, %	−0.13 ***	−0.14 ***
Insulin, μU/mL	−0.09 **	~
Glucose, mmol/L	−0.07 *	~
Laboratory measurements		
Leukocyte concentration, $x \times 10^9$/L	−0.03	
hs-CRP, mg/L	−0.14 ***	−0.17 ***
Albumin, g/L	0.14 ***	~
Lipids		
Total cholesterol, mmol/L	0.05	
HDL cholesterol, mmol/L	0.12 ***	~
LDL cholesterol, mmol/L	0.07 *	~
Free fatty acids, μmol/L	−0.07	
Triglycerides, mmol/L	−0.09 **	~
Oxidative stress		
Uric acid, mmol/L	−0.14 ***	~
Malondialdehyde, μmol/L	0.01	
Gamma-glutamyl transpeptidase, U/L	−0.05	
Alkaline phosphatase, U/L	−0.18 ***	−0.15 ***
Kidney transplant and immunosuppressive therapy		
Dialysis vintage, months	−0.09 **	−0.09 **
Time since transplantation, years	0.18 ***	~
Donor type (living)	0.02	
Use of calcineurin inhibitor	−0.08 **	~
Cyclosporine	−0.03	
Tacrolimus	−0.06	
Use of antimetabolites	0.01	
Azathioprine	0.10 **	~
Mycophenolate acid	−0.09 **	~
Use of mammalian target of rapamycin inhibitors	−0.09 **	~
Cumulative dose of prednisolone, g	0.17 ***	~

* p Value < 0.1; ** p Value < 0.05; *** p Value < 0.01. † Linear regression analysis; adjusted for age and sex. § Stepwise backwards linear regression analysis; for inclusion and exclusion in this analysis, p Values were set at 0.1 and 0.05, respectively. ~ Excluded from the final model. Abbreviations: Std. β, standardized beta coefficient; hs-CRP, high-sensitive C reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HbA_{1C}, glycated hemoglobin.

3.2. Primary Prospective Analyses

At a median follow-up of 7.0 (IQR, 6.2–7.5) years, 131 (22%) patients died, of which 32 (24%) deaths were due to cancer (summary of types of cancer can be found in Table S1). Median time from kidney transplantation to cancer death was 12.0 (IQR, 6.2–20.0). In multivariable-adjusted Cox proportional hazards regression analyses, plasma vitamin C concentration was inversely associated with cancer mortality risk (HR 0.50; 95%CI 0.34–0.74; $p < 0.001$), independent of potential confounders including age, sex, smoking status, eGFR, dialysis vintage, time since transplantation, proteinuria, fruit and vegetable intake, diabetes mellitus, hs-CRP, prior history of cardiovascular disease, immunosuppressive therapy and transplantation era (Table 3, Models 1–6) (Figure 1). Full report of coefficient estimates for both the variable of interest plasma vitamin C as well as for potential confounders included in every multivariable model (Models 1–6) are shown in Table S2. Neither significant interaction of the association of vitamin C with cancer mortality was found for potential confounders (Table S3) nor for oxidative stress biomarkers. Results of interaction and subgroup analyses of oxidative stress biomarkers are presented in Figure 2.

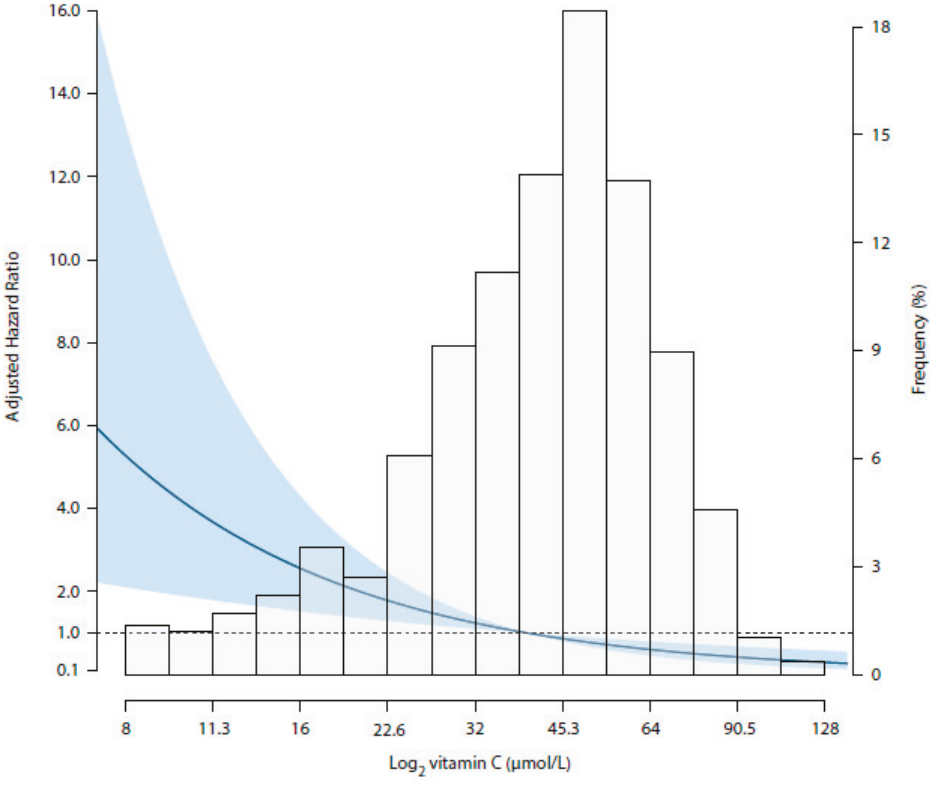

Figure 1. Association of plasma vitamin C with cancer mortality risk in 598 KTR. Data were fitted by a Cox proportional hazards regression model adjusted for age, sex, smoking status, estimated Glomerular Filtration Rate, dialysis vintage, time since transplantation, proteinuria, fruit and vegetable intake, diabetes mellitus, high-sensitivity C-reactive protein, and prior history of cardiovascular disease (Model 4). The gray areas indicate the 95% CIs. The line in the graph represents the hazard ratio.

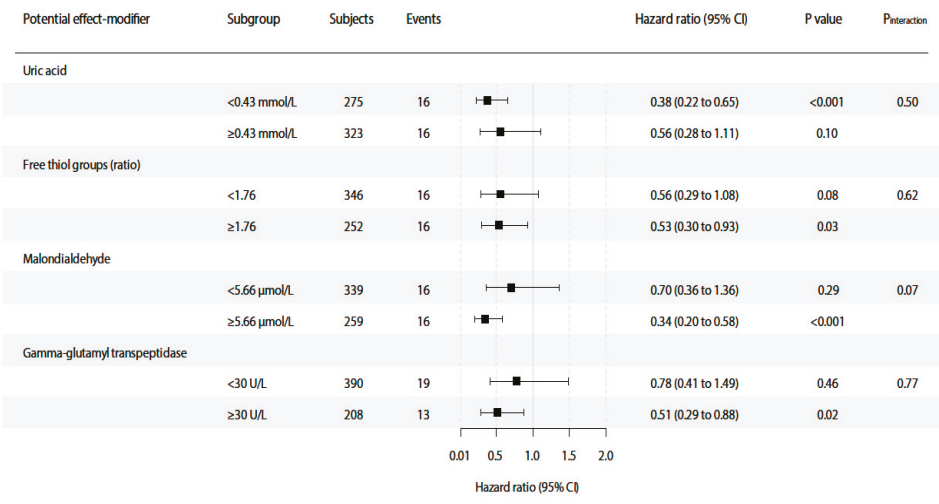

Figure 2. Interaction and subgroup analyses of the association of plasma vitamin C with cancer mortality. $P_{interaction}$ was calculated by fitting models which contain both main effects as continuous variables and their cross-product term. Hazard ratios were calculated with adjustment for age, sex, smoking status, estimated Glomerular Filtration Rate, dialysis vintage, time since transplantation, proteinuria, and fruit and vegetable intake, analogous to Model 3 of the overall prospective analyses. Abbreviations: CI, confidence interval; MDA, malondialdehyde; GGT, gamma-glutamyl transpeptidase.

Table 3. Association of plasma vitamin C with cancer mortality in 598 kidney transplant recipients.

Models	Vitamin C (Log_2), Continuous (µmol/L)		
	HR [a]	95% CI	*p* Value
Crude	0.63	0.43–0.92	0.016
Model 1	0.61	0.43–0.87	0.006
Model 2	0.52	0.35–0.75	0.001
Model 3	0.50	0.34–0.74	<0.001
Model 4	0.49	0.33–0.72	<0.001
Model 5	0.55	0.38–0.80	0.002
Model 6	0.47	0.32–0.70	<0.001

Cox proportional hazards regression analyses were performed to assess the association of plasma vitamin C with cancer mortality. Model 1: adjustment for age, sex and smoking status. Model 2: Model 1 + adjustment for estimated Glomerular Filtration Rate, dialysis vintage, time since transplantation and proteinuria. Model 3: Model 2 + adjustment for fruit and vegetable intake. Model 4: Model 3 + adjustment for diabetes mellitus, high-sensitivity C-reactive protein and prior history of cardiovascular disease. Model 5: Model 3 + adjustment for immunosuppressive therapy. Model 6: Model 3 + adjustment for transplantation era. Abbreviations: HR, hazard ratio; CI, confidence interval. [a] Each model hazard ratio is given per doubling of vitamin C concentration.

3.3. Secondary Prospective Analyses

In secondary analyses, at a median follow-up of 7.0 (IQR, 6.2–7.5) years, 131 (22%) patients died, of which 67 (49%) deaths were due to cardiovascular causes. Median time from kidney transplantation to cardiovascular death was 11.0 (IQR, 7.6–14.8). There was no significant association of plasma vitamin C with cardiovascular mortality (HR 1.16; 95%CI 0.83–1.62; $p = 0.40$) (Table 4). This finding remained unaltered after adjustment for potential confounders, analogous to Models 1 to 6 of the primary analyses.

Table 4. Association of plasma vitamin C with cardiovascular mortality in 598 kidney transplant recipients.

Models	Vitamin C (Log_2), Continuous (μmol/L)		
	HR	95% CI	*p* Value
Crude	0.97	0.70–1.33	0.83
Model 1	0.97	0.71–1.33	0.86
Model 2	1.04	0.75–1.44	0.83
Model 3	1.16	0.83–1.62	0.40
Model 4	1.31	0.92–1.86	0.13
Model 5	1.21	0.86–1.70	0.27
Model 6	1.15	0.82–1.61	0.41

Cox proportional hazards regression analyses were performed to assess the association of plasma vitamin C with cardiovascular mortality. Model 1: adjustment for age, sex, and smoking status. Model 2: Model 1 + adjustment for estimated Glomerular Filtration Rate, dialysis vintage, time since transplantation and proteinuria. Model 3: Model 2 + adjustment for fruit and vegetable intake. Model 4: Model 3 + adjustment for diabetes mellitus, high-sensitivity C-reactive protein and prior history of cardiovascular disease. Model 5: Model 3 + adjustment for immunosuppressive therapy. Model 6: Model 3 + adjustment for transplantation era. Abbreviations: HR, hazard ratio; CI, confidence interval.

3.4. Sensitivity Analyses

After performing graft failure-censored Cox proportional hazards regression analyses, our primary findings of the association of plasma vitamin C with both cancer mortality and cardiovascular mortality remained materially unchanged (Tables S4 and S5, respectively). After performing Cox proportional hazards regression analyses of the association of plasma vitamin C with cancer mortality with adjustment for HbA1c instead of diabetes mellitus the association remained materially unchanged (Table S6).

4. Discussion

In the current study, we show that cancer is a substantially prevalent individual cause of death after kidney transplantation, and that plasma vitamin C concentrations are inversely and independently associated with long-term cancer mortality risk in stable KTR. Secondary analyses did not reveal significant associations with cardiovascular mortality. To the best of our knowledge, this is the first study that provides prospective data supporting vitamin C as a potential risk factor for cancer mortality in KTR.

Our results are in line with previously reported cancer mortality risk data in KTR. Au et al. reported that 16.7% of deaths in a large cohort of KTR were due to cancer after a median follow-up of 6.3 (IQR, 2.3–12.0) years. Although cancer mortality has been previously described as an increasing and imperative problem in KTR [2,5,6,10], there is a paucity of studies exploring potential risk factors and underlying mechanisms leading to this increased cancer mortality in KTR. Immunosuppression following kidney transplant is the most accepted risk factor, specifically CNI [4,6,38,39]. In fact, there is extensive research focused on finding the best combination of immunosuppressants in order to reduce de novo malignancy incidence without increasing rejection rates, where m-TOR inhibitors could have a role in reducing cancer risk [6,40–42]. Noteworthy is that according to our findings, the association of plasma vitamin C concentrations with cancer mortality is independent of immunosuppressive therapies after a kidney transplant.

Low plasma vitamin C has been previously associated with gastric cancer risk in the general population. In this patient setting, mean plasma vitamin C concentration was 39.9 ± 25.2 μmol/L for cases and 41.5 ± 19.4 μmol/L for controls, both comparable to those from our study [14]. Likewise, in the general male population, low plasma vitamin C was linked to an increased risk of mortality with cancer playing a key role. In this study, median plasma vitamin C was 49.4 (IQR, 47.7–51.7) μmol/L [13], also comparable to our study. Furthermore, the anti-cancer properties from vitamin C and other antioxidants have drawn much attention in the oncology research field [43–46]. According to the results of cross-sectional analyses of our study, daily fruit intake was independently associated

with plasma vitamin C levels, congruent with evidence suggesting a diet high in fruits to be associated with decreased cancer risk in various patient settings, with antioxidants playing a key-role [47–53]. Surprisingly, our results show that the association of lower plasma vitamin C with cancer mortality risk is independent of fruit and vegetable intake, introducing vitamin C as a specific therapeutic target in this setting of patients.

A possible explanation for the association we found could be the important role that vitamin C plays as epigenetic modulator in health and disease [43–46], and specifically in cancer cell lines [54]. On the other hand, it is well known that oxidative stress can cause cancer [55,56], due to oxidative damage to deoxyribonucleic acid (DNA) [57]. This oxidative damage is usually counteracted by DNA repair enzymes, but in a pro-oxidant environment, e.g., chronic inflammation and uremic state [58,59], this defense-mechanism is held back [56,60,61]. It has been suggested that antioxidant treatment cannot prevent occurrence of gastrointestinal cancer and that it may even increase overall risk of mortality [55]. However, it has been described that kidney transplant recipients (KTR) have increased oxidative stress [19], which in turn can lead to increased oxidative damage to DNA [57]. Together with decreased immunological surveillance secondary to post-transplant immunosuppression, these phenomena can play a role in increased cancer mortality in KTR and an increased contribution of oxidative stress therein. It can therefore not be excluded that other than subjects of the general population, KTR could benefit from anti-oxidant treatment. High dosages of vitamin C supplementation have been linked to higher risk of development of oxalate kidney stones in male subjects of the general population [62,63]. Vitamin C supplementation may also enhance immunity, which could result in increased risk of rejection. Such effects could limit the utility of vitamin C supplementation in clinical practice and should be taken into account when considering vitamin C supplementation strategies in KTR. Of note, no significant interaction of the association of vitamin C with cancer mortality was found by oxidative stress biomarkers. In light of these results, it could be hypothesized that the inverse association of vitamin C with cancer mortality hereby reported may be explained by its potential role as epigenetic modulator rather than through its antioxidant properties. The latter may be further supported by the finding that plasma vitamin C was inversely associated with cancer mortality independently of fruit and vegetable intake, which suggests that the beneficial effect of vitamin C would not be fully related to the classic theory of dietary intake of natural antioxidants as anticarcinogens [53,57].

Our study has important strengths, including its large sample size of stable KTR, which were closely monitored during a considerable follow-up period by regular check-up in the outpatient clinic, without loss of participants to follow-up. Furthermore, data were extensively collected, allowing to adjust our findings for several potential confounders and predictors of the main results, including current or former smoking status. We acknowledge the study's limitations as the following. First, vitamin C was measured at baseline. Like the current study, most epidemiological studies use a single baseline measurement to predict outcomes, which adversely affects predictive properties of variables associated with outcomes [64–67]. If intra-individual variability of predictive biomarkers using repeated measurements is taken into account, this results in strengthening of predictive properties, particularly in case of markers with high intra-individual variation [64,67]. The lower the intra-individual variation from one measurement to the next would be, the more accurate the single measurement represents the usual level of the marker [64–67]. Noteworthy, evidence available for intra-individual variability of plasma vitamin C suggests that its concentrations relatively stable over time, with a single plasma vitamin C measurement being representative of an individual's status for long periods of time [65]. Moreover, previous epidemiological studies have used a baseline measurement of plasma vitamin C to predict clinical outcomes over a period of several years [68–70]. Second, we measured plasma vitamin C rather than leukocyte vitamin C, which could have provided assessment of tissue vitamin C, and therefore additional information on the role of vitamin C in disease prevention [71]. Third, initiation of vitamin C supplementation during follow-up was not recorded, which could have introduced bias that cannot be accounted for in our analyses. Fourth, incidence and types of non-fatal cancer were not documented, while this information would have been

of added value to the reported findings. With the presented data, we had no power to discriminate the association with cancer mortality by types of cancer, which does not necessarily imply that associations are similar for all types of cancer. Nevertheless, our results show, for the first time, a prospective association of plasma vitamin C with long-term risk of cancer mortality in stable kidney transplant recipients, which holds a plea for future studies in which data on incidence and types of non-fatal cancer are collected. To allow for such studies we have started a new large, long-lasting prospective cohort study in kidney transplant recipients in which collection of such data is included [72]. Another limitation is that history of cured skin cancer was not documented, it could therefore not be included in multivariable analyses. Finally, due to its observational design, conclusions on causality cannot be drawn from our results.

In conclusion, we show that cancer is a substantially prevalent individual cause of death after kidney transplantation, and that plasma vitamin C concentrations are inversely and independently associated with cancer mortality risk. Remarkably, our findings link for the first time plasma vitamin C concentrations with cancer mortality risk in KTR, which underscores that vitamin C may be a meaningful as yet overlooked modifiable risk factor of cancer mortality in KTR. Considering the relative increase in cancer mortality rates in kidney transplant recipients along with the decline in deaths due to cardiovascular causes, it is expected that novel risk management strategies are to emerge. Whether a novel vitamin C-targeted strategy may represent an opportunity to decrease the burden of cancer mortality in KTR requires further studies.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/8/12/2064/s1, Figure S1: Strobe flow diagram, Table S1: Death-related type of cancer, Table S2: Association of plasma vitamin C with cancer mortality, all models, Table S3: Interaction analyses for potential confounders on the association of vitamin C with cancer mortality, Table S4: Sensitivity analysis; association of plasma vitamin C with cancer mortality in 598 kidney transplant recipients, censored for graft-failure, Table S5: Sensitivity analysis; association of plasma vitamin C with cardiovascular mortality in 598 kidney transplant recipients, censored for graft-failure, Table S6: Sensitivity analysis; association of plasma vitamin C with cancer mortality in 598 kidney transplant recipients, with HbA1c instead of diabetes mellitus as potential confounder.

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Urinary Excretion of N^1-Methylnicotinamide, as a Biomarker of Niacin Status, and Mortality in Renal Transplant Recipients

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Abstract: Renal transplant recipients (RTR) commonly suffer from vitamin B_6 deficiency and its functional consequences add to an association with poor long-term outcome. It is unknown whether niacin status is affected in RTR and, if so, whether this affects clinical outcomes, as vitamin B_6 is a cofactor in nicotinamide biosynthesis. We compared 24-h urinary excretion of N^1-methylnicotinamide (N^1-MN) as a biomarker of niacin status in RTR with that in healthy controls, in relation to dietary intake of tryptophan and niacin as well as vitamin B_6 status, and investigated whether niacin status is associated with the risk of premature all-cause mortality in RTR. In a prospective cohort of 660 stable RTR with a median follow-up of 5.4 (4.7–6.1) years and 275 healthy kidney donors, 24-h urinary excretion of N^1-MN was measured with liquid chromatography-tandem mass spectrometry LC-MS/MS. Dietary intake was assessed by food frequency questionnaires. Prospective associations of N^1-MN excretion with mortality were investigated by Cox regression analyses. Median N^1-MN excretion was 22.0 (15.8–31.8) µmol/day in RTR, compared to 41.1 (31.6–57.2) µmol/day in healthy kidney donors ($p < 0.001$). This difference was independent of dietary intake of tryptophan (1059 ± 271 and 1089 ± 308 mg/day; $p = 0.19$), niacin (17.9 ± 5.2 and 19.2 ± 6.2 mg/day; $p < 0.001$), plasma vitamin B_6 (29.0 (17.5–49.5), and 42.0 (29.8–60.3) nmol/L; $p < 0.001$), respectively. N^1-MN excretion was inversely associated with the risk of all-cause mortality in RTR (HR 0.57; 95% CI 0.45–0.71; $p < 0.001$), independent of potential confounders. RTR excrete less N^1-MN in 24-h urine than healthy controls, and our data suggest that this difference cannot be attributed to lower dietary intake of tryptophan and niacin, nor vitamin B_6 status. Importantly, lower 24-h urinary excretion of N^1-MN is independently associated with a higher risk of premature all-cause mortality in RTR.

Keywords: urinary excretion of N^1-methylnicotinamide; kidney transplantation; mortality; niacin status; dietary intake; tryptophan; vitamin B_3

1. Introduction

Kidney transplantation is the preferred treatment for end-stage renal disease in terms of survival, quality of life and costs [1,2]. Advances in transplantation medicine have lifted the 1-year patient survival higher than 90% [3]. While short-term patient outcomes are continuing to improve, the long-term posttransplant survival has remained largely unchanged over the past few decades [4]. Compared with the general population, renal transplant recipients (RTR) are at highly increased risk of premature mortality [5]. Improving perspectives relies on the management of modifiable factors that impact long-term outcome in RTR, of which nutrition is increasingly acknowledged [6,7].

Recently, we found that RTR commonly suffer from vitamin B_6 deficiency and its functional consequences that add to an association with poor long-term outcomes [8]. As vitamin B_6 is an essential cofactor of key enzymes involved in de novo biosynthesis of nicotinamide from tryptophan [9], niacin deficiency might be lurking in these patients as well. Nicotinamide, nicotinic acid, and nicotinamide riboside are collectively referred to as niacin or vitamin B_3, and are precursors of the metabolically active NAD^+. Besides dietary intake of pre-formed niacin, the so-called tryptophan-nicotinamide pathway is critical to maintaining niacin status [10]. Ongoing NAD^+ supply from its metabolic precursors, collectively referred to as "niacin equivalents", is required to provide reducing equivalents for energy metabolism and substrates of NAD^+ consuming enzymes [11]. NAD^+ is catabolized to N^1-methylnicotinamide (N^1-MN) through methylation of nicotinamide in the liver, and the 24-h urinary excretion of N^1-MN is considered the most reliable biomarker of niacin status [12–14].

It is unknown whether niacin status is affected in RTR and, if so, whether this affects clinical outcomes. Hence, this study aims to compare 24-h urinary excretion of N^1-MN in RTR with that in healthy kidney donors, in relation to dietary intake of tryptophan and niacin as well as vitamin B_6 status, and to investigate whether niacin status is associated with the risk of premature all-cause mortality in RTR.

2. Materials and Methods

2.1. Study Population

This prospective study was conducted in a well-characterized, single-center cohort of 707 RTR (aged ≥18 years) with a functioning graft for at least 1 year who visited the outpatient clinic of the University Medical Center Groningen, Groningen, the Netherlands, between 2008 and 2011 [15–17]. As a control group, 367 healthy kidney donors were included who participated in a screening program before kidney donation. Signed informed consent was obtained from all participating subjects and the study protocol was approved by the institutional review board (METc 2008/186) adhering to the Declaration of Helsinki. Exclusion of subjects with missing biomaterial or niacin supplementation use left 660 RTR and 275 kidney donors eligible for statistical analyses (Figure S1).

2.2. Data Collection

All baseline measurements were obtained during a morning visit to the outpatient clinic. Participants were instructed to collect a 24-h urine sample on the day before their visit, and to fast overnight for 8 to 12 h. Urine samples were collected under oil, and chlorhexidine was added as an antiseptic agent. Fasting blood samples were drawn after completion of the urine collection. Blood was collected in a series of evacuated tubes with different additives (Vacutainer®; BD, Franklin Lakes, NJ, USA) for preparation of plasma and serum. Body composition and hemodynamic parameters were measured according to a previously described, strict protocol [15]. Serum parameters, including lipid, inflammation, and glucose homeostasis variables were measured with spectrophotometric-based routine clinical laboratory methods (Roche Diagnostics, Rotkreuz, Switzerland). Diabetes was diagnosed if fasting plasma glucose was ≥7.0 mmol/L or antidiabetic medication was used [15]. Plasma vitamin B_6 was determined as its principal, metabolically active form pyridoxal-5′-phosphate using a

HPLC method (Waters Alliance, Milford, MA, USA) with fluorescence detection (JASCO, Inc., Easton, MD, USA) [8].

Renal function was assessed by estimation of the glomerular filtration rate (eGFR) and detection of proteinuria. The eGFR was calculated using the combined creatinine and cystatin C-based Chronic Kidney Disease Epidemiology Collaboration equation [18], which has been shown to be the most accurate equation in RTR [19]. Proteinuria was diagnosed if total urinary protein excretion was ≥0.5 g/day as measured by a biuret reaction-based assay (MEGA AU510; Merck Diagnostica, Darmstadt, Germany).

Dietary intake including tryptophan and niacin intakes was assessed with a validated semi-quantitative food frequency questionnaire (FFQ) [20–22]. The self-administered questionnaire was filled out at home and inquired about 177 food items during the last month, taking seasonal variations into account. During the visit to the outpatient clinic, the FFQ was checked for completeness by a trained researcher and inconsistent answers were verified with the participant. The FFQ was validated for RTR as previously reported [16]. Dietary data were converted into daily nutrient intake using the Dutch Food Composition Table of 2006 [23]. Alcohol consumption and smoking behavior were assessed with a separate questionnaire [6]. Additional data on medical history and use of medication and vitamin supplements were obtained from medical records [6].

2.3. Assessment of N^1-MN Excretion

Measurement of N^1-MN concentration was performed with a validated liquid chromatography (Luna HILIC column; Phenomenex, Torrance, CA, USA) isotope dilution-tandem mass spectrometry (LC-MS/MS) (Quattro Premier; Waters, Milford, MA, USA) method, as described previously [24]. The 24-h urinary excretion of N^1-MN (μmol/day) was obtained after multiplying N^1-MN concentration (μmol/L) by total urine volume calculated from weight (L/day). The reference range of N^1-MN excretion in healthy individuals was previously established at 17.3–115 μmol/day [24].

2.4. Clinical Endpoints

The primary outcome of this study was all-cause mortality which was recorded until 30 September 2015 with no loss due to follow-up. RTR status was kept up-to-date through the continuous surveillance system of the outpatient program.

2.5. Statistical Analysis

Data are presented as the mean ± SD, median (IQR) and absolute number (percentage) for normally distributed, skewed, and nominal data, respectively. Assumptions for normality were checked by visual judgments of the corresponding frequency distribution and Q-Q plot.

Baseline characteristics of RTR and healthy kidney donors were compared by means of *t*, Mann-Whitney, and Chi-Square tests. Niacin status in RTR and healthy kidney donors was compared by linear regression analyses of 2-base log-transformed N^1-MN excretion, with subsequent cumulative adjustment for age and sex (model 1), eGFR (model 2) and intake of energy, tryptophan, and niacin and plasma vitamin B_6 (model 3).

RTR characteristics were divided into tertiles of N^1-MN excretion stratified by sex (T1, T2, and T3) and compared by means of ANOVA, Kruskal-Wallis, and Chi-Square tests.

For prospective analyses, a Cox proportional hazards regression model for all-cause mortality outcome was fitted to N^1-MN excretion as a sex-stratified tertile-based categorical variable, as well as a continuous variable adjusted for sex (model 1). Confounding was controlled for by including potential confounders as covariates in the regression model. Crude associations were adjusted cumulatively for age (model 2), smoking and body surface area (model 3) and, to prevent overfitting, additionally for intake of alcohol and energy and plasma vitamin B_6 (model 4), kidney function (model 5), medication use (model 6), and high-sensitivity C-reactive protein (hs-CRP) (model 7). Variables that could lie in the causal pathway of N^1-MN excretion and all-cause mortality were not adjusted for because this

might obscure otherwise existing associations unintentionally. Assumptions of proportionality of the hazard functions and the linearity of log-hazards were checked by visual judgements of Kaplan Meier plots of the survival and log-survival function entering the sex-stratified N^1-MN excretion tertile group variable.

In secondary analyses, effect modification was assessed by including the cross product term of each potential confounder and 2-base log-transformed N^1-MN excretion in the Cox regression model adjusted for age and sex (model 2). Subsequent stratified analyses were performed for subgroups of significant effect modifiers on the association of N^1-MN excretion with all-cause mortality.

For all statistical analyses, a two-sided p-value of less than 0.05 was considered to indicate statistical significance and SPSS Statistics version 23.0 (IBM, Armonk, NY, USA) was used as software.

3. Results

3.1. Baseline Characteristics and Comparison of N^1-MN Excretion

This study included 660 stable RTR (57% male; mean age 53.0 ± 12.7 years), at a median time of 5.6 (2.0–12.0) years after transplantation, and 275 healthy kidney donors (41% male; mean age 53.3 ± 10.7 years) (Table 1). Intake of tryptophan was similar in both groups (1059 ± 271 and 1089 ± 308 mg/day, respectively; $p = 0.19$), while intake of niacin was lower in RTR than in kidney donors (17.9 ± 5.2 and 19.2 ± 6.2 mg/day, respectively; $p = 0.01$). Taken together, intake of niacin equivalents was lower in RTR than in kidney donors (35.6 ± 9.2 mg/day and 37.4 ± 10.8, respectively; $p = 0.03$) (Figure 1). All RTR and kidney donors complied with the recommended daily intake that is set at a minimum of 6.6 niacin equivalents per 1000 kcal (≥ 9.6 and ≥ 11.7 mg/1000 kcal, respectively) [12]. As previously reported, RTR had significantly lower plasma vitamin B_6 compared to kidney donors (29.0 (17.5–49.5) and 42.0 (29.8–60.3) nmol/L, respectively; $p < 0.001$). Median N^1-MN excretion was 22.0 (15.8–31.8) µmol/day in RTR, compared to 41.1 (31.6–57.2) µmol/day in kidney donors ($p < 0.001$) (Figure 1). Furthermore, urinary excretion of N^1-MN was below the reference limit of 17.3 µmol/day in 202 (31%) RTR, against 4 (2%) kidney donors. The difference in N^1-MN excretion between RTR and kidney donors was independent of age, sex, eGFR, intake of energy, tryptophan, and niacin and plasma vitamin B_6 (Table 2). Cyclosporine, azathioprine, and anticonvulsants were used by, respectively, 253 (38%), 112 (17%) of 19 (3%) of RTR, and none of the controls received drugs that are known to potentially affect niacin status.

Table 1. Baseline characteristics of stable RTR compared to that in healthy kidney donors [1].

Variable	Donors $n = 275$	RTR $n = 660$	p-Value [2]
Age, years	53.3 ± 10.7	53.0 ± 12.7	0.68
Male, *n* (%)	112 (41)	379 (57)	0.001
Body surface area, m^2	1.9 ± 0.2	1.9 ± 0.2	0.90
Current smoker, *n* (%)	39 (14)	78 (12)	<0.001
Alcohol intake, g/day	6.7 (1.1–16.4)	3.1 (0.1–11.9)	<0.001
Energy intake, kcal/day	2295 ± 746	2182 ± 642	0.04
Niacin equivalents intake, mg/day [3]	37.4 ± 10.8	35.6 ± 9.2	0.03
Tryptophan intake, mg/day	1089 ± 308	1059 ± 271	0.19
Niacin intake, mg/day	19.2 ± 6.2	17.9 ± 5.2	0.01
N^1-MN excretion, µmol/day	41.4 (31.6–57.2)	22.0 (15.8–31.8)	<0.001
<17.3 µmol/day, *n* (%)	4 (2)	202 (31)	0.03
Plasma vitamin B_6 (nmol/L)	42.0 (29.8–60.3)	29.0 (17.5–49.5)	<0.001
Systolic blood pressure, mmHg	125.1 ± 13.9	135.8 ± 17.3	<0.001
Diastolic blood pressure, mmHg	75.6 ± 9.1	82.5 ± 11.0	<0.001
Triglycerides, mmol/L	1.2 (0.9–1.7)	1.7 (1.2–2.3)	<0.001
HbA1c, (%)	5.6 (5.4–5.8)	5.8 (5.5–6.2)	<0.001

Table 1. *Cont.*

Variable	Donors $n = 275$	RTR $n = 660$	p-Value [2]
eGFR, ml/min/1.73 m^2	91.0 ± 14.2	53.0 ± 20.0	<0.001
Acetylsalicylic acid, *n* (%)	4 (2)	127 (19)	<0.001
Proton pump inhibitor, *n* (%)	5 (2)	326 (49)	<0.001
Diuretic, *n* (%)	9 (3)	261 (40)	<0.001

[1] Data are presented as mean ± SD, median (IQR) and absolute number (percentage) for normally distributed, skewed and nominal data, respectively. [2] p-value for difference was tested by t and Mann-Whitney tests for normally and skewed distributed continuous variables, respectively, and Chi-Square tests for nominal variables. [3] Intake of niacin equivalents was calculated by adding up niacin and one-sixtieth of tryptophan intake. Subjects who were using niacin supplementation were excluded. eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; N^1-MN, N^1-methylnicotinamide; RTR, renal transplant recipients.

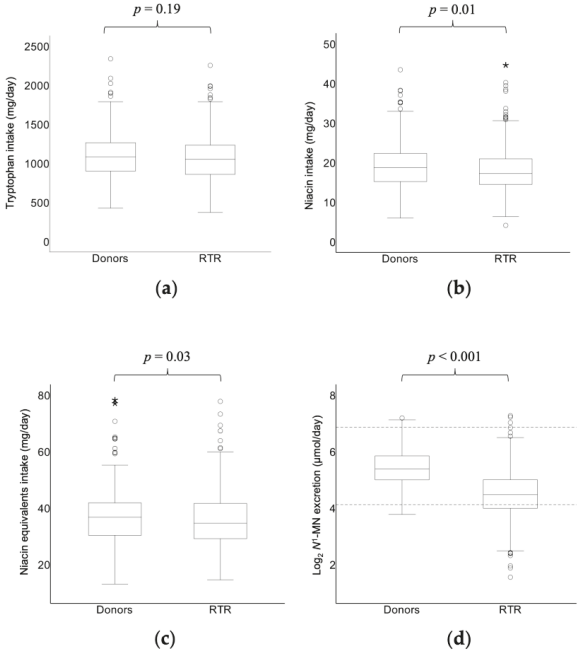

Figure 1. Box plots of dietary intake of (**a**) tryptophan, (**b**) niacin and (**c**) niacin equivalents and (**d**) $\log_2$ 24-h urinary excretion of N^1-MN in RTR compared to that in healthy kidney donors. Boxes, bars and whiskers represent IQRs, medians and values <1.5 × IQR, respectively, whereas outliers (1.5–3 × IQR) are indicated by circles and extreme outliers (>3 × IQR) by asterisks. Log_2 of the lower and upper bound of the reference range of N^1-MN excretion in healthy individuals (17.3–115.0) µmol/day [24] are indicated with dotted lines (**d**). p-value for difference between RTR and donors was tested by t and Mann-Whitney tests for normally and skewed distributed continuous variables, respectively. Intake of niacin equivalents was calculated by adding up niacin and one-sixtieth of tryptophan intake. N^1-MN, N^1-methylnicotinamide; RTR, renal transplant recipients.

Table 2. Association of RTR and healthy kidney donors grouping with N^1-MN excretion [1].

Variable	Model 1 [2]		Model 2 [3]		Model 3 [4]		Model 4 [5]	
	Std.β	*p*-Value	Std.β	*p*-Value	Std.β	*p*-Value	Std.β	*p*-Value
Grouping	−0.42	<0.001	−0.44	<0.001	−0.25	<0.001	−0.21	<0.001
Sex	-	-	−0.15	<0.001	−0.14	<0.001	−0.10	0.002
Age, years	-	-	−0.16	<0.001	−0.11	<0.001	−0.07	0.02
eGFR, ml/min/1.73 m^2	-	-	-	-	0.31	<0.001	0.29	<0.001
Energy intake, kcal/day	-	-	-	-	-	-	−0.10	0.08
Tryptophan intake, mg/day	-	-	-	-	-	-	0.007	0.91
Niacin intake, mg/day	-	-	-	-	-	-	0.25	<0.001
Plasma vitamin B_6, nmol/L	-	-	-	-	-	-	0.23	<0.001
R^2	0.18		0.23		0.28		0.37	

[1] Linear regression analyses were performed to investigate the association of RTR and healthy kidney donors as grouping variable with N^1-MN excretion, with adjustment for potential confounders. [2] Model 1: crude model. [3] Model 2: adjusted for age and sex. [4] Model 3: adjusted as for model 2 and for eGFR. [5] Model 4: adjusted as for model 3 and for intake of energy, tryptophan and niacin and plasma vitamin B_6. eGFR, estimated glomerular filtration rate; N^1-MN, N^1-methylnicotinamide; RTR, renal transplant recipients; std.β, standardized beta coefficient.

RTR characteristics across tertiles of sex-stratified N^1-MN excretion (M: <19.2, 19.2–28.8, >28.8 µmol/day; F: <16.1, 16.1–25.6, >25.6 µmol/day in T1, T2, and T3, respectively) are shown in Table 3. Age and the presence of acetylsalicylic acid, proton pump inhibitors, diuretics and post mortem donors were lower with increasing tertiles of N^1-MN excretion, while intake of alcohol, energy, tryptophan and niacin, plasma vitamin B_6, kidney function and the presence of proliferation inhibitors and primary glomerular disease were higher with increasing tertiles of N^1-MN excretion.

Table 3. Baseline characteristics of RTR across tertiles of N^1-MN excretion stratified by sex [1].

Variable	Tertiles of Sex-Stratified N^1-MN Excretion			*p*-Value [2]
	T1 *n* = 219	T2 *n* = 221	T3 *n* = 220	
Males, µmol/day	<19.2	19.2–28.8	>28.8	
Females, µmol/day	<16.1	16.1–25.6	>25.6	
Male, *n* (%)	126 (58)	127 (58)	126 (57)	-
Age, years	54.6 ± 12.7	53.7 ± 13.1	50.7 ± 12.1	0.004
BMI, kg/m^2	25.8 (22.7–29.4)	26.1 (23.3–29.0)	26.0 (23.6–29.6)	0.41
Body surface area, m^2	1.9 ± 0.2	1.9 ± 0.2	2.0 ± 0.2	0.13
Lifestyle				
Current smoker, *n* (%)	21 (10)	25 (11)	32 (15)	0.26
Alcohol consumption, g/day	0.5 (0.0–7.0)	3.2 (0.1–11.3)	6.7 (0.8–20.9)	<0.001
Vegetarian, *n* (%)	7 (3)	2 (1)	3 (1)	0.16
Dietary intake				
Energy, kcal/day	2065 ± 586	2197 ± 675	2285 ± 647	0.002
Tryptophan, mg/day	1001 ± 253	1063 ± 273	1112 ± 274	<0.001
Niacin, mg/day	16.6 ± 4.9	17.6 ± 4.8	19.5 ± 5.5	<0.001
Plasma vitamin B_6, nmol/L	20.3 (14.0–39.0)	29.5 (19.0–47.0)	39.0 (22.0–65.0)	<0.001
Hemodynamic				
Systolic blood pressure, mmHg	139 ± 18	134 ± 18	135 ± 16	0.01
Diastolic blood pressure, mmHg	83 ± 11	82 ± 12	83 ± 11	0.20
Mean arterial pressure, mmHg	109 ± 15	106 ± 15	106 ± 14	0.07
Heart rate, beats per minute	69 ± 11	68 ± 12	68 ± 12	0.52
Antihypertensive use, *n* (%)	199 (91)	193 (87)	189 (86)	0.26

Table 3. *Cont.*

Variable	Tertiles of Sex-Stratified N^1-MN Excretion			p-Value [2]
	T1 n = 219	T2 n = 221	T3 n = 220	
Lipids				
Total cholesterol, mmol/L	5.1 ± 1.2	5.2 ± 1.1	5.0 ± 1.1	0.36
HDL, mmol/L	1.3 (1.0–1.6)	1.3 (1.1–1.6)	1.3 (1.1–1.7)	0.06
LDL, mmol/L	3.0 ± 0.9	3.1 ± 0.9	2.9 ± 0.9	0.31
Triglycerides, mmol/L	1.7 (1.3–2.3)	1.7 (1.3–2.3)	1.6 (1.1–2.2)	0.03
Statin, *n* (%)	122 (56)	115 (52)	112 (51)	0.55
Glucose homeostasis				
Glucose, mmol/L	5.3 (4.8–6.0)	5.3 (4.8–5.9)	5.2 (4.7–6.2)	0.58
HbA1c, (%)	5.8 (5.5–6.3)	5.9 (5.6–6.1)	5.7 (5.4–6.1)	0.05
Diabetes, *n* (%)	58 (27)	44 (20)	50 (23)	0.26
Antidiabetic, *n* (%)	41 (19)	28 (13)	27 (12)	0.10
Other serum parameters				
Hs-CRP, mg/L	1.7 (0.8–5.3)	1.6 (0.6–3.8)	1.4 (0.7–4.6)	0.42
Phosphate, mmol/L	1.0 ± 0.2	1.0 ± 0.2	0.9 ± 0.2	0.01
Immunosuppressant medication				
Prednisolon dose, mg/day	10 (7.5–10)	10 (7.5–10)	10 (7.5–10)	0.18
Calcineurin inhibitor, *n* (%)	136 (62)	125 (57)	112 (51)	0.06
Cyclosporine, *n* (%)	87 (40)	82 (37)	84 (38)	0.85
Azathioprine, *n* (%)	35 (16)	36 (16)	41 (19)	0.72
Proliferation inhibitor, *n* (%)	171 (78)	186 (84)	191 (87)	0.04
Other medication				
Acetylsalicylic acid, *n* (%)	55 (25)	47 (21)	25 (11)	0.001
Anticonvulsant, *n* (%)	7 (3)	5 (2)	7 (3)	0.80
Proton pump inhibitor, *n* (%)	127 (58)	107 (48)	92 (42)	0.003
Diuretic, *n* (%)	104 (48)	79 (36)	78 (36)	0.01
Kidney function				
Serum creatinine, µmol/L	138 (104–189)	122 (101–153)	114 (94–140)	<0.001
Cystatin C, mg/L	2.0 (1.4–2.8)	1.6 (1.3–2.1)	1.4 (1.2–1.9)	<0.001
eGFR, ml/min/1.73 m^2	39.0 ± 18.7	45.8 ± 16.9	52.7 ± 18.0	<0.001
Proteinuria ≥ 0.5 g/day, *n* (%)	55 (25)	39 (18)	38 (17)	0.07
Kidney transplantation				
Time since transplantation, years	5.6 (1.7–12.9)	5.0 (1.5–11.0)	6.5 (2.9–12.3)	0.16
Donor				
Age, years	46 (33–54)	47 (29–57)	43 (29–53)	0.22
Male, *n* (%)	104 (48)	110 (50)	112 (51)	0.60
Post mortem status, *n* (%)	161 (74)	143 (65)	121 (55)	<0.001
Primary kidney disease				
Primary glomerular disease, *n* (%)	48 (22)	67 (30)	71 (32)	0.04
Glomerulonephritis, *n* (%)	12 (6)	17 (8)	21 (10)	0.27
Tubulointerstitial disease, *n* (%)	27 (12)	30 (14)	20 (9)	0.32
Polycystic renal disease, *n* (%)	52 (24)	45 (20)	40 (18)	0.35
Dysplasia and hypoplasia, *n* (%)	9 (4)	10 (5)	9 (4)	0.97
Renovascular disease, *n* (%)	17 (8)	8 (4)	11 (5)	0.15
Diabetic nephropathy, *n* (%)	15 (7)	7 (3)	13 (6)	0.20
Other or unknown cause, *n* (%)	39 (18)	36 (16)	35 (16)	0.85

[1] Data are presented as mean ± SD, median (IQR) and absolute number (percentage) for normally distributed, skewed and nominal data, respectively. [2] p-value for difference was tested by ANOVA and Kruskal-Wallis tests for normally and skewed distributed continuous variables, respectively, and Chi-Square tests for nominal variables. eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; hs-CRP, high-sensitivity C-reactive protein; N^1-MN, N^1-methylnicotinamide; RTR, renal transplant recipients.

3.2. N^1-MN Excretion and Mortality

During a median follow-up time of 5.4 (4.7–6.1) years, 143 (22%) RTR died. The risk of all-cause mortality increased with lower tertiles of N^1-MN excretion, as depicted by Kaplan-Meier curves (Figure 2). Cox regression analyses revealed an inverse association of N^1-MN excretion with all-cause mortality (Model 2: HR 0.57; 95% CI 0.45–0.71; $p < 0.001$), independent of potential confounders (Table 4). The same held for analyses across tertiles of sex-stratified N^1-MN excretion (Table 4). RTR in the lowest and middle tertiles were at higher risk of all-cause mortality compared to those in the highest tertile as reference (Model 2: HR 2.68; 95% CI 1.67–4.33; $p < 0.001$ and HR 2.04; 95% CI 1.25–3.34; $p = 0.004$, respectively), independent of potential confounders (Table 4).

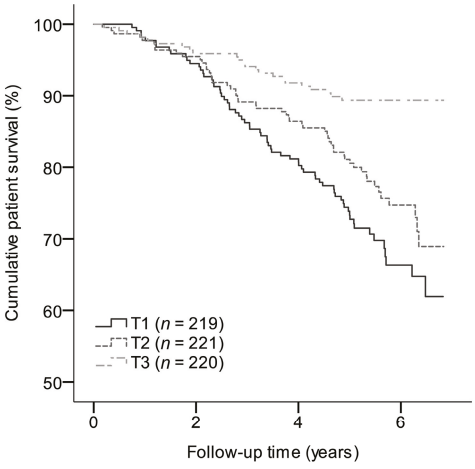

Figure 2. Survival curves for all-cause mortality in RTR according to tertiles of sex-stratified N^1-MN excretion. N^1-MN excretion was <19.2, 19.2–28.8, and >28.8 µmol/day for males, and <16.1, 16.1–25.6 and >25.6 µmol/day for females in T1, T2, and T3, respectively. N^1-MN, N^1-methylnicotinamide; RTR, renal transplant recipients.

Table 4. Association of N^1-MN excretion with risk of all-cause mortality in RTR [1].

Model	N^1-MN Excretion (log_2) As Continuous Variable n = 660		Tertiles of Sex-Stratified N^1-MN Excretion [2]				
			T1 n = 219		T2 n = 221		T3 n = 220
	HR (95% CI)	p-Value	HR (95% CI)	p-Value	HR (95% CI)	p-Value	Reference HR
1 [3]	0.53 (0.43–0.65)	<0.001	3.28 (2.04–5.26)	<0.001	2.41 (1.48–3.93)	<0.001	1.00
2 [4]	0.57 (0.45–0.71)	<0.001	2.68 (1.67–4.33)	<0.001	2.04 (1.25–3.34)	0.004	1.00
3 [5]	0.59 (0.47–0.74)	<0.001	2.65 (1.60–4.39)	<0.001	2.10 (1.25–3.52)	0.005	1.00
4 [6]	0.69 (0.53–0.90)	0.005	2.10 (1.17–3.78)	0.01	2.04 (1.15–3.63	0.02	1.00
5 [7]	0.75 (0.58–0.96)	0.02	1.86 (1.07–3.25)	0.02	1.80 (1.04–3.13)	0.04	1.00
6 [8]	0.65 (0.51–0.82)	<0.001	2.25 (1.35–3.75)	0.002	2.06 (1.23–3.46)	0.006	1.00
7 [9]	0.60 (0.48–0.76)	<0.001	2.59 (1.54–4.35)	<0.001	2.13 (1.26–3.61)	0.005	1.00
Events (n)	143		67		53		23

[1] Cox regression analyses were performed to investigate the association of N^1-MN excretion with risk of all-cause mortality in RTR, with adjustment for potential confounders. [2] N^1-MN excretion was <19.2, 19. 2–28.8, and >28.8 µmol/day for males, and <16.1, 16.1–25.6, and >25.6 µmol/day for females in T1, T2, and T3, respectively. [3] Model 1: not adjusted in tertiles of sex-stratified N^1-MN excretion, adjusted for sex in continuous analyses. [4] Model 2: adjusted as for model 1 and for age. [5] Model 3: adjusted as for model 2 and for smoking and body surface area. [6] Model 4: adjusted as for model 3 and for intake of alcohol and energy and plasma vitamin B_6. [7] Model 5: adjusted as for model 3 and for eGFR, proteinuria, donor status and primary glomerular disease. [8] Model 6: adjusted as for model 3 and for use of proliferation inhibitors, acetylsalicylic acid, proton pump inhibitors and diuretics. [9] Model 7: adjusted as for model 3 and for hs-CRP. eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; N^1-MN, N^1-methylnicotinamide; RTR, renal transplant recipients.

Secondary analyses exposed significant effect modification of hs-CRP on the association of N^1-MN excretion with all-cause mortality ($p = 0.05$), independent of age and sex. The inverse association of N^1-MN excretion with all-cause mortality was stronger for individuals in the subgroup with serum hs-CRP <2.4 mg/L (HR 0.47; 95% CI 0.35–0.64; $p < 0.001$), than in the subgroup with serum hs-CRP ≥2.4 mg/L (HR 0.70; 95% CI 0.50–0.96; $p = 0.03$) according to subsequent stratified analysis.

4. Discussion

In this large prospective cohort study, we showed that RTR excrete less N^1-MN in 24-h urine than healthy controls and our data suggest that this difference cannot be attributed to lower dietary intake of tryptophan and niacin, nor vitamin B_6 status. Furthermore, lower 24-h urinary excretion of N^1-MN as a biomarker of niacin status was independently associated with a higher risk of premature all-cause mortality in RTR.

To the best of our knowledge, niacin status has not been studied within the context of kidney transplantation and its concomitant long-term implications yet. In fact, prospective data on the urinary excretion of N^1-MN have been limited to one previous study in patients recovering from leukemia treatment [25]. Studies on niacin nutrition in relation to prospective outcomes are likewise scarce, as the prevailing intake of niacin equivalents is suggested to be not sufficiently low to compromise survival. Presumed health benefits of niacin are pharmacological rather than physiological [26–29], although higher survival with higher niacin intake in elderly has been reported previously [30] in congruence with our findings.

Niacin is considered the least critical vitamin to meet the recommendations through dietary intake in western societies [31], as niacin equivalents are found in a wide range of foods [12]. In line with this, dietary intake of niacin equivalents was sufficient according to WHO guidelines in all RTR and healthy kidney donors, while we found that urinary excretion of N^1-MN was commonly below the established reference bound in RTR. The observed disparity of N^1-MN excretion between RTR and healthy kidney donors could moreover not be explained by lower dietary intake of niacin equivalents in RTR in the present study.

The fact that we found a positive association of plasma vitamin B_6 concentration with N^1-MN excretion strengthens our hypothesis that inadequacies of this cofactor might affect niacin status in RTR. Adjustment for plasma vitamin B_6, however, neither did alter the discrepancy of N^1-MN excretion between RTR and healthy kidney donors. Therefore, one should consider other factors that could interfere with N^1-MN excretion as a biomarker of niacin status, and add to poor long-term outcome in RTR.

Whereas secondary dietary inadequacies may interrupt niacin metabolism, this also holds for certain medications including specific antituberculosis, anticonvulsant and antiproliferative drugs, as well as cyclosporine and azathioprine [32–34], which are common immunosuppressant drugs in RTR, although in our population those did not appear to affect N^1-MN excretion.

We can furthermore speculate on the presence of enhanced consumption of tryptophan for protein biosynthesis at the cost of niacin status in RTR. Interestingly, tryptophan is argued to be quantitatively the most important NAD^+ precursor, as it is more effective in elevating liver NAD^+ and urinary excretion of N^1-MN than the salvageable precursors [35–38]. The tryptophan-nicotinamide pathway is, however, mainly regulated by tryptophan intake rather than niacin status, since the generally accepted conversion ratio of 60:1 falls when dietary tryptophan is limiting [39]. Indeed, tryptophan is used primarily for protein biosynthesis and only after nitrogen balance has been achieved for the nicotinamide pathway [40]. This allows us to speculate on protein catabolism and negative protein balance as part of protein-energy wasting in RTR, engendered by metabolic derangement, systemic inflammation, acidemia, and the use of immunosuppressive drugs, to induce tryptophan consumption for protein synthesis in this population [41,42]. However, as our study was not designed to assess protein-energy wasting, we cannot conclusively address such an effect on N^1-MN excretion in RTR.

On the contrary, the tryptophan-nicotinamide pathway is implicated in disease states in which systemic inflammation is present, by the enhanced action of indoleamine 2,3-dioxygenase in response to inflammatory cytokines and mediators. This upregulation of tryptophan degradation towards nicotinamide is known to yield relativity large amounts of quinolinic acid to fuel NAD^+-consuming poly (ADP-ribose) polymerase (PARP) reaction in response to immune-related (oxidative) damage [35]. Although we observed lower serum hs-CRP levels as a low-grade inflammation biomarker with higher tertiles of N^1-MN excretion, this difference did not reach significance.

Finally, the renal clearance of N^1-MN itself can also be affected by several factors and not in the least by impaired kidney function. In fact, N^1-MN is eliminated almost exclusively by the kidneys, being partly excreted partly by glomerular filtration and partly by tubular secretion with negligible and saturable tubular reabsorption [43]. Whereas renal clearance of N^1-MN has been investigated as a model of renal secretory function [43] and to predict renal clearance of cationic drugs in renal insufficiency [44], plasma concentrations are suggested to be less sensitive to kidney function because of the contribution of aldehyde oxidase to N^1-MN clearance, yielding N^1-methyl-2-pyridone-5-carboxamide (2Py) [45]. Although our findings appeared independent of kidney function, future studies are warranted to rule out enhanced oxidative metabolism, causing a shift towards urinary excretion of 2Py in this population.

Regarding potential mechanisms for the association of N^1-MN excretion with mortality, NAD^+ homeostasis has been linked to increased resistance against a range of pathophysiological processes that are predominant and impact poor long-term outcome in RTR, including cardiovascular, inflammatory, malignant and metabolic disorders [46]. The availability of NAD^+ is determined by its production from niacin equivalents, as well as its degradation in NAD^+ consuming activities [47]. NAD^+ levels remain constant when used as a coenzyme, being recycled back and forth between its oxidized and reduced forms [11], but are depleted by three distinct classes of enzymes that consume NAD^+ as a substrate: PARP, cyclic ADP ribose synthases (CD38 and CD157), and sirtuins [48]. Excessive activation of PARP and CD38 is induced by stresses such as inflammation, oxidative stress and DNA damage that are predominant in in RTR [48,49]. As a result, NAD^+ availability might become limiting for beneficial sirtuin activities; in particular with lower niacin status. These beneficial effects of sirtuins have been described more specifically for renal diseases, including renoprotective effects by inhibition of renal cell apoptosis, inflammation, and fibrosis and regulation of mitochondrial function and glucose, lipid, and energy metabolism [50–53].

Whereas we did not find an association of N^1-MN excretion with hs-CRP, this low-grade inflammation biomarker appeared to affect the magnitude of the inverse association of N^1-MN excretion with all-cause mortality. Although we can only speculate on the underlying mechanism, earlier mentioned inflammation-related overconsumption of NAD^+ limiting its downstream beneficial activities might at least partly explain the lower protective effect of niacin status on mortality in the subgroup with higher serum hs-CRP levels.

The current study should be interpreted within its strengths and limitations. First, its observational nature prohibits causal inferences, but it also did not allow us to draw conclusions on underlying mechanisms of lower N^1-MN excretion in RTR and its contribution to worse survival. Second, the generalizability of our findings might be compromised by overrepresentation of Caucasian individuals from a single center, despite being controlled for by the inclusion of a large, representative control group. Third, the reliability of FFQ data is subject to sources of measurement error, including recall and social desirability biases and limitations in food composition databases [54]. Higher similarity in dietary sources could be achieved by including spouses as a control group. Finally, the present study is confined to the 24-h urinary excretion of N^1-MN as the recommended biomarker of niacin nutritional status by authorities, including the WHO and the European Food Safety Authority [12–14]. Future studies are, however, encouraged to elaborate on plasma concentrations of niacin and its metabolites, or NAD^+ and the ratio of NAD^+ to $NADP^+$ in erythrocytes as additional indices of niacin status. Although observational evidence is inherent to limitations, prospective cohort studies provide a strong design to address nutritional status and health outcome associations over a long period of

time. Strengths of our study include its large sample size, with a sufficient number of incident cases and no loss to follow-up, and therefore minimizing the risk of bias in the assessment of outcome. The extensive characterization of participants, moreover, allowed us to control for confounding and effect modification in estimates of the effect.

5. Conclusions

In conclusion, 24-h urinary excretion of N^1-MN as a biomarker of niacin status is lower in RTR than in healthy controls, and other factors than dietary intake of niacin equivalents and vitamin B_6 status appear to reinforce this discrepancy. Importantly, 24-h urinary excretion of N^1-MN is inversely associated with a higher risk of premature all-cause mortality in RTR and niacin status is therefore revealed as a potential target for nutritional strategies to improve long-term outcome after kidney transplantation. However, further research is warranted to unravel underlying mechanisms that potentially interfere with N^1-MN excretion in RTR, and to strengthen causal inferences for health outcomes to support dietary recommendation.

Supplementary Materials: The following is available online at http://www.mdpi.com/2077-0383/8/11/1948/s1, Figure S1: Flow of participants through study protocol.

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Validation of Identified Susceptible Gene Variants for New-Onset Diabetes in Renal Transplant Recipients

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Abstract: Genome-wide association studies (GWAS) and candidate gene approaches have identified single nucleotide polymorphisms (SNPs) associated with new-onset diabetes after renal transplantation (NODAT). We evaluated associations between NODAT and SNPs identified in previous studies. We genotyped 1102 renal transplant recipients from the Korean Organ Transplantation Registry (KOTRY) database; 13 SNPs were assessed for associations with NODAT (occurring in 254 patients; 23.0%), within one year after transplantation. The frequency of the T allele at *KCNQ1* rs2237892 was significantly lower in patients with NODAT compared to control patients (0.30 vs. 0.39; $p = 8.5 \times 10^{-5}$). The T allele at rs2237892 was significantly associated with decreased risk of NODAT after adjusting for multiple variables, compared to the C allele (OR 0.63, 95% CI 0.51–0.79; $p = 5.5 \times 10^{-5}$). Dominant inheritance modeling showed that CT/TT genotypes were associated with a lower risk for development of NODAT (OR 0.56, 95% CI 0.42–0.76; $p = 2.0 \times 10^{-4}$) compared to the CC genotype. No other SNPs were associated with NODAT. Our study validated the protective effect of T allele at *KCNQ1* rs2237892 on the development of NODAT in a large cohort of renal transplant recipients. Our findings on susceptibility variants might be a useful tool to predict NODAT development after renal transplantation.

Keywords: new onset diabetes after renal transplantation; single nucleotide polymorphisms; renal transplantation

1. Introduction

Development of new-onset diabetes after renal transplantation (NODAT) is a common complication in patients that have undergone transplantation. The cumulative incidence of NODAT is approximately 15%–30% at 1-year post-transplantation, and the annual incidence of NODAT is approximately 4%–6% [1–3]. This metabolic disorder induces a worse cardiovascular risk profile and results in a three-fold risk of cardiovascular morbidity [4,5]. In addition, NODAT is associated with a 1.5- to 3-fold risk of allograft loss and results in a 10%–20% reduction in long-term patient survival [1,6,7]. The accumulated health-care cost is also considerable, with an estimated cost of US $21,500 per new patient with diabetes in the second year after transplantation [8]. Therefore, NODAT is a critical burden of recipient care and a major clinical challenge for the longevity and survival of renal allograft patients.

The risk of developing NODAT is associated with several clinical factors, including the recipient age, BMI, use of tacrolimus and corticosteroid, acute rejection, hepatitis C virus, cytomegalovirus infection, autosomal dominant polycystic kidney disease, and hypomagnesemia [1,9–15]. However,

evidence suggests an increased incidence of NODAT despite the identification of clinical risk factors and the effort to mitigate the risk [16]. As current strategies have limited effectiveness in preventing NODAT, genetic risk stratification emerges as a key approach to address this problem.

Several studies have shown genetic predisposition as a risk factor for the development of NODAT. Genetic polymorphism studies on NODAT led to the identification of several candidate genes, derived from genome-wide association studies (GWAS) for type 2 diabetes [17,18]. Commonly evaluated genetic determinants included genes involving carbohydrate metabolism, insulin secretion, and insulin resistance [19]. In addition, genes that encode inflammatory cytokines correlated with type 2 diabetes and were also associated significantly with NODAT [20]. More recently, GWAS showed that genes involved in β-cell apoptosis are associated with the development of NODAT [21,22]. However, candidate gene approaches included only a few individuals with NODAT, leading to inconsistent results, and the significant genes identified in GWAS are not replicated in independent cohorts. Therefore, these limitations severely interrupt the development of prevention strategies against NODAT.

This study aimed to verify the association of previously identified genetic polymorphisms with NODAT in a large nationwide prospective cohort. We selected 17 single nucleotide polymorphisms (SNPs) on susceptibility loci and evaluated the effects of these independent SNPs on the risk of developing NODAT.

2. Materials and Methods

2.1. Study Population

The study population was selected from the Korean Organ Transplantation Registry (KOTRY), which is a prospective, multicenter, nationwide cohort study that includes transplantation information in Korea. Thirty-two representative national hospitals and transplantation centers participated in KOTRY. Recipients were enrolled consecutively upon undergoing a transplantation procedure and followed up accordingly from July 2014 to December 2018. The registry accumulated data on individual patients including demographics, comorbidities, laboratory data, induction and maintenance of the immunosuppressive regimen, and several other types of events. Our study was reviewed and approved by the Institutional Review Board of each transplantation center. All patients provided written informed consent before enrollment in the study.

Blood samples from 1826 patients were stored for genotyping and screened using the KOTRY database. The following patients were excluded: Renal transplant recipients with established diabetes (n = 503), patients followed up for less than one year (n = 107), non-functioning graft at one-year follow-up (n = 32), incomplete record of medical or laboratory findings (n = 65), missing information on human leukocyte antigen (HLA) typing (n = 2), and others (n = 15). In total, finally, 1102 patients were enrolled for this study.

2.2. Selection of SNPs and Genotyping

We conducted an extensive literature review for published variants that were significantly associated with NODAT in renal transplant recipients. We evaluated SNPs, which showed top-ranked associations with NODAT in individual studies. We selected seventeen SNPs that were significantly associated with NODAT from GWAS or well-established association studies of NODAT [18,20–22].

Blood samples (3 mL each) were collected in tubes containing RBC lysis solution. The blood sample from each study participant was centrifuged to obtain white blood cells. Genomic DNA was extracted from white blood cells using a DEX^{TM} II genomic DNA extraction kit (Intron, Sungnam, Korea). DNA samples were stored at −80 °C before analysis. Quality of stored DNA samples was evaluated using agarose gel electrophoresis to confirm sample integrity. SNPs were genotyped from these DNA samples using TaqMan-based QuantStudio OpenArray® (Life Technologies, Carlsbad, CA, USA). DNA from patients and controls was randomly transferred into 96-well plates and genotyped using a blinded method. The call rates for genotyping of the SNPs were >98%.

2.3. Data Collection and Definition

We collected the following baseline patient characteristics at the time of transplantation: Age, gender, body mass index (BMI), relevant comorbid conditions, information on human leukocyte antigen (HLA), blood typing, desensitization, and induction and maintenance of the immunosuppressive regimen. Laboratory data were collected at baseline and regularly followed up. Clinical events were identified, including diabetes, the occurrence of biopsy-proven acute rejection, all-cause graft loss, and patient death or follow-up loss.

The primary outcome was the evaluation of SNP impact on the risk of developing NODAT within the first year after transplantation. Based on the definition of the American Diabetes Association, NODAT was diagnosed when fasting blood sugar was higher than 126 mg/dL six months after transplantation, or when insulin or oral hypoglycemic agents were required for treatment [23]. The control group consisted of renal transplant recipients who did not meet NODAT criteria during the follow-up period.

2.4. Statistical Analysis

Continuous variables were presented as the mean ± standard deviation. Allelic frequencies were analyzed using a chi-squared test between the two groups. Student's t-tests and chi-squared tests were used to evaluate between-group differences for continuous and categorical variables, respectively. For all SNPs, minor allele frequency (MAF), compliance with Hardy-Weinberg equilibrium (HWE), linkage disequilibrium analysis, and the association between rs2237892 and NODAT in different genetic models were assessed using SNPstats software (https://www.snpstats.net/start.htm). A multivariate logistic regression model was used to investigate the confounding effects of clinical variables significantly associated with NODAT and SNP associations. We included clinical covariates according to their weights in univariate testing, and we included clinically fundamental parameters. The confounders used in this analysis were recipient age, recipient sex, BMI, HLA mismatch number, desensitization in HLA incompatibility, ABO incompatibility, use of tacrolimus, use of steroids, biopsy-proven acute rejection, donor age, and deceased donor. Bonferroni correction was used in the association analysis when multiple comparisons were performed. We used multiple inheritance models, including codominant (major allele homozygotes vs. heterozygotes vs. minor allele homozygotes), dominant (major allele homozygotes vs. minor allele homozygotes plus heterozygotes), recessive (major allele homozygotes plus heterozygotes vs. minor allele homozygotes), and log-additive (major allele homozygotes vs. heterozygotes vs. minor allele homozygotes) models. Statistical analyses were performed using SPSS for Windows software (version 20.0; SPSS, Chicago, IL, USA). The significance level was set at $p < 0.05$.

3. Results

3.1. Baseline Clinical Characteristics and SNP Information

The incidence of NODAT in this study population was 23.0% (254/1102 patients). Baseline characteristics of recipients are summarized in Table 1. Transplant recipients who developed NODAT were significantly older, tended to be male, and had higher BMI scores than those who did not develop NODAT. Donor age in the NODAT group was significantly higher than in the control group. Desensitization treatment for HLA incompatibility was used more frequently in the control group. There was no difference between the two groups in the incidence of biopsy-proven acute rejection, or the use of tacrolimus or steroids as maintenance immunosuppressant treatments.

We excluded *AGMAT* rs11580170 from further analysis because it was in strong linkage disequilibrium with *DNAJC16* rs7533125 ($r^2 = 0.99$). Of rs1494558 and rs2172749 in *IL7R*, only rs2172749 was analyzed, because these SNPs were also in linkage disequilibrium ($r^2 = 0.98$). Of the 15 SNPs tested, 14 were consistent with HWE ($p > 0.05$). While *DNAJC16* rs7533125 violated HWE in the control group ($p = 0.037$), minor allele frequency (MAF) did not deviate from that of the East

Asian population [24]. Therefore, we included *DNAJC16* rs7533125 in the genetic association test. We additionally excluded *TCF7L2* rs7903146 and *NPPA* rs198372 in the association test, because MAF was less than 0.05 (frequency of T allele at *TCF7L2* rs7903146, 0.02; and frequency of A allele at *NPPA* rs198372, 0.01).

Table 1. Baseline demographics and characteristics of the study population.

	NODAT (n = 254)	Controls (n = 848)	p
Recipient			
Age (years)	52.2 ± 10.4	45.1 ± 12.0	<0.001
Male (%)	152 (59.8)	445 (52.5)	0.039
Dialysis duration (months)	63.7 ± 72.0	59.1 ± 67.0	0.384
BMI	23.2 ± 3.3	22.3 ± 3.2	<0.001
Donor			
Age (years)	48.6 ± 12.5	45.7 ± 12.9	0.001
Male (%)	136 (53.5)	467 (55.1)	0.668
Deceased (%)	108 (42.5)	310 (36.6)	0.086
HLA mismatch number	3.4 ± 1.7	3.2 ± 1.7	0.083
Desensitization for HLA incompatibility (%)	42 (16.5)	189 (22.3)	0.048
ABO incompatibility (%)	18 (7.1)	75 (8.8)	0.377
Anti-thymocyte globulin induction (%)	64 (25.2)	196 (23.1)	0.493
Tacrolimus (%)	252 (99.2)	831 (98.0)	0.191
Steroid (%)	252 (99.2)	845 (99.6)	0.367
Biopsy-proven acute rejection (%)	35 (13.8)	96 (11.3)	0.288

BMI = body mass index; and NODAT = new-onset diabetes after renal transplantation.

3.2. Allelic Frequency and Association between SNPs and NODAT

The allele frequencies of the genetic polymorphisms in the NODAT and control groups are summarized in Table 2. The allelic frequency of the T allele at *KCNQ1* rs2237892 was significantly lower in patients with NODAT compared to that in the control group (0.30 vs. 0.39; $p = 8.5 \times 10^{-5}$). The C allele at *CDKAL1* rs10946398 had a higher frequency in the NODAT group, with marginal statistical significance (0.52 vs. 0.47; $p = 0.080$).

We examined the genetic association between SNPs and NODAT in an allele-specific pattern (Table 3). Univariate analyses showed that the T allele at *KCNQ1* rs2237892 was significantly associated with decreased risk of NODAT (odds ratio (OR) 0.66, 95% confidence interval (CI) 0.53–0.82; $p = 1.3 \times 10^{-4}$). The C allele at *CDKAL1* rs10946398 was associated with a 1.2-fold higher risk for development of NODAT (95% CI 0.98–1.46; $p = 0.078$). However, none of the other SNPs evaluated in this study (*ATP5F1P6* rs10484821, *DNAJC16* rs7533125, *CELA2B* rs2861484, *CASP9* rs2020902, *NOX4* rs1836882, *INPP5A* rs4394754, *IL7R* rs2172749, *IL17R* rs4819554, *IL17RB* rs1025689, *IL17RB* rs1043261, and *PLXDC1* rs72823322) were significantly associated with NODAT. The association between *KCNQ1* rs2237892 and NODAT was enhanced when evaluated using multivariate logistic regression analysis (OR 0.63, 95% CI 0.51–0.79; $p = 5.5 \times 10^{-5}$). However, no other SNPs were significantly associated with NODAT in the multivariate logistic regression analysis.

Table 2. Allele frequencies of polymorphisms previously associated with NODAT.

Gene	SNP	Chr: Position	Minor Allele	MAF			
				All	NODAT	Control	*p*
CDKAL1	rs10946398	6:20660803	C	0.48	0.52	0.47	0.080
KCNQ1	rs2237892	11:2818521	T	0.37	0.30	0.39	8.5×10^{-5}
ATP5F1P6	rs10484821	6:139547773	C	0.33	0.32	0.33	0.583
DNAJC16	rs7533125	1:15557249	C	0.07	0.07	0.07	0.747
CELA2B	rs2861484	1:15486170	T	0.07	0.07	0.06	0.599
CASP9	rs2020902	1:15507865	G	0.04	0.04	0.04	0.930
NOX4	rs1836882	11:89498993	C	0.27	0.28	0.27	0.587
INPP5A	rs4394754	10:132529558	T	0.09	0.10	0.09	0.632
IL7R	rs2172749	5:3585516	C	0.40	0.40	0.40	0.976
IL17R	rs4819554	22:17084145	G	0.43	0.45	0.42	0.256
IL17RB	rs1025689	3:53849695	C	0.45	0.47	0.44	0.226
IL17RB	rs1043261	3:53865249	T	0.10	0.11	0.10	0.256
PLXDC1	rs72823322	17:39130161	G	0.21	0.22	0.21	0.185

NODAT = new onset diabetes after renal transplantation; Chr = chromosome; MAF = minor allele frequency; and SNP = single nucleotide polymorphism.

Table 3. Allele-based incidence and risk of NODAT.

Gene	SNP	Allele	Crude		Adjusted *	
			OR (95% CI)	*p*	OR (95% CI)	*p*
CDKAL1	rs10946398	C (vs. A)	1.20 (0.98, 1.46)	0.078	1.22 (0.98, 1.50)	0.070
KCNQ1	rs2237892	T (vs. C)	0.66 (0.53, 0.82)	1.3×10^{-4}	0.63 (0.51, 0.79)	5.5×10^{-5}
ATP5F1P6	rs10484821	C (vs. T)	0.94 (0.76, 1.17)	0.583	0.96 (0.77, 1.20)	0.726
DNAJC16	rs7533125	C (vs. T)	0.94 (0.65, 1.37)	0.756	0.96 (0.65, 1.43)	0.855
CELA2B	rs2861484	T (vs. G)	1.11 (0.76, 1.62)	0.607	1.07 (0.71, 1.60)	0.751
CASP9	rs2020902	G (vs. A)	0.98 (0.61, 1.56)	0.933	0.92 (0.56, 1.50)	0.733
NOX4	rs1836882	C (vs. T)	1.06 (0.85, 1.33)	0.588	1.02 (0.80, 1.29)	0.904
INPP5A	rs4394754	T (vs. C)	1.08 (0.78, 1.51)	0.635	1.08 (0.76, 1.54)	0.657
IL7R	rs2172749	C (vs. G)	1.00 (0.81, 1.22)	0.976	1.07 (0.86, 1.33)	0.535
IL17R	rs4819554	G (vs. A)	1.12 (0.92, 1.37)	0.255	1.09 (0.88, 1.35)	0.415
IL17RB	rs1025689	C (vs. G)	1.13 (0.93, 1.38)	0.226	1.15 (0.93, 1.41)	0.204
IL17RB	rs1043261	T (vs. C)	1.21 (0.87, 1.67)	0.252	1.21 (0.86, 1.71)	0.265
PLXDC1	rs72823322	G (vs. A)	0.85 (0.66, 1.09)	0.190	0.84 (0.65, 1.10)	0.199

NODAT = new onset diabetes after renal transplantation; CI = 95% confidence interval; OR = odds ratio; and SNP = single nucleotide polymorphism. * Adjusted for recipient age, recipient sex, BMI, HLA mismatch number, desensitization in HLA incompatibility, ABO incompatibility, use of tacrolimus, use of steroids, biopsy-proven acute rejection, donor age, and deceased donor.

3.3. Genotype Distribution and Association between *KCNQ1* rs2237892 and NODAT

We tested the effect of *KCNQ1* rs2237892 genotype on NODAT using a multiple inheritance model as shown in Table 4). In the codominant model, the TT genotype at rs2237892 was associated with the lowest risk for development of NODAT, compared to the CC genotype (OR 0.41, 95% CI 0.25–0.67; $p = 4.7 \times 10^{-4}$). In the dominant model, the CT/TT genotype was also associated with a reduced risk for development of NODAT (OR 0.56, 95% CI 0.42–0.76; $p = 2.0 \times 10^{-4}$). The T allele significantly reduced the risk of NODAT compared to the CC genotype in the log-additive model. However, no significant differences were observed in the recessive model with Bonferroni correction.

Table 4. NODAT incidence and risk of *KCNQ1* rs2237892 in multiple inheritance models.

Model	Type	N (%)		OR (95% CI) *	*p*
		NODAT	Control		
Codominant	CC	128 (50.4)	317 (37.4)	Reference	
	CT	101 (39.8)	395 (46.6)	0.62 (0.45, 0.85)	2.8×10^{-3}
	TT	25 (9.8)	136 (16.0)	0.41 (0.25, 0.67)	4.7×10^{-4}
Dominant	CC	128 (50.4)	317 (37.4)	Reference	
	CT/TT	126 (49.6)	531 (62.6)	0.56 (0.42, 0.76)	2.0×10^{-4}
Recessive	CC/CT	229 (90.2)	712 (84.0)	Reference	
	TT	25 (9.8)	136 (16.0)	0.53 (0.33, 0.84)	0.0051
Log-additive	-			0.63 (0.51, 0.79)	$<1.0 \times 10^{-4}$

NODAT = new onset diabetes after renal transplantation; CI = 95% confidence interval; and OR = odds ratio. *Adjusted for recipient age, recipient sex, BMI, HLA mismatch number, desensitization in HLA incompatibility, ABO incompatibility, use of tacrolimus, use of steroids, biopsy-proven acute rejection, donor age, and deceased donor.

4. Discussion

In the present study, using samples from a large cohort of renal transplant recipients, we examined the association of 13 SNP pairs and candidate genes for risk of NODAT development. Of the studied variants, there was a significant difference in the frequency of the T allele at *KCNQ1* rs2237892 between the NODAT and control groups, and this allele showed an independent association with NODAT. The TT and CT genotypes of *KCNQ1* rs2237892 were associated with a significantly reduced risk for development of NODAT in codominant, dominant, and log-additive models. These findings suggested that the genetic variant of *KCNQ1* is a significant contributor to the development of NODAT in renal transplant recipients.

Although NODAT results from the combined effect of insulin resistance and β-cell dysfunction, several recent studies have shown that β-cell dysfunction is the main contributing factor for the development of NODAT [3,25,26]. *KCNQ1* rs2237892 and *CDKAL1* rs10946398 were identified as a susceptibility gene for type 2 diabetes in GWAS, and each of these genes is associated with β-cell dysfunction [27–31]. Previous studies with type 2 diabetes risk genes suggested an association between *KCNQ1* rs2237892 and NODAT [19]. Our study also validated that variant rs2237892 of the T allele was associated with decreased risk for development of NODAT compared to the C allele. Similarly, *CDKAL1* rs10946398 was also associated with NODAT, as reported in a study that used a candidate gene approach in patients who underwent transplantation [18,19]. However, our data did not confirm this association. These findings suggested that *KCNQ1* is a more robust and influential indicator of β-cell dysfunction in renal transplant recipients.

KCNQ1 encodes a subunit of the voltage-gated K^+ channel, which is expressed in pancreatic islets [32]. In the *KCNQ1*-overexpressing pancreatic β-cell line, the density of the K+ current increased significantly and affected the pancreatic cell membrane action potential [33]. Therefore, *KCNQ1* overexpression contributes to impairment of glucose-stimulated insulin secretion, and a specific *KCNQ1* blocker also stimulates insulin secretion [34]. In addition, allelic mutation of *KCNQ1* results in up-regulation of the neighboring gene, cyclin-dependent kinase inhibitor 1C, which encodes a cell cycle inhibitor and leads to reduction in pancreatic β-cell mass [35]. Therefore, we suggest that variant *KCNQ1* induces impaired β-cell function and reduced β-cell mass, and this biological function could be a potential underlying mechanism for the association between *KCNQ1* variants and increased risk for NODAT development.

Three types of *KCNQ1* SNPs were evaluated as potential risk factors for the development of NODAT in Spanish patients who received kidney transplants from deceased donors [17]. *KCNQ1* rs2237895, rs2237892, and rs8234 were genotyped, and SNP rs2237895, but not rs2237892, was found to be associated with an increased risk for development of NODAT in the first year after transplantation. This apparent discrepancy could be due to the allele frequencies of these SNPs. The T allele frequency at rs2237892 was reported to be 0.34–0.36 in the East Asian population, but only 0.04–0.08 in the European

population [36]. Consequently, lower MAF at rs2237892 was not significantly associated with NODAT in Spanish transplant recipients. Therefore, we suggest that different genetic backgrounds should be considered when attempting to determine the risk of development of NODAT using *KCNQ1* genetic variants as indicators.

In a recent GWAS, numerous variants were found to be associated with risk for the development of NODAT [21]. *ATP5F1P6*, *CELA2B*, *CASP9*, *NOX4*, and *INPP5A* were identified as risk genes in Caucasian renal transplant recipients. These genetic variants were implicated in β-cell apoptotic pathways, but not insulin resistance, suggesting that β-cell apoptosis was a critical component of NODAT pathogenesis. However, our study did not find a significant association between NODAT and any SNPs from this GWAS. Three possible factors might explain this inconsistency: First, the β-cell apoptotic pathways could be a weak contributor to the development of NODAT in Asian compared to Caucasian recipients of a renal transplant. Second, a different definition of NODAT phenotype might have resulted in dissimilar findings in the two studies. Third, the limited sample size in the GWAS may have less power to detect significant associations [37].

Inflammatory cytokines are involved in insulin action and insulin secretion. An SNP within the gene encoding the IL-7R chain was found to be associated with type 1 diabetes mellitus [38,39]. Moreover, our previous study showed that genetic variants of *IL-7R*, *IL-17R*, and *IL-17RB* were associated significantly with NODAT [14]. However, the relevant SNPs of these interleukin genes were not associated with NODAT in the exploratory GWAS analysis or the secondary verification analysis [17]. Furthermore, our validation study also showed no meaningful differences in allele frequencies. These findings suggested that the effects of interleukin gene polymorphisms on the risk for development of NODAT were inconclusive, and further studies are necessary to obtain precise results.

The present study had a few limitations. As data regarding family history of type 2 diabetes were not available, the association between family history and the development of NODAT could not be evaluated. In addition, the effect of BMI and weight gain after transplantation was not included in our analysis. Finally, we did not perform an oral glucose tolerance test or HbA1c estimation before kidney transplantation. Therefore, patients with prediabetes might have been included in our study.

In conclusion, our validation study showed a significant association between *KCNQ1* rs2237892 and development of NODAT in a large cohort. Our results suggest that *KCNQ1* might play a crucial role in the pathogenesis of NODAT following renal transplantation. *KCNQ1* variants might be a useful tool to predict NODAT development in renal transplant recipients, and help screen for patients at a higher risk for NODAT.

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A Low Tacrolimus Concentration/Dose Ratio Increases the Risk for the Development of Acute Calcineurin Inhibitor-Induced Nephrotoxicity

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Abstract: Fast tacrolimus metabolism is linked to inferior outcomes such as rejection and lower renal function after kidney transplantation. Renal calcineurin-inhibitor toxicity is a common adverse effect of tacrolimus therapy. The present contribution hypothesized that tacrolimus-induced nephrotoxicity is related to a low concentration/dose (C/D) ratio. We analyzed renal tubular epithelial cell cultures and 55 consecutive kidney transplant biopsy samples with tacrolimus-induced toxicity, the C/D ratio, C0, C2, and C4 Tac levels, pulse wave velocity analyses, and sublingual endothelial glycocalyx dimensions in the selected kidney transplant patients. A low C/D ratio (C/D ratio < 1.05 ng/mL×1/mg) was linked with higher C2 tacrolimus blood concentrations (19.2 ± 8.7 µg/L vs. 12.2 ± 5.2 µg/L respectively; $p = 0.001$) and higher degrees of nephrotoxicity despite comparable trough levels (6.3 ± 2.4 µg/L vs. 6.6 ± 2.2 µg/L respectively; $p = 0.669$). However, the tacrolimus metabolism rate did not affect the pulse wave velocity or glycocalyx in patients. In renal tubular epithelial cells exposed to tacrolimus according to a fast metabolism pharmacokinetic profile it led to reduced viability and increased Fn14 expression. We conclude from our data that the C/D ratio may be an appropriate tool for identifying patients at risk of developing calcineurin-inhibitor toxicity.

Keywords: calcineurin inhibitor nephrotoxcity; tacrolimus; C/D ratio; tacrolimus metabolism; kidney transplantation

1. Introduction

Although the calcineurin inhibitor (CNI) tacrolimus (Tac) is effective in preventing graft rejection after transplantation, its therapeutic window is narrow. Furthermore, Tac exhibits a high intra- and inter-individual variability in pharmacokinetics (PK) and pharmacodynamics [1]. Tac-related adverse effects are common even in patients with Tac trough levels within the intended therapeutic range, despite meticulous therapeutic drug monitoring. CNI-induced nephrotoxicity (CNIT) especially

remains a severe issue during CNI treatment [2]. While acute CNIT comprises isometric tubular vacuolization, acute arteriolopathy, and thrombotic microangiopathy, the features of chronic CNIT include interstitial fibrosis and tubular atrophy, arteriolar hyalinosis, tubular microcalcifications, and global glomerulosclerosis [2]. Unfortunately, there are no specific molecular markers of CNIT, but it was recently experimentally shown that, e.g., the TWEAK/Fn14 pathway, is critically involved in the pathogenesis of CNIT [3]. Although it is known that overexposure to Tac causes CNIT trough level-dependently, even patients presenting with Tac trough levels within the therapeutic range (5–15 µg/L) are vulnerable to developing both acute or chronic CNIT [4–9]. This indicates the possibility of additional causative factors.

Using the concentration/dose (C/D) ratio, a strong association between a fast Tac metabolism rate/fast oral Tac clearance (C/D ratio < 1.05 µg/L×1/mg) and reduced renal function within the first month following renal transplantation (RTx) can be demonstrated [9]. Other studies found comparable outcomes; even after liver transplantation [7,10–14]. However, this effect cannot be observed, if considerably higher C/D-ratio cut-offs are chosen [15]. The Tac metabolism effect on renal function was detectable even five years after RTx and was also associated with increased mortality in patients with a low C/D ratio [16].

What are the reasons for these findings? Apart from an increased susceptibility of fast metabolizers to BK virus infections, these patients more frequently required indication biopsies that revealed higher rates of rejections and acute CNIT [9,16,17]. Therefore, we hypothesized that the C/D ratio as a simple estimate of the Tac metabolism correlates with the severity of CNIT.

2. Experimental Section

2.1. Patients and Histology

At first, the study was conducted to answer the question if there is an association between histological findings of acute CNIT and the corresponding Tac C/D ratio at the time of biopsy. We hypothesized that a C/D ratio <1.05 ng/mL×1/mg is associated with acute CNIT. To prove the hypothesis, we performed a histological reevaluation of all for-cause RTx-biopsy samples that showed acute CNIT in our center between 2007 and 2016. Only samples with definite histological signs of acute CNIT (isometric vacuolization of tubular epithelial cells) were included in the study. Biopsy samples with isometric vacuolization that could be attributed to other causes were excluded from the evaluation.

Two pathologists, independently and blinded, categorized the graft biopsies in the following groups: <10%, 10–25%, 25–50%, and ≥50% of tubules showing isometric vacuolization of the cytoplasm (Figure 1A–D). In case of different assessment of the pathologists, mean values were taken. Due to limited sample numbers, for final analysis two categories were considered: samples with <25% and with ≥25% affected tubular cells (n = 35 and n = 20, respectively).

The C/D ratio was calculated by the Tac blood trough concentrations and the corresponding Tac doses on the day of the renal biopsy. C/D ratio values < 1.05 ng/mL×1/mg defined patients as fast Tac metabolizers (patients with fast oral clearance), values ≥ 1.05 ng/mL×1/mg characterized slow metabolizers (patients with slow oral clearance) as published before [8,13]. Only 12 h Tac trough levels were used for this analysis.

After confirmation of our first hypothesis, we secondly designed a prospective part of the study to address the question, if CNIT could be related to Tac peak levels. We hypothesized, that patients with a fast oral Tac clearance develop higher Tac peak levels than patients with a slow oral Tac clearance. Therefore, C0 and C2 Tac levels were determined in an additional cohort of 56 RTx patients. Additionally, we assessed C4 levels and the area under the curve (AUC) in 25 of these 56 individuals. For C0, 12 h trough levels were assessed. C2 was assessed 2 h and C4 4 h after intake of the morning dose, respectively. Whole blood was analyzed for Tac (automated tacrolimus (TACR) assay; Dimension Clinical Chemistry System; Siemens Healthcare Diagnostic GmbH; Eschborn; Germany). In addition,

a cell culture model using supra-therapeutic Tac concentrations was used to mimic the different Tac profiles of patients with fast and slow oral Tac clearance (see below).

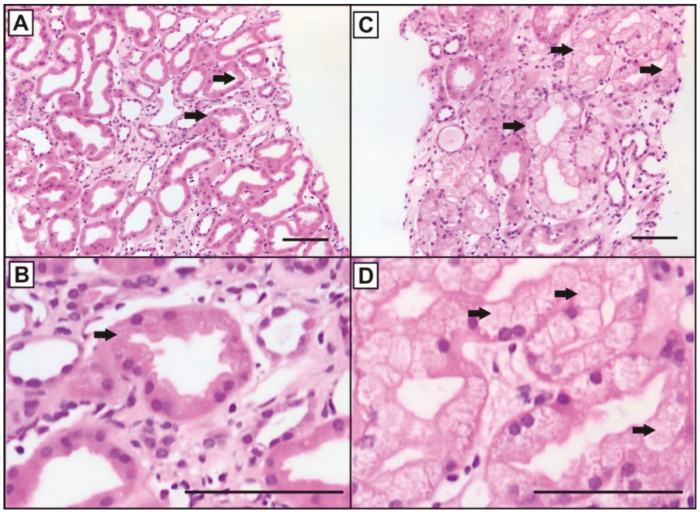

Figure 1. Examples of Hematoxylin and Eosin (HE)-stained sections of kidney transplant biopsies with different overall scores of isometric vacuolization (arrows) as a marker of calcineurin inhibitor-induced nephrotoxicity. (**A**): < 25% of the tubular epithelial cells, (**B**): magnification of A, (**C**) ≥ 25% of the tubular epithelial cells, and (**D**): magnification of C (bars: 100 µm).

All patients received an induction therapy with basiliximab or anti T-lymphocyte antibody and an immunosuppressive regimen containing immediate release tacrolimus (Prograf©), mycophenolate (CellCept©/Myfortic©), and prednisolone (Soludecortin H© /Decortin H©).

Patients' demographics were taken from the clinical hospital database and are presented in Table 1, and Tables S1 and S2.

Table 1. Patient characteristic: Histological analysis.

CNI Nephrotoxicity	x < 25% (*n* = 35)	x ≥ 25% (*n* = 20)	*p*-Value
Age (years, mean ± SD)	57.8 ± 12.4	50.2 ± 20.2	0.014 [a]
Male sex, *n* (%)	24 (68.6)	12 (60)	0.566 [b]
BMI (kg/m^2, mean ± SD)	25.5 ± 5.2	25.6 ± 5.3	0.981 [a]
Prednisolone dose (mg, mean ± SD)	10.0 ± 6.3	14.9 ± 17.5	0.239 [a]
Living donor transplantation, *n* (%)	26 (74.3)	14 (70)	0.761 [b]
ESP, *n* (%)	9 (25.7)	1 (5)	0.075 [b]
Combined RTx + liver Tx, *n* (%)	3 (8.6)	1 (5)	1 [b]
Previous Tx, *n* (%)	3 (8.6)	0	0.293 [b]
ABOi, *n* (%)	4 (11.4)	2 (10)	1 [b]
CIT (hours, mean ± SD)	9.2 ± 5.0	8.5 ± 5.0	0.669 [a]
WIT (min, mean ± SD)	32.5 ± 8.1	32.5 ± 5.4	0.418 [a]
DGF	11 (31.4)	2 (10)	0.107 [b]
Donor data			
Male donor sex, *n* (%)	13 (47,1)	14 (70)	0.026 [b]
Donor age (years, mean ± SD)	61.1 ± 15.7	52.2±14.7	0.073 [a]
Time from RTx to biopsy (days)	63 (3–2877)	223 (10–5057)	0.059 [c]

Patients with a CNI nephrotoxicity < 25% were observed to be older and received more female allografts; CNI, calcineurin inhibitor; BMI, body mass index; ESP, European senior program; RTx, renal transplantation, Tx, transplantation; ABOi, ABO incompatible transplantation; CIT, cold ischemia time, WIT, warm ischemia time, DGF, delayed graft function; [a] Student's *t*-test; [b] Fisher's exact test; [c] Mann–Whitney U test.

The study was performed in accordance with the Declaration of Helsinki and approved by the local ethics committee (Ethik Kommission der Ärztekammer Westfalen-Lippe und der Medizinischen Fakultät der Westfälischen Wilhelms-Universität, 2017-407-f-S). Prior to analysis, all patient data were anonymized. Written informed consent with regard to recording their clinical data was given by all participants at the time of transplantation or inclusion into the study. Recipients aged <18 years, pregnant women, or patients with uncontrolled infection, tumor, or hypertension were excluded from the study.

2.2. Assessment of Pulse Wave Velocity and Glycocalyx

Besides tubular changes tacrolimus toxicity comprises vascular effects (vasoconstriction, arteriolopathy) as well. After having linked tubular changes with the Tac oral clearance in patients and a cell culture model, respectively, we conducted a second prospective study to assess potential Tac metabolism-related vascular changes. Therefore, we measured pulse wave velocity (PVW) and the glycocalyx as surrogate parameters of endothelial dysfunction/arterial stiffness in 120 stable RTx outpatients (30 patients with a C/D ratio < 1.05 ng/mL×1/mg and 90 patients with a C/D ratio ≥ 1.05 ng/mL×1/mg). Arterial stiffness was assessed as pulse wave velocity (PWV) using cuff-based oscillometry (Mobil-O-Graph, IEM, Stolberg, Germany) [18,19]. Subjects rested for 10 min at 23 °C before the baseline hemodynamic measurements were performed. Initially, brachial systolic blood pressure (mmHg) was measured. Two sequential measurements separated by a 5-min interval were obtained. The mean PWV was used for the analysis only if the PWV difference between the assessments was <0.5 m/sec. Otherwise, a third measure was conducted and the median of all values was calculated as published before [20]. An experienced single operator performed the measurements.

Furthermore, we prospectively assessed the dynamic lateral red blood cell movement into the glycocalyx that is expressed as the perfused boundary region (PBR) (in μm) in a subset of 28 (14 fast metabolizer) stable and matched RTx patients using bedside real-time intravital microscopy [21]. The sublingual microvasculature was visualized and examined with the use of GlycoCheckTM Software, coupled to a sidestream dark field (SDF) camera (CapiScope HVCS, KK Technology, Honiton, UK) by an experienced single operator, as thoroughly described before [21]. Briefly, the SDF camera uses stroboscopic diodes (540 nm) to detect the hemoglobin of the red blood cells (RBC). The GlycoCheck software allows automatic video recording when predefined image quality criteria (motion, intensity, focus) are met. The software automatically identifies all available micro-vessels with a diameter between 5 to 25 μm and marks vascular segments every 10 μm along the assessed microvasculature. Before further analysis of the videos, it performs an automatic quality check (Figure 2C). Invalid vascular segments are marked with yellow and automatically discarded, while all valid vascular segments (green lines) are subjected to further analysis. The software measures the PBR (in μm); an inverse parameter of glycocalyx dimensions. Specifically, the dynamic lateral RBC movement towards the endothelial wall is assessed in an average of about 3000 different vascular segments with a diameter from 5 to 25 μm (Figure 2B,C). An impaired endothelial glycocalyx allows RBCs to penetrate more deeply towards the endothelium, which translates into higher PBR values.

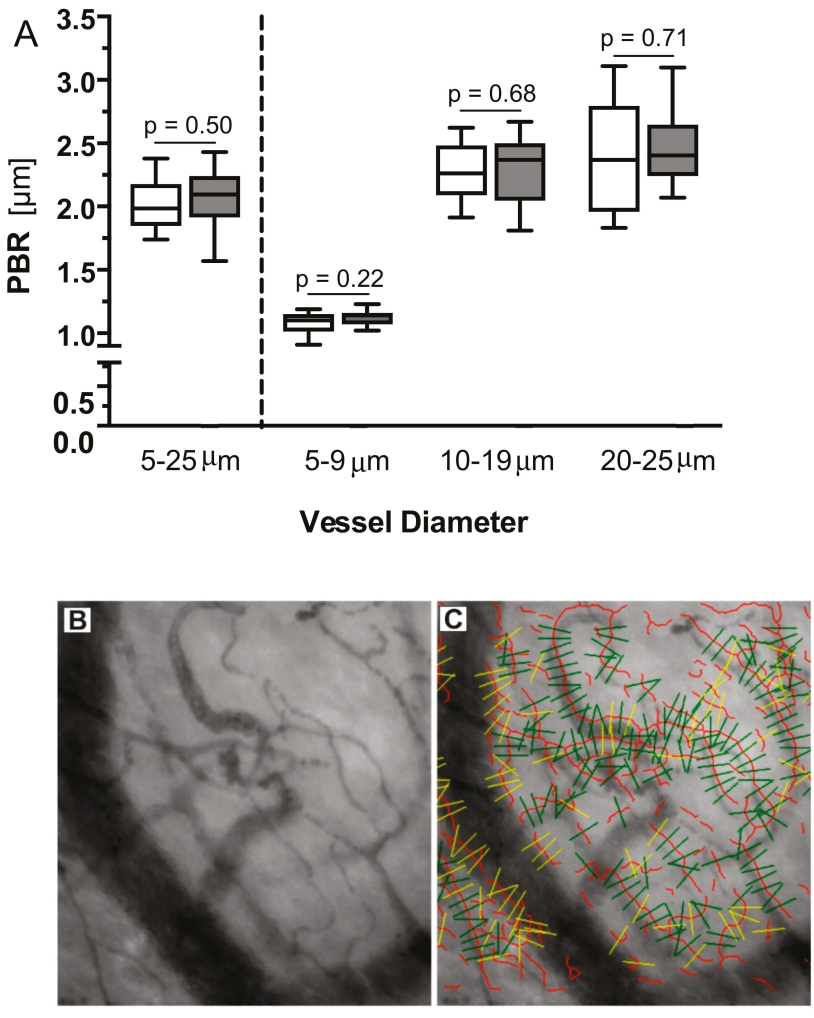

Figure 2. (**A**): Boxplots of PBR values of patients with a high C/D ratio (white) and low C/D ratio (grey) based on the different microvascular diameter ranges. No difference was detected between the groups. (**B**): Representative image of the sublingual mucosa acquired with the SDF camera in a kidney transplant patient. (**C**): Exemplary picture of a video recording showing the automatic identification of all available micro-vessels with a diameter between 5 to 25 µm. Vascular segments are marked every 10 µm along the assessed microvasculature (red lines) by the GlycoCheck software (green lines: valid segments for further analysis, yellow lines: discarded by quality check).

2.3. Cell Culture

Tubular epithelial cells (NRK-52E; ATCC) were cultivated in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Darmstadt, Germany) supplemented with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1% antibiotics (Pen/Strep) and L-Glutamine (PAA; Cölbe, Germany), and were cultured at 37 °C and 5% CO_2. NRK-52E cells were grown in 12-well or 96-well plates until 80% confluence followed by treatment with tacrolimus (Prograf®i.v., Astellas, Munich, Germany) diluted in 0.9% sodium chloride) or medium only as a control over 12 h. Tac working solutions were freshly

prepared by appropriate dilution of stock solution in the culture medium. Due to the inherent robustness of rat cells, titration series were conducted to determine the optimal tacrolimus concentrations that induce an appropriate reduction in cell viability. Culture medium was changed every hour using the indicated Tac concentrations between 6 and 20 μg/mL that were based on our titration studies and previous studies by Lamoureux et al. (Figure S1) [22]. After the incubation period of 12 h, the culture medium was removed and cells were washed three times with PBS and then prepared for quantitative Western blot analysis or MTT assay as described below. All samples were tested in triplicate wells. Data are representative of three different experiments.

2.4. Lysate Preparation and Western Blot Analysis

As fibroblast growth factor-inducible 14 (Fn14) is involved in the pathogenesis of CNIT we analyzed its expression in Tac-treated NRK cells using primary antibodies against α-actinin 4 and Fn14, respectively [3].

Preparation and quantitative Western blot analysis of cell lysates have been described previously [23]. Briefly, for quantitative Western blotting, cells were grown on dishes and then scraped into 1x LaemmLi (4% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue, 0.0625 M Tris-HCl; pH 6.8). Samples were shaken at 1000 rpm for 2 h and then subjected to ultrasound bath treatment for 15 min. After being boiled for 5 min, equal volumes of cell lysates were separated onto 10% SDS-PAGE gels (Bio-Rad). Proteins were transferred to a PVDF membrane (Millipore) and incubated for 1 h at room temperature in blocking buffer (5% skim milk powder dissolved in TBS containing 0.05% Tween-20). A primary antibody against Fn14 (Cell Signaling, Danvers, MA, USA) was used in a 1:1000 dilution in TBS–Tween-20 and incubated at 4 °C overnight. After being washed three times with TBS–Tween-20, the membrane was incubated with horseradish peroxidase–coupled secondary antibodies (Jackson Immunoresearch, via Dianova, Hamburg, Germany) diluted 1:5000 in blocking buffer for 45 min at room temperature. After three washes, the Western blot was developed using a chemiluminescence detection reagent (Roche). For normalization of band density following chemiluminescence detection the samples were equalized using α-actinin (Enzo, Loerrach, Germany) as the loading control. All samples were tested in triplicate wells and three different experiments.

2.5. MTT Test

Cell viability was assessed by a colorimetric assay, which is based on the conversion of dissolved yellow 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenases of living cells as previously described [24,25]. Therefore, this MTT assay offers precise quantification of cell viability in mammalian cell cultures. Briefly, after Tac treatment for 12 h, the medium was carefully removed and replaced by 200 μL of fresh complete cell culture medium. 10 μL of MTT solution containing 5 mg/mL of the dye were added to each well, and the cells were again incubated for 3 h. The medium was then removed and 100 μL of lysis buffer containing 10% (w/v) sodium dodecyl sulfate and 40% (v/v) dimethylformamide was added to each well. The plates were shaken for 10 min to destroy the cell structure and dissolve the blue formazan dye. Finally, the absorbance was measured at 590 nm using an automated microtiter plate reader (Infinite M200; Tecan, Männedorf, Switzerland). The percentage of viable cells in the untreated controls was compared to that for the respective Tac treatments.

2.6. Statistical Analysis

Statistical analysis were performed using IBM SPSS®Statistics 25 for Windows (IBM Corporation, Somers, NY, USA) or GraphPad Prism version 4.0 (GraphPad Sofware, La Jolla, CA, USA). Normally distributed continuous variables are shown as mean ± standard deviation (SD) or as mean ± SEM and non-normally distributed continuous variables as median and first and third quartiles (interquartile range, IQR). Absolute and relative frequencies have been given for categorical variables. Pairs of independent groups were compared using the Student's t-test for normally distributed data,

Mann–Whitney U test for non-normal data, and Fisher's exact test for categorical variables. To compare paired data, we used the Wilcoxon test for continuous variables and the McNemar test for categorical variables. Comparison among groups in Western blot experiments was performed by one-way ANOVA along with post-hoc Tukey test. p-values < 0.05 were considered as statistically noticeable.

3. Results

3.1. Histology

The histological re-analysis of 55 consecutive kidney transplant biopsy samples from patients (low C/D ratio, n = 27) with evidence of CNIT indicated by the presence of the characteristic isometric vacuolization of the tubular epithelial cells in < 10% (n = 20), 10–24% (n = 15), 25–49% (n = 12) and eight biopsies ≥ 50% of affected tubular cells. For further comparison, samples were regrouped according to < 25% (n = 35) or ≥ 25% (n = 20) tubular isometric vacuolization (Figure 3, Table 1).

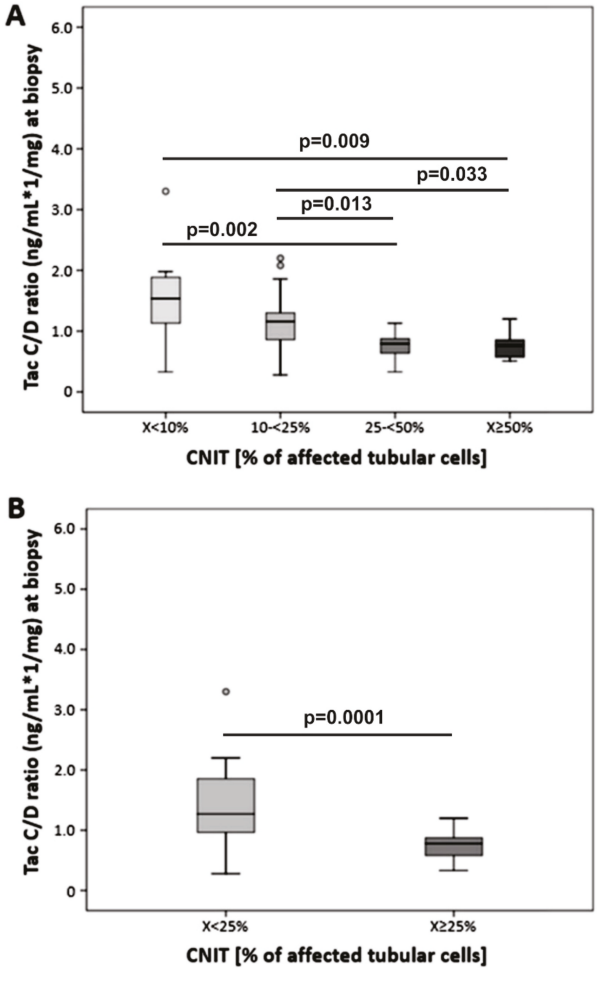

Figure 3. Histological analysis of calcineurin inhibitor-induced nephrotoxicity (CNIT) in kidney transplant biopsies, assessing the degree percentage of tubular cells with isometric vacuolization of the cytoplasm. The C/D ratio indicated a strong negative association with the severity of CNIT.

Although the trough levels at the time of biopsy were similar for both groups (Table 2), the degree of CNIT indicated a strong negative association to the C/D ratio values (Figure 3). Trough levels in 56 additional patients were comparable between patients with a low and high C/D ratio (6.3 ± 2.4 µg/L vs. 6.6 ± 2.2 µg/L respectively; $p = 0.669$). However, patients with a low C/D ratio displayed significantly higher C2 levels (19.2 ± 8.7 µg/L vs. 12.2 ± 5.2 µg/L, respectively; $p = 0.001$, Figure 4A). In a subgroup of 25 patients, C0 levels (6.3 ± 3.2 µg/L vs. 6.2 ± 2.3 µg/L, respectively; $p = 0.620$) and C4 (11.3 ± 5.8 µg/L vs. 9.0 ± 2.7 µg/L, respectively; $p = 0.466$) were comparable between groups. However, C2 levels of patients with a low C/D ratio were increased (20.2 ± 10.3 µg/L vs. 9.8 ± 4.2 µg/L, respectively; $p = 0.004$, Figure 4B).

Table 2. Tac trough level and dose of two calcineurin inhibitor toxicity groups.

	x < 25% (*n* = 35)	x ≥ 25% (*n* = 20)	*p*-Value
Tac trough level (ng/mL ± SD)	6.0 (3.1–15.1)	5.8 (2.4–12.5)	0.431
Tac dose (mg, mean ± SD)	5.0 (1.0–18.0)	8.0 (3.0–16.0)	0.009
C/D ratio, ng/mL×1/mg, median (min-max)	1.27 (0.28–5.03)	0.78 (0.33–1.20)	< 0.001

Tac, tacrolimus; Mann–Whitney U test.

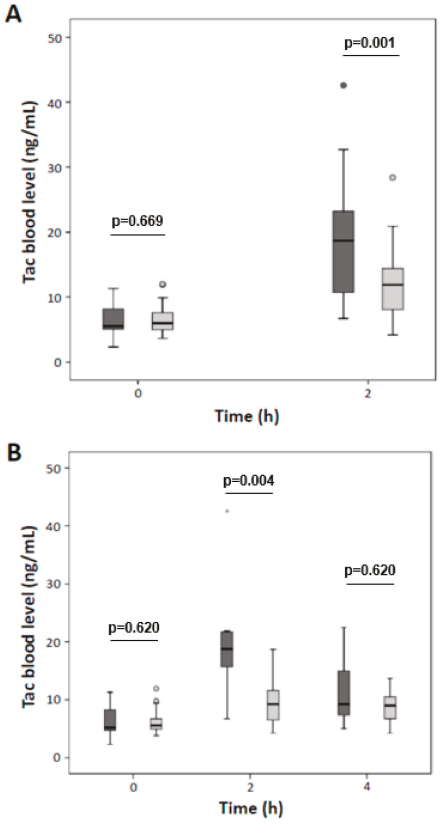

Figure 4. Presented are C0, C2 ($n = 56$) (**A**), and C4 ($n = 25$) (**B**) Tac levels in stable kidney transplanted patients. While the trough level (C0) and the C4 level were comparable between patients with a high (dark grey bars) and low (light grey bars) C/D ratio, C2 levels were significantly increased in patients with a low C/D ratio.

3.2. Pulse Wave Velocity Analysis

PWV correlated with age and systolic blood pressure (SBP) but not with the C/D ratio (Table S1, Figure 5).

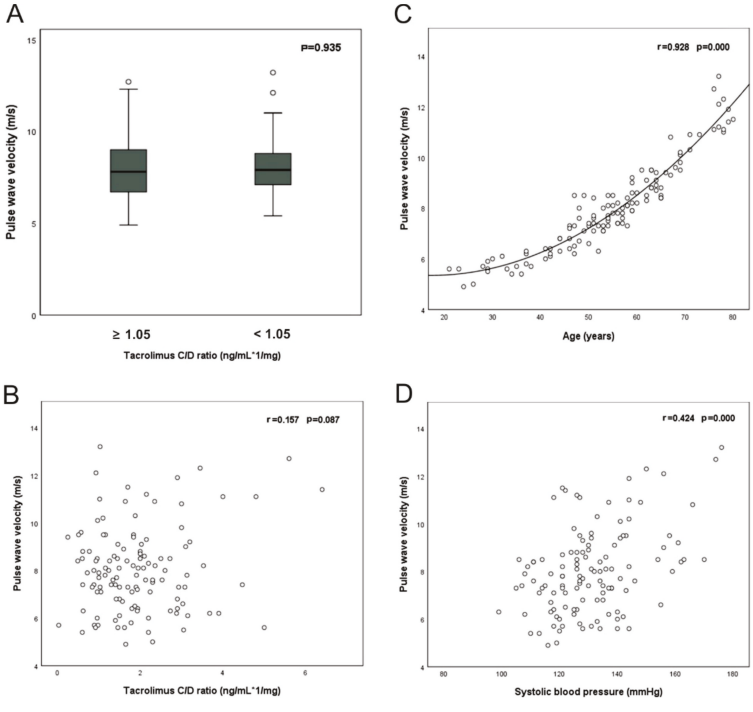

Figure 5. Pulse wave analysis of kidney transplanted patients. No correlation was observed between the C/D ratio and pulse wave velocity (**A**,**B**). The scatter plot in (**C**) indicates a strong quadratic relation of age and pulse wave velocity. Systolic blood pressure showed a moderately strong correlation to pulse wave velocity (**D**). R: Pearson correlation coefficient.

3.3. Glycocalyx Analysis

The PBR, an inverse parameter of endothelial glycocalyx dimensions in sublingual vessels, was comparable between the patients with a low and a high C/D ratio (Figure 2).

3.4. Cell Culture

Titration series revealed a tacrolimus concentration between 6 µg/mL and 20 µg/mL to induce an appropriate reduction of NRK cell viability in MTT assays. Tac exposure decreased the viability of NRK cells (Figure 6A) the most in cells that were treated with Tac corresponding to the PK profiles of fast metabolizers (6 µg/mL to 19 µg/mL) (77.3%) compared to cells that were exposed to continuous Tac treatment (8.5 µg/mL) or to Tac corresponding to the PK profiles of slow metabolizers (6 µg/mL to 12 µg/mL, respectively (81.3% vs. 84.7%, Figure 6A). Accordingly, these cells showed the highest Fn14 expression (Figure 6B).

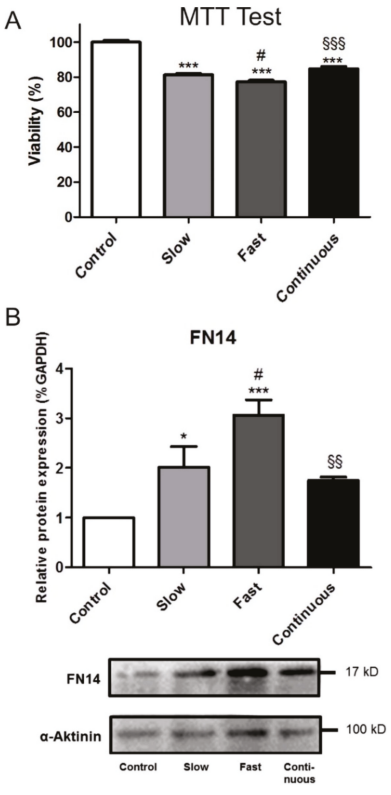

Figure 6. The viability of tubular epithelial cells (NRK-52E; ATCC) assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test (**A**). Cells treated for 12 h with Tac according to the pharmakokinetic profile of fast metabolizers (Fast) showed the most reduced viability (*** $p < 0.001$ vs. Control, # $p < 0.05$ vs. Tac slow, §§§ $p < 0.001$ vs. Tac fast. Western blot analysis of Fn14 expression in NRK cells (**B**), showed higher Fn14 expression levels in NRK cells when compared to the other groups (* $p < 0.05$ vs. Control, *** $p < 0.001$ vs. Control, # $p < 0.05$ vs. Tac slow. §§ $p < 0.01$ vs. Tac fast). An exemplary Western blot is presented below.

4. Discussion

CNIT is a frequent complication of Tac exposure and is associated with reduced renal function and kidney graft loss. So far, no specific treatment of CNIT is available. Therefore, approaches to minimize its occurrence and identify the patients at risk are required.

The C/D ratio is a simple estimate of the Tac metabolism rate and is therefore useful to stratify patients' risk [1]. Since we identified a strong negative association between the C/D ratio and degree of acute CNIT observed in RTx biopsies, we describe in this study for the first time that a low C/D ratio (defined as a C/D ratio < 1.05 µg/L×1/mg) is linked to CNIT severity (Figure 3).

We previously observed that a low C/D ratio is associated with an inferior renal function after transplantation [1]. This effect persisted in a five-year follow-up, and a low C/D ratio was identified as an independent risk factor for a decreased graft and patient survival [16]. In an earlier study, the indication biopsy rate, that histologically showed more frequently CNIT in patients with a low C/D ratio, was higher than in patients with a high C/D ratio [9]. However, the sample size in this study was low and no information was available on the severity of the CNIT lesions. To fill these

gaps, we performed the presented combined retro- and prospective studies to further investigate the influence of fast Tac metabolism on CNIT occurrence and severity.

Despite higher daily Tac dosages, patients with a low C/D ratio do not usually display higher trough concentrations, AUCs, and Tac metabolites compared to patients with a high C/D ratio (Table 2) [5–7,9,26]. Patients' PK profiles, including the peak level concentration, must consequently differ—a finding that we can confirm (Figure 4) [27]. In a study on the PK Tac profiles of stable RTx patients, Miura et al. presented in Figure 1 12 h PK profiles from their patients whose Tac concentration sharply increases to an early, high peak after Tac intake followed by a rapid decrease of the Tac blood level (suggestive of a low C/D ratio). In contrast, other patients exhibited a slow increase of Tac levels to a lower peak level after Tac intake that was followed by a slower decrease of Tac concentration to the trough level (suggestive of a high C/D ratio) [28]. In this regard, a randomized, prospective crossover study that assessed 24 h Tac PK profiles in genotyped, kidney transplanted African Americans provided informative insights [29]. African Americans, who are predominantly CYP3A5 expressors and therefore fast metabolizers, required double doses of weight-normalized immediate release (IR) Tac as compared to CYP3A5 non-expressors. Despite comparable Tac AUCs and a similar total exposure of fast and slow metabolizers to the compound, PK profiles and the exposure to Tac at different time points after intake differ. This is important because peak level concentrations that presumably cause temporary Tac overexposure are linked, e.g., to neurotoxicity [30].

In our cell culture model, the viability of NRK tubular epithelial cells significantly decreased when cells were incubated with Tac according to the PK profiles of fast metabolizers (Figure 6). These most affected cells notably expressed the highest amount of Fn14, a receptor protein known to be involved in the pathogenesis of CNIT [3]. It has to be considered that due to the robustness of NRK cells we applied Tac concentrations that are supra-therapeutic compared to Tac blood concentrations that are observed in patients and a direct translation into clinical practice and exact modelling of patients' Tac exposure, which is much more complex and influenced by many factors in vivo, is limited. However, by using this rather simple in vitro system we herein provide insights into the pathophysiologic effects of the different Tac PK profiles. Since endothelial dysfunction is essentially involved in the pathogenesis of acute CNIT, we exemplarily analyzed the functioning of the glycocalyx and the PWV; such analysis has been recently shown to be of additive value for the assessment of vessel function [2,31]. However, no differences were observed between the matched patients with low and high C/D ratios. The underlying causes for this could be the small sample size or a relatively small impact of Tac on the vessels [19]. Tac, in contrast to cyclosporine, does not reduce renal plasma flow, GFR, or blood pressure—at least not in healthy subjects [32].

Our study has limitations. First, Tac metabolites have not been measured and the analysis of Tac peak levels was not performed in the same group of RTx patients in whom the association of C/D ratio and histological CNIT was investigated since this part of the study was of a retrospective nature and could therefore only be hypothesis generating. Assessment of Tac metabolism is complex as it includes different processes such as uptake, metabolism in the intestine, liver, blood, and kidneys as well as its elimination. All these steps underlie many influencing factors (e.g., genetics, albumin level, hematocrit, differences in absorption and compliance). As we did not assess Tac metabolites, the C/D ratio can only serve as an estimate (sum of all effects that affect Tac metabolism in vivo) of the true Tac metabolism rate. Rather, the C/D ratio constitutes a simple tool to describe the stable condition between uptake and elimination of Tac in the blood. Nevertheless, a correlation between the C/D ratio and several CYP3A subtypes has already been shown by others (e.g. Reference [33]). To note, in terms of clinical outcomes it has been very recently demonstrated by Jouve et al. that e.g., genetics, were in contrast to the C/D ratio not suitable to predict the outcome of patients [13]. Despite the aforementioned limitations, the C/D ratio can serve as a simple estimate of the metabolism rate which is practical, cost-effective and can assist physicians in the daily routine for risk assessment and to individualize their patients' immunosuppressive therapy. Second, the vascular parameters PBR and PWV have not been analyzed directly in the kidney but are usually extrapolated from measures at other body sides. Therefore, local

effects of Tac on the renal endothelial might have been missed using our approach. Moreover, there are further parameters that can impact on the glycocalyx and the vascular stiffness such as diabetes or hypertension (frequently present in RTx patients and also potentially related to Tac) which have not been investigated in our study. Third, the sample size of our single center study is limited.

Despite these limitations, we demonstrated that a low C/D ratio is associated with significantly higher Tac C2 levels and more severe CNIT. We also showed that systemic markers of endothelial (dys-)function were not associated with the C/D ratio and NRK tubular epithelial cells in vitro were most affected when exposed to Tac according to a fast metabolism PK profile. The C/D ratio may, therefore, be an appropriate tool for identifying patients at risk of developing CNIT.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/8/10/1586/s1. Figure S1: PK profiles used for NRK cell incubation, Table S1: Patient characteristics: Pulse wave analysis, Table S2: Patient demographics: Glycocalyx assessment.

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