Pre-transplant anti-LG3 autoantibodies in kidney transplantation: Impact on the occurrence and prognosis of early acute vascular rejection.

Habib Mawad

Department of Epidemiology, Biostatistics and Occupational Health

McGill University, Montreal

December 2017

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science in Epidemiology

TABLE OF CONTENTS

ABSTRACT

Background – The role of autoimmunity in transplantation is an area of growing interest. Pre-transplant autoantibodies to the LG3 fragment of perlecan (anti-LG3) have recently been associated with acute vascular rejection (AVR) and delayed graft function (DGF) in kidney transplant recipients (KTR). Preliminary evidence suggests that their deleterious effects are enhanced by endothelial damage which is thought to expose cryptic antigenic targets.

Objectives – The ***main objective*** of this work is to validate the association between positive pre-transplant anti-LG3 titers and early AVR, and investigate factors related to endothelial damage as potential effect modifiers for this association. As a ***secondary objective***, I will evaluate the impact of positive pre-transplant anti-LG3 titers on 1-year graft function (glomerular filtration rate: GFR) and on the change in graft function between 6 and 12 months post-transplant (delta-GFR). AVR will be examined as an effect modifier for this relationship.

Methods – This was a retrospective cohort study including consecutive KTR from 2008 to 2014 in 2 Canadian adult transplant centers. Pre-transplant anti-LG3 titers were measured using a locally-developed ELISA and are expressed in optical density (OD) units (x1000). Titers were dichotomized at 130 OD units, the median value of the distribution. For the ***main objective***, I used Cox regression models with biopsy proven AVR as the outcome of interest. Patients who did not develop AVR were censored at death, transfer, non-immune graft loss or 6 months after transplant. An additional analysis was performed using any biopsy-proven acute rejection (AR) as the outcome. For the ***secondary objective***, I assessed the relationships between anti-LG3 and each of 1-year GFR and delta-GFR using separate linear regression models. For ***both objectives***, potential effect modifiers were investigated by including an interaction term in the multivariate model and through exploratory stratified analyses.

Results – There were 444 study subjects with available pre-transplant serum. For the ***main objective***, multivariate Cox regression showed no significant association between positive pre-transplant anti-LG3 and time to first AVR [hazard ratio (HR): 1.25, 95% confidence interval (CI): 0.59; 2.66] or AR (HR: 1.03, 95% CI: 0.68; 1.58). None of the interaction terms were statistically significant, although the point estimates of the adjusted HRs were consistently higher in KTR with characteristics known to be associated with endothelial damage (i.e., DGF, prolonged ischemia, extended-criteria donors/donors after cardiac-arrest). For the ***secondary objective***, positive anti-LG3 titers were not significantly associated with 1-year GFR (β: 0.27 ml/min, 95% CI: -4.00; 4.53), nor was there any significant interaction with early AVR. Findings were similar when considering delta-GFR as the outcome. Exploratory stratified analysis revealed a statistically significant association between positive anti-LG3 titers and delta-GFR in KTR with AVR (β: -7.56 ml/min, 95% CI: -14.0; -1.08). Positive anti-LG3 were not associated with a significant delta-GFR in KTR without AVR (β: -0.02 ml/min, 95% CI: -2.40; 2.35).

Conclusions – Firm conclusions cannot be drawn from this study. In contrast to previous studies, we did not observe a significant association between positive pre-transplant anti-LG3 titers and AVR. These findings may be explained by stronger immunosuppressive protocols and enhanced sensitivity of screening techniques for the evaluation of the pre-transplant immunological risk in the current study compared to previous work. Due to a limited sample size, results were also inconclusive with regards to the hypothesis that endothelial damage enhances the association between pre-transplant anti-LG3 and AVR, and with regards to the role of AVR as an effect modifier for the association between anti-LG3 and delta-GFR. Larger studies are needed to evaluate these hypotheses.

RÉSUMÉ

Contexte – Dans les dernières années, un nombre croissant d'études s'intéresse au rôle de l'auto-immunité en transplantation rénale. La présence en pré-greffe d'auto-anticorps contre le fragment LG3 du perlecan (anti-LG3) a récemment été associée avec un risque accru de rejet aigu vasculaire (RAV) et de retard de reprise de fonction (RRF). L'effet délétère des anti-LG3 semble être amplifié par la sévérité du dommage endothélial qui exposerait des antigènes cryptiques.

Objectifs – L'***objectif principal*** de cette thèse est de valider l'association entre la présence en pré-greffe d'anti-LG3 et la survenue de RAV précoce. Je vais également investiguer si cette association est modifiée par la présence de facteurs reliés au dommage endothélial. Le ***deuxième objectif*** est d'évaluer l'impact des anti-LG3 pré-greffe sur la fonction du greffon à 1 an (débit de filtration glomérulaire : DFG) et sur le changement de fonction entre 6 et 12 mois post-greffe (delta-DFG). La présence de RAV dans les 6 premiers mois sera évaluée en tant que modificateur d'effet pour ces deux associations.

Méthodes – J'ai mené une étude de cohorte rétrospective incluant les patients greffés de 2008 à 2014 dans 2 centres canadiens de transplantation rénale adulte. Les niveaux d'anti-LG3 pré-greffe ont été mesurés par ELISA et sont exprimés en unités de densité optique (UDO, x1000). Ils ont été dichotomisés à 130 UDO ce qui correspond à la valeur médiane dans cette cohorte. Pour l'***objectif principal***, j'ai réalisé une analyse par régression de Cox avec RAV comme issue. Une analyse supplémentaire considérant tout type de rejet aigu (RA) comme issue a également été effectuée. Pour le ***deuxième objectif***, j'ai entrepris une analyse par régression linéaire en considérant successivement DGF à 1 an et delta-DGF comme issues. Pour les **deux objectifs**, les modificateurs d'effets ont été évalués par l'inclusion d'une variable d'interaction dans les modèles multivariés et par des analyses stratifiées exploratoires.

Résultats – Il y avait 444 sujets ayant du sérum pré-greffe. Pour l'***objectif principal***, l'analyse multivariée par régression de Cox n'a pas démontré d'association significative entre la présence d'anti-LG3 pré-greffe et le premier épisode de RAV [hazard ratio (HR): 1.25, intervalle de confiance (IC) à 95%: 0.59; 2.66] ou le premier épisode de RA (HR: 1.03, 95% IC: 0.68; 1.58). Aucune des interactions investiguées n'était statistiquement significative. Cependant, les estimations ponctuelles des HR étaient toujours plus élevées en présence de facteurs reliés au dommage endothélial (c'est-à-dire RRF, ischémie prolongée, donneur à critères élargis et/ou donneur après arrêt cardiaque). Pour le ***deuxième objectif***, la présence d'anti-LG3 pré-greffe n'était pas associée au DFG à 1 an (β: 0.27 ml/min, IC 95%: -4.00; 4.53) et il n'y avait pas d'interaction significative avec la présence de RAV dans les 6 premiers moins. Les trouvailles sont similaires lorsque delta-DFG est considéré comme issue. Dans l'analyse stratifiée exploratoire, il y avait une association statistiquement significative entre la présence d'anti-LG3 pré-greffe et le delta-DFG dans le sous-groupe avec RAV dans les 6 premiers mois (β: -7.56 ml/min, IC 95%: -14.0; -1.08). Ce n'était pas le cas en l'absence de RAV (β: -0.02 ml/min, IC 95%: -2.40; 2.35).

Conclusions – Il est impossible de tirer des conclusions définitives de cette étude. L'absence d'association entre les anti-LG3 pré-greffe et la survenue de RAV diffère des trouvailles antérieures, ce qui pourrait s'expliquer par une immunosuppression plus forte et de meilleures techniques de stratification du risque immunologique dans cette étude. A cause d'une taille d'échantillon limitée, nous ne pouvons pas conclure sur le rôle du dommage endothélial comme modificateur d'effet dans cette association, ni sur le rôle du RAV comme modificateur d'association entre les anti-LG3 pré-greffe et le DFG à 1-an ou le delta-DFG. Des études supplémentaires sont nécessaires pour évaluer ces hypothèses.

AKNOWLEDGEMENTS

I would like to express my sincere gratitude to my thesis supervisors Dr. Bethany J. Foster and Dr. Héloïse Cardinal for their continued support and guidance. Specifically, I would like to acknowledge Dr. Cardinal's expertise in autoimmunity which was instrumental to the completion of this thesis; and Dr. Foster's invaluable advice and insight which helped surmount many of the challenges encountered. I would also like to thank Julie Boucquemont for her precious contributions to all aspects of this work and specifically for her help in the statistical analysis.

I would like to thank my parents for their limitless love and unconditional support throughout all my years of education. I would like to thank my brother Wadi for his encouragements and for being an example of hard work and determination. Finally, I would like to thank my wife Dalia for her endless love and for being a constant source of strength and inspiration.

PREFACE

This thesis has been written in the traditional monograph style. The **background** provides an overview of fundamental concepts of kidney transplantation with emphasis on transplant immunology; in particular the role of autoimmunity. First, I review the data on patient and kidney graft survival, and show how they are related to acute rejection and 1-year graft function, which are the outcomes of interest in the subsequent analyses. Second, I review the available literature on anti-LG3 antibodies and other auto-antibodies in solid organ transplantation. Lastly, I present selected covariates that can potentially act as confounders and/or effect modifiers in my analyses. In the following sections, I explore two separate objectives which are presented in parallel in the methods and results section. The ***first objective*** was to validate the association between pre-transplant anti-LG3 auto-antibodies and early acute vascular rejection. As part of this first objective, I also looked at the association with first acute rejection, regardless of histologic subtype. The ***second objective*** was to evaluate the association between pre-transplant anti-LG3 auto-antibodies and graft function at 1 year, and with the change in graft function between months 6 and 12 post-transplant.

CONTRIBUTION of AUTHORS

Habib Mawad (HM) conducted the literature review and was responsible for drafting all sections of the thesis. Comments and suggestions from Bethany J. Foster (BJF), Héloïse Cardinal (HC) and Julie Boucquemont (JB) were incorporated by HM into the final version of the thesis. All authors contributed to the conception and design of the study. HM performed the statistical analyses under the guidance of BJF, HC and JB.

LIST of TABLES

Appendix

LIST of FIGURES

Appendix

ABBREVIATIONS and ACRONYMS

ABMR: Antibody-Mediated Rejection

APCs: Antigen Presenting Cells

AR: Acute Rejection

AVR: Acute Vascular rejection

AT1R: Angiotensin II type 1 receptor

CDC: Complement-Dependent Cytotoxicity

CHUM: Centre Hospitalier de l'Université de Montréal

CIT: Cold Ischemia Time

CKD: Chronic Kidney Disease

CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration

CORR: Canadian Organ Replacement Registry

CTS: Collaborative Transplant Study

DD: Deceased-Donor

DBD: Donation after Brain Death

DCD: Donation after Cardiac Death

DGF: Delayed Graft Function

DSA: Donor Specific Antibodies

DWFG: Death with a Functioning Graft

ECD: Expanded criteria donors

ELISA: Enzyme-Linked Immunosorbent Assay

ESRD: End-Stage Renal Disease

GFR: Glomerular Filtration Rate

HLA: Human Leukocyte Antigen

HMR: Hôpital Maisonneuve-Rosemont

IVIG: Intravenous Gamma-globulins

LD: Living-Donor

MHC: Major Histocompatibility Complex

OD units: Optical Density units

PRA: Panel Reactive Antibody

RRT: Renal Replacement Therapy

SCD: Standard Criteria Donor

sICAM-1: Soluble Intercellular Adhesion Molecule 1

SRTR: Scientific Registry of Transplant Recipients

sVCAM-1: Soluble Cell Vascular Adhesion Molecule 1

TMA: Thrombotic Microangiopathy

TCMR: T Cell-Mediated Rejection

USRDS: United States Renal Data System

INTRODUCTION

For most patients with end-stage renal disease (ESRD), kidney transplantation is the preferred modality of renal replacement therapy (RRT), with many studies showing improved survival[1-3] and quality of life[4] compared to maintenance dialysis while being more cost-effective.[4,5] The Canadian Institute for Health Information (CIHI) estimates that the one-time cost for a kidney transplant is approximately $23,000, plus $6,000 per year for immunosuppression and follow-up.[5] In contrast, the estimated cost for hemodialysis treatment is approximately $60,000 per patient per year.[5] Unfortunately, the availability of donor kidneys has not been able to keep up with the ever-growing ESRD population. In Canada, the number of patients receiving RRT has nearly tripled over the last 20 years.[6] An estimated 5,333 Canadians initiated some form of RRT in 2013.[6] According to a cross-sectional report from the Canadian Organ Replacement Registry (CORR), about 42,000 Canadians were living with ESRD in 2014.[6] Approximately 42% had a functioning kidney transplant and the remaining 58% were receiving some form of maintenance dialysis. At the start of 2014, about 3,500 Canadians were registered on the waiting list for a deceased donor kidney transplant.[7] Despite considerable efforts made by organ procurement networks, the number of kidney transplants performed that year was only about 1,400 including approximately 120 retransplants. **The scarcity of donor kidneys highlights the importance of maximizing allograft survival.** Indeed, patients who have experienced a previous allograft failure compete for the same rare resource as transplant-naïve ESRD patients. Moreover, a return to dialysis after transplantation has been associated with significant morbidity and mortality.[8] A successful kidney transplant is therefore not only beneficial for the individual recipient, but also for all wait-listed ESRD patients.

Much of the success of kidney transplantation is attributable to a better understanding of **alloimmunity**, or the immunological reaction of transplant recipients to **'non-self' antigens** detected in the allograft. This has led to the development of increasingly more potent immunosuppressive regimens to prevent and treat episodes of acute rejection.[9,10] Despite remarkable improvements, a considerable number of kidney transplant recipients still experience rejection and immune-mediated graft loss.[11,12] Over the last decade, there has been a growing interest in the interaction between alloimmunity and autoimmunity. **Autoimmunity** refers to the immunological processes triggered by **'self' antigens**. The presence of autoantibodies (i.e. antibodies directed against 'self' antigens) has classically been associated with pathogenesis of disease.[13] For instance, agonistic antibodies to the angiotensin II type I receptor (anti-AT1R antibodies) have been associated with scleroderma[14] and pre-eclampsia[15]. In contrast, other naturally occurring autoantibodies seem to be involved in physiological processes such as the clearance of cellular debris.[16-18] Although never associated with disease in transplant-naïve patients, autoantibodies such as **anti-LG3 antibodies** may become clinically relevant after transplantation.[19] There is accumulating evidence that anti-LG3 autoantibodies contribute to vascular damage in kidney transplant recipients.[19] Their effects seem to be enhanced by endothelial damage which can occur as a result of the ischemia-reperfusion injury inherent to any transplant procedure or in the setting of alloimmune mediated vascular injury. [19,20]

The main objective of this thesis is to validate the association between pre-transplant anti-LG3 autoantibodies and early acute vascular rejection of the kidney allograft, and to assess whether factors related to endothelial damage act as potential effect modifiers for this association. As a secondary objective, I will evaluate the association of anti-LG3 autoantibodies with 1-year graft

function, an established surrogate marker for long term-graft survival. First, I will present known determinants of graft outcome and review the available evidence on the role of autoimmunity. Second, I will present my own work on pre-transplant anti-LG3 autoantibodies considering potential confounders and effect modifiers.

BACKGROUND

1. Outcomes in kidney transplantation

The main outcomes of kidney transplantation that will be examined in the subsequent work are **acute rejection and 1-year graft function. Although the most clinically relevant post-transplant outcomes are patient and graft survival, we could not use these as the main outcomes given the relatively short follow-up time and low number of events in our cohort. Hence, we chose outcomes that are clinically relevant as they are strongly linked to graft survival, but that are more frequent and occur relatively early in the post-transplant course.** I will first review the data on patient and kidney graft survival, and show how they are related to acute rejection and 1-year graft function, our outcomes of interest.[21]

1.1. Patient survival

Survival of kidney transplant recipients is still significantly lower than that of the general population. In the latest report from the United States Renal Data System (USRDS), mortality rates among kidney transplant recipients aged 65 to 74 years were 2.5 to 3.3 times higher than the aged-matched general Medicare population.[22] The differences in mortality rates likely reflect various contributions of traditional and transplant-specific risk factors. **Traditional risk factors** include age, sex and comorbid conditions such as diabetes, hypertension and smoking.[23,24] **Transplant-specific factors** include donor source, level of immunosuppression and graft function. For recipients of living donor (LD) transplants, the survival rates at 1 and 5 years post-transplantation

are respectively 98% and 91%.[25] For recipients of deceased donor (DD) transplants, these rates are at best 96% and 83%.[25]

The three leading causes of mortality among kidney transplant recipients are infection, cardiovascular disease, and malignancy.[26] Infection risk is associated with the overall level of immunosuppression used for induction, maintenance and **treatment of rejection.**[27] As a result, infection is much more frequent in the early post-transplant period. Beyond the first year post-transplant, cardiovascular disease is the leading cause of death among adult transplant recipients. The risk of cardiovascular mortality increases with recipient age, presence of diabetes and, importantly, with **poorer kidney graft function**.[28] Malignancy may occur at any time and appears to be precipitated by unchecked replication of oncogenic viruses and deficient tumour surveillance mechanisms[29], both of which are influenced by immunosuppression, including that given for the treatment of **acute rejection.**

1.2. Graft survival

Because of high mortality rates in adult transplant recipients, death with a functioning graft (DWFG) is common and accounts for more than 40% of all graft losses.[11,30] Hence, graft survival is typically captured in two different ways: **i) overall graft survival**, defined as the time from kidney transplantation until death, return to dialysis, or retransplantation, or **ii) death-censored graft survival**, defined as return to dialysis or retransplantation, with observation censored at death. Overall graft survival is believed to offer a more comprehensive picture of the determinants of graft outcome including the patient's global health. The same factors that predict patient survival

also predict overall graft survival, because DWFG is the single most common cause of graft loss.[11,30] On the other hand, death-censored graft survival is thought to better reflect the transplant-specific determinants of graft failure including immunologic risk factors.[31] However, the difference is not straightforward and it is therefore recommended to report both measures whenever possible.[32]

Graft survival is often separated into **short-term** and **long-term** because the causes of early and late graft failure are different. Among adult transplant recipients, the most common causes of graft loss in the first year following transplantation are primary non-function, surgical complications, **acute rejection** and DWFG.[11] Over the last two decades, there has been considerable improvement in short-term graft survival. According to the Scientific Registry of Transplant Recipients (SRTR), all-cause graft failure at 1-year decreased from 16.1% to 4.4% for DD transplants and from 7.2% to 2.7% for LD transplants.[33] For the most part, these improvements are attributable to the introduction of more potent immunosuppressive agents and better management of immunological risk factors.[9] In fact, over that same period of time, there was an equally impressive reduction in the 1-year cumulative incidence of acute rejection from 30-40% to 10-15%.[9,34] Although these improvements are very encouraging, **acute rejection remains one of the most important causes of graft failure in the first year of transplantation**.[11] **Moreover, patients with a history of acute rejection, especially if it occurred in the first 6 months post-transplant, have shorter long-term allograft survival**.[12,31,35,36]

Beyond the first year, the most common causes of graft loss are DWFG, glomerular disease (recurrent or *de novo*) and chronic allograft injury.[11] Chronic allograft injury encompasses both

immune-mediated injury (i.e. chronic active rejection) and **non-immune injury**. Non-immune injury may be caused by chronic hypertension, calcineurin inhibitor toxicity, chronic obstruction or infectious pyelonephritis.[37] Although there has been considerable improvement in short-term graft survival, long-term graft survival remains disappointing. All-cause graft failure at 10-years is estimated at 51.2% for DD transplants and 34.1% for LD transplants.[33]

1.3. Graft function

Short-term graft survival is excellent, with the latest 1 –year overall graft survival estimated at 96-98%.[33] Long-term graft survival, however, is much worse with 10-year overall graft survival at only 49-66%.[33] This discrepancy precludes the use of short-term graft survival as a surrogate for long-term graft survival. On the other hand, **early graft *function* has been shown to be a powerful predictor of long-term graft survival.**[38,39] Graft function is most commonly reported as the glomerular filtration rate (GFR), which is estimated using equations based on the endogenous filtration marker creatinine. In a large North American study of over 100,000 DD and LD transplants, the hazard ratio for graft failure was 1.63 (95% CI: 1.61 to 1.65) with each 88.4 μmol/L increment of the 1 year serum creatinine.[38] The hazard ratio for graft failure increased to 2.26 (95% CI: 2.20 to 2.31) for each 44.2 μmol/L increase in serum creatinine between month 6 and 12 post-transplant.[40] In another study, selected risk factors, including **acute rejection** and donor type (DD vs LD), were shown to be associated with 5-year estimated glomerular filtration rate (GFR) exclusively through their association with 1-year estimated GFR.[39] **These findings illustrate the usefulness of early graft function, in a clinical or research setting, as a surrogate for the effect of acute rejection and other risk factors on long-term graft outcome.**

1.4. Acute rejection

Episodes of acute rejection are defined as **T cell-mediated (TCMR)** or **antibody-mediated (ABMR)** based on the Banff Working Classification of Renal Allograft Pathology.[41,42]

TCMR is characterized by a lymphocytic infiltrate in the allograft. When this infiltrate is limited to the tubules and the interstitium, the rejection is classified as Banff grade I and is sensitive to corticosteroid treatment in 60-70%[43,44] and reversible in terms of graft function in 80-90% of cases.[45] In contrast, when TCMR involves small-sized arteries, the rejection is classified as Banff grade II or III, or **acute vascular rejection (AVR),** which is associated with increased resistance rates to first-line treatment and increased risk of graft loss.[43,46]

Classically, **ABMR** has been described in association with anti-HLA antibodies directed against the donor's HLA. These donor-specific antibodies (DSA) bind to their antigenic targets on the vascular endothelium and produce injury to the graft microcirculation (peritubular capillaritis and glomerulitis) through both complement-dependent and complement-independent mechanisms. Complement-dependent responses give rise to C4d-positive ABMR[47], in which tissue stains diffusely for the marker C4d in peritubular capillaries. C4d is a by-product of the classical complement pathway, which is activated by the antibody-antigen immune complex; it is therefore a marker of antibody-mediated damage. Diffuse C4d staining in peritubular capillaries has been identified as a predictor of poor prognosis.[48,49] As DSA can also injure the graft through antibody-dependent cell-mediated cytotoxicity, which is complement-independent, the most recent Banff classification also recognizes the existence of C4d-negative ABMR.[41] Recent data show that Banff grade II and grade III TCMR vascular lesions, also called endarteritis, often coexist with features

of ABMR such as C4d deposition or the presence of DSA.[50] **Vascular involvement in the rejection process, whether part of ABMR and/or TCMR, whether affecting small and/or large vessels, has been consistently associated with adverse graft outcomes.**[46,51-53] Hence, in the present work, we define **AVR as rejection affecting either the graft arteries (Banff grade II - III TCMR) and/or the graft microcirculation (ABMR).**

Acute rejection is a significant risk factor for both short-term and long-term allograft loss.[11,12,35] In addition to the severity of histological findings, the degree of recovery of graft function after treatment is also a powerful predictor of the long-term impact of an acute rejection episode.[35,54,55]

2. Determinants of Graft Outcome

The graft outcomes outlined above are determined by both immunologic and non-immunologic risk factors. This thesis will primarily focus on the immunologic component which includes alloimmune and autoimmune processes. Below, the role of variables that can potentially act as confounders and/or effect modifiers in the relationship between anti-LG3 and rejection/ graft function is discussed, so that the reader can appreciate why these variables were included in my analyses.

2.1. Alloimmunity

In the absence of adequate immunosuppression, an alloimmune response is orchestrated by the recipient's immune system when it recognizes a foreign antigen as "non-self". The end result is the rejection of the allograft. Several types of alloantigens have been recognized including ABO blood group antigens and the major histocompatibility complex (MHC) molecules. The MHC molecules are a group of highly polymorphic cell surface proteins coded by the human leukocyte antigen (HLA) genes on chromosome 6. The MHC class I genes include: HLA-A, HLA-B and HLA-C. The MHC class II genes include: HLA-DP, HLA-DQ and HLA-DR. These genes are not only highly polymorphic, but they are also co-dominantly expressed which means that each locus expresses both alleles. These characteristics allow MHC molecules to be different enough for each individual that they are able to distinguish "self" from "non-self".[56]

Antigen presenting cells (APCs) are a group of specialized immune cells capable of activating T-cells. APCs process surrounding antigens and express them on surface MHC molecules. The MHC-antigen complex is recognized by T-cell receptor as either "self" or "non-self". In the latter case, T cells become activated and undergo clonal expansion and differentiation. In the setting of kidney transplantation, the resulting effector T-cells infiltrate the allograft and recruit other mononuclear cells giving rise to TCMR.[10,21] When exposed to a given MHC alloantigen (through transfusion, pregnancy or previous transplantation)[57], an individual may also develop specific alloantibodies. These are called **anti-HLA antibodies**. Before going through with a transplantation, the potential recipient is screened to rule out the presence of circulating preformed antibodies specific to the donor's HLA. In that case, transplantation is said to be incompatible because of the presence of **donor specific antibodies (DSA)**. DSAs can cause accelerated acute rejection leading to early graft loss. Therefore, they are considered a contra-indication to transplantation.[58,59] *De novo* DSA also occur after transplantation in up 15-20% of kidney transplant recipients and are associated with ABMR and poorer graft survival[60,61] although in certain cases their significance is unclear.[62] In recent years, alloantibodies directed against other antigenic targets such as the polymorphic MHC class I-related chain A (MICA) and chain B (MICB) have also been associated with acute kidney graft rejection and poorer graft survival in some[63], but not in all studies[64].

If there are preformed anti-HLA antibodies not specific to the donor's HLA, transplantation can go ahead but immunosuppression is usually adjusted accordingly. The recipient's degree of immune sensitization is reported as the **panel reactive antibody (PRA).** The PRA is expressed as a percentage and represents the proportion of the population to which the transplant candidate is

expected to react via preformed antibodies. Some[65,66], but not all[67] studies suggest that the degree of immune sensitization is associated with acute rejection and reduced allograft survival even in the absence of pre-transplant DSA.[68]

Another important element is the degree of **HLA matching** between donor and recipient with respect to HLA-A, HLA-B and HLA-DR. Better HLA matching translates into superior graft survival particularly for HLA-B and HLA-DR.[69,70] **The number of HLA mismatches has also been associated with increased risk of acute rejection**.[71,72] Given that each HLA gene expresses both alleles, there is potential for a maximum of 2 mismatches per HLA.

The **ABO blood group antigens** are not only expressed on red blood cells but also on the endothelium lining the vast network of capillaries in the kidney. This makes them problematic in ABO-incompatible kidney transplants because of naturally occurring DSA (i.e. anti-ABO antibodies). For the same reasons as HLA-incompatibility, these transplants are not routinely performed in North America outside of dedicated programs having developed an expertise in this area.

Currently, the pre-transplant assessment of immunological risk (the risk of rejection) thus comprises the pre-transplant PRA level, screening for pre-transplant DSA, ABO and HLA typing of both donors and recipients, history of previous sensitizing events such as pregnancy, transfusions or transplantations, as well as recipient age, race and sex.[73-76] Assessing this risk is important both in terms of the decision to accept or refuse an organ, but also in selecting the appropriate immunosuppressive regimen for each patient. **As rejections still occur in up to 15% of patients despite this assessment, there is an unmet need for improving the pre-transplant risk stratification procedures.**

2.2. Autoimmunity

In the past decade, **an emerging body of evidence shows that autoantibodies may also participate in and/or enhance the severity of rejection in heart, lung and kidney transplant recipients**.[77] Understanding the interaction between the alloimmune and the autoimmune response in mediating graft damage is a new and rapidly evolving field.

Tolerance is the ability of an individual's immune system to ignore specific antigens recognized as "self". **Central tolerance** is accomplished by negative selection of self-reactive T-cells and B-cells during their development in central lymphoid organs (i.e. thymus and bone marrow). **Peripheral tolerance** refers to the combined processes that prevent circulating self-reactive cells from triggering an immune response (i.e. autoimmune response).[56] When there is a break in tolerance, **autoimmune disease** may occur. Specific circulating autoantibodies are recognized in a wide range of disease states such as systemic lupus erythematous, Grave's hyperthyroidism and multiple sclerosis.[13] Although classically associated with pathogenicity, some autoantibodies are now believed to also fulfill a physiological role as an integral part of the **innate immune system.**[13,16] This shift in paradigm is based on the occurrence of natural autoantibodies in otherwise perfectly healthy individuals.[13,16,17] These autoantibodies are abundant in normal human serum and directed against self-antigens in organs and tissues from all over the body.[18] Their diversity and quantity are associated with age, sex, and concurrent disease.[18] It has been suggested that these naturally occurring autoantibodies play a role in the clearance of cellular debris from apoptotic cells.[16,18]

There has been growing interest in the role of autoimmunity in kidney transplantation. In the immediate post-transplant period, there is inherent ischemia-reperfusion injury to the allograft endothelium, which occurs during organ procurement and transplantation.[78] The injured endothelium recruits non-specific immune cells into the allograft as part of the **innate immune response.**[78] The resulting inflammatory response is greater if the ischemic time is prolonged. In the absence of adequate immunosuppression, the **adaptive immune system**[79] will also be activated and cause subsequent TCMR[80] or ABMR.[81] Endothelial injury is thought to expose cryptic antigenic targets to circulating auto-antibodies. **It has been suggested that the presence of selected auto-antibodies pre-transplant may synergize with ischemia-reperfusion injury to enhance the alloimmune mediated organ damage**.[13,20,82] **Allograft vascular endothelial injury is not limited to the peri-transplant period. In the setting of acute rejection affecting the vasculature, there is also potential for interaction between alloimmune and autoimmune processes**. Although other transplant-relevant autoantibodies have been described, we will mostly focus on anti-LG3 antibodies.

Anti-LG3 antibodies

LG3 is a truncated C-terminal fragment of perlecan, a proteoglycan of the vascular basement membrane whose production is triggered by endothelial apoptosis.[83] Apoptosis refers to the process of programmed cell death. Antibodies directed against LG3 have been identified in kidney transplant recipients. In a retrospective case-control study, although there was some overlap between groups, patients with acute vascular rejection were found to have higher pre-transplant and post-transplant levels of anti-LG3 compared to matched controls.[19] This association was

independent of DSA status. Interestingly, there were cases of C4d-positive (i.e. classical pathway activation of complement system) acute vascular rejection without any circulating DSA but with elevated anti-LG3 levels.[19] This suggests that anti-LG3 antibodies may act directly to cause complement dependent vascular injury in the absence of any alloimmune process. Also, episodes of DSA-positive acute vascular rejection were associated with worse allograft survival if pre-transplant anti-LG3 were elevated, **suggesting that DSA-associated alloimmune vascular injury created permissive conditions for anti-LG3 to meet their antigenic match and enhance vascular damage** .[19]

The concept that anti-LG3 antibodies are not only biomarkers but enhancers/effectors of vascular rejection is supported by animal data. In a murine model of vascular rejection, passive transfer of anti-LG3 was shown to increase the severity of histological findings on graft biopsy compared to control non-specific intra-venous gamma-globulins.[19] However, infusion of anti-LG3 only enhanced the severity of rejection when the transplanted vessel had undergone ischemia prior to transplantation.[19] Taken together, **these observations raise the possibility that anti-LG3 antibodies accelerate vascular damage to the allograft when combined with other permissive factors, such as ischemia or DSA.**

Other Relevant Autoantibodies in Solid Organ Transplantation

Anti-AT1R antibodies

Angiotensin II regulates numerous physiological responses such as vasoconstriction of smooth muscle cells, aldosterone secretion by the adrenal cortex and sodium reabsorption in the

proximal tubule.[78] Angiotensin II type 1 receptor (AT1R) is the principal mediator of angiotensin II's effects. Agonistic autoantibodies binding AT1R were first described in patients with DSA-negative, C4d-negative, refractory acute vascular rejection with malignant hypertension.[84] Infusion of anti-AT1R antibodies in an animal model of kidney transplantation was shown to induce similar histological findings only in the allograft, without any damage to native kidneys.[85] **In another animal model, the vasoconstrictive impact of anti-AT1R antibodies on isolated renal artery rings was enhanced in the presence of ischemic preconditioning.[86]**

Pre-transplant anti-AT1R is associated with poor prognosis independent of classical immunological risk factors.[87] In a prospective study of 107 consecutive kidney transplantations, those with pre-transplant AT1R antibody levels above 9 U/ml experienced more frequent and more severe episodes of acute rejection.[87] Compared with patients with pre-transplant levels <9 U/ml, the 25 (23%), patients with levels above this cut-off had significantly lower allograft function at 1-year (serum creatinine 151 $\pm$ 44 vs. 122 $\pm$ 35 μmol/l, $p<0.05$) and more graft loss (11.1% vs. 1.1%, $p<0.05$).[87] Similar findings were reported in another study where 47.2 % of kidney recipients had anti-AT1R levels above 10 U/ml before transplantation.[88] These patients were shown to have higher risks of acute rejection and graft failure.[88] Another study showed a significant association between de novo anti-AT1R and abnormal biopsy findings including rejection.[89] **Episodes of acute rejection where both anti-HLA and anti-AT1R were detected were associated with the worse allograft survival, again suggesting a synergistic effect between alloantibodies and autoantibodies.[89,90]**

Anti-Vimentin

Antibodies directed against vimentin were initially described in heart transplant recipients in whom they were associated with cardiac allograft vasculopathy.[91] In a murine model of heart transplantation, presensitization with anti-vimentin antibodies was associated with accelerated acute heart rejection.[92] Vimentin is a cytoskeletal protein that can be found within the cytosol of fibroblasts, leukocytes and endothelial cells. There is also evidence that supports cell surface expression of vimentin especially when there is tissue damage.[93] Kidney transplant recipients appear to produce significantly higher levels of anti-vimentin antibodies if the allograft came from a deceased donor after cardiac death rather than after brain death.[94] Anti-vimentin antibodies have been associated with chronic graft vasculopathy in kidney transplant recipients but their significance in acute rejection has never been assessed.[94]

Polyreactive Antibodies to apoptotic cells

Polyreactive antibodies to apoptotic cells were initially identified in the serum of a kidney transplant recipient with ABMR.[95] Isolated B-cell clones were found to be reactive to both auto-antigens and allo-antigens.[95] A subsequent study showed that these antibodies reacted almost exclusively to apoptotic cells and spared viable cells.[96] It also demonstrated that serum concentrations were significantly higher in patients experiencing ABMR compared to other kidney transplant recipients. These antibodies were also shown to have complement-activating properties. Similar to anti-LG3 and anti-AT1R, antibodies polyreactive to apoptotic cells have also been found before transplantation; the presence of pre-transplant polyreactive antibodies were associated with a higher risk of rejection and reduced long-term allograft survival.[97]

Anti-Fibronectin and Anti-Collagen Type IV

Collagen type IV is the one of the major components of the glomerular basement membrane.[78] Fibronectin is the most abundant glycoprotein in the mesangial matrix of the glomerulus.[78] Pre-transplant and *de novo* anti- fibronectin and anti-collagen IV were shown to increase the risk of chronic allograft rejection in kidney transplantation.[98]

The mechanisms of generation of these auto-antibodies remain to be elucidated. Anti-AT1R have been detected in pregnant women with preeclampsia[15] and in cases of systemic sclerosis[14] . However, they do not seem to be associated with normal pregnancy precluding allosensitization as the trigger for antibody production.[99] Anti-LG3 do not seem to be associated with pregnancy either.[99] Anti-vimentin antibodies have been identified in patients with autoimmune diseases such as lupus and rheumatoid arthritis.[93]

2.3 Other Determinants of Graft Outcome

Having outlined the major immunologic determinants of short-term and long-term allograft outcome, I will now briefly review donor-related and recipient-related non-immunologic risk factors.[21,78] **These factors also represent potential effect modifiers and/or confounders in my analyses**.

Donor-related factors

LD transplants have consistently been associated with greater short-term and long-term graft survival compared to DD transplants.[6,33] The difference is maintained even when the LD is unrelated to the recipient, which suggests that the benefit is not solely immune mediated.[100] In order to minimize the risks of live donation, LD candidates are carefully selected after undergoing thorough medical evaluation.[101] Consequently, they make up a healthier subset of the general population than do DD, which often translates to healthier kidneys.[102] The difference in outcomes may also be attributed to the elective nature of live donation, which allows for better preparation for any foreseeable complication, as well as to a shorter cold ischemic time (described below). LD transplant recipients also spend less time on dialysis which has been shown to improve post-transplant outcomes possibly by reducing exposure to chronic inflammation.[103] When it comes to acute rejection, the SRTR shows similar rates in the first year of transplantation for DD and LD.[104]

DD transplants are further categorized into **donation after cardiac death (DCD)** and **donation after brain death (DBD)**. Although DCD has been associated with an increased

incidence of delayed onset of graft function (DGF), DCD is not apparently associated with worse long-term survival compared to DBD.[105-107] Furthermore, DCD is associated with a lower risk of acute rejection than DBD, possibly attributable to the absence of cytokine storm described in DBD. [108,109] Indeed, after brain death there is rapid upregulation of inflammatory mediators in the peripheral organs including the kidney. [109] This increases host immune responsiveness to the allograft which translates clinically into higher rates of acute rejection. [108,109]

DD are also categorized as either **expanded criteria donors (ECD)** or standard criteria donor (SCD)**.** ECD are either deceased donors older than 60 years or deceased donors older than 50 years with two of the following three criteria: terminal creatinine $\geq 132\ \mu$mol/l, history of hypertension or death from a cerebrovascular accident. When these criteria are not met, the deceased donor is referred to as a standard criteria donor (SCD). ECD have been associated with greater risk of graft failure compared to SCD.[104,110] However, in a select group of older patients with comorbidities such as diabetes and hypertension, ECD offer a survival advantage compared to remaining on the waiting list.[111,112] ECD have been associated with increased risk of rejection in some[113,114] but not all studies.[115]

The time elapsed between organ recovery and transplantation has also been associated with allograft survival. ***Cold*** **ischemia time (CIT)** refers to the period of cold storage or hypothermic machine perfusion of the allograft.[56] Prolonged CIT is associated with worse allograft survival, particularly when it exceeds 24h.[116,117] CIT has also been recognized as an independent risk factor for acute rejection.[118] Compared with DBD, DCD entails longer *warm* ischemia which refers to the period of time between cardio circulatory arrest and start of CIT. Although warm ischemia

has not been associated specifically with acute rejection, it has been associated with adverse long-term patient and graft survival. [119]

Delayed graft function (DGF) commonly refers to the need for dialysis in the first week after transplantation.[120] There are several other definitions including i) failure of serum creatinine to decrease by more than 10% in the first 3 days post-op, and ii) failure of serum creatinine to decrease below 250 μmol/l in the first 5 days post-op.[120,121] Occurrence of DGF depends on the interplay between the severity of the ischemia-reperfusion injury and the vulnerability of the graft to such injury. The most important risk factors for the development of DGF include advanced donor age, donor history of hypertension, donor source and ischemic times.[122-125] Immunological risk factors for DGF include elevated peak panel reactive antibody (PRA) and poor human leukocyte antigen (HLA) match. **DGF is associated with reduced long-term allograft survival[126,127] and with a higher risk of acute rejection.**[128]

These three risk factors (DD, prolonged CIT and DGF) have the greatest potential to modify the effect of auto-antibodies on allograft outcome. **Auto-antibodies are believed to act in synergy with ischemia-reperfusion injury to enhance alloimmune organ damage.** The effect of these three risk factors on allograft outcome is mostly mediated by prolonged ischemia and consequently more severe ischemia-reperfusion injury.

Donor age is another well-recognized prognostic factor for both LD and DD transplants.[129] Renal senescence refers to the expected loss of nephrons with aging. Grafts from older donors therefore have fewer functioning nephrons. Age-related comorbidities such as hypertension and atherosclerosis[130] may also influence the quality of graft. It has also been suggested that kidneys from older donors are more immunogenic which translates into a greater risk of acute rejection compared to young donors.[131,132] Moreover, episodes of rejection in kidneys from older donors seem to have worse prognostic impact compared to younger donors.[131] This is likely due to reduced renal reserve and impaired mechanisms of tissue repair.[131] The reasons behind the increased immunogenicity of older kidney donors are not yet fully understood. It has been suggested that age-related fibrosis of the kidney may promote infiltration of immune cells.[132]

Recipient-related factors

In addition to donor-related factors, there are also well recognized recipient-related factors which predict clinical outcomes after transplantation. Retrospective studies have shown that greater **time spent on dialysis** prior to transplantation is associated with **acute rejection**, early graft loss and increased mortality.[103,133,134] There is also evidence that pre-emptive LD transplantation (i.e. without a period of dialysis) is associated with a lower risk of acute rejection in the first six months compared to LD transplantation after a period of dialysis.[134]

Recipient age, race and sex have been associated with rejection and graft survival. Elderly patients experience fewer episodes of acute rejection than younger recipients but when they reject, they rarely return to baseline function.[73] African American recipients have worse allograft

survival, and experience more frequent and more severe episodes of acute rejection compared to Caucasians.[74] There have been conflicting reports with regards to the effect of sex on allograft outcome. The SRTR shows better graft survival in male recipients[104]. Other studies suggest that female recipients may be at greater risk for acute rejection due to a higher degree of sensitization.[76]

In the previous section, I have outlined the main determinants of graft outcome with emphasis on acute rejection as this will be the focus of the subsequent work. First, I reviewed mechanisms of alloimmunity and autoimmunity in the context of solid organ transplantation. Second, I presented donor- and recipient-related factors that may modify immunogenicity of kidney transplantation. These factors represent potential effect modifiers in the relationship between autoantibodies and acute rejection.

RESEARCH QUESTIONS

Research Questions

1. Are pre-transplant anti-LG3 autoantibodies independently associated with AVR or with acute rejection of any type in the first 6 months following transplantation?
    - Are ischemic time, donor type or DGF effect modifiers in the relationship between pre-transplant anti-LG3 autoantibodies and AVR?

2. Are pre-transplant anti-LG3 autoantibodies independently associated with graft function at 1 year post-transplant or with the change in graft function between 6 and 12 months post-transplant?
    - Is AVR in the first 6 months an effect modifier in the relationship between pre-transplant anti-LG3 autoantibodies and graft function at 1 year post-transplant or the change in graft function between 6 and 12 months post-transplant?

Rationale

The incidence of acute rejection in kidney transplantation has decreased considerably thanks to better stratification of alloimmune risk factors and use of more potent immunosuppressive regimens.[9,34] Despite notable improvements, acute rejection still occurs in up to 15% of kidney transplant recipients[33] and is a common cause of graft loss in the first year after transplantation.[11] Acute rejection in the first 6 months[12] and involvement of the graft vasculature[46] are associated with worse short-term and long-term outcomes. **Hence, improving risk**

stratification for acute rejection, especially acute vascular rejection, is an unmet need in kidney transplantation. There is evidence to support an association of pre-transplant autoantibodies against LG3[19,20] with **more frequent episodes of acute vascular rejection.** However, previous work on the topic was done in patients **with the less potent immunosuppression and less refined screening techniques for pre-transplant DSA** used in the 1990's and early 2000's. Furthermore, despite the strong association between anti-LG3 and AVR, **overlapping values in anti-LG3 titers were seen in patients who went on to develop AVR and those who did not.** Endothelial damage resulting from ischemia-reperfusion or alloimmune-mediated injury is believed to expose cryptic antigenic targets (such as LG3) which become available for binding by circulating autoantibodies (such as anti-LG3). Hence, we undertook this study to validate the association between anti-LG3 and AVR in the current era of transplantation care, and to assess whether some clinical conditions associated with endothelial damage made these antibodies more relevant in terms of risk stratification for AVR and 1-year graft function. **If our hypotheses are confirmed, the detection of anti-LG3 autoantibodies pre-transplant could improve risk stratification and guide preventive measures. This could have important clinical applications with regards to organ allocation, choice of immunosuppression and post-transplant surveillance.**

Hypotheses

For the ***first question***, I expect that anti-LG3 autoantibodies will be associated with a higher risk of acute rejection affecting the vasculature, but not other types of rejection. In particular, I do not expect to find an association with acute rejection of any type, irrespective of histology. I also hypothesize that the relationship with acute rejection affecting the vasculature will be stronger in the presence of permissive factors related to endothelial damage (donor type, prolonged ischemia or DGF). In recipients with no such risk factors, I hypothesize that anti-LG3 antibodies will not be associated with acute vascular rejection.

The identification of these three potential effect modifiers is based on the following rationale:

1. **Donor type**
   - The prolonged ischemia associated with **DCD** may increase endothelial damage and consequently expose cryptic antigenic LG3 targets.
   - By definition, **ECD** encompasses older donors with some degree of vascular disease and/or kidney damage. Therefore, it is reasonable to assume that the allograft endothelium would be more susceptible to circulating auto-antibodies.
2. **Prolonged ischemia**
   - Prolonged ischemia entails more severe ischemia-reperfusion injury which is hypothesized to expose cryptic LG3 endothelial targets.
3. **DGF**
   - DGF results from severity of ischemia-reperfusion injury and vulnerability of the allograft. The endothelium is therefore vulnerable to circulating autoantibodies.

For the ***second question***, I hypothesize that pre-transplant anti-LG3 autoantibodies will not be associated with 1-year graft function or change in graft function unless patients experienced AVR. The endothelial damage resulting from alloimmune injury will expose LG3 antigenic targets and allow for binding by circulating autoantibodies. As previous animal studies show that anti-LG3 transfer can enhance microvascular injury, peritubular capillary drop-off and fibrosis,[19] I expect to find lower 1-year GFR and a larger delta-GFR in patients with AVR and positive pre-transplant anti-LG3 compared to those with AVR and low pre-transplant anti-LG3. I expect to find higher 1-year GFR in patients without AVR than in patients with AVR, and no relationship between pre-transplant anti-LG3 levels and 1-year GFR or delta GFR in patients without AVR.

METHODS

Study design and patients

I performed a retrospective cohort study using the University of Montreal Renal Transplant Biobank. The biobank aims at providing biological material and data to researchers working in the field of kidney transplantation locally, nationally and internationally. This database gathers clinical and biological data on kidney transplant recipients from the Centre Hospitalier de l'Université de Montréal (CHUM) and Hôpital Maisonneuve-Rosemont (HMR) and their donors. The number of recruited subjects is approximately 70 to 80 participants per year at CHUM and approximately 50 at HMR. The biobank started in June of 2008 and is still accruing participants. Patients are asked to participate in this observational study, with an enrollment rate > 95%. There are currently over 700 patients registered in this database. This bank includes biological material (recipient serum, plasma, urine, DNA, ARN/protein, graft renal tissue; donor sera, spleen and kidney graft tissue) that is collected pre- and post-transplantation (1, 6, 12, 24 months and at the time of any graft biopsy). The biological samples can be linked (using identification codes) to a bank of clinical data. The biological material is stored at -80°C in freezers located at the CHUM Research Center (CRCHUM) and HMR.

The funding for this bank has been secured by the Université de Montréal's nephrology research consortium via the *Vice-décanat à la recherche de la Faculté de médecine de l'Université de Montréal,* Hôpital Maisonneuve-Rosemont Research Center, CHUM Research Center, Hôpital du Sacré-Coeur Research Center, Roche Canada, and Astellas Canada. The biobank is not for profit, is dedicated to academic users only, and requests for use are examined by a scientific

committee that follows pre-established criteria to grant access. The biobank is approved by the CHUM and HMR local ethics committees.

For the purpose of this work, I included all patients enrolled in the University of Montreal Renal Transplant Biobank from June 1st 2008 to August 1st 2014. If a patient had more than one kidney transplantation recorded in the database, only the most recent episode was included. Patients with positive pre-transplant DSA were excluded.

Outcomes

For the ***first question***, I performed two separate survival analyses:

1. In the first, the primary outcome was biopsy-proven **acute vascular rejection (AVR) in the first 6 months after transplantation**, defined as any histological evidence of immune mediated micro- or macro-vascular injury. This included antibody mediated rejection (ABMR), T-cell mediated rejection (TMCR) Banff grade II or III, immune-mediated thrombotic microangiopathy (TMA) or glomerulitis.
2. In the second analysis, the primary outcome was **first biopsy-proven acute rejection** regardless of histological subtype.

For both survival analyses, patients who did not develop the outcome were censored at death, transfer, non-immune graft loss or 6 months after transplantation. Biopsies in the first 6 months may be either clinically indicated or part of a routine surveillance program. In one of the participating transplant centers, routine surveillance biopsies are usually performed between 3 and

9 months post-transplant. Episodes of rejection were consecutively recorded in the database along with detailed histologic findings. We reviewed all episodes of rejection in the first 6 months and classified them as AVR or not according to the previous definition.

For the ***second question***, the GFR was estimated at 6 and 12 months post-transplant using the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation.[135] If there was graft failure before these time points, a GFR of 0 ml/min per 1.73m^2 was assigned. The change in graft function between 6 and 12 months post-transplant, subsequently referred to as "delta-GFR", was calculated by subtracting the GFR at 12 from that at 6 months. As a result, a negative delta-GFR represents a loss in graft function.

Exposure

Anti-LG3 titers were measured on the banked pre-transplant sera using ELISA and are expressed in optical density (OD) units (x1000). Outcome status was not known to the laboratory technician performing the ELISA. The ELISA was developed locally at the CHUM and has been described previously.[19] There are no definitive cut-offs for positivity of anti-LG3 antibodies. I dichotomized results at 130 OD units which corresponds to the median in our cohort, and is very close to the cut-off used in previous work (140 OD units), which was the median level observed in that cohort of patients.[19] Dichotomization of anti-LG3 titers may result in some loss of information but it simplifies interpretation of results which facilitates decision-making.

Covariates

Known associations between anti-LG3 antibodies, AVR and other covariates were incorporated into a directed acyclic graph (DAG). Missing arrows usually indicate a strong assumption of the absence of an association between two variables. For anti-LG3, it is hard to make these assumptions for most variables because of the scarcity of current data. Anti-LG3 autoantibodies have only recently been described and therefore, underlying associations have yet to be fully defined. I have therefore illustrated only the known associations to avoid overcrowding the graph (figure A1 in Appendix). This was meant to identify potential confounders for the main question based on current literature and avoid conditioning on common effects (i.e. colliders).

Potential confounders were also investigated by examining associations within the study cohort. These included recipient baseline characteristics (e.g., age, race, sex) as well as donor characteristics (e.g., age, donor type). I also explored immunologic factors such as recipient PRA, degree of HLA mismatch and prior allosensitization (i.e., previous transplantation, transfusion or pregnancy).

Donor type was modeled as a 3-level variable with LD being the reference category. Deceased donors could not be categorized as ECD according to traditional criteria because terminal creatinine was not available. Instead, I introduced a modified ECD variable (mECD) based on the available criteria (deceased donor older than 60 years or deceased donor older than 50 years with hypertension and death from a cerebrovascular accident). mECD and DCD were grouped together in a single category. The third category included DBD who did not meet criteria for mECD (i.e. standard criteria DBD).

PRA was measured using complement-dependent cytotoxicity (CDC). HLA mismatch was reported as a continuous variable from 0 to 4 for the number of mismatches in HLA-B and HLA-DR. Total ischemia time was used rather than cold ischemia time because of fewer missing values. Total ischemia time was analyzed as a continuous variable in hours as well as a dichotomous variable using the top quartile of 15 hours as the cut-off.

Pre-transplant anti-LG3 autoantibodies have been associated with DGF[20], which is a known risk factor for acute rejection.[128] As such, DGF could be on the causal pathway between exposure and outcome. Adjusting for a variable on the causal pathway is commonly used to estimate the direct effect of the exposure on outcome, i.e. the effect not mediated through the intermediate variable. However, this approach becomes problematic when there is an unmeasured common cause of the intermediate variable and the outcome.[136,137] In this case, adjusting for the intermediate variable (i.e. including DGF in the multivariate model) could introduce a spurious association between exposure and the unmeasured confounder and by the same token between exposure and outcome.[136,137] Mediation analysis is a more reliable technique to assess the relative magnitude of each pathway.[138,139] As I cannot confidently assume the absence of any unmeasured confounders, and I did not use any mediation analysis, DGF was not included as a covariate in the final models looking at the association between anti-LG3 and acute rejection. Although residual confounding is likely, leaving out DGF is preferred as a more conservative approach.

DGF was defined as the need for dialysis within the first week after transplantation, failure of serum creatinine to decrease by more than 10% on the first 3 post-operative days, or serum creatinine levels >250 μmol/L on post-operative day 5 in the presence of scintigraphic evidence of acute tubular necrosis. [120,121]

Statistical analysis

For categorical variables, I report the number of observations alongside the corresponding percentage of available data. Continuous variables that were normally distributed were expressed as mean ± standard deviation (SD); otherwise, they were reported as median with interquartile range (IQR). Characteristics of patients without available pre-transplant serum were also reported in order to compare with those included.

A distribution curve and normal Q-Q plot were produced for all available pre-transplant autoantibodies. The Shapiro-Wilk normality test was also used to assess anti-LG3 distribution. Using anti-LG3 as a continuous variable, I compared titers across levels of each categorical variable using the Kruskal-Wallis test. If the Kruskal-Wallis test showed statistical significance, pairwise comparison was performed using Dunn's test. I also looked for correlations with other continuous variables by examining scatterplots and calculating Spearman's correlation coefficients. I then used anti-LG3 as a dichotomous variable and compared proportions of positive results across levels of categorical variables using Chi-square tests and Fisher's exact tests.

For the ***first question***, I performed two separate survival analyses using Cox proportional hazards models looking at each outcome of interest: **i) biopsy-proven AVR** and **ii) first biopsy-proven acute rejection of any type.** In order to select the most relevant covariates for multivariate models, I first ran a multivariate Cox proportional hazard model including all covariates of potential importance (based on the literature review). I then ran the same model excluding a single variable at a time and evaluated the relative change in the point estimate for the main exposure (i.e. anti-LG3> 130 OD units). If the change was greater than or equal to ± 5%, the variable was considered relevant and was kept in the model; otherwise it was excluded. Once the final model

was obtained, I tested the proportional hazards assumption using the Schoenfeld Residuals Test and graphical inspection.

For each potential effect modifier, I included an interaction variable in the multivariate model and assessed statistical significance using $p<0.05$ as the cut-off. I also ran the multivariate model within strata of each potential effect modifier and compared the hazard ratios (HR) for anti-LG3 >130 OD units. These exploratory analyses are not likely to yield statistically significant results because of limited power, but they may still be informative and help guide subsequent studies. If my hypotheses are correct, the HRs will be greater in the stratum with more endothelial damage.

Finally, I performed a sensitivity analysis using a slightly different definition of AVR which was used in a previously published work, to facilitate comparison. In this additional survival analysis, AVR was defined as biopsy-proven Banff grade II or III TCMR. [19]

For the ***second question***, I used linear regression to evaluate the association between pre-transplant anti-LG3 antibodies and 1-year GFR. Covariates found to be statistically significant on univariate analysis were included in the multivariate model. The same steps were followed to evaluate the association between pre-transplant anti-LG3 antibodies and delta-GFR. In order to assess whether AVR in the first 6 months was an effect modifier in the relationship between anti-LG3 antibodies and graft function, an interaction term was included in the multivariate models. I also ran the multivariate models in KTR with and without AVR. I verified that the linear assumption was reasonable by examining scatterplots of continuous variables included in the final

models. I also graphed the residuals of each model using a histogram to verify the normality assumption.

All analyses were performed using R version 3.2.3 (The R Foundation for Statistical Computing, Vienna, Austria) and Stata version 14.2 (StataCorp, College Station, Texas)

Ethics approval

The data used in this study were obtained as part of **the University of Montreal Renal Transplant Biobank.** The project was reviewed and approved by the local research ethics board.

RESULTS

Study population

From June 1st, 2008 to August 1st, 2014, a total of 553 incident kidney transplant recipients were enrolled in the University of Montreal Renal Transplant Biobank. After exclusion of participants with positive pre-transplant DSA (n=2) and absent pre-transplant serum (n=106), the final study cohort included 444 patients from both transplant centers (Figure 1). Three patients had multiple kidney transplants within the study period but only the most recent transplant was considered.

Recipient, donor, and transplant characteristics were available in nearly all study participants (Table 1). The mean age at transplantation was 50.5 ± 13.6 years (Table 1). Study participants were mostly male (64%) with no prior history of kidney transplantation (89%). There were 115 LD transplants which accounted for a quarter of all participants. Among the remaining 328 DD transplants, about 40% met criteria for mECD and/or DCD (n=129). A detailed breakdown of donor types is available in the appendix (Table A1). Basiliximab was the most commonly used immunosuppressive agent for induction (77%). Intravenous gamma-globulins were administered in a small number of participants (7%). Typical maintenance immunosuppression included tacrolimus (96%) and mycophenolate (99%). The median total ischemic time was 9.5 hours. In 25% of cases, total ischemic time exceeded 15 hours (i.e. top quartile). A total of 163 patients (37%) met criteria for DGF.

Excluded patients

For the 106 patients without available pre-transplant serum, the rate and features of rejection episodes were similar to those included in the study cohort (Table A2 in the Appendix). There were 18 episodes of AR (17%) of which 5 (5%) met the histologic definition of AVR. Recipient, donor and transplant characteristics were also similar to those of included patients (Table A3 in the Appendix). However, there were fewer LD transplants (10% vs. 26%) and more standard criteria DBD (62% vs. 45%).

Anti-LG3 autoantibodies

The median level of pre-transplant anti-LG3 was 130.0 OD units with an interquartile range of 86.9 to 214.0 (Table 2). The distribution of pre-transplant anti-LG3 within the study cohort was skewed to the right (Figure A2 in the Appendix). The normal QQ-plot and the Shapiro-Wilks test confirm that anti-LG3 titers are not normally distributed.

Positive anti-LG3 antibodies (dichotomized at the median) were not associated with any recipient, donor, or transplant characteristics (Tables A4 and A5 in the Appendix). When treated as a continuous variable, anti-LG3 levels were found to be significantly higher in black patients compared to all others (198.8 OD units, IQR 99.1-329.1 vs. 128.0 OD units, IQR 85.6-203.5, p=0.007). Anti-LG3 levels were also slightly higher in patients receiving thymoglobulin (ATG, which is more often given to black patients because of their higher immunological risk) compared to basiliximab and in transplants with at least one mismatch in HLA-DR compared with no mismatch (Table A5 in the Appendix). There were no other statistically significant associations (Tables A4 to A6 and Figure A3 in the Appendix).

Pre-transplant anti-LG3 and acute vascular rejection (AVR)

There were 33 episodes of biopsy-proven rejection that met the histologic definition of AVR (Table 3). More than half of these were classified as TCMR Banff grade II or III which is the definition used for the sensitivity analysis. Biopsies showing AVR were done for clinical indications in most cases (i.e. not surveillance) and were performed on average 1.90 ± 1.88 months post-transplantation (Table 3). For most patients not experiencing AVR, follow-up was available for at least 6 months (Figure 1). Only 41 patients were censored less than 6 months after transplantation. This was because they were either transferred to another hospital (n=31), had non-immune graft loss (n=5) or died within the first 6 months (n=5).

Fifty-eight percent of patients who experienced AVR had positive anti-LG3 levels before transplantation, compared with 49% for those with no AVR (p-value = 0.4, Table 2). In the unadjusted Cox regression model, we could not detect a significant association between positive pre-transplant anti-LG3 titers and AVR in the first 6 months post-transplant (HR 1.40; 95% CI 0.70 to 2.80). After adjusting for recipient age, intravenous gamma-globulins and total ischemia time, the HR associated with positive anti-LG3 was 1.25 with a 95% CI of 0.59 to 2.66 (Table 4). The results were similar in the sensitivity analysis, using a slightly different definition of AVR (Table A8 in the Appendix). Testing of the proportional hazards assumption (Figure A4) and the change-in-estimate procedure used for variable selection (Table A9) are detailed in the appendix.

None of the interaction variables were statistically significant (Table 5). However, the point estimate for the association between anti-LG3 and AVR was higher when total ischemic time was >15h (HR 2.06, 95% CI: 0.36-11.81) compared to ≤15h (HR 1.36, 95% CI: 0.58-3.17). Similarly,

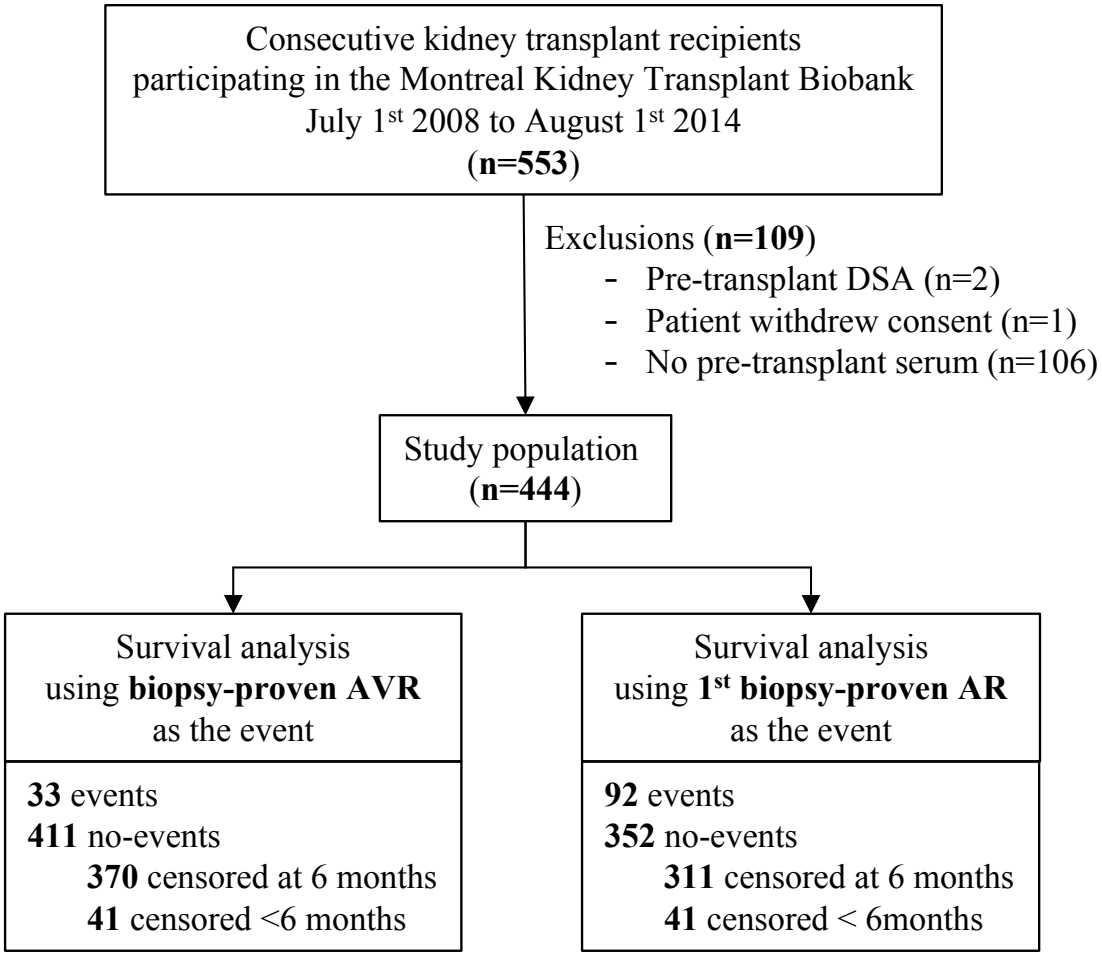
the point estimate was higher in KTR with DGF (HR 1.56, 95% CI: 0.58-4.15) compared to KTR without DGF (HR 1.09, 95% CI: 0.33; 3.60). In addition, the HR was higher in transplants from mECD and/or DCD (HR 3.01 95% CI: 0.68; 13.36) compared to standard criteria DBD (HR 0.67, 95% CI: 0.18; 2.47). Although our stratified analyses suggest that factors promoting endothelial damage may indeed potentiate the relation between pre-transplant anti-LG3 and AVR, the large confidence intervals preclude definitive conclusions.

Pre-transplant anti-LG3 and 1st acute rejection of any type (1st AR)

There were 92 kidney transplant recipients with at least one episode of biopsy proven AR of any type. The most important histologic subtype was borderline rejection accounting for 45% of all 1st AR episodes (Table 3). The mean time to 1st AR was 2.32 ± 1.69 months (Table 3).

When considering AR of any type, there was no appreciable difference in the proportion of patients with AR who had positive pre-transplant anti-LG3 compared with patients without AR (Table 3). The unadjusted Cox regression model showed a HR of 1.08 with a 95% CI of 0.72 to 1.63 associated with positive aniti-LG3 (Table 6). As shown in Table A10 in the appendix, none of the considered covariates appeared to substantially confound the relationship between anti-LG3 and AR. Therefore, the same covariates were included as for the model with AVR as the outcome. The adjusted models showed no significant relationship between anti-LG3 and AR in the first 6 months post-transplant [HR 1.03; 95% CI 0.68 to 1.58 (Table 6)]. Testing of the assumption of proportional hazards is detailed in the appendix (Figure A5).

Figure 1. Patient flow chart `



DSA: donor specific antibodies; **AVR**: acute vascular rejection; **AR**: acute rejection

Table 1. Recipient, Donor and Transplant Characteristics of **study population**

Characteristics	**n**	
Recipient Characteristics		
Age (years)	444	50.5 ± 13.6
Race (black)	444	34 (8%)
Sex (male)	444	283 (64%)
Baseline Renal Disease	444	
− Glomerular or Auto-immune		177 (40%)
− Diabetes or Hypertension		92 (21%)
− Other		175 (39%)
Comorbidities		
− Coronary disease	440	80 (18%)
− Diabetes	443	96 (22%)
− Smoking history	441	252 (57%)
Previous Kidney Transplantation	444	48 (11%)
Previous Pregnancy	444	117 (26%)
Previous Transfusion	441	187 (42%)
PRA CDC >20%	443	14 (3%)
Donor Characteristics		
Age (years)	444	49.0 ± 14.7
Comorbidities		
− Diabetes	420	36 (9%)
− Hypertension	423	101 (24%)
Donor type	443	
− Live donor		115 (26%)
− Deceased donor		328 (74%)
− mECD and/or DCD		129 (29%)
− Standard criteria DBD		199 (45%)
Transplant Characteristics		
Center (CHUM)	444	233 (53%)
HLA Mismatches in DR and B	444	2.2 ± 1.1
Induction IS (Basiliximab)	443	342 (77%)
Intravenous Gammaglobulins	444	31 (7%)
Maintenance IS (Tacrolimus)	442	425 (96%)
Adjuvant IS (Mycophenolate)	440	437 (99%)
Total Ischemia time (hours)	433	9.5 (4.9; 14.8)
Total Ischemia time > 15h	433	106 (24%)
Delayed Graft Function	442	163 (37%)

Column "n" contains the number of available observations for each variable.
Categorical variables are reported as count (%).
Continuous variables are reported as mean ± standard deviation if normal distribution. Otherwise, they are reported as median (IQR: Q1; Q3);
PRA CDC: Panel Reactive Assay Complement-Dependent Cytotoxicity; **mECD**: modified expanded criteria donor; **DCD**: donor after cardiac death; **DBD**: donor after brain death; **HLA**: Human Leukocyte Antigen; **IS**: immunosuppression

Table 2. Pre-transplant anti-LG3 according to **AVR** and **1st AR**

	Anti-LG3 titers (OD units**)**	**Anti-LG3 > 130** OD units
444 patients (all patients)	130.0 (86.9; 214.0)	220 (50%)
33 with outcome AVR 411 without AVR	143.5 (86.0; 222.0) 129.5 (87.0; 213.2)	19 (58%) 201 (49%)
92 with outcome AR 352 without AR	131.5 (86.4; 243.6) 129.0 (87.0; 209.8)	47 (51%) 173 (49%)
Median (IQR: Q1; Q3) is reported for anti-LG3 as a continuous variable **Count (% of total available observation)** is reported for anti-LG3 as a dichotomous variable.		

Table 3. Characteristics of rejection episodes in **study participants**

Characteristics	**n**	**AVR 33 (7%)**	**n**	**1st AR 92 (21%)**
Time of Rejection (months after KTx)	33	1.90 ± 1.88	92	2.32 ± 1.69
Histology	33		92	
– Borderline		2 (6%)*		41 (45%)
– IA		1 (3%)*		14 (15%)
– IB		0		8 (9%)
– IIA		18 (55%)		17 (18%)
– IIB		1 (3%)		1 (1%)
– III		1 (3%)		1 (1%)
– HCAPI		7 (21%)		7 (8%)
– HEND		2 (6%)		2 (2%)
– TMA		1 (3%)		1 (1%)
Peritubular capillary **c4d** staining >50%	33	7 (21%)	91	7 (8%)
Positive **DSA** at the time of rejection	27	5 (19%)	71	10 (14%)
Indication for biopsy	33		92	
– Surveillance		4 (12%)		24 (26%)
– Clinical		29 (88%)		68 (74%)
Column "n" contains the number of available observations for each variable. * The 2 cases with borderline histology classified as AVR had glomerulitis. The case with IA histology categorized as AVR had thrombotic microangiopathy; **AVR:** acute vascular rejection; **AR:** acute rejection; **KTx:** kidney transplantation; **HCAPI**: humoral rejection with capillaritis and glomerulitis; **HEND**: humoral rejection with endarteritis; **TMA**: thrombotic microangiopathy; **DSA:** donor specific antibodies				

Table 4. Multivariate Cox proportional hazards models: **AVR as the outcome**

Variables	Reference category	HR (95% CI)	
		Unadjusted	Multivariate
Anti-LG3 > 130 OD units	≤130	**1.40 (0.70; 2.80)**	**1.25 (0.59; 2.66)**
Recipient Age (years)	per 10-year increment	-	0.76 (0.95; 1.00)
Gammaglobulins – Yes	No	-	3.64 (1.53; 8.67)
Total ischemia time	per 1-hour increment	-	0.97 (0.91; 1.04)

Table 5. Stratified analyses – Multivariate **Cox** models with **AVR as the outcome**

Variable	Levels	n	HR (95% CI) for **anti-LG3 >130** OD units	p-value for interaction term
Total ischemia	**≤15 hours**	327	1.32 (0.57; 3.10)	0.85
	>15 hours	106	1.80 (0.34; 9.60)	
DGF	**No**	279	1.09 (0.33; 3.60)	0.69
	Yes	163	1.56 (0.58; 4.15)	
Donor type	**Live donor**	115	2.13 (0.54; 8.41)	
	mECD and/or DDC/SCD	129	3.03 (0.69; 13.33)	0.85
	Standard criteria DBD	199	0.58 (0.153; 2.17)	0.22

A multivariate model was used in each level of the potential effect modifiers:
- For **total ischemia**: AVR ~ Anti-LG3 + Recipient age + Gamma IV
- For **DGF**: AVR ~ Anti-LG3 + Recipient age + Gamma IV + ischemia (hours)
- For **Donor type**: AVR ~ Anti-LG3 + Recipient age + Gamma IV + ischemia (hours)

The p-value corresponds to the interaction variable when included in the multivariate model.
DGF: delayed graft function; **mECD**: modified expanded criteria donor;
DCD: donor after cardiac death; **DBD**: donor after brain death.

Table 6. Multivariate Cox proportional hazards models: **1st AR as the outcome**

Variables	Reference category	HR (95% CI)	
		Unadjusted	Multivariate
Anti-LG3 > 130 OD units	≤130	**1.08 (0.72; 1.63)**	**1.03 (0.68; 1.58)**
Recipient Age (years)	per 10-year increment	-	0.85 (0.72; 0.99)
Gammaglobulins – Yes	No	-	1.44 (0.72; 2.89)
Total ischemia time	per 1-hour increment	-	1.00 (0.97; 1.04)

Association of pre-transplant anti-LG3 and 1-year GFR

Among the 444 patients included the study cohort, 383 had 1-year GFR available. Among these, 19 were assigned a 1-year GFR of 0 ml/min per 1.73m^2 because they either died (n=6) or lost the graft (n=13) within the first year of transplantation. The 61 patients without 1-year GFR were either transferred to another hospital (n=32) or were transplanted less than a year before data collection (n=29). The mean 1-year GFR was 55.7 ± 23.8 ml/min with values ranging from 0 to 116 ml/min per 1.73m^2 (Figure 2 A).

On univariate analysis, 1-year GFR was similar in KTR with positive pre-transplant anti-LG3 compared to those with negative pre-transplant anti-LG3: the difference in 1-year GFR between those with positive and negative pre-transplant anti-LG3 was –0.91 ml/min (95% CI: –5.71; 3.88). In order to identify potential confounders, I looked at other factors associated with 1-year GFR within the study cohort. Using simple linear regression, AVR in the first 6 months was associated with significantly lower 1-year GFR (difference in 1-year GFR: –12.13 ml/min, 95% CI: –20.96; –3.30). Recipient age, induction with thymoglobulin and DGF were also associated with significantly lower 1-year graft function in unadjusted analyses (Table A11 in the Appendix). One-year GFR was 15.48 ml/min lower in mECD and/or DCD transplants than in LD transplants (95% CI: 9.40; 21.55). mECD incorporates information on donor age and donor hypertension. These two characteristics were also individually associated with lower 1-year GFR.

In the multivariate model (Table 7), adjusted for these potential confounders (i.e., AVR, recipient age, induction immunosuppression, DGF and donor type), there was no significant difference in 1-year GFR associated with positive anti-LG3 antibodies, compared with negative

anti-LG3 (difference: 0.27 ml/min (95% CI: –4.00; 4.53). The association between anti-LG3 and 1-year GFR did not differ appreciably when there was AVR in the first 6 months compared with when there was no AVR (Table 8).

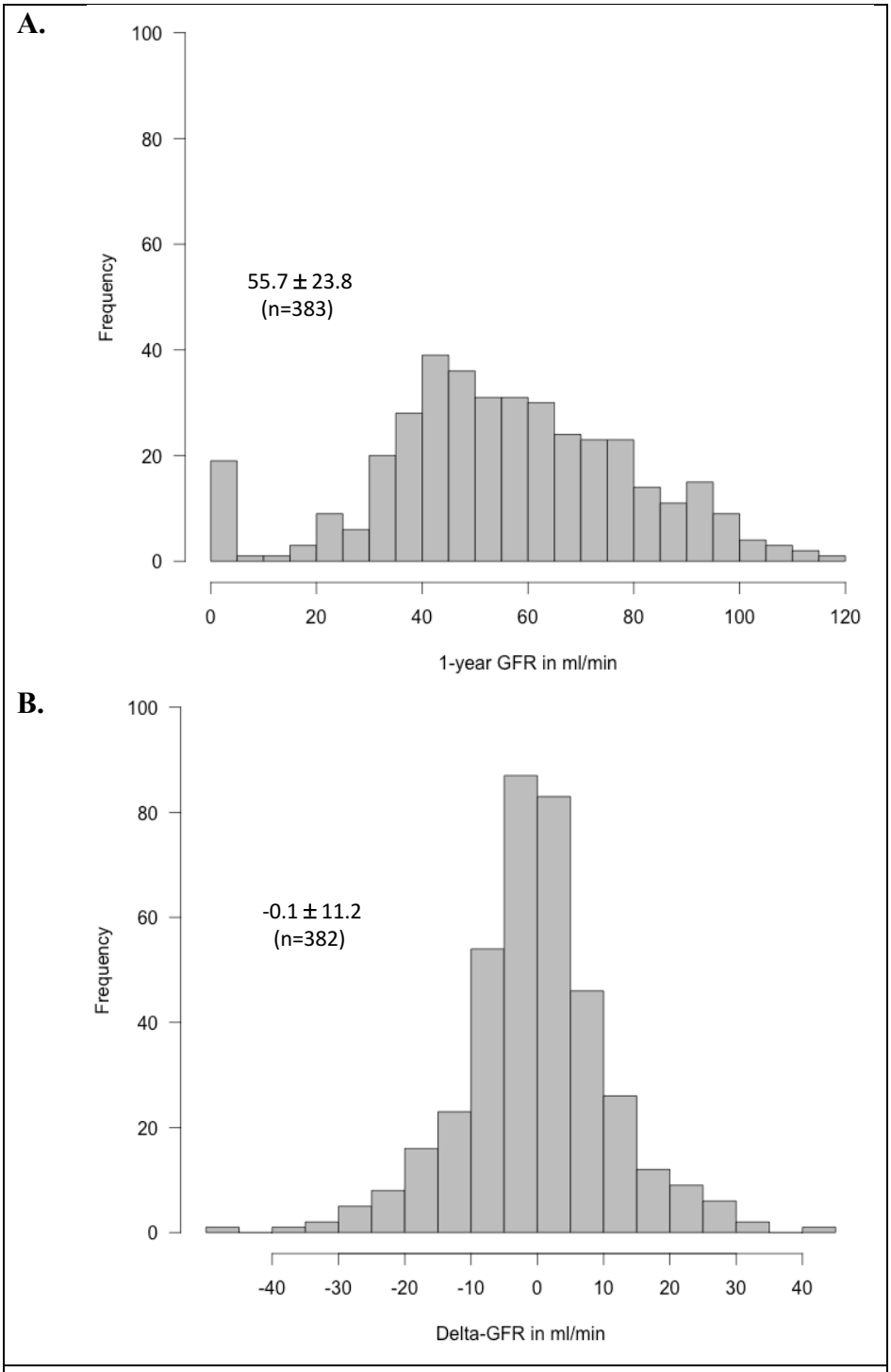
Association of pre-transplant anti-LG3 and delta-GFR

The change in graft function between 6 and 12 months post-transplant (delta-GFR; 12-month minus 6-month GFR), was available in 382 patients. All but one of the 383 patients with 1-year GFR also had an available GFR at 6 months post-transplant. The mean delta-GFR was -0.1 ± 11.2 ml/min per 1.73m^2 (Figure 2 B).

There were no statistically significant associations between positive pre-transplant anti-LG3 antibodies and delta-GFR for the full cohort. In the unadjusted analysis, the decrease in GFR was 1.15 ml/min (95% CI: –1.12; 3.41) larger in KTR with positive pre-transplant anti-LG3 compared to those with negative pre-transplant anti-LG3 (not statistically significant, Table 9). The only covariates associated with delta-GFR on univariate analyses were race and donor type (Table A11 in the Appendix). Black patients experienced a significantly larger decrease in graft function than all other races (β: –4.50 ml/min; 95% CI: –8.61; –0.38). Moreover, standard criteria DBD transplants had a small, statistically significant increase in GFR between months 6 and 12. These two variables were therefore included in the multivariate model (Table 9). In this adjusted model (Table 9), the decrease in GFR was 0.65 ml/min per 1.73m^2 (95% CI: –1.59; 2.90) greater for KTR with positive pre-transplant anti-LG3 compared to those with negative pre-transplant anti-LG3 (not statistically significant).

There was a statistically significant interaction between pre-transplant anti-LG3 positivity and AVR for delta-GFR ($p = 0.04$) in the unadjusted model (Table 10). This suggests that the presence of AVR in the first 6 months modifies the effect of positive pre-transplant anti-LG3 on delta-GFR. In the adjusted model, the p-value for the interaction term between AVR and positive pre-transplant anti-LG3 was 0.05. Among patients with AVR, pre-transplant positive anti-LG3 status was associated with a significantly larger decrease GFR than negative pre-transplant anti-LG3 status (difference in delta-GFR: –7.56 ml/min, 95% CI: –14.0; –1.08). In patients without AVR, pre-transplant anti-LG3 status was not associated with delta GFR (difference in delta-GFR: –0.02 ml/min for patients with positive versus negative pre-transplant anti-LG3, 95% CI: –2.40; 2.35).

Figure 2. Distribution of **1-year GFR** and **Delta-GFR**



Histogram **A.** shows the distribution of 1-year GFR. Histogram **B.** shows the delta-GFR. In both cases, the mean ± standard deviation is reported along with the number of available observations. The width of each bar corresponds to 5 ml/min.

Table 7. Multivariate linear regression model: **1-year GFR as outcome**

Variables	Reference category	Coefficients (95% CI)	
		Unadjusted	Multivariate
Anti-LG3 > 130 OD units	≤130	-0.91 (-5.71; 3.88)	0.27 (-4.00; 4.53)
Recipient Age (years)	per 10-year increment	-	-0.31 (-0.49; -0.14)
Donor type mECD and/or DCD Standard criteria DBD	Live donor	-	-5.85 (-12.22; 0.51) 7.11 (1.71; 12.51)
Induction IS – ATG	Basiliximab	-	-6.41 (-11.51; -1.30)
DGF – Yes	No	-	-13.43 (-18.11; -8.76)
AVR in first 6 months	No	-	-10.0 (-18.03; -1.96)

mECD: modified expanded criteria donor; **DCD**: donor after cardiac death; **DBD**: donor after brain death; **ATG**: thymoglobulin; **IS:** immunosuppression; **DGF**: Delayed Graft Function; **AVR**: acute vascular rejection

Table 8. Stratified analyses – **1-year GFR as the outcome**

	AVR in first 6 months	n	Coef (95% CI) for **anti-LG3 >130** OD units	p-value for interaction term
Unadjusted model	**No**	353	-0.15 (-4.95; 4.64)	0.68
	Yes	30	-3.94 (-28.6; 20.8)	
Multivariate model	**No**	353	0.21 (-4.00; 4.42)	0.83
	Yes	30	-0.03 (-25.3; 25.2)	

The following multivariate model was used for subsets with AVR and without:
- **1-year GFR** ~ Anti-LG3 + Donor type + Induction IS + DGF

The p-value corresponds to the interaction variable when included in the model.
AVR: acute vascular rejection; **IS**: immunosuppression; **DGF:** delayed graft function;

Table 9. Multivariate linear regression model: **Delta-GFR as outcome**

Variables	**Reference category**	**Coefficients (95% CI)**	
		Unadjusted	**Multivariate**
Anti-LG3 > 130 OD units	≤130	-1.15 (-3.41; 1.12)	-0.65 (-2.90 1.59)
Race – Black	Not black	-	-5.15 -9.28; -1.02)
Donor type mECD and/or DCD Standard criteria DBD	Live donor	-	1.32 (-1.68; 4.32) 3.89 (1.12; 6.65)
mECD: modified expanded criteria donor; **DCD**: donor after cardiac death; **DBD**: donor after brain death; **ATG**: thymoglobulin; **IS:** immunosuppression; **DGF**: Delayed Graft Function; **AVR**: acute vascular rejection			

Table 10. Stratified analyses – **Delta-GFR as the outcome**

	AVR in first 6 months	**n**	**Coef (95% CI) for anti-LG3 >130** OD units	**p-value for interaction term**
Unadjusted model	**No**	352	-0.45 (-2.84; 1.94)	0.04
	Yes	30	**-9.44 (-15.9; -2.96)**	
Multivariate model	**No**	352	-0.02 (-2.40; 2.35)	0.05
	Yes	30	**-7.56 (-14.0; -1.08)**	
The following multivariate model was used for subsets with AVR and without: - **Delta-GFR** ~ Anti-LG3 + Race + Donor type The p-value corresponds to the interaction variable when included in the multivariate model. **AVR**: acute vascular rejection;				

DISCUSSION

Although its incidence has been decreasing in the past decades, acute kidney graft rejection remains associated with short- and long-term graft loss. [11,12,35] Kidney graft rejections that target the vasculature are more likely to be resistant to treatment and to be associated with poorer graft survival, especially when mediated by antibodies. [43,46] Although alloantibodies against the donor HLA have been classically associated with rejections affecting the kidney transplant vasculature, a large body of evidence has now linked auto-antibodies to both acute and chronic rejection in kidney transplant patients [19,85,97,98] as well as in heart and lung transplant recipients.[91,140]

I undertook a retrospective cohort study to validate the previously described association between one of these autoantibodies, anti-LG3 antibodies, and acute vascular rejection. Because existing data suggested that ischemia and vascular damage could enhance the effect of anti-LG3[19] and other autoantibodies, [86,141] I identified clinical factors associated with endothelial damage and evaluated their role as effect modifiers for the relationship between anti-LG3 and AVR. As previous work suggested that positive pre-transplant anti-LG3 was associated with poorer graft survival in AVR patients with positive DSA,[19] I assessed whether pre-transplant anti-LG3 was associated with lower 1-year eGFR and with the change in eGFR between months 6 and 12 post-transplant, both surrogate markers for long-term graft survival. Based on previous results, [19] I had hypothesized that this relationship would only exist in patients with AVR given the vascular damage associated with alloimmune injury.

Pre-transplant anti-LG3, acute vascular rejection (AVR) and 1st acute rejection (1st AR)

In this retrospective cohort study, the number of overall rejection episodes was within the range reported by others. [33] There were 33 episodes of AVR which corresponds to 7% of the study population and is also consistent with previous reports. [52] I was unable to demonstrate any significant association between pre-transplant anti-LG3 antibodies and acute rejection, whether I considered AVR or 1st AR of any type. As expected, the point estimate for the HR was greater for AVR compared to first AR of any type. However, large confidence intervals preclude a definite conclusion to be drawn on the association between anti-LG3 and AVR.

The present results conflict with a previously published case-control study in which Cardinal *et al.* observed a strong and significant association between pre-transplant anti-LG3 antibodies above the median (>140 OD units) and AVR, with an odds ratio greater than 6.00.[19] Several different factors may have contributed to the discrepant findings. First, the previous work included patients transplanted in earlier eras (1985-2008). The immunosuppressive agents used in the past had weaker potency than current regimen.[142] Hence, the deleterious impact of pre-transplant anti-LG3 may be weaker in the presence of stronger immunosuppression. Consistent with that hypothesis, the time to rejection in the earlier study was 3 days, while AVR occurred at a median time of 23 days in the present cohort. Second, the screening techniques for DSAs used in the past were not as sensitive as the solid-phase assays that are currently used. Hence, in the previous study, some patients had unrecognized pre-transplant positive DSAs that were discovered a posteriori when measured through solid-phase assays for research purposes. These patients were not excluded in the previous study: the presence of pre-transplant DSA was accounted for in the multivariate model, but was not studied as a potential effect modifier. There may have been a

synergistic effect of DSA and anti-LG3 on the risk of AVR which had not been accounted for in the Cardinal study. In support of this hypothesis, a synergistic effect between DSA and anti-ATR1 on graft survival has been described previously.[89,90] In the present study, I excluded patients with positive pre-transplant DSA to target the isolated impact of anti-LG3. This may also have contributed to our discrepant findings.

I also used a definition of AVR that was slightly different from the previous study. In the previous work, AVR was defined as Banff grade II and III TCMR only, which considers only macrovascular injury (i.e., endarteritis). Because antibodies, including DSA, can also affect the microvasculature, I included any macro- or microvascular injury as AVR in the present work. I do not believe this explains the difference in observed results as I performed sensitivity analyses using the same definition as was used in the past and my current results were unchanged.

Potential effect modifiers for the relationship between pre-transplant anti-LG3 and AVR

I had hypothesized that clinical factors associated with vascular and endothelial damage (ischemic time, the presence of DGF and donors with a fragile vasculature) would potentiate the effect of anti-LG3 on the risk of AVR. This was based on animal data showing that ischemia-reperfusion increased the adverse impact of anti-LG3 and other auto antibodies on vascular damage in aortic and lung transplantation. [19,141] Although none of the interaction variables were statistically significant, the HR were consistently numerically higher among patients with clinical risk factors associated with endothelial/vascular damage (long versus short cold ischemic time,

DGF versus no DGF, mECD/DCD versus not mECD/DCD). This suggests that the hypothesis that vascular/endothelial damage potentiates the effect of pre-transplant anti-LG3 on AVR deserves further study.

There are several possible explanations for the observed lack of statistically significant interactions. First, the large confidence intervals suggest that the sample size may be inadequate to show significant associations even if they truly exist. Second, it is possible that the degree of endothelial damage captured by the interaction variables tested was not severe enough to demonstrate any conclusive effect modification. For example, because the donor terminal creatinine was not available, donor type could not be characterized as ECD according to traditional criteria. Instead, a more liberal definition of ECD was used: mECD. This may have led to misclassification of donor type, with some donors not classified as mECD who would have been classified as ECD if their terminal creatinine was considered. Therefore, the severity of endothelial damage may not have been as prominent among mECD as defined in this study compared to traditional ECD. Had I been able to use the traditional definition of ECD, the interaction between anti-LG3 and ECD may have been significant. Similarly, the total ischemic times were rather short in the study population and it is possible that longer ischemic times are necessary to demonstrate the hypothesized interaction. The short cold ischemic times likely reflect the over-representation of LD transplants in the study population: 26% of patients with pre-transplant serum had a LD transplant compared with only 10% of those without pre-transplant serum. As opposed to DD transplants, LD transplants are scheduled ahead of time which allows for greater opportunities to collect pre-transplant serum and increase the likelihood of inclusion in the study.

It is possible that biomarkers (e.g., sVCAM-1, sICAM-1, endothelial microparticles, etc.) may be more sensitive than clinical factors in revealing the extent of endothelial damage associated with the transplantation process and therefore provide a better method of stratifying risk. [143,144] Future studies may assess the association between positive anti-LG3 and AVR in patients with and without endothelial damage identified using these biomarkers.

Pre-transplant anti-LG3, 1-year GFR and delta-GFR

In this cohort, the average observed 1-year eGFR was consistent with other reports in the literature [145] (55.7 ± 23.8 ml/min per 1.73m^2). Factors associated with 1-year GFR were also those expected based on the literature.[39] My hypothesis was that alloimmune injury, in the form of AVR, exposes cryptic antigenic targets which allows for circulating anti-LG3 antibodies to cause further damage to the kidney vasculature which should translate into worse 1-year GFR and faster decline of kidney function (delta-GFR). I could not demonstrate any association with 1-year GFR, but as expected, among patients with AVR, positive pre-transplant anti-LG3 titers were associated with a significantly larger decrease in GFR than negative pre-transplant anti-LG3 titers. In contrast, among patients without AVR, delta-GFR did not differ based on pre-transplant anti-LG3 status. The p-value for the interaction term for anti-LG3 and AVR was 0.05 in the multivariate model. These findings partly support the hypothesis that anti-LG3 auto-antibodies worsen the damage incurred by alloimmune injury affecting the vasculature, but larger studies are needed. To better characterize this interaction, I am planning in future studies to measure anti-LG3 antibodies at the time of rejection. Hypothetically, anti-LG3 levels may vary in the post-transplant period according to the level of immunosuppression, among other factors. At the time when cryptic endothelial LG3

targets are exposed due to the endothelial damage caused by AVR, the positivity of anti-LG3 autoantibodies may have changed compared to the pre-transplant period.

The mechanisms of generation of anti-LG3 auto-antibodies remain unknown. [99] In this cohort, positive pre-transplant anti-LG3 antibodies were not found to be associated with any recipient or transplant characteristics. When considered as a continuous variable, anti-LG3 levels were found to be significantly higher in black patients compared to all others (198.8 OD units, IQR 99.1-329.1 vs. 128.0 OD units, IQR 85.6-203.5, p=0.007). Ethnic disparities in specific autoimmune diseases have been well documented. [146] Although this was not the objective of this study, the association of anti-LG3 levels with black race is a novel finding and deserves further investigation.

This study has several strengths and limitations. There was a high rate of enrollment into the University of Montreal Renal Transplant Biobank, minimizing the risks of selection bias. Clinical characteristics were available for all participants with very few missing data. Although a significant proportion of participants (106/553, 19%) did not have pre-transplant serum available and therefore could not be included, measured characteristics were similar for patients with and without pre-transplant serum. However, the possibility of selection bias remains if included and excluded patients differed based on characteristics not measured that are also linked to the exposure and the outcome. This is unlikely because the most frequent reason for exclusion was missing pre-transplant serum, which occurred mostly at the inception of the biobank, when the study personnel were not entirely familiar with the biobanking process. Techniques such as

multiple imputation or inverse probability weighting could have been considered to address this issue. This would have helped improve statistical power which is a major limiting factor in this study. A priori power calculation should be considered in subsequent studies. Another possible improvement would be the use of sensitivity analyses to account for competing risks. Moreover, I did not use any multiple testing correction for any of the analyses but this could certainly be considered in subsequent studies if the number of statistical tests performed is larger. For the purpose of this study, the dataset could be further enriched with additional information such as donor terminal creatinine for ECD categorization and cold ischemic time. Repeating this study in a couple of years using an improved dataset would also allow for a bigger sample size by enrolling more recent transplant recipients. The immune characteristics of transplant recipients and the immunosuppressive agents used in both transplant centers is a good representation of North American practices. However, the generalizability of this dataset may be limited by the under-representation of black transplant recipients.

CONCLUSION

Firm conclusions regarding the association between pre-transplant anti-LG3 antibodies and acute rejection in the first 6 months after transplantation cannot be drawn from this study. Although I was able to control for many potential confounders, residual confounding remains an important consideration. I found no significant association between positive anti-LG3 antibodies and AVR or first episode of rejection of any type. A larger sample would be necessary to properly investigate the association of anti-LG3 and AVR. The proportion of patients experiencing AVR was as expected, but the number of patients without available pre-transplant serum was higher than expected. I found no significant modifying effect of endothelial damage as defined by the presence of DGF, prolonged ischemia and DCD and/or mECD donor, on the relation between positive anti-LG3 antibodies and AVR. The trends towards larger HR in the setting of endothelial damage in the stratified analysis favor the hypothesis that vascular/endothelial damage potentiates the effect of pre-transplant anti-LG3 on AVR. This interaction deserves further study with better characterization of endothelial damage, possibly using novel biomarkers.

The presence of pre-transplant anti-LG3 antibodies was not significantly associated with either 1-year GFR or delta-GFR. However, in the stratified analysis, positive pre-transplant anti-LG3 antibodies were associated with a significantly larger decrease in GFR from months 6 to 12 in a subgroup of patients with AVR. This was not the case in KTR that did not experience AVR. This finding supports the hypothesis that alloimmune injury affecting the vasculature potentiates the effects of anti-LG3. Further studies are needed to confirm this finding and should assess anti-LG3 positivity at the time of rejection rather than pre-transplant.

Recent research has revealed an emerging role for autoimmunity in kidney transplantation. This work specifically evaluated pre-transplant anti-LG3 autoantibodies. Although the sample size was not large enough to draw definitive conclusions, there are trends in support of the clinical relevance of pre-transplant anti-LG3. The questions investigated in this work deserve further study.

REFERENCES

1. Wolfe RA, Ashby VB, Milford EL, et al. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. *The New England journal of medicine.* 1999;341(23):1725-1730.

2. Oniscu GC, Brown H, Forsythe JLR. Impact of cadaveric renal transplantation on survival in patients listed for transplantation. *J Am Soc Nephrol.* 2005;16(6):1859-1865.

3. Medin C, Elinder CG, Hylander B, Blom B, Wilczek H. Survival of patients who have been on a waiting list for renal transplantation. *Nephrol Dial Transplant.* 2000;15(5):701-704.

4. Laupacis A, Keown P, Pus N, et al. A study of the quality of life and cost-utility of renal transplantation. *Kidney Int.* 1996;50(1):235-242.

5. Canadian Institute for Health I. Canadian Organ Replacement Register Annual Report: Treatment of End-Stage Organ Failure in Canada, 2001 to 2010. Ottawa, ON2011.

6. Canadian Institute for Health I. *Canadian Organ Replacement Register Annual Report: Treatment of End-Stage Organ Failure in Canada, 2004 to 2013*. Ottawa, ON2015.

7. Canadian Institute for Health I. *Canadian Organ Replacement Register Annual Report: Treatment of End-Stage Organ Failure in Canada, 2005 to 2014.* Ottawa, ON2016.

8. Kassakian CT, Ajmal S, Gohh RY, Morrissey PE, Bayliss GP. Immunosuppression in the failing and failed transplant kidney: optimizing outcomes. *Nephrol Dial Transplant.* 2016;31(8):1261-1269.

9. Zand MS. Immunosuppression and immune monitoring after renal transplantation. *Seminars in dialysis.* 2005;18(6):511-519.

10. Halloran PF. Immunosuppressive drugs for kidney transplantation. *The New England journal of medicine.* 2004;351(26):2715-2729.

11. El-Zoghby ZM, Stegall MD, Lager DJ, et al. Identifying specific causes of kidney allograft loss. *Am J Transplant.* 2009;9(3):527-535.

12. Meier-Kriesche HU, Ojo AO, Hanson JA, et al. Increased impact of acute rejection on chronic allograft failure in recent era. *Transplantation.* 2000;70(7):1098-1100.

13. Wang L, Wang F-S, Gershwin ME. Human autoimmune diseases: a comprehensive update. *J Intern Med.* 2015;278(4):369-395.

14. Riemekasten G, Philippe A, Näther M, et al. Involvement of functional autoantibodies against vascular receptors in systemic sclerosis. *Ann Rheum Dis.* 2011;70(3):530-536.

15. Irani RA, Zhang Y, Blackwell SC, et al. The detrimental role of angiotensin receptor agonistic autoantibodies in intrauterine growth restriction seen in preeclampsia. *The Journal of experimental medicine.* 2009;206(12):2809-2822.

16. Panda S, Ding JL. Natural antibodies bridge innate and adaptive immunity. *J Immunol.* 2015;194(1):13-20.

17. Carroll MC, Holers VM. Innate autoimmunity. *Adv Immunol.* 2005;86:137-157.

18. Nagele EP, Han M, Acharya NK, DeMarshall C, Kosciuk MC, Nagele RG. Natural IgG autoantibodies are abundant and ubiquitous in human sera, and their number is influenced by age, gender, and disease. *PLoS One.* 2013;8(4):e60726-e60726.

19. Cardinal H, Dieudé M, Brassard N, et al. Antiperlecan antibodies are novel accelerators of immune-mediated vascular injury. *Am J Transplant.* 2013;13(4):861-874.

20. Yang B, Dieudé M, Hamelin K, et al. Anti-LG3 Antibodies Aggravate Renal Ischemia-Reperfusion Injury and Long-Term Renal Allograft Dysfunction. *Am J Transplant.* 2016.

21. Taal M, Chertow G, Marsden P, Skorecki K, Yu A, Brenner B. *Brenner and Rector's The Kidney.* 2012.

22. United States Renal Data S. 2015 USRDS annual data report: Epidemiology of kidney disease in the United States. *National Institute of Diabetes and Digestive and Kidney Diseases.* 2015.

23. Wu C, Evans I, Joseph R, et al. Comorbid conditions in kidney transplantation: association with graft and patient survival. *J Am Soc Nephrol.* 2005;16(11):3437-3444.

24. Arend SM, Mallat MJ, Westendorp RJ, van der Woude FJ, van Es LA. Patient survival after renal transplantation; more than 25 years follow-up. *Nephrol Dial Transplant.* 1997;12(8):1672-1679.

25. McCullough KP, Keith DS, Meyer KH, Stock PG, Brayman KL, Leichtman AB. Kidney and pancreas transplantation in the United States, 1998-2007: access for patients with diabetes and end-stage renal disease. *Am J Transplant.* 2009;9(4 Pt 2):894-906.

26. Briggs JD. Causes of death after renal transplantation. *Nephrol Dial Transplant.* 2001;16(8):1545-1549.

27. Rubin RH. Infectious disease complications of renal transplantation. *Kidney Int.* 1993;44(1):221-236.

28. Meier-Kriesche H-U, Baliga R, Kaplan B. Decreased renal function is a strong risk factor for cardiovascular death after renal transplantation. *Transplantation.* 2003;75(8):1291-1295.

29. Kasiske BL, Snyder JJ, Gilbertson DT, Wang C. Cancer after kidney transplantation in the United States. *Am J Transplant.* 2004;4(6):905-913.

30. Ojo AO, Hanson JA, Wolfe RA, Leichtman AB, Agodoa LY, Port FK. Long-term survival in renal transplant recipients with graft function. *Kidney Int.* 2000;57(1):307-313.

31. Humar A, Hassoun A, Kandaswamy R, Payne WD, Sutherland DE, Matas AJ. Immunologic factors: the major risk for decreased long-term renal allograft survival. *Transplantation.* 1999;68(12):1842-1846.

32. EBPG Expert Group on Renal Transplantation. European best practice guidelines for renal transplantation. Section IV: Long-term management of the transplant recipient. IV.13 Analysis of patient and graft survival. *Nephrol Dial Transplant.* 2002;17 Suppl 4:60-67.

33. Hart A, Smith JM, Skeans MA, et al. OPTN/SRTR 2014 Annual Data Report: kidney. *Am J Transplant.* 2016;16 Suppl 2:11-46.

34. Vincenti F. A decade of progress in kidney transplantation. *Transplantation.* 2004;77(9 Suppl):S52-61.

35. Pallardó Mateu LM, Sancho Calabuig A, Capdevila Plaza L, Franco Esteve A. Acute rejection and late renal transplant failure: risk factors and prognosis. *Nephrol Dial Transplant.* 2004;19 Suppl 3:iii38-42.

36. Cole EH, Johnston O, Rose CL, Gill JS. Impact of acute rejection and new-onset diabetes on long-term transplant graft and patient survival. *Clin J Am Soc Nephrol.* 2008;3(3):814-821.

37. Solez K, Colvin RB, Racusen LC, et al. Banff '05 Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). *Am J Transplant.* 2007;7(3):518-526.

38. Hariharan S, McBride MA, Cherikh WS, Tolleris CB, Bresnahan BA, Johnson CP. Post-transplant renal function in the first year predicts long-term kidney transplant survival. *Kidney Int.* 2002;62(1):311-318.

39. Salvadori M, Rosati A, Bock A, et al. Estimated one-year glomerular filtration rate is the best predictor of long-term graft function following renal transplant. *Transplantation.* 2006;81(2):202-206.

40. Hariharan S, McBride MA, Cherikh WS, Tolleris CB, Bresnahan BA, Johnson CP. Post-transplant renal function in the first year predicts long-term kidney transplant survival. *Kidney Int.* 2002;62(1):311-318.

41. Haas M, Sis B, Racusen LC, et al. Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. *Am J Transplant.* 2014;14(2):272-283.

42. Solez K, Colvin RB, Racusen LC, et al. Banff 07 classification of renal allograft pathology: Updates and future directions. *Am J Transplant.* 2008;8(4):753-760.

43. Palomar R, Ruiz JC, Zubimendi JA, et al. Acute rejection in the elderly recipient: influence of age in the outcome of kidney transplantation. *Int Urol Nephrol.* 2002;33(1):145-148.

44. Gray D, Shepherd H, Daar A, Oliver DO, Morris PJ. Oral versus intravenous high-dose steroid treatment of renal allograft rejection. The big shot or not? *Lancet.* 1978;1(8056):117-118.

45. Wu K, Budde K, Lu H, et al. The severity of acute cellular rejection defined by Banff classification is associated with kidney allograft outcomes. *Transplantation.* 2014;97(11):1146-1154.

46. Mueller A, Schnuelle P, Waldherr R, van der Woude FJ. Impact of the Banff '97 classification for histological diagnosis of rejection on clinical outcome and renal function parameters after kidney transplantation. *Transplantation.* 2000;69(6):1123-1127.

47. Sis B, Halloran PF. Endothelial transcripts uncover a previously unknown phenotype: C4d-negative antibody-mediated rejection. *Current opinion in organ transplantation.* 2010;15(1):42-48.

48. Sis B, Jhangri GS, Bunnag S, Allanach K, Kaplan B, Halloran PF. Endothelial gene expression in kidney transplants with alloantibody indicates antibody-mediated damage despite lack of C4d staining. *Am J Transplant.* 2009;9(10):2312-2323.

49. Herzenberg AM, Gill JS, Djurdjev O, Magil AB. C4d deposition in acute rejection: an independent long-term prognostic factor. *J Am Soc Nephrol.* 2002;13(1):234-241.

50. Sis B, Einecke G, Chang J, et al. Cluster analysis of lesions in nonselected kidney transplant biopsies: microcirculation changes, tubulointerstitial inflammation and scarring. *Am J Transplant.* 2010;10(2):421-430.

51. Nankivell BJ, Allen RD, O'Connell PJ, Chapman JR. Renal dysfunction in acute rejection. Effect of HLA typing, therapy, and histology. *Transplantation.* 1995;60(1):28-36.

52. Lefaucheur C, Loupy A, Vernerey D, et al. Antibody-mediated vascular rejection of kidney allografts: a population-based study. *Lancet.* 2013;381(9863):313-319.

53. Bates WD, Davies DR, Welsh K, Gray DW, Fuggle SV, Morris PJ. An evaluation of the Banff classification of early renal allograft biopsies and correlation with outcome. *Nephrol Dial Transplant.* 1999;14(10):2364-2369.

54. Vereerstraeten P, Abramowicz D, de Pauw L, Kinnaert P. Absence of deleterious effect on long-term kidney graft survival of rejection episodes with complete functional recovery. *Transplantation.* 1997;63(12):1739-1743.

55. Madden RL, Mulhern JG, Benedetto BJ, et al. Completely reversed acute rejection is not a significant risk factor for the development of chronic rejection in renal allograft recipients. *Transpl Int.* 2000;13(5):344-350.

56. Danovitch G. *Handbook of Kidney Transplantation.* 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins, Wolters Kluwer; 2010.

57. Rees L, Kim JJ. HLA sensitisation: can it be prevented? *Pediatr Nephrol.* 2015;30(4):577-587.

58. Patel R, Terasaki PI. Significance of the positive crossmatch test in kidney transplantation. *The New England journal of medicine.* 1969;280(14):735-739.

59. Akalin E, Pascual M. Sensitization after kidney transplantation. *Clin J Am Soc Nephrol.* 2006;1(3):433-440.

60. Willicombe M, Brookes P, Sergeant R, et al. De novo DQ donor-specific antibodies are associated with a significant risk of antibody-mediated rejection and transplant glomerulopathy. *Transplantation.* 2012;94(2):172-177.

61. Wiebe C, Gibson IW, Blydt-Hansen TD, et al. Evolution and clinical pathologic correlations of de novo donor-specific HLA antibody post kidney transplant. *Am J Transplant.* 2012;12(5):1157-1167.

62. Everly MJ, Rebellato LM, Haisch CE, et al. Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. *Transplantation.* 2013;95(3):410-417.

63. Zou Y, Stastny P, Süsal C, Döhler B, Opelz G. Antibodies against MICA antigens and kidney-transplant rejection. *The New England journal of medicine.* 2007;357(13):1293-1300.

64. Lemy A, Andrien M, Lionet A, et al. Posttransplant major histocompatibility complex class I chain-related gene A antibodies and long-term graft outcomes in a multicenter cohort of 779 kidney transplant recipients. *Transplantation.* 2012;93(12):1258-1264.

65. Cinti P, Pretagostini R, Lai Q, et al. Alloantibodies and outcomes of deceased donor kidney allografts. *Hum Immunol.* 2009;70(8):651-654.

66. Domingues EMFL, Matuck T, Graciano ML, et al. Panel reactive HLA antibodies, soluble CD30 levels, and acute rejection six months following renal transplant. *Clin Transplant.* 2010;24(6):821-829.

67. Aubert V, Venetz J-P, Pantaleo G, Pascual M. Low levels of human leukocyte antigen donor-specific antibodies detected by solid phase assay before transplantation are frequently clinically irrelevant. *Hum Immunol.* 2009;70(8):580-583.

68. Scornik JC, Bromberg JS, Norman DJ, Bhanderi M, Gitlin M, Petersen J. An update on the impact of pre-transplant transfusions and allosensitization on time to renal transplant and on allograft survival. *BMC Nephrol.* 2013;14:217-217.

69. Held PJ, Kahan BD, Hunsicker LG, et al. The impact of HLA mismatches on the survival of first cadaveric kidney transplants. *The New England journal of medicine.* 1994;331(12):765-770.

70. Opelz G. Correlation of HLA matching with kidney graft survival in patients with or without cyclosporine treatment. *Transplantation.* 1985;40(3):240-243.

71. Wissing KM, Fomegne G, Broeders N, et al. HLA mismatches remain risk factors for acute kidney allograft rejection in patients receiving quadruple immunosuppression with anti-interleukin-2 receptor antibodies. *Transplantation.* 2008;85(3):411-416.

72. Lim WH, Chadban SJ, Clayton P, et al. Human leukocyte antigen mismatches associated with increased risk of rejection, graft failure, and death independent of initial immunosuppression in renal transplant recipients. *Clin Transplant.* 2012;26(4):E428-437.

73. Huang E, Segev DL, Rabb H. Kidney transplantation in the elderly. *Semin Nephrol.* 2009;29(6):621-635.

74. Neylan JF. Effect of race and immunosuppression in renal transplantation: three-year survival results from a US multicenter, randomized trial. FK506 Kidney Transplant Study Group. *Transplant Proc.* 1998;30(4):1355-1358.

75. Zeier M, Döhler B, Opelz G, Ritz E. The effect of donor gender on graft survival. *J Am Soc Nephrol.* 2002;13(10):2570-2576.

76. Meier-Kriesche HU, Ojo AO, Leavey SF, et al. Gender differences in the risk for chronic renal allograft failure. *Transplantation.* 2001;71(3):429-432.

77. Dragun D, Catar R, Philippe A. Non-HLA antibodies in solid organ transplantation: recent concepts and clinical relevance. *Current opinion in organ transplantation.* 2013;18(4):430-435.

78. Floege J, Johnson R, Feehally J. *Comprehensive Clinical Nephrology.* 2010.

79. Salvadori M, Rosso G, Bertoni E. Update on ischemia-reperfusion injury in kidney transplantation: Pathogenesis and treatment. *World journal of transplantation.* 2015;5(2):52-67.

80. Pratt JR, Basheer SA, Sacks SH. Local synthesis of complement component C3 regulates acute renal transplant rejection. *Nat Med.* 2002;8(6):582-587.

81. Fuquay R, Renner B, Kulik L, et al. Renal ischemia-reperfusion injury amplifies the humoral immune response. *J Am Soc Nephrol.* 2013;24(7):1063-1072.

82. McCullough JW, Renner B, Thurman JM. The role of the complement system in acute kidney injury. *Semin Nephrol.* 2013;33(6):543-556.

83. Cailhier J-F, Sirois I, Laplante P, et al. Caspase-3 activation triggers extracellular cathepsin L release and endorepellin proteolysis. *The Journal of biological chemistry.* 2008;283(40):27220-27229.

84. Dragun D, Bräsen JH, Schönemann C, et al. Patients with steroid refractory acute vascular rejection develop agonistic antibodies targeting angiotensin II type 1 receptor. *Transplant Proc.* 2003;35(6):2104-2105.

85. Dragun D, Müller DN, Bräsen JH, et al. Angiotensin II type 1-receptor activating antibodies in renal-allograft rejection. *The New England journal of medicine.* 2005;352(6):558-569.

86. Lukitsch I, Kehr J, Chaykovska L, et al. Renal ischemia and transplantation predispose to vascular constriction mediated by angiotensin II type 1 receptor-activating antibodies. *Transplantation.* 2012;94(1):8-13.

87. Banasik M, Boratyńska M, Kościelska-Kasprzak K, et al. The influence of non-HLA antibodies directed against angiotensin II type 1 receptor (AT1R) on early renal transplant outcomes. *Transpl Int.* 2014;27(10):1029-1038.

88. Giral M, Foucher Y, Dufay A, et al. Pretransplant sensitization against angiotensin II type 1 receptor is a risk factor for acute rejection and graft loss. *Am J Transplant.* 2013;13(10):2567-2576.

89. Taniguchi M, Rebellato LM, Cai J, et al. Higher risk of kidney graft failure in the presence of anti-angiotensin II type-1 receptor antibodies. *Am J Transplant.* 2013;13(10):2577-2589.

90. Tinckam K, Campbell P. Angiotensin II Type 1 receptor antibodies: great expectations? *Am J Transplant.* 2013;13(10):2515-2516.

91. Jurcevic S, Ainsworth ME, Pomerance A, et al. Antivimentin antibodies are an independent predictor of transplant-associated coronary artery disease after cardiac transplantation. *Transplantation.* 2001;71(7):886-892.

92. Mahesh B, Leong H-S, McCormack A, Sarathchandra P, Holder A, Rose ML. Autoantibodies to vimentin cause accelerated rejection of cardiac allografts. *The American journal of pathology.* 2007;170(4):1415-1427.

93. Rose ML. Role of anti-vimentin antibodies in allograft rejection. *Hum Immunol.* 2013;74(11):1459-1462.

94. Carter V, Shenton BK, Jaques B, et al. Vimentin antibodies: a non-HLA antibody as a potential risk factor in renal transplantation. *Transplant Proc.* 2005;37(2):654-657.

95. Porcheray F, DeVito J, Helou Y, et al. Expansion of polyreactive B cells cross-reactive to HLA and self in the blood of a patient with kidney graft rejection. *Am J Transplant.* 2012;12(8):2088-2097.

96. Porcheray F, Fraser JW, Gao B, et al. Polyreactive antibodies developing amidst humoral rejection of human kidney grafts bind apoptotic cells and activate complement. *Am J Transplant.* 2013;13(10):2590-2600.

97. Gao B, Moore C, Porcheray F, et al. Pretransplant IgG reactivity to apoptotic cells correlates with late kidney allograft loss. *Am J Transplant.* 2014;14(7):1581-1591.

98. Angaswamy N, Klein C, Tiriveedhi V, et al. Immune responses to collagen-IV and fibronectin in renal transplant recipients with transplant glomerulopathy. *Am J Transplant.* 2014;14(3):685-693.

99. Hönger G, Cardinal H, Dieudé M, et al. Human pregnancy and generation of anti-angiotensin receptor and anti-perlecan antibodies. *Transpl Int.* 2014;27(5):467-474.

100. Terasaki PI, Cecka JM, Gjertson DW, Takemoto S. High survival rates of kidney transplants from spousal and living unrelated donors. *The New England journal of medicine.* 1995;333(6):333-336.

101. Delmonico F. A Report of the Amsterdam Forum On the Care of the Live Kidney Donor: Data and Medical Guidelines. *Transplantation.* 2005;79(6 Suppl):S53-66.

102. Delmonico FL, Dew MA. Living donor kidney transplantation in a global environment. *Kidney Int.* 2007;71(7):608-614.

103. Meier-Kriesche HU, Port FK, Ojo AO, et al. Effect of waiting time on renal transplant outcome. *Kidney Int.* 2000;58(3):1311-1317.

104. Matas AJ, Smith JM, Skeans MA, et al. OPTN/SRTR 2013 Annual Data Report: kidney. *Am J Transplant.* 2015;15 Suppl 2:1-34.

105. Singh RP, Farney AC, Rogers J, et al. Kidney transplantation from donation after cardiac death donors: lack of impact of delayed graft function on post-transplant outcomes. *Clin Transplant.*25(2):255-264.

106. Gok MA, Buckley PE, Shenton BK, et al. Long-term renal function in kidneys from non-heart-beating donors: A single-center experience. *Transplantation.* 2002;74(5):664-669.

107. Nicholson ML, Metcalfe MS, White SA, et al. A comparison of the results of renal transplantation from non-heart-beating, conventional cadaveric, and living donors. *Kidney Int.* 2000;58(6):2585-2591.

108. Sanchez-Fructuoso AI, Prats D, Marques M, et al. Does donor brain death influence acute vascular rejection in the kidney transplant? *Transplantation.* 2004;78(1):142-146.

109. Pratschke J, Wilhelm MJ, Kusaka M, et al. Accelerated rejection of renal allografts from brain-dead donors. *Ann Surg.* 2000;232(2):263-271.

110. Metzger RA, Delmonico FL, Feng S, Port FK, Wynn JJ, Merion RM. Expanded criteria donors for kidney transplantation. *Am J Transplant.* 2003;3 Suppl 4:114-125.

111. Rao PS, Merion RM, Ashby VB, Port FK, Wolfe RA, Kayler LK. Renal transplantation in elderly patients older than 70 years of age: results from the Scientific Registry of Transplant Recipients. *Transplantation.* 2007;83(8):1069-1074.

112. Chavalitdhamrong D, Gill J, Takemoto S, et al. Patient and graft outcomes from deceased kidney donors age 70 years and older: an analysis of the Organ Procurement Transplant Network/United Network of Organ Sharing database. *Transplantation.* 2008;85(11):1573-1579.

113. Frei U, Noeldeke J, Machold-Fabrizii V, et al. Prospective age-matching in elderly kidney transplant recipients--a 5-year analysis of the Eurotransplant Senior Program. *Am J Transplant.* 2008;8(1):50-57.

114. Filiopoulos V, Boletis JN. Renal transplantation with expanded criteria donors: Which is the optimal immunosuppression? *World J Transplant.* 2016;6(1):103-114.

115. Diet C, Audard V, Roudot-Thoraval F, Matignon M, Lang P, Grimbert P. Immunological risk in recipients of kidney transplants from extended criteria donors. *Nephrol Dial Transplant.* 2010;25(8):2745-2753.

116. Simpkins CE, Montgomery RA, Hawxby AM, et al. Cold ischemia time and allograft outcomes in live donor renal transplantation: is live donor organ transport feasible? *Am J Transplant.* 2007;7(1):99-107.

117. Salahudeen AK, Haider N, May W. Cold ischemia and the reduced long-term survival of cadaveric renal allografts. *Kidney Int.* 2004;65(2):713-718.

118. Mikhalski D, Wissing KM, Ghisdal L, et al. Cold ischemia is a major determinant of acute rejection and renal graft survival in the modern era of immunosuppression. *Transplantation.* 2008;85(7 Suppl):S3-9.

119. Tennankore KK, Kim SJ, Alwayn IP, Kiberd BA. Prolonged warm ischemia time is associated with graft failure and mortality after kidney transplantation. *Kidney Int.* 2016;89(3):648-658.

120. Yarlagadda SG, Coca SG, Garg AX, et al. Marked variation in the definition and diagnosis of delayed graft function: a systematic review. *Nephrol Dial Transplant.* 2008;23(9):2995-3003.

121. Humar a, Ramcharan T, Kandaswamy R, Gillingham K, Payne WD, Matas aJ. Risk factors for slow graft function after kidney transplants: a multivariate analysis. *Clin Transplant.* 2002;16:425-429.

122. Halloran PF, Hunsicker LG. Delayed graft function: state of the art. *Am J Transplant.* 2001;1:115-120.

123. Chapal M, Le Borgne F, Legendre C, et al. A useful scoring system for the prediction and management of delayed graft function following kidney transplantation from cadaveric donors. *Kidney Int.* 2014;86(6):1130-1139.

124. Jeldres C, Cardinal H, Duclos A, et al. Prediction of delayed graft function after renal transplantation. *Canadian Urological Association journal.* 2009;3(5):377-382.

125. Irish WD, Ilsley JN, Schnitzler MA, Feng S, Brennan DC. A risk prediction model for delayed graft function in the current era of deceased donor renal transplantation. *Am J Transplant.* 2010;10(10):2279-2286.

126. Shoskes DA, Cecka JM. Deleterious effects of delayed graft function in cadaveric renal transplant recipients independent of acute rejection. *Transplantation.* 1998;66(12):1697-1701.

127. Ojo AO, Wolfe RA, Held PJ, Port FK, Schmouder RL. Delayed graft function: risk factors and implications for renal allograft survival. *Transplantation.* 1997;63(7):968-974.

128. Wu WK, Famure O, Li Y, Kim SJ. Delayed graft function and the risk of acute rejection in the modern era of kidney transplantation. *Kidney Int.* 2015;88(4):851-858.

129. Takemoto S, Terasaki PI. Donor age and recipient age. *Clin Transpl.* 1988:345-356.

130. Collins AJ, Foley RN, Gilbertson DT, Chen S-C. United States Renal Data System public health surveillance of chronic kidney disease and end-stage renal disease. *Kidney international supplements.* 2015;5(1):2-7.

131. de Fijter JW, Mallat MJ, Doxiadis, II, et al. Increased immunogenicity and cause of graft loss of old donor kidneys. *J Am Soc Nephrol.* 2001;12(7):1538-1546.

132. Reutzel-Selke A, Jurisch A, Denecke C, et al. Donor age intensifies the early immune response after transplantation. *Kidney Int.* 2007;71(7):629-636.

133. Goldfarb-Rumyantzev A, Hurdle JF, Scandling J, et al. Duration of end-stage renal disease and kidney transplant outcome. *Nephrol Dial Transplant.* 2005;20(1):167-175.

134. Mange KC, Joffe MM, Feldman HI. Effect of the use or nonuse of long-term dialysis on the subsequent survival of renal transplants from living donors. *The New England journal of medicine.* 2001;344(10):726-731.

135. Levey AS, Stevens LA, Schmid CH, et al. A new equation to estimate glomerular filtration rate. *Ann Intern Med.* 2009;150(9):604-612.

136. Cole SR, Hernan MA. Fallibility in estimating direct effects. *Int J Epidemiol.* 2002;31(1):163-165.

137. Hernan MA, Hernandez-Diaz S, Werler MM, Mitchell AA. Causal knowledge as a prerequisite for confounding evaluation: an application to birth defects epidemiology. *Am J Epidemiol.* 2002;155(2):176-184.

138. Richiardi L, Bellocco R, Zugna D. Mediation analysis in epidemiology: methods, interpretation and bias. *Int J Epidemiol.* 2013;42(5):1511-1519.

139. VanderWeele TJ. Mediation Analysis: A Practitioner's Guide. *Annu Rev Public Health.* 2016;37:17-32.

140. Bharat A, Saini D, Steward N, et al. Antibodies to self-antigens predispose to primary lung allograft dysfunction and chronic rejection. *Ann Thorac Surg.* 2010;90(4):1094-1101.

141. Subramanian V, Ramachandran S, Banan B, et al. Immune response to tissue-restricted self-antigens induces airway inflammation and fibrosis following murine lung transplantation. *Am J Transplant.* 2014;14(10):2359-2366.

142. Kaufman DB, Shapiro R, Lucey MR, Cherikh WS, R TB, Dyke DB. Immunosuppression: practice and trends. *Am J Transplant.* 2004;4 Suppl 9:38-53.

143. Amabile N, Guerin AP, Leroyer A, et al. Circulating endothelial microparticles are associated with vascular dysfunction in patients with end-stage renal failure. *J Am Soc Nephrol.* 2005;16(11):3381-3388.

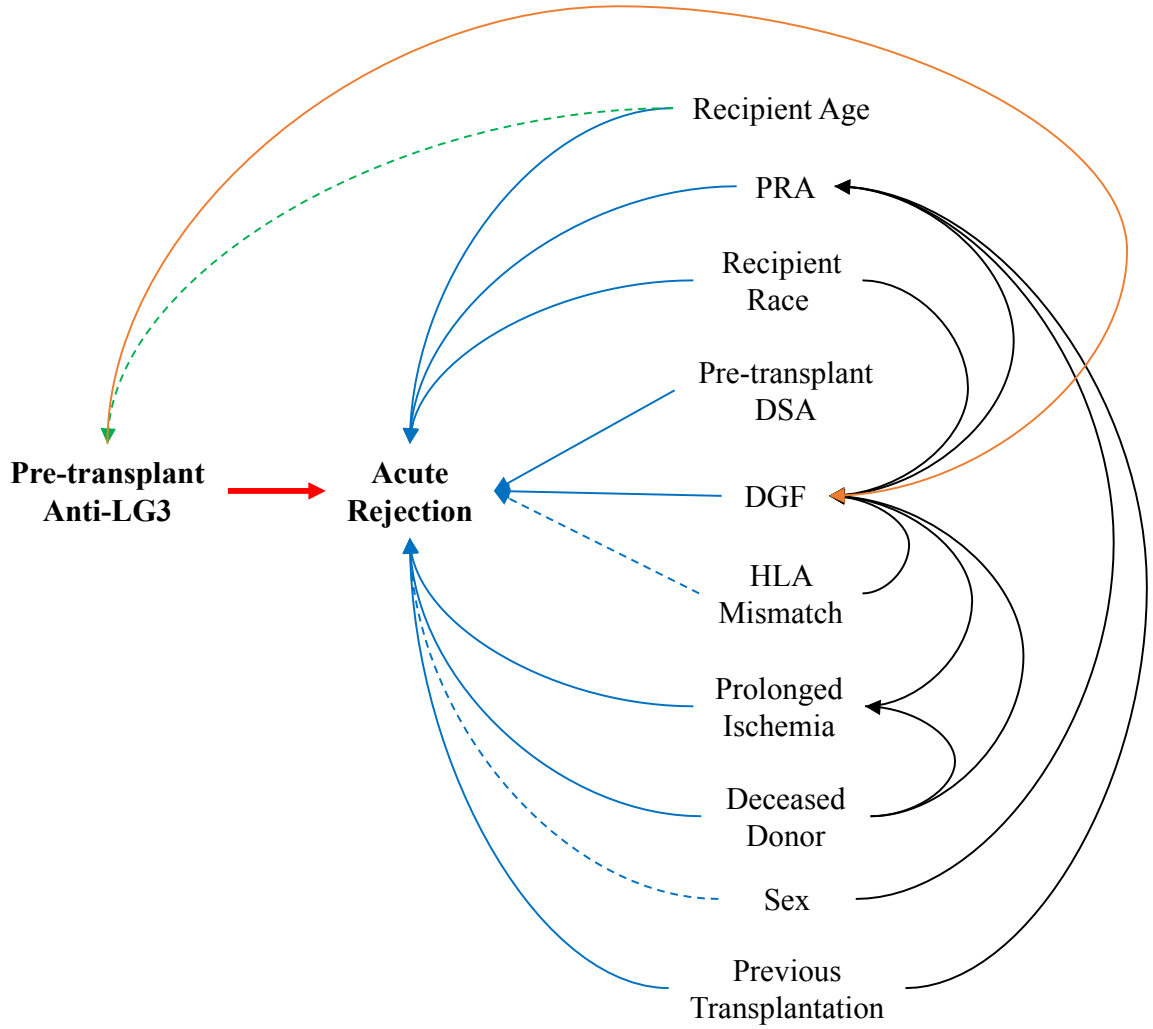
144. Chen J, Hamm LL, Mohler ER, et al. Interrelationship of Multiple Endothelial Dysfunction Biomarkers with Chronic Kidney Disease. *PLoS One.* 2015;10(7):e0132047.

145. Huang Y, Tilea A, Gillespie B, et al. Understanding Trends in Kidney Function 1 Year after Kidney Transplant in the United States. *J Am Soc Nephrol.* 2017.

146. Cooper GS, Stroehla BC. The epidemiology of autoimmune diseases. *Autoimmun Rev.* 2003;2(3):119-125.

APPENDIX – Supplementary figures and tables

Figure A1. Directed acyclic graph (DAG)



Dotted lines represent lower level of evidence compared to solid lines. The red arrow indicates the research question. Blue arrows are related to outcome (acute rejection) and green and orange arrows to exposure (pre-transplant anti-LG3). Note that only known associations have been illustrated. The absence of an arrow does not necessarily mean that there is no association.

Table A1. Categories of donors for transplant recipients **included** in the study

Live donor	115 (26%)
Deceased donor	328 (74%)
– Standard criteria donor (SCD)	228 (51%)
– Donor after brain death (DBD)	199 (45%)
– Donor after cardiac death (DCD)	29 (7%)
– Modified expanded criteria donor (mECD)	100 (23%)
– Donor after brain death (DBD)	89 (20%)
– Donor after cardiac death (DCD)	11 (2%)
The percentages represent the proportions of the total number of patients in the study cohort with available data on donor type	

Table A2. Characteristics of rejection episodes among **excluded** patients (n=106)

Characteristics	**n**	**AVR 5 (5%)**	**n**	**1st AR 18 (17%)**
Time of Rejection (months after KTx)	5	0.27 ± 0.20	18	2.05 ± 1.65
Histology	5		18	
– Borderline		0		8 (44%)
– IA		1 (20%)*		5 (28%)
– IB		0		1 (6%)
– IIA		3 (60%)		3 (17%)
– IIB		0		0
– III		0		0
– HCAPI		1 (20%)		1 (6%)
– HEND		0		0
– MAT		0		0
Peri tubular capillary **c4d** staining >50%	5	2 (40%)	18	2 (11%)
Positive **DSA** at the time of rejection	5	1 (20%)	16	2 (13%)
Indication for biopsy	5		18	
– Surveillance		0		7 (39%)
– Clinical		5 (100%)		11 (61%)

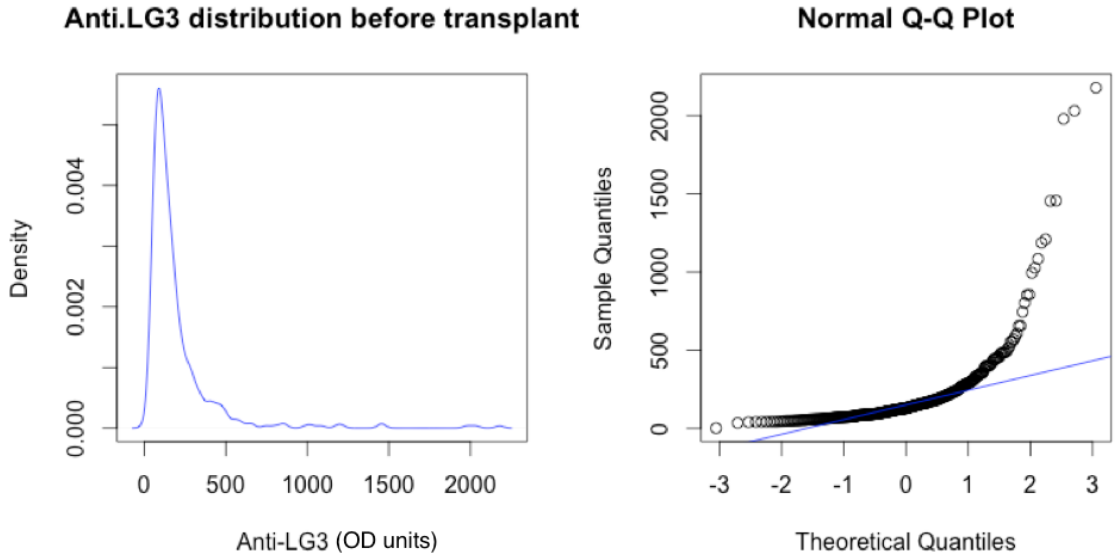
Column "n" contains the number of available observations for each variable.
***** 1 case of AVR showing IA rejection with glomerulitis;
AVR: acute vascular rejection; **AR:** acute rejection; **KTx:** kidney transplantation; **HCAPI**: humoral rejection with capillaritis and glomerulitis; **HEND**: humoral rejection with endarteritis; **TMA**: thrombotic microangiopathy; **DSA:** donor specific antibodies

Table A3. Characteristics of patients **with** and **without** pre-transplant serum

Characteristics	**No pre-transplant serum** (n=106)	**Pre-transplant serum** (n=444)
Recipient Characteristics		
Age (years)	48.0 ± 13.0	50.5 ± 13.6
Race (black)	7 (7%)	34 (8%)
Sex (male)	68 (64%)	283 (64%)
Baseline Renal Disease		
− Glomerular or Auto-immune	44 (42%)	177 (40%)
− Diabetes or Hypertension	25 (24%)	92 (21%)
− Other	37 (35%)	175 (39%)
Comorbidities		
− Coronary disease	14 (14%)	80 (18%)
− Diabetes	30 (29%)	96 (22%)
− Smoking history	47 (45%)	252 (57%)
Previous Kidney Transplantation	12 (11%)	48 (11%)
Previous Pregnancy	24 (23%)	117 (26%)
Previous Transfusion	44 (42%)	187 (42%)
PRA CDC >20%	3 (3%)	14 (3%)
Donor Characteristics		
Age (years)	45.5 ± 15.4	49.0 ± 14.7
Comorbidities		
− Diabetes	5 (5%)	36 (9%)
− Hypertension	23 (25%)	101 (24%)
Donor type		
− Live donor	10 (10%)	115 (26%)
− Deceased donor	95 (90%)	328 (74%)
− mECD and/or DCD	30 (29%)	129 (29%)
− Standard criteria DBD	65 (62%)	199 (45%)
Transplant Characteristics		
Center (CHUM)	102 (96%)	233 (53%)
HLA Mismatches in DR and B	2.0 ± 1.1	2.2 ± 1.1
Induction IS (Basiliximab)	75 (71%)	342 (77%)
Intravenous Gammaglobulins	9 (8%)	31 (7%)
Maintenance IS (Tacrolimus)	103 (97%)	425 (96%)
Adjuvant IS (Mycophenolate)	104 (100%)	437 (99%)
Total Ischemia time (hours)	10.6 (7.9; 14.9)	9.5 (4.9; 14.8)
Total Ischemia time > 15h	25 (24%)	106 (24%)
Delayed Graft Function	41 (39%)	163 (37%)

Column "n" contains the number of available observations for each variable. Categorical variables are reported as count (%). Continuous variables are reported as mean ± standard deviation if normal distribution. Otherwise, they are reported as median (IQR: Q1; Q3);
PRA CDC: Panel Reactive Assay Complement-Dependent Cytotoxicity; **mECD**: modified expanded criteria donor; **DCD**: donor after cardiac death; **DBD**: donor after brain death; **HLA**: Human Leukocyte Antigen; **IS**: immunosuppression

Figure A2. Anti-LG3 distribution before transplantation and normal Q-Q Plot



* p-value < 2.2e-16 using Shapiro-Wilk normality test
(excludes null hypothesis of normal distribution)

Table A4. Anti-LG3 according to recipient characteristics

Recipient Characteristics	n	Anti-LG3 (OD units) (continuous)	p-value	Anti-LG3 > 130 OD units	p-value
Race black	34	198.8 (99.1-329.1)	**0.007**	21 (62%)	ns
Race not black	410	128.0 (85.6-203.5)		199 (49%)	
Sex Male	283	130.0 (92.0-213.0)	ns	141 (50%)	ns
Female	161	130.0 (84.5-214.0)		79 (49%)	
Baseline Renal Disease					
− Glomerular or Auto-immune	177	126.5 (86.0-206.5)	ns	85 (48%)	ns
− Diabetes or Hypertension	92	145.2 (85.6-253.1)		51 (55%)	
− Other	175	129.5 (87.8-209.8)		84 (48%)	
Previous KTx	48	122.8 (84.6-177.6)	ns	21 (44%)	ns
No previous KTx	396	132.8 (86.0-220.5)		199 (50%)	
Previous Pregnancy	117	125.5 (90.5-207.5)	ns	56 (48%)	ns
No previous pregnancy	327	132.0 (85.8-217.0)		164 (50%)	
Previous Transfusion	187	130.5 (79.8-226.2)	ns	94 (50%)	ns
No previous transfusion	254	128.0 (89.5-201.5)		123 (48%)	
PRA CDC: 0-20%	429	129.5 (86.5-219.0)	ns	212 (49%)	ns
>20%	14	130.2 (97.8-199.6)		7 (50%)	

Column "n" indicates the number of observations for levels of each variable. Median (IQR) is reported for anti-LG3 as a continuous variable**;** Count (%) is reported for anti-LG3 as a dichotomous variable. Only p-values <0.2 are reported; p-values <0.05 are identified in bold;
Krukal-Wallis test was used to compare anti-LG3 titres across levels of categorical variables. If Kruskal-Wallis showed statistical significance, pairwise comparison was performed using Dunn's test.
Chi-square test and Fisher's exact test (when cell count≤5) were used to compare proportion of anti-LG3 >130 OD units across levels of categorical variables.

Table A5. Anti-LG3 according to transplant and donor characteristics

Characteristics	n	Anti-LG3 (OD units) (continuous)	p-value	Anti-LG3 > 130 OD units	p-value
Center: CHUM	233	132.0 (87.0-213.0)	ns	117 (50%)	ns
HMR	211	127.5 (87.3-217.5)		103 (49%)	
HLA Mismatches in DR: 0	158	122.2 (78.0-199.4)	**0.03**	73 (46%)	ns
1 or 2	286	137.8 (91.3-228.0)		147 (51%)	
HLA Mismatches in B: 0	49	122.5 (86.0-175.0)	ns	21 (43%)	ns
1 or 2	394	132.2 (87.8-218.0)		199 (51%)	
Induction IS: Basiliximab	342	125.2 (83.0- 203.5)	**0.03**	162 (47%)	0.09
Thymoglobulin	101	143.0 (99.0-258.5)		58 (57%)	
Gammaglobulins	31	157.0 (91.8-224.2)	ns	20 (65%)	0.10
No gammaglobulins	413	127.5 (86.0-212.0)		200 (48%)	
Maintenance IS: Tacrolimus	425	130.0 (87.0-213.5)	ns	210 (49%)	ns
Cyclosporine	16	136.5 (78.9-222.8)		8 (50%)	
Total ischemia time >15h	106	118.0 (83.3-184.0)	ns	44 (42%)	0.06
Total ischemia time ≤15h	327	138.0 (88.5-228.5)		171 (52%)	
Delayed Graft Function	163	137.0 (87.8-248.8)	ns	85 (52%)	ns
No Delayed Graft Function	279	124.5 (85.5-196.0)		134 (48%)	
Donor type					
Live donor	115	140.0 (97.8-214.0)	ns	59 (51%)	ns
Deceased donor					
mECD and/or DCD	129	130.0 (76.5-225.5)		64 (50%)	
Standard criteria DBD	199	127.5 (87.0-212.5)		96 (48%)	

Column "n" indicates the number of observations for levels of each variable. Median (IQR) is reported for anti-LG3 as a continuous variable**;** Count (%) is reported for anti-LG3 as a dichotomous variable. Only p-values <0.2 are reported; p-values <0.05 are identified in bold;

Krukal-Wallis test was used to compare anti-LG3 titres across levels of categorical variables. If Kruskal-Wallis showed statistical significance, pairwise comparison was performed using Dunn's test.

Chi-square test and Fisher's exact test (when cell count≤5) were used to compare proportion of anti-LG3 >130 OD units across levels of categorical variables.

Figure A3. Correlation matrix of Anti-LG3 and other continuous variables

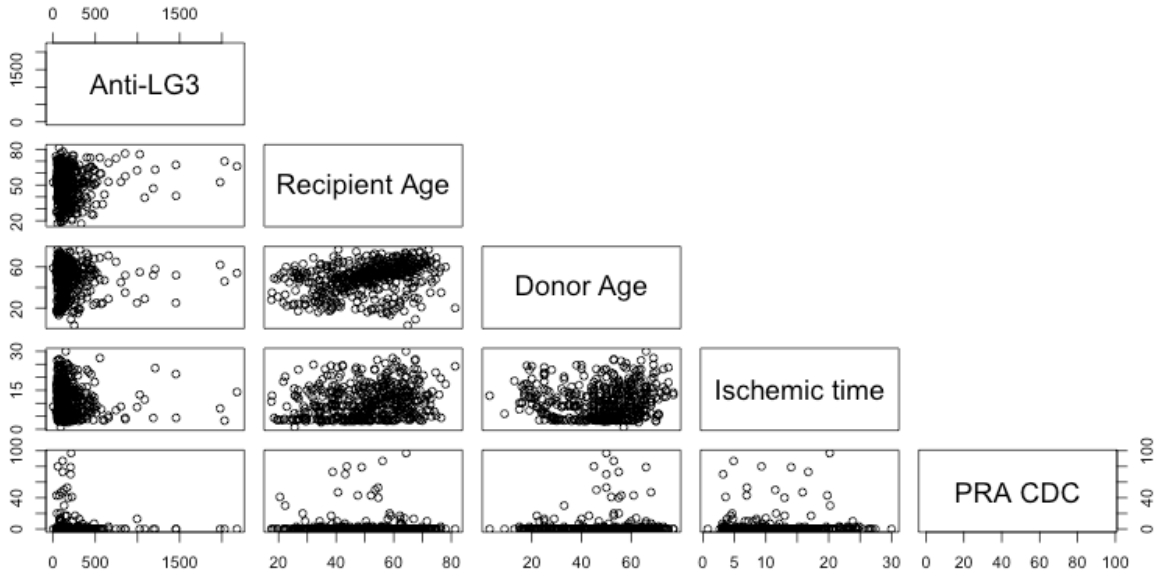


Table A6. Spearman's correlation coefficients for anti-LG3 and other continuous variables

Anti-LG3 (OD units)				
- 0.033 ns	**Recipient Age (years)**			
- 0.016 ns	0.374 **p <0.05**	**Donor Age (years)**		
- 0.086 ns	0.208 **p <0.05**	0.085 ns	**Ischemic time (hours)**	
0.004 ns	- 0.046 ns	0.034 ns	0.006 ns	**PRA CDC (%)**

Table A7. Univariate Cox proportional hazards models: **AVR** and **1st AR as the outcomes**

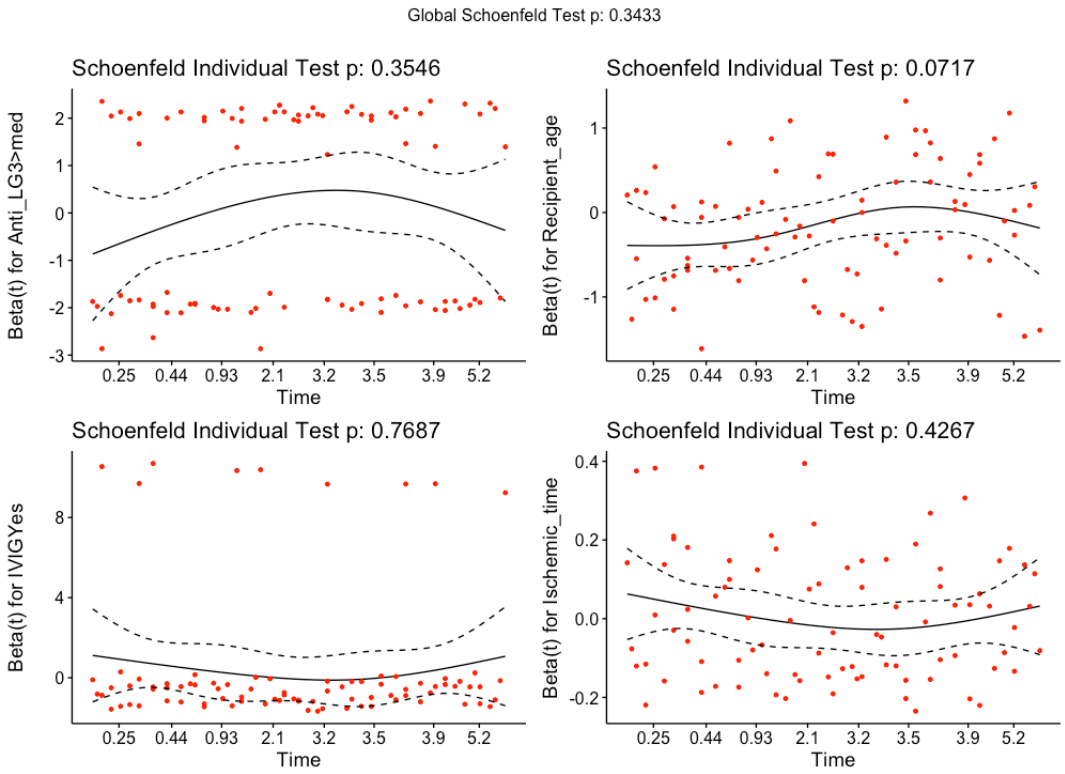
Variables	Reference category	AVR as event		1st AR as event	
		HR (95% CI)	p-value	HR (95% CI)	p-value
Auto-antibodies					
Anti-LG3 (OD units)	per 100 OD units increment	0.98 (0.84; 1.15)	-	1.02 (0.92; 1.13)	-
Anti-LG3 > 130 OD units	≤130	1.40 (0.70; 2.80)	-	1.08 (0.72; 1.63)	-
Recipient Characteristics					
Age (years)	per 10 years increment	0.72 (0.56; 0.92)	**0.009**	0.85 (0.73 ; 0.98)	**0.030**
Race – Black	Not black	1.30 (0.40; 4.30)	-	1.51 (0.76 ; 3.00)	-
Sex – Male	Female	0.77 (0.39; 1.50)	-	1.23 (0.79 ; 1.90)	-
Previous KTx – Yes	No	1.49 (0.58; 3.86)	-	1.27 (0.69 ; 2.34)	-
Previous Pregnancy – Yes	No	1.19 (0.57; 2.49)	-	0.77 (0.47; 1.25)	-
Previous Transfusion – Yes	No	1.40 (0.70; 2.81)	-	1.31 (0.87; 1.97)	-
PRA CDC	per 1% increment	1.01 (0.99; 1.03)	-	0.99 (0.96; 1.01)	-
Donor Characteristics					
Age (years)	per 10 years increment	1.03 (0.82; 1.31)	-	1.02 (0.89; 1.18)	-
Donor type					
mECD and/or DCD	Live donor	0.69 (0.30; 1.58)	-	1.21 (0.71; 2.06)	-
Standard criteria DB		0.44 (0.19; 1.01)	0.052	0.86 (0.51; 1.44)	-
Diabetes – Yes	No	0.37 (0.05; 2.71)	-	0.36 (0.11; 1.15)	0.084
Hypertension – Yes	No	1.18 (0.53; 2.66)	-	1.17 (0.72; 1.90)	-
Transplant characteristics					
Center – HMR	CHUM	1.38 (0.69; 2.73)	-	1.08 (0.71; 1.62)	-
HLA MM in B and DR	per 1 MM increment	1.38 (1.02; 1.88)	**0.039**	1.25 (1.04; 1.50)	**0.017**
Induction IS – ATG	Basiliximab	2.01 (0.99; 4.09)	0.053	1.39 (0.89; 2.19)	0.150
Gammaglobulins – Yes	No	3.68 (1.60; 8.48)	**0.002**	1.46 (0.73; 2.90)	-
Total Ischemia time (hours)	per 1 hour increment	0.95 (0.89; 1.01)	0.130	1.00 (0.96; 1.03)	-
Total Ischemia time >15h	≤15h	0.80 (0.33; 1.97)	-	0.94 (0.57; 1.54)	-
DGF – Yes	No	2.52 (1.26; 5.03)	**0.009**	1.89 (1.26; 2.85)	**0.002**

Only p-values <0.2 are reported; p-values <0.05 are identified in **bold**
KTx: kidney transplantation; **PRA CDC**: Panel Reactive Assay Complement-Dependent Cytotoxicity; **mECD**: modified expanded criteria donor; **DCD**: donor after cardiac death; **DBD**: donor after brain death; **HLA**: Human Leukocyte Antigen; **ATG**: thymoglobulin; **IS:** immunosuppression; **DGF**: Delayed Graft Function.

Table A8. Multivariate Cox regression models: **AVR as the outcome (Sensitivity Analysis)**

Variables	Reference category	HR (95% CI)	
		Unadjusted	Multivariate
Anti-LG3 > 130 OD units	≤130	**0.84 (0.34; 2.02)**	**0.78 (0.30; 2.07)**
Recipient Age (years)	per 10-year increment	-	0.71 (0.49; 1.02)
Gammaglobulins – Yes	No	-	3.65 (1.17; 11.4)
Total ischemia time	per 1-hour increment	-	0.97 (0.89; 1.06)
In this analysis, AVR is defined as Banff grade II and III TCMR (T-cell mediated rejection).			

Figure A4. Proportional hazards assumption test: Cox model with **AVR as the outcome**



Refers to the multivariate Cox model in Table 4:
AVR ~ Anti-LG3 + Recipient age (10 years) + Gamma IV + ischemia (hours)

Table A9. Change-in-estimate procedure for variable selection (**AVR as outcome**)

Variables in full model	Reference category	HR for Anti-LG3 > 130 OD units	% HR
Recipient			
Age (years)	per 10-year increment	1.23 (0.58; 2.62)	**-5.3%**
Race – Black	Not black	1.30 (0.61; 2.78)	-0.1%
Sex – Male	Female	1.30 (0.61; 2.79)	+0.4%
Previous KTX– Yes	No	1.31 (0.61; 2.79)	+0.9%
PRA CDC	per 1% increment	1.29 (0.60; 2.76)	-0.9%
Donor type			
mECD or DCD SCD/DBD	Live donor	1.28 (0.60; 2.73)	-1.6%
Transplant			
HLA MM in B and DR	Per 1 MM increment	1.29 (0.60; 2.76)	-0.6%
Gamma – Yes	No	1.39 (0.65; 2.96)	**+7.0%**
Induction IS – ATG	Basiliximab	1.29 (0.61; 2.75)	-0.6%
Ischemia time	per 1-hour increment	1.24 (0.61; 2.53)	**-4.5%**
Delayed Graft function – Yes	No	1.22 (0.57; 2.61)	**-6.4%**

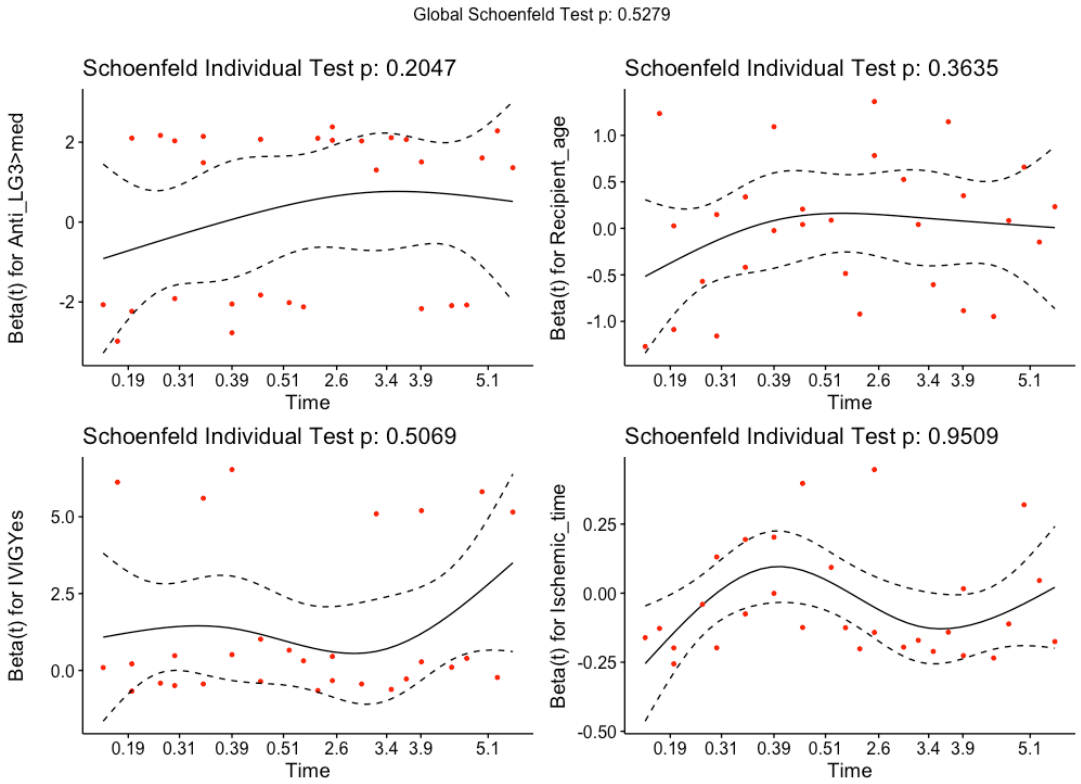
The point estimate of the HR for anti-LG3 >130 OD units in the initial models is 1.30
Column "**HR for Anti-LG3 > 130 OD units"** refers to the HR for anti-LG3>130 OD units when the variable in the corresponding row is removed from the initial full model.
Column "**%HR"** refers to the percent change in the HR for anti-LG3 >130 OD units compared to the initial model.

Table A10. Change-in-estimate procedure for variable selection (**1st AR as outcome**)

Variables in full model	Reference category	HR for Anti-LG3 > 130 OD units	% HR
Recipient			
Age (years)	per 10-year increment	0.93 (0.60; 1.43)	-0.6%
Race – Black	Not black	0.95 (0.62; 1.46)	+1.8%
Sex – Male	Female	0.93 (0.60; 1.44)	-0.4%
Previous KTX– Yes	No	0.94 (0.61; 1.44)	+0.2%
PRA CDC	per 1% increment	0.94 (0.61; 1.45)	+0.6%
Donor type			
mECD or DCD SCD/DBD	Live donor	0.94 (0.61; 1.44)	+0.2%
Transplant			
HLA MM in B and DR	Per 1 MM increment	0.95 (0.62; 1.46)	+1.8%
Gamma – Yes	No	0.95 (0.62; 1.47)	+2.1%
Induction IS – ATG	Basiliximab	0.94 (0.61; 1.45)	+0.5%
Ischemia time	per 1-hour increment	0.96 (0.63; 1.46)	+2.6%
Delayed Graft function – Yes	No	0.94 (0.61; 1.46)	+1.0%

The point estimate of the HR for anti-LG3 >130 OD units in the initial models is 0.93
Column "**HR for Anti-LG3 > 130 OD units"** refers to the HR for anti-LG3>130 OD units when the variable in the corresponding row is removed from the initial full model.
Column "**%HR"** refers to the percent change in the HR for anti-LG3 >130 OD units compared to the initial model.

Figure A5. Proportional hazards assumption test: Cox model with **AR as the outcome**



Refers to the multivariate Cox model in Table 6:
AR ~ Anti-LG3 + Recipient age (10 years) + Gamma IV + ischemia (hours)

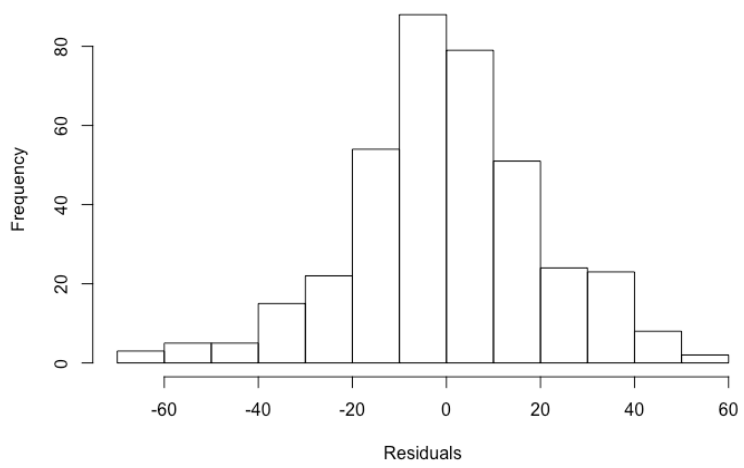
Table A11. Univariate linear regression models: **1-year GFR** and **Delta-GFR as the outcomes**

Variables	Reference category	1-year GFR as outcome		Delta-GFR as outcome	
		Coef (95% CI)	**p-value**	**Coef (95% CI)**	**p-value**
Anti-LG3 > 130 OD units	≤130	-0.91 (-5.71; 3.88)	ns	-1.15 (-3.41; 1.12)	ns
AVR in first 6 months	No	-12.13 (-20.96; -3.30)	**0.0072**	-0.40 (-4.60; 3.81)	ns
Recipient Characteristics					
Age (years)	per 10-year increment	-3.78 (-5.53; -2.02)	**3e-05**	0.07 (-0.78; 0.91)	ns
Race – Black	Not black	-8.52 (-17.26; 0.22)	0.056	-4.50 (-8.61; -0.38)	**0.032**
Sex – Male	Female	4.27 (-0.70; 9.23)	0.092	2.21 (-0.14; 4.55)	0.065
Previous KTx – Yes	No	-1.11 (-8.70; 6.48)	ns	0.47 (-3.10; 4.05)	ns
PRA CDC	per 1% increment	0.09 (-0.11; 0.29)	ns	-0.03 (-0.12; 0.06)	ns
Donor Characteristics					
Age (years)	per 10-year increment	-6.48 (-7.97; -5.00)	**2.4e-16**	-0.14 (-0.91; 0.62)	ns
Donor type mECD and/or DCD Standard criteria DBD	Live donor	 -15.48 (-21.55; -9.40) 2.08 (-3.51; 7.67)	 **8.4e-07** ns	 0.94 (-2.06; 3.94) 3.49 (0.73; 6.24)	 ns **0.013**
Diabetes – Yes	No	-2.82 (-12.44; 6.80)	ns	-3.26 (-7.83; 1.31)	0.16
Hypertension – Yes	No	-10.32 (-16.06; -4.57)	**0.0005**	0.16 (-2.63 2.95)	ns
Transplant characteristics					
Center – HMR	CHUM	-0.90 (-5.73; 3.92)	ns	-0.17 (-2.45; 2.11)	ns
HLA MM in B and DR	per 1 MM increment	-0.75 (-2.88; 1.38)	ns	0.29 (-0.72; 1.29)	ns
Induction IS – ATG	Basiliximab	-8.00 (-13.60; -2.41)	**0.0052**	-0.17 (-2.83; 2.50)	ns
Gammaglobulins – Yes	No	-0.91 (-9.82; 8.01)	ns	-1.83 (-6.03; 2.37)	ns
Total Ischemia (hours	per 1-hour increment	-0.35 (-0.74; 0.03)	0.074	0.09 (-0.09; 0.28)	ns
Total Ischemia >15h	≤15h	-2.12 (-7.67; 3.43)	ns	1.16 (-1.49; 3.82)	ns
DGF – Yes	No	-17.06 (-21.69; -12.42)	**2.6e-12**	-0.30 (-2.64; 2.05)	ns

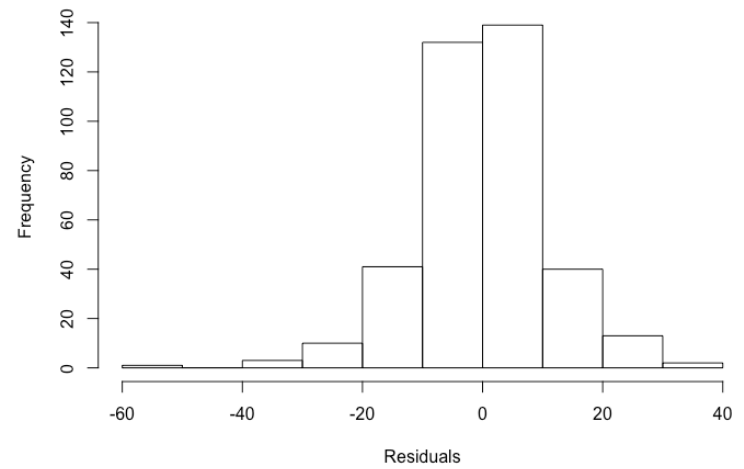
p-values <0.05 are identified in **bold;**
KTx: kidney transplantation; **PRA CDC**: Panel Reactive Assay Complement-Dependent Cytotoxicity; **mECD**: modified expanded criteria donor; **DCD**: donor after cardiac death; **DBD**: donor after brain death; **HLA**: Human Leukocyte Antigen; **ATG**: thymoglobulin; **IS:** immunosuppression; **DGF**: Delayed Graft Function; **AVR**: acute vascular rejection;

Figure A6. Histograms of residuals of linear regression models

A.



B.



Histogram **A.** shows the distribution residuals for the multiple linear regression model with 1-year GFR as the outcome (Table 7)
Histogram **B.** shows the distribution residuals for the multiple linear regression model with Delta-GFR as the outcome (Table 9)